Draft Assessment Report

Evaluation of Active Substances

Plant Protection Products

Prepared according to **assimilated Regulation No 1107/2009**
as it applies in Great Britain

Inpyrfluxam

Volume 3 – B.9 (AS)

Ecotoxicology Data

Great Britain

March 2026

Version History

When	What
November 2025	Initial DAR
March 2026	Updates made after ECP

Contents

B.9 Ecotoxicology Data

B.9.1 Effects on birds and other terrestrial vertebrates

B.9.1.1 Effects on birds

Summary of active substance endpoints

The following table provides a summary of the endpoints generated from the studies conducted with the active substance inpyrfluxam for birds. Full study summaries and evaluation can be found in Section B.9.1.1.

Table B.9.1 – 1: Toxicity endpoints for the risk assessment of birds for 'Inpyrfluxam' ('S 2399 TG')

Species	Substance	Exposure system	Results	Reference
Acute toxicity				
Northern bobwhite (*Colinus virginianus)*	Inpyrfluxam	Acute, 14d oral (single dose)	LD_{50}[a] = >2250 mg a.s./kg b.w.	CA 8.1.1.1/01 [redacted] and [redacted] 2014
Mallard (*Anas platyrhychos)*	Inpyrfluxam	Acute, 14d oral (single dose)	LD_{50}[b] = >1350 mg a.s./kg b.w.	CA 8.1.1.1/02 [redacted] and [redacted] L. 2016
Short-term dietary toxicity				
Northern bobwhite (*Colinus virginianus)*	Inpyrfluxam	Short-term, dietary (5 days)	LC_{50} >5620 ppm (>1348 mg a.s./kg b.w./d)	CA 8.1.1.2/01 Hibbard, P.M., [redacted] and [redacted] 2014a
Mallard (*Anas platyrhychos)*	Inpyrfluxam	Short-term, dietary (5 days)	LC_{50} >5620 ppm (>2136 mg a.s./kg b.w./d)	CA 8.1.1.2/02 [redacted] and [redacted] 2014b
Zebra Finch (*Taeniopygia guttata)*	Inpyrfluxam	Short-term, dietary (5 days)	**$LD0$[c] =** 253 ppm **(38 mg a.s./kg b.w./d)**	CA 8.1.1.2/03 [redacted] and

Species	Substance	Exposure system	Results	Reference
			NOEL = 80ppm **(19 mg a.s./kg b.w./d)**	[redacted] 2017
Reproductive toxicity				
Northern Bobwhite (*Colinus virginianus)*	Inpyrfluxam	Reproduction, dietary (21 weeks)	NOED[d] = 44.3 mg a.s./kg b.w./d	CA 8.1.1.3/01 [redacted] and 2015a
Mallard (*Anas platyrhychos)*	Inpyrfluxam	Reproduction, dietary (20 weeks)	NOEC[d] = 1000 ppm (130 mg a.s./kg b.w./day)	CA 8.1.1.3/02 [redacted] and 2015b

[a] Calculation of $LD_{10/20}$ not possible due to study design (limit test)
[b] $LD_{10/20}$ values could not be obtained with the mortality and regurgitation pattern observed during the test
[c] A definitive LD_{50} value could not be calculated due to a lack of clear dose response. As a worst-case, the lower value of the range (38-50 mg a.s./kg b.w./day) taken from the raw data was used for risk assessment purposes. There was no mortality at the lower value (38 mg a.s./kg b.w./day) which was also the LD_0
[d] $EC_{10/20}$ values could not be calculated as no effects on mortality or reproduction were seen in the study
Endpoints highlighted in **bold** used in the risk assessment

No avian studies with metabolites were submitted.

B.9.1.1.1 Acute oral toxicity to birds

Reference:	KCA 8.1.1.1/01
Report Title:	S-2399 TG: An acute oral toxicity study with the Northern Bobwhite
Author(s) & year:	[redacted] and [redacted] (2014)
Document No, Authority registration No:	[redacted] Report No.TPW-0001
Substance used:	Inpyrfluxam S-2399 TG (13CG0617G, 95%)
Method of analysis:	Not required
Guideline(s):	U.S. EPA OCSPP Number 850.2100 and JMAFF Test Guidelines 12 Nohsan
Deviations:	Yes, see HSE comments section

GLP or GEP:	GLP
Acceptability:	Yes
Study relied upon:	No. No longer part of data requirements

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material Inpyrfluxam (S-2399 TG)
Lot/Batch: 13CG0617G
Purity: 95.0 % (certified by certificate of analysis)
Description: Solid
Expiration date: 23 July 2016

B. TREATMENTS

Test concentrations: 0 and 2250 mg a.s./kg body weight
Controls: Negative control – one empty gelatine capsule (Torpac gelatine capsule size 00)

C. TEST ORGANISMS

Species: Northern Bobwhite (*Colinus virginianus*)
Age: Approximately 24 weeks
Acclimation period: Approximately 11 weeks
Treatment for disease: Beginning three days following arrival in the test facility, test birds were given water soluble antibiotics in their drinking water for eight consecutive days to prevent disease outbreak caused by the stress of shipping and handling. The birds received no form of antibiotic medication for approximately eight weeks prior to and during the test
Source: [redacted]
All birds were from the same hatch, pen-reared and phenotypically indistinguishable from wild birds
Fasting: Approximately 16.5 hours prior to dosing
Diet: Game bird ration formulated to [redacted] specifications

Water (town of Easton public water supply)

Bird mass:	188-241 grams at test initiation

D. TEST DESIGN

Test type:	Limit test
Housing:	78 x 51 cm floor pens constructed of galvanised wire with side walls of galvanised sheeting. Floors were sloped so ceiling height ranged from 20 to 25cm.
Replication:	Two pens per dosing group, 5 birds per pen (one pen with five males, the other with five females)
Dosing:	Oral insertion of one capsule (containing test substance or empty for treatment and control group respectively), coated with corn oil, into the crop/proventriculus of each bird
Post-dosing observation period:	14 days

E. TEST CONDITIONS

Test temperature:	22.4 - 23.6°C (mean = 23.1°C ± 0.3°C (SD))
Relative humidity:	22 - 68% (mean = 39 % ± 13 % (SD))
Photoperiod:	8 hours light, 16 hours darkness (average of approximately 208 lux of illumination)

STUDY DESIGN AND METHODS

Experimental dates: Hatch – May 5, 2013; Acclimation – August 9 2013 to October 22 2013; Experimental Start (OECD) – October 21, 2013; Experimental Start (EPA) – October 22, 2013; Experimental Termination – November 5, 2013

Test organism

Colinus virginianus was selected as a suitable species for this study due to the availability of captive bred individuals, alongside their low background mortality and low propensity to regurgitate.

Dosage preparation and dosing

The dosages were adjusted to 100% active substance. All dosages and the LD_{50} value are reported as milligrams of active substance per kilogram of body weight. Each bird was individually weighed and dosed on the basis of milligrams of active substance per kilogram of body weight.

Housing and environmental conditions

Each pen was identified with a unique number, and groups of pens were identified by project number and dosage. Individual birds within each pen were identified by coloured leg bands.

The light source was fluorescent lights that closely approximate noonday sunlight. The air handling system in the study room was designed to vent up to 15 room air volumes every hour and replace them with fresh air. Housing and husbandry practices were conducted so as to adhere to the guidelines established by the National Research Council[1].

Observations and measurements

During acclimation, all birds were observed daily. Birds exhibiting abnormal behaviour or physical injury were not used. On the day of dosing (Day 0), all birds were observed continually for at least sixty-four minutes following the completion of dosing to determine the onset of clinical signs of toxicity and any indication of regurgitation, if applicable. Following the initial period of observation, all birds were observed six additional times on the day of dosing. All birds were observed twice daily for the remainder of the test. A record was maintained of all signs of toxicity and abnormal behaviour.

Body weights were measured individually one day prior to initiation of the test and on Days 3, 7 and 14 of the test. Feed consumption was determined by pen for approximately 24-hour intervals from Day 0 to Day 1, Day 1 to Day 2 and Day 2 to Day 3. Average feed consumption was then determined by pen from Day 3 to Day 7 and from Day 7 to Day 14. Feed consumption was determined by measuring the change in the weight of the feed presented to the birds over a given period of time. The accuracy of feed consumption values may have been affected by the unavoidable wastage of feed by the birds.

A gross necropsy was performed on all surviving birds from treatment group and the control group at test termination. A gross necropsy included, but was not limited to, a general examination of the exterior of the bird and an examination of the thoracic and abdominal cavities, including cardiovascular and respiratory systems, liver, spleen, gastro-intestinal tract, and urogenital system.

Statistics

There were no mortalities observed in this study. Therefore, it was not possible to perform the calculation of an LD_{50} with 95% confidence intervals. In this study, the LD_{50} value was determined to be greater than the highest dosage tested.

II. RESULTS AND DISCUSSION

Mortality

There were no mortalities or evidence of regurgitation following dosing in either the control or treatment group.

Sub-lethal effects

Clinical observations

[1] National Research Council. 1996 Guide for the Care and Use of Laboratory Animals. Washington, D.C. National Academy Press. 125 pp

In the 2250 mg a.s./kg treatment group, one male was noted with a ruffled appearance on Day 4 of the test and two males were noted with a slight ruffled appearance on Day 5 of the test. For all other observations, all birds were normal in appearance and behaviour throughout the test.

Necropsy

There were no remarkable findings for the birds in the control group. In the treatment group two birds had remarkable findings. One bird was noted with bruising on the head and another bird was noted with a small and slightly pale spleen. The findings were not considered to be treatment related by the study conductor.

Body weight and feed consumption

Mean body weight and mean feed consumption for each treatment group and sex are presented in Tables B.9.1.1.1-1 and B.9.1.1.1-2

Table B.9.1.1.1-1: Mean body weights (g)

Treatment (mg a.s./kg)	Sex	Mean body weights in grams (± Standard Deviation)							
		Day -1	Change[a]	Day 3	Change[a]	Day 7	Change[a]	Day 14	Total Change[a]
Control	M	219(13)	-6(3)	213(13)	1(4)	214(11)	4(3)	219(11)	0(4)
	F	206(18)	-8(5)	198(16)	4(2)	202(17)	1(4)	203(20)	-3(5)
2250	M	217(6)	-20(8)	197(4)	4(5)	201(8)	5(7)	207(7)	-10(6)
	F	217(14)	-31(13)	187(12)	12(7)	198(13)	11(4)	210(11)	-8(6)

[a]The mean change is calculated separately from the mean body weights using the individual changes in body weights

Table B.9.1.1.1-2: Mean feed consumption (g/bird/day)

Treatment (mg a.s./kg)	Sex	Estimated feed consumption (g/bird/day)				
		Days 0-1	Days 1-2	Days 2-3	Days 3-7	Days 7-14
Control	M	17	15	15	14	15
	F	14	12	13	14	13
2250	M	11	9	8	18	17
	F	5	4	5	15	17

When compared to the control group, there was an apparent treatment related loss of mean body weight from Day -1 to Day 3 of the test in the 2250 mg a.s./kg treatment group (Table B.9.1.1.1-1). From Day 3 to Day 7 and Day 7 to Day 14, the treatment group had mean body

weight gains that were greater than the control. However, the treatment group showed an overall mean body weight loss that was greater than the control group.

When compared to the control group, there was a slight reduction in mean feed consumption for the treatment group during the feeding intervals from Day 0 to Day 3 of the test (Table B.9.1.1.1-2). For the feeding intervals from Day 3 to Day 7 and Day 7 to Day 14 of the test, the feed consumption of the treatment group was comparable to the control group feed consumption.

Validity criteria
The validity criteria for the study were met according to OECD 223 (2016) and OCSPP 850.2100 (2012) (Table B.9.1.1.1-3).

Table B.9.1.1.1-3: Compliance with OECD 223 (2016) and OCSPP 850.2100 (2012) validity criteria

Validity criterion	Required	Obtained
Control mortality (OECD 223 (2016) & OCSPP 850.2100 (2012))	≤ 10 %	0 %
Bird assignment to test pens (OCSPP 850.2100 (2012))	Random	Random
Birds per dose level (OCSPP 850.2100 (2012))	10	10
Administration route (OCSPP 850.2100 (2012))	Oral (gavage or capsule)	Oral (capsule)

III. CONCLUSION

The acute oral LD_{50} value for northern bobwhite exposed to 'Inpyrfluxam' as a single oral dose was determined to be > 2250 mg a.s./kg b.w., the highest dosage tested. The no-mortality level was 2250 mg a.s./kg b.w.

HSE COMMENTS
The study was carried out according to OCSPP 850.2100 (2012) and evaluated against OCSPP 850.2100 (2012), as well as the validity criteria of OECD 223 (2016). All validity criteria were met.

The following deviations from OCSPP 850.2100 (2012) were noted:

OCSPP 850.2100 (2012) (e)3(vii)(A) details the recommended nutritional values for game bird feed. crude protein - 27 to 29 %, crude fibre - 3.5 to 5.0 %, crude fat - 2.5 to 7.0 %, calcium - 2.6 to 3.6 % and phosphorus - 0.9 to 1.1 % are recommended. The feed used had 1.12 % calcium and 0.77 % phosphorous. There was no mortality or sub-lethal effects reported throughout the study or in the test population during the 14 days preceding the test, indicating the feed provided was suitable. HSE considers this a minor deviation.

OCSPP 850.2100 (2012) (e)7(ii)(A) specifies the required dimensions for test pens. It states, "*pens … should be at least 24 centimetres (cm) (approximately 9.5 inches) high for northern bobwhite*". Floors were sloped so ceiling height ranged from 20 to 25cm. According to the guidelines the minimum ceiling height should have been 24 cm. This minor deviation will have not impacted the study results and HSE consider it acceptable.

OCSPP 850.2100 (2012) (e)8(ii) provides a relative humidity range of 45 to 70 %. The relative humidity for the test ranged from 22 to 68 %. The guideline does state that humidity is not as critical as other variables. To support this, no mortality or sub-lethal effects were recorded in any control individuals, which suggests environmental conditions were acceptable. HSE considers this a minor deviation.

OCSPP 850.2100 (2012) (e)8(iii) recommends a photoperiod of 10 hours light and 14 hours dark. The study used an 8 hour light, 16 hours dark regime. This regime, however, served the same purpose of preventing birds from coming into the reproductive condition. HSE considers this a minor deviation.

OCSPP 850.2100 (2012) (f)(1)(iii) covers the treatment of results for bodyweight. The guideline requires the report to contain plots for mean body weight change and standard error by observation interval and mean total body weight change and standard error to assess effects on the pattern of weight change. These were not provided. An appreciation of weight change over time was possible through scrutiny of the tables included in this study evaluation. HSE consider this a minor deviation.

OCSPP 850.2100 (2012) (f)(1)(iv) recommends a plot of mean food consumption by treatment level and observation period. This was not provided but, as above, could be viewed in the table provided in this study evaluation. HSE consider this a minor deviation.

OCSPP 850.2100 (2012) (f)(3)(ii) covers reporting the proportion of mortality and associated confidence intervals. This information is important because, "*for assessing risks, the confidence in the estimated proportion impacted is considered in determining acute effects at environmental exposure doses. If the uncertainty in the estimate of the true proportion of mortality ($\hat{p}$) is high at the limit concentration, and the expected environmental exposure concentration is close to the limit concentration, risks to threatened and endangered species*

may not be able to be discounted". Although the $\hat{p}$ = 0 and the 95 % confidence interval = 0 to 0.31 is known from the guidelines, this was not reported in the study. As this information is readily accessible in the guideline HSE considers this a minor deviation.

Finally, there were two remarkable findings found during the necropsy in the treatment group (slightly small, pale spleen and bruising on the head). There is a possibility these were treatment related. This will be considered at the risk assessment stage using a margin of safety approach.

The above study was conducted to GLP and considered valid.

The agreed endpoint for consideration in risk assessment is LD_{50} > 2250 mg a.s./kg body weight.

Reference:	KCA 8.1.1.1/02
Report Title:	S-2399 TG: An Acute Oral Toxicity Study with the Mallard [redacted] Project No. 263B-178, Sumitomo Chemical Co., Ltd. Report No. TPW-0040
Author(s) & year:	[redacted] and [redacted] (2016)
Document No, Authority registration No:	[redacted] Report No. TPW-0040
Substance used:	Inpyrfluxam S-2399 TG (13CG0617G, 95.0%)
Method of analysis:	Not required
Guideline(s):	U.S. EPA OCSPP Number 850.2100
Deviations:	Yes, see HSE comments section
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

I. MATERIALS AND METHODS

A.MATERIALS

1. Test material: S-2399 TG
Description: Solid
Lot/Batch: 13CG0617G

Purity: 95.0%
Reference item: None
Expiry date: 23rd July 2016
Solvent: None

B.STUDY DESIGN AND METHODS

1. Test animals: Mallard (*Anas platyrhychos*)
Age/growth stage: 23 weeks old at dosing
Source: [redacted]
Acclimation: 17 days prior to dosing
Analysis of concentrations: 0, 292, 486, 810, 1350, and 2250 mg a.s./kg body weight
Replication: 10 birds per concentration
No. of birds/pen: 2 or 3
Diet: Game bird ration *ad libitum* during acclimation and during the test, except periods of fasting prior to testing (approximately 17 hours) and approximately two hours after dosing

Housing: Pens measuring 75 × 90 cm with a ceiling height of 45 cm. External walls, ceilings and floors were constructed of vinyl-coated wire grid

Environmental conditions:
Temperature: 16.5 - 20.9°C
Relative humidity: 43 - 70%
Photoperiod: 8 hours light, 16 hours darkness

A summary of environmental conditions is shown in Table B.9.1.1.1-4 below.

Table B.9.1.1.1-4: Summary of environmental conditions obtained in study of acute oral toxicity of Mallards exposed to Inpyrfluxam

Variable	Required OCSPP 850.2100 (2012)	Obtained
Temperature	15˚C – 27˚C	16.5˚C – 20.9˚C
Humidity	45 - 70%	43-70 %
Photoperiod	10 hours light and 14 hours dark	8 hours light and 16 hours darkness
Ventilation	10 – 15 exchanges per hour	15 room air volumes every hour

Study dates: 11th – 25th March 2016

5. Animal assignment and treatment:

The test consisted of five treatment groups and a control group. There were 10 birds (5 males and 5 females), randomly assigned to pens per treatment and control. There were four pens per treatment, with two or three birds in each. At the experimental start, following a fasting period of 17 hours, a single dose of the test substance in a capsule with corn oil was administered into the crop or proventriculus of each bird. The control birds received an empty capsule coated with corn oil. Following dosing, the birds were observed for a period of 14 days. Food and water were provided *ad libitum*.

6. Dose preparation:

The test substance was weighed into a tared capsule bottom. Individual doses were prepared based on bird body weights to provide a constant weight to body weight dosage for all treatment birds within a level. Nominal dosages used in this study were 0, 292, 486, 810, 1350 and 2250 mg a.s./kg b.w.

7. Measurements and observations:

Following dosing, multiple cage-side observations were performed on Day 0 of the test. All birds were observed at least twice daily during the test. Body weights were measured individually on the day prior to dosing (Day -1) and on Days 3, 7, and 14 of the test. Feed consumption was determined by pen for approximately 24-hour intervals from Day 0 to 1, Day 1 to 2, and Day 2 to 3. Average daily feed consumption was then determined from Days 3 to 7 and from Days 7 to 14. A gross necropsy was performed on all mortalities and on three birds from each group at test termination.

8. Statistics:

There were no levels with greater than 30% mortality so an LD_{50} value could not be calculated. The LD_{50} value was determined to be greater than the highest dosage tested with 20% or less regurgitation and no mortality. Body weight data were compared by Dunnett's multiple-comparison test using TOXSTAT®.

II. RESULTS AND DISCUSSION

A. MORTALITY

There were no mortalities in the control group or the 292, 486, 810 and 1350 mg a.s./kg b.w. groups. There was 30% mortality in the 2250 mg a.s./kg b.w. group and a 38% mortality among those birds retaining the dosage (non-regurgitating). Mortality data is presented in Table B.9.1.1.1-5.

Table B.9.1.1.1-5: Mortality of Mallard Duck following exposure to Inpyrfluxam

Treatment (mg a.s./kg)	Mortality after 14 days exposure to S-2399 TG	
	Total mortality (%)	Total mortality (%) from birds retaining dosage
Control	0	0
292	0	0
486	0	0
810	0	0
1350	0	0
2250	30	38

B. SUBLETHAL EFFECTS

All birds in the control group and in the 292 and 486 mg a.s./kg b.w. were normal in appearance and behaviour throughout the duration of the test. No regurgitation was noted for the control group or the 292 and 486 mg a.s./kg b.w. groups. The 810, 1350 and 2250 mg a.s./kg b.w. groups had two birds each noted as regurgitating.

In the 810 mg a.s./kg b.w. treatment group, one male and one female regurgitated within 30 minutes of dosing. Four birds in this treatment showed signs of toxicity (loss of coordination (ataxia) and lower limb weakness). All birds were normal in appearance from approximately two hours following treatment to test termination.

In the 1350 mg/kg b.w. treatment group, two females regurgitated within approximately 30 minutes of dosing. The first signs of toxicity were approximately 25 minutes after dosing. Eight birds in this treatment group showed signs of toxicity (ruffled appearance, loss of coordination (ataxia), lower limb weakness, prostrate posture, and depression). All birds were normal in appearance from approximately six hours and 30 minutes following dosing to test termination.

In the 2250 mg a.s./kg b.w. treatment group, two males regurgitated within 20 minutes of dosing. The first signs of toxicity were noted approximately 20 minutes after dosing. All birds in this treatment group showed signs of toxicity (ruffled appearance, loss of coordination (ataxia), lower limb weakness, prostrate posture, loss of righting reflex, minor muscle fasciculation, and depression). All surviving birds were normal in appearance from Day 1 to test termination.

When compared to the control group, there were no statistically significant differences in mean body weights or mean changes in body weights. A summary of mean body weight is presented in Table B.9.1.1.1-6.

Feed consumption values were variable even within the control group. A summary of mean feed consumption is presented in Table B.9.1.1.1-7. Given the high variability in feed consumption values and the lack of a statistical body weight effect the differences in feed consumption were not considered to be adverse effects.

A gross necropsy was performed on three birds from each group at test termination. There were no remarkable findings for any of the birds necropsied at test termination for the control group and the 486, 810, and 2250 mg a.s./kg dosage levels. At the 292 mg a.s./kg dosage level of the three birds necropsied, two were noted with pale kidneys and one was noted with a pale spleen. At the 1350 mg a.s./kg dosage level, of the three birds necropsied, one was noted with an enlarged spleen and one was noted with a pale spleen.

Table B.9.1.1.1-6: Mean Body Weights (g)

Treatment (mg a.s./kg)	Sex	Mean body weights in grams (± Standard Deviation)							
		Day -1	Change[a]	Day 3	Change[a]	Day 7	Change[a]	Day 14	Total Change[a]
Control	M	1109 (85)	-17 (37)	1092 (100)	19 (39)	1111 (70)	12 (21)	1123 (61)	14 (47)
	F	1011 (60)	-12 (16)	999 (73)	-15 (34)	984 (83)	2 (30)	986 (61)	-25 (12)
292	M	1094 (177)	-22 (44)	1072 (158)	27 (14)	1099 (155)	-14 (10)	1086 (150)	-8 (61)
	F	933 (80)	-43 (54)	890 (59)	21 (26)	911 (70)	21 (27)	931 (67)	-2 (98)
486	M	1075 (93)	-33 (26)	1042 (85)	33 (11)	1075 (87)	-2 (21)	1073 (102)	-2 (27)
	F	1017 (62)	-29 (11)	988 (53)	29 (18)	1017 (53)	15 (31)	1032 (68)	15 (47)
810	M	1044 (115)	17 (63)	1061 (56)	40 (31)	1101 (47)	28 (23)	1129 (53)	86 (102)
	F	1015 (106)	-52 (44)	963 (135)	44 (31)	1007 (109)	27 (15)	1033 (107)	18 (21)
1350	M	1086 (46)	-17 (40)	1069 (20)	31 (41)	1099 (50)	22 (9)	1121 (41)	36 (19)
	F	1019 (80)	2 (8)	1021 (86)	10 (10)	1031 (89)	21 (20)	1052 (102)	33 (32)
2250	M	1097 (126)	-22 (21)	1099 (140)	-2 (36)	1097 (125)	26 (9)	1122 (125)	1 (34)
	F	935 (41)	-34 (57)	903 (50)	35 (89)	939 (62)	13 (16)	951 (77)	14 (42)

[a]The mean change is calculated separately from the mean body weights using the individual changes in body weights

Table B.9.1.1.1-7: Estimated Mean Feed Consumption (g/bird/day)

Treatment (mg a.s./kg)	Sex	Days 0-1	Days 1-2	Days 2-3	Days 3-7	Days 7-14
Control	M	81	91	116	141	120
	F	71	112	123	89	108
292	M	88	118	147	135	140
	F	41	68	79	108	105
486	M	48	102	107	112	120
	F	42	83	88	88	114
810	M	77	152	178	164	135
	F	54	69	91	98	116
1350	M	45	125	172	166	153
	F	52	97	100	95	97
2250	M	26	119	127	122	155
	F	13	59	33	117	128

C.VALIDITY CRITERIA

As mortality was less than 10% in the controls (actual value 0%) the study was considered valid.

Table B.9.1.1.1-8 below shows the validity criteria as per OCSPP 850.2100 (2012) as these are the guidelines the study was conducted to but will not be used to determine validity for UK usage.

Table B.9.1.1.1-8: Validity criteria of OCSPP 850.2100 (2012) guidelines

Criteria	Required OCSPP 850.2100 (2012)	Obtained
Treatment and control assignment	Birds randomly assigned	Birds randomly assigned
Control mortality	<10%	<10%
Test animals	Minimum of 10 birds per dose level of test substance and control	10 birds per dose level of test substance and control
Substance administration	Oral via capsule or gavage	Capsule

Criteria	Required OCSPP 850.2100 (2012)	Obtained
Dosage levels	Minimum of 5 dose levels plus control	5 dosage levels and a control group

III. CONCLUSION

The acute oral LD_{50} value for mallard exposed to S-2399 TG as a single oral dose was determined to be > 1350 mg a.s./kg b.w., based on 30% mortality and 20% regurgitation occurring at the highest dosage tested of 2250 mg a.s./kg b.w. The no-mortality level was 1350 mg a.s./kg b.w. and the no-observed-effect level was 492 mg a.s./kg b.w.

HSE comments:

This study was completed under GLP adhering to U.S. EPA OCSPP Number 850.2100 and has been assessed against U.S EPA OCSPP 850.2100 (2012) guidance. OECD 223 (2016) guidance has been used for the validity criteria to determine applicability in the UK.

The photoperiod requirements in OCSPP Number 850.2100 (2012) are 10 hours light and 14 hours darkness. The study obtained a photoperiod of 8 hours light and 16 hours darkness, which does adhere to OCSPP Number 850.2100 (1996) and OECD 223 (2016) guidance. No adverse observations were made that could be attributed to the difference in photoperiod and all the validity criteria were met; therefore, the study is considered valid.

An LD_{50} could not be determined in this study as no concentration of S-2399TG used resulted in 50% mortality. A range-finding experiment was conducted under non-GLP conditions to establish the concentrations for experimental testing. No further information was provided on the range-finding experiment, so it's suitability for determining sensitivity cannot be commented on. There was also regurgitation in two birds for each of the 810, 1350 and 2250mg a.s./kg b.w. group, which should be avoided. All validity criteria were met, so the study is considered valid.

No statistical analysis was conducted on the mortality data. Body weight data were compared by Dunnett's multiple-comparison test using TOXSTAT®.

The endpoints to use in risk assessment are:

- **LD_{50} >1350mg a.s./kg b.w. based on 30% mortality and 20% regurgitation at the highest tested dose (2250 mg a.s./kg b.w.)**

B.9.1.1.2 Short-term dietary toxicity to birds

Reference:	KCA 8.1.1.2/01
Report Title:	S-2399 TG: A dietary LC_{50} study with the northern bobwhite
Author(s) & year:	[redacted] and [redacted] (2014a)
Document No, Authority registration No:	[redacted] Report No. TPW-0008
Substance used:	Inpyrfluxam S-2399 (13CG0617G, 95.0%%)
Method of analysis:	HPLC-VWD
Guideline(s):	OCSPP Number 850.2200, OECD Guideline 205 and JMAFF Test Guideline 12 NohSan No. 8147
Deviations:	Yes – see HSE comments
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	No – no longer part pf core data requirements

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: Inpyrfluxam S-2399 TG
Description: Solid
Lot/Batch: 13CG0617G
Purity: 95.0%
Reference item: None
Expiry date: 23rd July 2016
Solvent/Carrier: None

B. STUDY DESIGN AND METHODS

1. Test animals: Northern Bobwhite (*Colinus virginianus*)
Age/growth stage: 12 days old at test initiation (19-26 g b.w.)
Source: [redacted]
Acclimation: From day of hatch until test initiation (12 days)
Nominal test concentrations: 0, 562, 1000, 1780, 3160 and 5620 ppm a.s.

LOQ:	31.3 ppm a.s.
2. Diet:	Game bird ration formulated to [redacted] specifications
3.Housing:	Brooding pens measuring 72 cm x 90 cm x 23 cm
No. of birds/pen:	2 pens of 5 chicks/per concentration
4.Environmental conditions:	
Temperature:	38.4°C ± 1.6°C (within brooding compartments) 26.3˚C ± 1.3˚C (under test conditions)
Relative humidity:	20.2% ± 5.9%
Photoperiod:	16 hours light, 8 hours darkness (352 lux)

A summary of environmental conditions is shown in Table B.9.1.1.2 -1 below:

Table B.9.1.1.2 - 1: A summary of environmental conditions for short-term dietary toxicity of northern bobwhite exposed to Inpyrfluxam

Variable	Required OECD 205 (1984)	Obtained
Temperature	Age 0-7 days = 35˚C – 39˚C Age 8-14 days = 30˚C – 32˚C Age > 14 days = 25˚C – 28˚C	26.3°C ± 1.3°C (during testing) Min: 23.4°C Max: 27.7°C
Relative humidity	50% - 75%	20.2% ± 5.9%
Lighting (lux)	12 to 16 hours of light per day	16 hours light, 8 hours darkness (352 lux)
Ventilation	Not stated	15 room air volumes every hour

Study dates:
19th Feb 2014 – 27th Feb 2014

5. Animal assignment and treatment:
Birds were assigned to five test groups and one control group. Each treatment group contained 10 chicks and the control group contained 30 chicks. Birds were housed in brooding pens containing five birds each. The birds used in the study were immature and could not be differentiated by sex. Diets were presented to the birds at initiation of the test and were offered *ad libitum* during the 5-day treatment period. No other food was available during this period. Following the exposure, birds were fed with untreated basal diet for three days.

6. Dose preparation:

Test diets were prepared on the first day of dosing by mixing the test substance into the feed containing 2% corn oil. An amount of diet sufficient to last the exposure period was prepared on the first day of exposure. Dietary concentrations were corrected for purity of the test substance. Nominal concentrations were 0, 562, 1000, 1780, 3160 and 5620 ppm.

7. Measurements and observations:

Samples of the test diets were collected to confirm stability and homogeneity of the test substance in the diets. Homogeneity was confirmed from the 562 and 5620 ppm test diets on Day 0 (preparation day), with collection from the top, middle and bottom of the left and right sections of the mixing vessel. These samples also served as verification samples for those concentrations. One verification sample was collected from the control diet and two were collected from each remaining treatment on Day 0. On Day 5, samples were collected from feed troughs of the control group and treatment groups to assess stability. Concentrations of S-2399 TG were determined by HPLC (High performance liquid chromatography).

Birds were observed at least twice daily during the test, for all signs of toxicity and abnormal behaviours. Individual body weights were measured at test initiation (Day 0), on Day 5 and at test termination (Day 8). Average feed consumption values were determined daily during the exposure period (Days 0-5) and during the post-exposure observation period (Days 6-8) for each treatment group and control.

At termination, a gross necropsy was performed on the one mortality and on three birds from each test concentration. The LOQ was 31.3 ppm a.s.

8. Statistics:

There were no treatment-related mortalities observed in this study; therefore it was not possible to perform the calculation of an LC_{50}. The LC_{50} value was determined to be greater than the highest concentration tested. Body weight data were compared by Dunnett's t-test using TOXSTAT®.

II. RESULTS AND DISCUSSION

A. MORTALITY

There were no mortalities in the control group or in the 562, 1000, 1780 and 5620 ppm treatment groups. There was a single mortality in the 3160 ppm test concentration. The mortality was incidental and not treatment related. The mortality data for the study is displayed in Table 8.1.1.2/01-2.

Table B.9.1.1.2 - 2: Summary of the cumulative mortality data of northern bobwhite quail exposed to Inpyrfluxam under short-term dietary exposure conditions

Experimental Group (ppm a.s.)	No. Dead Per No. Exposed. Exposure period						No. Dead Per No. Exposed. Post-Exposure Period.		
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Control 0	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30	0/30
Treatment 562	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
1000	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
1780	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
3160	0/10	0/10	1/10[a]	1/10	1/10	1/10	1/10	1/10	1/10
5620	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
The LC_{50} value was determined to be greater than 5620 ppm a.s., the highest test concentration. [a] Bird euthanised due to injury, mortality not treatment related.									

Note that the mortalities recorded for the 3160 ppm concentration on days 3-8 is representing the single mortality that occurred on day 2.

B. SUBLETHAL EFFECTS

There was no evidence of treatment-related effects on food consumption at all test concentrations. There were no treatment related effects on mean body weight or mean body weight change at the 562, 1000 and 1780 ppm concentrations. At the 3160 ppm concentration there was a treatment related reduction in mean body weight gain from Day 0 to Day 5. At the 5620 ppm concentration the birds had a slightly lower initial mean body weight, with difference to the control group being significant. Mean body weight increase and food consumption are presented in Table 8.1.1.2/01-3. No abnormalities were observed in the gross necropsy at study termination.

Table B.9.1.1.2 - 3: Mean body weight increase and food consumption following dietary exposure to Inpyrfluxam

Treatment (ppm)	Days of study				
	Body weight increase (g) (SD)[a]			Group mean food consumption (g/bird/day) (SD)	
	0 to 5	5 to 8	Total change	0 to 5	5 to 8
0 (control)	13(2)	9(4)	22(5)	7(0)	10(2)
562	13(2)	10(1)	23(3)	7	9
1000	12(2)	10(3)	22(4)	7	10
1780	12(2)	11(3)	22(4)	6	8
3160	10*(3)	9(2)	19(5)	7	9
5620	8**(3)	8(4)	15**(5)	6	8

[a] Mean change is calculated separately from the mean body weights using individual body weights
SD = Standard Deviation
*Statistically significant difference from the control group at $p < 0.05$ (Dunnett's t-test)
** Statistically significant difference from the control group at $p < 0.05$ (Dunnett's t-test)

C. DIET ANALYSIS

Analysis of the control samples did not show any presence of the test substance or the presence of a co-eluting substance at the characteristic retention time of the test substance. Diet samples from the 562 and 5620 ppm concentrations analysed for homogeneity recorded mean concentrations of 566 (± 18.9 ppm) and 6460 (± 414 ppm), respectively.

Samples collected on Day 0 for the 1000, 1780 and 3160 ppm test concentrations to verify test concentrations had mean concentrations of 1000, 2000 and 3740 ppm, respectively. These values represented 100%, 112% and 118% of nominal concentrations. Analysis of diet samples collected from feeders after being held in ambient temperature for five days averaged 90%, 107%, 85%, 98% and 92% of the Day 0 values for the 562, 1000, 1780, 3160 and 5620 ppm concentrations, respectively. The results from the analysis of diet samples collected from feeders after being held at ambient temperature are presented in the table below.

Table B.9.1.1.2 – 4: Summary of analytical results

Nominal concentration (ppm)	Day 0[a]		Day 5	
	Mean Measured (ppm)	Mean % of nominal	Mean measured (ppm)	Mean % of nominal
0	n.d	n.a	n.d	n.a
562	566	101	506	90
1000	1000	100	1070	107
1780	2000	112	1690	85
3160	3740	118	3680	98
5620	6460	115	5960	92

[a] Day 0 values are from homogeneity samples
n.d = not detectable as below limit of quantitation (LOQ)
n.a = not applicable

D.VALIDITY CRITERIA

The validity criteria of the OECD 205 (1984) guideline is shown in Table 8.1.1.2/01-5 below. Additional validity criteria are required as part of the OCSPP 850.2200 (2012) guidelines, but these are not required in addition to OECD validity criteria.

Table B.9.1.1.2 – 5: OECD 205 (1984) validity criteria

Criteria	Required by OECD 205 (1984)	Obtained
Mortalities in control	<10%	0%
Concentrations maintained	>80% of nominal concentration	>85%
Toxic effects	Lowest treatment level should not result in compound-related mortality observable toxic effects	No mortalities or sublethal effects in lowest concentration

III. CONCLUSION

The dietary LC_{50} value for northern bobwhite exposed to Inpyrfluxam was determined to be > 5620 ppm (> 1348 mg a.s./kg b.w./day), the highest concentration tested. The no-treatment-related mortality concentration was 5620 ppm (1348 mg a.s./kg b.w./day). The no-observed-effect concentration (NOEC) was determined to be 1780ppm (396 mg a.s./kg b.w./day) due to a body weight effect at the 3160 ppm concentration (808 mg a.s./kg

b.w./day).

HSE comments:

This study was conducted under GLP and has been assessed against OCSPP 850.2200 (2012) and OECD 205 (1984).

There are several protocol deviations to note. The first deviation is that the temperature obtained during testing dropped below the minimum for birds over 14 days old. OECD 205 (1984) guidance states that northern bobwhite under 14 days old (days day 1 and 2 of study) should be kept in temperatures of 30-32˚C and birds over 14 days should be kept in temperatures of 25-28˚C. The study obtained an average temperature of 26.3°C ± 1.3°C with a minimum temperature of 23.4°C and a maximum temperature of 27.7°C. This study was also conducted in line with OCSPP 850.2200 (2012) guidance, which states that there should be a temperature gradient of 22-38˚C and the study would meet these requirements. As there were no mortalities or sublethal effects and all validity criteria were met, the study is considered valid.

It is also noted that the humidity is outside of the range required in OECD 205 (1984). The relative humidity obtained in the study was 20.2% ± 5.9%, which is considerably below the OECD 205 (1984) recommendation of 50% - 75% and the OCSPP 850.2200 (2012) recommendation of 45% - 70%. However, as the validity criteria have been met, this does not invalidate the study.

The study obtained a photoperiod of 16 hours light and 8 hours darkness, which does adhere to OECD 205 (1984) guidance, but does not adhere to OCSPP 850.2200 (2012) guidelines that require 14 hours light and 10 hours darkness. This will not have a significant impact on the study and as all the validity criteria has been met, the study is considered valid.

In OECD 205 (1984), the highest recommended treatment level is 5000ppm. This is the same for OCSPP 850.2200 (2012), depending on the maximum expected environmental concentration. There were no comments on the maximum expected environmental concentration and no range finding test appears to have been conducted. It is therefore unclear why the highest nominal concentration (5620ppm) was used in this study.
It is worth noting that the homogeneity for the highest concentration tested on day 0 was significantly higher than the nominal concentration stated. The nominal test concentration of 5620 ppm recorded a mean concentration of 6460 (±414) when analysed for homogeneity. Ultimately, this is within ± 20% and nominal test values can be used. The chemistry conclusions on the methods of analysis are presented below.

As the above study is not required for the risk assessment, the analytical methods have not been evaluated by HSE Chemistry.

In terms of sub-lethal effects, the 5620 ppm concentration the birds had a slightly lower initial mean body weight, with difference to the control group being significant. As no mortalities were recorded in this group and birds appeared normal, it is not likely to have had a significant effect on the study and as all validity criteria were met, the study is considered valid.

It is also worth noting that, whilst the materials for pen design adhered to OECD 205 (1984) and OCSPP 850.2200 (2012) one bird in the control group and one bird in the 3160 ppm test group caught their legs in the caging, resulting in one bird being euthanised (in the 3160 ppm group). As the study had followed guideline requirements for test design and as all birds were observed daily, this does not affect the validity of the study as all validity criteria have been met.

As no treatment-related mortalities were recorded in this study, no statistics or dose-response curve has been produced relating to mortality. Body weight data were compared by Dunnett's t-test using TOXSTAT®.

The endpoints to use in risk assessment are:

- **LC_{50} > 5620 ppm (based on nominal concentrations of S-2399TG)**

Reference:	KCA 8.1.1.2/02
Report Title:	S-2399 TG: A dietary LC_{50} study with the mallard
Author(s) & year:	[redacted] [redacted] and [redacted] (2014b)
Document No, Authority registration No:	[redacted] Report No: TPW-0009
Substance used:	Inpyrfluxam S-2399TG (13CG0617G, 95%)
Method of analysis:	HPLC-VWD
Guideline(s):	OCSPP Number 850.2200 and OECD Guideline 205 and JMAFF Test Guideline 12 NohSan No. 8147
Deviations:	See HSE comments section
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	No. No longer part of core data requirements.

I. MATERIALS AND METHODS

A. MATERIALS

1.Test material	Inpyrfluxam S-2399 TG
Lot/Batch:	13CG0617G
Purity:	95.0 % (certified by certificate of analysis)
Description:	Solid
Expiration date:	23 July 2016

B. TREATMENTS

1.Test concentrations:	Nominal test concentrations: 0 and 562, 1000, 1780, 3160 and 5620 ppm a.s.
Controls:	Negative control – unspiked feed
Chemical analysis:	Yes, for homogeneity in feed (Day 0) and concentration verification (Day 0 and 5). Determined using High Performance Liquid Chromatography (HPLC-VWD). LOQ = 31.3 ppm a.s.

C.TEST ORGANISMS

1.Species:	Mallard duck (*Anas platyrhychos*)
Age:	5 days (good health at test initiation, immature, mixed sex)
Acclimation period:	5 days (from day of hatch until test initiation)
Treatment for disease:	Birds received no form of antibiotic medication during acclimation or the test.
Source:	[redacted] All birds were from the same hatch, pen-reared and phenotypically indistinguishable from wild birds
Diet:	Game bird ration formulated to [redacted] specifications by [redacted] Water (town of Easton public water supply)
Bird mass:	75 – 104 grams at test initiation

D.TEST DESIGN

1.Housing:	Brooding pens (Safeguard Products, Inc, 62 x 92 x 26 cm)
Replication:	Control – four pens, five ducklings per pen Treatment levels – two pens, five ducklings per pen

Exposure:	Spiked feed for five days (post-exposure observation for three days)
E.TEST CONDITIONS	
1.Test temperature:	20.0 to 22.6 °C (mean =21.6 °C ± 0.8 °C (SD), room) mean = 30.3 ± 1.1 °C (SD) (brooding compartments)
Relative humidity:	27 to 59 % (mean = 43 % ± 8 % (SD))
Photoperiod:	16 hours light, 8 hours darkness (average of approximately 221 lux of illumination)

STUDY DESIGN AND METHODS

Experimental dates: Study Initiation – September 23, 2013. Experimental Termination – March 10, 2014

Test organism

Anas platyrhynchos was selected as a suitable species for this study as it is easy to rear and widely available throughout the year.

Dosage preparation and dosing

Test diets were prepared by mixing the test substance into the feed which had 2% corn oil incorporated, with a blender and Hobart mixer. An amount of diet sufficient to last the exposure period was prepared on the first day of exposure. Diets were presented to the birds at initiation of the test. Dietary test concentrations were corrected for purity of the test substance.

Diet sampling

Homogeneity of the test substance in the diet was evaluated by collecting six samples from the 562 and 5620 ppm a.s. test diets at preparation on Day 0. Homogeneity samples were collected from the top, middle and bottom of the left and right sections of the mixing vessel. The homogeneity samples also served as verification samples for those concentrations. Concentrations of S-2399 in extracts of the samples were determined by high performance liquid chromatography using a Waters Alliance high performance liquid chromatograph (HPLC) equipped with a Waters 2489 variable wavelength detector (VWD). Chromatographic separations were achieved using a YMC-PACK ODS-AM analytical column.

Observations and measurements

During acclimation, all birds were observed daily. Birds exhibiting abnormal behaviour or physical injury were not used. All birds were observed at least twice daily throughout the test. A record was maintained of all signs of toxicity and abnormal behaviours.

Individual body weights were measured at the initiation of the test (Day 0), on Day 5 and at termination of the test on Day 8. Average feed consumption values were determined daily during the exposure period (Days 0-5) and during the post-exposure observation period (Days 6-8) by pen for each treatment group and the control group. Feed consumption was determined by measuring the change in the weight of the feed presented to the birds over a given period of time and dividing it by bird days (number of birds and number of days). The accuracy of feed consumption values may have been affected by the unavoidable wastage of feed by the birds.

Estimated test substance intakes, or daily dietary dose, for mallard were calculated by treatment group during the exposure period using the following formula:

$$Daily\ Dietary\ Dose\ (mg\ a.s./kg\ body\ weight/day) = \frac{Test\ Concentration\ (ppm\ a.i.)\ \times\ Mean\ Feed\ Consumption\ (g/bird/day)}{Mean\ Body\ Weight\ (g/bird)}$$

Body weight and feed consumption values were averaged over Day 0 through Day 5. The accuracy of the estimated mean daily dietary dosage may have been impacted by differences in individual feed consumption, both within and between pens, and feed wastage.

A gross necropsy was performed on three birds from each test concentration at test termination. A gross necropsy included, but was not limited to, a general examination of the exterior of the bird and an examination of the thoracic and abdominal cavities, including cardiovascular and respiratory systems, liver, spleen, gastro-intestinal tract, and urogenital system.

Statistics

There were no mortalities observed in this study. Therefore, it was not possible to perform LC_{50} calculations. The LC_{50} value was determined to be greater than the highest dietary concentration tested. Body weight data were compared by Dunnett's t-test using TOXSTAT[2].

III. RESULTS AND DISCUSSION

Mortality

There were no mortalities in either the control or treatment groups.

Sub-lethal effects

Clinical observations

[2] West, Inc. and D.D. Gulley. 1996. TOXSTAT® Release 3.5 Western Ecosystems Technology, Inc. Cheyenne, Wyoming.

All birds in the control and treatment groups were normal in appearance and behaviour for the duration of the test.

Necropsy

There were no remarkable findings for the birds necropsied at test termination except for two birds being noted with a slightly small spleen, one from the 1780 ppm a.s. test concentration and another from the 3160 ppm a.s. test concentration. These findings were not considered to be treatment related by the study conductor.

Body weight and feed consumption

Mean body weight increase and feed consumption for the control and treatment groups are presented in Table B.9.1.1.2-6.

Table B.9.1.1.2 – 6: Mean body weight increase and food consumption following dietary exposure to Inpyrfluxam

Treatment (ppm)	Days of study				
	Body weight increase[a] (g) (SD)			Group mean food consumption (g/bird/day) (SD)	
	0 to 5	5 to 8	Total change	0 to 5	5 to 8
0 (control)	102 (10)	84 (13)	186 (18)	60 (7)	85 (10)
562	105 (14)	94 (8)	199 (19)	63	96
1000	104 (19)	93 (13)	196 (29)	59	91
1780	114 (10)	95 (12)	208* (17)	56	94
3160	108 (11)	96* (9)	205 (18)	64	95
5620	91 (9)	93 (8)	183 (14)	50	84

[a] Mean change is calculated separately from the mean body weight using individual body weights
*Statistically significant difference from the control group at $p < 0.05$ (Dunnett's t-test)
SD = standard deviation

There was no evidence of treatment-related effects on food consumption or mean body weight in the 562, 1000, 1780 and 3160 ppm treatment groups. There were slight increases in body weight change from Day 0 to Day 8 at the 1780 ppm test concentration and from Day 5 to Day 8 at the 3160 ppm concentration, which were statistically significant. As the changes were positive, slight, transient and not concentration responsive, they were not considered treatment related by the study conductor.

However, while not statistically significant, at the 5620 ppm test concentration there was a reduction in mean body weight gain from Day 0 to Day 5 and in the mean body weight relative to the control on Day 5 (control = 188 g, 5620 ppm a.s. = 176 g), which was considered to be treatment related by the study conductor. This was accompanied by a reduction in feed consumption for all five exposure days, relative to the control, for the 5620 ppm a.s. treatment level. Note that no statistical testing was performed on the feed consumption data.

Diet analysis

A summary of the analytical results is presented in Tables B.9.1.1.2-7 and B.9.1.1.2-8. Measured concentrations stayed within ± 20 % of the nominal concentration through the exposure period. The Coefficients of Variation (CVs) demonstrate that spiked feed was prepared correctly with an even distribution of test substance throughout.

Table B.9.1.1.2 – 7: Summary of analytical results

Nominal concentration (ppm)	Day 0[a]		Day 5		Mean measured concentration Day 0-5 (% of nominal)[c]
	Mean measured (ppm)	Mean % of nominal	Mean measured (ppm)	Mean % of nominal[b]	
0	n.d	n.a	n.d	n.a	n.a
562	566	101	483	86	524.5 (93)
1000	1000	100	886	89	943 (94)
1780	2000	112	1840	103	1920 (108)
3160	3740	118	3410	108	3575 (113)
5620	6460	115	5830	104	6145 (109)

[a] Day 0 values for nominal concentrations of 562 and 5620 are from homogeneity samples
[b] Calculated by HSE using figures provided in the table
[c] Calculated by HSE using figures provided in the study report
n.d = not detectable as below limit of quantitation (LOQ)
n.a = not applicable

Table B.9.1.1.2 – 8: Homogeneity of Inpyrfluxam in avian diet

Nominal Concentration (ppm a.s.)	Sample I.D. Number (166-207-)	Location Sampled in Mixing Vessel	Concentration of S-2399 (ppm a.s.)[3] Measured[1] (ppm a.s.)	Mean Measured (ppm a.s.) Standard Deviation (SD) Coefficient of Variation (CV)	Mean Percent of Nominal
562	2	Top Left	595	x= 566 SD = 18.9 CV = 3.34%	101
	3	Top Right	538		
	4	Middle Left	566		
	5	Middle Right	574		
	6	Bottom Left	557		
	7	Bottom Right	563		
5620	14	Top Left	6470	x = 6460 SD = 414 CV = 6.41%	115
	15	Top Right	6120		
	16	Middle Left	6150		
	17	Middle Right	6430		
	18	Bottom Left	7250[2]		
	19	Bottom Right	6320		

n.d = not detectable as below limit of quantitation (LOQ)

n.a = not applicable

[1] Measured values were not corrected for mean procedural recoveries based on sample sets

[2] The sample was reanalysed in duplicate due to high initial recoveries and had values of 7400 and 7090 ppm a.s. (132% and 126%, respectively). The mean value of the reanalysed samples is being reported.

[3] Measured values are for total isomer content and nominal values were corrected for content of S-2399 (95.0% of S-2399 TG).

Daily dietary dose

The estimated daily dietary doses associated with each treatment level are provided in Table B.9.1.1.2-9. Note this was calculated using the nominal test concentrations.

Table B.9.1.1.2 – 9: Estimated daily dietary dose (mg a.s./kg b.w./day)

Nominal Test Concentration (ppm a.s.)	Mean Measured Concentration Day 0-5	Mean Body Weight (g)[a]	Mean Feed Consumption (g/bird/day)	Estimated Daily Dietary Dose* (mg a.s./kg b.w./day) (Nominal)	Estimated Daily Dietary Dose (mg a.s./kg b.w./day) (Mean Measured Concentration)[b]
0	0	137.5	59.7	0	0
562	524.5	136.5	62.9	259	242
1000	943	136.8	58.8	430	405
1780	1920	141.1	56.4	712	767
3160	3575	140.8	63.7	1431	1617
5620	6145	130.8	49.7	2136	2335

[a] Mean from Day 0 and Day 5 body weights.

[b] Calculated by HSE

Validity criteria

The validity criteria for the study were met according to OECD 205 (1984) (Table B.9.1.1.2-10).

Table B.9.1.1.2-10: Compliance with OECD 205 (1984) validity criteria

Validity criterion	Required	Obtained
Control mortality	≤ 10 %	0 %
Maintenance of concentration	≥ 80 % of nominal	86 – 118 %
Effect of lowest treatment level	No compound-related mortality or other observable toxic effects	No compound-related mortality or other observable toxic effects

III.CONCLUSION

The dietary LC_{50} value for mallards exposed to 'inpyrfluxam' was determined to be greater than 5620 ppm a.s., the highest nominal test concentration tested. The no-mortality concentration was 5620 ppm a.s. (2136 mg a.s./kg b.w./day) and, based upon effects on both body weight and feed consumption at the 5620 ppm a.s. test concentration, the no-observed-effect concentration (NOEC) was 3160 ppm a.s. (1431 mg a.s./kg b.w./day).

HSE COMMENTS

The study was performed according to OED 205 (1984). All validity criteria were met and the study was conducted in compliance with GLP standards. HSE notes several points of interest and minor deviations below.

OECD 205 (1984) recommends an age of 10 – 17 days for Mallard duck on study initiation. The study conductor initiated the study when ducklings were 5 days old. The age of ducklings at study initiation likely impacts their sensitivity to pesticides. The degree and directionality of the difference in pesticide sensitivity between 5 days old and 10 - 17 days old individuals is unclear. The possible impact of this deviation is discussed further during risk assessment.

Related to the above point, due to the age of the ducklings at study initiation, it was not possible to acclimate them to the facilities and basal diet for seven days. Birds were acclimated to the caging and facilities from the day of hatch until initiation of the test, which HSE finds acceptable.

During the test, the mean temperature in the brooding compartments was 30.3 ± 1.1 °C (SD). This is lower than the 32 - 35 °C quoted for ducklings aged 0 – 7 days in OECD 205 (1984). Therefore, for the first three days of the test, temperatures were lower than those recommended. Given that control mortality was 0 % throughout the study and all replicates

were subjected to the same temperature, HSE considers this an acceptable, minor deviation.

The humidity throughout the study was also lower (43% ± 8% (SD)) than the 60 – 85 % range specified for mallards in OECD 205 (1984). Given that control mortality was 0 % throughout the study and all replicates were subjected to the same humidity, HSE considers this an acceptable, minor deviation.

HSE confirms the recommended pen space per bird was met. Ducklings had 1140.8 cm^2/bird, roughly double the recommended conditions (600 cm^2/bird).

HSE recalculated the daily dietary dose (DDD) values based on mean measured concentrations for Days 0 – 5. This resulted in a 2335 mg a.s./kg b.w./day DDD for the top concentration tested.

HSE notes that the highest tested concentration (5620 ppm nominal) is slightly higher than the maximum recommended 5000 ppm treatment concentration. Why this higher concentration was selected in unclear but ultimately does not impact the study or subsequent risk assessment. Therefore, HSE deems this alteration acceptable.

HSE agrees with the interpretations of the results by the applicant, outlined in the above study summary. All statistical methods were appropriate or not possible due to a lack of mortality. The applicant reported a NOEC = 3160 ppm a.s. (NOED = 1431 mg a.s./kg b.w./day), which equates to a **NOEC = 3575 ppm a.s. (NOED = 1617 mg a.s./kg b.w./day)** based on mean measured concentrations.

As the study is not required for the risk assessment, the analytical methods have not been evaluated by HSE Chemsitry.

The endpoint suitable for use in risk assessment is LD_{50} > 2335 mg a.s./kg b.w./day based on mean measured concentrations.

Reference:	KCA 8.1.1.2/03
Report Title:	S-2399: A dietary LC_{50} study with the zebra finch
Author(s) & year:	[redacted] and [redacted] (2017)
Document No, Authority registration No:	[redacted] Report No: TPW-0071
Substance used:	S-2399 TG (13CG0617G, 95%)

Method of analysis:	HPLC
Guideline(s):	OCSPP Number 850.2200
Deviations:	See HSE comments section
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: S-2399 TG
Description: Solid
Lot/Batch: 13CG0617G
Purity: 95.5 %

B. STUDY DESIGN AND METHODS

1. Test animals: Zebra finch (*Taeniopygia guttata*)
Age/growth stage: 7 to 8 months at test initiation (11.0-17.3 g b.w. for males and 10.3-17.3 g b.w. for females)
Source: [redacted]
Acclimation: 7 Weeks
Diet: Pelleted diet (ZuPreem FruitBlend Flavour). Grit (Kaytee Hi Cal Grit) was provided to aid digestion
Housing: Pens measuring 29 x 26 cm with a ceiling height of 31 cm, constructed of coated wire and separated from other pens by a fiberglass barrier. Each pen contained perches and one cuttle bone.

4.Environmental conditions:
Temperature: 21.3° C ± 0.2° C
Relative humidity: 72 % ± 7 %
Photoperiod: 8 hours light, 16 hours darkness (277 lux)

5. Animal assignment and treatment:
Birds were assigned to five test groups and one control group. Each treatment group and the control group contained 10 zebra finches (five males and five females). Birds were housed individually in pens. Diets were presented to the birds during the exposure period. Following the five-day exposure period all groups were provided untreated basal diet for three additional days.

6. Dose preparation:

Test diets were prepared by mixing the test substance with acetone and then into the feed. An amount of diet sufficient to last the exposure period was prepared three days prior to exposure. The acetone was allowed to volatize before the diet was stored frozen. The control diet was prepared by mixing acetone into the diet. Dietary concentrations were corrected for purity of the test substance (95.5 %). Nominal concentrations were 0, 80, 142, 253, 450 and 800 ppm a.s.

7. Measurements and observations:

Samples of the test diets were collected to verify the test concentrations administered and to confirm stability and homogeneity of the test substance in the diets. Homogeneity was evaluated by collecting six samples from the 80 and 800 ppm test diets on Day -3 (preparation day), with collection from the top, middle and bottom of the left and right sections of the mixing vessel. These samples also served as verification samples for those concentrations. One verification sample was collected from the control diet and two were collected from each remaining treatment on Day -3. On Day 5, samples were collected from feed troughs of the control group and treatment groups to assess stability. Concentrations of S-2399 were determined by HPLC (high performance liquid chromatography).

Birds were observed at least twice daily during the test, for all signs of toxicity and abnormal behaviours. Individual body weights were measured at test initiation (Day 0), on Day 1, at the end of the exposure period on Day 5 and at test termination (Day 8). Average feed consumption values were determined daily for three days prior to the exposure period (Day -3 to Day 0), during the exposure period (Days 0-5), and daily for the post-exposure observation period by pen for each treatment group and control (Days 5-8).

At termination, a gross necropsy was performed on three birds from each test concentration and on all mortalities.

8. Statistics:

Using the mortality data LC_{50}, LC_{20} and LC_{10} values were calculated by probit analysis, using the SAS System for Windows. Body weight data were compared by Dunnett's multiple-comparison test using TOXSTAT®.

II. RESULTS AND DISCUSSION

A. MORTALITY

There were no mortalities in the control group or in the 80, 142, and 253 ppm treatment groups. There was a 70 % mortality at the 450 ppm test concentration and 100 % mortality at the 800 ppm test concentration.

B. SUBLETHAL EFFECTS

All birds in the control and 80 ppm treatment groups were normal in appearance and behaviour. At the 142 ppm test concentration, three birds were noted with clinical signs and one bird was noted with a ruffled appearance on Day 1. Two birds were recorded with loss of coordination on Day 4 and one of those birds showed a slight ruffled appearance on Day 5. In the 253 ppm test concentration, all birds were noted with clinical signs on Days 0 to 7, including a ruffled appearance, wing droop, lethargy, reduced reaction, lower limb weakness and loss of coordination. All birds were normal in appearance on Day 8. At the 450 ppm test concentration, all birds were noted with clinical signs on Days 0 to 6, which included a ruffled appearance, wing droop, lethargy, reduced reaction, lower limb weakness, loss of coordination and depression. All surviving birds were normal in appearance from the afternoon of Day 6. At the 800 ppm test concentration, all birds were noted with clinical signs including ruffled appearance, wing droop, lethargy, reduced reaction, lower limb weakness, loss of coordination, prostrate posture and depression.

There were no treatment-related effects on food consumption or mean body weight in the 80 and 142 ppm treatment groups. A statistically significant ($p < 0.01$) loss in mean body weight was observed from Day 0 to Day 1 at the 253, 450, 800 ppm test concentrations. A statistically significant ($p < 0.01$) loss in mean body weights, at the 450 ppm test concentration, was observed from Day 1 to Day 5 of the test. The mean body weight on Day 5 for birds in the 253 and 450 ppm test concentration was statistically less ($p < 0.05$) than the control group. A statistically significant ($p < 0.01$) gain in mean body weights, at the 253 and 450 ppm test concentrations, was observed from Day 5 to Day 8 of the test. Body weights or body weight changes following Day 1 for the 800 ppm test concentration could not be compared to the control group due to complete mortality of birds in this treatment group.

When compared to the control group, there appeared to be no treatment related effects on mean feed consumption at the 80 and 142 ppm test concentrations during the exposure period. Mean feed consumption for birds in the 253, 450, and 800 ppm test concentrations during the exposure period days, appeared to be reduced when compared to the control group and the pre-exposure values for birds in these treatment groups. The mean post-exposure feed consumption for birds in all surviving test concentration groups appeared to be comparable to the control group. Mean body weight increase and food consumption data are presented in B.9.1.1.2-11 and B.9.1.1.2-12.

Common findings in the gross necropsy performed on the mortalities were loss of muscle mass and a small pale spleen. Gross necropsy performed on three birds from the control and each treatment group at test termination had no remarkable findings.

Table B.9.1.1.2-11: Mean body weight for *Taeniopygia guttata* following dietary exposure to Inpyrfluxam

Treatment (ppm)	Days of study							
	Body weight (g) (SD)							
	0	Change[a]	1	Change[a]	5	Change[a]	8	Total change
0 (control)	14.4 (1.6)	0.4 (0.4)	14.8 (1.7)	-0.5 (0.5)	14.3 (1.5)	0.4 (0.3)	14.7 (1.5)	0.3 (0.5)
80	13.3 (1.5)	0.4 (0.3)	13.7 (1.5)	-0.4 (0.4)	13.3 (1.5)	0.3 (0.5)	13.6 (1.7)	0.3 (0.8)
142	13.2 (1.7)	0.1 (0.4)	13.3 (1.8)	-0.2 (0.3)	13.2 (1.8)	0.6 (0.5)	13.7 (1.8)	0.5 (0.6)
253	13.4 (1.8)	-0.4 (0.4)**	13.0 (1.7)	-0.9 (0.7)	12.2* (1.6)	1.9 (0.8)**	14.0 (1.9)	0.6 (0.7)
450	13.8 (1.1)	-1.0 (0.3)**	12.8* (1.4)	-2.5 (0.5)**	11.4* (0.2)	2.5 (0.6)**	14.0 (0.5)	-0.7 (0.9)
800	14.0 (1.7)	-1.4 (0.4)	12.8 (1.7)	-	-	-	-	-

[a] Mean change is calculated separately from the mean body weight using individual body weights
*Statistically significant difference from the control group at p ≤ 0.05 (Dunnett's t-test)
**Statistically significant difference from the control group at p ≤ 0.01 (Dunnett's t-test)
Negative values indicate a reduction in body weight
(-) No data available due to mortality
SD = standard deviation

Table B.9.1.1.2-12: Mean food consumption for *Taeniopygia guttata* following dietary exposure to Inpyrfluxam

Treatment (ppm)	Group mean food consumption (g/bird/day) (SD)	
	Days 0 to 4	Days 5 to 7
0 (control)	3.3 (0.4)	3.5 (0.4)
80	3.3 (0.3)	3.3 (0.7)
142	3.0 (0.5)	3.5 (0.6)
253	1.9 (0.5)	3.6 (0.6)
450	1.5 (0.3)	3.4 (0.4)
800	0.7 (0.3)	-

C. DIET ANALYSIS

Analysis of the control samples did not show any presence of the test substance or the presence of a co-eluting substance at the characteristic retention time of the test substance. Diet samples from the 80 and 800 ppm concentrations analysed for homogeneity recorded mean concentrations of 71 (± 3.15 ppm) and 735 (± 78.8 ppm),

respectively. Samples collected on Day 0 to verify test substance concentrations for the 80, 142, 253, 450 and 800 ppm diets had mean concentrations of 71, 126, 223, 407 and 735 ppm, respectively. These values represented 89%, 89%, 88%, 90% and 92% of nominal concentrations. The results from the analysis of diet samples are presented in Table B.9.1.1.2 - 13. Analysis of diet samples collected from feeders after being held in ambient temperature for 24 hours after frozen storage averaged 100%, 100%, 105%, 95% and 101% of the Day 0 values for the 80, 142, 253, 450 and 800 ppm concentrations, respectively.

Table B.9.1.1.2-13: Summary of analytical results

Nominal concentration (ppm)	Day 0[a]		Day 5	
	Mean measured (ppm)	Mean % of nominal	Mean measured (ppm)	Mean % of nominal
0	<LOQ	-	-	-
80	71	89	71	89
142	126	89	126	89
253	223	88	235	93
450	407	90	387	86
800	735	92	744	93

[a] Day 0 values for nominal concentrations of 0, 142, 253 and 450 ppm are from verification samples. Day 0 values for 80 and 800 ppm concentrations are from homogeneity samples

D. VALIDITY CRITERIA

As test conditions followed validity criteria and mortality in the controls at test end was < 10 % (actual value 0 %), the test was considered valid.

III. CONCLUSION

The dietary LC_{50} value for zebra finch exposed to S-2399 was calculated to be 432 ppm, with a 95 % confidence interval of 407 to 460 ppm. The dietary LC_{20} value was calculated to be 406 ppm with a 95 % confidence interval of 382 to 432 ppm. The LC_{10} value was 393 ppm with a confidence interval of 369 to 418 ppm. The no-mortality test concentration was 253 ppm (38 mg a.s./kg b.w./day or a cumulative dose of 190 mg a.s./kg b.w.). The No-Observed-Effect Concentration (NOEC) was 80 ppm (19 mg a.s./kg b.w./day or a cumulative dose of 97 mg a.s./kg b.w.) based on the observation of clinical signs in the 142 ppm test concentration (32 mg a.s./kg b.w./day or a cumulative dose of 161 mg a.s./kg b.w.).

HSE evaluator comments

The study was conducted to GLP and predominantly in line with guideline EPA 850.2200 (2012) for testing avian dietary toxicity in terms of definitive testing. The authors verified the test substance was maintained in the test diet through the test at over 80 % (89-83 %) of the nominal concentration, as specified in the guidelines. The analytical method has a LOQ of 50.0 ppm a.s. and recoveries of 70-110 %, which is acceptable in avian diet. HSE Chemistry have concluded the following:

The analytical method is not fully validated according to SANTE/2020/12830 rev. 1 for the determination of the active substance inpyrfluxam in avian feed from the short-term dietary study on zebra finch as the matrix effects have not been determined and the stability of standards and extracts has not been addressed. However, the studies were generated prior to the implementation of SANTE 2020/12830 rev.1 and SANCO 3029 rev.4 did not require matrix effects and the stabilities of standards and extracts to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose.

Overall, the study is considered to be valid, however there are some minor points are noted for reference:

The selection of study species was based on a non-GLP range finders for zebra finch and canary. Where Zebra finch was the most sensitive species with 50 % mortality and 100 % regurgitation at lowest concentration.

EPA 850.2200 suggest chicks or young individuals for the test. The zebra finches used in the study were 8 months old with mature plumage which is not recommended according to the guidelines. However, there is not guidelines for the use of passerines in dietary toxicity. The bird oral acute toxicity guidelines EPA 850.2100 indicates that young adults not-yet mated should be used in the test, and for the case of passerines recommends submitting a protocol prior test initiation. Although EPA 850.2100 states the use of older individuals for acute toxicity, in the study no withholding of food prior testing was performed, contrasting with EPA 850.2100. In addition, there is not data supporting that the animals cannot survive for five days without eating. This generates uncertainty in the study, because instead of evaluating substrate toxicity, the observe outcome is avoidance behaviour. However, the 100 % mortality in the 800 ppm treatment group and the survival of the control group indicates that the age of Zebra finch does not impact the outcome of the study.

It is also noted that one bird was not observed twice daily during one of the test days. HSE do not consider this to affect the interpretation of results, and this does not impact the validity of the study. No explanation for this deviation is presented.

The study had an acclimation period of 7 weeks contrasting with what is stated in the guideline EPA 850.2200 but fulfilling the criteria of EPA 850.2100. The authors highlight that

for the test healthy birds were used, with normal behaviours and the absence of mortality in the control group shows that this has no adverse effect on the study outcome.

There are clear differences between the test and the guideline, the study temperature was below recommended throughout all the study. It is also noted that there was no temperature gradient provided. There was higher humidity overall, 72 % ± 7 instead of the range of 45 – 70 % recommended by the guideline. The photoperiod increased the dark hours (16 hours) and decreased the light hours (8 hours) in reference to the guideline (14 hours light and 10 hours dark). This change had no adverse effect on the study outcome based on the survival of the control group and the behavioural sub-chronic endpoints. As the validity criteria were otherwise met it is not considered to have had an effect on the endpoints.

It is noted that it was not possible to monitor twice the feed consumption in one replicate at 800 ppm a.s. test concentration on day 2 of the test. The bird was monitored on the morning of day 2 and on morning of day 3 with the same observation. Data was obtained for all other timepoints, and the 100 % mortality for the mentioned test concentration indicates that the missing data point has not impact on the study results.

The statistical analysis used in the study has also been considered and are deemed appropriate according to the guideline (probit fit and Dunnett test).

Conversion of the LD_{50} (432 ppm) to mg/kg b.w./d is not considered to be appropriate due to the demonstrated concentration-related food avoidance (SANCO/4145/2000 – final)[3], therefore, the highest concentration with no effect on adults has been selected as the lowest endpoint.

Therefore, the agreed endpoint to consider in risk assessment is:

- **5-day LC_{50} = 432 ppm (95 % C.I 407 - 460 ppm)**
- **5-day LC0 = 253 ppm (equivalent to 38 mg a.s./kg b.w./d)**
- **5-dat NOEL = 80ppm (equivalent to 19 mg a.s./kg b.w./d)**

B.9.1.1.3 Sub-chronic toxicity and reproduction to birds

Reference:	KCA 8.1.1.3/01
Report Title:	S-2399 TG: A reproduction study with the northern bobwhite
Author(s) & year:	[redacted] and [redacted] (2015a)

[3] European Commission, 2002c. Guidance Document on Risk Assessment for Birds and Mammals Under CouncilDirective 91/414/EEC. SANCO/4145/2000

Document No, Authority registration No:	[redacted] Report No: TPW-0018
Substance used:	S-2399 TG (13CG0617G, 95%)
Method of analysis:	HPLC
Guideline(s):	OECD 206, U.S. EPA OCSPP Number 850.2300, FIFRA Subdivision E, Section 71-4 and ASTM Standard E1062-86
Deviations:	Yes – see HSE comments
GLP or GEP:	GLP
Acceptability:	Yes
Study relied upon:	Yes

I. MATERIALS AND METHODS

A. MATERIALS

Test material S-2399 TG
Lot/Batch: 13CG0617G
Purity: 95.0 % (certified by certificate of analysis)
Description: Solid
Expiration date: 23 July 2016

B. TREATMENTS

Test concentrations: Nominal test concentrations: 0, 250, 500 and 1000 ppm a.s.
Controls: Negative control – unspiked feed
Chemical analysis: Yes, for homogeneity in feed (Day 0, Week 1), stability (Day 7 of Weeks 1, 12 and 20) and verification (Day 0 of Weeks 4, 12 and 20). Determined using High Performance Liquid Chromatography (HPLC) with a Waters Alliance variable wavelength detector (VWD). LOQ = 31.3 ppm a.s.

C.TEST ORGANISMS

Species: Northern bobwhite (*Colinus virginianus*)
Age: 18 weeks at test initiation
Acclimation period: 5 weeks
Treatment for disease: Acclimation phase: medicated water

	Exposure phase: antibiotic ointment as required for treatment of lesions Pre-incubation: formaldehyde fumigation of eggs for two hours
Source:	[redacted] All birds were from the same hatch, healthy, phenotypically indistinguishable from wild birds and approaching their first breeding season
Diet:	Basal ration = ≥ 27% protein and 2% crude fat, and ≤ 5% crude fibre ([redacted]) Additional 5% (w/w) of limestone (approximately 38.0% Ca) was added to the basal diet for the adults. Approximately 3 % Ca overall Water (town of Easton public water supply) Offspring received water-soluble vitamin and electrolyte mix in their water Feed and water (provided *ad libitum*) were analysed periodically in accordance with Wildlife International Standard Operating Procedures
Bird mass:	172 -222 grams at test initiation
D.TEST DESIGN	
Housing:	Pens (adults) measuring approximately 25 x 51 cm, with a ceiling height of 20 to 26 cm (sloping floor, Georgia Quail Farm Manufacturing Model No. 0330) Pens (hatchlings) measuring approximately 72 X 90 X 23 cm high (Beacon Steel Company Model B735Q). Sisal rope added to each pen for animal enrichment
Replication:	Control and treatment levels - 18 pairs of birds with one male and one female per pen
Exposure:	Spiked feed for 21 weeks (adults), all offspring were fed unspiked feed
E.TEST CONDITIONS	
Test temperature:	Adult pens: 20.6 ± 1.2 °C (17.8 – 23.4 °C) Egg storage: 13.2 ± 0.2 °C Incubator: 37.4 ± 0.0 °C Hatcher: 37.3 ± 0.0 °C Hatchling pens: 25.5± 2.4 °C
Relative humidity:	Adult pens: 38 ± 15 % (15 – 79 %)

	Egg storage: 65 ± 8 % Incubator: 55 ± 0 % Hatcher: 58 ± 0 % Hatchling pens: 17 ± 6 %
Photoperiod:	Acclimation until Week 8: 8 hours light: 16 hours darkness Week 9 until end of egg production: 17 hours light: 7 hours darkness Hatchlings: 16 hours light: 8 hours darkness Light intensity: 466 lux during pre-photo stimulation, 756 lux post-photo stimulation and 371 lux during egg-laying phase
Air replacement:	15 room air volumes every hour

STUDY DESIGN AND METHODS

Experimental dates:
Study Initiation – Study Initiation – July 14, 2014
Experimental Termination – March 30, 2015

Test organism

Colinus virginianus was selected as a suitable species for this study as it is easy to rear and widely available throughout the year. Bird sex was determined by visual examination of plumage.

Dosage preparation and dosing

Test diets were prepared by mixing the test substance into premixes which had approximately 2% corn oil incorporated, with a blender and stand mixer. These were subsequently mixed with the appropriate mass of ration (and limestone where appropriate) to yield the desired a.s. concentrations. Control diet and treated diets were prepared weekly and presented to the birds on Tuesday of each week. Dietary concentrations were adjusted for purity of the test substance.

Diet sampling

Homogeneity of the test substance in the diet was evaluated by collecting six samples of each test diet and one sample from the control at preparation on Day 0 of Week 1. Homogeneity samples were collected from the top, middle and bottom of the left and right sections of the mixing vessel. The homogeneity samples also served as verification samples for Week 1.

Observations and measurements

During acclimation, all birds were observed daily. Birds exhibiting abnormal behaviour or debilitating physical injury were not used. All birds were observed daily throughout the test for signs of toxicity and abnormal behaviours. Additionally, all offspring were observed daily from hatching until 14 days of age.

Adult body weights were measured at test initiation, at the end of Weeks 2, 4, 6, 8, and at adult termination. Body weights were not measured during egg laying because of the possible adverse effects handling may have on egg production.

Feed consumption for each pen was measured weekly throughout the test. An attempt was made to minimize feed wastage by the birds by using externally mounted feeders designed with a “feed-saver” lip. Feed wastage was further reduced by placement of a piece of wire grid on the top of the feed. The wire grid allowed birds to feed unencumbered but prevented the birds from “scooping” feed out of the feeder. The amount of feed wasted by the birds was not quantified, since the wasted feed was normally scattered and mixed with water and excreta. Therefore, feed consumption is presented as an estimate of total feed consumption.

Estimated test substance intakes, daily dietary dose, for northern bobwhite were calculated by treatment group for the pre-egg production period, the egg production period and the overall adult period using the following formula:

$$\text{Daily Dietary Dose} = \frac{\text{Test Concentration (mg a.s./kg) x Daily Feed Consumption (g/bird/day) (mg a.s./kg body weight/day)}}{\text{Body Weight (g/bird)}}$$

The mean body weight value is the mean of both male and female body weights. For the pre-egg production interval the body weights were averaged over Weeks 0, 2, 4, 6 and 8. For the egg-production interval body weights were averaged over Weeks 8 and 21 (adult termination). The accuracy of the estimated mean daily dietary dose may be impacted by differences in individual feed consumption, both within and between pens, and feed wastage.

Eggs were collected daily from all pens, when available. The eggs were stored in a cold room until incubation. All eggs laid in a weekly interval were considered as one lot.

At the end of the weekly interval, all eggs were removed from the cold room, counted and eggs selected by indiscriminate draw for egg shell thickness measurement. The remaining eggs were candled with an egg-candling lamp (Speed King Model No. 32) to detect egg shell cracks or abnormal eggs. Cracked or abnormal eggs were recorded and discarded. All eggs not discarded or used for egg shell thickness measurements were placed in an

incubator (NatureForm Model No. NMC 4000). Eggs were candled on Day 11 of incubation to determine embryo viability and on Day 21 to determine embryo survival.

In order to prevent adhesion of the embryo to the shell membrane, the incubator was also equipped with an automatic egg rotation device, designed to rotate the eggs from 45° off of vertical in one direction to 45° off of vertical in the opposite direction (total arc of rotation was 90°) every hour through Day 21 of incubation.

On Day 21 of incubation, the eggs were placed in an incubator configured for hatching (NatureForm Model No. 2340) and allowed to hatch. Hatchlings were separated by parental pen of origin. All hatchlings, unhatched eggs, and egg shells were removed from the hatcher on Day 25 or 26 of incubation. The body weights of surviving hatchlings were recorded and the average body weight by pen was determined. Hatchlings were housed according to the appropriate parental concentration grouping in brooding pens until 14 days of age. At 14 days of age, the individual body weight of each surviving hatchling was recorded. The chicks were euthanized with carbon dioxide and disposed of by incineration.

Weekly throughout the egg laying period, one egg was collected, when available, from each of the odd-numbered pens during odd numbered weeks (1,3,5, 7 & 9) and from each of the even-numbered pens during the even numbered weeks (2,4,6, 8 & 10). The eggs were opened, the contents removed, and the shells thoroughly rinsed with water. The shells were then allowed to air dry for at least one week at room temperature. The average thickness of the dried shell plus the membrane was determined by measuring five points around the waist of the egg using a micrometre (to the nearest 0.002 mm).

Adult birds that died or were euthanized during the course of the study were subjected to a gross necropsy. At the conclusion of the exposure period, all surviving adult birds were euthanized with carbon dioxide gas, necropsied, and disposed of by incineration.

Statistics

Upon completion of the test, an analysis of variance (ANOVA) was performed to determine statistically significant differences among groups. Dunnett's multiple comparison procedure was used to compare the four treatment means with the control group mean and assess the statistical significance of the observed differences. Sample units were the individual pens within each experimental group, except adult body weights where the sample unit was the individual bird. Percentage data were examined using Dunnett's method following arcsine square root transformation for reproductive parameters. Each of the following parameters was analysed statistically:

1. Adult Body Weight - Individual body weight was measured at test initiation, Weeks 2, 4, 6, 8, and at adult termination. Statistical comparisons were made between the control group and each treatment group at each weighing interval by sex.
2. Adult Feed Consumption - Feed consumption expressed as grams of feed per bird per day was examined by pen weekly during the test. Statistical comparisons were made between the control and each treatment group.
3. Eggs/Hen/Day - The number of eggs laid per female divided by the total number of days of egg production.
4. Eggs Cracked of Eggs Laid - The number of eggs determined by candling to be cracked divided by the number of eggs laid, per pen.
5. Fertile Eggs of Eggs Set – The number of fertile eggs at the Day 11 candling was divided by the number of eggs set, per pen.
6. Viable Embryos of Eggs Set - The number of viable embryos at the Day 11 candling was divided by the number of eggs set, per pen.
7. Live 3-Week Embryos of Viable Embryos - The number of live embryos at the Day 21 candling was divided by the number of viable embryos, per pen.
8. Hatchlings of 3-Week Embryos - The number of hatchlings removed from the hatcher was divided by the number of live 3-week embryos, per pen.
9. 14-Day Old Survivors of Hatchlings - The number of 14-day old survivors was divided by the number of hatchlings, per pen.
10. Hatchlings of Eggs Set - The number of hatchlings was divided by the number of eggs set, per pen.
11. Hatchlings of Fertile Eggs – The number of hatchlings was divided by the number of fertile eggs, per pen.
12. 14-Day Old Survivors of Eggs Set - The number of 14-day old survivors was divided by the number of eggs set, per pen.
13. Hatchlings/Pen/Day - The number of hatchlings per female divided by the total number of days of egg production.
14. 14-Day Old Survivors/Pen/Day - The number of 14-day old survivors per pen divided by the total number of days of egg production.
15. Egg Shell Thickness - The average egg shell thickness of indiscriminately selected eggs per pen was measured.
16. Offspring's Body Weight - The average body weights of surviving hatchlings and 14-day old survivors were measured by parental pen group.

II. RESULTS AND DISCUSSION

Analytical results

A summary of the analytical results is presented in Table B.9.1.1.3-1. Test concentrations in feed remained with ± 20 % of the nominal concentration.

Table B.9.1.1.3 – 1: Summary of analytical results. Day 0 and 7 values represent verification and stability values respectively

Week	Day	Mean measured values (ppm) (%)				
		0	125	250	500	1000
1[a,b]	0	< LOQ	126 (101)	240 (96)	532 (106)	1050 (105)
1	7	< LOQ	108 (86)	247 (99)	528 (106)	1040 (104)
4	0	< LOQ	142 (114)	266 (107)	537 (108)	1110 (111)
12	0	< LOQ	105 (84)	240 (96)	544 (109)	982 (98)
12	7	< LOQ	113 (90)	258 (103)	550 (110)	1090 (109)
20	0	< LOQ	120 (96)	243 (97)	532 (106)	1060 (106)
20	7	< LOQ	134 (107)	299 (120)	546 (109)	1170 (117)

[a] Week 1, Day 0 values are from homogeneity samples

[b] Day 0 values are percent of nominal, Day 7 values are percent of nominal calculated by HSE using rounded figures in table

A homogeneous distribution of S-2399 in feed was demonstrated for each treatment level (Table B.9.1.1.3-2).

Table B.9.1.1.3 – 2: Summary of S-2399 homogeneity in feed samples for all treatment levels on Day 0, Week 1

Nominal treatment level (ppm)	Mean measured concentration (ppm)	Standard deviation	Coefficient of variation (%)
125	126	17	13.5
250	240	16.8	7.01
500	532	20.8	3.92
100	1050	59.7	5.67

Mortality

Two mortalities occurred during the test, one in the control group and one in the 250 ppm a.s. treatment group. The first was a male of the control group that suffered a compound leg fracture on Day 6 of Week 15 and was subsequently euthanized. At necropsy, the male weighed 193 g and had a compound fracture in the right tarsometatarsus. Internally the male's liver and kidneys were slightly pale, the gastrointestinal tract was primarily empty and the caecal contents were firm. Necropsy of the male's pen mate was unremarkable.

The second mortality was a female of the 250 ppm a.s. treatment group that was found dead on Day 6 of Week 16. Prior to being found dead the female was normal in appearance and behaviour. At necropsy, the hen weighed 256 g and had feather loss on the head and petechial haemorrhaging at the base of the skull. Internally the kidneys were pale, there was frank blood throughout the abdominal cavity and the gastrointestinal tract was primarily empty. Necropsy of the female's pen mate indicated feather loss but the bird was otherwise unremarkable.

No other mortalities occurred during the study. The study conductor considered the mortality in the 250 ppm a.s. treatment group not to be treatment related due to the nature of the lesions.

Clinical observations
A summary of the clinical observations is provided in Table B.9.1.1.3-3.

Table B.9.1.1.3 – 3: Summary of clinical observations

Nominal concentration (ppm a.s.)	0	125	250	500	1000
Number of Birds	34	36	34	36	36
Description of clinical observation (number of affected birds, typically transient)	feather loss (1) foot lesions (2) head lesion (1) lameness (1)	foot lesions (4) head lesion (2) eye picked (1) lameness (1)	foot lesions (2) right leg fracture (1) ruffled appearance (1)	feather loss (1) foot lesions (2) head lesion (1) lameness (1)	foot lesions (3) lameness (1)
Number displaying any clinical signs:	4	6	3	3	3

Most of the clinical observations listed in Table B.9.1.1.3-3 are normally associated with injuries and penwear. Such signs included foot and head lesions, feather loss, and eye picking. In addition, however, lameness and a ruffled appearance were also observed.

Gross necropsy

A summary of the gross pathological observations for both sexes is provided in Table B.9.1.1.3-4.

Table B.9.1.1.3 – 4: Summary of Gross Pathological Observations from a Northern Bobwhite Reproduction Study with Inpyrfluxam Birds Euthanized at Test Termination

Male					
Nominal concentration (ppm a.s.)	**0**	**125**	**250**	**500**	**1000**
Number of birds	17	18	17	18	18
External – feather loss	4	0	0	1	1
External – foot lesions, missing digits and/or swelling	1	3	2	0	2
External – head lesion	0	0	0	0	0
Spleen - > 1.2 x 1.0 cm	3	2	0	0	1
Gastrointestinal tract – caecal contents firm	0	0	0	0	0
Reproductive – right testis small, < 1.4 cm	1	6	3	6	6
Reproductive – left testis small, < 1.4 cm	0	2	0	2	0
Reproductive – testes small, < 1.4 cm	1	0	2	0	1
Not remarkable	9	8	10	11	9
Female					
Nominal concentration (ppm a.s.)	**0**	**125**	**250**	**500**	**1000**
Number of birds	17	18	17	18	18
External – feather loss	3	5	1	2	1
External – foot lesions, missing digits and/or swelling	3	7	1	5	0
External – head lesion	1	0	0	0	0
Spleen - > 1.2 x 1.0 cm	0	0	0	0	0
Abdominal cavity – egg remnants	0	0	1	0	1
Abdominal cavity – old egg yolk peritonitis	0	0	1	0	3
Abdominal cavity – slight egg yolk peritonitis	0	0	1	0	0
Abdominal cavity – egg yolk peritonitis	0	1	1	0	0
Abdominal cavity – extensive egg yolk peritonitis	1	1	1	0	0
Gastrointestinal tract – caecal contents firm	0	0	1	0	0

Male					
Nominal concentration (ppm a.s.)	**0**	**125**	**250**	**500**	**1000**
Reproductive – cystic follicle	0	1	0	0	0
Reproductive – ovary regressing	1	1	0	0	1
Reproductive – ovary regressed	1	1	0	0	0
Not remarkable	10	8	12	12	14

All findings observed were considered unrelated to treatment by the study conductor.

Food consumption and body weight
Adult and offspring body weight changes over the treatment period were similar in all groups and there was no evidence of any statistically significant treatment-related effects.

There were no apparent treatment-related effects upon feed consumption at the 125, 250, 500 and 1000 ppm test concentrations. There were slight, but statistically significant reductions in mean feed consumption at the 125 ppm test concentration during Weeks 10, 14 and 15, at the 250 ppm test concentration during Week 1, at the 500 ppm test concentration during Weeks 8, 14 and 15 and at the 1000 ppm test concentration during Week 8, 14, 15 and 17 of the test. The reductions in mean feed consumption were not considered to be related to treatment by the study conductor since they were slight and neither consistent over time nor concentration responsive.

Mean adult body weight and food consumption measurements body weight and food consumption measurements, plus the corresponding daily dietary doses, are presented in Table B.9.1.1.3-5.

Table B.9.1.1.3 – 5: Mean body weight, food consumption and daily dietary dose of 'Inpyrfluxam'

Test interval (test weeks)	Test concentration (ppm)	Mean body weight (g)	Mean food consumption (g/bird/day)	Estimated daily dietary dose (mg a.s./kg b.w./day)
Pre-egg production (weeks 1 – 10)	0	202	16	0
	125	206	16	9.45
	250	203	15	18.9
	500	203	16	38.5
	1000	201	15	76.0
Egg production (weeks 11 – 21)	0	221	22	0.0
	120	222	21	11.7
	250	219	22	24.9
	500	223	22	48.5
	1000	219	21	94.9
Overall (weeks 1 – 21)	0	207	19	0.0
	125	210	18	10.8
	250	207	18	22.2
	500	209	18	44.3
	1000	206	18	87.0

Reproductive results

A summary of the reproductive endpoints is presented in Table B.9.1.1.3 – 6.

Table B.9.1.1.3 – 6: Summary of reproductive performance in northern bobwhite exposed to 'Inpyrfluxam'

Reproductive parameter	Experimental Group (ppm)				
	Control	125	250	500	1000
	(% reduction from control where applicable)[d]				
Number Surviving Replicates	17	18	17	18	18
Eggs Laid[a]	876	682	796	764	692

Reproductive parameter	Experimental Group (ppm)				
	Control	125	250	500	1000
	(% reduction from control where applicable)[d]				
Eggs Laid/Hen	52	38	47	42	38
Eggs Laid/Pen/Day[b]	0.57	0.42* **(26)**	0.51 **(11)**	0.47 **(18)**	0.42* **(26)**
Eggs Cracked/Eggs Laid (%)[c]	1	2	2	3	2
Fertile Eggs/Eggs Set (%)	88	96	94	98	97
Viable Embryos/Eggs Set (%)	86	91	93	92	91
Live 3-week Embryos/Viable Embryos (%)	99	99	98	99	99
Hatchlings/Live 3-week Embryos (%)	94	93	88	86	82
14-Day Old survivors/Hatchlings (%)	86	85	83	86	88
Hatchlings/Egg Set (%)	81	84	80	80	76
Hatchlings/Fertile Eggs (%)	86	87	85	81	78
14-Day Old Survivors/Eggs Set (%)	70	73	67	69	67
Hatchlings/Pen/Day[b]	0.41	0.30 **(27)**	0.36 **(12)**	0.32 **(22)**	0.28 **(32)**
14-Day Old Survivors/Pen/Day[b]	0.36	0.27 **(25)**	0.31 **(14)**	0.29 **(19)**	0.25 **(31)**

[a] The total number of eggs laid in each group

[b] Based on 91 days of egg production

[c] Percent values represent replicate means for each experimental group

[d] Calculated by HSE using rounded values in table

*Significantly different from control at $p < 0.05$ (Dunnett's t-test)

There were two statistically significant reductions for reproductive endpoints: the number of eggs laid per hen per day reduced for the 125 and 1000 ppm nominal treatment levels in relation to the control. The study conductor, however, did not deem these results treatment related. Their justification was the mean egg production for the control group was 52 eggs per hen, which exceeded the mean value from 15 of the most recent [redacted] studies (45 + 5). They also argued that the lack of a clear-concentration response did not support the conclusion that the effects were treatment related.

In addition, there were no statistically significant effects upon egg shell thickness at any of the concentrations tested.

Validity criteria

The validity criteria for the study were met according to OECD 206 (1984) (Table B.9.1.1.3-7).

Table B.9.1.1.3 – 7: Compliance with OECD 206 (1984) validity criteria

Validity criterion	Required	Obtained
Control mortality	≤ 10 %	6 %
Maintenance of concentration	≥ 80 % of nominal	86 – 123 %
Average number of 14-day-old survivors per hen in control	≥ 12	32
Control egg shell thickness	≥ 0.19 mm	0.226 mm

III. CONCLUSION

The study conductor concluded that there were no treatment-related adult mortalities, overt signs of toxicity or treatment-related effects upon body weight or feed consumption at any of the concentrations tested. Additionally, they concluded that there were no treatment-related effects upon any of the reproductive parameters measured at the 125, 250, 500 or 1000 ppm test concentrations. The No-Observed-Effect Concentration (NOEC) for northern bobwhite exposed to ‘inpyrfluxam’ in the diet during the study was determined to be 1000 ppm (87.0 mg a.s./kg b.w./day), the highest concentration tested, by the study conductor.

HSE COMMENTS

The study was carried out and evaluated against OECD 206 (1984). All validity criteria were met. The study was conducted to GLP and considered valid. Periodic analysis of water and feed for potential contaminants were not performed, however, according to GLP standards. They were performed using a certified laboratory and standard US EPA analytical methods. This is a minor component of the study and HSE considers it acceptable for these periodic analyses to not conform to GLP.

The following deviations from OECD 206 (1984) were noted:

OECD 206 (1984) recommends a 15 – 30-minute transition period at dawn and dusk for lighting. This was not detailed in the study report. Control individuals displayed behaviours and signs typical for this study type, which suggests the lack of a lighting transition period had minimal effect on the study outcome. HSE considers this a minor, acceptable deviation.

OECD 206 (1984) Table 1 sets out the recommended conditions for adult birds. The study deviated from the recommended age (18 weeks vs 20 – 24 weeks) and minimum pen floor area per pair (0.128 m^2 vs 0.25 m^2) for the bobwhite quail. The effect of selecting individuals younger than the specified range is unclear and HSE will consider this during the risk assessment. However, OCSPP 850.2300, the equivalent EPA guidance, stipulates the use of birds ≥ 16 weeks. Regarding floor area per pair, birds were allocated approximately half the required floor area. This will have caused unnecessary stress and likely contributed to the recorded clinical observations in all treatment groups. To prevent additional vertebrate testing, HSE considers this an acceptable deviation. However, HSE requests that the study conductor amends this experimental error in future studies.

OECD 206 (1984) provides recommended housing and feeding conditions. It states that adult birds should be exposed to 50 to 75 % relative humidity. The mean relative humidity in adult pens was 38 ± 15 %. As the validity criteria relating to control mortality were met, adult housing conditions were demonstrably fit for purpose. Therefore, HSE considers this a minor deviation.

OECD 206 (1984) Table 2 further details temperature and relative humidity requirements throughout offspring development. Table B.9.1.1.3 - 8 quotes the recommended and realised conditions and highlights where they diverged.

Table B.9.1.1.3 - 8: Discrepancies between recommended and realised rearing conditions in study. Divergences are highlighted in bold

Stage	Target temperature (°C)	Mean actual temperature (°C)	Target relative humidity (%)	Mean actual relative humidity (%)
Storage	15 - 16	**13.2**	55 - 75	65
Incubation	37.5	**37.4**	50 - 65	55
Hatching	37.5	**37.3**	70 - 75	**58**
Young. first week	35 - 38	**25.5**	50 - 75	**17**
Young, second week	30 -32	**25.5**	50 - 75	**17**

Although the study deviated from the recommended environmental rearing conditions, particularly for hatchlings, offspring development still displayed acceptable success rates at key checkpoints for the control group. This is demonstrated in Table B.9.1.1.3-9, which outlines certain control group developmental validity criteria for OCSPP 850.2300 and normal values for reproductive parameters for OECD 206 (1984), which would have been affected if rearing environmental conditions were inappropriate. These developmental validity criteria were met, which suggests that environmental rearing conditions were appropriate. HSE, therefore, considers these deviations acceptable.

Table B.9.1.1.3 - 9: OCSPP 850.2300 control group validity criteria related to development and OECD 206 (1984) normal values for reproduction parameters

Validity criteria	Target OCSPP (%)	Target OECD (%)	Actual (%)
Viable embryos of eggs set at ~ Day 14	≥ 80	75 – 90	86
Live 3-week embryos of viable embryos at ~ Day 21	≥ 97	-	99
Hatchlings of live 3-week embryos	≥ 85	-	94
Hatchlings of eggs set for incubation	≥ 71	50 - 90	81
14-day old survivors of hatchlings	≥ 77	75 - 90	86

OECD 206 (1984) states that food consumption of young should be recorded in the first and second week after hatching. This was not recorded or not reported. The mean body weight of hatchlings and 14-day old survivors was recorded, however, and found to be similar

between the control and all treatment groups. The impact of this recording and reporting omission will be considered during risk assessment.

OECD 206 (1984) covers data and reporting requirements. It requests the reporting of the percentage of hens laying eggs. This was not fully reported although the raw data included in the study report appendices shows this is 100 % for every treatment group and the control.

The final point discussed is not a deviation from OECD 206 (1984) but the interpretation of the results by the study conductor. For Eggs Laid/Pen/Day, the 10.8 and 87.0 mg a.s./kg b.w./day treatment groups were significantly reduced compared to the concurrent control (26 % reduction for both), and all doses displayed > 10 % reductions. The study conductor considered the statistically significant reductions not to be treatment-related due to the lack of a dose-response and high mean egg production in the control group compared to historical control data. Based on these arguments, the reported NOED = 87.0 mg a.s./kg b.w./day.

Mean egg production in the experimental control group (52 eggs per hen) exceeded the mean value from 15 of the most recent [redacted] studies (45 ± 5 eggs per hen). To explore this, HSE expressed the mean of the historical control values (45 eggs per hen) as Eggs Laid/Pen/Day (45 Eggs/Pen ÷ 91 Egg Production Days = 0.495 Eggs Laid/Pen/Day) and compared this value to the egg-laying rates in the treatment groups (Table B.9.1.1.3 – 10).

Table B.9.1.1.3 – 10: Comparison of historical control mean for egg laying rate to inpyrfluxam treatment groups

Treatment group (mg a.s./kg b.w./day)	Eggs Laid/Pen/Day	Reduction (%)
Historical Control Mean	0.495	-
10.8	0.42	15.1
22.2	0.51	-3.1
44.3	0.47	5.0
87.0	0.42	15.1

Reductions of 15 % are still present for the 10.8 and 87.0 mg a.s./kg b.w./day treatment groups even when compared to the historical control baseline. Commission Regulation (EU) No 283/2013 data point 8.1.1.3. outlines the preference for an ED_{10} to be reported. From this, it can be inferred that effects of ≥ 10 % are of interest. Therefore, HSE does not consider the observed pattern of results to be supportive of non-treatment-related effects. Instead,

HSE deemed them open to interpretation, with the possibility that the true ED_{10} for Eggs Laid/Pen/Day is lower than 87.0 mg a.s./kg b.w./day.

To address this uncertainty, HSE consulted the most recent Risk assessment for Birds and Mammals Guidance (EFSA 2023)[4] and associated literature (Green *et al.* (2022)[5]) for scientific advice about an approach to setting endpoints for hard-to-interpret data. Using Figure 6 of EFSA (2023), a decision chart for determining whether data is suitable for ED_{10} (BMD_{10}) or NOED estimation, HSE concluded that, due to the non-monotonic dose-response, the Eggs Laid response variable was suitable only for NOED estimation. To confirm this, HSE attempted BMD modelling for the Eggs Laid variable, which, as expected, was undermined by the non-monotonic dose-response, as well as negative skew in the Eggs Laid response variable.

In absence of a robust ED_{10} and associated CIs, HSE has set the NOED endpoint using the Eggs Laid/Pen/Day variable with the historical control mean as a baseline. The 15.1 % reduction at 10.8 mg a.s./kg b.w./day is clearly not part of a dose response. Nothing else in the data set for this active substance indicates low dose effects as likely, so on balance this effect can be discounted based on < 10 % effects at 22.2 and 44.3 mg a.s./kg b.w./day. At 87 mg a.s./kg b.w./day, a 15.1 % reduction relative to the historical control mean is present. It is possible this is a treatment related effect given the dose-response between 22.2 and 87.0 mg a.s./kg b.w./day and the lack of data at a higher concentration to either support or refute biological relevance. Based on this trend analysis, which incorporates historical control data, HSE has decided to lower the NOED from 87.0 mg a.s./kg b.w./day to 44.3 mg a.s./kg b.w./day.

HSE Chemistry conclusion is as follows (see section B5, section B.5.1.2):

The analytical method is not fully validated according to SANTE/2020/12830 rev. 1 for the determination of the active substance inpyrfluxam in avian feed from the Mallard and Northern Bobwhite reproduction studies as the matrix effects have not been determined and the stability of standards and extracts has not been addressed. However, the studies were generated prior to the implementation of SANTE 2020/12830 rev.1 and SANCO 3029 rev.4 did not require matrix effects and the stabilities of standards and extracts to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose.

[4] EFSA (European Food Safety Authority), Aagaard A, Berny P, Chaton PF, Antia AL, McVey E, Arena M, Fait G, Ippolito A, Linguadoca A, Sharp R, Theobald A and Brock T, 2023. Guidance on the risk assessment for Birds and Mammals. EFSA Journal 2023;21(2):7790, 300 pp. https://doi.org/10.2903/j.efsa.2023.7790

[5] Green, J.W., Foudoulakis, M., Fredricks, T., Bean, T., Maul, J., Plautz, S., Valverde, P., Schapaugh, A., Sopko, X. and Gao, Z. (2022). Statistical analysis of avian reproduction studies. Environmental Sciences Europe, 34(1). doi:https://doi.org/10.1186/s12302-022-00603-5.

The endpoint for consideration in the risk assessment is NOED = 44.3 mg a.s./kg b.w./day

Reference:	KCA 8.1.1.3/02
Report Title:	S-2399 TG: A reproduction study with the mallard
Author(s) & year:	[redacted] and [redacted] (2015b)
Document No, Authority registration No:	[redacted] Report No: TPW-0019
Substance used:	S-2399TG (13CG0617G, 95%)
Method of analysis:	HPLC
Guideline(s):	OECD 206, U.S. EPA OCSPP Number 850.2300, FIFRA Subdivision E, Section 71-4 and ASTM Standard E1062-86
Deviations:	See HSE comments section
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: S-2399 TG
Description: Solid
Lot/Batch: 13CG0617G
Purity: 95.0%
Reference item: None
Expiry date: 23rd July 2016
Solvent: None
Test concentrations: 0, 125, 250, 500, and 1000 ppm a.s.
LOQ: 31.3 ppm a.s.
Analysis of concentrations: Waters Alliance high performance liquid chromatograph (HPLC)

B. STUDY DESIGN AND METHODS

1. Test animals: Mallard duck (*Anas platyrhynchos*)
Age/growth stage: Approximately 28 weeks old at test initiation (888-1392 g b.w.)

Source: [redacted]
Acclimation: 9 weeks prior to testing

2. Diet:
Basal feed diet formulated to [redacted] specifications by [redacted] (1.12% calcium in the basal diet was increased by 38% (5% w/w of limestone) for breeding birds for egg shell formation). Off spring received basal diet without test substance or the additional 5% supplemental limestone.

3. Housing:
Adults: cages containing a pair of birds. 75 x 90 x 45 cm constructed of vinyl-coated wire mesh. Sisal rope was added to each pen for animal enrichment.
Hatchlings: brooding pens measuring 62 x 92 x 25.5 cm constructed of vinyl-coated wire mesh.

4. Environmental conditions:
A summary of environmental conditions is shown in Table B.9.1.1.3 - 11 below.

Table B.9.1.1.3 - 11: Summary of environmental conditions obtained in reproductive study of mallard exposed to 'Inpyrfluxam'.

Variable	Required OECD 206 (1984)	Obtained
Temperature	**Adult pens:** 22 ± 5°C **Egg storage:** 14 – 16˚C (13-16 in EPA guidelines) **Incubator:** 37.5˚C +/- 1˚C **Hatcher:** 37.5˚C **Hatchling pens:** 32 – 35˚C	**Adult pens:** 20.4 ± 1.0°C **Egg storage:** 13.2 ± 0.2°C **Incubator:** 37.4 ± 0.0°C **Hatcher:** 37.3 ± 0.0°C **Hatchling pens:** 21.9 ± 1.5°C
Humidity	**Adult pens:** 50 – 75% **Egg storage:** 60 – 85% **Incubator:** 60 – 75% **Hatcher:** 75-85% **Hatchling pens:** 60 – 85%	**Adult pens:** 47 ± 15% **Egg storage:** 68 ± 7% **Incubator:** 55 ± 0% **Hatcher:** 60 ± 0% **Hatchling pens:** 40 ± 7%
Photoperiod	**Up to test week 8:** 7-8hrs light: 17-16hrs dark **Week 8 – egg production:** 16-18hrs light: 8-6hrs dark **Hatchlings:** approx. 14hrs light:10hrs dark	**Arrival – Week 9:** 8hrs light: 16hrs dark **Week 10 – end of egg production:** 17hrs light: 7hrs dark **Hatchlings:** 16hrs light: 8hrs dark
Light intensity	None in OECD **U.S EPA**: 10-65 lux for adults	**Pre-photo stimulation:** 418 lux **Egg-laying:** 356 lux

Variable	Required OECD 206 (1984)	Obtained
		Post-photo stimulation: 342 lux
Ventilation	15 air volumes per hour	15 air volumes per hour

Study dates: 22nd September 2014 – 26th March 2015

5. Animal assignment and treatment:

Each treatment and control group contained 18 pairs of birds with one male and one female per pen. Four treatment groups plus a control group were fed diets containing either 0, (control group), 125, 250, 500 or 1000 ppm of S-2399 TG for 20 weeks.

The photoperiod was increased at the beginning of week 10 to induce egg production and maintained until adult birds were euthanised (Week 20). Following the start of egg production, eggs were collected daily from all pens and stored in a cold room until incubation. All eggs laid in a weekly interval were considered as one lot. At the end of the weekly interval, all eggs were removed from the cold room, counted and eggs selected by indiscriminate draw for egg shell thickness measurement.

All remaining eggs were candled prior to incubation to detect egg shell cracks or abnormal eggs. Cracked or abnormal eggs were recorded and discarded. All eggs not discarded or used for egg shell thickness measurements were placed in an incubator. All eggs to be incubated were washed in a commercial egg washer with a chlorine based detergent, to reduce the possibility of pathogen contamination. The eggs were then stored in a cold room until incubation. All eggs laid in a weekly interval were considered as one lot.

The incubator was equipped with a pulsator fan and blades to produce a mild breathing air movement to eliminate intracabinet temperature and humidity variation. The incubator was also equipped with an automatic egg rotation device. Eggs were candled on day 14 to determine embryo viability and day 20 to determine embryo survival. On day 24 of incubation, the eggs were placed in an incubator configured for hatching and allowed to hatch. Eggs were not rotated in the hatcher. Once hatching was completed, hatchlings were removed from the hatcher into hatching pens, until the birds were 14 days of age. All hatchlings, unhatched eggs and egg shells were removed on day 27 or day 28.

All adult birds and their offspring were given feed and water *ad libitum*.

6. Dose preparation:

Test diets were prepared by mixing S-2399 TG into a premix that was used for weekly preparation of the final diet. Dietary concentrations were adjusted for purity of the test substance.

7. Measurements and observations:

Prior to start of the study, six samples of each of the test diets were collected to determine homogeneity of the test substance in the diets and one sample from the control diet (day 0). Samples were collected from the top, middle and bottom of the left and right sections of the mixing vessel. Control and treatment samples were collected from the bin feeders on day 7 of Weeks 1, 12 and 20 to assess stability. A sample was taken from the control and treatment group diets on day 0 during Weeks 4, 12 and 20 to verify test concentrations. Analysis was conducted using HPLC. The LOQ was 31.3 ppm a.s.

Adult birds and chicks were observed daily for mortalities and clinical signs. Individual adult body weights were recorded on Weeks 0, 2, 4, 6, 8 and at adult termination (Week 20). Body weights were not measured during egg-laying because the possible adverse effects of handling may have on egg production. Food consumption was measured weekly throughout the test.

Eggs were collected daily from all pens, when available and stored until incubation. All eggs laid in a weekly interval were considered as one lot. At the end of the week, all eggs were removed from the cold room, counted and eggs selected by indiscriminate draw for egg shell thickness measurement. The remaining eggs were candled with an egg-candling lamp to detect egg shell cracks or abnormal eggs, prior to incubation. Eggs were candled again on day 14 of incubation to determine embryo viability and on day 20 to determine embryo survival.

Eggs were measured weekly to determine egg shell thickness. During odd numbered weeks one egg was collected from each odd-numbered pen and from the even-numbered pens on the even numbered weeks. Eggs were opened at the waist, contents removed, and the shells thoroughly rinsed with water. The shells were then allowed to air dry for at least one week at room temperature. The average thickness of the shell plus membrane was determined by measuring five points around the waist of the egg using a micrometre, to the nearest 0.002 mm.

On day 24, eggs were placed in an incubator configured for hatching and allowed to hatch. The number of hatchlings were recorded, body weights determined, and survival recorded up to 14 days of age. At 14 days of age, the individual body weight of each surviving hatchling was recorded.

All surviving adults and birds that died or were euthanised during the course of the study were subjected to gross necropsy follow termination.

8. Statistics:

An ANOVA was performed to determine statistically significant differences among groups. Dunnett’s multiple comparison procedure was used to compare the four treatment means

with the control group mean and assess the statistical significance of observed differences. Sample units were the pens within each group, except adult body weights where the sample unit was the individual bird. Percentage data were examined using Dunnett's method following arcsine square root transformation for reproductive parameters.

II. RESULTS AND DISCUSSION

A. MORTALITY

A single incidental mortality occurred in the 500 ppm treatment group, though this was not considered to be treatment-related. One female mortality found dead on day 3 of week 16 that had been normal in appearance and behaviour prior to mortality. Necropsy revealed small head nodule, air sacculitis, and right heart ventricle appeared flaccid.

B. SUBLETHAL EFFECTS

Clinical observations

Clinical observations and *post mortem* changes were consistent with long-term housing of birds in cages, e.g. foot and head lesions, feather loss and feather picking. Lameness, unkempt appearance and wing droop were also observed. Clinical observations and gross necropsy were both considered unrelated to treatment.

Food consumption and body weight

Adult and chick body weight changes over the treatment period were similar in all groups and there was no evidence of any statistically significant treatment-related effect. There were no apparent treatment-related effects upon feed consumption at any test concentration. Slight increases in mean feed consumption were determined at the 125 ppm concentration during Week 17 and at the 250 and 500 ppm concentrations during Week 18, which were statistically significant. The observed increases were not considered treatment-related since they were neither consistent over time nor concentration responsive.

Mean adult body weight and food consumption measurements body weight and food consumption measurements, plus the corresponding daily dietary doses, are presented in Table B.9.1.1.3 - 12.

Table B.9.1.1.3 - 12: Mean body weight, food consumption and daily dietary dose of 'Inpyrfluxam'

Test interval (test weeks)	Test concentration (ppm)	Mean body weight (g)	Mean food consumption (g/bird/day)	Estimated daily dietary dose (mg a.s./kg b.w./day)
	0	1097	106	0.0

Test interval (test weeks)	Test concentration (ppm)	Mean body weight (g)	Mean food consumption (g/bird/day)	Estimated daily dietary dose (mg a.s./kg b.w./day)
Pre-egg production (weeks 1 – 10)	125	1080	113	13.1
	250	1074	111	25.8
	500	1108	109	49.0
	1000	1080	108	100.4
Egg production (weeks 11 – 20)	0	1121	167	0.0
	125	1112	189*	21.2
	250	1100	184*	41.9
	500	1129	185*	81.8
	1000	1124	176	156.6
Overall (weeks 1 – 20)	0	1105	136	0.0
	125	1091	151	17.3
	250	1083	147	34.0
	500	1114	147	65.8
	1000	1094	142	130.1

*statistically significant increase compared to control

Reproductive results

Compared to the control, there were no treatment-related effects on the reproductive parameters of the number of eggs, fertility of the eggs, normal development (including viability and survival of embryos), hatchability, offspring survival and egg shell thickness at any of the concentrations tested.

A summary of the reproductive data is presented in Table B.9.1.1.3 - 13.

Table B.9.1.1.3 - 13: Summary of reproductive performance in Mallard ducks exposed to 'Inpyrfluxam'.

Reproductive parameter		Experimental Group (ppm)			
	Control	125	250	500	1000
Number Surviving Replicates	18	18	18	17	18
Eggs Laid[a]	872	911	964	945	965
Eggs Laid/Pen/day[b]	0.63	0.66	0.70	0.72	0.70

Reproductive parameter	Control	Experimental Group (ppm)			
		125	250	500	1000
Eggs Cracked/Eggs Laid (%)[c]	0	0	1	0	0
Fertile Eggs/Eggs Set	98	99	90	95	95
Viable Embryos/Eggs Set (%)	94	94	84	93	92
Live 3-week Embryos/Viable Embryos (%)	99	99	98	98	100
Hatchlings/Live 3-week Embryos (%)	83	87	83	79	85
14-day Old survivors/Hatchlings (%)	99	99	99	99	99
Hatchlings/Egg Set (%)	77	81	69	72	78
Hatchlings/Fertile Eggs (%)	79	82	76	75	82
14-day Old Survivors/Eggs Set (%)	77	80	68	72	77
Hatchlings/Pen/Day[b]	0.44	0.49	0.44	0.46	0.48
14-day Old Survivors/Pen/Day[b]	0.44	0.48	0.43	0.46	0.48

[a] The total number of eggs laid in each group

[b] Based on 77 days of egg production

[c] Percent values represent replicate means for each experimental group.

Differences between the control group and each of the treatment groups were not significant ($p > 0.05$, Dunnett's t-test)

C. DIET ANALYSIS

Analysis of the control samples did not show any indication of the presence of the test substance or a co-eluting substance at the characteristic retention time of the test substance. Mean concentrations and standard deviations for the four test concentrations 125, 250, 500 and 100 ppm tested for homogeneity were 126 (101% nominal), 240 (96% nominal), 532 (106% nominal) and 1050 (105% nominal) ppm, respectively. The results from the chemical analysis of diet samples are presented in Table B.9.1.1.3 – 14.

Samples collected to verify test substance concentrations for the 125, 250, 500 and 1000 ppm diets had mean concentrations and standard deviations of 122 (98% nominal), 250 (100% nominal), 538 (100% nominal) and 1050 (105% nominal) ppm, respectively.

Table B.9.1.1.3 - 14: Summary of analytical results

Week	Day	Mean measured values (ppm) (%)			
		125	250	500	1000
1[a,b]	0	126 (101)	240 (96)	532 (106)	1050 (105)
1	7	124 (98)	246 (103)	543 (102)	994 (95)
4	0	142 (114)	266 (107)	537 (108)	1110 (111)
12	0	105 (84)	240 (96)	544 (109)	982 (98)
12	7	121 (115)	240 (100)	472 (87)	947 (96)
20	0	120 (96)	243 (97)	532 (106)	1060 (106)
20	7	134 (112)	244 (100)	496 (93)	1060 (100)

[a] Day 0 values are from homogeneity samples

[b] Day 0 values are percent of nominal, Day 7 values are percent of values on Day 0.

D.VALIDITY CRITERIA

The following criteria were met; therefore the test is considered valid:

1. Test concentrations were maintained at ≥80% of nominal
2. Mortality was ≤ 10%
3. The average number of eggs laid per hen was ≥ 29
4. The proportion of viable eggs set at ~ Day 14 was ≥ 80%
5. The number of live 3-Weekembryos pf viable embryos at ~ Day 21 was ≥ 94
6. The number of hatchlings of live 3-week embryos was≥ 52%
7. The number of hatchlings of eggs set for incubation was ≥ 44%
8. The proportion of 14-day old survivors of hatchlings was ≥94%
9. Average egg shell thickness was ≥ 0.340 mm
10. The proportion of cracked eggs of eggs laid was ≤ 13%

The OECD 206 (1984) validity criteria are shown in Table B.9.1.1.3 - 15.

Table B.9.1.1.3 - 15: OECD 206 (1984) validity criteria for avian reproductive study

Criteria	Required OECD 206 (1948)	Obtained
Mortality in controls	<10%	<0%
14-day-old survivors per hen in controls	>14	≥94%
Egg shell thickness in control	>0.34mm	≥ 0.34 mm
Concentrations	Recommended concentration scheme	Concentrations tested are in line with

Criteria	Required OECD 206 (1948)	Obtained
	followed and there is no effect on reproduction	recommendations. No effect on reproduction

III. CONCLUSION

There were no treatment-related adult mortalities, overt signs of toxicity or treatment-related effects upon body weight or feed consumption at any of the concentrations tested. Additionally, there were no treatment-related effects upon any of the reproductive parameters measured at the 125, 250, 500 or 1000 ppm test concentrations. The no-observed-effect concentration (NOEC) for mallard ducks exposed to S-2399 TG in the diet during the study was 1000 ppm (130.0 mg a.s./kg b.w./day), the highest concentration tested.

HSE COMMENTS:
This study was conducted under GLP and followed OECD 206 (1984) and OCSPP 850.2300 (2012) and has been assessed against these guidelines.

There are several deviations to protocol to note for this study. The first significant deviation relates to the age of the test animals. The animals were 28 weeks old at test initiation. OECD 206 (updated amendment, 2008) requires that birds be between 9 and 12 months old (approx. >45 weeks) for testing. This indicates that the test animals do not meet the required guidelines. U.S EPA (OCSPP 850.2300, 2012) states that birds must be at least 16 weeks old for testing. This means the study would adhere to these requirements. As the validity criteria have been met, this study is considered valid.

The temperature of the incubator, hatcher and hatching pen environments did not meet OECD 206 (1984) guidelines. All of these were below the requirements outlined on OECD 206 (1984). The temperatures recorded for the hatching pens in particular were much below the recommended levels. Thermostats in the brooding compartment of each pen were set to maintain a temperature of approximately 38° C from the time of hatching until the birds were five to seven days of age, when the temperature was adjusted to maintain a temperature of approximately 29˚C. The average ambient room temperature was 21.9 ± 1.5°C (SD). This may suggest that the thermostats were not working correctly. There were no adverse sublethal effects observed amongst the hatchlings, and the validity criteria relating to 14-day-old survivors per hen were met; therefore, the study is considered valid.

The next deviation to protocol relates to the humidity of the incubator, hatcher and hatching pen environments. All of these were notably lower than the requirements in

OECD 206 (1984) guidance. However, as no adverse sub-lethal effects were observed and all the validity criteria were met, the study is considered valid.

The light intensity obtained at each measured stage of the study also exceeded the value in the available OCSPP 850.2300 (2012) guidance by a significant amount. However, as no adverse behavioural effects were observed and the validity criteria have been met, the results are considered valid.

Another point to highlight is that some of the values obtained for the analytical results of test concentrations in the diet were quite a bit higher than the nominal concentration (e.g., week 4, day 0 of the 1000ppm a.s.). No carrier was used to help supply the test substance to the diet. This is unlikely to have a significant impact on the validity of the study as all test concentrations remained above 80% of the nominal concentration and no substance-related mortalities were recorded.

Another point to note is that there were multiple incidental observations made throughout the study across control and exposure groups. These relate to foot lesions, lameness, reduced reaction, wing droop, prostrate posture, lower limb weakness, unkempt, feather picking and feather loss). These are not unusual in reproductive avian studies but may indicate an unsuitable environment.

The statistics that were used were suitable for this study and adhere to OECD 206 (1984) guidance. The Daily Dietary Dose calculations were checked using excel and several values are slightly different to the ones presented in the study report but will not affect the reliability of results or validity of the study.

HSE Chemistry conclusion is as follows (see section B5, section B.5.1.2):

The analytical method is not fully validated according to SANTE/2020/12830 rev. 1 for the determination of the active substance inpyrfluxam in avian feed from the Mallard and Northern Bobwhite reproduction studies as the matrix effects have not been determined and the stability of standards and extracts has not been addressed. However, the studies were generated prior to the implementation of SANTE 2020/12830 rev.1 and SANCO 3029 rev.4 did not require matrix effects and the stabilities of standards and extracts to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose.

The endpoints to use in risk assessment are:

NOEC = 1000ppm a.s. (based on nominal concentration of Inpyrfluxam supplied in diet to mallard in reproductive study)

B.9.1.2 Effects on terrestrial vertebrates other than birds

Summary of active substance endpoints

The following tables provide a summary of the endpoints generated from the studies conducted with the active substance, inpyrfluxam, for mammals. Full study summaries and evaluation can be found in Volume 3 - B6 of the Review Assessment Report.

Table 9.1.2 -1: Toxicity endpoints for the acute risk assessment of mammals for 'Inpyrfluxam' ('S-2399 TG')

Technical a.s. (% purity)	Species (♂ or ♀ if relevant[1])	LD50 value (mg a.s./kg b.w.)[2,3]	MamTox section reference
Acute			
Inpyrfluxam	Wistar rats (♀)	50 < LD_{50} < 300 mg/kg b.w.	Vol. 3 CA B.6.2.1 [redacted] (2015a)
Inpyrfluxam	Wistar rats (♀)	**LD_{50} = 180 mg/kg b.w.**	Vol. 3 CA B.6.2.1 [redacted] (2017a)

Endpoints in **bold** for use in risk assessment

Table 9.1.2 - 2: Toxicity endpoints for the reproductive risk assessment of mammals for 'Inpyrfluxam'

Endpoint	NOAEL (mg a.s./kg b.w./d)	Reference	Studies to check
Body weight change[1],	31.7 mg/kg b.w./day (500 ppm in diet)	90 day study in rats Vol. 3 CA B.6.3.2 [redacted] (2016)	Repeated dose 28-day oral toxicity study in rodents (OECD 407) Sub-chronic oral toxicity study-rodent 90 day study (OECD 408) Multi-generation study (OECD 416) Developmental studies (OECD 414)
	27.8 mg/kg b.w./day (500 ppm in the diet) Body weight effects on adults and offspring	Two-generation reproductive toxicity study in rat Vol. 3 CA B.6.6.1 [redacted] (2017)	
	25 mg/kg b.w./day Body weights in dams and foetuses	Developmental toxicity study in rat Vol. 3 CA B.6.6.2 [redacted] (2017a)	

Endpoint	NOAEL (mg a.s./kg b.w./d)	Reference	Studies to check
	60 mg/kg b.w./day Body weights in does	Developmental toxicity study in rabbits Vol. 3 CA B.6.6.2 [redacted] (2017c)	
Behavioural effects and systemic toxicity[2]	30 mg/kg b.w. reduced body temperature and motor activity	Acute neurotoxicity study in rats Vol. 3 CA B.6.7.1 [redacted] (2016b)	Repeated dose 28-day oral toxicity study in rodents (OECD 407) Sub-chronic oral toxicity study-rodent 90-day study (OECD 408) Multi-generation study (OECD 416) Developmental studies (OECD 414)
Indices of gestation, litter size, pup and litter weight[3]	27.8 mg/kg b.w./day (500 ppm in diet) litter weight	Two-generation reproductive toxicity study in rat Vol. 3 CA B.6.6.1 [redacted] (2017)	Multi-generation study (OECD 416) Developmental studies (OECD 414)
	25 mg/kg b.w./day **Foetus weight**	Developmental toxicity study in rat Vol. 3 CA B.6.6.2 [redacted] (2017a)	
Indices of viability, pre- and post-implantation loss	No effects 86 mg/kg b.w./day (top dose)	Two-generation reproductive toxicity study in rat Vol. 3 CA B.6.6.1 [redacted] (2017)	Multi-generation study (OECD 416) Developmental studies (OECD 414)
	No effects 80 mg/kg b.w./day (top dose)	Developmental toxicity study in rat Vol. 3 CA B.6.6.2 [redacted] (2017a)	

Endpoint	NOAEL (mg a.s./kg b.w./d)	Reference	Studies to check
Embryo/foetal toxicity including teratological effects	No effects		Multi-generation study (OECD 416) Developmental studies (OECD 414)
Number aborting and number delivering early	No effects 86 mg/kg b.w./day (top dose)	Two-generation reproductive toxicity study in rat Vol. 3 CA B.6.6.1 [redacted] (2017)	Multi-generation study (OECD 416) Developmental studies (OECD 414)
	60 mg/kg b.w./day Abortions	Developmental toxicity study in rabbits Vol. 3 CA B.6.6.2 [redacted] (2017c)	
Systemic toxicity and effects on adult body weight	See above		Multi-generation study (OECD 416) Developmental studies (OECD 414)
Indices of post-natal growth[4], indices of lactation and data on physical landmarks	No effects		Multi-generation study (OECD 416) Developmental studies (OECD 414)
Survival and general toxicity up to sexual maturity	No effects		Multi-generation study (OECD 416) Developmental studies (OECD 414)

Endpoints in **bold** suitable for use in risk assessment

Table 9.1.2 -3 Toxicity endpoints from metabolites of 'Inpyrfluxam'

Species	Substance	Exposure System	Results	Reference
Acute active substance				
Wister rats	3'-OH-S-2840 (99.6% purity)	Toxic class method	LD50[a)] >2000 mg/kg b.w.	Vol. 3 CA B.6.8.1 [redacted] (2017b)
Wister rats	1'-COOH-S-2840 (99.8% purity)	Toxic class method	LD50[a)] >2000 mg/kg b.w.	Vol. 3 CA B.6.8.1 [redacted] (2017c)

a) For the acute toxic class (OECD 423) method where only a range is provided, an 'LD50 cut-off' value can be obtained from the charts in Annex 2 of the Test Guideline.

B.9.1.2.1 Acute oral toxicity to mammals

All relevant studies are provided in Volume 3 B6 of the Review Assessment Report.

B.9.1.2.2 Long-term and reproduction toxicity to mammals

All relevant studies are provided in Volume 3 B6 of the Review Assessment Report.

B.9.1.3 Active substance bioconcentration in prey of birds and mammals

According to the data requirements, substances with a log Pow > 3 have potential for bioaccumulation and should be assessed for the risk of bioaccumulation in the prey of birds and mammals. The log Pow of the active substance Inpyrfluxam is 3.65 (see Volume 3CA B2) and therefore consideration of the risk from bioaccumulation to fish-eating birds and mammals has been conducted, see Volume 3CP B9.

A new fish bioconcentration study is available and the summary is provided in Section B.9.2.

B.9.1.4 Other data on effects of terrestrial vertebrate wildlife (birds, mammals, reptiles and amphibians)

No active substance data was submitted for this data point.

B.9.1.5 Potential for endocrine disruption

The scientific criteria for determining endocrine disrupting properties in the context of pesticide regulation[6] have been finalised and published. Under this amendment to the EU regulation for pesticides a substance shall be considered as having endocrine disrupting properties that might cause adverse effects on non-target organisms if it meets the following criteria, unless there is evidence demonstrating that the adverse effects identified are not relevant at the (sub)population level for non-target organisms:

1. it shows an adverse effect in non-target organisms, which is a change in the morphology, physiology, growth, development, reproduction or life span of an organism, system, or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress or an increase in susceptibility to other influences.
2. it has an endocrine mode of action, i.e. it alters the function(s) of the endocrine system.
3. the adverse effect is a consequence of the endocrine mode of action.

On the basis of these criteria there is a need to further consider the potential for the active substance inpyrfluxam to have endocrine disrupting properties in relation to non-target organisms according to such criteria, which are supported by a modern guidance document: Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009[7].

The assessment for inpyrfluxam is detailed below. Firstly all available data has been considered (literature review and submitted studies) followed by an ecotoxicology assessment for each of the non-target organism groups in relevant sections (B.9.1.5 and B.9.2.4).

Literature review

One of the databases searched by the applicant as the ‘Endocrinology Abstracts’ database; but no further consideration of endocrine disruption was provided by the applicant. No results were found in the literature search.

HSE ecotoxicology comments:

No consideration of endocrine disruption was provided in the literature review provided by the applicant, with no results found in the database ‘Endocrinology Abstracts’. Therefore, no further consideration of the literature review is provided by HSE ecotoxicology.

[6] COMMISION REGULATION (EU) 2018/605 of 19 April 2018 ammending Annex II to Regulation (EC) No 1107/2009 by setting out scientific criteria for the determination of endocrine disrupting properties

[7] EFSA Journal 2018;16(6):5311

Summary of studies submitted relevant to endocrine disruption

Beyond the literature review several studies were submitted that can be considered in the endocrine disruption assessment. In accordance with EFSA/ECHA guidance, only reliable studies have been considered further by HSE for the endocrine disruption ecotoxicology assessment. No unreliable studies were considered to have been submitted and therefore, all studies have been evaluated.

A summary of all submitted ecotoxicology studies suitable for consideration of endocrine disruption are shown in the table below, with the exception of mammalian toxicology data.

Table B.9.1.5-1: Studies for endocrine disruption hazard assessment of inpyrfluxam in non-target organisms other than mammals

Study ID	Study type	Species	Guideline	Author (year)*
19	Avian reproduction test	Northern bobwhite quail (*Colinus virginianus*)	OECD 206 US EPA OCSPP 850.2300	[redacted] and [redacted] (2015a)
20	Avian reproduction test	Mallard (*Anas platyrhynchos)*	OECD 206 US EPA OCSPP 850.2300	[redacted] and [redacted] (2015b)
26	Amphibian metamorphosis assay (AMA)	African clawed frog (*Xenopus laevis*)	OECD 231	[redacted] (2021)
27	Fish short term reproduction assay (FSTRA)	Fathead minnow (*Pimephales promelas*)	OECD 229	[redacted] (2021)
21	Early life stage test (32 d)	Fathead minnow (*Pimephales promelas*)	OECD 210	[redacted] (2014)

Study ID	Study type	Species	Guideline	Author (year)*
22	Early life stage test (34 d)	Sheepshead minnow *(Cyprinodon variegatus)*	OECD 2010	[redacted] (2014)

*Study evaluations provided in the relevant sections of the Volume 3CA dossier

Where appropriate the studies have been considered in the relevant sections for the different non-target organism groups (section B.9.1.5 for birds/mammals/reptiles and section B.9.2.3 for aquatic organisms).

Birds

A summary of the results has been provided below. The format is in accordance with EFSA/ECHA guidance i.e. appendix E.

Table B.9.1.5-2: Reporting the lines of evidence for adverse effects from avian reproduction studies

Study ID Matrix	Effect classification	Effect target	Effect description	Species	Exposure*	Route	Lowest Effect dose	Doses	Dose unit	Assessment of each line of evidence
19	Sensitive to but not diagnostic of, EATS	Body weight (bird)	(g)	*Colinus virginianus*	21 weeks	Oral	n.a.	0, 125, 250, 500,1000	ppm	No indication
20	Sensitive to but not diagnostic of, EATS	Body weight (bird)	(g)	*Anas platyrhynchos*	20 weeks	Oral	n.a.	0, 125, 250, 500,1000	ppm	No indication
19	Sensitive to but not diagnostic of, EATS	Eggshell thickness	(mm)	*Colinus virginianus*	21 weeks	Oral	n.a.	0, 125, 250, 500,1000	ppm	No indication
20	Sensitive to but not diagnostic of, EATS	Eggshell thickness	(mm)	*Anas platyrhynchos*	20 weeks	Oral	n.a.	0, 125, 250, 500,1000	ppm	No indication

Study ID Matrix	Effect classification	Effect target	Effect description	Species	Exposure*	Route	Lowest Effect dose	Doses	Dose unit	Assessment of each line of evidence
19	Sensitive to but not diagnostic of, EATS	Cracked eggs	Cracked eggs of eggs laid (%)	*Colinus virginianus*	21 weeks	Oral	n.a.	0, 125, 250, 500,1000	ppm	No indication
20	Sensitive to but not diagnostic of, EATS	Cracked eggs	Cracked eggs of eggs laid (%)	*Anas platyrhynchos*	20 weeks	Oral	n.a.	0, 125, 250, 500,1000	ppm	No indication
19	Sensitive to but not diagnostic of, EATS	Egg production	Eggs laid; eggs laid per female (per day)	*Colinus virginianus*	21 weeks	Oral	1000	0, 125, 250, 500,1000	ppm	Decrease in mean egg production at highest tested concentration for bobwhite quail (15.1% reduction compared to historical control mean) but no effects on this parameter for mallard duck.
20	Sensitive to but not diagnostic of, EATS	Egg production	Eggs laid; eggs laid per female (per day)	*Anas platyrhynchos*	20 weeks	Oral	n.a.	0, 125, 250, 500,1000	ppm	

Study ID Matrix	Effect classification	Effect target	Effect description	Species	Exposure*	Route	Lowest Effect dose	Doses	Dose unit	Assessment of each line of evidence
19	Sensitive to but not diagnostic of, EATS	Egg fertility (embryonic day 8)	-	*Colinus virginianus*	21 weeks	Oral	n.a.	0, 125, 250, 500,1000	ppm	No indication
20	Sensitive to but not diagnostic of, EATS	Egg fertility (embryonic day 8)	-	*Anas platyrhynchos*	20 weeks	Oral	n.a.	0, 125, 250, 500,1000	ppm	No indication
19	Sensitive to but not diagnostic of, EATS	Embryo viability (embryonic day 15)	-	*Colinus virginianus*	21 weeks	Oral	n.a.	0, 125, 250, 500,1000	ppm	No indication
20	Sensitive to but not diagnostic of, EATS	Embryo viability (embryonic day 15)	-	*Anas platyrhynchos*	20 weeks	Oral	n.a.	0, 125, 250, 500,1000	ppm	No indication
19	Sensitive to but not diagnostic of, EATS	Hatchability	% hatching of viable embryo	*Colinus virginianus*	21 weeks	Oral	n.a.	0, 125, 250, 500,1000	ppm	No indication
20	Sensitive to but not	Hatchability	% hatching of viable embryo	*Anas platyrhynchos*	20 weeks	Oral	n.a.	0, 125, 250, 500,1000	ppm	No indication

Study ID Matrix	Effect classification	Effect target	Effect description	Species	Exposure*	Route	Lowest Effect dose	Doses	Dose unit	Assessment of each line of evidence
	diagnostic of, EATS									
19	Sensitive to, but not diagnostic of, EATS	No. of 14 day old survivors	The number of 14-day old survivors was divided by the number of eggs set, per pen.	*Colinus virginianus*	21 weeks	Oral	n.a.	0, 125, 250, 500,1000	ppm	No indication
20	Sensitive to, but not diagnostic of, EATS	No. of 14 day old survivors	The number of 14-day old survivors was divided by the number of eggs set, per pen.	*Anas platyrhynchos*	20 weeks	Oral	n.a.	0, 125, 250, 500,1000	ppm	No indication
19	Systemic toxicity	Mortality	Survival	*Colinus virginianus*	21 weeks	Oral	n.a.	0, 125, 250, 500,1000	ppm	No indication

Study ID Matrix	Effect classification	Effect target	Effect description	Species	Exposure*	Route	Lowest Effect dose	Doses	Dose unit	Assessment of each line of evidence
20	Systemic toxicity	Mortality	Survival	*Anas platyrhynchos*	20 weeks	Oral	n.a.	0, 125, 250, 500,1000	ppm	No indication

* Duration of exposure refers to the adult generation, eggs and offspring were not exposed
b Body weight of adults could also be considered under 'systemic toxicity'
a.s. : active substance
n.a. not applicable – no effects observed

The applicant's consideration for birds is provided in italics below:

'In the study with the bobwhite quail there were no treatment-related mortalities, overt signs of toxicity or treatment-related effects upon body weight or feed consumption at any of the concentrations tested. Additionally, there were no treatment-related effects upon any of the reproductive parameters measured. The NOEL for northern bobwhite exposed to S-2399 TG in the diet during the study was 1000 ppm (87.0 mg a.s./kg/day), the highest concentration tested. At 125 and 1000 ppm test concentrations, there were significant reductions in the number of eggs laid per hen per day, however, the reductions were influenced by the inordinately high productivity of the control group. The mean egg production for the control group was 52 eggs per hen, which exceeded the mean value from 15 of the most recent studies (45 ± 5) at the CRO. Since the differences in egg production were slight and not concentration responsive, they were not considered to be related to treatment.

In the study with the mallard there were no treatment-related mortalities, overt signs of toxicity or treatment-related effects upon body weight or feed consumption at any of the concentrations tested. Additionally, there were no treatment-related effects upon any of the reproductive parameters measured. The NOEL for mallard exposed to S-2399 TG in the diet during the study was 1000 ppm (130 mg a.s./kg/day), the highest concentration tested.

The avian reproduction test (OCED 206) is not considered a full life cycle test, only adults are exposed to the test item and offspring are monitored for 14 days. However, results from the two tests available showed no adverse effects on the adults and egg production was within the normal range for the species tested.'

HSE ecotoxicology conclusion for birds

When considering reproductive toxicity, HSE considers an NOEL of 44.3 mg a.s./kg b.w./d in the study with *C. virginianus* more appropriate due to potential effects observed in mean egg production. This set the LOEC at 87 mg a.s./kg b.w./d (1000 ppm). The effects on mean egg production observed at 125ppm were not considered to be treatment related however, it was concluded that there was insufficient information available to definitively conclude that the effects demonstrated at 1000ppm were not treatment related. Further consideration is detailed in section B.9.1.1.

There is no clear dose-response in the effects observed for mean egg production and therefore it is difficult to attribute the effects seen to a specific ED effect. Currently there are no further tests available for assessing endocrine activity in birds and therefore HSE do not consider further testing to be required at this stage. This is supported by the EFSA/ECHA 2018 guidance which states that '*for birds, only a limited number of standardised in vivo methods are available, and little information*

can be gained from those guidelines concerning potential ED-related effects. In general, little is known of the impact of endocrine disruptors in birds compared to other species and more research is needed to develop responsive parameters and in vitro and in vivo protocols to specifically address the differences between birds and other vertebrate taxa.'

No treatment-related effects on mortality, body weight or feed consumption were noted in the reproductive mallard study (and 2015b) The LOEC was set at >1000 ppm.

In accordance with EFSA/ECHA 2018 guidance the gross pathology findings should be reported. This was the case for the submitted avian studies and no treatment related effects were observed.

Overall on the basis of the current dataset and EFSA/ECHA 2018 guidance document, it is not possible to fully conclude for 'inpyrfluxam' against ED criteria when considering birds.

The wild mammal endocrine disruption assessment is shown below:

Ecotoxicology assessment of wild mammals:

As an initial step the Toxicology conclusions have been considered to inform the assessment of ED for mammals (as non-target organisms). Currently, if a substance is considered to meet the criteria for human health then they will also be met for mammals as non-target organisms so long as:

'The adverse effects on reproduction, growth/development, and other relevant adverse effects are likely to impact on (sub)populations' – as detailed in the implementing regulation (EU) 2018/605.

The applicant's consideration for wild mammals is provided in italics below:

'Overall, S-2399 TG has some effects on the endocrine related organs such as ovary, thyroid and adrenal glands, but these are not indicative of specific endocrine disrupting potential nor of endocrine-related adversity. Although thyroid hypertrophy (increase in weight and follicular cell hypertrophy) was observed in rats, mice and dogs, this was not associated with clear decrease in T4 levels indicating no potential concern for effects on neurodevelopment. There were no adverse effects on apical end points such as reproductive performance and thyroid tumorigenesis. Therefore, it is considered that S-2399 TG does not possess any endocrine disrupting potential of relevance for human health. The data base is sufficient to conclude that there is no endocrine-related adversity in mammals according to Scenario 1a in Section 3.4.4.1 of the Guidance.

The mammalian dataset is considered complete and sufficiently investigated in vitro and in vivo to conclude that the ED criteria are not met in mammals for the EAS-modalities. As the endocrine system is highly conserved across mammalian species, as confirmed through testing a range of mammalian species, the conclusion that the ED criteria are not met in non-target mammals other than humans is equally applicable. The data base for other non-target vertebrates is sufficient to conclude that there is no endocrine-related adversity in non-target vertebrates according to Scenario 1a in Section 3.4.4.1 of the Guidance. Therefore, it is concluded that S-2399 TG does not possess any endocrine disrupting potential'.

An exhaustive and detailed discussion of endocrine disruption for inpyrfluxam has been provided in the Volume 3CA, Section 6 dossier Part II (B.6.8.3) and the relevance of this to non-target mammals, as opposed to humans, is considered below.

Overall conclusions for EAS modalities (toxicology):

The following conclusion was reached in the toxicology section (see Volume 3CA, Section 6 dossier (B.6.8.4)): *"In all species investigated (rat, mouse, dog) there were no specific adverse effects on reproductive organs and other endocrine organs related to the EAS modalities following repeated exposure to inpyrfluxam. In addition, there were no specific adverse effects on reproduction in the rat and on development in the rat and rabbit. Overall, there was no clear and specific pattern of adversity for the EAS modalities. In addition, there was no evidence of EAS activity in a steroidogenesis assay and in a hERα or hAR transactivation test".*

HSE concludes that for the EATS-modalities, inpyrfluxam is not an ED and its ED potential has been sufficiently investigated, with no further information required.

In the standard dietary mammalian risk assessment, the following parameters are considered population relevant by HSE (noting this is not an exhaustive list):

- Effects on body weight change, behavioural effects and mortality.
- Effects on indices of gestation, litter size, pup and litter weight.
- Effects on indices of viability, pre- and post-implantation loss.
- Embryo/foetal toxicity including teratological effects.
- Effects on number aborting and number delivering early.
- Effects on adult body weight.
- Effects on indices of post-natal growth, indices of lactation and data on physical landmarks.
- Effects on survival and general toxicity observed up to sexual maturity.

No EAS-mediated reproductive and developmental effects were observed in either the F0 or F1 generations in the 2-generation rat study ([redacted] 2017; Study ID

Matrix-9). Inpyrfluxam had no effect on male or female fertility or reproductive performance up to the top dose of 113/86 mg/kg b.w./day; gestation duration, oestrus cycle and spermatogenic endpoints were also unaffected by treatment. There was also no effect on litter size, sex ratio, pup survival and developmental landmarks.

In the developmental toxicity studies in rats or rabbits, there were no effects on the mean number of live foetuses, percentage incidence of resorptions, foetal deaths, sex ratio, variations or malformations. ((2017b), study matrix ID-11; (2017c), study matrix ID-14). Overall, there were no EAS-mediated reproductive and developmental adverse effects in the available studies in rats and rabbits.

In the 28-day repeat dose study in rats, significant systemic toxicity was observed at and above 264.4/263 mg/kg b.w./day (decreased body weights and body weight gains (-23,1% and -25.4% in males and females at 246.4/263 mg/kg b.w./day). Clinical-chemistry parameters indicative of liver damage and disruption in lipid metabolism, changes in liver weight, and histopathological findings of the liver and bone marrow) with the MTD (Maximum Tolerated Dose) being reached or exceeded at 246.4/263 mg/kg b.w./day.

In the 90-day repeat dose study in rats significant general toxicity was observed at and above 144 mg/kg b.w./day (decreased body weights and body weight gains (-21% in females at 144 mg/kg b.w./day), clinical-chemistry parameters indicative of liver damage, changes in liver weight (with associated hypertrophy), and histopathological findings of the liver), with the MTD being reached or exceeded at 144 mg/kg b.w./day.

In the 90-day repeat dose study in dogs, significant systemic toxicity was noted at and above 160 mg/kg b.w./day in males and at the highest doses of 750/500 mg/kg b.w./day in females (vomiting, signs of anaemia, changes in clinical-chemistry parameters indicative of liver damage, increased liver weight and histopathological findings in the liver, gall bladder, kidney and optic nerve). These signs of systemic toxicity were noted from the 30 mg/kg be/d in the one-year repeat dose study in dogs.

In the 28-day repeat dose study in rats, decreases in ovary weights and uterine weights (absolute and/or relative) were seen from 263 mg/kg b.w./day. These changes were associated with vacuolation of the interstitial gland of the ovary and atrophy of the uterus. In the 90-day repeat dose study in rats, decrease in ovary weights at 292 mg/kg b.w./day and vacuolation of the interstitial gland of the ovary from 144 mg/kg b.w./day was noted.

Similar effects (and any possible association with effects on fertility) were not seen in the rat 2-generation study (2017; Study ID Matrix-9), possibly due to the lower

dose levels used in this study. Overall, the changes observed in the ovary and uterus of female rats occurred above the MTD and therefore they do not raise concerns regarding endocrine disruption.

HSE Toxicology confirmed that none of the other additional effects listed above were observed in the studies.

HSE (Ecotoxicology) considers that, based on the Toxicology conclusion, the ED criteria are not met for mammals as non-target organisms when considering EAS modalities and that these modalities have been sufficiently investigated. Further consideration of EAS modalities for wild mammals is not required.

Overall conclusions for the T modality (toxicology):

The following conclusion was reached in the toxicology section (see Volume 3CA, Section 6 dossier (B.6.8.3)): *'Overall, the changes observed in the thyroid gland in rats, mice and dogs occurred at or above the MTD/limit dose and therefore they do not raise concerns regarding endocrine disruption. In addition, whilst such effects occurred in the short-term studies, they were not replicated in the long-term studies up to doses causing significant toxicity. Overall, therefore, inpyrfluxam does not present a clear and specific pattern of adversity for the T modality in relation to effects on the thyroid gland. In addition, mechanistic information suggests that the thyroid effects might be secondary to liver effects and that inpyrfluxam does not have any effect on hTRα -mediated transactivation and TPO activity'.*

HSE (ecotoxicology) considers that based on the toxicology conclusion the ED criteria are not met for mammals as non-target organisms when considering the T modality and that this modality has been sufficiently investigated. No further consideration of the T modality for wild mammals is required.

Reptiles:

No publications or studies assessing the effect of 'inpyrfluxam' on reptiles have been submitted. Currently, investigation of ED properties in these taxa is hampered by a lack of test methods investigating endocrine specific endpoints. Indeed, the joint EFSA/ECHA 2018 guidance document sets as a recommendation for future research work to gain a better understanding of the endocrinology of reptiles and whether extrapolations from other vertebrate groups can be scientifically justified. As such HSE judges that no conclusion can be drawn with regards to the ED properties of 'inpyrfluxam' in relation to reptiles.

Overall HSE ecotoxicology conclusion for terrestrial vertebrates (endocrine disruption):

Overall, HSE concludes that based on current EFSA/ECHA 2018 guidance it is not possible to reach a conclusion for birds or reptiles when considering endocrine disruption.

For non-target wild mammals, HSE (ecotoxicology) concludes that based on the Toxicology conclusions above, the ED criteria are not met for mammals as non-target organisms when considering EATS modalities and that these modalities have been sufficiently investigated.

B.9.2 Effect on Aquatic organisms

B.9.2.1 Summary of toxicity data

The data available to address the toxicity of Inpyrfluxam are summarised below (Table 9.2.1-1).

Table 9.2.1-1: Endpoints relevant for Inpyrfluxam (S-2399)

Test substance	Test organism	Test system	Endpoint		Reference
Acute toxicity to fish					
Inpyrfluxam S-2399	*Oncorhynchus mykiss*	96-hour, static	LC_{50}	0.031 mg a.s./L (t.w.a)	KCA 8.2.1/01 [redacted] 2014a
Inpyrfluxam S-2399	*Lepomis macrochirus*	96-hour, static	LC_{50}	0.054 mg a.s./L (t.w.a)	KCA 8.2.1/02 [redacted] 2014b
Inpyrfluxam S-2399	*Pimephales promelas*	96-hour, static	LC_{50}	0.050 mg a.s./L (t.w.a)	KCA 8.2.1/03 [redacted] 2014c
Inpyrfluxam S-2399	*Cyprinus carpio*	96-hour, static	LC_{50}	0.067 mg a.s./L (t.w.a)	KCA 8.2.1/04 [redacted] 2014d
Inpyrfluxam S-2399	*Cyprinodon variegatus*	96-hour, static	LC_{50}	0.15 mg a.s./L (m.m)	KCA 8.2.1/05 [redacted] 2014e

Test substance	Test organism	Test system	Endpoint		Reference
Inpyrfluxam S-2399	*Poecilia reticulata*	96-hour, static	LC_{50}	0.35 mg a.s./L (t.w.a)	KCA 8.2.1/06 [redacted] 2016c
Inpyrfluxam S-2399	*Oryzias latipes*	96-hour, static	LC_{50}	0.79 mg a.s./L (t.w.a)	KCA 8.2.1/07 [redacted] 2016a
Inpyrfluxam S-2399	*Danio rerio*	96-hour, static	LC_{50}	0.30 mg a.s./L (t.w.a)	KCA 8.2.1/08 [redacted] 2016b
Chronic toxicity to fish					
Inpyrfluxam S-2399	*Pimephales promelas*	32-day, flow-through	LC_{10}	0.0066 mg a.s./L (m.m)	KCA 8.2.2.1/01 [redacted] 2014
Inpyrfluxam S-2399	*Cyprinodon variegatus*	34-day, flow-through	NOEC[a]	0.009 mg a.s./L (m.m)	KCA 8.2.2.1/02 [redacted] 2017
Bioconcentration in fish					
Inpyrfluxam S-2399	*Lepomis macrochirus*	31-day, flow-through	Lipid normalised, growth corrected, kinetic bioconcentration factor ($BCF_{kgL,TRR}$)	215.4 L/kg (Total 14C residue basis)	KCA 8.2.2.3/01 [redacted] 2015
			Lipid normalised steady state bioconcentration factor ($BCF_{SSL,\ S\text{-}2399}$)	38.4 L/kg (S-2399)	
Acute toxicity to invertebrates					

Test substance	Test organism	Test system	Endpoint		Reference
Inpyrfluxam S-2399	*Daphnia magna*	48-hour, static	EC_{50}	1.1 mg a.s./L (t.w.a)	KCA 8.2.4.1/01 [redacted] 2014f
Inpyrfluxam S-2399	*Americamysis bahia*	48-hour, static	LC_{50}	1.1 mg a.s./L (m.m)	KCA 8.2.4.2/01 [redacted] 2014g
Long-term toxicity to invertebrates					
Inpyrfluxam S-2399	*Daphnia magna*	21-day, static-renewal	NOEC	0.13 mg a.s./L (t.w.a)	KCA 8.2.5.1/01 [redacted] 2014h
			EC_{10} (reproduction)	0.21 mg a.s./L (t.w.a)	
Inpyrfluxam S-2399	*Americamysis bahia*	28-day, flow through	NOEC	Not determined	KCA 8.2.5.2/01 [redacted] 2016 **To be used as supporting information only**
Toxicity to sediment-dwelling organisms					
Inpyrfluxam S-2399	*Chironomus dilutus*	62-day, static renewal	NOEC	Not determined	KCA 8.2.5.4/01 [redacted] 2015 **Not suitable for use in risk assessment**

Test substance	Test organism	Test system	Endpoint		Reference
Inpyrfluxam S-2399	*Hyalella azteca*	42-day, static spiked sediment (with surface water renewal)	NOEC	Not determined	KCA 8.2.5.4/02 [redacted] 2016 **Not suitable for use in risk assessment**
Inpyrfluxam S-2399	*Leptocheirus plumulosus*	28-day, static renewal	NOEC[a]	10.26 mg a.s./kg sediment (t.w.a)	KCA 8.2.5.4/03 [redacted] 2017
Toxicity to algae					
Inpyrfluxam S-2399	*Pseudokirchneriella subcapitata*	96-hour, static	E_rC_{50} (72 h)	>23 mg a.s./L (m.m)	KCA 8.2.6.1/01 [redacted] 2015a
Inpyrfluxam S-2399	*Navicula pelliculosa*	96-hour, static	E_rC_{50} (72 h)	10.1 mg a.s./L (m.m)	KCA 8.2.6.2/01 [redacted] 2015b
Inpyrfluxam S-2399	*Anabaena flos-aquae*	96-hour, static	E_rC_{50} (72 h)	Not determined	KCA 8.2.6.2/02 [redacted] 2015 **Not suitable for use in risk assessment**
Inpyrfluxam S-2399	*Skeletonema costatum*	96-hour, static	E_rC_{50} (96 h)	1.28 mg a.s./L (m.m)	KCA 8.2.6.2/03 [redacted]

Test substance	Test organism	Test system	Endpoint		Reference
					K.A. 2015d
Toxicity to aquatic macrophytes					
Inpyrfluxam S-2399	*Lemna gibba*	7-day, semi-static	E_rC_{50}	> 24 mg a.s./L (m.m)	KCA 8.2.7/01 [redacted] 2016
Further testing on aquatic organisms					
Inpyrfluxam S-2399	*Crassostrea virginica*	96-hour, -flow through	EC_{50}	>0.99 mg a.s./L (m.m)	KCA 8.2.8/01 [redacted] A. 2016

nom. = nominal; m.m. = arithmetic mean measured; g.m. = geometric mean measured; t.w.a = time-weighted average; i.m = initial measured.

[a] Due to the lack of model fit robust EC_{10} and EC_{20} values could not be generated.

The data available to address the toxicity of the metabolite of Inpyrfluxam are summarised below (Table 9.2.1-2).

Table 9.2.1-2: Summary of toxicity data related to the metabolites of Inpyrfluxam

Test substance	Test organism	Test system	Endpoint (mg met./L)		Reference
Acute toxicity to fish					
3'-OH-S-2840	*Oncorhynchus mykiss*	96-hours, static	LC_{50}	> 6.2 (m.m.)	CA 8.2.1/10 [redacted] 2016a
1'-COOH-S-2840	*Oncorhynchus mykiss*	96-hours, static	LC_{50}	> 50 (m.m.)	CA 8.2.1/11 [redacted] 2016b

m.m. = mean measured

B.9.2.2 Acute toxicity to fish

B.9.2.2.1 Active substance: Inpyrfluxam

Reference:	KCA 8.2.1/01
Report Title:	S-2399 TG - Acute Toxicity Test with Rainbow Trout (*Oncorhynchus mykiss*) Under Static Conditions
Author(s) & year:	[redacted] (2014a)
Document No, Authority registration No:	[redacted] Study No. 13048.6776
Substance used:	S-2399 TG (13CG0617G, 95.0%)
Method of analysis:	LC/MS/MS
Guideline(s):	OECD 203 (1992)
Deviations:	Yes, see HSE comments section.
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

MATERIALS AND METHODS

A. MATERIALS

1. **Test material:** S-2399 TG
   Lot/Batch: 13CG0617G
   Purity: 95.0%
   Reference item: None
   Solvent carrier: Acetone

B. STUDY DESIGN AND METHODS

1. **Test animals:** Rainbow trout (*Oncorhynchus mykiss*)
   Wet weight: Mean: 0.46 g (range: 0.33 – 0.59 g)
   Length: Mean: 40 mm (range: 33 – 44 mm)
   Source: [redacted]
   Acclimation: 14 days
   Diet: Dry commercial fish food, Trout Chow, *ad libitum* daily (not fed during the 48 hours prior to test initiation or during the exposure period)

   Treatment of disease: None
   Test concentrations: Nominal concentrations: 0.023, 0.037, 0.059, 0.094

		and 0.15 mg S-2399 /L. Mean measured concentrations 0.020, 0.036, 0.064, 0.10, 0.16 mg S-2399TG/L
	Statistical Analysis:	Linear regression and Spearman-Kaber statistic test using CETIS™ Version 1.8
2.	**Dilution water:**	Well water
	Hardness:	52 mg $CaCO_3$/L
	Alkalinity:	20 mg $CaCO_3$/L
	pH:	7.3
3.	**Test vessels:**	39 x 20 x 25 cm glass aquaria, each containing 15 L of test solution
	Fish per tank:	10 (20 per concentration, 10 per replicate)
	Mean loading weight:	0.31g biomass/L of test solution
	Exposure regime:	Static
	Chemical analysis:	Samples collected at 0 and 96 hours from each treatment level and control
4.	**Environmental conditions:**	
	Temperature:	12 -14°C
	pH:	6.9 - 7.3
	Dissolved oxygen:	8.1 - 9.9 mg/L 60% of saturation is 6.5 mg/L at 12 °C 60% of saturation is 6.2 mg/L at 14 ºC.
	Photoperiod:	16 hours light: 8 hours darkness (460 to 740 lux)

The test aquaria were impartially placed in a temperature-controlled water bath designed to maintain exposure solution temperatures at 12 ± 2 °C. Each aquarium was constructed of glass and silicone adhesive and measured 39 x 20 x 25 centimetres (L x W x H). The test was illuminated to a light intensity of 43 to 69 footcandles (460 to 740 lux) using fluorescent bulbs. A 16-hour light, 8-hour dark photoperiod was maintained with an automatic timer. A summary of environmental conditions is shown in Table 9.2.2-1.

Table 9.2.2-1: Summary of environmental conditions

Variable	Required OECD 203 (2019)	Obtained
Temperature	10 ° C – 14 ° C	12 ° C – 14 ˚C
pH	6.0 to 8.5	7.3
Dissolved oxygen concentration	≥ 60 % of air saturation value	8.1-9.9 mg / L
Photoperiod	12-16 hours daily	16 hours

Lighting intensity	n/a	460 – 740 lux
Hardness of dilution water	40 – 250 mg $CaCO_3$ / L preferably < 80	54 $CaCO_3$ / L
Alkalinity of dilution water	n/a	20 mg $CaCO_3$ / L
Conductivity of dilution water	≤ 10 µS/cm	390 µS/cm
Total organic Carbon (TOC) mg/L	n/a	0.43mg/L

STUDY DESIGN AND METHODS
Study dates: 27 – 31 January 2014

5. Animal assignment and treatment:
20 fish per test item concentration (10 per replicate), as well as the control and solvent control, were impartially selected and distributed to each aquarium. The mean organism loading rate was 0.31 g of biomass per litre of test solution. The nominal test concentrations were 0.023, 0.037, 0.059, 0.094 and 0.15 mg a.s./L.

6. Dose preparation:
Prior to exposure initiation, a 30 mg a.s./mL primary stock solution was prepared by placing 0.7881 g of S-2399 TG (0.7487 g active substance) in a volumetric flask and bringing it to volume with 25 mL of acetone. The stock solution was clear and colourless with no visible undissolved test substance following multiple inversions. The primary stock solution was used to prepare secondary stock solutions, which were subsequently used to prepare the exposure solutions. Exposure solutions were mixed thoroughly using a glass rod for approximately a minute. All solutions were clear and colourless with no visible undissolved test substance. A solvent control was prepared by bringing 3.2 mL of acetone to a final volume of 32 L with dilution water. A control solution was also established containing only dilution water. Each solution was divided into two replicate aquaria, each containing approximately 15 L of solution.

7. Measurements and observations:
All aquaria were examined after 0, 6, 24, 48, 72 and 96 hours for mortality (dead fish were removed), biological observations, including sublethal effects (e.g., lethargy, loss of equilibrium) and observations of the physical characteristics of the test solutions (e.g. presence of precipitate, film on the solution's surface) were made and recorded. Effects were based on death, defined as the lack of movement by the exposed organisms (i.e., absence of gill movement and reaction to gentle prodding).

The pH, dissolved oxygen concentration and temperature were measured at 0, 24, 48, 72 and 96 hours in each replicate of each treatment and control. Continuous temperature monitoring was performed in one replicate vessel.

At exposure initiation (0 hour) and exposure termination (96 hour), one sample was

collected from each treatment level and the controls for analysis of S-2399 TG concentration by LC/MS/MS. At exposure initiation, samples were removed from the intermediate mixing vessel, prior to division into replicate vessels. At exposure termination, samples were removed from a composite of each replicate of each concentration and controls. The mean measured concentrations (reported as "time-weighted average concentrations") of S-2399 TG were calculated for each treatment level. The LOQ was set at 0.600 µg/L and MDL was 0.200 µg/L.

8. Statistics:

The 24-, 48-, 72- and 96-hour LC_{50} values and 95% confidence intervals were estimated using the mean measured concentrations and the corresponding mortality data. If at least one concentration resulted in ≥ 50% mortality, then the computer program CETIS™v.1.8 was used to calculate the LC_{50} values. Based on the data, LC_{50} values could be determined by either linear regression or Spearman-Kärber estimates. The No-Observed-Effect Concentration (NOEC) was defined as the highest mean measured concentration tested at and below which there were no toxicant-related mortality or physical and behavioural abnormalities (e.g., lethargy), with respect to the control organisms.

II. RESULTS AND DISCUSSION

At test termination (96 hours), 5 % and 70 % mortality was observed among fish exposed to the 0.020 and 0.036 mg a.s./L treatment levels, respectively (mean measured concentrations). Three surviving fish exposed to the 0.020 mg a.s./L treatment level exhibited a partial loss of equilibrium and darkened pigmentation. Two surviving fish exposed to the 0.036 mg a.s./L treatment group exhibited a darkened pigmentation and were observed at the bottom of the test vessel, two surviving fish were observed on the bottom of the test vessel, one surviving fish exhibited a darkened pigmentation and a complete loss of equilibrium and one surviving fish exhibited a complete loss of equilibrium. No mortality or adverse effects were observed among fish in the control or solvent control. A summary of the cumulative percent mortalities and observations is presented in Table 9.2.2-2 below.

Table 9.2.2-2: Summary of mortalities and observations following exposure to S-2399 TG

Mean measured concentration (mg a.s./L)	Mean cumulative mortality (%)				
	6 hour	24 hour	48 hour	72 hour	96 hour
Control	0	0	0	0	0
Solvent control	0	0	0	0	0
0.020	0^{a}	0^{ef}	0^{fj}	0^{jm}	5^{nop}
0.036	10^{bc}	60^{ghi}	70^{kl}	70^{d}	70^{mqrs}
0.064	85^{d}	100	100	100	100

0.10	100	100	100	100	100
0.16	100	100	100	100	100

[a] Three fish exhibited a complete loss of equilibrium.
[b] Four surviving fish exhibited a complete loss of equilibrium.
[c] Fourteen surviving fish were observed on the bottom of the test vessel.
[d]All surviving fish were observed on the bottom of the test vessel.
[e.]Five fish exhibited a partial loss of equilibrium.
[f] Five fish exhibited a complete loss of equilibrium.
[g] Five surviving fish exhibited a complete loss of equilibrium and a darkened pigmentation.
[h] One surviving fish exhibited a partial loss of equilibrium and a darkened pigmentation.
[i] Two surviving fish exhibited a darkened pigmentation and were observed on the bottom of the test vessel.
[j] Six fish exhibited a partial loss of equilibrium.
[k] Four surviving fish exhibited a darkened pigmentation and were observed on the bottom of the test vessel.
[l] Two surviving fish exhibited a complete loss of equilibrium and a darkened pigmentation.
[m] One fish exhibited a complete loss of equilibrium.
[n] Three surviving fish exhibited a partial loss of equilibrium and a darkened pigmentation.
[o] Four surviving fish exhibited a partial loss of equilibrium.
[p] One surviving fish exhibited a darkened pigmentation.
[q] Two surviving fish were observed on the bottom of the test vessel.
[r] Two surviving fish exhibited a darkened pigmentation and were observed on the bottom of the test vessel.
[s] One surviving fish exhibited a complete loss of equilibrium and a darkened pigmentation

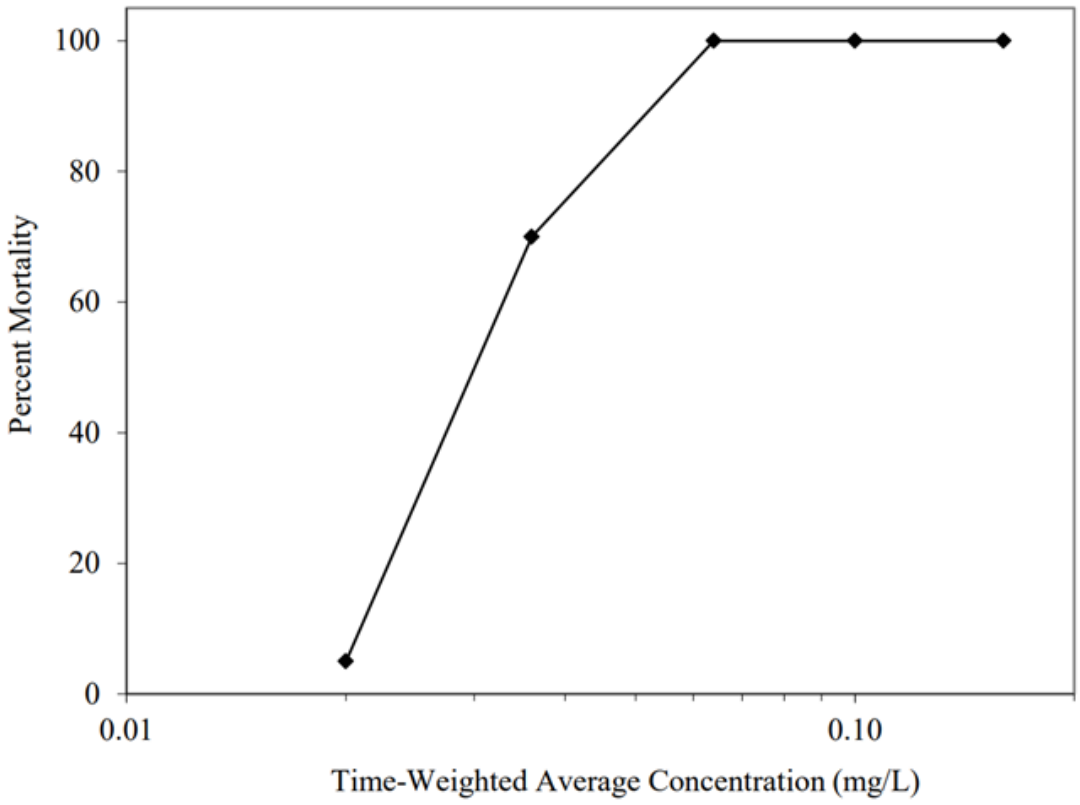


Figure 9.2-1: Concentration-response (mortality) curve for rainbow trout (*Oncorhynchus mykiss*) exposed to S-2399TG under static conditions

A summary of the toxicity endpoints determined from the study is presented in Table 9.2.2-3.

Table 9.2.2-3: Summary of endpoints

Time	LC_{50} (mg a.s./L)	95% confidence interval	
		Lower (mg a.s./L)	Upper (mg a.s./L)
24-hour [a]	0.033	0.033	0.033
48-hour [a]	0.031	0.026	0.039
72-hour [a]	0.031	0.026	0.039
96-hour [a]	0.031	0.023	0.040
96 hour NOEC = < 0.020 mg a.s./L			
Highest concentration producing 0% mortality = < 0.020 mg a.s./L			
Lowest concentration producing 100% mortality = 0.064 mg a.s./L			

[a] LC_{50} values and 95% confidence intervals were estimated by linear interpolation

B. ANALYSIS

Measured concentrations decreased between intervals, but the expected concentration gradient was maintained. Mean measured concentrations ranged from 88 to 110% of nominal concentrations and defined the treatment levels tested as 0.020, 0.036, 0.064, 0.10 and 0.16 mg a.s./L.

Table 9.2.2-4: Measured concentrations of S-2399 TG in the exposure solutions

Nominal concentration (mg a.s./L)	Measured concentration (mg a.s./L)		Mean measured concentration (mg a.s./L) [a]	Percent of nominal (%) [a]
	0 h	96 h		
Control	<0.0020 [b]	<0.0020 [b]	n.a.	n.a.
Solvent control	<0.0020 [b]	<0.0020 [b]	n.a.	n.a.
0.023	0.026	0.015	0.020	88
0.037	0.042	0.031	0.036	98
0.059	0.070	0.057	0.064	110
0.094	0.11	0.096	0.10	110
0.15	0.17	0.15	0.16	110

[a] Mean measured concentrations (referred to in the Study Report as “Time-weighted average concentrations”) and percent of nominal were calculated using the actual analytical (unrounded) results and not the rounded (two significant figures) values presented in this table.

[b] Concentrations expressed as less than values were below the minimum detectable

limit (MDL).
n.a. = not applicable

C.VALIDITY CRITERIA
As there were no mortalities in the controls and dissolved oxygen remained above 60%, the study was considered valid.

Table 9.2.2-5: Validity criteria as per OECD 203 (2019) guidance

Validity criterion	Required	Obtained
Mortality in the control(s)	≤ 10 %	No mortality
Dissolved oxygen concentration	≥ 60 % of air saturation value	Maintained ≥ 60 % saturation for temperature range.
Analytical measurement of test concentrations	Analysis of the highest and lowest test concentration and a concentration around the expected LC_{50} is considered the minimum requirement	At exposure initiation and exposure termination. One sample was collected from each treatment level and the controls for analysis for static study. Mortality data for each concentration considered. Recovery range of nominal concentrations were 88% - 110%.

III. CONCLUSION
Based on the results of this laboratory study, the 96hour LC_{50} to rainbow trout was estimated to be 0.031 mg a.s./L and the NOEC was determined to be < 0.020 mg a.s./L. The results are based on mean measured concentrations.

HSE COMMENTS
The study was carried out in accordance with GLP and the OECD 203 (1992) guideline available at the time and has been checked against current OECD 203 guideline (2019).
The concentrations of the test item were maintained between 80-120% of the nominal value throughout the test. Results should be based on nominal concentrations but have been expressed as mean measured concentrations. The method of analysis used in the study was evaluated by HSE Chemistry. The conclusion of their evaluation is reproduced below. Please see Volume 3 CA, section B5 for more details.

"*The analytical method is not fully validated according to SANTE/2020/12830 rev. 1 for the determination of the active substance inpyrfluxam in freshwater as the matrix effects have not been determined and the stability of standards and extracts has not been addressed. However, the studies were generated prior to the implementation of*

SANTE 2020/12830 rev.1 and SANCO 3029 rev.4 did not require matrix effects and the stabilities of standards and extracts to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose."

The applicant states there was slight deviation in the length of fish used in the study; however, the mean length and minimum and maximum lengths were in line with guidance for both OECD 203 (1992) and still meets updated OECD (2019) protocol. As fish used in this study were selected at random, this is likely to explain the variation in length. HSE do not think that this had any impact on exposure. All validity criteria required as per OECD 203 (1992) and OECD 203 (2019) were met for this study.

It is also worth noting that, whilst not a requirement of OECD 203 (1992) or OECD (2019), no reference item was used in this study, so no comparison can be made against an existing approved active substance.

The test design used silicone adhesive as part of the test structure conditions. OECD 203 (2019) guidance states that silicone should not be used in reference to flow-through studies. It should be considered whether it is possible this had any impact on concentrations given its strong capacity to absorb lipophilic chemicals. As the mean concentrations remained to be a > 80 % of the nominal concentration, it is unlikely to have affected the study.

All aquaria were examined after 0, 6, 24, 48, 72 and 96 hours for mortality, but latest guidance recommends a minimum of 2 observations should be conducted within the first 24 hours of the study with preferably at least 3 hours between observations. However, this wasn't part of the guidance available at the time in OECD 203 (1992). There is discrepancy between measurements and observations, with the pH, dissolved oxygen concentration and temperature were measured at 0, 24, 48, 72 and 96 hours, but examined after 0, 6, 24, 48, 72 and 96 hours. This implies no pH or dissolved oxygen concentration data was available for mortality examinations at 6 hours and is not included in the variable data for the test. This should be noted given the high percentage mortalities occurring after 6 hours at higher concentrations but is unlikely to have affected the validity of the data given the consistency of the variables shown between 24-96 hours.

High percentages of nominal concentrations were observed for each concentration after study termination (96 hours) and therefore should be noted that S-3299TG/L has the potential to exist in freshwater for long periods of time.

Spearman-Kärber statistical analysis was conducted on these data and is a suitable test when looking at mean data points and is line with OECD 203 (2019 guidance). However, guidance states this should mainly be used in situations where only one or no concentrations result in partial mortality, which does not apply to this study. As this guidance was not available at the time in OECD 203 (1992) and the statistical results correspond with the data presented, it is still valid for this study. A more suitable form of statistical analysis would have been the classical maximum likelihood methods for

fitting probit or logit models. Further studies to determine an exact NOEC value would be beneficial for future use given the very low concentration value presented.

The agreed endpoints for use in risk assessment are:

- **96-hour LC_{50} = 0.031 mg S-2399TG/L (based on mean measured concentrations).**

Reference:	KCA 8.2.1/02
Report Title:	S-2399 TG - Acute Toxicity Test with Bluegill Sunfish (*Lepomis macrochirus*) Under Static Conditions
Author(s) & year:	[redacted] (2014b)
Document No, Authority registration No:	[redacted] Study No. 13048.6806
Substance used:	S-2399 TG (13CG0617G, 95.0%)
Method of analysis:	LC/MS/MS
Guideline(s):	OECD 203 (1992)
Deviations:	Yes, see HSE comments section.
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

MATERIALS AND METHODS

MATERIALS	
Test material	S-2399 TG
Lot/Batch #:	13CG0617G
Purity:	95.0% (verified according to the certificate of analysis)
Description:	Not stated
Stability of test compound:	Not stated
Reanalysis/expiry date:	23 July 2016
Density:	Not applicable
TREATMENTS	
Test concentrations:	Dilution water control, solvent control (0.10 mL acetone/L), nominal concentrations of 0.023, 0.037, 0.059, 0.094 and 0.15 mg S-2399/L, time-weighted average measured concentrations of 0.022, 0.035, 0.058, 0.10 and 0.17 mg S-2399/L

Solvent:	Acetone (CAS No. 67-64-1)
Toxic reference	None
Analysis of test concentrations:	Yes, at 0 and 96 hours (all treatment levels and the dilution water and solvent controls) based on analysis of S-2399 using LC-MS/MS. The limit of quantification (LOQ) was set at 0.600 µg/L, the lowest validated concentration and the minimum detectable limit (MDL) was 0.200 µg/L for S-2399
TEST ORGANISMS	
Species:	Bluegill Sunfish (Lepomis macrochirus)
Source:	[redacted]
Acclimatisation period:	14 days
Treatment for disease:	None reported
Weight and length of a representative sample of fish (n = 30):	Mean wet weight: 0.37 g (range: 0.14 to 0.66 g) Mean length: 26 mm (range 20 to 35 mm)
Feeding:	Commercial fish food, [redacted] Mixed Prime Flakes (not fed during the 48 hours prior to test initiation or during the exposure period)
TEST DESIGN	
Test vessels:	39 x 20 x 25 cm (L x W x H) glass aquaria, each containing 15 L of test solution
Test medium:	Well water
Replication:	Two replicate aquaria were established for each treatment level, control and solvent control
No of fish per tank:	10
Exposure regime:	Static
Duration:	96 hours
TEST CONDITIONS	
Test temperature:	21 – 23 °C
pH:	6.9 – 7.6
Dissolved oxygen:	5.6 – 9.4 mg/L (60 % of saturation is 5.3 mg/L at 21 °C and 60 % of saturation is 5.1 mg/L at 23 ºC)
Hardness of dilution water:	48 – 52 mg/L as $CaCO_3$
Lighting:	820-950 lux photoperiod of 16 hours light and 8 hours darkness

STUDY DESIGN AND METHODS

Experimental dates: the 96-hour definitive exposure was conducted from 23 to 27 January 2014.

Test organism and acclimatisation

The Bluegill Sunfish (*Lepomis macrochirus*, juvenile), commonly used in freshwater

acute toxicity tests, was selected as the test species. Prior to testing, fish were acclimatised for 14 days under species appropriate conditions similar to those used for the definitive test. During acclimatisation, the fish were fed commercially prepared fish food, at least once daily. No mortality was observed among the test fish population during the 48-hour period prior to testing.

Test water
The dilution water (well water) used during this study was from the same source as the water used during acclimatisation. The dilution water conformed to the chemical characteristics defined as acceptable in OECD 203 (2019) for this species. Representative samples of the dilution water source were analysed periodically for the presence of pesticides, PCBs and toxic metals. In addition, samples were analysed monthly for Total Organic Carbon (TOC) concentration (0.43 mg/L for January 2014).

Definitive test and dose preparation
A static test system was employed. A 30 mg a.s./mL primary stock solution was prepared by placing 0.7881 g of S-2399 TG (0.7487 g active substance) in a volumetric flask and bringing it to volume with 25 mL of acetone. The mass of test substance was adjusted to account for its 95% purity. The stock solution was clear and colourless with no visible undissolved test substance following multiple inversions. The primary stock solution was used to prepare secondary stock solutions, which were subsequently used to prepare the exposure solutions. Exposure solutions were mixed thoroughly using a glass rod for approximately a minute. All solutions were clear and colourless with no visible undissolved test substance. A 0.10 mL/L solvent control was prepared by bringing 3.2 mL of acetone to a final volume of 32 L with dilution water. A control solution was also established containing only dilution water. Each solution was divided into two replicate aquaria, containing approximately 15 L of solution.

At the start of the test 20 fish, ten per replicate aquarium, were randomly allocated to each test concentration and the controls. The resulting test organism loading concentration was 0.25 g of biomass per litre of solution per aquarium per day. The test aquaria were impartially placed in a temperature-controlled water bath designed to maintain exposure solution temperatures at 22 ± 1 °C.

Measurements and observations
Observations for mortalities and symptoms of toxicity were made at 0, 6, 24, 48, 72 and 96 hours. Mortality (dead fish were removed), biological observations, including sublethal effects (e.g., lethargy, loss of equilibrium) and observations of the physical characteristics of the test solutions (e.g., presence of precipitate, film on the solution's surface) were made and recorded. Effects for this study were based on mortality, defined as the lack of movement by the exposed organisms (i.e., absence of gill movement and reaction to gentle prodding).

The pH, dissolved oxygen concentration and temperature were measured at 0, 24, 48, 72 and 96 hours in each replicate of each treatment and control. Continuous temperature monitoring was performed in one replicate vessel.

The pH was measured with a Yellow Springs Instrument (YSI) pH100 pH meter and

dissolved oxygen concentration and daily temperature were measured with a YSI 550A or Pro20 dissolved oxygen meter/temperature probe. Temperature was continuously monitored throughout this study in one replicate of the 0.059 mg /L nominal treatment level using a VWR minimum/maximum thermometer.

At exposure initiation (0 hours) and exposure termination (96 hours), one sample was collected from each treatment level and the controls for analysis of S-2399 TG concentration by liquid chromatography/mass spectrometry (LC/MS/MS). At exposure initiation, samples were removed from the intermediate mixing vessel, prior to division into replicate vessels. At exposure termination, samples were removed from a composite of each replicate of each concentration and controls. The time-weighted average concentrations of S-2399 TG were calculated for each treatment level using the formula specified in the JMAFF Guideline (13 SeiSan, Notification 3986, including the Notification No. 18-Shouan-14852):

$$C_{twa} = \frac{\left[\frac{C_{D0} - C_{D4}}{\ln(C_{D0}) - \ln(C_{D4})} \times 4\ Days\right]}{Total\ Days}$$

where:

C_{twa} = time-weighted average concentration
C_{D0} = measured concentration at day 0
C_{D4} = measured concentration at day 4
$\ln$ = natural log
$Total\ Days$ = total number of days, 4

Statistical analysis

The median lethal concentration (LC_{50}) was defined as the concentration resulting in 50 % mortality of the fish in the time period specified. The 24-, 48-, 72- and 96-hour LC_{50} values and 95% confidence intervals were estimated using the time-weighted average concentrations and the corresponding mortality data. If at least one concentration resulted in ≥ 50 % mortality, then the computer program CETIS™v.1.8 was used to calculate the LC_{50} values. If no concentration resulted in ≥ 50% mortality, the LC_{50} was empirically estimated to be greater than the highest time-weighted average concentration. The No-Observed-Effect Concentration (NOEC) was defined as the highest time-weighted average concentration tested at and below which there were no toxicant-related mortality or physical and behavioural abnormalities (e.g., lethargy), with respect to the control organisms.

RESULTS AND DISCUSSION

Mortality and sub-lethal effects

At test termination (96 hours), 60 %, 100 % and 100 % mortality was observed among fish exposed to the 0.058, 0.10 and 0.17 mg a.s./L treatment levels, respectively. One fish exposed to the 0.058 mg a.s./L treatment level exhibited a complete loss of equilibrium. No mortality or adverse effects were observed among fish in the 0.022, 0.035 mg a.s./L treatment levels, the control or solvent control. The cumulative percent mortalities and observations of each replicate and their mean is presented in Table 9.2.2-6.

Table 9.2.2-6: Summary of mortalities and observations following exposure to S-2399 TG. 0.058 mg a.s./L replicates are highlighted to draw attention to the large discrepancy in mortality between them

Time-weighted average concentration (mg a.s./L)	Replicate	Cumulative mortality (%)				
		6 hour	24 hour	48 hour	72 hour	96 hour
Control	A	0	0	0	0	0
	B	0	0	0	0	0
	Mean	0	0	0	0	0
Solvent control	A	0	0	0	0	0
	B	0	0	0	0	0
	Mean	0	0	0	0	0
0.022	A	0	0	0	0	0
	B	0	0	0	0	0
	Mean	0	0	0	0	0
0.035	A	0	0	0	0	0
	B	0	0	0	0	0
	Mean	0	0	0	0	0
0.058	A	10	100	100	100	100
	B	0	0	0	20	20
	Mean	5 [abc]	50 [bfg]	50 [hi]	60 [jk]	60 [l]
0.10	A	10	100	100	100	100
	B	10	100	100	100	100
	Mean	10 [ad]	100	100	100	100
0.17	A	100	100	100	100	100
	B	90	100	100	100	100
	Mean	95 [e]	100	100	100	100

[a] Eleven fish exhibited a complete loss of equilibrium.
[b] Two fish exhibited a partial loss of equilibrium.
[c] Five fish were observed to be lethargic.
[d] Seven fish were observed on the bottom of the test vessel.
[e] All surviving fish exhibited a complete loss of equilibrium.
[f] Five fish exhibited a complete loss of equilibrium.
[g] Three fish were observed on the bottom of the test vessel.
[h] Six fish were observed on the bottom of the test vessel.
[i] Four fish exhibited a partial loss of equilibrium.
[j] Five fish were observed on the bottom of the test vessel.

[k]Three fish exhibited a partial loss of equilibrium.
[l] One fish exhibited a complete loss of equilibrium.

A summary of the toxicity endpoints determined from the study is presented in Table 9.2.2-7 and the 96-hour concentration response visualised below.

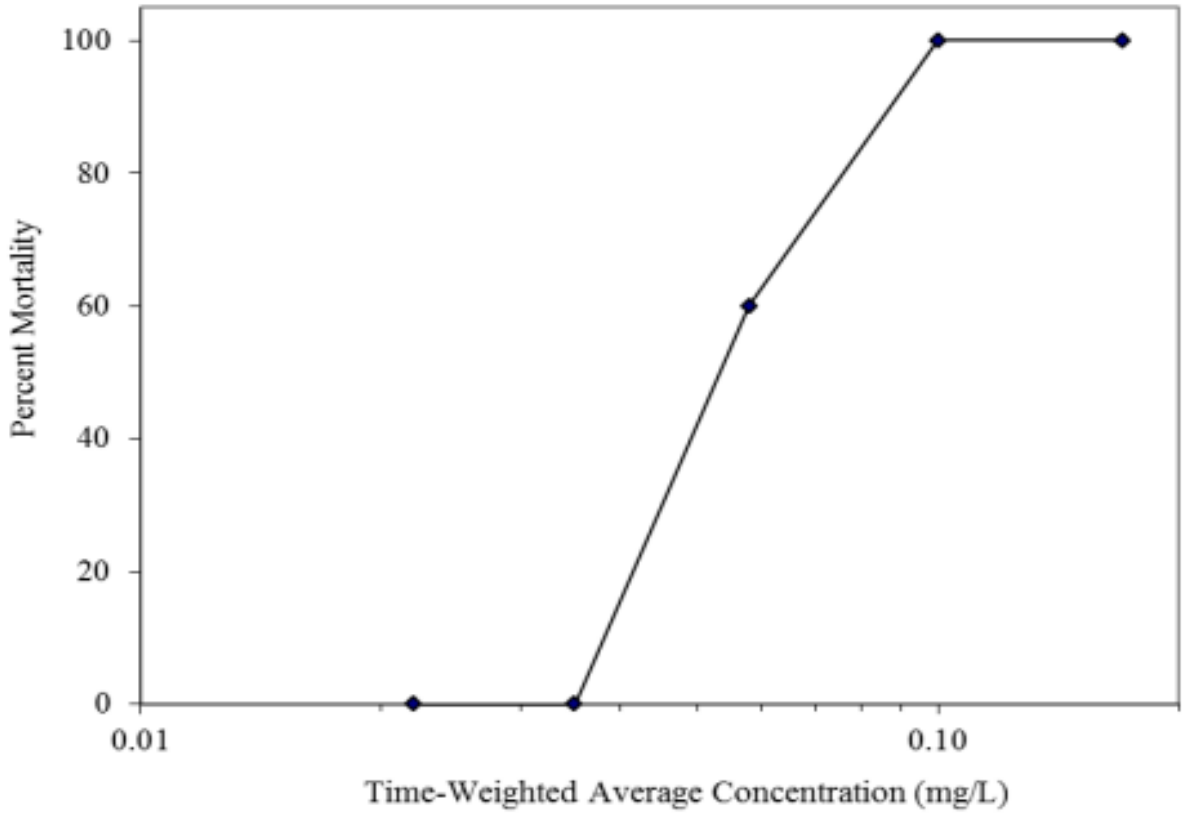


Figure 9.2-2: The 96-hour concentration-response (mortality) curve for bluegill sunfish (Lepomis macrochirus) exposed to S-2399 under static conditions.

Table 9.2.2-7: Summary of endpoints

Time	LC_{50} (mg a.s./L) [a]	95 % confidence interval	
		Lower (mg a.s./L)	Upper (mg a.s./L)
24-hour [a]	0.058	0.011	0.14
48-hour [a]	0.058	0.011	0.14
72-hour [a]	0.054	0.023	0.13
96-hour [a]	0.054	0.023	0.13
96-hour NOEC = 0.035 mg a.s./L			
Highest concentration producing 0 % mortality = 0.035 mg a.s./L			
Lowest concentration producing 100 % mortality = 0.10 mg a.s./L			

[a] LC_{50} values and corresponding 95 % confidence intervals were estimated by linear interpolation.

Analysis
Measured concentrations of S-2399 TG (
Table 9.2.2-8) decreased between intervals. Time-weighted average concentrations ranged from 93 to 110 % of nominal concentrations. Individual measured concentrations, however, ranged from 70 % (96-hour 0.037 mg a.s./L) to 122 % (0-hour 0.037 mg a.s./L) of the nominal concentrations.

Table 9.2.2-8: Measured concentrations of S-2399 TG in the exposure solutions

Nominal concentration (mg a.s./L)	Measured concentration (mg a.s./L)[a]			Percent of nominal (%)		
	0 Hour	96 Hour	Time-weighted average	0 Hour[c]	96 Hour[c]	Time-weighted average[a]
Control	<0.0020 [b]	<0.0020 [b]	n.a.	n.a.	n.a.	n.a.
Solvent control	<0.0020 [b]	<0.0020 [b]	n.a.	n.a.	n.a.	n.a.
0.023	0.027	0.018	0.022	117	78	96
0.037	0.045	0.026	0.035	122	70	93
0.059	0.069	0.048	0.058	117	81	98
0.094	0.11	0.092	0.10	117	98	110
0.15	0.18	0.15	0.17	120	100	110

[a] Time-weighted average concentrations and mean percent of nominal were calculated using the actual analytical (unrounded) results and not the rounded (two significant figures) values presented in this table.
[b] Concentrations expressed as less than values were below the minimum detectable limit (MDL).
[c] Calculated by HSE based on rounded values provided.

n.a = not applicable.

Validity criteria
The validity criteria for the study were met according to OECD 203 (1992) and OECD 203 (2019).

Table 9.2.2-9: Compliance with OECD 203 validity criteria

Validity criterion	Required	Obtained
Mortality in the control(s)	≤ 10 %	0 %

Validity criterion	Required	Obtained
Test conditions	Constant conditions	A static conditions design was selected. Constant conditions were maintained.
Dissolved oxygen concentration	At least 60 % of the air saturation value throughout the test	Dissolved oxygen concentration > 60 % of the air saturation throughout the test.
Concentration of substance	Analytical measurement of test concentrations is compulsory. At least 80 % of the nominal concentration throughout the test. If the deviation from the nominal concentration is greater than 20 % results should be based on the measured concentration.	Measured concentrations in individual replicates ranged from 70 - 122 % of nominal. Results based on time-weighted averages, which closely tracked nominal concentrations.

CONCLUSIONS
The results of the laboratory study demonstrate the 96-hour LC_{50} of S-2399 TG to bluegill sunfish to be 0.054 mg a.s./L (95 % confidence intervals of 0.023 to 0.13 mg a.s./L) and the NOEC to be 0.035 mg a.s./L, based on time-weighted average concentrations.

HSE COMMENTS
The study was carried out according to GLP and follows OECD 203 (1992), the guideline available at the time of experimentation. The study was evaluated against the most recent OECD 203 guideline (2019). All validity criteria outlined in OECD 203 (2019) were satisfactorily met for the duration of the study. There were no significant deviations to the guideline.

The following deviations were noted:
OECD 203 (2019) paragraph (§) 9 states that the study report method include a description of the apparatus used to carry out the test and appropriate documentation to validate equipment functionality. The equipment used to measure illuminance, water hardness and TOC was not reported. Appropriate documentation to validate equipment functionality was also omitted. This requirement was not present in OECD 203 (1992) and its omission is not expected to affect the study outcome considering the up-to-date GLP certification of the study laboratory. HSE considers this a minor deviation.

OECD 203 (2019) § 10 states, "*the use of silicone tubing in flow-through studies and use of silicone seals in contact with water should be minimised*", due to the strong binding capacity of silicone for lipophilic chemicals. Study aquaria were constructed of glass and silicone adhesive. In addition, when test vessels are placed in the test area

they should be shielded from excessive noise, vibration and light. The lack of excessive noise, vibration and light was not documented. The reporting omissions are acceptable due to these requirements not being outlined in OECD 203 (1992). The use of silicone sealant is also not covered in OECD 203 (1992). Its use was unlikely to have impacted the study outcome due to the close alignment of the nominal and time-weighted test concentrations. HSE consider this a minor deviation.

OECD 203 (2019) § 12 describes the age and size of fish to be used. For juvenile *L. macrochirus*, length should range between 1.0-3.0 cm. In the study, fish length ranged from 2.0-3.5 cm. This protocol deviation was highlighted in test report results section. Although the length of some fish exceeded the recommended range of 1–3 cm, the overall variation of 1.5 cm is less than the 2 cm recommended, suggesting that age matching was appropriate. All test organisms were from the same life stage, control mortality was < 10 % and the acceptable total length variation indicates appropriate age matching. Therefore, HSE consider this a minor deviation which is not expected to impact the study outcome.

OECD 203 (2019) § 14 outlines the criteria for test fish population mortality and health during the acclimatisation period. For the test fish batch to be accepted, less than 5 % mortality should be observed in seven days. Or, if mortality is between 5-10 %, acclimatisation is continued for seven additional days; and less than 5% mortality must be observed during the second seven-day period. This is also a requirement of OECD 203 (1992). The study did not provide this level of detail, only reporting no mortality in the test fish population 48 hours prior to testing. Furthermore, no information was provided detailing signs of disease, stress, malformations or treatments against disease or parasites within 14 days prior to testing. Throughout the exposure period there was no mortality or sub-lethal effects recorded in the test dilution water controls indicating the health of the test fish population was acceptable. HSE consider this a minor deviation which is not expected to impact the study outcome.

OECD 203 (2019) § 16 details required chemical testing of dilution water. Within this paragraph it states, "*analyses of nitrate and chlorine should be performed on each batch of dilution water to demonstrate that the limits specified in Annex 3 are not exceeded*". This was not reported in the study. This was not a requirement in OECD 203 (1992) and the lack of control mortality suggests its omission is not expected to impact the study outcome. HSE considers this a minor deviation.

OECD 203 (2019) § 17 outlines test solution preparation requirements. It states, *"the use of solvents should be avoided and only used as a last resort in order to produce a suitably concentrated stock solution".* The study conductor dissolved the test substance in acetone when preparing the primary stock solution. The solubility of S2399 in water was not provided, which precludes determining whether solvent use necessary. Within OECD 203 (1992) the requirement to minimise solvent use was not present. The lack of mortality in the solvent control indicates the use of acetone is not expected to impact the study outcome. HSE considers this a minor deviation.

OECD 203 (2019) § 20 details sample size and replication requirements. It states, "*a minimum of 7 fish must be used at each test concentration and in the control(s)… no test tank replication is required*". The study used 20 fish for each concentration and control, comprising of ten fish in two replicates. In line with ethical considerations

regarding the reduction of fish used in ecotoxicological testing, this degree of replication was unnecessary. Moreover, due to the mortality disparity between the two replicates for 0.058 mg/L, the single concentration that resulted in partial mortality after 96 hours, the larger than recommended sample size did not result in less uncertainty about the LC_{50}. Furthermore, the use of two replicates was not required under the guidance of OECD 203 (1992) either. Although this deviation did not impact study outcome, HSE notes the unnecessarily large sample size conflicts with the aim to reduce the number of animals used in vertebrate testing.

OECD 203 (2019) § 21 concerns integrating existing sources of information into test concentration selection if such information is available. The study provided no evidence of this approach being attempted. This was not a requirement under OECD 203 (1992) and this deviation is considered minor with respects to study outcome.

OECD 203 (2019) § 23 profiles control requirements. It states, "*the dilution water control can be omitted, and the test conducted and evaluated with a solvent control only, provided it is appropriate when considering the needs for these data and the requirements of the relevant regulatory authorities*". It could be argued that the water dilution control was useful for demonstrating that test conditions did not induce stress in the test subjects, especially when considering the incomplete reporting of mortality in the test fish population during the acclimatisation stage. However, the lack of effect of 0.1mL/L acetone for fish is well described. It is the opinion of HSE that the need to minimise vertebrate testing outweighs the additional information the dilution water control provided. Therefore, the dilution water control was not required. This was, however, not covered under OECD 203 (1992), which stipulates the requirement of a solvent control if a solvent is used. Therefore, this deviation is acceptable.

OECD 203 (2019) § 24 outlines the requirements regarding the analytical determination and measurement of the test substance. It states, "*there must be evidence that the concentration of the chemical being tested has been satisfactorily maintained, and preferably it should be at least 80 % of the nominal concentration throughout the test*". For both the initial and final measured concentrations, deviations of more than 20 % were present. However, for all concentrations the initial measured concentration was higher than the nominal concentration and the final measured concentration lower or equivalent. This resulted in the time-weighted averages closely aligning with the nominal concentrations. The time-weighted average concentrations were used to calculate the LC_{50}.

OECD 203 (2019) § 26, covering the frequency of biological observation and recording, states, "t*o the extent feasibly possible, a minimum of 2 observations should be conducted within the first 24 hours of the study"*. The study only performed one observation within the first 24 hours (6-hour). Also, "*on days 2-4 of the test, all vessels with living fish should be inspected twice per day*". The study only performed one observation period a day for days 2-4. Under the enacted observation programme, however, some transient sub-lethal effects may have been omitted. Again, these requirements were not present in OECD 203 (1992) making these deviations acceptable.

OECD 203 (2019) § 28, concerning the fate of test fish, was not addressed in the study report. Furthermore, § 29 requires fish to be measured within one week of test

commencement. When fish were measured, in relation to the start of the test, was not detailed. These requirements were not present within OECD 203 (1992) and their omission is deemed acceptable.

OECD 203 (2019) § 32 sets out the appropriate statistical methods to be followed when estimating LC_{50}s. The study performed linear interpolation to estimate LC_{50}s, a method not detailed within § 32. Consequently, the slope of curve was not estimated. OECD 203 (1992) did not require slope estimation and there was a lack of clarity on what the appropriate statistical methods were. Therefore, the use of linear interpolation is acceptable.

OECD 203 (2019) § 33 outlines the requirements for the test report, including the reporting of test substance physico-chemical properties. The study report did not provide the required physico-chemical properties or a structural formula. A purity of 95 % for the test substance was given. However, the chemical identity of the 5 % impurities was not provided. Analytical measurement at exposure initiation and completion confirmed the presence of the test substance in the test solution indicating the omission of the physico-chemical properties did not impact test integrity. Furthermore, nominal and time-weighted average concentrations were in close agreement. HSE considers this deviation acceptable.

OECD 203 (2019) § 33 outlines the requirements for the test report, including the reporting of results. The study report did not discuss the large mortality variation between the two 0.058 mg a.s./L replicates, the only concentration that elicited partial mortality, after 96 hours. The relevant rows, highlighted in Table 9.2.2-6, show that mortality was 100 % in one replicate and 20 % in the other. Even after 24 hours, this pattern was evident. The large degree of variation between replicates for the sole concentration resulting in partial mortality led to greater uncertainty around the LC_{50} estimate.

The method of analysis used in the study was evaluated by HSE Chemistry. The conclusion of their evaluation is reproduced below. Please see Volume 3 CA, section B5 for more details.

"*The analytical method is not fully validated according to SANTE/2020/12830 rev. 1 for the determination of the active substance inpyrfluxam in freshwater as the matrix effects have not been determined and the stability of standards and extracts has not been addressed. However, the studies were generated prior to the implementation of SANTE 2020/12830 rev.1 and SANCO 3029 rev.4 did not require matrix effects and the stabilities of standards and extracts to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose.*"

The above study was conducted to GLP and considered valid.

The agreed endpoint suitable for use in the risk assessment is:
96-hour LC_{50} = 0.054 mg / S-2399/L*.

*noting the uncertainty due to the large variation between the two 0.058 mg a.s./L replicates.

Reference:	KCA 8.2.1/03
Report Title:	S-2399 - Acute Toxicity Test with Fathead Minnow (*Pimephales promelas*) Under Static Conditions Following OECD Guideline #203, OPPTS Draft Guideline 850.1075, JMAFF 12 NohSan, No. 8147 Fish, Acute Toxicity Test (2-7-1-1) and The Official Journal of the European Communities, L383A, Method C.1, Acute Toxicity for Fish
Author(s) & year:	[redacted] (2014c)
Document No, Authority registration No:	[redacted] Study No. 13048.6777
Substance used:	S-2399 TG (13CG0617G, 95.0%)
Method of analysis:	LC/MS/MS
Guideline(s):	OECD 203 (1992)
Deviations:	Yes, see HSE comments section.
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test material:** S-2399 TG
   Description: Not described
   Lot/Batch: 13CG0617G
   Purity: 95.0 %

2. **Solvent control:** Acetone (25 mL)

3. **Reference item:** Not tested

B. STUDY DESIGN AND METHODS

1. **Test animals:** *Pimephales promelas*
   Phenotype: Not stated
   Size at start of test: Range 20 – 35 mm

	Diet:	Dry commercial flake fish food, [redacted] Mixed Prime Flakes, daily during culture
	Source:	Cultures maintained at [redacted]
	Acclimation:	Two weeks. Vessels: 500 L fiberglass tanks. Photoperiod: 16 hours light and 8 hours dark; Medium: well water; Water total hardness: 48 to 52 mg/L ($CaCO_3$); Conductivity: 340 to 390 µS/cm; Dissolved oxygen saturation: 100 – 110 % saturation; Temperature: 23 °C; pH: 7.4 – 7.7
	Loading:	0.29 g of biomass per litre of test solution
2.	**Dilution water:**	Well water
	Hardness:	52 mg/L (as $CaCO_3$)
	Alkalinity:	20 mg/L
	pH:	7.3
	Conductivity:	390 µS/cm
	Total organic content:	0.43 mg/L for January 2014
3.	**Test vessels:**	15 L glass aquaria with silicone adhesive 39 x 20 x 25 cm
4.	**Environmental conditions:**	
	Temperature:	20 °C – 24 °C
	pH:	7.0 – 7.3
	Photoperiod:	16 hours light and 8 hours darkness
	Light intensity:	610 – 1000 lux using fluorescent bulbs
	Aeration:	Gentle oil-free aeration was initiated at the 48-hour interval to raise and maintain dissolved oxygen levels at or above 60 % of saturation.
	Oxygen Saturation:	5.7 – 9.6 mg/L (60 % of saturation is 5.1 mg/L at 23 °C)

5. Animal assignment and treatment:

A range-finding test was conducted under static conditions at nominal concentrations of 0.0032, 0.010, 0.032, 0.10 and 0.32 mg/L, a dilution water control and a solvent (acetone) control. Ten fathead minnow were exposed to each treatment level and control. Following 24 hours of exposure, 70 % and 100 % mortality was observed among fish exposed to the 0.10 and 0.32 mg/L treatment levels, respectively. Following 96 hours of exposure, 90 % mortality was observed among fish exposed to the 0.10 mg/L treatment level. No mortality or adverse effects were observed among fish exposed to the remaining treatment levels tested (0.032, 0.010 and 0.032 mg/L), the control or the solvent. Based on these results nominal concentrations of 0.023, 0.037, 0.059, 0.094 and 0.15 mg/L, a dilution water control and a solvent (acetone) control were selected for the definitive exposure.

The definitive test was conducted under static conditions for a duration of 96-hours. Each test vessel contained 20 fish per treatment and control, impartially selected and allocated. Fish were added two at a time until the aquarium

contained 10 fish. The test vessels were impartially placed in a temperature-controlled water bath designed to maintain the solution at 22 ± 1 °C.

6. Dose preparation:

Based on the range-finding test the following nominal concentrations were tested in the definitive test: 0.023, 0.037, 0.059, 0.094 and 0.15 mg/L, with a solvent (acetone) and dilution control.

Prior to exposure initiation, a 30 mg/mL primary stock solution was prepared by placing 0.7881 g (0.7487 g as active ingredient) of S-2399 in a volumetric flask and bringing it to volume with 25 mL acetone. This stock solution was observed to be clear and colourless with no visible undissolved test substance following multiple inversions. The 30 mg/mL primary stock solution was used to prepare the secondary stock solutions as presented in Table 9.2.2-10.

Table 9.2.2-10: Primary Stock solution preparation of S-2399

Primary Stock Concentration [mg/mL]	Volume of Stock Used [mL]	Total Volume with Acetone [mL]	Secondary Stock Concentration [mg/mL]
30	5.0	100	1.5
1.5	15.67	25	0.94
1.5	9.83	25	0.59
1.5	6.17	25	0.37
1.5	3.83	25	0.23

Table 9.2.2-11: Secondary Stock Solution Preparation of S-2399

Stock Concentration [mg/mL]	Volume of Stock Used [mL]	Total Volume with Dilution Water [L]	Nominal concentration [mg/L]
1.5	3.2	32	0.15
0.94	3.2	32	0.094
0.59	3.2	32	0.059
0.37	3.2	32	0.037
0.23	3.2	32	0.023

Exposure solutions were mixed thoroughly using a glass rod for approximately one minute. Following mixing, all solutions were observed to be clear and colourless with no visible undissolved test substance. Each test solution was divided into two replicate aquaria, each containing approximately 15 L of solution.

A solvent control was prepared by bringing a 3.2 mL of acetone to a final volume of 32 L with dilution water. A control solution was also established containing only dilution

water. The control and solvent control solutions were each divided into two replicate vessels containing approximately 15 L of solution.

7. Measurements and observations:
All aquaria were examined after 0, 6, 24, 48, 72 and 96 hours of exposure. At each observation mortalities were recorded and the dead fish removed. Death was defined as a lack of movement including absence of gill movement or reaction to gentle prodding. Biological observations were carried out including sublethal effects relating to lethargy, loss of equilibrium etc. Observations of the physical characteristics of the test solution such as precipitates, film on the solution's surface etc, were made and recorded if applicable.

The pH, dissolved oxygen concentration and temperature were measured at 0, 24, 48, 72 and 96 hours in each replicate of each treatment and control. Continuous temperature monitoring was performed in replicate B of the 0.059 mg/L concentration throughout the exposure period.

Analytical samples were taken at exposure initiation (0 hours) and exposure termination (96 hours). One sample was collected from each treatment level and the controls for analysis of S02399 concentration. Three quality control samples (QC) were prepared in dilution water at each sampling interval at nominal concentrations approximating the test concentration range along with 3 archive QC samples which were later frozen. QC samples were prepared from 0.100, 1.00 and 10.0 mg/L stock solutions using the test substance,

All exposure solutions and QC samples were analysed for S-2399 by liquid chromatography with mass spectrometry (LC/MS/MS). The method validation study established an average recovery of 107 ± 3.55 % for S-2399 from freshwater (reconstituted for hardness). Acceptance criteria for mean recoveries of samples prepared during the method validation was 70.0 to 110 % based on SANCO and TNsG on Data Requirements criteria. The acceptable range for evaluating individual QC sample recovery was set at 80.0 % to 120 %.

8. Statistics:

The time-weighted average concentrations tested and the corresponding mortality data derived from the definitive toxicity test were used to estimate the 24-, 48-, 72- and 96-hour median lethal concentrations (LC_{50}) and 95 % confidence intervals. If at least one test concentration caused mortality of ≥ 50 % of the test population, then a computer program, CETIS™ Version 1.8 was used to calculate the LC_{50} values and 95 % confidence intervals. Based on the data, LC_{50} values could be determined by either linear regression or Spearman-Kärber estimates. CETIS™ can determine the most appropriate statistical method based on the data set.

The NOEC during the 96-hour exposure period was also determined along with the highest concentration resulting in 0 % mortality and the lowest concentration resulting in 100 % mortality.

RESULTS AND DISCUSSION

Experimental start and end dates: 27th – 31st January 2014

A. VALIDITY CRITERIA

Control mortality: The mortality in the control should not exceed 10 %, one fish, at the end of the test. In this study no mortality or adverse effects were observed among the fish the in the dilution and solvent controls.

Oxygen saturation: The dissolved oxygen saturation in control and test vessels should be at least 60 % of the air saturation value throughout the test. In this test, the dissolved oxygen ranged from 5.6 – 9.6 mg/L where 60 % of saturation at 21 – 23 °C is 5.3 – 5.1 mg/L, respectively. The temperature range across all test and control vessels was 20 – 24 °C

Analytics: Analytical measurements of test concentrations are compulsory. Results of the analytical measurements are listed below.

B. BIOLOGICAL EFFECTS

At 96 hours after exposure, 20 %, 55 % and 100 % mortality was observed among fish exposed to the 0.028, 0.054 and 0.10 mg/L treatment levels, respectively. All surviving fish exposed to the 0.028 mg/L treatment level were observed to be lethargic. Four surviving fish exposed to the 0.054 mg/ treatment level exhibited a complete loss of equilibrium, one surviving fish was observed to be lethargic and four surviving fish exhibited a partial loss of equilibrium. No mortality or adverse effects were observed among fish exposed to the remaining treatment level (0.017 mg/L). These results are presented in Table 9.2.2-12

Table 9.2.2-12: Time-weighted average concentrations tested, corresponding mortalities and observations made during the 96-hour static toxicity exposure of fathead minnow (*Pimephales promelas*) to S-2399

Time-Weighted Average Concentration [mg/L]	Cumulative Percent Mortality					
	Replicate	6 hours	24 hours	48 hours	72 hours	96 hours
Control	A B Mean	0 (0) 0 (0) 0	0 (0) 0 (0) 0	0 (0) 0 (0) 0	0 (0) 0 (0) 0	0 (0) 0 (0) 0
Solvent	A	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Time-Weighted Average Concentration [mg/L]	Cumulative Percent Mortality					
	Replicate	6 hours	24 hours	48 hours	72 hours	96 hours
Control	B Mean	0 (0) 0	0 (0) 0	0 (0) 0	0 (0) 0	0 (0) 0
0.017	A B Mean	0 (0) 0 (0) 0	0 (0) 0 (0) 0	0 (0) 0 (0) 0	0 (0) 0 (0) 0	0 (0) 0 (0) 0
0.028	A B Mean	0 (0) 0 (0) 0 [b c]	10 (1) 0 (0) 5 [e g]	20 (2) 0 (0) 10 [k l m]	20 (2) 10 (1) 15 [o p]	20 (2) 20 (2) 20 [s]
0.054	A B Mean	0 (0) 30 (3) 15 [c d e]	60 (6) 50 (5) 55 [h i]	60 (6) 50 (5) 55 [i n]	60 (6) 50 (5) 55 [q r]	60 (6) 50 (5) 55 [g t u]
0.10	A B Mean	40 (4) 40 (4) 40 [f]	90 (9) 100 (10) 95 [i]	90 (9) 100 (10) 95 [f]	90 (9) 100 (10) 95 [f]	100 (10) 100 (10) 100
0.17	A B Mean	70 (7) 30 (3) 50 [f]	100 (10) 100 (10) 100	100 (10) 100 (10) 100	100 (10) 100 (10) 100	100 (10) 100 (10) 100

[a] Actual number of mortalities is presented in parentheses
[b] Two fish exhibited a partial loss of equilibrium
[c] One fish was observed on the bottom of the test vessel
[d] Seven surviving fish exhibited a complete loss of equilibrium
[e] Nine surviving fish exhibited a partial loss of equilibrium
[f] All surviving fish were observed on the bottom of the test vessel
[g] Four surviving fish exhibited a complete loss of equilibrium
[h] Three surviving fish were observed on the bottom of the test vessel
[i] Six surviving fish exhibited a complete loss of equilibrium
j All surviving fish exhibited a complete loss of equilibrium
[k] Seven surviving fish exhibited a partial loss of equilibrium
[l] Four surviving fish were observed to be lethargic
[m] Two surviving fish exhibited a complete loss of equilibrium
[n] Three surviving fish exhibited a partial loss of equilibrium
[o] Six surviving fish exhibited a partial loss of equilibrium
[p] Nine surviving fish were observed to be lethargic
[q] Eight surviving fish exhibited a complete loss of equilibrium
[r] One surviving fish exhibited a partial loss of equilibrium
[s] All surviving fish were observed to be lethargic
[t] Four surviving fish exhibited a partial loss of equilibrium
[u] One surviving fish was observed to be lethargic

The dose-response curve for mortality is presented below.

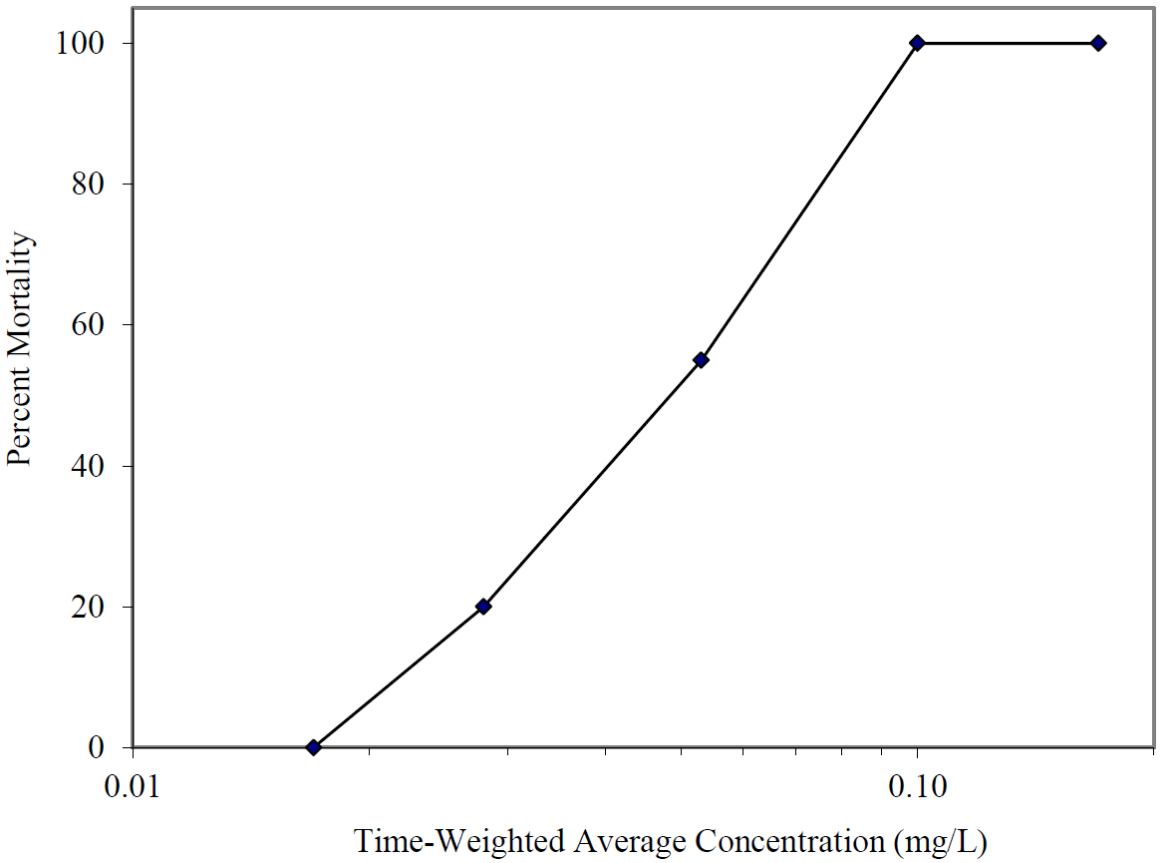


Figure 9.2-3: Concentration-response (mortality) curve for fathead minnow (*Pimephales promelas*) exposed to S-2399 under static conditions

The time-weighted average concentrations tested and the corresponding mortality data derived from the definitive toxicity test were used to estimate the 24, 48, 72 and 96-hour LC_{50} and 95 % confidence limits.

If at least one test concentration cause mortality of ≥ 50 % of the test population, then a computer program, CETIS™ Version 1.8 was used to calculate the LC_{50} values and 95 % confidence intervals. Based on the data, LC_{50} values could be determined by either linear regression or Spearman-Kärber estimates. The NOEC during the 96-hour exposure period was also determined.

A summary of the endpoints is presented in Table 9.2.2-13.

Table 9.2.2-13: The LC50 values (corresponding to 95 % confidence intervals) and NOEC established during the 96-hour static acute exposure of fathead minnow (*Pimephales promelas*) to S-2399

	LC50 [mg/L]	95 % Confidence Intervals	
		Lower [mg/L]	Upper [mg/L]
24-hour [a]	0.051	0.041	0.062
48-hour [a]	0.051	0.037	0.063
72-hour [a]	0.051	0.038	0.064
96-hour [a]	0.050	0.039	0.065
NOEC through 96 hours = 0.017 mg/L			
Highest concentration producing 0 % mortality = 0.017 mg/L			
Lowest concentration producing 100 % mortality = 0.10 mg/L			

[a] LC_{50} value and corresponding 95 % confidence intervals were estimated by linear interpolation

C. ANALYSIS

The content S-2399 in test solutions was analytically determined to confirm the correct application of the test item. HSE has calculated the measured content of S-2399 in fresh test solutions to be between 108 – 119 % of the nominal concentration. The measured content of S-2399 in aged test solutions (96 hours) was between 45 - 113 %.

Since the measured concentrations of S-2399 in all test solutions were not maintained within ± 20 % (80 – 120 %) of the nominal concentration, the biological endpoints will be evaluated using the measured concentrations.

A summary of the analysed concentrations is presented in Table 9.2.2-14.

Table 9.2.2-14: Concentrations measured in the exposure solutions during the 96-hour static acute exposure of fathead minnow (*Pimephales promelas*) to S-2399

Nominal Concentration [mg/L]	Measured concentration [mg/L]		Time-Weighted Average [mg/L]	Percent of Nominal [%]
	0-hour	96-hour		
Control	< 0.0020 [b]	< 0.0020	NA [c]	NA
Solvent Control	< 0.0020	< 0.0020	NA	NA

Nominal Concentration [mg/L]	Measured concentration [mg/L]		Time-Weighted Average [mg/L]	Percent of Nominal [%]
	0-hour	96-hour		
0.023	0.025	0.011	0.017	72
0.037	0.044	0.017	0.028	77
0.059	0.068	0.042	0.054	91
0.094	0.11	0.095	0.10	110
0.15	0.17	0.17	0.17	110

[a] Time-weighted average concentrations and percent of nominal were calculated using the actual analytical (unrounded) results and not the rounded (two significant figures) values presented in this table.
[b] Concentrations expressed as less than values were below the minimum detectable limit (MDL). The MDL is dependent upon the lowest concentration calibration standard used and the dilution factor of the controls (i.e., 0.000100 mg/L x 20.0 = 0.0020 mg/L).
[c] NA = Not applicable
[d] QC = Quality Control sample. Percent recovery for each QC sample is presented in parentheses.

III. CONCLUSION

Based on time-weighted average concentrations, the 96-hour LC50 value for *Pimephales promelas* and S-2399 was estimated by linear interpolation to be 0.050 mg/L, with 95 % confidence intervals of 0.039 to 0.065 mg/L. The NOEC was determined to be 0.017 mg/L.

The highest concentration producing 0 % toxicant related mortality was 0.017 mg/L. The lowest concentration producing 100 % mortality was 0.10 mg/L.

HSE COMMENTS

This study was conducted to GLP standard and in accordance with the OECD 203 (1992) guidance. However, HSE have evaluated the study in accordance with the OECD 203 (2019) guidance being the most up to date version. As the study was conducted in 2014 the latest guidance would not have been available. According to the OECD 203 (2019) there are no significant deviations. All validity criteria were satisfactorily met.

The length of the fish used in this study ranged from 2.0 to 3.5 cm. The OECD 203 (2019) guidelines recommend a range of between 1 – 3 cm and the 1992 guidelines recommend a length of 2.0 ± 1.0 cm. Therefore, the size of the fish minimally exceeds the maximum length. Given that all the validity criteria were met this is not deemed a significant deviation.
The OECD 203 (2019) recommend temperature range for *Pimephelas promelas* was between 21 – 23 °C and the 1992 guidelines recommend a temperature range of between 21 – 25 °C. Within this study the temperature range was between 20 – 24 °C according to the maximum and minimum readings taken. As this is in line with the

1992 guidelines available at the time of testing, no detrimental effect was observed in the dilution control fish and all validity criteria were met this is not considered a significant deviation.

The recovery analysis showed that the measured concentrations were not maintained within ± 20 % (80 – 120 %) of the nominal concentrations. The applicant has used the time weighted average concentrations which is commonly associated with a flow-through or semi-static design. For a static system test the appropriate measurement would be the geometric mean measured concentrations. However, in this case, HSE has calculated that the time weighted average concentrations are remarkably close to the geomean concentrations, so the calculation of the endpoints is unlikely to differ substantially. Therefore, HSE accepts the use of the time weighted average concentrations for agreed endpoints.

No mortality or adverse effects were observed in the dilution or solvent control or in the test item concentration 0.017 mg/L. Between 20 – 100 % mortality was observed in the 3 test item concentrations tested (0.028, 0.054 and 0.10 mg/L respectively). Adverse effects were observed in the surviving fish at concentrations of 0.028 and 0.054 mg/L. The LD_{50} and 95 % confidence limits were calculated using linear regression or Spearman-Kärber which is appropriate for the data set obtained.

The method of analysis used in the study was evaluated by HSE Chemistry. The conclusion of their evaluation is reproduced below. Please see Volume 3 CA, section B5 for more details.

"*The analytical method is not fully validated according to SANTE/2020/12830 rev. 1 for the determination of the active substance inpyrfluxam in freshwater as the matrix effects have not been determined and the stability of standards and extracts has not been addressed. However, the studies were generated prior to the implementation of SANTE 2020/12830 rev.1 and SANCO 3029 rev.4 did not require matrix effects and the stabilities of standards and extracts to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose.*"

The agreed endpoint suitable for use in the risk assessment is:
LC_{50} (96-h) = 0.050 mg S-2399/L

Reference:	KCA 8.2.1/04
Report Title:	S-2399 TG - Acute Toxicity Test with Common Carp (*Cyprinus carpio*) Under Static Conditions
Author(s) & year:	[redacted] (2014d)
Document No, Authority registration No:	[redacted] Study No. 13048.6778
Substance used:	S-2399 TG (13CG0617G, 95.0%)

Method of analysis:	LC/MS/MS
Guideline(s):	OECD 203 (1992)
Deviations:	Yes, see HSE comments section.
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test material:** S-2399 TG
Description: Not stated
Lot/Batch: 13CG0617G
Purity: 95.0%
Reference item: None
Solvent carrier: Acetone

B. STUDY DESIGN AND METHODS

1. **Test animals:** Common carp (*Cyprinus carpio*)
Wet weight: Mean: 0.83 g (range: 0.38 to 1.6 g)
Length: Mean: 34 mm (range: 25 to 48 mm)
Source: [redacted]
Acclimation: 14 days
Diet: Commercial fish food, [redacted] Mixed Prime Flakes daily (not fed during the 48 hours prior to test initiation or during the exposure period)

Treatment of disease: None
Statistical Analysis: Based on the data, LC_{50} values could be determined by linear regression or Spearman-Kärber estimates.
Nominal concentrations: 0.023, 0.037, 0.059, 0.094 and 0.15 mg a.s./L
LOQ = 0.600 µg/L, MDL = 0.200 µg/L

2. **Dilution water:** Well water
Hardness: 52 mg $CaCO_3$/L
Alkalinity: 20 $CaCO_3$/L
pH: 7.5

3. **Test vessels:** 39 x 20 x 25 cm glass aquaria, each containing 15 L of test solution

Fish per tank : 10 (20 per concentration replicate)
Mean loading weight: 0.55g biomass / L test solution

Exposure regime : Static
Chemical analysis : Sample collected at 0 and 96 hours from each treatment level and control

4. Environmental conditions:

Continuous temperature monitoring was performed in replicate B of the 0.059 mg/L concentration throughout the exposure period. [redacted] Study No. 13048.6778 Page 16 The pH was measured using a Yellow Springs Instrument (YSI) Model pH 100 pH meter. The dissolved oxygen concentration and daily temperature were measured with either a YSI Model 550A or Model Pro20 dissolved oxygen meter/temperature probe. Temperature was continuously monitored throughout this exposure using a VWR minimum-maximum thermometer.

Table 9.2.2-15: Summary of environmental conditions

Variable	Required OECD 203 (2019)	Obtained
Temperature	20˚C - 24˚C	22˚C – 23˚C
pH	6.0 – 8.5	6.8 – 7.5
Dissolved oxygen concentration	≥ 60 % of air saturation value	4 % - 90% (see deviations)
Photoperiod	12 – 16 hours	16 hours light, 8 hours dark
Lighting intensity	n/a	410 – 730 lux
Hardness of dilution water	40 – 250, preferably <180	52 mg $CaCO_3$ / L
Alkalinity of dilution water	n/a	20 $CaCO_3$ / L
Conductivity of dilution water	≤ 10 µS/cm	370 µS/cm
Total organic Carbon (TOC) mg/L	n/a	0.43mg/L

STUDY DESIGN AND METHODS
Study dates: 23 to 27 January 2014

5. Animal assignment and treatment:

20 fish per test item concentration (10 per replicate), as well as the control and solvent control, were impartially selected and distributed to each aquarium. The mean organism loading rate was 0.55 g of biomass per litre of test solution. The nominal test concentrations were 0.023, 0.037, 0.059, 0.094 and 0.15 mg a.s./L.

6. Dose preparation:

Prior to exposure initiation, a 30 mg a.s./mL primary stock solution was prepared by placing 0.7881 g of S-2399 TG (0.7487 g active substance) in a volumetric flask and

bringing it to volume with 25 mL of acetone. The stock solution was clear and colourless with no visible undissolved test substance following multiple inversions. The primary stock solution was used to prepare secondary stock solutions, which were subsequently used to prepare the exposure solutions. Exposure solutions were mixed thoroughly using a glass rod for approximately a minute. All solutions were clear and colourless with no visible undissolved test substance. A solvent control was prepared by bringing 3.2 mL of acetone to a final volume of 32 L with dilution water. A control solution was also established containing only dilution water. Each solution was divided into two replicate aquaria, each containing approximately 15 L of solution.

7. Measurements and observations:

All aquaria were examined after 0, 6, 24, 48, 72 and 96 hours for mortality (dead fish were removed), biological observations, including sublethal effects (e.g. lethargy, loss of equilibrium) and observations of the physical characteristics of the test solutions (e.g. presence of precipitate, film on the solution's surface) were made and recorded. Effects were based on death, defined as the lack of movement by the exposed organisms (i.e. absence of gill movement and reaction to gentle prodding).

The pH, dissolved oxygen concentration and temperature were measured at 0, 24, 48, 72 and 96 hours in each replicate of each treatment and control. Continuous temperature monitoring was performed in one replicate vessel. Gentle, oil-free aeration was initiated at the 24-hour interval to raise and maintain dissolved oxygen levels at or above 60% of air saturation.

At exposure initiation (0 hour) and exposure termination (96 hour), one sample was collected from each treatment level and the controls for analysis of S-2399 TG concentration by LC/MS/MS. At exposure initiation, samples were removed from the intermediate mixing vessel, prior to division into replicate vessels. At exposure termination, samples were removed from a composite of each replicate of each concentration and controls. The mean measured concentrations (reported as "time-weighted average concentrations") of S-2399 TG were calculated for each treatment level. The LOQ was set at 0.600 µg/L and MDL minimum detectable limit (MDL) was 0.200 µg/L.

8. Statistics:

The 24-, 48-, 72- and 96-hour LC_{50} values and 95% confidence intervals were estimated using the mean measured concentrations and the corresponding mortality data. If at least one concentration resulted in ≥ 50% mortality, then the computer program CETIS™v.1.8 was used to calculate the LC_{50} values. Based on the data, LC_{50} values could be determined by linear regression or Spearman-Kärber estimates. The No-Observed-Effect Concentration (NOEC) was defined as the highest mean measured concentration tested at and below which there were no toxicant-related mortality or physical and behavioural abnormalities (e.g., lethargy), with respect to the control organisms.

A. MORTALITY AND SUBLETHAL EFFECTS

At test termination (96 hours), 5% mortality was observed among fish exposed to the 0.043 mg a.s./L treatment level (mean measured concentrations). Four surviving fish exposed to the 0.043 mg a.s./L treatment level exhibited a partial loss of equilibrium, two surviving fish exhibited a complete loss of equilibrium and thirteen surviving fish

were observed to be lethargic. No mortality or adverse effects were observed among fish exposed to the remaining treatment levels (0.015 and 0.027 mg a.s./L) the control or solvent control. A summary of the cumulative percent mortalities and observations is presented in Table 9.2.2-16 below.

Table 9.2.2-16: Summary of mortality and observations following exposure to S-2399 TG

Mean measured concentration (mg a.s./L)	Mean Cumulative percent mortality (%)				
	6 hour	24 hour	48 hour	72 hour	96 hour
Control	0	0	0	0	0
Solvent control	0	0	0	0	0
0.015	0	0	0	0	0
0.027	0	0	0	0	0
0.043	0^{ab}	0^{gh}	0^{lm}	5^{gm}	5^{opq}
0.095	0^{cd}	60^{ij}	95^{n}	100	100
0.16	50^{ef}	70^{ik}	90^{n}	100	100

[a] Twelve fish exhibited a complete loss of equilibrium.
[b] Eight fish exhibited a partial loss of equilibrium.
[c] Eight fish exhibited a complete loss of equilibrium.
[d] Twelve fish were observed to be on the bottom of the test vessel.
[e] Four fish exhibited complete loss of equilibrium.
[f] Six fish were observed to be on the bottom of the test vessel.
[g] Nine fish exhibited a complete loss of equilibrium.
[h] Eleven fish exhibited a partial loss of equilibrium.
[i] Five fish were observed to be on the bottom of the test vessel.
[j] Three fish exhibited complete loss of equilibrium.
[k] One fish exhibited complete loss of equilibrium.
[l] Ten fish exhibited a complete loss of equilibrium.
[m] Ten fish exhibited a partial loss of equilibrium.
[n] All surviving fish were observed to be on the bottom of the test vessel.
[o] Two fish exhibited a complete loss of equilibrium.
[p] Four fish exhibited a partial loss of equilibrium.
[q] Thirteen fish were observed to be lethargic

The dose-response curve for the calculated 96-hour LD_{50} is shown in the figure below:

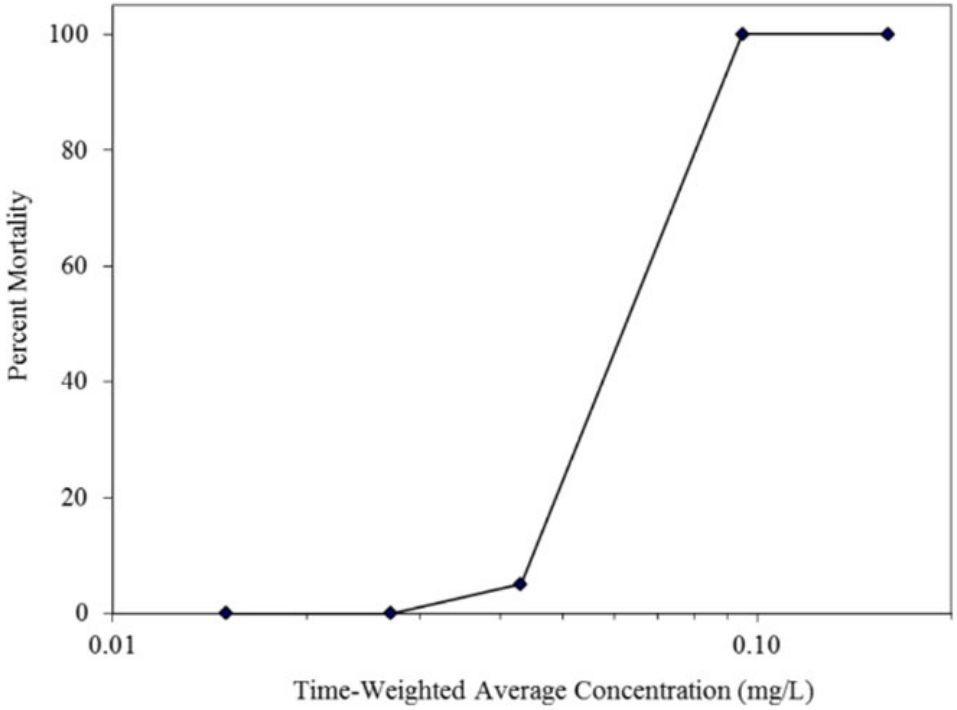


Figure 9.2-4: The 96-Hour Concentration-Response (Mortality) Curve for the static Acute Exposure of Common Carp (*Cyprinus carpio*) to S- 2399TG

A summary of the toxicity endpoints determined from the study is presented in Table 9.2.2-17.

Table 9.2.2-17: Summary of endpoints

Time	LC_{50} (mg a.s./L)	95% confidence interval	
		Lower (mg a.s./L)	Upper (mg a.s./L)
24-hour [a]	0.086	0.061	0.12
48-hour [a]	0.071	0.068	0.074
72-hour [a]	0.067	0.061	0.073
96-hour [a]	0.067	0.061	0.073
96 hour NOEC = 0.027 mg a.s./L			
Highest concentration producing 0% mortality = 0.027 mg a.s./L			
Lowest concentration producing 100% mortality = 0.095 mg a.s./L			

[a] LC_{50} value and corresponding 95% confidence intervals were estimated by linear interpolation

B. ANALYSIS

Measured concentrations decreased between intervals, but the expected concentration gradient was maintained. Mean measured concentrations ranged from 64 to 110% of nominal concentrations and defined the treatment levels tested as 0.015, 0.027, 0.043, 0.095 and 0.16 mg a.s./L.

Table 9.2.2-18: Measured concentrations of S-2399 TG in the exposure solutions

Nominal concentration (mg a.s./L)	Measured concentration (mg a.s./L)		Mean measured concentration (mg a.s./L)	Percent of nominal[a] (%)
	0 Hour	96 Hour		
Control	<0.0020 [b]	<0.0020 [b]	n.a.	n.a.
Solvent control	<0.0020 [b]	<0.0020 [b]	n.a.	n.a.
0.023	0.027	0.0068	0.015	64
0.037	0.044	0.015	0.027	72
0.059	0.070	0.024	0.043	73
0.094	0.11	0.081	0.095	100
0.15	0.18	0.15	0.16	110

[a] Mean measured concentrations (referred in the study report as "time-weighted average concentration") and percent of nominal were calculated using the actual analytical (unrounded) results and not the rounded (two significant figures) values presented in this table.
[b] Concentrations expressed as less than values were below the minimum detectable limit (MDL).
n.a. = not applicable

C. VALIDITY CRITERIA

A summary of the validity criteria are shown in Table 9.2.2-19 below.

Table 9.2.2-19: Validity criteria

Validity criterion	Required	Obtained
Mortality in the control(s)	≤ 10%	No mortality in control or solvent control
Dissolved oxygen concentration	≥ 60% of air saturation	4 – 90% (protocol deviation)
Analytical measurement of test concentrations	Analysis of the highest and lowest test concentration and a concentration around the expected LC_{50} is considered the minimum	At exposure initiation and exposure termination. One sample was collected from each treatment level and the controls for analysis for

	requirement	static study. Mortality data for each concentration evaluated. Recovery rates range from 64% to 110%.

III. CONCLUSION

The results of the laboratory study demonstrate the 96-hour LC_{50} value for common carp exposed to S-2399 TG to be 0.067 mg a.s./L and the No-Observed-Effect Concentration (NOEC) value to be 0.027 mg a.s./L, based on mean measured concentrations.

HSE COMMENTS:
The study was carried out in accordance with GLP under OECD 203 (1992) guidance and has been assessed against OECD 203 (2019) guidance.

The concentrations of the test item were not maintained between 80-120% of the nominal value throughout the test. Concentrations obtained ranged from 64-110% of nominal. Results should be based on mean measured concentrations as the applicant has presented. The method of analysis used in the study was evaluated by HSE Chemistry. The conclusion of their evaluation is reproduced below. Please see Volume 3 CA, section B5 for more details.

"*The analytical method is not fully validated according to SANTE/2020/12830 rev. 1 for the determination of the active substance inpyrfluxam in freshwater as the matrix effects have not been determined and the stability of standards and extracts has not been addressed. However, the studies were generated prior to the implementation of SANTE 2020/12830 rev.1 and SANCO 3029 rev.4 did not require matrix effects and the stabilities of standards and extracts to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose.*"

The first significant deviation to protocol to note in the study is the drop in dissolved oxygen concentrations to 4% in replicate B of test concentration 0.094 mg/L due to the pump malfunction at hour 72. It is stated that gentle oil-free aeration was initiated at 24 hours under protocol deviation but then claims to have been started at 48 hours in the measurements and observations section. As it is also stated in the full study that aeration was initiated at 24 hours, HSE have taken 24 hours to be the time of initiation. A steady decline in dissolved oxygen concentration can still be observed following aeration, so is unclear if aeration was successful at keeping dissolved oxygen levels above 60% given the pump failure that occurred at 72 hours. OECD 203 (1992, 2019) guidance states that aeration can be used provided that it does not lead to a significant loss of test chemical as verified by analytical measurements of test concentrations. As the percentage of nominal concentration for 0.094 mg/L was recorded at 100% at study termination, it can be determined that aeration did not result in significant loss of test chemical.

The applicant has stated that whilst the dissolved oxygen concentrations recorded do not meet validity criteria, as 100% mortality was observed in replicate A of this

concentration, the mortalities in replicate B cannot be attributed to the low oxygen concentrations. HSE agree that the validity criteria for oxygen concentrations have not been met in this study, but as the mortality data of the unaffected replicate demonstrates clear toxicant effects at this concentration. At 48 hours, prior to the dissolved oxygen concentration dropping below acceptable levels, there was already 90% mortality in replicate B. There is only one additional mortality from 28 to 72 hours, so it is unlikely that the low dissolved oxygen concentration had any adverse effect. The study can be accepted in order to minimise further vertebrate testing.

The second deviation to protocol relates to the length of fish used in the study. OECD 203 (2019) guidance states a maximum length of 40mm. This is the same for OECD 203 (1992) guidance. The longest fish was reported at 48mm. As the mean length (34mm) was still within the recommended values for this species (20-40mm) in OECD 203 (1992, 2019), it can be determined that it had no significant impact on the study. All validity criteria for study were met.

All aquaria were examined after 0, 6, 24, 48, 72 and 96 hours for mortality, but latest guidance recommends a minimum of 2 observations should be conducted within the first 24 hours of the study with preferably at least 3 hours between observations. However, this wasn't part of the guidance available at the time in OECD 203 (1992). There is discrepancy between measurements and observations, with the pH, dissolved oxygen concentration and temperature were measured at 0, 24, 48, 72 and 96 hours, but examined after 0, 6, 24, 48, 72 and 96 hours. This implies no pH or dissolved oxygen concentration data was available for mortality examinations at 6 hours and is not included in the variable data for the test.

It is also worth noting that, whilst not a requirement of OECD 203 (1992) or OECD (2019), no reference item was used in this study; therefore, species sensitivity is uncertain.

The test design used silicone adhesive as part of the test structure conditions. OECD 203 (2019) guidance states that silicone should not be used in reference to flow-through studies. It should be considered whether it is possible this had any impact on test concentrations given its strong capacity to absorb lipophilic chemicals in this static environment. As nominal concentrations at 0.023, 0.037 and 0.059mg/L recorded values of ≤ 80% after study termination (96 hours), this could be a potential area of concern. OECD 203 (2019) guidance states that if the concentrations are expected to decline by more than 20%, then all test concentrations should be measured, and more frequent analyses are recommended (e.g., 48 hours). In this study, one sample was collected from each treatment level (and the controls) for analysis at only exposure initiation (hour 0) and exposure termination (hour 96). OECD 203 (1992) guidance that was available at the time states that if the deviation from the nominal concentration is greater than 20 per cent, results should be based on the measured concentration. Mortality data is recorded against mean measured concentration, so this adheres to guidance available at the time.

Spearman-Kärber statistical analysis was conducted on these data and is a suitable test when looking at mean data points and is line with OECD 203 (2019 guidance) where only one concentration results in partial mortality. As this applies to the data obtained in this study, the statistical analysis is deemed suitable.

The agreed endpoints for use in risk assessment are:

- **96-hour LC_{50} = 0.067 mg S-2399TG/L (based on mean measured values)**

Reference:	KCA 8.2.1/05
Report Title:	S-2399 TG: Acute Toxicity to Sheepshead Minnow (*Cyprinodon variegatus*) Under Static Conditions, Following OCSPP Draft Guideline 850.1075 and OECD Guideline #203
Author(s) & year:	[redacted] (2014e)
Document No, Authority registration No:	[redacted] Study No. 12709.6360
Substance used:	S-2399 TG (13CG0617G, 95.0%)
Method of analysis:	LC/MS/MS
Guideline(s):	OECD 203 (1992)
Deviations:	Yes, see HSE comments section.
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

MATERIALS AND METHODS

MATERIALS

Test material S-2399 TG

Lot/Batch #: 13CG0617G

Purity: 95.0% (verified according to the certificate of analysis)

Description: Not stated

Stability of test compound: Not stated

Reanalysis/expiry date: 23 July 2016

Density: Not applicable

TREATMENTS

Test concentrations: Dilution water control, solvent control (0.10 mL acetone/L), nominal concentrations of 0.063, 0.13, 0.25, 0.50 and 1.0 mg S-2399/L, mean measured concentrations of 0.065, 0.11, 0.22, 0.48 and 0.89 mg S-2399/L

Solvent:	Acetone (CAS No. 67-64-1)
Toxic reference	None
Analysis of test concentrations:	Yes, at 0 and 96 hours (all treatment levels and the dilution water and solvent controls) based on analysis of S-2399 using LC-MS/MS. The limit of quantification (LOQ) was set at 0.600 µg/L, the lowest validated concentration and the minimum detectable limit (MDL) was 0.200 µg/L for S-2399

TEST ORGANISMS

Species:	Sheepshead Minnow (*Cyprinodon variegatus*)
Source:	[redacted]
Acclimatisation period:	14 days
Treatment for disease:	None reported
Weight and length of a representative sample of fish (n = 30):	Mean wet weight: 0.14 g (range: 0.090 to 0.20 g) Mean length: 17 mm (range: 12 to 23 mm)
Feeding:	Commercial flake fish food, [redacted] Mixed Prime Flakes daily (not fed during the 48 hours prior to test initiation or during the exposure period)

TEST DESIGN

Test vessels:	39 x 20 x 25 cm (L x W x H) glass aquaria, each containing 15 L of test solution
Test medium:	Natural, filtered seawater
Replication:	Two replicate aquaria were established for each treatment level, control and solvent control
No of fish per tank:	10
Exposure regime:	Static
Duration:	96 hours

TEST CONDITIONS

Test temperature:	21 – 23 °C
pH:	7.6 – 8.1
Salinity	20‰ (promille)
Dissolved oxygen:	5 – 7.5 mg/L (60 % saturation is 4.7 mg/L at 21 °C and 20‰ salinity, and 4.6 mg/L at 23 ºC and 20‰ salinity)
Hardness of dilution water:	Not applicable
Lighting:	320-490 lux photoperiod of 16 hours light and 8 hours darkness

STUDY DESIGN AND METHODS

Experimental dates: the 96-hour definitive exposure was conducted from 17 to 21 June 2014.

Test organism and acclimatisation
The Sheepshead minnow (*Cyprinodon variegatus*), juvenile), commonly used in acute saltwater toxicity tests, was selected as the test species. Prior to testing, fish were acclimatised for 14 days under species appropriate conditions (25°C, 20‰ salinity). During acclimatisation, the fish were fed commercially prepared fish food, at least once daily. No mortality was observed among the test fish population during the seven-day period prior to testing.

Test water
The dilution water (filtered natural seawater) used during this study was from the same source as the water used during acclimatisation. The dilution water was prepared by filtering seawater through a series of polypropylene core filters (20- and 5-micron) and adjusting the salinity to 20 ± 3‰ with laboratory well water. Representative samples of the dilution water source were analysed periodically for the presence of pesticides, PCBs and toxic metals. None of these compounds were detected at concentrations considered toxic in any of the water samples analysed, in agreement with ASTM (2002)[8] standard practice. In addition, samples were analysed monthly for Total Organic Carbon (TOC) (0.96 mg/L for June 2014).

Definitive test and dose preparation
A static test system was employed. A 10 mg a.s./mL primary stock solution was prepared by placing 0.2666 g of S-2399 TG (0.2533 g active substance) in a volumetric flask and bringing it to volume with 25 mL of acetone. The mass of test substance was adjusted to account for its 95% purity. The stock solution was clear and colourless with no visible undissolved test substance following multiple inversions. The primary stock solution was used to prepare secondary stock solutions, which were subsequently used to prepare the exposure solutions. Exposure solutions were mixed thoroughly using a glass rod for approximately a minute. All solutions were clear and colourless with no visible undissolved test substance. A 0.10 mL/L solvent control was prepared by bringing 3.2 mL of acetone to a final volume of 32 L with dilution water. A control solution was also established containing only dilution water. Each solution was divided into two replicate aquaria, containing approximately 15 L of solution.
At the start of the test 20 fish, ten per replicate aquarium, were randomly allocated to each test concentration and the controls. The resulting test organism loading concentration was 0.093 g of biomass per litre of solution per aquarium per day. The test aquaria were impartially placed in a temperature-controlled water bath designed to maintain exposure solution temperatures at 22 ± 1 °C.

Measurements and observations
Observations for mortalities and symptoms of toxicity were made at 0, 6, 24, 48, 72 and 96 hours. Mortality (dead fish were removed), biological observations, including sublethal effects (e.g., lethargy, loss of equilibrium) and observations of the physical characteristics of the test solutions (e.g., presence of precipitate, film on the solution's surface) were made and recorded. Effects for this study were based on mortality, defined as the lack of movement by the exposed organisms (i.e., absence of gill

[8] ASTM, 2002. Standard practice for conducting acute toxicity tests with fishes, macroinvertebrates and amphibians. Standard E729-96. American Society for Testing and Substances, 100 Barr Harbor Road, West Conshohocken, PA 19428.

movement and reaction to gentle prodding).

The pH, dissolved oxygen concentration, temperature and salinity were measured at 0, 24, 48, 72 and 96 hours in each replicate of each treatment and control. Continuous temperature monitoring was performed in one replicate vessel.

The pH was measured with a Yellow Springs Instrument (YSI) pH100 pH meter, dissolved oxygen concentration and daily temperature with a YSI 550A or Pro20 dissolved oxygen meter/temperature probe, and salinity with a VitalSine SR-6 refractometer. Temperature was continuously monitored throughout this study in one replicate of the 0.13 mg /L nominal treatment level using a VWR minimum/maximum thermometer.

At exposure initiation (0 hours) and exposure termination (96 hours), one sample was collected from each treatment level and the controls for analysis of S-2399 TG concentration by liquid chromatography/mass spectrometry (LC/MS/MS). At exposure initiation, samples were removed from the intermediate mixing vessel, prior to division into replicate vessels. At exposure termination, samples were removed from a composite of each replicate of each concentration and controls. The mean measured concentrations of S-2399 TG were calculated for each treatment level.

Statistical analysis

The median lethal concentration (LC_{50}) was defined as the concentration resulting in 50 % mortality of the fish in the time period specified. The 24-, 48-, 72- and 96-hour LC_{50} values and 95% confidence intervals were estimated using the mean measured concentrations and the corresponding mortality data. If at least one concentration resulted in ≥ 50 % mortality, then the computer program CETIS™v.1.8 was used to calculate the LC_{50} values. If no concentration resulted in ≥ 50% mortality, the LC_{50} was empirically estimated to be greater than the highest mean measured concentration. The No-Observed-Effect Concentration (NOEC) was defined as the highest mean measured concentration tested where there were no toxicant-related mortality or physical and behavioural abnormalities (e.g., lethargy), with respect to the control organisms.

RESULTS AND DISCUSSION

Mortality and sub-lethal effects

At exposure termination (96 hours), 10% mortality was observed among fish exposed to 0.11 mg/L, and 100% mortality was observed for fish exposed to the 0.22 mg/L and all higher concentrations. No adverse effects were observed among fish exposed to 0.065 mg/L and controls. A summary of mortalities and sub-lethal observations is presented in Table 9.2.2-20.

Table 9.2.2-20: Summary of mortalities and observations following exposure to S-2399 TG

Mean measured concentration (mg a.s./L)	Cumulative percent mortality (%)				
	6 hour	24 hour	48 hour	72 hour	96 hour
Control	0	0	0	0	0

Mean measured concentration (mg a.s./L)	Cumulative percent mortality (%)				
	6 hour	24 hour	48 hour	72 hour	96 hour
Solvent control	0	0	0	0	0
0.065	0	0	0	0	0
0.11	0	0	0	5	10
0.22	0[ab]	25[ef]	65[gh]	100	100
0.48	80[cd]	100	100	100	100
0.89	100	100	100	100	100

[a] Two fish exhibited a complete loss of equilibrium.
[b] Eighteen surviving fish were observed to be lethargic.
[c] One surviving fish were observed on the bottom of the test vessel.
[d] Three surviving fish exhibited a partial loss of equilibrium.
[e] Fourteen surviving fish observed to be lethargic.
[f] One surviving fish exhibited a complete loss of equilibrium.
[g] Two surviving fish were observed on the bottom of the test vessel.
[h] Five surviving fish exhibited a complete loss of equilibrium.

A summary of the toxicity endpoints determined from the study is presented in Table 9.2.2-21 and the 96-hour concentration response visualised below.

Table 9.2.2-21: Summary of endpoints

Time	LC_{50} (mg a.s./L)	95% confidence interval	
		Lower (mg a.s./L)	Upper (mg a.s./L)
24-hour [a]	0.27	0.23	0.31
48-hour [a]	0.20	0.17	0.24
72-hour [a]	0.15	0.14	0.16
96-hour [a]	0.15	0.13	0.16
96 hour NOEC = 0.065 mg a.s./L			
Highest concentration producing 0% mortality = 0.065 mg a.s./L			
Highest concentration producing 100% mortality = 0.22 mg a.s./L			

[a] LC_{50} values and corresponding 95% confidence intervals were estimated by Spearman-Kärber estimates.

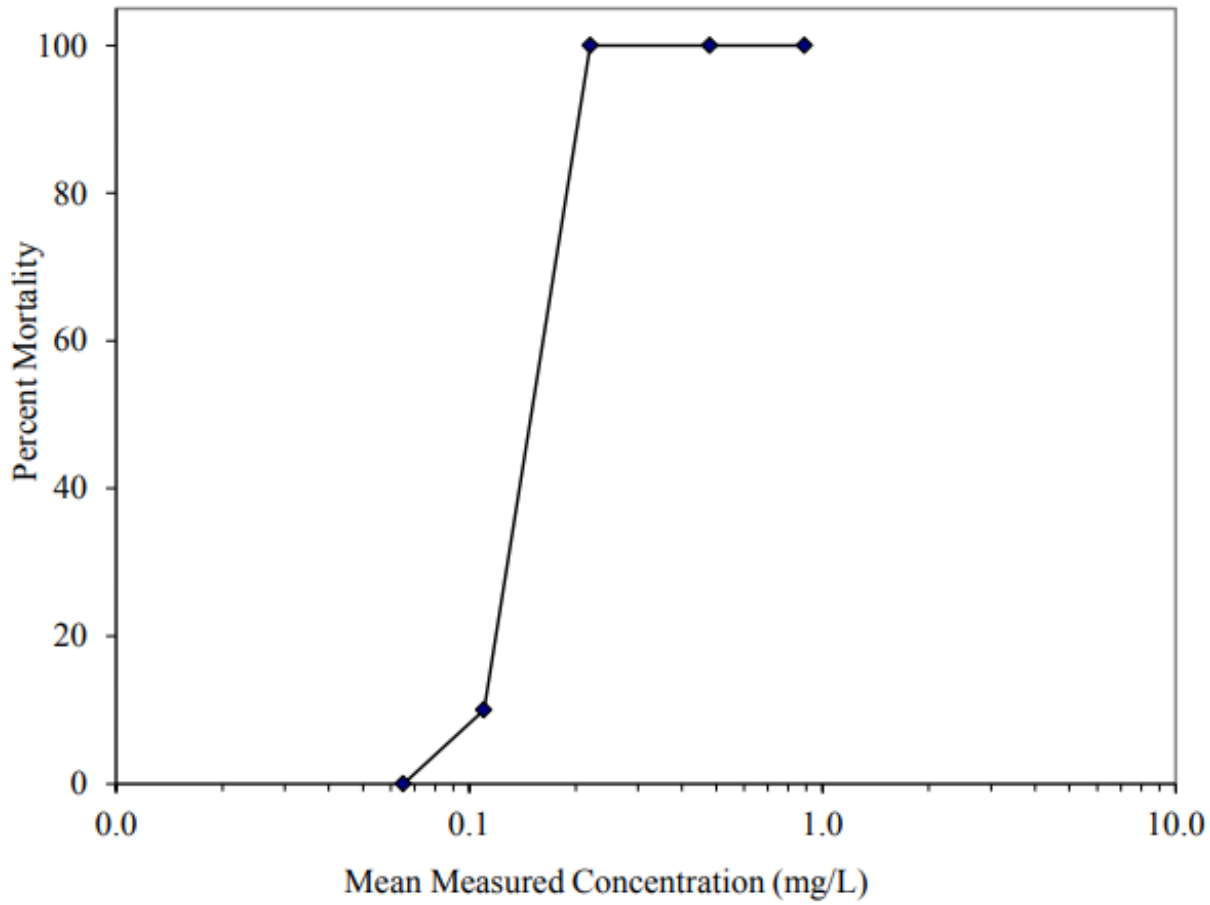


Figure 9.2-5: The 96-hour concentration-response (mortality) curve for sheepshead minnow (*Cyprinodon variegatus*) exposed to S-2399 under static conditions

Chemical analysis

Measured concentrations (Table 9.2.2-22) were generally consistent between sampling intervals and closely approximated nominal concentrations. Mean measured concentrations ranged from 87 to 100% of nominal concentrations.

Table 9.2.2-22: Measured concentrations of S-2399 TG in the exposure solutions

Nominal concentration (mg a.s./L)	Measured concentration (mg a.s./L)[a]			Percent of nominal (%)		
	0 Hour	96 Hour	Mean (SD)	0 Hour[c]	96 Hour[c]	Mean[a]
Control	<0.0050[b]	<0.0050[b]	n.a.	n.a.	n.a.	n.a.
Solvent control	<0.0050[b]	<0.0050[b]	n.a.	n.a.	n.a.	n.a.
0.063	0.061	0.069	0.065 (5.36)	97	110	100
0.13	0.12	0.11	0.11	92	85	87

Nominal concentration (mg a.s./L)	Measured concentration (mg a.s./L)[a]			Percent of nominal (%)		
	0 Hour	96 Hour	Mean (SD)	0 Hour[c]	96 Hour[c]	Mean[a]
			(4.18)			
0.25	0.23	0.21	0.22 (1.32)	92	84	90
0.50	0.48	0.47	0.48 (1.03)	96	94	95
1.0	0.82	0.97	0.89 (1.02)	82	97	89

[a] Mean measured concentrations and mean percent of nominal were calculated using the actual analytical (unrounded) results and not the rounded (two significant figures) values presented in this table.
[b] Concentrations expressed as less than values were below the minimum detectable limit (MDL).
[c] Calculated by HSE based on rounded values provided.
n.a. = not applicable

Validity criteria
The validity criteria for the study were met according to OECD 203 (1992) and OECD 203 (2019) (Table 9.2.2-23).

Table 9.2.2-23: Compliance with OECD 203 validity criteria

Validity criterion	Required	Obtained
Mortality in the control(s)	≤ 10 %	0 %
Test conditions	Constant conditions	A static conditions design was selected. Constant conditions were maintained.
Dissolved oxygen concentration	At least 60 % of the air saturation value throughout the test	Dissolved oxygen concentration > 60 % of the air saturation throughout the test.
Concentration of substance	Analytical measurement of test concentrations is compulsory. At least 80 % of the nominal concentration throughout the test. If the deviation from the nominal concentration is greater than 20 % results should	Measured concentrations in individual replicates ranged from 82 - 110 % of nominal. Results based on mean measurement concentrations, which closely tracked nominal concentrations.

	be based on the measured concentration.	

CONCLUSIONS
The results of the laboratory study demonstrate the 96-hour LC50 of S-2399 TG to sheepshead minnow to be 0.15 mg a.s./L (95% confidence intervals of 0.13 to 0.16 mg a.s./L) and the NOEC to be 0.065 mg a.s./L, based on mean measured concentrations.

HSE COMMENTS
The study was carried out according to GLP and follows OECD 203 (1992), the guideline available at the time of experimentation. The study was evaluated against the most recent OECD 203 guideline (2019). All validity criteria outlined in OECD 203 (2019) were satisfactorily met for the duration of the study. There were no significant deviations to the guideline.

The following deviations were noted:
OECD 203 (2019) paragraph (§) 9 states that the study report method include a description of the apparatus used to carry out the test and appropriate documentation to validate equipment functionality. The equipment used to measure illuminance and TOC was not reported. Appropriate documentation to validate equipment functionality was also omitted. This requirement was not present in OECD 203 (1992) and its omission is unlikely to have affected the study outcome considering the up-to-date GLP certification of the study laboratory. HSE considers this a minor deviation.

OECD 203 (2019) § 10 states, "*the use of silicone tubing in flow-through studies and use of silicone seals in contact with water should be minimised*", due to the strong binding capacity of silicone for lipophilic chemicals. Study aquaria were constructed of glass and silicone adhesive. In addition, when test vessels are placed in the test area they should be shielded from excessive noise, vibration and light. The lack of excessive noise, vibration and light was not documented. The reporting omissions are acceptable due to these requirements not being outlined in OECD 203 (1992). The use of silicone sealant is also not covered in OECD 203 (1992). Its use was unlikely to have impacted the study outcome due to the close alignment of the nominal and time-weighted test concentrations. HSE considers this a minor deviation.

OECD 203 (2019) § 12 describes the age and size of fish to be used. For juvenile *C. variegatus*, length should range between 1.0-2.0 cm. In the study, fish length ranged from 1.2-2.3 cm. Recommended length was not present for this species in OECD 203 (1992). Although the length of some fish exceeded the recommended range of 1.0–2.0 cm, the overall variation of 1.1 cm is comparable to the 1 cm recommended, suggesting that age matching was appropriate. All test organisms were from the same life stage, control mortality was < 10 % and the acceptable total length variation indicates appropriate age matching. HSE consider this a minor deviation, which is not expected to impact the study outcome.

OECD 203 (2019) § 14 outlines the criteria for test fish population mortality and health during the acclimatisation period. No information was provided detailing signs of disease, stress, malformations or treatments against disease or parasites within 14 days prior to testing. This point was not present in OECD 203 (1992). The study conductors reported no mortality for the seven days prior to exposure initiation, as well as no mortality or sub-lethal effects during the exposure period in the test dilution water controls. indicating the health of the test fish population was acceptable. HSE consider this a minor deviation, which is not expected to impact the study outcome.

OECD 203 (2019) § 16 details required chemical testing of dilution water. Within this paragraph it states, "*analyses of nitrate and chlorine should be performed on each batch of dilution water to demonstrate that the limits specified in Annex 3 are not exceeded*". This was not reported in the study. This was not a requirement in OECD 203 (1992) and the lack of control mortality suggests its omission is not expected to impact the study outcome. HSE considers this a minor deviation.

OECD 203 (2019) § 17 outlines test solution preparation requirements. It states, *"the use of solvents should be avoided and only used as a last resort in order to produce a suitably concentrated stock solution".* The study conductor dissolved the test substance in acetone when preparing the primary stock solution. The solubility of S2399 in water was not provided, which precludes determining whether solvent use necessary. Within OECD 203 (1992) the requirement to minimise solvent use was not present. The lack of mortality or sub-lethal effects in the solvent control indicates the use of acetone is not expected to impact the study outcome. HSE considers this a minor deviation.

OECD 203 (2019) § 19 concerns the conditions of exposure. It states, *"temperature…should be within the temperature ranges specified for the test species (Annex 2)"*. The temperature range stated for *C. variegatus* (23-27°C) differs from that used during the exposure period (21-23°C). This deviation stems from the omission of *C. variegatus* from the recommended species list in OECD 203 (1992). The lack of mortality or sub-lethal effects in the controls suggest this deviation is not expected to impact the study outcome. HSE considers this a minor deviation.

OECD 203 (2019) § 20 details sample size and replication requirements. It states, "*a minimum of 7 fish must be used at each test concentration and in the control(s)… no test tank replication is required*". The study used 20 fish for each concentration and control, comprising of ten fish in two replicates. In line with ethical considerations regarding the reduction of fish used in ecotoxicological testing, this degree of replication was unnecessary. Furthermore, the use of two replicates was not required under the guidance of OECD 203 (1992) either. Although this deviation did not impact study validity, HSE notes the unnecessarily large sample size conflicts with the aim to reduce the number of animals used in vertebrate testing.

OECD 203 (2019) § 21 concerns integrating existing sources of information into test concentration selection if such information is available. The study provided no evidence of this approach being attempted. This was not a requirement under OECD 203 (1992) and this deviation is not expected to impact the study outcome. HSE considers this a minor deviation.

OECD 203 (2019) § 23 profiles control requirements. It states, "*the dilution water control can be omitted, and the test conducted and evaluated with a solvent control only, provided it is appropriate when considering the needs for these data and the requirements of the relevant regulatory authorities*". It could be argued that the water dilution control was useful for demonstrating that test conditions did not affect the test subjects. However, the lack of effect of 0.1 mL/L acetone in fish is well described. It is the opinion of HSE that the need to minimise vertebrate testing outweighs the additional information the dilution water control provided. Therefore, the dilution water control was not strictly necessary. This was, however, not covered under OECD 203 (1992), which stipulates the requirement of a solvent control if a solvent is used. Therefore, this deviation is acceptable.

OECD 203 (2019) § 26, covering the frequency of biological observation and recording, states, "t*o the extent feasibly possible, a minimum of 2 observations should be conducted within the first 24 hours of the study"*. The study only performed one observation within the first 24 hours (6-hour). Also, "*on days 2-4 of the test, all vessels with living fish should be inspected twice per day*". The study only performed one observation period a day for days 2-4. Under the enacted observation programme, however, some transient sub-lethal effects may have been omitted. Again, these requirements were not present in OECD 203 (1992) making these deviations acceptable.

OECD 203 (2019) § 28, concerning the fate of test fish, was not addressed in the study report. Furthermore, § 29 requires fish to be measured within one week of test commencement. When fish were measured, in relation to the start of the test, was not detailed. These requirements were not present within OECD 203 (1992) and their omission is deemed acceptable.

OECD 203 (2019) § 33 outlines the requirements for the test report, including the reporting of test substance physico-chemical properties. The study report did not provide the required physico-chemical properties or a structural formula. A purity of 95 % for the test substance was given. However, the chemical identity of the 5 % impurities was not provided. Analytical measurement at exposure initiation and completion confirmed the presence of the test substance in the test solution indicating the omission of the physico-chemical properties did not impact test validity. Furthermore, nominal and mean measured concentrations were in close agreement. HSE considers this a minor deviation.

Although not a deviation HSE notes two initial definitive exposures were initiated and terminated due to high analytical recoveries of one concentration at each exposure initiation (0.13 and 0.25 mg/L respectively).

The method of analysis used in the study was evaluated by HSE Chemistry. The conclusion of their evaluation is reproduced below. Please see Volume 3 CA, section B5 for more details.

"*The analytical method is not fully validated according to SANTE/2020/12830 rev. 1 for the determination of the active substance inpyrfluxam in freshwater as the matrix effects have not been determined and the stability of standards and extracts has not*

been addressed. However, the studies were generated prior to the implementation of SANTE 2020/12830 rev.1 and SANCO 3029 rev.4 did not require matrix effects and the stabilities of standards and extracts to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose."

The above study was conducted to GLP and considered valid.

The agreed endpoint suitable for use in the risk assessment is: 96-hour LC_{50} = 0.15 mg S-2399/L.

Reference:	KCA 8.2.1/06
Report Title:	Acute Toxicity Study of S-2399 TG with Guppy (*Poecilia reticulata*)
Author(s) & year:	[redacted] (2016c)
Document No, Authority registration No:	[redacted] Study No. 1603EFAG
Substance used:	S-2399 TG (13CG0617G, 95.0%)
Method of analysis:	Reversed-phase HPLC
Guideline(s):	OECD 203 (1992)
Deviations:	Yes, see HSE comments section.
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

II. MATERIALS AND METHODS

A. MATERIALS

1. **Test material:** S-2399 TG
   Description: White solid
   Lot/Batch: 13CG0617G
   Purity: 95.5 %

2. **Toxic reference:** Not tested

3. **Solvent:** N,N-dimethylformamide (DMF) 100 µL/L

B. STUDY DESIGN AND METHODS

1. **Test animals:** Guppy (*Poecilia reticulata*)
   Size at start

	Of test:	21 – 25 mm (2.1 – 2.5 cm)
	Diet:	Commercial feed, Tetramin ([redacted]) 5 times a week (i.e. daily from Monday to Friday). The amount of feed supplied was ca 5 % of body weight/day. No feeding 48 hours prior to test item exposure)
	Source:	In-house culture
	Acclimation:	12 days in the same water conditions as the test
	Loading:	10 individuals per vessel
	Culture Conditions:	Listed as the same as test conditions
	Culture mortality:	0 % during 7 days prior to exposure.
2.	**Dilution water:**	Tap water (Takarazuka City, Hyogo) dechlorinated with charcoal filter. Residual chlorine less than the limit of detection (< 0.05 ppm).
	Hardness:	39 mg $CaCO_3$/L
	Alkalinity:	57 mg/L
	Total organic Content:	< 0.05 mg/L
	Specific Conductivity:	24 mS/m
3.	**Test vessels:**	5 L whole glass aquarium (inside size ca. 21 D x 16 W x 23 H cm); covered with a transparent plastic lid
4.	**Environmental conditions:**	
	Temperature:	23.1 – 23.5 °C
	pH:	7.8 – 8.2
	Photoperiod:	16 hours light/8 hours dark
	Light intensity:	Not stated
	Aeration:	Not conducted
	Oxygen Saturation:	6.8 – 8.0 mg/L
	Feeding:	None

5. Animal assignment and treatment:

The study was conducted in a static system with 5 concentrations of 0.19, 0.28, 0.42, 0.61 and 0.90 mg S-2399 TG/L, a solvent control and a dilution control. Each treatment had 2 replicates with 20 individuals tested per concentration. These were divided into two separate vessels containing 10 individuals per vessel. The duration of the test was 96 hours.

No feeding occurred during testing.

6. Dose preparation:

Prior to the definitive test, a range-finding test was conducted at nominal concentrations of 0.10, 0.30 and 0.90 mg S-2399 TG/L. The environmental conditions were similar to the definitive test with 5 individuals per treatment group.

Based on the results of the range-finding test, the following nominal concentrations were selected for the definitive test: 0.19, 0.28, 0.42, 0.61 and 0.90 mg S-2399 TG/L. There was also a control group of dilution water only, and a solvent control with dilution water containing DMF at the same concentration as the test item treatment groups (100 µL/L). No mortality or toxic symptoms were observed at 0.10 mg S-2399/L and 100 % mortality was observed at 0.90 mg S-2399/L.

A 0.2095 g aliquot of the test substance was weighed into a 10 mL volumetric flask and diluted to the volume with DMF to prepare a 20 mg S-2399/mL stock solution. This stock solution was further diluted down with DMF to prepare stock solutions of 9.0, 6.1, 4.2, 2.8 and 1.9 mg S-2399/mL. A 0.50 mL aliquot of each stock solution was spiked into a 5.0 L of the dilution water under stirring to prepare each test solution. The appearance of the test solutions was recorded just after preparation.

7. Measurements and observations:

Fish were observed for mortality and toxic symptoms at 6, 24, 48, 72 and 96 hours after initiation of exposure. When dead fish were found, they were removed immediately to prevent water quality deterioration. Fish were defined as being dead if no breathing movements were visible and touching of the caudal peduncle produced no reaction. Behaviour of the fish was compared between the solvent control and the dilution water control to confirm the effect of the solvent.

Dissolved oxygen, temperature and pH of the test solutions in all groups were measured once daily. At the same time, the appearance of the test solutions was recorded.

At termination of the definitive exposure, 20 surviving fish were taken out and their total lengths and body weights measured individually.

Analytical samples were taken from all test groups and measured at initiation and termination of the definitive exposures. The same quantity of test solution of each replicate was combined and analysed per test group (n = 1). Quality Control (QC) samples were taken at the initiation and termination of definitive exposure to validate the analytical procedure. QC samples were prepared by fortifying the solvent control test solution with the standard solutions and their concentrations were the lower (0.0200 mg S-2399/L at the initiation of exposure) and the upper (5.00 mg S-2399/L at the termination of exposure) limits of quantification.

8. Statistics:

Appropriate calculation methods were selected based on mortality. A computer program (StatLight regression analysis, version 2.00, Yukms Co. Ltd.) was used to perform the statistical analysis including Probit, Moving average or Binominal method. Where these three methods were not applicable, the Doudoroff method of statistical analysis was used.

II. RESULTS AND DISCUSSION

Definitive experimental start and end dates: 25th July – 29th July 2016

A. VALIDITY CRITERIA

Control mortality: The mortality in the control groups should not exceed 10 % at the exposure termination. During this study, the mortality at test termination in the dilution control was 0 % and 0 % in the solvent control.

Dissolved oxygen Concentration: The dissolved oxygen concentration should be at least 60 % of the air saturation value throughout the exposure (saturation value at 23.0 °C = 8.39 mg/L). During this study the dissolved oxygen concentration ranged from 6.8 – 8.0 mg/L.

The validity criteria of the study were all met.

B. BIOLOGICAL EFFECTS

Mortalities (%) were calculated for each test group by dividing the number of dead fish by the number of fish tested.

Cumulative mortalities were 0, 0, 65, 100 and 100 % in the guppy exposed to S-2399 TG for 96 hours at the mean measured concentrations of 0.16, 0.24, 0.37, 0.59 and 0.87 mg S-2399/L. As toxic symptoms, slow swimming, stimulation swimming, loss of equilibrium and lethargic were observed at, and above, 0.24 mg S-2399/L.

Cumulative mortalities and toxic symptoms at each observation are presented in Table 9.2.2-24. The concentration-mortality curve is presented below.

Table 9.2.2-24: Cumulative mortalities and toxic symptoms of guppy exposed to S-2399 TG

Nominal concentration [mg S-2399 TG/L]	Replicate	Cumulative mortality [%] (Toxic symptom)				
		6-hour	24-hour	48-hour	72-hour	96-hour
Dilution water control	(1)	0 (NA:10)	0 (NA:10)	0 (NA:10)	0 (NA:10)	0 (NA:10)
	(2)	0 (NA:10)	0 (NA:10)	0 (NA:10)	0 (NA:10)	0 (NA:10)
	Mean	0	0	0	0	0
Solvent control #1	(1)	0 (NA:10)	0 (NA:10)	0 (NA:10)	0 (NA:10)	0 (NA:10)
	(2)	0 (NA:10)	0 (NA:10)	0 (NA:10)	0 (NA:10)	0 (NA:10)
	Mean	0	0	0	0	0
0.19 [0.16]	(1)	0 (NA:10)	0 (NA:10)	0 (NA:10)	0 (NA:10)	0 NA:10)

Nominal concentration [mg S-2399 TG/L]	Replicate	Cumulative mortality [%] (Toxic symptom)				
		6-hour	24-hour	48-hour	72-hour	96-hour
	(2)	0 (NA:10)	0 (NA:10)	0 (NA:10)	0 (NA:10)	0 (NA:10)
	Mean	0	0	0	0	0
0.28 [0.24]	(1)	0 (NA:10)	0 (NA:10)	0 (NA:10)	0 (NA:10)	0 (NA:10)
	(2)	0 (NA:10)	0 (NA:8, SS:2)	0 (NA:8, SS:1, LE:1)	0 (NA:8, SS:1, LE:1)	0 (NA:9, SS:1,LE:1)
	Mean	0	0	0	0	0
0.42 [0.37]	(1)	0 (SS:3, ST:1, LT:6)	30 (SS:2, ST:1, LT:4)	60 (SS:3, LT:1)	60 (SS:3, LT:1)	60 (SS:3, LT:1)
	(2)	0 (SS:4, ST:4, LT:2)	30 (SS:4, LT:3)	60 (SS:2, LT:2)	70 (SS:2, LT:1)	70 (SS:2, LT:1)
	Mean	0	30	60	65	65
0.61 [0.59]	(1)	100 (-)	100 (-)	100 (-)	100 (-)	100 (-)
	(2)	90 (LT:1)	100 (-)	100 (-)	100 (-)	100 (-)
	Mean	95	100	100	100	100
0.90 [0.87]	(1)	100 (-)	100 (-)	100 (-)	100 (-)	100 (-)
	(2)	100 (-)	100 (-)	100 (-)	100 (-)	100 (-)
	Mean	100	100	100	100	100

[ ]: Mean measured concentration
#1: DMF 100 µL
Number of organisms tested: 20 individuals/test group (10 individuals/vessel)
Category of toxic symptoms:
NA: Normal; SS: Slow swimming; ST: Stimulation swimming; LE: Loss of equilibrium; LT: Lethargic

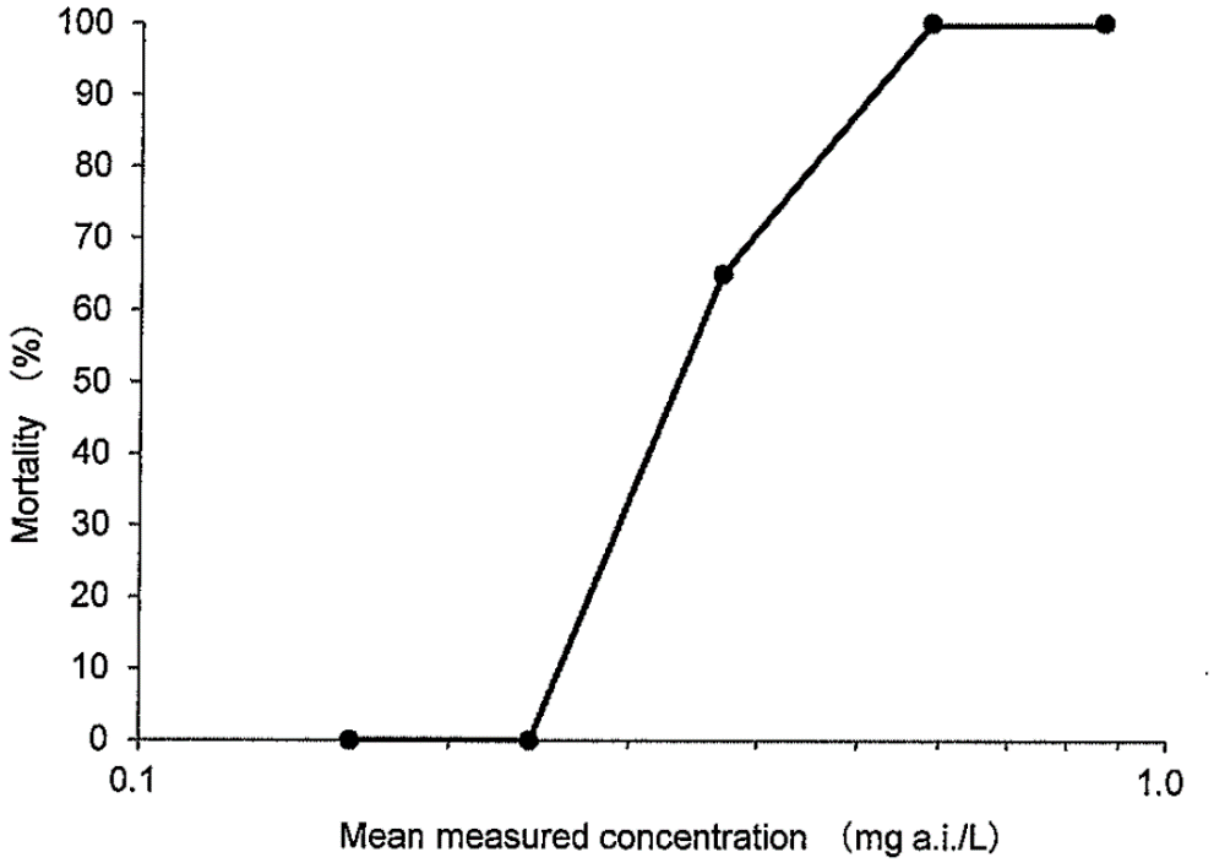


Figure 9.2-6: Concentration-mortality curve of guppy exposed to S-2399 TG at termination of exposure

C. ANALYSIS

Measured concentrations of S-2399 TG were 96 – 98 % and 72 – 96 % of the nominal concentrations at the initiation and termination of exposure, respectively. Mean measured concentrations were 0.16, 0.24, 0.37, 0.59 and 0.87 mg S-2399/L which were 84 – 97 % of the nominal concentrations. Recoveries of 0.0200 and 5.00 mg S-2399/L Quality Control samples were 96.0 and 102 %, respectively.

The measured concentrations in the dilution water and the solvent controls were less than the quantification limit (0.0200 mg S-2399/L) with no detectable peak at the elution position of S-2399.

S-2399 TG concentrations in the test solutions at the initiation and termination of exposure are shown in Table 9.2.2-25.

Table 9.2.2-25: Concentrations of S-2399 in the test solutions

Nominal concentration [mg S-2399 TG/L]	Measured concentration [mg S-2399 TG/L][#2]		
	0-hour	96-hour	Mean
Dilution water	< 0.0200[#1]	< 0.0200[#1]	-

Nominal concentration [mg S-2399 TG/L]	Measured concentration [mg S-2399 TG/L]#2		
	0-hour	96-hour	Mean
control			
Solvent control #3	< 0.0200#1	< 0.0200#1	-
0.19	0.19 [98]	0.14 [74]	0.16 [85]
0.28	0.28 [98]	0.20 [72]	0.24 [84]
0.42	0.41 [97]	0.34 [81]	0.37 [89]
0.61	0.60 [98]	0.59 [96]	0.59 [97]
0.90	0.86 [96]	0.87 [96]	0.87 [96]

[ ] : Percentage to the nominal concentration (%)
#1: Less than quantification limit
#2: Measured concentrations and percent of nominal concentrations in the raw data have 3 significant figures. The values were rounded off to 2 significant figures to present in this table
#3: DMF 100 µL
Recoveries of 0.0200 and 5.00 mg S-2399/L Quality Control samples were 96.0 and 102 %, respectively.

III. CONCLUSION

The maximum concentration that produced no mortality (LC_0) and the concentration that produced 100 % mortality (LC_{100}) were determined. In addition, the highest concentration that showed no difference in sublethal or lethal effects from the controls was determined as the NOEC.

The LC_{50} value of S-2399 TG for guppy after 96 hours of exposure is as follows on the basis of the mean measured concentrations: LC_{50} (96-hours) = 0.35 mg S-2399 TG/L (95 % confidence limit: 0.32 – 0.39 mg S-2399 TG/L); LC_0 = 0.24 mg S-2399 TG/L; LC_{100} = 0.59 mg S-2399 TG/L; NOEC = 0.16 mg S-2399 TG/L.

HSE COMMENTS

This study was conducted to GLP standard and in accordance with the OECD 203 (1992) guidance. However, HSE have evaluated the study in accordance with the OECD 203 (2019) guidance being the most up to date version. As the study was conducted in 2014 the latest guidance would not have been available. According to the OECD 203 (2019) there are no significant deviations. All validity criteria were satisfactorily met.

The length of the fish used in this study ranged from 2.1 to 2.5 cm. The OECD 203

(2019) guidelines recommend a range of between 1 – 3 cm and the 1992 guidelines recommend a length of 2.0 ± 1.0 cm. Therefore, the size of the fish under the guidelines available at the time of testing is acceptable. Given that all the validity criteria were met this is not deemed a significant deviation.

The tap water used as dilution water was analysed for contaminants and water quality. The analysis would have been conducted against the 1992 guidelines which requires a water hardness of between 10 and 250 mg $CaCO_3$ (actual 39 mg $CaCO_3$) and a pH of between 6.0 – 8.5 (actual 7.7). The 2019 guidelines require more detailed analysis of which some of the results do not meet the required levels. However, as the 1992 guideline requirements are met and there is no mortality in either control group it can be assumed that the water was of a suitable quality.

The recovery analysis showed that the measured concentration was not maintained within ± 20 % (80 – 120 %) of the nominal concentration and therefore the endpoints will be based on the mean measured concentrations.

Mortalities were observed in all test concentrations except the lowest concentration tested, 0.16 mg S-2399/L. Adverse effects were observed in all concentrations at, and above, 0.24 mg S-2399/L. The LC_{50} was determined using probit analysis which is an appropriate statistical method for this data set. The confidence limits were within a range that indicates the LC_{50} is reliable and is a good fit for the dose-response curve.

The method of analysis used in the study was evaluated by HSE Chemistry. The conclusion of their evaluation is reproduced below. Please see Volume 3 CA, section B5 for more details.

"*The analytical method is not fully validated according to SANTE/2020/12830 rev. 1 for the determination of the active substance inpyrfluxam in water as the matrix effects have not been determined and the stability of standards has not been addressed. However, the studies were generated prior to the implementation of SANTE 2020/12830 rev.1 and SANCO 3029 rev.4 did not require matrix effects and the stabilities of standards to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose.*"

The agreed endpoint suitable for use in the risk assessment is:
LC_{50} (96-hours) = 0.35 mg S-2399/L (mean measured)

Reference:	KCA 8.2.1/07
Report Title:	Acute Toxicity Study of S-2399 TG with Japanese medaka (*Oryzias latipes*)
Author(s) & year:	[redacted] (2016a)
Document No, Authority registration No:	[redacted] Study No. 1603EFAM

Substance used:	S-2399 TG (13CG0617G, 95.0%)
Method of analysis:	Reversed-phase HPLC
Guideline(s):	OECD 203 (1992)
Deviations:	Yes, see HSE comments section.
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test material:** S-2399 TG
   Description: White solid
   Lot/Batch: 13CG0617G
   Purity: 95.5%
   Reference item: None
   Solvent carrier:` N-N dimethylformamide (DMF)

B. STUDY DESIGN AND METHODS

1. **Test animals:** Japanese medaka (*Oryzias latipes*)
   Wet weight: Mean: 0.060g (range: 0.048 to 0.092 g)
   Length: Mean: 20 mm (range: 18 to 21 mm)
   Source: In-house culture
   Acclimation: 12 days, under the same conditions as during the test
   Diet: Commercial feed, Tetramin 5 times a week ca 5% of body weight/day (not fed during the 48 hours prior to test initiation or during the exposure period)
   Treatment of disease: None
   Test concentrations: Nominal concentrations: 0.43, 0.63, 0.93, 1.4, 2.0 mg a.s./L
   LOQ = 0.0200 mg a.s./L. LOD = 0/0125mg a.s./L.

   Statistical analysis: Computer program StatLight regression analysis v.2.00 was used to perform statistical analysis including Probit, Moving average or Binomial method

2. **Dilution water:** Dechlorinated tap water
   Hardness: 40 mg $CaCO_3$/L
   Alkalinity: 57 mg $CaCO_3$/L
   pH: 7.7

3. **Test vessels:** 5 L volume whole glass aquarium (21D x 16W x

	23H cm) covered with a transparent plastic lid
Fish per tank:	10 (20 fish per concentration)
Mean loading weight:	None
Exposure regime:	Static
Chemical analysis:	S – 2399TG concentrations in all tested groups were measured at initiation (0 hours) and termination (96 hours) of definitive exposure.

4. Environmental conditions:

Requirements for environmental variables from OECD 203(2019) and a summary of values obtained in this study are outlined in Table 9.2.2-26.

Table 9.2.2-26: Summary of environmental conditions

Variable	Required OECD 203 (2019)	Obtained
Temperature	23˚C – 27˚C	23.1℃– 23.5°C
pH	6.0 – 8.5	7.8 – 8.2
Dissolved oxygen concentration	≥60% of the air saturation value	6.7 – 8.0 mg/L
Photoperiod	12-16 hours	16 hours light: 8 hours darkness
Lighting intensity	n/a	n/a
Hardness of dilution water	40 – 250 mg/L CaCO3 (preferably <180)	40 mg $CaCO_3$/L
Alkalinity of dilution water	n/a	57 mg $CaCO_3$/L
Conductivity of dilution water	≤ 10 µS/cm	2.4 µS/cm
Toral organic Carbon (TOC) mg/L	<2mg/L	< 0.5mg/L

STUDY DESIGN AND METHODS:

Study dates: July 25th 2016 – July 29th 2016 (definitive exposure).

5. Animal assignment and treatment:

20 fish were impartially selected and distributed to two replicate test vessels (10 per replicate) for each test item concentration, as well as for the control and solvent control. The fish were exposed for 96 hours under static conditions to nominal test concentrations 0.43, 0.63, 0.93, 1.4 and 2.0 mg a.s./L.

6. Dose preparation:

At exposure initiation, a 20 mg a.s./mL primary stock solution was prepared by placing 0.2095 g of S-2399 TG in 10 mL of dimethylformamide (DMF). This stock solution was further diluted with DMF to prepare stock solutions of 4.3, 6.3, 9.3, and 14 mg a.s./L. A 0.50 mL aliquot of each stock solution was spiked into 5.0 L of the dilution water

under stirring to prepare each test solution. Appearance of the test solutions were recorded just after preparation. The control group contained only dilution water and the solvent control consisted of dilution water containing DMF at the same concentration as the exposure groups (100µL/L).

7. Measurements and observations:

All aquaria were examined after 6, 24, 48, 72 and 96 hours for mortality (dead fish were removed) and toxic symptoms. Fish were defined as being dead if no breathing movements were visible and touching of the caudal peduncle produced no reaction. Behaviour of fish was compared between the solvent control and the dilution water control to confirm the effect of solvent.

At exposure termination, the surviving fish were taken out and their total lengths and body weights were measured individually.

The pH, dissolved oxygen concentration and temperature were measured daily in each replicate of each treatment and control. At the same time, appearance of the test solutions was recorded.

At exposure initiation (0 hour) and exposure termination (96 hour), test solution was taken from mid-depth of each vessel without agitation. The same quantity of each replicate was combined and analysed per test group. Samples were analysed for S-2399 TG concentration by reversed-phase HPLC. LOQ = 0.0200 mg a.s./L. LOD = 0/0125mg a.s./L.

8. Statistics:

The LC_{50} values and 95% confidence intervals, if appropriate, were calculated using the mean measured concentrations and the corresponding mortality data. If at least one concentration resulted in ≥ 50% mortality, then the computer program StatLight regression analysis v.2.00 was used to perform statistical analysis including Probit, Moving average or Binomial method. The maximum concentration that produced no mortality (LC_0) and the minimum concentration that produced 100% mortality (LC_{100}) were determined. In addition, the highest test concentration that showed no difference in sublethal or lethal effects from the controls was determined as NOEC.

II. RESULTS AND DISCUSSION

A. MORTALITY AND SUBLETHAL EFFECTS

Following 24 hours of exposure, 100% mortality was observed in 2.0 mg a.s./L treatment level. At exposure termination (96 hours), 50% mortality was observed among fish exposed to the 0.93 mg a.s./L treatment level, and 100% mortality was observed among fish exposed to the 1.4 and 2.00 mg/L treatment levels. No mortality was observed in the 0.63 mg a.s./L treatment group but slow swimming was noted in two fish. No mortality or adverse effects were observed among fish exposed to the remaining treatment level (0.43 mg a.s./L) or the controls. A summary of the cumulative percent mortalities and observations is presented in Table 9.2.2-27 below.

Table 9.2.2-27: Summary of mortalities and observations of *Oryzias latipes* following exposure to S-2399 TG

Mean measured concentration (mg a.s./L)	Cumulative percent mortality (%)				
	6 hour	24 hour	48 hour	72 hour	96 hour
Control	0 NA:20	0 NA:20	0 NA:20	0 NA:20	0 NA:20
Solvent control	0 NA:20	0 NA:20	0 NA:20	0 NA:20	0 NA:20
0.35	0 NA:20	0 NA:20	0 NA:20	0 NA:20	0 NA:20
0.52	0 NA:20	0 NA:18, SS:2	0 NA:18, SS:2	0 NA:18, SS:2	0 NA:18, SS:2
0.79	0 SS:20	15 SS:15, LT:1, LE:1	45 SS:10, LT:1	50 SS:10	50 SS:10
1.3	20 SS:2, LE:11 LT:3	95 LT:1	100	100	100
1.9	100	100	100	100	100

NA: Normal
SS: Slow swimming
LE: Loss of equilibrium
LT: Lethargic

The curve showing percentage mortality against mean measured concentration (mg as/L) is shown below.

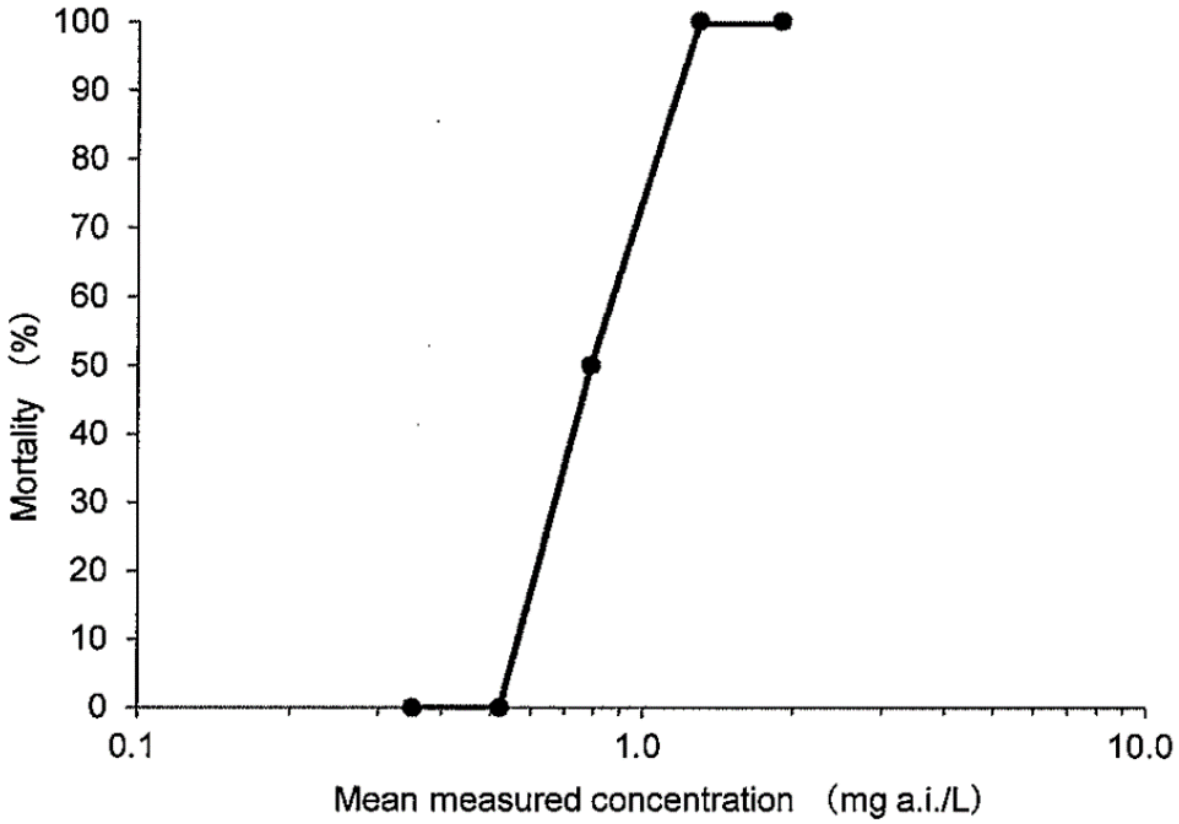


Figure 9.2-7: Percentage mortality against mean measured concentration (mg a.s./L) of Japanese Medaka (*Oryzias latipes*) exposed to S-2399TG under static conditions S-2399TG under static conditions

A summary of the toxicity endpoints determined from the study is presented in Table 9.2.2-28.

Table 9.2.2-28: Summary of endpoints for *Oryzias latipes* following exposure to S-2399 TG

Time	LC_{50} (mg a.s./L)	95% confidence interval	
		Lower (mg a.s./L)	Upper (mg a.s./L)
24-hour	0.96	0.86	1.1
48-hour	0.81	0.73	0.89
72-hour	0.79	0.72	0.87
96-hour	**0.79**	0.72	0.87
96 hour NOEC = 0.35 mg a.s./L			
Highest concentration producing 0% mortality = 0.52 mg a.s./L			
Lowest concentration producing 100% mortality = 1.3 mg a.s.			

B. ANALYSIS

Mean measured concentrations ranged from 81 to 95% of nominal concentrations and defined the treatment levels tested as 0.35, 0.52, 0.79, 1.3 and 1.9 mg a.s./L. The results of the analysis of the exposure solutions for S-2399 TG are presented in Table 9.2.2-29.

Table 9.2.2-29: Measured concentrations of S-2399 TG in the exposure solutions

Nominal concentration (mg a.s./L)	Measured concentration (mg a.s./L)[a]			Percent of nominal[a] (%)
	0 Hour	96 Hour	Mean (SD)	
Control	<0.0200 [b]	<0.0200 [b]	n.a.	n.a.
Solvent control	<0.0200 [b]	<0.0200 [b]	n.a.	n.a.
0.43	0.41	0.29	0.35	81
0.63	0.61	0.43	0.52	82
0.93	0.87	0.71	0.79	85
1.4	1.3	1.3	1.3	92
2.0	1.9	1.9	1.9	95

[a] Mean measured concentrations and percent of nominal in the raw data have 3 significant figures. The values were rounded off to 2 significant figures to present in this table.
[b] Concentrations expressed as less than values were below the minimum detectable limit (MDL).
n.a. = not applicable

C. VALIDITY CRITERIA

As there were no mortalities in the controls and dissolved oxygen remained above 60%, the study was considered valid.

Validity criteria for study are detailed in Table 9.2.2-30.

Table 9.2.2-30: Validity criteria as per OECD 203 (2019) guidance

Validity criterion	Required	Obtained
Mortality in the control(s)	<10 %	No mortality
Dissolved oxygen concentration	≥ 60% of air saturation value	Maintained ≥ 60% saturation for temperature range
Analytical measurement of test concentrations	Analysis of the highest and lowest test concentration and a concentration around the expected LC50 is considered the minimum	S – 2399TG concentrations in all tested groups were measured at initiation (0 hours) and termination (96 hours) of

	requirement	definitive exposure. Recovery range of nominal concentrations were 81 – 95%.

II. CONCLUSION

The results of the laboratory study demonstrate the 96-hour LC_{50} of S-2399 TG to the Japanese medaka to be 0.79 mg a.s./L (95% confidence intervals of 0.72 to 0.87 mg a.s./L) and the NOEC to be 0.35 mg a.s./L, based on mean measured concentrations.

HSE COMMENTS:
The study was carried out in accordance with GLP under OECD 203 (1992) guidance available at the time and has been checked against current OECD 203 guidance (2019).

The concentrations of the test item were maintained between 80-120% of the nominal value throughout the test. The method of analysis used in the study was evaluated by HSE Chemistry. The conclusion of their evaluation is reproduced below. Please see Volume 3 CA, section B5 for more details.

"*The analytical method is not fully validated according to SANTE/2020/12830 rev. 1 for the determination of the active substance inpyrfluxam in water as the matrix effects have not been determined and the stability of standards has not been addressed. However, the studies were generated prior to the implementation of SANTE 2020/12830 rev.1 and SANCO 3029 rev.4 did not require matrix effects and the stabilities of standards to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose.*"

There are no stated deviations from guidance available at the time of study in OECD 203 (1992), but there are some areas to highlight against the newest guidance in OECD 203 (2019). The first minor consideration is the length of fish used in the study. OECD 203 (1992) gives the recommended length of Japanese medaka (*Oryzias latipes)* as 1-3cm, whereas newest guidance in OECD 203 (2019) gives a recommended length for this species as 1-2cm. The largest fish recorded in this study was 2.1cm. As the mean length was still within recommended guidelines and the largest fish being only 1mm longer than recommended, it should not have had a significant impact on the study.

It is also worth noting that no data could be found relating to the loading weight of fish used in this study. However, as the mean wet weight per tank works out at around 0.6g in total (mean wet weight = 0.006g x 10 fish per 5L tank), this should not exceed the loading recommendations in OECD 203 (2019) of 0.8g wet weight fish / L.

Whilst not a requirement of OECD 203 (1992) or OECD (2019), no reference item was used in this study; therefore, species sensitivity is uncertain.

This study used dechlorinated tap water, which is permitted under OECD 203 (2019)

guidance: however, there is a requirement to demonstrate that there was no impact on the survival, growth and reproduction of the test organism and they exhibited no other signs of stress. It is also required that analyses of nitrate and chlorine should be performed on each batch of dilution water. It is not stated that these factors were checked specifically, but as there were no mortalities or observable effects in the control groups and residual chlorine in water analysis was less than the maximum concentration in OECD 203 (2019) guidelines, this should be satisfactory as this guidance on dechlorinated water was not available at the time of study in OECD 203 (1992). No data is available for total organic chlorine or nitrate for this study.

All aquaria were examined after 0, 6, 24, 48, 72 and 96 hours for mortality, but latest guidance recommends a minimum of 2 observations should be conducted within the first 24 hours of the study with preferably at least 3 hours between observations. However, this wasn't part of the guidance available at the time in OECD 203 (1992).

There is discrepancy between measurements and observations, with the pH, dissolved oxygen concentration and temperature were measured at 0, 24, 48, 72 and 96 hours, but examined after 0, 6, 24, 48, 72 and 96 hours. This suggests that no pH or dissolved oxygen concentration data was available for mortality examinations at 6 hours and is not included in the variable data for the test.

Computer program StatLight regression analysis v.2.00 was used to perform statistical analysis including Probit, Moving average or Binomial method. OECD 203 (2019) suggests using Moving average (ISO, 1996) or Binomial method ((USEPA, 2002) where an experiment results in only one concentration with partial mortality or no concentration with partial mortality. As only one concentration resulted in partial mortality, these methods of statistical analysis are deemed suitable.

The agreed endpoints for use in risk assessment are:

- **96-hour LC_{50} = 0.79 mg S-2399TG/L (based on mean measured values)**
- **96-hour NOEC = 0.35 mg S-3299TG/L (based on mean measured concentrations)**

Reference:	KCA 8.2.1/08
Report Title:	Acute Toxicity Study of S-2399 TG with Zebrafish (*Danio rerio*)
Author(s) & year:	[redacted] (2016b)
Document No, Authority registration No:	[redacted] Study No. 1603EFAZ
Substance used:	S-2399 TG (13CG0617G, 95.0%)
Method of analysis:	Reversed-phase HPLC
Guideline(s):	OECD 203 (1992)
Deviations:	Yes, see HSE comments section.

GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

III. MATERIALS AND METHODS

C. MATERIALS

1. Test material: S-2399 TG
Description: White solid
Lot/Batch: 13CG0617G
Purity: 95.5 %

2. Toxic reference: Not tested

3. Solvent: *N,N*-dimethylformamide (DMF)

D. STUDY DESIGN AND METHODS

1. Test animals: Zebrafish (*Danio rerio*)
Size at start of test: 22 – 26 mm (2.2 – 2.6 cm)
Diet: Newly hatched brine shrimp nauplii or commercial feed Tetramin ([redacted]) 5 times a week (i.e. daily from Monday to Friday). The amount of commercial feed supplied was ca 5 % of body weight/day (not fed for 48 hours prior to exposure).
Source: In-house culture, originally obtained from [redacted]
Acclimation: Test organisms were acclimated for at least 12 days in the same conditions (water qualities, water temperature, light etc.) as the test.
Loading: 10 individuals per vessel, 20 per treatment group
Culture conditions: See environmental conditions
Culture mortality: Mortality during 7 days prior to exposure was 0 %

2. Dilution water: Tap water (Takarazuka City, Hyogo) dechlorinated with charcoal filter was aerated well and adjusted to test temperature before use as dilution water. Residual chlorine was confirmed to be less than the detection limit (< 0.05 ppm)
Hardness: 42 mg $CaCO_3$/L
Total organic content: < 0.5 mg/L

Specific conductivity: Not reported

3. Test vessels: 5 L volume whole glass aquarium (inside size ca. 21 D x 16 W x 23 H cm) covered with a transparent plastic lid

4. Environmental conditions:

Temperature:	23.0 – 23.2 °C
pH:	7.8 – 8.2
Photoperiod:	16 hour light/8 hours darkness
Light intensity:	Room lighting
Aeration:	Not conducted
Oxygen saturation:	6.9 – 8.2 mg/L ≥ 60 % of saturation
Feeding:	None during testing

5. Animal assignment and treatment:

The study was conducted in a static system with 5 nominal concentrations of 0.10, 0.18, 0.32, 0.56 and 1.0 mg S-2399/L, a dilution water control and a solvent control containing dilution water and DMF at the same concentration as the test item groups (100 µL/L). Each treatment had two separate vessels containing 10 individuals per vessel. The duration of the test was 96 hours.

No feeding occurred during testing.

6. Dose preparation:

Prior to the definitive test a range finding test was conducted under static conditions at nominal concentrations of 0.10, 0.30 and 0.90 mg S-2399/L, a dilution water control and a solvent control (DMF). The environmental conditions were similar to the definitive test with 5 individuals per vessel/per treatment group. No mortality or toxic symptoms was observed at 0.10 mg S-2399/L and 100 % mortality was observed at 0.90 mg S-2399/L.

Based on the results of the range-finding test, the following nominal concentrations were selected for the definitive test: 0.10, 0.18, 0.32, 0.56 and 1.0 mg S-2399/L.

A 0.1047 g aliquot of the test substance was weighed into a 5 mL volumetric flask and diluted to the volume with DMF to prepare a 20 mg S-2399/L stock solution. This stock solution was further diluted with DMF to prepare stock solutions of 10, 5.6, 3.2, 1.8 and 1.0 mg S-2399/mL. A 0.50 mL aliquot of each stock solution was spiked into 5.0 L of the dilution water under stirring to prepare each test solution. Appearance of the test solutions was recorded just after preparation.

7. Measurements and observations:

Fish were observed for mortality and toxic symptoms at 6, 24, 48, 72 and 96 hours after initiation of exposure. When dead fish were found, they were removed immediately. Fish were defined as being dead if no breathing movements were visible and touching of the caudal peduncle produced no reaction. Behaviour of fish was compared between the solvent and control and the dilution water control to confirm the effect of the solvent.

Dissolved oxygen, temperature and pH of the test solutions in all groups were measured once daily. At the same time, the appearance of the test solutions was recorded.

At termination of definitive exposure, 20 surviving fish were taken out and their total lengths and body weights were measured individually.

Analytical samples were taken from all test item groups at initiation (0 h) and termination (96 h) of the definitive exposure. Test solution samples were taken from mid-depth of each vessel without agitation. The same quantity of test solution of each replicate was combined and analysed per test group (n = 1). Sub-samples were collected at the same time and frozen for potential further analysis.

Quality Control (QC) sample measurements were conducted at the initiation and termination of definitive exposure to validate the analytical procedure. QC samples were prepared by fortifying the solvent control test solution with standard solutions and their concentrations were the lower (0.0200 mg S-2399.L at the initiation of exposure) and the upper (5.00 mg S-2399/L) at the termination of exposure) limits of quantification.

8. Statistics:

Appropriate calculation methods were selected based on mortality. A computer program (StatLight regression analysis, version 2.00, Yukms Co., Ltd.) was used to perform the statistical analysis including Probit, Moving average or Binominal method. Where these three methods were not applicable, the Doudoroff method of statistical analysis was used.

II. RESULTS AND DISCUSSION

Definitive experimental start and end dates: 11th July 2016 – 2nd September 2016

A. VALIDITY CRITERIA

Control mortality: The mortality in the control groups should not exceed 10 % at the exposure termination. During this study the

mortality at test termination in the dilution control was 0 % and 0 % in the solvent control.

Dissolved oxygen Concentration: The dissolved oxygen concentration should be at least 60 % of the air saturation value throughout the exposure (saturation value at 23.0 °C = 8.39 mg/L). During this study the dissolved oxygen concentration ranged from 6.9 – 8.2 mg/L

All validity criteria were met.

B. BIOLOGICAL EFFECTS

Mortalities were calculated for each test group by dividing the number of dead fish by the number of fish tested.

Cumulative mortalities were 0, 0, 15, 100 and 100 % in the zebrafish exposed to S-2399 TG for 96 hours at the mean measured concentrations of 0.075, 0.14, 0.24, 0.49 and 0.94 mg S-2399/L. Slow swimming was observed at 0.14 and 0.24 mg S-2399/L. Loss of equilibrium was observed at 0.24 mg S-2399/L, and lethargy was observed at and above 0.24 mg S-2399/L.

Cumulative mortalities and toxic symptoms at each observation are presented in Table 9.2.2-31. The concentration-mortality curve is presented below.

Table 9.2.2-31: Cumulative mortalities and toxic symptoms of zebrafish exposure to S-2399 TG

Nominal concentration [mg S-2399 TG/L]	Replicate	Cumulative mortality [%] (Toxic symptom)				
		6-hour	24-hour	48-hour	72-hour	96-hour
Dilution water control	(1)	0 (NA:10)	0 (NA:10)	0 (NA:10)	0 (NA:10)	0 (NA:10)
	(2)	0 (NA:10)	0 (NA:10)	0 (NA:10)	0 (NA:10)	0 (NA:10)
	Mean	0	0	0	0	0
Solvent control #1	(1)	0 (NA:10)	0 (NA:10)	0 (NA:10)	0 (NA:10)	0 (NA:10)
	(2)	0 (NA:10)	0 (NA:10)	0 (NA:10)	0 (NA:10)	0 (NA:10)
	Mean	0	0	0	0	0
0.10 [0.075]	(1)	0 (NA:10)	0 (NA:10)	0 (NA:10)	0 (NA:10)	0 (NA:10)
	(2)	0 (NA:10)	0 (NA:10)	0 (NA:10)	0 (NA:10)	0 (NA:10)

Nominal concentration [mg S-2399 TG/L]	Replicate	Cumulative mortality [%] (Toxic symptom)				
		6-hour	24-hour	48-hour	72-hour	96-hour
	Mean	0	0	0	0	0
0.18 [0.14]	(1)	0 (NA:9; SS:1)	0 (NA:10)	0 (NA:10)	0 (NA:10)	0 (NA:10)
	(2)	0 (NA:10)	0 (NA:10)	0 (NA:10)	0 (NA:10)	0 (NA:10)
	Mean	0	0	0	0	0
0.32 [0.24]	(1)	0 (SS:6; LE:2; T:2)	0 (SS:8; LE:1; LT:1)	20 (SS:7; LT:1)	20 (SS:5; LE:3)	20 (SS:5; LE:3)
	(2)	0 (SS:7; LE:2; T:1)	0 (SS:9; LE:1)	0 (SS:9; LT:1)	0 (SS:8; LE:2)	10 (SS:8; LT:1)
	Mean	0	0	10	10	15
0.56 [0.49]	(1)	0 (LT:10)	20 (LT:8)	90 (LT:1)	100 (-)	100 (-)
	(2)	20 (LT:8)	40 (LT:6)	80 (LT:2)	100 (-)	100 (-)
	Mean	10	30	85	100	100
1.0 [0.94]	(1)	80 (LT:2)	100 (-)	100 (-)	100 (-)	100 (-)
	(2)	100 (-)	100 (-)	100 (-)	100 (-)	100 (-)
	Mean	90	100	100	100	100

[ ]: Mean measured concentration
#1: DMF 100 µL/L
Number of organisms tested: 20 individuals/test group (10 individuals/vessel)
Category of toxic symptoms: NA: Normal; SS: Slow swimming; LE: Loss of equilibrium; LT: Lethargic

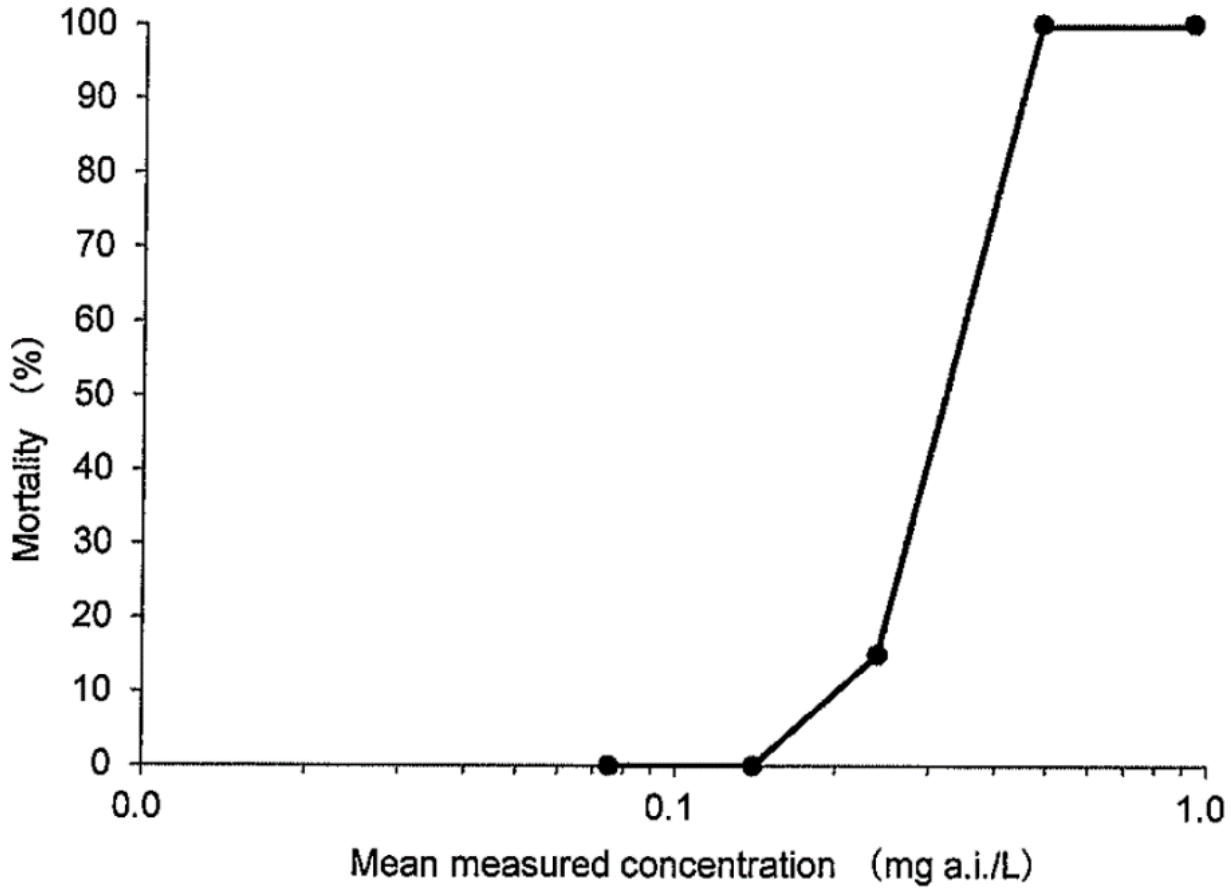


Figure 9.2-8: Concentration-mortality curve of zebrafish exposed to S-2399 TG at termination of exposure

C. ANALYSIS

Measured concentrations of S-2399 TG in the test solution were 93 - 96 % and 58 – 94 % of the nominal concentrations at the initiation and termination of exposure, respectively. Mean measured concentrations were 0.075, 0.14, 0.24, 0.49 and 0.94 mg S-2399/L which were 75 – 94 % of the nominal concentrations. Recoveries of 0.0200 and 5.00 mg S-2399/L QC samples were 95.5 and 102 %, respectively.

The measured concentrations in the dilution water and the solvent controls were less than the quantification limit (0.0200 mg S-2399/L) with no detectable peak at the elution position of S-2399.

S-2399 TG concentrations in the test solutions at the initiation and termination of exposure are shown in Table 9.2.2-32.

Table 9.2.2-32: Concentrations of S-2399 in the test solutions

Nominal concentration [mg S-2399 TG/L]	Measured concentration [mg S-2399 TG/L] #2		
	0-hour	96-hour	Mean
Dilution water control	< 0.0200 #1	< 0.0200 #1	-
Solvent control #3	< 0.0200 #1	< 0.0200 #1	-
0.10	0.095 [95]	0.058 [58]	0.075 [75]
0.18	0.17 [96]	0.11 [59]	0.14 [76]
0.32	0.30 [95]	0.19 [60]	0.24 [76]
0.56	0.52 [93]	0.46 [83]	0.49 [88]
1.0	0.93 [93]	0.94 [94]	0.94 [94]

[ ]: Percentage to the nominal concentration (%)

#1: Less than quantification limit

#2: Measured concentrations and percent of nominal concentrations in the raw data have 3 significant figures. The values were rounded off to 2 significant figures to present in this table.

#3: DMF 100 µL/L

Recoveries of 0.0200 and 5.00 mg S-2399/L QC samples were 95.5 and 102 % respectively

III. CONCLUSION

The maximum concentration that produced no mortality (LC_0) and the concentration that produced 100 % mortality (LC_{100}) were determined. In addition, the highest concentration that showed no difference in sublethal or lethal effects from the controls was determined as the NOEC.

The LC_{50} value of S-2399 TG for zebrafish after 96 hours of exposure is as follows on the basis of mean measured concentrations: LC_{50} (96-hours) = 0.30 mg S-2399/L (95 % confidence limit: 0.27 – 0.33 mg S-2399/L); LC_0 = 0.14 mg S-2399/L; LC_{100} = 0.49 mg S-2399/L; NOEC = 0.16 mg S-2399/L

HSE COMMENTS

This study was conducted to GLP standard and in accordance with the OECD 203 (1992) guidance. However, HSE have evaluated the study in accordance with the OECD 203 (2019) guidance being the most up to date version. As the study was conducted in 2014 the latest guidance would not have been available. According to the OECD 203 (2019) there are no significant deviations. All validity criteria were satisfactorily met.

The length of the fish used in this study ranged from 2.2 – 2.6 cm. The OECD 203 (2019) guidelines recommend a range of between 1 – 3 cm and the 1992 guidelines recommend a length of 2.0 ± 1.0 cm. Therefore, the size of the fish minimally exceeds the maximum length. Given that all the validity criteria were met this is not deemed a significant deviation.

The recovery analysis showed that the measured concentration was not maintained within ± 20 % (80 – 120 %) of the nominal concentration and therefore the endpoint will be based on the mean measured concentrations.

Mortalities were observed in all test concentrations at and above 0.24 mg S-2399/L. At the two highest test concentrations of 0.49 and 0.94 mg S-2399/L 100 % mortality was observed at 96-hours. Adverse effects were observed in all test concentrations at and above 0.14 mg S-2399/L. The LC_{50} was determined using probit analysis which is an appropriate statistical method for this data set. The confidence limits were within a range than indicates the LC_{50} is reliable and is a good fit for the dose-response curve.

The method of analysis used in the study was evaluated by HSE Chemistry. The conclusion of their evaluation is reproduced below. Please see Volume 3 CA, section B5 for more details.

"*The analytical method is not fully validated according to SANTE/2020/12830 rev. 1 for the determination of the active substance inpyrfluxam in water as the matrix effects have not been determined and the stability of standards has not been addressed. However, the studies were generated prior to the implementation of SANTE 2020/12830 rev.1 and SANCO 3029 rev.4 did not require matrix effects and the stabilities of standards to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose.*"

The agreed endpoint suitable for use in the risk assessment is:
LC_{50} (96-hours) = 0.30 mg S-2399/L (mean measured)

B.9.2.2.2 Metabolite: 3'-OH-S-2840

Reference:	KCA 8.2.1/10
Report Title:	Acute Toxicity Study of 3'-OH-S-2840 with Rainbow Trout (*Oncorhynchus mykiss*)
Author(s) & year:	[redacted] (2016a)
Document No, Authority registration No:	[redacted] Study No. 1512EFAR
Substance used:	3'-OH-S-2840, 15SC8508366, 99.5%
Method of analysis:	Reversed-phase HPLC
Guideline(s):	OECD 203 (1992)
Deviations:	Yes, see HSE comments section.
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test material:** 3'-OH-S-2840
   Description: Crystalline powder
   Lot/Batch: 15SC8508366
   Purity: 99.5%
   Reference item: None
   Solvent carrier: N,N-dimethylformamide

B. STUDY DESIGN AND METHODS

1. **Test animals:** Rainbow trout (*Oncorhynchus mykiss*)
   Wet weight: Mean: 0.77 g (0.55 – 1.00 g)
   Length: Mean: 46 mm (43 – 51 mm)
   Source: [redacted])
   Acclimation: ≥ 12 days (0.16% mortality in 7 days prior to exposure)
   Diet: Dry commercial feed, formula feed for trout 1CDX 5 times a week at *ca.* 2% body weight/day. Fish were not fed during the 48 hours prior to test initiation or during the exposure period
   Treatment of disease: None
   Test concentrations: Nominal test concentrations: 1.0, 1.6, 2.6, 4.3 and

		7.0 mg a.s./L LOD = 0.0625mg a.s./L. LOQ = 0.100 mg a.s./L
	Statistical analysis:	None
2.	**Dilution water:**	Tap water (dechlorinated)
	Hardness:	42 mg $CaCO_3$/L
	Alkalinity:	Not stated
	pH:	7.7
3.	**Test vessels:**	20L glass aquaria, 30 x 30 x 30 cm, with a transparent plastic lid and a volume of 10L test solution
	Fish per tank:	10 (20 per concentration replicate)
	Mean loading weight:	None
	Exposure regime:	Static
	Chemical analysis:	QC sample measurements taken at initiation and termination of definitive exposure
	Observations:	Appearance of test solutions clear through preparation of test solutions to termination of exposure. Precipitation observed at 48 hours for 7.0 mg a.s./L and at termination of exposure for 2.6 and 4.3 mg a.s./L. Floating materials observed at 24 hours for 7.0 mg a.s./L.

4. Environmental conditions:

Dissolved oxygen, water temperature and pH of the test solution in all groups were measured once daily and the appearance of test solutions was recorded. A summary of environmental conditions is shown in Table 9.2.2-33. Temperature was controlled with a water bath (FRP tank with controlled heater and cooler). An electronic balance was used for the test substance (XP205V Mettler-Toledo International Inc.) and for weighing the test fish (PB3002-S Mettler-Toledo International Inc.). Dissolved oxygen was measured with Model 58 (YSI/Nanotech Inc.). Water temperature meter (SK-250WP Sato Keiryoki Mfg. Co., Ltd). pH was measured with D-51 (Horiba Ltd.) and water hardness was tested with HA-DT (HACH Company Ltd.). A micrometre calliper CD-15, 500 Series (Mitsutoyo Corporation).

Table 9.2.2-33: Summary of environmental conditions

Variable	Required OECD 203 (2019)	Obtained
Temperature	10 ° C – 14 ° C	13.9 ° C – 14.4 ° C
pH	6.0 to 8.5	7.7 – 8.4
Dissolved oxygen concentration	≥ 60 % of air saturation value	7.8 – 9.0 mg / L
Photoperiod	12-16 hours daily	16 hours

Lighting intensity	n/a	n/a
Hardness of dilution water	40 – 250 mg $CaCO_3$ / L preferably < 80	42 $CaCO_3$ / L
Alkalinity of dilution water	n/a	n/a
Conductivity of dilution water	≤ 10 µS/cm	n/a
Total organic Carbon (TOC) mg/L	n/a	n/a

Study dates: 11th April 2016 – 15th April 2016

5. Animal assignment and treatment:
20 fish per test item concentration (10 per replicate), as well as the control and solvent control, were impartially selected and distributed to each aquarium. Nominal test concentrations were 1.0, 1.6, 2.6, 4.3 and 7.0 mg a.s../L.

6. Dose preparation:
Stock solutions were prepared by weighing a defined amount of test substance and diluting it in a defined volume of Dimethylformamide (DMF). A 1.0 mL aliquot of each stock solution was spiked into 10 L of the dilution water under stirring to prepare each test solution. A control group with only dilution water and a solvent group with dilution water containing DMF at the same concentration as the exposure group (100 µL/L) were included. Test solutions were observed to be clear at preparation.

7. Measurements and observations:
Mortality (absence of gill movement and reaction to gentle prodding) and sub-lethal effects were recorded at 6, 24, 48, 72 and 96 hours of exposure. Fish were defined as being dead if no breathing movements were visible and touching of the caudal peduncle produced no reaction.

Dissolved oxygen concentration, pH and temperature measurements were made once daily in the treatment and control aquaria. At the same time, observations of the physical characteristics of the test solutions were recorded.

At exposure initiation (0 hour) and exposure termination (96 hour), one sample was collected from each treatment level and the controls for analysis of 3'-OH-S-2840 concentration by HPLC. Samples from each replicate were combined and analysed per test group. LOD = 0.0625mg a.s./L. LOQ = 0.100 mg a.s./L.

8. Statistics:
The 24-, 48-, 72- and 96-hour median lethal concentrations (LC_{50}) were empirically estimated to be greater than the highest concentration tested. The highest concentration tested at which no significant mortality and sub-lethal effects from the controls were determined as the No-Observed-Effect Concentration (NOEC).

II. RESULTS AND DISCUSSION

A. MORTALITY AND SUBLETHAL EFFECTS

At test termination (96 hours), no mortality was observed among fish exposed to any of the treatment levels tested or the controls. Effects on swimming were observed in the highest concentration, with some of the fish displaying slow swimming. A summary of the cumulative percent mortalities and sublethal effects is presented in Table 9.2.2-34 below.

Table 9.2.2-34: Summary of mortalities following exposure to 3'-OH-S-2840

Mean measured concentration (mg a.s./L)	Mean cumulative mortality (%)				
	6 hour	24 hour	48 hour	72 hour	96 hour
Control	0 NA: 20	0 NA: 20	0 NA: 20	0 NA: 20	0 NA: 20
Solvent control	0 NA: 20	0 NA: 20	0 NA: 20	0 NA: 20	0 NA: 20
0.92	0 NA: 20	0 NA: 20	0 NA: 20	0 NA: 20	0 NA: 20
1.5	0 NA: 20	0 NA: 20	0 NA: 20	0 NA: 20	0 NA: 20
2.4	0 NA:20	0 NA: 20	0 NA: 20	0 NA: 20	0 NA: 20
3.9	0 NA: 20	0 NA: 20	0 NA: 20	0 NA: 20	0 NA: 20
6.2	0 NA: 20	0 SS: 20	0 SS: 20	0 NA:2,SS:18	0 NA:2,SS:18

SS: Slow swimming: NA: Normal

A summary of the toxicity endpoints determined from the study is presented in Table 9.2.2-35.

Table 9.2.2-35: Summary of endpoints

Time	LC_{50} (mg a.s./L) [a]	95% confidence interval	
		Lower (mg a.s./L)	Upper (mg a.s./L)
24-hour[a]	> 6.2	n.a	n.a
48-hour[a]	> 6.2	n.a	n.a
72-hour[a]	> 6.2	n.a	n.a
96-hour[a]	**> 6.2**	n.a	n.a
96 hour NOEC = 3.9 mg a.s./L			

Time	LC_{50} (mg a.s./L) [a]	95% confidence interval	
		Lower (mg a.s./L)	Upper (mg a.s./L)
Highest concentration producing 0% mortality = 6.2 mg a.s./L			
Lowest concentration producing 100% mortality = > 6.2 mg a.s./L			

[a] LC_{50} values were empirically estimated to be greater than the highest mean measured concentration
n.a = not applicable

B. ANALYSIS
Precipitation of the test solutions was observed in the 7.0 mg a.s./L (nominal) treatment level from the 48-hour observation, and at 2.6 and 4.3 mg a.s./L at exposure termination. Floating materials were observed at 7.0 mg a.s./L from the 24-hour observation.

Measured concentrations in the test solutions ranged from 89 to 92% of the nominal concentrations. The results of the analysis of the exposure solutions for 3'-OH-S-2840 are presented in Table 9.2.2-36.

Table 9.2.2-36: Measured concentrations of 3'-OH-S-2840 in the exposure solutions

Nominal concentration (mg a.s./L)	Measured concentration (mg a.s./L)[a]			Percent of nominal (%)[a]
	0 h	96 h	Mean	
Control	<0.100[b]	<0.100[b]	n.a.	n.a.
Solvent control	<0.100[b]	<0.100[b]	n.a.	n.a.
1.0	0.95	0.89	0.92	92
1.6	1.5	1.4	1.5	91
2.6	2.5	2.3	2.4	91
4.3	4.1	3.8	3.9	91
7.0	6.7	5.7	6.2	89

[a] Measured concentrations and percent of nominal have three significant figures. The values were rounded off to two significant figures to present in this table.
[b] Concentrations expressed as less than values were below the quantification limit.
n.a. =Not applicable

C.VALIDITY CRITERIA
As there were no mortalities in the controls and dissolved oxygen remained above 60% saturation, the study was considered valid. Details of validity criteria according to OECD 203 (2019) are shown in Table 9.2.2-37 below.

Table 9.2.2-37: Validity criteria as per OECD 203 (2019)

Validity criterion	Required	Obtained
Mortality in the control(s)	≤ 10 %	No mortality
Dissolved oxygen concentration	≥ 60 % of air saturation value	Maintained ≥ 60 % saturation for temperature range.
Analytical measurement of test concentrations	Analysis of the highest and lowest test concentration and a concentration around the expected LC_{50} is considered the minimum requirement	At exposure initiation and exposure termination. One sample was collected from each treatment level and the controls for analysis for static study. Mortality data for each concentration considered. Recovery range of nominal concentrations were 89% - 92%.

III. CONCLUSION

Based on the results of this laboratory study, the 96hour LC_{50} to rainbow trout was empirically estimated to be > 6.2 mg a.s./L, the highest mean measured concentration tested. The NOEC was determined to be 3.9 mg a.s./L. Results are based on mean measured concentrations.

HSE COMMENTS

This study was carried out in accordance with GLP and under OECD 203 (1992) guidance available at the time and has been checked against current OECD 203 (2019 guidance).

The concentrations of the test item were maintained between 80-120% of the nominal value throughout the test. Results should be based on nominal concentrations but have been expressed by the applicant as mean measured concentrations. The method of analysis used in the study was evaluated by HSE Chemistry. The conclusion of their evaluation is reproduced below. Please see Volume 3 CA, section B5 for more details.

"*The analytical method is not fully validated according to SANTE/2020/12830 rev. 1 for the determination of 3'-OH-S2840 in aquatic test solutions as the matrix effects have not been determined and the stability of standards has not been addressed. However, the studies were generated prior to the implementation of SANTE 2020/12830 rev.1 and SANCO 3029 rev.4 did not require matrix effects and the stabilities of standards to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose.*"

There is a deviation to protocol noted for feeding that is not declared by the study. OECD 203 (1992) and OECD 203 (2019) guidance recommend feeding daily or three

times weekly (or to satiety in OECD 203 (2019)), but in this study fish were fed five times per week. As the loading weight of fish per tank is less than the maximum loading value given in OECD 203 (2019), it is unlikely to have significantly impacted the study. No value has been given for loading weight, but this has been worked out to be approximately 0.77 g/L of test solution:

Mean wet weight × number of fish per tank ÷ volume of test solution
0.77 x 10 ÷ 10 = 0.77 g biomass/L (maximum loading weight in OECD 203 (2019) = 0.8 g/L)

0.16% mortality was recorded during the 7-day acclimation period, which is within the 5 % mortality limit for batch acceptance.

There is discrepancy between measurements and observations, with the pH, dissolved oxygen concentration and temperature measured at 0, 24, 48, 72 and 96 hours, but examined for mortality after 0, 6, 24, 48, 72 and 96 hours. This suggests that no pH or dissolved oxygen concentration data was available for observations at 6 hours and is not included in the variable data for the test. HSE does not consider this to have an impact on the results given the consistency of the variables shown between 24-96 hours and that no mortalities were recorded.

All validity criteria for OECD 203 (1992) and OECD 203 (2019) were met for this study. It is also worth noting, that whilst not a requirement of OECD 203 (1992) or OECD 203 (2019), no reference item was used for this study; therefore, cannot be compared to an existing approved substance.

The applicant has not provided details on any statistical analysis that may have been conducted. As there were no mortalities, this is not considered to impact the results of the study.

The method of analysis used in the study was evaluated by HSE Chemistry. The conclusion of their evaluation is reproduced below. Please see Volume 3 CA, section B5 for more details.

"*The analytical method is not fully validated according to SANTE/2020/12830 rev. 1 for the determination of 3'-OH-S2840 in aquatic test solutions as the matrix effects have not been determined and the stability of standards has not been addressed. However, the studies were generated prior to the implementation of SANTE 2020/12830 rev.1 and SANCO 3029 rev.4 did not require matrix effects and the stabilities of standards to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose.*"

The agreed endpoints for use in risk assessment are:

- **96-hour LC_{50} > 6.2 mg met./L (based on highest mean measured concentration)***
- **96-hour NOEC = 3.9 mg met./L (based on mean measured concentrations)**

*No mortalities recorded in the study, therefore no exact LC_{50} value determined for risk assessment

B.9.2.2.3 Metabolite: 1'-COOH-S-2840

Reference:	KCA 8.2.1/11
Report Title:	Acute Toxicity Study of 1'-COOH-S-2840 with Rainbow Trout (*Oncorhynchus mykiss*)
Author(s) & year:	[redacted] (2016b)
Document No, Authority registration No:	[redacted] Study No. 1513EFAR
Substance used:	1'-COOH-S-2840, 16SC8508359, 100 %
Method of analysis:	Reversed-phase HPLC
Guideline(s):	OECD 203 (1992)
Deviations:	Yes, see HSE comments section.
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

MATERIALS AND METHODS

MATERIALS

Test material 1'-COOH-S-2840

Lot/Batch #: 16SC8508359

Purity: 100% (verified according to the certificate of analysis)

Description: White solid

Stability of test compound: Not stated

Reanalysis/expiry date: 31 January 2019

Density: Not applicable

TREATMENTS

Test concentrations: Dilution water control, solvent control (0.10 mL N, N-dimethylformamide (DMF)/L), nominal concentrations of 60 mg 1'-COOH-S-2840/L, mean measured concentrations of 50 mg 1'-COOH-S-2840/L

Solvent: DMF (CAS No. 68-12-2)

Toxic reference None

Analysis of test concentrations: Yes, at 0 and 96 hours (treatment level and the dilution water and solvent controls) based on analysis of 1'-COOH-S-2840 using reversed phased high performance

	liquid chromatography (HPLC). The limit of detection (LOD) was set at 0.625 mg a.s./L. The limit of quantification (LOQ) (the lowest concentration in the recovery test) was 1.00 mg a.s./L.
TEST ORGANISMS	
Species:	Rainbow trout (*Oncorhynchus mykiss*)
Source:	[redacted])
Acclimatisation period:	≥ 12 days
Treatment for disease:	None reported
Weight and length of a representative sample of fish (n = 20):	Mean weight: 0.77 g (range: 0.57 to 0.99 g) Mean length: 47 mm (range: 43 to 51 mm)
Feeding:	Dry commercial feed, formula feed for trout 1CDX 5 times a week at *ca* 2% body weight/day. Fish were not fed during the exposure period and 48 hours prior to start.
TEST DESIGN	
Test vessels:	20 L whole glass aquaria (10 L/vessel) (inside size ca. 30D x 30W x 30H cm; covered with a transparent plastic lid)
Test medium:	Dechlorinated tap water
Replication:	Three replicate aquaria were established for each treatment level, dilution water control and solvent control
No of fish per tank:	10
Exposure regime:	Static
Duration:	96 hours
TEST CONDITIONS	
Test temperature:	13.9 – 14.4 °C
pH:	7.6 – 8.4
Salinity	Not applicable
Dissolved oxygen:	8.1 – 9 mg/L (> 60 % saturation)
Hardness of dilution water:	42 mg $CaCO_3$/L
Lighting:	Photoperiod of 16 hours light and 8 hours darkness (lux not reported)

STUDY DESIGN AND METHODS

Experimental dates: the 96-hour definitive exposure was conducted from 11 to 15 April 2016.

Test organism and acclimatisation

The Rainbow trout (*Oncorhynchus mykiss*), juvenile), commonly used in acute freshwater toxicity tests, was selected as the test species. Prior to testing, fish were acclimatised for ≥ 12 days under the same conditions as the definitive test. During

acclimatisation, the fish were fed commercially prepared fish food five times a week (Monday – Friday). Test fish population mortality during the seven-day period prior to testing was 0.16%.

Test water
The dilution water (dechlorinated tap water) used during this study was from the same source as the water used during acclimatisation. The dilution water was prepared by dechlorinating tap water with a charcoal filter. Residual chlorine was below the limit of detection (LOD) (<0.05 ppm) in the dechlorinated tap water. Representative samples of the dilution water source were analysed periodically for the presence of pesticides, PCBs and toxic metals. None of the compounds listed in Annex 3 of OECD 203 (2019) were above the Limit Of Detection (LOD). In addition, samples were periodically analysed monthly for Total Organic Carbon (TOC) (<0.5 mg/L for February 2016).

Definitive test and dose preparation
A static test system was employed. The definitive test was conducted as a limit test at a nominal concentration of 60 mg a.s./L.

A 600 mg a.s./mL stock solution was prepared in DMF. A 1.0 mL aliquot of this stock solution was spiked into 10 L of the dilution water under stirring to prepare the 60 mg a.s./L test solution. Test solutions were observed to be clear at preparation. The test solution was kept standing for a few minutes. Undissolved material was observed as adhered above the water surface and precipitated at the bottom. The adhered material was wiped off and the precipitations were collected using a glass pipette. The collected precipitations were added with the test solution from the same vessel and were dissolved by sonication. After sonication, the solution was returned to the test vessel. A control group with only dilution water and a solvent group with dilution water containing DMF at the same concentration as the exposure group (0.1 mL/L) were included. Each solution was added to a replicate aquarium, containing 10 L of solution.

At the start of the test 30 fish, ten per replicate aquarium, were randomly allocated to each test concentration and the controls. The resulting test organism loading concentration was not reported, although it could be approximated from the mean fish mass (0.77 g), number of fish per aquarium (10) and litres of water per aquarium (10 L) to be 0.77 g/L. Exposure solutions were maintained at 14 ± 1 °C by placing aquaria within a temperature-controlled water bath (FRP tank, controlled with heater and cooler).

Measurements and observations
Observations for mortalities and symptoms of toxicity were made at 0, 6, 24, 48, 72 and 96 hours. Mortality (dead fish were removed), biological observations, including sublethal effects (e.g., lethargy, loss of equilibrium) and observations of the physical characteristics of the test solutions were made and recorded. Mortality was defined as the lack of movement by the exposed organisms (i.e., absence of gill movement and reaction to gentle prodding of the caudal peduncle).

The pH, dissolved oxygen concentration, temperature and salinity were measured at 0, 24, 48, 72 and 96 hours in each replicate of each treatment and control.

Apparatus used for this test included: electronic balance for weighing test substance (XP205V, Mettler-Toledo International Inc.), electronic balance for weighing fish (PB3002-S, Mettler-Toledo International Inc.), dissolved oxygen meter (Model 58, YSI/Nanotech Inc.), water temperature meter (SK-250WP, Sato Keiryoki Mfg. Co., Ltd.), pH meter (D-51, Horiba Ltd), Total hardness test kit (HA-DT, HACH COMPANY Ltd.) and micrometre calliper (CD-15, 500 Series, Mitsutoyo Corporation).

At exposure initiation (0 hours) and exposure termination (96 hours), one sample was collected from each treatment level and the controls for analysis of 1'-COOH-S-2840 concentration by reversed phased HPLC. Samples were taken from the mid-depth of each vessel without agitation. An equivalent volume of test solution from each replicate was combined and analysed per test group. The mean measured 1'-COOH-S-2840 concentrations were calculated for each treatment level using the following equation:

$$MC = \frac{(A - B)}{\ln(A) - \ln(B)}$$

where:
$A = measured\ concentration\ at\ initiation\ of\ exposure$
$B = measured\ concentartion\ at\ termination\ of\ exposure$
$\ln = natural\ logarithm$

Statistical analysis
The 24-, 48-, 72- and 96-hour median lethal concentrations (LC50) were empirically estimated to be greater than the highest concentration tested. The highest concentration tested at which no significant mortality and sub-lethal effects from the controls were determined as the No-Observed-Effect Concentration (NOEC).

RESULTS AND DISCUSSION
Mortality and sub-lethal effects
At test termination (96 hours), no mortality was observed among fish exposed to the exposure group or the controls. No sublethal effects were observed in any group.

A summary of the toxicity endpoints determined from the study is presented in Table 9.2.2-38.

Table 9.2.2-38: Summary of endpoints for 1'-COOH-S-2840

Time	LC_{50} (mg a.s./L) [a]	95% confidence interval	
		Lower (mg a.s./L)	Upper (mg a.s./L)
24-hour[a]	>50	n.a.	n.a.
48-hour[a]	>50	n.a.	n.a.
72-hour[a]	>50	n.a.	n.a.
96-hour[a]	>50	n.a.	n.a.
96 hour NOEC = 50 mg a.s./L			

Time	LC_{50} (mg a.s./L) [a]	95% confidence interval	
		Lower (mg a.s./L)	Upper (mg a.s./L)
Highest concentration producing 0% mortality = 50 mg a.s./L			
Highest concentration producing 100% mortality > 50 mg a.s./L			

[a] LC_{50} values were empirically estimated to be greater than the highest mean measured concentration
n.a. = not applicable

Analysis
Appearance of the test solutions was clear and with no undissolved material observed throughout the exposure. The mean measured concentration of 1'-COOH-S-2840 in the test solution was 84% of the nominal concentrations. The results of the analysis of the exposure solutions for 1'-COOH-S-2840 are presented in Table 9.2.2-39.

Table 9.2.2-39: Measured concentrations of 1'-COOH-S-2840 in the exposure solutions

Nominal concentration (mg a.s./L)	Measured concentration (mg a.s./L)[a]			Mean percent of nominal (%)[a]
	0 h	96 h	Mean	
Control	< 1.00[b]	< 1.00[b]	n.a.	n.a.
Solvent control	< 1.00[b]	< 1.00[b]	n.a.	n.a.
60	50	50	50	84

[a] Measured concentrations and percent of nominal in the raw data have three significant figures. The values were rounded off to two significant figures to present in this table.
[b] Concentrations expressed as less than values were below the minimum detectable limit (MDL).
n.a. = not applicable

Validity criteria
The validity criteria for the study were met for the duration of the study according to OECD 203 (1992) and OECD 203 (2019) (Table 9.2.2-40).

Table 9.2.2-40: Compliance with OECD 203 validity criteria

Validity criterion	Required	Obtained
Mortality in the control(s)	≤ 10 %	0 %
Test conditions	Constant conditions	A static conditions design was selected. Constant conditions were maintained.

Validity criterion	Required	Obtained
Dissolved oxygen concentration	At least 60 % of the air saturation value throughout the test	Dissolved oxygen concentration > 60 % of the air saturation throughout the test.
Concentration of substance	Analytical measurement of test concentrations is compulsory. At least 80 % of the nominal concentration throughout the test. If the deviation from the nominal concentration is greater than 20 % results should be based on the measured concentration.	Measured concentrations was 84 % of nominal. Results based on mean measured concentrations.

CONCLUSIONS
Based on the results of this laboratory study, the 96-hour LC50 to rainbow trout was empirically estimated to be > 50 mg a.s./L and NOEC was determined to be 50 mg a.s./L, based on mean measured concentrations, for 1'-COOH-S-2840.

HSE COMMENTS
The study was carried out according to GLP and follows OECD 203 (1992), the guideline available at the time of experimentation. The study was evaluated against the most recent OECD 203 guideline (2019). All validity criteria outlined in OECD 203 (2019) were satisfactorily met for the duration of the study. There were no significant deviations to the guideline.

The following deviations were noted:
OECD 203 (2019) paragraph (§) 9 states that the study report method include a description of the apparatus used to carry out the test and appropriate documentation to validate equipment functionality. The equipment used to measure illuminance was not reported. Appropriate documentation to validate equipment functionality was also omitted. This requirement was not present in OECD 203 (1992) and its omission is unlikely to affect study outcome considering the up-to-date GLP certification of the study laboratory.

OECD 203 (2019) § 10 states, *"test vessels should be randomly positioned in the test area and shielded from unwanted disturbance (excessive noise, vibration, light)"*. This was not detailed in the study report. However, this point was not covered in OECD 203 (1992). Therefore, HSE considers this deviation acceptable.

OECD 203 (2019) § 13 details the conditions for the holding of fish. It states that the holding temperature should be appropriate for the species. Annex II of OECD 203 (2019) recommends 10-14 °C for *Oncorhynchus mykiss*. The study selected 14±1°C. This minor deviation stems from the different recommended temperature range for

this species in OECD 203 (1992) (13-17 °C) and HSE considers this acceptable.

OECD 203 (2019) § 14 outlines the criteria for test fish population mortality and health during the acclimatisation period. No detailed information was provided detailing signs of disease, stress, malformations or treatments against disease or parasites within 14 days prior to testing. This point was not present in OECD 203 (1992). The study conductors reported low mortality for the seven days prior to exposure initiation (0.16 %), as well as no mortality or sub-lethal effects during the exposure period in the test dilution water controls, indicating the health of the test fish population was acceptable. HSE consider this a minor deviation, which is not expected to impact the study outcome.

OECD 203 (2019) § 15 presents the chemical characteristic requirements of water used during the study. It states, *"any water which conforms to the chemical characteristics of acceptable dilution water as listed in Annex 3 is suitable as a test water".* The study report attached a representative analytical report detailing the levels of toxic metals and other elements within test water. The (analytical sensitivity) for many elements were not sensitive enough to determine whether the water conformed to the chemical characteristics detailed in Annex III of OECD 203 (2019). Specific maximum concentration values were not included within OECD 203 (1992). This, combined with the lack of mortality or sub-lethal effects within the dilution water control, indicates the water is not expected to have impacted the outcome of the study. Therefore, HSE considers this a minor deviation.

OECD 203 (2019) § 16 details required chemical testing of dilution water. Within this paragraph it states, "*analyses of nitrate and chlorine should be performed on each batch of dilution water to demonstrate that the limits specified in Annex 3 are not exceeded*". The study reported chlorine levels in the dechlorinated water was < 50 µg/L. Annex III defines a maximum concentration of 10 µg/L. Therefore, the analytical sensitivity of the selected method was too low. Therefore, it is not possible to determine whether the recommended concentration was met. Again, the lack of mortality or sub-lethal effects in the dilution water control suggests residual chlorine levels are not expected to have impacted the outcome of the study. Therefore, HSE considers this a minor deviation.

OECD 203 (2019) § 17 outlines test solution preparation requirements. It states, *"the use of solvents should be avoided and only used as a last resort in order to produce a suitably concentrated stock solution".* The study conductor dissolved the test substance in DMF when preparing the primary stock solution. The solubility of S2399 in water was not provided, which precludes determining whether solvent use necessary. Within OECD 203 (1992) the requirement to minimise solvent use was not present. The lack of mortality or sub-lethal effects in the solvent control indicates the use of DMF is not expected to have impacted the outcome of the study. Therefore, HSE considers this a minor deviation.

OECD 203 (2019) § 19 concerns the conditions of exposure. It states, *"temperature…should be within the temperature ranges specified for the test species (Annex 2)"*. The temperature range stated for *O. mykiss* (10-14 °C) differs from that used during the exposure period (13-15 °C). This deviation stems OECD 203 (1992) recommending a different temperature range (13-17 °C), which the study conformed

to. The lack of mortality or sub-lethal effects in the controls suggest this deviation is not expected to have impacted the outcome of the study. Therefore, HSE considers this a minor deviation.

OECD 203 (2019) § 19 states, "*Loading: for freshwater fish, maximum loading of 0.8 g wet weight fish/L for static and semi-static renewal testing is recommended*". This was not reported. Using the fish mean mass (0.77 g), the number of fish (10) and the volume of water per aquarium (10 L), an approximate loading of 0.77 g/L can be estimated. This is close to recommended 0.8 g/L limit. In OECD 203 (1992), however, this limit was higher (1 g/L). The approximate loading level suggests loading levels were appropriate.

OECD 203 (2019) § 19 states, *"Light: should be within the photoperiod ranges specified for the test species (Annex 2) and with an intensity of 10-20 µE/m2 /s, 540-1000 lux, or 50-100 ft-c (ambient laboratory levels)"*. Although a specific light intensity range was not reported, the study conductor did state the experiment was performed with "room lighting", which is equivalent to ambient laboratory levels. HSE deems this acceptable.

OECD 203 (2019) § 20 details sample size and replication requirements. It states, "*a minimum of 7 fish must be used at each test concentration and in the control(s)… no test tank replication is required*". The study used 30 fish for each concentration and control, comprising of ten fish in three replicates. In line with ethical considerations regarding the reduction of fish used in ecotoxicological testing, this degree of replication was unnecessary. Furthermore, the use of three replicates was not required under the guidance of OECD 203 (1992) either. Although this deviation did not impact study outcome, HSE notes the unnecessarily large sample size conflicts with the aim to reduce the number of animals used in vertebrate testing.

OECD 203 (2019) § 21 concerns integrating existing sources of information into test concentration selection if such information is available. The study provided no evidence of this approach being attempted. This was not a requirement under OECD 203 (1992) and this deviation is considered minor with respects to study outcome.

OECD 203 (2019) § 23 profiles control requirements. It states, "*the dilution water control can be omitted, and the test conducted and evaluated with a solvent control only, provided it is appropriate when considering the needs for these data and the requirements of the relevant regulatory authorities*". Given the use of dechlorinated tap water and the inappropriate analytical sensitivity for methods defining water characteristics, the use of a dilution water control demonstrated the low toxicity of the test water. Therefore, HSE consider the use of a water dilution control acceptable.

OECD 203 (2019) § 26, covering the frequency of biological observation and recording, states, "t*o the extent feasibly possible, a minimum of 2 observations should be conducted within the first 24 hours of the study"*. The study only performed one observation within the first 24 hours (6-hour). Also, "*on days 2-4 of the test, all vessels with living fish should be inspected twice per day*". The study only performed one observation period a day for days 2-4. Under the enacted observation programme, however, some transient sub-lethal effects may have been omitted. Again, these requirements were not present in OECD 203 (1992) making these deviations

acceptable.

OECD 203 (2019) § 28, concerning the fate of test fish, was not addressed in the study report. This requirement was not present within OECD 203 (1992) and its omission is deemed acceptable.

OECD 203 (2019) § 29 requires fish to be measured prior to the initiation of exposure. Fish were measured after exposure termination. Specific details regarding the measurement of fish in relation to study timings were not available in OECD 203 (1992). Furthermore, fish length corresponded to the recommended range outlined in Annex II of OECD 203 (2019). Therefore, HSE consider this deviation acceptable.

OECD 203 (2019) § 33 outlines the requirements for the test report, including the reporting of test substance physico-chemical properties. The study report did not provide the required physico-chemical properties or a structural formula. Analytical measurement at exposure initiation and completion confirmed the presence of the test substance in the test solution indicating the omission of the physico-chemical properties did not impact test integrity. Furthermore, mean measured concentrations were within the 80%-120% acceptable range of the nominal concentration. Therefore, HSE considers this a minor deviation.

The method of analysis used in the study was evaluated by HSE Chemistry. The conclusion of their evaluation is reproduced below. Please see Volume 3 CA, section B5 for more details.

"*The analytical method is not fully validated according to SANTE/2020/12830 rev. 1 for the determination of the 1'-COOH-S2840 in in aquatic test solutions as the matrix effects have not been determined and the stability of standards has not been addressed. However, the studies were generated prior to the implementation of SANTE 2020/12830 rev.1 and SANCO 3029 rev.4 did not require matrix effects and the stabilities of standards to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose.*"

The above study was conducted to GLP and considered valid.

The agreed endpoint suitable for use in the risk assessment is: 96-hour LC_{50} > 50 mg 1'-COOH-S-2840/L.

B.9.2.3 Long-term and chronic toxicity to fish

B.9.2.3.1 Fish early life stage toxicity tests

Reference:	KCA 8.2.2.1/01
Report Title:	S-2399 TG – Early Life-Stage Toxicity Test with Fathead Minnow, *Pimephales promelas*, Following OECD Guideline #210 and OPPTS Draft Guideline 850.1400
Author(s) & year:	[redacted] (2014)

Document No, Authority registration No:	[redacted] Study No. 13048.6781
Substance used:	S-2399 TG, 13CG0617G, 95.0%
Method of analysis:	LC/MS/MS
Guideline(s):	OECD 210 (2013)
Deviations:	Yes, see HSE comments section.
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: S-2399 TG
Description: Not stated
Lot/Batch: 13CG0617G
Purity: 95.0%
Reference item: None
Solvent/carrier: Acetone

B. STUDY DESIGN AND METHODS:

1. Test animals:
Species: Fathead minnow (*Pimephales promelas*) embryos
Source: [redacted] culture
Age: Approximately 23 hours old
Diet: From day 5 (day 1 post-hatch) larvae were fed live brine shrimp nauplii (*Artemia salina*) three times daily, *ad libitum*. Larvae were not fed during the 24 hours prior to study termination.
Acclimatisation period: Embryos were set in a container in a 25 °C heated water bath and allowed to acclimate to the exposure temperature for approximately 30 minutes
Treatment for disease: None
Biomass: Did not exceed 0.0559 g/L of flowing test solution per day or 0.395 g/L at any time in any replicate exposure aquarium.

2. Test design
System: Flow-through
Duration: 32 days (28 days post-hatch)
Test vessels: Glass test aquarium (30 x 14.5 x 25 cm) with

silicone sealant and a 12.5 cm high side drain to maintain a constant exposure solution volume of approximately 5.5 L.
Embryo incubation cups were round glass jars (5 cm in diameter, 8 cm in height) with screen bottoms (475-micron mesh) that were gently oscillated.

Number of eggs per tank: 30
Number of fish per tank: 20 (impartially selected larvae on test day 4, day 3 post exposure)

Dilution water: Laboratory well water
Hardness: 56 to 68 mg $CaCO_3$/L
Alkalinity: 20 to 24 mg $CaCO_3$/L
Conductivity: 270 to 380 µS/cm
pH: 6.6 to 7.7

3. **Test concentration:** 1.4, 2.4, 4.2, 7.1 and 12 µg a.s./L (nominal)
1.6, 2.7, 4.6, 7.5 and 13 µa.s./L (mean measured)

4. **Environmental conditions:**
Temperature: 24 – 26 °C
pH: 6.9 – 7.7
Specific conductance: 340 – 480 µS/cm
Total hardness: 52 – 64 mg/L as $CaCO_3$
Total alkalinity: 20 – 22 mg/L as $CaCO_3$
Dissolved oxygen: 5.0 – 8.2 mg/L (61 to 99% saturation)
Photoperiod: 16 hours light: 8 hours darkness at 40 to 69 footcandles (430 to 740 lux).

A summary of environmental conditions obtained throughout the test is shown in Table 9.2.3-1 against required standards as per OECD 210 (2013).

Table 9.2.3-1: Summary of environmental conditions

Variable	Required OECD 210 (2013)	Obtained
Temperature	25 ± 1.5	24 – 26 °C
pH	Not stated	6.9 – 7.7
Dissolved oxygen concentration	>60% of the air saturation value throughout the test	5.0 – 8.2 mg/L (61 to 99% saturation)
Photoperiod	16 hours light	16 hours light
Hardness of dilution water	Not stated	56 to 68 mg $CaCO_3$/L
Alkalinity of dilution	Not stated	20 – 22 mg/L as $CaCO_3$

Variable	Required OECD 210 (2013)	Obtained
water		
Conductivity of dilution water	Not stated	270 to 380 µS/cm
Total organic carbon (toc) mg/l	Not stated	0.46 and 0.34 mg/L for the months of May and June 2014

Definitive study dates: 15 May to 16 June 2014

5. Animal assignment and treatment:
The test was conducted using an exposure system consisting of an intermittent-flow proportional diluter, a temperature-controlled water bath and a set of 28 exposure aquaria. The exposure system was designed to provide five concentrations of the test substance, a dilution water control and a solvent control to four replicate exposure aquaria. Flow-splitting cells were employed to equally distribute the solutions to the four replicate aquaria for each concentration and the control group at a rate of 250 mL of test solution per vessel per cycle. Flow splitting accuracy of the diluter cells was within ± 5% of the nominal value. The diluter system delivered the control and exposure solutions to the exposure aquaria at a rate sufficient to provide approximately 7 aquarium volumes per 24-hour period, with a 90% replacement time of approximately 8 hours

The exposure of fathead minnow to S-2399 TG was initiated when the 28 embryo incubation cups, each containing 30 embryos, were impartially distributed to each of the test aquaria. At study initiation, the embryos were approximately 23 hours old.

Completion of hatch was considered to be day 4 when all viable embryos in the control and all treatment level incubation cups were observed to be hatched. The 28-day post-hatch survival was initiated on the day of hatch (day 4). On test day 4, the surviving larvae in each incubation cup were thinned to 20 organisms per replicate (80 per treatment level or control) and placed into each respective aquarium with the exception of replicates C and D of the 13 µg/L dose, which due to a protocol deviation had only two replicates.

6. Dose preparation:
A 3 mg a.s./mL diluter stock solution was prepared prior to exposure initiation and as needed throughout the exposure by adding, for example, 0.1583 g (0.1504 g) of test substance to a volumetric flask and diluting to a final volume of 50 mL with acetone. A 30 µL/mL solvent control stock solution was prepared by diluting 30 mL of acetone to 1000 mL reagent grade water. Both solutions were clear and colourless.

Prior to test initiation, a pump in conjunction with a 10-mL gas-tight syringe was calibrated to deliver 0.00904 mL/cycle of the diluter stock solution (3.0 mg a.s./mL) into the diluter's chemical mixing chamber, which received 2.26 L of dilution water per cycle. The mixing chamber was positioned over a magnetic stir plate and partially submerged within an ultrasonic water bath which aided in mixing the stock solution

with the dilution water. The concentration of S-2399 TG in the solution contained within the mixing chamber was equivalent to that of the highest nominal test concentration (12 µg a.s./L) and was proportionally diluted by a constant factor of approximately 1.7 to provide the remaining nominal exposure concentrations (7.1, 4.2, 2.4, 1.4 µg a.s./L).The concentration of acetone in the mixing chamber and the highest concentration constituted the highest acetone concentration (18 µL/L). A pump was calibrated to deliver 0.61 mL/cycle of the 30 µL/mL solvent stock solution to 1.0 L of dilution water per cycle, which was subsequently delivered to the solvent control aquaria. Control aquaria contained the same dilution water but contained no S-2399 TG.

Calibration of the diluter system was conducted prior to exposure initiation and confirmed at exposure termination by measuring delivery volumes of toxicant and dilution water. The function of the diluter (e.g., flow rate, stock solution consumption) was monitored daily and a visual check of the system's operation was performed twice daily. The exposure system was operating properly for at least 48 hours prior to exposure initiation to allow equilibration of the test substance in the diluter apparatus and exposure aquaria.

7. Measurements and observations:

At exposure initiation, a subsample of embryos (N = 30) was collected to determine the embryonic stage of development at the start of the study. After the acclimation period, the embryos were assessed for fertilization under appropriate magnification. For this assessment, fertilized embryos (those displaying embryonic development) were kept and unfertilized embryos were discarded. Observations of the viability of the embryos used in the study were made daily, with dead and live embryos recorded until the day of hatch (Day 4). Any dead embryos were removed when observed.

During the post-hatch exposure period, dead larvae were removed when observed and behaviour and appearance of the larval fish were recorded daily. Larval survival was recorded daily. On Day 28 post-hatch exposure, surviving larvae were counted and euthanised, measured and weighed individually to determine the total length and wet weight.

Dissolved oxygen concentration, pH and temperature were measured in all aquaria on Day 0. Daily thereafter, these parameters were measured in a single replicate of each concentration and control. Replicates were successively alternated each day. Total hardness, total alkalinity and conductivity were measured on Day 0 and weekly thereafter in one replicate of the control, low and high test concentrations. Replicates were successively alternated each week.

One replicate of each treatment level and control solutions were sampled prior to the start of the exposure and analysed for S-2399 TG. A sample of diluter stock solution was also analysed. During the in-life phase, water samples were removed from alternating replicates of each treatment level and the control on test Days 0, 4, 13, 20, 27 and 32, from the midpoint of the aquarium using a pipette. A sample of diluter stock solution was also analysed at each sampling interval. All exposure solution analysis was conducted using LC/MS/MS.

8. Statistics:

At the termination of the early life-stage exposure, data obtained on embryo hatching success, the percentage of embryos producing live normal larvae at hatch and larval survival, total length and wet weight at exposure termination were compared to pooled control organisms. Analyses were performed using the mean organism response in each replicate aquarium rather than individual response values. All statistical analyses (Student's t-test, Shapiro Wilks and William's test) were conducted at the 95% level of certainty except in the case of Bartlett's Tests, in which the 99% level of certainty was applied. CETIS™ was used to perform the statistical computations. Fisher's Exact Test with Bonferroni-Holm's adjustment was used to determine treatment effects for the endpoints with quantal responses (percent survival, percent hatch success and percent live, normal larvae at hatch). Shapiro-Wilk's Test for normality was used to compare the observed sample distribution with a normal distribution for growth (length and wet weight). Bartlett's Test was conducted to evaluate the homoscedasticity of the data. As all data met the assumptions for normal distribution and homogeneity of variance, data was evaluated using Dunnett's Multiple Comparison Tests, to establish treatment effects. LOQ was set at 0.600 µg/L and the MDL was 0.200 µg/L.

II. RESULTS AND DISCUSSION

A. BIOLOGICAL OBSERVATIONS

No significant differences were observed between the negative and solvent controls; therefore the data were pooled for all subsequent analyses.

From assessment of the subsample of embryos collected to determine the embryonic stage, the median and mean stages were 10 (high blastula) and 8 (32-celled blastodisc), respectively. Developmental stages ranged from 3 to 30 (one-celled blastodisc to high blastula).

No delays in hatch were observed at any treatment level; all viable embryos in all treatment and control embryo incubation cups began hatching on day 3 and completed hatching on day 4. Embryo hatching success and percentage of embryos that produce live normal larvae are presented in Table 8.2.4/01-2 A significant difference in embryo hatching success in the 4.6 µg a.s./L treatment level was noted. A significant difference was noted in the percent of live normal larvae in the 13 µg a.s./L treatment.

Following the 28-day post-hatch exposure (Day 32), there were significant differences in larval survival among organisms exposed to the 2.7, 4.6 and 13 µg a.s./L treatments.

A significant difference in length and wet weight was determined in the 13 µg a.s./L treatment group, compared to the pooled control. No significant differences were determined between any other treatment and the pooled control.

A summary of the biological results for the early life stage parameters assessed are presented in Table 9.2.3-2.

Table 9.2.3-2: Summary of effects on embryo viability, survival of organisms at hatch and larval survival, total length, wet weight and dry weight of fathead minnow (*Pimephales promelas*) during the early life-stage exposure of S-2399 TG technical

Mean measured concentration (µg a.s./L)	Embryo hatching success (%)	Live normal larvae at hatch (%)	Larvae (28 days post-hatch)		
			Larval survival (%)	Mean total length (SD) (mm)	Mean wet weight (SD) (g)
Control	93	95	96	22.81 (0.61)	0.1009 (0.0075)
Solvent Control	93	92	99	23.78 (0.55)	0.1163 (0.0120)
Pooled Control	93	93	98	23.30 (0.749)	0.1086 (0.0124)
1.6	94	88	94	23.87 (0.61)	0.1196 (0.0064)
2.7	93	90	90*	24.01 (0.14)	0.1200 (0.0026)
4.6	83*	95	88*	23.94 (0.50)	0.1222 (0.0074)
7.5	87	90	93	22.53 (0.40)	0.1067 (0.0053)
13	87	79*	75*	19.19** (0.52)	0.0800** (0.0055)

* Significantly reduced compared to the pooled control, based on Fisher's Exact Test with Bonferroni-Holm's Adjustment
** Significantly reduced compared to the pooled control, based on Dunnett's Multiple Comparison Test
SD = Standard deviation

Based on the results of this study, the study conductor determined a NOEC = 7.5 µg a.s./L (mean measured concentration) and LOEC = 13 µg a.s./L (mean measured concentration). A summary of the endpoints are presented in Table 9.2.3-3.

Table 9.2.3-3: Summary of endpoints

Parameter	NOEC (µg a.s./L)	LOEC (µg a.s./L)	EC_{10} (95% Confidence Interval) (µg a.s./L)	EC_{20} (95% Confidence Interval) (µg a.s./L)
Hatching success (%)	13	> 13	> 13 (n.a.)	>13 (n.a.)

Parameter	NOEC (µg a.s./L)	LOEC (µg a.s./L)	EC_{10} (95% Confidence Interval) (µg a.s./L)	EC_{20} (95% Confidence Interval) (µg a.s./L)
Percent of live normal larvae (%)	7.5	13	7.5-13 (n.a.)	> 13 (n.a.)
Survival (%)	Please see 'HSE Comments' and 'RAI and response' sections below			
Total length (mm)	7.5	13	9.7 (9.1-10)	> 13 (n.a.)
Body weight (g)	7.5	13	7.5-13 (n.a.)	11 (9.4-12)

n.a.= not applicable. EC value was empirically estimated, therefore corresponding 95% confidence intervals could not be calculated

B. ANALYSIS

Results of the analyses established that the measured concentrations were generally consistent between sampling intervals and maintained the expected concentration gradient. Mean measured concentrations were approximately 110% of the nominal levels and defined the treatment levels tested as 1.6, 2.7, 4.6, 7.5 and 13 µg a.s./L. Results are detailed in Table 9.2.3-4.

Table 9.2.3-4: Measured concentrations of S-2399 TG in the exposure solutions

Nominal concentration (µg a.s./L)	Mean measured (µg/L) (SD)[a]	% CV	Percent of nominal[a](%)
Control	n.a.	n.a.	n.a.
Solvent control	n.a.	n.a.	n.a.
1.4	1.6 (0.11)	7.1	110
2.4	2.7 (0.15)	5.6	110
4.2	4.6 (0.28)	6.1	110
7.1	7.5 (0.34)	4.5	110
12	13 (0.44)	3.5	110

[a] Mean measured values and percent of nominal were calculated using the actual analytical results and not the rounded values (two significant figures) presented in this table.
n.a. = not applicable
SD = standard deviation
CV = co-efficient of variation

C. VALIDITY CRITERIA

All control organisms and water quality parameters met the performance criteria, therefore the study was considered valid.

1. Hatching success was ≥ 70% (actual 93%)
2. Larvae survival was ≥ 75% (actual control 96%, solvent control 99%)
3. Dissolved oxygen was ≥ 60% (actual value 61-99%)
4. Water temperature remained at ± 1.5^{0}C throughout the exposure and between test chambers.
5. The solubilising agent had no effect on survival
6. The concentrations of the test substance remained ± 20% of mean measured values

III. CONCLUSION

Based on the most sensitive indicators of toxicity (i.e. live normal larvae, larval survival, length and wet weight), a NOEC = 7.5 µg a.s./L (mean measured concentration) and LOEC = 13 µg a.s./L (mean measured concentration) were determined by the study conductor. Whether HSE Ecotoxicology agrees with this is discussed in the HSE Comments and RAI and response sections below.

HSE COMMENTS:

This study was carried out under GLP and under guidance of OECD 210 (1992). It has been assessed against updated OECD (2013) guidance. Study commenced following release of 2013 guidance but planning and preparation for study will have been carried out prior to OECD 210 (2013) availability.

The concentrations of the test item were maintained between 80-120% of the nominal value throughout the test. Results were expressed as mean measured concentrations by the applicant.

The method of analysis used in the study was evaluated by HSE Chemistry. The conclusion of their evaluation is reproduced below. Please see Volume 3 CA, section B5 for more details.

"*The analytical method is not fully validated according to SANTE/2020/12830 rev. 1 for the determination of the active substance inpyrfluxam in freshwater as the matrix effects have not been determined and the stability of standards and extracts has not been addressed. However, the studies were generated prior Ito the implementation of SANTE 2020/12830 rev.1 and SANCO 3029 rev.4 did not require matrix effects and the stabilities of standards and extracts to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose.*"

A statistically significant reduction in embryo hatching success was detected at the 4.6 µg a.s./L treatment level. The applicant has stated that this was not biologically relevant due to a lack of effect at the two higher treatment levels. HSE agrees with this assessment considering the < 10 % effects at 7.5 and 13.0 µg a.s./L are strong evidence to support the lack of clear concentration response for this response variable.

For larval survival (28 days post-hatch), however, HSE Ecotoxicology did not agree with the NOEC = 7.5 µg a.s./L. This topic was raised during a RAI from the applicant. The RAI and its response are presented in a separate section below.

One protocol deviation reported is that only 2 replicates are available for the concentration 13 µg/L (measured). Larvae were inadvertently not transferred to the respective exposure vessels in replicates C and D of this dose during the completion of hatch. The applicant has stated that whilst this is a deviation from protocol, the validity criteria for the study have still been met and it is not expected to have a negative impact on the study since the exposure met expectations; the remaining replicates of the high dose appropriately illustrated an effect in the growth and survival endpoints. HSE note that having the correct number of replicates is a requirement for adequate statistical analysis in determining the significance of the effects at this concentration compared to the pooled control. This issue was addressed through the estimation of an EC_{10} (see RAI and response section below).

There is a protocol deviation for the light intensity during the exposure period. On the final day of exposure, multiple light intensity readings fell fellow 50 footcandles (range 40-49), which the applicant has presented as a deviation from OECD 210 guidelines. There is no requirement for light intensity presented in OECD 210 (1992) or OECD 210 (2013) guidelines. HSE do not consider this to have had any significant impact on the study as all other environmental conditions were adequately maintained throughout the test and no adverse behavioural observations were made in these groups.

A notable deviation is the use of silicone sealant in this study. The use of silicone is discouraged in OECD 210 (2013) as it is known to have a strong capacity to absorb lipophilic substances in flow-through systems. As the concentrations recorded at the end of this study were ≥ 80% of the nominal concentration, it is not likely to have had any effect in this instance.

OECD 210 (2013) guidance states that test reports should include the incidence, description and number of morphological abnormalities and incidence, description and number of behavioural effects; if any. The applicant stated in the study report that they recorded the appearance and behaviour of larvae daily, but this information has not been presented; therefore, morphological and behaviours effects of S-2399TG on early life stages of *Pimephales promelas* cannot be determined. The high percentage of hatching success and larval survival suggests that it is unlikely any effect of S-2399TG on morphology and behaviour would be significant. The was provided after a RAI (see section below).

Feed was provided on day 1 post-hatch in this study. This complies with OECD 210 (1992) guidelines but is in deviation of OECD (2013) guidance that states first feed for *Pimephales promelas* should be given at 2 days post-hatch. This is unlikely to have affected the validity of the study as the mean wet weights and length of larvae were within guideline values at the end of study.

Whilst not a requirement of OECD 210 (2013), no reference item was used for this study; therefore, species sensitivity is undetermined.

All methods for statistics used in this study were recommended methods in OECD 210 (2013), apart from Bartlett's Test that was used to evaluate the homoscedasticity of the data. However, as this was recommended for use by U.S EPA (2002), it is still a

suitable method for statistical analysis.

RAI and response:
HSE Ecotoxicology did not agree with the NOEC = 7.5 µg a.s./L set by the study conductor for larval survival. This was raised with the applicant during an RAI, reproduced below:

"Uncertainty in the reported NOEC for the *P. promelas* ELS fish study (KCA 8.2.2.1/01): the NOEC for larval survival was set at 7.5 µg a.s./L despite evidence for a lower NOEC of 1.6 µg a.s./L. The LC_{10} for larval survival was empirically estimated. Please attempt concentration-response modelling for the larval survival LC_{10} endpoint to avoid the use of NOEC = 1.6 µg a.s./L in risk assessment."

"**Uncertainty in the reported NOEC for the *P. promelas* ELS fish study (KCA 8.2.2.1/01).** HSE does not agree with the 7.5 µg a.s./L NOEC set for the larval survival response variable. Larval survival presented a relatively well described concentration-response, except for 7.5 µg a.s./L. Rather than discounting two statistically significant results at 2.7 and 4.6 µg a.s./L as not treatment related, HSE raises the possibility that the higher larval survival at 7.5 µg a.s./L was a result of sampling error. HSE deems the more conservative 1.6 µg a.s./L NOEC to be suitable for risk assessment. Commission Regulation (EC) 283/2013 Data point 8.2.2.1 states, "*the EC_{10} and EC_{20} shall be reported together with the NOEC. Where EC_{10} and EC_{20} cannot be estimated, an explanation shall be provided*". Only empirically estimated EC_{10} and EC_{20}s were reported. From the study report, it is unclear if concentration-response modelling was attempted for larval survival. The statistical section of Appendix 4 reports a STP 2x2 Contingency Tables analysis for larval survival (Page 172) but no non-linear regression. No clear explanation for why concentration-response modelling was not possible was presented in the study report. HSE suggests that concentration-response modelling for larval survival should be considered further to facilitate an LC_{10} estimate. This could then be used in risk assessment in place of the 1.6 µg a.s./L NOEC set by HSE."
In response, an additional non-linear regression analysis was presented by the laboratory in charge of the ELS study (Annex 2 of RAI response). The analysis was performed in CETIS (v 1.9.7) and a full statistical report was presented. For the 2-parameter exponential model fitted (binomial error distribution), a significant model F-test demonstrated that the model compared favourably to a null mode. Also, the lack of fit F-test is non-significant, which indicates that the variability not explained by the model is attributable to the variability within replicates. In other words, the variability of replicates around model predictions is similar to the variability of replicates around their means. The model fit with 95 % confidence intervals and replicate values are presented below. A visual appraisal by HSE Ecotoxicology concluded an acceptable model fit, supporting the formal statistical tests.

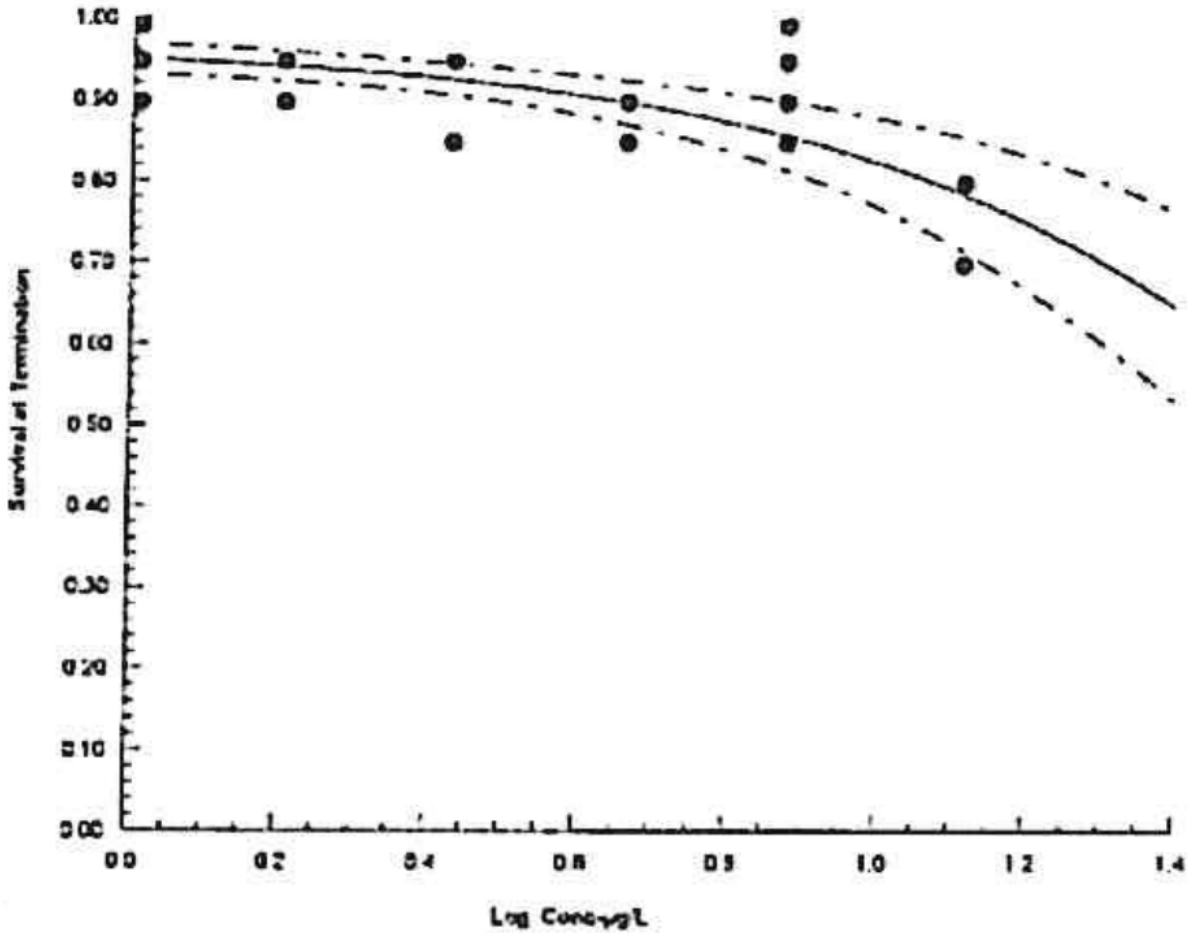


Figure 9.2-9: Model prediction with 95 % confidence intervals and replicate data for larval survival against Log(concentration).

The LC_{10} = 6.556 µg a.s./L (3.961 to 9.264). The LC_{20} = 13.89 µg a.s./L (8.196 to 20.15). For completeness, HSE Ecotoxicology used Appendix F of EFSA Supporting publication 2015:EN-924 to assess the reliability of the reported EC_{10}. The normalised width (NW) of the confidence interval = 0.809, which corresponds to a 'Fair' rating. A 'Fair' rating was the most frequently achieved rating when the NW classification criteria were applied to a comprehensive endpoint database (Azimonti et al. (2015))[9]. A classification can also be determined based on the relationship between the LC_{10} and LC_{20} lower confidence limit. Here, the LC_{10} is lower than the $LC_{20, low}$, demonstrating that there is a high certainty in the protection level (> 95 % probability that the true effect at 6.556 µg a.s./L is below 20 %). Taken together, the classification results and model fit tests demonstrate that the estimated **LC_{10} = 6.556 µg a.s./L** is reliable and suitable for use in risk assessment.

[9] Comparison of NOEC values to EC10/EC20 values, including confidence intervals, in aquatic and terrestrial ecotoxicological risk assessment. (2015). EFSA Supporting Publications, 12(12). doi:https://doi.org/10.2903/sp.efsa.2015.en-906.

The applicant provided additional non-linear regression analyses in ToxRat (v 3.3.0). All non-linear regressions performed had a significant lack of fit test. When this occurs, the recommendation is to identify a more appropriate dose-response function. A suitable dose-response function was identified in the analysis presented in Annex 2 of the RAI response, described in detail above. Consequently, no further discussion of the non-linear regression analyses submitted by the applicant in the main RAI response document is required.

Further arguments were presented relating to the larval survival at 2.7 and 4.6 µg a.s./L residing within the range of historical control data. HSE Ecotoxicology does not accept the arguments concerning historical control data as the concurrent control is the most appropriate background response level. For this study, no experimental concerns were raised relating to the concurrent control, and, consequently, no alternative background reference level is appropriate.

To conclude, after an RAI response HSE Ecotoxicology has selected a LC_{10} = 6.556 µg a.s./L as a suitable endpoint for use in risk assessment.

HSE Ecotoxicology also requested a "**Tabular summary of larval behaviour and appearance daily observations for the *P. promelas* ELS fish study (KCA 8.2.2.1/01):** please provide a summary table for the larval behaviour and appearance daily observations data.". This was provided and reported no consistent sub-lethal effects for treatment groups below the LC_{10} = 6.556 µg a.s./L. Consistent sub-lethal effects were only recorded for the 13 µg a.s./L treatment group.

The agreed endpoint for use in risk assessment is:

- **LC_{10} = 6.556 µg a.s./L (mean measured concentration).**

Reference:	KCA 8.2.2.1/02
Report Title:	S-2399 TG - Early life-stage toxicity test with sheepshead minnow, *Cyprinodon variegatus*
Author(s) & year:	[redacted] (2017)
Document No, Authority registration No:	12709.6373
Substance used:	S-2399 TG, 13CG0617G, 95.0%
Method of analysis:	LC/MS/MS
Guideline(s):	OECD 210 (2013)
Deviations:	Yes, see HSE comments section.

GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

MATERIALS

Test Material	S-2399 TG
Description:	Not stated
Lot/Batch #:	13CG0617G
Purity:	95.0% (verified according to the certificate of analysis)
Stability of test compound:	Not stated
Reanalysis/expiry date:	23 July 2016
Treatments	
Test concentrations:	Nominal concentrations of 7.5, 15, 30, 60 and 120 µg a.s./L, mean measured concentrations of 9.0, 15, 34, 63 and 130 µg a.s./L.
Control:	Dilution water control, solvent control (13 µL acetone/L),
Solvent:	Acetone
Analysis of test concentrations:	Yes on days 0, 6, 13, 20, 25, 28 and 34 by liquid chromatography with tandem mass spectrometry (LC/MS/MS). The limit of quantification (LOQ) was set at 0.600 µg/L, the lowest validated concentration and the minimum detectable limit (MDL) was 0.200 µg/L for S-2399
Test animals	
Species:	Sheepshead minnow (*Cyprinodon variegatus*)
Source:	Brood stock maintained at [redacted] (approximately six months old)
Acclimatisation period:	None
Treatment for disease:	None
Feeding:	Beginning on day 7 (day 1 post-hatch), larvae were fed live brine shrimp nauplii (*Artemia salina*) three times daily. Larvae were not fed during the 24 hours prior to study termination. Food was screened for the presence of pesticides, PCBs and toxic metals. None of the compounds were detected at concentrations considered toxic to the test organism.
Test design	
Exposure regime:	Flow-through (Mount & Brungs intermittent-flow proportional diluter system).
Aeration:	None required due to high test solution turnover rate

	of flow-through system.
Replication:	For embryonic exposure there were four replicates containing 30 embryos per treatment level. On hatching, if >20 hatched, 20 larvae were indiscriminately selected per replicate to enter the 28-day post-hatch larval exposure stage.
Test vessels:	Aquaria (glass and silicone sealant, 30 x 14.5 20 cm, 5.5 L exposure solution volume). Within the aquaria embryo incubation cups were placed (round glass jars (5 cm in diameter, 8 cm in height) with screen bottoms (475-micron mesh), gently oscillated)
No of eggs per tank:	Pre hatch: 30 eggs, post hatch: 20 larvae impartially chosen
Duration:	28 days post-hatch (34 days exposure)
Environmental conditions	
Test temperature:	24 – 27 °C
pH:	7.3 – 7.8
Dissolved oxygen:	4.84 – 7.56 mg/L (67 to 100% saturation)
Salinity of dilution water:	20 to 21 ‰
Lighting:	16 hours light, 8 hours darkness, 15-30 min transition period (540 to 650 lux)

STUDY DESIGN AND METHODS

Definitive test dates: 8 March to 11 April 2016

Test organism
Sheepshead minnow (*Cyprinodon variegatus*), a commonly used estuarine fish in early life-stage toxicity studies, was selected as the test organism. The brood stock used for this exposure was approximately six months old. No mortality was observed among the brood stock during the 48 hours prior to testing. The water flowing to the culture unit was from the same source as the dilution water used during the early life-stage exposure. The brood stock was maintained at 20 ± 2°C unless the fish were induced to spawn, typically 24 hours prior to exposure initiation. The temperature was raised to approximately 25 °C at the end of the working day on 7 March 2016 to induce spawning. On the morning of 8 March 2016, sheepshead minnow embryos were obtained that were ≤ 20 hours old. During the 14 days prior to exposure initiation, the culture temperature ranged from 19 to 20 ºC, except when the fish were induced to spawn, in which case the temperature was raised to 25 to 27 ºC for approximately 24 hours. Dissolved oxygen ranged from 70 to 91% of saturation.

Test water
The dilution water used during this study was prepared by filtering natural seawater collected from the Cape Cod Canal, Bourne, Massachusetts. Prior to use in this study, the seawater was adjusted with laboratory well water to a salinity range of 20 to 21‰

and the pH range of 7.5 to 7.8, filtered through a series of polypropylene core filters as fine as 5-µm and bag filters as fine as 1-µm, and heated to the required test temperature.

Representative samples of the dilution water were analysed periodically for the presence of pesticides, PCBs and toxic metals by GeoLabs, Inc., Braintree, Massachusetts. None of these compounds have been detected at concentrations that are considered toxic in any of the water samples analysed, in agreement with ASTM (2007)[10] standard practice. In addition, representative samples of the dilution water were analysed monthly for total organic carbon (TOC) concentration. The TOC concentration in the seawater was 1.2 mg/L for both March and April 2016.

Dose preparation
A 9 mg a.s./mL diluter stock solution was prepared prior to exposure initiation and as needed throughout the exposure by adding 0.9484 g (0.9010 g as active substance) of test substance to a final volume of 100 mL with acetone. A 100 µL/mL solvent control stock solution was prepared by diluting 100 mL of acetone to 1000 mL reagent grade water. Both solutions were clear and colourless. Prior to test initiation, a pump in conjunction with a 100-mL gas-tight syringe was calibrated to deliver 0.0258 mL/cycle of the diluter stock solution (9.0 mg/mL) into the diluter's chemical mixing chamber, which received 1.94 L of dilution water per cycle. The mixing chamber was positioned over a magnetic stir plate and partially submerged within an ultrasonic water bath which aided in mixing the stock solution with the dilution water. The solution contained within the mixing chamber was equivalent to that of the highest nominal test concentration (120 µg/L) and was proportionally diluted (50%) to provide the remaining nominal exposure concentrations (7.5, 15, 30 and 60 µg a.s./L). These treatment solutions were subsequently delivered to the respective treatment aquaria. The highest concentration of acetone present a treatment solution was 13 µL/L (120 µg/L treatment level) and the solvent control acetone concentration matched this. A pump was calibrated to deliver 0.68 mL/cycle of the 100 µL/mL solvent stock solution to 5.07 L of dilution water per cycle, which was subsequently delivered to the solvent control aquaria. Dilution water control aquaria contained the same dilution water but contained no S-2399 TG or acetone.

Flow-splitting cells were employed to equally distribute the solutions to the four replicate aquaria for each concentration and the control groups, at a rate of 250 mL of test solution per vessel per cycle. The diluter system delivered the control and exposure solutions to the exposure aquaria at a rate sufficient to provide approximately 13 aquarium volumes per 24-hour period, with a 90% replacement time of approximately 4 hours.

Animal assignment and treatment
The exposure system was designed to provide five concentrations of the test substance, a negative control and a solvent control to four replicate exposure aquaria.

Embryos were transferred from the brood unit to the exposure system on the day of

[10] ASTM, 2007. Standard practice for conducting acute toxicity tests with fishes, macroinvertebrates and amphibians. Standard E729-96. American Society for Testing and Materials, 100 Barr Harbor Drive, West Conshohocken, Pennsylvania

initiation. Embryos were set in a glass dish in a 25 °C heated water bath and allowed to water harden for approximately 60 minutes. After the hardening period, the embryos were assessed for fertilization under appropriate magnification. For this assessment, fertilized, viable embryos (those displaying embryonic development) were maintained and unfertilized embryos were discarded.

The exposure of fathead minnow to S-2399 TG was initiated when the embryo incubation cups, each containing 30 embryos, were impartially distributed to the respective test aquaria. At study initiation, the embryos were approximately 21 hours old.

At exposure initiation, a subsample of embryos (N= 30) was collected to determine embryonic stage of development. The embryo subsample was preserved in Stockard's solution for at least 24 hours prior to assessment. Embryonic stage was determined on each individual embryo within the subsample using guidance in the Pre-Hatching Development of the Sheepshead Minnow *Cyprinodon variegatus* Rafinesque (U.S. EPA, 1996a)[11].

Completion of hatch was considered to be Day 6 when all viable embryos in the control and all treatment level incubation cups were observed to be hatched. The 28-day post-hatch survival was initiated on the day of hatch (Day 6). On test Day 6, the surviving larvae in each incubation cup were thinned to 20 organisms per replicate (80 per treatment level or control) and placed back into each respective aquarium.

Measurement and observations
Observations of the viability of the embryos used in the study were made daily, with dead and live embryos recorded until the day of hatch (Day 6). Any dead embryos were removed when observed.

During the post-hatch exposure period, larval survival, behaviour and appearance were recorded daily. Dead larvae were removed. On Day 28 post-hatch exposure, surviving larvae were counted and euthanised. These larvae were measured and weighed individually to determine the total length and wet weight.

Dissolved oxygen concentration, pH, salinity and temperature were measured in all aquaria on Day 0. Daily thereafter, these parameters were measured in a single replicate of each concentration and control. Replicates were successively alternated each day. Exposure solution temperature was continuously monitored in replicate A of the negative control using a VWR minimum/maximum thermometer. Dissolved oxygen concentrations and daily temperature were measured using a Yellow Springs Instrument (YSI) 550A dissolved oxygen meter/temperature probe. The pH was measured using a YSI pH100A pH meter. Salinity was measured with a YSI Pro 30 salinity and conductivity meter.

One replicate of each treatment level and control solutions were sampled prior to the start of the exposure and analysed for S-2399 TG. A sample of diluter stock solution

[11] U.S. EPA, 1996a. Prehatching Development of the Fathead Minnow *Pimephales promelas* Rafinesque. Office of Research and Development. U.S. Environmental Protection Agency, Washington, D.C. EPA/60/R-96/079. July 1996.

was also analysed. During the in-life phase, water samples were taken from alternating replicates of each treatment level and the control on test Days 0, 6, 13, 20, 25, 28 and 34. Archive samples were also collected at each sampling interval and stored frozen for possible future analysis and were discarded if not analysed. A sample of diluter stock solution was also analysed at each sampling interval. Three quality control (QC) samples were prepared at each sampling interval at nominal concentrations approximating the test concentration range and remained with the exposure solution samples throughout the analytical process. An additional set of QC samples was prepared in the same manner for frozen archival with the test samples with no further additions or processing. Analysis of the QC samples was used to judge the precision and quality control maintained during the analytical process. All exposure solution analysis was conducted using LC/MS/MS.

Statistical analysis

At the termination of the early life-stage exposure, data obtained on embryo hatching success, the percentage of embryos producing live, normal larvae at hatch and larval survival, total length and wet weight at exposure termination were analysed to identify significant reductions in the treatment organisms compared to the control organisms. Analyses were performed using the mean organism response in each replicate aquarium rather than individual response values. All statistical analyses were conducted at the 95% level of certainty except in the case of Shapiro-Wilks' Test (U.S. EPA, 2002)[12] and Bartlett's Test (U.S. EPA, 2002)[12], in which the 99% level of certainty was applied.

The following procedures were used:

1. Solvent control and negative control data were first compared using Mann-Whitney U Two-Sample Test (U.S. EPA, 2002)12. If no significant difference was established between the two control data sets, the negative control was used for comparison of the treatment responses. For this study, negative control and solvent control data were statistically similar for the hatching success, percent live, normal larvae at hatch, and larval survival at termination endpoints. A statistically significant difference between the two control groups was detected in growth endpoints (length and wet weight). Therefore, length and wet weight data were compared to both the negative control and solvent control means independently to demonstrate if there was a difference in statistical determinations for these endpoints, and if the growth enhancement in the solvent control had an effect on the derived No-Observed-Effect Concentration or Lowest-Observed-Effect Concentration.

2. Statistical analysis of percent hatching success, percent live normal larvae at hatch and percent survival was performed. Since the data for percent hatching success and percent live normal larvae at hatch were non-monotonic, Fisher’s Exact Test with Bonferroni-Holm’s Adjustment (U.S. EPA, 2002)[12] was used to determine treatment effects for these endpoints. Cochran-Armitage’s Trend Step-Down Test (U.S. EPA, 2002)[12] was used to evaluate percent survival data since this endpoint data exhibited a monotonic dose response.

[12] U.S. EPA, 2002. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. Fourth Edition. Office of Water, U.S. Environmental Protection Agency, Washington, D.C. EPA/821/R-02-013.

3. Shapiro Wilks' Test for normality (U.S. EPA, 2002)[12] was conducted to evaluate the distribution of the growth data. For this study, data for total length and wet weight were normally distributed when treatment data were evaluated.

4. Bartlett's Equality of Variance Test (U.S. EPA, 2002)[12] was conducted to evaluate the homoscedasticity of the growth data. For this study, data for total length and wet weight met the assumption of homogeneity of variance when treatment data were evaluated.

5. Since total length and wet weight data met the assumptions for normal distribution and homogeneity of variance, Dunnett's Multiple Comparison Test (U.S. EPA, 2002)[12], a parametric procedure, was used to establish treatment effects for this endpoint. CETIS™ Version 1.8 (Ives, 2013)[13] was used to perform the statistical computations. The lowest mean measured concentration that elicited a statistically significant effect on organism performance (Lowest-Observed-Effect Concentration, LOEC) and the highest mean measured concentration that did not elicit a statistically significant difference between the exposed organisms and the appropriate control (No-Observed-Effect Concentration, NOEC) were determined. Determination of these levels is based on the performance criteria evaluated (e.g., embryo hatching success, percentage of embryos producing live, normal larvae at hatch, survival and larval growth at termination (total length and wet weight).

RESULTS AND DISCUSSION

Test conditions

The results of the water quality determinations made during the 34-day definitive exposure are presented in Table 9.2.3-5. Dissolved oxygen, salinity and pH were at acceptable levels throughout. Daily measurements for temperature were also within the 25 ± 1.5 ºC range specified within OECD 210 (2013). Continuous temperature monitoring in replicate A of the negative control, however, demonstrated that the temperature ranged from 24 to 27 ºC throughout the exposure. On test days 0 through 10, the maximum temperature reading from the minimum/maximum thermometer, located in replicate A of the negative control, had a reading of 27 ºC. The minimum/maximum thermometer temperature ranges during test days 0 through 10 remained at 26 to 27 ºC and did not differ by more than 1.0 ºC at any time.

Table 9.2.3-5: Early life-stage exposure of sheepshead minnow (*Cyprinodon variegatus*) to S-2399 T.G. – Water quality determinations

Nominal concentration (µg/L)	Ranges				
	Dissolved oxygen[a]		Temperature[ab] (ºC)	Salinity[a] (‰)	pH[a]
	mg/L	% of saturation			
Negative control	5.3 - 7.6	74 - 100	24 - 26	20 - 22	7.6 - 7.8

[13] Ives, M., 2013. CETIS, Comprehensive Environmental Toxicity Information SystemTM. User's Guide. Tidepool Scientific Software, McKinleyville, California

Nominal concentration (µg/L)	Ranges				
	Dissolved oxygen[a]		Temperature[ab] (ºC)	Salinity[a] (‰)	pH[a]
	mg/L	% of saturation			
Solvent control	5.4 - 7.5	75 - 100	24 - 26	20 - 21	7.4 - 7.8
7.5	5.3 - 7.5	73 - 100	25 - 26	20 - 21	7.3 - 7.7
15	5.1 – 7.3	71 - 99	25 - 26	20 - 21	7.3 - 7.7
30	4.9 - 7.1	67 - 97	25 - 26	20 - 21	7.3 - 7.7
60	4.8 - 7.1	67 - 97	25 - 26	20 - 21	7.3 - 7.7
120	5.5 - 7.0	77 - 96	25 - 26	20 - 21	7.4 - 7.7

[a] Negative control, solvent control, 7.5, 15, and 30 µg/L treatment levels, n = 38. 60 µg/L treatment level, n = 18. 120 µg/L treatment level, n = 11.
[b] Continuous temperature monitoring of the negative control (replicate A) established a temperature range of 24 to 27 ºC throughout the exposure period

During the 28-day post-hatch exposure period, biomass loading based on the negative control organism wet weight at test termination, did not exceed 0.018 g/L under the exposure's flow-through conditions or 0.24 g/L at any time in any replicate exposure aquarium.

Analytical results
The diluter system which prepared and delivered the exposure solutions to the exposure aquaria functioned properly during the pre-test period and throughout the definitive exposure. No undissolved test substance (e.g., precipitate) was observed in the diluter system (e.g., mixing chamber and chemical cells) or exposure solutions during the pre-test period or throughout the exposure period. The results of the analysis of exposure solutions for S-2399 T.G. during the definitive study are presented in Table 9.2.3-6.

Table 9.2.3-6: Analysis of test solutions throughout the exposure window

Nominal Conc. (µg/L)	Measured concentration (µg/L) (Percent of nominal[e])							Mean measured (SD)[a]	Percent of nominal[a]	%CV
	Day 0	Day 6	Day 13	Day 20 (FA)[d]	Day 25	Day 28	Day 34			
Negative control	< 1.0[b] (NA[c])	< 1.0 (NA)	< 1.0 (NA)	< 1.0 (NA)	< 1.0 (NA)	< 1.0 (NA)	< 1.0 (NA)	NA (NA)	NA	NA
Solvent control	< 1.0 (NA)	< 1.0 (NA)	< 1.0 (NA)	< 1.0 (NA)	< 1.0 (NA)	< 1.0 (NA)	< 1.0 (NA)	NA (NA)	NA	NA
7.5	8.7 (116)	8.6 (115)	8.6 (115)	11 (147)	8.4 (112)	8.9 (119)	9.3 (124)	9.0 (0.85)	120	9.4

Nominal Conc. (µg/L)	Measured concentration (µg/L) (Percent of nominal[e])							Mean measured (SD)[a]	Percent of nominal[a]	%CV
	Day 0	Day 6	Day 13	Day 20 (FA)[d]	Day 25	Day 28	Day 34			
15	15 (100)	14 (93)	14 (93)	18 (120)	13 (87)	15 (100)	14 (93)	15 (1.6)	99	11
30	33 (110)	31 (103)	31 (103)	39 (130)	30 (100)	36 (120)	36 (120)	34 (3.6)	110	11
60	58 (97)	64 (107)	56 (93)	74 (123)	54 (90)	70 (117)	66 (111)	63 (7.6)	105[f]	12
120	130 (108)	130 (108)	120 (100)	170 (142)	120 (100)	120 (100)	130 (108)	130 (19)	108[f]	14

[a] Mean measured concentrations, standard deviation (SD) and percent of nominal were calculated using actual analytical results and not the rounded values (two significant figures) presented in this table.
[b] Concentrations expressed as less than values were below the minimum detectable limit (MDL). The MDL is dependent upon the lowest concentration calibration standard used and the dilution factor derived from the sample volume of the controls, i.e., (0.100 µg/L × 10 = 1.0 µg/L).
[c] NA = Not Applicable.
[d] Day 20 frozen archive (FA) samples yielded a consistent recovery trend across the concentration gradient and provide an accurate representation of S-2399 T.G. concentrations in the exposure system. Therefore, the original Day 20 results were excluded from mean measured concentrations.
[e] Percent of nominal values for individual replicate measurements were calculated by HSE using the provided rounded figures.
[f]These values were incorrect in the original table and corrected by HSE using the rounded figures provided.

Measured concentrations of S-2399 were generally consistent between sampling intervals; however, exposure solution samples from day 20 of exposure yielded S-2399 T.G. concentrations out of trend with previous results; measured concentrations were approximately 20 to 40% higher than previous samples, and the 30 µg/L treatment level replicate D sample measured approximately 184% nominal (55 µg/L). The diluter system was subsequently checked and proper function was confirmed. Therefore, all day 20 frozen archive samples were analysed. Frozen archive sample concentrations were consistent with original day 20 sample concentrations, with one exception: the 30 µg/L sample was measured at 39 µg/L. Since the frozen archive sample concentrations were similar to the original samples for all treatment levels except 30 µg/L, and the 30 µg/L sample was more in line with the nominal concentration (39 µg/L as opposed to 55 µg/L), the study conductor decided that the frozen archive concentrations were a more accurate representation of S-2399 T.G. concentrations on day 20 of exposure. Therefore, original day 20 analysis results were excluded from the mean measured concentrations and the results of the archive samples were used in calculation of mean measured concentrations. This timepoint is highlighted in Table 9.2.3-6 to draw attention to the use of the frozen archive

samples.

Biological observations
From assessment of a subsample of embryos collected to determine the embryonic stage, both the median and mean stages were 15 (three-quarter epiboly), and the range of developmental stages was 15 to 16 (three-quarter epiboly to closure of the germ ring). The range of developmental stages was consistent with expectations for sheepshead minnow embryos that were approximately 21 hours old.

All viable embryos in the 9.0, 15, and 34 µg/L treatment levels and control incubation cups were hatched by day 6 of exposure. A dose dependent delay in hatch was observed at the 63 and 130 µg/L treatment levels; less than 90% of embryos in all replicates of the 63 µg/L treatment level were hatched on day 6 of exposure. All replicates of this treatment level completed hatch on day 7. Since a reduced number of hatched larvae were observed in all replicates on this day (i.e., less than 20 larvae), the observed number of hatched larvae were released into each replicate aquarium for this treatment level. Three dead hatched larvae were observed in the 130 µg/L treatment level on day 6. On day 7 of exposure, all remaining embryos in the 130 µg/L treatment level had failed to hatch. Therefore, they were considered dead embryos and were appropriately discarded. Embryo hatching success and percent of embryos that produce live, normal larvae for the definitive study are presented in Table 9.2.3-7.

Table 9.2.3-7: Early life-stage exposure of sheepshead minnow (*Cyprinodon variegatus*) to S-2399 T.G. – Endpoint summary

Mean Measured Concentration (µg/L)		Embryo Hatching Success (%)	Live, Normal Larvae at Hatch (%)	28 Days Post-Hatch		
				Larval Survival (%)	Total Length (SD[a]) (mm)	Wet Weight (SD) (g)
Negative Control	A	97	100	100	16.61 (1.22)	0.0618 (0.0150)
	B	93	96	100	16.78 (1.23)	0.0627 (0.0139)
	C	80	96	100	16.19 (1.61)	0.0580 (0.0188)
	D	82	91	95	16.66 (1.12)	0.0599 (0.0119)
	Mean	88	96	99	16.56 (0.26)	0.0606 (0.0021)
Solvent Control	A	83	96	90	17.81 (1.34)	0.0717 (0.0151)
	B	83	96	95	17.96 (1.56)	0.0658 (0.0163)
	C	80	100	100	17.56 (1.01)	0.0695

Mean Measured Concentration (µg/L)		Embryo Hatching Success (%)	Live, Normal Larvae at Hatch (%)	28 Days Post-Hatch		
				Larval Survival (%)	Total Length (SD[a]) (mm)	Wet Weight (SD) (g)
						(0.0120)
	D	90	96	100	17.67 (1.08)	0.0713 (0.0147)
	Mean	84	97	96	17.75 (0.17)	0.0696 (0.0027)
9.0	A	97	97	95	18.31 (1.39)	0.0760 (0.0134)
	B	83	100	95	17.93 (0.99)	0.0767 (0.0214)
	C	70	100	95	17.65 (1.09)	0.0711 (0.0156)
	D	70	100	95	18.11 (0.87)	0.0758 (0.0143)
	Mean	80	99	95	18.00 (0.28)	0.0749 (0.0026)
15	A	80	100	95	18.29 (1.13)	0.0764 (0.0110)
	B	86	100	85	18.53 (1.28)	0.0830 (0.0192)
	C	93	100	85	18.33 (1.32)	0.0818 (0.0177)
	D	80	100	100	18.17 (1.00)	0.0744 (0.0128)
	Mean	85	100	91[d]	18.33 (0.15)	0.0789 (0.0041)
34	A	73	95	100	17.50 (1.09)	0.0660 (0.0136)
	B	67	100	95	18.17 (1.20)	0.0776 (0.0142)
	C	97	100	65	19.42 (1.12)	0.0903 (0.0129)
	D	93	96	65	18.58 (3.59)	0.0947 (0.0381)
	Mean	82	98	81[e]	18.42 (0.81)	0.0822 (0.0130)

Mean Measured Concentration (µg/L)		Embryo Hatching Success (%)	Live, Normal Larvae at Hatch (%)	28 Days Post-Hatch		
				Larval Survival (%)	Total Length (SD[a]) (mm)	Wet Weight (SD) (g)
63	A	23	0	0	NA (NA)	NA (NA)
	B	17	0	0	NA (NA)	NA (NA)
	C	24	0	0	NA (NA)	NA (NA)
	D	23	0	0	NA (NA)	NA (NA)
	Mean	22[b]	0	0	NA (NA)	NA (NA)
130	A	0	NA[c]	0	NA (NA)	NA (NA)
	B	10	0	0	NA (NA)	NA (NA)
	C	0	NA[c]	0	NA (NA)	NA (NA)
	D	0	NA[c]	0	NA (NA)	NA (NA)
	Mean	3[b]	0	0	NA (NA)	NA (NA)

[a] SD = Standard Deviation.
[b] Significant reduction compared to the negative control, based on Fisher's Exact Test with Bonferroni-Holm's Adjustment. Treatment level was excluded from further statistical analysis.
[c] NA = Not Applicable. No embryos were observed in this replicate.
[d] Significant reduction compared to the negative control, based on Cochran-Armitage's Trend Step-Down Test.
[e] Significant reduction compared to the negative control, based on Cochran-Armitage's Trend Step-Down Test.

At the completion of hatch (day 6), hatching success in the negative control and solvent control averaged 88 and 84%, respectively. Embryo hatching success in the 9.0, 15, 34, 63 and 130 µg/L treatment levels averaged 80, 85, 82, 22 and 3%, respectively. Fisher's Exact Test with Bonferroni-Holm's Adjustment determined a significant difference in embryo hatching success among fish exposed to the 63 and 130 µg/L treatment levels compared to the negative control (88%). Based on the hatching success effects at 63 and 130 µg/L, these treatment levels were excluded from further statistical analysis.

At the completion of hatch, the percent of live, normal larvae in the negative control and solvent control averaged 96 and 97%, respectively. Percent of live, normal larvae in the 9.0, 15, 34, 63 and 130 µg/L treatment levels averaged 99, 100, 98, 0 and 0%, respectively. Fisher's Exact Test with Bonferroni-Holm's Adjustment determined a significant difference in percent of live, normal larvae among fish exposed to the 63 and 130 µg/L treatment levels compared to the negative control (96%).

Survival data is presented in Table 9.2.3-7. At exposure termination (34 days, 28 days

post-hatch), larval survival in the negative control and solvent control averaged 99 and 96%, respectively. Larval survival in the 9.0, 15, 34, 63 and 130 µg/L treatment levels averaged 95, 91, 81, 0 and 0%, respectively. Cochran-Armitage's Trend Step-Down Test determined a significant reduction in larval survival among fish exposed to the 15 and 34 µg/L treatment levels compared to the negative control (99%). The study conductor compared the 91% larval survival rate for the 15 µg/L treatment level to historical control larval survival rates (mean = 96%, standard deviation = 5.6%, coefficient of variation = 5.9%, range = 80 - 100%, n = 36) and observed that it falls within the range of historical control survival. Furthermore, they argued this statistically significant result was likely a product of the high negative control mean (99%) and low variability (coefficient of variation = 2.5%). On the basis of these two points the study conductor concluded the significant reduction at the 15 µg/L treatment level was not indicative of a true dose response and considered biologically relevant.

The study conductor argued further that the statistical significance of the 34 µg/L treatment level is also not biologically meaningful due to it being a product of the low mean larval survival in replicates C and D (65%). They draw attention to the fact that replicates C and D are out of trend with replicates A and B (100 and 95%). They go further by comparing the larval survival of replicates C and D to replicates from two earlier attempts at the definitive exposure study and the range finding study (Table A). Together, replicates C and D of the final definitive test are the only two replicates to have a larval survival < 80%. They support this argument by stating that the larval mean survival for 34 µg/L of 81% is within the range of historical lab control performance.

For the total length and wet weight, the study conductor reported no significant differences for any of the treatment groups (9, 15 and 34 µg/L) compared to either the solvent or dilution water control (Table 9.2.3-7).

EC_x conditions were not met for any endpoint; all available models in CETIS™ displayed a significant lack of fit and did not meet homogeneity of variance or normality criteria. Therefore, EC_x values were not reported.

Table 9.2.3-8 presents a summary of the NOEC/LOEC endpoints as reported by the study conductor.

Table 9.2.3-8: Summary of endpoints as concluded by study conductor

Endpoint:	NOEC (µg/L)	LOEC (µg/L)
Hatch Success	34	63
Percent, Live Normal Larvae at Hatch	34	63
Percent Survival at Termination	34	63
Total Body Length	34	> 34
Wet Weight	34	> 34

VALIDITY CRITERIA

The study partially meets the validity criteria of guideline OECD 210 (2013) as follows:

Table 9.2.3-9: Compliance with OECD 210 validity criteria

Validity criterion	Required	Obtained
Dissolved oxygen concentration	Should remain >60 % of air saturation value throughout the test	67 to 100 % ASV
Water temperature	Should not differ by ± 1.5 °C between test chambers or successive days at any time during test. Should remain within temperature range specified for test species, which is 25 ± 1.5 °C for *C. variegatus*.	Remained within ± 1.5 °C between test chambers and successive days during the test. Daily temperature measurements remained within 25 ± 1.5 °C (24 – 26 °C) Continuous temperature monitoring in one replicate established a range of (24 – 27 °C)
Analytical measurements of test concentration	Must be carried out using validated method. If measured concentrations are ± 20 % of nominal, results should be based on arithmetic mean (flow-through studies) or geometric mean (semi-static studies).	Results are based on arithmetic mean measured concentrations. Analytical measured concentrations varied from 87 – 147 % of nominal.
Overall survival of fertilized eggs and post-hatch success in control(s)	Should be minimum 75 % hatching success and 80 % post-hatch success for *C. variegatus*.	Hatching success for dilution water control = 88 % (SD = 8.29 %) and solvent control = 84 % (SD = 4.24 %). Survival (28 days post-hatch success) for dilution water control = 99 % (SD = 2.50 %) and solvent control = 96 % (SD = 4.79 %).

CONCLUSION
Based on the mean measured concentrations and percent survival at termination (the most sensitive indicator of toxicity) of S-2399 TG to the early life-stages of sheepshead minnow, the No-Observed-Effect Concentration (NOEC) was determined by the study conductor to be 34 µg a.s./L. The Lowest-Observed-Effect Concentration (LOEC) was determined by the study conductor to be 63 µg a.s./L.

EC_x conditions were not met for any endpoint; all available models in CETISTM displayed a significant lack of fit and did not meet homogeneity of variance or

normality.

HSE COMMENTS

The study was carried out according to GLP and follows OECD 210 (2013). The validity criterion regarding temperature staying within the range specified for the test species (*C. variegatus*, 25 ± 1.5 °C) was not met according to the continuous measurement in one replicate. HSE consider this a small deviation with minimal impact on study validity for a number of reasons: 1) temperature remained within ± 1.5 °C between test chambers and successive days during the test, and daily temperature measurements remained within 25 ± 1.5 °C (24 – 26 °C); 2) temperatures of 26 to 27 ºC are suitable for sheepshead minnow embryos and larvae, and are recommended by sheepshead minnow full life-cycle guideline literature (U.S. EPA 1996b)[14]; 3) hatching success and larval survival exceeded the acceptability criteria for both controls; and 4) all other validity criteria were met.

The following deviations were noted:
OECD 210 (2013) paragraph (§) 6 concerns available information on the test chemical and provides a list of useful properties including water solubility, pK_a, P_{ow}, and stability in water and light. OECD 210 (2013) paragraph (§) 34 states that water solubility and additional relevant physicochemical properties should be reported and they were not. This reporting omission will not have impacted the study results. HSE consider this a minor deviation.

OECD 210 (2013) paragraph (§) 8 states, "*effects on survival, hatch or growth occurring in the solvent control, when compared to the negative control, should be reported and discussed in the context of the reliability of the test data*". The solvent control had a small stimulatory effect on growth data when compared to the dilution water control. Therefore, the treatment analyses were performed against the dilution water and solvent control in separate analyses. The study conductor reported no effect between either control (dilution water and solvent) and the 9, 15 and 34 µg/L treatment levels using Dunnett tests for total length or wet weight. HSE re-ran these tests after noticing the ANOVA performed by the study conductor when using the dilution water control was significant. Every treatment level was significant compared to the dilution water control but non-significant compared to the solvent control for both total length and wet weight (Table 9.2.3-10). The interpretation of this result is the solvent control had a small stimulatory effect on growth, and the presence of solvent in the treatment levels also produced this effect. The test substance itself had no effect on growth when appropriately compared to the solvent control. This error did not impact the study conclusions (the test substance had no effect on growth up to 34 µg/L) and HSE considers this a minor deviation.

[14] U.S. EPA, 1996b. Office of Chemical Safety and Pollution Prevention. Ecological Effects Test Guideline, OCSPP 850.1500. Fish Life Cycle Toxicity. "Public Draft". EPA 712-C-96-122. April 1996. U.S. Environmental Protection Agency. Washington, D.C

Table 9.2.3-10: Recalculated Dunnett test values for comparing total length and wet weight treatment levels to the dilution water control.

Comparison[a]	Difference	Lower confidence interval	Upper confidence interval	p-value
Total length				
Control vs 9 µg/L	1.44	0.585	2.30	0.00186
Control vs 15 µg/L	1.77	0.915	2.63	0.000350
Control vs 34 µg/L	1.86	1.00	2.71	0.000350
Wet weight				
Control vs 9 µg/L	0.0143	0.000996	0.0276	0.0348
Control vs 15 µg/L	0.01830	0.00500	0.0316	0.0080
Control vs 34 µg/L	0.0216	0.00825	0.0349	0.0027

[a]Calculated using DunnettTest() function within DescTools package in R (4.3.2).

OECD 210 (2013) paragraph (§) 9 states, "*the use of silicone tubing in flow-through studies and use of silicone seals in contact with water should be minimised*". The diluter system and exposure aquaria were fabricated of glass and silicone sealant. The final definitive experiment did not suffer from unexpected loss of the active substance, indicating that the use of silicone sealant did not overly impact experimental integrity. HSE consider this a minor deviation. OECD 210 (2013) paragraph (§) 9 also says, "*the test system should preferably be conditioned with concentrations of the test chemical for a sufficient duration to demonstrate stable exposure concentrations prior to the introduction of test organisms*". Several initial definitive exposures were performed but were either terminated prior to 28 days post-hatch or were determined to be unacceptable due to variable measured concentrations at all treatment levels. The failure of two definitive exposures on the grounds of highly variable analytical results indicates the demonstration of stable exposure concentrations prior to the introduction of the test organism was not performed. This resulted in an avoidable increase in vertebrate testing. HSE accept that this deviation did not impact the final definitive exposure regime and consider it minor.

OECD 210 (2013) paragraphs (§) 11 and 21 address the feeding requirements of brood stock fish and test fish, respectively. The study conductor did not report a feeding regime for brood fish. Furthermore, during the test larvae were fed live brine shrimp nauplii. This did not change from newly hatched to 48h old brine shrimp nauplii as the larvae developed in juveniles, as specified in Annex III. Although the food provided did not adhere to the specified age food was live throughout, which provided the intended source of environmental enrichment. This is a minor deviation from the

guidance, which is not expected to have impacted the study results.

OECD 210 (2013) paragraph (§) 18 defines the preferred developmental stage of fertilised eggs. It states, "*the test should start as soon as possible after the eggs have been fertilised and preferably being immersed in the test solutions before cleavage of the blastodisc commences, or as close as possible after this stage*". This equates to developmental stage 9 or before, according to (U.S. EPA 1996a)[2]. This study used embryos at stage 15-16. Although older than preferred they were well age matched. It is, however, possible that some effects were overlooked due to the majority of embryonic development being completed before exposure initiation. HSE consider this a minor deviation but notes it may have had a small impact on the study results.

OECD 210 (2013) paragraph (§) 19 concerns the number of fertilised eggs included at the start of the test. The study conductor used 30 eggs per replicate, which is sufficient. After hatching success and % healthy larvae were determined the surviving larvae, if > 20, were thinned to 20 per replicate. There is no justification for why this was done. It was, however, performed indiscriminately. It is, therefore, unlikely to have impacted the study outcome and HSE consider this an acceptable modification.

OECD 210 (2013) Annex V outlines statistical guidance for NOEC determination. It states, "*if a solvent is used, then both a dilution water control and a solvent control should be included. The two controls should be compared for each response and combined for statistical analysis if no significant difference is found between the controls. Otherwise, the solvent control should be used for NOEC determination or ECx estimation and the water control is not used*". The study conductor compared treatment levels to the negative control (dilution water control) for responses where the two control were not statistically different. This is a more conservative approach, which is not expected to have impacted the integrity of the study.

The remaining points of discussion are not deviations from OECD 210 (2013) *per se*. Instead, these relate to three decisions made by the study conductor concerning the selection of analytical samples and the biological relevance of statistically significant results.

1) The first is the exclusion of the original measured concentration samples for Day 20. The study conductor reported that the original measured concentration samples were all approximately 20-40% higher than previous samples, with the 30 µg/L treatment level deviating even further (55 µg/L, 184 % of nominal). To investigate these unexpected results they analysed the frozen archive samples. These confirmed the elevated concentrations for all treatment levels at Day 20 (Table 9.2.3-6). However, the 30 µg/L nominal treatment level frozen sample had a less elevated concentration of 39 µg/L. Although not clearly reported, for both the original and frozen batches of analytical measurements it seems like the quality control samples were within the 70% - 120% acceptable range, ruling out a technical issue with the LC/MS/MS procedure. Substituting in the 55 µg/L value into the arithmetic mean concentration calculation changes the mean measured concentration from 34 to 36 µg/L.

   There is one clear line of evidence to support the 39 µg/L frozen archive sample value being a more accurate reflection of the test concentration at Day 20: the

fact that the frozen archive samples for all treatment levels are elevated by a more consistent percentage when compared to the original samples. This is in keeping with the experimental design, where the most concentrated test solution is prepared first, then serially diluted to create the other treatment levels. With this methodology it would be expected that an error in dosing would be proportionally similar in terms of the nominal concentration across all treatment levels, which is the pattern approximately present in the frozen archive samples. Nevertheless, whichever samples are used there is a clear increase in the test chemical concentration for all treatment groups at this timepoint, which suggests that test subjects were exposed to an unplanned concentration spike. This lack of a consistent concentration throughout the exposure period may have impacted study integrity. HSE propose a protective approach and the use of the frozen archive samples for the calculation of mean measured concentrations. This approach associates statistically significant effects with a lower concentration of the test substance. HSE note the potential unplanned concentration spike should be clearly highlighted as this may have impacted larval survival (see below).

2) Next, the conclusion of the study conductor that the statistically significant result for larval survival at 15 µg/L treatment level was not biologically relevant. Their argument is based on two points: the 91% larval survival value is within the range of historical control performance (mean = 96%, standard deviation = 5.6%, coefficient of variation = 5.9%, range = 80 - 100%, n = 36) and that the statistically significant result was likely a product of the unusually high negative control mean (99%) and low variability (coefficient of variation = 2.5%). On interrogating the historical control performance (Table 9.2.3-11) it becomes clear that high larval survival and low variability is not exceptional. In the 9 studies presented, 4 other studies had a mean larval survival of ≥ 98 % and CV ≤ 3 %. In fact, it could be argued that studies H and I were unusual in their relatively high variability and the presence of replicates with relatively low control larval survival. There are numerous explanations for the historical control variability that would not impact the control test subjects within the current study (variation in handling by study conductors, subtle differences in brood and test conditions, difference in inherent survivability of different brood stocks). All of these variables would be matched within the same study, hence the lower variability in the controls within study compared to between study. There is nothing to say that if the 15 µg/L S-2399 treatment level were carried out within any of the other studies there would not be an effect of the same magnitude (8%). Most importantly, within the confines of this study, where it is known that the aforementioned sources of variation are controlled for, there was a significant difference between the controls and the 15 µg/L treatment level. Therefore, HSE considers this is of biological relevance.

Table 9.2.3-11: Historical control performance of sheepshead minnow larval survival during early life-stage exposure studies

Study[a]	Replicate Mean (%)	Study Mean (%)	STDEV[b] (%)	CV[c] (%)
A	100	96	4.8	5

Study[a]	Replicate Mean (%)	Study Mean (%)	STDEV[b] (%)	CV[c] (%)
	90			
	95			
	100			
B	100	98	2.9	3
	100			
	95			
	95			
C	100	98	2.9	3
	95			
	95			
	100			
D	95	96	2.5	2.6
	100			
	95			
	95			
E	100	100	0	0
	100			
	100			
	100			
F	95	99	2.5	2.5
	100			
	100			
	100			
G	95	94	2.5	2.7
	90			
	95			
	95			
H	80	93	8.7	9.4
	100			
	95			
	95			
I	95	89	10.3	11.6
	100			

Study[a]	Replicate Mean (%)	Study Mean (%)	STDEV[b] (%)	CV[c] (%)
	80			
	80			
2-Year Variability	Mean Survival	96	5.6	5.9
	Minimum (%)	80		
	Maximum (%)	100		

[a]Summarised data compiles control larval survival from nine sheepshead minnow (Cyprinodon variegatus) early life-stage exposures dating back two years at [redacted]
[b]STDEV = Standard Deviation
[c]CV = Coefficient of Variation

3) Finally, the conclusion of the study conductor that the statistically significant result for larval survival at 30 µg/L treatment level was not biologically relevant. This conclusion was based on, alongside the above arguments, the observation that survival in replicates C and D (65 %) was lower compared to replicates A and B, replicates from the range finding test and replicates from the two failed definitive tests (Table 9.2.3-12). For the replicates from the range finding test and the two failed definitive tests to be valid, measured concentrations across the exposure period should closely follow the nominal concentration. The range finding test did not perform any analytical measurements, making the confirmation of the nominal concentration impossible. It is likely the concentration was lower than the nominal as the study conductor reported that testing prior to the final definitive study utilised a turnover rate of 10 aquarium replacements per day, instead of 13. This was implemented to combat S-2399 recovery losses suggesting that all studies before the final definitive study would have suffered from lower-than-expected measured concentrations for certain periods through the exposure window. This point can also be applied to replicates from the two failed definitive tests. The study conductor provided mean measured concentrations of 26 and 27 µg/L for the two failed definitive exposures, respectively. Mean measured concentrations, however, have no appreciation for the continuity of exposure and the study conductor reported that the previous definitive exposure attempts failed due to variable measured concentrations at all treatment levels. Therefore, unless the complete set of all analytical measurements for the failed definitive exposures are provided, the robustness of these replicates cannot be determined.

Table 9.2.3-12: 28-day post-hatch larval survival of sheepshead minnow exposed to 30 µg/L S-2399.

Test ID	Replicate ID	Survival (%)	SD (%)	CV (%)
Definitive 1	A	100	9.5	10
	B	100		
	C	95		
	D	80		

Test ID	Replicate ID	Survival (%)	SD (%)	CV (%)
Definitive 2	A	90	4.1	4.3
	B	95		
	C	100		
	D	95		
Range-Finder	A	95	5.8	6.4
	B	85		
	C	95		
	D	85		
Final	A	100	18.9	23.2
	B	95		
	C	65		
	D	65		

In the absence of additional data there is no strong evidence to support the claim that replicates C and D of the 30 µg/L treatment level are outliers with respect to larval survival when the nominal concentration is closely tracked throughout the exposure period. It could be argued that the potential concentration spike at Day 20 for the 30 µg/L treatment level was responsible for the lower larval survival in replicates C and D. If this were true it would raise a new question: why did replicates A and B not also present lower larval survival? Given the uncertainty surrounding multiple aspects of the analytical measurements at the 30 µg/L treatment level, both in the final definitive study and the previous failed definitive attempts, it is not possible to reject the biological relevance of the statistically significant reduction in larval survival. Therefore, HSE consider this is of biological relevance.

One final point to note is the replicates generated in this study are not completely independent. The solution preparation procedure involved preparing the treatment solution once, then flow-splitting cells were employed to equally distribute the solution to the four replicate aquaria. This means the replicates were pseudo-replicates. HSE notes that the spatial constraints with the laboratory may have precluded true replicates. It does, however, lead to issues in test chemical dosing affecting all replicates. Moreover, the solution preparation procedure also meant that replicates between treatment levels were also dependent on one another. This issue is clearly captured on Day 20, when an incorrectly high dosing whilst preparing the highest concentration affected all subsequent concentrations, due to the serial dilution approach. Overall, this experimental design resulted in the propagation of concentration errors for replicates between and within treatment levels, which in turn made the determination of NOEC and LOEC concentrations challenging.

The method of analysis used in the study was evaluated by HSE Chemistry. The conclusion of their evaluation is reproduced below. Please see Volume 3 CA, section

B5 for more details.

"*The analytical method is not fully validated according to SANTE/2020/12830 rev. 1 for the determination of the active substance inpyrfluxam in freshwater as the matrix effects have not been determined and the stability of standards and extracts has not been addressed. However, the studies were generated prior Ito the implementation of SANTE 2020/12830 rev.1 and SANCO 3029 rev.4 did not require matrix effects and the stabilities of standards and extracts to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose.*"

The agreed endpoints suitable for use in the risk assessment are:
NOEC (34 days) = 9 µg S-2399/L for larval survival
NOEC = 34 µg S-2399/L for hatch success
NOEC = 34 µg S-2399/L for live, normal larvae at hatch
NOEC = 34 µg S-2399/L for total body length
NOEC = 34 µg S-2399/L for wet weight

Therefore, based on the most sensitive indicator of toxicity the NOEC is 9 µg S-2399/L. The LOEC is 15 µg S-2399/L.

B.9.2.3.2 Fish full life stage toxicity test

No full fish life stage toxicity test was submitted.

B.9.2.3.3 Bioconcentration in fish

Reference:	KCA 8.2.2.3/01
Report Title:	Amended Report [^{14}C]S-2399 – Flow-Through Bioconcentration and Metabolism Study with Bluegill Sunfish (*Lepomis macrochirus*)
Author(s) & year:	[redacted] / [redacted] (2015/2020)
Document No, Authority registration No:	[redacted] Study No. 13048.6863
Substance used:	[pyrazolyl-4-^{14}C]-S-2399, CFQ41802, 99.3 %
Method of analysis:	HPLC/RAM
Guideline(s):	OECD 305 (2012)
Deviations:	Yes, see HSE comments section.
GLP or GEP:	Yes
Acceptability:	Yes

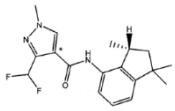
Study relied upon:	Yes

MATERIALS

Test Materials

Radiolabelled test material

[pyrazolyl-4-^{14}C]-S-2399



* denotes ^{14}C label position

Lot/Batch #: CFQ41802

Radioactive purity: 99.3% (based on HPLC analysis, verified by the certificate of analysis)

Specific activity: 57 mCi/mmol, 377,307 dpm/µg

Non-radiolabelled test material: S-2399 AS

Lot/Batch #: YT3424G

Purity: 99.9%

Solubility: 16.4 mg/L at 20 °C (water)

Log P_{ow} 3.65 at 25 °C

Treatments

Test concentrations: 0.2 and 0.6 µg a.s./L

Control: Solvent control

Solvent: Acetone

Analysis of test water concentrations: Day 0, 1, 3, 7, 14, 21 and 28 of exposure. Day 0, 1 and 3 of depuration. Analysed for [^{14}C]S-2399 using high performance liquid chromatography with radiometric detection (HPLC/RAM) and liquid scintillation counting (LSC) (LOD = 0.00603 to 0.00648 µg/L, LOQ = 0.0251 to 0.0264 µg/L)

Analysis of fish tissues: Day 1, 3, 7, 14, 21 and 28 (uptake)
Day 0, 1 and 3 (depuration)
(LOD = 0.177 µg/kg, LOQ = 0.53 µg/kg)

Test animals

Species: Bluegill Sunfish (*Lepomis macrochirus*)

Source: [redacted]

Length: Mean: 57 mm (52-63 mm) (n = 30)

Wet weight:	Mean: 2.82 g (2.31-3.45 g) (n = 30)
Acclimatisation period:	14 days prior to test initiation
Diet:	Commercial pelletised food daily at a rate of 1.5% (w/w) of biomass. Uneaten food and faecal matter were removed (by siphon) from each aquarium within one hour after feeding.

Test design

Exposure regime:	Flow-through
Aeration:	None
Replication:	5 aquaria (2 exposure (low and high concentration), 2 depuration, 1 control) 145 fish per treatment level. Five fish taken per sampling time point and pooled for analysis of fish tissue (10 time points) Additional fish from treated and control aquaria were collected and stored in a freezer.
Test vessels:	Clear glass aquaria with silicone adhesive measuring 75 × 39 × 30 cm (L × W × H) containing approximately 73 L. The aquaria were placed in a temperature-controlled water bath designed to maintain the temperature at 23 ± 2°C
Dilution water:	Well water (drawn from a 100-meter bedrock well) Hardness ($CaCO_3$) = 52 to 68 mg/L Alkalinity ($CaCO_3$) = 20 to 22 mg/L pH = 6.5 – 7.0 Conductivity = 322 to 462 µS/cm Total Organic Carbon (TOC) = 0.51 to 1.2 mg/L Representative samples of the dilution water source were analysed for the presence of pesticides, PCBs and toxic metals. None of these compounds were detected in any of the water samples analysed. Several species of daphnids are cultured and maintained in water from the same source as the dilution water utilized during this study and have successfully survived and reproduced over multiple generations.

Duration:	28 days exposure, 3 days depuration
Environmental conditions	
Test temperature:	19 – 25 °C (min max readings) 22 – 24 °C (daily measurement)
pH:	6.7 – 7.5
Dissolved oxygen:	5.3 to 8.1 mg/L (exposure aquaria) 7.0 to 8.5 mg/L (depuration aquaria) (60% of dissolved oxygen saturation value at 22 ºC is 5.2 mg/L)
Hardness:	44 to 48 mg/L
Lighting:	16 hours light, 8 hours darkness
Total Organic Carbon	4.750 to 6.084 mg/L (exposure), 1.522 to 1.911 mg/L (depuration)

STUDY DESIGN AND METHODS

Exposure and depuration period dates: 12 May 2015 to 9 June 2015 (exposure), 9 June 2015 to 14 June 2015 (depuration)

Test organism
Bluegill sunfish (*Lepomis macrochirus*; juvenile) was selected as the test species since it is recommended in OECD 305 (2012) and equivalent EPA literature for fish bioconcentration studies. Prior to testing fish were held in a 600 L fiberglass tank containing water from the same source as the test water (acclimation water quality – pH = 6.5 to 7.4, dissolved oxygen = 5.3 to 8.4 mg/L, flow rate = eight tank volumes/day, temperature = 22 to 24 ºC). No fish were observed to be dead in the test-fish population during the 48 hours prior to testing. The 435 fish from the test population had a mean wet weight of 2.82 g (range 2.31 to 3.45 g) and a subsample of that group (n = 30) had a mean length of 57 mm (range 52 to 63 mm).

Representative samples of the food were analysed for the presence of pesticides, PCBs and toxic metal. All analyte concentrations were below levels of concern.

Dosing stock preparation and test system
The primary stock solution (0.203 mg a.s./mL) was diluted with the appropriate volume of acetone to prepare the low (0.2 µg a.s./L) and high (0.6 µg a.s./L) concentration stock solutions. These were analysed by LSC to confirm concentrations of 0.0164 mg a.s./mL and 0.0498 mg a.s./mL, respectively.

The test substance delivery system consisted of two syringe pumps. One pump was used for the high and low concentration stocks, with two 50 mL gas-tight syringes, and the other was used for the solvent stock, also with a gas-tight syringe. Both pumps

were calibrated to deliver 5.04 µL/min of the appropriate stock solution into the appropriate mixing cell. These cells also received a flow of 420 mL/min of dilution water, providing a turnover rate equivalent to 8.3 aquarium volumes per 24 hours and a 90% aquarium volume replacement every 6-hour period. The concentration of acetone was equivalent to the solvent concentration in the exposure aquaria (0.01 mL/L).

The diluter system was in operation for at least fourteen days prior to test initiation. During this period, test solutions were sampled and analysed for [^{14}C]S-2399 on Day -2 to determine whether the appropriate test substance concentrations were being delivered and maintained in the aquaria.

Test initiation and monitoring

At test initiation, the total biomass per aquarium was 2.82 g (0.68 g/L of the 24-hour flow-through volume of the aquaria). Daily observations were made on the appearance and behaviour of the fish and the physical appearance of the test solution. Temperature, dissolved oxygen concentration and pH were measured daily in each test aquarium. Total organic carbon (TOC) was measured in each treatment level test aquarium twice prior to addition of fish. In all aquaria, TOC was measured weekly during the in-life phase. Total hardness (expressed as CaCO3) was measured at test initiation in each aquarium.

Exposure of bluegill to [^{14}C] S-2399 at nominal concentration of 0.2 and 0.6 µg/L established a steady state tissue residue concentration. Steady state is defined as three consecutive sampling intervals of at least two days for which the measured concentrations of test substance in fish did not differ by more than 20% of each other and when the tissue concentration, plotted against time, becomes parallel to the time axis. Once equilibrium was achieved and the fish had been exposed for 28 days, the remaining fish (59 fish for both the 0.2 and 0.6 µg/L treatment level) from each exposure aquarium were transferred to corresponding depuration aquaria. Dilution water was introduced at a rate equal to the flow rate maintained during the exposure period (420 mL/min).

Determination of [^{14}C] residues in water

To monitor the concentration of ^{14}C residues in the exposure water, triplicate 10-mL water samples were taken from the 0.2 and 0.6 µg a.s./L nominal exposure tanks on Day 1, 3, 7, 14, 21 and 28. Triplicate 10-mL water samples were collected from the 0.2 and 0.6 µg a.s./L aquaria on Days 0, 1 and 3 of depuration, respectively.

Exposure water was transferred into separatory funnel, dichloromethane added and vigorously shaken for approximately one minute. The layers were allowed to separate and the organic layer was placed into a graduate cylinder to measure the volume. The aqueous layer was partitioned a second time with dichloromethane using the above procedure. The organic layers were combined, volume recorded and analysed by

LSC. The organic extract was then concentrated to dryness under reduced pressure using a rotary evaporator. The flask was rinsed with 2 mL of 1:1 acetonitrile:methanol (v:v), sonicated for approximately two minutes and analysed by LSC. A portion of the concentrated aliquot was centrifuged at 10,000 rpm for five minutes and aliquot of the supernatant was analysed by LSC and HPLC/RAM.

Determination of Total Radioactive Residues (TRR) and metabolites in fish

To quantify the total [^{14}C] residues in the tissue of bluegill, five fish were collected from each exposure aquaria on Day 1, 3, 7, 14, 21 and 28 of exposure and on Day 0, 1 and 3 of depuration.

Calculations

LSC Calculations

The concentration of total ^{14}C residues (TRR) as S-2399 equivalents in each water and tissue sample was calculated using the formulae:

$$Net\ dpm\ =\ [(subsample\ dpm)\ /\ (subsample\ size)]\ \times\ (total\ sample\ size)$$

$$TRR\ =\ (Net\ dpm)\ /\ (Effective\ Specific\ Activity\ _{diluter\ stock\ solution})\ \times\ (total\ sample\ size)$$

where:

$Net\ dpm\ =$
$Disintegrations\ per\ minute\ calculated\ by\ the\ LSC\ after\ background\ subtraction$
$Effective\ specific\ activity\ _{diluter\ stock\ solution}\ =\ Specific\ activity\ of\ [14C]S-2399\ \times\ \%\ radioactivity\ of\ the\ diluter\ stock\ solution\ (dpm/\mu g)$
$Subsample\ Size\ =$
$Subsample\ weight\ (g)\ or\ subsample\ volume\ (mL)\ for\ LSC\ analysis$
$Sample\ Size\ =\ Sample\ wet\ weight\ (g)\ or\ sample\ volume\ (mL)$

Fish Lipid Content Calculations

The weight of the whole fish lipid sample was divided by the original whole fish tissue wet weight to determine the fraction of wet weight lipid content. The weight of the whole fish lipid sample was divided by the final dry tissue weight to determine the fraction of dry weight lipid content.

Bioconcentration Factor Computation

BCF_{TRR} – Based on total radioactive residues at steady state was determined by dividing the mean measured equilibrium (steady state) ^{14}C tissue concentration by the mean measured water concentration of total ^{14}C residue during the exposure phase. The mean measured equilibrium ^{14}C residue tissue concentrations were calculated for each concentration using pooled whole fish tissue measurements starting from the interval at which steady state was established (Day 14) through exposure termination

(Day 28) for the 0.2 and 0.6 µg/L exposure aquaria.

$BCF_{S\text{-}2399}$ – Based on parent [^{14}C] S-2399 at steady state was determined by dividing the measured equilibrium (steady state) [^{14}C] S-2399 concentration of fish tissue by the mean measured water concentration of [^{14}C] S-2399 during the exposure phase.

$BCF_{L,TRR}$ and $BCF_{L,S\text{-}2399}$ – Based on the lipid-normalised fish with a 5% lipid content using the following calculation:

$$BCFL = BCF \times 5\% / Lipid\ content\ at\ steady\ state\ (\%)$$

Uptake and Depuration Rate Constants

The uptake rate constant (Ku) and depuration rate constant (Kd) were determined by obtaining the best fit of the water concentration during uptake and tissue concentrations during the uptake and depuration phases simultaneously with the following equations describing tissue accumulation and depuration:

For uptake phase:

$$C_t/C_w = (K_u/K_d)\,[1 - e^{(-K_d t)}]$$

For depuration phase:

$$C_t = C_{t,0}\, e^{(-K_d t)}$$

(C_w is assumed to be zero for the depuration phase)

where:

$C_t = tissue\ concentration\ at\ time\ t$
$K_u = uptake\ constant\ (day - 1\ )$
$K_d = depuration\ constant\ (day - 1\ )$
$C_w = mean\ water\ concentration\ during\ uptake\ phase\ (\mu g/L)$
$C_{t,0} = tissue\ concentration\ at\ the\ start\ of\ depuration\ period\ (\mu g/kg)$
$t = time\ in\ days$

A nonlinear regression was used to determine the parameters K_u and K_d that minimized the sum of the squared differences between the observed and fitted values. The software curve-fitting program SigmaPlot® (Version 10.0, SPSS Inc., Chicago, Illinois), employing the described algorithm, was used to solve for the values for K_u and K_d.

The depuration half-life was calculated for whole fish using first order kinetics and the following equation:

$$Half\ life\ =\ ln\,(2)\ /\ depuration\ rate\ constant\ (K_d)$$

Kinetic Bioconcentration Factor ($BCF_{K,\ TRR}$)

Calculated using the ratio of K_u and K_d and expressed on the basis of the total radioactive residues. [^{14}C]S-2399 based BCF_K was not calculated due to rapid depuration below the limit of detection.

BCF_{KG} and BCF_{KGL}

The BCF_K was also corrected for growth by subtraction of the growth rate constant (K_g) from K_d (K_{2g}, which in turn gives BCF_{KG}), which was further normalised to a 5% lipid content (to give BCF_{KGL}).

RESULTS AND DISCUSSION

Throughout the study, no undissolved test substance was observed in the dilution system or the test aquaria. At test termination no fish were observed to be dead during the test period (uptake/depuration phase) for the control, low- and high-level concentrations, respectively. All surviving fish appeared to be healthy and exhibited normal behaviour throughout the study.

Fish weight and lipid content

Table 9.2.3-13 Table 9.2.3-14 present fish tissue weights and lipid content in fish tissue during the exposure and depuration phase, respectively. These values were used for the growth rate and lipid content BCF corrections (Table 9.2.3-19).

Table 9.2.3-13: Fish tissue weights during the exposure and depuration phase

Stage	Sampling Day	Weight (g) Control[a]	Weight (g) Low Dose[a]	Weight (g) High Dose[a]
Exposure	0	14.7323	15.1193	13.6217
	1	15.6615	15.528	14.9012
	3	17.1094	14.0643	16.8387
	7	18.9555	16.5354	17.3587
	14	15.2704	18.8178	18.4536
	21	20.7636	20.4856	14.6654
	28	24.1975	19.8678	20.6769
Depuration	1	25.2022	22.1318	25.4628
	3	24.2654	22.2578	20.5803

[a] The weights are the composite weight of 5 fish sampled at each time point.

Table 9.2.3-14: Lipid content in fish tissue during the exposure and depuration phase

Sample Day / Treatment Group	Total Fish Tissue Wet Weight (g)	Lipids Weight (g)	Total Fish Tissue Dry Weight (g)	Lipid Content Wet (%)[a]	Lipid Content Dry (%)[b]
Exposure Day 0					
Control	14.7323	0.6435	2.9627	4.37	21.72
0.2 µg/L	15.1193	0.5148	3.0544	3.4	16.85
0.6µg/L	13.6217	0.5022	2.6665	3.69	18.83
Exposure Day 1					
Control	15.6615	0.6548	3.0221	4.18	21.67
0.2	15.528	0.6413	3.036	4.13	21.12
0.6	14.9012	0.612	2.8624	4.11	21.38
Exposure Day 3					
Control	17.1094	0.7759	3.3546	4.53	23.13
0.2	14.0643	0.5922	2.8449	4.21	20.82
0.6	16.8387	0.6936	3.7876	4.12	18.31
Exposure Day 7					
Control	18.9555	0.8723	3.7111	4.6	23.51
0.2	16.5354	0.7597	3.3559	4.59	22.64
0.6	17.3587	0.8387	3.4508	4.83	24.3
Exposure Day 14					
Control	15.2704	0.739	2.9326	4.84	25.2
0.2	18.8178	0.8774	3.7477	4.66	23.41
0.6	18.4536	0.9205	3.558	4.99	25.87
Exposure Day 21					
Control	20.7636	0.9319	4.1358	4.49	22.53
0.2	20.4856	0.8818	4.1938	4.3	21.03
0.6	14.6654	0.6788	3.0308	4.63	22.4
Exposure Day 28					
Control	24.1975	1.0664	4.6593	4.41	22.89
0.2	19.8678	0.9013	4.3091	4.54	20.92

Sample Day / Treatment Group	Total Fish Tissue Wet Weight (g)	Lipids Weight (g)	Total Fish Tissue Dry Weight (g)	Lipid Content Wet (%)[a]	Lipid Content Dry (%)[b]
0.6	20.6769	0.9487	4.5778	4.59	20.72
Depuration Day 1					
Control	25.2022	1.2545	5.8489	4.98	21.45
0.2	22.1318	1.0389	4.9384	4.69	21.04
0.6	25.4628	1.2377	5.1863	4.86	23.86
Depuration Day 3					
Control	24.2654	1.2038	4.5115	4.96	26.68
0.2	22.2578	1.0795	4.1884	4.85	25.77
0.6	20.5803	0.9962	4.5929	4.84	21.69

[a] (%) wet weight lipid = total lipids weight (g) ÷ total fish tissue weight wet (g) × 100
[b] (%) dry weight lipid = total lipids weight (g) ÷ final fish weight dry (g) × 100

Chemical analysis

Determination of [^{14}C] residues in water

Throughout the exposure phase, only [^{14}C] S-2399 was detected in the exposure water, and concentrations of measured S-2399 ranged from 0.185 to 0.222 µg a.s./L and 0.527 to 0.636 µg a.s./L for the low and high concentrations, respectively. The concentration of S-2399 was maintained within ± 20% of the mean measured values during uptake phase. Furthermore, the analysis showed that the test substance was not detected in the low and high tank water samples during the depuration phase. The results of the analysis in the water samples during the uptake phase are summarised in the Table 9.2.3-15.

Table 9.2.3-15: Determination of [^{14}C] S-2399 concentration in water during the exposure phase

Nominal concentration (µg a.s./L)	Mean measured exposure concentration (µg a.s./L)						
	Day 0	Day 1	Day 3	Day7	Day 14	Day 21	Day 28
Total ^{14}C concentration (µg/L) (% of nominal) by Direct LSC[a]							
Control	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
0.2	0.190 (94.99)	0.185 (92.66)	0.212 (105.99)	0.221 (110.40)	0.204 (102.12)	0.222 (110.98)	0.213 (106.67)
0.6	0.602 (100.39)	0.527 (87.77)	0.629 (104.89)	0.636 (105.94)	0.575 (95.78)	0.581 (96.83)	0.579 (96.48)

Nominal concentration (µg a.s./L)	Mean measured exposure concentration (µg a.s./L)						
	Day 0	Day 1	Day 3	Day7	Day 14	Day 21	Day 28
Measured concentration of [^{14}C] S-2399[b] (µg a.s./L) (% of nominal) by HPLC/RAM							
Control	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
0.2	0.190 (94.99)	0.185 (92.66)	0.212 (105.99)	0.221 (110.40)	0.204 (102.13)	0.222 (110.98)	0.213 (106.67)
0.6	0.602 (100.39)	0.527 (87.77)	0.629 (104.89)	0.636 (105.94)	0.575 (95.78)	0.581 (96.83)	0.579 (96.48)

[a] The concentration of radioactivity in the water samples was determined by LSC analysis of triplicate (10 mL) aliquots
[b] The concentration of S-2399 in the water samples was determined by duplicate analysis of HPLC/RAM.
n.d.= not detected

Determination of Total Radioactive Residues (TRR) and [^{14}C] S-2399 in fish

The concentrations of [^{14}C] residues measured during the exposure and depuration are presented in Table 9.2.3-15.

Table 9.2.3-16: Concentrations of total radioactivity residues fish tissue during the exposure and depuration phase

Nominal concentration (µg a.s./L)	Measured exposure concentration (µg a.s./kg)									
	Exposure Phase						Depuration Phase[b]			
	Day 1	Day 3	Day 7	Day 14	Day 21	Day 28	Day 1	% reduction from steady state[c]	Day 3	% reduction from steady state[c]
0.2	34.9	36.2	37.8	44.1[a]	34.5[a]	42.9[a]	2.19	94.6	0.697	98.3
0.6	73.2	97.5	88.2	97.0[b]	111[b]	92.0[b]	10.2	89.8	8.87	91.1

[a] The steady state concentration in fish tissue of the low dose (Average of Day 14, Day 21, Day 28) was determined to be 40.5 µg/kg
[b] The steady state concentration in fish tissue of the low dose (Average of Day 14, Day 21, Day 28) was determined to be 100 µg/kg
[c] Calculated by HSE using rounded figures provided in table.

The distribution of extractable and non-extractable radioactivity in the fish tissues demonstrates that the extractability generally remained consistent over the exposure ranging from 95.8 to 96.8% TRR and 95.8 to 97.6% TRR in the low and high

concentrations, respectively. During depuration phase, extractability was 100% for the low concentration and 93.2 and 92.4% on depuration Day 1 and Day 3 for the high concentration.

Metabolic pathway of S-2399 in Bluegill Sunfish

[^{14}C] S-2399 was metabolised by hydroxylation, oxidation and sulphate conjugation to form *N*-des-Me-1'-CH_2OH-S-2840 and 1'-CH_2OH-S-2840A, 1'-COOH-S-2840A or 1'-CH_2OH-S-2840B-sulfate. No isomerisation of a.s. was observed. The distribution of [^{14}C] residues in fish during the exposure and depuration stage is presented in Table 9.2.3-17 (low concentration) and Table 9.2.3-18 (high concentration).

Table 9.2.3-17: Distribution of ^{14}C residues in fish samples (test concentration 0.2 µg/L) during the study

Exposure	Day 1		Day 3		Day 7		Day 14		Day 21		Day 28		Dep Day 1		Dep Day 3	
	µg/kg	%	µg/kg	%	µg/kg	%	µg/kg	%	µg/kg	%	µg/kg	%	µg/kg	%	µg/kg	%
Whole fish	34.93	100.0	36.18	100.0	37.80	100.0	44.07	100.0	34.54	100.0	42.89	100.0	2.19	100.0	0.70	100.0
Extract-able	33.48	95.9	34.86	96.4	36.53	96.7	42.59	96.6	33.09	95.8	41.52	96.8	2.19	100.0	0.70	100.0
Unextractable	1.45	4.1	1.32	3.6	1.26	3.3	1.48	3.4	1.45	4.2	1.36	3.2	n.d.	n.d.	n.d.	n.d.
S-2399	6.88	19.7	7.11	19.7	7.10	18.8	6.61	15.0	6.05	17.5	5.97	17.3	n.d.	n.d.	n.d.	n.d.
MET 1	26.60	76.1	15.64	43.2	22.06	58.4	28.42	64.5	12.17	35.2	16.96	39.5	0.81	36.9	n.d.	n.d.
MET 2	n.d.	n.d.	12.11	33.5	6.05	16.0	6.45	14.6	10.71	31.0	13.55	31.6	0.62	28.4	n.d.	n.d.
MET 3	n.d.	n.d.	n.d.	n.d.	1.33	3.5	1.11	2.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MET 4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.16	12.0	5.04	11.7	0.41	18.9	n.d.	n.d.
Acetone	<LOQ	n.a.	<LOQ	n.a.	<LOQ	n.a.	<LOQ	n.a.	<LOQ	n.a.	<LOQ	n.a.	<LOQ	n.a.	<LOQ	n.a.

n.d. = not detected
n.a. = not applicable
LOQ = limit of quantitation
MET 1 = 1'-COOH-S-2840A-sulfate/1'-CH2OH-S-2840B-sulfate, MET 2 = *N*-des-Me-

1'-CH2OH-S-2840, MET 3 = Not identified, MET 4 = 1'-CH2OH-S-2840A
Note: Calculations were performed using the actual unrounded analytical data and not the rounded values presented in this table

Table 9.2.3-18: Distribution of ^{14}C residues in fish samples (test concentration 0.6 µg/L) during the study

Exposure	Day 1		Day 3		Day 7		Day 14		Day 21		Day 28		Dep Day 1		Dep Day 3	
	µg/kg	%	µg/kg	%	µg/kg	%	µg/kg	%	µg/kg	%	µg/kg	%	µg/kg	%	µg/kg	%
Whole fish	73.16	100.0	97.47	100.0	88.15	100.0	96.95	100.0	111.05	100.0	92.03	100.0	10.22	100[a]	8.87	100.0
Extract-able	70.99	97.0	94.16	96.6	86.08	97.6	93.32	96.3	106.38	95.8	88.99	96.7	9.53	93.2[a]	8.19	92.4
Unextractable	2.17	3.0	3.32	3.4	2.07	2.4	3.64	3.7	4.67	4.2	3.04	3.3	0.69	6.75[a]	0.68	7.6
S-2399	17.98	24.6	18.10	18.6	17.81	20.2	15.65	16.1	18.69	16.8	16.77	18.2	n.d.	n.d.	n.d.	n.d.
MET 1	53.01	72.5	41.76	42.8	49.82	56.5	55.32	57.1	36.30	32.7	32.89	35.7	3.24	31.7	2.37	26.7
MET 2	n.d.	n.d.	34.29	35.2	13.46	15.3	16.79	17.3	34.82	31.4	27.54	29.9	3.75	36.7	3.74	42.2
MET 3	n.d.	n.d.	n.d.	n.d.	5.00	5.7	5.55	5.7	3.47	3.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MET 4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	13.11	11.8	11.78	12.8	1.35	13.3	1.77	20.0
Acetone	<LOQ	n.a.	<LOQ	n.a.	<LOQ	n.a.	<LOQ	n.a.	<LOQ	n.a.	<LOQ	n.a.	<LOQ	n.a.	<LOQ	n.a.

n.d. = not detected
n.a. = not applicable
LOQ = limit of quantitation
MET 1 = 1'-COOH-S-2840A-sulfate/1'-CH_2OH-S-2840B-sulfate, MET 2 = *N*-des-Me-1'-CH_2OH-S-2840, MET 3 = Not identified, MET 4 = 1'-CH_2OH-S-2840A
Note: Calculations were performed using the actual unrounded analytical data and not the rounded values presented in this table
[a] Corrected by HSE and calculated using rounded values provided in table

Bioconcentration factors
A summary of the bioconcentration factors are presented in the Table 9.2.3-19.

Table 9.2.3-19: Calculated BCF values and associated endpoints

	Low concentration (0.2 µg a.s./L, nominal)	High concentration (0.6 µg a.s./L, nominal)
Total ^{14}C residue basis		
Average ^{14}C water concentration (Total Radioactive Residues (TRR), µg/L)	0.207	0.590
Steady state tissue concentration (TRR, µg/kg)	40.5	100
Bio-Concentration Factor (BCF) based on TRR (BCF_{TRR}, L/kg)	196	170
BCF_{TRR} normalised for 5 % lipid content ($BCF_{L,TRR}$, L/kg)	218	179
Uptake constant ($K_{u,TRR}$, L/kg/day)	465	294
Depuration constant ($K_{d,TRR}$, day^{-1})	2.55	1.77
Growth rate (K_g)	0.0134	0.0134
Growth corrected depuration constant (K_{2g})	2.54	1.75
Kinetic BCF_{TRR} ($BCF_{K,TRR}$, L/kg)	183	166
Growth corrected $BCF_{K,TRR}$ ($BCF_{KG,TRR}$, L/kg)	184	168
Lipid normalised $BCF_{KG,TRR}$ ($BCF_{KGL,TRR}$, L/kg)	210	185
Depuration half-life of total residue (days)	0.27	0.39
[^{14}C] S-2399 basis		

	Low concentration (0.2 µg a.s./L, nominal)	High concentration (0.6 µg a.s./L, nominal)
Steady state [^{14}C] S-2399 Water concentration (µg/L)	0.207	0.590
Steady state [^{14}C] S-2399 Tissue concentration (µg/kg)	6.21	17.1
$BCF_{S\text{-}2399}$ (L/kg)	30.0	29.0
$BCF_{L,S\text{-}2399}$ (L/kg)	33.4	30.5

Calculated values presented in this table have been rounded and may not match calculations performed using unrounded data.
The S-2399 based depuration kinetics was not conducted due to no S-2399 being detected during the depuration phase.
K_g = growth rate constant
$K_{2g} = K_{d,\ TRR} - K_g$
$BCF_{KG} = K_{U,\ TRR} \div k_{2g}$
BCF_{KGL} = growth-lipid-corrected kinetic BCF. 5% lipid normalization was done using average lipid value
throughout exposure/depuration period; Low: 4.37%, High: 4.52%.

Validity criteria
The study partially meets the validity criteria of guideline OECD 305 (2012) as follows:

Table 9.2.3-20: Compliance with OECD 305 (2012) validity criteria

Validity criterion	Required	Obtained
Temperature	Variation < ± 2°C	22 – 24 ºC (daily measurements) 19 – 25 ºC (minimum/maximum)
Dissolved oxygen	> 60 % saturation	5.3 to 8.1 mg/L (exposure aquaria) 7.0 to 8.5 mg/L (depuration aquaria) (60% of dissolved oxygen saturation value at 22 ºC is 5.2 mg/L)
Test substance concentration	Within ± 20% of the mean of the measured values	1.31 – 10.5 %[a]

Validity criterion	Required	Obtained
	during the uptake phase	
Test substance concentration in relation to solubility limit	Below limit of effective solubility	Yes
Mortality or other adverse effects/disease in control and test fish	< 10 % at test termination	0 % mortality. Healthy and normal behaviour

[a] Calculated by HSE using the rounded values provided in Table A

CONCLUSION

The bioconcentration factor for total radioactive residues (BCF_{TRR}) in the low concentration medium (0.2 µg a.s./L) was 196 L/kg and the corresponding value for S-2399 (BCF_{S-2399}) was 30.0 L/kg. The bioconcentration factor for total radioactive residues ($BCFT_{RR}$) in the high concentration medium (0.6 µg a.s./L) was 170 L/kg and the corresponding value for S-2399 (BCF_{S-2399}) was 29.0 L/kg.

The lipid-normalised bioconcentration factor for total radioactive residues ($BCF_{L,TRR}$) was 218 L/kg, and the corresponding value for S-2399 ($BCF_{L,S-2399}$) was 33.4 L/kg for the low exposure concentration. The lipid-normalised bioconcentration factor for total radioactive residues ($BCF_{L,TRR}$) was 179 L/kg, and the corresponding value for S-2399 ($BCF_{L,S-2399}$) was 30.5 L/kg for the high exposure concentration.

The uptake rate constants (K_{u-TRR}) based on total ^{14}C were 465 and 294 day^{-1} for the low and high concentration, respectively.

The depuration rate constants (K_{d}-$_{TRR}$) based on total ^{14}C were 2.55 and 1.77 day^{-1} for the low and high concentration, respectively. The half-life for depuration based on the total residues was 0.27 and 0.39 days for the low and high concentration, respectively. After 3 days of depuration, more than 98.3% and 91.1% of radioactivity present in whole fish at steady-state of the low and high concentrations, respectively, was eliminated. The half-life based on S-2399 was not calculated due to rapid depuration below the limit of detection.

The kinetic bioconcentration factors for total radioactive residues ($BCF_{K,TRR}$) in the low and high concentration medium were 183 and 166 L/kg, respectively. Once corrected for growth and lipid content $BCF_{KGL,TRR}$ = 210 and 185 L/kg for the low and high concentration treatment groups, respectively. The kinetic $BCF_{KGL,\ TRR}$ values of [^{14}C] residues were very similar to measured $BCF_{L,\ TRR}$.

HSE COMMENTS:
The study was carried out according to GLP and follows OEC 305 (2012). All validity criteria were met except the criterion involving temperature variation. On Day 16 the minimum/maximum thermometer read 19.4 ºC, which was outside the lower range (23 ºC ± 2°C). This was associated with a scheduled power outage that lasted for approximately one hour. This deviation from the defined temperature range lasted for a short period of time, as evidenced by the acceptable daily temperature measurements on Day 16. Furthermore, fish were observed to be stress free in the time period directly after the temperature deviation. Therefore, HSE considers this a minor deviation, which is unlikely to have impacted study validity.

The following deviations to OECD 305 (2012) were noted:

OECD 305 (2012) paragraph (§) 19 states, "*the uptake rate constant, the depuration (loss) rate constant (or constants, where more complex models are involved), the bioconcentration factor (steady-state and/or kinetic), and where possible, the confidence limits of each of these parameters are calculated from the model that best describes the measured concentrations of test substance in fish and water*". Confidence limits around parameters were not reported. The impacts of this omission are discussed in more detail in relation to § 78 below.

OECD 305 (2012) § 38 states, "*a steady-state is reached in the plot of test substance in fish (C_f) against time when the curve becomes parallel to the time axis and three successive analyses of C_f made on samples taken at intervals of at least two days are within ± 20 % of each other, and there is no significant increase of C_f in time between the first a last successive analysis*".
For the low concentration, the steady state was calculated as the average of C_f for Day 14, 21 and 28. The Day 14 and 21 C_f concentrations are not within 20 % of one another (44.1 – 34.5 = 9.6; (9.6/44.1)*100 = 21.8% decrease). Therefore, steady state was not met according to the guideline for these three time points. Furthermore, when pooled samples are analysed, at least four successive concentrations within ± 20 % of each other are required. Technically the low concentration did not reach steady state, with no four successive concentrations within ± 20 % of each other. This, however, was likely due to variation in the data associated with the use of one pooled sample at each timepoint, rather than a true failure to reach steady state. For example, between Day 1 to 14 the TRR in fish tissue increased by 26.4 % from 34.9 to 44.1 µg/kg, then decreased to 34.5 µg/kg on Day 21. The comparable concentration for Day 1 and Day 21 supports the interpretation that the 26.4 % increase between Day 1 to 14 was due to experimental variation rather than a failure to reach steady state. Therefore, HSE Ecotoxicology has taken a pragmatic approach and defined steady state as between Day 1 and 14 for the low concentration, despite the exceedance of the 20 % threshold. The average concentration within this time frame is 38.3 µg TRR/kg.

For the high concentration, a similar situation arises. Steady state based on the four successive timepoint definition is not reached. However, Day 3 to 21 appears a suitable candidate for steady state. Between Day 3 and 21, the largest difference is between Day 7 (88.2 µg/kg) and Day 21 (111 µg/kg), a 25.9 % increase. This is followed by a reduction to 92 µg/kg on Day 28, again suggesting that experimental variation explains the pattern of results between Day 3 and 21. Consequently, Day 3 to 21 is selected as the steady state. The average concentration within this time frame is 98.4 µg TRR/kg.

OECD 305 (2012) § 39 states, "*for substances following first order kinetics, a period of half the duration of the uptake phase is usually sufficient for an appropriate (e.g. 95%) reduction in the body burden of the substance to occur*". The study conductor selected a 3-day depuration phase, which resulted in a 91.1 % reduction in steady state concentration for the high treatment level. This is below the 95 % reduction deemed appropriate. Why such a short depuration phase was selected is unclear. Nevertheless, the generated data allowed for the calculation of K_d for the high treatment level. For this reason, HSE consider this a minor deviation with low impact on the study output.

OECD 305 (2012) § 40 and 66 concern the analysis of test concentration in fish. They state, "*fish should only be pooled if analysis of single fish is not feasible*" and "*the concentration of the test substance should usually be determined for each weighed individual fish. If this is not possible, pooling of the samples on each sampling occasion may be done*". The study pooled fish at each unique time point and test concentration combination for analysis. This justification for this approach was not outlined. This point is discussed further in the RAI response detailed below.

OECD 305 (2012) § 41 states, "*it should also be reported whether male or female, or both are used in the experiment. If both sexes are used, differences in growth and lipid content between sexes should be documented to be non-significant before the start of the exposure, in particular if it is anticipated that pooling of male and female fish will be necessary*". The study does not report the sex of the fish used but does report that they were juveniles. Historically, HSE Ecotoxicology has accepted the lack of sex characterisation for juvenile fish if they are a comparable weight and length. This is the case here (mean wet weight of 2.82 g (range 2.31 to 3.45 g), n = 435; mean length of 57 mm (range 52 to 63 mm), n = 30) so to be consistent with previous submissions, this omission is considered acceptable.

OECD 305 (2012) § 42 states, "*since weight and age of a fish may have a significant effect on BCF values (12) these details should be recorded accurately*". For age, fish were reported to be approximately the same age. The study did report that the smallest fish was within two-thirds the weight of the largest, all test fish were taken from the same population and their weight was accurately determined before the test. Weights for each treatment group were within 10 % of one another on Day 0, which

indicates that fish were sufficiently matched at the beginning of the experiment. The lack of detailed information on age is unlikely, therefore, to have impacted the study outcome.

OECD 305 (2012) § 48 describes the requirements for the type and characteristics of illumination. The study inadvertently omitted a light intensity measurement. Considering the lack of abnormal fish behaviour throughout the study this is considered a minor reporting omission.

OECD 305 (2012) § 51 details the selection of appropriate test concentrations. 0.2 µg/L appears to have been appropriately selected as it is an order of magnitude (ten times) above the LOQ for test water and 270 times smaller than the 96-hour LC_{50} of 54 µg/L. § 51 states, "*if a second concentration is used, it should differ from the one above by a factor of ten*". Concentrations only differed by a factor of 3. In the absence of chronic toxicity data, the guideline suggests concentrations should be below 1 % of the acute asymptotic LC_{50}. This was not reported, which prevents its comparison with 2 µg/L (the high concentration that should have been used if the guideline was adhered to). It is possible that 0.6 µg/L was selected as it is approximately 1 % of the 96-hour LC_{50} and using a tenth of this concentration for the lower concentration was prohibited by the requirement of the concentration being ten times greater than the LOQ.

OECD 305 (2012) § 61(16) states, "*in addition to weight, total length should be recorded because comparison of the extent of length increase during the test is a good indicator of whether an adverse effect has occurred*". Total length was not recorded throughout the study. It is possible that some adverse effects, expressed as a change in length but not mass, may have been overlooked. Measuring length may have helped determine if potential difference in mass between treatment groups at the end of the exposure period were due to the adverse effects of S-2399 or a product of natural variation in mass. This omission of length data is unlikely to have been impactful due to the lack of treatment effect on growth rate, detailed in the RAI response below. No further consideration is required.

OECD 305 (2012) § 69 explains how the BCF_{SS} should be calculated. It states, "*if the curve has reached a plateau, that is, become approximately asymptotic to the time axis, the steady-state BCF (BCF_{SS}) should be calculated from: C_f at steady state (mean) / C_w at steady state (mean)".* The study instead performed the calculation: C_f at steady state (mean) / C_w whole exposure period (mean). Furthermore, a simple arithmetic mean was used to calculate C_w instead of TWA. HSE has recalculated the BCF_{TRR} (BCF_{SS}) for the low and high treatment groups using only concentrations from the steady-state phase. A TWA equation from OECD 23 (2019), suitable for flow-through exposure systems, was used for C_w calculations in the table below. $BCF_{L,TRR}$ was calculated according to the guideline, which states, "*If lipid analysis was not conducted on all sampled fish, a mean lipid value is used to normalise the BCF. For*

the steady-state BCF, the mean value recorded at the end of the uptake phase in the treatment group should be used.". This is 4.26 % (an average of all lipid wet weights across the exposure period) for the 0.2 µg/L treatment group and 4.42 % for the 0.6 µg/L treatment group.

Table 9.2.3-21: Recalculated $C_{f,\ TRR}$, $C_{w,\ TRR}$, BCF_{TRR} and $BCF_{L,TRR}$

Concentration (µg TRR/L)	$C_{f,TRR}$ at steady-state (arithmetic mean, µg TRR/kg)	$C_{w,TRR}$ at steady-state (twa mean, µg TRR/L)	BCF_{TRR} (L/kg fish)	$BCF_{L,TRR}$ (L/kg fish)
0.2	38.25	0.212	180.42	211.77
0.6	98.43	0.600	164.05	185.58

The same is done for $C_{f,\ S\text{-}2399}$.

Table 9.2.3-22: Recalculated $C_{f,\ S\text{-}2399}$, $C_{w,\ S\text{-}2399}$, $BCF_{S\text{-}2399}$ and $BCF_{L,\ S\text{-}2399}$

Concentration (µg S-2399/L)	$C_{f,S\text{-}2399}$ at steady-state (arithmetic mean, µg S-2399/kg)	$C_{w,S\text{-}2399}$ at steady-state (twa mean, µg S-2399/L)	$BCF_{S\text{-}2399}$ (L/kg fish)	$BCF_{L,S\text{-}2399}$ (L/kg fish)
0.2	6.93	0.212	32.73	38.42
0.6	17.58	0.600	29.25	33.09

OECD 305 (2012) § 71, 72 and 73 discuss correcting K_d with growth rate K_g and how this should be performed. The first step is to correctly calculate K_g. The study report did this by regressing the natural logarithm of all fish masses together against time in days. Annex 5 recommends this approach only if no significant differences have been detected between treatment groups and/or experimental phases. Testing for these potential differences was either not performed or reported. This may have resulted in an inappropriate growth rate correction. Moreover, not performing these comparisons prevented any potential differences in growth rate between treatment groups being detected (OECD 305 (2012) § 77). Again, it is possible that adverse treatment effects were not detected, which may have interfered with the experiment. This point is addressed within the RAI response detailed below.

OECD 305 (2012) § 78 and Annex 5 set out model fitting best practices and target variation for specific parameter estimates. For the fitted models it states, "*the result of a χ^2 goodness-of-fit-test should show a good fit (i.e. small measurement error*

percentage (32) for the bioaccumulation model, so that the rate constants can be considered reliable". Such a test was either not performed or reported. Furthermore § 78 says, "*if more than one test concentration is used, the variation in uptake/depuration constants between the test concentrations should be less than 20 %. If not, concentration dependence could be indicated. Observed significant differences in uptake/depuration rate constants between the applied test concentrations should be recorded and possible explanations given*". Both K_1 and K_2 vary by more than 20 % between concentration levels, which could indicate concentration dependence and a deviation from first order kinetics. This issue was not discussed in the study report and alternative approaches (more complex models or sequential fitting methods) were not explored to determine if estimates of K_1 and K_2 significantly changed. These issues, combined with the lack of confidence intervals or standard errors for any model parameter and BCF_k estimates, make it challenging to assess the reliability of the reported model parameters and BCF_k estimates. This point is addressed in more detail during the RAI response below.

In addition to the above deviations from the guideline there were a number of test reporting omissions (§ 81):

The sex of the test subjects was not reported.

For mean measured concentrations in test water, a time-weighted average across the whole exposure period was not provided. Also, for each concentration, although the mean measured concentration was provided, the associated standard deviation was not.

Relating to feed, its composition (lipid and protein content) was not provided.

Methods used for treatment randomization and assignment of fish to test vessels were not reported.

The test report should include the date of introduction of test organisms to test solutions and test duration. Bluegills were added to the test aquaria on 15 April 2015 but test initiation was not until 13 May 2015. This point is clarified in the RAI response below.

The test report should include a number of graphs, which were not provided. These include the uptake and depuration of the test chemical in the fish (on one graph), natural logarithm transformed concentration vs. uptake time, natural logarithm transformed concentration (ln concentration) vs. depuration time (including the derived depuration rate constant k2). This information is provided within the RAI response below.

Finally, there were a number of reporting errors:

There was some discrepancy relating to the beginning of exposure. The study reported exposure beginning on both 12 May 2015 and 13 May 2015.

The depuration period was specified as lasting for three days. It was stated, however, to begin on 9 June 2015 and finish on 14 June 2015, which is 5 days.

Table D contained errors relating to the percentage of TRR concentration present in the whole fish and how much was extractable and unextractable, for depuration Day 1. The whole fish concentration percentage was incorrectly inputted as 93.2 %, when it should, by definition, have been 100 %. Following from this, the extractable fraction was 93.2 % of the whole and unextractable fraction was 6.75 % of the whole (calculated using rounded values provided in Table D). This was corrected by HSE.

RAI and response:
HSE Ecotoxicology submitted a RAI to the applicant, requesting clarification on four areas. For each issue, the request is reproduced and a summary of the applicant's response and HSE's final conclusion is provided.

a) the omission of confidence limits for k_1, k_2 and BCF_K
"*OECD 305 (2012) paragraph (§) 19 states, "the uptake rate constant, the depuration (loss) rate constant (or constants, where more complex models are involved), the bioconcentration factor (steady-state and/or kinetic), and where possible, the confidence limits of each of these parameters are calculated from the model that best describes the measured concentrations of test substance in fish and water". Confidence limits around parameters were not reported. The lack of confidence intervals or standard errors makes it challenging to assess the reliability of the reported model parameters and BCFK estimates. Please explain why confidence limits were not reported and, if it was not possible to estimate confidence limits, please explain why.*"

The applicant submitted an updated analysis, using the bcmfR R package, which conformed to the Guidance Document on Aspects of OECD TG 305 on Fish Bioaccumulation (OECD, 2017). HSE Ecotoxicology presents the model outputs for the low and high concentrations below.

```
##
## Formula: cfish.data ~ RunModel_Aqueous(time.data, cwater, tdepur, fitk1,
##     fitk2)
##
## Parameters:
##       Estimate Std. Error t value Pr(>|t|)
## fitk1 460.8011   144.8704   3.181   0.0191 *
## fitk2   2.5239     0.8121   3.108   0.0209 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Residual standard error: 3.469 on 6 degrees of freedom
##
## Number of iterations to convergence: 4
## Achieved convergence tolerance: 7.039e-06
```

```
##          Estimate Std.Error      2.5%    97.5%         Unit
## k1       460.8    144.87    176.86    744.75   L/kgFish/day
## k2         2.5239   0.81213   0.93212   4.1157        1/day
## k2g        2.5105   0.81213   0.91874   4.1023        1/day
## BCFK     182.57     6.9482  168.96     196.19        L/kgFish
## BCFKg    183.55     7.0708  169.69     197.41        L/kgFish
## tHalfg     0.2761   0.08932   0.10104   0.4512         day
## BCFKgLip 203.94     7.8565  188.54     219.34        L/kgFish
```

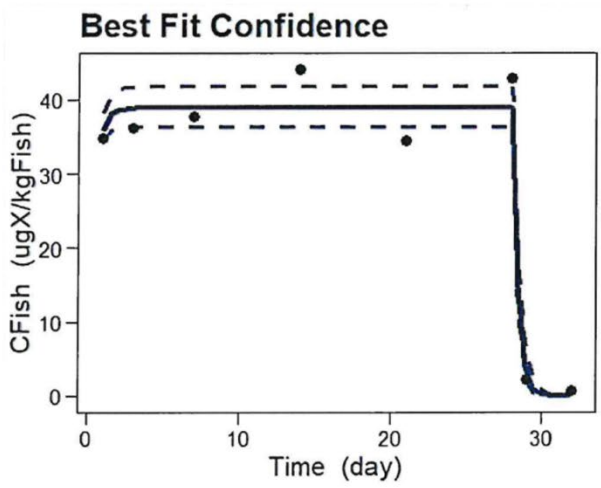


Figure 9.2-10: Parameter estimates and model prediction with 95 % confidence limits for the low concentration (0.2 µg/L) treatment group (untransformed model).

```
##
## Formula: cfish.data ~ RunModel_Aqueous(time.data, cwater, tdepur, fitk1,
##     fitk2)
##
## Parameters:
##       Estimate Std. Error t value Pr(>|t|)
## fitk1 289.1346    60.7531   4.759  0.00313 **
## fitk2   1.7411     0.3746   4.648  0.00351 **
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Residual standard error: 8.741 on 6 degrees of freedom
##
## Number of iterations to convergence: 4
## Achieved convergence tolerance: 2.369e-07
```

```
##           Estimate Std.Error      2.5%    97.5%         Unit
## k1       289.13     60.753   170.06    408.21   L/kgFish/day
## k2         1.7411    0.37463   1.0068    2.4754        1/day
## k2g        1.7277    0.37463   0.99345   2.462         1/day
## BCFK     166.06      6.4884  153.35     178.78        L/kgFish
## BCFKg    167.35      6.6052  154.4      180.3         L/kgFish
## tHalfg     0.40119   0.08699   0.23069   0.5717         day
## BCFKgLip 176.9       6.9822  163.22     190.59        L/kgFish
```

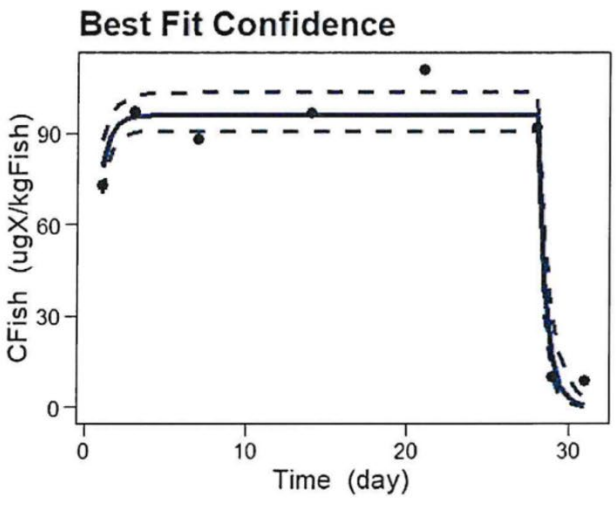


Figure 9.2-11: Parameter estimates and model prediction with 95 % confidence limits for the high concentration (0.6 µg/L) treatment group (untransformed model).

For both the low and high concentration treatment groups, Shapiro-Wilk tests for normality of residuals and Runs tests for error independence in combination with model diagnostic plots both indicate the assumptions of the model (normality and independence of residuals) are not violated. In line with the guidance, untransformed, ln-transformed and boxcox transformed models were presented. For both treatment groups, the untransformed model provided the best fit to the data. Consequently, only the untransformed model outputs are reported. The analysis report submitted within the RAI response (Annex 1), addresses all the original reporting omissions. Further reference will be made to analysis report in the subsequent sections as required.

b) no justification was provided for the pooling of fish at each time point for the analysis of test concentrations

"*OECD 305 (2012) § 40 and 66 concern the analysis of test concentration in fish. They state, "fish should only be pooled if analysis of single fish is not feasible" and "the concentration of the test substance should usually be determined for each weighed individual fish. If this is not possible, pooling of the samples on each sampling occasion may be done". The study pooled fish at each unique time point and test concentration combination for analysis. The justification for this approach was not outlined. Please provide an explanation for why this was done as this decision markedly reduced the sample size.*"

The CRO provided a detailed response in the RAI response Annex 1, reproduced in full below.

"*As indicated in the Total Radioactive Residue (TRR) data and the S-2339 concentration in fish tissue during the depuration stage in the report, the test substance depurated rapidly, reaching levels below the detection limit if individual fish were analysed. Even with pooled fish, S-2339 was not detected on depuration day 3 (TRR 0.697 µg/kg) in the low treatment with values just above the LOQ (0.53 µg/kg). Additionally, during the uptake phase, the parent concentration in fish were 6.0—7.1 µg/kg (ppb) at the low exposure rate and 15.7—18.7 µg/kg at the high exposure rate. For low-dose exposure, a 3-4 g fish would contain approximately 6,800—9,100 dpm/fish of the parent compound, which is further divided into extractable and bound residues. And since only a certain portion of the extract can be injected into HPLC apparatus for chromatographic analysis due to fish matrices, the radioactivity is insufficient for HPLC analysis, even with a pool of five fish. Given these low TRR, the detection of the parent chemical and especially minor metabolites (e.g., < 10% TRR level) would be technically challenging and likely below the method's sensitivity.*"

The applicant added, "*If individual fish had been used, the volume of each fraction would have been significantly reduced and subsequently the LOQ/LOD of the method significantly increased, potentially leading to non-quantifiable results, especially for metabolites at low levels*".

HSE Ecotoxicology accepts the explanation provided. As acceptable BCF_{SS} and BCF_k could be estimated, this reduction in sample size did not markedly impact the study outcome. No further consideration is required.

c) the calculation of K_g, used to correct BCF_K, did not explore the possibility of treatment-related differences in K_g

"*OECD 305 (2012) § 71, 72 and 73 discuss correcting K_2 with growth rate K_g and how this should be performed. The first step is to correctly calculate K_g. The study report did this by regressing the natural logarithm of all fish masses together against time in*

days. Annex 5 recommends this approach only if no significant differences have been detected between treatment groups and/or experimental phases. Testing for these potential differences was either not performed or reported. This may have resulted in an inappropriate growth rate correction. Moreover, not performing these comparisons prevented any potential differences in growth rate between treatment groups from being detected (OECD 305 (2012) § 77). Therefore, it is possible that adverse treatment effects were not detected, which may have interfered with the experiment. Please perform the growth rate correction according to Annex 5 Section 7 to demonstrate that growth between control and treatment groups and different experimental phases were not significantly different. HSE notes that this point is only applicable if first-order kinetics (see point c below) do apply."

A statistical analysis was submitted by the CRO that tested the difference in growth rates between the treatment groups. Linear regressions of the natural logarithm of fish weight over time were performed for each treatment group and the slopes (growth rates) estimated. A pairwise comparison of the groups detected no significant differences between their growth rates ($p > 0.95$ for all comparisons). Therefore, the pooling of growth data across treatment groups is valid for the calculation of an overall growth rate. This analysis also indicates that there were no adverse effects for treatments on growth rate. No further consideration is required.

<u>d) not investigating the potential concentration dependence indicated by the > 20 % deviations for uptake/depuration rate constants between tested concentrations</u>

"*OECD 305 (2012) § 78 and Annex 5 set out model-fitting best practices and target variation for specific parameter estimates. For the fitted models it states, "the result of a χ^2 goodness-of-fit-test should show a good fit (i.e. small measurement error percentage (32) for the bioaccumulation model, so that the rate constants can be considered reliable". Such a test was either not performed or reported. Furthermore § 78 says, "if more than one test concentration is used, the variation in uptake/depuration constants between the test concentrations should be less than 20 %. If not, concentration dependence could be indicated. Observed significant differences in uptake/depuration rate constants between the applied test concentrations should be recorded and possible explanations given". Both K_1 and K_2 vary by more than 20 % between concentration levels, which could indicate concentration dependence and a deviation from first-order kinetics. This issue was not discussed in the study report and alternative approaches (more complex models or sequential fitting methods) were not explored to determine if estimates of K_1 and K_2 significantly changed. Please explore alternative approaches to address this apparent concentration dependence. For these approaches, please report all relevant goodness-of-fit tests.*"

The aspects of this question relating to goodness-of-fit can be considered addressed by the analysis report referenced in point a). Regarding the divergence of k1 and k2 by > 20 %, the applicant replied,

"*The kinetics in this study were calculated using non-linear regression in either SigmaPlot or subsequently R Studio (Annex 1). While the variation in uptake and depuration values between the 2 test treatments exceeds 20%, the evaluation of concentration dependence should focus on the BCF_K values for the low and high treated fish, which show less than a 20% difference.*"

No explanation of why considerations of concentration dependence should focus on BCF_K estimates rather than k_1 and k_2 estimates was provided. Within the "Guidance Document on Aspects of OECD TG 305 on Fish Bioaccumulation" (OECD, 2017), Annex 2 reports a distribution profiling the differences between BCF estimates for high and low tested concentrations from 40 bioaccumulation studies. This distribution was created in part to determine "*how large a difference between BCF estimates from two exposure concentrations must be before we decide that a single BCF value cannot adequately describe the bioconcentration potential of a chemical.*". I.e., concentration dependence. The focus of Annex 2 on BCF estimates suggests that BCF is the primary measure of interest when investigating concentration dependence. Therefore, based on the precedence set in the Guidance document for OECD 305, HSE Ecotoxicology has accepted the argument put forward by the applicant, despite the lack of explanation. The BCF_k = 182.57 (low concentration) and 166.06 L/kg fish (high concentration) estimates are within ± 10 % of one another. Although not a perfect comparison[15], a deviation of < ± 10 % falls within Q1 and Q3 of the calculated BCF difference distribution in Annex 2 of the Guidance document, indicating that such a deviation between high and low concentration BCF estimates is commonplace. To conclude, HSE Ecotoxicology accepts the presented BCF_k estimates and how their < ± 10 % deviation supports a lack of concentration dependence.

<u>e) uncertainty surrounding exposure start</u>

"*One final point, which relates to the overall study design, is why were test individuals added to aquaria on 15 April 2015 but test initiation did not occur until 13 May 2015? Why was the exposure period not defined as when the fish were added to the aquaria containing the test solutions? This unusual approach made the results more difficult to interpret.*"

The applicant expanded upon the experimental design, reproduced in full below.

"*Final point. The CRO expected the delivery of a chiral column, required for chiral analysis in water and fish tissue, to arrive on schedule (about one week) after adding the fish to the tanks on 15 April. However, the delivery was delayed. For clarification, the fish were added to clean tanks in the test area on 15 April in readiness for the exposure start which was expected to be approximately one week later when the chiral column was received. These tanks were the same tanks that were to be used for the depuration phase of the study receiving clean dilution water only and fish were moved to the uptake/exposure tanks at the exposure start. Therefore the fish had an extended*

[15] Due to the selection of the "best" BCF value from BCF_k and BCF_{SS} in the calculated BCF difference distribution in Annex 2 of the Guidance document.

pre-incubation time which would have allowed the fish to better acclimate to the test conditions in the test area before the treatment fish were moved to the exposure tanks on 13 May. The dilutor feeding the exposure tanks was previously in operation for approx. 14 days and test substance concentrations were confirmed at day -2."

This allowed HSE Ecotoxicology to confirm that exposure began as stated (13th May) and the rapid achievement of steady state was due to the properties of inpyrfluxam rather than an incorrectly specified exposure initiation date.

HSE Conclusion

To summarise, after a RAI, the applicant provided a detailed analysis report that outlined all model outputs. This allowed HSE Ecotoxicology to fully evaluate the performed analysis. The final BCF_{SS} and BCF_k estimate for the low and high concentrations are reported in the table below. All estimates are corrected for lipid content (4.26 % for 0.2 µg/L and 4.42 % for 0.6 µg/L) and growth rate (0.0134 day^{-1}), where appropriate.

Table 9.2.3-23: Agreed HSE estimates for $BCF_{SSL, TRR}$, $BCF_{kgL, TRR}$, $BCF_{SSL, S-2399}$, and $t_{1/2g}$

Treatment group (nominal concentration, µg/L)	$BCF_{SSL, TRR}$ (L/kg fish)	$BCF_{kgL, TRR}$ (L/kg fish)	$BCF_{SSL, S-2399}$ (L/kg fish)	$t_{1/2g}$ (days)
0.2	211.8	215.4	38.4	0.276
0.6	185.6	189.3	33.1	0.401

The steady states for the 0.2 and 0.6 µg/L treatment groups were Between Days 1 to 14 and 3 to 21, respectively. The defined steady states are in broad agreement with the model prediction plots in Figure 9.2-10 and Figure 9.2-11. Due to the rapid achievement of steady state, the sampled timepoints do not fully describe the uptake curve. This, however, did not prevent the fitted models from providing BCF_k estimates with 95 % confidence limits < 10 % of the derived BCF_k. Also, due to the rapid depuration of S-2399 below the LOD, a $BCF_{k, S-2399}$ was not estimated. The steady state and kinetic BCF estimates are in close agreement within treatment. HSE Ecotoxicology has selected the largest BCF estimate of the four, 215.4 L/kg for use in risk assessment.

Lipid normalized, growth corrected, kinetic bioconcentration factor for total radioactive residues ($BCF_{kgL, TRR}$) = 215.4 L/kg

Lipid normalized, steady state bioconcentration factor for inpyrfluxam (S-2399) = 38.42 L/kg

B.9.2.4 Potential for endocrine disruption

B.9.2.4.1 EAS modality endocrine assessment for non-target organisms

FSTRA study summary ([redacted] 2021):

Under this study, the maximum tolerated concentration (MTC) was 20.0 mg a.s./L, which is in line with the test guideline which states 'for the purposes of this test, the highest test concentration should be set by the maximum tolerated concentration (MTC) determined from a range finder or from other toxicity data, or 10 mg/L, or the maximum solubility in water (1.64×10^{-2}), whichever is lowest.' The MTC was considered acceptable by HSE (further details available in the study evaluation above).

The maximum tolerated concentration (MTC) is defined in the EFSA/ECHA 2018 guidance[16] as the highest test concentration of the chemical which results in less than 10 % mortality. The guideline OECD 229 (2012) states: '*for the purposes of this test, the highest test concentration should be set by the maximum tolerated concentration (MTC) determined from a range finder or from other toxicity data, or 10 mg/L, or the maximum solubility in water, whichever is lowest*.' In the range finding test, there was 40% mortality at 40 µg/L in one replicate, but 0% mortality in the second replicate at this concentration. There was 90% and 80% mortality in replicates A and B of the 80 µg/L concentration of the range-finding test. Based on these data and the guideline recommendations, an MTC of slightly above 4 µg/L is expected. However, no mortality was observed in the 20 µg/L concentration. Based on this, there is an argument that concentrations > 20 µg/L could have been used in the definitive test. Despite this, different results were seen in the definitive test, with 12% mortality at 20 µg/L, which is not uncommon with the use of more replicates. Additionally, the EC_{10} for survival in the fish ELS study on the same species was determined to be between 7.5 and 13 µg/L. Ultimately, HSE conclude that the definitive test has been carried out at a sufficiently high concentration.

The study and its results can be considered reliable, and results have been considered further in the endocrine disruption hazard assessment for inpyrfluxam.

This study considered vitellogenin levels and secondary sexual characteristics and used the fathead minnow as the test species (see study in section B.9.2.4). Samples were collected for histopathology analysis, but these tests were not conducted and have not been presented in the study report. The other measured parameters are sensitive to, but not diagnostic of 'EAS' modalities. The results from the FSTRA 21-

[16]Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009

day flow-through GLP study with inpyrfluxam are shown in the tables below for the parameters assessed.

Table 9.2.4-1: Biological results during the in-life phase of the study

Parameters measured		Nominal concentration [µg S-2399/L]			
		Control	2.0	6.3	20.0
		Mean measured concentration [µg S-2399/L]			
		Control	2.0	6.1	18.0
Total egg no. per day and female [n] [A]	Mean SD % CV	19 6.8 35.8	23 3.9 17	24 5.5 22.9	12 3.6 30
Wet weight males at study termination [g] [A]	Mean SD	5.2432 0.2193	4.9299 0.4405 (5.97 % decrease compared with control)	4.6809 0.7101 (10.7 % decrease compared with control)	4.9048 0.2144 (6.45 % decrease compared with control)
Wet weight females at study termination [g] [A]	Mean SD	2.4493 0.2400	2.4492 0.2775 (0.004 % decrease compared with control)	2.5685 0.1507 (4.9 % increase compared with control)	2.4783 0.1682 (1.1 % increase compared with control)
VTG [ng/mL]; males at study termination (including outliers)[A]	Mean SD	42 42	300 10 (614.3 % increase compared with control)	140 54 (233 % increase compared with control)	36 42.6 (14.2 % decrease compared with control)
	Range	4.7 - 92	20 - 1100	68 - 180	8.1 - 71
VTG [ng/mL]; females at study	Mean SD	$1.8x10^7$ $2.1x10^6$	$1.1x10^7$ $3.3x10^6$ (38.9 % decrease	$1.4x10^7$ $1.8x10^6$ (22.2% decrease	$1.5x10^7$ $7.0x10^5$ (16.7 % decrease

Parameters measured		Nominal concentration [µg S-2399/L]			
		Control	2.0	6.3	20.0
		Mean measured concentration [µg S-2399/L]			
		Control	2.0	6.1	18.0
termination (including outliers) [A]			compared with control)	compared with control)	compared with the control)
	Range	9.5x10^6 – 2.3x10^7	7.0x10^5 - 1.4x10^7	6.8x10^6 – 1.7x10^7	8.5x10^6 – 2.4x10^7
VTG [ng/mL]; males at study termination (excluding outliers)	Mean SD	42 42	31 10 (26.1% decrease compared with control)	140* 54 (233.3% increase compared with control)	36 26 (14.2% decrease compared with control)
	Range	4.7 - 149	102 – 65.5	4.7 - 350	8.05 - 116
VTG [ng/mL]; females at study termination (excluding outliers)	Mean SD	2.0x10^7 2.1x10^6	1.1x10^7* 3.3x10^6 (45% decrease compared with control)	1.5x10^7 1.8x10^6 (25% decrease compared with control)	1.9x10^7 7.0x10^5 (5% decrease compared with control)
	Range	1.33x10^7 - 2.53x10^7	2.47x10^5- 2.08x10^7	3.62x10^6 – 2.23x10^7	1.16x10^5 – 7.0x10^7
Tubercule score (Male)	Mean SD Size	39 3.3 4 x 2 (enlarged)	34 1.9 4 x 2 (enlarged)	34 4.5 1 x 1 (present) 3 x 2 (enlarged)	37 5.7 4 x 2 (enlarged)
Tubercule score (Female)	Mean SD	0 0	0 0	0 0	0 0

SD = Standard deviation; CV = Coefficient of Variation; VTG = Vitellogenin.
[A]: No statistically significant difference between controls and treatments, according to Dunnett's Multiple Comparison Test
*Statistically significantly different compared to control (males show statistically significant increase, whereas females show statistically significant decrease)
Chemical analysis LOQ: (0.100 µg/L): 110 ± 1.52% (RSD: 1.39%)

Egg production and wet weight results:

There were no significant treatment-related effects on reproduction in fathead minnow as there was no statistically significant difference in eggs produced per female per productive day in comparison to the control for any concentration. There was a decrease in the number of eggs produced at the highest test concentration compared with the control, although it is noted that there was overlap between the standard deviations of the control and highest mean measured test concentration. Additionally, there was a statistically significant reduction in fertilisation success at the highest mean measured concentration. Wet weight and egg production are “sensitive to” EAS but are not indicative of in vivo mechanistic or EAS-mediated effects. These parameters will be used in the hazard assessment as additional information to the key mechanistic parameter of vitellogenin analysis and the key EAS-mediated parameter of secondary sexual characteristics. There were no significant treatment-related effects on total length or wet weight among male or female fathead minnow during the 21-day test.

Consideration of vitellogenin results:

Consideration the vitellogenin results is limited as no histopathology data has been provided. The vitellogenin data has been considered for males and females below.

As stated in OECD 229 and EFSA/ECHA guidance (2018), vitellogenin levels can be impacted by general toxicity, non-endocrine toxic modes of action, and confounding factors such as diet or infection. The ranges of VTG levels are shown in Table 9.2.4-1. For male fish, when including outliers, there were no statistically significant effects following vitellogenin analysis. When considering results excluding outliers, there was ~~a~~ statistically significant increase in males at the middle test concentration. In the OECD validation report for 21 day fish screening assay (2007) the following is stated: ‘*there is typically high variability of VTG values (high SD’s) but true responses are sensitive and dramatic, thus high enough to easily reach statistical significance*’. The VTG levels in males showed no clear concentration response, with a significant increase in VTG at the middle concentration, which then decreased at the highest tested concentration. Overall, there was a lack of dose response for vitellogenin levels for males. The measured VTG concentrations were in line with the OECD 229 guidelines which states ‘*as a guide, the VTG levels in control groups of males and females should be distinct and separated by about three orders of magnitude in fathead minnow and zebrafish*’. The applicant has stated that, despite being statistically significant, the results in these groups were within the range of historical control data. Historical data was not evaluated by HSE, but it was detailed in the applicant’s hazard assessment report that male VTG levels range from 2.0 ×

10^1 ng/mL to 7.9 × 10^3 ng/mL (mean = 1.57 × 10^3 ng/mL) in data from March 2019 to December 2020. Thus, the elevated levels seen in males at 6.1 µg a.s./L (1.4 × 10^2 ng/mL) are within the boundaries of historical control data. Additionally, the increase in male VTG in the definitive test does not reach levels of control females. Therefore, it is unlikely that the increase is ED-mediated when considering male VTG levels. This is further supported by the lack of effects for other measured parameters, including secondary sexual characteristics. From the data is does appear that three individuals (2B2, 2C1 and 2D2) were the reason for the high VTG levels recorded at this concentration out of the total of 7 males. This concentration also had 1 fewer replicate due the instance of male mortality in one replicate compared to the control and other test concentrations.

For females, when including outliers, there were no statistically significant effects following vitellogenin analysis. When considering results excluding outliers, there was a statistically significant reduction in vitellogenin concentrations in females at the lowest test concentration, but this was not observed at the middle test concentration and returns approximately to control levels at the highest test concentration. Overall, there was a lack of dose response for vitellogenin levels for females. The measured VTG concentrations were in line with the OECD 229 guidelines which states '*as a guide, the VTG levels in control groups of males and females should be distinct and separated by about three orders of magnitude in fathead minnow and zebrafish*'. The applicant has stated that, despite being statistically significant, the results in these groups were within the range of historical control data. Historical data was not evaluated by HSE, but it was detailed in the applicant's hazard assessment report that female VTG levels range from 6.17 × 10^6 ng/mL to 2.04 × 10^7 ng/mL (mean = 1.13× 10^7 ng/mL) in data from March 2019 to December 2020. Thus, the decreased levels seen in females at 2.0 µg a.s./L (1.1 × 10^7 ng/mL) are within the boundaries of historical control data. The lowest test concentration for females had one individual (3D5) that recorded much lower VTG than other individuals within this group and across all other test concentrations and likely contributed towards the statistically significant decrease in VTG seen at this concentration when outliers are removed.

For both males and females, when outliers for each test concentration and control were included, VTG levels for both males and females were comparable to the controls with no statistically significant difference. It is noted that the range of VTG in the control is quite narrow when outliers were excluded.

The study report states that *"individually, these results can potentially be an indication of endocrine activity; however, these results are contradictory to a specific mode of action."* The ED guidance states that *"induction of VTG production in male is a biomarker used to detect estrogenic compounds, whereas reduction of VTG in female may be indicative of sexual steroid synthesis modulation. VTG modulation can also be triggered by chemicals that interfere with the AR-mediated pathway".*

Graphs showing VTG levels in males and females have been presented in the figures below.

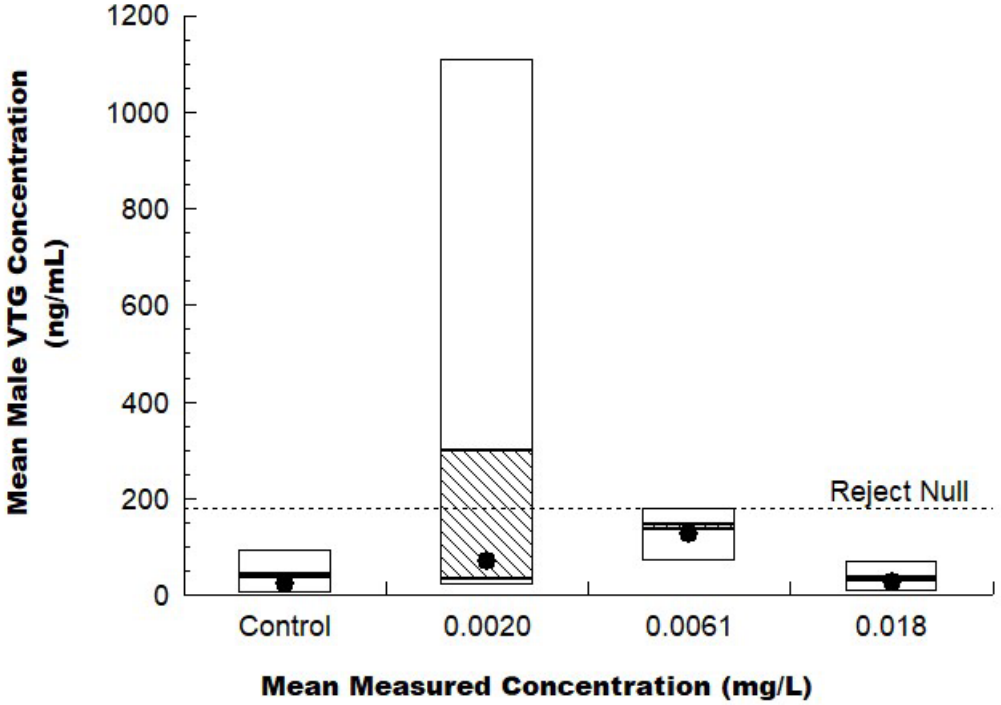


Figure 9.2-12: Mean male VTG concentration (including outliers)

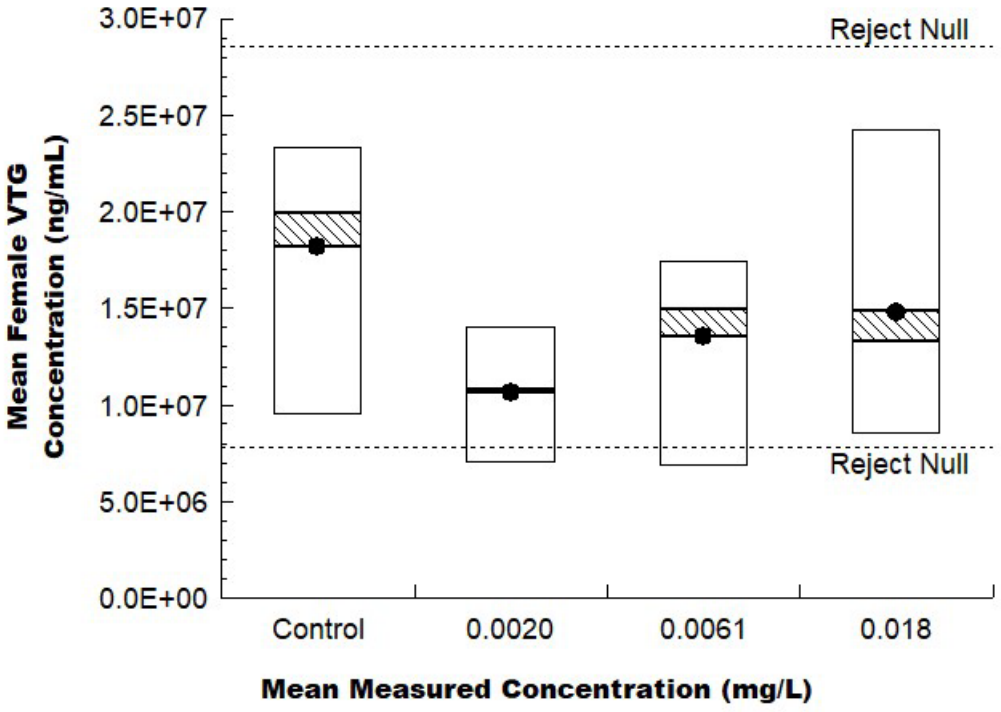


Figure 9.2-13: Mean female VTG concentration (including outliers)

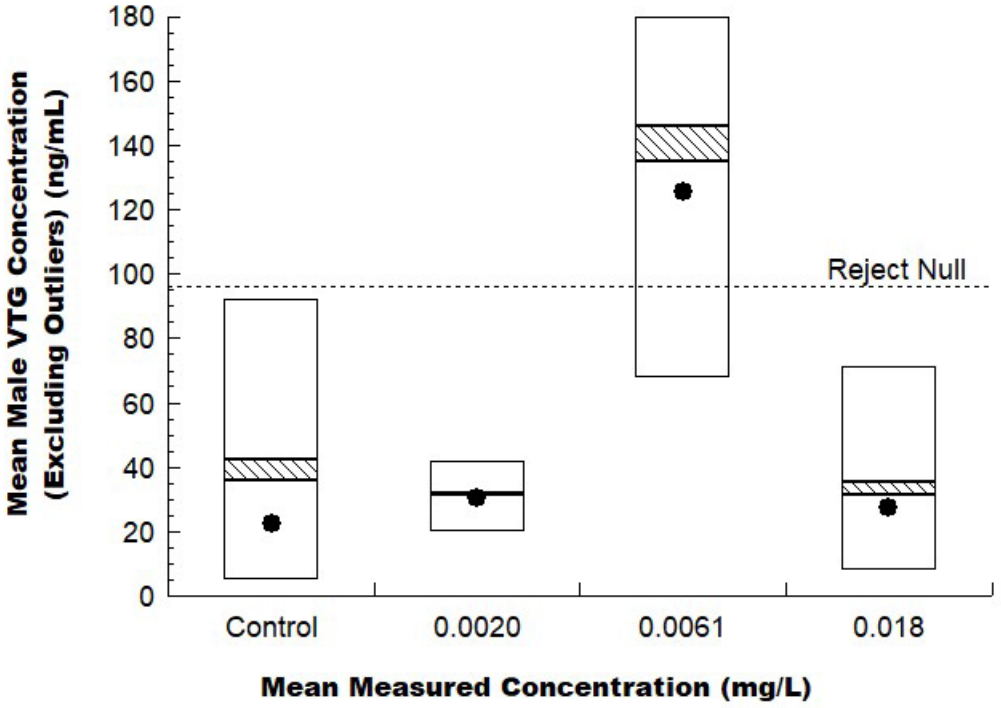


Figure 9.2-14: Mean male VTG concentration (excluding outliers)

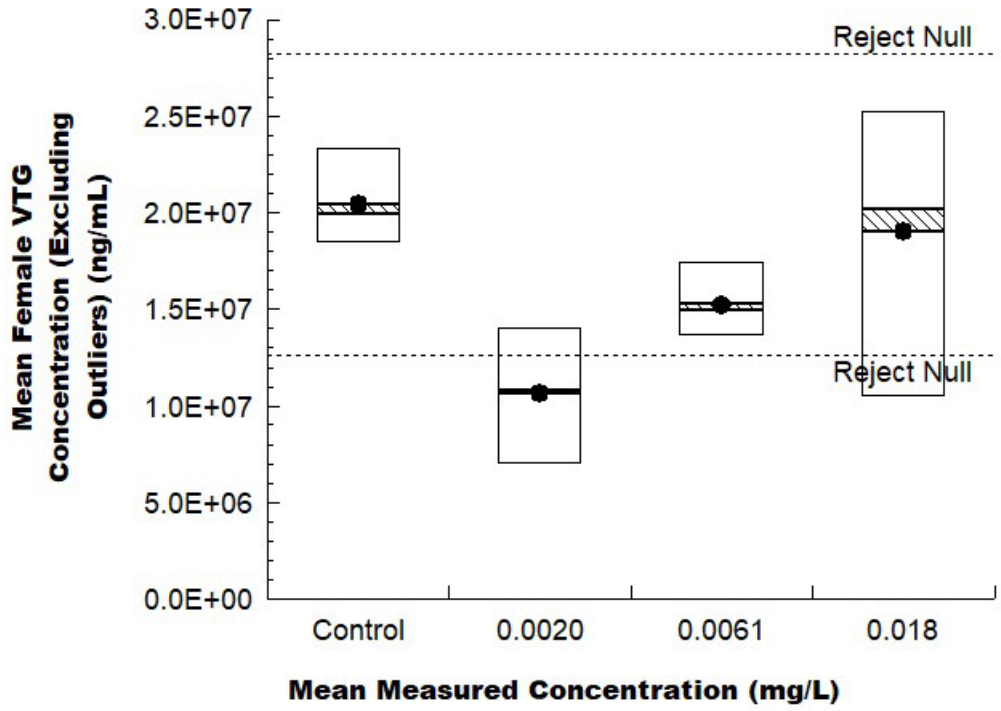


Figure 9.2-15: Mean female VTG concentration (excluding outliers)

Overall, HSE agrees that there are no clear treatment related effects of S-2399 on endocrine activity for the EAS modalities at the highest concentration tested based on the submitted FSTRA study.

Secondary sexual characteristics:

Male secondary sex characteristics were evaluated to determine if reduced nuptial tubercle scores occurred as a result of S-2399 exposure. No statistically significant difference in nuptial tubercle scores of male fish was observed among fish exposed to any of the treatment levels; however, the majority of male replicates displayed enlarged tubercles in both the control and treatment groups. The presence of nuptial tubercles or dorsal nape pads was not observed on females at any point for any treatment level, including the control, during the exposure.

Histological analysis

No histological data was submitted to or considered by HSE. A request for additional information (RAI) was sent to the applicant for histopathology data to be provided if available. Ultimately, the data was not provided. This has been commented on further in the HSE EAS conclusion below.

Fertility and fecundity

There were decreases in both fertility and fecundity at the highest tested concentration, with the reduction in fertilisation being statistically significant. The reduced fertility is primarily seen in replicates B and C, with one instance each. There is no obvious reduction in fertility across all replicates. Fecundity showed a general reduction across all replicates at the highest tested concentration. These effects were limited to the highest tested concentration above the MTC, where clear systemic toxicity for female fish is demonstrated.

EAS summary of parameters:

A summary of the results from the FSTRA study has been provided in the table below. The format is in accordance with EFSA/ECHA guidance i.e., appendix E. The results summarised are for the EAS modalities.

Table 9.2.4-2: Reporting the lines of evidence for adverse effects from fish studies (EAS modalities) on Fathead minnow *(Pimephales promelas)*, note the effect classification for parameters is in-line with EFSA/ECHA guidance 2018

Effect classification	Effect target	Study type	Species	Duration of exposure (days)	Exposure route	Lowest effect dose (µg a.s./L)	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence	Modality
In vivo mechanistic	Vitellogenin (VTG) in females (including outliers)	FSTRA	Fathead minnow	21	Flow-through	20.0	No effect	No indication	Uncertainty whether results for both males and females are endocrine mediated. Contradictory to a specific mode of action	EAS
	Vitellogenin (VTG) in females (excluding outliers)	FSTRA	Fathead minnow	21	Flow-through	2.0	Decrease	Reduced VTG but not dose responsive		EAS
	Vitellogenin (VTG) in males (including outliers)	FSTRA	Fathead minnow	21	Flow-through	20.0	No effect	No indication		EAS
	Vitellogenin (VTG) in males (excluding outliers)	FSTRA	Fathead minnow	21	Flow-through	6.3	Increase	Increased VTG but not dose responsive		EAS
EATS-mediated	Tubercle score (females)	FSTRA	Fathead minnow	21	Flow-through	20.0	No effect	The presence of nuptial	No indication of EATS-	EAS

Effect classification	Effect target	Study type	Species	Duration of exposure (days)	Exposure route	Lowest effect dose (µg a.s./L)	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence	Modality
								tubercles or dorsal nape pads was not observed on females at any point for any treatment level, including the control.	mediated effects.	
	Tubercle score (males)	FSTRA	Fathead minnow	21	Flow-through	20.0	No effect	No statistically significant difference in nuptial tubercle scores of male fish. Majority of male replicates displayed enlarged tubercles in both the control and treatment groups.		EAS

Effect classification	Effect target	Study type	Species	Duration of exposure (days)	Exposure route	Lowest effect dose (µg a.s./L)	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence	Modality
Sensitive to, but not diagnostic of, EATS	Behaviour and morphological abnormalities (fish)	FSTRA	Fathead minnow	21	Flow-through	20.0	No abnormal observations noted	No abnormal observations. Male territorial behaviour observed daily in all replicates at all treatment levels and control. Normal appearance of ovaries and testes at all treatment levels at end of test.	Some evidence of potential ED due to statistically significant decrease in fertilisation at 20 µg a.s./L concentration and non-statistically significant decrease in fecundity at 20 µg a.s./L, showing sensitivity to, but non-diagnostic of EATS modality.	N
	Body weight (female fish)	FSTRA	Fathead minnow	21	Flow-through	20.0	No abnormal observations noted	Body weight not statistically analysed.		N
	Body weight (male fish)	FSTRA	Fathead minnow	21	Flow-through	20.0	No abnormal observations noted	Body weight not statistically analysed.		N
	Length (female fish)	FSTRA	Fathead minnow	21	Flow-through	20.0	No effect	Not statistically analysed.		N

Effect classification	Effect target	Study type	Species	Duration of exposure (days)	Exposure route	Lowest effect dose (µg a.s./L)	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence	Modality
	Length (male fish)	FSTRA	Fathead minnow	21	Flow-through	20.0	No effect	Not statistically analysed.		N
	Morphological abnormalities	FSTRA	Fathead minnow	21	Flow-through	20.0	No effect	No abnormal morphological effects observed.		N
	Reproduction (fecundity, fertility)	FSTRA	Fathead minnow	21	Flow-through	20.0	Effect	There was a statistically significant decrease in fertilisation success at the highest test concentration, as well as a decrease in fecundity (not statistically significant). The OECD 229 (2012) guideline states that: *Although not endocrine specific, fecundity, due*		Sensitive to, but not diagnostic of, EATS

Effect classification	Effect target	Study type	Species	Duration of exposure (days)	Exposure route	Lowest effect dose (µg a.s./L)	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence	Modality
								to its demonstrated sensitivity across known endocrine active substances, is an important endpoint to include because when it and other endpoints are unaffected one is more confident that a compound is not likely endocrine active. However, when fecundity is affected it will contribute heavily in weight of		

Effect classification	Effect target	Study type	Species	Duration of exposure (days)	Exposure route	Lowest effect dose (µg a.s./L)	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence	Modality
								evidence inferences.		
	Embryo time-to-hatch	ELS	Fathead minnow	32 days	Flow-through	13	No effect	All viable embryos in all treatment and control embryo incubation cups began hatching on day 3 and completed hatching on day 4	Potential for some sensitivity to EATS indicated by the statistically significant reduction in hatching success at 4.6 µg a.s./L, but this is less certain as there were no statistically significant effects at the highest two concentrations. Statistically significant	N
	Embryo time-to-hatch	ELS	Sheepshead minnow	34 days	Flow-through	63	Effect	Dose dependent delay in hatching observed at 63 µg/L and 130 µg/L (two highest tested concentrations).		

Effect classification	Effect target	Study type	Species	Duration of exposure (days)	Exposure route	Lowest effect dose (µg a.s./L)	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence	Modality
	Hatching success	ELS	Fathead minnow	32 days	Flow-through	2.7	Decrease	Statistically significant reduction in hatching success recorded at 4.6 µg a.s./L, but no statistically significant effects at the highest two concentrations.	reductions in length and wet at the highest mean measured concentration was also observed. Morphological differences were noted in the fathead minnow study at the highest tested concentration. Statistically significant effects on hatching success, normal larvae and survival were noted in the ELS study with	
	Hatching success	ELS	Sheepshead minnow	34 days	Flow-through	63	Decrease	Effects at the second-highest tested concentration (63 µg/L) where a statistically significant reduction in hatching success was observed.		

Effect classification	Effect target	Study type	Species	Duration of exposure (days)	Exposure route	Lowest effect dose (µg a.s./L)	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence	Modality
	Morphological abnormalities / behaviour	ELS	Fathead minnow	32 days	Flow-through	13	Effect	Effects at the highest tested concentration (13 µg/L) where all remaining live fry were noted as “small” between days 9 and 27 post-hatch. This was not noted on the final day.	sheepshead minnow, but all had higher LOECs than the fathead minnow.	
	Morphological abnormalities/behaviour	ELS	Sheepshead minnow	34 days	Flow-through	63	Effect	Statistically significant difference in percentage of live normal larvae at 63 and 130 µg/L		
	Body weight (fish)	ELS	Fathead minnow	32 days	Flow-through	7.5	Decrease	Statistically significant reduction in mean wet weight at the highest mean measured test		N

Effect classification	Effect target	Study type	Species	Duration of exposure (days)	Exposure route	Lowest effect dose (µg a.s./L)	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence	Modality
								concentration (0.013 mg a.s./L).		
	Body weight (fish)	ELS	Sheepshead minnow	34 days	Flow-through	>34	No effect	No significant differences at highest tested concentration with live normal larvae		
	Length (fish)	ELS	Fathead minnow	32 days	Flow-through	7.5	Decrease	Statistically significant reduction in length at highest mean measured concentration (0.013 mg a.s./L)		N
	Length (fish)	ELS	Sheepshead minnow	34 days	Flow-through	>34	No effect	No significant differences at highest tested concentration with live normal larvae		

Effect classification	Effect target	Study type	Species	Duration of exposure (days)	Exposure route	Lowest effect dose (µg a.s./L)	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence	Modality
Systemic toxicity	Survival (fish)	FSTRA	Fathead minnow	21	Flow-through	20.0	Decrease	Statistically significant reduction in survival of females (and male/female combined survival) at the highest tested concentration (0.018 mg/L).	Some possible signs of systemic toxicity.	N/A
		ELS	Fathead minnow	32 days	Flow-through	1.6	Decrease	Statistically significant reduction in larval survival at three of the four highest test concentrations.		N/A
		ELS	Sheepshead minnow	34 days	Flow-through	15	Decrease	Statistically significant reduction in survival at test termination		N/A

E = Estrogen, A = Androgen, S = Steroidogenesis, N = Not assignable to a specific modality, N/A = not applicable, stat sig = statistically Significant

HSE EAS conclusion:

A FSTRA study ([redacted] 2021) was provided that investigated the EAS activity of inpyrfluxam. Histopathology was not measured within this study as it was listed as an optional parameter as per Table 15 in the ED guideline. As a result, the study was based solely on VTG levels and SSCs, and effects sensitive to, but not diagnostic of EATS.

In terms of vitellogenin analysis, with outliers excluded, there was a statistically significant increase in vitellogenin in males at the middle test concentration, and a statistically significant decrease in vitellogenin in females at the lowest test concentration. There is a lack of dose-response demonstrated, and the applicant has stated that the VTG values are within historical control data. In addition, the effects observed are contradictory to a single mode of action. Overall, in terms in VTG analysis, the data are not indicative of endocrine disruption via EAS modalities. No effects were observed in terms of the tubercules for males or females. The majority of male replicates displayed enlarged tubercles in both the control and treatment groups, but none of the scores were statistically significant. This indicates a lack of endocrine-disrupting effects. No effects were observed in relation to systemic toxicity for behaviour, morphology, body weight or length for males and females in the FSTRA study. In terms of reproduction, there was a statistically significant decrease in fertilisation success at the highest test concentration, as well as a decrease in fecundity (not statistically significant). However, the reductions in fecundity and fertility also occur at the highest tested concentration, where statistically significant decrease in female survival was also observed. This indicates that the effects are not likely to be EAS mediated.

In the ELS study conducted on the same species, there were statistically significant reductions in larval survival were observed at three of the four highest test concentrations, but there was a lack of clear dose-response. Similarly, a statistically significant reduction was recorded for hatching success, but no clear dose-response was demonstrated. Body weight and length recorded statistically significant reductions compared to the control at the highest tested concentration. Overall, the results from the ELS study show some evidence of systemic toxicity, but the lack of clear dose-responses for multiple parameters indicate a lack of endocrine disruption. The lowest observed effect concentration for the *P. promelas* study was 13 µg/L (EC10), including survival. This indicates that effects seen in the FSTRA study at 20 µg/L are a result of systemic toxicity and not EAS mediated.

The applicant has concluded the following for the FSTRA:

"Statistical analysis determined an increase in male VTG at the 6.1 µg/L treatment level and a decrease in female VTG at the 2.0 µg/L treatment level. Individually, these results can potentially be an indication of endocrine activity; however, these results are contradictory to a specific mode of action. These differences are not considered to be biologically relevant because there were no dose dependent response and the mean values of male VTG concentration in the 6.1 µg/L treatment level and female VTG

concentration in the 2.0 µg/L treatment level are within the lab historical values (Appendix 8). Since no clear related effects were also observed in other inspections, it is considered to be incidental".

"No significant difference in nuptial tubercle scores of male fish was observed among fish exposed to any of treatment levels. The presence of nuptial tubercles or dorsal nape pads was not observed on females at any point during the exposure. No abnormal behaviour or notable changes in secondary sex characteristics were observed in either sex throughout the 21-day study. No other abnormal observations (e.g., body colour (light or dark), coloration patterns, body shape, size of dorsal nape pad in males, or ovipositor size in females) were observed during the exposure period or at study termination in any of the treatment levels or the control"

"The presence of behavioural and morphological abnormalities and reductions in survival and fecundity were evaluated to determine effects sensitive to, but not diagnostic of, EAS mediated effects. A significant reduction in female survival as well as male/female combined survival was observed among fish exposed to the 18 µg/L treatment level".

"No abnormal observations in behaviour such as hyperventilation, loss of equilibrium, uncoordinated swimming, atypical quiescence, and or feeding abstinence were noted in any of the treatment levels tested or the control during daily observations. Normal male territorial behaviour was observed daily in all replicates from all treatment levels and the control".

"Macroscopic examination of the female fish in all treatment levels and the control at test termination confirmed normal appearance of ovaries; examination of the males confirmed normal appearance of testes. No significant reduction in fecundity was observed among fish exposed to any of the treatment levels tested. A significant reduction in fertilization success was observed in the 18 µg/L treatment level compared to the control. However, the percentage of the fertilisation success in the 18 µg/L treatment level is within the historical data".

"The significant reduction in fertilisation success that occurred at the 18 µg/L treatment level is sensitive to, but not diagnostic of an EAS-mediated modality. Furthermore, this significant response only occurred at the high concentration where significant mortality was observed, indicating systemic toxicity (i.e., above the Maximum Tolerated Concentration (MTC)).
Therefore, these effects cannot be directly attributed to endocrine disruption".

Despite the applicants arguments, HSE still had concerns for the potential for endocrine disruption given the statistically significant effects seen on vitellogenin. As such, an RAI was requested, noting that samples were taken for histopathological analysis and queried

the applicants decision not to conduct the analysis given that effects were seen for several parameters, including VTG.

The following response was received:

" Histopathology examination of the gonads is an optional measurement in the OECD 229 TG. While this may be needed in cases of equivocal results, the Applicant considered that the results of the study clearly indicate no EAS mediated effects of the test substance in a fully valid study. No effects were observed for the EAS mediated endpoint of secondary sex characteristics and Sensitive To But Not Diagnostic Endpoints (STBNDE) of fecundity, gonadal somatic index. For the in vivo mechanistic biomarker VTG endpoints, results are known to be intrinsically variable, (Brown et al,. (2023). Are Changes in Vitellogenin Concentrations in Fish Reliable Indicators of Chemical-Induced Endocrine Activity? Ecotoxicology and Environmental Safety 266: 115563) and taken in isolation should not be used to define an EAS mediated effect. The significant increase in VTG in males at the middle concentration alone (outliers removed) does not follow a dose response and is considered to be within the Historical Control Data (HCD) of the performing lab as discussed and presented in the test report. Equally the significant reduction in female VTG at the lower concentration alone (outliers removed) does not follow a dose response and is considered to be within the HCD of the performing lab. Together both reported changes in VTG if considered to be accurate and EAS-mediated would contradict each other in terms of any postulated Mode of Action. No VTG effects are observed at the highest test concentration. The reported significant reduction of the STBNDE fertilisation success is observed in the highest concentration only where significant mortality was observed (12%). This concentration is therefore clearly above the Maximum Tolerated Concentration (MTC) which should not be above 10% mortality and the observed effect on fertilisation success is likely a result of the onset of systemic toxicity. It is also noted that the reported reduction in fertilisation at the highest concentration is also within the HCD of the performing laboratory. Given that, the FSTRA is considered to be sufficiently acceptable to investigate the ED properties of S-2399 through the EAS-modalities, without the need of the optional measurement of histopathological examination of the gonads".

Whilst HSE agree that performing histopathology is an optional assessment in the guidance, the provision of histopathology data would have provided more certainty on the conclusion of endocrine disruption given that statistically significant effects were reported where outliers were removed from the data for males and females. However, as this data has not been provided, it cannot be used to form part of the conclusion on endocrine disruption.

Additionally, HSE Toxicology have considered the potential for endocrine disruption and EAS modalities. The following conclusion was reached in the toxicology section (see Volume 3CA, Section 6 dossier (B.6.8.4)):

"In all species investigated (rat, mouse, dog) there were no specific adverse effects on reproductive organs and other endocrine organs related to the EAS modalities following repeated exposure to inpyrfluxam. In addition, there were no specific adverse effects on reproduction in the rat and on development in the rat and rabbit. Overall, there was no clear and specific pattern of adversity for the EAS modalities. In addition, there was no evidence of EAS activity in a steroidogenesis assay and in a hERα or hAR transactivation test".

Ultimately, due to the lack of dose response, no effects at the MTC, data being within historical control levels, and no indication of endocrine disruption within other parameters, HSE can conclude that inpyrfluxam is likely not to be endocrine disrupting. This is further supported by the HSE Toxicology conclusion that no adverse EAS mediated effects were noted.

In accordance with current guidance (EFSA/ECHA, 2018) HSE concludes that inpyrfluxam does not meet the endocrine disruption criteria for EAS-modalities in aquatic organisms.

B.9.2.4.2 T modality endocrine assessment for non-target organisms

AMA study overview

An Amphibian Metamorphosis Assay (*Xenopus laevis*), conducted in line with the OECD 231 (2009) guideline and OECD 82 (2007) AMA histology guidance, was submitted by the applicant to support the T modality endocrine assessment. The study was evaluated by HSE and found to be valid with only minor deviations.

Nominal concentrations for the amphibian metamorphosis assay with African clawed frog (Xenopus laevis) were selected based on a 96-hour preliminary range-finding exposure. The maximum tolerable concentration (MTC) was approximated as 1/3 of the acute LC50 value (140 µg a.s./L / 3 = 46.7 µg a.s./L). 45 µg a.s./L was selected as the highest concentration for the definitive assay. This process was in line with OECD 231 (2009).

A detailed summary and evaluation for the AMA study is included at the end of B.9.2.4. A brief overview of the results is included here to provide context for the subsequent T modality ED assessment.

Table 9.2.4-3: Summary of Endpoints (Developmental Stage, Snout-Vent Length, Hind Limb Length, and Whole Body Wet Weight) Following 21 Days of Exposure after 7 and 21 days of exposure

Mean Measured Concentration (µg/L)	Mean of 4 replicates	Developmental Stage[a]			Snout-Vent Length (mm)[b]				Hind Limb Length (mm)[c]			
		Min	Median	Max	Mean	Median	SD	% CV	Mean	Median	SD	% CV
Control	Day 7	52	54	55	17.38	17.37	0.57	3.3	2.39	2.42	0.16	6.6
3.7	Day 7	53	54	55	16.25	16.29	0.71	4.4	2.22	2.23	0.11	5.0
1.4	Day 7	53	55	55	16.31	16.38	0.96	5.9	2.25	2.30	0.24	11
43	Day 7	53	54	55	15.34[d]	15.38	0.82	5.3	2.14	2.18	0.08	3.5
Control	Day 21	57	59	61	27.40	27.19	0.89	3.3	15.44	15.90	0.89	5.8
3.7	Day 21	57	59	61	26.12[e]	25.73	0.72	2.8	14.94	15.37	0.79	5.3
14	Day 21	57	58	60	26.44	26.36	0.44	1.6	14.23	14.71	0.33	2.3
43	Day 21	56	58[d]	60	25.24[f]	25.34	0.89	3.5	12.37	12.03	2.14	17

Mean Measured Concentration (µg/L)	Mean of 4 replicates	Hind Limb Length (mm) (Normalized by SVL)[b]				Whole Body Wet Weight (g)[b]			
		Mean	Median	SD	% CV	Mean	Median	SD	% CV
Control	Day 7	0.137	0.137	0.005	4.0	0.3822	0.3902	0.0487	13
3.7	Day 7	0.136	0.136	0.001	0.97	0.3297	0.3310	0.0494	15
14	Day 7	0.138	0.138	0.007	5.3	0.3412	0.3395	0.0576	17
43	Day 7	0.139	0.140	0.004	2.9	0.2643[d]	0.2608	0.0347	13
Control	Day 21	0.564	0.551	0.036	6.4	1.5201	1.5101	0.1287	8.5
3.7	Day 21	0.572	0.571	0.015	2.6	1.3620	1.3248	0.1240	9.1
14	Day 21	0.537	0.539	0.018	3.3	1.3798	1.3899	0.0498	3.6
43	Day 21	0.487[f]	0.464	0.070	14.4	1.2389[f]	1.2538	0.1378	11.1

SD = Standard Deviation

CV = Coefficient of Variation

N = replicate count

[a] Analysis performed using the median organism response in each replicate. All tadpoles (i.e., late-stage and non-late-stage) are included.

[b] Analysis performed using mean organism response in each replicate. Late-stage tadpoles (i.e., >60 NF) are excluded.
[c] Not statistically analysed as it was not required by study protocol or applicable guidance.
[d] Significantly reduced compared to the control, based on Jonckheere-Terpstra's Step-Down Test.
[e] Significantly reduced compared to the control, based on Dunnett's Multiple Comparison Test. However, due to the lack of statistical significance at the next higher treatment level (i.e., 14 µg/L), this significance was determined to not be treatment related.
[f] Significantly reduced compared to the control, based on Dunnett's Multiple Comparison Test.

On Day 7, snout-vent length (SVL) and whole-body wet weight were significantly reduced for the highest concentration 43 µg/L. On Day 21, developmental stage, SVL, normalised hind limb length (HLL), and whole-body wet weight were significantly reduced for 43 µg/L. There was also a statistically significant reduction in SVL on Day 21 for 3.7 µg/L. HSE concluded that this effect was not treatment related due to the lack of a clear concentration-response, with the 14 µg/L treatment group only displaying a non-significant 3.5 % reduction relative to the control. An analysis of the distribution profiles of developmental stages confirmed the developmental delay at 43 µg/L with the 20th to 60th percentiles, inclusive, significantly reduced compared to the control. Reductions in the 20th and 60th percentiles were also observed for 14 µg/L.

No treatment related effects on survival and behaviour or deformity, malformation and lesion prevalence were observed.

A summary of the thyroid gland histology results is given in Table 9.2.4-4.

Table 9.2.4-4: Summary of thyroid gland histology

Prevalence and Severity of Thyroid Findings					
S-2399 Treatment Group (µg/L), Mean Measured Concentrations		**0.0 (Control)**	**3.7**	**14**	**43**
Number Examined		20	20	20	20
Thyroid	Follicular cell hyperplasia mild	4	6	2	5
		4	6	2	5
	Follicular cell hypertrophy mild	14	15	13	16
		14	15	13	16

There were no treatment-related histopathologic findings in this study. Most frogs exhibited follicular cell hypertrophy (mild), while follicular cell hyperplasia (mild) was less common.

The prevalence and severity of both hypertrophy and hyperplasia in S-2399 treated frogs were comparable to the negative controls. This conclusion will be discussed in more detail in the following ED assessment.

ED assessment

The applicant performed a T modality ED assessment for non-target organisms according to the ECHA/EFSA Guidance (2018)[17] for endocrine disruptors. They provided a table summarising the evidence for endocrine adversity and activity in non-target organisms related to the thyroid modality. The submitted table has been reduced to include response variables from the AMA study only (Table 9.2.4-5). This was done to simplify the assessment as the results of the AMA study alone are sufficient for regulatory decision-making in this instance. The format is in accordance with ECHA/EFSA Guidance (2018) Appendix E..

[17] ECHA (European Chemicals Agency) and EFSA (European Food Safety Authority) with the technical support of the Joint Research Centre (JRC), Andersson N, Arena M, Auteri D, Barmaz S, Grignard E, Kienzler A, Lepper P, Lostia AM, Munn S, Parra Morte JM, Pellizzato F, Tarazona J, Terron A and Van der Linden S, 2018. Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009. EFSA Journal 2018;16(6):5311, 135 pp. https://doi.org/10.2903/j.efsa.2018.5311. ECHA-18-G-01-EN.

Table 9.2.4-5: Adverse effects and endocrine activity in non-target organisms related to the thyroid modality

Integrated line of evidence	Study ID Matrix	Effect classification	Effect target	Species	Duration of exposure	Duration unit	Route of administration	Lowest Effect dose	Dose unit	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
Adversity	26	EATS-mediated	Developmental stage	Xenopus laevis	21	Days	Uptake from water	>43	µg ai/L	No effect	Day 7. No effects	No effects	There are significant reductions in NF Stage and HLL (normalised for SVL). The negative histopathology results suggest that these effects were caused indirectly by other factors such as systemic toxicity and/or inanition. Systemic toxicity could have also led to the observed significant decrease in body weight and SVL in the highest dose group. Therefore, these effects cannot be directly attributed to	T
	26a	EATS-mediated	Developmental stage	Xenopus laevis	21	Days	Uptake from water	43	µg ai/L	Decrease	Day 21, Significant reduction in median score at 43 µg a.s./L (58) compared with control (59) (P < 0.05)	Not considered endocrine driven as no histopathological changes of note		
	26	EATS-mediated	Hind limb length	Xenopus laevis	21	Days	Uptake from water	>43	µg ai/L	No effect	Day 7 (HLL normalised for SVL)	No effects		
	26a	EATS-mediated	Hind limb length	Xenopus laevis	21	Days	Uptake from water	43	µg ai/L	Decrease	Day 21 (HLL normalised for SVL). Significant reduction at 43 µg a.s./L (0.487 mm) compared with control (0.564 mm) (P < 0.05)	Reductions in growth alone not considered thyroid driven		

Integrated line of evidence	Study ID Matrix	Effect classification	Effect target	Species	Duration of exposure	Duration unit	Route of administration	Lowest Effect dose	Dose unit	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	26	EATS-mediated	Thyroid histopathology (amphibian)	Xenopus laevis	21	Days	Uptake from water	>43	µg ai/L	No effect	No effects	No effects	endocrine disruption.	
	26	Sensitive to, but not diagnostic of, EATS	Body weight (amphibian)	Xenopus laevis	21	Days	Uptake from water	43	µg ai/L	Decrease	Day 7. Significant reduction at 43 µg a.s./L (0.2643 g) compared with control (0.3822 g) (P < 0.05)	Reductions in growth alone not considered thyroid driven		
	26a	Sensitive to, but not diagnostic of, EATS	Body weight (amphibian)	Xenopus laevis	21	Days	Uptake from water	43	µg ai/L	Decrease	Day 21. Significant reduction at 43 µg a.s./L (1.289 g) compared with control (1.5201 g) (P < 0.05)	Reductions in growth alongside impaired development not considered thyroid driven		

Integrated line of evidence	Study ID Matrix	Effect classification	Effect target	Species	Duration of exposure	Duration unit	Route of administration	Lowest Effect dose	Dose unit	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	26	Sensitive to, but not diagnostic of, EATS	Malformations	Xenopus laevis	21	Days	Uptake from water	>43	µg ai/L	No effect	No effects	No effects		
	26	Sensitive to, but not diagnostic of, EATS	Snout-vent length/growth	Xenopus laevis	21	Days	Uptake from water	43	µg ai/L	Decrease	Day 7. Significant reduction in mean snout vent length at 43 µg a.s./L (15.34 mm) compared with control (17.38 mm) ($P < 0.05$)	Reductions in growth alongside impaired development not considered thyroid driven		
	26a	Sensitive to, but not diagnostic of,	Snout-vent length/growth	Xenopus laevis	21	Days	Uptake from water	43	µg ai/L	Decrease	Day 21. Significant reduction in SVL at 43 µg a.s./L (25.24 mm) compared with control (27.40 mm) ($P < 0.05$). Significant reduction at 3.7 µg	Reductions in growth alongside impaired development not consider		

Integrated line of evidence	Study ID Matrix	Effect classification	Effect target	Species	Duration of exposure	Duration unit	Route of administration	Lowest Effect dose	Dose unit	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
		EATS									a.s./L (26.12 mm) due to lack of dose response not considered treatment related.	ed thyroid driven		

With the lines of evidence for endocrine activity and adversity assembled, assessed, integrated and reported (Table 9.2.4-5), HSE Ecotoxicology has used Figure 1 of the ED Guidance (2018) for ED assessment.

An initial analysis of evidence is first performed to determine whether EATS-mediated parameters have been sufficiently investigated. For the T modality, ECHA/EFSA Guidance (2018) Section 3.4.1 states, "*In principle, to have the T-mediated adversity with regard to other non-target organisms sufficiently investigated the results from all the 'T-mediated' parameters foreseen to be investigated in the Larval amphibian growth and development assay (LAGDA; OECD TG 241 (OECD, 2015d)) would be needed. However, if the T-mediated parameters foreseen to be investigated in an amphibian metamorphosis assay (AMA, OECD TG 231 (OECD, 2009c)) are negative, this would be sufficient to support that T-mediated adversity is unlikely because no T-related endocrine activity has been observed*". As no LAGDA study has been submitted, the dataset has not sufficiently investigated T-mediated adversity.

This leads to the next question, "*Has 'EATS-mediated' adversity been observed?*". For the AMA study on Day 21, T-mediated parameters developmental stage and normalised HLL were reduced at 43 µg/L. As discussed in OECD 231 (2009) (§ 61), however, developmental delay can also be a result of indirect toxicity. Mild developmental delays accompanied by mild reductions in growth (wet weight and SVL) are indicative of non-specific toxic effects. This was the case for the submitted AMA study, with statistically significant reductions in wet weight and SVL at 43 µg/L on Day 21. EFSA Guidance (2018) goes further and states that "*delayed development is not by itself an indicator of anti-thyroidal activity and needs to be confirmed by histopathological analysis of the thyroid*". In the submitted AMA study there was a lack of thyroid histopathological alterations in all treatment groups compared to the negative controls. For the 43 µg/L treatment group, an 80 % and 25 % prevalence for mild hypertrophy and mild hyperplasia, compared to 70 % and 20 % in the control, was recorded. There was no increase in the severity of either hypertrophy or hyperplasia between the control and the top concentration tested (43 µg/L). These results can be contrasted to the marked increase in hypertrophy and hyperplasia severity associated with perchlorate exposure, a known thyroid hormone disruptor tested in the validation report for OECD 231 (2009)[18]. Therefore, when the observed reductions for T-mediated parameters developmental stage and normalised HLL are interpreted in conjunction with other results from OECD 231 (2009), particularly the lack of histopathological effects, they do not support a conclusion of T-mediated adversity.

As T-mediated adversity has not been observed, the following question is "*has endocrine activity been observed?*". The thyroid histopathology results from the submitted AMA study,

[18] FINAL REPORT OF THE VALIDATION OF THE AMPHIBIAN METAMORPHOSIS ASSAY: PHASE 2 – MULTI-CHEMICAL INTERLABORATORY STUDY, ENV/JM/MONO(2007)24

as discussed above, do not present any evidence of endocrine activity for inpyrfluxam. This is supported by the *in vitro* mechanistic assays findings presented in Document B6 (3CA).

This leads to the final question from Figure 1 of the ED Guidance (2018), "*Has endocrine activity been sufficiently investigated?*". Section 3.4.2. states that for non-target organisms, T-modality endocrine activity has been sufficiently investigated if an AMA study has been submitted. This is the case for inpyrfluxam.

In conclusion, using the decision tree provided in the ED Guidance (2018), Scenario 2a(ii) – No endocrine activity observed, but sufficiently investigated, is applicable and inpyrfluxam does not meet the T modality ED criteria for non-target organisms. No further consideration is required.

B.9.2.4.3 FSTRA study evaluation

Reference:	KCA 8.2.3/01
Report Title:	S-2399 - Fish Short-Term Reproduction Assay with Fathead Minnow (*Pimephales promelas*)
Author(s) & year:	[redacted] (2021)
Document No, Authority registration No:	[redacted] Study No. 13048.7149 Sumitomo Chemical Co., Ltd. Report No: TPW-0137
Substance used:	S-2399 TG, 13CG0617G, 95.0%
Method of analysis:	LC-MS/MS.
Guideline(s):	OECD 229 (2012) and OCSPP 890.1350 (2009)
Deviations:	Yes, see HSE Comments
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

I. MATERIALS AND METHODS

1. Test animals:

Species: Fathead minnow (*Pimephales promelas*)

Source: In-house culture ([redacted] culture). The population was housed in a continuous flow-through culture unit and was maintained at the same photoperiod as the pre-exposure and definitive exposure periods (16 hours light: 8 hours dark, with a 15- to 30-minute transition period);

	water quality was similar to that of the pre-exposure and definitive exposure periods.
Age:	Approximately 24 weeks old at the initiation of the pre-exposure period
Wet weight:	Males: 4.0 – 5.9 g; females: 2.3 – 3.4 g (prior to pre-exposure period)
Diet:	Frozen brine shrimp twice daily and once daily with fish flake food, with the exception of weekends where two daily feedings (one of brine shrimp and one of flake fish food) was sufficient. Fish were not fed during the 24-hour period prior to test termination to clear the intestinal tract for necropsy.

2. Test design

Test item:	S-2399 TG
Lot No.:	13CG0617G
Purity:	95.2%
Expiry date:	13 May 2022
System:	Flow-through
Dilution water:	The dilution water used during this study was laboratory well water and was from the same source as the culture water. Dilution water was considered to be of acceptable quality. In addition, representative samples of the dilution water source were analysed monthly for total organic carbon (TOC) concentration.
Duration:	Pre-exposure period: 14 days; exposure period: 21 days. No mortality occurred during the 2 weeks prior to pre-exposure initiation, and fish did not receive treatment for disease at any time prior to the spawning pre-qualification period or during the exposure period.
Test vessels:	Glass test aquarium: 39 x 20 x 25 cm with a 13 cm high side drain to maintain a constant exposure solution volume of approximately 10 L. Substrate to deposit adhesive embryos: 80-mm length of semi-circular (“arch”) made of polyvinyl chloride (PVC). Lipped piece of PVC tray approximately 100 mm in length (to capture embryos which did not adhere to the PVC arch).
Endpoints evaluated:	Survival, fecundity (expressed as eggs per female per reproductive day), percent fertilization success, blood plasma vitellogenin (VTG) concentration, gonadosomatic index (GSI), and nuptial tubercle score.
No. of replicates/test group:	4
No. of organisms/concentration:	24
No. of organisms/replicate:	4 female and 2 male fish

Dilution water: Laboratory well water
Hardness: 64 to 76 mg $CaCO_3$/L
Alkalinity: 22 to 24 mg $CaCO_3$/L
Conductivity: 380 to 510 µS/cm
pH: 6.8 to 7.1

3. Test solutions
Test concentration: 2.0, 6.3 and 20 µg/L (nominal). Spacing factor: approx. 3.15
2.0, 6.1 and 18 µg/L (mean measured). Spacing factor: approx. 2.9
LOQ (0.100 µg/L): 110 ± 1.52% (RSD: 1.39%)
High (100 µg/L): 108 ± 0.956% (RSD: 0.882%)

Sampling for chemical analysis:
Prior to the start of the definitive exposure, samples from two replicates of each treatment level and the control, as well as the saturator column stock solutions, were collected and analysed for S-2399 concentration. During the in-life phase of the definitive study, exposure solution samples were removed from alternating replicates of each treatment level and the control at exposure initiation and weekly thereafter. In addition, samples of the saturator column stock solutions were also analysed at each sampling interval during the exposure period. Additional subsamples of the test solutions and saturator column effluents were also collected at each sampling interval and stored frozen as archive samples. Instrumentation: LC-MS/MS.

Selection of concentrations:
A 14-day preliminary exposure was conducted at [redacted] from 20 July to 3 August 2020 exposing fathead minnows to S-2399 under flow-through conditions to nominal concentrations of 5.0, 10, 20, 40, and 80 µg/L, and a control. Two replicate aquaria each containing 10 fathead minnows (5 males and 5 females) was established for each treatment level and the control. The use of 5 males and 5 females differs slightly from the spawning groups utilized in the definitive exposure (i.e., 2 males and 4 females) and is meant to ensure that tolerability differences among the sexes is evaluated. The results of the preliminary exposure are shown in the table below.

Table 9.2.4-6: Results of the preliminary exposure of *Pimephales promelas* exposed to S-2399 TG in flow-through conditions.

Concentration (µg/L)	Replicate	Sex of Fish	Day 14		Total % effects
			Lethal	Sub-lethal	
Control	A	Male Female	0 0	0 0	0
	B	Male Female	0 0	0 0	0

Concentration (µg/L)	Replicate	Sex of Fish	Day 14		Total % effects
			Lethal	Sub-lethal	
5.0	A	Male Female	0 0	0 0	0
	B	Male Female	0 0	0 0	0
10	A	Male Female	0 0	0 0	0
	B	Male Female	0 0	0 0	0
20	A	Male Female	0 0	0 0	0
	B	Male Female	0 0	0 0	0
40	A	Male Female	4 0	0 0	40
	B	Male Female	0 0	0 0	0
80	A	Male Female	4 5	1 CLE NA	100
	B	Male Female	4 4	1 CLE 1 CLE	100

CLE = Complete Loss of Equilibrium
N/A = Not Applicable; no surviving fish were observed

4. Environmental conditions:
A summary of the environmental conditions is shown in the table below.

Table 9.2.4-7: Environmental conditions obtained in the Fish Short-Term Reproduction Assay with *Pimephales promelas* exposed to S-2399 TG in flow-through conditions.

Variable	Required OECD 229 (2012)	Obtained
Temperature	25 ± 2 ℃	25 – 26 ˚C
pH	6.5-9.0	6.8 – 7.4
Specific conductance	Not stated	450 – 540 µS/cm
Total hardness	Not stated	64 – 76 mg/L as $CaCO_3$
Total alkalinity	> 20 mg/L as $CaCO_3$	20 – 24 mg/L as $CaCO_3$
Dissolved oxygen	> 4.9 mg/L (> 60% saturation)	6.34 – 8.19 mg/L (77.2 to 99.3% saturation)
Photoperiod	16 h light, 8 h dark (15-30 minute transition period)	16 hours light: 8 hours darkness
Light intensity	540-1000 lux, or 50-100	52 to 96 footcandles (560 to 1030 lux)

Study dates: 14 October to 4 November 2020

5. Animal assignment and treatment:

Prior to exposure initiation, the adult fish were housed in aquaria within a pre-exposure system to evaluate reproductive performance over a 14-day period. The pre-exposure phase was conducted under test conditions identical to those used during the chemical exposure. No mortality occurred during the 2 weeks prior to exposure.

Based on the results of preliminary testing, nominal concentrations selected for the definitive assay were 2.0, 6.3 and 20 µg/L, with an untreated control group tested in parallel. There were four replicate aquaria per treatment group, each containing four females and two males. The exposure was maintained for a period of 21 days.

6. Dose preparation:

Stock solution preparation

A glass wool saturator column was used to deliver S-2399 to the exposure system. The glass columns were packed with glass wool and then coated with the test substance. Each column was designed to provide a constant flow of saturated aqueous solutions (21 mg/L) of S-2399 to the diluter system. To coat a column, approximately 6 g of S-2399 was dissolved in 25 mL of acetone, the solution was poured into the glass column then a vacuum pump was used to draw the solution throughout the column to uniformly coat the glass wool with the test substance and evaporate the remaining acetone.

The column was then attached to a pump, which continuously delivered dilution water through the column at 5.7 mL/min into an overflow glass reservoir where excess stock drained through a glass standpipe. A second pump was used to deliver the column effluent from the reservoir to the mixing chamber. Based on the functional solubility of S-2399 (21 mg/L), a flow rate of 0.48 mL/min was required to achieve the highest test concentration to dose the system during the definitive exposure. This chamber also received 2.82 L per cycle and was positioned over a magnetic stir plate, which aided in homogenisation of the exposure solutions.

Exposure system

The study was conducted using an exposure system consisting of a 2-L intermittent-flow proportional diluter and a 2-tiered water bath, consisting of an upper and lower-level water bath (one positioned over the other). The upper bath contained replicates A and C and the lower bath contained replicates B and D.

The solution in the mixing chamber (described above) was equivalent to that of the highest nominal test concentration (20 µg/L) and was proportionally diluted by a constant factor of 3.2 to produce the remaining nominal test concentrations (6.3 and 2.0 µg/L). The control vessels contained the same dilution water and was maintained under the same conditions as the treatment level vessels but contained no S-2399.

Flow-splitting chambers were used between the diluter cells and the four replicate test vessels to promote mixing of the S-2399 solution and diluent water, and to equally split the

test solution between the test vessels. During the test, 0.50 L per cycle of test solution was delivered to each aquarium at a rate of approximately 260 cycles per 24 hours. This flow provided a turnover rate of approximately 13 volume replacements every 24 hours, or a 90% replacement time of approximately 4 hours.

7. Measurements and observations:

During the exposure period, the appearance of the fish (e.g. coloration patterns or bands, differences in body shape in head and pectoral region), behaviour, fecundity (eggs produced per females), and fertilisation success were assessed daily. An assessment of survival was conducted daily throughout the exposure. At test termination, observations were first made on the behaviour and secondary sex characteristics of the fish (i.e. presence of tubercles, coloration patterns or vertical bands, dorsal nape in males, as well as presence of ovipositors in females).

After euthanasia, standard length and wet weight were measured. Blood samples were then taken for plasma vitellogenin (VTG) analysis. The correlation co-efficient values for VTG calibration curves were >0.996. Mean measured S-2399 concentrations ranged from 90 to 100% of nominal concentration and defined the treatment levels tested as 2.0, 6.1 and 18 µg/L. The gonads were preserved for potential histological analyses. Fish carcasses were also preserved for subsequent tubercle scoring.

At exposure initiation and weekly thereafter, total hardness, total alkalinity, and conductivity were measured and recorded in one replicate of the high and low treatment levels and the control alternating between treatment levels and replicate vessels each week. Dissolved oxygen, pH, and temperature were recorded in each concentration and control vessel at exposure initiation and subsequently in alternating replicates daily. Test solution temperature was continuously monitored during the exposure period in the upper and lower water baths.

Prior to the start of the definitive exposure, samples from two replicates of each treatment level and the control, as well as the saturator column stock solutions were collected and analysed for S-2399 concentration to confirm the diluter was functioning properly prior to test initiation.

During the in-life phase of the definitive study, exposure solution samples were removed from alternating replicates of each treatment level and the control at exposure initiation and weekly thereafter. In addition, samples of the saturator column stock solutions were also analysed at each sampling interval during the exposure period. All samples were analysed using liquid chromatography with tandem mass spectrometry detection (LC-MS/MS).

8. Statistics:

All statistical conclusions were made at the 95% level of certainty except in the case of the basic assumption tests, e.g., Shapiro-Wilks' Test (normal distribution) and Bartlett's Test (homogeneity of variance), in which the 99% level of certainty was applied. The following procedures were used:

1. Significant differences in the percent male survival were determined using one-tailed Fisher's Exact Test with Bonferroni-Holm's Adjustment since mortality followed a non-

monotonic concentration response. Significant differences in percent female survival and percent combined male and female survival were determined using one-tailed Cochran Armitage's Trend Test since mortality followed a monotonic concentration response.

2. Shapiro-Wilks' Test for normality was conducted on the continuous quantitative endpoint data and compared to the observed sample distribution with a normal distribution. If the data was not normally distributed, then a non-parametric procedure was used for subsequent analyses. For this study, all quantitative endpoint data were normally distributed.

3. Bartlett's Equality of Variance Test was conducted on the continuous quantitative endpoint data to evaluate the homogeneity of the data. For this study, all quantitative endpoint data passed the equality of variance qualifying test, with the exception of fertilisation success.

4. For the fecundity endpoint, the data did not resemble a monotonic concentration response. Therefore, the performance of the treatment organisms was compared with the performance of the control using a one-tailed Dunnett's Multiple Comparison Test.

5. For the fertilisation success endpoint, the data resembled a monotonic concentration response. Therefore, the performance of the treatment organisms was compared with the performance of the control using a one-tailed Jonckheere-Terpstra's Step-Down Test.

6. For the GSI endpoint, both male and female GSI data resembled a non-monotonic concentration response. Therefore, the performance of the male and female treatment organisms were compared with the performance of the male and female control organisms using a two-tailed Dunnett's Multiple Comparison Test.

7. A two-tailed Jonckheere-Terpstra's Step-Down Test was used for the male tubercle score endpoint.

8. Male and female plasma VTG data were analysed for outliers and statistical analysis was conducted on each dataset with and without suspected outliers. Outliers were identified as values that exceeded the mean plus three times the interquartile range (i.e. the difference between the 75^{th} and 25^{th} percentiles).

9. Since decreased or increased female plasma VTG concentrations can be indicative of endocrine activity, a two-tailed test was used to compare treatment and control organism performance. Since the data response for both female VTG including and excluding outliers was non-monotonic, Dunnett's Multiple Comparison Test was used.

10. Increased male plasma VTG concentrations can be indicative of endocrine activity. Reduction in male VTG is generally not considered to be indicative of endocrine activity. Male data was log-transformed prior to outlier analysis. Since the data was non-monotonic and passed both qualifying tests, a one-tailed Dunnett's Multiple Comparison Test was used to compare the treatment and control organism performance for datasets with outliers.

CETIS Version 1.9 was used to perform all statistical computations.

II. RESULTS AND DISCUSSION

A. BIOLOGICAL OBSERVATIONS

Endpoints sensitive to, but not diagnostic of, EAS modalities

Behavioural/morphological observations
No abnormal observations in behaviour were noted in the control or test item treatment levels during daily observations. Normal male territorial behaviour was observed in all replicates. Appearance of all fish throughout the exposure was within expectations for adult, spawning fathead minnow. Macroscopic examination of the females in all treatment levels at test termination confirmed normal appearance of ovaries; examination of the males confirmed normal appearance of testes.

Survival
There was a statistically significant difference in female survival and survival among male and female combined exposure at the highest tested concentration (18 µg/L). No other statistically significant effects on survival were observed. A summary of the results for survival at test termination is presented in the following table.

Table 9.2.4-8: Summary of survival at test termination

Mean measured concentration (µg/L)	Male survival % (SD)	Female survival % (SD)	Male/female combined survival % (SD)
Control	100 (0)	100 (0)	100 (0)
2.0	100 (0)	100 (0)	100 (0)
6.1	88 (25)	100 (0)	96 (8)
18	100 (0)	81 (24) *	88 (16) *

SD: standard deviation
* Significantly reduced compared to the control, based on one-tailed Cochran-Armitage's Trend Test

The reduction in female survival was primarily seen in replicates B and D from days 14 and 16, respectively. At test termination there were 3 surviving females in replicate B and 2 surviving females in replate D out of the 4 females at test initiation. The reduction in male survival noted at the median concentration was seen in replicate A on day 7, with 1 surviving male from the starting pair.

Fertilisation and fecundity
There was a significant reduction in the percentage of fertilised eggs among fish exposed to the 18 µg/L mean measured treatment level compared to control data. No other statistically significant effects on fertilisation success or fecundity were observed. A summary of the results for fertilisation success and fecundity is presented in the following table.

Table 9.2.4-9: Summary of fertilisation success and fecundity

Mean measured concentration (µg/L)	Fertilisation success (% fertilised) (SD)	Fecundity (number of eggs per female per reproductive day) (SD)
Control	99.7 (0.038)	19 (6.8)
2.0	99.6 (0.18)	23 (3.9)
6.1	99.5 (0.25)	24 (5.5)
18	98.6 (1.2) *	12 (3.6)

SD: standard deviation
* Significantly reduced compared to the control, based on one-tailed Jonckheere-Terpstra's Step-Down Test.

The reduction in fecundity is seen more generally across all replicates at the highest concentration. Reduction in fertility can be seen in each replicate over the course of the test but is limited to more isolated instances with no general trend.

Gonadal somatic index (GSI)
Statistical analysis determined no significant difference in mean male or female GSI among fish exposed to any of the treatment levels tested compared to the control data.

Summaries of results for length, weight and GSI are presented in the following tables for males and females, respectively.

Table 9.2.4-10: Male termination endpoint summary

Mean measured concentration (µg/L)	Standard length (mm) (SD) [a]	Wet body weight (g) (SD) [a]	GSI (%) (SD) [b]
Control	57.56 (0.42)	5.2432 (0.2193)	2.4 (0.51)
2.0	56.76 (3.01)	4.9299 (0.4405)	2.2 (0.23)
6.1	56.73 (3.68)	4.6809 (0.7101)	2.5 (0.44)
18	58.53 (0.87)	4.9048 (0.2144)	2.8 (0.35)

SD: standard deviation
[a] Length and body weight not statistically analysed for differences from the control. Length and weight are not endpoints in this study and therefore, statistical analysis was not required by protocol.
[b] GSI: gonad weight/body weight × 100

Table 9.2.4-11: Female termination endpoint summary

Mean measured concentration (µg/L)	Standard length (mm) (SD) [a]	Wet body weight (g) (SD) [a]	GSI (%) (SD) [b]
Control	47.39 (0.97)	2.4493 (0.2400)	16 (1.6)
2.0	47.81 (1.20)	2.4992 (0.2775)	18 (1.3)
6.1	48.17 (0.20)	2.5685 (0.1507)	18 (2.4)
18	47.61 (0.49)	2.4782 (0.1628)	17 (4.1)

SD: standard deviation
[a] Length and body weight not statistically analysed for differences from the control. Length and weight are not endpoints in this study and therefore, statistical analysis was not required by protocol.
[b] GSI: gonad weight/body weight × 100

EAS-mediated endpoints

Secondary sex characteristics

Tubercles and the presence of dorsal nape pads were not observed in females at any concentration during the test or in preserved females after termination. No abnormal behaviour or notable changes in secondary sex characteristics were observed in either sex throughout the 21-day study. No other abnormal observations were observed during the exposure period. There was no significant difference in mean male tubercle score in fish exposed to any of the treatment levels tested compared to the control data.

A summary of the results for male and female tubercle scores is presented in the following table.

Table 9.2.4-12: Male and female tubercle score

Mean measured concentration (µg/L)	Tubercle Score: male (SD)	Tubercle Size: male (replicate x size)	Tubercle Score: female (SD)
Control	39 (3.3)	4 x 2 (enlarged)	0 (0)
2.0	34 (1.9)	4 x 2 (enlarged)	0 (0)
6.1	34 (4.5)	1 x 1 (present) 3 x 2 (enlarged)	0 (0)
18	37 (5.7)	4 x 2 (enlarged)	0 (0)

SD: standard deviation

Gonad histopathology

Gonad histopathology was not performed. Following an RAI for this to be completed, the applicant has provided the following reasoning for not conducting histopathology analysis:

"Histopathology examination of the gonads is an optional measurement in the OECD 229 TG. While this may be needed in cases of equivocal results, the Applicant considered that the results of the study clearly indicate no EAS mediated effects of the test substance in a fully valid study. No effects were observed for the EAS mediated endpoint of secondary sex

characteristics and Sensitive To But Not Diagnostic Endpoints (STBNDE) of fecundity, gonadal somatic index. For the in vivo mechanistic biomarker VTG endpoints, results are known to be intrinsically variable, (Brown et al,. (2023). Are Changes in Vitellogenin Concentrations in Fish Reliable Indicators of Chemical-Induced Endocrine Activity? Ecotoxicology and Environmental Safety 266: 115563) and taken in isolation should not be used to define an EAS mediated effect. The significant increase in VTG in males at the middle concentration alone (outliers removed) does not follow a dose response and is considered to be within the Historical Control Data (HCD) of the performing lab as discussed and presented in the test report. Equally the significant reduction in female VTG at the lower concentration alone (outliers removed) does not follow a dose response and is considered to be within the HCD of the performing lab. Together both reported changes in VTG if considered to be accurate and EAS-mediated would contradict each other in terms of any postulated Mode of Action. No VTG effects are observed at the highest test concentration. The reported significant reduction of the STBNDE fertilisation success is observed in the highest concentration only where significant mortality was observed (12%). This concentration is therefore clearly above the Maximum Tolerated Concentration (MTC) which should not be above 10% mortality and the observed effect on fertilisation success is likely a result of the onset of systemic toxicity. It is also noted that the reported reduction in fertilisation at the highest concentration is also within the HCD of the performing laboratory. Given that, the FSTRA is considered to be sufficiently acceptable to investigate the ED properties of S-2399 through the EAS-modalities, without the need of the optional measurement of histopathological examination of the gonads.".

HSE determine that the provision of histopathology results would have been useful to assist with the interpretation of VTG given that there were statistically significant effects observed The implications of this are discussed in the hazard assessment section.

In-vivo mechanistic, EAS-mediated endpoints

Blood plasma vitellogenin concentration

Statistical analysis determined a significant increase in mean male VTG among fish exposed to the 6.1 µg/L mean measured treatment level compared to the control data (with calculated outliers excluded). There was also a significant reduction in mean female VTG among fish exposed to the 2.0 µg/L mean measured treatment level compared to the control data (with calculated outliers excluded). The results for male and female VTG analysis using all data points (excluding outliers) are presented in the following table.

Table 9.2.4-13: Vitellogenin (VTG) analysis (excluding outliers)

Mean measured concentration (µg/L)	Male VTG concentration (ng/mL) (SD)	Female VTG concentration (ng/mL) (SD)
Control	4.2×10^1 (4.2×10^1)	2.0×10^7 (2.1×10^6)
2.0	3.1×10^1 (1.0×10^1)	1.1×10^7 (3.3×10^6) *
6.1	1.4×10^2 (5.4×10^1) *	1.5×10^7 (1.8×10^6)
18	3.6×10^1 (2.6×10^1)	1.9×10^7 (7.0×10^6)

SD: standard deviation
* Statistical analysis determined a statistically significant increase in male VTG at the 6.1 µg/L treatment level and a statistically significant decrease in female VTG at the 2.0 µg/L treatment level.

Table 9.2.4-14: Vitellogenin (VTG) analysis (including outliers)

Mean measured concentration (µg/L)	Male VTG concentration (ng/mL) (SD)	Female VTG concentration (ng/mL) (SD)
Control	4.2×10^1 (4.2×10^1)	1.8×10^7 (2.1×10^6)
2.0	3.0×10^2 (1.0×10^1)	1.1×10^7 (3.3×10^6)
6.1	1.4×10^2 (5.4×10^1)	1.4×10^7 (1.8×10^6)
18	3.6×10^1 (2.6×10^1)	1.5×10^7 (7.0×10^6)

SD: standard deviation

Summary of biological results
An overall summary of the biological results observed is presented in the following table.

Table 9.2.4-15: Overall summary of biological results

Endpoints	Mean measured concentration (µg/L)		
	2.0	6.1	18
Male Survival	-	-	-
Female Survival	-	-	↓
Combined Male and Female Survival	-	-	↓
Fecundity	-	-	-
Fertilisation Success	-	-	↓
Nuptial Tubercle Score	-	-	-
Male Gonadal Somatic Index (GSI)	-	-	-
Female GSI	-	-	-
Male VTG, outliers removed	-	*	-
Female VTG, outliers removed	*	-	-

- Endpoint not statistically different from control.
↓ Statistical analysis determined endpoint to be significantly reduced compared to the control.
* Statistical analysis determined an increase in male VTG at the 6.1 µg/L treatment level and a decrease in female VTG at the 2.0 µg/L treatment level.

B. ANALYSIS

Results of the S-2399 analyses demonstrated that the measured concentrations approximated nominal concentration, were relatively consistent for the duration of the exposure period and maintained the expected concentration gradient. Mean measured S-2399 concentrations ranged from 90 to 100% of nominal concentration and defined the treatment levels tested as 2.0, 6.1 and 18 µg/L. The coefficient of variance (% CV) for the measured concentrations ranged from 9.7 to 16%. A summary of the analytical results is presented in the following table.

Table 9.2.4-16: Summary of analytical results

Nominal concentration (µg/L)	Measured concentration (µg/L)				Mean measured concentration (SD) (µg/L)	Percent of nominal (%)	% CV
	Day 0	Day 7	Day 14	Day 21			
Control	< 0.29	< 0.29	< 0.29	< 0.29	NA (NA)	NA	NA
	< 0.29	< 0.29	< 0.29	< 0.29			
2.0	1.9	2.0	1.4	2.1	2.0 (0.32)	100	16
	2.2	2.3	1.8	2.4			
Mean	**2.0**	**2.1**	**1.6**	**2.3**			
6.3	6.3	6.3	5.2	6.8	6.1 (0.59)	97	9.7
	6.4	6.0	5.2	6.5			
Mean	**6.4**	**6.1**	**5.2**	**6.6**			
20	19	20	15	18	18 (2.1)	90	11.7
	19	19	14	19			
Mean	**19**	**20**	**15**	**19**			

Concentrations expressed as less than values were below the method detection limit (MDL)
SD: standard deviation; CV: coefficient of variation; NA: not applicable

C. VALIDITY CRITERIA

The validity criteria in the following table were used to determine whether the test was sufficient to assess the required endpoints, in line with OECD 229 (2012) and OCSPP 890.1350 (2009). The table illustrates the criteria, the acceptable limits listed in the test guidelines, and the performance of this study. All validity criteria were met.

Table 9.2.4-17: Summary of validity criteria

Required	Obtained	Criterion met (Yes/No)
Test concentrations maintained at ≤ 20% CV (variability of measured test concentration) over the 21-day test, per treatment level.	CV values ranged from 9.7 to 16%.	Yes
Control mortality ≤ 10%.	Mean control survival at exposure termination was 100% (i.e. control mortality of 0%).	Yes

Required	Obtained	Criterion met (Yes/No)
Evidence that fish are actively spawning in all replicates prior to initiating chemical exposure and in control replicates during the test	Prior to exposure initiation: ≥5 spawns, ≥ 22 eggs/female/reproductive day/replicate. During the exposure: 19 eggs/female/reproductive day during the exposure.	Yes
Control fertilisation > 95%	Mean control fertilisation success was 99.7%.	Yes
Dissolved oxygen ≥ 60% air saturation for the duration of testing.	Dissolved oxygen was maintained at ≥ 77.2% of air saturation.	Yes
Water temperature must not differ by more than ± 1.5 °C between test chambers or between successive days at any time during the exposure.	At no point in the exposure were test chambers measured to differ more than ± 1 °C or between successive days.	Yes
Water temperature should be maintained within a range of 2°C within the temperature ranges specified for the test species (25˚C)	Daily water temperature measurements and continuous temperature monitoring of the control established a temperature range of 25 to 26 °C throughout the exposure period.	Yes

III. CONCLUSION

Statistical analysis determined an increase in male VTG at the 6.1 µg/L treatment level and a decrease in female VTG at the 2.0 µg/L treatment level.

Secondary sex characteristics were evaluated and no effects indicative of ED-parameters were noted: No significant difference in nuptial tubercle scores of male fish was observed among fish exposed to any of treatment levels. The presence of nuptial tubercles or dorsal nape pads was not observed on females at any point during the exposure. No abnormal behaviour or notable changes in secondary sex characteristics were observed in either sex throughout the 21-day study. No other abnormal observations (e.g., body colour (light or dark), coloration patterns, body shape, size of dorsal nape pad in males, or ovipositor size in females) were observed during the exposure period or at study termination in any of the treatment levels or the control.

A significant reduction in female survival as well as male/female combined survival was observed among fish exposed to the 18 µg/L treatment level. In addition, a significant reduction in fertilisation success was observed in the 18 µg/L treatment level compared to the control. No other significant effects of the test item compared to the control were observed.

The significant reduction in fertilisation success that occurred at the 18 µg/L treatment level is sensitive to, but not diagnostic of an EAS-mediated modality. Furthermore, this significant response only occurred at the high concentration where significant mortality was observed.

HSE COMMENTS

This study has been conducted to OECD 229 (2012) and OCSPP 890.1350 (2009) guidelines and has been assessed against these same guidelines. The study has been conducted to GLP and all the validity criteria have been met.

Mean measured concentrations at the test start and end were within ± 20 % of the nominal test concentrations. However, the applicant has based results on mean measured concentrations. This is acceptable as the mean measured concentrations are slightly below the nominal, so there is no overestimation of results.

Consideration of the MTC is detailed in the hazard assessment in section B.9.2.4.4.

It is noted that both the male and female fish exceeded the weight recommendations in the guideline, which states that males should be 2.5 g ± 20%, and females should be 1.5 g ± 20%. This would take the maximum weight of males to be 3 g, and for females to be 1.8 g. In the study, males weighed up to 5.9 g and females weighed up to 3.4 g. Whilst this is a deviation to the guidelines, this does not impact the validity of the study. However, it does introduce some uncertainty as weight can have impacts on fecundity. Additionally, the age of the fish exceeds the recommended age range in the OECD 229 (2012) guidelines, which states that fathead minnow should be 20 (±2) weeks, but the fish used in this study were approximately 24 weeks. This is not ideal and introduces some uncertainty as age of the fish is important for correct development stage for assessing endocrine effects.

The method of analysis used in the study was evaluated by HSE Chemistry. The conclusion of their evaluation is reproduced below. Please see Volume 3 CA, section B5 for more details.

"*The analytical method is not fully validated according to SANTE/2020/12830 rev. 1 for the determination of inpyrfluxam in aqueous solutions from the fish short-term reproduction assay with Fathead Minnow (Pimephales promelas) and the amphibian metamorphosis assay with African Clawed Frog (Xenopus laevis) studies due to the stability of standards and stock solutions has not being addressed. However, the studies were generated prior to the implementation of SANTE 2020/12830 rev.1 and SANCO 3029 rev.4 did not require stabilities of standards and extracts to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose.*"

The use of statistics is acceptable and in line with OECD 229 (2019). Standard deviations have been provided along with measured parameters.

The study and its results can be considered reliable, and results have been considered further in the endocrine disruption hazard assessment for S-2399.

B.9.2.4.4 AMA study evaluation

Reference:	KCA 8.1.4/01
Report Title:	Amphibian Metamorphosis Assay with African Clawed Frog (*Xenopus laevis*)
Author(s) & year:	[redacted] (2021)
Document No, Authority registration No:	[redacted] [redacted] Study No. 13048.7147, Sumitomo Chemical Co., Ltd. Report No.: TPW-0138.
Substance used:	S-2399 TG, 13CG0617G, 95.0%
Method of analysis:	LC-MS/MS
Guideline(s):	OECD 231 (2009)
Deviations:	Yes, see HSE Comments
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

MATERIALS

Test Material S-2399 TG

Description: Not stated

Lot/Batch #: 13CG0617G

Purity: 95.2% (verified according to the certificate of analysis)

Stability of test compound: Not stated

Reanalysis/expiry date: 13 May 2022

Treatments

Test concentrations: Nominal concentrations of 4.1, 14 and 45 µg a.s./L, mean measured concentrations of 3.7, 14, and 43 µg a.s./L.

Control: Dilution water control

Solvent: Acetone (evaporated off after glass wool coating using vacuum pump)

Analysis of test concentrations: Yes

	Prior to the start of the definitive exposure, samples from each treatment level and the control, as well as the saturator column effluent. During the in-life phase of the definitive study, Day 0, 7, 14 and 21. Liquid chromatography with tandem mass spectrometry detection (LC-MS/MS). The limit of quantification (LOQ) was set at 0.100 µg/L
Test animals	
Species:	African Clawed Frog (*Xenopus laevis*, Stage 51)
Source:	The tadpoles originated from adult brood stock maintained at [redacted] The brood stocks were obtained as adult, proven breeders from [redacted] and maintained as in-house breeders for at least 3 months prior to generating tadpoles for use in this exposure.
Test design	
Exposure regime:	Flow-through (Mount & Brungs intermittent-flow proportional diluter system).
Aeration:	None required due to high test solution turnover rate of flow-through system.
Replication:	Four test vessels per concentration, 20 tadpoles per vessel.
Test vessels:	9.5-L exposure aquarium (30 × 14.5 × 20 cm) with a 12.5-cm high side drain that maintained a constant exposure solution volume of approximately 5.5 L
Duration:	15 days pre-exposure, 21 days exposure
Dilution water:	
Source:	Mixture of unadulterated on-site well water and de-chlorinated Town of Wareham well water
pH:	7.0 – 7.3
Conductivity	400 - 500 µS/cm
Total hardness as $CaCO_3$	64 - 68 mg/L
Total alkalinity as $CaCO_3$	23 - 26 mg/L
Environmental conditions	
Test temperature:	20 – 22 °C
pH:	6.3 – 7.4

Dissolved oxygen: 3.3 to 8.9 mg/L (37 to 100% of saturation)
Lighting: 12 hours light at a light intensity range of 60 to 110 footcandles (650 to 1200 lux) and 12 hours dark

STUDY DESIGN AND METHODS

Definitive test dates: 9 to 30 December 2020

Test organism
Adult brood stock frogs were maintained in a continuous flow-through culture unit in 20-L aquaria. The culture water was approximately 22 °C laboratory well water. Brood and test organisms were cultured and tested in water from the same source. The culture unit was maintained at the same photoperiod as the definitive exposure (12 hours light: 12 hours dark).

Feeding
Tadpoles were fed [redacted] tadpole food ([redacted]) twice a day. During the pre-exposure period, tadpoles were fed at the rates outlined in Table 9.2.4-18.

Table 9.2.4-18: Feeding rates during pre-exposure period

Pre-Exposure Day	Daily Food Ration (mg/tadpole)	Tank Density	Total Fed/Day (mg)	Food Concentration (g/L)	Feeding Rate (mL/day)	Feeding Rate (mL/feeding)
3 - 6	2.7	100	267	21	13	6.3
7 - 10	5.3	100	533	21	25	13
11 - 14	11	100	1067	21	50	25
15 - 19	16	100	1600	21	76	38

During the exposure period, tadpoles were fed at the rates outlined in Table 9.2.4-19.

Table 9.2.4-19: Feeding rates during exposure period

Exposure Day	Daily Food Ration (mg/tadpole)	Tank Density	Total Fed/Day (mg)	Food Concentration (g/L)	Feeding Rate (mL/day)	Feeding Rate (mL/feeding)
0 - 4	16	20	320	21	15	7.6
5 - 7	21	20	427	21	20	10
8 - 10	27	15	400	21	19	9.5
11 - 14	37	15	560	21	26	13
15 - 20	43	15	640	21	30	15

Representative samples of the food source were analysed for the presence of pesticides, PCBs, and toxic metals by [redacted]. None of these compounds were detected in the food samples at concentrations considered toxic to the test organisms.

Test water

The on-site well water is obtained from a 100-meter bedrock well. The Town of Wareham well water is de-chlorinated by use of ultraviolet (UV) treatment and activated carbon filtration. The two sources of water pass individually through 1-µm polypropylene bag filters, a degasser, and is then mixed in a 5700-L polyethylene tank. The resulting dilution water is continuously circulated through a degassing chamber to ensure proper equilibration of dissolved gases with the laboratory atmosphere.

Representative samples of the dilution water were analysed biannually for the presence of pesticides, PCBs, and toxic metals in agreement with ASTM (2007)[19] standard practice. Analysis was conducted by Eurofins Lancaster Laboratories Environmental, Lancaster, Pennsylvania, using standard methods (U.S. EPA, 1997)[20]. None of these compounds were detected at concentrations that are considered toxic in any of the water samples analysed. In addition, representative samples of the dilution water were analysed monthly for total organic carbon (TOC) concentration. The TOC of the dilution water was 0.98 mg/L for December 2020. The TOC was measured using a Shimadzu Model TOC-L TOC analyser.

[19] ASTM, 2007. Standard Practice for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates and Amphibians. Standard E729-96. American Society for Testing and Materials, 100 Barr Harbor Drive, West Conshohocken, PA 19428, pp. 65 – 86.

[20] U.S. EPA, 1997. Office of Waste. Test Methods for Evaluating Solid Waste, Physical/Chemical Methods (SW-846). February 2007. U.S. Environmental Protection Agency, Washington, D.C.

Iodide concentration was analysed in December 2020 in one representative sample of the dilution water by Galbraith Laboratories, Inc., Knoxville, Tennessee. A sample was taken at initiation and at termination of the definitive exposure (day 0 and 21). Iodide concentration was measured to range from 7.7 to 8.2 µg/L. The testing guidelines include an ideal iodide concentration range of 0.5 to 10 µg/L for the dilution water.

Test concentration selection

Nominal concentrations for the amphibian metamorphosis assay with African clawed frog (*Xenopus laevis*) were selected based on a 96-hour preliminary range-finding exposure. The maximum tolerable concentration (MTC) was approximated as 1/3 of the acute LC_{50} value (140 µg a.s./L / 3 = 46.7 µg a.s./L). 45 µg a.s./L was selected as the highest concentration for the definitive assay.

Dose preparation

A glass wool saturator column was used to deliver S-2399 to the exposure system, similar to that described in Kahl et al., 1999[21] and OECD, 2018[22]. The glass columns (30 cm × 2.5 cm, 15% of column packed with wool) were packed with wool, and then coated with the test substance. Each column was designed to provide a constant flow of saturated aqueous solutions (21 mg/L) of S-2399 to the diluter system without the use of a carrier solvent. The columns were constructed entirely of chemical inert materials (glass and Teflon). To coat a column, approximately 6 grams of S-2399 was dissolved into 25 mL of acetone (CAS No. 67-64-1) and poured into the glass column. After the solution was added, the column was attached to a vacuum pump, used to draw the solution evenly throughout the column to uniformly coat the glass wool with the test substance and evaporate the remaining acetone. After solution evaporation, the column attached to a FLUID Metering, Inc. (FMI) pump, which continuously delivered dilution water through the column at 5.7 mL/min. A second FMI pump was used to deliver the column effluent from the reservoir to the mixing chamber.

Exposure system

The exposure system was constructed of glass, silicone sealant, and nylon. All test chambers were brushed and siphoned at least twice weekly to remove detritus and uneaten food during the test.

Based on the functional solubility of S-2399 (21 mg/L), a flow rate of 0.46 mL/min (approximately 3.0 mL/cycle) was required to achieve the high-test concentration to dose the system during the definitive exposure. This chamber also received 1.395 L per cycle and was positioned over a magnetic stir plate. The solution in the mixing chamber was

[21] Kahl, M.D., Russom, C.L., DeFoe, D.L. and Hammermeister, D.E., 1999. Saturation Units for Use in Aquatic Bioassays. Chemosphere 39: 539-551.

[22] OECD, 2018. Revised Guidance Document 150 on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption, OECD Series on Testing and Assessment, OECD Publishing, Paris. https://doi.org/10.1787/9789264304741-en

equivalent to that of the highest nominal test concentration (45 µg/L) and was serially diluted by a factor of 3.3 to produce the remaining nominal test concentrations (14 and 4.1 µg/L).

Flow-splitting chambers were used between the diluter cells and the 4 replicate test vessels to promote mixing of the S-2399 solution and diluent water, and to equally split the test solution between the test vessels. During the test, 0.25 L per cycle of test solution was delivered to each aquarium at a rate of approximately 216 cycles per 24 hours. This flow provided a turnover rate of approximately 9.8 volume replacements every 24 hours, or a 90 % replacement time of approximately 5 hours (Sprague, 1969)[23].

Calibration of the diluter system was conducted prior to exposure initiation and confirmed at test termination (day 21) by measuring delivery volumes of toxicant and dilution water. Proper diluter function (e.g., dilution water flow rate, stock solution consumption) was checked twice daily. The exposure system was operating properly for 21 days prior to exposure initiation to allow equilibration of the test substance in the diluter apparatus and exposure aquaria.

Exposure initiation

Pre-exposure period

On pre-exposure day 0, three pairs of adult male and female *X. laevis* were induced to breed. Each frog was provided a primary injection of human chorionic gonadotropin (hCG, 250 International Units (I.U.) for both males and females) and a secondary injection in the afternoon (500 I.U. for males and 750 I.U. for females). Subsequently, each pair was placed into a separate 20-L breeding aquarium. Each breeding aquarium contained a plastic false bottom, which allowed the egg masses to fall to the bottom of the aquarium and remain undisturbed. The following morning (pre-exposure day 1), the adult frogs and false bottoms were carefully removed from the breeding aquaria. The embryos remained in their respective breeding aquaria until all viable embryos had hatched and all tadpoles had reached feeding stage (6 days post-fertilization). During embryo incubation, the number of live and dead embryos as well as the number of hatched tadpoles was approximated for each spawn. The spawn selected for use in the exposure yielded approximately 4000 embryos (minimum number of embryos criterion = 1500; OECD 231, 2009), and the embryo survival from this spawn was estimated at > 90%.

Once all tadpoles were at the feeding stage, larvae from the highest quality spawn, based on the number of embryos and hatching success, were transferred to ten 10-L rearing tanks. One hundred tadpoles were transferred to each rearing tank for a larval density of 10 tadpoles/L. One of the ten rearing tanks was designated as a replacement tank. These tadpoles were used as replacements in the event that mortalities occurred in the rearing

[23] Sprague, J.B., 1969. Measurement of pollutant toxicity to fish. 1. Bioassay methods for acute toxicity. Water Research 3: 793-821.

tanks during the next week of rearing. This procedure was performed to maintain consistent organism density and thereby reduce developmental divergence during the pre-exposure period.

During this pre-exposure period, tadpoles were maintained under flow-through conditions similar to that of the actual exposure. Flow rates and temperature were monitored daily; dissolved oxygen and pH were measured at least three times per week. Temperature was monitored continuously in a representative rearing tank using a minimum/maximum thermometer and recorded daily. During this period, flow rates were maintained at 25 mL/min, daily aquarium temperature ranged from 18 to 23 °C, dissolved oxygen ranged from 76 to 110 % saturation, and pH ranged from 6.5 to 7.4.

Tadpoles were evaluated daily for mortalities, proper development, and overall health. Excess food and debris were siphoned from the rearing tanks as needed during the rearing period. Care was taken to minimize stress on the tadpoles during inspection and cleaning of aquaria.

Definitive exposure

On pre-exposure day 15 (day 15 post-fertilization), the majority of tadpoles had reached NF stage 51 (Nieuwkoop and Faber, 1994)[24]. All healthy tadpoles from each rearing vessel were carefully removed and pooled into a vessel containing dilution water maintained at approximately 22 °C. For selection, normal, healthy-looking tadpoles were removed from the pooled group, transferred to a deep well Petri dish containing dilution water, and observed under a binocular dissection microscope to determine the developmental stage. Staging was conducted on each individual tadpole according to the Normal Table of *Xenopus laevis* (Nieuwkoop and Faber, 1994). The primary developmental landmark for staging the tadpoles was hind limb morphology. Once an appropriate number of stage 51 tadpoles were collected, tadpoles were randomly distributed to assigned test vessels. Tadpoles were distributed to the test vessels five at a time until each vessel contained 20 tadpoles.

Measurement and observations

Day 7 measurements

On day 7 of the exposure, five tadpoles were randomly selected from each test vessel for growth metrics. For this procedure, a unique set of random numbers was generated in the range of 1 to 20 for each replicate concentration. The first 5 numbers generated per set represented the tadpole to be selected for termination. For each replicate vessel, all tadpoles were removed and placed into a secondary container containing dilution water at test temperature. Each tadpole was then removed from the secondary container and counted in ascending order. When the appropriate random number was reached, that

[24] Nieuwkoop, P.D. and Faber, J., 1994. Normal Table of Xenopus laevis. Garland Publishing, New York.

tadpole was selected for termination. This method ensured that every tadpole was netted and handled and gave each tadpole equal probability of being selected for termination measurements. Tadpoles selected for measurement were euthanized in a separate vessel containing a neutrally buffered solution of tricaine methane sulfonate (MS-222) and dilution water. Tadpoles not selected for measurement were returned to their respective exposure vessels.

Euthanized tadpoles were then removed from the MS-222 solution, rinsed with water, and gently blotted dry. Developmental stage (Nieuwkoop and Faber, 1994) was then determined for each tadpole using a binocular dissection microscope. Digital images were then taken of each tadpole for snout-vent length and hind limb length measurements (to the nearest 0.01 mm) (Zeiss Stemi-2000 microscope, Zeiss AxioCam ICc 5 camera, Carl Zeiss Zen 2011 Blue Edition image analysing software). Whole body wet weight was then determined on a calibrated analytical balance to the nearest 0.1 mg.

Day 21 measurements

At test termination (day 21), the remaining tadpoles were removed and euthanized with buffered MS-222. Developmental stage (Nieuwkoop and Faber, 1994) was then determined for each tadpole using a binocular dissection microscope. Digital images were taken of each tadpole for snout-vent length and hind limb length measurements using the equipment and procedures described above. Each tadpole was then weighed to the nearest 0.1 mg. Following these procedures, each tadpole was transferred to a storage container with an appropriate volume of Davidson's fixative. Tadpoles remained submerged, as whole-body samples, in Davidson's fixative for approximately 48 hours. Samples were then rinsed with 70% reagent grade ethanol and stored in 10% neutral buffered formalin.

For snout-vent length measurements, the cranial aspect of the vent was used as the caudal limit of the measurement.

For hind limb length measurements, the left hind limb was used and all length measurements originated at the body wall and followed the limb midline. On day 21, natural bends in the limb occur due to the development of the tadpoles. Therefore, these length measurements followed the limb midline through any natural angular deviations. Hind limb length is reported as the total length, as described above, and as a normalized value. Hind limb length is normalized by snout-vent length by calculating the ratio of the hind limb length to snout-vent length. Normalized hind limb length is the measurement used for statistical evaluation as recommended by the appropriate guideline (OECD 231, 2009).

Thyroid gland histology

For histological analyses, a total of five tadpoles was impartially selected from each replicate test concentration at day 21. If possible, stage-matched tadpoles would have been selected so that all selected tadpoles matched the median stage of the control (stage 59).

However, for this exposure, tadpoles in the treatments were not able to stage match to the median stage of the control (stage 59). Since the guideline recommends sampling the next lower stage if the chemical treatment is associated with a retardation of development, and in consultation with the Study Sponsor, stage 58 was selected for histopathology examination. If stage 58 tadpoles were not available in a treatment level, then tadpoles at the next stage down (stage 57) were selected. Tadpoles at the lower developmental stage were selected in this manner, when necessary, based on guideline recommendations (OECD 231, 2009). The histological processing and analyses were performed by Experimental Pathology Laboratories (EPL), Sterling, Virginia according to the OECD Guidance Document on Amphibian Thyroid Histopathology (OECD 2007)[25].

Analytical verification

Analytical verification of the test item concentrations was performed on days 0, 7, 14 and 21. On test days 0 (exposure initiation) and 14, samples were removed from replicates C and D; on test days 7 and 21 (exposure termination), samples were removed from replicates A and B. Samples of the saturator column effluent were also analysed at each sampling interval during the exposure period.

Other measurements

All test vessels were examined daily for survival and behavioural assessment. Dead animals were removed from the test tank and recorded when observed. Observations of abnormal behaviour, such as floating on the surface, lying on the bottom of the tank, irregular swimming, etc., or differences in food consumption, visible gross malformations, or lesions were also recorded.

Dissolved oxygen concentration, pH, and temperature measurements were taken in all vessels on day 0 and in one replicate of each concentration and the controls each day thereafter; replicates were alternated each day. Total hardness, total alkalinity, and conductivity were measured in replicate A of the controls, low, and high test concentrations on day 0 and in sequentially alternating replicates weekly thereafter.

Statistical analysis

At test termination, data obtained on developmental stage, snout-vent length, hind limb length, and whole-body wet weight were analysed to identify significant reductions or enhancements in the treatment organisms compared to the control organisms. Statistical analyses of the data essentially followed the procedures described in the OECD Guideline for the Testing of Chemicals. No. 231. If any procedures were not appropriate for the respective data set, then the OECD Current Approaches in the Statistical Analysis of Ecotoxicity Data “A Guidance to Application” were consulted (OECD 2006). All statistical conclusions were made at the 95% level of certainty except in the case of the basic

[25] OECD, 2007. Guidance Document on Amphibian Thyroid Histology. OECD Environment Health and Safety Publications. ENV/JM/MONO(2007)31. Paris, France.

assumption tests, e.g., Shapiro-Wilks' Test (normal distribution) and Bartlett's Test (homogeneity of variance) in which the 99% level of certainty was applied. CETIS Version 1.9 was used to perform the following statistical computations:

1. Survival did not resemble a monotonic concentration response. Therefore, the replicate means were analysed with a one-tailed (C > T) Fisher's Exact Test with Bonferroni-Holm's Adjustment.
2. Developmental stage was analysed at day 7 and day 21 using a one-tailed (C > T) Jonckheere-Terpstra's Step-Down Test on the replicate medians.
3. Day 7 and 21 snout-vent length, day 7 and 21 wet weight, and day 7 and 21 hind limb length normalized by snout to vent length did not resemble a monotonic concentration response. Therefore, these endpoints were analysed using a one-tailed (C > T) Dunnett's Multiple Comparison Test, a parametric procedure, on the replicate means. This endpoint data met the assumptions of normal distribution and homogeneity of variance using the assumption tests described above.
4. The multi-quantal Jonckheere-Terpstra's Step-Down Test from the 20th to 80th percentile was used to evaluate day 7 and day 21 developmental stage effects among the distribution profile.

Histopathology results were evaluated qualitatively.

RESULTS AND DISCUSSION

Biological observations

Survival

No mortalities or sub-lethal effects were observed in the control or any of the treatment levels tested.

Overt systemic toxicity

No signs of overt systemic toxicity were observed in tadpoles in the control or any of the treatment levels tested. Tadpoles in all treatment levels and the control exhibited normal behaviour throughout the exposure period. No noticeable differences in food consumption between treatments were observed. Also, no gross malformations or lesions were observed.

Deformities

On day 7 (N = 5 tadpoles observed per replicate), no spinal deformities (e.g., scoliosis, bent tail) were observed in any of the treatment levels tested or the control. On day 21 (N = 15 tadpoles observed per replicate), no spinal deformities were observed in the control, and in 3, 2, and 0% of tadpoles exposed to the 3.7, 14, and 43 µg/L treatment levels, respectively. For the entire exposure (N = 20 tadpoles per replicate), no spinal deformities were observed for control animals, and for 3, 1, and 0% of tadpoles exposed to the 3.7, 14, and 43 µg/L

treatment levels, respectively. Overall, the incidence of spinal deformities was unrelated to treatment with S-2399 and did not impact any endpoint collected for this assay.

Growth rates

A summary of the four main growth experimental endpoints (developmental stage, snout-vent length, hind limb length and whole-body wet weight) following 7- and 21-days exposure are presented in Table 9.2.4-20 and Table 9.2.4-21.

Table 9.2.4-20: Summary of Endpoints (Developmental Stage, Snout-Vent Length, Hind Limb Length, and Whole Body Wet Weight) Following 21 Days of Exposure after 7 and 21 days of exposure

Mean Measured Concentration (µg/L)	Mean of 4 replicates	Developmental Stage[a]			Snout-Vent Length (mm)[b]				Hind Limb Length (mm)[c]			
		Min	Median	Max	Mean	Median	SD	% CV	Mean	Median	SD	% CV
Control	Day 7	52	54	55	17.38	17.37	0.57	3.3	2.39	2.42	0.16	6.6
3.7	Day 7	53	54	55	16.25	16.29	0.71	4.4	2.22	2.23	0.11	5.0
1.4	Day 7	53	55	55	16.31	16.38	0.96	5.9	2.25	2.30	0.24	11
43	Day 7	53	54	55	15.34[d]	15.38	0.82	5.3	2.14	2.18	0.08	3.5
Control	Day 21	57	59	61	27.40	27.19	0.89	3.3	15.44	15.90	0.89	5.8
3.7	Day 21	57	59	61	26.12[e]	25.73	0.72	2.8	14.94	15.37	0.79	5.3
14	Day 21	57	58	60	26.44	26.36	0.44	1.6	14.23	14.71	0.33	2.3
43	Day 21	56	58[d]	60	25.24[f]	25.34	0.89	3.5	12.37	12.03	2.14	17

SD = Standard Deviation

CV = Coefficient of Variation

[a] Analysis performed using median organism response in each replicate.

[b] Analysis performed using mean organism response in each replicate.

[c] Not statistically analysed as it was not required by study protocol or applicable guidance.

[d] Significantly reduced compared to the control, based on Dunnett's Multiple Comparison Test.

[e] Significantly reduced compared to the control, based on Dunnett's Multiple Comparison Test. However, due to the lack of statistical significance at the next higher treatment level (i.e., 14 µg/L), this significance was determined to not be treatment related by the applicant.

[f] Significantly reduced compared to the control, based on Dunnett's Multiple Comparison Test.

Table 9.2.4-21: Summary of Endpoints (Developmental Stage, Snout-Vent Length, Hind Limb Length, and Whole Body Wet Weight) Following 21 Days of Exposure after 7 and 21 days of exposure (Continued)

Mean Measured Concentration (µg/L)	Mean of 4 replicates	Hind Limb Length (mm) (Normalized by SVL)[b]				Whole Body Wet Weight (g)[b]			
		Mean	Median	SD	% CV	Mean	Median	SD	% CV
Control	Day 7	0.137	0.137	0.005	4.0	0.3822	0.3902	0.0487	13
3.7	Day 7	0.136	0.136	0.001	0.97	0.3297	0.3310	0.0494	15
14	Day 7	0.138	0.138	0.007	5.3	0.3412	0.3395	0.0576	17
43	Day 7	0.139	0.140	0.004	2.9	0.2643[d]	0.2608	0.0347	13
Control	Day 21	0.564	0.551	0.036	6.4	1.5201	1.5101	0.1287	8.5
3.7	Day 21	0.572	0.571	0.015	2.6	1.3620	1.3248	0.1240	9.1
14	Day 21	0.537	0.539	0.018	3.3	1.3798	1.3899	0.0498	3.6
43	Day 21	0.487[f]	0.464	0.070	14.4	1.2389[f]	1.2538	0.1378	11.1

SD = Standard Deviation
CV = Coefficient of Variation
N = replicate count

[a] Analysis performed using the median organism response in each replicate. All tadpoles (i.e., late-stage and non-late-stage) are included.

[b] Analysis performed using mean organism response in each replicate. Late-stage tadpoles (i.e., >60 NF) are excluded.

[c] Not statistically analysed as it was not required by study protocol or applicable guidance.

[d] Significantly reduced compared to the control, based on Jonckheere-Terpstra's Step-Down Test.

[e] Significantly reduced compared to the control, based on Dunnett's Multiple Comparison Test. However, due to the lack of statistical significance at the next higher treatment level (i.e., 14 µg/L), this significance was determined to not be treatment related.

[f] Significantly reduced compared to the control, based on Dunnett's Multiple Comparison Test.

After NF stage 60, tadpoles show a reduction in size and weight due to tissue resorption and reduction of absolute water content. Thus, measurements of snout-vent length and hind-limb length normalized by snout-vent length cannot appropriately be used in statistical analyses for differences in growth rates. Since the total number of organisms observed to be at late stage in the control and all treatment levels were <20%, the animals >60 NF (Nieuwkoop and Faber stage) were censored and not used in calculations or statistical analysis of means/medians for the growth metrics presented (i.e., snout-vent length, hind limb length normalized by snout-vent length, and wet body weight).

Developmental stage: At day 7 the Jonckheere-Terpstra's Step-Down Test determined no significant difference in developmental stage among tadpoles exposed to any of the treatment levels tested compared to the control. At day 21 a significant reduction in developmental stage among tadpoles exposed to the 43 µg/L treatment level compared to the control was determined. The distribution profiles of developmental stages were also examined by applying the multi-quantal Jonckheere-Terpstra's Step-Down Test to the 20th through the 80th percentiles for all treatment levels compared to the control for Day 7 and 21. The overall multi-quantal procedure determined no significant difference in day 7 percentile developmental stage at any treatment level. For Day 21, the overall multi-quantal procedure determined a significant difference in Day 21 percentile developmental stage at 43 µg/L for the 20^{th} through the 60^{th} percentiles compared to the control but not the 70^{th} and 80^{th} percentiles. For 14 µg/L, the 20^{th} and 60^{th} percentiles were significantly different to the control but not other percentiles.

Snout-Vent length: Dunnett's Multiple Comparison Test determined a significant reduction in day 7 snout-vent length among tadpoles exposed to the 43 µg/L treatment level compared to the control and a significant reduction in tadpoles exposed to the 3.7 and 43 µg/L at day 21. Due to the lack of statistical significance at the next middle treatment level (i.e., 14 µg/L), the significance detected at the 3.7 µg/L treatment level at day 21 was determined to not be treatment related by the applicant.

Hind limb length: Dunnett's Multiple Comparison Test determined no significant difference in day 7 hind limb length normalized by SVL among tadpoles exposed to any of the treatment levels tested compared to the control. At day 21 a significant reduction in hind limb length normalized by SVL among tadpoles exposed to the 43 µg/L treatment level compared to the control was determined.

Whole body wet weight: Dunnett's Multiple Comparison Test determined a significant reduction in day 7 and day 21 whole body wet weight among tadpoles exposed to the 43 µg/L treatment level compared to the control.

Thyroid gland histology

There were no treatment-related histopathologic findings in this study. Most frogs exhibited follicular cell hypertrophy (mild), while follicular cell hyperplasia (mild) was less common, but the prevalence of both findings in S-2399 treated frogs were comparable to the negative controls.

Table 9.2.4-22: Summary of thyroid gland histology

Prevalence and Severity of Thyroid Findings					
S-2399 Treatment Group (µg/L), Mean Measured Concentrations		**0.0 (Control)**	**4.1**	**14**	**45**
Number Examined		20	20	20	20
Thyroid	Follicular cell hyperplasia mild	4	6	2	5
		4	6	2	5
	Follicular cell hypertrophy mild	14	15	13	16
		14	15	13	16

An overview of statistically significant results across the measured endpoints and selected timeframes is presented in Table 9.2.4-23.

Table 9.2.4-23: Overview of findings of the AMA exposure with S-2399

Endpoints		Mean Measured Concentration (µg/L)		
		3.7	14	43
Day 7	Developmental Stage	-	-	-
	Hind Limb Length (Normalized by Snout-Vent Length; SVL)	-	-	-
	Snout-Vent Length	-	-	↓
	Whole Body Wet Weight	-	-	↓
Termination	Survival	-	-	-
	Developmental Stage	-	-	↓**
	Hind Limb Length (Normalized by SVL)	-	-	↓
	Snout-Vent Length	*	-	↓
	Whole Body Wet Weight	-	-	↓
	Thyroid Gland Histology	NF	NF	NF

- Endpoint not impacted by the concentration of S-2399 compared to the control.
↓ Endpoint significantly reduced based on Jonckheere-Terpstra's Step-Down Test compared to the control median.**
↓ Endpoint significantly reduced based on Dunnett's Multiple Comparison Test compared to the control mean.
*** Endpoint significantly reduced based on Dunnett's Multiple Comparison Test compared to the control mean. However, due to the lack of statistical significance at the next higher treatment level (i.e., 14 µg/L), the significance detected at the 3.7 µg/L treatment level was determined to not be treatment related by the applicant.**
NF – No histological findings

Analytical results

A summary of the analytical results is presented in Table 9.2.4-24.

Table 9.2.4-24: Summary of analytical results

Nominal Concentration (µg/L)	Measured Concentration (µg/L)				Mean Measured Concentration (SD)[c]	Percent of Nominal (%)[c]	% CV[c]
	Day 0[a]	Day 7[b]	Day 14[a]	Day 21[b]			
Control	<0.59[d] <0.59	<0.59 <0.59	<0.59 <0.59	<0.59 <0.59	NA[e] (NA)	NA	NA
Mean	**NA**	**NA**	**NA**	**NA**			
4.1	3.7 3.7	3.1 3.0	3.9 4.2	4.1 4.1	3.7 (0.48)	91	13
Mean	**3.7**	**3.0**	**4.1**	**4.1**			
14	13 14	12 12	15 15	15 15	14 (1.3)	99	9.5
Mean	**14**	**12**	**15**	**15**			
45	42 42	37 36	46 46	49 48	43 (4.8)	96	11
Mean	**42**	**37**	**46**	**48**			

[a] Analytical samples were removed from replicates C and D of each treatment level and the control.

[b] Analytical samples were removed from replicates A and B of each treatment level and the control.

[c] Mean measured values, standard deviations (SD), percent of nominal, and % coefficient of variation (CV) were calculated using the actual (unrounded) analytical results and not the rounded values (two significant figures) presented in this table.

[d] Concentrations expressed as less than values were below the method detection limit (MDL). The MDL is dependent upon the lowest concentration calibration standard and the dilution factor of the controls (i.e., 0.0250 µg/L × 23.5 = 0.59 µg/L).

[e] NA = Not Applicable

Prior to the start of the definitive exposure, samples from alternating replicates of each treatment level and control solution were collected and analysed for S-2399 concentration. In addition, a sample of the saturator column stock solution was also analysed. Results of the pretest analyses were used to verify that sufficient quantities of S-2399 were being delivered and maintained in the exposure aquaria prior to test initiation. The diluter system,

which prepared and delivered the test solutions to the exposure aquaria functioned properly throughout the exposure, and all exposure solutions were observed to be clear and colourless. No undissolved test substance was observed in the diluter system.

Results of the S-2399 analyses demonstrated that the measured concentrations approximated nominal concentration, were relatively consistent for the duration of the exposure period and maintained the expected concentration gradient. Mean measured S-2399 concentrations ranged from 91 to 99% of nominal concentration and defined the treatment levels tested as 3.7, 14, and 43 µg/L. The coefficient of variance (% CV) for the measured concentrations ranged from 9.5 to 13%.

VALIDITY CRITERIA

The study fulfilled the validity and performance criteria of guideline OECD 231 (2009) as follows:

Table 9.2.4-25: Compliance with OECD 231 (2009) validity criteria in a test determined to be negative for thyroid activity

Criterion	Required	Obtained	Criterion Met (Yes/No)
Treatment/control mortality	For any given treatment (including controls), mortality should not exceed 10%. For any given replicate, mortality cannot exceed three tadpoles, otherwise the replicate is considered compromised	No mortality was observed in any of the treatment levels tested or the control	Yes
Treatment levels analysed	At least two treatment levels, with four uncompromised replicates, will be used for analysis	Three treatments levels with four uncompromised replicates.	Yes
Test concentrations (non-control) without overt toxicity	≥2	Overt toxicity was not observed in any of the treatment levels tested	Yes

Table 9.2.4-26: Compliance with OECD 231 (2009) performance criteria

Criterion	Required	Obtained	Criterion Met (Yes/No)
Test concentrations	Maintained at ≤ 20% CV over 21 days	9.5 - 13 %	Yes
Minimum median developmental stage of controls at end of test	57	57	Yes
Spread of development stage in control group	The 10th and the 90th percentile of the development stage distribution should not differ by more than 4 stages	57.3 – 60.1	Yes
Dissolved oxygen	≥ 40% air saturation	37 – 100 %	No
pH	6.5 – 8.5 The inter-replicate/inter-treatment differentials should not exceed 0.5	6.3 – 7.4	No
Water temperature	22 ± 1 ºC The inter-replicate/inter-treatment differentials should not exceed 0.5 ºC	20 – 22 ºC	No

CONCLUSION

The effects of S-2399 TG on the thyroid system of the African clawed frog (*Xenopus laevis*) were assessed in an amphibian metamorphosis assay over 21 days. Results are based on nominal test item concentrations. The absence of histopathologic effects in the current study is consistent with the developmental stage among frogs examined microscopically, including negative controls and those treated with S-2399. Although perturbation of the thyroid axis might be suggested by the relative decreases in normalised hind limb length and delayed developmental stage after 21 days in animals that received S-2399 when compared to controls, the negative histopathology results suggest that these effects were caused indirectly by other factors such as systemic toxicity, which was indicated by the reduction in

SVL and wet weight. Therefore, the applicant did not attribute these effects to thyroid mediated endocrine disruption. HSE has provided an overall conclusion on ED effects, including the AMA endpoints, within the ED assessment at the beginning of this section.

HSE COMMENTS
The study was carried out according to GLP and follows OECD 231 (2009). All four validity criteria were met.

The study conductor determined the maximum tolerated concentration (MTC) through a 96-h range finding experiment. From this, a LC_{50} = 0.14 mg/L was approximated. The MTC was estimated as a 1/3 of the LC_{50}, in line with OECD 231 (2009), which is 46.7 µg/L. The top concentration selected for the definitive assay was 45 µg/L, in line with the MTC.

Several minor deviations from OECD 231 (2009) were noted.

First, there were four protocol deviations identified by the study conductor:
On day 7 of the exposure, the daily temperature for replicate C of the 43 µg/L treatment level was recorded as 20 °C. Temperature readings for all other days and replicates were within 22 ± 1 °C range quoted in OECD 231 (2009). HSE considers this minor deviation from the specified range acceptable.

On test day 20, the dissolved oxygen in replicate D of the control was measured as 37.4%. Reduced water flow into the replicate was observed and the headboard siphon was adjusted to resolve the issue. Later that day the dissolved oxygen was re-measured and found to be > 50%. OECD 231 (2009) quotes an air saturation threshold of ≥ 40 %. There were no abnormal behavioural observations or increases in mortality, and growth/development was within expectations in that replicate suggesting that the temporary decrease in dissolved oxygen did not have lasting impacts. As this deviation lasted for less than a day and was accompanied by no observable impacts HSE considers it minor and acceptable.

On test day 15. The pH measured in the 3.7, 14 and 43 µg/L treatment levels was 6.4, 6.3, and 6.4, respectively. Also, the initial measured pH value in replicate D of the control on day 20 was 6.4. When this value was re-measured after correcting the water flow to the test aquaria (see previous dissolved oxygen deviation), the pH was 6.6. Given the very slight and temporary excursions from the pH range (6.5 - 8.5) in the protocol, HSE considers these deviations unlikely to have negatively impacted the study.

On test day 8, 16 tadpoles, instead of 15, were observed in the 43 µg/L replicate B aquarium. Immediately following the discovery of the loading error, one tadpole was indiscriminately selected and removed from the replicate of interest at the Study Director’s discretion. The growth and development of this replicate was comparable to the other replicates in the 43 µg/L treatment level. Empirically, none of the endpoints of interest (i.e., normalized hind limb

length, developmental stage, and wet weight) showed a difference from the other replicates in the treatment group at either day 7 or day 21. This suggests that the slightly increased density of one additional tadpole in the 43 µg/L replicate B aquarium for approximately one week of exposure did not substantially impact access to food or swimming space of the tadpoles. HSE considers this is a minor and acceptable study deviation.

Further to the four deviations identified in the study report, HSE notes the following minor deviations:

OECD 231 (2009) § 13 outlines the feeding regime through the pre-exposure and exposure periods. The guideline recommends Sera Micron® and provides the amount that should be provided to each tadpole a day throughout the experiment. The study selected a different food, [redacted], and reduced the feeding rates. This was based on prior experience of running AMA experiments at [redacted] with this food. Tadpoles reached stage 51 within 15 days, which is within the 17 days threshold mentioned in § 12 and 21 of the guideline, supporting the suitability of the feeding regime. HSE consider this an acceptable modification.

OECD 231 (2009) § 29 to 32 detail the selection process of tadpoles for histological assessment. It states "*animals selected for histopathology (n=5 from each replicate) should be matched to the median stage of the controls (pooled replicates) whenever possible*", "*if there are replicate tanks with less than five larvae at the appropriate stage, then randomly selected individuals from the next lower or upper developmental stage should be sampled to reach a total sample size of five larvae per replicate*", and "*in cases of severe alterations of tadpole development due to treatment with a test chemical, there might be no overlap of the stage distribution in the chemical treatments with the calculated control median developmental stage. In only these cases, the selection process should be modified by using a stage different from the control median stage to achieve a stage-matched sampling of larvae for thyroid histopathology*". According to the guideline, only if there is no overlap between the treatment levels and the median control growth stage should a growth stage other than the median control growth stage be selected. For every treatment replicate there was at least one tadpole of growth stage 59, the control median growth stage. Therefore, if the guideline were strictly followed, growth stage 59 individuals would have been selected where possible and the remaining individuals selected from growth stage 58 or 60. Instead, the study conductor decided to select tadpoles from growth stage 58 and, if five individuals in this growth stage were not available per replicate, growth stage 57. Although an explicit justification for this deviation was not provided, HSE assumes this approach was taken to reduce the overall spread of growth stages for tadpoles entering histological analysis (60 – 58 vs 58 – 57). If this were indeed the case, reducing the growth stage variation would have improved follicular cell height comparisons between treatment groups as it is dependent on growth stage. Conditional on this interpretation being correct, HSE considers this an acceptable deviation.

There were several reporting errors in the study report relating to statistical significance. The study report stated, "*the overall multi-quantal procedure determined a significant difference in day 21 percentile developmental stage at 43 µg/L for the 20th through the 80th distribution profiles compared to the control*". This was incorrect. Appendix 7, containing the raw statistical analysis, showed that only the 20th through to the 60th percentiles were significantly different from the control. The 70th to the 80th percentiles were not. The statistical analyses were also incorrectly presented in Table 6, with only the 20th and 30th percentiles indicated as statistically significant. Furthermore, the 20th and 60th percentiles of the 14 µg/L treatment level were also statistically significant, and this was not reported anywhere in the study report apart from Appendix 7. Regarding the 43 µg/L treatment level, the majority of the multi-quantal analyses do corroborate the overall finding that developmental stage is delayed. For the 14 µg/L treatment level, two of the seven percentile analyses were significant. This is evidence for delayed general toxicity at 14 µg/L.

Regarding the interpretation of the statistically significant reductions, HSE agrees with the decision to not categorise the statistically significant reduction in snout-vent length after 21 days in the 3.7 µg/L treatment level as treatment related. HSE agrees with the study coordinator's reasoning that there was no clear dose response due to the 14 µg/L treatment group not showing a statistically significant reduction (3.5 % reduction relative to the control).

Although not a deviation from the guideline, HSE notes that the decision logic flowchart in Figure 3 of OECD 231 (2009) was used to conclude that S-2399 has no thyroid activity. This was based on the lack of advanced development, asynchronous development and histological thyroid effects. These endpoints are weighed more heavily than delayed development, snout-vent length and wet body weight, which were statistically reduced/delayed by S-2399, because they can be affected by general toxicity. To further support this decision tree, OECD 231 (2009) states *"developmental retardation, in the absence of toxicity, is a strong indicator of anti-thyroid activity, but the developmental stage analysis is less sensitive and less diagnostic than the histopathological analysis of the thyroid gland".* There was also a reduction in normalised hind limb length. This scenario is not covered in OECD 231. Taken together, the presence of delayed development concurrent with reduced growth and unaltered thyroid histopathology suggests no thyroid activity for inpyrfluxam. This is expanded upon during the ED assessment.

The method of analysis used in the study was evaluated by HSE Chemistry. The conclusion of their evaluation is reproduced below. Please see Volume 3 CA, section B5 for more details.

"*The analytical method is not fully validated according to SANTE/2020/12830 rev. 1 for the determination of inpyrfluxam in aqueous solutions from the fish short-term reproduction assay with Fathead Minnow (Pimephales promelas) and the amphibian metamorphosis*

assay with African Clawed Frog (Xenopus laevis) studies due to the stability of standards and stock solutions has not being addressed. However, the studies were generated prior to the implementation of SANTE 2020/12830 rev.1 and SANCO 3029 rev.4 did not require stabilities of standards and extracts to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose."

The results of the study are considered suitable to inform the weight-of-evidence assessment and are discussed further in the T modality ED assessment.

B.9.2.5 Acute toxicity to aquatic invertebrates

B.9.2.5.1 Acute toxicity to *Daphnia magna*

Reference:	KCA 8.2.4.1/01
Report Title:	S-2399 TG – Acute Toxicity to Water Fleas (*Daphnia magna*) Under Static Conditions, Following OECD Guideline #202, OPPTS Draft Guideline 850.1010, JMAFF 12 NohSan, No. 8147 Daphnia Acute Immobilization Test (2-7-2-1) and The Official Journal of the European Communities L383A, Method C.2, Acute toxicity for Daphnids
Author(s) & year:	[redacted] (2014f)
Document No, Authority registration No:	Smithers Viscient Study No. 13048.6779
Substance used:	S-2399 TG, 13CG0617G, 95 %
Method of analysis:	LC/MS/MS
Guideline(s):	OECD 202 (2004)
Deviations:	Yes, see HSE comments section.
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test material:** S-2399 TG
   Description: Not stated
   Lot/Batch: 13CG0617G.

Expiration date: 23rd July 2016
Purity: 95.0%
Reference item: Potassium dichromate
Solvent: Acetone

B. STUDY DESIGN AND METHODS

1. Test animals: *Daphnia magna*
Age: < 24 hours at test initiation
Source: Smithers Viscient culture
Diet: *Ankistrodesmus falcatus* green algae and a suspension of YCT (yeast, cereal and flaked fish food). Daphnids were not fed over the duration of the study.
Acclimation conditions: None
Analysis of conc.: Yes. Exposure initiation and exposure termination. LOQ- 0.600 µg/L
Water sampling: Fortified well water
Exposure regime: Static
Concentrations tested: 0.094, 0.19, 0.38, 0.75, 1.5 and 3.0 mg/L
LOQ = 0.600 µg/L, MDL = 0.200 µg/L

2. Dilution water: Fortified well water
Hardness: 180 mg $CaCO_3$/L
Alkalinity: 88 mg $CaCO_3$/L
pH: 8.1
Specific conductivity: 680 mS/cm

3. Test vessels: Beakers containing 200 mL of solution

4. Environmental conditions :

A summary of the environmental conditions obtained in this study is shown in Table 9.2.5-1 below, with values compared to the required conditions as per OECD 202 (2004).

Table 9.2.5-1: Environmental conditions obtained in study of acute toxicity to *Daphnia magna* under static conditions

Variable	Required OECD 202 (2004)	Obtained
Temperature	range of 18˚C and 22˚C	19˚C - 21°C
pH	6-9	8.2 - 8.3
Dissolved oxygen concentration	≥ 3 mg/L	8.5 - 9.4 mg/L
Photoperiod	16-hour light and 8-hour dark cycle is recommended	16 hours light: 8 hours darkness
Lighting intensity	30 to 100 footcandles	27 to 130 footcandles

Hardness of dilution water	Between 140 and 250 mg/l	180 mg $CaCO_3$/L
Alkalinity of dilution water	Not stated	88mg $CaCO_3$/L
Conductivity of dilution water	Not stated	680 mS/cm

Study test dates: 7th to 9th January 2014

5. Animal assignment and treatment:
Daphnids, < 24 hours old at test initiation, were impartially selected and distributed so each beaker contained five daphnids (four replicates per treatment). Daphnids were exposed to six concentrations of S-2399 TG (0.094, 0.19, 0.38, 0.75, 1.5 and 3.0 mg a.s./L) in addition to a dilution water control and solvent control. The exposure was initiated when each beaker of daphnids was added to each respective test vessel. The exposure duration was 48 hours. Daphnids were not fed during exposure.

A reference test was conducted prior to the start of the definitive phase, using potassium dichromate as the toxicant to evaluate the sensitivity of *Daphnia magna*.

6. Dose preparation:
A 30 mg a.s./mL primary stock solution was prepared by placing 0.7881g (0.7487 g a.s.) of S-2399 TG in a volumetric flask and bringing it to volume with 25 mL of acetone. This solution was clear and colourless. The primary stock solution was used to prepare secondary stock solutions which were then used to prepare the exposure solutions (0.094, 0.19, 0.38, 0.75, 1.5 and 3.0 mg a.s./L). Exposure solutions were mixed thoroughly using a glass rod for approximately 1 minute. Following mixing, all solutions were clear and colourless. A solvent control was prepared by bringing 0.10 mL acetone to a final volume of 1.0 mL with dilution water. A control solution containing dilution water only was also established. Each test solution was divided into four replicate vessels, each containing approximately 200 mL of solution.

7. Measurements and observations:
The number of immobilised daphnids in each replicate test vessel was recorded at 24 and 48 hours of exposure. Immobilisation was defined as those animals not able to swim within 15 seconds after gentle agitation of the test vessel. Biological observations and observations of the physical characteristics of each replicate test solution were also made and recorded at 0, 24 and 48 hours. The pH, dissolved oxygen concentration and temperature were measured at 0, 24 and 48 hours in a single replicate of each treatment level and control. Continuous temperature monitoring was performed in a satellite vessel within the test chamber.

At test initiation and test termination, one sample was removed from the exposure solutions and controls for analysis of test substance concentrations. At exposure termination, samples were removed from a composite of the replicates of each concentration prior to analysis. All samples were analysed by LC/MS/MS and mean measured concentrations (reported as “time-weighted average concentrations”) were calculated. The LOQ was set at 0.600 µg/L and the MDL was 0.200 µg/L.

8. Statistics:

The EC_{50} values and 95% confidence limits for immobilisation were estimated with the computer program CETIS™v.1.8, using Spearman-Karber estimates, linear, and non-linear regression. The No-Observed-Effect Concentration (NOEC) was defined as the highest mean measured concentration tested at and below which there were no toxicant-related immobilisation or physical and behavioural abnormalities (e.g. lethargy), with respect to the control organisms.

II. RESULTS AND DISCUSSION

A. BIOLOGICAL EFFECTS

Following 48 hours of exposure, immobilisation of 20, 95 and 100% was observed in the 0.85, 1.7 and 3.4 mg a.s./L treatment levels, respectively. Thirteen daphnids exposed to the 0.85 mg a.s./L treatment level were observed to be lethargic. No immobilisation or adverse effects were observed among daphnids exposed to the remaining levels tested (0.096, 0.20 and 0.41 mg a.s./L) or the controls. A summary of the cumulative percent of immobilised daphnids is presented in Table 9.2.5-2.

Table 9.2.5-2: Summary of cumulative percent of immobilised organisms

Mean measured concentration (mg a.s./L)	Cumulative percent of immobilised organisms	
	24 hour	48 hours
Control	0	0
Solvent control	0	0
0.096	0	0
0.20	0	0
0.41	0	0
0.85	0[a]	20[b]
1.7	35[c]	95[c]
3.4	45[c]	100

[a] Seventeen daphnids were observed to be lethargic
[b] Thirteen daphnids were observed to be lethargic
[c] All surviving daphnids were observed to be lethargic

The concentration-response (immobilisation) curve for Daphnia magna exposed to S-2399 is shown below.

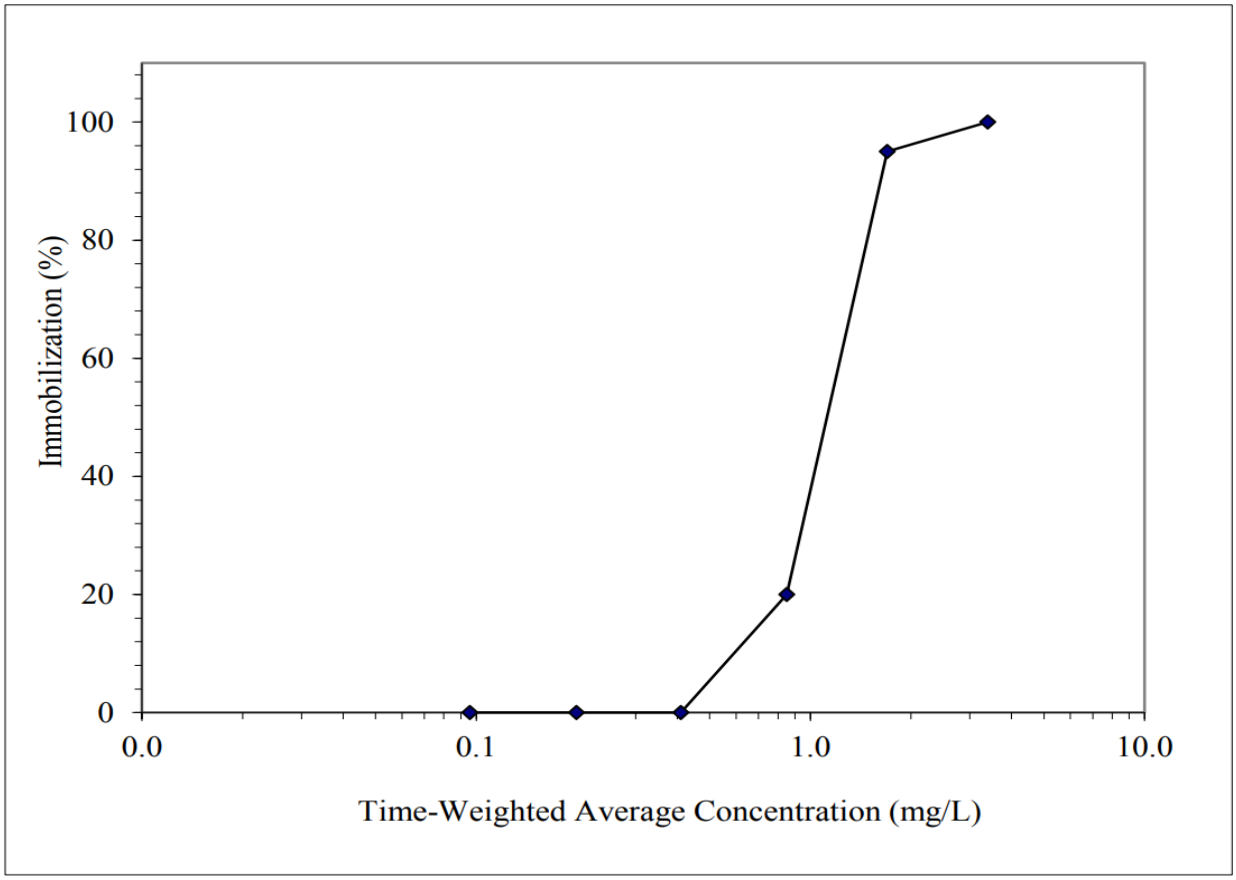


Figure 9.2-16: Concentration-response (immobilisation) curve for daphnids (*Daphnia magna*) exposed to S-2399 for 48 hours

The 48-hour EC_{50} value for S-2399 TG and *Daphnia magna* was estimated to be 1.1 mg a.s./L, with 95% confidence levels of 0.93 to 1.2 mg a.s./L. The No-Observed-Effect Concentration (NOEC) was determined to be 0.41 mg a.s./L. A summary of the endpoints is presented in Table 9.2.5-3.

Table 9.2.5-3: Summary of endpoints (based on mean measured concentrations)

Time scale	EC_{50} (mg a.s./L)	95% Confidence intervals	
		Lower (mg a.s./L)	Upper (mg a.s./L)
24 Hours	>3.4	n.a.	n.a.
48 Hours [a]	1.1	0.93	1.2
48-hour NOEC = 0.41 mg a.s./L			
Highest concentration producing 0% immobilisation = 0.41 mg a.s./L			

Time scale	EC_{50} (mg a.s./L)	95% Confidence intervals	
		Lower (mg a.s./L)	Upper (mg a.s./L)
Lowest concentration producing 100% immobilisation = 3.4 mg a.s./L			

n.a. = not applicable. EC_{50} value was empirically estimated; therefore, 95% confidence intervals could not be calculated

[a] EC_{50} value determined by Spearman-Kärber Estimates

B. ANALYSIS

Results of the analyses established that the measured concentrations were generally consistent between sampling intervals and maintained the expected concentration gradient. The mean measured concentrations ranged from 100 to 120% of nominal concentration and defined the treatment levels as 0.096, 0.20, 0.41, 1.7 and 3.4 mg a.s./L. (Table 9.2.5-4)

Table 9.2.5-4: Measured concentrations of S-2399 in the exposure solutions

Nominal concentration (mg a.s./L)	Measured concentration (mg a.s./L)			Percent of nominal [a] (%)
	0 hour	48 hours	Mean measured average [a]	
Control	< 0.0076 [b]	< 0.0076 [b]	n.a.	n.a.
Solvent control	< 0.0076 [b]	< 0.0076 [b]	n.a.	n.a.
0.094	0.098	0.095	0.096	100
0.19	0.20	0.20	0.20	110
0.38	0.41	0.41	0.41	110
0.75	0.86	0.84	0.85	110
1.5	1.7	1.7	1.7	110
3.0	3.5	3.4	3.4	120

[a] Mean measured concentration (referred to as " time-weighted average concentrations" in the report) and percent of nominal were calculated using actual analytical data and not the rounded (2 significant figures) data presented in this table. Standard deviations could not be calculated for time-weighted averages.

[b] Concentrations expressed as less than values were below the minimum detectable limit (MDL).

n.a. not applicable

C. VALIDITY CRITERIA

Table 9.2.5-5 below shows the validity requirements as per OECD 202 (2004) and the values obtained in the study.

Table 9.2.5-5: Validity criteria for this study

Validity criteria	Requirements OECD 202 (2004)	Obtained
Control immobilisation	< 10% immobilisation	No immobilisation in control or solubilising agent control
Dissolved oxygen concentration	≥ 3mg/L in control and test vessels	8.5 - 9.4 mg/L

The 24-hour reference test established an EC_{50} of 2.4 mg a.s./L, which was within the expected range for *D. magna* exposed to potassium dichromate (2.3 ± 0.24 mg a.s./l, historically) and demonstrates that the test conditions were reliable.

CONCLUSION
The 48-hour EC_{50} value for S-2399 TG and *Daphnia magna* was calculated to be 1.1 mg a.s./L, with 95% confidence levels of 0.93 to 1.2 mg a.s./L. The No-Observed-Effect Concentration (NOEC) was determined to be 0.41 mg a.s./L. All endpoints have been based on mean measured concentrations.

HSE COMMENTS:
This study was conducted under GLP and OECD 202 (2004) and has been assessed against OECD 202 (2004) guidelines.

The concentrations of the test item were maintained between 80-120% of the nominal value throughout the test. Results should be based on nominal concentrations but have been expressed by the applicant as mean measured concentrations.

The method of analysis used in the study was evaluated by HSE Chemistry. The conclusion of their evaluation is reproduced below. Please see Volume 3 CA, section B5 for more details.

"*The analytical method is not fully validated according to SANTE/2020/12830 rev. 1 for the determination of the active substance inpyrfluxam in freshwater as the matrix effects have not been determined and the stability of standards and extracts has not been addressed. However, the studies were generated prior Ito the implementation of SANTE 2020/12830 rev.1 and SANCO 3029 rev.4 did not require matrix effects and the stabilities of standards and extracts to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose.*"

The only protocol deviation to note for this study is the light intensity, which ranged both above and below the recommended values. Guidelines allow for light intensity from 30 – 100 footcandles, but this study obtained 27 – 130 footcandles. HSE do not consider this to have a significant impact on the results as test concentrations were maintained between 80-120% of the nominal value, no abnormal behaviour or mortality was recorded for the control groups, and there were no anomalous results throughout the exposure data. All validity criteria for this study were met; therefore, the study remains valid.

There was no acclimation period for this study, but the values used in the culture medium were similar to the values used in the definitive exposure study. This therefore meets the requirements of OECD 202 (2004).

Total organic carbon (TOC) levels in the well water of this study met the requirements in OECD 202 (2004) guidelines. No data are available for other parameters in annex 2 of OECD 202 (2004), but the study states that representative samples of the dilution water source were analysed periodically for the presence of pesticides, PCBs and toxic metals. None of these compounds have been detected at concentrations considered toxic in any of the water samples analysed.

The EC_{50} values and 95% confidence limits for immobilisation were estimated with the computer program CETIS™v.1.8, using Spearman-Karber estimates, linear, and non-linear regression. These are suitable statistical methods for the data obtained in the study as more than one study recorded partial immobilisation.

The endpoints for use in risk assessment are:

- **EC_{50} value = 1.1 mg a.s./L (based on mean measured concentrations)**

B.9.2.5.2 Acute toxicity to an additional aquatic invertebrate species

Reference:	KCA 8.2.4.2/01
Report Title:	S-2399 TG: Acute Toxicity to Mysids (*Americamysis bahia*) Under Static Conditions, Following OCSPP Draft Guideline 850.1035
Author(s) & year:	[redacted] (2014g)
Document No, Authority registration No:	Smithers Viscient Study No. 12709.6361
Substance used:	S-2399 TG, 13CG0617G, 95 %
Method of analysis:	LC/MS/MS
Guideline(s):	OCSPP Draft Guideline 850.1035 (1996)
Deviations:	Yes, see HSE comments section.
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: S-2399 TG

Description: Not stated
Lot/Batch: 13CG0617G
Expiry date: 23 July 2016
Purity: 95.0%
Reference item: None
Solvent carrier: Acetone

B. STUDY DESIGN AND METHODS

1. Test animals: *Americamysis bahia*
Age: ≤ 24 hours old at test initiation
Source: In-house culture
Diet: Live brine shrimp (*Artemia salina*) nauplii, once daily during the exposure period.
Analysis of conc.: At exposure initiation and exposure termination
LOQ was set at 0.600 µg/L. MDL was 0.200 µg/L for S 2399
Concentrations used: Nominal concentrations of 0.19, 0.38, 0.75, 1.5 and 3.0 mg/L
Mean measured concentrations: 0.18, 0.36, 0.68, 1.4 and 2.9 mg/L
Acclimation: None

2. Dilution water: Natural filtered seawater
pH: 7.9
Salinity: 20 – 21‰

3. Test vessels: 1.0 L glass beakers, each containing 0.90 L of test solution
Exposure regime: Static

4. Environmental conditions:
Temperature: 25°C
pH: 7.5 – 8.0
Dissolved oxygen: 3.8 – 7.4 mg/L
Salinity: 20 – 21‰
Photoperiod: 16 hours light: 8 hours darkness (42 to 50 footcandles)

5. Animal assignment and treatment:
Mysids, ≤ 24 hours old at test initiation, were impartially selected and distributed to each respective test vessel (ten mysids per replicate vessel, 20 per treatment level and control), for the control, solvent control, and treatment groups. The nominal test concentrations were 0.19, 0.38, 0.75, 1.5 and 3.0 mg a.s./L. The exposure duration was 96 hours. Mysids were fed once daily during the exposure period.

A summary of the environmental conditions obtained in this study against the required values outlined in OCSPP 850.1035 (2016) is shown in Table 9.2.5-6 below.

Table 9.2.5-6: Environmental conditions obtained in S-2399 TG: Acute Toxicity to Mysids study

Variable	Required (OCSPP 850.1035, 2016)	Obtained
Temperature	25 °C	25 ˚C
pH	7.5 - 8.5	7.5-8.0
Dissolved oxygen concentration	Between 60 and 100% saturation	3.8-7.4[a]
Photoperiod	16 hours light:8 hours dark	16 hours light, 8 hours dark
Lighting Intensity	50-100 footcandles	42 to 50 footcandles
Salinity of dilution water	20 ppt (constant within ±2 ppt during the test.)	20-21%
Conductivity of dilution water	Not stated	Not stated
Total Organic Carbon	≤ 2 mg/L	0.99 mg/L

[a] 60% of saturation value at a temperature of 25 ºC with a salinity of 20 to 21‰ is 4.4 mg/L. 105% of saturation value at a temperature of 25 ºC with a salinity of 20 to 21‰ is 7.7 mg/L

Study dates: 16th to 20th May 2014

6. Dose preparation:

A 30 mg a.s./mL primary stock solution was prepared by adding 0.7824 g of S-2399 TG (0.7433 g as active substance) to 25-mL of acetone and mixing by inversion. The resulting stock solution was observed to be clear and colourless with no visible undissolved test substance. The primary stock solution was used to prepare the secondary stock solution, diluted in acetone. The resulting secondary stock solutions were observed to be clear and colourless with no visible undissolved test substance following mixing by inversion. Both primary and secondary stock solutions were used to prepare the exposure solutions. A solvent control solution was prepared by bringing 0.20 mL of acetone to a final volume of 2.0 L with dilution water. A control solution was also established, containing dilution water only.

Exposure solutions were mixed with a glass rod for approximately one minute prior to division into replicate exposure vessels. Following mixing, the test solutions were observed to be clear and colourless with no visible undissolved test substance.

7. Measurements and observations:

Biological observations and observations of the physical characteristics of the test solutions (e.g., precipitate, film on solution's surface) were recorded at exposure initiation and at each subsequent 24-hour interval until exposure termination (96 hours). Mortality (absence of mobility and reaction to gentle prodding) was recorded at 0, 24, 48, 72 and 96 hours of exposure.

The pH, dissolved oxygen concentration, temperature and salinity were measured daily in each replicate of the treatment levels and the controls. Continuous temperature monitoring was conducted in one replicate in one treatment level only.

One sample was removed from the exposure and control solutions at exposure initiation and termination for analysis of S-2399 TG concentration. At exposure initiation, samples were removed from the intermediate mixing vessel, prior to division into replicate vessels. At exposure termination, samples were removed from the approximate mid-point of the vessels by pipette. All samples were analysed for S-2399 TG using LC/MS/MS. LOQ was set at 0.600 µg/L and the MDL was 0.200 µg/L for S-2399.

8. Statistics:

The 24-, 48-, 72- and 96-hour median lethal concentrations (LC_{50}) were either determined by the computer program CETIS™ v 1.8, if at least one concentration resulted in ≥ 50% mortality, or they were empirically estimated, if no concentration resulted in ≥ 50% mortality. The No-Observed-Effect Concentration (NOEC) was defined as the highest concentration tested at and below which there were no toxicant-related mortality or physical and behavioural abnormalities (e.g., lethargy, loss of equilibrium), with respect to the control organisms. Spearman-Kärber was the statistical method used in this study.

II. RESULTS AND DISCUSSION

A. BIOLOGICAL EFFECTS

At 48 hours 100% mortality was observed in the 2.9 mg a.s./L treatment group. Surviving mysids in the 1.4 and 2.9 mg a.s./L treatment groups were observed to be lethargic after 48 and 24 hours, respectively. A summary of the cumulative percent mortality and observations is presented in Table 9.2.5-7.

Table 9.2.5-7: Summary of cumulative percent of mortality

Mean measured concentration (mg a.s./L)	Mean cumulative percent mortality (%)[a]			
	24 hour	48 hours	72 hours	96 hours
Control	0	0	0	5
Solvent control	0	0	0	0
0.18	0	0	0	0
0.36	0	0	0	0
0.68	0	0	0	0
1.4	5	75[b]	80[b]	80[b]
2.9	35[b]	100	100	100

[b] All surviving mysids were observed to be lethargic

The figure below shows the concentration-response (mortality) curve for mysids (*Americamysis bahia*) exposed to S-2399 TG under static conditions

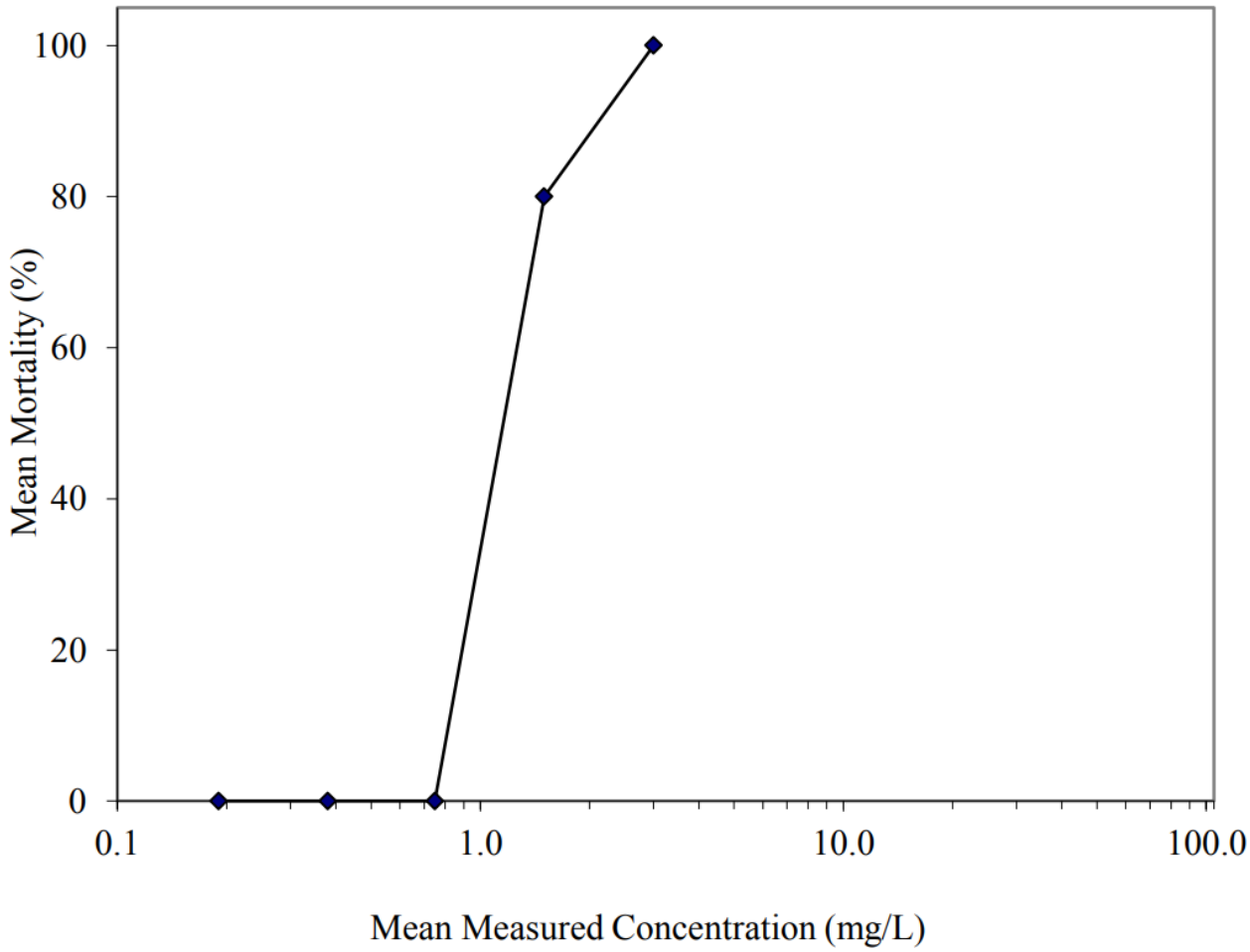


Figure 9.2-17: Concentration-response (mortality) curve for mysids (*Americamysis bahia*) exposed to S-2399TG under static conditions after 96 hours

Based on mean measured concentrations, the 96-hour LC_{50} for S-2399 TG and *Americamysis bahia* was determined to be 1.1 mg a.s./L, with 95% confidence intervals of 0.99 to 1.3 mg a.s./L. The NOEC was determined to be 0.68 mg a.s./L. A summary of the endpoints is presented in Table 9.2.5-8.

Table 9.2.5-8: Summary of endpoints (based on mean measured concentrations)

Time scale	LC_{50} (mg a.s./L)	95% Confidence intervals	
		Lower (mg a.s./L)	Upper (mg a.s./L)
24 Hour	> 2.9[a]	n.a.	n.a.
48 Hour [b]	1.2	1.0	1.3
72 Hour [b]	1.1	0.99	1.3
96 Hour [b]	1.1	0.99	1.3

Time scale	LC_{50} (mg a.s./L)	95% Confidence intervals	
		Lower (mg a.s./L)	Upper (mg a.s./L)
96 hour NOEC = 0.68 mg a.s./L			
Highest concentration producing 0% mortality = 0.68 mg a.s./L			
Lowest concentration producing 100% mortality = 2.9 mg a.s./L			

[a] LC_{50} value was empirically estimated, therefore, corresponding 95% confidence intervals could not be determined

[b] LC_{50} value and corresponding 95% confidence intervals were determined by Spearman-Kärber estimates

n.a. = not applicable.

B. ANALYSIS

Measured concentrations of S-2399 TG were between 91 and 96% of nominal. Concentrations were generally consistent between sampling intervals, maintained the expected concentration gradient and closely approximated nominal concentrations. Mean measured concentrations defined the treatment levels as 0.18, 0.36, 0.68, 1.4 and 2.9 mg a.s./L (Table 9.2.5-9).

Table 9.2.5-9: Measured concentrations of S-2399 TG in the exposure solutions

Nominal concentration (mg a.s./L)	Measured concentration (mg a.s./L)			Percent of nominal (%)
	0 hour	96 hours	Mean (SD)	
Control	< 0.015[a]	< 0.015 [a]	n.a.	n.a.
Solvent control	< 0.015 [a]	< 0.015 [a]	n.a.	n.a.
0.19	0.17	0.18	0.18 (0.0037)	93
0.38	0.36	0.36	0.36 (0.000042)	94
0.75	0.71	0.65	0.68 (0.047)	91
1.5	1.4	1.4	1.4 (0.042)	92
3.0	2.7	3.0	2.9 (0.21)	96

[a] Concentrations expressed as less than values were below the minimum detectable limit (MDL)

n.a. = not applicable

C. VALIDITY CRITERIA

As mortality was less than 10% in the control organisms (actual: 5%), the test was considered valid.

The validity criteria required in OCSPP 850.1035, 2016 and the values obtained in the study are shown in Table 9.2.5-10.

Table 9.2.5-10: Validity criteria

Criteria	Required (OCSPP 850.1035, 2016)	Obtained
Test chambers	All test vessels (and retention chambers) were identical	All vessels identical
Treatments	Treatments were randomly or indiscriminately assigned to individual test vessel locations, or individual test organisms were randomly or indiscriminately assigned to test vessels	Treatments and organisms randomly assigned to test vessels
Controls	A dilution water control (and vehicle (solvent) control, if a vehicle was used) was included in the test.	Dilution water control and solvent control
Mortality in controls	Less than 10% of the organisms in either the dilution water or vehicle (solvent) controls showed signs of disease, stress (e.g., discoloration, unusual behaviour, immobilization), and/or death	Less than 10% Control: 5% Solvent control: 0%
Surfactants	A surfactant or dispersant was not used in the preparation of a stock or test solution	None used

III. CONCLUSION

Based on mean measured concentrations, the 96-hour LC_{50} for mysids (*Americamysis bahia*) exposed to S-2399 TG was determined to be 1.1 mg a.s./L with 95% confidence intervals of 0.99 to 1.3 mg a.s./L. The No-Observed-Effect Concentration (NOEC) was determined to be 0.68 mg a.s./L.

HSE COMMENTS:

This study was done under GLP and under OPPTS 850.1035 (1996) guidelines. It has been assessed against OCSPP 850.1035 (2016).

The concentrations of the test item were maintained between 80-100% of the nominal value throughout the test.

The method of analysis used in the study was evaluated by HSE Chemistry. The conclusion of their evaluation is reproduced below. Please see Volume 3 CA, section B5 for more details.

"*The analytical method is not fully validated according to SANTE/2020/12830 rev. 1 for the determination of the active substance inpyrfluxam in freshwater as the matrix effects have not been determined and the stability of standards and extracts has not been addressed. However, the studies were generated prior Ito the implementation of SANTE 2020/12830 rev.1 and SANCO 3029 rev.4 did not require matrix effects and the stabilities of standards and extracts to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose.*"

There are several deviations to protocol to note in this study. The first one to note is the drop in dissolved oxygen concentrations below 60% (at the 48-hour interval) in replicate B of the 3.0 mg/L (nominal) test solution. As 100% mortality was recorded at this concentration in both replicates, and lower concentrations were already recording high levels of mortality, mortalities in this replicate are not attributed to low dissolved oxygen concentrations by the regulator.

The total organic carbon (TOC) in this study is within OCSPP 850.1035 (2016) guidelines and the filter size used in this study was correct.

The light intensities obtained in this study also differ from OCSPP 850.1035 (2016) guidance. The guidelines suggest light intensities of 50-100 footcandles, but this study obtained values of 42-50 footcandles. There is no value given in OCSPP 850.1035 (1996) on suitable light intensities. However, given low percentage mortalities and observed effects in the controls and lowest 3 concentrations, this is not considered to have impacted the results of the study.

No acclimation period was conducted prior to definitive exposure, but as the 14 day culture conditions were similar to the conditions in definitive exposure; this meets the study requirements.

No reference item was used for this study, so species sensitivity is uncertain. However, given the low mortality in controls and lowest concentration values, this is unlikely to have affected the validity of the study.

The statistics used in this study are suitable for the data as at least one group recorded ≥ 50% mortality. No specific guidance on statistics are given in OPPTS 850.1035 (1996) and (2016).

The endpoints for use in risk assessment are:

- **96 hour LC_{50} = 1.1 mg a.s./L (based on mean measured concentrations)**

B.9.2.6 Long-term and chronic toxicity to aquatic invertebrates

B.9.2.6.1 Reproductive and development toxicity to *Daphnia magna*

Reference:	KCA 8.2.5.1/01
Report Title:	S-2399 TG - Full Life-Cycle Toxicity Test with Water Fleas, Daphnia magna, Under Static-Renewal Conditions, Following OECD Guideline #211 and OPPTS Draft Guideline 850.1300, JMAFF 12 NohSan, No. 8147 Daphnia Reproduction Test (2-7-2-3) and The Official Journal of the European Communities L383A, Method C.20, Daphnia magna Reproduction Test
Author(s) & year:	[redacted] (2014h)
Document No, Authority registration No:	Smithers Viscient Study No. 13048.6780
Substance used:	S-2399 TG, 13CG0617G, 95 %
Method of analysis:	LC/MS/MS
Guideline(s):	OECD 211 (2012)
Deviations:	Yes, see HSE comments section.
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: S-2399 TG

Description: Not stated
Lot/Batch: 13CG0617G
Expiration date: 23 July 2016
Purity: 95.0%
Reference item: None
Solvent: Acetone

B. STUDY DESIGN AND METHODS

1. Test animals: *Daphnia magna*
Age: < 24 hours old
Source: Smithers Viscient culture
Diet: Not fed during exposure

2. Dilution water: Fortified well water

Hardness: 160 - 180 mg $CaCO_3$/L
Alkalinity: 88 - 90 mg $CaCO_3$/L
pH: 8.0 - 8.1
Conductivity: 530 - 680 mS/cm

Test vessels: 100 mL clear glass beakers, each containing 80 mL of test solution
Animals per vessel: 10 individually held replicates per control and concentration
Exposure regime: Semi-static
Duration: 21 days
Analysis of conc.: 0.031, 0.063, 0.13, 0.25 and 0.50 mg/L. LOQ = 0.600 µg/L

4. Environmental conditions:

A summary of environmental conditions is shown in Table 9.2.6-1 below:

Table 9.2.6-1: A summary of environmental conditions

Variable	Required OECD 211 (2012)	Obtained
Temperature	18-22°C	19-21˚C
pH	6 - 9	7.7 – 7.8
Dissolved oxygen concentration	Above 3 mg/l	5.2 – 11 mg/L
Photoperiod	16 hours light	16 hours daylight, 8 hours darkness
Lighting intensity	Not exceeding 15-20 µE m^{-2} s^{-1}	2.0 to 14 µE m^{-2} s^{-1}
Hardness of dilution water	Hardness above 140 mg/L	160 - 180 mg $CaCO_3$/L
Alkalinity of dilution water	Not stated	88 - 90 mg $CaCO_3$/L
Conductivity of dilution water	Not stated	530 - 680 µS/cm

Study dates: 8 to 29 January 2014

5. Animal assignment and treatment:

Individual daphnids, < 24 hours old at test initiation, were transferred from an impartially selected intermediate beaker of ten daphnids to each test vessel. During the exposure, the food was introduced daily at a rate of 200µL of algal suspension, and 50 µL of YCT suspension. Fresh exposure solutions were prepared at exposure initiation and at 48- to 72-hour intervals thereafter. On renewal days, daphnids were carefully transferred from the aged solution into the freshly prepared test solutions and the food solutions were then added. The exposure duration was 21 days.

6. Dose preparation:
A 30 mg a.s./mL primary stock solution was prepared by placing 0.7881g (0.7487 g a.s.) of S-2399 in a volumetric flask and bringing it to volume with 25 mL of acetone. This solution was clear and colourless. The primary stock solution was used to prepare secondary stock solutions which were then used to prepare the exposure solutions, at exposure initiation and at each renewal interval (every 48 or 72 hours). Exposure solutions were mixed thoroughly using a glass rod for approximately 1 minute. Following mixing, all solutions were clear and colourless. A solvent control was prepared by bringing 0.15 mL acetone to a final volume of 1.5 mL with dilution water. A control solution containing dilution water only was also established. Each test solution was divided into ten replicate vessels, each containing approximately 80 mL of solution.

7. Measurements and observations:
The number of immobilised daphnids and observations of abnormal behaviour were recorded daily. Immobilisation was defined as those animals not able to swim within 15 seconds after gentle agitation of the test vessel. Numbers of offspring were determined upon the first brood release in any vessel and daily throughout the remainder of the test. Offspring were removed, counted and discarded at each observation interval. In addition, immobilised offspring and the time to first brood release were recorded for each treatment level and controls. Observations of the physical characteristics of test solutions were recorded, if applicable, whenever the test organisms were observed.

At exposure termination (day 21), the total body length of each surviving adult daphnid was measured, as well as the dry weight. Daphnids were measured from the apex of the head to the base of the carapace spine. They were also dried at approximately 100^0C for 24 hours to determine the dry weight.

The pH, dissolved oxygen concentration and temperature were measured in each test item and control solution at the beginning (new solutions) and end (aged solutions) of each renewal period. Aged samples consisted of a composite sample of all ten replicates from each treatment and control solution. Temperature in the environmental chamber was continuously monitored throughout the study. Total hardness, alkalinity and conductivity were measured and recorded in the freshly prepared solutions of the highest nominal test concentration (0.50 mg/L) and the control at exposure initiation and weekly thereafter. On day 21 (exposure termination), all measurements were taken from aged solutions.

A sample of newly prepared test solutions (days 0, 2, 16 and 19) was removed from each treatment level, control and solvent control prior to division into replicate test vessels. Aged (48 or 72 hours old) test solutions were also sampled and analysed on test days 2, 5, 19 and 21. Samples from aged test solutions were removed from a composite of all available vessels. The mean measured concentrations (reported as “time-weighted average concentrations”) of S-2399 TG were calculated for each treatment level. LOQ was set at 0.600 µg/L. MDL was 0.200 µg/L.

8. Statistics:
Fisher’s Exact Test was conducted to statistically compare control to the solvent control survival data. An equal Variance Two Sample t-Test was conducted to statistically compare control to the solvent control reproduction and growth data. Cochran-Armitage Trend Step-

Down Test was used to determine significant differences in the percent survival. Shapiro-Wilk's Test for normality was used to compare the observed sample distribution with a normal distribution for reproduction and growth data. Bartlett's Test was used to check on the assumption of homogeneity of variance on reproduction and growth data. As reproduction and growth data met the assumptions for normal distribution and homogeneity of variance, data was evaluated using Dunnett's Multiple Comparison Test. CETIS™ v. 1.8 was used for statistical computations, including calculation of EC values and 95% confidence intervals. EC values were determined by Spearman Kärber, liner or non-linear regression.

II. RESULTS AND DISCUSSION

A. BIOLOGICAL EFFECTS

After 21 days of exposure, survival among the dilution water control and solvent control organisms was 100%. The mean number of offspring released per female organism in the dilution water control and solvent control during the 21-day test was 167 and 172 offspring per female, respectively. There was no statistically significant difference between the control and solvent control survival data, therefore the treatment data for survival was compared to the pooled control data.

There was a statistically significant difference between the control and solvent control data. Therefore, the treatment data for total body length was compared to the solvent control data. A significant reduction in total body length was found in daphnids exposed to the 0.54 mg a.s./L treatment level compared to the solvent control data.

First brood release occurred on test day 8 in the controls and all treatments levels tested, with the exception of the 0.27 mg a.s./L treatment level, where first brood release occurred on day 11 and the 0.54 mg a.s./L treatment level, where no offspring were released during the exposure period.

A summary of the results is presented in Table 9.2.6-2.

Table 9.2.6-2: Summary of effects on survival, reproduction, total body length and dry weight of parental *Daphnia* during the 21-day static-renewal exposure to S-2399 TG

Mean measured concentration (mg a.s./L)	Mean percent survival (%)	Mean cumulative number of offspring produced per female daphnid (± SD)	Mean (SD) total body length (mm)	Mean (SD) dry weight (mg)
Control	100	167 (18)	4.91 (0.19)	0.98 (0.11)
Solvent control	100	172 (10)	5.06 (0.10)	1.09 (0.12)
Pooled controls	100	170 (14)	n.a.	1.04 (0.13)
0.031	80	182 (11)	5.00 (0.12)	1.03 (0.15)

Mean measured concentration (mg a.s./L)	Mean percent survival (%)	Mean cumulative number of offspring produced per female daphnid (± SD)	Mean (SD) total body length (mm)	Mean (SD) dry weight (mg)
0.065	100	175 (17)	4.99 (0.19)	1.01 (0.09)
0.13	100	181 (11)	4.97 (0.10)	1.08 (0.08)
0.27	90	146* (19)	4.99 (0.16)	1.21 (0.12)
0.54	80	0* (0)	4.45** (0.20)	0.68* (0.12)

SD =standard deviation
n.a=not applicable
* Significantly reduced compared to the pooled control, based on Dunnett's Multiple Comparison Test
** Significantly reduced compared to the solvent control, based on Dunnett's Multiple Comparison Test

Based on reproduction as the most sensitive indictor of toxicity, the 21-day NOEC for S-2399 and *Daphnia was* determined to be 0.13 mg a.s./L. A summary of endpoints are presented in Table 9.2.6-3. The MATC is equal to the geometric mean of the limits set by the lowest test concentration that elicited a statistically significant effect on organism performance (LOEC) and the highest test concentration that elicited no statistically significant difference between the exposed organisms and the appropriate control (NOEC).

Table 9.2.6-3: Summary of endpoints following exposure to S-2399 TG

	NOEC (mg a.s./L)	LOEC (mg a.s./L)	MATC (mg a.s./L)	mg a.s./L (95% Confidence Intervals)		
				EC_{10}	EC_{20}	EC_{50}
21-Day Survival	0.54	>0.54	-- [a]	0.27a (0.023 - n.d.)	0.54 (0.23 - n.d.)	>0.54 (n.a.)
21-Day Reproduction	0.13	0.27	0.19	0.21 (0.18 - 0.27)	0.28 (0.24 - 0.30)	0.37 (0.36 - 0.38)
21-Day Total Body Length	0.27	0.54	0.38	0.49 (0.43 - n.d.)	>0.54 (n.a.)	>0.54 (n.a.)
21-Day Dry Weight	0.27	0.54	0.38	0.34 (0.32 - 0.36)	0.42 (0.39 - 0.47)	>0.54 (n.a.)

[a] MATC (Maximum-Acceptable Toxicant Concentration) could not be determined for this endpoint
n.d.= not determined

n.a.= not applicable

B. ANALYSIS

Results of the analyses established that the measured concentrations were generally consistent between sampling intervals and maintained the expected concentration gradient. The mean measured concentrations ranged from 100 to 110% of nominal concentration and defined the treatment levels as 0.031, 0.065, 0.13, 0.27 and 0.54 mg a.s./L (Table 9.2.6-4).

Table 9.2.6-4: Measured concentrations of S-2399 TG in the exposure solutions

Test Day	Replicate	Nominal concentration (mg a.s./L)						
		Control	Solvent Control	0.031	0.063	0.13	0.25	0.50
0	New	< 0.0025 [a]	< 0.0025 [a]	0.027	0.058	0.13	0.24	0.50
2	Aged	< 0.0025 [a]	< 0.0025 [a]	0.025	0.057	0.12	0.23	0.48
2	New	< 0.0025 [a]	< 0.0025 [a]	0.029	0.059	0.13	0.24	0.53
5	Aged	< 0.0025 [a]	< 0.0025 [a]	0.027	0.059	0.14	0.24	0.51
16	New	< 0.0025 [a]	< 0.0025 [a]	0.036	0.074	0.13	0.30	0.62
19	Aged	< 0.0025 [a]	< 0.0025 [a]	0.035	0.067	0.13	0.29	0.58
19	New	< 0.0025 [a]	< 0.0025 [a]	0.036	0.076	0.16	0.29	0.55
21	Aged	< 0.0025 [a]	< 0.0025 [a]	0.034	0.070	0.14	0.29	0.54
Mean measured [b]		n.a.	n.a.	0.031	0.065	0.13	0.27	0.54
Percent of nominal (%) [b]		n.a.	n.a.	100	100	100	110	110

[a] Concentrations expressed as less than values were below the minimum detectable limit (MDL).

[b] Mean measured concentrations (referred to as “time-weighted average concentrations” in the report) and percent of nominal were calculated using the actual analytical (unrounded) results and not the rounded (two significant figures) values presented in this table.

n.a.= not applicable

C. VALIDITY CRITERIA

As mortality of the parent animals in the controls did not exceed 20% at the end of the test and the mean number of living offspring per parent surviving at the end of the test was ≥ 60, the study was considered valid.

The validity criteria of the study is shown in Table 9.2.6-5 below.

Table 9.2.6-5: Validity criteria

Validity criteria	Required OECD 211 (2012)	Obtained
Parent mortality	< 20%	Control: 0% Solvent control: 0%
Mean offspring survival (per parent)	≥ 60	Control: 167 Solvent control: 172

III. CONCLUSION

Based on mean measured average concentrations, the 21-day NOEC for S-2399 and *D. magna* was determined to be 0.13 mg a.s./L, based on the most sensitive parameter, reproduction. The 21-day LOEC value was determined to be 0.27 mg a.s./L, based on reproduction.

HSE COMMENTS

This study was completed under GLP and OECD 211 (2012) guidelines and has been assessed against these same OECD 211 (2012) guidelines.

The method of analysis used in the study was evaluated by HSE Chemistry. The conclusion of their evaluation is reproduced below. Please see Volume 3 CA, section B5 for more details.

"*The analytical method is not fully validated according to SANTE/2020/12830 rev. 1 for the determination of the active substance inpyrfluxam in freshwater as the matrix effects have not been determined and the stability of standards and extracts has not been addressed. However, the studies were generated prior Ito the implementation of SANTE 2020/12830 rev.1 and SANCO 3029 rev.4 did not require matrix effects and the stabilities of standards and extracts to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose.*"

There were 3 listed deviations to protocol noted with this study report from the applicant. The first relates to the dissolved oxygen concentrations obtained in this study. OECD 211 (2012) guidelines state that dissolved oxygen concentrations should be between 60 and 105%. The dissolved oxygen concentrations obtained in the study ranged from 56% to 127%. HSE find that this is unlikely to have had a significant impact on the validity of the study as there was no mortality or adverse effects recorded in the control or solvent control groups and no anomalous results in the study.

The next protocol deviation relates to the sampling intervals of the different concentrations. OECD 211 (2012) recommends samples taken at day 0 and days 2, 5, 14 and 19 for new solutions, and 2, 5, 7, 16 and test termination (day 21) for aged solutions, but for new solutions, samples were taken at: 0, 2, 16 and 19 days and aged samples were taken at 2, 5, 19 and 21. HSE find that this is unlikely to have affected the findings of the study as the percentage of nominal concentrations were ≥ 100% at all concentrations.

The next listed deviation from protocol relates to measurements of daphnids within the study. Protocol guidelines recommend measuring daphnid length to the nearest 0.01mm, but in the study they were measured to the nearest 0.05mm. HSE conclude that. as there is no recommended length guidelines in OECD 211 (2012) and all test subjects were treated equally, this is unlikely to have affected the results of the study.

It is worth noting that in Table 9.2.6-3 there is overlap between the EC_{10} and EC_{20} 21-day reproduction confidence limits. No concentration-response curve is available in this study to visually check the fit of this data. This reduces the reliability of this indicator, but the regulator does not consider this to be significant as these values are not required for risk assessment.

A comprehensive number of statistical tests were done for this study and are all in line with OECD 211 (2012) guidelines.

The agreed endpoints for use in risk assessment are:

- **21 day EC_{10} = 0.21 mg a.s./L (0.18 – 0.27 with 95% confidence limits) based on reproduction (mean measured concentrations)**
- **21 day NOEC = 0.13 mg a.s./L (based on reproduction (mean measured concentrations)**

B.9.2.6.2 Reproductive and development toxicity to an additional aquatic invertebrate species

Reference:	KCA 8.2.5.2/01
Report Title:	S-2399 TG - Life-Cycle Toxicity Test with Mysids (*Americamysis bahia*)
Author(s) & year:	[redacted] (2016/2020)
Document No, Authority registration No:	Smithers Viscient Study No. 12709.6374
Substance used:	S-2399 TG, 13CG0617G, 95 %
Method of analysis:	LC/MS/MS
Guideline(s):	US EPA OCSPP 850.1350 (1996) and Standard Guide for Conducting Life-Cycle Toxicity Tests with Saltwater Mysids
Deviations:	Yes, see HSE comments section.
GLP or GEP:	Yes
Acceptability:	Supplementary
Study relied upon:	Yes

MATERIALS

Test Material	S-2399 TG
Lot/Batch #:	13CG0617G
Purity:	95.0% (verified by certificate of analysis)
Description:	Not provided
Expiration date:	23 July 2016

STUDY DESIGN AND METHODS

Treatments

Test concentrations:	Nominal: 0.022, 0.044, 0.088, 0.18 and 0.35 mg/L Mean measured: 0.023, 0.053, 0.081, 0.18 and 0.36 mg/L
Controls:	Dilution water control and solvent control (14 µL acetone/L)
Solvent:	Acetone
Analysis of test concentrations:	Yes, analysis at days 0, 7, 14, 21 and 28 using liquid chromatography with tandem mass spectroscopy (LC/MS/MS). The Limit of Quantification (LOQ) was set at 0.600 µg/L. The minimum detectable limit (MDL) was 0.200 µg/L for S-2399 TG

Test organisms

Species:	*Americamysis bahia*, SMV Lot No. 11A168
Source:	MBL Aquaculture (Sarasota, Florida). Maintained in house for 49 months prior to use
Feeding:	Live brine shrimp (*Artemia salina*) nauplii, ≤ 48 hours old (post-hydration), twice daily (once daily enriched with Selco®). Rations for F0 retention chambers (containing 20 mysids each) were approximately 90 nauplii/mysid on test days 0 to 3, 135 nauplii/mysid on test days 4 to 6, 180 nauplii/mysid on test days 7 to 9 and 225 nauplii/mysid on test day 10 to pairing. From day of pairing until test termination, the F0 pairing chambers were fed approximately 450 nauplii/mysid and the retention chambers were fed approximately 3600 nauplii/chamber. F1 chambers were fed a ration of approximately 90 nauplii/mysid/feeding.
Dilution water:	Dilute, filtered, natural seawater
Life stage:	Juvenile (≤ 24 hours old)

Test design

Test vessels:	Glass test aquaria (30 x 15 x 20 cm with a 10-cm high side drain) with a constant exposure solution volume of

	4.5 L. For the first 12 days of exposure, each aquarium contained a retention chamber (10 cm x 2 cm glass petri dishes with a 14 cm high 350-µm mesh collar), partially submerged. During reproductive phase of the exposure, each exposure aquarium contained one retention chamber and a maximum of 5 pairing chambers (6 cm diameter petri dishes, with a 14 cm high 350 µm mesh size opening attached).
Test medium:	Dilute, filtered, natural seawater
Replication:	Pre-sexual maturity: four replicate vessels per test group, with 20 mysids per vessel Post-sexual maturity: four replicate vessels per test group, each containing up to five pairing chambers (one mature male and female per pairing chamber).
Exposure regime :	Flow-through
Aeration:	From day 20
Duration:	F_0 mysids: 28 days F_1 mysids: 96 hours
ENVIRONMENTAL CONDITIONS	
Test temperature:	26 to 27 °C (intermittent measurement) 25 to 28 °C (continuous measurement)
pH:	7.4 to 7.9
Salinity:	19 to 21‰
Dissolved oxygen:	52 to 100 % air saturation value (ASV)
Lighting:	16 hours light: 8 hours darkness (270 to 460 lux, 15 to 30 minute transition period)

STUDY DESIGN AND METHODS

Definitive exposure dates: 24 February to 23 March 2016

Test organism

Mysids were cultured in six recirculating 80-L glass aquaria containing dilute, natural seawater. The seawater in the aquaria was characterized as having a salinity range of 22 to 23‰, a dissolved oxygen range of 90 to 95% of saturation, and a pH of 7.8 during the 14-day period prior to exposure initiation. Culture solution temperature ranged from 25 to 26 °C. Brood stock and test organisms were cultured and tested in seawater from the same source. The area in which the mysids were cultured received a regulated photoperiod of 16 hours of light at an intensity range of 26 to 30 footcandles and 8 hours of darkness. The mysids used to initiate the life-cycle exposure were approximately 23.5 hours old.

Test water

Natural seawater was diluted to a salinity of 20 ± 3‰ with laboratory well water and filtered

through 20- µm, 5-µm and 1-µm polypropylene core filters. The seawater used for this study had a salinity range of 20 to 21‰ and a pH range of 7.2 to 7.7. Routine analyses were conducted periodically on representative samples of the water source for the presence of pesticides, PCBs, and toxic metals by GeoLabs, Inc., Braintree, Massachusetts. None of these compounds were detected at concentrations that are considered toxic in any of the water samples analysed in agreement with ASTM (2007)[26] standard practice. The TOC concentration of the dilution water was 1.1 and 1.2 mg/L for February and March 2016, respectively.

Dose preparation and exposure system

A 25 mg/mL diluter stock solution was prepared prior to exposure initiation by placing, for example, 2.6499 g of S-2399 T.G. (2.5174 g as active ingredient) in a 100-mL volumetric flask and bringing it to volume with acetone. The resulting stock solution was observed to be clear and colourless. Prior to exposure initiation through day 19 of exposure, a Harvard Apparatus Pump in conjunction with a 100-mL Hamilton gas-tight syringe was calibrated to deliver 0.02713 mL/cycle of the 25 mg/mL diluter stock solution to the diluter system's mixing chamber which also received 1.938 L of dilution water each cycle. On day 20 of exposure, the stock solution delivery to the mixing cell was intentionally increased by 20 percent to 0.03256 mL/cycle to offset an anticipated decline of S-2399 T.G. in the day 21 exposure concentrations, as a result of gentle aeration implemented on day 20. The volume of dilution water added to the mixing chamber at each cycle remained unchanged (1.938 L/cycle). The solution contained in the mixing chamber constituted the highest nominal test concentration (0.35 mg/L) and was subsequently diluted (50%) to provide the remaining nominal exposure concentrations (0.18, 0.088, 0.044, and 0.022 mg/L). The concentration of acetone in the mixing chamber was adjusted to match the concentration found in the highest T.G. treatment level (14 µL acetone/L before day 20, 17 µL acetone/L afterwards). This resulted in all treatment levels and the solvent control having the same solvent concentration. A set of dilution water control vessels were also established.

The life-cycle toxicity test was conducted using an exposure system consisting of an intermittent-flow proportional diluter (Mount and Brungs, 1967)[27], a temperature-controlled water bath, and a set of 28 exposure aquaria. The exposure system was in proper operation for 4 days prior to exposure initiation to allow equilibration of the test substance in the diluter apparatus and exposure vessels. During the study, the diluter provided the exposure solutions to each test vessel at a rate of approximately 11 aquarium volume additions per day to provide a 90% test solution replacement rate of approximately 5 hours. The function of the diluter system (e.g., dilution water flow rates) was monitored daily and a visual check was performed twice daily. In addition, weekly analysis of the exposure solutions was used to verify exposure concentrations and proper operation of the diluter system.

In addition to the definitive test, an initial attempt was performed using higher S-2399 T.G. concentrations (0.40 and 0.80 mg/L). However, several attempts to dose the diluter system at these concentrations resulted in the accumulation of considerable undissolved material

[26] ASTM, 2007. Standard practice for conducting acute toxicity test with fishes, macroinvertebrates and amphibians. Standard E729-96. American Society for Testing and Materials, 100 Barr Harbor Drive, West Conshohocken, Pennsylvania.

[27] Mount, D.I. and W.A. Brungs, 1967. A simplified dosing apparatus for fish toxicological studies. Water Research. 1: 21-29.

(e.g., precipitate) in the diluter mixing chamber and chemical cells, indicating that this dosing was above the functional solubility of S-2399 T.G. under test conditions. This hindered the description of a dose-response relationship.

Animal assignment

F_0 generation

Mysids (≤ 24 hours old at test initiation) were impartially selected and distributed to each respective test vessel (four replicate vessels per test group, with 20 mysids per vessel), for the control, solvent control and treatment groups. When sexual maturity was reached (day 13) one mature male and one mature female were assigned to each of the pairing chambers with a maximum of five male/female pairs per replicate. The unpaired mysids remained in the initial retention chambers until they were paired or until test termination.

When three or fewer unpaired mysids remained in a retention chamber, they were transferred to a separate pairing chamber. Male mysids from this pool were used to replace dead males from the paired (male/female) groups. Females that died in the pairing chambers were replaced at the Study Director's discretion. For example, if a female within a pairing chamber died prior to that replicate's median first brood release and a female was available that had not reproduced, the dead female could be replaced.

F_1 generation

During the reproductive phase, groups of 10 offspring per replicate (40 per treatment level) were collected and evaluated. Offspring were removed from adult mysid chambers in each replicate vessel and placed in separate pairing chambers within that replicate. Chambers were established based on the number of available juvenile F_1 mysids; therefore, each chamber was not necessarily initiated on the same day. One F_1 group was established and monitored for each replicate vessel. The chambers with F_1 mysids were established to monitor survival up to 96 hours post-release. This period ensured equal observation timing across all treatment levels and the control prior to exposure termination.

Measurements and observations

For the F_0 mysids, survival, and abnormal appearance or behaviour were recorded daily throughout the test (28 days). After males and females had been paired (day 13) number of offspring was also recorded daily. At test termination, all surviving mysids were recorded for total body length and dry weight. Digital photographs were taken of each mysid using a binocular dissection microscope for individual body length measurements. Individual body length was measured to the nearest 0.01 mm. Images were obtained using the following equipment: a Zeiss Stemi-2000 CS microscope, a Zeiss AxioCam ICc 5 camera, and Zeiss Zen 2011 (version 1.0.1.0) image analysing software. Male and female mysids were transferred to aluminium pans, dried in an oven at 98 to 100 °C for approximately 24 hours and then placed in a desiccator. Individual dry body weight to the nearest 0.01 mg was determined using an analytical balance. For the F_1 mysids, observations of stress, abnormal behaviour (discoloration, immobilization and inability to maintain position in the water column) and survival were recorded daily for 96 hours. Mortality was defined as lack of movement after gentle prodding with a pipette.

The pH, dissolved oxygen concentration, temperature and salinity were measured in each replicate on day 0 and alternated between replicates daily thereafter throughout the exposure period, for each treatment level and the controls. Continuous temperature

monitoring was also conducted in one control replicate.

Prior to exposure initiation, samples were removed from one replicate of each treatment level and the controls for analysis of S-2399 TG concentration. On days 0, 7, 14, 21 and 28, samples were removed form alternating replicates of each treatment level and the controls. All samples were analysed for S-2399 TG concentration using LC/MS/MS.

Statistical analysis

The endpoints used for determination of significant adverse effects on F_0 organisms included 28-day survival, male and female survival (post-pairing), growth (average dry body weight and average total body length) of both male and female mysids and reproduction (number of young released per female). F_1 survival was also analysed.

Briefly, Equal Variance Two-Sample t-Tests were used to compare negative control and solvent control performance. For this study, no significant difference was detected between the negative and solvent controls for all endpoints. Therefore, comparison of treatment data for all endpoints was performed using the negative control data. Shapiro-Wilk's Tests were deployed for normality checks. Homogeneity of variance was checked using Barlett's Tests, Variance Ratio F Tests or Levene's Equality of Variance Test. Data for the survival endpoints were analysed using Fisher's Exact Test with Bonferroni-Holm's Adjustment.

All continuous data endpoints (i.e., growth and offspring per female) displayed normality, homogeneous variance and were non-monotonic; therefore, Dunnett's Multiple Comparison Tests were used to evaluate the data.

Lethal and sublethal responses were statistically assessed and compared with the negative control values to determine the No-Observed-Effect Concentration (NOEC) and Lowest-Observed-Effect Concentration (LOEC) values. The NOEC defined as the highest mean measured concentration that did not exhibit a statistically significant difference ($p < 0.05$) between the exposed organisms and the negative control mean, was determined. The LOEC, defined as the lowest mean measured concentration that exhibited a statistically significant difference ($p < 0.05$) from the negative control mean, was also determined.

CETIS™ was used to perform these statistical analyses.

RESULTS AND DISCUSSION

Test conditions

Water quality parameters measured during the 28-day exposure period are presented in Table 9.2.6-6. For two treatment levels (0.088 and 0.35 mg/L) dissolved oxygen concentration on day 19 dropped below the 60% saturation limit outlined in OPPTS 850.1350 (1996). To correct this, oil-free aeration was initiated from day 20. Measurements on day 20 confirmed that dissolved oxygen levels were restored to above 60% of saturation. Daily measurements for temperature and salinity were within the ranges quoted within OPPTS 850.1350 (1996). Continuous temperature measurements within replicate A of the control, however, established a maximum of 28 ºC on day 18.

During the 28-day exposure period, maximum biomass loading did not exceed 0.0025 g/L flowing solution per day or 0.02 g/L of solution at any time, in any replicate exposure aquarium. Calculations of biomass loading are based on conservative culture estimate of

the typical average wet weight of an adult mysid (0.0045 g).

Table 9.2.6-6: Life-cycle exposure of mysids (Americamysis bahia) to S-2399 T.G. – Summary of water quality parameters

Nominal concentration (mg/L)	Ranges				
	Dissolved oxygen[a]		Temperature[ab] (°C)	Salinity[a] (‰)	pH[a]
	mg/L	% of saturation			
Control	5.17–7.36	72–100	26–27	19–21	7.6–7.9
Solvent Control	4.53–7.04	64–99	26–27	20–21	7.4–7.6
0.022	4.82–7.16	68–100	26–27	20–21	7.4–7.6
0.044	4.32–6.91	61–97	26–27	20–21	7.4–7.8
0.088	3.69–6.72	52[c]–94	26–27	20–21	7.4–7.8
0.18	4.39–6.91	62–97	26–27	20–21	7.4–7.8
0.35	4.14–6.66	58[c]–92	25–27	20–21	7.4–7.8

[a] N = 32 for all treatment levels, the control and solvent control, with the exception of the 0.088 and 0.35 mg/L treatment levels that were N = 33.
[b] Continuous temperature monitoring of the control (replicate A) established a temperature range of 25 to 28 ºC throughout the exposure period.
[c] Dissolved oxygen concentrations re-measured within 24 hours of original measurements after gentle, oil-free aeration was employed to all exposure solutions on test day 20. Re-measurements confirmed dissolved oxygen concentrations >60% saturation.
NOTE: Values presented in the table have been rounded to two significant figures with the exception of dissolved oxygen, which is presented as three significant figures.

Analytical results
The analytical measurements for S-2399 T.G. during the in-life portion of the definitive test are presented in Table 9.2.6-7. Mean measured concentrations ranged from 92 to 120 % of nominal concentrations.

Table 9.2.6-7: Measured concentrations of S-2399 TG in the exposure solutions

Nominal concentration (mg a.s./L)	Measured concentration (mg a.s./L) (% of nominal[b])						Mean percent of nominal (%)
	Day 0	Day 7	Day 14	Day 21	Day 28	Mean (SD)	
Control	< 0.0017[a] (n.a.)	< 0.0017[a] (n.a.)	< 0.0017[a] (n.a.)	< 0.0017[a] (n.a.)	< 0.0017[a] (n.a.)	n.a.	n.a.
Solvent control	< 0.0017[a] (n.a.)	< 0.0017[a] (n.a.)	< 0.0017[a] (n.a.)	< 0.0017[a] (n.a.)	< 0.0017[a] (n.a.)	n.a.	n.a.
0.022	0.022 (100)	0.021 (95)	0.024 (109)	0.025 (114)	0.020 (91)	0.023 (0.0023)	100
0.044	0.053 (120)	0.051 (116)	0.052 (118)	0.057 (130)	0.049 (111)	0.053 (0.0030)	120
0.088	0.073 (83)	0.069 (78)	0.088 (100)	0.097 (110)	0.075 (85)	0.081 (0.011)	92
0.18	0.18 (100)	0.18 (100)	0.19 (106)	0.19 (106)	0.16 (89)	0.18 (0.012)	100
0.35	0.37 (106)	0.35 (100)	0.38 (109)	0.42 (120)	0.29 (83)	0.36 (0.049)	100

[a] Control and solvent control samples showed concentrations below the minimum detectable limit (MDL) of 0.0017 mg/L
[b] Calculated by HSE using rounded figures provided in table.
n.a. = not applicable

Biological observations

A summary of F_0 mysid survival and reproduction endpoints are presented in Table 9.2.6-8. For the F_0 generation, survival of males and females during the reproductive phase and for the mysids following 28 days of exposure was not statistically significantly different between any of the treatment levels and the negative control. The reproductive success (mean number of offspring per female) was only statistically significantly different from the negative control for the 0.081 mg a.s./L treatment group. Due to the lack of a significant difference for the two higher treatment levels, the effect observed at 0.081 mg a.s./L was not considered to be toxicant related nor biologically relevant by the study conductor. For the mysids in the F_0 generation no behavioural abnormalities were observed during the exposure period.

Table 9.2.6-8: Survival and reproductive success of F0 generation *Americamysis bahia* exposed to S-2399 TG

Mean measured concentration (mg a.s./L)	Mean survival (% ± SD)			Mean # females producing offspring (% ± SD)	Number of offspring per female (± SD)	Number of offspring per female (% reduction compared to negative control)[b]	Number of offspring per female (% reduction compared to solvent control)[b]
	Males[c] Females[c]	Post-pairing	28-Day[d]				
Negative control	86 ± 11 98 ± 5	90 ± 3	80 ± 7	100 ± 0	17.6 ± 3.4	0	- 9.3
Solvent control	90 ± 14 98 ± 5	94 ± 8	82 ± 5	100 ± 0	16.1 ± 2.7	8.5	0
0.023	81 ± 24 98 ± 5	89 ± 9	68 ± 15	100 ± 0	14.1 ± 3.1	19.9	12.4
0.053	87 ± 12 85 ± 30	87 ± 8	72 ± 14	100 ± 0	14.0 ± 2.9	20.5	13.0
0.081	71 ± 17 85 ± 20	77 ± 17	65 ± 13	100 ± 0	11.2 ± 1.9[a]	36.4	30.4
0.18	90 ± 13 94 ± 7	90 ± 8	73 ± 8	100 ± 0	12.5 ± 2.9	29.0	22.4
0.36	91 ± 6 90 ± 13	90 ± 9	77 ± 13	100 ± 0	15.6 ± 3.7	11.4	3.1

[a] Significantly reduced compared to the negative control using Dunnett's multiple comparison test.
[b] These values were calculated by HSE using the rounded values in the table provided.
[c] Calculations of male and female survival began after pairing.
[d] Calculations of survival are of both male and female mysids combined.
SD = Standard deviation

For body length of the F_0 generation (Table 9.2.6-9), a statistically significant difference to the negative control was only observed for the males at the highest treatment level of 0.36 mg a.s./L. For body weight, only females exposed to the lowest treatment level of 0.023 mg a.s./L showed a statistically significant difference to the negative control. This effect was not considered to be toxicant related nor biologically relevant by the study conductor, due to the lack of a significant differences for the four higher treatment levels.

Table 9.2.6-9: Total body length and dry body weight and of F_0 generation *Americamysis bahia* exposed to S 2399 TG

Mean measured concentration (mg a.s./L)	Total body length (mm) (mean ± SD)		Dry body weight (mg) (mean ± SD)	
	Males	Females	Males	Females
Control	7.66 ± 0.19	7.84 ± 0.19	0.98 ± 0.06	1.36 ± 0.13
Solvent control	7.48 ± 0.17	7.59 ± 0.13	0.93 ± 0.04	1.28 ± 0.06
0.023	7.49 ± 0.29	7.78 ± 0.08	0.89 ± 0.09	1.13 ± 0.11[a]
0.053	7.36 ± 0.17	7.62 ± 0.20	0.94 ± 0.05	1.29 ± 0.14
0.081	7.50 ± 0.16	7.64 ± 0.26	0.94 ± 0.07	1.19 ± 0.11
0.18	7.56 ± 0.12	7.64 ± 0.08	0.94 ± 0.05	1.32 ± 0.04
0.36	7.18 ± 0.24[a]	7.72 ± 0.08	0.88 ± 0.07	1.33 ± 0.06

[a] Significantly reduced compared to the negative control using Dunnett's multiple comparison test (16.9 % and 11.7% reduction compared to control and solvent control respectively).
SD = Standard deviation

Following the 96-hour observation period, the F_1 generation in the negative control and solvent control groups showed 98% and 100% survival, respectively. Mean survival of 95%, 95%, 93%, 90% and 93% was observed for the mysids exposed to 0.023, 0.053, 0.081, 0.18 and 0.36 mg a.s./L treatment levels, respectively. No statistically significant differences were determined for the mysids in the treatment groups compared to the control group.

Based on mean measured concentrations, the NOEC was determined to be 0.18 mg a.s./L by the study conductor. The LOEC was established at 0.36 mg a.s./L. A summary of the endpoints is presented in Table 9.2.6-10.

Table 9.2.6-10: NOEC and LOEC values for survival, reproduction and growth of Americamysis bahia exposed to S-2399 TG

Endpoint	NOEC (mg a.s./L)[a]	LOEC (mg a.s./L)[a]
Male survival (from pairing to day 28)	0.36	> 0.36
Female survival (from pairing to day 28)	0.36	> 0.36
28-day survival	0.36	> 0.36
Offspring per female	0.36	> 0.36
Male length	0.18	0.36
Female length	0.36	> 0.36
Male body weight	0.36	> 0.36

Endpoint	NOEC (mg a.s./L)[a]	LOEC (mg a.s./L)[a]
Female body weight	0.36	> 0.36
F_1 survival at 96-hours post-release	0.36	> 0.36

[a] Based on mean measured concentrations

VALIDITY CRITERIA

The validity criteria for the test were met according to OCSPP 850.1350 (1996) (Table 9.2.6-11).

Table 9.2.6-11: Compliance with validity criteria

Validity criterion	Required	Obtained
Percentage of reproductive females in the control	> 75 %	100 %
Average number of offspring produced per female	> 3[a]	Negative control = 17.6 Solvent control = 16.1
28-day control survival[b]	> 70 %	Negative control = 80 % Solvent control = 82 %

[a] This is incorrectly stated as > 3 per day in OCSPP 850.1350 (1996).

[b] Criterion not specified in OCSPP 850.1350 (1996)

CONCLUSION

Based on mean measured concentrations, the No-Observed-Effect Concentration (NOEC) for mysids (*Americamysis bahia*) exposed to S-2399 TG was determined to be 0.18 mg a.s./L by the study conductor. The Lowest-Observed-Effect Concentration (LOEC) was determined to be 0.36 mg a.s./L.

HSE COMMENTS

The study was carried out according to GLP and follows OCSPP 850.1350 (1996). All validity criteria involving control reproduction were met.

The following deviations were noted:

OCSPP 850.1350 (1996) paragraphs (d)(1)(iii), (d)(3)(v) and (f)(8) outline the requirement for a maximum-acceptable-toxicant-concentration (MATC) to be reported. Although possible, this was not performed for male length. The MATC for male length is 0.255 mg/L.

OCSPP 850.1350 (1996) paragraph (d)(3)(ii) states, "*mysids should be physically separated into replicate groups of no more than eight individuals when most of the mysids reach sexual*

maturity (usually 10–14 days after the beginning of the test". The study took a different approach by creating five mysid pairs per replicate that were each housed individually. Therefore, there were only two mysids in each physically separated replicate group. Although this approach potentially deviates from the intended methodology outlined in the guidelines, it allowed for the accurate quantification of offspring per female. Therefore, HSE consider this potential deviation minor.

OCSPP 850.1350 (1996) paragraph (d)(3)(v) states, "*body length (as measured by total midline body length, from the anterior tip of the carapace to the posterior margin of the uropod) should be recorded for males and females at the time when sex can be determined simultaneously for all mysids in control and treatment groups*" (also covered by (f)(7)(i)). This was not performed. This prevented growth effects being associated with either the mysid maturation or sexually mature stage, or the detection of possible transient growth effects. Nevertheless, growth effects were still tracked in both sexes. HSE notes this omission and will consider it during the risk assessment.

OCSPP 850.1350 (1996) paragraph (d)(3)(v) also states, "*if available prior to termination of the test, observations on the mortality, number of males and females and male and female body length should be recorded for the G2 mysids*" (also covered by paragraph (f)(7)(iv)). Number of males and females and male and female body length was not recorded for G2 mysids. Consequently, some effects on the progeny may have been undetected. HSE note this omission. This raises uncertainties around the completeness of the results and will be considered in the risk assessment.

OCSPP 850.1350 (1996) paragraph (d)(3)(viii) states, "*the concentration of the test substance in the chambers should be measured as often as is feasible during the test. The measured concentration of the test substance should not vary more than 20 percent among replicate test chambers of a treatment concentration*". The 0.044 mg/L treatment level was 130% of the nominal concentration on day 21. As mean measured concentrations were used to calculate NOECs and LOECs this deviation from the specified range was reflected in the results. Therefore, HSE consider this a minor deviation.

OCSPP 850.1350 (1996) paragraphs (e)(2)(i)(A)(4) and (e)(3)(iv) outline the requirement for the provision of a 14–h light and 10–h dark photoperiod with a 15– to 30–min transition period. The study used a 16–h light and 8–h dark photoperiod with a 15– to 30–min transition period. The use of a longer light photoperiod is supported in other regulatory standards documentation (ASTM, 2008)[28]. Furthermore, cultures reared under this photoperiod were reported as healthy and control survival in the definitive test was > 80 %. Therefore, HSE considers this a minor deviation.

OCSPP 850.1350 (1996) paragraph (e)(2)(iii)(A) states, "*materials and equipment that contact test solutions should be chosen to minimize sorption of test chemicals from the dilution water and should not contain substances that can be leached into aqueous solution in quantities that can affect the test results*". The exposure system contained silicone, which

[28] ASTM, 2008. Committee E-47 on Biological Effects and Environmental Fate. Standard Guide for Conducting Life-Cycle Toxicity Tests with Saltwater Mysids. ASTM Designation E 1191-03a (re-approved 2008). American Society for Testing and Materials, 100 Barr Harbor Drive, West Conshohocken, PA 19428.

can lead to the sorption of chemicals. Mean measured concentrations were within 20% of the nominal concentrations throughout the study, indicating that sorption had minimal impact on study robustness. HSE consider this a minor deviation.

OCSPP 850.1350 (1996) paragraph (e)(3)(i) states, "*the test temperature should be 25 °C. Excursions from the test temperature should be no greater than ± 2 °C*". On test day 18, continuous temperature monitoring established a maximum temperature of 28 ºC. This was the only time throughout the duration of the study that the quoted temperature range was exceeded. This, combined with the negative and solvent control survival and reproduction exceeding the acceptability criteria suggest that this deviation had minimal impact on study integrity. HSE consider this an acceptable deviation.

OCSPP 850.1350 (1996) paragraph (e)(3)(ii) specifies that DOC should be maintained between 60 and 105 % saturation and if aeration is required this should be done before the addition of the test substance. DOC did fall below 60 % saturation for the 0.088 and 0.35 mg/L (nominal) treatment levels on day 19. DOC levels were restored after gentle, oil-free aeration was added to all aquaria. To counter the expected decline in S-2399 T.G. concentrations measured within the experimental system after the implementation of oil-free aeration, the stock solution delivery to the mixing cell was intentionally increased by 20 percent. This maintained the measured concentrations at the desired concentrations. There is a possibility that the reduced DOC levels in the 0.088 mg/L nominal treatment level led to the non-concentration-response relationship between S-2399 T.G. and number of offspring per female. For this endpoint there was a negative effect at the 0.088 mg/L treatment level that was not witnessed at higher concentrations. Although DOC reductions also occurred at 0.35 mg/L, reducing the plausibility of this explanation for reduced reproductive output at 0.088 mg/L. HSE note this uncertainty and will consider it during the risk assessment.

OCSPP 850.1350 (1996) paragraph (e)(3)(iii) states, "*the number of mysids placed in a test solution should not be so great as to affect results of the test. Loading requirements for the test will vary depending on the flow rate of dilution water. The loading should not cause the DOC to fall below the recommended levels*". There is a possibility that increased loading may have resulted in the reduced DOC levels for the two treatment levels (0.081 mg/L and 0.36 mg/L) on day 19. As discussed above, DOC reductions were short-lived and only affected two treatment levels. This is not expected to have overly impacted the integrity of the defined NOEC. For this reason, HSE consider this a minor deviation.

OCSPP 850.1350 (1996) paragraph (f)(2) states, "*reporting of test data should include the following…detailed information about the test organisms, including the scientific name and method of verification, average length, age, source, history, observed diseases, treatments, acclimation procedures, and food used*". An average length is not provided. This makes it challenging to assess whether the batch of mysids used in this study were representative of their culture population. Brood stock and test organisms were determined to be in good health at the start of the exposure phase and control survival was within the acceptability criteria defined by the study conductor. This deviation is not expected to have impacted study integrity. HSE consider this deviation acceptable.

Finally, OCSPP 850.1350 (1996) paragraphs (d)(3)(i), (d)(3)(ii), (d)(3)(v), (d)(4)(ii)(A), (d)(4)(ii)(B) and (f)(9) relate to the determination of a concentration-response curve. This was hindered by the solubility limit of the test substance in the test system. An initial attempt

at the definitive exposure study using 0.050, 0.10, 0.20, 0.40, and 0.80 mg/L resulted in the accumulation of considerable undissolved material (e.g., precipitate) in the diluter mixing chamber and chemical cells. This resulted in inconsistent system dosing and variable analytical recoveries. Consequently, a maximum concentration of 0.35 mg/L was selected as this was found to be compatible with the flow-through system in a preliminary test. The 0.35 mg/L treatment level did not result in significant negative effects for any endpoint apart from F_0 total male length.

Another deviation from OCSPP 850.1350 (1996) was mentioned in the method, "*females that died in the pairing chambers were replaced at the Study Director's discretion. For example, if a female within a pairing chamber died prior to that replicate's median first brood release and a female was available that had not reproduced, the dead female could be replaced*". This approach is not recommended in the guideline. By taking this approach, the offspring produced per female variable is as independent from F_0 mortality as possible. HSE consider this acceptable and similar to the approach taken in other areas of risk assessment, including NTAs.

There were two additional treatment level/endpoint combinations that resulted in significant negative effects: 0.023 mg/L (mean measured concentration) for dry female body weight and 0.081 mg/L (mean measured concentration) for number of offspring per female. In both cases, higher concentrations of S-2399 T.G had no significant negative effects on the respective endpoints. The lack of a clear concentration response in both cases suggests the observed significant effects may not have been toxicant related.

The lower dry female body weight at 0.023 mg/L (16.9 % and 11.7% reduction compared to control and solvent control respectively) is unconvincing when considering the four higher treatment levels all displaying no significant effect. In this case, HSE consider the lack of a clear concentration response as evidence that the observed effects were not toxicant related and agrees with the reported NOEC/LOEC levels.

For the number of offspring per female, there was a > 19% reduction for all treatment groups except 0.36 mg/L when compared to the control (> 12 % reduction for the solvent control). There was no clear concentration-response and no clear NOEC level. Due to the lack of interpretability of the results, HSE has decided to exclude them. This decision will be explored at the risk assessment stage by comparing RACs to the concentrations used in this study.

The method of analysis used in the study was evaluated by HSE Chemistry. The conclusion of their evaluation is reproduced below. Please see Volume 3 CA, section B5 for more details.

"*The analytical method is not fully validated according to SANTE/2020/12830 rev. 1 for the determination of the active substance inpyrfluxam in freshwater as the matrix effects have not been determined and the stability of standards and extracts has not been addressed. However, the studies were generated prior Ito the implementation of SANTE 2020/12830 rev.1 and SANCO 3029 rev.4 did not require matrix effects and the stabilities of standards and extracts to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose.*"

As a NOEC for a key reproductive endpoint, offspring per female, could not be determined, HSE cannot report a conclusive study NOEC. Consequently, HSE has decided to accept this study as supplementary information only.

B.9.2.6.3 Development and emergence in *Chironomus riparius*

No study was submitted for development and emergence in *Chironomus riparius*.

B.9.2.6.4 Sediment dwelling organisms

Reference:	KCA 8.2.5.4/01
Report Title:	Amended Report: Life-Cycle Toxicity Test Exposing Midges (*Chironomus dilutus*) to S-2399 TG Applied to Sediment Under Static-Renewal Conditions Following EPA Test Methods
Author(s) & year:	[redacted] (2015/2020)
Document No, Authority registration No:	Smithers Study No. 12709.6364
Substance used:	S-2399 TG, 13CG0617G, 95 %
Method of analysis:	LC/MS/MS
Guideline(s):	OCSPP Draft Guideline 850.1760. and EPA Test Method 100.5 (2000).
Deviations:	Yes, see HSE comments section.
GLP or GEP:	Yes
Acceptability:	Supplementary
Study relied upon:	No, not used in aquatic risk assessment

MATERIALS

Test Material S-2399 TG

Lot/Batch #: 13CG0617G
Purity: 95.0 % (verified by certificate of analysis)
Description: Not provided
Expiration date: 23 July 2016

STUDY DESIGN AND METHODS

Treatments

Test concentrations:	Nominal sediment: 1.2, 3.6, 11, 33 and 100 mg a.s./kg Mean measured sediment: 1.3, 3.2, 9.9, 28 and 92 mg a.s./kg Mean measured pore water: 0.034, 0.12, 0.44, 1.4 and 5.3 mg a.s./L
Controls:	Negative and solvent control
Solvent:	Acetone
Analysis of test concentrations:	Yes in overlying water, pore water and sediment at Day 0, 20 and 62. Analysed by liquid chromatography with mass spectrometry detection (LC/MS/MS). Limit of quantitation (LOQ) of 200 µg/kg and the minimum detectable limit (MDL) was 16.0 µg/kg for sediment. The limit of quantitation (LOQ) was 0.6 µg/L and the minimum detectable limit (MDL) was 0.2 µg/L for overlying and pore water.

Test organisms

Species:	*Chironomus dilutus*
Source:	Smithers Viscient cultures
Feeding:	Finely-ground suspension of flaked fish food (4.0 mg/mL), daily during rearing. During the exposure, the food was introduced at a rate of 1.5 mL of flaked fish food suspension per test vessel per day.
Age:	Three days old at test initiation (first instar)
Overlying water	Laboratory well water
Hardness:	56 - 76 mg $CaCO_3$/L
Alkalinity:	14 - 26 mg $CaCO_3$/L
Conductivity:	430 - 450 µS/cm
pH:	6.3 - 7.5
Ammonia:	≤ 0.10 – 1.1 mg/L
Sediment:	Artificial sediment prepared according to OECD Guideline 218 (2004)
Percent organic carbon:	2. 1%
Particle size distribution:	78 % sand, 5 % silt, 17 % clay
pH:	6.6

Percent solids:	64.4 %
Test design	
Test vessels:	300-mL clear glass beakers, each containing a 147 g of sediment wet weight (94.6 g dry weight) and 175 mL of overlying water
Test medium:	Dilute, filtered, natural seawater
Replication:	Biological observations: 12 replicate vessels containing 12 midge larvae each (144 midges per treatment level or control) Analytical measurements: four replicate vessels Pore water quality measurements: three replicate vessels (control group only) Auxiliary male production: four replicate vessels The additional replicates set up for analytical measurements, pore water quality measurements and auxiliary male production were maintained under the same conditions and contained test organisms (organisms were not added to replicates that were sacrificed at test initiation for analytical sampling and pore water quality measurements) but were not used to evaluate the biological response of the test organisms.
Exposure regime :	Static-renewal
Duration:	62 days
ENVIRONMENTAL CONDITIONS	
Test temperature:	21 – 24°C
pH:	6.3 – 7.5
Dissolved oxygen:	2.5 – 8.6 mg/L
Lighting:	16 hours light: 8 hours darkness (240 – 600 lux)

STUDY DESIGN AND METHODS

Definitive exposure dates: 6 February to 9 April 2015

Test organism

The dipteran midge (*Chironomus dilutus*) was selected as the test organism because it is sensitive to a variety of chemical substances, is easily cultured and has a relatively short life cycle (approximately 30 days at 25 ºC). The sensitivity of the organism was verified

periodically using a reference toxicant (potassium chloride).

Prior to exposure initiation, midge egg masses were placed in crystallizing dishes containing approximately 200 mL of laboratory well water at a temperature of 23 ± 1 ºC. On hatching, midge larvae were transferred to a shallow glass bowl containing approximately 1 L of culture water (laboratory well water) and 2.5 mL of *Ankistrodesmus falcatus*, a unicellular green algae (4×10^7 cells/mL), to serve as a substrate. Midge larvae were reared under static conditions in laboratory well water with gentle oil-free aeration (23 ºC and 6.6 to 7.2 mg/L) for three days to produce first instar larvae. These were confirmed to be 3 days old at exposure initiation.

Overlying water
Representative samples of the overlying water source were analysed periodically for the presence of pesticides, PCBs and toxic metals by GeoLabs, Inc., Braintree, Massachusetts. None of these compounds have been detected at concentrations that are considered toxic in any of the water samples analysed, in agreement with ASTM (2002)[29] standard practice. In addition, representative samples of the overlying water source were analysed monthly for total organic carbon (TOC) concentration. The TOC concentration of the overlying water source ranged from 0.52 to 0.89 mg/L for February through April 2015. Midges were maintained in water from the same source as the overlying water utilised in this study and have successfully survived and reproduced over multiple generations.

Sediment
Artificial sediment was prepared by mixing the following components (based on dry weight): 2.8 kg sphagnum peat, 11 kg kaolin clay and 43 kg fine sand (i.e., approximately 5.0, 20 and 75%, respectively). Prior to being used in the sediment preparation, the pH of the peat was increased from 3.3 to 5.5 by mixing in 140 g of $CaCO_3$ and soaking in laboratory well water for approximately one week. A total of 11 L of water from the same source used in the toxicity test was also added to the batch of sediment components during the mixing process. Representative samples of the sediment were analysed periodically for the presence of pesticides, PCBs and toxic metals by GeoLabs, Inc., Braintree, Massachusetts. None of these compounds have been detected at concentrations that are considered toxic in any of the samples analysed, in agreement with ASTM (2002)[29] standard practice.

Dose and test vessel preparation
A 25 mg a.s./mL primary stock solution was prepared by placing 1.31675 g of S-2399 TG (1.25091 g as active substance) in a 50-mL volumetric flask and dissolving the material by bringing it to volume with acetone (CAS No. 67-64-1). The resulting stock solution was observed to be clear and colourless with no visible undissolved test substance following preparation. The primary stock solution was used to create five individual dosing stock solutions.

[29] ASTM, 2002. Standard practice for conducting acute toxicity tests with fishes, macroinvertebrates and amphibians. Standard E729-96. American Society for Testing and Materials, 100 Barr Harbor Drive, West Conshohocken, Pennsylvania 19428.

A jar-rolling technique was used to apply the test substance to the sediment (Ditsworth et al., 1990)[30]. A 10-mL volume of each dosing stock solution was applied to 0.050 kg of fine silica sand placed in glass Petri dishes, which was mixed with a metal spatula for approximately two minutes. The solvent was allowed to evaporate off the sand for 35 minutes. The dry sand, containing the test substance, was then added to 3.5 kg of wet sediment (2.2540 kg dry weight of test sediment based on a percent solids value of 64.40% with the 0.050 kg of dry sand for a total of 2.3040 kg) in individual glass jars to yield the desired treatment level concentrations.

The jars were sealed and positioned horizontally on a rolling mill. Each jar was then rolled for four hours at room temperature at approximately 15 rpm. On completion, sediments were allowed to equilibrate for a 14-day period in the dark at 2 to 8 ºC. Once a week during the 14-day equilibration period and again prior to addition into the replicate test vessels, the jars were mixed on the rolling mill for an additional two hours at room temperature to ensure the sediment was homogeneous. A 14-day equilibration period was deemed acceptable for sediment toxicity testing with S-2399 TG based on the results of a sediment-pore water equilibration trial conducted prior to this study.

A solvent control was prepared using the method described above but without the addition of the test substance or the solvent, respectively. The negative control sediment consisted of 3.5 kg of unadulterated wet sediment without the addition of solvent, test substance or the additional 0.050 kg of fine silica sand.

One day prior to test initiation (day -1), the treated and control sediments (100 mL per vessel) were allocated to the replicate vessels. 175 ml of overlying water was gently added to each vessel with the aid of a turbulence reducer, used to minimise sediment layer disturbance, and the vessels were placed under the renewal system.

On test day 14, emergence traps were placed over the test vessels to trap emergent flies for the remainder of the test.

Water Renewal

During the 62-day study, the overlying water was renewed by adding two volume additions of water (i.e., 350 mL) per test vessel per day using an intermittent delivery system in combination with a calibrated water distribution system (Zumwalt et al., 1994)[31]. As decreasing dissolved oxygen levels were observed during the early portion of the exposure, the water delivery system was adjusted on test day 12 to deliver four overlying volume replacements per vessel per day in order to maintain water quality levels within an acceptable range. The calibration of the overlying water renewal system was checked prior to exposure initiation and confirmed at test termination. During the test, the renewal system

[30] Ditsworth, G. R., D. W. Schults, J. K. P. Jones, 1990. Preparation of Benthic Substrates for Sediment Toxicity Testing. Environmental Toxicology and Chemistry. Vol. 9, pp. 1523−1529.

[31] Zumwalt, D.C., F.J. Dwyer, I.E. Greer and C.G. Ingersoll, 1994. A water-renewal system that accurately delivers small volumes of water to exposure chambers. Environmental Toxicology and Chemistry. pgs. 1311-1314.

was visually inspected at least twice daily. A complete check of intermittent delivery system function was made once daily.

Measurements and observations

Water quality

At exposure initiation, day 10, day 20 and test termination, dissolved oxygen concentration, temperature and pH were measured in the overlying water of each replicate vessel of each treatment level and control used for biological monitoring. On the remaining test days, dissolved oxygen and temperature were measured in one alternating replicate of each treatment level and control each day. In addition, the temperature was continuously monitored in an auxiliary vessel in the temperature controlled water bath used to house the test vessels throughout the study. Total hardness, alkalinity, conductivity and ammonia concentration of the overlying water were monitored at test initiation, day 10, day 20 and test termination in each treatment level and control solution from a composite sample taken from the biological replicates.

At toxicity exposure initiation, day 20 and test termination, pH and ammonia (as nitrogen) concentration were measured in a pore water sample of the control group only.

Biological observations

All vessels were examined at exposure initiation and at 24-hour intervals thereafter, until test termination (day 62). Daily observations of mortality (larvae or pupae on the sediment surface) and abnormal behaviour (sediment avoidance) were made and the physical characteristics of the test solutions were recorded.

At test Day 20, four replicate vessels from each treatment level and control were selected to determine midge larval survival and growth. Midge larval survival and growth were determined in these test vessels by sieving the sediment to remove all surviving midges. The growth (as ash-free dry weight (AFDW)) was determined by pooling the surviving midge larvae in each replicate vessels and drying at 60 ± 5°C for approximately 24 hours in an oven. The pooled, dried midge larvae were weighed and ashed at 550 ± 50°C for approximately two hours, prior to reweighing (to the nearest 0.01 mg).

Starting on Day 15 and daily thereafter, the number of male and female midges emerged from each replicate test vessel was observed and recorded. Complete emergence occurs when a midge has shed the pupal exuviae completely and escapes the surface tension of the water. If complete emergence occurs but the midge has not escaped the surface tension, it will die within 24 hours. Therefore, midges that had completely emerged but had not escaped the surface tension were observed after an additional 24 hours before recording as an emerged midge or dead midge.

The observed emergence days were used to calculate mean development times, which were in turn used to calculate an emergence rate (1 / development time). OECD 218 (2004) the use of emergence rate over development time due to its more favourable statistical properties. This was calculated according to the equation defined in OECD 218 (2004)

paragraph 51.

Starting on Day 15 and daily thereafter, the emerged males and females were collected on a replicate basis from the emergence traps and placed in reproductive/oviposit chambers. Adult survival and produced egg masses were recorded daily, alongside time to ovipositing.

The number of eggs produced in each primary egg mass were counted the day the mass was laid using the ring method. Using a dissecting microscope, five rings of eggs in each egg mass were selected at approximately equal distances along the length of the egg mass and the number of eggs in each of these five rings were then counted. The mean number of eggs per ring was then multiplied times the number of rings in the egg mass to estimate the total number of eggs. The number of unhatched eggs was counted following six days of incubation (23 ± 1 °C), and hatching success determined.

Analytical measurements

Dosed sediments were sampled during the mixing/equilibrium period, prior to the allocation of the sediments into the replicate exposure vessels. In addition, subsamples of the dosing stock solutions were also analysed for test substance concentration. All pore water, overlying water and sediment samples from one replicate per treatment level and control were removed and analysed for S-2399 TG concentrations on Day 0, Day 20 and Day 62. Sediment and aqueous samples were analysed for S-2399 TG using LC/MS/MS.

Statistics

The adverse effects on percent survival, percent emergence and percent hatch were determined after transformation (e.g. arcsine square-root percentage).

An Unequal Variance Two-Sample t-Test, an Equal Variance Two-Sample t-Test or Wilcoxon’s Rank Sum Two-Sample Test was used to compare the performance of the control organisms with that of the solvent control organisms in order to determine if there were any statistically significant positive or negative effects.

Both percent hatch and male emergence rate in the solvent control group was significantly greater than the negative control group. As the solvent control and negative control data was similar for the majority of endpoints and the differences referenced above were within natural variability for this species, it was considered that the addition of solvent did not have a significant impact and therefore all statistical analyses were performed comparing treatment data to the negative control data.

Shapiro-Wilk’s Test for normality was conducted to compare the observed sample distribution with a normal distribution for all endpoints. Analysis of the data for all endpoints with the exception of percent emergence, male emergence rate, egg hatchability and time to oviposition met the assumption for normality.

The assumption of homogeneity of variance was analysed with Modified Levene's Test or Barlett's Test. Analysis of the data for all endpoints, with the exception of egg hatchability met the assumption of homogeneity.

Steel's Many-One Rank Sum Test was used to establish effects for percent emergence. Wilcoxon's Test with Bonferroni's Adjustment was used to establish treatment effects for egg hatchability, male emergence rate and time to oviposition. Dunnett's Multiple Comparison Test was used to establish treatment effects for 20-day survival and egg masses per female. Bonferroni's Adjusted t-Test was used to establish treatment effects for all remaining endpoints. CETIS™ v.188 was used to perform the computations.

The results were used to establish the highest test concentration that showed no statistically significant effect (No-Observed-Effect Concentration, NOEC) and the lowest test concentration that showed a statistically significant effect (Lowest-Observed-Effect Concentration, LOEC) from the appropriate control data.

If no concentration tested during this study resulted in ≥ 50% reduction in any of the endpoints (other than survival) at test Day 20 or test termination, then the EC_{50} value was empirically estimated to be greater than the highest mean concentration tested. If no concentration tested during this study resulted in ≥ 50% mortality, then the LC_{50} value was empirically estimated to be greater than the highest mean concentration tested.

RESULTS AND DISCUSSION

Test conditions

Dissolved oxygen was maintained at or above 2.5 mg/L throughout the exposure. Daily measurements of the temperature in the overlying water of each test vessel ranged from 21 to 24 ºC. Continuous monitoring in an auxiliary vessel established a temperature range of 21 to 24 ºC throughout the definitive study.

Total ammonia (as nitrogen) in pore water samples measured in the negative control was 1.8, 0.75 and ≤ 0.10 mg/L, respectively, on days 0, 20 and 62. Pore water pH measured in the negative control was 6.2, 6.5 and 6.3, respectively, on days 0, 20 and 62.

Analytical results

Analysis of the dosing stock solutions resulted in measured concentrations ranging from 92 to 110% of nominal concentration. Analysis of dosed sediment samples after mixing and prior to allocation into the test vessels resulted in recoveries ranging from 89 to 120% of nominal concentrations. These results indicate that the appropriate amount of test substance was initially introduced to the sediment during the dosing procedure.

Mean measured concentrations of S-2399 TG in sediment ranged from 84 to 110% of nominal and defined the treatment levels tested as 1.3, 3.2, 9.9, 28 and 92 mg a.s./kg.

Based on the analytical results of sediment, pore water and overlying water during this study, the majority of S-2399 TG applied remained associated with the sediment throughout the exposure and no significant degradation was observed throughout the sediment phase of the exposure.

The calculated partition coefficient (K_d) for days 0, 20 and 62 are presented in Table 9.2.6-12. Overall partition coefficient (K_d) values for the 1.2, 3.6, 11, 33 and 100 mg a.s./kg treatments were 40, 30, 23, 25 and 24, respectively.

Table 9.2.6-12: Calculated partition coefficient (Kd) values during the chronic exposure of midge (Chironomus dilutus) to S-2399 TG

Nominal sediment concentration (mg a.s./kg)	K_d			
	Day 0	Day 20	Day 62	Overall Mean
1.2	29	47	42	40
3.6	19	30	40	30
11	20	24	24	23
33	12	44	20	25
100	10	15	48	24

$K_d = C_{sediment} / C_{pore\ water}$

A summary of the concentrations of S-2399 TG in the overlying water, pore water and sediment are presented in Table 9.2.6-13.

Table 9.2.6-13: Measured concentrations of S-2399 TG measured in overlaying water, pore water and sediment samples

Nominal conc (mg a.s./kg dw)	Measured concentration											
	Overlying water (mg a.s./L)				Pore water (mg a.s./L)				Sediment (mg a.s./kg)			
	Day 0	Day 20	Day 62	Mean[b]	Day 0	Day 20	Day 62	Mean	Day 0 (% recovery)[b]	Day 20 (% recovery)[b]	Day 62 (% recovery)[b]	Mean (% recovery)
Control	< 0.00020[a]	< 0.00020[a]	< 0.00020[a]	n.a.	< 0.020[a]	< 0.020[a]	< 0.020[a]	n.a.	<0.10[a] (n.a.)	<0.10[a] (n.a.)	<0.10[a] (n.a.)	n.a. (n.a.)
Solvent	< 0.00020	< 0.0002	< 0.0002	n.a.	< 0.020	< 0.020	< 0.020	n.a.	<0.10[a] (n.a.)	<0.10[a] (n.a.)	<0.10[a] (n.a.)	n.a. (n.a.)

Nominal conc (mg a.s./kg dw)	Measured concentration											
	Overlying water (mg a.s./L)				Pore water (mg a.s./L)				Sediment (mg a.s./kg)			
	Day 0	Day 20	Day 62	Mean[b]	Day 0	Day 20	Day 62	Mean	Day 0 (% recovery)[b]	Day 20 (% recovery)[b]	Day 62 (% recovery)[b]	Mean (% recovery)
control	[a]	0 [a]	0 [a]		[a]	[a]	[a]					
1.2	0.0028	0.00041	0.00029	0.0017	0.043	0.027	0.032	0.034	1.3 (108)	1.3 (108)	1.3 (108)	1.3 (110)
3.6	0.013	0.0020	0.00078	0.0053	0.18	0.090	0.091	0.12	3.4 (94)	2.7 (75)	3.7 (103)	3.2 (90)
11	0.061	0.0043	0.0032	0.023	0.53	0.42	0.37	0.44	11 (100)	10 (91)	8.7 (79)	9.9 (90)
33	0.15	0.016	0.0084	0.058	2.2	0.75	1.2	1.4	26 (79)	33 (100)	24 (73)	28 (84)
100	0.58	0.025	0.033	0.21	8.3	5.2	2.4	5.3	81 (81)	80 (80)	120 (120)	92 (92)

[a] Concentrations expressed as less than values were below the minimum detectable limit (MDL)
[b] Calculated by HSE using rounded values provided in table.
n.a. = not applicable

Biological observations

The effects of exposure on midge survival and larval growth after 20 days are presented in Table 9.2.6-14. Survival is described in terms of midges not larvae as some individuals by day 20 had developed into adults.

Table 9.2.6-14: Mean percent midge survival and mean larval AFDW (after 20 days)

Mean measured concentration (mg a.s./kg sediment dw)	Mean percent survival (SD)	Mean AFDW per larvae in mg (SD)
Control	85 (4)	2.35 (0.66)
Solvent control	92 (0)	2.02 (0.34)
1.3	90 (4)	1.96 (0.47)
3.2	88 (5)	3.04 (0.55)
9.9	81 (8)	1.86 (0.35)
28	88 (8)	2.36 (0.49)
92	71 (14)	2.59 (1.10)

SD = standard deviation

The effects of exposure on midge emergence, emergence rate and days to death are summarised in Table 9.2.6-15. A significant difference was determined in percent emergence among midges exposed to the 3.2 mg/kg weight treatment level compared to the control. However, due to the lack of a clear dose response, this observation was not considered to be toxicant related by the study conductor.

Table 9.2.6-15: Percent emergence, mean emergence rate and mean days to death (after 62 days)

Mean measured concentration (mg a.s./kg sediment dw)	Mean percent emerged (SD)	Mean percent emerged, reduction in relation to control (%)	Mean percent emerged, reduction in relation to solvent control (%)	Mean male emergence rate (SD)	Mean female emergence rate (SD)	Mean male days to death (SD)	Mean female days to death (SD)
Control	80 (14)	0.0	-10	0.0508 (0.0051)	0.0451 (0.0036)	3.5 (0.97)	3.1 (0.61)
Solvent control	72 (10)	10.0	0	0.0561 (0.0030)	0.0462 (0.0029)	3.0 (0.86)	2.8 (0.73)
1.3	64 (18)	20.0	11.1	0.0523 (0.0025)	0.0463 (0.0039)	3.4 (1.3)	3.1 (0.40)
3.2	59* (16)	26.3	18.1	0.0508 (0.0079)	0.0435 (0.0046)	2.5 (0.76)	2.9 (0.38)
9.9	61 (17)	23.8	15.3	0.0523 (0.0065)	0.0451 (0.0062)	3.7 (1.1)	3.5 (0.50)
28	70 (10)	12.5	2.8	0.0547 (0.0051)	0.0471 (0.0041)	3.6 (1.7)	3.2 (0.75)
92	60 (25)	25	16.7	0.0535 (0.0036)	0.0494 (0.0020)	3.9 (1.2)	3.3 (0.67)

SD = standard deviation

*Significant reduction compared to the control, based on Steel's Many-One Rank Sum Test. This observation was not considered to be toxicant related due to the lack of a clear dose response

The number of egg masses, eggs per egg mass, eggs per mated female, percent hatch and days to oviposition are presented in Table 9.2.6-16. No significant effect was determined for any reproduction associated tracked variable.

Table 9.2.6-16: Mean number of egg masses per mated female, eggs per mass, number of eggs per mated female, percent hatch and days to oviposition

Mean measured concentration (mg a.s./kg sediment dw)	Mean egg masses per mated female (SD)	Mean eggs per egg mass (SD)	Mean number of eggs per mated female (SD)	Mean percent hatch (SD)	Mean days to oviposition (SD)
Control	0.67 (0.26)	894 (175)	593 (267)	84 (16)	1.1 (0.20)
Solvent control	0.62 (0.32)	1112 (267)	696 (384)	97 (1)	1.0 (0)
1.3	0.78 (0.21)	860 (239)	677 (260)	91 (4)	1.1 (0.19)
3.2	0.55 (0.39)	1017 (140)	750 (260)	86 (12)	1.0 (0)
9.9	0.48 (0.40)	1022 (254)	677 (451)	84 (12)	1.1 (0.20)
28	0.45 (0.27)	903 (160)	448 (174)	76 (34)	1.2 (0.38)
92	0.42 (0.33)	1384 (223)	749 (281)	91 (5)	1.1 (0.12)

SD = standard deviation

A summary of the established endpoints for this study according to the applicant are presented in Table 9.2.6-17.

Table 9.2.6-17: Summary of defined endpoints for tracked variables

Endpoint	Mean measured sediment (mg a.s./kg)	Mean measured pore water (mg a.s./kg)
Midge larval survival (20 days)		
LOEC	> 92	> 5.3
NOEC	92	5.3
LC_{50}	> 92	> 5.3
Midge larval growth (20 days)		
LOEC	> 92	> 5.3
NOEC	92	5.3
EC_{50}	> 92	> 5.3
Percent emergence (62 days)		
LOEC	> 92	> 5.3
NOEC	92	5.3

Endpoint	Mean measured sediment (mg a.s./kg)	Mean measured pore water (mg a.s./kg)
EC_{50}	> 92	> 5.3
Male emergence rate (62 days)		
LOEC	> 92	> 5.3
NOEC	92	5.3
EC_{50}	> 92	> 5.3
Female emergence rate (62 days)		
LOEC	> 92	> 5.3
NOEC	92	5.3
EC_{50}	> 92	> 5.3
Males days to death (62 days)		
LOEC	> 92	> 5.3
NOEC	92	5.3
EC_{50}	> 92	> 5.3
Females days to death (62 days)		
LOEC	> 92	> 5.3
NOEC	92	5.3
EC_{50}	> 92	> 5.3
Days to oviposition (62 days)		
LOEC	> 92	> 5.3
NOEC	92	5.3
EC_{50}	> 92	> 5.3
Egg masses per mated female (62 days)		
LOEC	> 92	> 5.3
NOEC	92	5.3
EC_{50}	> 92	> 5.3
Eggs per mated female (62 days)		
LOEC	> 92	> 5.3
NOEC	92	5.3

Endpoint	Mean measured sediment (mg a.s./kg)	Mean measured pore water (mg a.s./kg)
EC_{50}	> 92	> 5.3
Eggs per egg mass (62 days)		
LOEC	> 92	> 5.3
NOEC	92	5.3
EC_{50}	> 92	> 5.3
Percent hatch (62 days)		
LOEC	> 92	> 5.3
NOEC	92	5.3
EC_{50}	> 92	> 5.3

VALIDITY CRITERIA

The validity criteria for the test were met according to EPA Test Method 100.5 (2000) (Table 9.2.6-18). The study was also checked against OECD 218 (2004) and OECD 233 (2010). There were three deviations from the OECD 218 (2004) and OECD 233 (2010) validity criteria: 1) on Day 45 overlying water temperature in replicate A of the control was recorded below the acceptable range; 2) dissolved oxygen was frequently below the 60 % ASV threshold; and 3) time to emergence for males was < 20 days.

Table 9.2.6-18: Compliance with validity criteria for EPA Test Method 100.5 and OECD 218 (2004)

Validity criterion	Required	Obtained
Midge survival (20 days) (EPA Test Method 100.5)	≥ 70 %	Control = 85 % Solvent control = 92 %
Midge growth (20 days) (EPA Test Method 100.5)	≥ 0.48 mg/larvae (AFDW)	Control = 2.35 mg/larvae Solvent control = 2.02 mg/larvae
Emergence (EPA Test Method 100.5, OECD 218 (2004) & OECD 233 (2010))	≥ 50 % (EPA Test Method 100.5) ≥ 70 %	Control = 80 % Solvent control = 72 %

Validity criterion	Required	Obtained
	(OECD 218 (2004) & OECD 233 (2010))	
Number of eggs / egg case (EPA Test Method 100.5)	≥ 800	Control = 894 Solvent control = 1112
Percent hatch (EPA Test Method 100.5)	≥ 80 %	Control = 84 % Solvent control = 97 %
Dissolved oxygen in overlying water (EPA Test Method 100.5, OECD 218 (2004) & OECD 233 (2010))	≥ 2.5 mg/L (EPA Test Method 100.5) ≥ 60 % ASV (OECD 218 (2004) & OECD 233 (2010))	2.5 – 8.6 mg/L 29.1 % - 100 %
Time to emergence (OECD 218 (2004) & OECD 233 (2010))	20 - 65 days	Control mean male emergence length = 18.6 days[a] Control mean female emergence length = 20.3 days[a]
pH (OECD 218 (2004) & OECD 233 (2010))	6 - 9	6.4 – 7.5
Overlying water temperature (OECD 218 (2004) & OECD 233 (2010))	22 – 24 °C	21 – 24 °C
Sex ratio OECD 233 (2010)	0.4 ≥ sex ratio ≤ 0.6	Not reported
Egg masses per female OECD 233 (2010)	0.6	Control = 0.67 Solvent control = 0.62

Validity criterion	Required	Obtained
Egg masses per reproductive chamber	0.6	Not reported

[a]Calculated using the reciprocal of mean male/female emergence rate.

CONCLUSION

Based on all measured parameters and the mean measured concentrations of S-2399 TG in the sediment, the No-Observed-Effect Concentration (NOEC) for the midge (*Chironomus dilutus*) was determined to be 92 mg a.s./kg by the study conductor. The Lowest-Observed-Effect Concentration (LOEC) was determined to be > 92 mg a.s./kg.

Since no concentration tested resulted in ≥ 50% reduction of midge survival, growth and reproduction when compared to the control data, both LC_{50} and EC_{50} values were empirically estimated to be > 92 mg a.s./kg, the highest concentration tested.

HSE COMMENTS

The study was carried out according to GLP and follows OCSPP Draft Guideline 850.1760. and EPA Test Method 100.5 (2000). It is noted that some reference to OECD 218 (2004) was made. Therefore, the study was checked against EPA Test Method 100.5 (2000), OECD 218 (2004) and OECD 233 (2010). All validity criteria for EPA Test Method 100.5 (2000) were met that were compatible with the test method carried out.

As outlined in the validity criteria section there were several deviations from the OECD 218 (2004) and OECD 233 (2010) validity criteria:

On Day 45 overlying water temperature in replicate A of the control was recorded below the acceptable range. Acceptable survival and reproduction in the control suggest this temperature deviation did not impact the study outcome. HSE consider this a minor deviation.

Dissolved oxygen was frequently below the 60 % ASV threshold. However, this study was conducted according to EPA Test Method 100.5 (2000), which had a lower dissolved oxygen limit. *C. dilutus* has been shown to display normal levels of survival, dry weight and biomass in response to 2.5 mg/L dissolved oxygen levels over 10 days in a study investigating the effects of reduced dissolved oxygen levels on sediment dwelling organisms (Mattson et al., 2008)[32]. Sub-lethal effects were only observed at ≤ 1.67 mg/L dissolved oxygen. In keeping with this, control individuals met all reported EPA Test Method 100.5, OECD 218 (2004) and

[32] Mattson, V.R., Hockett, J.R., Highland, T.L., Ankley, G.T., Mount, D.R., 2008. Effects of low dissolved oxygen on organisms used in freshwater sediment toxicity tests. Chemosphere. Vol 79, Iss 10, pgs. 1840-1844.

OECD 233 (2010) survival, reproduction and development-based validity criteria except time to emergence, discussed below. For these reasons, HSE consider this an acceptable deviation.

The third deviation is the shorter than expected time to emergence for male midges (18.6 days). This is close to the 20-day minimum emergence length stated in OECD 218 (2004) and OECD 233 (2010) and this deviation could be a product of methodological differences between EPA Test Method 100.5 (2000) and OECD 218 (2004). HSE considers this a minor deviation.

The final validity criteria deviations were with respect to OECD 233 (2010). Two validity criteria (sex ratio and egg masses per reproductive chamber) were not reported within the study. Therefore, it was not possible to assess the results against the specified criteria. This is acceptable as the study followed EPA Test Method 100.5 (2000).

The following deviations from EPA Test Method 100.5 (2000) not relating to the validity criteria were noted:
Table 15.1 of EPA Test Method 100.5 (2000) outlines the required test conditions, which includes a temperature range of 23 ± 1 °C for the daily mean test temperature (also covered by section 15.3.5.1). One daily measurement in the overlying water was recorded outside of this range, on day 45 (21.4 °C). Temperatures measured in the overlying water in the test vessels were still within acceptable ranges for the rest of the exposure and were within the tolerance range of the organisms for all test days. Therefore, this temperature deviation is not expected to have impacted the results of the study. HSE consider this an acceptable deviation.

Table 15.1 of EPA Test Method 100.5 (2000) also states the age of larvae at exposure initiation as < 24 hours (also covered by section 15.3.2). The study used three-day old larvae, which is acceptable according to OECD 218 (2004) paragraph 25. Most importantly, midge larvae were confirmed to be three days old at exposure initiation, indicating that age matching was achieved. HSE consider this a minor deviation, which is not expected to have impacted the study results.

Table 15.1 of EPA Test Method 100.5 (2000) further outlines the conditions for test duration (also covered in section 15.3.9). It states, "*each treatment is ended separately when no additional emergence has been recorded for seven consecutive days*". For this study, all vessels were terminated on day 62. No emergence was recorded for any replicate in any treatment level from day 52 to 62. This demonstrates that all emergence events were captured. HSE consider this a minor deviation with minimal impact on the study results.

Table 15.1 of EPA Test Method 100.5 (2000) covers the endpoints that should be tracked and reported. Adult mortality is included yet not reported by the study conductor.

Table 15.2 of EPA Test Method 100.5 (2000) describes a general activity schedule for the long-term sediment toxicity test. After the addition of the larvae to the test vessel it recommends that the beaker is left to sit outside the test system for one hour. This was either not performed or not reported. Given the acceptable control survival, growth and reproduction criteria this minor deviation is unlikely to have impacted the study results. HSE consider this deviation acceptable.

Table 15.2 of EPA Test Method 100.5 (2000) states, "*in preparation for weight determinations, ash weigh pans at 550°C for 2 h. Note that the weigh pans should be ashed before use to eliminate weighing errors due to the pan oxidizing during ashing of samples*" (also covered by section 15.3.8.3.3). This was either not reported or not performed, which may have introduced weighing errors that were not shared equally across treatments and replicates (replicates and treatments ashed first would have lost more weight if the same pans were reused for later replicates). HSE notes this uncertainty and will consider it during the risk assessment if necessary.

Table 15.3 of EPA Test Method 100.5 (2000) states, "*hardness, alkalinity, and ammonia in the overlying water typically should not vary by more than 50% during the test, and dissolved oxygen should be maintained above 2.5 mg/L in the overlying water*" (also covered by section 15.3.4.1). Ammonia varied between ≤ 0.10 – 1.1 mg/L and alkalinity from 14 to 26 mg/L. For ammonia, high levels were found for all treatment groups at day 10 that markedly decreased by day 20 due to the increased rate of water renewal. Due to the weakly basic nature of ammonia, relatively high alkalinity reading were also reported on day 10. Considering the acceptable control survival, growth and reproductive capacity, the transient increase in ammonia and alkalinity is unlikely to have impacted the study results. Furthermore, as high ammonia and alkalinity was present for every treatment level any potential effects were likely shared equally across treatment levels. HSE consider this a minor deviation.

Section 15.3.1 of EPA Test Method 100.5 (2000) details the production and collection of egg cases during the rearing of larval midges used for the experiment. This level of detail was not provided in the study report. The appropriateness of the deployed rearing method was demonstrated by the approximate 80 % percent hatch rate of egg masses from which experimental larvae were derived. HSE consider this a minor reporting omission and acceptable.

Section 15.3.4.1 of EPA Test Method 100.5 (2000) addresses the renewal of overlying water. It recommends two volume additions of overlying water should be delivered to the test chambers daily. From day 12 this study performed four volume additions to increase DOC levels and reduce ammonia concentrations. This modification improved water quality conditions test individuals were exposed to. HSE consider this an acceptable deviation.

One minor reporting omission pertaining to Section 15.3.4.1 of EPA Test Method 100.5 (2000) was the lack of flow rate variation. The guideline states, "*at any particular time during the test, flow rates through any two test chambers should not differ by more than 10%*". Calibration of the renewal system was checked at test initiation and termination. Furthermore, a complete check of the renewal and water delivery systems were made daily. HSE consider this reporting omission minor as it is unlikely to have impacted the study results.

Section 15.3.8.4.1 of EPA Test Method 100.5 (2000) recommends that emergence traps be placed on reproductive replicates from day 20. The study did this on day 14. HSE consider this an acceptable deviation.

Section 15.4.6.4 of EPA Test Method 100.5 (2000) reports typical coefficient of variation (CV) values for various endpoints in the *C. dilutus* life-cycle test. CVs of < 20 % and < 10 % were stated for reproduction as mean eggs per female and percent hatch, respectively. In the study reproduction as mean eggs per female and percent hatch had CVs of 45 % and 19 %. HSE notes the unusually high variation in these endpoints and will take this into account during the risk assessment if necessary.

For one endpoint, percent emergence, one treatment level displayed a statistically significant reduction (3.2 mg a.s./kg sediment dw) compared to the control. The three higher treatment levels, however, did not result in a statistically significant reduction. Due to the lack of a clear dose-response the study conductor concluded that this statistically significant effect was not toxicant related or biologically relevant. However, if the mean percent reduction for each treatment level is expressed in terms of percentage reduction in comparison to the control, every treatment level bar one (28 mg a.s./kg sediment dw) displays a > 20 % emergence reduction. If the solvent control is used as a reference, then each treatment level, except 28 mg a.s./kg sediment dw, displays a > 10 % emergence reduction. Such data patterns are challenging to interpret with any confidence. Due to the lack of a concentration-response and high degree of variation (CV = 13.8 to 41.7 % across treatment groups) combined with effects frequently > 10 % compared to either control for treatment groups, HSE has decided to not determine a NOEC for 62-day percent emergence.

For two endpoints (male emergence rate and percent hatch) a significant difference was detected between the control and solvent control. In both cases the solvent control had a stimulatory effect. The study conductor used the control as the reference level in both cases, justifying this approach by stating it was in accordance with current EPA guidance. As the solvent control had a stimulatory effect when compared to the control this approach was not precautionary and could have potentially underestimated the test substance effect. For example, for percent hatch, if the control is used as a reference the largest treatment % reduction is 9.5 % (28 mg a.s./kg sediment dw). If, however, the solvent control is selected

as a reference this % reduction increases to 21.6 %. The two variables, however, did not present a clear dose response, making interpretation challenging. HSE notes this decision and will consider its impact during the risk assessment if necessary.

Finally, for 20-day midge survival the 92 mg/kg treatment group presented a 16.5 % and 22.8 % reduction relative to the control and solvent control, respectively. This variable followed an approximate concentration response, although the level of variation in the 92 mg/kg treatment group was higher than other experimental groups (SD = 14 vs next highest SD = 8). As a precautionary measure, HSE has set the NOEC to 28 mg/kg for this variable.

Due to the failure to identify a NOEC for a key endpoint, 62-day percent emergence, HSE concludes that this study is not reliable and not suitable for use in risk assessment.

Reference:	KCA 8.2.5.4/02
Report Title:	42-Day Toxicity Test Exposing Freshwater Amphipods (*Hyalella azteca*) to S-2399 TG Applied to Sediment Under Static-Renewal Conditions Following EPA Test Methods
Author(s) & year:	[redacted] (2016)
Document No, Authority registration No:	12709.6365
Substance used:	S-2399 TG, 13CG0617G, 95 %
Method of analysis:	LC/MS/MS
Guideline(s):	Draft OCSPP Guideline 850.1770, EPA Test Method 100.4 (2000)
Deviations:	Yes, see HSE comments section.
GLP or GEP:	Yes
Acceptability:	Supplementary
Study relied upon:	No, not used in aquatic risk assessment

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: S-2399 TG
Description: Not stated
Lot/Batch: 13CG0617G

Purity:	95.0%
Expiration date:	23 July 2016
Reference item:	None
Solvent:	Solvent

B. STUDY DESIGN AND METHODS

1.	**Test animals:**	Freshwater amphipod (*Hyalella azteca*)
	Age:	8 Days
	Source:	In-house culture
	Acclimation:	9 Days
	Diet:	During acclimation, amphipods were fed a combination of yeast, cereal leaves and flaked fish food suspension (YCT) on a daily basis. During the exposure, each replicate vessel received 1.5 mL of YCT and 0.50 mg of flaked fish food, daily.
	Nominal concentrations:	6.3, 13, 25, 50 and 100 mg a.s./kg. The LOQ was 200 µg/kg and the MDL was 16.0 µg/kg.
	Test duration:	42 days
	Organisms per vessel:	10
	Exposure regime:	Semi-static
2.	**Overlying water:**	Laboratory well water
	Hardness:	60 to 64 mg $CaCO_3$/L
	Alkalinity:	20 to 24 mg $CaCO_3$/L
	pH:	6.5 to 7.1
	Conductivity:	290 to 470 µS/cm
	Sediment:	Artificial sediment prepared according to OECD Guideline 218
	Percent organic carbon:	2.6%
	Particle size distribution:	80% sand, 3% silt, 17% clay
	pH:	6.9
	Percent solids:	64.95%
	Water holding capacity:	39.3%
3.	**Test vessels:**	300 mL glass vessels containing 100 mL of sediment
4.	**Environmental conditions:**	
	Temperature:	20 – 24°C
	pH:	5.8 – 7.4
	Dissolved oxygen:	2.5 – 8.1 mg/L
	Photoperiod:	16 hours light: 8 hours darkness (210– 880 lux)

5. Animal assignment and treatment:

One day prior to test initiation (Day $^{-1}$), the treated and control sediments were allocated to the twelve replicate vessels per treatment or control (100 mL per vessel). Three additional replicates were established for the treated and solvent control sediments while six additional

replicates were established for the negative control. In the case of the negative control, additional replicates were also established for water quality measurements of pore water. Overlying water was gently added to each vessel and each vessel was placed in the renewal system.

At test initiation, 10 amphipods (8 days old) were impartially added to each test vessel. Each replicate vessel for the biological response contained 10 amphipods, a total of 120 amphipods per concentration or control. The additional replicates for analytical and pore water quality measurements were maintained under the same conditions and contained test organisms but were not used to evaluate the biological response of the test organisms. Amphipods were exposed to the treatment for 28 days, when four of the twelve replicates were randomly selected to determine survival and growth. The remaining eight replicates were removed from the test vessels and placed in water-only vessels containing a thin layer of silica sand, with reproduction and survival measured on Days 35 and 42. Additionally, at Day 28 any offspring were counted and recorded. At Day 42 growth (length) and sex ratio were also determined.

During the 42-day study, the overlying water was renewed by adding two volume additions of water per test vessel per day using an intermittent delivery system in combination with a calibrated water-distribution system. The intermittent delivery system was calibrated to provide 1L of water per cycle to the water-distribution system, which subsequently provided 50 mL of water per cycle to each vessel. The system cycled approximately 7 times per day, providing approximately 350 mL per vessel every 24 hours.

7. Dose preparation:

A 25 mg a.s./mL primary stock solution was prepared by placing 1.31531 g of S-2399 TG (1.24954 g a.s.) in a 50 mL volumetric flask and bringing it to volume with acetone. The resulting stock solution was observed to be clear and colourless with no visible undissolved test substance following preparation.

Five individual dosing stock solutions were prepared from the primary stock solution with 25 mL of acetone for application of the test substance to the sediment. All dosing solutions were observed to be clear and colourless with no visible undissolved test substance following preparation.

A 10 mL volume of each dosing stock solution was applied to 0.050 kg of fine silica sand and placed in petri dishes. Following evaporation of the solvent (25 min), the entire 0.050 kg of sand/test substance was added to 2.75 kg of wet sediment (1.7860 kg dry weight of test sediment based on a percent solids value of 64.95% with the 0.050 kg of dry sand for a total of 1.8360 kg) in individual glass jars. The jars were sealed and positioned horizontally on a rolling mill. Each jar was rolled at room temperature at approximately 15 rpm for four hours. The sediments were allowed to equilibrate for a 14-day period in the refrigerator. Once a week during this equilibrium period, the jars were rolled for two hours to ensure the sediment was homogeneous.

A solvent control was prepared in the same manner as the treated sediment by adding 10.0 mL of acetone, containing no test substance, to 0.050 kg of fine silica sand and the solvent was allowed to evaporate. The sand was then added to 2.75 kg wet weight of sediment and processed in the same manner as the treated sediments. The negative control sediment

group was prepared using only untreated sediment (2.75 kg wet weight).

8. Measurements and observations:

Daily observations of organisms' behaviour (i.e. adverse effects) were made and the physical characteristics of the sediment water system were recorded.

Dissolved oxygen concentration, temperature and pH were measured in the overlying water of each replicate vessel of each treatment level and control used for biological monitoring at Days 0 (exposure initiation), 28, 29 and 42 (test termination). On the remaining days, dissolved oxygen and temperature were measured in one alternating replicate of each treatment level and control each day. In addition, the temperature was continuously monitored in an auxiliary vessel. Total hardness, alkalinity, conductivity and ammonia concentration of the overlying water were monitored at Days 0, 28, 29 and 42 in each treatment level and control solution from the composite sample taken from the biological replicates.

At exposure initiation, Day 14 and Day 28, pH and ammonia (as nitrogen) concentration were measured in one pore water sample from each of three replicates of the control group only.

Prior to Day 28, four of the twelve replicate vessels were randomly selected. Amphipod survival and growth (length) were determined in these test vessels on Day 28 by sieving the sediment to remove all surviving amphipods through a fine mesh net with an opening of approximately 0.25 mm. The adults were preserved in sugar formalin solution for up to two weeks before taking images for length determination. Length was measured from the base of the first antenna to the tip of the third uropod along the curve of the dorsal surface to the nearest 0.01 mm. Any offspring observed at the end of the sediment exposure phase (Day 28) were counted and recorded. Amphipods in the remaining eight replicates, following determination of survival and growth, were placed in 300 mL water-only vessels containing a thin layer (approximately 5 mL) of silica sand. Reproduction and survival were measured on Days 35 and 42. On Day 35, adults were enumerated to assess Day 35 survival and returned to their respective test vessels after reproduction had been assessed. At test termination (Day 42), the adult amphipods were enumerated to assess Day 42 survival and preserved in sugar formalin solution for up to two weeks prior to taking images for length determination. The numbers of male and female amphipods were also determined.

Dosed sediments were sampled during the mixing/equilibrium period, prior to the allocation of the sediments into the replicate exposure vessels, In addition, subsamples of the dosing stock solutions were also analysed for test substance concentration. All pore water, overlying water and sediment samples from one replicate per treatment level and control were removed and analysed for S-2399 TG concentrations on Day 0, Day 14 and Day 28. Sediment and aqueous samples were analysed for S-2399 TG using LC/MS/MS. The LOQ was 200 µg/kg and the MDL was 16.0 µg/kg.

9. Statistics:

The adverse effects on percent survival, percent emergence and percent hatch were determined after transformation (e.g., arcsine square-root percentage). An equal variance two sample t-test was conducted on 28-day growth, 35-day reproduction, 42-day survival, 42-day growth and 42-day reproduction data to compare the performance of control

organisms to that of the solvent control. An Unequal Variance Two Sample t-Test was conducted on male:female ratio to compare control and solvent control organisms. Wilcoxon's Rank Sum Two-Sample Test was conducted on 28-day and 35-day survival data to compare control, and solvent control organisms. All remaining statistical analyses were performed on control data.

Shapiro-Wilk's Test for normality was conducted to compare the observed sample distribution with a normal distribution for all endpoints. Analysis of the data for all endpoints with the exception of 28- and 35-day survival, 35-and 42-day reproduction and 42-day male:female ratio data, met the assumption for normality.

The assumption of homogeneity of variance was analysed with Barlett's Test. Analysis of the data for all endpoints, with the exception of 28-day survival, 35-day reproduction and 42-day male:female ratio met the assumption of homogeneity.

Steel's Many-One Rank Sum Test was used to establish effects for 28- and 35-day survival. Wilcoxon's Test with Bonferroni's Adjustment was used to establish treatment effects for 35- and 42-day reproduction and 42-day male:female ratio. Dunnett's Multiple Comparison Test was used to establish treatment effects for the remaining endpoints (28- and 42-day length and 42-day survival). CETIS™ v.1.8 was used to perform the computations.

The results were used to establish the highest test concentration that showed no statistically significant effect (No-Observed-Effect Concentration, NOEC) and the lowest test concentration that showed a statistically significant effect (Lowest-Observed-Effect Concentration, LOEC) from the appropriate control data.

If the data was not appropriate for the calculation of a point estimate or no concentration tested during this study resulted in ≥ 50% reduction in any of the sublethal endpoints at Days 28, 35 and 42, then the EC_{50} value was empirically estimated to be greater than the highest mean sediment concentration tested. If no concentration tested during this study resulted in ≥ 50% mortality, then the LC_{50} value was empirically estimated to be greater than the highest mean concentration tested.

II. RESULTS AND DISCUSSION

A. BIOLOGICAL EFFECTS

A summary of survival and growth of the amphipod after 28 days is presented in Table 9.2.6-19.

Survival and reproduction determined on Day 35 are presented in Table 9.2.6-20. A significant difference was determined in survival among amphipods exposed to the 45 mg a.s./kg treatment level compared to the control and in the mean number of offspring per female in the 5.6 mg a.s./kg treatment level compared to the control (based on mean measured concentration). Since no concentration resulted in ≥ 50% mortality when compared to the control data, the 35-day LC_{50} value for survival was empirically estimated to be > 93 mg a.s./kg, the highest mean measured sediment concentration.

Survival, growth, reproduction and sex ratio on Day 42 are presented in Table 9.2.6-21. A significant difference in survival among amphipods exposed to the 22 and 45 mg a.s./kg

treatment levels compared to the control. Since no concentration resulted in ≥ 50% mortality when compared to the control data, the 42-day LC_{50} value for survival was empirically estimated to be > 93 mg a.s./kg, the highest mean measured sediment concentration. No significant difference in length or male:female ratio among any treatment levels was determined. Since no concentration resulted in ≥ 50% mortality when compared to the control data, the 42-day EC_{50} value for growth and male:female ratio was empirically estimated to be > 93 mg a.s./kg, the highest mean measured sediment concentration. No significant difference in reproduction among any treatment levels was determined.

Table 9.2.6-19: Mean percent survival and mean length per amphipod on Day 28

Mean measured sediment concentration (mg a.s./kg)	Mean percent survival (± SD)	Mean length per amphipod (mm) (±SD)
Control	99 (3)	5.09 (0.16)
Solvent control	96 (5)	5.27 (0.24)
5.6	97 (5)	5.20 (0.18)
12	96 (9)	5.31 (0.10)
22	95 (5)	5.07 (0.26)
45	92 (14)	4.97 (0.36)
93	94 (7)	5.17 (0.28)

SD = standard deviation

Table 9.2.6-20: Mean percent survival and mean number of offspring released per female on Day 35

Mean measured sediment concentration (mg a.s./kg sediment dw)	Mean percent survival (± SD)	Mean number of offspring released per female (±SD)
Control	98 (7)	3.7 (1.3)
Solvent control	95 (8)	3.1 (3.2)
5.6	95 (5)	1.5 (1.5) **
12	89 (14)	3.0 (2.4)
22	91 (8)	2.1 (3.0)
45	84 (15) *	3.0 (2.2)
93	93 (7)	6.5 (5.1)

*Significantly reduced compared to the negative control, based on Steel's Many-One Rank Sum Test.

**Significantly reduced compared to the negative control, based on Wilcoxon's Test with Bonferroni's Adjustment.
SD = standard deviation
n.a. = not applicable

Table 9.2.6-21: Mean percent survival of adult amphipods, mean growth (length), mean number of offspring released per female and mean male:female ratio on Day 42

Mean measured sediment concentration (mg a.s./kg)	Mean percent survival (± SD)	Mean length per amphipod (mm) (± SD)	Mean number of offspring released per female (± SD)
Control	96 (7)	5.49 (0.25)	8.7 (6.2)
Solvent control	88 (14)	5.94 (0.29)	7.5 (6.8)
5.6	94 (7)	5.77 (0.29)	3.9 (3.6)
12	85 (15)	5.75 (0.25)	5.9 (3.5)
22	83 (13) *	5.70 (0.26)	4.3 (4.9)
45	80 (19) *	5.99 (0.31)	6.1 (5.7)
93	90 (9)	5.78 (0.34)	12 (11)

SD = standard deviation
* Significantly reduced compared to the negative control, based on Dunnett's Multiple Comparison Test.

Table 9.2.6-22: Survival (Day 28)

Endpoint	Mean measured sediment (mg a.s./kg)	Mean measured pore water (mg a.s./L)
LOEC	>93	>6.1
NOEC	93	6.1
LC_{50} (95% Confidence Intervals)	>93 (n.a.)	>6.1 (n.a.)

n.a. = not applicable. LC/EC_{50} value was empirically estimated. Therefore, corresponding 95% confidence intervals could not be determined

Table 9.2.6-23: Growth (Day 28)

Endpoint	Mean measured sediment (mg a.s./kg)	Mean measured pore water (mg a.s./L)
LOEC	>93	>6.1
NOEC	93	6.1

Endpoint	Mean measured sediment (mg a.s./kg)	Mean measured pore water (mg a.s./L)
EC_{50} (95% Confidence Intervals)	>93 (n.a.)	>6.1 (n.a.)

n.a. = not applicable. LC/EC_{50} value was empirically estimated. Therefore, corresponding 95% confidence intervals could not be determined

Table 9.2.6-24: Survival (Day 35)

Endpoint	Mean measured sediment (mg a.s./kg)	Mean measured pore water (mg a.s./L)
LOEC	>93	>6.1
NOEC	>93	6.1
LC_{50} (95% Confidence Intervals)	>93 (n.a.)	>6.1 (n.a.)

n.a. = not applicable. LC/EC_{50} value was empirically estimated. Therefore, corresponding 95% confidence intervals could not be determined

Table 9.2.6-25: Reproduction (Day 35)

Endpoint	Mean measured sediment (mg a.s./kg)	Mean measured pore water (mg a.s./L)
LOEC	>93	>6.1
NOEC	>93	6.1
EC_{50} (95% Confidence Intervals)	>93 (n.a.)	>6.1 (n.a.)

n.a. = not applicable. LC/EC_{50} value was empirically estimated. Therefore, corresponding 95% confidence intervals could not be determined

Table 9.2.6-26: Survival (Day 42)

Endpoint	Mean measured sediment (mg a.s./kg)	Mean measured pore water (mg a.s./L)
LOEC	>93	>6.1
NOEC	93	6.1
LC_{50} (95% Confidence Intervals)	>93 (n.a.)	>6.1 (n.a.)

n.a. = not applicable. LC/EC_{50} value was empirically estimated. Therefore, corresponding 95% confidence intervals could not be determined

Table 9.2.6-27: Growth (Day 42)

Endpoint	Mean measured sediment (mg a.s./kg)	Mean measured pore water (mg a.s./L)
LOEC	>93	>6.1
NOEC	93	6.1
EC_{50} (95% Confidence Intervals)	>93 (n.a.)	>6.1 (n.a.)

n.a. = not applicable. LC/EC_{50} value was empirically estimated. Therefore, corresponding 95% confidence intervals could not be determined

Table 9.2.6-28: Reproduction (Day 42)

Endpoint	Mean measured sediment (mg a.s./kg)	Mean measured pore water (mg a.s./L)
LOEC	>93	>6.1
NOEC	93	6.1
EC_{50} (95% Confidence Intervals)	>93 (n.a.)	>6.1 (n.a.)

n.a. = not applicable. LC/EC_{50} value was empirically estimated. Therefore, corresponding 95% confidence intervals could not be determined

Table 9.2.6-29: Male:Female Ratio (Day 42)

Endpoint	Mean measured sediment (mg a.s./kg)	Mean measured pore water (mg a.s./L)
LOEC	>93	>6.1
NOEC	93	6.1
EC_{50} (95% Confidence Intervals)	>93 (n.a.)	>6.1 (n.a.)

n.a. = not applicable. LC/EC_{50} value was empirically estimated. Therefore, corresponding 95% confidence intervals could not be determined

B. ANALYSIS

Analysis for S-2399 TG concentration of the dosing stock solution samples ranged from 94 to 110% of the nominal concentrations.

Table 9.2.6-30 summarises the measured concentrations of S-2399 TG in the overlying, pore water and sediment samples at the end of the exposure period (28 days).

Table 9.2.6-30: Measured concentrations of S-2399 TG measured in overlaying water, pore water and sediment samples

Nominal concentration (mg a.s./kg dw)	Measured concentration		
	Overlying water (mg a.s./L)	Pore water (mg a.s./L)	Sediment (% nominal) (mg a.s./kg dw) [a]
	Day 28	Day 28	Mean
Control	< 0.00020	< 0.010	n.a. (n.a.)
Solvent control	< 0.00020	< 0.010	n.a. (n.a.)
6.3	0.0036	0.18	5.6 (89)
13	0.0069	0.41	12 (91)
25	0.019	0.97	22 (89)
50	0.041	2.1	45 (91)
100	0.096	4.7	93 (93)

[a] Mean and percent recovery values were calculated using the actual analytical results and not the rounded values (two significant figures) presented in this table
n.a. = not applicable

C. VALIDITY CRITERIA

The following criteria were included based on the OCSPP Guideline 850.1770:

1. During the 28 day exposure period, amphipod survival and growth in both the control and solvent control was ≥ 80% and ≥ 3.2 mm per amphipod, respectively (actual: 99 and 96% for control, and solvent control survival, respectively, 5.09 and 5.27 mm per amphipod for control and solvent control, respectively).
2. Cumulative reproduction between Days 28 and 42 was ≥ 2 offspring per control female (actual: 8.7 and 7.5 for control and solvent control, respectively).

The study was therefore considered to be valid.

As the OCSPP Guideline 850.1770 is not yet available, the Test Acceptability Requirements from the EPA_600_R99_064 (2000) have also been displayed in Table 9.2.6-31:

Table 9.2.6-31: Test Acceptability Requirements from EPA_600_R99_064 (2000)

Validity criteria	Required EPA_600_R99_064 (2000)	Obtained
Age of organism at start of test	7 to 8 days	8 days
28 day survival	Average ≥ 80%	80-96% for controls and test concentrations

Validity criteria	Required EPA_600_R99_064 (2000)	Obtained
Water quality variability	Hardness, alkalinity and ammonia in overlying water should not very by more than 50%	60-64% hardness 20 to 24 mg/L alkalinity ≤ 0.10 to 0.69mg/L ammonia
Dissolved oxygen concentration	>2.5mg/L in overlying water	>2.5mg/L
Length	>3.2mm/individual	>3.2mm/individual
Reproduction*	>2 young/per female between day 28 and 42	3.9 – 12

*Reproduction was more variable within and among laboratories; hence, more replicates might be needed to establish statistical differences among treatments with this endpoint.

III. **CONCLUSION**

Based on all measured parameters and mean measured concentrations of S-2399 TG, the No-Observed-Effect Concentration (NOEC) for the sediment-dwelling freshwater amphipod *(Hyalella azteca*) was determined to be 93 mg a.s./kg by the applicant. The Lowest-Observed-Effect Concentration was determined to be > 93 mg a.s./kg. Since no concentration tested resulted in ≥ 50% reduction in survival, growth and reproduction when compared with the control data, the LC_{50} and EC_{50} values were empirically estimated to be > 93 mg a.s./kg, the highest mean measured sediment concentration tested.

HSE COMMENTS:

This study was conducted under GLP and under OCSPP Guideline 850.1770 and has been assessed against EPA_600_R-99_064 (2000) Test Method 100.4 as OCSPP Guideline 850.1770 is not yet publicly available.

The concentrations of the test item were maintained between 80-120% of the nominal value throughout the test. Results should be based on nominal concentrations but have been expressed by the applicant as mean measured concentrations.

A statistically significant difference in survival was determined after 35 days in the 45mg a.s./kg treatment level compared to the control, and in the mean number of offspring per female in the 5.6mg a.s./kg treatment level compared to the control based on mean measured concentrations. The applicant has stated that, due to a lack of clear dose response and relatively high survival throughout the concentration range, these results are not considered to be toxicant-related. For 35-day survival, there is no clear dose-response and comparable survival for the highest concentration tested compared to the control (5.1 % reduction) and solvent control (2.1 % reduction). HSE agrees with the applicant that the effect at 45 mg a.s./kg is likely not toxicant related and agrees with the reported NOEC. For 35-day mean number of offspring released per female, there is no clear dose-response but also high degree of variation across treatment groups (CV = 35.1 to 142.8 %). Such high variation calls into to the question the interpretability of this endpoint. This also affected the 42-day mean number of offspring released per female variable. Consequently, HSE has decided to not determine NOECs for these endpoints.

A statistically significant difference in survival after 42 days in the 22 and 45 mg a.s./kg treatment levels was also observed. The applicant has stated that, due to a lack of a clear dose response and relatively high survival throughout the concentration range, these results were not considered to be toxicant-related. The top concentration (93 mg a.s./kg) presented similar survival to both the control (6.25 % decrease) and solvent control (2.27 % increase). HSE disagrees with this interpretation. Between 5.6 and 45 mg/kg there is a clear dose-response, which is followed by 93 mg/kg with equivalent survival to the controls. Such a pattern of results is inconclusive as it raises the possibility that the 93 mg/kg treatment group is an outlier. Consequently, HSE has decided to not determine a NOEC for this endpoint.

There were 2 protocol deviations noted in the study report. The first deviation reported is temperature falling below the 23 ± 1°C on test day 8 and above on test day 34. On test day 8, the water temperature within the test vessels was below the acceptable temperature with a range of 19.7 to 20.1 °C. On test day 34, the water bath maximum temperature recorded was above the acceptable range at 25 °C. HSE conclude that this deviation is unlikely to have had a significant affect on the results as no adverse behavioural observations were made during these deviations in the control groups and there were no anomalous results in the exposure groups.

The second deviation to protocol is that measurements of dissolved oxygen and temperature were not recorded on day 7. The protocol states that at test initiation (test day 0), test days 28 and 29 and test termination (day 42), temperature, dissolved oxygen concentration and pH will be measured in the overlying water and recorded for each test vessel. On the remaining test days, temperature and dissolved oxygen will be measured and recorded in one alternating replicate each day. The regulator can conclude that, since the renewal system was observed to be working properly on test day 7 and water quality was measured and recorded during the remainder of the exposure, this deviation is unlikely to have had a negative impact on the results or interpretation of the study.

Another deviation from protocol that was not reported in this study is the food provided in this study. The EPA_600_R-99_064 (2000) guidelines recommend 1.0ml/L of YCT fed daily from day 0 to day 42, but this study provided 1.5 mL of YCT and 0.50 mg of flaked fish food, daily. HSE conclude that, as water quality parameters, length and survival met the validity criteria of this study, it is unlikely to have had a significant impact on the study.

All of the validity criteria were met for this study. The study report uses OCSPP Guideline 850/1770 validity criteria, but as these are not publicly available at this time, EPA_600_R-99 (2000) guidelines have also been used to assess the validity criteria. OECD 218 (2004) guidelines relate to a different species; therefore, are not suitable for evaluating this study.

There was an extensive use of statistical analysis in this study that all meet the requirements of EPA_600_R-99_064 (2000) guidelines.

HSE was not able to determine NOECs for three response variables, which precludes the confident determination of a study-wide NOEC. Consequently, HSE has categorised this study as not reliable and not suitable for use in risk assessment.

Reference:	KCA 8.2.5.4/03
Report Title:	S-2399 - 28-Day Toxicity Test Exposing Estuarine Amphipods (*Leptocheirus plumulosus*) to a Test Substance Applied to Sediment Following EPA Test Methods
Author(s) & year:	[redacted] (2017)
Document No, Authority registration No:	12709.6366
Substance used:	S-2399 TG, 13CG0617G, 95 %
Method of analysis:	LC/MS/MS
Guideline(s):	EPA Test Methods 2001 (EPA/600/R-01/020) and OCSPP850.1780
Deviations:	Yes, see HSE comments section.
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

MATERIALS

Test Material S-2399 TG

Lot/Batch #: 13CG0617G

Purity: 95.5% (verified by certificate of analysis)

Description: Not provided

Expiration date: 29 June 2019

STUDY DESIGN AND METHODS

Treatments

Test concentrations: Nominal sediment: 0.76, 2.4, 7.8, 25, and 80 mg a.s./kg (dry weight basis)
Mean measured sediment: 0.45, 1.5, 4.2, 14, and 47 mg a.s./kg (dry weight basis)
Mean measured pore water: 0.011, 0.042, 0.13, 0.46, and 1.8 mg/L

Controls: Negative and solvent control

Solvent: Acetone

Analysis of test concentrations: Yes, in pore water, overlying water and sediment samples from one replicate per treatment level and

	control on Day 0, Day 14 and Day 28. Sediment and aqueous samples were analysed for S-2399 using liquid chromatography with tandem mass spectrometry detection (LC/MS/MS). The limit of quantitation (LOQ) was 200 µg/kg and the minimum detectable limit (MDL) was 16.0 µg/kg for sediment. The limit of quantitation (LOQ) was 0.6 µg/L and the minimum detectable limit (MDL) was 0.2 µg/L for overlying and pore water.
Test organisms	
Species:	*Leptocheirus plumulosus*
Source:	Chesapeake Cultures, Hayes, Virginia
Feeding:	During acclimation, amphipods were fed once with 1 mL of 100 mg/mL flaked fish food suspension. During the exposure, each replicate vessel received 1.0 mL of flaked fish food suspension daily, consisting of 9.0mg of flaked food on Days 0 to 6, 14 mg on Days 7 to 13, 20mg on Days 14 to 20 and 30 mg on Days 21 to 27. Representative samples of the food source used during testing were analysed periodically for the presence of pesticides, PCBs and toxic metals. None of these compounds have been detected at concentrations considered toxic in any of the samples analysed.
Age:	Approximately 7 to 14 days old (juvenile organisms passing through a 0.60-mm sieve and retained on a 0.25-mm sieve).
Acclimation:	48 hours
Overlying water	Natural, filtered seawater (salinity adjusted with laboratory well water)
pH:	7.5 to 7.7
Salinity:	20 ± 2 ‰ The water quality ranges referenced above encompass measurements of the source water during the test duration and are indicative of overall source water characteristics.
Sediment:	Marine sediment collected from Sequim Bay, Sequim,

	Washington, SMV Batch No. 070116
Total organic carbon:	2.9 %
Particle size distribution:	33 % sand, 47 % silt, 20 % clay
pH:	7.8
Percent solids:	38.56 %
Water holding capacity (percent moisture at 1/3 bar)	80.6 %
Pore water ammonia (as mg nitrogen/L) prior to use in testing	6.9 mg/L

Test design

Test vessels:	1 L glass jars containing 120 mL (wet weight averaged 157 g) of sediment and 600 mL overlying water
Replication:	Six replicate vessels were used to evaluate the biological response of the test organisms used to evaluate chronic survival, growth, and reproduction. Five replicates were also established and designated for chemical analysis and pore water quality measurements. Each biological response replicate vessel contained 20 amphipods, a total of 120 amphipods per concentration or control for the replicates maintained for monitoring the biological response. The additional replicates were maintained under the same conditions and contained test organisms (with the exception of the replicates that were sacrificed at exposure initiation for analytical and pore water quality measurements), but were not used to evaluate the biological response of the test organisms
Exposure regime:	Static-renewal
Duration:	28 days

EXPERIMENTAL CONDITIONS

Test temperature:	24 – 26°C (overlying water), 21 – 22° (pore water)
pH:	7.2 – 8.3 (overlying water), 6.0 – 7.3 (pore water)
Dissolved oxygen:	5.3 – 7.8 mg/L (overlying water)
Salinity:	20 – 22 ‰ (overlying water), 19 –26 ‰ (pore water)
Ammonia:	0.44 –1.8mg/L (overlying water), 1.3 – 13 mg/L

	(pore water)
Lighting:	16 hours light: 8 hours darkness (540 – 990 lux)

STUDY DESIGN AND METHODS
Definitive exposure dates: 4 November to 2 December 2016

Test organism
Leptocheirus plumulosus was selected to test the toxicity of estuarine and marine sediment. The choice of this amphipod species as a test organism is based on its sensitivity to sediment-associated contaminants, availability and ease of collection and culturing, tolerance of environmental conditions (e.g., temperature, salinity, grain-size), ease of handling in the laboratory, and ease of measuring test endpoints.

On receipt from the supplier the temperature of the water used to transport the amphipods was 20 °C. During the acclimation period, the water used had a salinity of 20 ‰ and a temperature of 24 °C. No mortality was observed in the test population 48 hours prior to exposure initiation.

On the day of exposure initiation, three subsets of 20 neonate amphipods were selected from the same population used to initiate the exposure. These amphipods were dried at 55 to 60 ºC for approximately 24 hours in an oven. Each subset of 20 dry amphipods were then weighed on an analytical balance to the nearest 0.01 mg. The mean value of these three subsets resulted in an initial dry weight of 0.0333 mg dry weight per amphipod, which was used to determine growth rate at termination of the exposure. This initial weight is within the expected initial weight range of 0.030 to 0.060 mg/amphipod provided in the test method.

Overlying water
Seawater was sourced from Cape Cod Canal, Bourne, Massachusetts. Representative samples of the overlying water source were analysed for the presence of pesticides, PCBs and metals by GeoLabs, Inc., Braintree, Massachusetts. None of these compounds were detected in any of the water samples analysed in accordance with ASTM (2007)[33] guidelines. In addition, representative samples of the overlying water source were analysed monthly for total organic carbon (TOC) concentration. The TOC concentration of the overlying water source was 1.2 and 1.7 mg/L for the months of November and December 2016, respectively.

Sediment
Prior to characterization and use in testing, the sediment was wet pressed through a 0.25-mm sieve to remove large particles and indigenous organisms. The sediment pore water ammonia concentration was within the expected range based on historical data for marine

[33] ASTM, 2007. Standard practice for conducting acute toxicity tests with fishes, macroinvertebrates and amphibians. Standard E729-96. American Society for Testing and Materials, 100 Barr Harbor Drive, West Conshohocken, Pennsylvania 19428.

sediment from this source and below 60 mg/L (water-only ammonia NOEC for *L. plumulosus*).

Representative samples of the sediment were analysed periodically for the presence of pesticides, PCBs and toxic metals by GeoLabs, Inc., Braintree, Massachusetts. None of these compounds have been detected at concentrations that are considered toxic in any of the samples analysed, in agreement with ASTM (2007)[33] standard practice.

Dose and test vessel preparation
An 8.11 mg/mL primary stock solution was prepared by dissolving 0.42470 g of S-2399 (0.40559 g as active substance in 50 mL of acetone (CAS No. 67-64-1). The resulting stock solution was observed to be clear and colourless with no visible undissolved test substance following preparation. The primary stock solution was used to create five individual dosing stock solutions. All solutions obtained were clear colourless and with no visible undissolved test substance following preparation.

A jar-rolling technique was used to apply the test substance to the sediment (Ditsworth et al., 1990)[34]. A 10-mL volume of each dosing stock solution was applied to 0.050 kg of fine silica sand placed in glass Petri dishes, which was mixed with a metal spatula for approximately two minutes. The solvent was allowed to evaporate off the sand for 30 minutes. The dry sand, containing the test substance, was then added to 2.5 kg of wet sediment (1.014 kg total dry weight based on a percent solid value of 38.56% and including the 0.050 kg of fine silica sand) in individual glass jars to yield the desired treatment level concentrations.

The jars were sealed and positioned horizontally on a rolling mill. Each jar was then rolled for four hours at room temperature at approximately 15 rpm. On completion, sediments were allowed to equilibrate for a 14-day period in the dark at 2 to 8 ºC. Once a week during the 14-day equilibration period and again prior to addition into the replicate test vessels, the jars were mixed on the rolling mill for an additional two hours at room temperature to ensure the sediment was homogeneous. A 14-day equilibration period was deemed acceptable for sediment toxicity testing with S-2399 TG based on the results of a sediment-pore water equilibration trial conducted prior to this study.

A solvent and negative control were also prepared using the method described above but without the addition of the test substance or the solvent, respectively.

One day prior to test initiation (day -1), the treated and control sediments (120 mL per vessel) were allocated to the replicate vessels. 600 ml of overlying water was gently added to each vessel with the aid of a turbulence reducer, used to minimise sediment layer disturbance. Aeration with oil-free air was provided to each replicate with a constant flow of bubbles from a 1-mL glass pipette. The vessels were established on day -1 to allow for

[34] Ditsworth, G.R., D.W. Schults, J.K. P. Jones, 1990. Preparation of Benthic Substrates for Sediment Toxicity Testing. Environmental Toxicology and Chemistry. Vol. 9, pp. 1523-1529.

equilibration of the sediment and overlying water under in situ test conditions.

Water Renewal
During the 28-day study, the overlying water was renewed by adding two volume additions of water per test vessel per day using an intermittent delivery system in combination with a calibrated water-distribution system (Zumwalt et al., 1994)[35]. The intermittent delivery system was calibrated to provide 500 mL of water per cycle to the water-distribution system, which subsequently provided 100 mL of water per cycle to each replicate test vessel. The water delivery system cycled approximately 12 times per day, providing approximately 1200 mL per vessel every 24 hours (i.e., approximately two overlying volume replacements per vessel per day).

The calibration of the overlying water renewal system was checked prior to exposure initiation and confirmed at exposure termination. During the test, the renewal system was visually inspected daily. A complete check of intermittent delivery system function was made once daily.

Measurements and observations
Water quality
At exposure initiation and termination, dissolved oxygen concentration, salinity, temperature and pH were measured in the overlying water of each remaining replicate vessel of each treatment level and control used for biological monitoring during the 28-day exposure. On test days 1 through 27, dissolved oxygen, salinity, pH and temperature were measured in one alternating replicate of each treatment level and control. In addition, the temperature was continuously monitored in an auxiliary vessel in the temperature-controlled water bath used to house the test vessels throughout the study. Ammonia concentration (as nitrogen) of the overlying water was monitored at exposure initiation and termination in each treatment level and control from a composite sample taken from the remaining replicates.

In addition, at exposure initiation and exposure termination, salinity, pH, temperature and ammonia concentrations (as nitrogen) were measured in a pore water sample of each treatment level and control.

Biological observations
All vessels were examined at exposure initiation and at daily intervals thereafter, until exposure termination (day 28). Observations of organism mortality and abnormal behaviour were made and the physical characteristics of the sediment:water system were recorded.

At exposure termination (day 28), the total number of surviving adult and young amphipods was determined in each test vessel by sieving the sediment through a 0.60-mm and 0.25-mm sieve. According to the test method, adult amphipods recovered at exposure termination were defined as those individuals that were retained on the 0.60 mm sieve. Young

[35] Zumwalt, D.C., F.J. Dwyer, I.E. Greer and C.G. Ingersoll, 1994. A water-renewal system that accurately delivers small volumes of water to exposure chambers. Environmental Toxicology and Chemistry. pp. 1311-1314.

amphipods (offspring) were defined according to the test method as those individuals that were retained on the 0.25-mm sieve.

At exposure termination, adult amphipods were preserved to allow for identification of gender at a later date i.e., six days following exposure termination. The gender of the surviving adults was determined by the presence or absence of eggs in the brood pouch or further morphological characteristics described in the EPA test method (U.S. EPA, 2001)[36].

Growth was determined after first pooling the surviving adult amphipods (separated by gender) from each replicate vessel and drying at 61 ºC for approximately 24 hours in an oven. Amphipods were rinsed gently with deionized water to remove any residual salt deposits and blotted dry prior to being dried in the oven. The pooled, dry amphipods were then weighed on an analytical balance to the nearest 0.01 mg. Growth rate for both males and female individuals was calculated using the following equation:

mg gain/amphipod/day = (mg/amphipod at termination – mg/amphipod at initiation)/28 days

The offspring were preserved and stained for enumeration at a later date, i.e., four to seven days following exposure termination. Reproduction was determined as the number of young per surviving female amphipod in each replicate vessel.

Analytical measurements

Dosed sediments were sampled during the mixing/equilibration period prior to the allocation of the sediments into the replicate exposure vessels. In addition, subsamples of the dosing stock solutions used to dose the sediments were also analysed for test substance concentration. These were in addition to the measurements performed during the in-life phase of the definitive study outlined in the table above.

Pore water samples were collected by removing the entire sediment sample from each test vessel and centrifuging for 30 minutes at approximately 10,000 g. The resulting pore water was removed from the centrifuge tube and its volume measured. Following centrifugation and removal of the pore water sample, sediment samples were collected from each centrifuge tube with a stainless steel spatula and mixed thoroughly.

The analysis of sediment and pore water samples was utilized to calculate a partition coefficient (K_d) at each sampling interval as well as a mean partition coefficient for each test concentration.

Statistics

At the termination of the study, data obtained on amphipod survival, growth and reproduction were statistically analysed to identify significant treatment-related effects. The lowest test

[36] U.S. EPA, 2001. Methods for Assessing the Chronic Toxicity of Marine and Estuarine Sediment-Associated Contaminants with the Amphipod Leptocheirus plumulosus. Office of Research and Development. Washington, D.C. EPA/600/R-01/020.

concentration that showed a statistically significant effect (Lowest-Observed-Effect Concentration, LOEC) and the highest test concentration that showed no statistically significant effect (No-Observed-Effect Concentration, NOEC) were determined.

A Two-Sample t-Test (U.S. EPA, 2002)[37] was used to compare the performance of the negative control organisms with that of the solvent control organisms in order to determine if there were any statistically significant positive or negative effects. For this study, negative control and solvent control data were statistically similar for male growth rate, female growth rate and reproduction. There was a significant difference observed between the negative control and solvent control for the survival endpoint. The study conductor stated this difference was not attributable to solvent interference as the solvent was evaporated off. All the remaining statistical analyses were conducted comparing the treatment level data to the negative control based on current EPA guidance.

Shapiro-Wilks' Test for normality (U.S. EPA, 2002)[37] was conducted to compare the observed sample distribution with a normal distribution. Day 28 survival, female growth rate and reproduction data met this assumption of normal distribution. Male growth rate data did not meet the assumption of normal distribution.

As a check on the assumption of homogeneity of variance implicit in parametric statistics, data were analysed using Bartlett's Test (U.S. EPA, 2002)[37]. Day 28 survival and female growth rate data met the assumption of homogeneous variance. Male growth rate and reproduction data did not meet the assumption homogeneous variance.

Survival and female growth rate data met the assumptions for both normal distribution and homogeneity of variance; therefore, Dunnett's Multiple Comparison Test (U.S. EPA, 2002)[37], a parametric statistical procedure, was used to establish treatment effects. Wilcoxon's Test with Bonferonni-Holm's Adjustment and Steel's Many-One Rank Sum Test, non-parametric statistical procedures, were used to establish treatment effects for male growth rate and reproduction, respectively.

The EC/LC_{50} is the estimated concentration of the test substance which produces 50% effect/mortality in the test population at exposure termination. If applicable, an appropriate statistical method within CETIS™ Version 1.8 (Ives, 2013)[38] was used to calculate the EC/LC_{50} values. If no concentration resulted in a reduction in growth/reproduction/survival of ≥ 50% when compared to the negative control at test day 28, the EC/LC_{50} was empirically estimated to be greater than the highest concentration included in the test.

CETIS™ Version 1.8 (Ives, 2013)[38] was used to perform all statistical analyses.

[37] U.S. EPA, 2002. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms. Fourth Edition. EPA-821-R-02-013. Office of Water (4303T), U.S. Environmental Protection Agency, Washington, D.C.

[38] Ives, M., 2013. CETIS, Comprehensive Environmental Toxicity Information System™, User's Guide. Tidepool Scientific Software, McKinleyville, California

RESULTS AND DISCUSSION

Test conditions

Dissolved oxygen was maintained above 50% saturation (i.e., > 3.6 mg/L at 25 ºC and 20 ‰ salinity) during the study. Daily measurements of the temperature in the overlying water of each test vessel established a temperature range of 24 to 26 ºC. Continuous monitoring in an auxiliary vessel in the water bath used to house the test vessels established a temperature range of 23 to 27 ºC throughout the definitive study. Total ammonia, salinity, temperature and pH were measured in pore water samples on test days 0 and 28. Pore water salinity was elevated (> 20 ± 2‰) at exposure initiation which is reflective of the use of natural sediment collected from a marine environment with higher salinity.

Analytical results

Analysis of the dosing stock solutions (0.0772, 0.244, 0.792, 2.54, and 8.11 mg/mL) resulted in measured concentrations ranging from 97.0 to 113% of the nominal concentrations. Analysis of the dosed sediment samples after mixing and prior to allocation into the test vessels resulted in measured concentrations of the 0.68, 2.2, 6.0, 20, and 69 mg/kg in the 0.76, 2.4, 7.8, 25, and 80 mg/kg nominal concentration groups, respectively. Measured concentrations resulted in recoveries ranging from 77 to 90% of nominal concentrations. Based on the sediment pretest analysis, these results established that the appropriate concentrations were achieved in order to generate a reasonable concentration gradient

Table 9.2.6-32, Table 9.2.6-33 and Table 9.2.6-34 present the concentration of S-2399 T.G. in sediment, pore water and overlying water throughout the study respectively.

Table 9.2.6-32: Concentrations of S-2399 in sediment samples during the 28-day toxicity test with amphipods (Leptocheirus plumulosus)

Nominal concentration (mg/kg sediment dry weight)	Measured sediment concentration; mg/kg sediment dry weight (% of the nominal concentration)					Mean percent of nominal[b]
	Pretest[a]	Day 0	Day 14	Day 28	Mean[b] (SD)	
Negative Control	< 0.063[c] (NA)	< 0.062 (NA)	< 0.062 (NA)	< 0.062 (NA)	NA[d] (NA)	NA
Solvent Control	< 0.063 (NA)	< 0.062 (NA)	< 0.062 (NA)	< 0.062 (NA)	NA (NA)	NA
0.76	0.68 (89)	0.74 (98)	0.41 (54)	0.19 (25)	0.45 (0.28)	59
2.4	2.2 (90)	2.5 (110)	1.3 (53)	0.62 (26)	1.5 (0.97)	62

Nominal concentration (mg/kg sediment dry weight)	Measured sediment concentration; mg/kg sediment dry weight (% of the nominal concentration)					Mean percent of nominal[b]
	Pretest[a]	Day 0	Day 14	Day 28	Mean[b] (SD)	
7.8	6.0 (77)	7.3 (93)	4.0 (51)	1.5 (19)	4.2 (2.9)	54
25	20 (79)	25 (100)	12 (48)	3.6 (14)	14 (11)	54
80	69 (86)	81 (101)	38 (47)	23 (28)	47 (30)	59

[a] Results presented for pretest analysis were not included in the calculation of mean measured concentrations, standard deviations and percent recoveries.
[b] Mean measured and percent recovery values were calculated using the actual analytical results and not the rounded values (two significant figures) presented in this table.
[c] Concentrations expressed as less than values were below the minimum detectable limit (MDL).
[d] NA = Not Applicable

Table 9.2.6-313: Concentrations of S-2399 in pore water samples during the 28-day toxicity test with amphipods (*Leptocheirus plumulosus*)

Nominal concentration (mg/kg sediment dry weight)	Measured pore water concentration (mg/L)			Mean[a] (SD)
	Day 0	Day 14	Day 28	
Negative Control	< 0.010[b]	< 0.0010	< 0.0010	NA[c] (NA)
Solvent Control	< 0.010	< 0.0010	< 0.0010	NA (NA)
0.76	0.021	0.0084	0.0051	0.011 (0.0083)
2.4	0.079	0.027	0.019	0.042 (0.032)
7.8	0.25	0.099	0.052	0.13 (0.10)
25	1.1	0.23	0.10	0.46 (0.52)
80	3.5	1.1	0.79	1.8

Nominal concentration (mg/kg sediment dry weight)	Measured pore water concentration (mg/L)			Mean[a] (SD)
	Day 0	Day 14	Day 28	
				(1.5)

[a] Mean measured values were calculated using the actual analytical results and not the rounded values (two significant figures) presented in this table.
[b] Concentrations expressed as less than values were below the minimum detectable limit (MDL).
[c] NA = Not Applicable

Table 9.2.6-324: Concentrations of S-2399 in overlying water samples during the 28-day toxicity test with amphipods (*Leptocheirus plumulosus*)

Nominal concentration (mg/kg sediment dry weight)	Measured overlying water concentration (mg/L)		
	Day 0	Day 14	Day 28
Negative Control	< 0.0010[a]	< 0.0010	< 0.0010
Solvent Control	< 0.0010	< 0.0010	< 0.0010
0.76	0.0031	< 0.0010	< 0.0010
2.4	0.0088	0.0018	0.0016
7.8	0.019	0.0050	0.0057
25	0.092	0.013	0.012
80	0.43	0.077	0.051

[a] Concentrations expressed as less than values were below the minimum detectable limit (MDL).

The calculated partition coefficient (Kd) for days 0, 14, and 28 as well as the overall mean partition coefficients are presented in Table 9.2.6-34.

Table 9.2.6-335: Calculated partition coefficient (Kd) values during the chronic exposure of amphipods (*Leptocheirus plumulosus*) to S-2399

Nominal concentration (mg/kg sediment dry weight)	K_d[a]			
	Day 0	Day 14	Day 28	Overall Mean
0.76	35	49	38	41
2.4	32	47	32	37

Nominal concentration (mg/kg sediment dry weight)	K_d[a]			
	Day 0	Day 14	Day 28	Overall Mean
7.8	29	40	28	32
25	23	52	36	37
80	23	35	29	29

[a]$K_d = C_{sediment} / C_{pore\ water}$

Biological observations

A summary of survival and growth of the amphipod after 28 days is presented in Table 9.2.6-35. No sub-lethal or behavioural effects were noted among the amphipods in any treatment group.

Table 9.2.6-346: Mean percent survival and mean length per amphipod on Day 28

Mean measured sediment concentration (mg a.s./kg sediment dry weight)	Mean percent survival (SD)	Mean male growth rate mg/day (SD)	Mean female growth rate mg/day (SD)	Mean number of offspring released per surviving female (SD)
Control	94 (3.8)	0.093 (0.013)	0.056 (0.0043)	14 (1.6)
Solvent control	83 (11)	0.098 (0.0091)	0.059 (0.0038)	15 (8.6)
0.45	90 (9.5)	0.088 (0.0062)	0.060 (0.0036)	14 (2.8)
1.5	63 (19)[a]	0.077 (0.015)	0.046 (0.0096)[a]	16 (3.4)
4.2	90 (7.1)	0.13 (0.050)	0.053 (0.0053)	10 (2.9)[c]
14	86 (9.2)	0.085 (0.0093)	0.062 (0.0096)	20 (6.3)
47	83 (22)	0.075 (0.0031)[b]	0.049 (0.0071)	14 (8.9)

SD = standard deviation

[a] Significantly reduced compared to the negative control, based on Dunnett's Multiple Comparison Test.

[b] Significantly reduced compared to the negative control, based on Wilcoxon's Test with Bonferroni's Adjustment.

[c] Significantly reduced compared to the negative control, based on Steel's Many-One Rank Sum Test.

Statistical analysis (Dunnett's Multiple Comparison Test) determined a significant reduction in survival among amphipods. exposed to the 1.5 mg/kg treatment level compared to the negative control (94%). This was not considered to be treatment-related by the study conductor due to a lack of a clear dose-response and the lack of any significant reduction at the three higher treatment levels.

Statistical analysis (Wilcoxon's Test with Bonferroni-Holm's Adjustment) determined a significant reduction in growth rate among male amphipods exposed to the 47 mg/kg treatment level compared to the negative control (0.093 mg per amphipod per day).

Statistical analysis (Dunnett's Multiple Comparison Test) determined a significant reduction in growth rate among female amphipods exposed to the 1.5 mg/kg treatment level compared to the negative control (0.056 mg per amphipod per day). This is not considered to be treatment related by the study conductor due to a lack of a clear dose response and the lack of any significant reduction at the three higher treatment levels.

Statistical analysis (Steel's Many-One Rank Sum Test) demonstrated a significant reduction in reproduction among amphipods exposed to the 4.2 mg/kg treatment level compared to the survival of the negative control (14 offspring per female amphipod). This is not considered to be treatment-related by the study conductor due to a lack of a clear dose response and the lack of any significant reduction at the two higher treatment levels.

Due to the non-monotonicity of the male/female growth rate and reproduction data sets and the lack of a defined dose response across the concentration range, it was not feasible to generate robust EC10 and EC20 values for use in a risk assessment framework.

Table 9.2.6-37 presents the NOEC and LOEC values for each endpoint as determined by the study conductor.

Table 9.2.6-357: Summary of NOEC and LOEC endpoints for *Leptocheirus plumulosus* after 28 day exposure to S-2399

Endpoint	Mean measured sediment (mg a.s./kg)	Mean measured pore water (mg a.s./L)
Survival		
LOEC	>47	>1.8
NOEC	47	1.8
LC_{50} (95% Confidence Intervals)	>47 (n.a[a].)	>1.8 (n.a.)
Male growth		
LOEC	47	1.8
NOEC	14	0.46

Endpoint	Mean measured sediment (mg a.s./kg)	Mean measured pore water (mg a.s./L)
LC_{50} (95% Confidence Intervals)	>47 (n.a.)	>1.8 (n.a.)
Female growth		
LOEC	>47	>1.8
NOEC	47	1.8
LC_{50} (95% Confidence Intervals)	>47 (n.a.)	>1.8 (n.a.)
Reproduction		
LOEC	>47	>1.8
NOEC	47	1.8
LC_{50} (95% Confidence Intervals)	>47 (n.a.)	>1.8 (n.a.)

[a]n.a. = not applicable. LC/EC_{50} value was empirically estimated. Therefore, corresponding 95% confidence intervals could not be determined

VALIDITY CRITERIA

The validity criteria for the test that were performed were met according to EPA Test Method, "Methods for Assessing the Chronic Toxicity of Marine and Estuarine Sediment-Associated Contaminants with the Amphipod *Leptocheirus plumulosus*" (U.S. EPA, 2001)[36] (Table 9.2.6-38). For salinity, no instantaneous monitoring was performed.

Table 9.2.6-368: Compliance with validity criteria

Validity criterion	Required	Obtained
Neonate *L. plumulosus*, size-selected.	Retained between 0.25-mm and 0.6-mm screens	Retained between 0.25-mm and 0.6-mm screens
Average survival of amphipods in the negative control sediment	≥ 80 %, with no single replicate ≤ 60 %	Negative control = 90 -100 %
Growth and reproduction in negative control	Measurable in all replicates	Male growth rate = 0.075 to 0.111 mg/amphipod/day Female growth rate = 0.050 – 0.061 mg/amphipod/day 12.6 – 16.8 offspring per surviving female

Validity criterion	Required	Obtained
Overlying water temperature	Time-weighted average of daily temperature readings must be within ± 2 °C of 25 °C Instantaneous temperature must always be within ± 3 °C of 25 °C	Daily temperature reading = 24 - 26 °C Instantaneous temperature = 23 - 27 °C
Overlying water salinity	Time-weighted average of daily salinity readings must be 20 ‰ ± 2 ‰ Instantaneous salinity readings must always be 5‰ ±3‰ or 20‰ ± 3‰.	Daily salinity readings = 20 – 22 ‰ Instantaneous salinity readings not performed

CONCLUSION

Based on all measured parameters and mean measured concentrations of S-2399, the No-Observed-Effect Concentration (NOEC) for the sediment-dwelling estuarine amphipod *(Leptocheirus plumulosus*) was determined to be 47 mg a.s./kg for amphipod survival and reproduction and female growth by the study conductor. The NOEC for male growth was 14 mg a.s./kg. The Lowest-Observed-Effect Concentration was determined to be > 47 mg a.s./kg for amphipod survival and reproduction and female growth. The LOEC for male growth was 47 mg a.s./kg. Since no concentration tested resulted in > 50% reduction in survival, growth and reproduction when compared with the control data, the LC_{50} and EC_{50} values were empirically estimated to be > 47 mg a.s./kg, the highest mean measured sediment concentration tested.

HSE COMMENTS

The study was carried out according to GLP and follows EPA Test guideline , “Methods for Assessing the Chronic Toxicity of Marine and Estuarine Sediment-Associated Contaminants with the Amphipod *Leptocheirus plumulosus*” (U.S. EPA, 2001)[36]. All validity criteria for EPA Test guideline were met apart from continuous salinity monitoring, which was not performed. Considering daily salinity readings were within the recommended range throughout the study HSE consider the omission of instantaneous salinity measurements as minor.

The following deviations were noted:
According to (U.S. EPA, 2001) paragraph 11.3.1.2, 175 mL of sediment and 725 mL of overlying seawater are the recommended volumes for each test vessel. 120 mL of sediment

and 600 mL of overlying water were used. This will have made the results not directly comparable to viability criteria surrounding control growth, survival and reproduction, although the effect of this modification is most likely small. HSE consider this an acceptable deviation.

(U.S. EPA, 2001) paragraph 11.2.2 details the use of five replicate test containers per treatment. Six replicate vessels per treatment were used. In addition, replicate E of the 4.2 mg/kg mean measured concentration treatment level was found to have 21 individuals present on day 28. Results were updated to reflect the increase in number of individuals for this replicate. These deviations did not negatively impact study outcomes and HSE considers them minor.

(U.S. EPA, 2001) paragraph 11.2.2 also details feeding frequency. It states that feeding is performed three times a week, whereas the study did this daily. Furthermore, feeding rates were increased more gradually over more stages (four vs two). Each subsequent feeding rate roughly increased the amount of food provided by a factor of 1.5. This modification is not expected to have negatively impacted control survival, growth or survival. This is supported by the 90 – 100 % negative control survival.

(U.S. EPA, 2001) paragraph 11.3.6.4.1 discusses the selection of feeding rate during the development of the guideline and its impact on the endpoint sensitivity. It refers to a polychlorinated biphenyls (PCB) study where decreased reproduction was witnessed at lower concentrations for lower feeding rates (20 mg/40 mg and 10 mg/20 mg feeding regimes displayed reproductive effects at 120ppm, where 30 mg/60 g feeding regime only displayed reproductive effects at 240 ppm). If the feeding rate used in this study and the recommended feeding rate are expressed in terms of mg food per week (Table 9.2.6-39), it is clear the deployed feeding rate is considerably higher than recommended for 75 % of the study duration (Day 7 – 28). This could have reduced the sensitivity of the reproduction endpoint. For the aforementioned PCB study, a higher feeding regime halved the sensitivity of test individuals to reproductive effects. If the same difference was present in this study due to the elevated feeding rate, then the NOEC set from the male growth rate would still be protective of this modified reproductive endpoint. For this reason, HSE considers this deviation to have had minimal impact on the reported endpoints.

Table 9.2.6-379: Deviation of study and guideline feeding regime

Days	Study feeding regime (mg food per week)	Guideline feeding regime (mg food per week)
0 – 6	63	60
7 - 13	98	60
14 - 20	140	120

Days	Study feeding regime (mg food per week)	Guideline feeding regime (mg food per week)
21 - 28	210	120

(U.S. EPA, 2001) Table 11.1 outlines the test conditions for conducting a 28-d Sediment Toxicity Test with *Leptocheirus plumulosus*. It recommends renewing overlying water three times per week by siphoning off and replacing 400 mL. The water renewal regime deployed performed approximately two overlying volume replacements per vessel per day (1200 mL). This approach is consistent with other, complementary sediment toxicity testing guidelines (U.S. EPA, 1996)[39] and will not have negatively impacted control survival. This modification does, however, complicate comparisons with validity criteria relating to growth, survival and reproduction that were developed according to the method described in (U.S. EPA, 2001)[36].

Table 11.2 describes a general activity schedule for conducting 28-d sediment toxicity tests with *Leptocheirus plumulosus*, including a pretest preparation instruction regarding culture initiation. It states, "*start or renew cultures approximately 6 to 8 weeks in advance of test initiation. Increase culture water temperature to about 25°C approximately 2 weeks in advance of test initiation".* Cultures were purchased from a commercial breeder and details on the date of culture initiation in relation to test initiation were not provided. Furthermore, amphipods were only acclimated to test conditions for 48 hours prior to testing (on arrival shipment water = 20 °C, test temperature = 25 °C). Relating to this, paragraph 11.3.4.2 states, "*a change in temperature or salinity not exceeding 3 °C per 24-h period is strongly recommended*". Test organisms experienced a 5 °C increase when the acclimation period began. Although no mortality was observed in the test population in the 48 hours leading up to the test, it is possible that test subjects were not fully acclimated to test conditions or experienced stress due to the greater than recommended temperature increase on arrival from the commercial supplier. HSE will consider this during the risk assessment.

Table 11.3 lists performance-based criteria for culturing *L. plumulosus* including the performance of periodic 96-h water-only reference-toxicity tests. This was not performed by the study conductor as cultures were not maintained in-house, making culture organism sensitivity is uncertain. However, cultures were sourced from a reputable supplier, who would be expected to perform reference toxicity tests. HSE notes this uncertainty and will consider it during the risk assessment stage.

One further comment not relating to any specific section of the guideline is the marked reduction in S-2399 sediment concentration as the study progressed. Using OECD 23 (2019), a non-study specific guideline with recommendations on best practices, it states, "*if*

[39] U.S. EPA, 1996a. Office of Chemical Safety and Pollution Prevention. Ecological Effects Test Guideline, OCSPP 850.1735. Whole Sediment Acute Toxicity Invertebrates, Freshwater. "Public Draft" EPA 712-C-96-354 April 1996. U.S. Environmental Protection Agency, Washington D.C.

measured concentrations in samples do not remain within 80-120% of nominal, the effect concentration should be expressed relative to the measured concentrations. In this situation, for both flow-through and static-renewal exposure systems, effects concentrations may be determined and expressed relative to the time-weighted mean measured concentrations". Sediment concentrations were expressed as arithmetic mean measured concentration for this study. HSE re-calculated the mean measured concentrations as geometric means using the formula provided in Annex 2 of OECD 23 (2019), presented below:

Table 9.2.6-40: Recalculation of geometric mean measured concentration

Nominal concentration (mg a.s./kg sediment)	Geometric mean measured concentration (mg a.s./kg sediment)[a]
0.76	0.39
2.4	1.26
7.8	3.52
25	10.26
80	41.37

[a] Calculated by HSE using values presented in Table 4 of the study report.

The NOECs reported below for use in risk assessment will be expressed according to the geometric mean measured concentration.

Finally, there were three instances of statistically significant reductions across all tracked endpoints, which the study conductor did not deem toxicant related. These were: a reduction in survival for the 1.5 mg/kg treatment level (33% compared to negative control and 24% compared to solvent control), a reduction in female growth rate for the 1.5 mg/kg treatment level (18% compared to negative control) and a reduction in number of offspring per female for the 4.2 mg/kg treatment level (29 % compared to negative control). In all cases there were no statistically significant reductions at higher tested concentrations and no clear concentration-response relationship.

For survival, the negative control was chosen as the reference, which was a conservative approach given the statistically significant negative effect of the solvent control. Compared to the solvent control four of the five treatment levels have equal to or greater survival, which strongly suggests the effects at 1.5 mg/kg are not toxicant related.

For both female growth rate and number of offspring per female there is a treatment level with a greater concentration than the statistically significant level that displays greater than 100 % negative control activity. Furthermore, only at the 47 mg/kg treatment level is there a greater than 10% reduction for either female growth rate or the number of offspring, except

for the statistically significant treatment levels. HSE agrees with the study conductor that in this case these statistically significant effects are likely not toxicant related.

The method of analysis used in the study was evaluated by HSE Chemistry. The conclusions of their evaluation are reproduced below. Please see Volume 3 CA, section B5 for more details.

"*The analytical method is not fully validated according to SANTE/2020/12830 rev. 1 for the determination of the active substance inpyrfluxam in freshwater as the matrix effects have not been determined and the stability of standards and extracts has not been addressed. However, the studies were generated prior Ito the implementation of SANTE 2020/12830 rev.1 and SANCO 3029 rev.4 did not require matrix effects and the stabilities of standards and extracts to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose*."

"*The analytical method is not fully validated according to SANTE/2020/12830 rev. 1 for the determination of the active substance inpyrfluxam in sediment as the matrix effects have not been determined and the stability of extracts and standards has not been addressed. However, the studies were generated prior to the implementation of SANTE 2020/12830 rev.1 and SANCO 3029 rev.4 did not require matrix effects and the stabilities of extracts and standards to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose.*"

The agreed endpoints suitable for use in the risk assessment, expressed in terms of sediment concentration, are:
NOEC 28-day amphipod survival = 41.37 mg a.s./kg
NOEC 28-day male amphipod growth rate = 10.26 mg a.s./kg
NOEC 28-day female amphipod growth rate = 41.37 mg a.s./kg
NOEC 28-day amphipod reproduction = 41.37 mg a.s./kg

The LC_{50} for every endpoint was empirically estimated to be > 41.37 mg a.s./kg

Therefore, based on the most sensitive indicator of toxicity the NOEC is 10.26 mg a.s./kg. The LOEC is 41.37 mg a.s./kg.

B.9.2.7 Effects on algal growth

B.9.2.7.1 Effects on growth of green algae

Reference:	KCA 8.2.6.1/01
Report Title:	S-2399 TG – 96-Hour Toxicity Test with the Freshwater Green Alga, *Pseudokirchneriella subcapitata*
Author(s) & year:	[redacted] (2015a)
Document No, Authority registration No:	Smithers Viscient Study No. 13048.6860
Substance used:	S-2399 TG, 13CG0617G, 95 %
Method of analysis:	HPLC/UV method
Guideline(s):	OECD 201 (2011)
Deviations:	Yes, see HSE comments section.
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: S-2399 TG
Description: Not stated
Lot/Batch: 13CG0617G
Purity: 95.0%
Reference item: Zinc chloride
Expiry date: 22 May 2016
Solvent: Dimethylformamide

B. STUDY DESIGN AND METHODS

1. Test organism: *Pseudokirchneriella subcapitata*
Growth stage: Inoculum-four days since previous transfer
Source: Smithers Viscient culture
Culture conditions: Algal Assay Procedure (AAP) medium prepared with sterile, deionized water
Initial cell density: 1.0 x 10^4 cells/mL
Strain: 1648
Analysis of concentrations: 1.9, 3.8, 7.5, 15, and 30 mg/L. Measured every 24 hours. The LOQ was set at 0.600 µg/L. The MDL was 0.200µg/L

2. Growth medium: Algal Assay Procedure (AAP) Medium
pH: 7.5 ± 0.1

3. Test vessels: Sterile 250 ml conical flasks, containing 100 ml of test solution
Exposure regime: Static. Solution was sonicated for approximately 20 minutes, and mixed for approximately two hours with a Teflon®-coated stir bar and magnetic stir plate

4. Environmental conditions:
A summary of the environmental conditions obtained in this study against the required OECD 201 (2011) conditions is displayed in Table 9.2.7-1 below.

Table 9.2.7-1: Summary of environmental conditions

Variable	Required OECD 201 (2011)	Obtained
Temperature	21 to 24°C	22˚C – 23˚C
pH	Not stated	7.0 – 9.6
Photoperiod	Not stated	Continuous illumination
Lighting intensity	60-120 µE·m-2 s -1	60 to 80 µE/m2 /S
Conductivity of dilution water	Not stated	91-99 µS/cm

Study dates:
4th – 8th November 2014

5. Test organism set up and treatment:
Four replicates for each treatment level and control were prepared. Eight replicates were prepared for the solvent control. Each replicate consisted of a sterile 250 mL Erlenmeyer flask test vessel. After solutions were added to the flasks, a 0.632 mL inoculum of *Pseudokirchneriella subcapitata* cells, at a density of approximately 158 × 10^4 cells/mL, was aseptically introduced to each flask. This inoculum provided the required initial (0-hour) cell density of approximately 1.0 × 10^4 cells/mL.

Nominal test concentrations of S-2399 TG for the 96-hour test were 0.0 (control), 0.0 (solvent control), 1.9, 3.8, 7.5, 15 and 30 mg a.s./L. The test was conducted in an environmental chamber, with all flasks continuously agitated at a rate of 100 ± 10 rpm.

A reference test was conducted prior to the start of the definitive phase, using zinc chloride as the toxicant to evaluate the sensitivity of *Pseudokirchneriella subcapitata*.

6. Dose preparation:
A 300 mg a.s./mL primary stock solution was prepared prior to test initiation by adding 7.8946 g of S-2399 TG to 25 mL of dimethylformamide (DMF). The resulting stock solution was observed to be clear and light brown with no visible undissolved test substance. A 30 mg a.s./L secondary stock solution was prepared by bringing 0.20 mL of the 300 mg/mL

primary stock solution to volume with AAP medium in a 2 L flask. This solution appeared clear and colourless with a large amount of undissolved test substance following preparation. The solution was sonicated for approximately 20 minutes and mixed for approximately two hours. After mixing and sonication, a small amount of undissolved test substance remained. The solution was centrifuged at 3000 rpm for approximately 10 minutes. The supernatant of the 30 mg a.s./L solution was observed to be clear and colourless with a small amount of undissolved test substance. After filtering with a 0.45 µm filter the solution appeared clear and colourless with no undissolved test substance. The filtrate was used as the exposure solution for the highest concentration and to prepare the remaining test concentrations.

A solvent control, prepared from AAP medium and DMF was also established. Additional untreated AAP medium was used to prepare the control.

Following preparation, all test item and control solutions were observed to be clear and colourless with no visible undissolved test substance.

In order to estimate the impact of the presence of algal blooms on the test substance, an additional flask of the 7.5 mg a.s./L solution was prepared, which was not inoculated with algae.

7. Measurements and observations:

At each subsequent 24-hour interval, cell counts were conducted on every replicate treatment and control vessel using a hemacytometer and compound microscope. Observations of health were also made and recorded.

The subculture was examined microscopically every other day to determine whether cell growth has resumed. The subculture was discontinued after a substantial increase in cell density (i.e. > 10 ×) was observed.

Temperature was measured continuously. The photosynthetically-active radiation (PAR) of the test area was measured at test initiation at each 24-hour interval during the exposure period. Light intensity was also measured in lux at 0 hour. Following each observation interval, the test flasks were placed to new random positions. Water quality parameters, pH and conductivity, were measured at test initiation and pH and conductivity were also measured at the termination of the exposure period.

A single sample was removed from each test concentration and the controls at exposure initiation (0 hours) and test termination (96 hours) to determine S-2399 TG concentrations. Samples analysed at 0 hours were removed from the test item and controls solutions prior to division into the replicate test vessels. Samples analysed at 96 hours were removed from individually composited replicate solutions of the treatment levels and controls. All test samples were centrifuged prior to analysis to remove algal biomass. At 96 hours, two samples were removed from a replicate of the 7.5 mg a.s./L test concentration which did not contain algae. One was analysed for S-2399 concentration and one was retained for possible future analysis. The result of the analysis was compared with that obtained for the 96-hour analysis of the 7.5 mg a.s./L solution containing algae to assess the impact that algae had on S-2399 TG concentration. Measurements of S-2399 TG were determined using a validated HPLC/UV method. The LOQ was set at 0.600 µg/L and the MDL was

0.200 µg/L.

8. Statistics:

The EC values were determined by linear interpolation of response (percent inhibition of endpoint as compared with appropriate control) using the IC_p method. If the data sets passed the test for homogeneity and normality, Williams' Multiple Comparison Test was used to determine the NOEC and LOEC. If the data did not pass the tests for homogeneity and normality, then the NOEC and LOEC were determined using an appropriate non-parametric statistical test. CETIS™ v 1.8 was used to perform all statistical analyses.

II. RESULTS AND DISCUSSION

A. BIOLOGICAL EFFECTS

A summary of the effects on cell density, growth rate, biomass and area under the growth curve (AUGC) are presented in Table 9.2.7-2 to 8.Table 9.2.7-4, followed by a summary of the endpoints in Table 9.2.7-5.

Table 9.2.7-2: Summary of effects on cell density and growth rate after 96 hours exposure of *Pseudokirchneriella subcapitata* S-2399 TG

Mean measured concentration (mg a.s./L)	Mean cell density (× 10^4 cells/ml)[a] (SD)				Growth rate (0 - 72 hours)		Growth rate (0 – 96 hours)	
	24 Hours	48 Hours	72 Hours	96 Hours	Growth rate ($days^{-1}$)[a]	% Inhibition[ad]	Growth rate ($days^{-1}$)[a]	% Inhibition[ad]
Control	4.75 (1.02)	16.06 (2.13)	74.81 (9.63)	287.75 (20.12)	1.47 (0.04)	n.a	1.45 (0.02)	n.a
Solvent control	4.72 (0.73)	18.84 (4.36)	71.06 (11.66)	285.63 (25.07)	1.45 (0.06)	n.a	1.45 (0.02)	n.a
1.3	4.13 (0.52)	18.19 (5.83)	71.94 (4.14)	275.88 (14.48)	1.46 (0.02)	-1	1.44 (0.01)	1
3.1	4.13 (0.66)	16.25 (2.92)	63.88 (5.34)	252.50 (24.68)	1.42 (0.03)	2	1.42* (0.03)	2
6.2	4.13 (0.85)	16.44 (1.16)	48.75 (16.05)	156.92 (15.88)	1.32* (0.11)	10	1.29* (0.03)	11
11	3.56 (0.43)	12.56 (1.25)	41.56 (11.58)	95.88 (12.02)	1.27* (0.09)	13	1.17* (0.03)	19
23	2.25 (0.46)	7.63 (1.45)	24.19[b] (1.82)	56.56[bc] (4.70)	1.09* (0.03)	25	1.03* (0.02)	29

[a] Mean, standard deviation (SD) and percent inhibition are calculated from original raw data, not from the rounded values presented in this table
[b] Cell fragments observed
[c] Cells appeared to be bloated
[d] Percent inhibition compared to the solvent control
* Significantly reduced compared to the solvent control, based on William's Multiple

Comparison Test
n.a. = not applicable

Table 9.2.7-3: Biomass (expressed as yield) of *Pseudokirchneriella subcapitata* after 72 and 92 hours of exposure to S-2399 TG

Mean measured concentration (mg a.s./L)	Biomass 72 hours		Biomass 96 hours	
	Yield (× 10^4 cells days/ml) [b]	% Inhibition [ab]	Yield (× 10^4 cells days/ml) [b]	% Inhibition [ab]
Control	73.81 (9.63)	n.a.	286.75(20.12)	n.a
Solvent Control	70.06 (11.66)	n.a.	284.63(25.07)	n.a
1.3	70.94 (4.14)	-1	274.88(14.48)	3
3.1	62.88 (5.34)	10	251.50* (24.68)	12
6.2	47.75* (16.05)	32	155.92* (15.88)	45
11	40.56* (11.58)	42	94.88* (12.02)	67
23	23.19* (1.82)	67	55.56* (4.70)	80

[a] Percent inhibition relative to the solvent control
[b] Mean, standard deviation (SD) and percent inhibition are calculated from original raw data, not from rounded values presented in this table
* Significantly reduced compared to the solvent control, based on Williams' Multiple Comparison Test
n.a. = not applicable

Table 9.2.7-4: Calculated area under the growth curve (AUGC) after exposure of Pseudokirchneriella subcapitata to S-2399TG

Mean measured concentration (mg a.s./L)	Area under the growth curve (AUGC) (x 10^4 cells/ml)[b]							
	0-24 Hours	24-48 Hours	48-72 Hours	72-96 Hours	Total area (72 Hours)	Total area (96 Hours)	% inhibition [ab] (72 Hours)	% inhibition [ab] (96 Hours)
Control	1.74 (0.47)	9.41 (1.07)	44.28 (4.77)	176.53 (11.75)	55.4 (5.69)	231.95 (16.23)	n.a	n.a
Solvent Control	1.72 (0.34)	10.78 (2.31)	43.80 (6.56)	173.65 (15.82)	56.31 (8.28)	229.95 (22.61)	n.a	n.a
1.3	1.45 (0.24)	10.16 (2.92)	43.91 (4.14)	169.30 (8.41)	55.51 (7.00)	224.82 (13.62)	1	2
3.1	1.45	9.19	38.93	153.91	49.56	203.48*	12	12

Mean measured concentration (mg a.s./L)	Area under the growth curve (AUGC) (x 10^4 cells/ml)[b]							
	0-24 Hours	24-48 Hours	48-72 Hours	72-96 Hours	Total area (72 Hours)	Total area (96 Hours)	% inhibition [ab] (72 Hours)	% inhibition [ab] (96 Hours)
	(0.31)	(1.14)	(4.11)	(14.66)	(4.94)	(19.59)		
6.2	1.45 (0.4)	9.28 (0.30)	31.48 (8.32)	99.71 (13.84)	42.21* (8.00)	141.93* (21.29)	25	38
11	1.19 (0.20)	7.06 (0.52)	25.97 (6.20)	66.31 (9.74)	34.22* (6.26)	100.53* (15.23)	39	56
23	0.58 (0.21)	3.94 (0.62)	14.85 (1.25)	38.55 (1.73)	19.37* (1.54)	57.93* (2.58)	66	75

[a] Percent inhibition relative to the control
[b] Mean, Standard Deviation (SD) and percent inhibition are calculated from original raw data, not the rounded values presented in this table
*Significantly reduced compared to the control, based on Williams' Multiple Comparison Test
n.a = not applicable

The concentration-response curves for 72-hour and 96-hour growth rate inhibition are shown in the figures below.

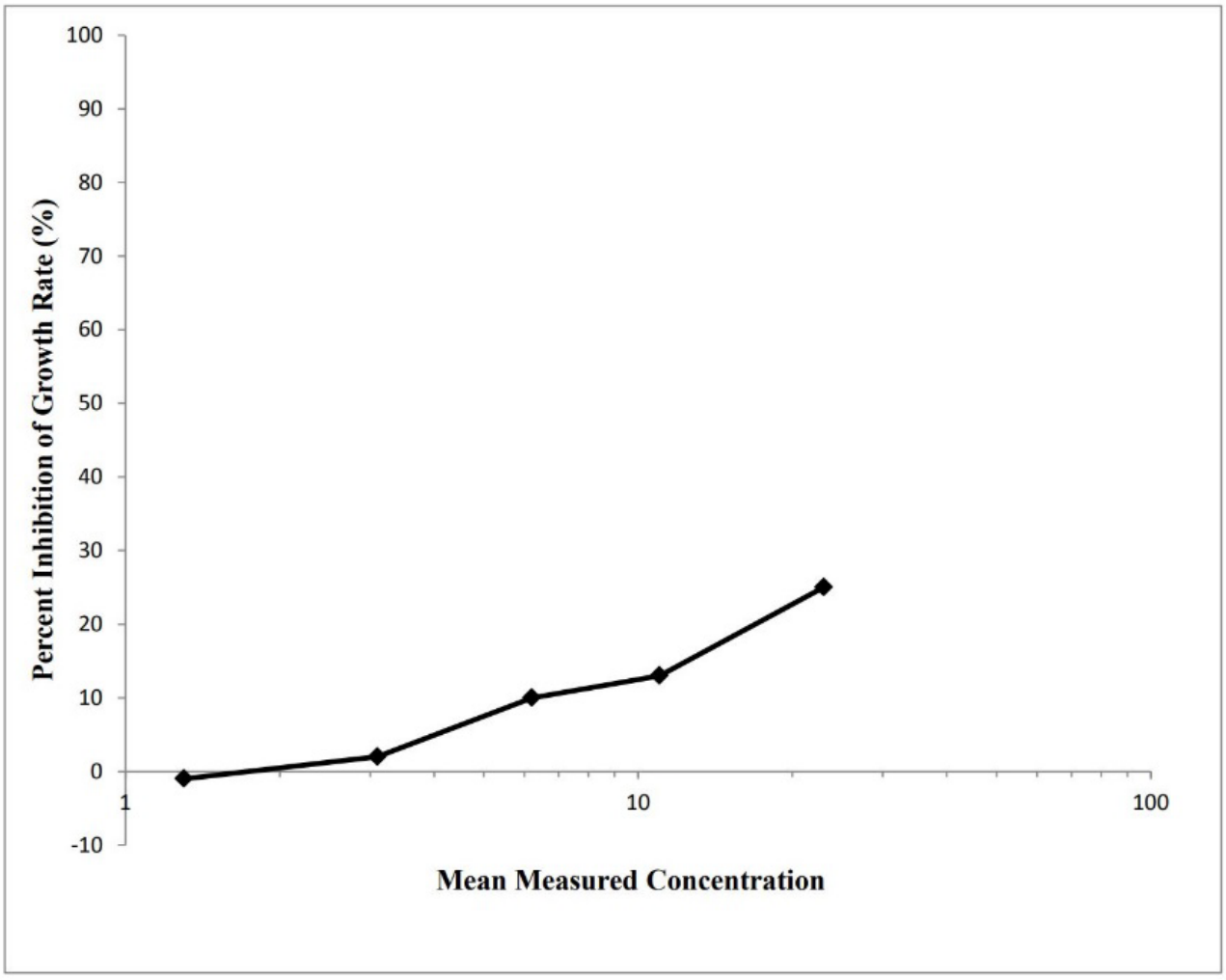


Figure 9.2-18: Percent inhibition of average growth rate (0- to 72-hour) for *Pseudokirchneriella subcapitata* exposed to S-2399 T.G.

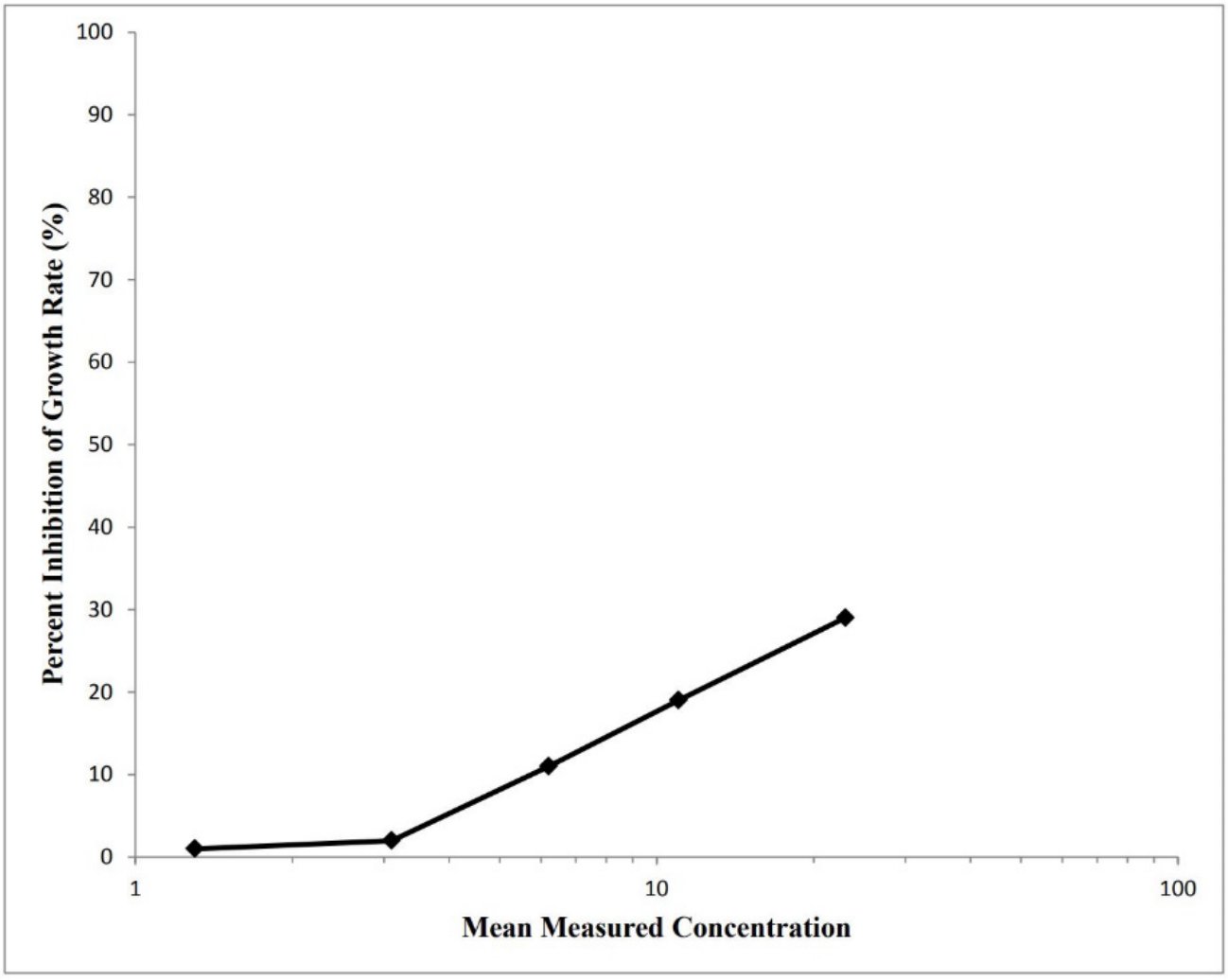


Figure 9.2-19: Percent inhibition of average growth rate (0- to 96-hour) for *Pseudokirchneriella subcapitata* exposed to S-2399 T.G.

Table 9.2.7-5: Summary of endpoints (based on mean measured concentrations)

Endpoint	Based on mean measured concentrations (mg a.s./L)				
	NOEC	LOEC	EC_{10} (95% Confidence Limits)	EC_{20} (95% Confidence Limits)	EC_{50} (95% Confidence Limits)
72-Hour Yield	3.1	6.2	2.9 (0.31-4.5)	4.2 (2.1-7.8)	14 (3.7-19)
72-Hour Growth Rate	3.1	6.2	6.4 (3.5-15)	17 (12-20)	>23 (n.a.)
72-Hour AUGC	3.1	6.2	2.7 (n.d-5.9)	4.8 (1.4-9.2)	15 (10-19)
96-Hour Yield	1.3	3.1	-*	-	7.1 (5.6 – 8.2)

Endpoint	Based on mean measured concentrations (mg a.s./L)				
	NOEC	LOEC	EC_{10} (95% Confidence Limits)	EC_{20} (95% Confidence Limits)	EC_{50} (95% Confidence Limits)
96-Hour Growth Rate	1.3	3.1	-	-	>23 (n.a)
96-Hour AUGC	1.3	3.1	-	-	9.0 (6.5 – 12)

n.a = not applicable. EC value is empirically estimated, therefore 95% confidence limits could not be determined
n.d = not determined. Corresponding lower 95% confidence limit could not be determined
*No data provided

B. ANALYSIS
Mean measured concentrations of S-2399 TG ranged from 68 to 83% of nominal concentrations. Results of the analyses demonstrated that the mean measured concentrations closely approximated the nominal concentrations and while generally consistent, did show a small decrease in the four highest treatments over time. Results were based on mean measured concentrations.

A summary of the measured concentrations of S-2399 TG is presented in Table 9.2.7-6.

Table 9.2.7-6: Measured concentrations of S-2399 TG in the exposure solutions

Nominal concentration (mg a.s./L)	Measured concentration (mg a.s./L)			% Nominal[a]
	0-Hour	96-Hour[d]	Mean[a]	
Control	<0.13[b]	<0.16[b]	n.a.	n.a.
Solvent Control	<0.13[b]	<0.16[b]	n.a.	n.a.
1.9	1.3	1.3	1.3	68
3.8	3.4	2.8	3.1	81
7.5	7.0	5.3/6.2[c]	6.2	83
15	12	9.3	11	71
30	25	21	23	78

[a] Mean measured concentrations and percent of nominal concentrations were calculated using actual analytical data
[b] Concentrations expressed as less than values were below the minimum detectable limit (MDL)
[c] Result of the addition sample without algae present to determine biological uptake/degradation
[d] As the study was conducted to US-EPA requirements, no analytical results were available at 72-h. However, the 96-h results are considered as conservative
n.a. = not applicable

C. VALIDITY CRITERIA

The reference test 96-hour EC_{50} value based on cell density was determined to be 0.085 mg Zn/L. Previous reference testing determined a mean EC_{50} value of 0.068 mg Zn/L. The EC_{50} value closely approximates the results determined in previous testing.

Table 9.2.7-7: Validity criteria for the untreated control

Test guideline	Criterion	Required Result		Result Obtained
		OECD 201	OCSPP 850.4500	
OECD 201 (2011) and OCSPP 850.4500 (2012)	Biomass increase in the control cultures by 72 (OECD) or 96 (OCSPP) hours	Increase by a factor of 16	Increase by a factor of 30	Increased by a factor of 73.81 (72 hours) and 70.06 (96 hours)
	Mean coefficient of variation for section-by-section specific growth rates in the control cultures	≤ 35 %	-	18.9% (72 hours) and 16.5% (96 hours)
	Coefficient of variation of average specific growth rates during the whole test period in the control cultures	≤ 10 %	≤ 15 %	2.8% (72 hours) and 1.3% (96 hours)
Test guideline	Criterion	Required Result		Result Obtained
		OECD 201	OCSPP 850.4500	
OECD 201 (2011) and OCSPP 850.4500 (2012)	Biomass increase in the control cultures by 72 (OECD) or 96 (OCSPP) hours	Increase by a factor of 16	Increase by a factor of 30	Increased by a factor of 286.75 (72 hours) and 284.63 (96 hours)
	Mean coefficient of variation for section-by-section specific growth rates in the control cultures	≤ 35 %	-	17.1% (72 hours) and 15.9% (96 hours)
	Coefficient of variation of average specific growth rates during the whole test period in the control cultures	≤ 10 %	≤ 15 %	4.0% (72 hours) and 1.6% (96 hours)

III. CONCLUSION

The results of the laboratory study on the effects of S-2399 TG on *Pseudokirchneriella subcapitata* demonstrate the 72 hour EC_{50} values yield, specific growth rate and area under the growth curve were 14, > 23 and 15 mg a.s./L, respectively. The 72-hour NOEC and LOEC were estimated to be 3.1 and 6.2 mg a.s./L, respectively. All endpoints were based on mean measured concentrations.

HSE COMMENTS:
This study was conducted under GLP and under OECD 201 (2011) and U.S. EPA OCSPP 850.4500 guidelines and has been assessed against OECD 201 (2011) guidelines.

The concentrations of the test item were not maintained between 80-120% of the nominal value throughout the test. Results should be based on mean measured concentrations, as has been expressed by the applicant. The method of analysis used in the study was evaluated by HSE Chemistry. The conclusions of their evaluation are reproduced below. Please see Volume 3 CA, section B5 for more details.

"*The analytical method is not fully validated according to SANTE/2020/12830 rev. 1 for the determination of the active substance inpyrfluxam in freshwater as the matrix effects have not been determined and the stability of standards and extracts has not been addressed. However, the studies were generated prior Ito the implementation of SANTE 2020/12830 rev.1 and SANCO 3029 rev.4 did not require matrix effects and the stabilities of standards and extracts to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose.*"

There are two deviations to protocol to note. The first is the 2.3-unit increase in the pH of the control medium that was recorded after 96 hours. OECD 201 (2011) guidance states that the pH of the control medium should not increase by more than 1.5 units during the test. No adverse observations were made for the control group and all validity criteria relating to the control group were met, therefore; the study is considered valid.

In the analysis of this study it is noted that the analytical result of the 96-hour sample from the 7.5 mg a.s./L nominal treatment levels, with algae present, was 5.3 mg a.s./L. The equivalent test solution without algae present resulted in a recovery of 6.2 mg a.s./L and demonstrated that the presence of algae had an impact on the concentration of S-2399 TG in the test solution. The concentrations of S-2399 TG ranged from 68 to 83% of nominal concentrations, therefore; mean measured concentrations were used for analysis of results.

The use of statistics in this study is in line with OECD 201 (2011) guidance.

The endpoints for use in risk assessment are:
96-hour E_rC_{50} > 23mg/L based on mean measured concentrations

B.9.2.7.2 Effects on growth of an additional algal species

Reference:	KCA 8.2.6.2/01
Report Title:	S-2399 TG: Toxicity Test with the Freshwater Diatom, *Navicula pelliculosa*
Author(s) & year:	[redacted] (2015b)
Document No, Authority registration No:	12709.6370
Substance used:	S-2399 TG, 13CG0617G, 95 %
Method of analysis:	LC/MS/MS method
Guideline(s):	OECD 201 (2011)
Deviations:	Yes, see HSE comments section.
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test material:** S-2399 TG
   Description: not stated
   Lot/Batch: 13CG0617G
   Purity: 95.0%
   Expiration date: 23rd July
   Reference item: Zinc chloride
   Solvent: Dimethylformamide

B. STUDY DESIGN AND METHODS

1. **Test organism:** *Navicula pelliculosa*
   Strain: 661
   Growth stage: Inoculum- three days since previous transfer
   Source: In-house culture
   Initial cell density: 1.0 x 10^4 cells/mL
   Analysis of concentrations: 0.028, 0.090, 0.29, 0.92, 2.9, 9.4 and 30 mg/L. LOQ was set at 0.600 µg/L. MDL was 0.200 µg/L
   Exposure regime: Static
   Mixing: Teflon®-coated stir bar and magnetic stir plate for two hours

2. **Growth medium:** Algal Assay Procedure (AAP) Medium

pH: 7.5 ± 0.1

3. Test vessels: Sterile 250 ml conical flasks, containing 100 ml of test solution

4. Environmental conditions:

A summary of environmental conditions is shown in Table 9.2.7-8 below.

Table 9.2.7-8: Summary of environmental conditions

Variable	Required OECD 201 (2011)	Obtained
Temperature	21 to 24°C	23˚C – 24˚C
pH	Not stated	7.2 – 8.5
Photoperiod	Continuous illumination	Continuous illumination
Lighting intensity	60-120 µE·m-2 s -1	60-76µE/m^2/s
Conductivity of dilution water	Not stated	270 to 280 µS/cm

Study dates:
22 June to 2 July 2015

5. Test organism set up and treatment:
Four replicate, 250 mL glass Erlenmeyer flasks for each treatment level and solvent control were prepared. Eight replicate flasks were prepared for the control, with 100 mL of the appropriate exposure solution. After the test solutions were added to the flasks, a 1.275 mL inoculum of *Navicula pelliculosa* cells, at a density of approximately 78.42 × 10^4 cells/mL, was introduced into each flask to provide the required initial cell density of 1.0 × 10^4 cells/mL.

Nominal test concentrations of S-2399 TG for the 96-hour test were 0.0 (control), 0.0 (solvent control), 0.028, 0.090, 0.29, 0.92, 2.9, 9.4 and 30 mg a.s./L. The test was conducted in an environmental chamber, with all flasks being agitated once daily by hand shaking.

A reference test was conducted prior to the definitive test, using zinc chloride as the toxicant to evaluate the sensitivity of *Navicula pelliculosa*.

6. Dose preparation:
A 300 mg a.s./mL primary stock solution was prepared prior to test initiation by placing 7.8947 g of S-2399 TG (7.5000g a.s.) in a 25 mL volumetric flask and bringing it to volume with dimethylformamide (DMF). The resulting stock solution was observed to be clear and tan in colour with no visible, undissolved test substance following approximately two minutes of sonication. A 30 mg a.s./L secondary stock solution was prepared by diluting 0.20 mL of the primary stock solution to a final volume of 2.0 L with AAP medium. The resulting stock solution was clear and colourless with a small amount of visible undissolved test substance

following sonication for 20 minutes and mixing for two hours. The stock solution was filtered using a 0.45 µm filter and then filtered a second time using a 0.22 µm filter to remove undissolved test substance The twice filtered stock solution was observed to be clear and colourless with no visible undissolved test substance. The filtrate of the 30 mg a.s./L stock solution was used as the highest nominal test concentration. The remaining test concentrations were prepared from dilutions of this stock solution.

In order to estimate the impact that the presence of algal biomass had on the test substance concentration, an additional replicate vessel of the 0.92 mg a.s./L (nominal) test solution was prepared.

A control was prepared from untreated AAP medium and a solvent control was prepared with 0.1 mL/L DMF.

7. Measurements and observations:

Due to the nature of *Navicula pelliculosa* cells to cluster together, the test item and control solutions were vigorously pipetted prior to each observation to disperse cells and provide a homogeneous suspension of cells for counting. At each subsequent 24-hour interval, cell counts were conducted on every replicate treatment and control vessels using a hemacytometer and compound microscope. Observations of health of the algal cells were also made and recorded. A visual check of the test solutions occurred daily.

Temperature was measured continuously with maximum and minimum temperatures recorded daily. The PAR of the test area was measured at test initiation at each 24-hour interval during the exposure period. Light intensity was also measured in lux at four locations around the perimeter of the test solutions. Following each observation interval, the test flasks were agitated by hand shaking and placed in new random positions. Water quality parameters, pH and conductivity, were measured at test initiation and pH was also measured at the termination of the exposure period.

A single sample was removed from each test concentration and the controls at exposure initiation (0 hours) and test termination (96 hours) to determine S-2399 TG concentrations. Samples analysed at 0 hours were removed from the test item and control solutions prior to filling the individual test vessels. Samples analysed at 96 hours were removed from individually composited replicate solutions of the treatment levels and controls. At 96 hours, two samples were removed from the additional replicate flask which did not contain algae. One was analysed for S-2399 TG concentration to assess the impact that algae had on test substance concentration. The other sample was retained for possible future analysis. Measurements of S-2399 TG were determined using a validated LC/MS/MS method. The LOQ was set at 0.600 µg/L and the MDL was 0.200 µg/L.

8. Statistics:

The EC values were determined by linear interpolation of response (percent reduction compared with the control) using the IC_p method. If the data sets passed the test for homogeneity and normality, Williams's Multiple Comparison Test was used to determine the NOEC and LOEC. If the data did not pass the tests for homogeneity and normality, then the NOEC and LOEC were determined using an appropriate non-parametric statistical test. CETIS™ v 1.8 was used to perform all statistical analyses.

II. RESULTS AND DISCUSSION

A. BIOLOGICAL EFFECTS

A summary of the effects on cell density, biomass, growth rate and area under the growth curve (AUGC) is presented in Table 9.2.7-9 to Table 9.2.7-11, followed by a summary of the endpoints in Table 9.2.7-12.

Table 9.2.7-9: Summary of effects on cell density and growth rate of Navicula pelliculosa following exposure to S-2399 TG

Initial measured concentration (mg a.s./L)	Mean cell density (× 10^4 cells/ml)[a] (SD)				Growth rate (0 – 72 hours)		Growth rate (0-96 hours)	
	24 Hours	48 Hours	72 Hours	96 Hours	Growth Rate (days^{-1}) [a]	% Inhibition[a] [d]	Growth Rate (days $^{-1}$)	% Inhibition[a] [d]
Control	2.09 (0.55)	10.56 (1.21)	48.91 (6.31)	92.94 (10.47)	1.33 (0.05)	n.a	1.17 (0.03)	n.a
Solvent control	2.44 (0.77)	12.81 (0.90)	51.63 (11.33)	96.00 (14.02)	1.35 (0.07)	n.a	1.17 (0.04)	n.a
0.029	2.13 (0.60)	13.00 (0.74)	57.13 (7.80)	99.75 (9.30)	1.39 (0.05)	-4	1.18 (0.03)	-2
0.085	1.75 (0.35)	12.81 (2.02)	56.13 (4.31)	93.25 (11.06)	1.38 (0.03)	-4	1.17 (0.03)	0
0.29	1.88 (1.01)	13.63 (1.27)	49.75 (4.39)	97.69 (10.07)	1.34 (0.03)	-1	1.18 (0.03)	-1
0.90	1.94 (0.75)	12.44 (1.30)	47.63 (8.23)	73.88 (7.85)	1.32 (0.06)	1	1.11 (0.03)	5
2.8	2.31 (0.24)	7.13 (1.81)	32.81 (6.64)	71.00 (6.21)	1.19* (0.07)	11	1.10[e] (0.02)	6
9.9	1.63 (0.32)	4.38 (1.05)	10.44 (1.97)	17.69 (3.58)	0.80* (0.07)	40	0.74[e] (0.05)	37
32	1.13 (0.25)	1.63 (0.85)	1.38 (0.32)	1.31 (0.43)	0.10* (0.08)	92	0.06[e] (0.09)	95

[a] Mean, standard deviation (SD) and percent inhibition are calculated from original raw data, not from the rounded values presented in this table
[b] Cell fragments observed
[c] Cells appeared to be bloated
[d] Percent inhibition compared to the solvent control
[e] Significantly reduced compared to the control, based on Dunnett's Multiple Comparison Test
*Significantly reduced compared to the solvent control, based on William's Multiple Comparison Test
** Significantly reduced compared to the solvent control, based on Dunnett's Multiple Comparison Test
n.a = not applicable

The 72-hour and 96-hour concentration-response curves are shown in figures below.

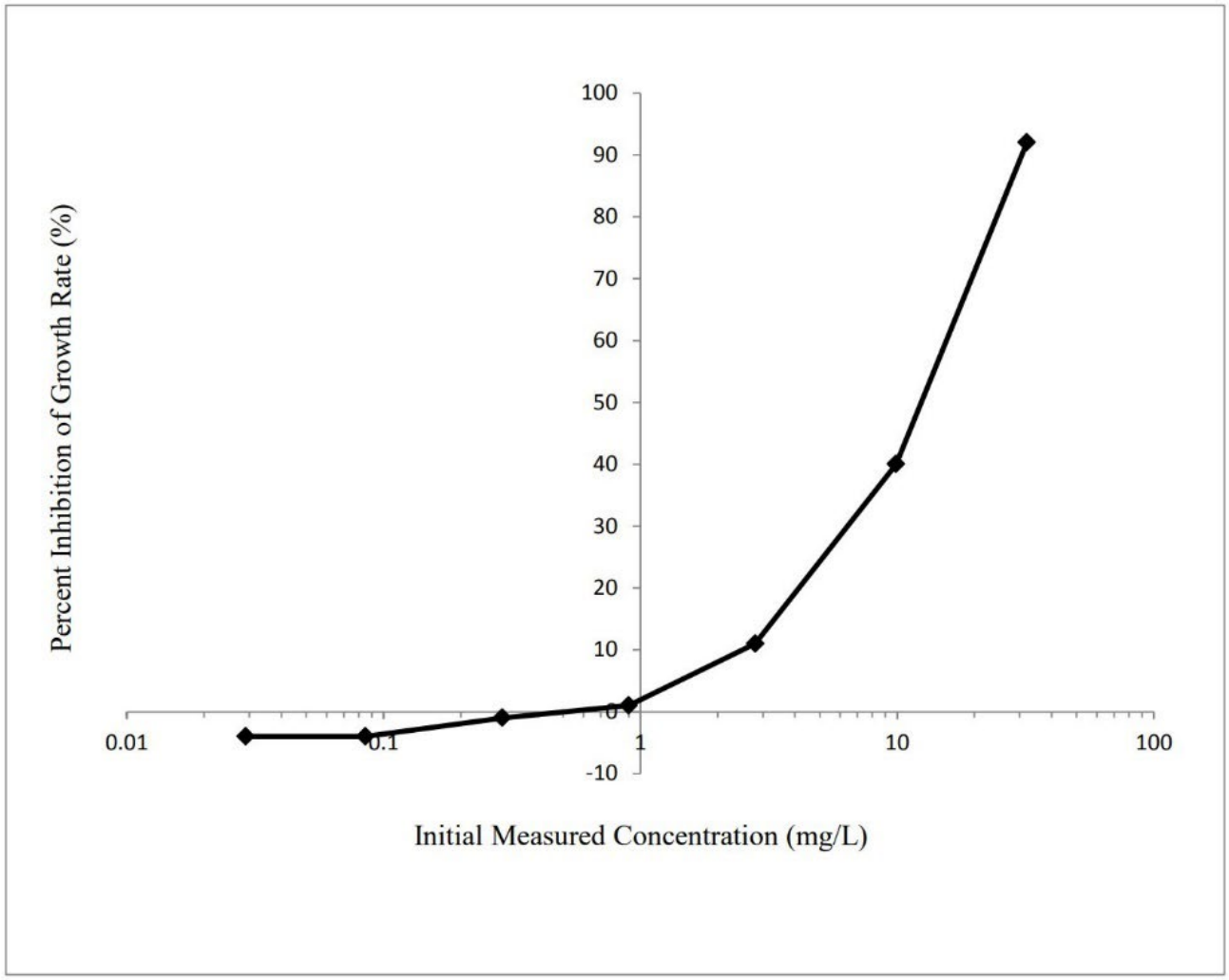


Figure 9.2-20: Percent inhibition of average growth rate (0- to 72-hour) for *Navicula pelliculosa*

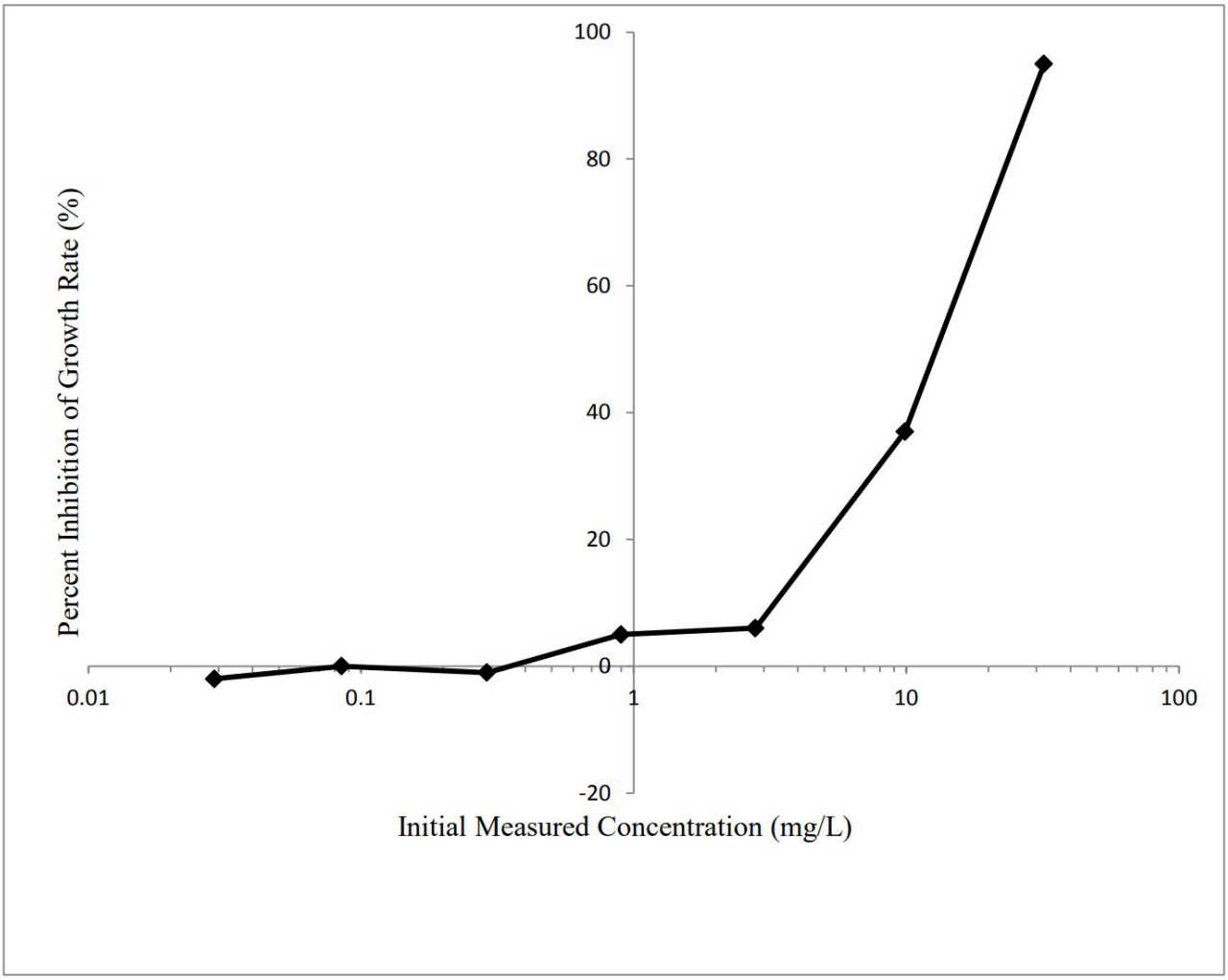


Figure 9.2-21: Percent inhibition of average growth rate (0- to 96-hour) for *Navicula pelliculosa*

Table 9.2.7-10: Biomass (expressed as yield) of *Navicula pelliculosa* after 72 hours of exposure to S-2399 TG

Initial measured concentration (mg a.s./L)	Biomass 0-72 hours		Biomass 0-96 hours	
	Yield (× 10^4 cells days/ml) [b]	% Inhibition [ab]	Yield (x10^4 cells days/ml)[b]	% Inhibition[ab]
Control	47.91 (6.31)	n.a.	91.94 (1.21)	n.a
Solvent Control	50.63 (11.33)	n.a.	95.00 (14.02)	n.a
0.029	56.13 (7.80)	-17	98.75 (9.30)	-7
0.085	55.13 (4.31)	-15	92.25 (11.06)	0
0.29	48.75 (4.39)	-2	96.69 (10.07)	-5

Initial measured concentration (mg a.s./L)	Biomass 0-72 hours		Biomass 0-96 hours	
	Yield (× 10^4 cells days/ml) [b]	% Inhibition [ab]	Yield (x10^4 cells days/ml)[b]	% Inhibition[ab]
0.90	46.63 (8.23)	3	72.88[c] (7.85)	21
2.8	31.81** (6.64)	34	70.00[c] (6.21)	24
9.9	9.44** (1.97)	80	16.69[c] (3.58)	82
32	0.38** (0.32)	99	0.31[c] (0.43)	100

[a] Percent inhibition relative to the solvent control
[b] Mean, standard deviation (SD) and percent inhibition are calculated from original raw data, not from rounded values presented in this table
[c] Significantly reduced compared to the control based on Dunnett's Multiple Comparison Test
* Significantly reduced compared to the solvent control, based on Dunnett's Multiple Comparison Test
** Significantly reduced compared to the solvent control, based on Jonckheere-Terpstra Step-Down Test
n.a. = not applicable

Table 9.2.7-11: Calculated area under the growth curve (AUGC) after exposure of *Navicula pelliculosa* to S -2399 TG

Initial measured concentration (mg a.s./L)	Area under the growth curve (AUGC) (× 10^4 cells/ml)[b]							
	0-24 Hours	24-48 Hours	48-72 Hours	72-96 Hours	72 Hours Total area	96 Hours Total area	72-Hour % inhibition [ab]	96-Hour % inhibition
Control	0.50 (0.25)	5.28 (0.50)	28.81 (3.33)	67.98 (6.83)	34.60 (3.59)	102.58 (9.68)	n.a.	n.a.
Solvent Control	0.66 (0.35)	6.57 (0.72)	31.31 (5.77)	70.79 (3.93)	38.53 (5.69)	109.32 (7.61)	n.a.	n.a.
0.029	0.51 (0.27)	6.51 (0.41)	34.16 (4.04)	75.29 (7.68)	41.18 (3.67)	116.47 (11.25)	-19	-14
0.085	0.34 (0.16)	6.23 (1.16)	33.56 (2.77)	71.64 (6.41)	40.13 (3.72)	111.77 (9.02)	-16	-9
0.29	0.40 (0.46)	6.69 (0.46)	30.77 (1.60)	70.70 (5.41)	37.87 (2.00)	108.57 (7.02)	-9	-6
0.90	0.43 (0.34)	6.14 (0.46)	29.11 (3.80)	58.09 (5.25)	35.68 (4.16)	93.77 (9.00)	-3	9
2.8	0.60 (0.11)	3.69 (0.91)	19.02(3.41)	49.49 (5.40)	23.31* (3.92)	72.80* (8.62)	33	29

Initial measured concentration (mg a.s./L)	Area under the growth curve (AUGC) (× 10^4 cells/ml)[b]							
	0-24 Hours	24-48 Hours	48-72 Hours	72-96 Hours	72 Hours Total area	96 Hours Total area	72-Hour % inhibition [ab]	96-Hour % inhibition
9.9	0.29 (0.15)	1.98 (0.47)	6.42 (1.16)	12.70 (2.22)	8.69* (1.60)	21.39 * (3.72)	75	79
32	0.06 (0.11)	0.37 (0.54)	0.50 (0.31)	0.33 (0.33)	0.93* (0.94)	1.26* (0.85)	97	99

[a] Percent inhibition relative to the control
[b] Mean, standard deviation (SD) and percent inhibition are calculated from original raw data, not the rounded values presented in this table
* Significantly reduced compared to the control, based on Williams' Multiple Comparison Test
n.a. = not applicable

Table 9.2.7-12: Summary of endpoints (based on initial measured concentrations) after 72 hours exposure

Endpoint	Based on initial measured concentrations (mg a.s./L)				
	NOEC	LOEC	EC_{10} (95% Confidence Limits)	EC_{20} (95% Confidence Limits)	EC_{50} (95% Confidence Limits)
72-Hour Yield	0.90	2.8	0.55 (0.0038-1.6)	1.3 (0.36-2.1)	3.9 (2.0-5.3)
72-Hour Average Specific Growth Rate	0.90	2.8	2.1 (1.2-3.5)	4.0 (2.8-4.9)	12 (11-14)
72-Hour AUGC	0.90	2.8	1.0 (0.19-1.5)	1.5 (0.95-2.1)	4.1 (2.3-5.3)
96-Hour Yield	0.29	0.90	-*	-	4.9 (4.1 - 5.5)
96-Hour Average Specific Growth Rate	0.90	2.8	-	-	13 (12-14)
96-Hour AUGC	0.90	2.8	-	-	4.5 (3.4 – 5.3)

∗ No data provided

B. ANALYSIS
Mean measured concentrations of S-2399 TG ranged from 79 to 95% of nominal concentrations. Concentrations closely approximated the nominal concentrations at 0 hour

and declined slightly during the exposure but maintained the concentration gradient. The results were based on initial (0 hour) measured concentrations.

The analytical result of the 96-hour sample from the 0.92 mg a.s./L nominal treatment levels, with algae present was 0.67 mg a.s./L. The equivalent test solution without algae present resulted in a recovery of 0.75 mg a.s./L and demonstrated that the presence of algae had a slight impact on the concentration of S-2399 T.G. in the test solution.

A summary of the measured concentrations of S-2399 TG is presented in Table 9.2.7-13.

Table 9.2.7-13: Measured concentrations of S-2399 TG in the exposure solutions

Nominal concentration (mg a.s./L)	Measured concentration[a] (mg a.s./L)			% Nominal[a]
	0-Hour	96-Hour[d]	Mean	
Control	<0.0020[b]	<0.0020[b]	n.a	n.a
Solvent Control	<0.0020[b]	<0.0020[b]	n.a	n.a
0.028	0.029	0.020	0.024	85.9
0.090	0.085	0.058	0.071	78.5
0.29	0.29	0.22	0.25	86.8
0.92	0.90	0.67/0.75[c]	0.78	84.3
2.9	2.8	2.3	2.5	87.4
9.4	9.9	7.8	8.7	93.1
30	32	25	28	94.9

[a] Measured concentrations and percent of nominal concentrations were calculated using actual analytical data
[b] Concentrations expressed as less than values were below the minimum detectable limit (MDL)
[c] Result of the addition sample without algae present to determine biological uptake/degradation
[d] As the study was conducted to US-EPA requirements, no analytical results were available at 72-h. However, the 96-h results are considered as conservative
n.a. = not applicable

C. VALIDITY CRITERIA

Table 9.2.7-14: Validity criteria for the untreated control

Test guideline	Criterion	Required Result		Result Obtained
		OECD 201	OCSPP 850.4500	
OECD 201 (2011) and OCSPP 850.4500 (2012)	Biomass increase in the control cultures by 72 (OECD) or 96 (OCSPP) hours	Increase by a factor of 16	Increase by a factor of 30	Increased by a factor of 47.01 **(72 hours)** and 50.63 **(96 hours)**
	Mean coefficient of variation for section-by-section specific growth rates in the control cultures	≤ 35 %	-	43.1 % **(72 hours)** and 50.1 % **(96 hours)**
	Coefficient of variation of average specific growth rates during the whole test period in the control cultures	≤ 10 %	≤ 15 %	3.4 % **(72 hours)** and 2.6 % **(96 hours)**

Table 9.2.7-15: Validity criteria for the untreated solvent control

Test guideline	Criterion	Required Result		Result Obtained
		OECD 201	OCSPP 850.4500	
OECD 201 (2011) and OCSPP 850.4500 (2012)	Biomass increase in the control cultures by 72 (OECD) or 96 (OCSPP) hours	Increase by a factor of 16	Increase by a factor of 30	Increased by a factor of 91.94 **(72 hours)** and 95.00 **(96 hours)**
	Mean coefficient of variation for section-by-section specific growth rates in the control cultures	≤ 35 %	-	32.6 % **(72 hours)** and 45.6 % **(96 hours)**
	Coefficient of variation of average specific growth rates during the whole test period in the control cultures	≤ 10 %	≤ 15 %	5.6 % **(72 hours)** and 3.5% **(96 hours)**

The reference test 96-hour EC_{50} value based on cell density was determined to be 0.091 mg Zn/L. Previous reference testing determined a mean EC_{50} value of 0.11 mg Zn/L. The EC_{50} value approximates the results determined in previous testing, therefore, the test conditions are reliable.

III. CONCLUSION

The results of the laboratory study on the effects of S-2399 TG on *Navicula pelliculosa* demonstrate the 72-hour EC_{50} for yield, average specific growth rate and AUGC to be 3.9, 12 and 4.1 mg a.s./L, respectively. The 72-hour NOEC was estimated to be 0.90 mg a.s./L and the LOEC was 2.8 mg a.s./L. All endpoints were based on initial measured concentrations.

HSE COMMENTS:
This study was conducted under GLP and under OCSPP Guideline 850.4500 and OECD 201 (2011) guidance and has been assessed against OECD 201 (2011) guidance.

The concentrations of the test item were not maintained between 80-120% of the nominal value throughout the test. Results were based on initial measured concentrations but should have been based on mean measured concentrations. As there was no inhibition of growth rate recorded at the concentration that had a mean measured concentration of <80%, HSE consider it unlikely that even if the results had been based on mean measured concentrations that this would alter the ErC_{50} derived. The method of analysis used in the study was evaluated by HSE Chemistry. The conclusions of their evaluation are reproduced below. Please see Volume 3 CA, section B5 for more details.

"*The analytical method is not fully validated according to SANTE/2020/12830 rev. 1 for the determination of the active substance inpyrfluxam in freshwater as the matrix effects have not been determined and the stability of standards and extracts has not been addressed. However, the studies were generated prior Ito the implementation of SANTE 2020/12830 rev.1 and SANCO 3029 rev.4 did not require matrix effects and the stabilities of standards and extracts to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose.*"

The validity criterion for the mean CV of section-by-section specific growth rate in the control group after 72 hours was not met. The value obtained (40%) exceeds the validity criterion in the OECD 201 (2011) guidance (<35%). The applicant has stated that the section-by-section growth rate CV for the solvent control was within the guideline requirement at 31%. Additionally, all remaining criteria have been met for the control for both OECD and OSCPP guidelines, and the dose response clearly defines the toxicity of S-2399 TG to *Navicula pelliculosa*. The applicant states that the missed criterion is considered to have had no negative impact on the results or interpretation of this study and it is considered valid. As the percentage inhibition has been calculated from the solvent control, which meets the validity criteria, HSE consider the 72-hour endpoint suitable for use for risk assessment.

Whilst not a deviation from protocol or validity criteria, it is worth noting that the analytical result of the 96-hour sample from the 0.92 mg a.s./L nominal treatment levels, with algae present was 0.67 mg a.s./L. The equivalent test solution without algae present resulted in a recovery of 0.75 mg a.s./L and demonstrated that the presence of algae had a slight impact on the concentration of S-2399 T.G. in the test solution.

It is also worth noting that there is overlap in the 95% confidence limits of the E_rC_{10} and the E_rC_{20} values, which may limit the reliability of these endpoint values. These endpoints will not be used in the risk assessment.

The use of statistics in this study is suitable and meets requirements of OECD 201 (2011) guidance.

RAI and response:
From Table 9.2.7-13, there is evidence that the concentration of the substance was not kept within ± 20 % of the nominal concentration (78.5 % of nominal concentration for 0.090 mg a.s./L treatment group). In such cases, endpoints should be calculated using geometric mean measured concentrations. This endpoint re-expression was requested in a RAI. The RAI response calculated the geometric mean measured concentrations and used these to recalculate E_rC_x values (Table 9.2.7-16), using ToxRat (version 3.3.0). Negative and solvent controls were pooled as there were no significant difference between them. A probit model was fitted.

Table 9.2.7-16: Recalculated E_rC_x values for KCA 8.2.6.2/01

Time period	mg a.s./L		
	ErC10 (95% CI)	ErC20 (95% CI)	ErC50 (95% CI)
72 hours	3.437 (2.745 - 4.071)	4.979 (4.225 - 5.655)	10.113 (9.253 - 11.053)
96 hours	4.524 (3.825 - 5.132)	6.102 (5.418 - 6.695)	10.817 (10.127 - 11.597)

Based on these recalculated endpoints, the **Endpoint to be used for risk assessment is:**

- **72-hour E_rC_{50} = 10.113 mg a.s./L (based on mean measured concentrations)**

Reference:	KCA 8.2.6.2/02
Report Title:	S-2399 TG: Toxicity Test with the Freshwater Cyanobacterium, *Anabaena flos-aquae*
Author(s) & year:	[redacted] (2015c)
Document No, Authority registration No:	12709.6369
Substance used:	S-2399 TG, 13CG0617G, 95 %

Method of analysis:	LC/MS/MS method
Guideline(s):	OECD 201 (2011)
Deviations:	Yes, see HSE comments section.
GLP or GEP:	Yes
Acceptability:	No
Study relied upon:	No, due to unacceptable deviations from the validity criteria

MATERIALS AND METHODS

MATERIALS

Test material S-2399 TG

Lot/Batch: 13CG0617G

Purity: 95.0 % (certified by certificate of analysis)

Description: Not stated

Expiration date: 23 July 2016

TREATMENTS

Test concentrations: Nominal test concentrations: 1.9, 3.8, 7.5, 15 and 30 mg/L

Mean measured test concentrations: 1.5, 3.1, 6.1, 12 and 27 mg/L

Controls: Negative control - Algal Assay Procedure (AAP) medium
Solvent control - DMF (0.1 mL/L)

Toxic reference Zinc Chloride 96-hour EC_{50} = 0.043 mg Zn/L (95% confidence intervals of 0.037 to 0.045 mg Zn/L) (conducted 12 to 16 December 2014). Comparable to mean EC_{50} value of 0.048 mg Zn/L for *A. flos-aquae* from previous testing.

Analysis of test concentrations: Yes, at 0 and 96 hours (all treatment levels and control) based on analysis of S-2399 using liquid chromatography with mass spectrometry detection (LC/MS/MS). The limit of quantification (LOQ) of the analytical method was 0.600 S-2399 µg/L and minimum detectable limit (MDL) was 0.200 S-2399 µg/L.

TEST ORGANISMS

Species: *Anabaena flos-aquae* (strain 67)

Growth stage: Inoculum - three days since previous transfer

Stock cultures: Daily agitation, 24 to 26 ºC, illumination of 30 µE/m^2/S ± 4 (1830 to 2470 lux). The inoculum used to initiate the toxicity test with S-2399 TG was taken from a stock culture that had been transferred to fresh medium three days before testing.

Inoculation rate:	1 × 10^4 cells/mL
Source:	In-house culture (originally from the Canadian Phycological Culture Centre for Algae, Cyanobacteria, and Lemna at the University of Waterloo)
TEST DESIGN	
Test vessels:	Sterile glass 250 mL Erlenmeyer (conical) flasks filled with 100 mL of test or control medium and covered with stainless steel caps
Test medium:	AAP Medium (pH 7.5 ± 0.1)
Replication:	Four replicates per treatment and solvent control. Eight replicates for the negative control.
Exposure regime:	Static test conditions with no renewal of the test media
Duration:	96 hours
TEST CONDITIONS	
Test temperature:	23 - 24 °C
pH:	Test start: 7.2 Test end: 8.4 – 9.1
Lighting:	Continuous light at a light intensity at a photosynthetically-active radiation (PAR) of 27 – 32 µE/m^2/S ± 4 (2100 to 2300 lux)
Shaking:	Hand shaking once daily

STUDY DESIGN AND METHODS

Experimental dates: The exposure phase of the definitive test was conducted between 8 and 12 June 2015.

Test organism and culturing

The test organism, *Anabaena flos-aquae*, is a freshwater cyanobacterium of the class Cyanophyceae. The AAP culturing medium was prepared using deionised water and analysed periodically for the presence of pesticides, PCBs and toxic metals using U.S. EPA standard methods (U.S. EPA, 1997)[40]. None of these compounds were detected at concentrations considered toxic in any of the water samples analysed in agreement with ASTM Guidelines (2007)[41]. Furthermore, a representative sample of AAP medium was analysed monthly for total organic carbon (TOC) concentration (June 2015 = 0.38 mg/L).

Test solution preparation

A 300 mg/mL primary stock solution was prepared by placing 7.8947 g of S-2399 TG (7.5000 g as active ingredient) in a 25-mL volumetric flask and bringing it to volume with dimethylformamide (DMF, CAS No.: 68-12-2). The resulting stock solution was observed to be clear and tan in colour with no visible, undissolved test substance following

[40] U.S. EPA, 1997. Office of Waste. Test Methods for Evaluating Solid Waste, Physical/Chemical Methods (SW-846). February 2007. U.S. Environmental Protection Agency, Washington, D.C.

[41] ASTM, 2007. Standard Practice for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates and Amphibians. Standard E729-96. American Society for Testing and Materials, 100 Barr Harbor Drive, West Conshohocken, PA 19428, pp. 65 – 86.

approximately two minutes of sonication.

A 30 mg/L secondary stock solution was prepared by diluting 0.200 mL of the 300 mg/mL primary stock solution to a final volume of 2.0 L with AAP medium. This secondary stock solution was filtered three times to remove undissolved test substance. The filtrate of the 30 mg/L stock solution was used as the highest nominal test concentration and serial dilutions yielded the other test concentrations. All exposure solutions were observed to be clear and colourless with no visible undissolved test substance. All test solutions contained 0.1 mL DMF/L thereby matching the solvent control DMF concentration. The negative control contained untreated AAP medium only.

In order to estimate the impact that the presence of cyanobacterial biomass had on the test substance concentration, an additional replicate test vessel of the 7.5 mg/L (nominal) test solution was prepared. This test vessel, which was not inoculated with cyanobacteria, was analysed at 96 hours of exposure for S-2399 TG concentration. The result of this analysis was compared with the result for the 7.5 mg/L (nominal) solution containing cyanobacteria.

Definitive exposure

Cyanobacterial growth

Exposure initiation began when a 1.91 mL inoculum of *A. flos-aquae* cells, at a density of approximately 52.35 × 10^4 cells/mL, was aseptically introduced into each flask containing 100 mL of test solution.

Every 24 hours cell counts were conducted on the replicate treatment and control vessels using a hemacytometer (Neubauer Improved) and compound microscope. Prior to cell density determination, due to the filamentous structure of this cyanobacterium, it was necessary to vigorously pipette test solutions to produce an approximate homogeneous cell distribution. Two samples were removed from each flask and a single cell count was made on each sample. The two cell counts were averaged, and the average was used as the cell count for that flask at the specified time interval. Visual observations of the health of the cyanobacterium cells were also made and recorded at each 24-hour interval.

Test conditions

Temperature was measured continuously with a VWR minimum/maximum thermometer located in an additional flask of water adjacent to the test flasks in the environmental chamber. Minimum and maximum temperatures were recorded daily. The PAR of the test area was measured at exposure initiation and at each 24-hour interval during the exposure period using a Licor Model LI-189 Photometer and Model LI-190SA radiation sensor probe. Light intensity was also measured in lux at four locations around the perimeter of the test solutions with a VWR Traceable light meter at exposure initiation. Test flasks were randomly placed on a shelf in an environmentally controlled chamber at exposure initiation based on computer-generated random numbers. Following each observation interval, the test flasks were agitated by hand shaking (once daily) and placed in new random positions.

At exposure initiation, pH and conductivity were measured in the test solution remaining in each of the mixing flasks after the individual test flasks for each treatment had been filled. At termination of the 96-hour exposure period, after cell counts were completed, samples were removed from the replicate flasks of each treatment level, control and solvent control and were respectively composited for pH measurements. Test solution pH was measured

with a Yellow Springs International (YSI) pH meter and conductivity was measured with a YSI 3100-115V conductivity meter.

Analytical measurements
Samples analysed at 0 hour were removed from the test and control solutions prior to division into the replicate test vessels. Samples analysed at 96 hours were removed from individually composited replicate solutions of the treatment levels and the controls.

Prior to processing and analysis, all 96-hour test solution samples were centrifuged to remove cyanobacteria cells. Samples were removed from the test solutions using pre-conditioned sampling pipets and were centrifuged in Teflon®-coated Nalgene centrifuge tubes (that had been pre-conditioned with test solution prior to use) at 3000 rpm for 15 minutes.

Data processing and statistical analysis
The algal cell concentration data were evaluated using three approaches: area under the growth curve (AUC), average specific growth rate and final yield.

The AUC was calculated using the formula:

$$A = \frac{N_1 - N_0}{2} \times (t_1) + \frac{N_1 + N_2 - 2N_0}{2} \times (t_2 - t_1) + \frac{N_{n-1} + N_n - 2N_0}{2} \times (t_n - t_{n-1})$$

The average specific growth rate (μ) was calculated using the formula:

$$\mu = \frac{\ln N_n - \ln N_0}{t_n - t_0}$$

where:
N_0 = initial measured cell concentration at time t_0 (cells/mL)
N_1 = measured cell concentration at time t_1 (cells/mL)
N_2 = measured cell concentration at time t_2 (cells/mL)
N_n = measured cell concentration at time t_n (cells/mL)
t_1 = time of first measurement after the beginning of the test (days)
t_2 = time of second measurement after the beginning of the test (days)
t_n = time of nth measurement after the beginning of the test (days)

The final yield was calculated using the formula:

$$Y = Final\ time\ point\ cell\ concentration\ (cells/mL) - Initial\ (inoculum)\ cell\ concentration\ (cells/mL)$$

The percentage inhibition (I_A %) of cell growth was calculated using the following equation:

$$I_A(\%) = \frac{A_c - A_s}{A_c} \times 100$$

where:
A_c = mean values of Y, A or μ determined for the control treatment
A_s = mean values for Y, A or μ determined for each treatment concentration

The average specific growth rate (μ_{ave}) for each replicate was calculated for the entire span of the test period (e.g., 0 to 96 hours) and this data was used for statistical analyses. The section-by-section growth rates during the course of the test (0- to 24-, 24- to 48-, 48- to 72-, and 72- to 96-hours) were calculated for each replicate and are included with the study results.

Since a solvent was used as a carrier for the test substance, an Equal Variance Two-Sample t-Test ($p \leq 0.05$) was used to compare the results of the solvent control to the results of the control. If the data were similar, the negative (blank) control was used for further analyses per U.S. EPA Guideline (2012)[42].

Based on the results of statistical analysis performed for 72- and 96-hour yield, area under the growth curve and average specific growth rate, the No-Observed-Effect Concentration (NOEC, the highest test concentration which demonstrated no statistically adverse effects ($p \leq 0.05$) when compared to the control data) and the Lowest-Observed-Effect Concentration (LOEC, the lowest test concentration which demonstrated a statistically adverse effect ($p \leq 0.05$) when compared to the control data) for each endpoint were determined.

The data were first checked for normality using Shapiro-Wilk's Test (U.S. EPA, 2002)[43] and for homogeneity of variance using Bartlett's Test (U.S. EPA, 2002)[43]. If the data sets passed the tests for homogeneity and normality, then Dunnett's Multiple Comparison Test or Williams' Multiple Comparison Test (U.S. EPA, 2002)[43] was used to determine the treatment-related effects. If the data did not pass the tests for homogeneity and normality, then an appropriate non-parametric statistical test, e.g., Jonckheere-Terpstra's Test (U.S. EPA, 2002)[43], was used to determine treatment-related effects. All statistical determinations were made at the 95% level of certainty, except in the case of Shapiro-Wilk's and Bartlett's Tests, where the 99% level of certainty was applied.

The 72-hour EC_{10} and EC_{20} and the 72- and 96-hour EC_{50} values and the 95% confidence intervals were also determined by linear interpolation of response (percent reduction compared with the control) versus the initial (0 hour) measured concentration using the ICp method (Norberg-King, 1993)[44]. The EC values for yield were denoted as E_yC_{10}, E_yC_{20} and E_yC_{50}, respectively, and the EC values for average growth rate were denoted as E_rC_{10}, E_rC_{20} and E_rC_{50}, respectively. The EC values for AUC are reported as EC_{10}, EC_{20} and EC_{50}, respectively. If less than the required response was observed (i.e., < 10, 20 or 50%

[42] U.S. EPA, 2012. Office of Chemical Safety and Pollution Prevention. Ecological Effects Test Guideline, OCSPP 850.4550: Cyanobacteria (Anabaena flos-aquae) Toxicity. EPA 712-C-005. U.S. Environmental Protection Agency. Washington, D.C.

[43] U.S. EPA, 2002. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to marine and estuarine organisms. Third edition. EPA/821/R-02/014. Office of Water, U. S. Environmental Protection Agency, Washington, DC.

[44] Norberg-King, T.J., 1993. Linear Interpolation Method for Sublethal Toxicity: The Inhibition Concentration (ICp) Approach (Version 2.0). National Effluent Toxicity Assessment Center, Technical Report 03-93. Environmental Protection Agency, Environmental Research Laboratory-Duluth, Duluth, Minnesota.

response), the E_yC, E_rC or EC value was empirically estimated to be greater than the highest mean measured concentration tested. CETIS™ Version 1.8 (Ives, 2013)[45] was used to perform the statistical computations.

RESULTS AND DISCUSSION

Chemical analysis

Concentrations of S-2399 TG ranged from 81.1 to 89.2% of nominal concentrations at test termination. Concentrations remained generally consistent throughout the exposure and maintained the expected concentration gradient. Results were based on mean measured concentrations.

The analytical result of the 96-hour sample from the 7.5 mg a.s./L nominal treatment levels, with cyanobacteria present was 5.6 mg a.s./L. The equivalent test solution without cyanobacteria present resulted in a recovery was 6.1 mg a.s./L demonstrating that the presence of cyanobacteria had minimal impact on the concentration of S-2399 TG in the test solution.

A summary of the measured concentrations of S-2399 TG is presented in Table 9.2.7-17.

Table 9.2.7-17: Measured concentrations of S-2399 TG in the exposure solutions

Nominal concentration (mg a.s./L)	Measured concentration[ab] (mg a.s./L)			% Nominal[a]
	0-Hour	96-Hour[e]	Mean	
Control	<0.15[c] (n.a.)	<0.15[c] (n.a.)	n.a.	n.a.
Solvent Control	<0.15[c] (n.a.)	<0.15[c] (n.a.)	n.a.	n.a.
1.9	1.7 (89.8)	1.4 (72.4)	1.5	81.1
3.8	3.5 (91.8)	2.8 (73.4)	3.1	82.6
7.5	6.6 (87.9)	5.6/6.1[d] (75.2/81.6)	6.1	81.5
15	14 (91.4)	11 (74.8)	12	83.1
30	29 (98.1)	24 (80.4)	27	89.2

[a] Measured concentrations and percent of nominal concentrations were calculated using actual analytical data, not the rounded values presented in this table
[b] Percent of nominal presented in parentheses

[45] Ives, M., 2013. CETIS, Comprehensive Environmental Toxicity Information System™, User's Guide. Tidepool Scientific Software, McKinleyville, California.

[c] Concentrations expressed as less than values were below the minimum detectable limit (MDL). The MDL is dependent upon the lowest concentration calibration standard used and the dilution factor derived from the sample volume of the controls, i.e. (0.000100 mg/L × 1540 = 0.15 mg/L)
[d] Result of the addition sample without algae present to determine biological uptake/degradation
[e] As the study was conducted to US-EPA requirements, no analytical results were available at 72-h. However, the 96-h results are considered as conservative
n.a. = not applicable

Biological effects
A summary of the effects on cell density, yield, growth rate and area under the growth curve (AUC) are presented in Table 9.2.7-18 to Table 9.2.7-20.

No statistically significant difference was detected between the control and solvent control ($p \leq 0.05$) at 72 or 96 hours for yield, growth rate or area under the growth curve (AUC).

Table 9.2.7-18: Summary of effects on cell density and growth rate following exposure of *Anabaena flos-aquae* to S-2399 TG

Mean measured concentration (mg a.s./L)	Mean cell density (× 10^4 cells/ml)[a] (SD)[a]				Growth rate (0 – 72 hours)		Growth rate (0 – 96 hours)	
	24 Hours	48 Hours	72 Hours	96 Hours	Growth Rate ($days^{-1}$) (SD) [a]	% Inhibition[ab]	Growth Rate ($days^{-1}$) (SD) [a]	% Inhibition[ab]
Control	2.52 (1.47)	14.92 (8.58)	28.02 (13.10)	146.71 (26.61)	1.08 (0.19)	n.a.	1.26 (0.05)	n.a.
Solvent control	1.44 (1.52)	11.41 (4.62)	22.22 (7.51)	144.33 (24.20)	1.02 (0.12)	n.a.	1.26 (0.04)	n.a.
1.5	3.19 (3.22)	13.16 (2.84)	43.78 (22.32)	149.31 (18.67)	1.23 (0.19)	-14	1.27 (0.03)	-1
3.1	2.38 (2.00)	13.13 (3.41)	54.22 (26.42)	126.46 (27.25)	1.29 (0.22)	-20	1.22 (0.06)	3
6.1	2.38 (2.41)	11.19 (6.97)	32.78 (10.43)	125.16 (36.84)	1.16 (0.11)	-7	1.22 (0.08)	4
12	3.59 (2.55)	16.22 (5.17)	31.75 (9.73)	111.95 (30.65)	1.15 (0.10)	-7	1.19 (0.08)	6
27	5.66 (2.09)	6.22 (1.71)	33.63 (8.56)	80.13 (20.62)	1.17 (0.09)	-9	1.10* (0.07)	12

[a] Mean, standard deviation (SD) and percent inhibition are calculated from original raw data, not from the rounded values presented in this table
[b] Percent inhibition compared to the negative control
* Significantly reduced compared to the solvent control, based on Dunnett's Multiple Comparison Test
n.a. = not applicable

Table 9.2.7-19: Biomass (expressed as yield) of *Anabaena flos-aquae* after 72 and 96 hours of exposure to S-2399TG

Mean measured concentration (mg a.s./L)	Biomass 0-72 hours		Biomass 0-96 hours	
	Yield (× 10^4 cells days/ml) [b]	% Inhibition [ab]	Yield (× 10^4 cells days/ml) [b]	% Inhibition [ab]
Control	27.02 (13.10)	n.a.	145.71 (26.61)	n.a.
Solvent Control	21.22 (7.51)	n.a.	143.33 (24.2)	n.a.
1.5	42.78 (22.32)	-58	148.31 (18.67)	-2
3.1	53.22 (26.42)	-97	125.46 (27.25)	14
6.1	31.78 (10.43)	-18	124.16 (36.84)	15
12	30.75 (9.73)	-14	110.95* (30.65)	24
27	32.63 (8.56)	-21	79.13* (20.62)	46

[a] Percent inhibition relative to the solvent control
[b] Mean, standard deviation (SD) and percent inhibition are calculated from original raw data, not from rounded values presented in this table
*Significantly reduced compared to the control, based on Williams' Multiple Comparison Test
n.a. = not applicable

Table 9.2.7-20: Calculated area under the growth curve (AUC) after exposure to S-2399 TG

Mean measured concentration (mg a.s./L)	Area under the growth curve (AUC) (× 10^4 cells/ml) (SD)[b]							
	0-24 Hours	24-48 Hours	48-72 Hours	Total area (72-hour)	72-Hour % inhibition [ab]	72-96 Hours	Total area (96-hour)	96-Hour % inhibition [ab]
Control	0.68 (0.65)	8.38 (5.29)	20.61 (9.30)	29.67 (14.29)	n.a.	83.06 (16.17)	112.73 (28.02)	n.a.
Solvent Control	0.19 (0.68)	5.88 (3.15)	15.92 (4.85)	22.00 (7.55)	n.a.	79.13 (12.47)	101.13 (16.23)	n.a.
1.5	0.97 (1.43)	7.78 (2.77)	27.66 (10.00)	36.41 (7.59)	-23	91.9 (18.41)	128.31 (25.91)	-14

Mean measured concentration (mg a.s./L)	Area under the growth curve (AUC) (× 10^4 cells/ml) (SD)[b]							
	0-24 Hours	24-48 Hours	48-72 Hours	Total area (72-hour)	72-Hour % inhibition [ab]	72-96 Hours	Total area (96-hour)	96-Hour % inhibition [ab]
3.1	0.61 (0.89)	7.32 (0.77)	32.90 (14.82)	40.83 (14.71)	-38	85.93 (12.54)	126.76 (22.97)	-12
6.1	0.61 (1.07)	6.27 (4.79)	21.13 (1.97)	28.01 (4.75)	6	74.99 (12.79)	103.00 (16.58)	9
12	1.16 (1.14)	9.66 (2.67)	23.14 (2.71)	33.96 (4.11)	-14	68.14 (11.14)	102.10 (11.17)	9
27	2.07 (0.93)	5.36 (1.83)	19.05 (3.55)	26.48 (2.62)	11	53.74 (9.49)	80.22* (11.75)	29

[a] Percent inhibition relative to the control
[b] Mean, standard deviation (SD) and percent inhibition are calculated from original raw data, not the rounded values presented in this table
* Significantly reduced compared to the control, based on Williams' Multiple Comparison Test
n.a. = not applicable

The 72-hour growth rates for individual control replicates and their coefficient of variation (CV), are presented in Table 9.2.7-21.

Table 9.2.7-21: 72-hour growth rates for individual control replicates

Vessel number	Section-by-section growth rates (day^{-1})
	0 – 72 hours
Control V1	1.08
Control V2	1.29
Control V3	1.22
Control V4	1.05
Control V5	0.94
Control V6	1.25
Control V7	0.70
Control V8	1.08
Mean	1.08
Standard deviation	0.19
Coefficient of variation (%)	18

Table 9.2.7-22 presents the section-by-section growth rates for the control replicates and the CV between individual time frames within a replicate, as well as the mean CV (part of the validity criteria below).

Table 9.2.7-22: Section-by-section growth rate of *Anabaena flos-aquae* in the control

Vessel Number	Section-by-section growth rates			Mean	Standard deviation	Coefficient of variation (%)
	0-24 hours	24-48 hours	48-72 hours			
Control V1	0.78	1.17	1.26	1.07	0.26	24
Control V2	1.08	1.1	1.68	1.29	0.34	26
Control V3	0.71	2.22	0.59	1.17	0.91	78
Control V4	0.07	2.11	0.77	0.98	1.04	106
Control V5	0.25	1.95	0.47	0.89	0.92	103
Control V6	1.96	1.6	0.23	1.26	0.91	72
Control V7	1.23	1.22	-0.32	0.71	0.89	125
Control V8	1.08	1.77	0.33	1.06	0.72	68
Mean coefficient of variation (%)						75

Growth curves for *Anabaena flos-aquae* during the definitive test are visualised in the figure below.

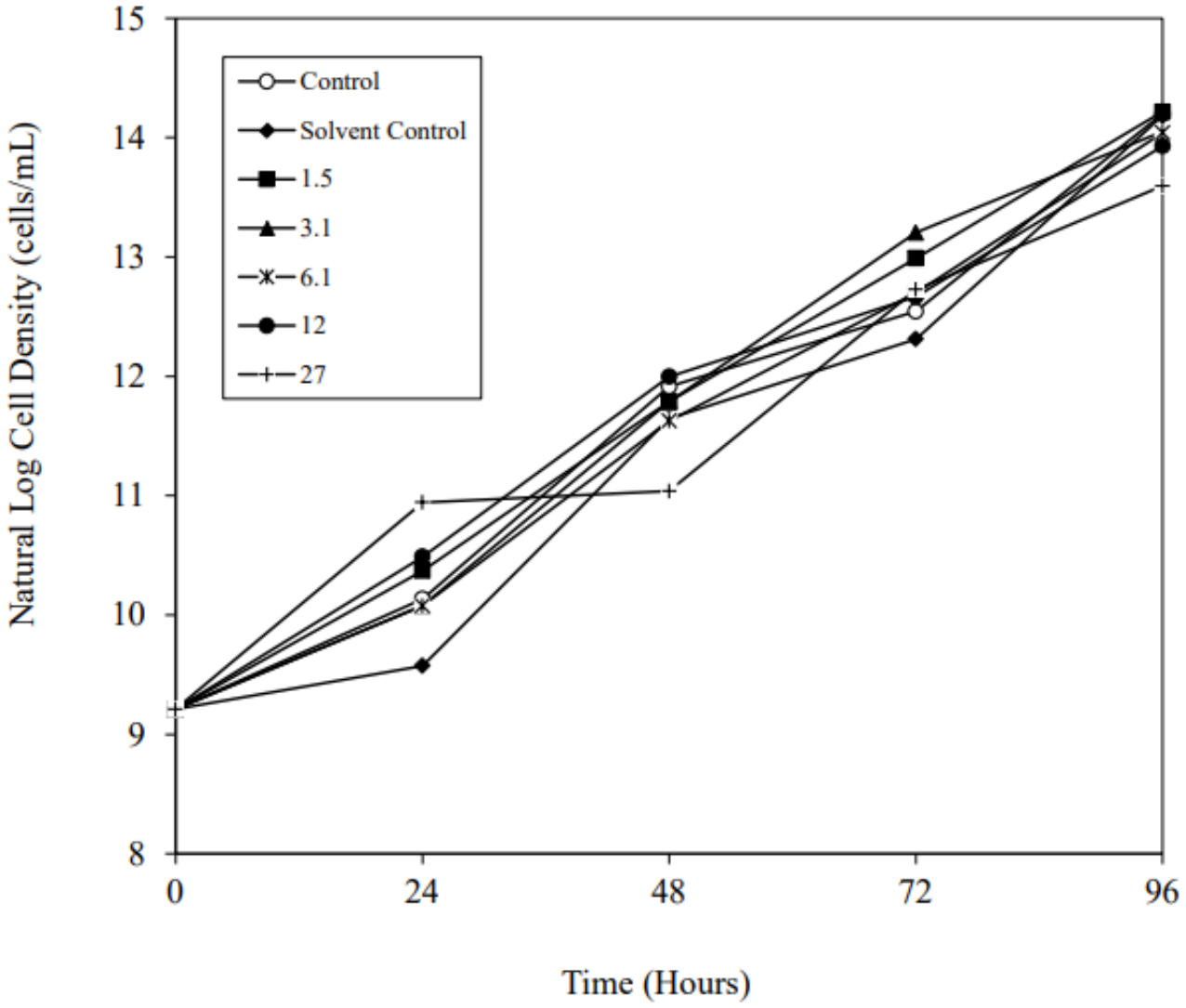


Figure 9.2-22: Growth curves for *Anabaena flos-aquae* during the definitive test for each treatment level

The figure below depicts the concentration-response relationship between S-2399 and yield, growth rate and AUC respectively after 72 hours.

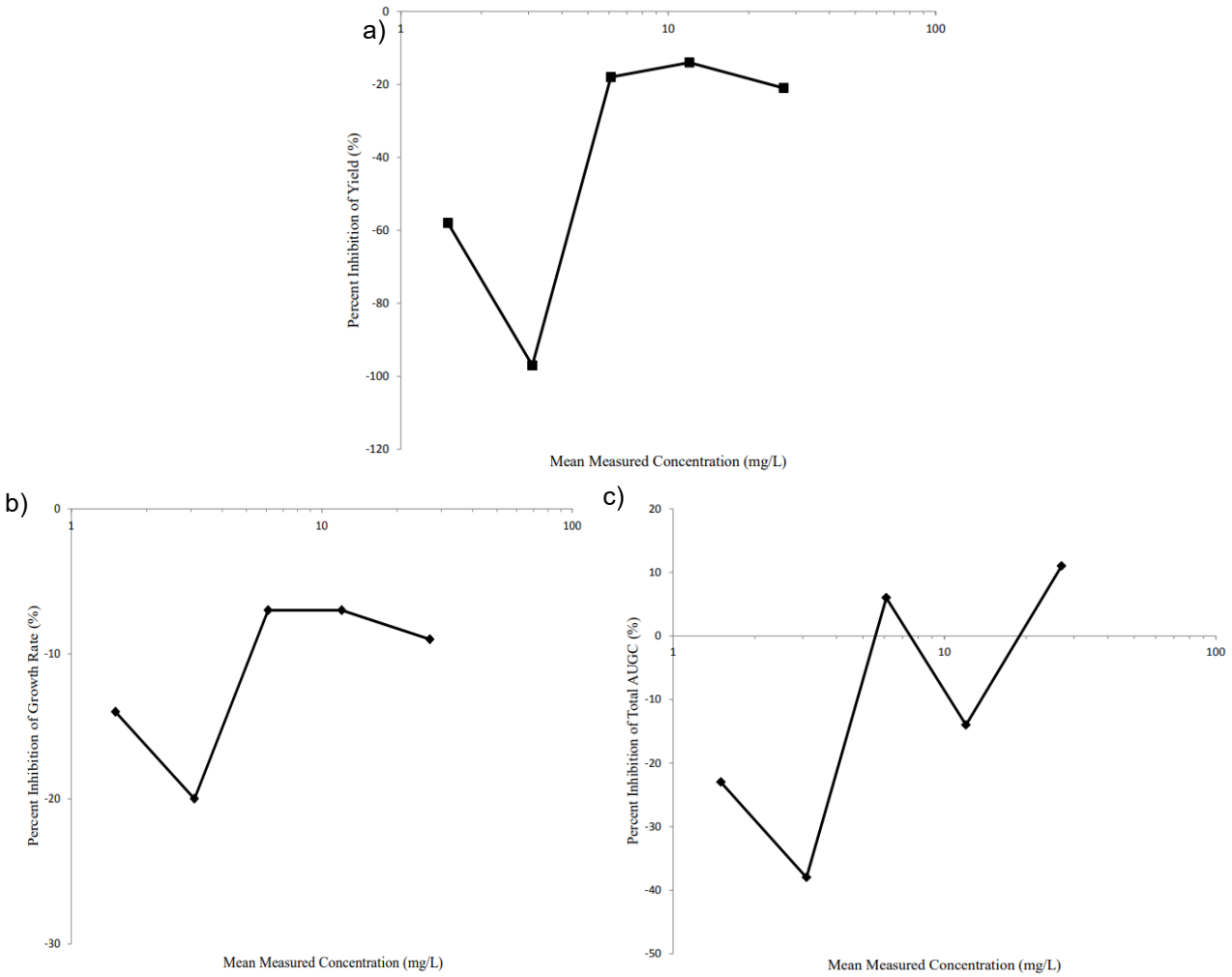


Figure 9.2-23: Percent inhibition of a) yield, b) growth rate and c) AUC for *Anabaena flos-aquae* after 72 hours of exposure to S-2399 TG.

Figure 9.2-24 depicts the concentration-response relationship between S-2399 and yield, growth rate and AUC respectively after 96 hours. In Figure 9.2-24c the 27 mg a.s./L treatment level is omitted and 12 mg a.s./L is incorrectly labelled as significant. This is reflected in Table 9.2.7-23.

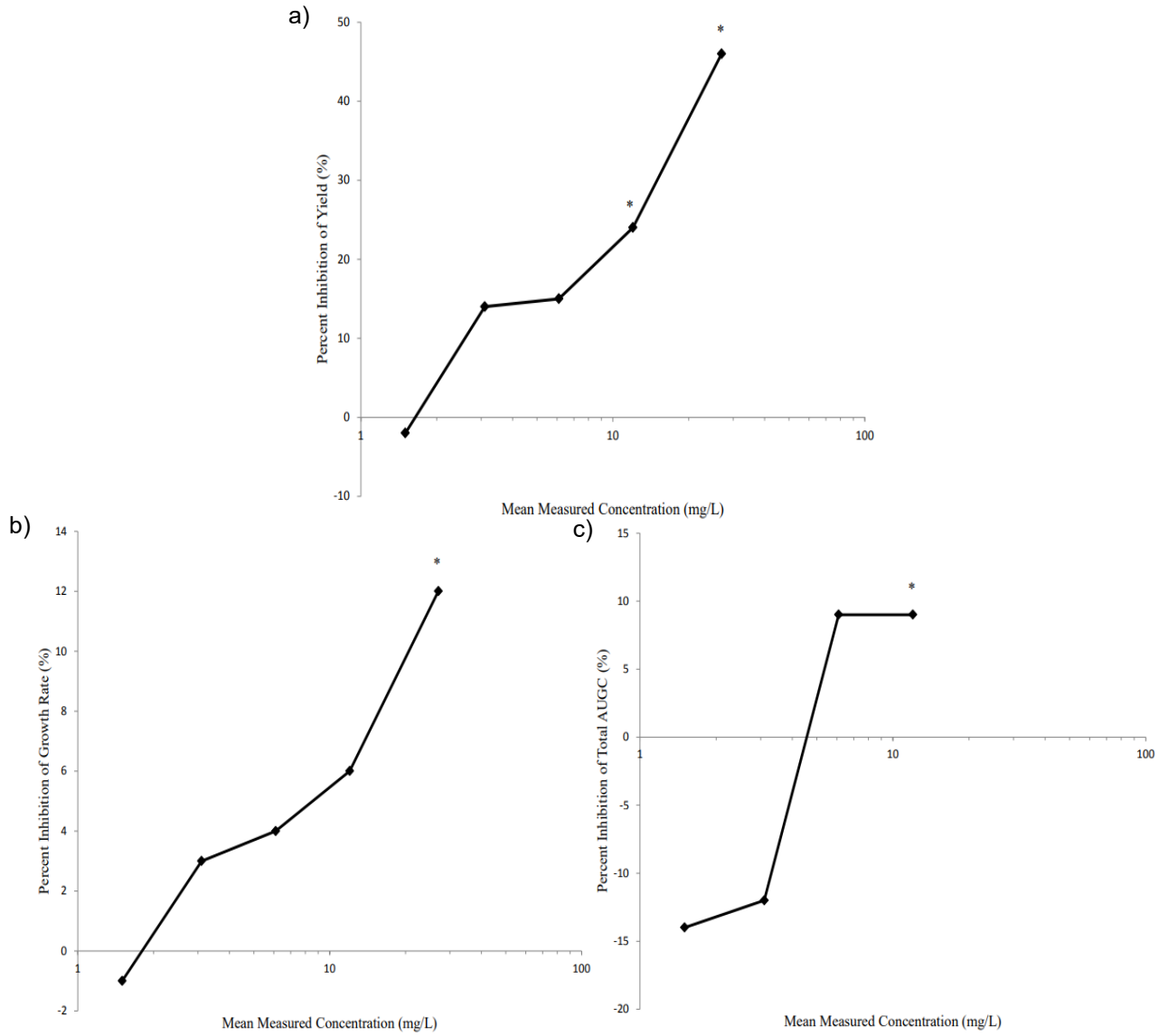


Figure 9.2-24: Percent inhibition of a) yield, b) growth rate and c) AUC for Anabaena flos-aquae after 96 hours of exposure to S-2399 TG. Stars signify treatment levels significantly reduced compared to the negative control

Endpoints for yield, AUC and growth rate are summarised in Table 9.2.7-23.

Table 9.2.7-23: Summary of endpoints (based on initial measured concentrations) after 72 and 96 hours exposure

Endpoint	Based on mean measured concentrations (mg a.s./L)				
	NOEC	LOEC	EC_{10} (95% Confidence Limits)	EC_{20} (95% Confidence Limits)	EC_{50} (95% Confidence Limits)
72-Hour Yield	27	>27	>27 (n.a.)	>27 (n.a.)	>27 (n.a.)
72-Hour Average Specific Growth Rate	27	>27	>27 (n.a.)	>27 (n.a.)	>27 (n.a.)
72-Hour AUC	27	>27	>27 (n.a.)	>27 (n.a.)	>27 (n.a.)
96-Hour Yield	6.1	12	-[a]	-	>27 (n.a.)
96-Hour Average Specific Growth Rate	12	27	-	-	>27 (n.a.)
96-Hour AUC	12	27	-	-	>27 (n.a.)

n.a.= not applicable. EC value was empirically estimates; therefore, corresponding 95% confidence limits could not be calculated

[a] 96-hour EC_{10} and EC_{20} values not calculated by study conductor

Validity criteria

The validity criteria for the study were not met according to OECD 201 (2011) (Table 9.2.7-24).

Table 9.2.7-24: Compliance with OECD 201 (2011) validity criteria

Validity criterion	Required	Obtained
Mean cell count (cells/mL)) control increase (biomass surrogate)	Increase by a factor of at least 16	28
Coefficient of variation of average specific growth rates at 72h for control	≤ 10 %	18 %
Mean coefficient of variation for section-by-section specific growth rates for control (individual replicates – 0-24, 24- 48, 48-72 hours)	≤ 35 %	75 %

CONCLUSIONS

The results of the laboratory study on the effects of S-2399 TG on *Anabaena flos-aquae* demonstrate the 72-hour EC_{50} for yield, average specific growth rate and AUC to be >27 mg a.s./L. The 72-hour NOEC was estimated to be 27 mg a.s./L and the LOEC was >27 mg a.s./L. All endpoints were based on mean measured concentrations.

The 96-hour EC_{50} for yield, average specific growth rate and AUC is estimated to be >27 mg a.s./L. The 72-hour NOEC was estimated to be 6.1 mg a.s./L and the LOEC was 12 mg a.s./L. All endpoints were based on mean measured concentrations.

HSE COMMENTS

The study was carried out according to U.S. EPA OCSPP 850.4550 (2012) and OECD 201 (2011) guidelines and evaluated against the OECD 201 guideline (2011). Neither of the validity criteria relating to growth rate variation outlined in OECD 201 (2011) were met. In both cases, deviation considerably exceeded the specified threshold and therefore HSE consider this study unsuitable for risk assessment purposes. Therefore, no further HSE comments are provided.

The above study was conducted to GLP and considered invalid.

Due to the unacceptable deviations from the validity criteria, HSE Ecotoxicology conclude the study is unreliable and unsuitable for use in risk assessment.

Reference:	KCA 8.2.6.2/03
Report Title:	S-2399 TG: Toxicity Test with the Marine Diatom, *Skeletonema costatum*
Author(s) & year:	[redacted] (2015d)
Document No, Authority registration No:	12709.6371
Substance used:	S-2399 TG, 13CG0617G, 95 %
Method of analysis:	LC/MS/MS method
Guideline(s):	OECD 201 (2011) and OCSPP 850.4500 (2012)
Deviations:	Yes, see HSE comments section.
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: S-2399 TG
Description: Not stated
Lot/Batch: 13CG0617G
Purity: 95.0%
Reference item: zinc chloride
Expiry date: 22 May 2016

B. STUDY DESIGN AND METHODS

1. Test organism: *Skeletonema costatum*
Strain: CCMP 1332
Growth stage: Inoculum- three days since previous transfer
Source: In-house culture
Initial cell density: 1.0 x 10^4 cells/mL
Nominal test concentrations: 0.30, 0.67, 1.5, 3.3 and 7.3 mg/L
LOQ = 0.600 µg/L
MDL = 0.200 µg/L

2. Growth medium: Artificially Enriched Seawater (AES) Medium
pH: 8.0 ± 0.1
Salinity: 30 ± 2 g/L

3. Test vessels: Sterile 250 ml conical flasks, containing 100 ml of test solution

4. Environmental conditions:

A summary of environmental conditions is shown in Table 9.2.7-25.

Table 9.2.7-25: Summary of environmental conditions

Variable	Required OCSPP 850.4500 (2012)[a]	Obtained
Temperature	20˚C (± 2˚C)	18˚C – 20˚C
pH	Not stated	7.2 – 8.5
Photoperiod	14 hours of light, 10 hours of darkness	14 hours of light, 10 hours of darkness
Lighting intensity	Approx. 4300 lux	3700 to 4300 lux
Conductivity	Not stated	43 to 45 mS/cm

[a] Environmental conditions not given for *S. costatum* in OECD 201 (2011), so U.S E.P.A OCSPP Guideline 850.4500 used.

Study dates:
15 to 25 June 2015 (including recovery period)

5. Test organism set up and treatment:
Four replicate, 250 mL glass Erlenmeyer flasks for each treatment level and solvent control were prepared. And eight replicate flasks were prepared for the control. Each flask contained 100 mL of the appropriate exposure solution. After the test solutions were added to the flasks, a 0.653 mL inoculum of *Skeletonema costatum* cells, at a density of approximately 153.11 × 10^4 cells/mL, was introduced into each flask to provide the required initial cell density of 1.0 × 10^4 cells/mL.

Nominal test concentrations of S-2399 TG for the 96-hour test were 0.0 (control), 0.0 (solvent control), 0.30, 0.67, 1.5, 3.3 and 7.3 mg a.s./L. The test was conducted in an environmental chamber, with all flasks being agitated once daily by hand shaking.

A reference test was conducted prior to the definitive test, using zinc chloride as the toxicant to evaluate the sensitivity of *Skeletonema costatum*.

6. Dose preparation:
A 300 mg a.s./mL primary stock solution was prepared prior to test initiation by placing 7.8947 g of S-2399 TG (7.5000 g a.s.) in a 25 mL volumetric flask and bringing it to volume with dimethylformamide (DMF). The resulting stock solution was observed to be clear and tan in colour with no visible, undissolved test substance following approximately two minutes of sonication. A 73 mg/mL secondary stock solution was prepared by diluting 6.1 mL of the primary stock solution to a final volume of 25 mL with DMF. The resulting stock solution was clear and very light tan with no visible undissolved test substance following preparation. A 7.3 mg/L secondary stock solution was prepared by diluting 0.20 mL of the 73 mg/mL stock solution to a final volume of 2.0 L with AES medium. This solution was observed to be clear and light yellow with a large amount of visible undissolved test substance. Following mixing for approximately one hour and shaking by inversion, the resulting stock solution was observed to be clear and light yellow with no visible undissolved material. Nominal concentrations were prepared from the 7.3 mg a.s./L solution. Following mixing by shaking and inversion of the flasks, all exposure solutions were observed to be clear and light yellow with no visible undissolved material. The light yellow colour is resultant of the AES medium. In order to estimate the impact that the presence of algal biomass had on the test substance concentration, an additional replicate vessel of the 1.5 mg a.s./L (nominal) test solution was prepared.

A control was prepared from untreated AES medium and a solvent control was prepared with 0.10 mL/L DMF.

7. Measurements and observations:
Due to the nature of *Skeletonema costatum* cells to cluster together, the test item and control solutions were vigorously pipetted prior to each observation to distribute the cells for counts and observations. At each subsequent 24-hour interval, cell counts were conducted on every replicate treatment and control vessels using a hemacytometer and compound microscope. Observations of health of the algal cells were also made and recorded. A visual check of the test solutions occurred daily.

Temperature was measured continuously with maximum and minimum temperatures recorded daily. The PAR of the test area was measured at test initiation at each 24-hour

interval during the exposure period. Light intensity was also measured in lux at four locations around the perimeter of the test solutions. Following each observation interval, the test flasks were placed in new random positions. Water quality parameters, pH and conductivity, were measured at test initiation and pH was also measured at the termination of the exposure period.

A single sample was removed from each test concentration and the controls at exposure initiation (0 hours) and test termination (96 hours) to determine S-2399 TG concentrations. Samples analysed at 0 hours were removed from the test item and control solutions prior to filling the individual test vessels. Samples analysed at 96 hours were removed from individually composited replicate solutions of the treatment levels and controls. At 96 hours, two samples were removed from the additional replicate flask which did not contain algae. One was analysed for S-2399 TG concentration to assess the impact that algae had on test substance concentration. The other sample was retained for possible future analysis. Measurements of S-2399 TG were determined using a validated LC/MS/MS method. The LOQ was set at 0.600 µg/L. The MDL was 0.200 µg/L.

8. Statistics:

The EC values were determined by linear interpolation of response (percent reduction compared with the control) using the IC_p method. If the data sets passed the test for homogeneity and normality, Williams's Multiple Comparison Test was used to determine the NOEC and LOEC. If the data did not pass the tests for homogeneity and normality, then the NOEC and LOEC were determined using an appropriate non-parametric statistical test. CETIS™ v 1.8 was used to perform all statistical analyses.

II. RESULTS AND DISCUSSION

A. BIOLOGICAL EFFECTS

A summary of the effects on cell density, biomass, growth rate and area under the growth curve (AUGC) is presented in Table 9.2.7-26 to Table 9.2.7-28, followed by a summary of the endpoints in Table 9.2.7-29.

Table 9.2.7-26: Summary of effects on cell density and growth rate following exposure of *Skeletonema costatum* to S-2399 TG

Initial measured concentration (mg a.s./L)	Mean cell density (× 10^4 cells/ml)[a] (SD)				Growth rate (0 - 72 hours)		Growth rate (0 - 96 hours)	
	24 Hours	48 Hours	72 Hours	96 Hours	Growth Rate ($days^{-1}$) [a]	% Inhibition[ab]	Growth Rate ($days^{-1}$) [a]	% Inhibition[ab]
Control	2.50 (0.63)	7.19 (1.14)	21.33 (2.62)	72.56 (5.32)	1.04 (0.04)	n.a.	1.06 (0.02)	n.a
Solvent control	2.06 (0.94)	7.13 (1.11)	21.81 (2.84)	73.75 (9.48)	1.04 (0.04)	n.a.	1.06 (0.03)	n.a
0.32	2.94 (0.97)	6.50 (0.91)	22.69 (2.55)	78.69 (7.19)	1.06 (0.04)	-2	1.08 (0.02)	-2

Initial measured concentration (mg a.s./L)	Mean cell density (× 10^4 cells/ml)[a] (SD)				Growth rate (0 - 72 hours)		Growth rate (0 - 96 hours)	
	24 Hours	48 Hours	72 Hours	96 Hours	Growth Rate ($days^{-1}$) [a]	% Inhibition[ab]	Growth Rate ($days^{-1}$) [a]	% Inhibition[ab]
0.63	3.19 (1.72)	5.38 (0.60)	16.50 (2.74)	28.81 (4.46)	0.95* (0.05)	8	0.83* (0.04)	22
1.6	2.50 (0.61)	2.81 (0.66)	4.13 (0.88)	6.81 (1.60)	0.48* (0.07)	54	0.47* (0.07)	56
3.3	1.38 (0.75)	1.56 (0.63)	1.81 (0.24)	2.13 (0.66)	0.20* (0.05)	81	0.18* (0.08)	83
8.0	0.94 (0.24)	1.50 (0.35)	1.69 (0.72)	1.88 (0.66)	0.15* (0.18)	86	0.14* (0.09)	86

[a] Mean, standard deviation (SD) and percent inhibition are calculated from original raw data, not from the rounded values presented in this table
[b] Percent inhibition compared to the solvent control
* Statistically significant inhibition compared to the control, based on William's Multiple Comparison Test
n.a = not applicable

The 72-hour and 96-hour concentration-response curves for the effect of S-2399TG on *Skeletonema costatum* are shown in the figures below.

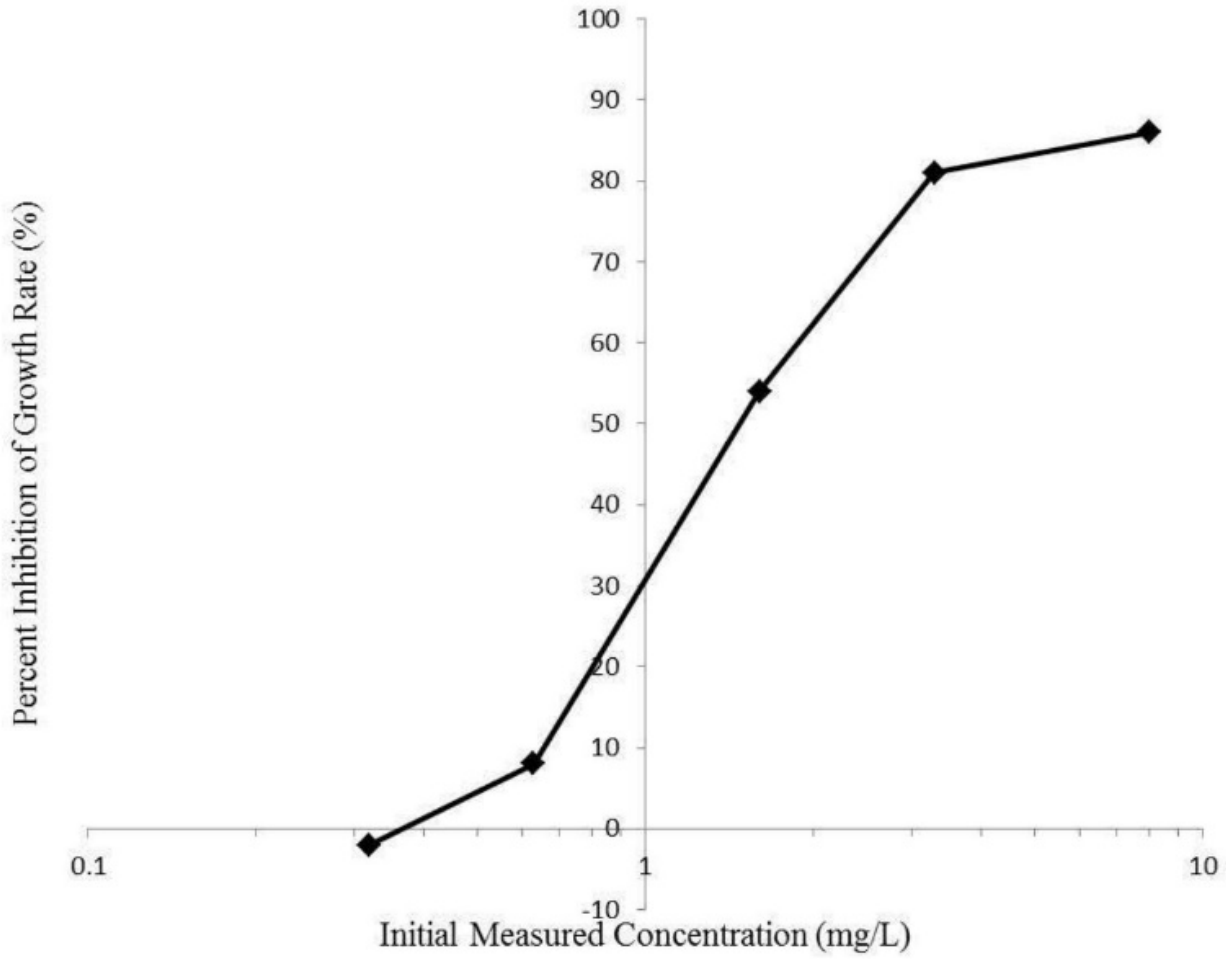


Figure 9.2-25: Percent inhibition of average growth rate (0- to 72-hour) for *Skeletonema costatum* exposed to S-2399 TG

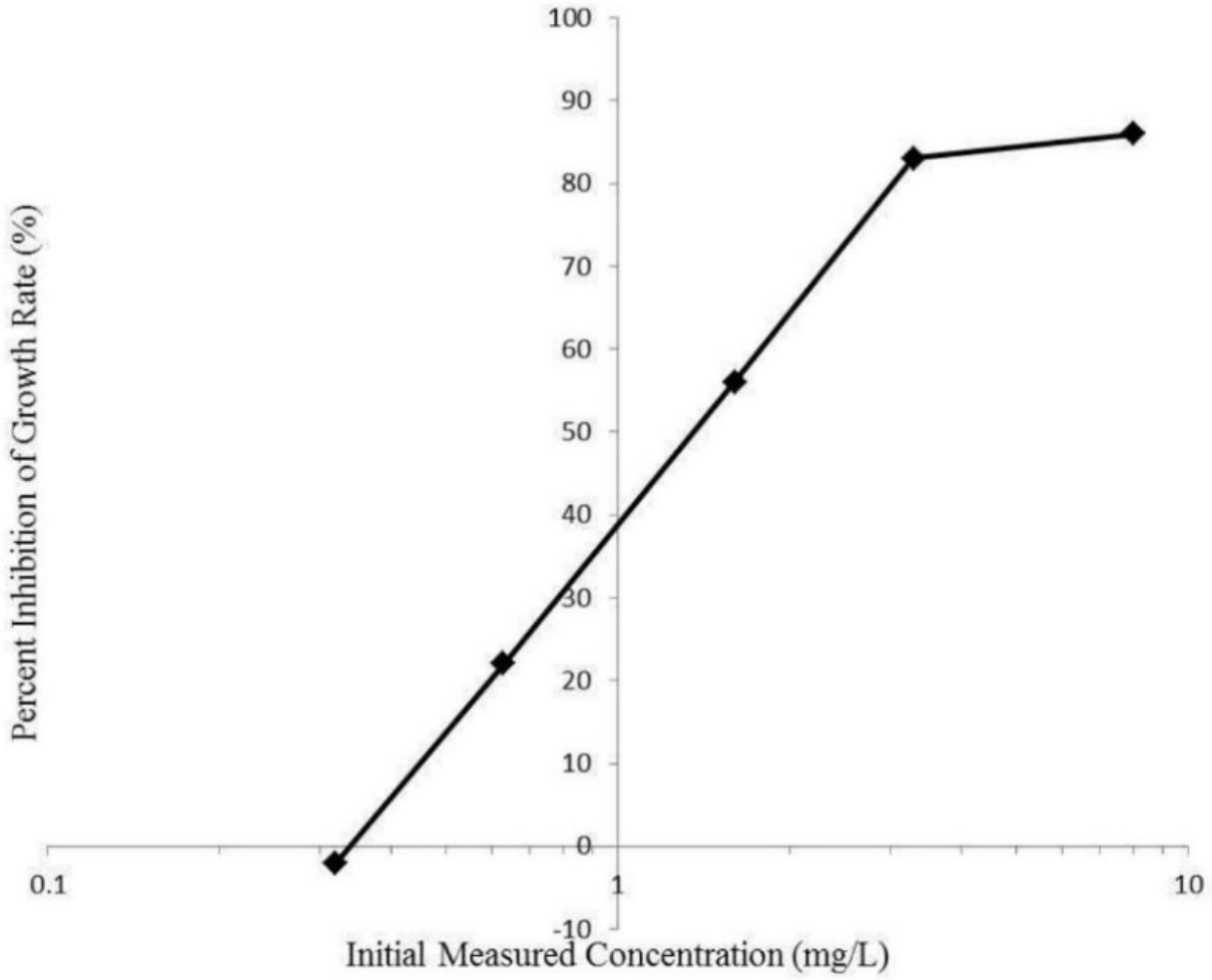


Figure 9.2-26: Percent inhibition of average growth rate (0- to 72-hour) for *Skeletonema costatum* exposed to S-2399 TG

Table 9.2.7-27: Biomass (expressed as yield) of *Skeletonema costatum* after 72 and 96 hours of exposure to S-2399 TG

Initial measured concentration (mg a.s./L)	Biomass 0 - 72 hours		Biomass 0 - 96 hours	
	Yield (× 10^4 cells days/ml) [b]	% Inhibition [ab]	Yield (× 10^4 cells days/ml) [b]	% Inhibition [ab]
Control	20.33 (2.62)	n.a.	71.56 (5.32)	n.a
Solvent Control	20.81 (2.84)	n.a.	72.75(9.48)	n.a
0.32	21.69 (2.55)	-7	77.69(7.19)	-9
0.63	15.50 (2.74)	24	27.81[c] (4.46)	61
1.6	3.13* (0.88)	85	5.81[c] (1.60)	92

Initial measured concentration (mg a.s./L)	Biomass 0 - 72 hours		Biomass 0 - 96 hours	
	Yield (× 10^4 cells days/ml) [b]	% Inhibition [ab]	Yield (× 10^4 cells days/ml) [b]	% Inhibition [ab]
3.3	0.81* (0.24)	96	1.13[c](0.66)	98
8.0	0.69* (0.72)	97	0.88[c](0.66)	99

[a] Percent inhibition relative to the solvent control
[b] Mean, standard deviation (SD) and percent inhibition are calculated from original raw data, not from rounded values presented in this table
[c] Significantly reduced compared to the control, based on Jonckheere-Terpstra's Step-Down Test.
*Significantly reduced compared to the control, based on Williams' Multiple Comparison Test
** Significantly reduced compared to the control, based on Jonckheere-Terpstra Step-Down Test
n.a. = not applicable

Table 9.2.7-28: Calculated area under the growth curve (AUGC) after exposure of *Skeletonema costatum* to S-2399 TG

Initial measured concentration (mg a.s./L)	Area under the growth curve (AUGC) (× 10^4 cells/ml)[b]						
	0-24 Hours	24-48 Hours	48-72 Hours	0-72 Hours	0-96 Hours	72-Hour % inhibition [ab]	96-Hour % inhibition [ab]
Control	0.82 (0.34)	3.65 (0.64)	11.95 (1.38)	16.42 (2.00)	50.38 (3.04)	n.a.	n.a
Solvent Control	0.58 (0.52)	3.41 (0.88)	12.14 (1.55)	16.14 (2.42)	51.3 (6.47)	n.a.	n.a
0.32	1.06 (0.53)	3.53 (0.85)	12.25 (0.99)	16.85 (1.78)	54.48 (5.08)	-3	-7
0.63	1.2 (0.94)	3.12 (0.97)	8.96 (1.41)	13.27* (2.50)	23.75 (3.92)	19	45
1.6	0.82 (0.34)	1.57 (0.2)	2.23 (0.38)	4.62* (0.57)	4.9 (1.26)	72	86
3.3	0.21 (0.41)	0.45 (0.43)	0.62 (0.33)	1.27* (0.96)	1.06 (0.47)	92	97
8.0	-0.03 (0.13)	0.21 (0.2)	0.54 (0.36)	0.71* (0.34)	0.86 (0.28)	96	98

[a] Percent inhibition relative to the control
[b] Mean, standard deviation (SD) and percent inhibition are calculated from original raw data, not the rounded values presented din this table
[c] No statistically significant reduction was determined compared to the controls, based on Jonckheere-Terpstra Step-Down Test. Although 45% inhibition of AUGC was observed at

this treatment level, based on the specific nature of this dataset no statistically significant reduction was detected.
*Significantly reduced compared to the control, based on Williams' Multiple Comparison Test
**Significantly reduced compared to the control, based on Jonckheere-Terpstra Step-Down Test
n.a. = not applicable

Table 9.2.7-29: Summary of endpoints (based on initial measured concentrations) after 72 hours exposure

Endpoint	Based on mean measured concentrations (mg a.s./L)				
	NOEC	LOEC	EC_{10} (95% Confidence Limits)	EC_{20} (95% Confidence Limits)	EC_{50} (95% Confidence Limits)
72-Hour Yield	0.63	1.6	0.43 (0.33-0.60)	0.55 (0.44-0.78)	0.97 (0.79-1.1)
72-Hour Average Specific Growth Rate	0.32	0.63	0.64 (0.48-0.76)	0.82 (0.71-0.94)	1.5 (1.3-1.8)
72-Hour AUGC	0.32	0.63	0.47 (0.24-0.78)	0.63 (0.38-0.89)	1.1 (0.87-1.3)
96-Hour Yield	0.32	0.63	-*	-	0.56 (0.53-0.60)
96-Hour Average Specific Growth Rate	0.32	0.63	-	-	1.4 (1.2 – 1.6)
96-Hour AUGC	0.63	1.6	-	-	0.70 (0.55 – 0.90

* No data provided

B. ANALYSIS
Concentrations of S-2399 TG ranged from 83.3 to 107.5% of nominal concentrations. Concentrations closely approximated the nominal concentrations at 0 hour and declined slightly during the exposure but maintained the concentration gradient. The results were based on initial (0 hour) concentrations.

The analytical result of the 96-hour sample from the 1.5 mg a.s./L nominal treatment levels, with algae present was 1.2 mg a.s./L. The equivalent test solution without algae present resulted in a recovery of 1.3 mg a.s./L and demonstrated that the presence of algae had minimal impact on the concentration of S-2399 TG in the test solution.

A summary of the measured concentrations of S-2399 TG is presented in Table 9.2.7-30.

Table 9.2.7-30: Measured concentrations of S-2399 TG in the exposure solutions

Nominal concentration (mg a.s./L)	Measured concentration[ad] (mg a.s./L)			
	0-Hour	96-Hour[f]	Mean	% nominal[e]
Control	<0.025[b]	<0.025 [b]	n.a.	n.a.
Solvent Control	<0.025 [b]	<0.025 [b]	n.a.	n.a.
0.30	0.32 (107)	0.18 (61.5)	0.25	83.3
0.67	0.63 (94.4)	0.53 (79.7)	0.58	86.6
1.5	1.6 (106)	1.2 (82.0)	1.4	93.3
1.5[c]	n.a. (n.a.)	1.3 (83.8)	1.3	86.6
3.3	3.3 (98.8)	3.2 (95.9)	3.25	98.5
7.3	8.0 (109)	7.7 (106)	7.85	107.5

[a] Measured concentrations were calculated using actual analytical data
[b] Concentrations expressed as less than values were below the minimum detectable limit (MDL)
[c] Result of the additional sample without algae present to determine biological uptake/degradation
[d] Percent of nominal in parentheses
[e] Percent nominal values were calculated using values presented in the table, not actual analytical data
[f] As the study was conducted to US-EPA requirements, no analytical results were available at 72-h. However, the 96-h results are considered as conservative
n.a.= not applicable

C. VALIDITY CRITERIA

Table 9.2.7-31: Validity criteria for the untreated control

Test guideline	Criterion	Required Result		Result Obtained
		OECD 201	OCSPP 850.4500	
OECD 201 (2011) and OCSPP 850.4500 (2012)	Biomass increase in the control cultures by 72 (OECD) or 96 (OCSPP) hours	Increase by a factor of 16	Increase by a factor of 30	Increased by a factor of 20.33 **(72 hours)** and 20.81 **(96 hours)**
	Mean coefficient of variation for section-by-section specific growth rates in the control cultures	≤ 35 %	-	27.7% **(72 hours)** and 25.3 % **(96 hours)**

Test guideline	Criterion	Required Result		Result Obtained
		OECD 201	OCSPP 850.4500	
	Coefficient of variation of average specific growth rates during the whole test period in the control cultures	≤ 10 %	≤ 15 %	4.2 % **(72 hours)** and 1.7 % **(96 hours)**

Table 9.2.7-32: Validity criteria for the untreated solvent control

Test guideline	Criterion	Required Result		Result Obtained
		OECD 201	OCSPP 850.4500	
OECD 201 (2011) and OCSPP 850.4500 (2012)	Biomass increase in the control cultures by 72 (OECD) or 96 (OCSPP) hours	Increase by a factor of 16	Increase by a factor of 30	Increased by a factor of 71.56 **(72 hours)** and 72.75 **(96 hours)**
	Mean coefficient of variation for section-by-section specific growth rates in the control cultures	≤ 35 %	-	40.8 % **(72 hours)** and 35.4 % **(96 hours)**
	Coefficient of variation of average specific growth rates during the whole test period in the control cultures	≤ 10 %	≤ 15 %	4.3% **(72 hours)** and 2.9 % **(96 hours)**

III. CONCLUSION

The results of the laboratory study on the effects of S-2399 TG on *Skeletonema costatum* demonstrate the 72-hour EC_{50} for yield, average specific growth rate and AUGC to be 0.97, 1.5 and 1.1 mg a.s./L, respectively.

The 72-hour NOEC was estimated to be 0.63 mg a.s./L for yield and 0.32 mg a.s./L for average specific growth rate and AUGC. The 72-hour LOEC was 1.6 mg a.s./L for yield and 0.63 mg a.s./L for average specific growth rate and AUGC.

HSE COMMENTS

This study was conducted under GLP and under OCSPP Guideline 850.4500 and OECD Guideline 201 (2011) guidance. This has been assessed against OECD 201 (2011) guidance with environmental conditions assessed against OCSPP Guideline 850.4500.

The concentrations of the test item were maintained between 80-120% of the nominal value throughout the test. Results should be based on nominal concentrations, as has been

expressed by the applicant. The method of analysis used in the study was evaluated by HSE Chemistry. The conclusions of their evaluation are reproduced below. Please see Volume 3 CA, section B5 for more details.

"*The analytical method is not fully validated according to SANTE/2020/12830 rev. 1 for the determination of the active substance inpyrfluxam in freshwater as the matrix effects have not been determined and the stability of standards and extracts has not been addressed. However, the studies were generated prior to the implementation of SANTE 2020/12830 rev.1 and SANCO 3029 rev.4 did not require matrix effects and the stabilities of standards and extracts to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose.*"

There were no deviations to protocol reported for this study and all environmental conditions met the required criteria.

It is worth nothing that the analytical result of the 96-hour sample from the 1.5 mg a.s./L nominal treatment levels, with algae present was 1.2 mg a.s./L. The equivalent test solution without algae present resulted in a recovery of 1.3 mg a.s./L and demonstrated that the presence of algae had minimal impact on the concentration of S-2399 TG in the test solution.

In this study, the applicant has presented the data as percentage inhibition compared to the solvent control. The validity criteria for coefficient of variation for section-by-section specific growth rates in the solvent control were not met. However, as the growth rate in the normal control, which met validity criteria, is identical in the solvent control, and the failure to meet the validity criteria at 96-hours in the solvent control is only marginal, HSE will accept the 96-hour ErC_{50}.

The use of statistics in this study is suitable and meets the guidelines of OECD 201 (2011).

RAI and response:
From Table 9.2.7-30, there is evidence that the concentration of the substance was not kept within ± 20 % of the nominal concentration (for example, 61.5 % of nominal concentration for 0.3 mg a.s./L treatment group after 96 hours). In such cases, endpoints should be calculated using geometric mean measured concentrations. This endpoint re-expression was requested in a RAI. The RAI response calculated the geometric mean measured concentrations and used these to recalculate E_rC_x values (Table 9.2.7-33), using ToxRat (version 3.3.0). Negative and solvent controls were pooled as there were no significant difference between them. A probit model was fitted.

Table 9.2.7-33: Re-calculated E_rC_x values for KCA 8.2.6.2/03

Time period	mg a.s./L		
	ErC10 (95% CI)	ErC20 (95% CI)	ErC50 (95% CI)
72 hours	0.450 (0.273 – 0.609)	0.676 (0.469 - 0.854)	1.472 (1.221 - 1.772)
96 hours	0.34 (0.237 - 0.438)	0.536 (0.412 - 0.651)	1.28 (1.109 - 1.477)

Based on these recalculated endpoints, the endpoint to be used in risk assessment is:

- **96-hour E_rC_{50} = 1.28 mg/L (based on mean measured concentrations)**

B.9.2.8 Effects on aquatic macrophytes

Reference:	KCA 8.2.7/01
Report Title:	S-2399 TG: 7-Day Toxicity Test with Duckweed (*Lemna gibba*)
Author(s) & year:	[redacted] (2016)
Document No, Authority registration No:	12709.6368
Substance used:	S-2399 TG, 13CG0617G, 95 %
Method of analysis:	LC/MS/MS method
Guideline(s):	OECD 221 (2006)
Deviations:	Yes, see HSE comments section.
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

MATERIALS AND METHODS

MATERIALS

Test material S-2399 TG

Lot/Batch: 13CG0617G

Purity: 95.0 % (certified by certificate of analysis)

Description:	Not stated
Expiration date:	23 July 2016

TREATMENTS

Test concentrations:	Nominal test concentrations: 3.1, 6.3, 13, 25 and 50 mg/L Mean measured test concentrations: 1.4, 2.8, 5.8, 11 and 24 mg/L
Controls:	Negative control - 20X Algal Assay Procedure (AAP) medium Solvent control - DMF (0.1 mL/L)
Toxic reference	Zinc Chloride 96-hour EC_{50} = 0.69 mg Zn/L (95% confidence intervals of 0.24 to 0.88 mg Zn/L) (conducted 9 to 16 April 2015).
Analysis of test concentrations:	Yes, at days 0, 3, 5 and 7 (all treatment levels and control) based on analysis of S-2399 using liquid chromatography with mass spectrometry detection (LC/MS/MS). The limit of quantification (LOQ) of the analytical method was 0.600 S-2399 µg/L and minimum detectable limit (MDL) was 0.200 S-2399 µg/L.

TEST ORGANISMS

Species:	*Lemna gibba*
Growth stage:	Inoculum - nine days since previous transfer
Stock cultures:	24 ± 1 °C, illumination of 57 to 90 µE/m^2/S (fluorescent bulbs), 270-mL covered crystallizing dishes containing 100 mL of medium
Inoculation rate:	12 fronds (plants with three or four fronds each)
Source:	Canadian Phycological Culture Centre (CPCC)

TEST DESIGN

Test vessels:	270-mL glass crystallizing dishes filled with 100 mL of test or control medium and covered with inverted, sterile, glass Petri dish
Test medium:	20X AAP Medium (pH 7.5 ± 0.1) (same as culture medium)
Replication:	Four replicates per treatment and negative control. Eight replicates for the solvent control.
Exposure regime:	Semi-static
Duration:	7 days

TEST CONDITIONS

Test temperature:	23 - 24 °C
pH:	Test start: 8.0 – 8.2 Test end: 8.6 – 8.7 New media: 7.9 – 8.3 Aged media: 8.4 – 8.7

Lighting:	Continuous light at a light intensity of 60 – 79 µE/m^2/s (photosynthetically-active radiation (PAR)) (4300 to 5400 lux)

STUDY DESIGN AND METHODS

Experimental dates: The exposure phase of the definitive test was conducted between 10 to 24 July 2015 (including dry weight determination).

Test organism and culturing

The test organism, *L.gibba*, is a freshwater vascular plant selected for its ease of culturing and handling, as well as its sensitivity to a range of chemical substances.

The 20X AAP culturing medium was prepared using deionised water and analysed periodically for the presence of pesticides, PCBs and toxic metals using U.S. EPA standard methods (U.S. EPA, 1997)[46]. None of these compounds were detected at concentrations considered toxic in any of the water samples analysed in agreement with ASTM Guidelines (2007)[47]. Furthermore, a representative sample of 20X AAP medium was analysed monthly for total organic carbon (TOC) concentration (June 2015 = 2.4 mg/L).

Test solution preparation

A 50 mg/L primary stock solution was prepared prior to exposure initiation and at the beginning of each renewal period (days 3 and 5), by placing approximately 0.0526 g of S-2399 TG and 0.1 mL of dimethylformamide (DMF, CAS No. 68-12-2) into a 1.0-L volumetric flask and bringing it to volume with 20X AAP medium. The resulting stock solution was observed to be clear and colourless with a large amount of undissolved test substance clumped together. The stock solution was then sonicated for approximately 50 minutes and mixed for two hours with no change in observed characteristics. The stock solution was filtered twice using a 0.45-µm and 0.22-µm filter and the resulting stock solution was clear and colourless with no visible undissolved test substance. The filtrate of the 50 mg/L nominal stock solution was used as the highest nominal test concentration and serial dilutions yielded the other test concentrations. All exposure solutions were observed to be clear and colourless with no visible undissolved test substance. All test solutions contained 0.1 mL DMF/L thereby matching the solvent control DMF concentration. The negative control contained untreated 20X AAP medium only.

Definitive exposure

Exposure initiation

Exposure initiation began when a 12 frond inoculum of *L.gibba* was aseptically introduced into each flask. Test vessels were then randomly placed on a single shelf within an environmental chamber. Additionally, in order to determine initial biomass, four replicates of 12 fronds each were collected from the same source that was used to inoculate the test. These fronds were placed in pre-weighed aluminium pans and dried in a radiant oven at 60 to 70 °C for seven days prior to dry weight determination. The average plant dry weight

[46] U.S. EPA, 1997. Office of Waste. Test Methods for Evaluating Solid Waste, Physical/Chemical Methods (SW-846). February 2007. U.S. Environmental Protection Agency, Washington, D.C.

[47] ASTM, 2007. Standard practice for conducting acute toxicity tests with fishes, macroinvertebrates and amphibians. Standard E729-96. American Society for Testing and Materials, 100 Barr Harbor Drive, West Conshohocken, Pennsylvania.

from the representative 12-frond replicates at exposure initiation was 2.6 mg.

Test monitoring
On test days 3 and 5, fronds were transferred to newly prepared test solutions to maintain the solution concentrations and replenish nutrients used in plant growth. The exposure was conducted in a temperature-controlled environmental chamber with overhead fluorescent lights designed to maintain the test conditions specified in the table above. Temperature was measured continuously with a VWR minimum/maximum thermometer located in a flask of water adjacent to the test vessels. Photosynthetically-active radiation (PAR) was measured at initiation of the exposure phase and daily thereafter using a Li-Cor Photometer Model LI-189 and Model LI-190SA radiation sensor. Light intensity was measured with a VWR Traceable light meter at exposure initiation. The pH of the exposure solutions was measured in new solutions (at exposure initiation, and days 3 and 5) and in aged solutions (at days 3, 5 and 7). Test solution pH was measured with a Yellow Springs Instruments (YSI) Ecosense Model pH100A meter.

Biological monitoring
On days 3, 5 and at exposure termination (day 7), the number of frond were quantified (density). At exposure termination (day 7), after frond density determination, the fronds were removed from each vessel, blotted dry and dried as above in order to calculate frond dry weight (biomass).

Analytical measurements
At days 0 (exposure initiation), 3, 5 and 7 (exposure termination), a single algal medium sample was removed from each test concentration and the control and analysed for the concentration of S-2399 TG. At days 0 and 5, samples of newly prepared solutions were removed from the test and control solutions prior to division into the replicate test vessels. On days 3 and 7, samples of aged solutions were removed from individually composited replicate solutions of the treatment levels and controls.

Time-weighted average concentrations were calculated for each treatment level using the equation (U.S. EPA, 2012)[48]:

$$\frac{\left[\frac{C_{D0} - C_{D3}}{\ln(C_{D0}) - \ln(C_{D3})} \times 3\ days\right] + \left[\frac{C_{D5} - C_{D7}}{\ln(C_{D5}) - \ln(C_{D7})} \times 2\ days\right]}{total\ days\ (5\ days)}$$

where:

C_{twa} = time-weighted average concentration
C_{Dx} = measured concentration at day x
Total days = total number of days where measurements were taken (i.e. days 0 – 3 and days 5 – 7)

Data processing and statistical analysis

[48] U.S. EPA, 2012. Office of Chemical Safety and Pollution Prevention. Ecological Effects Test Guideline, OCSPP 850.4400. Aquatic Plant Toxicity Test Using Lemna spp. EPA 712-C-008, January 2012. U.S. Environmental Protection Agency. Washington, D.C.

Yield was calculated from frond density and dry weight biomass. The yield based on frond density was calculated from day 7 density minus the initial frond density at the start of the exposure. Yield based on dry weight was calculated as the dry weight at exposure termination minus the initial dry weight of a sample of the inoculum (e.g., 12 fronds per replicate) used to initiate the test.

Growth rate was also calculated from frond density and dry weight biomass. The growth rate (μ) for each replicate test vessel was calculated from frond density for the period from exposure initiation to each observation time and from the change in dry weight biomass from exposure initiation to termination using the following equation:

$$\mu = \frac{\ln N_n - \ln N_0}{t_n - t_0}$$

where:
μ = specific growth rate (days^{-1})
N_0 = frond density or dry weight at time t_0
N_n = frond density or dry weight at time t_n
t_1 = time of first measurement after the beginning of the test (days)
t_n = time of nth measurement after the beginning of the test (days)

Based on the results of statistical analysis performed for 7-day growth rate and yield (based on frond density and frond dry weight biomass), the No-Observed-Effect Concentration (NOEC), the highest test concentration which demonstrated no statistically adverse effect ($p \leq 0.05$) when compared to the control data, was determined. Additionally, the Lowest-Observed-Effect Concentration (LOEC), the lowest concentration tested with a statistically significant reduction relative to the control data, was determined.

The data were first checked for normality using Shapiro-Wilks' Test (U.S. EPA, 2002)[49] and for homogeneity of variance using Bartlett's Test (U.S. EPA, 2002)[49]. If the data sets passed the tests for homogeneity and normality, then Dunnett's Multiple Comparison Test (U.S. EPA, 2002)[49] was used to determine the NOEC and LOEC values. If the data did not pass the tests for homogeneity and/or normality, then Jonckheere-Terpstra's Step-Down Test (U.S. EPA, 2002)[49] was used to determine the NOEC and LOEC value. All statistical determinations were made at the 95% level of certainty, except in the case of Shapiro-Wilks' and Bartlett's Tests, where the 99% level of certainty was applied.

Since a solvent was used as a carrier for the test substance, an Equal Variance Two-Sample t-Test ($p \leq 0.05$) was used to compare the results of the solvent control to the results of the control. No significant difference was detected between the control and solvent control data for any endpoint; therefore, the results were compared to the negative control.

For all tests, EC_{10}, EC_{20} and EC_{50} values were calculated, when possible, for yield and average growth rate (based on frond density and dry weight biomass) at exposure

[49] U.S. EPA, 2002. Methods for measuring the acute toxicity of effluents to freshwater and marine organisms. Fifth edition. Office of Water, U.S. Environmental Protection Agency, Washington, D.C. 20460. EPA/821/R-02/012.

termination by linear interpolation of response (percent reduction of yield or growth rate, based on frond density or dry weight biomass as compared with the control) versus time-weighted average concentrations using the Linear Interpolation Method (ICp) method (Norberg-King, 1993)[50]. CETIS™ Version 1.8 (Ives, 2013)[51] was used to perform both the statistical (NOEC and LOEC determinations) and EC calculations. If no concentration resulted in a 10, 20, or 50% reduction, the EC values were empirically estimated to be greater than the highest time-weighted average concentration tested.

RESULTS AND DISCUSSION

Chemical analysis

Results from the chemical analysis are summarised in Table 9.2.8-1. The observation of undissolved test material made during the preparation of the 50 mg/L primary stock solution, in conjunction with the analytical recoveries of the new test solutions analysed on Day 0 and 5, indicates that the highest concentration tested closely approximates the functional limit of solubility of the test substance in the growth medium.

Table 9.2.8-1: Summary of analytical results

Nominal test concentration (mg a.s./L)	Measured concentration (mg a.s./L)[a] (% of nominal)[c]				Time-weighted average concentration (mg a.s./L) [a]	% of Nominal [a]
	Day 0 (new)	Day 3 (aged)	Day 5 (new)	Day 7 (aged)		
Control	<0.25[b] (n.a.)	<0.25 [b] (n.a.)	<0.25 [b] (n.a.)	<0.25 [b] (n.a.)	n.a.	n.a.
Solvent control	<0.25 [b] (n.a.)	<0.25 [b] (n.a.)	<0.25 [b] (n.a.)	<0.25 [b] (n.a.)	n.a.	n.a.
3.1	1.5 (48)	1.5 (48)	1.4 (45)	1.4 (45)	1.4	47
6.3	2.8 (44)	2.9 (46)	2.6 (41)	2.7 (43)	2.8	44
13	6.0 (46)	5.8 (45)	5.5 (42)	5.6 (43)	5.8	44
25	12 (48)	11 (44)	10 (40)	11 (44)	11	45
50	24 (48)	24 (48)	21 (42)	24 (48)	24	47

[a] Time-weighted average concentrations and percent of nominal concentrations were calculated using actual analytical data and not the rounded (2 significant figures) data presented in this table

[b] Concentrations expressed as less than values were below the minimum detectable limit (MDL)

[c] % of nominal values for individual timepoints were calculated by HSE using rounded values provided

[50] Norberg-King, Teresa J., 1993. A Linear Interpolation Method for Sublethal Toxicity: The Inhibition Concentration (ICp) Approach. National Effluent Toxicity Assessment Center, Environmental Research Laboratory – Duluth, U.S. Environmental Protection Agency, Duluth, Minnesota. Technical Report 03-93.

[51] Ives, M. 2013. CETIS, Comprehensive Environmental Toxicity Information System™, User's Guide. Tidepool Scientific Software, McKinleyville, California.

n.a. = not applicable

Biological effects

Frond density

A summary of the yield and growth rate results based on frond density is provided in Table 9.2.8-2.

Table 9.2.8-2: Mean values for yield (y) and growth rate (μ) based on frond density of *Lemna gibba* after 7 days of exposure

Time-weighted average concentration (mg a.s./L)	Fronds / replicate [a]	Yield (y) [a]	% inhibition (yield)	Growth rates (μ) ($days^{-1}$)[a]	% inhibition (growth rate)
Control	199 (14)	187 (14)	n.a.	0.41 (0.01)	n.a.
Solvent control	189 (32)	177 (32)	n.a.	0.40 (0.02)	n.a.
1.4	205 (20)	193 (20)	-3	0.41 (0.01)	-1
2.8	197 (8)	185 (8)	1	0.40 (0.01)	0
5.8	182 (18)	170 (18)	9	0.39 (0.01)	3
11	183 (10)	171 (10)	9	0.39 (0.01)	3
24	154 (14)	142 (14)	24 [b]	0.37 (0.01)	9 [b]

[a] Mean and standard deviation (SD)

[b] Significantly reduced compared to the control based on Dunnett's Multiple Comparison Test

n.a. = not applicable

For yield based on frond density, a significant difference was detected in the 24 mg/L treatment level compared to the control data. Therefore, the 7-day NOEC and LOEC values were determined to be 11 and 24 mg/L, respectively. The 7-day E_yC_{10}, E_yC_{20} values were determined to be 5.6 and 18 mg/L, respectively and the E_yC_{50} was empirically estimated to be > 24 mg/L, the highest time-weighted average concentration tested.

For growth rate based on frond density, a significant difference was detected in the 24 mg/L treatment level compared to the control data. Therefore, the 7-day NOEC and LOEC values for growth rate (based on frond density) were determined to be 11 and 24 mg/L, respectively. Since no concentration resulted in ≥ 10% inhibition, the 7-day E_rC_{10}, E_rC_{20} and E_rC_{50} values for growth rate (based on frond density) were empirically estimated to be > 24 mg/L, the highest time-weighted average concentration tested.

Dry weight (biomass)

A summary of the yield and growth rate results based on dry weight is provided in Table 9.2.8-3.

Table 9.2.8-3: Mean values for yield (y) and growth rate (μ) based on dry weights of *Lemna gibba after* 7 days of exposure

Time-weighted average concentration (mg a.s./L)	Dry weight (mg)[a]	Yield (mg)[a]	% Yield inhibition	Average growth rates (days^{-1}) [a]	% Growth rate inhibition
Control	23.9 (2.3)	21.3 (2.3)	n.a.	0.32 (0.01)	n.a.
Solvent Control	22.4 (4.0)	19.8 (4.0)	n.a.	0.31 (0.03)	n.a.
1.4	23.9 (1.6)	21.3 (1.6)	0	0.32 (0.01)	0
2.8	22.0 (0.8)	19.4 (0.8)	9	0.31 (0.01)	4
5.8	19.6 (1.4)	17.0 (1.4)	20 [b]	0.29 (0.01)	9 [b]
11	19.4 (2.2)	16.8 (2.2)	21 [b]	0.29 (0.02)	10 [b]
24	15.9 (1.4)	13.3 (1.4)	38 [b]	0.26 (0.01)	18 [b]

[a] Mean and standard deviation (SD)
[b] Significantly reduced compared to the control based on Williams' Multiple Comparison Test
n.a. = not applicable

For yield based on frond dry weight, A significant reduction was detected in the 5.8, 11 and 24 mg/L treatment levels compared to the control data. Therefore, the 7-day NOEC and LOEC values were determined to be 2.8 and 5.8 mg/L, respectively. The 7-day E_yC_{10} and the E_yC_{20} values were determined to be 3.0 and 5.7 mg/L, respectively and the E_yC_{50} value was empirically estimated to be > 24 mg/L, the highest time-weighted average concentration tested.

For growth rate based on frond dry weight, A significant reduction was detected in the 5.8, 11 and 24 mg/L treatment levels compared to the control data. Therefore, the 7-day NOEC and LOEC values were determined to be 2.8 and 5.8 mg/L, respectively. The 7-day E_rC_{10} value was determined to be 11 mg/L and the E_rC_{20} and E_rC_{50} values were empirically estimated to be > 24 mg/L, the highest time-weighted average concentration tested.

Summary
A summary of the endpoints for each variable is presented in Table 9.2.8-4.

Table 9.2.8-4: Summary of the endpoints based on mean measured concentrations

Biological Parameter	Based on Mean Measured Concentrations (mg a.s./L)				
	EC_{10}	EC_{20}	EC_{50}	NOEC	LOEC
7-Day Yield (Based on Frond Density)	5.6 (1.1-18)	18 (9.3-n.a.)	>24 (n.d.)	11	24
7-Day Growth rate	>24 (n.a.)	>24 (n.a.)	>24 (n.a.)	11	24

Biological Parameter	Based on Mean Measured Concentrations (mg a.s./L)				
	EC_{10}	EC_{20}	EC_{50}	NOEC	LOEC
(Based on Frond Density)					
7-Day Yield (Based on Dry Weight)	3.0 (0.85-5.9)	5.7 (2.8-19)	>24 (n.a.)	2.8	5.8
7-Day Growth Rate (Based on Dry Weight)	11 (0.48-16)	>24 (n.a.)	>24 (n.a.)	2.8	5.8

n.a. = not applicable. Corresponding 95% confidence limits could not be determined
n.d. = not determinable. EC value was empirically estimated, therefore, corresponding 95% confidence limits could not be determined

Validity criteria
The validity criteria for the study were met according to OECD 221 (2006) (Table 9.2.8-5).

Table 9.2.8-5: Compliance with OECD 201 (2011) validity criterion

Validity criterion	Required	Obtained
Frond number doubling time	< 2.5 days	Negative control = 1.7 days Solvent control = 1.8 days

CONCLUSIONS
The influence of S-2399 TG on the growth of duckweed, *Lemna gibba* was assessed in a semi-static 7-day test. Based on mean measured concentrations, the 7-day E_rC_{50} and E_yC_{50} values were calculated to be > 24 mg a.s./L for both frond density and dry weight. The 7-day NOEC for growth rate was 11 mg a.s./L based on frond density and 2.6 mg a.s./L based on frond dry weight. The 7-day NOEC for yield was 11 mg a.s./L based on frond density and 2.6 mg a.s./L based on frond dry weight. The 7-day LOEC for growth rate was 24 mg a.s./L based on frond density and 5.8 mg a.s./L based on frond dry weight. The 7-day LOEC for yield was 24 mg a.s./L based on frond density and 5.8 mg a.s./L based on frond dry weight.

HSE COMMENTS
The protocol was carried out according to U.S. EPA OCSPP 850.4400 (2012) and OECD 221 (2006) guidelines and evaluated against the OECD 221 guideline (2006). The single specified validity criterion was met.

HSE noted the following deviations from OECD 221 (2006):

OECD 221 (2006) paragraph (§) 13 covers the testing of a reference substance to assess the sensitivity of test cultures to toxicants. The study conductor used $ZnCl_2$ as a toxic reference and compared the estimated EC_{50} (0.69 mg Zn/L) to the mean of historical estimated EC_{50}s for this chemical (0.79 mg Zn/L) within the laboratory. The results obtained are in line with historical data, thereby confirming the sensitivity of the test system.

OECD 221 (2006) § 18 discusses test organism selection. It states, "*young, rapidly growing plants without visible lesions or discoloration (chlorosis) should be used*". The study report does not detail the appearance of plants selected for testing beyond the number of fronds. This is likely only a minor reporting omission. As the study met the validity criterion HSE consider this a minor deviation.

OECD 221 (2006) § 23 and Annex 4 cover the growth medium 20X AAP and its composition. The study conductor added an extra nutrient (Na_2SeO_4), which was required according to a personal communication. As the validity criterion relating to control growth was met HSE consider this an acceptable deviation.

OECD 221 (2006) § 35 details the light intensity requirements during incubation. A light intensity should be selected from a range of 85 – 135 µE/m^2/s. The light intensity ranged from 60 – 79 µE/m^2/s in the definitive experiment. Again, given control growth rates adhered to the validity criterion HSE consider this a minor deviation.

OECD 221 (2006) § 38 outlines frond-based observations. It states, "changes in plant development, e.g. in frond size, appearance, indication of necrosis, chlorosis or gibbosity, colony break-up or loss of buoyancy, and in root length and appearance, should be noted". Changes in appearance and morphology were not reported in the study. This could either be a reporting omission or failure to track these signs of stress. HSE notes this omission and will consider it during the risk assessment.

OECD 221 (2006) § 45 discusses the requirements for analytical measurements within a semi-static test design. It states, "*in semi-static tests where the concentration of the test substance is not expected to remain within ±20 % of the nominal concentration, it is necessary to analyse all freshly prepared test solutions and the same solutions at each renewal (see paragraph 33). However, for those tests where the measured initial concentration of the test substance is not within ±20% of nominal but where sufficient evidence can be provided to show that the initial concentrations are repeatable and stable (i.e. within the range 80 - 120 % of the initial concentration), chemical determinations may be carried out on only the highest and lowest test concentrations*". The study demonstrated that the test substance was stable under test conditions for two time periods (Days 0 - 3 and Days 5 – 7). The time-weighted average concentration, however, was not within ± 20 % of the nominal concentration. This was not due to stability issues within the experimental system but because the selected highest nominal concentration was above the limit of solubility in the experimental system. Nevertheless, according to the guidelines under this scenario it is necessary to analyse all freshly prepared test solutions and the same solutions at each renewal. The freshly prepared test solution for Day 3 was not analysed on Day 3 when fresh or Day 5 when aged. Given the clear stability of the test substance in the test system HSE consider this a minor deviation. It should also be noted that HSE considers the solubility issue dealt with appropriately as filtering was performed to remove any precipitate, yielding a clear and colourless stock solution.

OECD 221 (2006) § 55 delineates which growth rates should be calculated. It states, "*the section-by-section growth rate should be assessed in order to evaluate effects of the test substance occurring during the exposure period (e.g. by inspecting log-transformed growth curves). Substantial differences between the section-by-section growth rate and the average growth rate indicate deviation from constant exponential growth and that close examination*

of the growth curves is warranted". Section-by-section growth rates were not calculated for Day 3 – 5 and Day 5 – 7. Growth curves were also not reported using a logarithmic scale for frond number. Consequently, transient effects of the test substance may have been overlooked and the reported endpoints for growth rate may be an overestimate. HSE will consider this during the risk assessment stage.

There were a number of reporting omissions relating to OECD 221 (2006) § 65:

The test report must include, "*physical nature and physical-chemical properties, including water solubility limit*". This was not reported. This is a minor reporting omission, which HSE considers acceptable.

The test report must include, "*growth curves for each concentration (recommended with log transformed measurement variable, see paragraph 55)*". A growth curve was reported without the log transformation. This is a minor reporting deviation, which HSE considers acceptable.

The test report must include, "*if ANOVA has been used, the size of the effect which can be detected (e.g. the least significant difference)".* The size of effect that can be detected was not provided. As EC_x endpoints were provided, which are preferable for use in risk assessment, this is a minor reporting omission.

The test report must include, "*any stimulation of growth found in any treatment".* For yield based on frond density a small increase at the 1.4 mg/L time-weighted average concentration was present but not reported. Considering linear interpolation with bootstrapping was performed, which is an appropriate method for when growth stimulation is present (OECD 201, 2011), this is a minor reporting omission with no impact on the integrity of estimated endpoints. HSE considers this deviation acceptable.

The test report must include, "*any visual signs of phytotoxicity as well as observations of test solutions*". This was not reported, which, as discussed above, may have resulted in signs of phytotoxicity being overlooked, with a resulting overestimation of endpoint values.

Finally, there was an error in the equation used to calculate the time-weighted average concentration in the study report. In the second part of the numerator D_0 was incorrectly specified instead of D_5. This was corrected in the equation provided above.

The method of analysis used in the study was evaluated by HSE Chemistry. The conclusions of their evaluation are reproduced below. Please see Volume 3 CA, section B5 for more details.

"*The analytical method is not fully validated according to SANTE/2020/12830 rev. 1 for the determination of the active substance inpyrfluxam in freshwater as the matrix effects have not been determined and the stability of standards and extracts has not been addressed. However, the studies were generated prior to the implementation of SANTE 2020/12830 rev.1 and SANCO 3029 rev.4 did not require matrix effects and the stabilities of standards*

and extracts to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose."

The above study was conducted to GLP and considered valid.

The agreed endpoints suitable for use in the risk assessment and preferred by OECD 221 (2006) are:
7-day growth rate (frond density) $EC_{10/20/50}$ > 24 mg a.s./L
7-day growth rate (dry weight) $EC_{20/50}$ > 24 mg a.s./L

Therefore, based on the most sensitive indicator of toxicity, the E_rC_{50} is > 24 mg a.s./L

B.9.2.9 Further testing on aquatic organisms

Reference:	KCA 8.2.8/01
Report Title:	S-2399 TG: Acute Toxicity to Eastern Oyster (*Crassostrea virginica*) Under Flow-Through Conditions
Author(s) & year:	[redacted] A. (2016)
Document No, Authority registration No:	12709.6367
Substance used:	S-2399 TG, 13CG0617G, 95 %
Method of analysis:	LC/MS/MS method
Guideline(s):	OCSPP 850.1025 Guidelines (1996a) and OCSPP 850.1000 (1996b)
Deviations:	Yes, see HSE comments section.
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	No, not used in aquatic risk assessment

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test material:** S-2399 TG
   Description: Not stated
   Lot/Batch: 13CG0617G
   Purity: 95.0%
   Reference item: None
   Expiry date: 23rd July 2013

Solvent: Acetone

B. STUDY DESIGN AND METHODS

1. Test animals: *Crassostrea virginica*
Age: Similar age with a mean valve height of 40 ±4.0 mm
Source: Northside Shellfish Company, West Barnstable, Massachusetts, USA
Acclimation: 6 days
Diet: During the acclimation period, oysters were fed a commercially prepared supplementary algal mixture prepared in seawater from a concentrate.
During the exposure period, oysters received supplemental feedings of commercially prepared algal mix. Concentrated volumes of algal suspension (approx. 18mL in 1.0 L of seawater) were added to each test aquarium three times daily to maintain an average concentration of at least 2 x 10^5 cells/mL in the test solution during the exposure period.
Organisms per test vessel: 20
Flow rate: 5.25L per oyster per hour
Exposure regime: Flow-through

2. Dilution water: Natural, filtered seawater
pH: 7.7 to 7.9
Salinity: 20‰

3. Test vessels: Glass aquaria measuring 49.5 x 25.5 x 29 cm, with a 14-cm high side drain that maintained a constant exposure solution volume of 18 L.
Nominal test concentrations: 0.063, 0.13, 0.25, 0.50 and 1.0 mg/L
LOQ: 0.600 µg/L
MDL: 0.200 µg/L

4. Environmental conditions:
Temperature: 20 - 21°C
pH: 7.4 – 8.0
Dissolved oxygen: 5.0 – 7.1 mg/L
Salinity: 20‰
Photoperiod: 16 hours light: 8 hours darkness (720 to 1100 lux)

A summary of the environmental conditions obtained in this study is shown in Table 9.2.9-1 below.

Table 9.2.9-1: Summary of environmental conditions

Variable	Required (OCSPP 850.1025) 2016	Obtained
Temperature	20˚C (can be ± 5 °C for < 8 hour intervals)	20˚C – 21˚C
pH	7.5 – 8.5[a]	7.4 – 8.0

Variable	Required (OCSPP 850.1025) 2016	Obtained
Dissolved oxygen concentration	60-100%	5.0 - 7.1mg/L (>60%)
Salinity	>12 ppt	20 ppt
Photoperiod	From 12 hours light : 12 hours dark to 16 hours light : 8 hours dark[b]	16 hours light; 8 hours darkness
Light intensity	540 – 1080 lux[c]	720 to 1100 lux

[a] No pH values given in OCSPP 850.1025 (1996)
[b] No photoperiod given in OCSPP 850.1025 (1996)
[c] No light intensity value given in OCSPP 850.1025 (1996)
Study dates: 22nd – 26th June 2015

5. Animal assignment and treatment:
Prior to testing, 3 to 5 mm of the new peripheral shell growth of each oyster was removed by grinding the perimeter of the shell to a blunt edge. The study was initiated by impartially selecting and placing 20 oysters in each test aquarium (20 per treatment levels and controls). Oysters were spaced equidistant from one another with the left valve down, and valve inflow openings facing toward the flow of water from the Teflon® circulator tube. The nominal test concentrations were 0.063, 0.13, 0.25, 0.50 and 1.0 mg a.s./L. The exposure duration was 96 hours.

The flow of exposure solution (75 mL/min) provided approximately 6 solution volume replacements every 24 hours. In addition, the contents of each aquarium were continuously circulated at a rate of 1.75 L/min or about 5.25 L per oyster per hour.

6. Dose preparation:
A 10 mg a.s./L primary stock solution was prepared by bringing 5.3708 g of S-2399 TG (5.1023 g a.s.) to a volume of 500 mL with acetone. The resulting solution was observed to be clear and colourless with no visible undissolved test substance following mixing by multiple inversions. Stock solutions were prepared from the 10 mg/L primary stock solution to produce stock solutions of 0.63, 1.3, 2.5 and 5.0 mg a.s./mL. The resulting stock solutions were mixed by multiple inversions and were observed to be clear and colourless with no visible undissolved test substance following preparation.

A syringe of neat acetone was used to dose the solvent control solution (0.10 mL/L). The concentration of acetone in each test solution was equal to the acetone concentration in the solvent control. A control solution was established containing dilution water only.

Prior to exposure initiation, a Harvard Apparatus Pump in conjunction with a 50-mL Glenco® gas-tight syringe was calibrated to deliver 7.5 µL/min of the appropriate stock solution or solvent (acetone) to the diluter's chemical mixing chambers, which received approximately 75 mL/minute of dilution water from the water cells. The mixing chambers were continuously stirred. Glass jars (5 cm O.D., 8 cm high) with Nitex® screen bottoms (475-µm screen openings) containing filter floss were suspended over each aquaria with the delivery tube

placed over the jar to allow exposure solution entering the aquaria to be filtered and to minimize undissolved material from entering the exposure vessels. No undissolved material was observed in the mixing chambers or exposure vessels of any treatment level during this exposure with the exception of the 1.0 mg/L mixing chamber in which undissolved material was observed at the tip of the dosing stock delivery tube. The same pump delivered 7.5 µL/min of neat acetone to a mixing chamber receiving 75 mL/min of dilution water which was delivered to the solvent control vessel.

7. Measurements and observations:

Biological observations (visible abnormalities, such as excessive mucous production or a failure to siphon and feed, as evidence by a lack of faeces and pseudofaeces production) and observations of the physical characteristics of the test solutions, if applicable, were recorded at 0, 24, 48, 72 and 96 hours of exposure of exposure. Adverse effects were determined by comparison of the performance and appearance of the exposed oysters to that of the control oysters. After 96 hours of exposure, the oysters were removed from the test aquaria and the new shell growth was measured microscopically to the nearest 0.1 mm using a calibrated micrometre.

Observations of the physical characteristics of test solutions were recorded at the same time as the biological observations.

The pH, dissolved oxygen concentration, temperature and salinity were measured daily in each aquarium.

Prior to the start of the test, samples were removed from the low, mid, and high treatment levels and negative control and analysed for S-2399 TG. Samples of the diluted stock solutions were also analysed for S-2399 TG. During the 96-hour test, one sample from each treatment level, control and solvent control was collected and analysed for S-2399 TG at 0 hour and 96 hours. All samples were analysed for S-2399 TG using LC/MS/MS. The LOQ was set at 0.600 µg/L. MDL was 0.200 µg/L.

8. Statistics:

The EC_{50} values were calculated using the statistical analysis program CETIS™ v 1.8, with linear interpolation. If data did not meet the assumptions of the linear interpolation method, another appropriate point estimate model was used such as probit analysis, binomial probability, linear regression, non-linear regression and Spearman-Kärber analysis.

The No-Observed-Effect Concentration (NOEC) was defined as the highest concentration at and below which there was no statistically significant reduction in growth or survival. Statistical confirmation of the NOEC was determined using Wilcoxon's Test with Bonferroni-Holm's Adjustment.

II. RESULTS AND DISCUSSION

A. BIOLOGICAL EFFECTS

At test termination, no mortality or sub-lethal effects were observed in any test concentration, control, or solvent control. Growth among dilution water control and solvent control oysters at test termination averaged 2.4 and 2.2 mm, respectively.

Since there were no solvent related effects for measured response variables, the treatment responses were compared to the negative control. The effects of S-2399 TG on mean shell growth are presented in Table 9.2.9-2.

Table 9.2.9-2: Effects of S-2399 TG on the shell deposition of Eastern oysters after 96 hours of exposure

Mean measured concentration (mg a.s./L)	Mean shell deposition (mm) (SD)	Mean percent reduction (%)
Control	2.4 (0.7)	n.a.
Solvent control	2.2 (0.8)	n.a.
0.068	2.0 (0.4)	15
0.14	2.2 (1.0)	6
0.27	2.2 (0.6)	5
0.51	1.8 (0.8)	22
0.99	1.8 (1.3)	24

SD = standard deviation

The concentration-response (shell deposition) of Eastern oysters after 96 hours is shown below.

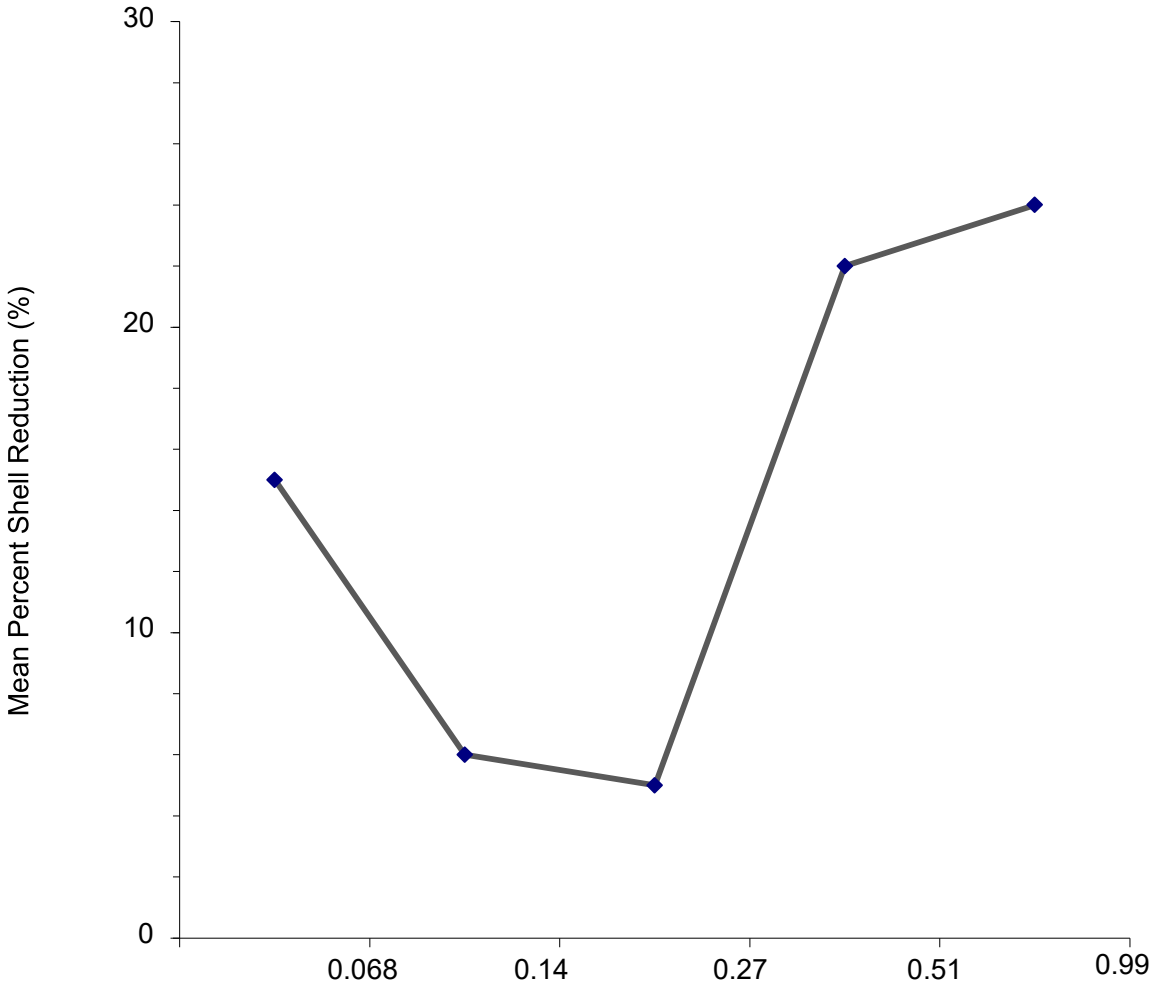


Figure 9.2-27: The concentration-response (shell deposition) of Eastern Oysters exposed to S-2399

A summary of the endpoints is presented in Table 9.2.9-3. Since no mortality was recorded in any test concentration, the 96-hour EC_{50} value was determined by linear interpolation to be > 0.99 mg/L (the highest test concentration).

Table 9.2.9-3: Summary of endpoints (based on mean measured concentrations)

Endpoint	96-Hour EC_{50} (mg a.s./L)	95% Confidence intervals	
		Lower (mg a.s./L)	Upper (mg a.s./L)
Growth	> 0.99	n.a	n.a.
96-Hour NOEC = 0.27 mg/L [a]			

[a] NOEC was determined using Wilcoxon's Test with Bonferroni-Holm's Adjustment
n.a. = not applicable. EC_{50} value was empirically estimated, therefore, 95% confidence intervals could not be determined

B. ANALYSIS

Based on the twice daily check of diluter function, it was determined that the diluter system functioned properly during the study. Mean measured concentrations were between 99 and 110% of nominal. Concentrations were consistent and maintained the expected concentration gradient. A summary of the results are presented in Table 9.2.9-4.

Table 9.2.9-4: Measured concentrations of S-2399 TG in the exposure solutions

Nominal concentration (mg a.s./L)	Measured concentration (mg a.s./L)[a]			Percent of nominal (%)[a]
	0 hour	96 hours	Mean (SD)	
Control	< 0.0050	< 0.0050	n.a. (n.a.)	n.a.
Solvent control	< 0.0050	< 0.0050	n.a. (n.a.)	n.a.
0.063	0.063	0.072	0.068 (0.0066)	110
0.13	0.13	0.15	0.14 (0.0091)	110
0.25	0.30	0.24	0.27 (0.043)	110
0.50	0.55	0.48	0.51 (0.047)	100
1.0	0.93	1.1	0.99 (0.083)	99

[a] Mean measured concentrations, standard deviations and percent of nominal were calculated using the actual analytical (unrounded) results and not the rounded (two significant figures) values presented in this table
n.a = not applicable
SD = standard deviation

No mortality or sub-lethal effects were detected during the 96-hour observation period.

C. VALIDITY CRITERIA

As mortality did not exceed 10% (actual: 0%) and a minimum of 2 mm of new shell growth was observed in the control groups (actual: 2.2 to 2.4 in the solvent control, and control, respectively), the study was considered valid.
Dissolved oxygen concentration remained above 60% throughout the test and the concentration of the test substance was satisfactorily maintained over the test period. Dissolved oxygen, temperature, salinity, and pH measurements should be made at the beginning and end of the test in each chamber.
A summary of the validity criteria as per OCSPP 850.1025 (2016) is shown in Table 9.2.9-5 below:

Table 9.2.9-5: Validity criteria as per OCSPP 850.1025 (2016)

Variable	Required (OCSPP 850.1025) 2016	Obtained
Test vessels	All identical	All identical

Variable	Required (OCSPP 850.1025) 2016	Obtained
Treatment assignment	Treatments indiscriminately assigned to test vessels and test organisms indiscriminately assigned to vessels	Treatments and test organisms randomly assigned to test vessels
Controls	Dilution water (and solvent) control included in the test	Dilution water control and solvent control included in the test
Mortality and behaviour	<10% organisms in dilution water (and solvent) control show signs 0f disease, stress and/or death	<10% mortality and distress in dilution water and solvent controls
Shell growth	Overall mean of at least 2mm new shell growth in control groups	2.4 mm mean in dilution control and 2.2 mm mean in solvent control
Surfactant	Surfactant or dispersant not used	Not used
Spawning	No spawning	Not reported

III. CONCLUSION

The 96-hour EC_{50} value of S-2399 TG to eastern oysters (*Crassostrea virginica*) was calculated by linear interpolation to be > 0.99 mg a.s./L The No-Observed-Effect Concentration (NOEC) was determined to be 0.27 mg a.s./L.

HSE COMMENTS

This study was conducted under GLP and under OCSPP 850.1025 Guidelines (1996a) and OCSPP 850.1000 (1996b). It has been assessed against OCSPP 850.1025 Guideline (2016).

The concentrations of the test item were maintained between 80-120% of the nominal value throughout the test. Results should be based on nominal concentrations, as has been expressed by the applicant.

There are several protocol deviations to note. The first is that the oysters were not given enough time in the holding and acclimatisation period. The OCSPP 850.1025 (2016) guidance recommends 10-12 days (12-15 days for OCSPP 850.1025 1996), but they were only given 6 days for this study. The applicant states that this is because oyster shell growth has historically been more variable when oysters are held in house for a longer duration (i.e., 12 days or longer). HSE conclude that the reasoning for this deviation is not acceptable due to lack of supporting evidence and failure to meet the updated guidance. Since the controls met acceptance criteria for growth and survival, and water quality measured during the culture six days prior to initiation was within the appropriate test ranges, HSE do not

consider this deviation to have a significant impact on the results or interpretation of the study. All validity criteria were met for this study; therefore, study is considered valid.

The next deviation to protocol is the lack of test replicates for individual controls. The latest guidance (OCSPP 850.1025, 2016) requires at least 2 replicates of 10 oysters each per concentration. This was not stated as a requirement in the guidance available at the time of study (OCSPP 850.1025, 1996). HSE conclude that, as all environmental conditions met the required criteria and there is already existing data on the sensitivity of eastern oysters, it is unlikely that additional replicates would have impacted the outcome of this study. All validity criteria were met, so the study is still considered to be valid.

Whilst not likely to have an effect on the study, there were a couple of environmental deviations to note. The pH obtained was 0.1 below the lower variable requirements outlined in OCSPP 850.1025 (2016), but this is not likely to have impacted the study. The light intensity obtained in this study also exceeds the upper limit for this variable given in OCSPP 850.1025 (2016) by 20 lux, but this is not likely to have impacted the study. OCSPP 850.1025 (2016) requires a 15-30 minute transition period between light and dark. It is stated in the study report that automatic timers were used to avoid sudden transitions between light and dark but does not state the duration of the transition period. However, as the change in light intensity was gradual this is not likely to have had a significant impact on the study.

OCSPP 850.1025 (1996) and OCSPP 850.1025 (2016) both require that no spawning be observed in the definitive exposure. There are no comments about spawning in the study report, but the study did determine that oysters were reproductively immature by confirming that no gametes were present. This should indicate that there was no spawning during the study, which adheres to the validity requirements of the study.

The use of statistics in this study are in line with OCSPP 850.1025 (2016) guidance. The NOEC was determined using Wilcoxon's Test with Bonferroni-Holm's Adjustment to give a value of 0.27 mg a.s./L, but this concentration was not tested under definitive exposure methods.

The method of analysis used in the study was evaluated by HSE Chemistry. The conclusions of their evaluation are reproduced below. Please see Volume 3 CA, section B5 for more details.

"*The analytical method is not fully validated according to SANTE/2020/12830 rev. 1 for the determination of the active substance inpyrfluxam in freshwater as the matrix effects have not been determined and the stability of standards and extracts has not been addressed. However, the studies were generated prior to the implementation of SANTE 2020/12830 rev.1 and SANCO 3029 rev.4 did not require matrix effects and the stabilities of standards and extracts to be addressed. As all other validation requirements have been met (including the minimum validation requirements outlined in SANTE 2020/12830 rev.1), the method is considered to be fit for purpose.*"

The endpoints to use in risk assessment are:

96-hour EC_{50} > 0.99 mg a.s./L (based on nominal test concentrations)

B.9.3 Effects on Arthropods

B.9.3.1 Summary of toxicity data

Table 9.3.1-1: Toxicity endpoints for Inpyrfluxam

Test Item	Study type	Species	Endpoint	Results	References
Acute adult					
Inpyrfluxam	48 h acute oral	*Apis mellifera*	LD_{50}	>111.3 µg a.s./bee	KCA 8.3.1.1.1/01 [redacted] 2015
Inpyrfluxam	48 h acute contact			>100 µg a.s./bee	
Inpyrfluxam	48 h acute oral	*Bombus terrestris*	LD_{50}	>95.1 µg a.s./bee	KCA 8.3.1.1.1/02 [redacted] 2016
Inpyrfluxam	48 h acute contact			>100 µg a.s./bee	
Larvae					
Inpyrfluxam	72 h acute	*Apis mellifera*	LD_{10}	Not determined	KCA 8.3.1.3/01 [redacted] 2017 **Does not address data requirements**
			LD_{20}		
			LD_{50}		
			NOED		

B.9.3.1.1 Acute toxicity to bees

Reference:	KCA 8.3.1.1.1/01
Report Title:	Effects of S-2399 TG (Acute Contact and Oral) on Honey Bees (*Apis mellifera* L.) in the Laboratory
Author(s) & year:	[redacted] (2015)
Document No, Authority registration No:	Project 94951035, TPW-0015

Substance used:	S-2399 TG, 13CG0617G, 95.0%
Method of analysis:	n/a
Guideline(s):	OECD 213 (1998) and 214 (1998)
Deviations:	Yes, see HSE Comments
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

I. MATERIAL AND METHODS

A. MATERIALS

1. Test material: S-2399 TG
Description: White, solid
Lot/Batch: 13CG0617G
Purity: 95.0%
Reference item: Perfekthion (BAS 152 11 I). Active Ingredient: dimethoate (400g/L)
Expiry date: July 23, 2016
Solvent: Acetone
Water solubility: 21.3 mg/L (20 °C)

2. Vehicle: Oral test: 50% w/v sucrose solution, with acetone (test substance vehicle)
Contact test: acetone (test substance vehicle)

B. STUDY DESIGN AND METHODS

1. Test organism
Species: Honey bees (*Apis mellifera* L.)
Age at test initiation: Young adult worker bees
Source: In-house culture
Food: Food (50 % w/v sucrose solution) was provided *ad libitum.*
Test concentration: Contact: Limit test 100.0 µg a.s. of S-2399 TG/bee
Oral test nominal: 100.0 µg a.is of S-2399 TG/bee
Oral test measured: 111.3 µg a.s. of S-2399 TG/bee
No. of bees/treatment: 50

2. Test units: Stainless steel cages 10 cm x 8.5 x 5.5 cm (length x height x width)

3. Environmental conditions

A summary of environmental conditions is shown in Table 9.3.1-2 below.

Table 9.3.1-2: Summary of environmental conditions of acute contact and oral toxicity to honeybees

Variable	Required OECD 213, 214 (1998)	Obtained
Temperature	24 - 25 °C	24-25˚C
Humidity	54 - 79 %	54-79%
Photoperiod	Darkness (except during observation)	Constant darkness

Study dates: 25th – 28th August 2014

4. Animal assignment and treatment:
The honey bees were collected in the morning of use. Individuals for the study were selected without conscious bias and any individual that was unhealthy or damaged was discarded.

The study comprised a water control containing water + 0.5% Adhäsit (contact test) or an untreated sucrose solution (oral test), as well as an acetone control (both tests), four doses of the toxic reference item (dimethoate 400g/L) and a limit dose of the test item (100 µg a.s./honey bee, nominal). Five replicates of ten bees per cage were used in each treatment. The duration of the test was 48 hours. Bees were starved for 15 minutes in the oral test for all treatment groups prior to exposure.

Contact test:
Bees were anaesthetised with CO_2 for approximately 20 seconds until they were completely immobilised. A single 5.0 µL droplet of S-2399 TG in acetone was placed on the dorsal thorax of the bees using a calibrated pipette. Control bees were dosed with the equivalent volume of a) tap water containing 0.5% Adhäsit and b) acetone. The reference item was also applied in a 5 µL droplet (dimethoate made up in tap water containing 0.5% Adhäsit). Adhäsit was used to improve adhesion of the droplet on the bee body.

Oral test:
The treated food was offered in syringes, which were weighed before and after introduction into the cages. After 2 hours 40 minutes, the syringes were removed, weighed and replaced by ones containing fresh, untreated food. Control bees were fed a solution of 50% w/v sucrose solution and for the solvent control 50% w/v sucrose solution with 5% acetone. The reference item was applied in 50% w/v sucrose solution.

5. Dose preparation:
For the contact test, the test item (100µg a.s./bee) and toxic reference item were diluted in acetone. An adjuvant (0.5% Adhäsit) was used in the toxic reference treatment and control groups to make the application of the solution to the thorax possible. The toxic reference nominal concentrations were 0.10, 0.15, 0.20 and 0.30 µg a.s./bee.

For the oral test, the dosing solution was prepared using an aliquot of the contact dose, diluted in 50% sucrose. There was one application at the limit dose of 100 µg a.s./bee. The toxic reference item was prepared by dissolving in acetone and then sucrose solution (at nominal concentrations of 0.05, 0.08, 0.15 and 0.30 µg a.s./bee).

6. Measurements/observations:
The number of dead bees was assessed after 4, 24 and 48 hours. Behavioural abnormalities were recorded over the same period.

7. Statistics:
The contact and oral LD_{50} values of the reference item were estimated using the binominal distribution, taking into account the mortality data corrected by control mortality using Abbott's formula. All statistical analysis was performed using ToxRat Professional, v 2.10.05, ® ToxRat Solutions GmbH.

II. RESULTS AND DISCUSSION

A. BIOLOGICAL OBSERVATIONS
Mortality data for the 48 hours contact and oral honey bee toxicity tests are provided in the following tables. No significant treatment-effects were observed, with mortality in the control and test item treatments not exceeding 5%.

There were no test item related behavioural affects in the bees throughout the study.

Table 9.3.1-3: Mortality and behavioural abnormalities of honey bee in the contact toxicity test (%)

Treatment (µg a.s./bee)	4 hours		24 hours		48 hours	
	Mortality	Behav. abnorm	Mortality	Behav. abnorm	Mortality	Behav. abnorm
Control	0.0	0.0	0.0	0.0	4.0	2.0
Solvent control	0.0	0.0	2.0	0.0	2.0	0.0
S-2399 TG 100	0.0	0.0	0.0	0.0	0.0	0.0
Dimethoate 0.10	0.0	2.0	18.0	0.0	32.0	0.0
0.15	0.0	8.0	44.0	0.0	44.0	0.0
0.20	0.0	18.0	82.0	4.0	88.0	0.0
0.30	0.0	50.0	92.0	2.0	94.0	0.0

Table 9.3.1-4: Mortality and behavioural abnormalities of honey bee in the oral toxicity test (%)

Consumed Treatment (µg a.s./bee)	4 hours		24 hours		48 hours	
	Mortality	Behav. abnorm	Mortality	Behav. abnorm	Mortality	Behav. abnorm
Control	0.0	0.0	0.0	0.0	2.0	2.0
Solvent control	0.0	0.0	0.0	0.0	2.0	0.0
S-2399 TG 111.3	0.0	0.0	0.0	0.0	0.0	0.0
Dimethoate 0.06	0.0	0.0	8.0	0.0	10.0	2.0
0.08	0.0	0.0	18.0	0.0	24.0	0.0
0.16	0.0	26.0	78.0	4.0	82.0	0.0
0.31	16.0	58.0	94.0	2.0	96.0	0.0

B. TOXICITY ENDPOINT

The toxicity endpoints are summarised in Table 9.3.1-5.

Table 9.3.1-5: Contact and oral toxicity endpoints to bees exposed to S-2399 TG

Test	48 hours toxicity endpoint	
	Test item	Reference item
Contact toxicity test	LD_{50} > 100 µg a.s./bee	0.16 µg a.s./bee (C.L. 0.10-0.20 µg a.s./bee)
Oral toxicity test	LD_{50} > 111.3 µg a.s./bee	0.11 µg a.s./bee (C.L. 0.08-0.16)

C.L. = 95% confidence limits

C. VALIDITY CRITERIA

The following criteria were included based on the OECD 213 and 214 guideline:

1. Control mortality was < 10% (actual: 4.0 and 2.0% for water and solvent, respectively) and the toxic reference LD_{50} was in the range of 0.10 – 0.30 µg/bee (actual: 0.16 µg/bee) in the contact test.
2. Control mortality was < 10% (actual: 2.0 and 2.0% for water and solvent, respectively) and the toxic reference LD_{50} was in the range of 0.10 – 0.35 µg/bee (actual: 0.11 µg/bee) in the oral test.

The study was considered valid.

III. CONCLUSION

The acute contact and oral toxicity of S-2399 TG was tested on honeybees under laboratory conditions. The 48 hour contact LD_{50} value was >100 µg a.s./bee, and the oral LD_{50} value was >111.3 µg a.s./bee.

HSE COMMENTS:
This study was conducted under GLP and under OECD 213 (1998) for oral toxicity and OECD 214 (1998) for contact toxicity.

Bees were only starved for 15 minutes prior to exposure in the oral test. OECD 213 (1998) guidelines recommend that bees are starved for up to 2 hours so that all bees are equal in terms of their gut content at the start of the test. No reason was provided by the applicant for the short starvation period. This is unlikely to have had a significant impact on the study as the oral uptake data in the study summary showed similar levels of intake across all groups. As the groups displayed differences in mortality and behaviour in the test, it indicates that there was sufficient uptake for any effects to be demonstrated.

A 5 µL droplet was chosen in deviation to the guideline recommendation of a 1 µL droplet since a higher volume ensured a more reliable dispersion of the test item. Higher volumes are suitable and no adverse effects on the outcome of the study are to be expected.

The use of statistics in these studies are acceptable. No dose-response curve is available as the studies for oral and contact toxicity were conducted as limit tests.

The endpoints to use in risk assessment are:

- **48-hour oral LD_{50} = >111.3 µg a.s./bee (based on measured limit test concentration)**
- **48-hour contact LD_{50} = > 100 µg a.s./bee (based on nominal limit test concentration)**

Reference:	KCA 8.3.1.1.1/02
Report Title:	S-2399 TG: Effects (Acute Contact and Oral) on Bumble Bees (*Bombus terrestris* L.) in the Laboratory
Author(s) & year:	[redacted] (2016)
Document No, Authority registration No:	113911105, TPW-0060
Substance used:	S-2399 TG, 13CG0617G, 95.0%
Method of analysis:	n/a
Guideline(s):	Based on Van der Steen (2001) OECD 213

	OECD 214 Ring test bumble bee acute oral toxicity (ICPR non-Apis group, 2015/2016), OECD proposals for new guidelines for bumblebees, acute contact and oral
Deviations:	Yes, see HSE Comments
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	No, not used in SANCO/10329/2002 risk assessment

MATERIALS AND METHODS

MATERIALS

Test material S-2399 TG

Description White, solid

Batch number: 13CG0617G

Active substance content: 95.5% (verified by certificate of analysis)

Storage on receipt: At 4 ± 4 °C, in the dark

Expiry date June 29 2019

TREATMENTS

Nominal test doses: Oral toxicity test: 100 µg a.s./bee (nominal), 95.1 µg a.s./bee (actual ingested)
Contact toxicity test: 100 µg a.s./bee (nominal)

Control: Oral toxicity test: 50 % w/v sucrose solution (negative control), 50 % w/v sucrose solution containing 5 % w/w acetone solution (solvent control).
Contact toxicity test: tap water containing 0.1 % v/v Triton X-100 (negative control), pure acetone (solvent control)

Toxic reference: BAS 152 11 I (Dimethoate, 420.3 g/L (analysed), 400 g/L (measured))

Toxic reference batch: FRE-001226

Wetting agent: Triton X-100 (Lot No. – K45384303 436), 0.1 % v/v

TEST ORGANISMS

Species: Bumble bees (*Bombus terrestris* L), adult worker bees

Source: Queen-right, healthy colonies obtained from Koppert B.V., Netherlands

Acclimatisation period:	Bees were collected from the upper non-nest area of the housing box under red light (no anaesthetic) one day before application. Contact test: 20 hours 30 minutes, Oral test: 15 hours 10 minutes.
Diet:	50 % w/v sucrose solution was provided *ad libitum*.

TEST DESIGN

Test cages:	Cylindrical, latticed plastic cages (7 cm length, 1.7 and 2.2 cm diameter each end)
Replication:	50 bees per treatment group, housed individually
Duration:	48 hours

TEST CONDITIONS

Test temperature:	Acclimation: 24 - 25 °C Exposure: 23 – 25 °C
Relative humidity:	Acclimation: 51 - 63 % Exposure: 50 -63 %
Lighting:	24 h darkness (except handling procedures, including treatment and observations)

STUDY DESIGN AND METHODS

Experimental dates: 05 October 2016 to 08 October 2016

Test organism and treatment

After collection from the colonies individuals of a middling size were visually selected and randomly distributed to treatment groups. Each bumble bee was weighed individually (anaesthetised with CO_2) to demonstrate appropriate weight matching between treatment groups (Table 9.3.1-6).

Table 9.3.1-6: Weight distribution of bumble bees among treatment groups

Treatment group	Contact toxicity test		Oral toxicity test	
	Mean weight (mg)	SD	Mean weight (mg)	SD
Test item	268	54.8	267	41.1
Water control	283	53.7	269	44.2
Solvent control	275	50.8	272	48.5
Reference item	267	49.5	272	46.4

Contact test

Bees were anaesthetised with CO_2 after which a 5.0 µL droplet was placed on the dorsal thorax of the bees using a calibrated pipette. The test item was tested at a concentration of

100 µg a.s./bee and the reference item was tested at a concentration of 10 µg dimethoate/bee.

Oral test
The bees were starved for 160 – 180 minutes prior to application. Hereafter, the treated food was offered in syringes, which were weighed before and after introduction into the cages. After 4 hours and 10 minutes, the syringes were removed, weighed and replaced by ones containing fresh, untreated food. The test item was tested at a nominal concentration of 100 µg a.s./bee and the reference item was tested at a nominal concentration of 4.0 µg dimethoate/bee. Consumption was calculated by subtracting the weight of syringes on removal from the weight of syringes on introduction to the cages.

Dose preparation
For the contact treatment, a stock solution was prepared by dissolving 209.03 mg of the test item in 10 mL of acetone. 100 µg a.s./bee dose was obtained when 5 µL of this stock solution was applied to the dorsal thorax of each bee.

For the oral treatment, 261.87 mg of the test item was dissolved in 5 g of acetone to form a stock solution. 0.5 g of this acetone stock solution was added to 9.5 g of 50 % w/v sucrose solution to form solution A. 40 mg of solution A, the consumption target of spiked solution for each bee, contained 100 µg of the test item S-2399 TG.

All calculations were adjusted to account for the 95.5 % purity of the test item.

The dose level of the toxic reference dimethoate was 10 µg/bee for the contact test and 4.6 µg/bee (measured) for the oral test.

Measurements and observations
The number of dead bees was assessed after 4, 24 and 48 hours. Behavioural abnormalities were recorded over the same period. These effects were either recorded as affected (bumble bees still upright and attempting to walk but showing signs of reduced coordination) or moribund (bumble bees cannot walk and show only very feeble movements of legs and antennae in response to stimulation).

Temperature and humidity were maintained using an incubator (RUMED 1301, Rubarth Apparate GmbH) and recorded continuously with appropriate, calibrated equipment.

Statistical analysis
The contact and oral mortality data of the test item group and reference item group were compared to the solvent control group using Fisher’s Exact test after Bonferroni-Holm (pairwise comparison, one-sided greater, $\alpha = 0.05$). All statistical analysis was performed using ToxRat Professional (v 3.2.1, ® ToxRat Solutions GmbH).

RESULTS AND DISCUSSION

Biological effects

Oral toxicity test

The results from the oral toxicity test are summarised in Table 9.3.1-7.

Table 9.3.1-7: Oral toxicity of S-2399 TG to bumble bees (*Bombus terrestris* L.)

Consumed Treatment (µg a.s./bee)	4 hours		24 hours		48 hours	
	Mortality (mean %)	Behav. Abnorm. [a] (mean %)	Mortality (mean %)	Behav. Abnorm. [a] (mean %)	Mortality (mean %)	Behav. Abnorm. [a] (mean %)
Control	0.0	0.0	2.0	0.0	2.0	0.0
Solvent control	0.0	0.0	0.0	0.0	0.0	0.0
S-2399 TG 95.1	0.0	0.0	0.0	0.0	0.0	0.0
Dimethoate 4.6	10.0	90.0	100.0	0.0	100.0	0.0

[a] Behavioural abnormalities

No statistically significant differences were observed for mortality in the test item group compared to the solvent control group. No test item related behavioural effects were observed for bees in the test item and the control groups.

In the oral test, the mean uptake of the test item solution was slightly lower than the target dose. Hence the dose level was estimated based on the mean measured food consumption of 95.1 µg a.s./bee.

Contact toxicity test

The results from the oral toxicity test are summarised in Table 9.3.1-8.

Table 9.3.1-8: Contact toxicity of S-2399 TG to bumble bees (*Bombus terrestris* L.)

Treatment (µg a.s./bee)	4 hours		24 hours		48 hours	
	Mortality (mean %)	Behav. Abnorm. [a] (mean %)	Mortality (mean %)	Behav. Abnorm. [a] (mean %)	Mortality (mean %)	Behav. Abnorm. [a] (mean %)
Control	0.0	0.0	0.0	0.0	0.0	0.0
Solvent control	0.0	0.0	0.0	0.0	0.0	0.0
S-2399 TG	2.0	0.0	2.0	0.0	4.0	0.0

Treatment (µg a.s./bee)	4 hours		24 hours		48 hours	
	Mortality (mean %)	Behav. Abnorm. [a] (mean %)	Mortality (mean %)	Behav. Abnorm. [a] (mean %)	Mortality (mean %)	Behav. Abnorm. [a] (mean %)
100						
Dimethoate 10	6.0	66.0	84.0	4.0	90.0	0.0

[a] Behavioural abnormalities

The toxicity endpoints are summarised in Table 9.3.1-9.

Table 9.3.1-9: Contact and oral toxicity endpoints for Bombus terrestris L. exposed to S-2399 TG

Test	48 hours toxicity endpoint	
	LD_{50}	NOED
Contact toxicity test	> 100 µg/bee	≥ 100 µg a.s./bee
Oral toxicity test	> 95.1 µg/bee	≥ 95.1 µg a.s./bee

Validity criteria

The validity criteria for the study were met according to OECD 246 (2017) and OECD 247 (2017) (Table 9.3.1-10).

Table 9.3.1-10: Compliance with OECD 246 and OECD 247 validity criteria

Validity criterion	Required	Obtained
OECD 246		
Control mortality	≤ 10 %	0 % (negative and solvent control)
Reference substance toxicity	≥ 50 %	90 %
OECD 247		
Control mortality	≤ 10 %	2 % (negative control) 0 % (solvent control)

Validity criterion	Required	Obtained
Reference substance toxicity	≥ 50 %	100 %

CONCLUSIONS

The acute contact and oral toxicity of S-2399 TG was tested on *Bombus terrestris* L. under laboratory conditions. The 48-hour contact LD_{50} value was > 100 µg a.s./bee and the oral LD_{50} value was > 95.1 µg a.s./bee.

HSE COMMENTS

The study was carried out according to OECD (2016), Proposal for new Guideline: Bumblebee, Acute Contact Toxicity Test, Draft August 2016 and OECD (2016), Proposal for new Guideline: Bumblebee, Acute Oral Toxicity Test, Draft August 2016. It was evaluated against OECD 246 (2017) and OECD 247 (2017), which are the final versions of the draft guidelines followed. All validity criteria outlined in OECD 246 (2017) and OECD 247 (2017) were satisfactorily met for the duration of the study. There were no significant deviations to the guidelines. Treatment with the toxic reference (dimethoate) indicated the sensitivity of the bees and reliability of the test system was appropriate for both oral and contact toxicity.

The following minor deviations were noted for OECD 246 (2017) and OECD 247 (2017):
OECD 246 (2017) and OECD 247 (2017) § 9 state that individuals should be sourced from medium sized colonies with approximately 60 to 80 workers. The size of the source colonies was not reported.

OECD 246 (2017) § 19 and 20 and OECD 247 (2017) § 21 and 22 refer to requirements for analytical verification of test chemical solutions. No analytical verification was performed during the study, possibly due to deviations between the finalised 2017 guidelines and the followed 2016 draft guidelines. At the time of undertaking, the draft guidelines were the most complete OECD documents. As this omission probably stemmed from the guidelines selected when performing the study HSE considers this an acceptable deviation.

OECD 246 (2017) § 23 and OECD 247 (2017) § 25 discuss the use of multiple colonies when selecting individual bees for the study and ensuring colonies are evenly distributed between treatment group. The word “colonies” is used when referring to the commercial source, suggesting multiple colonies were used. However, how bees from different colonies were evenly distributed between treatment groups, to avoid a possible colony effect, was not reported.

OECD 246 (2017) § 24 and OECD 247 (2017) § 26 outline the selection of a suitable reference level for statistical testing. The study only compared the test item to the solvent

control. The guideline recommends comparing the negative and solvent controls and, if there are no statistically significant differences, pooling them. This was not performed. There was no mortality in either control group, however, so this deviation from the guidelines would not have impacted the endpoints produced by the study.

OECD 246 (2017) § 33 and OECD 247 (2017) § 40 outline the requirements for test reporting. It states, "*all relevant information on colonies used for collection of test bumblebees, including health certificate, any adult disease, any pre-treatment, etc., if available*". No information on disease status was reported.

OECD 247 (2017) § 30 discusses the issue of "non-feeders" and measures taken to reduce their impact on the study. "non-feeders" were not reported in the study. On interrogation of the raw data in the appendix there were four "non-feeders" (individuals who consumed < 80 % of the 40 mg target consumption) present in the study, all in the test item treatment group. These individuals were not excluded before the derivation of endpoints. This means there may have been a small underestimation of the effect of the test item.

The above study was conducted to GLP and considered valid.

The agreed endpoints suitable for use in the risk assessment are: 48-hour oral LD_{50} > 95.1 µg a.s./bee and 48-hour contact LD_{50} > 100 µg a.s./bee.

B.9.3.1.2 Chronic toxicity to bees

No chronic toxicity studies were submitted for the active substance. Please refer to the DAR 21: Volume 3CP document for chronic effects associated with the representative formulation and justification for applying results from studies using the representative formulation to the active substance.

B.9.3.1.3 Effects on honeybee development and other honeybee life stages

Reference:	KCA 8.3.1.3/01
Report Title:	S-2399: Acute Survival of Honey Bee Larvae, *Apis mellifera* L., during an *In Vitro* Exposure
Author(s) & year:	[redacted] (2017)
Document No, Authority registration No:	12709.6372, TPW-0066
Substance used:	S-2399 TG, 13CG0617G, 95.0%
Method of analysis:	LC/MS/MS
Guideline(s):	OECD 237 (2013)

Deviations:	Yes, see HSE Comments
GLP or GEP:	Yes
Acceptability:	No, OECD 237 studies do not address Data point 8.3.1.3.
Study relied upon:	No, does not address Data point 8.3.1.3.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test material:** S-2399 TG
   Description: Not stated
   Lot/Batch: 13CG0617G
   Purity: 95.0%
   Water solubility: Not stated
   Molecular weight: Not stated

2. **Reference material:** Dimethoate PESTANAL®
   Description: Not stated
   Purity: 99.5%
   Lot/Batch: SZBC243XV

B. TREATMENTS

1. **Test concentrations:** 6.3, 13, 25, 50 and 100 µg a.s./larva
   LOQ was set at 0.600 µg/L
   MDL was set at 0.200 µg/L
   Solvent/carrier: Acetone
   Analysis of concentrations: Analysis of concentrations on dosing stock solutions and quality control samples

C. STUDY DESIGN AND METHODS

1. **Test animals:** *Apis mellifera* L.
   Age/growth stage: First instar larvae (≤ 24 hours old) at initiation of 3-day acclimation phase prior to the exposure
   Source: Woods Beekeeping Supply, Lincoln, Rhode Island. The hives were not exposed to pesticides for six months prior to the study
   Acclimation: 3 days
   Replicates: 36 larvae per test group

2. **Diet:** Royal jelly diet containing yeast extract, D-glucose and D-fructose

3. **Test vessel:** Sterile, 48-well cell culture plates (1.6 mL/well) each containing a plastic, grafting cell

4. **Environmental conditions:**

A summary of the environmental conditions obtained in this study is shown in Table 9.3.1-11 below.

Table 9.3.1-11: Environmental conditions obtained in the study of S-2399 toxicity to honeybee larvae

Variable	Required OECD 237 (2013)	Obtained
Temperature	34˚C - 35°C (Min. 23˚C, Max. 40˚C)	33˚C to 36°C
Relative Humidity	Not stated	99-100%
Photoperiod	Darkness	Darkness, except during observations and renewal of diet

Study dates:
Acclimation: 2 to 4 June 2015
Exposure: 5 to 8 June 2015

5. Animal assignment and treatment:
Frames containing brood cells were removed from each of the three hives. First instar honey bee larvae were removed from the brood frames and placed into the culture plates. Once each plate contained 12 larvae from each of the three different hives, for a total of 36 larvae per treatment and controls (remaining wells containing deionised water), the plate was placed in an incubator.

The initial phase involved a 3-day acclimation. On the first day of this acclimation phase (day -3), larvae were fed 20 µL of diet 1 , on Day -2 the larvae were not fed and on day -1 the larvae were fed 20 µL of diet 2 (refer to Table 8.3.1.3-01-2 for details of the diets used).

The exposure phase lasted 72 hours, with a treated diet only on the first day (day 0). On day 0, larvae were fed 30µL of diet 3, which contained increasing appropriate levels of test material or acetone only (solvent control) or was untreated (control). On days 1 and 2, untreated diet 3 was fed to all exposed and control larvae. All diets were warmed to 34 ± 2°C before being added to the plate wells.

The three different diets were prepared prior to test initiation and were frozen (-70 to -90°C) until use.

Table 9.3.1-12: Summary of diets fed to the honey bee larvae

Component	Diet 1	Diet 2	Diet 3
Sterile deionised water (mL)	111	67.0	60.0
D-glucose (g)	18.0	15.0	18.0
D-fructose (g)	18.0	15.0	18.0

Component	Diet 1	Diet 2	Diet 3
Yeast extract (g)	3.01	3.00	4.02
Royal jelly (g)	150	100	100

A reference test using dimethoate at a concentration of 8.8 µg a.s./larva was included to monitor the effectiveness of the test system.

6. Dose preparation:

A 151 mg a.s./mL primary stock solution was prepared by bringing 7.9618 g S-2399 TG to a volume of 50 mL with acetone. Dilutions of the primary stock solution were used to prepare the remaining stock solutions (9.5, 20, 38, 76 mg a.s./mL). All stock solutions were observed to be clear with no visible undissolved test substance following preparation. The treatment diets were dosed using the stock solutions and diet 3 to give the treatment diets (6.3, 13, 25, 50 and 100 µg a.s./larva). All treatment diets were observed to be viscous and yellow in colour. The solvent control was prepared by adding 0.2 mL of acetone to 10 g of diet 3. Untreated diet 3 was used for the control.

A 26.6 mg a.s./mL reference toxicant stock solution was prepared by bringing 0.2678 g of dimethoate to a volume of 10 mL with acetone. This stock solution (0.1 mL) was added to 10 g of diet 3. The resulting reference toxicant diet was observed to be viscous, yellow in colour with no undissolved material.

7. Measurements and observations:

Honey bee larvae mortality during the treatment phases was recorded daily. Death of larvae was defined as lack of reaction from the contact of a grafting tool. Percent survival during the exposure phase (Days 0 to 3) was based on the number of larvae surviving relative to the 36 larvae per treatment or control used to initiate the study. At test termination, observations of health were recorded.

Temperature and relative humidity in the incubator were monitored and recorded daily. LOQ was set at 0.600 µg/L. MDL was set at 0.200 µg/L

On the day of exposure, a sample of each dosing stock solution was collected and analysed for S-2399 TG concentration using liquid chromatography with mass spectrometry detection (LC/MS/MS).

8. Statistics:

The nominal concentration tested and the corresponding mortality data derived from the toxicity test was used to estimate the 72-hour mean lethal dose concentration (LD_{50}). If ≥ 50% mortality did not occur in any of the doses tested, the LD_{50} value was empirically estimated to be greater than the highest dose tested. CETIS™ v 1.8 was used to perform all statistical analyses.

II. RESULTS AND DISCUSSION

A. MORTALITY

No statistically significant differences in mortality were reported. A summary of mortality data

is detailed in Table 9.3.1-13.

Table 9.3.1-13: Summary of *Apis mellifera* mortality data exposed to S-2399 TG

Nominal dose (µg a.s./larva)	Cumulative percent mortality (number of dead larvae)			
	Day 0	Day 1	Day 2	Day 3
Control	0 (0)	0 (0)	0 (0)	3(1)
Solvent Control	0 (0)	0 (0)	0 (0)	0(0)[a]
6.3	0 (0)	0 (0)	0 (0)	0(0)[a]
13	0 (0)	0 (0)	0 (0)	0(0)
25	0 (0)	0 (0)	0 (0)	3(1)[b]
50	0 (0)	0 (0)	0 (0)	6(2)
100	0 (0)	0 (0)	0 (0)	39(14)
Toxic reference (8.8 µg dimethoate/larva)	0 (0)	0 (0)	0 (0)	64(23)

[a] Two larvae were observed to be discoloured
[b] One larvae was observed to be discoloured

The LD_{50} was determined to be >100 µg a.s./larva, based on nominal concentrations.

B. SUBLETHAL EFFECTS
At exposure termination, two larvae were observed to be discoloured among larvae exposed to the 6.3 µg a.s./larva treatment and solvent control and one larva was observed to be discoloured among larvae exposed to the 25 µg a.s./larva treatment. All remaining larvae were observed to be healthy.

C. ANALYSIS
Analysis of the dosing stock solutions resulted in measured concentrations of S-2399 TG of 11, 22, 45, 80 and 160 mg a.s./L, with related recoveries ranging from 100 to 120% of nominal concentration.

Table 9.3.1-14: Concentration of S-2399 TG measured in the dosing stock solution at test initiation

Nominal dosing stock concentration (µg a.s./larva)	Dosing stock concentration (mg a.s./mL)	Measured stock concentration (mg a.s./L)[a]	Percent of nominal[a]
Solvent control	0	<0.80[b]	n.a.
6.3	9.5	11	110
13	20	22	110
25	38	45	120

Nominal dosing stock concentration (µg a.s./larva)	Dosing stock concentration (mg a.s./mL)	Measured stock concentration (mg a.s./L)[a]	Percent of nominal[a]
50	76	80	110
100	150	160	100

[a] Measured stock and percent of nominal concentrations were calculated using the actual analytical (unrounded) results and not the rounded (two significant figures) values presented in this table
[b] Concentrations expressed as less than values were below the minimum detectable limit (MDL).
n.a. = not applicable

D. VALIDITY CRITERIA
As cumulative mortality in the control diet was < 15% (actual: 0% control, 3% solvent control) and the toxic reference mortality was > 50% (actual: 64%), the study was considered valid.

In addition, the 64% mortality observed among larvae exposed to the toxic reference substance demonstrated that the larvae were sensitive in this exposure system.

III. CONCLUSION

Since no dose resulted in ≥ 50% mortality, the 3-day LD_{50} for honey bee larvae, *Apis mellifera*, was determined to be >100 µg a.s./larva, the highest dose of S-2399 TG tested.

HSE COMMENTS
This study was conducted under GLP and under OECD 237 (2013) guidelines. It has been assessed against these same guidelines.

The concentrations of the test item were maintained between 80-120% of the nominal value throughout the test. Results should be based on nominal concentrations, as has been expressed by the applicant.

The study report has not commented on whether larvae had formed a “C” shape or not. OECD 237 (2013) guidelines state that larvae should not yet have formed a “C” shape. HSE conclude that, as the larvae used in this study were newly hatched, it is unlikely that they would have formed this shape. As this is not a validity criterion of the study, the study is still considered valid.

There is a minor observation related to the discolouration of larvae. 2 larvae in the solvent control and 6.3 µg a.s./larvae concentration groups, and 1 larva in the 25 µg/larvae group were observed to be discoloured. This is unlikely to be a result of the active substance given the groups in which this effect was observed. It does not state in which way the larvae were discoloured or which colony they were from. If these larvae were from the same colony, it may imply that it was not a healthy replicate group. As all environmental and dietary criteria were met and the validity criteria of the study were met, the study is considered valid.

The warming of the dietary substance was done to 34 ± 2°C . OECD 237 (2013) guidelines

state that diets should not be warmed above 35˚C in any case. No exact temperature recordings were provided in the study report, but it implies that some diets were warmed to 36˚C. This protocol deviation is unlikely to have a significant effect on the study but should still be noted given the low mortalities and high percentage of nominal concentrations recorded. As all validity criteria was met, the study was considered valid.

It has not been stated where the royal jelly component of the diet was obtained from. It does have a reference number relating to the test facility, but this does not indicate where the jelly originated from. It has also not been reported how much of the diet was consumed in each group; therefore, it is uncertain whether a lack of mortalities in some groups are a result of test substance consumed or low toxicity. It only states in the introduction to the study report that "laboratory testing provides greater certainty that bee larvae have actually consumed the test substance".

As ≥ 50% mortality did not occur in any of the doses tested, the LD_{50} value was empirically estimated. CETIS™ v 1.8 was used to perform all statistical analyses.

HSE does not consider OECD 237 (2013) studies adequate to address Data point 8.3.1.3 set out in COMMISSION REGULATION (EU) No 283/2013, which states, "*the bee brood study shall provide sufficient information to evaluate possible risks from the active substance on honeybee larvae*". The single exposure design of OECD 237 (2013) is not considered worst-case, unlike the multiple exposure design of OECD 239. HSE has identified a data gap and will address its impact at the risk assessment stage.

Due to the unsuitability of the test design to address Data point 8.3.1.3, no endpoints are suitable for use in risk assessment.

B.9.3.1.4 Sub-lethal effects

Please refer to B.9.3.1.1 for acute sub-lethal effects. Please refer to the DAR 21: Volume 3CP document for sub-lethal effects associated with the representative formulation and justification for applying results from studies using the representative formulation to the active substance.

B.9.3.2 Effects on non-target arthropods other than bees

No studies were submitted for non-target arthropods other than bees conducted with the active substance as a test item.

B.9.4 Effects on Non-target soil meso- and macrofauna

B.9.4.1 Summary of toxicity data

Table 9.4.1-1: Endpoints for Inpyrfluxam and its metabolites

Test item	Exposure system	Species	Endpoint	Results	References
S-2399 TG	Soil 14 d acute 10% peat content	*Eisenia fetida*	LC_{50}	Not determined	KCA 8.4/01 [redacted] 2015 **Does not address data requirements**
S-2399 TG	Soil 56 d chronic 5% peat content	*Eisenia fetida*	EC_{10}	21.5 mg a.s./kg soil dw	KCA 8.4.1/01 [redacted] 2016a
			NOEC	6.25 mg a.s./kg soil dw	
3'-OH-S-2840	Soil 56 d chronic 5% peat content	*Eisenia fetida*	EC_{10}	>100 mg/kg soil dw	KCA 8.4.1/02 [redacted] 2016a
			NOEC	100 mg/kg soil dw	
1'-COOH-S-2840	Soil 56 d chronic 5% peat content	*Eisenia fetida*	EC_{10}	52.4 mg/kg soil dw	KCA 8.4.1/03 [redacted] 2016b
			NOEC	50 mg/kg soil dw	
S-2399 TG	Soil 14 d, chronic 5% peat content	*Hypoaspis aculeifer*	EC_{10}	>100 mg a.s./kg soil dw	CA 8.4.2/04 [redacted] 2016c
			NOEC	100 mg a.s./kg soil dw	
S-2399 TG	Soil 28 d, chronic 5% peat content	*Folsomia candida*	EC_{10}	>100 mg a.s./kg soil dw	CA 8.4.2/01 [redacted] 2016b
			NOEC	100 mg a.s./kg soil dw	
3'-OH-S-	Soil	*Hypoaspis*	EC_{10}	>100 mg/kg soil dw	CA 8.4.2/05

Test item	Exposure system	Species	Endpoint	Results	References
2840	14 d, chronic 5% peat content	*aculeifer*	NOEC	100 mg/kg soil dw	[redacted] 2016e
3'-OH-S-2840	Soil 28 d, chronic 5% peat content	*Folsomia candida*	EC_{10}	>100 mg/kg soil dw	CA 8.4.2/02 [redacted] 2016c
			NOEC	100 mg/kg soil dw	
1'-COOH-S-2840	Soil 14 d, chronic 5% peat content	*Hypoaspis aculeifer*	EC_{10}	>100 mg/kg soil dw	CA 8.4.2/06 [redacted] 2016f
			NOEC	100 mg/kg soil dw	
1'-COOH-S-2840	Soil 28 d, chronic 5% peat content	*Folsomia candida*	EC_{10}	>100 mg/kg soil dw	CA 8.4.2/03 [redacted] 2016d
			NOEC	100 mg/kg soil dw	

Reference:	KCA 8.4/01
Report Title:	Acute Toxicity of S-2399 TG to the Earthworm *Eisenia fetida* in Artificial Soil
Author(s) & year:	[redacted] (2015)
Document No, Authority registration No:	94951021, TPW-0016
Substance used:	S-2399 TG, 13CG0617G, 95.0%
Method of analysis:	n/a
Guideline(s):	OECD 207 (1984)
Deviations:	Yes, see HSE Comments
GLP or GEP:	Yes

Acceptability:	No, does not form part of the current data requirements for active substances
Study relied upon:	No, does not form part of the current data requirements for active substances

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test material:** S-2399 TG
Description: White, solid
Lot/Batch: 13CG0617G
Purity: 95.0%
Reference item: 2-Chloroacetamide
Expiry date: 23rd July 2016
Solvent: No solvent – ground quartz sand used to make up concentrations
Solubility: In water: 21.3 mg/L (20 °C)
Control: Untreated control (the same amount of quartz sand as in the test item treated groups was added and moistened with deionised water)

B. STUDY DESIGN AND METHODS

Test animals: Earthworm, *Eisenia fetida*
Breeding information: Cultured under standardised conditions in IBACON laboratories in a breeding medium of cattle manure, peat, sand, calcium carbonate and straw
Age: 6 to 7 months old with clitellum.
Source: In-house culture
Weight: 300 to 600 mg
Acclimation: 1 day, in artificial soil, under test conditions
Diet: Feed with cattle manure during culture. Not fed during exposure.
Artificial soil: 10% sphagnum peat, 20% (air-dried and finely ground with no visible plant remains) kaolin clay, 69.5% quartz sand and 0.5% calcium carbonate.

Test units: 1 litre glass vessels with loosely covered glass lids 666.7g of prepared soil
Concentration tested: Control, 62.5, 125, 250, 500 and1000 mg a.s./kg soil dry weight

Number of replicates: 4x10 earthworms
Number of earthworms: 40
Maximum water holding capacity: 64% of the dry weight
Test duration: 14 day exposure

Environmental conditions:
A summary of the environmental conditions obtained in this study is shown in Table

9.4.1-2 below:

Table 9.4.1-2: A summary of the environmental conditions obtained in the acute toxicity study of S-2399TG on *Eisenia fetida*

Variable	Required OECD 207 (1984)	Obtained
Temperature	20° ± 2°C	18˚C – 22˚C
pH of soil	6.0 ± 0.5	5.8 – 6.5
Water content of soil	Approx 35%	34.3 – 35.3% of the dry weight at test initiation 32.6 -33.7% of the dry weight at test termination
Photoperiod	Continuous light	Continuous light
Light intensity	400 – 800 lux	400 to 800 lux

Study dates:
Experimental Starting Date: December 02, 2014.
Experimental Completion Date: December 17, 2014

Animal assignment and treatment:
Groups of ten earthworms were rinsed in dechlorinated water, dried, weighed and placed onto the soil surface of each test container. The containers were covered loosely with glass lids to retain moisture whilst allowing ventilation and were incubated under test conditions for 14 days. At test termination, on Day 14, worms were again rinsed in dechlorinated water, dried, re-weighed and the total bulk of soil from each treatment group was re-weighed to calculate the approximate moisture content.

The test comprised five test substance treatments (62.5, 125, 250, 500 and 1000 mg a.s./kg soil dw) and a control. Each treatment consisted of four replicates with ten worms each.

A reference test was conducted in a separate study with 2-Chloroacetamide at concentrations of 17.2, 21.5, 26.9, 33.6 and 42.0 mg a.s./kg soil dw.

Dose preparation:
Defined amounts of S-2399 TG were weighed and 20 g fine quartz sand was added and mixed to achieve a homogeneous distribution within the sand. The mixture was then added to artificial soil equivalent to 2030 g dry weight, resulting in the required nominal concentration levels. The control was treated with the same amount sand as the test item groups. While mixing the artificial soil in a laboratory mixer for approximately 5 minutes the soil of each treatment group was moistened with deionised water to achieve the required water content. Each group was treated in one batch and then split into 4 replicates.

Measurements and observations:
Weights of worms were recorded in treatment replicates immediately prior to treatment and on Day 14. Observations of any visible worms for behavioural (e.g., not burrowing into the

soil, lack of movement, rigidity, *etc*.) or pathological signs were made daily and mortality was assessed on Days 7 and 14. Artificial soil was emptied from the jars onto a tray and the number of live and dead earthworms in each replicate was assessed. Missing earthworms and earthworms failing to respond to gentle stimulation were considered as dead. Living earthworms and the soil were placed back into the test containers after the assessment on day 7.

Statistics:
An LC_{50} value and its 95% confidence-limits at Day 14 were calculated by applying Probit-Analysis. Mortality data were analysed for significance using Fisher's Exact Test (multiple comparison, one-sided greater, α = 0.05). Data was tested for normal distribution and homogeneity of variance using Shapiro-Wilk's test and Levene's test (α = 0.05), respectively. Data for body weight change were normally distributed and homogeneous, so the Williams t-test was used for comparison of the treated and control groups (multiple comparison, two-sided, α = 0.05). The software ToxRat Professional, Version 2.10.05, ® ToxRat Solutions GmbH was used for all statistical analysis.

II. RESULTS AND DISCUSSION

A. MORTALITY

No statistically significant increase in mortality was observed up to and including the concentration of 125 mg/kg soil dw. At 250 mg/kg soil dw the mortality was statistically significantly increased compared to the control. At 500 and 1000 mg/kg soil dw, all worms were dead after 14 days exposure.

Table 9.4.1-3: Effects of S-2399 TG on survival of earthworm *Eisenia fetida* (14 days of exposure)

Nominal concentration (mg a.s./kg soil dw)	Mortality (%)[a]	
	Day 7	Day 14
Control	0.0 (0.0)	0.0 (0.0)
62.5	0.0 (0.0)	2.5 (5.0)
125	0.0 (0.0)	0.0 (0.0)
250	0.0 (0.0)	60.0 (11.5)*
500	10.0 (8.2)	100.0 (0.0)*
1000	7.5 (5.0)	100.0 (0.0)*

[a] The tabulated results represent rounded results calculated on the exact raw data
*significantly different compared to the control, Fisher's Exact Test, α = 0.05, one-sided greater

The dose-response curve shows the effects of test concentrations of S-2399TG on mortality on *E. fetida*.

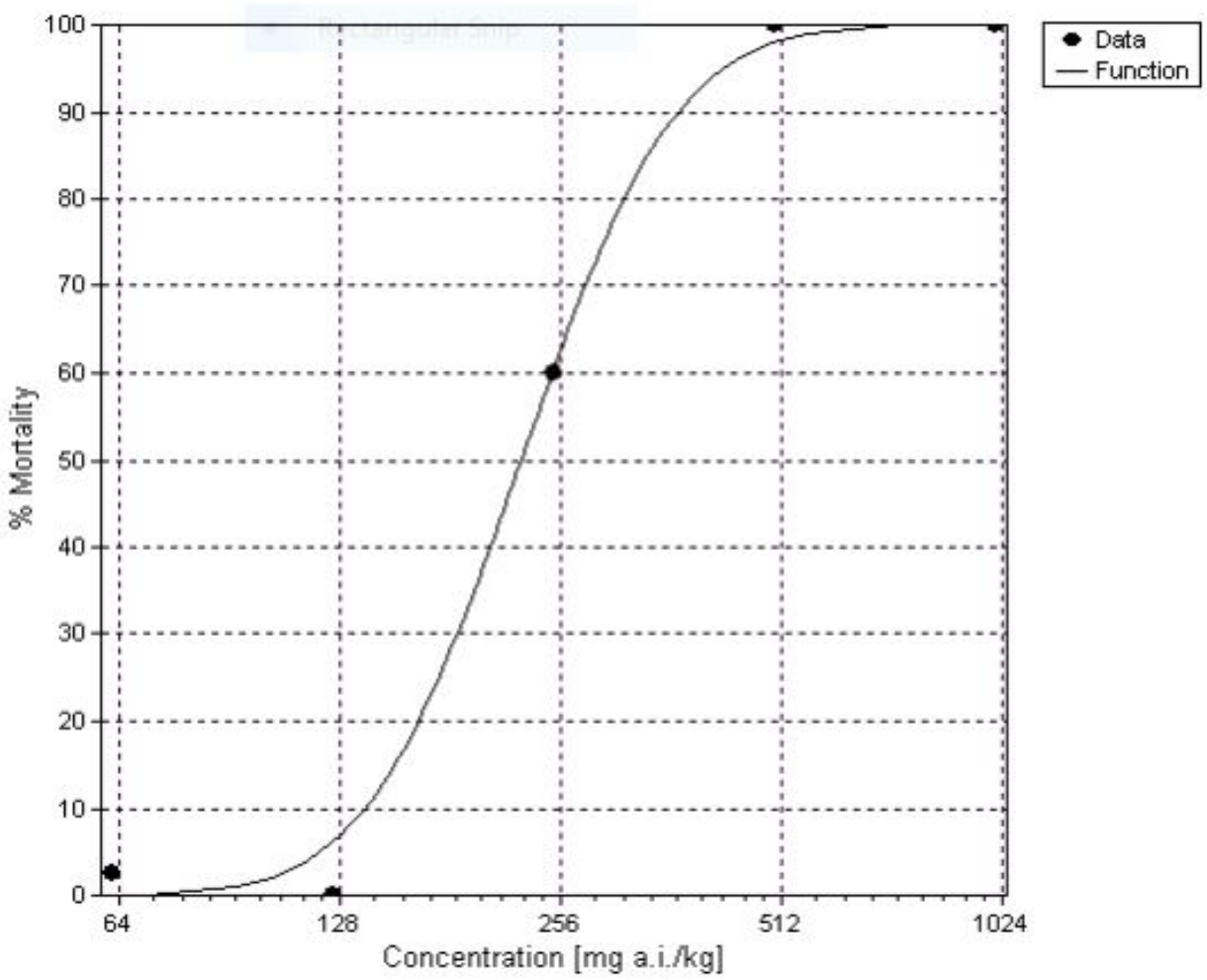


Figure 9.4-1: Dose-response curve showing effects of S-2399TG test concentrations of mortality of *E. fetida*

B. BODY WEIGHT AND BEHAVIOUR

Body weight changes were not statistically significant compared to the control up to and including 125 mg a.s./kg soil dw. At 250 mg a.s./kg soil dw and above, a statistically significant decrease in body weight was observed. Therefore the LOEC was determined to be 250 mg a.s./kg soil dw and the NOEC was determined to be 125 mg a.s./kg soil dw. A summary of the results is presented in Table 9.4.1-4 below.

After 7 days of exposure some worms at 500 mg a.s./kg soil dw showed constrained movement. No additional behavioural effects were observed.

Table 9.4.1-4: Effects of S-2399 TG on biomass of earthworm *Eisenia fetida* (14 days of exposure)

Nominal concentration (mg a.s./kg soil dw)	Group mean weight (mg/worm)		Weight change (%)[a]
	Day 0	Day 14	
Control	379.8	385.5	2.0

Nominal concentration (mg a.s./kg soil dw)	Group mean weight (mg/worm)		Weight change (%)[a]
	Day 0	Day 14	
62.5	385.6	376.6	-2.3
125	382.5	364.9	-4.5
250	384.6	316.9	-17.6*
500	378.6	/	/
1000	381.2	/	/

[a] The results represent rounded values
/ All the worms died
*Significantly different compared to the control, Williams t-test, α = 0.05, two-sided

The reference substance 2-Chloroacetamide was tested in a separate 14-day toxicity study. The 14-day LC_{50} was determined to be 25.3 mg/kg soil dw (95% confidence limits of 18.5-34.3 mg/kg soil dw).

C.VALIDITY CRITERIA
As there were no mortalities in the control treatment the validity criteria were met and the study was considered valid.

III. CONCLUSION
In this 14-day study assessing the acute toxicity of S-2399 TG to the earthworm (*Eisenia fetida*) the LC_{50} was estimated to be 225.9 mg a.s./kg soil dw. The LOEC was determined to be 250 mg a.s./kg soil dw and the NOEC was determined to be 125 mg a.s./kg soil dw.

HSE COMMENTS
This study was conducted under GLP and OECD 207 (1984) guidelines. The study has been assessed against these same guidelines. All validity criteria were met, so the study is considered valid.

The reference test was conducted using 2-Chloroacetamide and resulted in a 14-day LC_{50} of 25.3 mg/kg soil dw (95% confidence limits of 18.5-34.3 mg/kg soil dw). However, as there are no historical data to compare these results to, HSE are unable to conclude whether the test system is sufficiently sensitive.

There are some slight deviations to OECD 207 (1984) guidelines noted in relation to the dose preparation of concentrations. Sand quartz was used to make up the required concentrations in this study. OECD 207 (1984) guidelines state that 10g quartz should be added to the test item, but this study used 20g quartz. There is no data available regarding the mean measured concentration, so it is unclear if this had any impact on test concentrations. As the acute earthworm toxicity is study is not required as part of the current data requirements this study will not be used in the risk assessment and therefore the issue has not been considered further at this time.

Additionally, OECD 207 (1984) guidelines state that 750g weight of test medium should be placed into each glass container, but this study used 666.7g of test medium per container.

As there were no mortalities at the lowest test concentration, 100% mortality in the 500 mg a.s./kg soil dw group and the mean loss of biomass in controls did not exceed 20%, it is unlikely that this had a significant impact on the study. As the acute earthworm toxicity is study is not required as part of the current data requirements this study will not be used in the risk assessment and therefore the issue has not been considered further at this time.

The use of statistics are adequate for this study and meet OECD 207 (1984) guideline requirements. However, no 95% confidence limits have been provided for the LC_{50}. This introduces some uncertainty as to the reliability of this endpoint. It is unusual that the LOEC is greater than the LC_{50}, but the results are in line with the biological data. A dose-response curve has been provided, and the biological data fit the curve.

This study has been evaluated by HSE for completeness; however, acute earthworm toxicity data does not form part of the current data requirements for active substances (COMMISSION REGULATION (EU) No 283/2013).

Consequently, there are no agreed endpoints for use in risk assessment.

B.9.4.2 Earthworm – sub-lethal effects

B.9.4.2.1 Active substance: Inpyrfluxam

Reference:	KCA 8.4.1/01
Report Title:	S-2399 TG: Effects on Reproduction and Growth of Earthworms *Eisenia fetida* in Artificial Soil with 5% Peat
Author(s) & year:	[redacted] (2016a)
Document No, Authority registration No:	113911022, TPW-0052
Substance used:	S-2399 TG, 13CG0617G, 95.0%
Method of analysis:	n/a
Guideline(s):	OECD 222 (2004)
Deviations:	Yes, see HSE Comments
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

MATERIALS AND METHODS

MATERIALS

Test material	S-2399 TG
Description	White, solid
Batch number:	13CG0617G
Active substance content:	95.5% (verified by certificate of analysis)
Storage on receipt:	At 4 ± 4 °C, in the dark
Expiry date	June 29 2019

TREATMENTS

Nominal test doses:	6.276, 12.60, 25.10, 50.25 and 100.55 mg a.s./kg soil
Control:	Untreated control (the same amount of untreated quartz sand as in the test item treated groups was added and moistened with deionised water)
Toxic reference:	Carbendazim 600 g/L SC (separate study: 0.695, 1.00, 1.44, 2.08 and 3.00 mg a.s./kg soil dw treatment levels)

TEST ORGANISMS

Species:	*Eisenia fetida* (Savigny 1826), Adults
Age:	Approximately 8 months, with well-developed clitellum, age range between test individuals not differing by more than 4 weeks
Body weight:	307 mg to 586 mg
Source:	Bred under standardised conditions at ibacon laboratories in a breeding medium of cattle manure, peat, sand, calcium carbonate and straw, fed with cattle manure, stored at room temperature.
Acclimatisation period:	1 day, in artificial soil, under test conditions
Diet:	Finely ground cattle manure was used as food. 5 g/container was scattered on the soil surface at day 1 after application and was moistened with 5 g deionised water; 5 g/container (moistened with 2 - 3 g deionised water) was added each week for the first 4 weeks of the experiment, when the food of the previous week had almost been consumed. If the food was not quite fully consumed, the added amount of food was adjusted to replace the visually estimated consumption. Four weeks after application, the food was mixed into the substrate following removal of the adult worms. No further feeding took place during the remaining 4 weeks of the test.

TEST DESIGN

Test units:	Plastic boxes (18.3 cm x 13.6 cm x 6 cm, tapered towards the bottom, with a soil surface of approximately 16.5 cm x 11.5 cm = 189.75 cm²) with perforated transparent lids to enable exchange of air, to minimise evaporation from the artificial soil, and to prevent the worms from escaping. Each container was filled with 598.3 g of the prepared soil (500 g dry weight plus deionised water). The height of the soil layer in the containers was approximately 4 - 5 cm.
Replication:	8 per control, 4 per test item treated group, 10 individuals per replicate
Duration:	8 weeks (4 weeks exposure for adult worms, additional 4 weeks for offspring)

TEST CONDITIONS

Test temperature:	18 - 22 °C
Lighting:	16 h light: 8 h dark (400 lux to 800 lux)
pH:	Experimental start: 6.0 to 6.1 Experimental end: 5.9 to 6.3
Soil maximum water holding capacity:	38% of the dry weight of artificial soil
Water content:	Experimental start: 20.4 % to 20.7% (53.8% to 54.5% of the maximum water holding capacity). Experimental end: 23.1% to 26.4% (60.8% to 69.6% of the maximum water holding capacity)
Test substrate:	Based on OECD 222 but with reduced organic matter content: • 5% Sphagnum-peat, air-dried and finely ground (<2 mm, with no visible plant remains); (Floragard, Vertriebs GmbH für Gartenbau, 26138 Oldenburg, Germany) • 20% Kaolin clay (Erbslöh, 65558 Lohrheim, Germany) • 74.8% fine quartz-sand (F34) containing more than 50% by mass of particle size 0.05 mm to 0.2 mm; (Quarzwerke Frechen, Postfach 1780, 50207 Frechen, Germany) • 0.2% Calcium carbonate (CaCO3) was added to adjust pH to 6.0 ± 0.5 (Merck, 64293 Darmstadt, Germany). According to OECD 222 and EPPO (2003), 5 % of peat was used in the artificial soil to reduce the possibility of the test chemical adsorbing to the soil, thereby potentially increasing the availability of the test chemical to the worms. The artificial soil was moistened to approximately half of the final water content 1 day before the application. The additional water required to achieve the final water

content was added when applying the test item.

STUDY DESIGN AND METHODS
Experimental dates: 19 July 2016 to 14 September 2016

Dose preparation
S-2399 TG was weighed separately for each concentration using an analytical balance and 20 g fine quartz sand was added. After mixing with a spoon to reach a homogeneous distribution of the test item within the sand the mixture was added to artificial soil equivalent to 2080 g dry weight. While mixing the artificial soil in a laboratory mixer for approximately 5 min the soil of each treatment group was moistened with deionised water. Each group was treated in one batch (two in the control) which was then split into the replicates. Once a week the water content of the soil was checked by weighing each container and evaporated water was replenished. In this way, it was ensured that the difference in water content between experimental start and end was less than 10%.

Test organism and treatment
All worms were rinsed with tap water, dried with dry paper towels, weighed individually and randomly assigned to batches of 10 worms. The different batches were sorted into four classes on the basis of the total weight and one batch of each weight class was assigned to each treatment group (two batches for the control) to ensure weights were homogeneous. The earthworms were placed on the surface of the artificial soil after application.

Measurements and observations
After 4 weeks, the artificial soil was transferred to a tray and adult worms were counted, removed and weighed per replicate after being rinsed under tap water and dried on paper towels. Missing earthworms and earthworms that failed to respond to gentle stimulation were considered to be dead. Behavioural and morphological abnormalities were also quantified (e.g. lack of movement or rigidity). The remaining soil (without the adult worms) was then returned to the respective test containers.

After an additional 4 weeks, juveniles were removed by placing the test units in a water bath at 50 - 60 °C and counting all emerging worms. In addition, the soil of each container was emptied out onto a tray and checked visually for any remaining juvenile worms.

The cumulative amount of food added to each test container during the test period was quantified.

Statistical analysis
Mortality data were analysed for significance using Fisher’s Exact Binomial Test (multiple comparison, with Bonferroni correction, one-sided greater, $\alpha = 0.05$). Body weight change

was tested for normal distribution and homogeneity of variance using Shapiro-Wilk's test and Levene's test (α = 0.05), respectively. As the body weight data were normally distributed and homogeneous, the Williams t-test was used for comparison of the treated and control groups (multiple comparison, two-sided, α = 0.05). Reproduction data were tested for normal distribution and homogeneity of variance using Shapiro-Wilk's test and Bartlett's test (α = 0.05). As these data were normally distributed but not homogeneous, the Bonferroni-Welch t-test was used for comparison of the treated and control groups (multiple comparison, one-sided smaller, α = 0.05). The EC values and their 95% confidence limits were calculated using Probit-Analysis (Finney, 1971). The software ToxRat Professional, Version 2.10.05 and 3.2.1, ® ToxRat Solutions GmbH was used for all statistical analysis.

RESULTS AND DISCUSSION

Mortality

No statistically significant increase in mortality for the adult worms after four weeks of exposure was observed up to and including the highest test concentration of 100 mg a.s./kg soil dw. A summary of the results is presented in the

Table 9.4.2-1. The NOEC based on mortality was determined to be 100 mg a.s./kg soil dw and the LOEC was established as greater than 100 mg a.s./kg soil dw.

Body weight and behaviour

The body weight changes of the adult worms following four weeks of exposure were not statistically significantly different compared to the control for the test item treated groups up to and including the test concentration of 50 mg a.s./kg soil dw. However, for the highest test concentration of 100 mg a.s./kg soil dw, the body weight change was statistically significantly different compared to the control. A summary of the results is presented in

Table 9.4.2-1. The NOEC based on body weight changes was determined to be 50 mg a.s./kg soil dw and the LOEC was established as 100 mg a.s./kg soil dw.

The feeding activity and behaviour were similar between the earthworms in the treatment groups and the control group; the turnover of biomass of the earthworms exposed to the test item was comparable to the control, no behavioural abnormalities were observed in any test group and all worms burrowed into the soil within 15 minutes after introduction.

Reproduction

No statistically significant differences in reproduction rates were observed between the control group and the lowest test concentration of 6.25 mg a.s./kg soil dw. For all test concentrations higher than 6.25 mg a.s./kg soil dw the reproduction values were statistically significantly reduced. A summary of the results is presented in

Table 9.4.2-1. The NOEC based on reproduction was determined to be 6.25 mg a.s./kg soil dw and the LOEC was determined to be 12.5 mg a.s./kg soil dw. The EC_{10}, EC_{20} and EC_{50} values were calculated to be 21.5 mg a.s./kg soil dw (95% confidence intervals of 14.3 to 26.9 mg a.s./kg soil dw), 27.9 mg a.s./kg soil dw (95% confidence intervals of 20.7 to 33.2 mg a.s./kg soil dw) and 45.9 mg a.s./kg soil dw (95% confidence intervals of 39.7 to 53.1 mg a.s./kg soil dw), respectively.

Table 9.4.2-1: Mortality, body weight changes and reproductive success of *Eisenia fetida* following exposure to S-2399 TG

Nominal concentration of S-2399 TG (mg a.s./kg soil dw)	Mean mortality (% ± SD)	Mean body weight change per earthworm (% ± SD)	Reproduction	
			Mean number of juveniles/test unit (number ± SD)	% of control
Control	0.0 ± 0.0	63.3 ± 7.2	276 ± 37	n.a.
6.25	0.0 ± 0.0	58.6 ± 11.2	283 ± 20	102.6
12.5	0.0 ± 0.0	61.3 ± 8.2	224 ± 23 **	81.1
25	2.5 ± 5.0	64.9 ± 14.6	232 ± 19 **	84.0
50	0.0 ± 0.0	62.5 ± 9.9	138 ± 9 **	49.9
100	0.0 ± 0.0	48.1 ± 5.3 *	3 ± 2 **	0.9

SD = standard deviation
n.a. = not applicable
* Statistically significantly different to the control (Williams t-test, α = 0.05, two-sided)
** Statistically significantly different to the control (Bonferroni-Welch t-test, α = 0.05, one-sided)

The concentration-response curve for the number of juvenile worms found 8 weeks after treatment application is shown below.

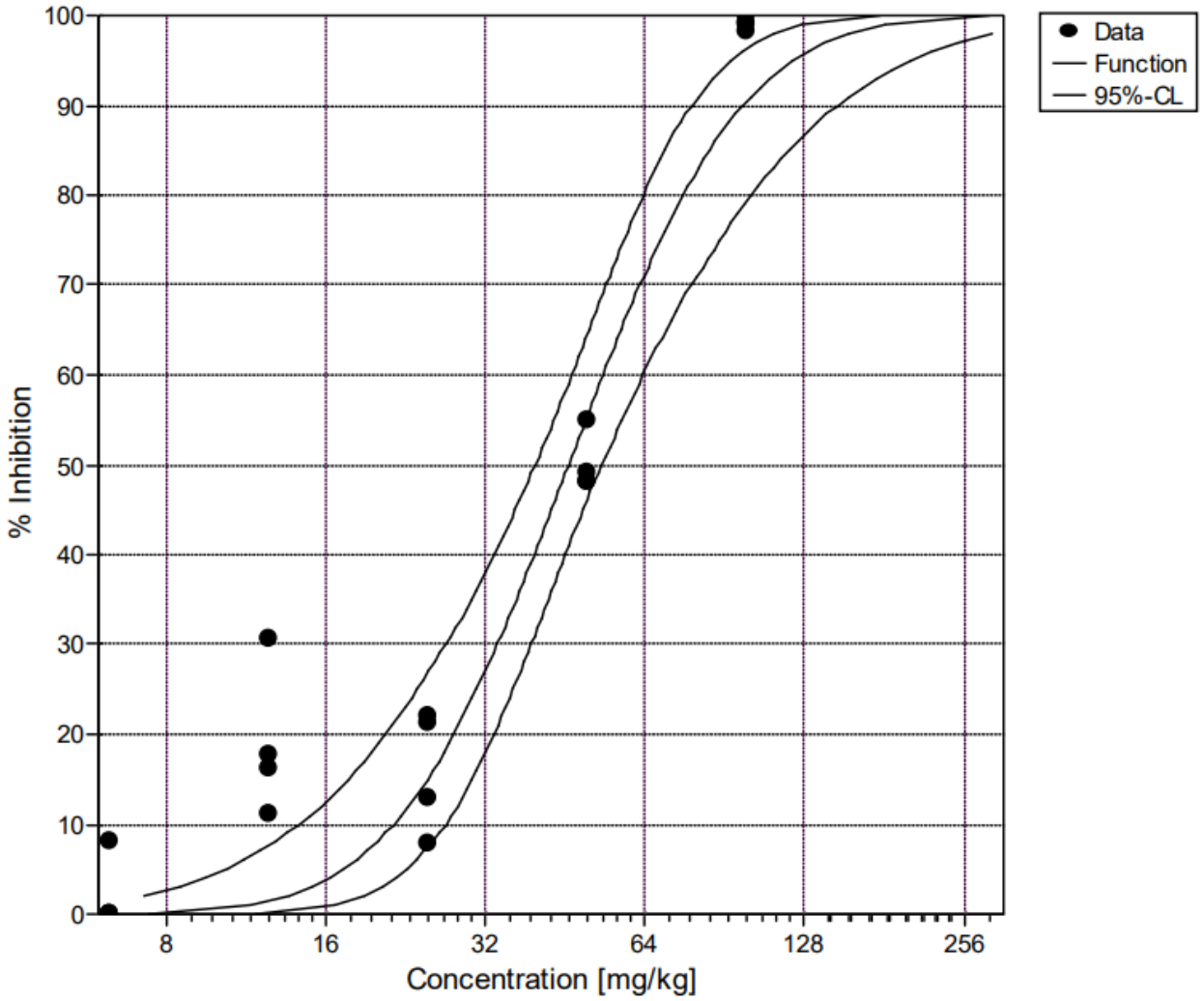


Figure 9.4-2: The concentration-response curve for reproduction after 56 days

For the reference material, carbendazim, there were statistically significant effects on reproduction at a concentration of 2.08 mg a.s./kg soil and higher. The EC_{50} for reproduction was calculated as 2.54 mg a.s./kg soil.

Validity criteria

The validity criteria for the study were met according to OECD 222 (2004), the guideline available on study initiation, and OECD 222 (2016), the most recent version of the guideline (Table 9.4.2-2).

Table 9.4.2-2: Compliance with OECD 222 validity criteria

Validity criterion	Required	Obtained
Control adult mortality	≤ 10 %	0 %
Control reproduction	≥ 30 juveniles	237 to 326 juveniles
Control reproduction Coefficient of Variation (CV)	≤ 30 %	13.4 %

CONCLUSIONS

In this 56-day study, the NOEC for *Eisenia fetida* exposed to S-2399 TG based on reproduction was determined to be 6.25 mg a.s./kg soil dw and the LOEC was established as 12.5 mg a.s./kg soil dw. The EC_{10}, EC_{20} and EC_{50} values were calculated to be 21.5 mg a.s./kg soil dw, 27.9 mg a.s./kg soil dw and 45.9 mg a.s./kg soil dw, respectively.

HSE COMMENTS

The study was carried out according to OECD 222 (2004). It was evaluated against OECD 222 (2004) and OECD 222 (2016), a newer version of the guideline that was adopted 10 days after the experimental phase of this study began. All validity criteria outlined in OECD 222 were satisfactorily met for the duration of the study. There were no significant deviations to the guidelines. Treatment with the toxic reference (carbendazim) yielded statistically significant effects on reproduction at a concentration of 2.08 mg a.s./kg soil and higher, which is in line with OECD 222 (2004) (effects should be observed between 1 and 5 mg a.s./kg soil). This demonstrated the appropriateness of the laboratory test conditions and sensitivity of the test individuals.

The following minor deviations were noted for OECD 222 (2004) and OECD 222 (2016):

OECD 222 (2004/2016) § 19 to 21 cover mixing the test substance into the soil. The study followed the procedure for test substances insoluble in water and organic solvents. S-2399 is soluble in acetone so it is uncertain why this approach was taken. Nevertheless, the approach selected was within the guidelines and resulted in an adequate preparation of treated soil. HSE considers this a minor and acceptable deviation.

Related to the point above, the study report method mentions acetone at one point. It states, "*the control was treated with the same amount of acetone and sand per g substrate as the test item groups*". Acetone is not mentioned anywhere else in the method and the study clearly follows the procedure for test substances insoluble in water and organic solvents.

For this reason, HSE considers this a minor, acceptable reporting omission.

OECD 222 (2004/2016) § 19 mentions that the final moisture content should be 40 to 60 % of the maximum water holding capacity. Although the study started within this range for the control and each treatment group, by the end of the study water content ranged from 60.8% to 69.6% of the maximum water holding capacity (control = 63 %). The suitable control mortality and reproduction, suggests that this minor deviation had minimal impact on the study outcome. HSE considers this a minor and acceptable deviation.

OECD 222 (2004/2016) § 31 and 32 lay out the food requirements for the study. Checks should be performed to ensure that manure used for food does not contain substances that could adversely affect worms during the test. To facilitate this, non-test worm cultures should be fed each fresh batch of food. The study did either not perform or report these checks. Control mortality and reproduction, however, adhered to the validity criteria and all food was consumed during the study, which suggests that the provided food was adequate. HSE considers this a minor and acceptable deviation.

The above study was conducted to GLP and considered valid.

The agreed endpoints suitable for use in the risk assessment are:
NOEC (reproduction) = 6.25 mg a.s./kg soil dw and EC_{10} (reproduction) = 21.5 mg a.s./kg soil dw.

B.9.4.2.2 Metabolite: 3'-OH-S-2840

Reference:	KCA 8.4.1/02
Report Title:	3'-OH-S-2840: Effects on Reproduction and Growth of Earthworms *Eisenia fetida* in Artificial Soil with 5% Peat
Author(s) & year:	[redacted] (2016a)
Document No, Authority registration No:	113861022, TPW-0050
Substance used:	3'-OH-S-2840, 15SC8508366, 99.5 %
Method of analysis:	n/a
Guideline(s):	OECD 222 (2004)
Deviations:	Yes, see HSE Comments
GLP or GEP:	Yes
Acceptability:	Yes

Study relied upon:	Yes

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test material:** 3'-OH-S-2840
   Description: White, solid
   Lot/Batch: 15SC8508366
   Purity: 99.5%
   Reference item: Carbendazim
   Solubility: Not stated
   Solvent: Not used. 20 g fine quartz sand to make up concentrations
   Expiry date: November 30, 2018

B. STUDY DESIGN AND METHODS

1. **Test animals:** Earthworm, *Eisenia fetida*
   Breeding information: Bred under standardised conditions in ibacon laboratories
   Age: Approximately 10 months old
   Source: In-house culture
   Breeding conditions: Bred under standardised conditions in ibacon laboratories in a breeding medium of cattle manure, peat, sand, calcium carbonate and straw, fed with cattle manure, stored at room temperature
   Weight: 300 to 596 mg
   Acclimation: 1 day, in artificial soil, under test conditions
   Diet: Cattle manure, scattered on the soils surface at day 1, and each week for the first 4 weeks. 5 g/container was scattered on the soil surface at day 1 after application and was moistened with 5 g deionised water; 5 g/container (moistened with 2 - 3 g deionised water). Four weeks after application, the food was mixed into the substrate following removal of the adult worms.

2. **Artificial soil:** 5% sphagnum peat, 20% kaolin clay, 74.8% quartz sand and 0.2% calcium carbonate. Maximum water holding capacity (MWHC) of the soil of 38%

3. **Test units:** Plastic boxes (18.3 x 13.6 x 6 cm) with perforated transparent lids with 599.2 g prepared soil
   Concentrations tested: Control, 6.25, 12.5, 25, 50 and 100 mg/kg soil dw
   Number of replicates: 4 (in test concentrations, 8 (in control group)
   Number of earthworms: 10 (in test concentrations), 10 (in control group)
   Maximum water holding capacity: 38% of the dry weight of artificial soil
   Test duration: 8 weeks

4. **Environmental conditions:**

A summary of the environmental conditions are shown in Table 9.4.2-3 below:

Table 9.4.2-3: Environmental conditions in study of *E. fetida* exposed to 3'-OH-S-2840

Variable	Required OECD 222 (2004)	Obtained
Temperature	20 ± 2 °C	18 ˚C - 22°C
pH	6.0 ± 0.5	Start: 6.1 End: 6.1-6.3
Water content of soil	Should not vary by more than 10 % from that at the start of the test	20.4 – 21.0% of the dry weight at test start (53.8% to 55.2% of WHC) 19.9 – 24.3% of the dry weight at test end (52.4% to 63.9% of WHC)
Photoperiod	16 hours light and 8 hours dark	16 hours light, 8hours dark
Light intensity	400 to 800 lux	400 to 800 lux

Study dates:
Experimental Starting Date: July 26, 2016
Experimental Completion Date: September 21, 2016

5. Animal assignment and treatment:
Groups of ten earthworms were individually weighed and then placed onto the soil surface of the respective test container. The test comprised five test substance treatments (6.25, 12.5, 25, 50 and 100 mg/kg soil dw). Each treatment consisted of four replicates with ten worms each (40 worms). A control was also included in the test, which consisted of 8 replicates with ten worms each (80 worms).
After 28 days, adult worms were removed from the soil and counted. The remaining soil (without adult worms) was then returned to the respective test containers, for an additional 28 days. At the end of the test, juvenile worms were removed from the soil and counted.

A reference test was conducted in a separate study in June 2016 with carbendazim 600 g/L SC at concentrations of 0.695, 1.00, 1.44, 2.08 and 3.00 mg a.s./kg soil dw.

6. Dose preparation:
Defined amounts of 3'-OH-S-2840 were weighed and then mixed with 20 g fine quartz sand, to achieve a homogeneous distribution. This mixture was then added to artificial soil to obtain a final net weight of 2080 g dry weight and mixed with a laboratory mixer to ensure a homogeneous distribution, with addition of deionised water. Each group was treated in one batch and then split into replicates. An untreated control was also prepared, containing the same amount of untreated quartz sand as in the test item treated groups.

7. Measurements and observations:

After four weeks, adult earthworms were counted and weighed per replicate. Missing worms and worms that failed to respond to gentle stimulation were considered to be dead. The surviving worms were checked for behavioural and morphological abnormalities. After an additional 28 days, juveniles were removed by placing the test units in a water bath (50 - 60ºC) and counting all emerging worms. In addition, the test units were emptied and the soil was visually checked for any remaining worms.

Water content and pH of the soil were measured at the start and end of the test.

8. Statistics:

Mortality data were analysed for significance using Fisher's Exact Binomial Test (multiple comparison, with Bonferroni correction, one-sided greater, α = 0.05). Body weight change and reproduction were tested for normal distribution and homogeneity of variance using Shapiro-Wilk's test and Levene's test (α = 0.05), respectively. As the data were normally distributed and homogeneous, the Williams t-test was used for comparison of the treated and control groups (multiple comparison, two-sided for weight and one-sided for reproduction, α = 0.05). The software ToxRat Professional, Version 2.10.05, ® ToxRat Solutions GmbH was used for all statistical analysis.

II. RESULTS AND DISCUSSION

The reference substance carbendazim was tested in a separate toxicity study. Significant effects were observed for a concentration of 2.08 mg a.s./kg soil dw and higher, and the NOEC was determined to be 1.44 mg a.s./kg soil dw, based on reproduction.

A. MORTALITY

No statistically significant increase in mortality for the adult worms after four weeks of exposure was observed up to and including the highest test concentration of 100 mg/kg soil dw. A summary of the results is presented in the table below. The NOEC based on mortality was determined to be 100 mg/kg soil dw, and the LOEC and LC_{50} values were established as greater than 100 mg/kg soil dw.

B. BODY WEIGHT AND BEHAVIOUR

The body weight changes of the adult worms following four weeks of exposure were not statistically significantly different compared to the control for the test item treated groups up to and including the highest test concentration of 100 mg/kg soil dw. A summary of the results is presented in the table below. The NOEC based on body weight changes was determined to be 100 mg/kg soil dw and the LOEC was established as greater than 100 mg/kg soil dw.

The feeding activity and behaviour for the earthworms in the treatment groups were similar to the control group; the turnover of biomass of the earthworms exposed to the test item was comparable to the control and no behavioural abnormalities were observed in any test group and all worms burrowed into the soil within 15 minutes after introduction.

C. REPRODUCTION

No statistically significant differences in reproduction rates were observed between the control group and any of the test item treated groups up to and including the highest test

concentration of 100 mg/kg soil dw. A summary of the results is presented in the table below. The NOEC based on reproduction was determined to be 100 mg/kg soil dw and the LOEC, EC_{10}, EC_{20} and EC_{50} values were estimated to be greater than 100 mg/kg soil dw.

Table 9.4.2-4: Mortality, body weight changes and reproductive success of Eisenia fetida following exposure to 3'-OH-S-2840

Nominal concentration of 3'-OH-S-2840 (mg/kg soil dw)	Mean mortality (% ± SD)	Mean body weight change per earthworm (% ± SD)	Reproduction	
			Mean number of juveniles/test unit (number ± SD)	% of control
Control	0.0 ± 0.0	39.4 ± 9.8	284 ± 35	n.a.
6.25	0.0 ± 0.0	41.3 ± 10.0	267 ± 47	93.8
12.5	0.0 ± 0.0	35.8 ± 4.0	295 ± 33	103.8
25	0.0 ± 0.0	41.3 ± 10.1	292 ± 36	102.6
50	2.5 ± 5.0	38.3 ± 4.4	280 ± 30	98.5
100	0.0 ± 0.0	41.6 ± 10.7	278 ± 35	97.7

SD = standard deviation
n.a. = not applicable

D.VALIDITY CRITERIA
As mortality in the control treatment did not exceed 10% (actual: 0%), the number of juveniles per replicate was ≥ 30 (actual: 256 – 338) and the coefficient of variation of reproduction in the control did not exceed 30% (actual: 12.3%), the validity criteria were met and the study was considered valid.

III. CONCLUSION

In this 56-day study, the NOEC for *Eisenia fetida* exposed to 3'-OH-S-2840 was determined to be 100 mg/kg soil dw, the highest concentration tested. The LOEC, EC_{10}, EC_{20} and EC_{50} values were estimated to be greater than 100 mg/kg soil dw.

HSE COMMENTS:
This study was conducted under GLP and OECD 222 (2004) guidelines. The study has been assessed against OECD 222 (2016) guidelines.

There is a notable deviation relating to the number of test concentrations used for this study. OECD 222 (2016) guidelines state that 8 concentrations should be tested with a geometric factor of ≤ 1.8 should be used to calculate the EC_{10} and NOEC values. This study used only 5 test concentrations with a spacing factor of 2. Ultimately, there were <10% effects at the highest concentration tested for both mortality and reproduction, so does not invalidate the study.

There are some slight deviations to OECD 222 (2016) guidelines notes in relation to the dose preparation of concentrations. Sand quartz was used to make up the required concentrations in this study. OECD 222 (2016) guidelines state that 10g quartz should be added to the test item, but this study used 20g quartz. There is no data available regarding the mean measured concentration, so it is unclear if this had any impact on test concentrations. OECD 222 (2016) guidelines recommend chemical analysis of the test substance at the start and the end of the test as the test where there is uncertainty in maintaining the nominal concentration. This is not referenced in the study report, so it cannot be determined whether the test concentrations were maintained over the duration of the study. However, as all validity criteria were met, the study is considered valid.

It is also noted that there is no reference to a range finding test in the full study report. As this study only used test concentrations up to 100mg/kg soil dw (considerably below the limit of 1000mg/kg soil dw) and no mortality or sub-lethal effects were observed, it could be considered whether the concentrations tested were suitable to produce any effects. As the reference item, tested in a different study, demonstrated significant effects from 2.08 mg a.s./kg soil and recorded a NOEC of 1.44mg a.s./kg soil dw, it remains indicative that 3'-OH-S-2840 has low toxicity to earthworms as their sensitivity has been established.

The results of the reference item test demonstrated that the test system was sufficiently sensitive, as significant effects were be observed between 1 and 5 mg a.s./kg soil dw (OECD 222) and the NOEC was determined to be 1.44 mg a.s./kg soil dw, based on reproduction.

The use of statistics are adequate for this study and meet OECD 222 (2016) guideline requirements. However, in this case, the EC_{10} values were not able to be statistically determined as there were <10% effects at the highest concentration.

The agreed endpoints for use in risk assessment are:

- **56-day NOEC = 100 mg/kg soil dw (based on nominal test concentrations)**
- **EC_{10}= >100mg/kg soil dw (based on nominal test concentrations)**

B.9.4.2.3 Metabolite 1'-COOH-S-2840

Reference:	KCA 8.4.1/03
Report Title:	1'-COOH-S-2840: Effects on Reproduction and Growth of Earthworms *Eisenia fetida* in Artificial Soil with 5% Peat
Author(s) & year:	[redacted] (2016b)
Document No, Authority registration No:	113871022, TPW-0058
Substance used:	1'-COOH-S-2840, 16SC8508359, 100 %
Method of analysis:	n/a

Guideline(s):	OECD 222 (2004)
Deviations:	Yes, see HSE Comments
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

MATERIALS AND METHODS

MATERIALS

Test material 1'-COOH-S-2840

- **Description** White, solid
- **Batch number:** 16SC8508359
- **Active substance content:** 100 % (verified by certificate of analysis, March 31, 2016)
- **Storage on receipt:** At 4 ± 4 °C, in the dark
- **Expiry date** January 31, 2019

TREATMENTS

- **Nominal test doses:** 6.238, 12.52, 25.0, 50.0 and 100.0 mg /kg soil
- **Control:** Untreated control (the same amount of untreated quartz sand as in the test item treated groups was added and moistened with deionised water)
- **Toxic reference:** Carbendazim 600 g/L SC (separate study: 0.695, 1.00, 1.44, 2.08 and 3.00 mg a.s./kg soil dw treatment levels)

TEST ORGANISMS

- **Species:** *Eisenia fetida* (Savigny 1826), Adults
- **Age:** Approximately 10 months, with well-developed clitellum, age range between test individuals not differing by more than 4 weeks
- **Body weight:** 302 mg to 598 mg
- **Source:** Bred under standardised conditions at ibacon laboratories in a breeding medium of cattle manure, peat, sand, calcium carbonate and straw, fed with cattle manure, stored at room temperature.
- **Acclimatisation period:** 1 day, in artificial soil, under test conditions
- **Diet:** Finely ground cattle manure was used as food. 5 g/container was scattered on the soil surface at day 1 after application and was moistened with 5 g deionised water; 5 g/container (moistened with 2 - 3 g deionised

water) was added each week for the first 4 weeks of the experiment, when the food of the previous week had almost been consumed. If the food was not quite fully consumed, the added amount of food was adjusted to replace the visually estimated consumption. Four weeks after application, the food was mixed into the substrate following removal of the adult worms. No further feeding took place during the remaining 4 weeks of the test.

TEST DESIGN

Test units: Plastic boxes (18.3 cm x 13.6 cm x 6 cm, tapered towards the bottom, with a soil surface of approximately 16.5 cm x 11.5 cm = 189.75 cm²) with perforated transparent lids to enable exchange of air, to minimise evaporation from the artificial soil, and to prevent the worms from escaping. Each container was filled with 598.9 g of the prepared soil (500 g dry weight plus deionised water). The height of the soil layer in the containers was approximately 4 - 5 cm.

Replication: 8 per control, 4 per test item treated group, 10 individuals per replicate

Duration: 8 weeks (4 weeks exposure for adult worms, additional 4 weeks for offspring)

TEST CONDITIONS

Test temperature: 18 - 22 °C

Lighting: 16 h light: 8 h dark (400 lux to 800 lux)

pH: Experimental start: 5.9 to 6.0
Experimental end: 6.1 to 6.3

Soil maximum water holding capacity: 38% of the dry weight of artificial soil

Water content: Experimental start: 20.6 to 21.0 % (54.2 % to 55.3 % of the maximum water holding capacity).
Experimental end: 20.2 to 25.5 % (53.1 % to 67.0 % of the maximum water holding capacity)

Test substrate: Based on OECD 222 but with reduced organic matter content:
• 5% Sphagnum-peat, air-dried and finely ground (<2 mm, with no visible plant remains); (Floragard, Vertriebs GmbH für Gartenbau, 26138 Oldenburg, Germany)
• 20% Kaolin clay (Erbslöh, 65558 Lohrheim, Germany)
• 74.8% fine quartz-sand (F34) containing more than 50% by mass of particle size 0.05 mm to 0.2 mm; (Quarzwerke Frechen, Postfach 1780, 50207 Frechen, Germany)
• 0.2% Calcium carbonate (CaCO3) was added to adjust

pH to 6.0 ± 0.5 (Merck, 64293 Darmstadt, Germany).

According to OECD 222 and EPPO (2003), 5 % of peat was used in the artificial soil to reduce the possibility of the test chemical adsorbing to the soil, thereby potentially increasing the availability of the test chemical to the worms. The artificial soil was moistened to approximately half of the final water content 1 day before the application. The additional water required to achieve the final water content was added when applying the test item.

STUDY DESIGN AND METHODS
Experimental dates: August 11, 2016 to October 07, 2016
Study initiation date: July 14, 2016

Dose preparation
1'-COOH-S-2840 was weighed separately for each concentration using an analytical balance and 20 g fine quartz sand was added. After mixing with a spoon to reach a homogeneous distribution of the test item within the sand the mixture was added to artificial soil equivalent to 2080 g dry weight. While mixing the artificial soil in a laboratory mixer for approximately 5 min the soil of each treatment group was moistened with deionised water. Each group was treated in one batch (two in the control) which was then split into the replicates.

Test organism and treatment
All worms were rinsed with tap water, dried with dry paper towels, weighed individually and randomly assigned to batches of 10 worms. The different batches were sorted into four classes on the basis of the total weight and one batch of each weight class was assigned to each treatment group (two batches for the control) to ensure weights were homogeneous. The earthworms were placed on the surface of the artificial soil after application.

Measurements and observations
After 4 weeks, the artificial soil was transferred to a tray and adult worms were counted, removed and weighed per replicate after being rinsed under tap water and dried on paper towels. Missing earthworms and earthworms that failed to respond to gentle stimulation were considered to be dead. Behavioural and morphological abnormalities were also quantified (e.g. lack of movement or rigidity). The remaining soil (without the adult worms) was then returned to the respective test containers.

After an additional 4 weeks, juveniles were removed by placing the test units in a water bath at 50 - 60 °C and counting all emerging worms. In addition the soil of each container was emptied out onto a tray and checked visually for any remaining juvenile worms.

The cumulative amount of food added to each test container during the test period was quantified.

Statistical analysis

Body weight change was tested for normal distribution and homogeneity of variance using Kolmogoroff-Smirnov test and Levene's test (α = 0.05), respectively. Reproduction data were tested for normal distribution and homogeneity of variance using Shapiro-Wilk's test and Levene's test (α = 0.05). As the data were normally distributed and homogeneous, the Williams t-test was used for comparison of the treated and control groups (multiple comparison, two-sided for body weight and one-sided smaller for reproduction, α = 0.05). The EC values and their 95% confidence limits were calculated using Logit Analysis. The software ToxRat Professional, Version 2.10.05, ® ToxRat Solutions GmbH was used for all statistical analysis.

RESULTS AND DISCUSSION

Mortality

No statistically significant increase in mortality for the adult worms after four weeks of exposure was observed up to and including the highest test concentration of 100 mg /kg soil dw. A summary of the results is presented in the Table 9.4.2-5. The NOEC based on mortality was determined to be 100 mg/kg soil dw and the LOEC was established as greater than 100 mg/kg soil dw.

Body weight and behaviour

The body weight changes of the adult worms following four weeks of exposure were not statistically significantly different compared to the control for the test item treated groups up to and including the highest test concentration of 100 mg/kg soil dw. A summary of the results is presented in Table 9.4.2-5. The NOEC based on body weight was determined to be 100 mg/kg soil dw and the LOEC was established as greater than 100 mg/kg soil dw.

The feeding activity (Table 9.4.2-5) and behaviour were similar between the earthworms in the treatment groups and the control group; the turnover of biomass of the earthworms exposed to the test item was comparable to the control, no behavioural abnormalities were observed in any test group and all worms burrowed into the soil within 15 minutes after introduction.

Reproduction

No statistically significant differences in reproduction rates were observed between the control group and the test concentration of 50 mg/kg soil dw and below. At the test concentrations of 100 mg/kg soil dw the reproductive success was statistically significantly reduced. A summary of the results is presented in Table 9.4.2-5. The NOEC based on reproduction was determined to be 50 mg/kg soil dw and the LOEC was determined to be

100 mg/kg soil dw. The EC_{10} and EC_{20} values were calculated to be 52.4 mg/kg soil dw (95% confidence intervals of 6.8 to 72.2 mg/kg soil dw) and 90.3 mg/kg soil dw (95% confidence intervals of 61.2 to 178 mg/kg soil dw), respectively.

Table 9.4.2-5: Mortality, body weight changes and reproductive success of *Eisenia fetida* following exposure to 1'-COOH-S-2840

Nominal concentration of 1'-COOH-S-2840 (mg/kg soil dw)	Mean mortality (% ± SD)	Mean body weight change per earthworm (% ± SD)	Reproduction		Food consumption (g ± SD)
			Mean number of juveniles/test unit (number ± SD)	% of control	
Control	0.0 ± 0.0	32.3 ± 3.4	217 ± 31	n.a.	22.0 ± 0.5
6.25	0.0 ± 0.0	36.7 ± 5.9	196 ± 13	90.2	22.5 ± 0.6
12.5	0.0 ± 0.0	37.3 ± 3.5	200 ± 10	92.1	22.5 ± 0.6
25	0.0 ± 0.0	39.2 ± 2.6	219 ± 27	100.9	22.0 ± 0.0
50	0.0 ± 0.0	38.6 ± 2.9	215 ± 23	99.1	23.0 ± 0.0
100	0.0 ± 0.0	38.4 ± 10.2	164 ± 18 *	75.7	20.0 ± 0.0

SD = standard deviation
n.a. = not applicable
* Statistically significantly different to the control (Williams t-test, α = 0.05, one-sided)

The concentration-response curve for the number of juvenile worms found 8 weeks after treatment application is shown below.

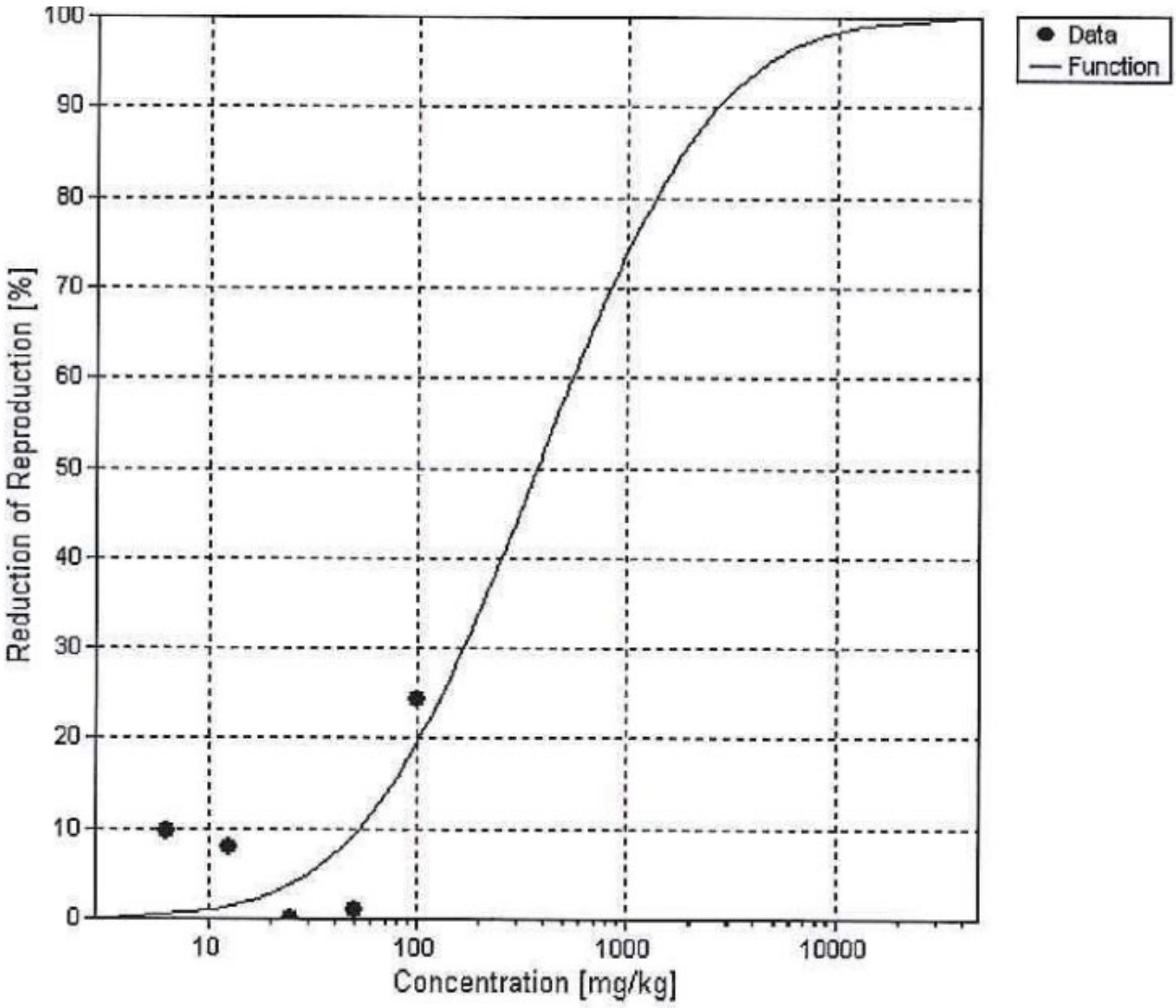


Figure 9.4-3: The concentration-response curve for reproduction after 56 days

For the reference material, carbendazim, there were statistically significant effects on reproduction at a concentration of 2.08 mg a.s./kg soil and higher. The EC_{50} for reproduction was calculated as 2.54 mg a.s./kg soil.

Validity criteria

The validity criteria for the study were met according to OECD 222 (2004), the guideline available on study initiation, and OECD 222 (2016), the most recent version of the guideline (Table 9.4.2-6).

Table 9.4.2-6: Compliance with OECD 222 validity criteria

Validity criterion	Required	Obtained
Control adult mortality	≤ 10 %	0 %
Control reproduction	≥ 30 juveniles	181 to 263 juveniles
Control reproduction Coefficient of Variation (CV)	≤ 30 %	14.3 %

CONCLUSIONS

In this 56-day study, the NOEC for *Eisenia fetida* exposed to 1'-COOH-S-2840 was determined to be 50 mg/kg soil dw. The LOEC was established as 100 mg/kg soil dw, and the EC_{10} and EC_{20} values were calculated to be 52.4 mg/kg soil dw and 90.3 mg/kg soil dw, respectively.

HSE COMMENTS

The study was carried out according to OECD 222 (2004). It was evaluated against OECD 222 (2004) and OECD 222 (2016), a newer version of the guideline that was adopted 15 days after the study was initiated. All validity criteria outlined in OECD 222 were satisfactorily met for the duration of the study. There were no significant deviations to the guidelines. Treatment with the toxic reference (carbendazim) yielded statistically significant effects on reproduction at a concentration of 2.08 mg a.s./kg soil and higher, which is in line with OECD 222 (2004) (effects should be observed between 1 and 5 mg a.s./kg soil). This demonstrated the appropriateness of the laboratory test conditions and sensitivity of the test individuals.

The following minor deviations were noted for OECD 222 (2004) and OECD 222 (2016):

OECD 222 (2004/2016) § 19 to 21 cover mixing the test substance into the soil. The study followed the procedure for test substances insoluble in water and organic solvents. 1'-COOH-S-2840 is soluble in acetone so it is uncertain why this approach was taken. Nevertheless, the approach selected was within the guidelines and resulted in an adequate preparation of treated soil. HSE considers this a minor and acceptable deviation.

OECD 222 (2004/2016) § 19 mentions that the final moisture content should be 40 to 60 % of the maximum water holding capacity (WHC). Although the study started within this range for the control and each treatment group, by the end of the study water content ranged from 53.1% to 67.0% of the maximum water holding capacity (control = 53.1 %). The 12.5 to 50 mg/kg soil dw treatment groups all had > 60 % maximum WHC and comparable mortality

and reproduction to the control. This suggests that having a water content > 60 % maximum WHC (up to 67.0 %) did not negatively affect individuals. Therefore, HSE considers this a minor and acceptable deviation.

OECD 222 (2004/2016) § 31 and 32 lay out the food requirements for the study. Checks should be performed to ensure that manure used for food does not contain substances that could adversely affect worms during the test. To facilitate this, non-test worm cultures should be fed each fresh batch of food. The study did either not perform or report these checks. Control mortality and reproduction, however, adhered to the validity criteria and all food was consumed during the study, which suggests that the provided food was adequate. HSE considers this a minor and acceptable deviation.

The above study was conducted to GLP and considered valid.

The agreed endpoints suitable for use in the risk assessment are:
NOEC (reproduction) = 50 mg/kg soil dw and EC_{10} (reproduction) = 52.4 mg/kg soil dw.

B.9.4.3 Effects on non-target soil meso- and macrofauna (other than earthworms)

B.9.4.3.1 Active substance: Inpyrfluxam (*Folsomia candida*)

Reference:	KCA 8.4.2/01
Report Title:	S-2399 TG: Effects on Reproduction of the Collembola *Folsomia candida* in Artificial Soil with 5% Peat
Author(s) & year:	[redacted] (2016b)
Document No, Authority registration No:	113911016, TPW-0044
Substance used:	S-2399 TG, 13CG0617G, 95 %
Method of analysis:	n/a
Guideline(s):	OECD 232 (2009)
Deviations:	Yes, see HSE Comments
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

MATERIALS AND METHODS

MATERIALS

Test material	S-2399 TG
Description	White, solid
Batch number:	13CG0617G
Active substance content:	95.5% (verified by certificate of analysis)
Storage on receipt:	At 4 ± 4 °C, in the dark
Expiry date	June 29 2019

TREATMENTS

Nominal test doses:	6.21, 12.73, 25.1, 50.3 and 100.6 mg a.s./kg soil
Control:	Untreated control (the same amount of untreated quartz sand as in the test item treated groups was added and moistened with deionised water)
Toxic reference:	Boric acid – 100.3 % (separate study: 30.5, 48.8, 78.1, 125 and 200 mg a.s./kg soil dw treatment levels)

TEST ORGANISMS

Species:	*Folsomia candida* (Willem 1902), Juveniles at experimental start
Age:	9 – 12 days
Source:	The synchronised individuals were bred at ibacon, fed granulated dry yeast and kept under breeding conditions until test start
Feeding:	After the introduction of the test organisms (day 0), and after 14 days, approximately 2 mg (half of a small spatula) of granulated dried yeast was spread over the soil surface.

TEST DESIGN

Test units:	Glass containers (volume: 100 ml; diameter: 5 cm), closed tightly to avoid water evaporation, filled with 30 g ± 1.0 g artificial soil fresh weight
Replication:	8 for the control, 4 for each test item group, 1 additional container per treatment to check the pH and water content of the test substrate after 28 days, 10 individuals per replicate
Exposure time:	28 days
Ventilation:	All vessels including the additional containers were ventilated on day 2, 5, 7, 9, 12, 14, 16, 19, 21, 23 and 26 by opening the lids for a short period.

TEST CONDITIONS

Test temperature:	18 - 22 °C
Lighting:	16 h light: 8 h dark (400 lux to 800 lux)
pH:	Experimental start: 6.0 to 6.2 Experimental end: 6.1
Soil maximum water holding capacity:	39% of the dry weight of artificial soil
Water content:	Experimental start: 20.1 % to 20.5 % (51.5 % to 52.5 % of the maximum water holding capacity). Experimental end: 19.3 % to 20.4 % (49.4 % to 52.4 % of the maximum water holding capacity)
Test substrate:	According to OECD 232: • 5% Sphagnum-peat, air-dried and finely ground (<2 mm, with no visible plant remains); (Floragard, Vertriebs GmbH fur Gartenbau, 26138 Oldenburg, Germany) • 20% Kaolin clay (Erbsloh, 65558 Lohrheim, Germany) • 74.8% fine quartz-sand (F34) containing more than 50% by mass of particle size 0.05 mm to 0.2 mm; (Quarzwerke Frechen, Postfach 1780, 50207 Frechen, Germany) • 0.2% calcium carbonate (CaCO3) was added to adjust pH to 6.0 ± 0.5 (Merck, 64293 Darmstadt, Germany). The artificial soil was moistened to approximately half of the final water content 2 days before the application. The additional water required to achieve the final water content was added when applying the test item

STUDY DESIGN AND METHODS

Experimental dates: 13 July 2016 to 11 August 2016

Dose preparation

Defined amounts of S-2399 TG were weighed and 75 g fine quartz sand was added and mixed to achieve a homogeneous distribution. This mixture was then added to artificial soil to obtain a final net weight of 300 g dry weight (600 g test substrate and 150 g sand for the 6.25 mg/kg soil group) and mixed with a laboratory mixer to ensure a homogeneous distribution, with addition of deionised water. Each group was treated in one batch and then split into replicates.

Test organism and treatment

The collembolans were collected with an aspirator, put into a small glass tube for counting, and 10 were placed onto the soil surface of the treated artificial soil of each replicate.

Measurements and observations

After 28 days, the contents of the test containers were suspended in water and the

suspensions were tinted with dark ink and stirred with a fine brush. The Collembola drifted to the surface. Adult animals were counted once visually; juvenile animals were counted at least twice under binocular microscopes. Two of the replicates were counted three times because the first two counts deviated more than 10% from their mean value.

The numbers of living adult Collembola at day 28 after application were recorded. Missing adult Collembola were recorded as dead as it is assumed that missing adult Collembola had died and degraded during the test period. Surviving Collembola were observed for any abnormal behaviour or conditions at day 28 after application. The number of juveniles was also quantified 28 days after application.

Water content was checked on day 14 after the application by reweighing the additional test containers. It was not necessary to compensate for loss of water as deviation did not exceed 2% of the initial water content.

Statistical analysis

Mortality data were statistically analysed using Fisher's Exact Binomial Test (multiple comparison, with Bonferroni Correction, α=0.05, one-side greater). Reproduction data were tested for normal distribution and Levene's test using Shapiro-Wilk's test and Levene's test (α=0.05). Further statistical evaluation was performed using William's t-test (multiple comparison, α=0.05, one-sided smaller). The determination of the NOEC and LOEC values was based on the results of the statistical evaluation.

The software ToxRat Professional, Version 2.10.05, ® ToxRat Solutions GmbH was used for all statistical analysis.

RESULTS AND DISCUSSION

Mortality

Mortality of the collembolans exposed to concentrations up to and including the highest test item concentration of 100 mg a.s./kg soil dw was not statistically significant different from the control. The results on mean mortality are shown in Table 9.4.3-1. The NOEC based on mortality was determined to be 100 mg a.s./kg soil dw. The LOEC and LC_{50} values were established as greater than 100 mg a.s./kg soil dw.

There were no behavioural abnormalities observed for the living collembolans in the test item treated groups and the control.

Reproduction

There were no statistically significant effects on reproduction up to and including the highest test item concentration of 100 mg a.s./kg soil dw, except for the lowest concentration of 6.25 mg a.s./kg soil dw. Since at all higher concentrations the reproduction values were not statistically significantly reduced, the reduction observed at 6.25 mg a.s./kg soil dw was not

considered to be test item related. The results for reproductive success are shown in Table 9.4.3-1.

The NOEC based on reproduction was determined to be 100 mg a.s./kg soil dw and the LOEC was established as greater than 100 mg a.s./kg soil dw. The EC_{10}, EC_{20} and EC_{50} values were also determined to be greater than 100 mg a.s./kg soil dw since no effects above 90% (% of control) were observed.

Table 9.4.3-1: Mortality and reproduction of *Folsomia candida* after 28 days of exposure to S-2399 TG

Nominal concentration of S-2399 TG (mg a.s./kg soil dw)	Mean mortality (% ± SD)	Reproduction	
		Mean number of juveniles/replicate (number ± SD)	% of control
Control	16 ± 15	654 ± 67	n.a.
6.25	20 ± 0	565 ± 52	86 *
12.5	5 ± 6	635 ± 65	97
25	10 ± 8	624 ± 60	96
50	15 ± 6	628 ± 19	96
100	10 ± 8	663 ± 54	101

SD = standard deviation
n.a. = not applicable
* Statistically significantly different compared to the control (Williams t-test (α=0.05), but not considered to be treatment related

For the reference material, boric acid, there were statistically significant effects on reproduction at a concentration of 78.1 mg a.s./kg soil and higher with a clear concentration-response relationship. The EC_{50} for reproduction was calculated as 94.0 mg a.s./kg soil.

Validity criteria
The validity criteria for the study were met according to OECD 232 (2009), the guideline available on study initiation, and OECD 232 (2016), the most recent version of the guideline (Table 9.4.3-2).

Table 9.4.3-2: Compliance with OECD 232 validity criteria

Validity criterion	Required	Obtained
Control adult mortality	≤ 20 %	16 %
Control reproduction	≥ 100 juveniles	566 to 790 juveniles
Control reproduction Coefficient of Variation (CV)	≤ 30 %	10.2 %

CONCLUSIONS

In this 28-day study, the NOEC for *Folsomia candida* exposed to S-2399 TG was determined to be 100 mg a.s./kg soil dw, the highest concentration tested. The LOEC, EC_{10}, EC_{20} and EC_{50} values were estimated to be greater than 100 mg a.s./kg soil dw.

HSE COMMENTS

The study was carried out according to OECD 232 (2009). It was evaluated against OECD 232 (2009) and OECD 232 (2016), a newer version of the guideline that was adopted 16 days after the experimental phase of this study began. All validity criteria outlined in OECD 232 were satisfactorily met for the duration of the study. There were no significant deviations to the guidelines. Treatment with the toxic reference (boric acid) yielded an EC_{50} (reproduction) of 94.0 mg a.s./kg soil, which is in line with OECD 232 (2009) (should reduce reproduction by 50 % at about 100 mg/kg dry weight soil). This demonstrated the appropriateness of the laboratory test conditions and sensitivity of the test individuals.

The following minor deviations were noted for OECD 232 (2009) and OECD 232 (2016):

OECD 232 (2009) and OECD 232 (2016) § 17 covers the selection and preparation of test animals. It states, "*the synchronous animals are selected randomly from the dishes and their health and physical condition is checked for each batch added to a replicate*". Random selection of individuals and health checks were either not reported in the study report or not performed. Given the validity criteria of the study were met, this suggests that there were no undetected health issues in the test population. HSE considers these minor deviations and acceptable.

OECD 232 (2009) and OECD 232 (2016) § 18-22 cover the various test item soil application methods. The study selected the soil application method suited for test items with poor water and organic solvent solubility. S-2399 is soluble in acetone. No justification for why this approach was selected was provided. The method is, however, valid and ensured a

homogeneous distribution of S-2399 in the soil. Therefore, HSE considers this an acceptable approach.

OECD 232 (2009) and OECD 232 (2016) § 31 and 46 details how the extraction and counting method for juveniles should be validated. The validity includes extraction efficiency of juveniles greater than 95%, e.g. by adding a known number to soil. This was not detailed within the study report. HSE notes, however, that measures were taken to maintain the integrity of the reproduction endpoint: when two juvenile counts diverged by > 10 % of their mean value, a third count was performed and the median of the three reported. HSE will consider this uncertainty during the risk assessment stage.

OECD 232 (2009) and OECD 232 (2016) § 46 describes test report requirements. It states that a description of breeding conditions should be provided. Breeding conditions were not included in the study report. HSE considers this a minor deviation as all control validity criteria were met.

OECD 232 (2009) and OECD 232 (2016) § 46 test results sections ask for the power of the actual test if hypothesis testing is performed and the minimal detectable difference (MDD) in relation to the NOEC. Power was not provided for the Williams test performed for reproduction or Fishers Exact test performed for mortality. Furthermore, no consideration of MDD was included. The study followed the experimental design (number of concentrations, replicates and concentration spacing) specified for NOEC/LOEC estimation, which suggests that statistical power was appropriate. HSE will consider these minor deviations during risk assessment.

The above study was conducted to GLP and considered valid.

The agreed endpoints suitable for use in the risk assessment are:
NOEC (reproduction) = 100 mg a.s./kg soil dw and EC_{10} (reproduction) > 100 mg a.s./kg soil dw.

B.9.4.3.2 Metabolite: 3'-OH-S-2840 (*Folsomia candida*)

Reference:	KCA 8.4.2/02
Report Title:	3'-OH-S-2840: Effects on Reproduction of the Collembola *Folsomia candida* in Artificial Soil with 5% Peat
Author(s) & year:	[redacted] (2016c)
Document No, Authority registration No:	113861016, TPW-0043
Substance used:	3'-OH-S-2840, 15SC8508366, 99.5%

Method of analysis:	n/a
Guideline(s):	OECD 232 (2009)
Deviations:	Yes, see HSE Comments
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test material:** 3'-OH-S-2840
   Description: White, solid
   Lot/Batch: 15SC8508366
   Purity: 99.5%
   Expiry date: 30th November 2018
   Reference item: Boric acid

B. STUDY DESIGN AND METHODS

1. **Test animals:** Collembola (*Folsomia candida*)
   Age: 10 - 12 days old
   Source: In-house culture
   Acclimation: Synchronised individuals kept under breeding conditions until test start
   Diet: 2 mg granulated dried yeast at test start and on day 14

2. **Artificial soil:** 5% sphagnum peat, 20% kaolin clay, 74.8% quartz sand and 0.2% calcium Carbonate. Maximum water holding capacity (MWHC) 39% of the dry weight

3. **Test units:** Glass containers (volume : 100 mL ; diameter : 5 cm), closed tightly to prevent water evaporation, filled with 30 g ± 1.0 g artificial soil fresh weight
   No. of replicates: 4 (for test groups), 8 (for control group)
   No. of mites per replicate: 10 (for test and control group)
   Study duration: 28 days

4. **Environmental conditions:**

   A summary of the environmental conditions is shown in Table 9.4.3-3 below.

Table 9.4.3-3: Environmental conditions in the study of *F. candida* exposed to 3'-OH-S-2840

Variable	Required OECD 232 (2016)	Obtained
Temperature	20 ± 2 °C	18 - 22°C
pH	6.0 ± 0.5	5.9 – 6.0 at test start and test end
Water content of soil	40-60% of the maximum WHC	19.8 – 20.1% at test initiation (50.7 – 51.5% of MWHC) 19.2 – 20.1% at test termination (49.2 – 51.5% of MWHC)
Photoperiod	16 hours light: 8 hours darkness	16 hours light, 8 hours dark
Light intensity	400 to 800 lux	400 to 800 lux

Study dates: Experimental start date: 25th July 2016. Experimental completion date: 23rd August 2016

5. Animal assignment and treatment:
There were four replicates with ten collembolans per replicate (40 collembolans) for the treatment groups and eight replicates (80 collembolans) for the control group. Organisms were exposed at concentrations of 6.25, 12.5, 25, 50 and 100 mg/kg soil dw containing 5% peat. The collembolans were placed onto the soil surface of the treated artificial soil. All vessels were ventilated on Days 2, 4, 7, 9, 11, 14, 16, 18, 21, 23 and 25 by opening the lids for a short period. After 28 days the collembolans were collected and counted.

A reference test was conducted in a separate study with boric acid at concentrations of 30.5, 48.8, 78.1, 125 and 200 mg/kg soil dw. The reference item test is performed at least once a year. The GLP conducted experiment was performed from November to December 2015.

6. Dose preparation:
Defined amounts of 3'-OH-S-2840 were weighed and 75 g fine quartz sand was added and mixed to achieve a homogeneous distribution. This mixture was then added to artificial soil to obtain a final net weight of 300 g dry weight (except for the 6.25 mg/kg soil where a final weight of 600 g was obtained) and mixed with a laboratory mixer to ensure a homogeneous distribution, with addition of deionized water. The nominal exposure concentrations of 6.25, 12.5, 25, 50 and 100 mg/kg soil dw correspond to 6.28, 12.56, 25.1, 50.3 and 100.5 mg test item/kg soil dw (based on a purity of 99.5%). Each group was treated in one batch and then split into replicates. An untreated control was also prepared, containing the same amount of untreated quartz sand as in the test item treated groups.

7. Measurements and observations:
After 28 days the content of the test vessels was suspended in water. These suspensions

were tinted with dark ink and stirred with a fine brush. The adult and juvenile collembolans that drifted to the surface were counted. Adult collembolans were counted once visually, and juveniles were counted twice under binocular microscopes. Missing collembolans were recorded as dead. The living collembolans were observed for any abnormal behaviour or conditions.

The pH and water content were determined at test start and test end.

Water content was checked on Day 14 by reweighing the additional tests containers. It was not necessary to compensate for loss of water.

8. Statistics:

Mortality data were statistically analysed using Fisher's Exact Binomial Test (multiple comparison, with Bonferroni Correction, α=0.05, one-side greater). Reproduction data were tested for normal distribution and Levene's test using Shapiro-Wilk's test and Levene's test (α=0.05). Further statistical evaluation was performed using William's t-test (multiple comparison, α=0.05, one-sided smaller). The determination of the NOEC and LOEC values was based on the results of the statistical evaluation.

The software ToxRat Professional, Version 2.10.05, ® ToxRat Solutions GmbH was used for all statistical analysis.

II. RESULTS AND DISCUSSION

A. MORTALITY

No statistically significant increase in mortality was observed up to and including the highest test item concentration of 100 mg/kg soil dw. The results for mean mortality are shown in the table below. The NOEC based on mortality was determined to be 100 mg/kg soil dw. The LOEC and LC_{50} values were established as greater than 100 mg/kg soil dw.
There were no behavioural abnormalities observed for the collembolans in the test item treated groups and the control.

B. REPRODUCTION

There were no statistically significant effects on reproduction up to and including the highest test item concentration of 100 mg/kg soil dw. The results for reproductive success are shown in the table below.

The NOEC based on reproduction was determined to be 100 mg/kg soil dw and the LOEC was established as greater than 100 mg/kg soil dw. The EC_{10}, EC_{20} and EC_{50} values were also determined to be greater than 100 mg/kg soil dw.

The reference substance boric acid was tested in a separate 28-day toxicity study and showed statistically significant treatment related effects on reproduction at a concentration of 78.1 mg/kg soil dw and above.

Table 9.4.3-4: Mortality and reproduction of *Folsomia candida* after 28 days of exposure to 3'-OH-S-2840

Nominal concentration of 3'-OH-S-2840 (mg/kg soil dw)	Mean mortality (% ± SD)	Reproduction	
		Mean number of juveniles/replicate (number ± SD)	% of control
Control	10 ± 8	477 ± 63	n.a.
6.25	10 ± 0	435 ± 25	91.2
12.5	5 ± 6	484 ± 26	101.4
25	20 ± 16	493 ± 52	103.3
50	13 ± 10	466 ± 46	97.5
100	10 ± 14	463 ± 48	97.0

SD = standard deviation
n.a. = not applicable

C.VALIDITY CRITERIA
As mean mortality in the control treatment was ≤ 20% (actual: 10%), control reproduction was ≥ 100 juveniles per replicate and the coefficient of variation of the control was ≤ 30% (actual: 13.2%), the validity criteria were met and the study is considered valid.

III. CONCLUSION

In this 28-day study, the NOEC *for Folsomia candida* exposed to 3'-OH-S-2840 was determined to be 100 mg/kg soil dw, the highest concentration tested. The LOEC, EC_{10}, EC_{20} and EC_{50} values were estimated to be greater than 100 mg/kg soil dw.

HSE COMMENTS
This study was conducted to GLP under OECD 232 (2009) guidelines. It has been assessed against the updated OECD 232 (2016) guidelines.

As this study was looking to determine both NOEC and ECx values, 8 test concentrations separated by a factor of ≤ 1.8 should have been tested in line with OECD 232 (2016), but this study only used 5 concentrations separated by a factor of 2. HSE do not consider this to be ideal, but as the validity criteria were met, this does not invalidate the study.

During dose preparation, the final net weight of the 6.25 mg/kg soil mixture was double the final net weight of all the other concentrations. No reason for this is supplied in the full study report. As the concentration was maintained and the same amount of soil applied to the test vessel as the other concentrations, this should not have affected the results of the study.

No extraction efficiency value appears to have been presented in the full study report. HSE will consider this uncertainty during the risk assessment stage.

The use of statistics is suitable for the study and is in line with OCD 232 (2016) guidelines.

As <10% effects on reproduction were observed, the EC_{10} and EC_{20} were unable to be statistically determined.

The reference item EC_{50} for reproduction was 94.0 mg/kg soil dw and demonstrated the test system was sufficiently sensitive (OECD 232: EC_{50} should be around 100 mg/kg soil dw).

The agreed endpoints for use in risk assessment are:
NOEC (reproduction) = 100 mg/kg soil dw
EC_{10} = >100mg/kg soil dw

B.9.4.3.3 Metabolite: 1'-COOH-S-2840 (*Folsomia candida*)

Reference:	KCA 8.4.2/03
Report Title:	1'-COOH-S-2840: Effects on Reproduction of the Collembola *Folsomia candida* in Artificial Soil with 5% Peat
Author(s) & year:	[redacted] (2016c)
Document No, Authority registration No:	113871016, TPW-0051
Substance used:	1'-COOH-S-2840, 16SC8508359, 100 %
Method of analysis:	n/a
Guideline(s):	OECD 232 (2009)
Deviations:	Yes, see HSE Comments
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

MATERIALS AND METHODS

MATERIALS

Test material 1'-COOH-S-2840
- **Description** White, solid
- **Batch number:** 16SC8508359
- **Active substance content:** 100% (verified by certificate of analysis)
- **Storage on receipt:** At 4 ± 4 °C, in the dark
- **Expiry date** January 31 2019

TREATMENTS

Nominal test doses:	6.25, 12.5, 25.0, 50.0 and 100.0 mg /kg soil
Control:	Untreated control (the same amount of untreated quartz sand as in the test item treated groups was added and moistened with deionised water)
Toxic reference:	Boric acid – 100.3 % (separate study: 30.5, 48.8, 78.1, 125 and 200 mg a.s./kg soil dw treatment levels)

TEST ORGANISMS

Species:	*Folsomia candida* (Willem 1902), Juveniles at experimental start
Age:	10 – 12 days
Source:	The synchronised individuals were bred at ibacon, fed granulated dry yeast and kept under breeding conditions until test start
Feeding:	After the introduction of the test organisms (day 0), and after 14 days, approximately 2 mg (half of a small spatula) of granulated dried yeast was spread over the soil surface.

TEST DESIGN

Test units:	Glass containers (volume: 100 ml; diameter: 5 cm), closed tightly to avoid water evaporation, filled with 30 g ± 1.0 g artificial soil fresh weight
Replication:	8 for the control, 4 for each test item group, 1 additional container per treatment to check the pH and water content of the test substrate after 28 days, 10 individuals per replicate
Exposure time:	28 days
Ventilation:	All vessels including the additional containers were ventilated on day 2, 4, 7, 9, 11, 14, 16, 18, 21, 23 and 25 by opening the lids for a short period.

TEST CONDITIONS

Test temperature:	18 - 22 °C
Lighting:	16 h light: 8 h dark (400 lux to 800 lux)
pH:	Experimental start: 6.0 Experimental end: 5.7 to 5.8
Soil maximum water holding capacity:	39% of the dry weight of artificial soil
Water content:	Experimental start: 19.2 % to 19.7 % (49.3 % to 50.5 % of the maximum water holding capacity). Experimental end: 18.5 % to 19.5 % (47.4 % to 49.9 % of the maximum water holding capacity)
Test substrate:	According to OECD 232: • 5% Sphagnum-peat, air-dried and finely ground (<2 mm,

with no visible plant remains); (Floragard, Vertriebs GmbH fur Gartenbau, 26138 Oldenburg, Germany)
• 20% Kaolin clay (Erbsloh, 65558 Lohrheim, Germany)
• 74.8% fine quartz-sand (F34) containing more than 50% by mass of particle size 0.05 mm to 0.2 mm; (Quarzwerke Frechen, Postfach 1780, 50207 Frechen, Germany)
• 0.2% calcium carbonate (CaCO3) was added to adjust pH to 6.0 ± 0.5 (Merck, 64293 Darmstadt, Germany). The artificial soil was moistened to approximately half of the final water content 2 days before the application. The additional water required to achieve the final water content was added when applying the test item

STUDY DESIGN AND METHODS
Experimental dates: 22 August 2016 to 20 September 2016

Dose preparation
Defined amounts of 1'-COOH-S-2840 were weighed and 80 g fine quartz sand was added and mixed to achieve a homogeneous distribution. This mixture was then added to artificial soil to obtain a final net weight of 320 g dry weight and mixed with a laboratory mixer to ensure a homogeneous distribution, with addition of deionised water. Each group was treated in one batch and then split into replicates.

Test organism and treatment
The collembolans were collected with an aspirator, put into a small glass tube for counting, and 10 were placed onto the soil surface of the treated artificial soil of each replicate.

Measurements and observations
After 28 days, the contents of the test containers were suspended in water and the suspensions were tinted with dark ink and stirred with a fine brush. The Collembola drifted to the surface. Adult animals were counted once visually; juvenile animals were counted at least twice under binocular microscopes. Two of the replicates were counted three times because the first two counts deviated more than 10% from their mean value.

The numbers of living adult Collembola at day 28 after application were recorded. Missing adult Collembola were recorded as dead as it is assumed that missing adult Collembola had died and degraded during the test period. Surviving Collembola were observed for any abnormal behaviour or conditions at day 28 after application. The number of juveniles was also quantified 28 days after application.

Water content was checked on day 14 after the application by reweighing the additional test containers. It was not necessary to compensate for loss of water as deviation did not exceed

2% of the initial water content.

Statistical analysis
Mortality data were statistically analysed using Fisher's Exact Binomial Test (multiple comparison, with Bonferroni Correction, α=0.05, one-side greater). Reproduction data were tested for normal distribution and Levene's test using Shapiro-Wilk's test and Levene's test (α=0.05). Further statistical evaluation was performed using William's t-test (multiple comparison, α=0.05, one-sided smaller). The determination of the NOEC and LOEC values was based on the results of the statistical evaluation.

The software ToxRat Professional, Version 2.10.05, ® ToxRat Solutions GmbH was used for all statistical analysis.

RESULTS AND DISCUSSION
Mortality
Mortality of the collembolans exposed to concentrations up to and including the highest test item concentration of 100 mg/kg soil dw was not statistically significant different from the control. The results on mean mortality are shown in Table 9.4.3-5. The NOEC based on mortality was determined to be 100 mg/kg soil dw. The LOEC and LC_{50} values were established as greater than 100 mg/kg soil dw.

There were no behavioural abnormalities observed for the living collembolans in the test item treated groups and the control.

Reproduction
There were no statistically significant effects on reproduction up to and including the highest test item concentration of 100 mg/kg soil dw. The results for reproductive success are shown in Table 9.4.3-5.

The NOEC based on reproduction was determined to be 100 mg/kg soil dw and the LOEC was established as greater than 100 mg/kg soil dw. The EC_{10}, EC_{20} and EC_{50} values were also determined to be greater than 100 mg/kg soil dw.

Table 9.4.3-5: Mortality and reproduction of Folsomia candida after 28 days of exposure to 1'-COOH-S-2840

Nominal concentration of 1'-COOH-S-2840 (mg/kg soil dw)	Mean mortality (% ± SD)	Reproduction	
		Mean number of juveniles/replicate (number ± SD)	% of control
Control	9 ± 10	456 ± 18	n.a.

Nominal concentration of 1'-COOH-S-2840 (mg/kg soil dw)	Mean mortality (% ± SD)	Reproduction	
		Mean number of juveniles/replicate (number ± SD)	% of control
6.25	13 ± 5	482 ± 20	106
12.5	5 ± 6	449 ± 47	98
25	5 ± 6	470 ± 43	103
50	18 ± 5	459 ± 37	101
100	13 ± 5	489 ± 42	107

SD = standard deviation
n.a. = not applicable

For the reference material, boric acid, there were statistically significant effects on reproduction at a concentration of 78.1 mg/kg soil and higher with a clear concentration-response relationship. The EC_{50} for reproduction was calculated as 94.0 mg/kg soil.

Validity criteria
The validity criteria for the study were met according to OECD 232 (2009), the guideline available on study initiation, and OECD 232 (2016), the most recent version of the guideline (Table 9.4.3-6).

Table 9.4.3-6: Compliance with OECD 232 validity criteria

Validity criterion	Required	Obtained
Control adult mortality	≤ 20 %	9 %
Control reproduction	≥ 100 juveniles	439 to 496 juveniles
Control reproduction Coefficient of Variation (CV)	≤ 30 %	3.9 %

CONCLUSIONS
In this 28-day study, the NOEC for *Folsomia candida* exposed to 1'-COOH-S-2840 was determined to be 100 mg/kg soil dw, the highest concentration tested. The LOEC, EC_{10}, EC_{20} and EC_{50} values were estimated to be greater than 100 mg/kg soil dw.

HSE COMMENTS

The study was carried out according to OECD 232 (2009). It was evaluated against OECD 232 (2009) and OECD 232 (2016), a newer version of the guideline adopted 15 days after the study initiated. All validity criteria outlined in OECD 232 were satisfactorily met for the duration of the study. There were no significant deviations to the guidelines. Treatment with the toxic reference (boric acid) yielded an EC_{50} (reproduction) of 94.0 mg/kg soil, which is in line with OECD 232 (2009) (should reduce reproduction by 50 % at about 100 mg/kg dry weight soil). This demonstrated the appropriateness of the laboratory test conditions and sensitivity of the test individuals.

The following minor deviations were noted for OECD 232 (2009) and OECD 232 (2016):

OECD 232 (2009) and OECD 232 (2016) § 17 covers the selection and preparation of test animals. It states, "*the synchronous animals are selected randomly from the dishes and their health and physical condition is checked for each batch added to a replicate*". Random selection of individuals and health checks were either not reported in the study report or not performed. Given the validity criteria of the study were met, this suggests that there were no undetected health issues in the test population. HSE considers these minor deviations and acceptable.

OECD 232 (2009) and OECD 232 (2016) § 31 and 46 details how the extraction and counting method for juveniles should be validated. The validity includes extraction efficiency of juveniles greater than 95%. This was not detailed within the study report. HSE notes, however, that measures were taken to maintain the integrity of the reproduction endpoint: when two juvenile counts diverged by > 10 % of their mean value, a third count was performed and the median of the three reported. HSE will consider this uncertainty during the risk assessment stage.

OECD 232 (2009) and OECD 232 (2016) § 46 describes test report requirements. It states that a description of breeding conditions should be provided. Breeding conditions were not included in the study report. HSE considers this a minor deviation as all control validity criteria were met.

OECD 232 (2009) and OECD 232 (2016) § 46 test results sections ask for the power of the actual test if hypothesis testing is performed and the minimal detectable difference (MDD) in relation to the NOEC. Power was not provided for the Williams test performed for reproduction or Fishers Exact test performed for mortality. Furthermore, no consideration of MDD was included. The study followed the experimental design (number of concentrations, replicates and concentration spacing) specified for NOEC/LOEC estimation, which suggests that statistical power was appropriate. HSE will consider these minor deviations during risk assessment.

The above study was conducted to GLP and considered valid.

The agreed endpoints suitable for use in the risk assessment are:
NOEC (reproduction) = 100 mg 1'-COOH-S-2840/kg soil dw and EC_{10} (reproduction) > 100 mg 1'-COOH-S-2840/kg soil dw.

B.9.4.3.4 Active substance: Inpyrfluxam (*Hypoaspis aculeifer*)

Reference:	KCA 8.4.2/04
Report Title:	S-2399 TG: Effects on Reproduction of the Predatory Mite *Hypoaspis aculeifer* in Artificial Soil with 5% Peat
Author(s) & year:	[redacted] (2016c)
Document No, Authority registration No:	113911089, TPW-0042
Substance used:	S-2399 TG, 13CG0617G, 95 %
Method of analysis:	n/a
Guideline(s):	OECD 226 (2008)
Deviations:	Yes, see HSE Comments
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

MATERIALS AND METHODS

MATERIALS

Test material S-2399 TG
- **Description** White, solid
- **Batch number:** 13CG0617G
- **Active substance content:** 95.5% (verified by certificate of analysis)
- **Storage on receipt:** At 4 ± 4 °C, in the dark
- **Expiry date** June 29 2019

TREATMENTS

- **Nominal test doses:** 6.21, 12.73, 25.1, 50.3 and 100.6 mg a.s./kg soil
- **Control:** Untreated control (the same amount of untreated quartz sand as in the test item treated groups was added and

	moistened with deionised water)
Toxic reference:	Perfekthion (BAS 152 11 I, dimethoate 420.3 g/L (analysed)) (separate study: 1.55, 2.24, 3.25, 4.70 and 6.80 mg a.s./kg soil dw)

TEST ORGANISMS

Species:	*Hypoaspis aculeifer (Canestrini 1883)*, Adult females (from a synchronized cohort)
Age:	Adults, approximately 12 days after reaching the adult stage (33 days after placing adult females in clean rearing vessels over a period of 3 days (start of egg laying in the synchronisation))
Source:	Cultured at ibacon
Feeding:	One spatula of cheese mites (*Tyrophagus putrescentiae* cultured by ibacon) at experimental start and on day 2, 5, 7, 9 and 12.

TEST DESIGN

Test units:	Glass containers (volume: 100 ml; diameter: 5 cm), tight screw top closure to avoid water evaporation, filled with approximately 20 g ± 1.0 g artificial soil dry weight.
Replication:	4 per treatment group and 8 for the control, 1 additional container per treatment to check the pH and water content of the test substrate after 14 days, 10 adult female mites per unit
Exposure time:	14 days
Ventilation:	All vessels including the additional containers were ventilated on days 2, 5, 7, 9 and 12 by opening the lids for a short period.

TEST CONDITIONS

Test temperature:	18 - 22 °C
Lighting:	16 h light: 8 h dark (400 lux to 800 lux)
pH:	Experimental start: 6.0 to 6.2 Experimental end: 5.7 to 6.0
Soil maximum water holding capacity:	39% of the dry weight of artificial soil
Water content:	Experimental start: 20.1 % to 20.5 % (51.5 % to 52.5 % of the maximum water holding capacity). Experimental end: 18.9 % to 19.8 % (48.4 % to 50.7 % of the maximum water holding capacity)
Test substrate:	According to OECD 226: • 5% Sphagnum-peat, air-dried and finely ground (<2 mm, with no visible plant remains); (Floragard, Vertriebs GmbH fur Gartenbau, 26138 Oldenburg, Germany)

• 20% Kaolin clay (Erbsloh, 65558 Lohrheim, Germany)
• 74.8% fine quartz-sand (F34) containing more than 50% by mass of particle size 0.05 mm to 0.2 mm; (Quarzwerke Frechen, Postfach 1780, 50207 Frechen, Germany)
• 0.2% calcium carbonate (CaCO3) was added to adjust pH to 6.0 ± 0.5 (Merck, 64293 Darmstadt, Germany). The artificial soil was moistened to approximately half of the final water content 2 days before the application. The additional water required to achieve the final water content was added when applying the test item

STUDY DESIGN AND METHODS
Experimental dates: 13 July 2016 to 29 July 2016

Dose preparation
Defined amounts of S-2399 TG were weighed and 75 g fine quartz sand was added and mixed to achieve a homogeneous distribution. This mixture was then added to artificial soil to obtain a final net weight of 300 g dry weight (600 g test substrate and 150 g sand for the 6.25 mg/kg soil group) and mixed with a laboratory mixer to ensure a homogeneous distribution, with addition of deionised water. Each group was treated in one batch and then split into replicates.

Test organism and treatment
The predatory mites were collected with a fine brush, put into a small glass tube, counted to ensure that 10 adult females are introduced and placed onto the surface of the treated artificial soil.

Measurements and observations
After 14 days exposure the soil was filled into Millipore pots with attached plastic containers for collecting the escaping mites. These extraction units were placed in a Kempson extractor. The soil including the mites was exposed to a temperature of approximately 25°C and 30°C for approximately 2 days. Escaping mites were collected in a fixing liquid, cooled at a temperature of approximately I6°C. The fixing liquid contained glycol and a detergent. Adult animals were counted once visually, juvenile animals were counted twice under binocular microscopes. Five of the replicates were counted three times because the first two counts deviated more than 10% from their mean value.

The extraction efficiency was checked separately in August 2015 by adding 39 mites in a first extraction unit and 45 mites in a second extraction unit containing untreated soil. The number of extracted animals was counted. 82 animals out of 84 were recovered giving an extraction efficiency of 97.6%.

Number of surviving adult female predatory mites 14 days after test initiation was recorded (counted after extraction). Missing adult predatory mites were recorded as dead as it was assumed they would have died and degraded during the test period. The living predatory mites were observed for differences in morphology or any abnormalities at experimental end. Number of juvenile mites at day 14 after application were counted after extraction.

Water content was checked on Day 7 after the application by reweighing the additional test containers. It was not necessary to compensate for loss of water as deviation did not exceed 2% of the initial water content.

Statistical analysis

Mortality data were statistically analysed using Fisher's Exact Binomial Test (multiple comparison, with Bonferroni Correction, α=0.05, one-side greater). The LC_{50} at Day 14 was not determined by statistical analysis as no mortality above 50% was observed. Reproduction data were tested for normal distribution and homogeneity of variance using Shapiro-Wilk's test and Levene's test (α=0.05). As data were normally distributed and homogeneous, the further statistical evaluation was performed using William's t-test (multiple comparison, α=0.05, one-sided smaller). The EC values at Day 14 were not determined by statistical analysis as no reduction in reproduction above 10% was observed.

The software ToxRat Professional, Version 2.10.05, ® ToxRat Solutions GmbH was used for all statistical analysis.

RESULTS AND DISCUSSION

Mortality

No statistically significant increase in mortality was observed up to and including the highest test item concentration of 100 mg/kg soil dw (Table 9.4.3-7). There were no behavioural abnormalities observed for the mites in the test item treated groups and the control. The NOEC based on mortality was determined to be 100 mg/kg soil dw and the LOEC was established as greater than 100 mg a.s./kg soil dw.

Reproduction

There were no statistically significant effects on reproduction up to and including the highest test item concentration of 100 mg a.s./kg soil dw (Table 9.4.3-7). The NOEC based on reproduction was determined to be 100 mg a.s./kg soil dw and the LOEC was established as greater than 100 mg a.s./kg soil dw. The EC_{10}, EC_{20} and EC_{50} values were also determined to be greater than 100 mg a.s./kg soil dw.

Table 9.4.3-7: Mortality and reproduction of *Hypoaspis aculeifer* after 14 days of exposure to S-2399 TG

Nominal concentration of S-2399 (mg a.s./kg soil dw)	Mean mortality (% ± SD)	Reproduction	
		Mean number of juveniles/replicate (number ± SD)	% of control
Control	13 ± 16	147 ± 18	n.a.
6.25	8 ± 10	177 ± 17	121
12.5	5 ± 6	186 ± 9	126
25	5 ± 6	182 ± 7	124
50	3 ± 5	177 ± 9	120
100	0 ± 0	165 ± 18	112

SD = standard deviation
n.a. = not applicable

For the reference material, dimethoate, there were statistically significant effects on mortality and reproduction at a concentration of 3.25 mg a.s./kg soil and higher with a clear concentration-response relationship. The LC_{50} = 2.97 (2.74 to 3.20) and $EC_{50\ (reproduction)}$ = 3.57 (3.46 to 3.69).

Validity criteria

The validity criteria for the study were met according to OECD 226 (2008), the guideline available on study initiation, and OECD 226 (2016), the most recent version of the guideline (Table 9.4.3-8).

Table 9.4.3-8: Compliance with OECD 226 validity criteria

Validity criterion	Required	Obtained
Control adult mortality	≤ 20 %	13 %
Control reproduction	≥ 50 juveniles	121 to 172 juveniles
Control reproduction Coefficient of Variation (CV)	≤ 30 %	12.2 %

CONCLUSIONS

In this 14-day study, the NOEC for *Hypoaspis aculeifer* exposed to S-2399 TG was determined to be 100 mg a.s./kg soil dw, the highest concentration tested. The LOEC, EC_{10}, EC_{20} and EC_{50} values were estimated to be greater than 100 mg a.s./kg soil dw.

HSE COMMENTS

The study was carried out according to OECD 226 (2008). It was evaluated against OECD 226 (2008) and OECD 226 (2016), a newer version of the guideline that was adopted 16 days after the experimental phase of this study began. All validity criteria outlined in OECD 226 were satisfactorily met for the duration of the study. There were no significant deviations to the guidelines. Treatment with the toxic reference (dimethoate) yielded an EC_{50} (reproduction) of 3.57 mg a.s./kg soil, which is in line with OECD 226 (2008) (between 3.0 and 7.0 mg a.s./kg soil (dw)). This demonstrated the appropriateness of the laboratory test conditions and sensitivity of the test individuals.

The following minor deviations were noted for OECD 226 (2008) and OECD 226 (2016):

OECD 226 (2008) and OECD 226 (2016) § 15-18 cover the various test item soil application methods. The study selected the soil application method suited for test items with poor water and organic solvent solubility. S-2399 is soluble in acetone. No justification for why this approach was selected was provided. The method is, however, valid and ensured a homogeneous distribution of S-2399 in the soil. Therefore, HSE considers this an acceptable approach.

OECD 226 (2016) § 32 states, "*at the end of the test, adults and juveniles are humanely euthanized, preferably by rapid freezing at -80°C or cryopreservation*". Euthanasia procedures were not detailed in the study report as OECD 226 (2008), the guideline available when the study was performed, did not include this requirement. HSE considers this an acceptable deviation.

OECD 226 (2008) and OECD 226 (2016) § 41 describes test report requirements. It states that a description of the test organism culturing conditions should be provided. Culturing conditions were not included in the study report. HSE considers this a minor deviation as all control validity criteria were met.

The above study was conducted to GLP and considered valid.

The agreed endpoints suitable for use in the risk assessment are:
NOEC (reproduction) = 100 mg a.s./kg soil dw and EC_{10} (reproduction) > 100 mg a.s./kg soil

B.9.4.3.5 Metabolite: 3'-OH-S-2840 (*Hypoaspis aculeifer*)

Reference:	KCA 8.4.2/05
Report Title:	S-2399 TG: Effects on Reproduction of the Predatory Mite *Hypoaspis aculeifer* in Artificial Soil with 5% Peat
Author(s) & year:	[redacted] (2016e)
Document No, Authority registration No:	113861089, TPW-0045
Substance used:	3'-OH-S-2840, 15SC8508366, 99.5 %
Method of analysis:	n/a
Guideline(s):	OECD 226 (2008)
Deviations:	Yes, see HSE Comments
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test material:** 3'-OH-S-2840
   Description: White, solid
   Lot/Batch: 15SC8508366
   Purity: 99.5%
   Reference item: Perfekthion (a.s. dimethoate 400g/L)

B. STUDY DESIGN AND METHODS

1. **Test animals:** Predatory mite **(***Hypoaspis aculeifer*)
   Age: Adult females (10 days after reaching adult stage)
   Source: In-house culture, from a synchronised cohort
   Acclimation: 31 days, in clean rearing vessels over a period of 3 days
   Diet: One spatula of cheese mites (*Tyrophagus putrescentiae*) at test start and on day 2, 4, 7, 9 and 11

2. **Artificial soil:** 5% sphagnum peat, 20% kaolin clay, 74.8% quartz sand and 0.2% calcium carbonate. Maximum water holding capacity (MWHC) of 39% of the dry weight

3. **Test units:** Glass containers (volume : 100 mL ; diameter : 5 cm), with a tight screw top closure to prevent water evaporation, filled with approximately 20 g ± 1.0 g artificial soil dry weight

Concentrations tested: 6.25, 12.5, 25, 50 and 100 mg a.s./kg soil dw
No. mites per unit: 10 adult females
No. of replicates: 4 (for test concentrations), 8 (for control groups)
Duration: 14 days

4. Environmental conditions:

A summary of the environmental conditions are shown in Table 9.4.3-9 below:

Table 9.4.3-9: Environmental conditions obtained in the study of H. aculeifer exposed to 3'-OH-S-2840

Variable	Required OECD 226 (2016)	Obtained
Temperature	20 ± 2 °C	18 - 22°C
pH of soil	6.0 ± 0.5	5.9 – 6.0 at test start and test end
Water content of soil	Maintained throughout the test	19.8 – 20.1% at test initiation (50.7% -51.5% of maximum water holding capacity) 19.6 – 20.0% at test end (50.3% - 51.4% of maximum water holding capacity)
Photoperiod	16 hours light and 8 hours dark	16 hours light: 8 hours darkness
Light intensity	400 to 800 lux	400 to 800 lux

Study dates: 25th July 2016 – 10th August 2016

5. Animal assignment and treatment:
There were four replicates with 10 females per replicate (40 mites) for the treatment groups and eight replicates (80 mites) for the control group. Organisms were exposed at concentrations of 6.25, 12.5, 25, 50 and 100 mg a.s./kg soil dw containing 5% peat. The mites were placed onto the soil surface of the treated artificial soil. All vessels were ventilated on Days 2, 4, 7, 9 and 11 by opening the lids for a short period. After 14 days, adult and juvenile mites were counted.

A reference test was conducted in a separate study with Perfekthion (BAS 152 11 I; 420.3 g dimethoate/L (analysed)) at concentrations of 1.55, 2.24, 3.25, 4.70 and 6.80 mg a.s./kg soil dw.

6. Dose preparation:
Defined amounts of 3'-OH-S-2840 were weighed and 75 g fine quartz sand was added and mixed to achieve a homogeneous distribution within the sand. The mixture was then added to artificial soil to obtain a final net weight of 300 g dry weight (except for the concentration 6.25 mg where a final net weight of 600 g was obtained) and mixed using

a laboratory mixer, with addition of deionised water. Each group was treated in one batch and then split into replicates. The nominal exposure concentrations of 6.25, 12.5, 25, 50 and 100 mg a.s./kg soil dw correspond to 6.28, 12.56, 25.1, 50.3 and 100.5 mg test item/kg soil dw (based on a purity of 99.5%). An untreated control was also prepared, containing the same amount of untreated quartz sand per g substrate as in the test item treated groups.

7. Measurements and observations:

After 14 days the soil was filled into Millipore pots with attached plastic containers, which were placed in a Kempson extractor. The soil including the mites was exposed to a temperature of 25 - 30°C for approximately 2 days. Escaping mites were collected in a fixing liquid and cooled at 16°C. Adult mites were counted once visually, and juveniles were counted twice under binocular microscopes. Missing mites were recorded as dead. The living mites were observed for differences in morphology or any abnormalities at experimental end.

The extraction efficiency was checked separately in August 2015. The extraction efficiency was 97.6%.

The pH and water content were determined at test start and test end. Water content was checked on Day 7 by reweighing the additional tests containers.

8. Statistics:

Mortality data were statistically analysed using Fisher's Exact Binomial Test (multiple comparison, with Bonferroni Correction, α=0.05, one-side greater). The LC_{50} at Day 14 was not determined by statistical analysis as no mortality above 50% was observed. Reproduction data were tested for normal distribution and homogeneity of variance using Shapiro-Wilk's test and Levene's test (α=0.05). As data were normally distributed and homogeneous, the further statistical evaluation was performed using William's t-test (multiple comparison, α=0.05, one-sided smaller). The determination of the NOEC and LOEC values was based on the results of the statistical evaluation. The EC_{50} at Day 14 was not calculated by statistical analysis as no effect above 50% was observed.

The software ToxRat Professional, Version 2.10.05, ® ToxRat Solutions GmbH was used for all statistical analysis.

II. RESULTS AND DISCUSSION

A. MORTALITY

No statistically significant increase in mortality was observed up to and including the highest test item concentration of 100 mg a.s./kg soil dw. The results on mean mortality are presented in the table below. The NOEC based on mortality was determined to be 100 mg a.s./kg soil dw and the LOEC was established as greater than 100 mg a.s./kg soil dw.

There were no behavioural abnormalities observed for the mites in the test item treated groups and the control.

B. REPRODUCTION

The reference substance Dimethoate was tested in a separate 14-day toxicity study and showed statistically significant treatment related effects on reproduction at a concentration of 3.25 mg a.s./kg soil and above.

There were no statistically significant effects on reproduction up to and including the highest test item concentration of 100 mg a.s./kg soil dw.

The NOEC based on reproduction was determined to be 100 mg a.s./kg soil dw and the LOEC was established as greater than 100 mg a.s./kg soil dw. The EC_{10}, EC_{20} and EC_{50} values were also determined to be greater than 100 mg/kg soil dw.

Table 9.4.3-10: Mortality and reproduction of Hypoaspis aculeifer after 14 days of exposure to 3'-OH-S-2840

Nominal concentration of 3'-OH-S-2840 (mg a.s./kg soil dw)	Mean mortality (% ± SD)	Reproduction	
		Mean number of juveniles/replicate (number ± SD)	% of control
Control	5 ± 8	174 ± 28	n.a.
6.25	0 ± 0	184 ± 19	105.4
12.5	5 ± 6	177 ± 18	101.3
25	13 ± 10	183 ± 38	104.8
50	10 ± 8	206 ± 49	118.4
100	5 ± 10	193 ± 35	110.8

n.a. = not applicable

C. VALIDITY CRITERIA

As mean mortality in the control treatment was ≤ 20% (actual: 5%), mean number of juveniles per replicate was >50 (actual mean number of juveniles in control replicates: 174 ± 28) and the coefficient of variation of the control was ≤ 30% (actual: 16.1%), the validity criteria were met and the study was considered valid.

III. CONCLUSION

In this 14-day study, the NOEC for *Hypoaspis aculeifer* exposed to 3'-OH-S-2840 was determined to be 100 mg a.s./kg soil dw, the highest concentration tested. The LOEC, EC_{10}, EC_{20} and EC_{50} values were greater than 100 mg a.s./kg soil dw.

HSE COMMENTS

This study was conducted to GLP under OECD 226 (2008). It has been assessed against OECD 226 (2016) guidelines.

There were no significant deviations to protocol to note but are some slight differences with the dose preparation. OECD 226 (2016) guidelines states that approximately 10g fine quartz should be used during dose preparation. This study used 75g fine quartz. This is considerably more than the example quantity given in the guidelines. No data is presented on the measured concentrations, so it is uncertain whether this had any affect on the exposure concentrations. HSE do not consider this to have an impact on the interpretation of results as the artificial soil was made to the correct measurements under acceptable conditions. As the validity criteria were met, this is unlikely to have had any impact on the study and it is considered valid.

During dose preparation, the final net weight of the 6.25 mg concentration was double that of the other concentrations. No explanation for this has been provided in the full study report. As the correct concentration has been reached and the same amount of soil was added to the test vessels, this is not considered to have any impact on the results.

As the study was looking to determine both NOEC and ECx values, 8 test concentrations should have been tested separated by a factor of ≤1.8 in line with OECD 226 (2016), yet only 5 concentrations separated by a factor of 2 were used. Whilst not idea, as the validity criteria were met, this does not invalidate the study.

It is also noted that the study report does not state how long after the test item was applied that the mites were introduced, but this does not invalidate the study.

The use of statistics is suitable for this study and adhered to OECD 226 (2016) guidance.

The reference item EC_{50} for reproduction was 3.57 mg a.s./kg soil and demonstrated the test system was sufficiently sensitive (OECD 226: EC_{50} should range from 3.0 to 7.0 mg a.s. /kg soil dw).

The agreed endpoints for use in risk assessment are:
NOEC (reproduction) = 100 mg metabolite/kg soil dw
EC_{10} = > 100 mg metabolite/kg soil dw

B.9.4.3.6 Metabolite: 1'-COOH-S-2840 (*Hypoaspis aculeifer*)

Reference:	KCA 8.4.2/06
Report Title:	1'-COOH-S-2840: Effects on Reproduction of the Predatory Mite *Hypoaspis aculeifer* in Artificial Soil with 5% Peat
Author(s) & year:	[redacted] (2016f)
Document No, Authority registration No:	113871089, TPW-0048
Substance used:	1'-COOH-S-2840, 16SC8508359, 100 %

Method of analysis:	n/a
Guideline(s):	OECD 226
Deviations:	Yes, see HSE Comments
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

MATERIALS AND METHODS

MATERIALS

Test material 1'-COOH-S-2840

- **Description** White, solid
- **Batch number:** 16SC8508359
- **Active substance content:** 100 % (verified by certificate of analysis)
- **Storage on receipt:** At 4 ± 4 °C, in the dark
- **Expiry date** January 31 2019

TREATMENTS

- **Nominal test doses:** 6.25, 12.5, 25.0, 50.0 and 100.0 mg/kg soil
- **Control:** Untreated control (the same amount of untreated quartz sand as in the test item treated groups was added and moistened with deionised water)
- **Toxic reference:** Perfekthion (BAS 152 11 I, dimethoate 420.3 g/L (analysed)) (separate study: 1.55, 2.24, 3.25, 4.70 and 6.80 mg a.s./kg soil dw)

TEST ORGANISMS

- **Species:** *Hypoaspis aculeifer* (Canestrini 1883), Adult females (from a synchronized cohort)
- **Age:** Adults, approximately 7 days after reaching the adult stage (28 days after placing adult females in clean rearing vessels (start of egg laying in the synchronisation))
- **Source:** Cultured at ibacon
- **Feeding:** Cheese mites (*Tyrophagus putrescentiae* cultured by ibacon) *ad libitum* at experimental start and on day 2, 4, 7, 9 and 11

TEST DESIGN

Test units:	Glass containers (volume: 100 ml; diameter: 5 cm), tight screw top closure to avoid water evaporation, filled with approximately 20 g ± 1.0 g artificial soil dry weight.
Replication:	4 per treatment group and 8 for the control, 1 additional container per treatment to check the pH and water content of the test substrate after 14 days, 10 adult female mites per unit
Exposure time:	14 days
Ventilation:	All vessels including the additional containers were ventilated on days 2, 4, 7, 9 and 11 by opening the lids for a short period.

TEST CONDITIONS

Test temperature:	18 - 22 °C
Lighting:	16 h light: 8 h dark (400 lux to 800 lux)
pH:	Experimental start: 6.0 Experimental end: 6.1
Soil maximum water holding capacity:	39% of the dry weight of artificial soil
Water content:	Experimental start: 19.2 % to 19.7 % (49.3 % to 50.5 % of the maximum water holding capacity). Experimental end: 19.2 % to 19.6 % (49.4 % to 50.3 % of the maximum water holding capacity)
Test substrate:	According to OECD 226: • 5% Sphagnum-peat, air-dried and finely ground (<2 mm, with no visible plant remains); (Floragard, Vertriebs GmbH fur Gartenbau, 26138 Oldenburg, Germany) • 20% Kaolin clay (Erbsloh, 65558 Lohrheim, Germany) • 74.8% fine quartz-sand (F34) containing more than 50% by mass of particle size 0.05 mm to 0.2 mm; (Quarzwerke Frechen, Postfach 1780, 50207 Frechen, Germany) • 0.2% calcium carbonate (CaCO3) was added to adjust pH to 6.0 ± 0.5 (Merck, 64293 Darmstadt, Germany). The artificial soil was moistened to approximately half of the final water content 2 days before the application. The additional water required to achieve the final water content was added when applying the test item

STUDY DESIGN AND METHODS

Experimental dates: 22 August 2016 to 07 September 2016

Dose preparation

Defined amounts of 1'-COOH-S-2840 were weighed and 80 g fine quartz sand was added

and mixed to achieve a homogeneous distribution. This mixture was then added to artificial soil to obtain a final net weight of 320 g dry weight and mixed with a laboratory mixer to ensure a homogeneous distribution, with addition of deionised water. Each group was treated in one batch and then split into replicates.

Test organism and treatment
The predatory mites were collected with a fine brush, put into a small glass tube, counted to ensure that 10 adult females are introduced and placed onto the surface of the treated artificial soil.

Measurements and observations
After 14 days exposure the soil was filled into Millipore pots with attached plastic containers for collecting the escaping mites. These extraction units were placed in a Kempson extractor. The soil including the mites was exposed to a temperature of approximately 25°C and 30°C for approximately 2 days. Escaping mites were collected in a fixing liquid, cooled at a temperature of approximately I6°C. The fixing liquid contained glycol and a detergent. Adult animals were counted once visually, juvenile animals were counted twice under binocular microscopes. One of the replicates were counted three times because the first two counts deviated more than 10% from their mean value.

The extraction efficiency was checked separately in July 2016 by adding 50 mites in a first extraction unit and 53 mites in a second extraction unit containing untreated soil. The number of extracted animals was counted. 97 animals out of 103 were recovered giving an extraction efficiency of 94.2%.

Number of surviving adult female predatory mites 14 days after test initiation was recorded (counted after extraction). Missing adult predatory mites were recorded as dead as it was assumed they would have died and degraded during the test period. The living predatory mites were observed for differences in morphology or any abnormalities at experimental end. Number of juvenile mites at day 14 after application were counted after extraction.

Water content was checked on Day 7 after the application by reweighing the additional test containers. It was not necessary to compensate for loss of water as deviation did not exceed 2% of the initial water content.

Statistical analysis
Mortality data were statistically analysed using Fisher’s Exact Binomial Test (multiple comparison, with Bonferroni Correction, α=0.05, one-side greater). Reproduction data were tested for normal distribution and homogeneity of variance using Shapiro-Wilk’s test and Levene’s test (α=0.05). As data were normally distributed and homogeneous, the further statistical evaluation was performed using Bonferroni-Welch t-test (multiple comparison, α=0.05, one-sided smaller). The determination of the NOEC and LOEC values was based

on the results of the statistical evaluation.

The software ToxRat Professional, Version 2.10.05, ® ToxRat Solutions GmbH was used for all statistical analysis.

RESULTS AND DISCUSSION

Mortality

No statistically significant increase in mortality was observed up to and including the highest test item concentration of 100 mg/kg soil dw (Table 9.4.3-11). There were no morphological abnormalities observed for the mites in the test item treated groups and the control. The NOEC based on mortality was determined to be 100 mg/kg soil dw and the LOEC was established as greater than 100 mg/kg soil dw.

Reproduction

There were no statistically significant effects on reproduction up to and including the highest test item concentration of 100 mg/kg soil dw (Table 9.4.3-11). The NOEC based on reproduction was determined to be 100 mg/kg soil dw and the LOEC was established as greater than 100 mg/kg soil dw. The EC_{10}, EC_{20} and EC_{50} values were also determined to be greater than 100 mg/kg soil dw.

Table 9.4.3-11: Mortality and reproduction of *Hypoaspis aculeifer* after 14 days of exposure to 1'-COOH-S-2840

Nominal concentration of 1'-COOH-S-2840 (mg a.s./kg soil dw)	Mean mortality (% ± SD)	Reproduction	
		Mean number of juveniles/replicate (number ± SD)	% of control
Control	8 ± 9	212 ± 21	n.a.
6.25	0 ± 0	205 ± 15	96
12.5	8 ± 10	193 ± 8	91
25	8 ± 10	203 ± 59	96
50	0 ± 0	202 ± 20	95
100	0 ± 0	200 ± 39	94

SD = standard deviation
n.a. = not applicable

For the reference material, dimethoate, there were statistically significant effects on mortality and reproduction at a concentration of 3.25 mg a.s./kg soil and higher with a clear concentration-response relationship. The LC_{50} = 2.97 (2.74 to 3.20) and $EC_{50\ (reproduction)}$ =

3.57 (3.46 to 3.69).

Validity criteria
The validity criteria for the study were met according to OECD 226 (2008), the guideline available on study initiation, and OECD 226 (2016), the most recent version of the guideline (Table 9.4.3-12).

Table 9.4.3-12: Compliance with OECD 226 validity criteria

Validity criterion	Required	Obtained
Control adult mortality	≤ 20 %	8 %
Control reproduction	≥ 50 juveniles	178 to 1240 juveniles
Control reproduction Coefficient of Variation (CV)	≤ 30 %	9.9 %

CONCLUSIONS
In this 14-day study, the NOEC for *Hypoaspis aculeifer* exposed to 1'-COOH-S-2840 was determined to be 100 mg/kg soil dw, the highest concentration tested. The LOEC, EC_{10}, EC_{20} and EC_{50} values were estimated to be greater than 100 mg/kg soil dw.

HSE COMMENTS
The study was carried out according to OECD 226 (2008). It was evaluated against OECD 226 (2008) and OECD 226 (2016), a newer version of the guideline that was adopted 15 days after the study initiated. All validity criteria outlined in OECD 226 were satisfactorily met for the duration of the study. There were no significant deviations to the guidelines. Treatment with the toxic reference (dimethoate) yielded an EC_{50} (reproduction) of 3.57 mg a.s./kg soil, which is in line with OECD 226 (2008) (between 3.0 and 7.0 mg a.s./kg soil (dw)). This demonstrated the appropriateness of the laboratory test conditions and sensitivity of the test individuals.

The following minor deviations were noted for OECD 226 (2008) and OECD 226 (2016):

OECD 226 (2008) and OECD 226 (2016) § 15-18 cover the various test item soil application methods. The study selected the soil application method suited for test items with poor water and organic solvent solubility. S-2399 is soluble in acetone. No justification for why this approach was selected was provided. The method is, however, valid and ensured a homogeneous distribution of S-2399 in the soil. Therefore, HSE considers this an acceptable

approach.

OECD 226 (2016) § 32 states, "*at the end of the test, adults and juveniles are humanely euthanized, preferably by rapid freezing at -80°C or cryopreservation*". Euthanasia procedures were not detailed in the study report as OECD 226 (2008), the guideline available when the study was performed, did not include this requirement. HSE considers this an acceptable deviation.

OECD 226 (2008) and OECD 226 (2016) § 41 describes test report requirements. It states that a description of the test organism culturing conditions should be provided. Culturing conditions were not included in the study report. HSE considers this a minor deviation as all control validity criteria were met.

The above study was conducted to GLP and considered valid.

The agreed endpoints suitable for use in the risk assessment are:
NOEC (reproduction) = 100 mg/kg soil dw and EC_{10} (reproduction) > 100 mg/kg soil dw.

B.9.5 Effects on Soil in Nitrogen Transformation

B.9.5.1 Summary of toxicity data

Table 9.5.1-1: Summary of endpoints for inpyrfluxam and its metabolites

Test Item	Exposure system	Results	References
S-2399 TG	28 d natural soil	Effects < 25% after 28 days at 0.27 and 1.33 mg a.s./kg soil dw	CA 8.5/01 [redacted] 2016a
3'-OH-S-2840	28 d natural soil	Effects < 25% after 28 days at 0.06 and 0.3 mg/kg soil dw	CA 8.5/02 [redacted] 2016b
1'-COOH-S-2840	28 d natural soil	Effects < 25% after 28 days at 0.1 and 0.5 mg/kg soil dw	CA 8.5/03 [redacted] 2016c

B.9.5.2 Active substance: Inpyrfluxam

Reference:	KCA 8.5/01
Report Title:	S-2399 TG: Effects on the Activity of the Soil Microflora in the

	Laboratory
Author(s) & year:	[redacted] (2016a)
Document No, Authority registration No:	113911080, TPW-0061
Substance used:	S-2399 TG, 13CG0617G, 95 %
Method of analysis:	n/a
Guideline(s):	OECD 216 (2000)
Deviations:	Yes, see HSE Comments
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

MATERIALS AND METHODS

MATERIALS

Test material S-2399 TG

Description White, solid

Batch number: 13CG0617G

Active substance content: 95.0 - 95.5% (verified by certificates of analysis)

Storage on receipt: At 4 ± 4 °C, in the dark

Expiry date July 23, 2016 (according to CoA from November 19, 2013)
June 29, 2019 (according to CoA from July 13, 2016)

TREATMENTS

Nominal test doses: Low Dose:
0.28 mg S-2399 TG/kg soil dry weight (0.27 mg a.s./kg soil dry
weight based on purity)

High Dose:
1.40 mg S-2399 TG/kg soil dry weight (1.33 mg a.s./kg soil dry
Weight based on purity)

	Concentrations derived from 200 g a.s./ha maximum application rate and five times the maximum application rate
Control:	Acetone treated quartz sand
Reference item:	Sodium Chloride (16 g/kg soil dry weight, separate study)

TEST SOIL

Test soil:	Loamy sand from a fallow grassland rented by the laboratory since 2004; no pesticides or organic or mineral fertilizer had been used on the soil for at least four years prior to test initiation
Source:	Darmstadt-Dieburg, 64380 Rossdorf, Germany, longitude [redacted] latitude [redacted]
Soil characteristics:	Clay 8.6 %, Silt 30.2 %, Sand 61.2%
pH:	6.5
Dry weight:	86.6% (nitrogen study), 85.87% (carbon study)
Total organic carbon (TOC):	0.95%
Nitrate content:	4.658 mg/kg dw (nitrogen study), 6.297 mg/kg dw (carbon study)
Ammonium content:	0.355 mg/kg dw
Nitrite content:	0.487 mg/kg dw
Total nitrogen:	0.107 %
Glucose conc. for soil respiration:	4 g/kg
Cation exchange capacity:	42 mmol Ba/kg dw
Max water holding capacity (WHC):	40.3 %
Microbial biomass (mg/kg):	292.0 mg C/kg dw (nitrogen study), 520.15 mg C/kg dw (carbon study)
Microbial biomass (% TOC):	3.1% of total organic carbon (nitrogen study), 5.48% (carbon study)

TEST DESIGN

Test units:	Plastic boxes filled up to 6 cm with perforated lids: Nitrogen study - 0.5 L (0.10 x 0.10 x 0.065 m), approximately 400 g soil (dry weight) Carbon study – 1 L (0.12 x 0.165 x 0.065 m) approximately 800 g soil (dry weight)
Replication:	3 replicates per treatment

Exposure time:	28 days (carbon and nitrogen transformation test)

TEST CONDITIONS

Test temperature:	20 ± 2 °C
Lighting:	In the dark
pH:	6.9 to 7.0 (carbon transformation) 6.6 to 6.7 (nitrogen transformation)
Water content:	51 to 53 % of WHC (carbon transformation) 45 to 49 % of WHC (nitrogen transformation) The soil water content was determined in one replicate of each treatment group of both tests at each sampling.

STUDY DESIGN AND METHODS

Experimental dates: 06 July 2016 to 01 October 2016

Soil and dose preparation

Soil batches were sampled (according to DIN 10381-6, sampling depth 0.05 to 0.2 m) and brought to the laboratory. In the laboratory it was air dried and sieved (mesh 2 mm) at room temperature. The soil was stored at 20 ± 2 °C with appropriate ventilation and periodical moisture adjustment until use. S-2399 TG was soluble in acetone. Therefore, a stock solution in acetone (21 mg S-2399 TG in 25 mL acetone) was prepared and applied onto quartz sand. After evaporation of the acetone, the quartz sand was mixed into the soil. For the nitrogen transformation test, an additional 0.5 % lucerne meal (related to soil dry weight, C:N ratio 15:1) was added. The test item was applied to the total soil amount for each treatment and soil from each treatment was divided into three replicates after application. Applications for the carbon transformation and nitrogen transformation tests were conducted separately.

To determine the amount of the test item to apply to the test system, the following assumptions were made:

- S-2399 TG is applied at 200 g a.s./ha (maximum application rate) and 1000 g a.s./ha (5 times the maximum application rate).
- The purity of the test item is 95.0%.
- The test item is applied to 1 ha (10000 m^2).
- The test item is assumed to be uniformly distributed in the top 5 cm of soil (penetration depth 0.05 m).
- The soil bulk density (ρ) is assumed to be 1.5 g dry weight of soil/cm^3. (1.5 g dry wt/cm^3 = 1.5 * 10^{-3} kg/10^{-6} m^3 = 1.5 * 10^3 kg/m^3).
- The penetrated soil volume/ha (V) is calculated to be 500 m^3/ha (500 m^3 = 0.05 m * 10,000 m^2).
- The calculated soil mass (V * ρ) is 750000 kg soil dry weight/ha.
- Concentration (mg per kg soil) = [application rate (g/ha)/soil mass (kg/ha)] x 1000

Measurements and observations

Nitrogen transformation study

The nitrate content was determined 6 hours and 7, 14 and 28 days after application, in each sample of treated and control soils. The soil (24 to 25 g) was suspended in a 100 mL 0.1 M potassium chloride solution and agitated for one hour, after which the suspensions were centrifuged and the extracts were used for nitrite and nitrate+nitrite determination. The concentrations of nitrate were calculated from measured values by subtracting the nitrite concentrations from the nitrate+nitrite concentrations. The nitrate formation rate was calculated using the difference between soil nitrate contents from two consecutive sampling dates.

Carbon transformation study

The glucose induced respiration rate was determined 6 hours and 7, 14 and 28 days after application, in each sample of treated and control soils. The soil samples (100g) were mixed with 4 g/kg glucose. The glucose amended soil samples were incubated at 20°C ± 2°C. Pressure decrease in the reaction vessels was measured up to 24 consecutive hours to calculate oxygen consumption and carbon dioxide release. The oxygen consumption was calculated by regression analysis of the linear part of the respiration curve over 12 hours (2 – 14 hours). Produced carbon dioxide was calculated using transformation factor 1.375.

Dry weight and water content of the soil were measured at each sampling date, and pH of the soil was measured at the start and end of the test. Water losses were compensated by adding pure water.

Calculation of results

Nitrogen transformation study

For the calculation of nitrate the following equation was used:

$$\frac{mg\ Nitrate}{kg\ dry\ weight} = \frac{A \times Val}{B}$$

Where:
A = Volume KCl-solution + water content extracted soil in mL
B = dry weight of extracted soil in g
Val = result of photometric determination of nitrate in mg/L

The following LOQ was determined for extracted soil: nitrate = 0.038 mg NO_3^-/kg soil dry matter

The nitrate formation rate was calculated using an incremental approach i.e. the difference between soil nitrate contents from two consecutive sampling dates. For example:

$$NO_3^-\ per\ day = \frac{NO_3^-\ (Day\ 14) - NO_3^-\ (Day\ 7)}{7\ days}$$

The amount of nitrate is presented as mg NO_3^-/kg soil dry weight.

Carbon transformation study

The amount of oxygen consumed by soil microorganisms was calculated based on the pressure decrease in the reaction vessel. For the calculation, the following equation was used:

$$SR = \frac{M(O_2)}{R \times T} \times \frac{V_{fr}}{S_{dw}} \times \Delta p$$

Where:
SR = soil respiration
$M(O_2)$ = molar weight oxygen (32000 mg/mol)
R = constant 83.144 L hPa mol^{-1} K^{-1}
T = temperature (293 °K)
V_{fr} = free volume of the reaction vessel
$S_{d.w.}$ = soil (dry weight) used for determination
Δp = change of pressure, slope of the pressure curve between 2 and 14 hours

The oxygen consumption was calculated by regression analysis of the linear part of the respiration curve over 12 hours. Produced carbon dioxide was calculated using transformation factor 1.375 (O2 → CO2). The results of the respiration test are reported as carbon dioxide produced (mg CO2/kg soil dry weight).

Statistical analysis

Data for short-term respiration and for soil nitrate contents and rate were tested for normality and homogeneity of variance using the R/S-Test (α = 0.01) and Levene´s test (α = 0.01), respectively. The Student t-test (pair wise comparison, two-sided, α = 0.05) was used for comparison of treated and control values. The software used to conduct the statistical analysis was ToxRat Professional, Version 3.2.1, ® ToxRat Solutions GmbH.

RESULTS AND DISCUSSION

Nitrate content

Statistically significant differences compared to the control were observed for the treatment group of 0.27 mg a.s./kg soil dw at day 7 and for the treatment group of 1.33 mg a.s./kg soil dw at day 7, 14 and 28. However, the differences were within the trigger range of 25% deviation to the control. Hence, no adverse effects of S-2399 TG exposure on nitrate content in soil were observed. The results are presented in the Table 9.5.2-1.

Table 9.5.2-1: Nitrate content for soil exposed to S-2399 TG for 28 days

Concentration of S-2399 TG (mg a.s./kg soil dw)	Mean nitrate levels (Day 0)		Mean nitrate levels (Day 7)		Mean nitrate levels (Day 14)		Mean nitrate levels (Day 28)	
	mg/kg soil dw[a]	% Dev. [a,b]	mg/kg soil dw [a]	% Dev. [a,b]	mg/kg soil dw [a]	% Dev. [a,b]	mg/kg soil dw [a]	% Dev. [a,b]
Control	6.107	-	1.838	-	10.372	-	26.738	-
0.27	6.151	0.72	1.513 *	-17.68	10.426	0.52	26.189	-2.05
1.33	6.044	-1.03	2.217 *	20.62	12.487 *	20.39	28.212 *	5.51

[a] Positive values indicate a stimulatory effect and negative value indicate an inhibitory effect

[b] % deviation from control

* Statistically significantly different from the control (Student-t-test, two-sided, α = 0.05)

Nitrate formation rate

Statistically significant differences compared to the control were observed for the treatment group of 0.27 mg a.s./kg soil dw at the intervals of 0 – 7 and 14 – 28 days. For the treatment group of 1.33 mg/kg soil dw statistically significant differences compared to the control were observed at all intervals. However, the differences were within the trigger range of 25% deviation to the control. Hence, no adverse effects of S-2399 TG exposure on nitrate formation rate in soil were observed. The results are presented in Table 9.5.2-2.

Table 9.5.2-2: Nitrate formation rate for soil exposed to S-2399 TG for 28 days

Concentration of S-2399 TG (mg a.s./kg soil dw)	Mean nitrate formation rate (Day 0 - 7)		Mean nitrate formation rate (Day 7 - 14)		Mean nitrate formation rate (Day 14 - 28)	
	mg/kg soil dw[a]	% Dev. [a,b]	mg/kg soil dw [a]	% Dev. [a,b]	mg/kg soil dw [a]	% Dev. [a,b]
Control	-0.610	-	1.219	-	1.169	-
0.27	-0.663 *	8.69	1.273	4.43	1.126 *	-3.68
1.33	-0.547 *	-10.33	1.467 *	20.34	1.123 *	-3.93

[a] Positive values indicate a stimulatory effect and negative value indicate an inhibitory effect

[b] % deviation from control
* Statistically significantly different from the control (Student-t-test, two-sided, α = 0.05)

For the reference material, Sodium Chloride, deviations from the control for the carbon and nitrogen studies are summarised in Table 9.5.2-3.

Table 9.5.2-3: Results from the reference test with Sodium Chloride

Results	Deviation from control	
	Day 28	Day 98
Carbon transformation test	- 71.80 %	- 65.23 %
Soil nitrate content	- 83.29 %	- 92.81 %
Soil nitrate formation rate[1]	- 100.43 %	-100.58 %

[1] incremental calculation (intervals days 14 - 28, days 86 - 98)

Validity criteria
The validity criteria for the study were met according to OECD 216 (2000) and OECD 217 (2000).

Table 9.5.2-4: Compliance with OECD 216 validity criteria

Validity criterion	Required	Obtained
Control Coefficient of Variation (CV) (Nitrogen)	≤ 15 %	0.61 to 3.66 %
Control CV (Carbon)	≤ 15 %	3.78 to 9.62 %

CONCLUSION
In this 28-day study, S-2399 TG had no adverse effect (≤ 25 % variation between the treatments and the control) on the microbial nitrogen and carbon transformation in soil exposed to S-2399 TG at 0.27 mg and 1.33 mg a.s./kg soil dw.

HSE COMMENTS
The study was carried out according to OECD 216 (2000) and OECD 217 (2000) and evaluated against OECD 216 (2000). The study was not evaluated against OECD 217 (2000) as carbon transformation is not required under data requirement 283/2013. The reasoning behind this is "*nitrate formation takes place subsequent to the degradation of carbon-nitrogen bonds. Therefore, if equal rates of nitrate production are found in treated*

and control soils, it is highly probable that the major carbon degradation pathways are intact and functional", taken from OECD 216 (2000). The validity criteria outlined in OECD 216 was satisfactorily met for the duration of the study. There were no significant deviations to the guidelines. The report included a reference material study (Sodium Chloride), which was not a requirement of OECD 216 (2000). The reference item had a retarding effect of more than ± 25% compared to the control at Day 28 and 98 after application. The results of the study demonstrate the sensitivity of the test system and adequacy of the laboratory test conditions.

The following minor deviations were noted for OECD 216 (2000):

OECD 216 (2000) § 29 states that "*particle-free liquid extracts can be stored prior to analysis at minus 20 ± 5 °C for up to six months*". Extracts were stored instead between -11 °C and -24 °C. The time period for storage between -11 °C and -14.5 °C was short (one day for a time period of 1.25 h, one day for a time period of 2.5 h). The study conductor stated that, based on laboratory experience, storage below -10°C has no influence on sample stability. Furthermore, both control and treatment samples were similarly affected. HSE considers this a minor deviation due to the brief time period that sample storage temperature deviated from the target temperature range.

Although not a deviation from the guideline, the soil concentration for the lower treatment level was estimated based on a 200 g/ha maximum application rate. The intended uses submitted to HSE use a 90 g/ha application rate. HSE considers this discrepancy acceptable as the concentration used in the study was based on an application rate higher than specified for the intended uses and is therefore more conservative.

The above study was conducted to GLP and considered valid.

The agreed endpoint suitable for use in the risk assessment is - mean nitrate formation rate (Day 14 – 28) < 25 % reduced at 1.33 mg a.s./kg soil dw

B.9.5.3 Metabolite: 3'-OH-S-2840

Reference:	KCA 8.5/02
Report Title:	3'OH-S-2840: Effects on the Activity of the Soil Microflora in the Laboratory (Nitrogen Transformation)
Author(s) & year:	[redacted] (2016b)
Document No, Authority registration No:	113861080, TPW-0046

Substance used:	3'-OH-S-2840, 15SC8508366, 99.5 %
Method of analysis:	n/a
Guideline(s):	OECD 216 (2000)
Deviations:	Yes, see HSE Comments
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test material:** 3'-OH-S-2840
   Description: White, solid
   Lot/Batch: 15SC8508366
   Purity: 99.5%
   Reference item: Sodium chloride
   Solvent: Acetone
   Carrier: Quartz sand

B. STUDY DESIGN AND METHODS

1. **Test soil:** Loamy sand from a fallow grassland rented by the laboratory since 2004; no pesticides or organic or mineral fertilizer had been used on the soil for at least four years prior to test initiation
   Soil classification: Loamy sand
   Source: Darmstadt-Dieburg, 64380 Rossdorf, Germany, longitude [redacted], latitude [redacted]
   Storage/pre-incubation: 6 days at 20 ± 2°C
   Soil characteristics: Clay 8.6 %, Silt 30.2 %, Sand 61.2%
   pH: 6.5
   Dry weight: 86.6%
   Total organic carbon: 0.95% based on soil dw
   Nitrate content: 4.658 mg/kg dw
   Ammonium content: 0.355 mg/kg dw
   Nitrite content: 0.487 mg/kg dw
   Total nitrogen: 0.107% based on soil dw
   Cation exchange capacity: 42 mmol Ba/kg dw
   Max. water holding capacity: 40.3%
   Microbial biomass: 292.0 mg C/kg dw (3.1% of total organic carbon)
   Lucerne meal: 6g per kg soil dw (C/N ratio: 15/1)
   Concentrations tested: 0.06, 0.3 mg metabolite/kg soil dw
   Duration: 28 days
   Test units: Plastic boxes with a size of 0.5 L (0.10 x 0.10 x 0.065 m)

filled up to 6 cm and covered by perforated lids

3. Environmental conditions:

A summary of the environmental conditions is shown in Table 9.5.3-1 below.

Table 9.5.3-1: Environmental conditions obtained in study of nitrogen transformation in soil dosed with 3'-OH-S-2840

Variable	Required OECD 216 (2000)	Obtained
Temperature	20 ± 2 °C	20 ± 2°C
pH of soil	5.5 - 7.5	6.6 – 6.7
Water content of soil	40 – 60 % of MWHC	45 - 49% of MWHC
Photoperiod	Continuous darkness	Continuous darkness

Study dates:
Experimental Starting Date: July 06, 2016
Experimental Completion Date: August 10, 2016

4. Soil assignment and treatment:
Soil batches were sampled, according to DIN 10381-6 with a sampling depth of 0.05 to 0.2 m and then air dried and sieved (mesh 2 mm). The soil was treated using treated quartz sand, which was mixed through the soil together with 0.5% lucerne meal (fine powdered Lucerne green grass meal, with a carbon to nitrogen ratio of 15 to 1) which was added to stimulate nitrogen transformation. The test comprised two test substance treatments of 0.06 and 0.3 mg a.s./kg soil dw. Each treatment consisted of three replicates. Additionally, a control (also with quartz sand and 0.5% lucerne meal added to it) was included in the test, which also consisted of three replicates. After application, soil samples were taken within 6 hours, and after 7, 14 and 28 days.

A reference test was conducted in a separate study with sodium chloride at a concentration of 16 g/kg soil dw.

5. Dose preparation:
A stock solution of 10 mg 3'-OH-S-2840 in 50 mL acetone was prepared and then added to quartz sand. After evaporation of the solvent, the treated sand (together with Lucerne meal) was mixed through the soil using a laboratory mixer. The nominal exposure concentrations of 0.06 and 0.3 mg a.s./kg soil dw correspond to 0.06 and 0.3 mg test item/kg soil dw (based on a purity of 99.5%). An untreated control was also prepared, containing the same amount of quartz sand (treated with acetone only) as in the test item treated groups.

6. Measurements and observations:
The nitrate content was determined within 6 hours, and after 7, 14 and 28 days in each sample of treated and control soils. The soil samples were suspended in a 0.1 M potassium chloride solution and agitated for one hour, after which the suspensions were

centrifuged and the extracts were used for nitrite and nitrate+nitrite determination.
The concentrations of nitrate were calculated from measured values by subtracting the nitrite concentrations from the nitrate+nitrite concentrations. Nitrate formation rate was calculated using the difference between soil nitrate contents from two consecutive sampling dates.

Dry weight and water content of the soil were measured at each sampling date, and pH of the soil was measured at the start and end of the test.

7. Statistics:
Data were tested for normality and homogeneity of variance using the R/S-Test (α = 0.01) and Levene's test (α = 0.01), respectively. The Student t-test (pair-wise comparison, two-sided, α = 0.05) was used for comparison of treated groups with the control group. The software ToxRat Professional, Version 3.2.1, ® ToxRat Solutions GmbH was used for all statistical analysis.

II. RESULTS AND DISCUSSION

A. NITRATE CONTENT
Statistically significant differences compared to the control were observed for the treatment group of 0.06 mg a.s./kg soil dw at day 7 and for the treatment group of 0.3 mg a.s./kg soil dw at day 7, 14 and 28. The differences were within the trigger range of 25% deviation to the control at day 28. The nitrate LoQ was 0.038 mg NO3 - /kg soil dry matter. The study duration was 28 days.

The results are presented in the table below.

Table 9.5.3-2: Nitrate content for soil exposed to 3'-OH-S-2840 for 28 days

Concentration of 3'-OH-S-2840 (mg a.s./kg soil dw)	Mean nitrate levels (Day 0)		Mean nitrate levels (Day 7)		Mean nitrate levels (Day 14)		Mean nitrate levels (Day 28)	
	mg/kg soil dw [a]	% Dev. [a,b]	mg/kg soil dw [a]	% Dev. [a,b]	mg/kg soil dw [a]	% Dev. [a,b]	mg/kg soil dw [a]	% Dev. [a,b]
Control	6.107	-	1.838	-	10.372	-	26.738	-
0.06	6.251	2.36	1.688 *	-8.16	10.493	1.17	26.826	0.33
0.3	6.244	2.24	2.650 *	44.18	13.199 *	27.26	29.808 *	11.48

[a] Positive values indicate a stimulatory effect and negative value indicate a inhibitory effect
[b] % deviation from control
* Statistically significantly different from the control (Student-t-test, two-sided, α = 0.05)

B. NITRATE FORMATION RATE
Statistically significant differences compared to the control were observed for the treatment group of 0.06 mg a.s./kg soil dw at the 0 – 7 days interval. For the treatment group of 0.3

mg a.s./kg soil dw statistically significant differences compared to the control were observed at the intervals of 0 – 7 and 7 – 14 days. The differences were within the trigger range of 25% deviation to the control and no statistically significantly differences from the control were observed at the 14 – 28 interval. The results are presented in the table below.

Table 9.5.3-3: Nitrate formation rate for soil exposed to 3'-OH-S-2840 for 28 days

Concentration of 3'-OH-S-2840 (mg a.s./kg soil dw)	Mean nitrate formation rate (Day 0 - 7)		Mean nitrate formation rate (Day 7 - 14)		Mean nitrate formation rate (Day 14 - 28)	
	mg/kg soil dw/day [a]	% Dev. [a,b]	mg/kg soil dw/day [a]	% Dev. [a,b]	mg/kg soil dw/day [a]	% Dev. [a,b]
Control	-0.610	-	1.219	-	1.169	-
0.06	-0.652 *	6.89	1.258	3.20	1.167	-0.17
0.3	-0.513 *	-15.90	1.507 *	23.63	1.187	1.54

[a] Positive values indicate a stimulatory effect and negative value indicate a inhibitory effect
[b] % deviation from control
* Statistically significantly different from the control (Student-t-test, two-sided, α = 0.05)

The reference substance sodium chloride was tested in a separate toxicity study. Significant effects were observed at the test concentration of 16 g a.s./kg soil dw, with deviations in soil nitrate content of -83.29% at day 28 and -92.81% at day 98, and deviations in soil nitrate formation rate of -100.43% at day 28 and -100.58% at day 98.

C.VALIDITY CRITERIA
As variation between replicates in the control treatment did not exceed 15% (actual: ranging from 0.61 to 3.66% over the 28 days) the validity criterion was met and the study was considered valid.

III. CONCLUSION

In this 28-day study, 3'-OH-S-2840 had no adverse effect (≤ 25 % variation between the treatments and the control) on the microbial nitrogen transformation in soil exposed to 3'-OH-S-2840 at 0.06 mg and 0.3 mg a.s./kg soil dw.

HSE comments:
This study was conducted to GLP under OECD 216 (2000) guidelines. It has been assessed against these same guidelines.

There are two deviations to protocol to note for this study:

The first protocol deviation relates to environmental temperature during the storage of soil extracts prior to analysis, which ranged from -11 to -24 ˚C and exceeds the guidelines temperatures of -20 ± 5 ˚C. The applicant has stated that the time outside of the

recommended temperature was short and should not have affected the study as samples remained frozen. HSE conclude that, as there is no evidence to show the time the samples spent out of temperature, the reasoning from the applicant cannot be supported. However, as the applicant has stated, as the samples remained frozen this deviation is unlikely to have an impact on the interpretation of results and does not invalidate the study.

It is also noted that acetone was used as the solvent. OECD 216 (2000) guidelines state that organic solvents should be avoided, as they can damage the microflora, and water or quartz should be used instead. The study report states that 3'-OH-S-2840 was soluble in acetone, so acetone solution was added to quartz sand for use in this study. After evaporation of the acetone, the quartz sand was mixed into the soil by means of a laboratory mixer. For the control, acetone treated quartz sand was mixed into the soil. HSE conclude that, whilst organic solvents should be avoided where possible, this method complies with OECD 216 (2000) guidelines for use of organic solvents and does not invalidate the study.

The use of statistics is in line with OECD 216 (2000) guidelines.

Agreed endpoint to use in risk assessment:

- **< 25% effects on nitrogen transformation at 0.3 mg met./kg soil dw**

B.9.5.4 Metabolite: 1'-COOH-S-2840

Reference:	KCA 8.5/03
Report Title:	1'COOH-S-2840: Effects on the Activity of the Soil Microflora in the Laboratory (Nitrogen Transformation)
Author(s) & year:	[redacted] (2016c)
Document No, Authority registration No:	113871080, TPW-0047
Substance used:	1'-COOH-S-2840, 16SC8508359, 100 %
Method of analysis:	n/a
Guideline(s):	OECD 216 (2000)
Deviations:	Yes, see HSE Comments
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

MATERIALS AND METHODS

MATERIALS

Test material	1'-COOH-S-2840
Description	White, solid
Batch number:	16SC8508359
Active substance content:	100 % (verified by certificates of analysis)
Storage on receipt:	At 4 ± 4 °C, in the dark
Expiry date	January 31, 2019 (according to CoA from March 31, 2016)

TREATMENTS

Nominal test doses:	Low Dose: 0.1 mg 1'-COOH-S-2840/kg soil dry weight High Dose: 0.5 mg 1'-COOH-S-2840/kg soil dry weight
Control:	Acetone treated quartz sand
Reference item:	Sodium Chloride (16 g/kg soil dry weight, separate study)

TEST SOIL

Test soil:	Loamy sand from a fallow grassland rented by the laboratory since 2004; no pesticides or organic or mineral fertilizer had been used on the soil for at least four years prior to test initiation
Source:	Darmstadt-Dieburg, 64380 Rossdorf, Germany, longitude [redacted], latitude [redacted]
Soil characteristics:	Clay 8.6 %, Silt 30.2 %, Sand 61.2%
pH:	6.5
Dry weight:	86.6%
Total organic carbon (TOC):	0.95%
Nitrate content:	4.658 mg/kg dw
Ammonium content:	0.355 mg/kg dw
Nitrite content:	0.487 mg/kg dw
Total nitrogen:	0.107 %
Cation exchange capacity:	42 mmol Ba/kg dw
Max water holding capacity (WHC):	40.3 %
Microbial biomass	292.0 mg C/kg dw (nitrogen study)

(mg/kg):	
Microbial biomass (% TOC):	3.1% of total organic carbon (nitrogen study)

TEST DESIGN

Test units:	250 g to 500 g soil (dry weight), box size approximately 0.5 L, filled up to 6 cm (dimensions: 0.10 m width x 0.10 m depth x 0.065 m height) The soil was filled loosely into the boxes, which were covered by perforated lids to allow air exchange to ensure aerobic incubation conditions.
Replication:	3 replicates per treatment
Exposure time:	28 days

TEST CONDITIONS

Test temperature:	20 ± 2 °C
Lighting:	In the dark
pH:	6.6 to 6.7
Water content:	46 to 49 % of WHC The soil water content was determined in one replicate of each treatment group of both tests at each sampling.

STUDY DESIGN AND METHODS

Experimental dates: 06 July 2016 to 10 August 2016

Soil and dose preparation

Soil batches were sampled (according to DIN 10381-6, sampling depth 0.05 to 0.2 m) and brought to the laboratory. In the laboratory it was air dried and sieved (mesh 2 mm) at room temperature. The soil was stored at 20 ± 2 °C with appropriate ventilation and periodical moisture adjustment until use. 1'-COOH-S-2840 was soluble in acetone. Therefore, a stock solution in acetone (15 mg 1'-COOH-S-2840 in 50 mL acetone) was prepared and applied onto quartz sand. After evaporation of the acetone, the quartz sand was mixed into the soil. An additional 0.5 % lucerne meal (related to soil dry weight, C:N ratio 15:1) was added. The test item was applied to the total soil amount for each treatment and soil from each treatment was divided into three replicates after application.

The concentration of 1'-COOH-S-2840 in soil was calculated using following parameters: the maximum PEC_{soil} of the parent S-2399 TG = 0.27 mg a.s./kg soil (corresponding to 200 g a.s./ha); the molecular mass ratio = 1.09; and the maximum occurrence of the metabolite in soil = 30.6%. Therefore, the PEC_{soil} of 1'-COOH-S-2840 in this study was determined to be 0.09 mg 1'-COOH-S-2840/kg soil. According to the guidance given by OECD 216 test guideline, the test concentrations will be the maximum estimated PEC_{soil} and 5 times the maximum estimated PEC_{soil}, considering the purity of the test item of 100%.

Measurements and observations

The nitrate content was determined 6 hours and 7, 14 and 28 days after application, in each sample of treated and control soils. The soil (24 to 25 g) was suspended in a 100 mL 0.1 M potassium chloride solution and agitated for one hour, after which the suspensions were centrifuged and the extracts were used for nitrite and nitrate+nitrite determination. Nitrate content was determined using a AA3 Continuous Flow Analyzer. The Limit of Quantification (LOQ) was 0.038 mg NO_3^-/kg soil dry matter. The concentrations of nitrate were calculated from measured values by subtracting the nitrite concentrations from the nitrate+nitrite concentrations. The nitrate formation rate was calculated using the difference between soil nitrate contents from two consecutive sampling dates.

Dry weight and water content of the soil were measured at each sampling date, and pH of the soil was measured at the start and end of the test. Water losses were compensated by adding pure water.

Calculation of results

Nitrogen transformation study

For the calculation of nitrate the following equation was used:

$$\frac{mg\ Nitrate}{kg\ dry\ weight} = \frac{A \times Val}{B}$$

Where:
A = Volume KCl-solution + water content extracted soil in mL
B = dry weight of extracted soil in g
Val = result of photometric determination of nitrate in mg/L

The following LOQ was determined for extracted soil: nitrate = 0.038 mg NO_3^-/kg soil dry matter

The nitrate formation rate was calculated using an incremental approach i.e. the difference between soil nitrate contents from two consecutive sampling dates. For example:

$$NO_3^-\ per\ day = \frac{NO_3^-\ (Day\ 14) - NO_3^-\ (Day\ 7)}{7\ days}$$

The amount of nitrate is presented as mg NO_3^-/kg soil dry weight.

Statistical analysis

Data for soil nitrate contents and rate were tested for normality and homogeneity of variance using the R/S-Test (α = 0.01) and Levene´s test (α = 0.01), respectively. The Student t-test

(pair wise comparison, two-sided, α = 0.05) was used for comparison of treated and control values. The software used to conduct the statistical analysis was ToxRat Professional, Version 3.2.1, ® ToxRat Solutions GmbH.

RESULTS AND DISCUSSION

Nitrate content

Statistically significant difference compared to the control was only observed for the treatment group of 0.1 mg a.s./kg soil dw at day 14. However, the difference was within the trigger range of 25% deviation to the control and no statistically significant difference was observed at day 28. Hence, no adverse effects of 1'-COOH-S-2840 exposure on nitrate content in soil were expected. The results are presented in Table 9.5.4-1.

Table 9.5.4-1: Nitrate content for soil exposed to 1'-COOH-S-2840 for 28 days

Concentration of 1'COOH-S-2840 (mg a.s./kg soil dw)	Mean nitrate levels (Day 0)		Mean nitrate levels (Day 7)		Mean nitrate levels (Day 14)		Mean nitrate levels (Day 28)	
	mg/kg soil dw [a]	% Dev. [a,b]	mg/kg soil dw [a]	% Dev. [a,b]	mg/kg soil dw [a]	% Dev. [a,b]	mg/kg soil dw [a]	% Dev. [a,b]
Control	6.107	-	1.838	-	10.372	-	26.738	-
0.1	6.193	1.41	1.976	7.51	11.047 *	6.51	27.490	2.81
0.5	6.031	-1.24	1.717	-6.58	10.775	3.89	26.578	-0.60

[a] Positive values indicate a stimulatory effect and negative value indicate an inhibitory effect
[b] % deviation from control
* Statistically significantly different from the control (Student-t-test, two-sided, α = 0.05)

Nitrate formation rate

No statistically significant differences compared to the control were observed for the treatment groups. Hence, no adverse effects of 1'-COOH-S-2840 exposure on nitrate formation rate in soil were expected. The results are presented in Table 9.5.4-2.

Table 9.5.4-2: Nitrate formation rate for soil exposed to 1'-COOH-S-2840 for 28 days

Concentration of 1'-COOH-S-2840 (mg a.s./kg soil dw)	Mean nitrate formation rate (Day 0 - 7)		Mean nitrate formation rate (Day 7 - 14)		Mean nitrate formation rate (Day 14 - 28)	
	mg/kg soil dw/day [a]	% Dev. [a,b]	mg/kg soil dw/day [a]	% Dev. [a,b]	mg/kg soil dw/day [a]	% Dev. [a,b]
Control	-0.610	-	1.219	-	1.169	-
0.1	-0.603	-1.15	1.296	6.32	1.175	0.51
0.5	-0.616	0.98	1.294	6.15	1.129	-3.42

[a] Positive values indicate a stimulatory effect and negative value indicate an inhibitory effect
[b] % deviation from control
* Statistically significantly different from the control (Student-t-test, two-sided, α = 0.05)

For the reference material, Sodium Chloride, deviations from the control are summarised in Table 9.5.4-3.

Table 9.5.4-3: Results from the reference test with Sodium Chloride

Results	Deviation from control	
	Day 28	Day 98
Soil nitrate content	- 83.29 %	- 92.81 %
Soil nitrate formation rate[1]	- 100.43 %	-100.58 %

[1] incremental calculation (intervals days 14 - 28, days 86 - 98)

Validity criteria
The validity criteria for the study were met according to OECD 216 (2000).

Table 9.5.4-4: Compliance with OECD 216 validity criteria

Validity criterion	Required	Obtained
Control Coefficient of Variation (CV)	≤ 15 %	0.61 to 3.66 %

CONCLUSION
In this 28-day study, 1'-COOH-S-2840 had no adverse effect (≤ 25 % variation between the

treatments and the control) on the microbial nitrogen transformation in soil exposed to 1'-COOH-S-2840 at 0.1 mg and 0.5 mg a.s./kg soil dw.

HSE COMMENTS
The study was carried out according and evaluated against OECD 216 (2000). The validity criteria outlined in OECD 216 was satisfactorily met for the duration of the study. There were no significant deviations to the guidelines. The report included a reference material study (Sodium Chloride), which was not a requirement of OECD 216 (2000). The reference item had a retarding effect of more than ± 25% compared to the control at Day 28 and 98 after application. The results of the study demonstrate the sensitivity of the test system and adequacy of the laboratory test conditions.

The following minor deviations were noted for OECD 216 (2000):

OECD 216 (2000) § 29 states that "*particle-free liquid extracts can be stored prior to analysis at minus 20 ± 5 °C for up to six months*". Extracts were stored instead between -11 °C and -24 °C. HSE considers this a minor deviation due to the temperature remaining below -10 °C at all times.

Although not a deviation from the guideline, the concentration of 1'-COOH-S-2840 in soil was calculated using a S-2399 TG PEC_{soil} (0.27 mg a.s./kg soil) ten times higher than the value quoted elsewhere in the submission pack (0.027 mg a.s./kg soil). This difference may have stemmed from the different application rates used to model the PEC_{soil} (200 vs 90 g a.s./ha). Alternatively, the PEC_{soil} may have been multiplied by ten to cover all scenarios. Either way, the PEC_{soil} selected was conservative and considered protective by HSE.

The above study was conducted to GLP and considered valid.

The agreed endpoint suitable for use in the risk assessment is - mean nitrate formation rate (Day 14 – 28) < 25 % reduced at 0.5 mg 1'-COOH-S-2840/kg soil dw

B.9.6 Effects on Terrestrial non-target higher plants

No studies were submitted for non-target higher plants conducted with the active substance as a test item, in line with the data requirement. Non-target higher plant studies with the formulation are available in the B9 3CP.

B.9.6.1 Summary of screening data

No studies submitted.

B.9.6.2 Testing on non-target plants

No studies submitted.

B.9.7 Effects on other Terrestrial organisms (Flora and Fauna)

No studies submitted.

B.9.8 Effects on Biological methods for sewage treatment

The endpoint for use in the activated sludge risk assessment is summarised below:

Table B.9.8-1: Endpoint for activated sludge exposed to inpyrfluxam

Species	Test system	Test substance	Endpoint	Results	References
Activated sludge microorganisms	3-hour aeration	S-2399 TG	EC_{50}	>100mg/L	[redacted] (2018)

Reference:	KCA 8.8/01
Report Title:	S-2399 –Activated Sludge Respiration Inhibition Test
Author(s) & year:	[redacted] (2018)
Document No, Authority registration No:	Smithers Viscient Study No. 13048.7112, TPW-0088
Substance used:	S-2399 TG, 13CG0617G, 95 %
Method of analysis:	n/a
Guideline(s):	OECD 209
Deviations:	Yes, see HSE Comments
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

I. MATERIALS AND METHODS

A.MATERIALS

1. Test material: S-2399 TG
Description: Not stated
Lot/Batch: 13CG0617G
Purity: 95.5%

2. Reference item: 3,5-Dichlorophenol
Description: Not stated
Lot/Batch: E20W012
Purity: 97.6%

B.STUDY DESIGN AND METHODS

1. Test organisms: Activated sludge microorganisms
Source: Wareham Waste Water Treatment Facility, Wareham, USA
Acclimation: Prior to use, sludge was fed with 200 mL synthetic sewage feed and kept aerated overnight at 20 ± 2°C

2. Test vessels: 1-L glass beakers

3.Environmental conditions:
A summary of the environmental conditions obtained in this study are shown in Table 9.8 - 2.

Table 9.8 - 2: Environmental conditions in the study of S-2399 TG on activated sludge

Variable	Required OECD 209 (2010)	Obtained
Temperature	20±2°C.	20 ± 2°C
pH	7.5 ± 0.5	7.47 (abiotic control), 7.92 - 7.99 (remaining samples)
Aeration	0.5 to 1 L/min	0.636 to 0.940 L/min

Study dates: 2 November 2018

4. Procedure:
The final inoculum contained a solids level of 3.06 g/L.

Three controls were tested in parallel, one abiotic control (synthetic sewage feed, laboratory well water with no microbial inoculum) and two controls (synthetic sewage feed, laboratory well water and microbial inoculum).

In parallel to the study with the test item, the reference item 3,5-dichlorophenol was tested at the nominal test concentrations 1.0, 3.0, 10 and 30 mg/L under otherwise identical test conditions.

For each replicate a test solution with a final volume of 500 mL was tested per treatment in a 1-L glass beaker. At time zero, 16 mL of synthetic sewage feed and an appropriate amount of test item or an appropriate volume of stock solution of the reference item were filled to 250 mL with laboratory well water. An aliquot of 250 mL of the microbial inoculum was added until the solution was at full volume (500 mL). Each beaker was aerated for three hours.

5. Dose preparation:

Appropriate amounts of the test item were directly weighed into the test vessels. The nominal test concentrations were 0.1, 10 and 100 mg test item/L.

To test the reference item, a stock solution of 3,5-dichlorophenol was prepared by placing 0.2562g (0.2501g active substance) of 3,5-dichlorophenol in 5.0 mL of 1 N sodium hydroxide (NaOH) and adding 10 mL purified reagent water. 2.0 mL of 1.0 N sulfuric acid (H_2SO_4) was added until a precipitate was observed. The solution transferred to a 500 mL volumetric flask and brought to volume with purified reagent water. The final pH was adjusted to 7.84 with 1 N sulfuric acid (H_2SO_4).

6. Measurements and observations:

For the measurement of the respiration rate, 23 mL of each test medium was transferred to a 50 mL cylinder and placed inside a 20 ± 1°C water bath placed over a stir plate. The cylinders received a stir bar and were fitted with an oxygen probes after receiving the sample. The samples were mixed continuously while oxygen measurements were taken. The oxygen consumption (in mg O_2/L) was determined from the most linear part of the respiration curve over a period of up to 10 minutes or in the range of 6.5 and 2.5 mg O_2/L.

The temperature of the environmental chamber was measured using a minimum/maximum thermometer. The pH was determined in each test vessel.

7. Statistics:

ECx values were derived by linear regression using Excel software. A paired t-test was conducted to compare respiration rates in the control and the highest concentration.

II. RESULTS AND DISCUSSION

A. BIOLOGICAL EFFECTS

A summary of the effects of S-2399 on respiration rate are presented in Table 9.8 - 3 below.

Table 9.8 - 3:Effects of S-2399 technical and the reference item 3, 5-dichlorophenol on respiration rate of activated sludge

Sample type	Nominal concentration (mg test item/L)	Respiration rate (mg O_2/L/hr)	Inhibition (%)[a]
Abiotic control	-	-1.6	n.a.
Control 1	-	65.6	n.a.
Control 2	-	58.6	n.a.
Mean (mg O_2/L/hr)		62.1	
Mean (mg O_2/g/hr)		41.4	

Sample type	Nominal concentration (mg test item/L)	Respiration rate (mg O_2/L/hr)	Inhibition (%)[a]
Test item (S-2399 Technical)	1.0	53.2	14.3
	10	46.9	24.5
	100	68.3	-10.0
	100	50.1	19.3
	100	44.9	27.7
EC_{10}, EC_{20}, EC_{50} & EC_{80}	>100 mg/L		
NOEC	100 mg/L		
3,5-dichlorophenol	1.0	45.2	27.2
	3.0	54.8	11.8
	10	27.2	56.2
	30	5.2	91.6
EC_{50}	6.4mg/L		

[a] Percent inhibition values were calculated with respiration rates adjusted with the abiotic control
n.a. = not applicable

B. VALIDITY CRITERIA

The oxygen uptake is greater than 20 mg O_2/g/hr (actual: 41.4 mg O_2/g/h, based on two controls) and the coefficient of variation of oxygen uptake in the control replicates should not be more than 30% at the end of the definitive test (actual: 19.5%).

The 3-hour EC_{50} of the reference item 3,5-dichlorophenol was 6.4 mg/L and therefore within the recommended range of 2 to 25 mg/L for total respiration, according to OECD 209 (2010).

II. CONCLUSION

As there were no statistically significant differences between the controls and three highest doses the EC_{10}, EC_{20}, EC_{50} and EC_{80} values were empirically estimated to be >100mg/L. The NOEC was also empirically estimated and set to 100 mg/L; the highest concentration tested.

HSE comments:
This study was conducted to GLP under OECD 209 (2010) guidelines. It has been assessed against these same guidelines.

For a definitive test, OECD 209 (2010) guidelines recommend that the test is conducted with six controls and 5 different test concentrations over a geometric series. This study used only three controls and 3 concentrations (over 5 groups). It is also not clear whether each of the groups had replicates (5 replicates per group are recommended in OECD 209, 2010 guidelines). Following an RAI, the applicant clarified that the test process had been conducted as a range-finding test in line with OECD 209 (2010) guidelines; therefore, triplicates of the highest tested concentration were used. However, it is noted that the

highest tested concentration (100 mg test item/L) is below the recommended concentration of 1000 mg/L.

It is noted that the preparation of the reference item differs slightly to the OECD 209 (2010) guidelines, but the resulting pH is in line with recommendations.

It is also noted that the percentage inhibition and respiration rate of the first 100 mg test item/L group resulted in anomalous results compared to the other two groups at this concentration. The applicant was asked to provide statistical analysis without the anomalous result in an RAI, and the response shows no effect at highest test concentration. The guideline says, *"If no statistically significant toxic effect occurs at this concentration, further testing at higher or lower concentrations is not necessary".* As there were no statistically significant effects at 100 mg a.s./L, HSE consider that it would not be necessary to test again at 1000 mg a.s./L. Less than 50% effects are clearly demonstrated for all replications of the 100 mg a.s./L group, but more replicates would be useful and reduced statistical power is noted.

The use of statistics is suitable for this study.
The agreed endpoints are:

- **3-hour EC_{50} = >100mg/L**
- **3-hour NOEC = >100mg/L**

B.9.9 Monitoring Data

No studies submitted.

B.9.10 Biological activity of metabolites potentially occurring in groundwater

B.9.10.1 Literature review summary

Article 8(5) of Regulation (EC) No 1107/2009 requires that applicants submitting dossiers for the approval of active substances of plant protection products under Regulation (EC) No 1107/2009 shall provide "*Scientific peer-reviewed open literature, […], on the active substance and its relevant metabolites dealing with side-effects on health, the environment and non-target species and published within the last ten years before the date of submission of the dossier…*". A literature review, fulfilling data points KCA Section 9 and KCP Section 11 of COMMISSION REGULATION (EU) No 283/2013 and COMMISSION REGULATION (EU) No 284/2013 respectively, was provided by the applicant. This review, conducted to comply with EFSA Guidance (2011)[52], is summarised below.

[52] European Food Safety Authority; Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009 (OJ L 309, 24.11.2009, p. 1-50). EFSA Journal 2011;9(2):2092. [49 pp.]. doi:10.2903/j.efsa.2011.2092. Available online: www.efsa.europa.eu

Database and search term selection and results retrieval

The literature review included inpyrfluxam (S-2399) and its known metabolites: 1'-COOH-S-2840, NDM-1'-COOH-S-2840, 3'-OH-S-2840, 1'-CH2OH-S-2840, DFPA, DFPA-CONH2, N-des-Me-S-2840, N-des-Me-1'-CH2OH-S-2840, N-des-Me-DFPA, and 1',1'-bis(CH2OH)-S-2840. A single-concept search strategy was selected using two database providers, STN and Dialog databases, which together included 40 databases (MEDLINE appeared in both database collections). A list of the selected databases is presented in Table 9.10.1-1.

Table 9.10.1-1: List of databases included in search

Databases	Frequency of updates
STN databases:	
ANABSTR (Analytical abstracts)	Updated weekly
BIOSIS (BIOSIS PREVIEWS®)	Updated weekly
BIOTECHNO (Elsevier Biotechno base)	Not updated: Closed in 2003
CABA (CA Abstracts)	Updated weekly
CAplus (Chemical abstracts plus)	Updated daily
CAplus (Toxicology focus)	Updated daily
CAplus (Analytical chemistry focus)	Updated daily
Chemical Abstracts REGISTRY	Updated daily
CROPU (Crop Protection)	Not updated: Closed in 2003
EMBASE (Excerpta Medica)	Updated daily
ESBIOBASE (Elsevier Current Research in Biology and BioScience)	Updated weekly
KOSMET (Cosmetic & Perfume Science & Technology)	Updated monthly
MEDLINE	Updated daily
NAPRALERT (Natural Products Alert)	Occasional updates
PASCAL (INIST's French National Research Council File)	Updated weekly
RTECS (Registry of Toxic Effects of Chemical Substances)	Updated quarterly
SCISEARCH (Science Citation Index)	Updated weekly
TOXCENTER (Toxicology Center produced by American Chemical Society CAS)	Updated weekly

Databases	Frequency of updates
STN databases:	
DIALOG Databases:	All PROQUEST databases are current and updated regularly
AGRICOLA	
AGRIS	
Aqualine	
Aquatic Science & Fisheries Abstracts (ASFA)	
Chemical Engineering and Biotechnology Abstracts	
CSA Life Sciences Abstracts	
Ecology Abstracts	
ENVIROLINE®	
Environment Abstracts	
Environmental Engineering Abstracts	
Environmental Sciences	
FLUIDEX	
FSTA®	
FOODLINE®	
GeoArchive	
GEOBASE	
MEDLINE®	
Meteorology & Geoastrophysical Abstracts.	
Pollution Abstracts	
ToxFile	
Toxicology Abstracts	
TOXLINE	
Water Resources Abstracts	

The single concept search, containing only inpyrfluxam, its synonyms and metabolites, allowed for the searching of open literature relevant to multiple data requirements. The main data categories of relevance, as given in Regulation (EC) No 1107/2009, for which scientific peer-reviewed open literature was searched for included:

1) Data requirements on chemical active substances (Commission Regulation (EU) No. 283/2013):
   a) Toxicological and metabolism studies on the active substance (CA Section 5)
   b) Residues in or on treated products, food and feed (CA Section 6)
   c) Fate and behaviour in the environment (CA Section 7)
   d) Ecotoxicological studies on the active substance (CA Section 8)
   e) Other data requirements for which information may have a direct or indirect effect on overall risk assessment (CA Sections 1, 2, 3 and 4) (only data requirements under these points having a direct impact on the risk assessment need to be considered)
2) Data requirements on plant protection products based on chemical preparations (Commission Regulation (EU) No. 284/2013):
   a) Toxicological studies on the plant protection product (CP Section 7)
   b) Residues in or on treated products, food and feed (CP Section 8)
   c) Fate and behaviour in the environment (CP Section 9)
   d) Ecotoxicological studies on the plant protection product (CP Section 10)
   e) Other data requirements for which information may have a direct or indirect effect on the overall risk assessment (CP Sections 1, 2, 3, 4, and 5) (only data requirements under these points having a direct impact on the risk assessment need to be considered)

Due to the broad nature of the open literature search, not all databases selected were directly related to ecotoxicology. Despite this, many databases covering ecotoxicology, biology, ecology, life sciences and agriculture were selected. A detailed justification and description of each database was provided in the literature review report.

The search terms used for each database provider, including the number of records retrieved are presented in Table 9.10.1-2.

Table 9.10.1-2: Reporting/Overview of the search process for scientific peer-reviewed open literature in bibliographic databases

	STN Toxicology Database Cluster	**Dialog**
Justification for choice of the database:	Provided for each database in the study report	
Date of the search:	21 September 2018	21 September 2018
Date of the latest database update	See Table 9.10.1-1	See Table 9.10.1-1

	STN Toxicology Database Cluster	Dialog
included in the search:		
Search strategies	(i) 1. RN: 1352994-67-2 2. AND Publication Year: 2008-current 3. NOT Document Type: conference 4. NOT Document Type: patent 5. 1H-Pyrazole-4-carboxamide, 3-(difluoromethyl)-N-[(3R)-2,3-dihydro-1,1,3-trimethyl-1H-inden-4-yl]-1-methyl- (CA INDEX NAME) 3-(Difluoromethyl)-N-[(3R)-2,3-dihydro-1,1,3-trimethyl-1H-inden-4-yl]-1-methyl-1H-pyrazole-4-carboxamide 3-(Difluoromethyl)-1-methyl-N-((3R)-1,1,3-trimethyl-2,3-dihydro-1H-inden-4-yl)-1H-pyrazole-4-carboxamide 3-(Difluoromethyl)-1-methyl-N-(1,1,3-trimethyl-2,3-dihydro-1H-inden-4-yl)-1H-pyrazole-4-carboxamide 3-(Difluoromethyl)-1-methyl-N-[(3R)-1,1,3-trimethyl-2,3-dihydro-1H-inden-4-yl]-1H-pyrazole-4-carboxamide 6. Inpyrfluxam 7. C18 H21 F2 N3 O	(i) 1. Inpyrfluxam OR INDIFLIN OR “S-2399” OR “3-(Difluoromethyl)-1-methyl-N-[(3R)-1,1,3-trimethyl-2,3-dihydro-1H-inden-4-yl]-1H-pyrazole-4-carboxamide” OR “3-(Difluoromethyl)-N-[(3R)-2,3-dihydro-1,1,3-trimethyl-1H-inden-4-yl]-1-methyl-1H-pyrazole-4-carboxamide” OR “1’-COOH-S-2840” OR “NDM-1’-COOH-S-2840” OR “3’-OH-S-2840” OR “1’-CH2OH-S-2840” OR DFPA OR “DFPA-CONH2” OR “N-des-Me-S-2840” OR “N-des-Me-1’-CH2OH-S-2840” OR “N-des-Me-DFPA” OR “1’,1’-bis(CH2OH)-S-2840 2. AND (pd(>20080901)) 3. AND (at.exact("Article" OR "Book Chapter" OR "Government & Official Document" OR "Case Study" OR "Technical Report" OR "Report"))
Search limitations	The time period was limited to studies published September 2008 to September 2018. Patents and conference papers were excluded.	

	STN Toxicology Database Cluster	Dialog
Number of summary records retrieved after removing duplicates	0	18
Total number of summary records retrieved after removing duplicates	18	

Relevancy criteria for ecotoxicology and rapid screen

As part of the determination of relevancy, the following criteria were considered to be fundamental by the applicant when considering the relevance of an open-literature study:

- Studies should address the data requirements detailed in Commission Regulations (EU) No. 283/2013 and 284/2013.
- Studies need to be performed with defined test material which is the appropriate active substance, metabolite or plant protection product.
- The test species in laboratory studies should preferably be relevant to the EU. However, studies which contain test species which are not found in the EU may in some cases be deemed relevant by expert judgement.
- Both the route of exposure and length of exposure of the test material should be appropriate. Studies which have exposure which is either too long or too short or via an inappropriate route are not considered to be relevant. Studies with *in vivo* or *ex vivo* exposure are considered relevant; *in vitro* tests may potentially be relevant and should be considered appropriately.
- Toxicity modelling (e.g. QSAR), literature review papers, meta-analysis papers, risk analysis papers and environmental monitoring papers are generally considered to not be relevant.
- Apart from mixture toxicity, other multi-stressor studies e.g. active substance and physic-chemical stress, are not considered to be relevant.
- Field studies should be performed to conditions which are relevant to the EU e.g. climate, crop species, test species.

The 18 studies retrieved from all databases searched underwent a rapid assessment for relevance, where their titles and, if available, abstracts were screened against the above relevancy criteria. The results of the literature review are presented in Table 9.10.1-3.

Table 9.10.1-3: Summary of results from each stage of the literature review

Total number of summary records retrieved after removing duplicates from all database searches	**18**
Number of summary records excluded after rapid assessment for relevance (by title/abstract)	18
Number of summary records of potential/unclear relevance assessed in further detail (by abstract/full-text)	0
Number of studies excluded from further consideration after detailed assessment for relevance (by abstract/full-text)	0
Number of studies not excluded for relevance after detailed assessment (i.e. relevant studies and studies of unclear relevance)	0
Number of relevant and reliable studies (Klimisch criteria 1-2) identified by the literature search and appraisal process	0

All retrieved studies were excluded at the rapid assessment stage. Due to the low number of total retrieved studies, an explanation was provided for each study exclusion. It was clear from titles and abstracts that none of the retrieved studies were related to inpyrfluxam. The majority of matches related to alternative uses of the DFPA acronym.

As no studies were retained after the rapid screening assessment stage, no detailed relevance or reliability assessments were performed.

Conclusion

A review of the published literature for Inpyrfluxam (S-2399) identified no articles that were both relevant and reliable with respect to the regulatory data package.

HSE Comments

Overall, HSE found the submitted literature review to be acceptable and in keeping with EFSA Guidance (2011). The breadth of relevant databases included resulted in a comprehensive literature pool to search. The search strategy and term were considered acceptable by HSE, with several minor alterations suggested by HSE below.

HSE identified one deviation relating to the literature review search dates. Searches of the two database providers were performed on 21 September 2018, to cover September 2008

to September 2018. Literature searches are required to be performed, or updated, within 6 months prior to the dossier submission date. HSE requested this updated search through an RAI (see RAI response section below).

HSE identified three deviations that may have impacted the breadth of the search term: 1) the applicant did not include CAS numbers for the active substance and metabolites, if available, in the search term, which may have reduced the number of matches; 2) one of the search limitations was the exclusion of conference papers. EFSA Guidance (2011) explicitly mentions conference proceedings (papers) as a potential valid form of scientific peer-review open literature. HSE would have preferred the inclusion of conference proceedings (papers) in the search term. HSE acknowledges, however, that it is often unclear from conference proceedings (papers) the degree of peer review, if any, that has been performed; and 3) the applicant did not outline how the metabolites included in the search term were selected (i.e. deemed relevant). EFSA Guidance (2011) states, "*for the purposes of this EFSA Guidance the scientific literature search should focus on metabolites, degradation products, or transformation products of an active substance formed either in organisms or in the environment, for which further assessment is required according to the data requirements and the Guidance documents applicable at the time of submitting the dossier*". A description of how each metabolite met the relevance criteria detailed in the above passage, and if all relevant metabolites were included in the literature review search term, is required. HSE will request these alterations and clarifications through an RAI.

There were several remaining inconsequential deviations from the guidance discussed below for completeness.

These relate to the specification of the relevancy criteria: 1) the relevancy criteria state that species selected in laboratory tests should preferably be endemic to the EU. GB is no longer part of the EU. In this case, EU should be interpreted as European. Even so, it is not appropriate to exclude species based on their geographic distribution as they still provide information on the overall distribution of species sensitivities; 2) when discussing the length of exposure, the applicant stated that exposure in relevant studies would be of an appropriate length, i.e. not too long or too short. No further detail was provided on what constitutes too long or too short exposure. It would have been preferable to provide specific timeframes for each organism group based on their biology and routes of exposure.; 3) literature reviews and meta-analyses were not considered relevant despite EFSA Guidance (2011) highlighting reviews (and by extension meta-analyses) as an alternative source of scientific peer-reviewed open literature other than databases; and 4) the relevancy criteria states that the test species of any field studies should be relevant to the EU. As with point 1 of this list, the GB (+NI) is the relevant geographical area. Furthermore, field studies conducted in similar climates that select UK relevant crops may still be informative to risk assessment despite species or community differences.

HSE notes that none of the aforementioned relevancy criteria deviations had any impact on the literature review outcome. All studies excluded at the rapid assessment stage did not select inpyrfluxam, its formulation or any of its metabolites as a test material, which made more specific relevancy criteria unnecessary. For this reason, HSE accepts the original relevancy criteria without amendment from the applicant.

RAIs and response:
The applicant submitted an up-to-date literature review report covering July 2013 up to July 2023. This covered the 10 years prior to the submission of the dossier for Inpyrfluxam (S-2399). The search term was updated to include common names and systematic nomenclature for all possible metabolites, regardless of their relevance. HSE Ecotoxicology agrees with this comprehensive approach. The full search term is included in the table below**.**

Table 9.10.1-4: Search term for updated literature review

Common name	Synonyms	Search engine	Fields searched
Inpyrfluxam	(*R*)-3-(difluoromethyl)-1-methyl-*N*-(1,1,3-trimethyl-2,3-dihydro-1*H*-inden-4-yl)-1*H*-pyrazole-4-carboxamide 1352994-67-2 S-2399	CAS (Sci-finder n) Proquest Dialogue	Full Text
3'-OH-S-2840	3-(difluoromethyl)-*N*-(3-hydroxy-1,1,3-trimethyl-2,3-dihydro-1*H*-inden-4-yl)-1-methyl-1*H*-pyrazole-4-carboxamide	CAS (Sci-finder n) Proquest Dialogue	Full Text

Common name	Synonyms	Search engine	Fields searched
1'-CH_2OH-S-2840	3-(difluoromethyl)-N-((1R,3S)-1-(hydroxymethyl)-1,3-dimethyl-2,3-dihydro-1H-inden-4-yl)-1-methyl-1H-pyrazole-4-carboxamide 3-(difluoromethyl)-N-((1S,3R)-1-(hydroxymethyl)-1,3-dimethyl-2,3-dihydro-1H-inden-4-yl)-1-methyl-1H-pyrazole-4-carboxamide	CAS (Sci-finder n) Proquest Dialogue	Full Text
1'-CH_2OH-S-2840	3-(difluoromethyl)-N-((1R,3R)-1-(hydroxymethyl)-1,3-dimethyl-2,3-dihydro-1H-inden-4-yl)-1-methyl-1H-pyrazole-4-carboxamide 3-(difluoromethyl)-N-((1S,3S)-1-(hydroxymethyl)-1,3-dimethyl-2,3-dihydro-1H-inden-4-yl)-1-methyl-1H-pyrazole-4-carboxamide	CAS (Sci-finder n) Proquest Dialogue	Full Text
N-des-Me-S-2840	3-(difluoromethyl)-*N*-(1,1,3-trimethyl-2,3-dihydro-1*H*-inden-4-yl)-1*H*-pyrazole-4-carboxamide	CAS (Sci-finder n) Proquest Dialogue	Full Text
N-des-Me-1'-CH_2OH-S-2840	3-(difluoromethyl)-N-((1R,3S)-1-(hydroxymethyl)-1,3-dimethyl-2,3-dihydro-1H-inden-4-yl)-1H-pyrazole-4-carboxamide	CAS (Sci-finder n)	Full Text

Common name	Synonyms	Search engine	Fields searched
	3-(difluoromethyl)-N-((1S,3R)-1-(hydroxymethyl)-1,3-dimethyl-2,3-dihydro-1H-inden-4-yl)-1H-pyrazole-4-carboxamide	Proquest Dialogue	
N-des-Me-1'-CH_2OH-S-2840	3-(difluoromethyl)-N-((1R,3R)-1-(hydroxymethyl)-1,3-dimethyl-2,3-dihydro-1H-inden-4-yl)-1H-pyrazole-4-carboxamide 3-(difluoromethyl)-N-((1S,3S)-1-(hydroxymethyl)-1,3-dimethyl-2,3-dihydro-1H-inden-4-yl)-1H-pyrazole-4-carboxamide	CAS (Sci-finder n) Proquest Dialogue	Full Text
7'-OH-S-2399	(*R*)-3-(difluoromethyl)-*N*-(7-hydroxy-1,1,3-trimethyl-2,3-dihydro-1*H*-inden-4-yl)-1-methyl-1*H*-pyrazole-4-carboxamide	CAS (Sci-finder n) Proquest Dialogue	Full Text
1'-CH_2OH-3'-OH-S-2840	*N*-[(1*RS*,3*RS*;1*RS*,3*SR*)-2,3-dihydro-1,3-dimethyl-3-hydroxy-1-(hydroxymethyl)-1*H*-inden-4-yl)]-1-methyl-3-(difluoromethyl)-1*H*-pyrazole-4-carboxamide	CAS (Sci-finder n) Proquest Dialogue	Full Text

Common name	Synonyms	Search engine	Fields searched
glucuronide of 1'-CH_2OH-3'-OH-S-2840	Glucuronide of *N*-[(1*RS*,3*RS*;1*RS*,3*SR*)-2,3-dihydro-1,3-dimethyl-3-hydroxy-1-(hydroxymethyl)-1*H*-inden-4-yl)]-1-methyl-3-(difluoromethyl)-1*H*-pyrazole-4-carboxamide	CAS (Sci-finder n) Proquest Dialogue	Full Text
ATMI	(*R*)-1,1,3-trimethyl-2,3-dihydro-1*H*-inden-4-amine 125349-37-3	CAS (Sci-finder n) Proquest Dialogue	Full Text
1'-COOH-S-2840	(1S,3R)-4-(3-(difluoromethyl)-1-methyl-1H-pyrazole-4-carboxamido)-1,3-dimethyl-2,3-dihydro-1H-indene-1-carboxylic acid (1R,3S)-4-(3-(difluoromethyl)-1-methyl-1H-pyrazole-4-carboxamido)-1,3-dimethyl-2,3-dihydro-1H-indene-1-carboxylic acid	CAS (Sci-finder n) Proquest Dialogue	Full Text
1'-COOH-S-2840	(1R,3R)-4-(3-(difluoromethyl)-1-methyl-1H-pyrazole-4-carboxamido)-1,3-dimethyl-2,3-	CAS (Sci-finder n)	Full Text

Common name	Synonyms	Search engine	Fields searched
	dihydro-1H-indene-1-carboxylic acid (1S,3S)-4-(3-(difluoromethyl)-1-methyl-1H-pyrazole-4-carboxamido)-1,3-dimethyl-2,3-dihydro-1H-indene-1-carboxylic acid	Proquest Dialogue	
1',1'-bis(CH_2OH)-S-2840	*N*-(1,1-bis(hydroxymethyl)-3-methyl-2,3-dihydro-1*H*-inden-4-yl)-3-(difluoromethyl)-1-methyl-1*H*-pyrazole-4-carboxamide	CAS (Sci-finder n) Proquest Dialogue	Full Text
N-des-Me-1',1'-bis(CH_2OH)-S-2840	*N*-(1,1-bis(hydroxymethyl)-3-methyl-2,3-dihydro-1*H*-inden-4-yl)-3-(difluoromethyl)-1*H*-pyrazole-4-carboxamide	CAS (Sci-finder n) Proquest Dialogue	Full Text
N-des-Me-1'-CH_2OH-3'-OH-S-2840	3-(difluoromethyl)-*N*-(3-hydroxy-1-(hydroxymethyl)-1,3-dimethyl-2,3-dihydro-1*H*-inden-4-yl)-1*H*-pyrazole-4-carboxamide	CAS (Sci-finder n) Proquest Dialogue	Full Text

Common name	Synonyms	Search engine	Fields searched
N-des-Me-1'-COOH-S-2840 NDM-1'-COOH-S-2840	4-(3-(difluoromethyl)-1*H*-pyrazole-4-carboxamido)-1,3-dimethyl-2,3-dihydro-1*H*-indene-1-carboxylic acid	CAS (Sci-finder n) Proquest Dialogue	Full Text
DFPA	3-(difluoromethyl)-1-methyl-1*H*-pyrazole-4-carboxylic acid 176969-34-9	CAS (Sci-finder n) Proquest Dialogue	Full Text
N-des-Me-DFPA	3-(difluoromethyl)-1*H*-pyrazole-4-carboxylic acid 151734-02-0	CAS (Sci-finder n) Proquest Dialogue	Full Text
DFPA-$CONH_2$	3-(difluoromethyl)-1-methyl-1*H*-pyrazole-4-carboxamide	CAS (Sci-finder n)	Full Text

Common name	Synonyms	Search engine	Fields searched
	925689-10-7	Proquest Dialogue	
1'-CH_2OH-S-2840-sulfate	(4-(3-(difluoromethyl)-1-methyl-1*H*-pyrazole-4-carboxamido)-1,3-dimethyl-2,3-dihydro-1*H*-inden-1-yl)methyl hydrogen sulfate	CAS (Sci-finder n) Proquest Dialogue	Full Text
Glu-1'-CH_2OH-S-2840	6-((4-(3-(difluoromethyl)-1-methyl-1*H*-pyrazole-4-carboxamido)-1,3-dimethyl-2,3-dihydro-1*H*-inden-1-yl)methoxy)-3,4,5-trihydroxytetrahydro-2*H*-pyran-2-carboxylic acid	CAS (Sci-finder n) Proquest Dialogue	Full Text
3'-OH-S-2840-dehydrate	3-(difluoromethyl)-1-methyl-*N*-(1,1,3-trimethyl-1*H*-inden-4-yl)-1*H*-pyrazole-4-carboxamide	CAS (Sci-finder n) Proquest Dialogue	Full Text

From this literature search, 352 studies were returned. 349 studies were excluded after a rapid assessment. One of these excluded studies was reference number 135 entitled "*Effects of fungicides on four native generalist phytoseiid species (Acari: Phytoseiidae)*". The abstract stated that inpyrfluxam was selected as a test item. HSE Ecotoxicology considered this reference potentially relevant to the effects on arthropods data requirements of Regulations 283/2013 and 284/2013.

HSE Ecotoxicology also had some concerns relating to the specification of the relevancy criteria: 1) the relevancy criteria state that species selected in laboratory tests should preferably be relevant to the EU (interpreted as European settings). The position of HSE Ecotoxicology is it is not appropriate to exclude species based on their geographic distribution as they provide information on the overall distribution of species sensitivities; and 2) the relevancy criteria stipulate that the route and length of exposure is appropriate. No further detail was provided on what constitutes appropriate exposure. Specific timeframes for each organism group based on their biology and routes of exposure should be defined if this relevancy criterion is to be included. These issues were outlined in the original evaluation but were not relevant due to the lack of search results.

A final point was in the originally submitted literature review, STN and DIALOG database providers were selected. In the newly submitted literature review, only DIALOG databases were selected.

A second RAI was sent out asking for clarification on the above points. The RAI response is provided below:

Justification for rapid screening assessment decision-making for reference number 135 and associated alteration to relevancy criteria for literature review:
From Appendix 6 of the updated literature review, reference number 135 entitled "Effects of fungicides on four native generalist phytoseiid species (Acari: Phytoseiidae)" was considered to be non-relevant after rapid assessment. Please provide further justification for this decision. From the abstract, it appears to be potentially relevant to the effects on arthropods data requirements of Regulations 283/2013 and 284/2013.

Applicant response (August 2025):
Under the EFSA Guidance (Journal 2011;9(2):2092) and the appendix to this guidance, a rapid assessment can be made on the abstract (as well as the title). The article was discounted via a rapid assessment with the abstract on the basis that inpyrfluxam was deemed harmless to the test species and as such data within would not contribute anything further to that already part of the data-package provided for inpyrfluxam on none target organisms

Related to this point, HSE has some concerns relating to the specification of the relevancy criteria: 1) the relevancy criteria state that species selected in laboratory tests should

preferably be relevant to the EU (interpreted as European settings). The position of HSE Ecotoxicology is it is not appropriate to exclude species based on their geographic distribution as they provide information on the overall distribution of species sensitivities; and 2) the relevancy criteria stipulate that the route and length of exposure is appropriate. No further detail was provided on what constitutes appropriate exposure. Please provide specific timeframes for each organism group based on their biology and routes of exposure if this relevancy criterion is to be included. Considering these relevancy criteria alterations, please check if any further references were excluded, which may now require further assessment.

Applicant response (August 2025):
The relevancy criteria listed within the literature review report are specifically ordered in a tiered approach (to build a body of evidence to make a final assessment). None of the articles discounted reached the higher tiers where geographical distribution or route and length of exposure were considered, and therefore, no further action is needed to address this point.

Justification for removal of STN databases from databases searched from literature review: In the originally submitted literature review, STN and DIALOG database providers were selected. In the newly submitted literature review, only DIALOG databases were selected. Please justify why STN databases were omitted from the literature search in the newly submitted literature review (31/03/25)

Applicant response (August 2025):
In between the original and new searches, the STN platform (STNeasy) has been discontinued and is no longer supported. On that basis, the updated search uses the PROOQUEST/DIALOG and SciFinder-n (CAS) collection of databases and the justifications for choosing these databases are outlined in Appendices 2 and 3 of this report.

HSE Ecotoxicology accept the reasoning behind the removal of the STN platform from the search process. Regarding the exclusion of the study, "*Effects of fungicides on four native generalist phytoseiid species (Acari: Phytoseiidae)*", HSE Ecotoxicology does not accept the justification put forward by the applicant. The relevancy criteria do not include any consideration of the magnitude or significance of effects associated with a treatment. At the rapid screening assessment, titles and abstracts are the only information available. From this information, it is not possible to determine the magnitude or significance of effects with any certainty. In this instance, the decision was based on inpyrfluxam being described as harmless in the abstract. Basing exclusion decision-making on author conclusions is susceptible to bias. To address this, HSE Ecotoxicology has included the study in the risk assessment process. A brief study summary is included in Section B.9.5.2 of the B9 3CP document and the results have been integrated into the NTA risk assessment in Section B.9.6.2 of the B9 3CP document.

Overall, the literature review identified one relevant study, although this was relevant to Environmental fate and behaviour. No further studies relevant to Ecotoxicology were identified. After checking the list of excluded references, HSE Ecotoxicology agrees with this decision.

In conclusion, HSE Ecotoxicology identified one relevant study according to the submitted relevancy criteria. This study has been added to the submission.

B.9.11 References Relied On

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation Y/N
KCA 8.1.1.2/03	[redacted] and [redacted]	2017	S-2399: A dietary LC_{50} study with the zebra finch [redacted]. Report No: TPW-0071 Yes Unpublished	Y	Y	Data for the first approval	Sumitomo Chemical Co., Ltd.	N
KCA 8.1.1.2/01	[redacted] and [redacted] and [redacted]	2014a	S-2399 TG: a dietary LC50 study with the northern bobwhite	Y	Y	Data for the first approval	Sumitomo Chemical Co., Ltd.	N

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation Y/N
			[redacted] Report No. TPW-0008 Yes Unpublished					
KCA 8.1.1.2/02	[redacted] and [redacted]	2014b	S-2399 TG: A dietary LC50 study with the mallard [redacted] Report No: TPW-0009 Yes Unpublished	Y	Y	Data for the first approval	Sumitomo Chemical Co., Ltd.	N

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation Y/N
KCA 8.2.1/01	[redacted]	2014	S-2399 TG - Acute Toxicity Test with Rainbow Trout (*Oncorhynchus mykiss*) Under Static Conditions [redacted] Study No. 13048.6776 Yes Unpublished	Y	Y	Data for the first approval	Sumitomo Chemical Co., Ltd.	N
KCA 8.2.1/02	[redacted]	2014	S-2399 TG - Acute Toxicity Test with Bluegill Sunfish (*Lepomis macrochirus*) Under Static Conditions	Y	Y	Data for the first approval	Sumitomo Chemical Co., Ltd.	N

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation Y/N
			[redacted] Study No.13048.6806 Yes Unpublished					
KCA 8.2.1/03	[redacted]	2014	S-2399 - Acute Toxicity Test with Fathead Minnow (*Pimephales promelas*) Under Static Conditions [redacted] Study No. 13048.6777 Yes	Y	Y	Data for the first approval	Sumitomo Chemical Co., Ltd.	N

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation Y/N
			Unpublished					
KCA 8.2.1/04	[redacted]	2014	S-2399 TG - Acute Toxicity Test with Common Carp (*Cyprinus carpio*) Under Static Conditions [redacted] Study No.13048.6778 Yes Unpublished	Y	Y	Data for the first approval	Sumitomo Chemical Co., Ltd.	N
KCA 8.2.1/05	[redacted]	2014	S-2399 TG: Acute Toxicity to	Y	Y	Data for the first approval	Sumitomo Chemical Co., Ltd.	N

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation Y/N
			Sheepshead Minnow (*Cyprinodon variegatus*) Under Static Conditions [redacted] Study No. 12709.6360 Sponsor Protocol/Project No. VP-38637 Yes Unpublished					
KCA 8.2.1/06	[redacted]	2016	Acute toxicity study of S-2399 TG with	Y	Y	Data for the first approval	Sumitomo Chemical Co., Ltd.	N

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation Y/N
			Guppy *(Poecilia reticulata)* [redacted] No. 1603EFAG Yes Unpublished					
KCA 8.2.1/07	[redacted]	2016	Acute Toxicity Study of S-2399 TG with Japanese medaka (*Oryzias latipes*) [redacted]	Y	Y	Data for the first approval	Sumitomo Chemical Co., Ltd.	N

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation Y/N
			[redacted] Study No. 1603EFAM Yes Unpublished					
KCA 8.2.1/08	[redacted]	2016	Acute Toxicity Study of S-2399 TG with Zebrafish (*Danio rerio*) [redacted] No. 1603EFAZ Yes Unpublished	Y	Y	Data for the first approval	Sumitomo Chemical Co., Ltd.	N

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation Y/N
KCA 8.2.1/10	[redacted]	2016	Acute Toxicity Study of 3'-OH-S-2840 with Rainbow Trout (*Oncorhynchus mykiss*) [redacted] Study No. 1512EFAR Yes Unpublished	Y	Y	Data for the first approval	Sumitomo Chemical Co., Ltd.	N
KCA 8.2.1/11	[redacted]	2016	Acute Toxicity Study of 1'-COOH-S-2840 with Rainbow Trout	Y	Y	Data for the first approval	Sumitomo Chemical Co., Ltd.	N

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation Y/N
			(*Oncorhynchus mykiss*) [redacted] Study No. 1513EFAR Yes Unpublished					
KCA 8.2.2.1/01	[redacted]	2014	S-2399 TG – Early Life-Stage Toxicity Test with Fathead Minnow, *Pimephales promelas*	Y	Y	Data for the first approval	Sumitomo Chemical Co., Ltd.	N

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation Y/N
			[redacted] Study No.13048.6781 Yes Unpublished					
KCA 8.2.2.1/02	[redacted]	2017	S-2399 TG - Early life-stage toxicity test with sheepshead minnow, *Cyprinodon variegatus* [redacted] Study No. 12709.6373 Yes	Y	Y	Data from the first approval	Sumitomo Chemical Co., Ltd.	N

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation Y/N
			Unpublished					
KCA 8.2.2.3/01	[redacted] / [redacted]	2015/2020	Amended Report [^{14}C]S-2399 – Flow-Through Bioconcentration and Metabolism Study with Bluegill Sunfish (*Lepomis macrochirus*) [redacted] Study No. 13048.6863 Yes Unpublished	Y	Y	Data from the first approval	Sumitomo Chemical Co., Ltd.	N

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation Y/N
KCA 8.1.4/01	[redacted] .	2021	Amphibian Metamorphosis Assay with African Clawed Frog (*Xenopus laevis*) [redacted] Study No. 13048.7147, Sumitomo Chemical Co., Ltd. Report No.: TPW-0138. Yes Unpublished	Y	Y	Data from the first approval	Sumitomo Chemical Co., Ltd.	N
KCA 8.2.3/01	[redacted] .	2021	S-2399 - Fish Short-Term Reproduction	Y	Y	Data from the first approval	Sumitomo Chemical Co., Ltd.	N

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation Y/N
			Assay with Fathead Minnow (*Pimephales promelas*) [redacted] Study No. 13048.7149 Yes Unpublished					
KCA 8.2.4.1/01	[redacted]	2014	S-2399 TG – Acute Toxicity to Water Fleas (*Daphnia magna*) Under Static Conditions Smithers Viscient Study No. 13048.6779	N	Y	Data from the first approval	Sumitomo Chemical Co., Ltd.	N

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation Y/N
			Yes Unpublished					
KCA 8.2.4.2/01	[redacted]	2014	S-2399 TG: Acute Toxicity to Mysids (*Americamysis bahia*) Under Static Conditions Smithers Viscient Study No. 12709.6361 Yes Unpublished	N	Y	Data from the first approval	Sumitomo Chemical Co., Ltd.	N

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation Y/N
KCA 8.2.5.1/01	[redacted]	2014	S-2399 TG - Full Life-Cycle Toxicity Test with Water Fleas, *Daphnia magna* Smithers Viscient Study No. 13048.6780 Yes Unpublished	N	Y	Data from the first approval	Sumitomo Chemical Co., Ltd.	N
KCA 8.2.5.2/01	[redacted]	2016/2020	S-2399 TG - Life-Cycle Toxicity Test with Mysids (*Americamysis bahia*)	N	Y	Data from the first approval	Sumitomo Chemical Co., Ltd.	N

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation Y/N
			Smithers Viscient Study No. 12709.6374 Yes Unpublished					
KCA 8.2.6.1/01	[redacted]	2015	S-2399 TG – 96-Hour Toxicity Test with the Freshwater Green Alga, *Pseudokirchneriella subcapitata* Smithers Viscient Study No. 13048.6860 Yes	N	Y	Data from the first approval	Sumitomo Chemical Co., Ltd.	N

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation Y/N
			Unpublished					
KCA 8.2.6.2/01	[redacted]	2015	S-2399 TG: Toxicity Test with the Freshwater Diatom, *Navicula pelliculosa* Smithers Viscient Study No. 12709.6370 Yes Unpublished	N	Y	Data from the first approval	Sumitomo Chemical Co., Ltd.	N
KCA 8.2.6.2/03	[redacted]	2015	S-2399 TG: Toxicity Test with the Marine Diatom, *Skeletonema costatum*	N	Y	Data from the first approval	Sumitomo Chemical Co., Ltd.	N

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation Y/N
			Smithers Viscient Study No. 12709.6371 Yes Unpublished					
KCA 8.3.1.1.1/01 and KCA 8.3.1.1.2/01	[redacted]	2015	Effects of S-2399 TG (Acute Contact and Oral) on Honey Bees (*Apis mellifera* L.) in the Laboratory Ibacon, Germany, Study No. 94951035 Sumitomo Chemical Co., Ltd.	N	Y	Data from the first approval	Sumitomo Chemical Co., Ltd.	N

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation Y/N
			Report No: TPW-0015 Yes Unpublished					
KCA 8.3.1.1.1/02 and KCA 8.3.1.1.2/02	[redacted]	2016	S-2399 TG: Effects (Acute Contact and Oral) on Bumble Bees (*Bombus terrestris* L.) in the Laboratory Ibacon, Germany, Study No. 113911105 Sumitomo Chemical Co., Ltd.	N	Y	Data from the first approval	Sumitomo Chemical Co., Ltd.	N

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation Y/N
			Report No: TPW-0060 Yes Unpublished					
KCA 8.4.1/01	[redacted]	2016a	S-2399 TG: Effects on Reproduction and Growth of Earthworms *Eisenia fetida* in Artificial Soil with 5% Peat Ibacon, Germany, Study No. 113911022 Sumitomo Chemical Co., Ltd.	N	Y	Data from the first approval	Sumitomo Chemical Co., Ltd.	N

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation Y/N
			Report No: TPW-0052. Yes Unpublished					
KCA 8.4.1/02	[redacted]	2016a	3'-OH-S-2840: Effects on Reproduction and Growth of Earthworms *Eisenia fetida* in Artificial Soil with 5% Peat Ibacon, Germany, Study No. 113861022 Sumitomo Chemical Co., Ltd.	N	Y	Data from the first approval	Sumitomo Chemical Co., Ltd.	N

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation Y/N
			Report No: TPW-0050. Yes Unpublished					
KCA 8.4.1/03	[redacted]	2016b	1'-COOH-S-2840: Effects on Reproduction and Growth of Earthworms *Eisenia fetida* in Artificial Soil with 5% Peat Ibacon, Germany, Study No. 113871022 Sumitomo Chemical Co., Ltd.	N	Y	Data from the first approval	Sumitomo Chemical Co., Ltd.	

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation Y/N
			Report No: TPW-0058. Yes Unpublished					
KCA 8.4.2/01	[redacted]	2016b	S-2399 TG: Effects on Reproduction of the Collembola *Folsomia candida* in Artificial Soil with 5% Peat Ibacon, Germany, Study No. 113911016 Sumitomo Chemical Co., Ltd. Report No: TPW-0044,	N	Y	Data from the first approval	Sumitomo Chemical Co., Ltd.	N

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation Y/N
			Yes Unpublished					
KCA 8.4.2/02	[redacted]	2016c	3'-OH-S-2840: Effects on Reproduction of the Collembola *Folsomia candida* in Artificial Soil with 5% Peat Ibacon, Germany, Study No. 113861016 Sumitomo Chemical Co., Ltd. Report No: TPW-0043,	N	Y	Data from the first approval	Sumitomo Chemical Co., Ltd.	N

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation Y/N
			Yes Unpublished					
KCA 8.4.2/03	[redacted]	2016d	1'-COOH-S-2840: Effects on Reproduction of the Collembola *Folsomia candida* in Artificial Soil with 5% Peat Ibacon, Germany, Study No. 113871016 Sumitomo Chemical Co., Ltd. Report No: TPW-0051, Yes	N	Y	Data from the first approval	Sumitomo Chemical Co., Ltd.	N

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation Y/N
			Unpublished					
KCA 8.4.2/04	[redacted]	2016c	S-2399 TG: Effects on Reproduction of the Predatory Mite *Hypoaspis aculeifer* in Artificial Soil with 5% Peat Ibacon, Germany, Study No. 113911089 Sumitomo Chemical Co., Ltd. Report No: TPW-0042, Yes Unpublished	N	Y	Data from the first approval	Sumitomo Chemical Co., Ltd.	N

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation Y/N
KCA 8.4.2/05	[redacted]	2016e	3'-OH-S-2840: Effects on Reproduction of the Predatory Mite *Hypoaspis aculeifer* in Artificial Soil with 5% Peat Ibacon, Germany, Study No. 113861089 Sumitomo Chemical Co., Ltd. Report No: TPW-0045, Yes Unpublished	N	Y	Data from the first approval	Sumitomo Chemical Co., Ltd.	N

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation Y/N
KCA 8.4.2/06	[redacted]	2016f	1'-COOH-S-2840: Effects on Reproduction of the Predatory Mite *Hypoaspis aculeifer* in Artificial Soil with 5% Peat Ibacon, Germany, Study No. 113871089 Sumitomo Chemical Co., Ltd. Report No: TPW-0048, Yes Unpublished	N	Y	Data from the first approval	Sumitomo Chemical Co., Ltd.	N

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation Y/N
KCA 8.5/01	[redacted]	2016a	S-2399 TG:Effects on the Activity of the Soil Microflora in the Laboratory Ibacon, Germany, Study No. 113911080 Sumitomo Chemical Co., Ltd. Report No: TPW-0061 Yes Unpublished	N	Y	Data from the first approval	Sumitomo Chemical Co., Ltd.	N
KCA 8.5/02	[redacted]	2016b	3'-OH-S-2840:Effects on the Activity of the Soil Microflora in the	N	Y	Data from the first approval	Sumitomo Chemical Co., Ltd.	N

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation Y/N
			Laboratory(Nitrogen Transformation) Ibacon, Germany, Study No. 113861080 Sumitomo Chemical Co., Ltd. Report No: TPW-0046, Yes Unpublished					
KCA 8.5/03	[redacted]	2016c	1'-COOH-S-2840: Effects on the Activity of the Soil Microflora in the Laboratory(Nitrogen Transformation)	N	Y	Data from the first approval	Sumitomo Chemical Co., Ltd.	N

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation Y/N
			Ibacon, Germany, Study No. 113871080 Sumitomo Chemical Co., Ltd. Report No: TPW-0047, Yes Unpublished					

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