



Draft Assessment Report

Evaluation of Active Substances

Plant Protection Products

Prepared according to **Regulation (EC) 1107/2009**
as it applies in Great Britain

Pydiflumetofen

Volume 3 – B.9 (AS)

Ecotoxicology Data

Great Britain

September 2023

Version History

When	What
October 2022	Initial DAR
June 2023	Post Expert Committee on Pesticides (ECP) Independent Scientific Advice (ISA)
September 2023	Update following consideration of new ED data for Ecotoxicology

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B.9. ECOTOXICOLOGY DATA

B.9.1. EFFECTS ON BIRDS AND OTHER TERRESTRIAL VERTEBRATES

B.9.1.1. Effects on birds

B.9.1.1.1. Acute oral toxicity to Birds

Report: K-CA 8.1.1.1, [REDACTED], [REDACTED]. (2013). SYN545974 - An Acute Oral Toxicity Study with the Northern Bobwhite Using a Sequential Testing Procedure, Report Number 528393. [REDACTED] (Syngenta File No. SYN545974_10062)

Guidelines

OECD Guideline for Testing of Chemicals, Method 223: Avian Acute Oral Toxicity Test. (2010)

GLP: Yes

Materials

Test Material SYN545974 tech.
Lot/Batch #: SMU2EP12007
Purity: 98.5 % w/w
Description: Off white powder
Stability of test compound: Stable under standard conditions
Reanalysis/Expiry date: 30 June 2016

Treatments

Test rates: Nominal concentrations; 0 and 2000 mg a.s./kg

Test organisms

Species: Northern Bobwhite (*Colinus virginianus*) 30 weeks old
Source: [REDACTED]
Acclimatisation period: Approximately 3 weeks
Treatment for disease: Beginning one day following arrival in the test facility, test birds were given water soluble antibiotics in their drinking water for eight consecutive days. The birds received no form of antibiotic medication during the test or in the 14 days preceding test initiation.
Weight: 196 – 243 g at test initiation

Test design

Replication: Five pens per group
No. of birds/pen : 1
Duration of test: Study phases;
 Acclimation to Test Caging - Approximately 3 weeks
 Fasting – Approximately 17 hours
 Dosing – Day of experimental start
 Post-dosing observation – 14 days.

Environmental test conditions

Temperature:	20.9 °C - 21.3 °C
Humidity:	21 % - 40 %
Photoperiod:	8 hr light : 16 hr dark, average illumination of approximately 136 lux

Study Design and Methods

Experimental dates: 15 to 30 November 2012

The test was designed as a limit test so consisted of one test concentration, alongside a control group. Five mixed-sex northern bobwhite quail, approximately 30 weeks old, and in good health were randomly assigned to the test group. The birds were housed individually in batteries of pens, each pen with a floor space of 25 x 51 cm which sloped so that ceiling heights ranged from 20 to 26 cm.

After a pre-test fasting period of approximately 17 hours, the nominal concentration of 2000 mg a.s./kg body weight was administered orally in a corn oil-coated gelatin capsule, which was inserted into the crop of each bird. Each bird was individually weighed and dosed on the basis of milligrams of active substance per kilogram of body weight. The control birds each received an empty gelatin capsule. During the test each bird was fed a game bird ration which, together with water from the town of [REDACTED] public water supply, was provided *ad libitum*.

Following dosing, multiple observations were performed on Day 0 of the test, with particular attention being paid for signs of regurgitation. All birds were observed at least twice daily for the remainder of the test. Individual body weights were measured at test initiation and on Days 3, 7 and 14 (test termination). Feed consumption was determined at approximately 24-hour intervals from Day 0 to Day 3, after which average feed consumption was determined from Day 3 to Day 7 and from Day 7 to Day 14.

Results and Discussion

Mortality and growth are summarised in the table below.

Table 9.1.1.1-1: Summary of effects of SYN545974 on mortality and growth of northern bobwhite (*Colinus virginianus*) following acute oral exposure

Treatment (mg a.s./kg bw)	Cumulative mortality	Mean weight change ¹ day 0-3 (SD) (g)	Mean weight change ¹ day 3-7 (SD) (g)	Mean weight change ¹ day 7- 14 (SD) (g)	Mean total weight change ¹ (SD) (g)
0	0/5	-2 (2)	2 (2)	-1 (1)	-1 (3)
2000	0/5	-4 (3)	1 (2)	-1 (3)	-4 (2)

No regurgitation was noted after dosing birds in both the control group and the 2000 mg a.s./kg treatment group. All birds in the control group were normal in appearance and behaviour for the duration of the test. In the 2000 mg a.s./kg treatment group one bird was noted displaying a ruffled appearance. This was short term and had no impact on the body weight or feed consumption for this bird. Therefore, it was not considered to be an adverse effect.

When compared to the control group, there was no apparent treatment related effect on mean body weight or change in mean body weight for the 2000 mg a.s./kg dosage group.

When compared to the control group, there was no apparent treatment related effect on mean feed consumption values for the 2000 mg a.s./kg dosage group.

Since no mortality occurred, it was not possible to complete statistical analysis.

Conclusions

(██████ and ██████, 2013)

Validity Criteria	Required	Obtained
Mortality in control group	< 10 %	0 %

- **14 day LD₅₀ = > 2000 mg a.s./kg body weight**

Report:	K-CA 8.1.1.1, [REDACTED], [REDACTED]. (2013a). SYN545974 - An Acute Oral Toxicity Study with the Canary Using a Sequential Testing Procedure, Report Number 528-394. [REDACTED] [REDACTED]. (Syngenta File No. SYN545974_10065)
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Test Material	SYN545974 tech.
Lot/Batch #:	SMU2EP12007
Purity:	98.5 % w/w
Description:	Off-white powder
Stability of test compound:	Stable under standard conditions
Reanalysis/Expiry date:	30 June 2016
Density:	Not applicable
Treatments	
Test rates:	2000 mg a.s./kg body weight (adjusted to 100 % active ingredient) and a gelatin capsule control
Test organisms	
Species:	Canary (<i>Serinus canaria</i>) approximately 8 months to 3 years old at time of receipt
Source:	Obtained from [REDACTED], on February 07, 2013
Acclimatisation period:	6 weeks to test facility and test caging

Treatment for disease:	None
Weight:	18.2 – 25.8 g
Test design	
Replication:	One control group of 10 birds, one test group of 10 birds (5 male and 5 female per group)
No. of birds/pen :	1
Duration of test:	14 days
Environmental test conditions	
Temperature:	Average: 22.5 °C ± 4.1 °C (SD) (Maximum: 25.6 °C; minimum: 21.8 °C)
Humidity:	Average: 23.0 % ± 6.3 % RH (SD) (Maximum: 36.9 %; minimum: 16.0 %)
Photoperiod:	8 hours daylight/15.5 hours darkness, with two 15-minute dim-light transition periods Fluorescent light, approximately 255 lux

Study Design and Methods

Experimental dates: 18 March to 2 April 2013

The test was designed as a limit test so consisted of one test concentration, alongside a control group. Five male and five female adult canaries, in good health, were randomly assigned to the test group. The birds were housed individually in batteries of pens. Each pen had a floor space of 29 x 26 cm, a ceiling height of approximately 31 cm, and external walls, ceilings and floors were constructed of coated wire.

After a pre-test fasting period of approximately 16.5 hours, the nominal concentration of 2000 mg a.s./kg body weight was administered orally in a corn oil-coated gelatin capsule, which was inserted into the crop of each bird. The control birds each received an empty gelatin capsule. During the test all birds were fed ZuPreem FruitBlend diet size xs which, together with water from the town of █████ public water supply, was provided *ad libitum*. Grit was provided to aid digestion.

The birds were observed at least twice daily for toxicological responses throughout the test. Particular attention was paid for signs of regurgitation. Individual body weights were measured one day prior to test initiation and on Days 3, 7 and 14 (test termination). Feed consumption was determined at approximately 24-hour intervals from Day 0 to Day 3, after which average feed consumption was determined from Day 3 to Day 7, from Day 7 to Day 10, and from Day 10 to Day 14.

Gross necropsies were performed on three birds from the control group and treatment group at test termination.

Results and Discussion

Results were reported in terms of the active substance (SYN545974). Mortality and growth are summarised in the table below.

Table 9.1.1.1-2: Summary of effects of SYN545974 on mortality and growth of canary (*Serinus canaria*) following acute oral exposure

Treatment (mg a.s./kg bw)	Sex	Cumulative mortality	Mean weight gain day 0-3 (SD) (g)	Mean weight gain day 3-7 (SD) (g)	Mean weight gain day 7-14 (SD) (g)	Mean total weight gain ¹ (SD) (g)
0	M	0/5	-0.1 (0.8)	0.3 (1.3)	0.8 (0.8)	1.1 (1.2)
	F	0/5	-0.8 (0.3)	-0.3 (0.3)	1.3 (0.5)	0.2 (0.7)
2000	M	0/5	-0.6 (0.3)	0.1 (0.5)	0.4 (0.6)	-0.1 (0.7)
	F	0/5	-1.2 (0.4)	-0.5 (0.2)	1.6 (1.8)	-0.1 (1.9)

¹ The mean and change is calculated separately from the mean body weights using the individual changes in body weight.

There were no mortalities in the control group and all birds in the control group were normal in appearance and behaviour for the duration of the test. One male in the 2000 mg a.s./kg body weight treatment group was noted with a slight ruffled appearance on Days 9 to 14 of the test. All other birds in the 2000 mg a.s./kg body weight treatment group were normal in appearance and behaviour for the duration of the test. There were no mortalities in the treatment group and no regurgitation was observed after dosing. There were no apparent treatment-related effects on mean body weight, mean body weight change, or in feed consumption in the treatment group compared to the control group, noting statistical analysis was not conducted.

No findings were noted for the three control birds that were necropsied. Of the three birds necropsied at the 2000 mg a.s./kg bw dosage level one bird was noted with no findings and another was noted with pale kidneys. The third bird, which had also been noted with a ruffled appearance, was noted as thin, with a prominent keel, pale spleen and pale liver.

Conclusions

The 14-day acute oral LD₅₀ for canary exposed to SYN545974 as a single oral dose was determined to be greater than 2000 mg a.s./kg body weight, the only concentration tested. The no mortality level was 2000 mg a.s./kg body weight.

(██████ and ██████, 2013a)

HSE evaluator comments

This study was conducted to GLP. The study followed OECD 223 (2010) guideline. However, the most recent guideline is OECD 223 (2016) so the study was assessed against this more recent version.

The study met the validity criteria of the guideline: there was no mortality in the control group ($\leq 10\%$ mortality is required).

The species using in this study, *Serinus canaria* (canary) is not specified as a data requirement, but sufficient justification is provided: the applicant states in their summary that this study was conducted to fulfil global registration requirements and is included for completeness.

Overall the study had no major deviations from the guideline. The following minor points are noted for reference but do not have an impact on the endpoint of the study:

- It is unclear whether the birds (obtained from a supplier) are cage-reared or wild-caught, and there is no breeding history provided. Cage-reared, genetically heterogenous birds with wild phenotypes are preferred in the guidelines. However, the long acclimatisation of 6 weeks used in the study (guideline minimum is 14 days) is adequate for both cage-reared or wild-caught birds. The authors do not provide a percentage survival in acclimation (guidelines state birds from batch should not be used if mortality is $> 5\%$ cage-reared or $> 10\%$ wild birds) but they do state that healthy birds are used in the test and any exhibiting abnormal behaviour or injury were not used.
- The age of the birds is described as adult plumage, with the age of the birds at 8 months to 3 years prior to acclimatisation. The guidelines state birds should be mature plumage but not breeding condition, and that cage-reared birds should be of similar age. The age range for the birds used in the study is relatively large, and breeding condition is not stated, generating some uncertainty.
- There were no mortalities in either control or treatment group. However, it is noted that two out of the three necropsied birds at the 2000 mg a.s./kg body weight treatment level had sub-lethal effects visible in organs, and one out of ten birds in the treatment level exhibited an abnormal ruffled appearance (and was one of the necropsied animals). These sublethal effects do not impact on the mortality endpoint but are noted for reference in risk assessment if required.
- The number of birds used was ten, whereas only five are required in OECD 223 guideline. The reason for using more birds than required is stated in the study report as at the request of the EPA. It is noted that US OCSP 850.2100 guideline recommends a minimum of ten birds per control/treatment group.

- Following dosing, the birds were monitored continuously 60 minutes, which is half the recommended time in the OECD 223 (2016) guideline. However, no signs of regurgitation were observed throughout the study so this is not an issue and would not invalidate the study. Additionally, 60 minutes is adequate in meeting the US EPA guideline requirements for post-dose monitoring (OCSPP 850.2100, 2012)

Statistical analysis has also been considered:

- As specified in the guidelines, if no mortality occurs in the dosed birds after 14 days, it can be concluded at the 95 % confidence level that the LD₅₀ is above the limit dose. Therefore, no statistical tests were required for mortality data in this study.
- It is noted that statistical analysis is not carried out on food consumption or body weight, however there did not appear to be any clear treatment related effects.

The agreed endpoint for consideration in risk assessment is:

- LD₅₀ > 2000 mg a.s./kg body weight

B.9.1.1.2. Short-term dietary toxicity to birds

Report:	K-CA 8.1.1.2 [REDACTED], [REDACTED], [REDACTED]. (2013). SYN545974 - A Dietary LC ₅₀ Study with the Northern Bobwhite, Report Number 528-391. [REDACTED] [REDACTED] (Syngenta File No. SYN545974_10063)
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Report:	K-CA 8.1.1.2, [REDACTED]. (2016) SYN545974 - Response to ANSES comments regarding the bird dietary toxicity studies Document No. VV-137213 , SYN545974_10459
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Guidelines

OECD Guidelines for Testing of Chemicals, Method 205: Avian Dietary Toxicity Test (1984)

US EPA Ecological Effects Test Guidelines, OPPTS 850.2200: Avian Dietary Toxicity Test (1996)

U.S. Environmental Protection Agency. Pesticide Assessment Guidelines, FIFRA Subdivision E, Hazard Evaluation: Wildlife and Aquatic Organisms, subsection 71-2 (1982)

GLP: Yes

Materials

Test Material	SYN545974 tech.
Lot/Batch #:	SMU2EP12007
Purity:	98.5 % w/w
Description:	Off white powder
Stability of test compound:	Stable under standard conditions
Reanalysis/Expiry date:	30 June 2016

Treatments

Test rates:	Nominal concentrations; 0, 562, 1000, 1780, 3160 and 5620 ppm a.s.
Analysis of test concentrations	Verification of dose tested day 0 at all test levels; homogeneity tested day 0 in samples from 562 and 5620 ppm SYN545974 test diet and stability tested in samples taken from all treatment groups on day 5.

Test organisms

Species:	Northern Bobwhite (<i>Colinus virginianus</i>) 13 days old
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Source:	Obtained from [REDACTED]
Acclimatisation period:	13 days
Treatment for disease:	The birds received no form of antibiotic medication during acclimation or the test
Weight:	19 – 29 g at test initiation
Test design	
Replication:	2 pens per treatment group, 6 pens per control
No. of birds/pen :	5
Duration of test:	Study phases: Acclimation – 13 days Exposure – 5 days Post-exposure observation – 3 days
Environmental test conditions	
Temperature:	Brooding compartment: 38.7 ± 1.6 °C Average ambient room temperature: 28.0 ± 0.5 °C
Humidity:	27.7 ± 9.2 %
Photoperiod:	16 hr light : 8 hr dark, average illumination of approximately 318 lux

Study Design and Methods

Experimental dates: 5 to 13 December 2012

All northern bobwhite (*Colinus virginianus*) were 13 days of age and appeared to be in good health at initiation of the test. Birds were randomly assigned to five test groups and a control group. Each treatment group contained ten chicks and the control group contained 30 chicks. Birds were housed in brooding pens containing five chicks each. Each pen had a floor space of approximately 72 x 90 cm, with a ceiling height of 23 cm. The birds used in this study were immature and could not be differentiated by sex.

Test diets were prepared by mixing the test substance directly into the feed using standard laboratory mixers. An amount of diet sufficient to last the five-day exposure period was prepared on the day of test initiation for each treatment and control group. Diets were presented to the birds at test initiation.

Dietary test concentrations were corrected to 100% active ingredient based upon the reported purity (98.5 %) of SYN545974. Nominal dietary test concentrations used in this study were 0, 562, 1000, 1780, 3160 and 5620 ppm SYN545974.

Test birds were observed four times on the day of test initiation, and twice daily throughout the remainder of the test. A record was maintained of all signs of toxicity and abnormal behaviour.

Individual body weights were measured at test initiation (Day 0), at the end of the exposure period 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. The accuracy of feed consumption values may have been affected by the unavoidable wastage of feed by the birds.

All birds at test termination were euthanized using carbon dioxide. Gross necropsies were performed on three birds from each of the levels at test termination.

There were no mortalities in this study. Therefore, it was not possible to perform the calculation of an LC₅₀ value. The LC₅₀ value was determined to be greater than the highest dosage tested. Body weight data were compared by Dunnett's t-test.

Results and Discussion

None of the control samples showed any indication of the presence of SYN545974 or of the presence of a co-eluting substance at the characteristic retention time of SYN545974. Diet samples were collected from the 562 and 5620 ppm SYN545974 test concentrations and were analysed to evaluate the homogeneity of SYN545974 in the diet. Mean and standard deviations for the two test concentrations were 561 ± 11.5 ppm SYN545974 and 5880 ± 97.4 ppm SYN545974, respectively. Samples collected on Day 0 to verify test substance concentrations for the 1000, 1780, and 3160 ppm active substance diets were found to be 100%, 102% and 101% of nominal concentrations, respectively. Analysis of diet samples collected from feeders after being held at ambient temperature for 5 days average 104 %, 105 %, 104 %, 105 % and 103 % of the Day 0 values for the 562, 1000, 1780, 3160 and 5620 ppm SYN545974 test concentrations, respectively.

Mortality and growth are summarised in the Table 9.1.1.2-1.

Table 9.1.1.2-1: Summary of effects of SYN545974 on mortality and growth of the northern bobwhite (*Colinus virginianus*) following acute oral exposure

Treatment (ppm a.s.)	Cumulative mortality	Mean weight change ¹ day 0-5 (SD) (g)	Mean weight change ¹ day 5-8 (SD) (g)	Total weight change (SD) (g)
0	0/30	16 (2)	11 (1)	27 (3)
562	0/10	13* (2)	11 (2)	24 (3)
1000	0/10	12* (1)	10 (3)	22* (4)
1780	0/10	13* (3)	9 (2)	22* (4)
3160	0/10	12* (2)	10 (3)	22* (4)
5620	0/10	12* (2)	10 (2)	22* (3)

¹ Mean change is calculated separately from the mean body weights using individual body weights.

* Difference from the control group statistically significant at $p < 0.05$ (Dunnett's t-test; TOXSTAT.)

There were no mortalities in the control group and no mortalities in the 562, 1000, 1780, 3160 and 5620 ppm SYN545974 treatment groups. All birds in the control group and all birds in the treatment groups were normal in appearance and behaviour throughout the test. In the 1000 ppm SYN545974 test concentration two birds were noted with injuries. One bird was noted with a laceration on the left foot/leg during the last observation on Day 0 and then on Day 1 of the test. Another bird was noted with picked toes (a form of pen-mate aggression) on Day 6 of the test. The birds' feet were bandaged for the remainder of the test. All other birds in the 1000 ppm SYN545974 test concentration were normal in appearance and behaviour for the duration of the test.

When compared to the control group, there was a slight, but statistically significant ($p < 0.05$), reduction in mean body weight gain from Day 0 to Day 5 of the test for all test concentrations. On Days 5 and 8, the mean body weights for birds in the 1000, 1780, 3160 and 5620 ppm SYN545974 test concentrations were less than the mean body weight of the control group and the difference observed was statistically significant at $p < 0.05$. There were statistically significant ($p < 0.05$) differences in the overall (Day 0 to Day 8) change in mean body weight for the 1000, 1780, 3160 and 5620 ppm SYN545974 test concentrations when compared to the control group.

The applicant has provided further consideration of weight gains and overall total weight observed in a report by [REDACTED], 2016. The applicant provides the following justification (shown in italics) for the weight differences in this study:

“Typically in five-day dietary toxicity studies with birds, body weight reduction is associated with reduced food consumption, though sometimes reductions are associated with mortality due to toxicity or starvation or both, confounding the estimate of the LC₅₀. For these reasons, body weight and food consumption are only measured and not considered as endpoints.

In the study conducted with bobwhite quail ([REDACTED] et al., 2013; SYN545974_10063) there were statistically significant reductions in mean body weight gain for all test concentrations. However, the reductions were not concentration related and only the untreated control was different. This may be explained by a small increase in food consumption by control birds (115%) compared to all five treatments levels.

Table 9.1.1.2-2: Mean food consumption (g/bird/day) from a bobwhite quail dietary LC₅₀ study with SYN545974

Experimental group (ppm a.s.)		Exposure period (days)					Mean	Post-exposure period (days)				Mean
		0-1	1-2	2-3	3-4	4-5	0-5	5-6	6-7	7-8	5-8	
Control	Mean	10	7	6	6	6	7	9	10	11	10	
	SD	1	1	0	0	0	0	1	1	1	0	
562	Mean	9	7	6	5	6	6	9	9	11	10	
1 000	Mean	9	6	5	5	5	6	9	9	11	10	
1 780	Mean	9	6	6	6	6	7	9	9	11	10	
3 160	Mean	9	8	6	5	5	6	10	10	14	11	
5 620	Mean	9	8	5	6	5	6	12	10	12	11	

Table 3 from original study report.

Mean values calculated using Excel in full-precision mode. Manual calculation may vary.

This is supported by the food consumption data from the bobwhite reproduction study (██████ et al., 2015; SYN545974_10130). The report concluded that there were no treatment-related effects upon food consumption at the 200, 1000 and 5000 ppm a.s. test concentrations over the course of 21 weeks. While there were statistically significant differences between the control group and each of the treatment groups, the differences were small, not concentration responsive and limited to one weekly interval in each treatment group.

As a consequence, we conclude the relevant endpoint for this study is the LC₅₀ > 5620 ppm (1258 mg a.s./kg/day) and that this is not confounded by starvation resulting from reduced food consumption and body weight change.

When compared to the control group, there were no apparent treatment related effects on feed consumption for any of the test concentrations.

Table 9.1.1.2-3: Daily Dietary Dose (mg a.s./kg bw/day) calculation from a Northern Bobwhite Dietary LC₅₀ Study with SYN545974

Treatment (ppm a.s.)	Mean body weight (g) (Day 0, Day 5, Day 8)	Mean food consumption (g/bird/day)	Estimated Daily Dietary Dose * (mg a.s./kg bw/day)
0	32.8	6.8	0
562	30.9	6.5	118
1000	30.8	6.1	199
1780	30.0	6.6	392
3160	30.6	6.5	668
5620	29.0	6.5	1258

* Calculated using unrounded data, calculations using data rounded to 1 decimal place may vary slightly.

Gross necropsies were performed on three birds from each of the levels a test termination. The findings for all birds were not remarkable except for one bird in the 562 ppm SYN545974 test concentration, which was noted with a retained yolk sac, an incidental finding unrelated to treatment.

Conclusions

The dietary LC₅₀ for northern bobwhites exposed to SYN545974 was determined to be greater than 5620 ppm a.s. (1258 mg a.s./kg body weight/day), the highest concentration tested. The no mortality concentration was 5620 ppm a.s.

(██████ et al., 2013)

(██████ 2016)

HSE evaluator comments

The applicant provided justification for this study which HSE accepts: 'Short term dietary studies are not required, as results from mammalian studies do not indicate a potential for the dietary LD50 measured by the short term dietary study to be lower than the LD50 based on an acute oral study. However, studies have been conducted to fulfil global registration requirements and are included for completeness.' The study has been evaluated by HSE to confirm whether it is adverse.

Validity criteria	Required	Obtained
Mortality in controls	< 10 %	0 %
Concentration of test substance	80 % of nominal	Within 5 %
Compound related mortality or observable toxic effects	0 % in lowest treatment level	0 %

The study was carried out according to GLP and follows OECD 205 (1984) with no significant deviations to the guideline or the study plan. All validity criteria outlined in OECD 205 (1984) have been satisfactorily met as shown in the table above.

It was noted that the average ambient temperature was not within recommended range for Bobwhite Quail in the age range of 8 – 14 days. OECD 205 (1984) recommends a temperature range of 30 – 32 °C, higher than the temperature of 28 ± 0.5 °C. Additionally, the relative humidity was lower than recommended in OECD 205 (1984) at 27.7 ± 9.2 %. This is not within the range of 50 – 75 %. As there were no mortalities or adverse effects reported during the study, and the validity criteria were all met, HSE does not consider this to have had an effect on the endpoints.

There was a discrepancy noted in the body weights reported. The body weight data provided in Table 4 of the report (██████████ *et al*, 2013) does not match with the Table 2 and Table 3 data from the report. If the body weight data from Table 2 and Table 3 in the report were used to calculate daily dietary dose, the daily dietary dose for the birds in the highest treatment level of 5620 ppm a.s. would be >1044 mg a.s./kg bw/day. It is considered that the discrepancy is due to the use of unrounded data. HSE accepts the stated daily dietary dose of 1258 mg a.s./kg bw/day because table 4 (study report) states it was calculated using unrounded data.

HSE has considered the additional report (██████████, 2016) and argumentation (shown in italics above) regarding the observed effects on body weight. Whilst there were statistically significant reductions in mean body weight gain for all test concentrations, HSE accepts that this was caused by an increase in food consumption in the control birds. HSE accepts that the reduction in body weight in the test treatments was not concentration responsive, and accepts the proposed endpoint.

As there was no resulting mortality during the study the data could not be statistically analysed to determine the LC₅₀. Body weight was compared to the control group using Dunnett's t-test. This is an appropriate statistical test in line with OECD 205 (1984).

The analytical method has been evaluated by HSE Chemistry specialists in Vol. 3 CA Part B5.1.2.6. The following was concluded for this method: "Acceptable method. LOQ: 120 ppm in avian diet". The measured concentration of test substance in the diet were within 5 % of the nominal concentrations. The agreed endpoints are:

8 day EC₅₀ = > 1258 mg a.s./kg body weight/day (nominal concentration)

Report: K-CA 8.1.1.2, ██████████, ██████████, ██████████. (2013a). SYN545974 - A Dietary LC₅₀ Study with the Mallard, Report Number 528-392. ██████████
██████████ (Syngenta File No. SYN545974_10064)

Report: K-CA 8.1.1.2, ██████████. (2016) SYN545974 - Response to ANSES comments regarding the bird dietary toxicity studies
Document No. VV-137213, SYN545974_10459

Guidelines

- OECD Guidelines for Testing of Chemicals, Method 205: Avian Dietary Toxicity Test (1984)
- US EPA Ecological Effects Test Guidelines, OPPTS 850.2200: Avian Dietary Toxicity Test (1996)
- U.S. Environmental Protection Agency, Pesticide Assessment Guidelines, FIFRA Subdivision E, Hazard Evaluation: Wildlife and Aquatic Organisms, subsection 71-2 (1982)

GLP: Yes

Materials

Test Material	SYN545974 tech.
Lot/Batch #:	SMU2EP12007
Purity:	98.5 % w/w
Description:	Off white powder
Stability of test compound:	Stable under standard conditions
Reanalysis/Expiry date:	30 June 2016

Treatments

Test rates:	Nominal concentrations; 0, 562, 1000, 1780, 3160 and 5620 ppm SYN545974
Analysis of test concentrations	Verification of dose tested day 0 at all test levels; homogeneity tested day 0 in samples from 562 and 5620 ppm test diet and stability tested in samples taken from all treatment groups on day 5.

Test organisms

Species:	Mallard (<i>Anas platyrhynchos</i>) 5 days old
Source:	Obtained from [REDACTED]
Acclimatisation period:	5 days
Treatment for disease:	The birds received no form of antibiotic medication during the test
Weight:	72 – 104 g at test initiation

Test design

Replication:	2 pens per treatment group, 4 pens per control
No. of birds/pen:	5
Duration of test:	Study phases: Acclimation – 5 days Exposure – 5 days Post-exposure observation – 3 days

Environmental test conditions

Temperature:	Brooding compartment: Day 1: 36.8 ± 1.7 °C Day 2 – 8: 30.6 ± 1.4 °C Average ambient room temperature: 21.9 ± 0.9 °C
Humidity:	49.3 ± 11.7 %
Photoperiod:	16 hr light : 8 hr dark, average illumination of approximately 203 lux

Study Design and Methods

Experimental dates: 5 to 13 December 2012

All mallard ducklings were 5 days of age and appeared to be in good health at initiation of the test. Birds were randomly assigned to five test groups and a control group. Each treatment group contained ten chicks and the control group contained 20 chicks. Birds were housed in brooding pens containing five chicks each. Each pen had a floor space of 62 x 92 cm, with a ceiling height of 25.5 cm. The birds used in this study were immature and could not be differentiated by sex.

Test diets were prepared by mixing the test substance directly into the feed using standard laboratory mixers. An amount of diet sufficient to last the five-day exposure period was prepared on the day of test initiation for each treatment and control group. Diets were presented to the birds at test initiation.

Dietary test concentrations were corrected to 100% active ingredient based upon the reported purity (98.5 %) of SYN545974. Nominal dietary test concentrations used in this study were 0, 562, 1000, 1780, 3160 and 5620 ppm SYN545974.

Test birds were observed four times on the day of test initiation, and twice daily throughout the remainder of the test. A record was maintained of all signs of toxicity and abnormal behaviour.

Individual body weights were measured at test initiation (Day 0), at the end of the exposure period 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. The accuracy of feed consumption values may have been affected by the unavoidable wastage of feed by the birds.

All birds at test termination were euthanized using carbon dioxide. Gross necropsies were performed on three birds from each of the levels at test termination.

There were no mortalities in this study. Therefore, it was not possible to perform the calculation of an LC₅₀ value. The LC₅₀ value was determined to be greater than the highest dosage tested. Body weight data were compared by Dunnett's t-test.

Results and Discussion

Analytical verification of test substance in diet

None of the control samples showed any indication of the presence of SYN545974. Diet samples were collected from the 562 and 5620 ppm SYN545974 test concentrations, and were analysed to evaluate the homogeneity of SYN545974 in the diet. Mean and standard deviations for the two test concentrations were 561 ± 11.5 ppm SYN545974 and 5880 ± 97.4 ppm SYN545974, respectively. Samples collected on Day 0 to verify test substance concentrations for the 1000, 1780, and 3160 ppm SYN545974 diets were found to be 100 %, 102 % and 101 % of nominal concentrations, respectively. Analysis of diet samples collected from feeders after being held at ambient temperature for 5 days average 95 %, 98 %, 99 %, 101 % and 96 % of the Day 0 values for the 562, 1000, 1780, 3160 and 5620 ppm SYN545974 test concentrations, respectively.

Mortality and growth (body weight)

Mortality and growth are summarised in the table below.

Table 9.1.1.2-4: Summary of effects of SYN545974 on mortality and growth of the mallard (*Anas platyrhynchos*) following acute oral exposure

Treatment (ppm a.s.)	Cumulative mortality	Mean weight change ¹ day 0-5 (SD) (g)	Mean weight change ¹ day 5-8 (SD) (g)	Total weight change (SD) (g)
0	0/20	114 (15)	108 (14)	223 (27)
562	0/10	107 (9)	103 (9)	210 (14)
1000	0/10	115 (19)	97 (13)	212 (31)
1780	0/10	114 (12)	100 (9)	214 (19)
3160	0/10	105 (16)	85* (16)	190* (31)
5620	0/10	101 (15)	97 (11)	198 (24)

¹ Mean change is calculated separately from the mean body weights using individual body weights.

* Statistically significant difference ($p < 0.05$) from the control group (Dunnett's t-test; TOXSTAT.)

There were no mortalities in the control group or in any of the treatment groups and all birds in the control group and treatment groups were normal in appearance and behaviour throughout the test.

On Day 0 of the test, all birds were randomized to the test pens. However, it was later determined that all test concentrations had a lower mean body weight than the control group. The Day 0 mean body weights of the birds from the 562 and 1780 ppm SYN545974 test concentrations were statistically different from the control group at $p < 0.05$. This initial difference at the 562 ppm SYN545974 test concentration also resulted in a slightly lower, but statistically significant ($p < 0.05$) difference in mean weight at Day 5.

However, at the 562, 1000 and 1780 ppm SYN545974 test concentrations, body weight change from Day 0 to Day 5 was comparable to the control group and slightly higher than the control group when expressed as a percentage value.

At the 3160 ppm SYN545974 test concentration, statistically significant ($p < 0.05$) reductions in Day 8 mean body weight and body weight change Day 5 to Day 8 and Day 0 to Day 8 were observed. At the 5620 ppm SYN545974 treatment level, statistically significant ($p < 0.05$) reductions in mean body weight were observed on Day 5 and Day 8.

The applicant has provided further consideration of weight gains and overall total weight observed in a report by [REDACTED], 2016. The applicant provides the following justification (shown in italics) for the weight differences in this study:

“In the study conducted with mallard ducks ([REDACTED] et al., 2013a; SYN545974_10064) there was no clear concentration related statistically significant effects on body weight gain and no significant effects on food consumption [HSE note: no statistical tests performed for food consumption].

It should be noted that the Day 0 mean body weights for all test concentrations had a lower mean body weight than the control group (two of the test concentrations were significantly lower). Statistically significant effects at Day 5 on body weight and body weight gain in the 562 and 1780 ppm treatments, respectively, were due to significantly reduced body weights at Day 0 and therefore not treatment related.

These two treatment levels effectively “caught up” to control levels by the end of the test period, as demonstrated by these two treatments levels not being significantly different from control for total body weight change.

Table 9.1.1.2-5: Mean body weights (g) from a mallard duck dietary LC₅₀ study with SYN545974

Experimental group (ppm a.s.)		Exposure period							Total change [Day 0-8]	Total % Change [Day 0-8]
		Day 0	Change [Day 0-5]	% Change [Day 0-5]	Day 5	Change [Day 5-8]	% Change [Day 5-8]	Day 8		
Control	Mean SD	93 7	114 15	123	207 18	108 14	52	316 30	223 27	240
562	Mean SD	80* 4	107 9	134	187* 8	103 9	55	290 15	210 14	262
1000	Mean SD	88 8	115 19	132	203 23	97 13	48	300 35	212 31	243
1780	Mean SD	82* 8	114 12	140*	196 19	100 9	51	295 26	214 19	263
3160	Mean SD	87 8	105 16	121	192 20	85* 16	44*	277* 35	190* 31	219
5620	Mean SD	87 6	101 15	116	188* 21	97 11	52	285* 29	198 24	229

Table 2 in original study report.

Mean change is calculated separately from the mean body weights using individual body weights

Change calculated as a percentage of the mean body weight at the start of the period.

* Statistically significant difference ($p < 0.05$) from the control group (Dunnett's *t*-test; TOXSTAT)

While mean body weight gain was lowest in the 3160 and 5620 ppm a.s. treatments, the total % change was not significantly less than the control. When body weight gain is expressed as % body weight gain of control, the 3160 and 5620 ppm a.s. treatments were 98 and 94 % of control at Day 5, respectively. The pattern of small differences

from the control is similar for total body weight change also, with the total body weight change of 91 and 95 % below the control weight at 3160 and 5620 ppm a.s., respectively.

Table 9.1.1.2-6: Mean body weights (g) from a Mallard dietary LC₅₀ study with SYN545974

Experimental group (ppm a.s.)	Mean body weight change compared to control Day 0-5	Mean body weight change compared to control Day 5-8	Mean body weight change compared to control Total (Day 0-8)
562	109 %	106 %	109 %
1000	107 %	92 %	101 %
1780	114 %	98 %	110 %
3160	98 %	85 %	91 %
5620	94 %	100 %	95 %

Change is calculated as a percentage of the mean body weight at the start of the period.

Despite the top two concentrations having similar starting weights, only the total body weight change at test termination in the second highest test concentration of 3160 ppm a.s. was significantly lower than the control. The effects on total body weight change were therefore not dose-dependent and there is no hypothesized mechanism to suggest a non-monotonic response.

HSE has considered the above argumentation in the comments section below.

Feed consumption

The food consumption and daily dietary dose are shown in the table below.

Table 9.1.1.2-7: Daily Dietary Dose (mg a.s./kg bw/day) calculation from a Mallard Dietary LC₅₀ Study with SYN545974

Treatment (ppm a.s.)	Mean body weight (g) across days 0-5	Mean food consumption (g/bird/day) across days 0-5	Estimated Daily Dietary Dose* (mg a.s./kg bw/day)
0	150.3	66.1	0
562	133.9	58.8	247
1000	145.1	52.3	361
1780	138.6	61.5	790
3160	139.7	58.7	1329
5620	137.1	59.5	2437

* Calculated using unrounded data, calculations using data rounded to 1 decimal place may vary slightly.

When compared to the control group, there was no clear dose response when considering the mean feed consumption during the exposure period for any treatment level. Gross necropsies were performed on three birds from each of the levels at test termination. The findings for all birds were not remarkable.

Validity Criteria

Validity criteria for the test were met:

- Birds were randomly assigned to control and treatment pens.
- The mortality in the control group did not exceed 10 %.
- Concentrations of the test substance in the diet were satisfactorily maintained (at least 80 % of nominal) throughout the exposure period.
- The test substance was administered in diet for five consecutive days (5 ~ 24 hr. periods).
- A minimum of ten birds were used for each control and treatment group.
- The test substance was administered in the diet.

- The definitive test of five concentration levels and a control group were tested.

Conclusions

The dietary LC₅₀ for mallards exposed to SYN545974 was determined to be greater than 5620 ppm SYN545974 (2437 mg a.s./kg body weight/day), the highest concentration tested. The no mortality concentration was 5620 ppm SYN545974. The no-observed-effect concentration was 1780 ppm SYN545974 (790 mg a.s./kg body weight/day), based on a body weight effect at the 3160 ppm SYN545974 (1329 mg a.s./kg body weight/day), test concentration.

(██████████ *et al.*, 2013a)
(██████████ 2016)

HSE evaluator comments

This study was conducted to GLP. This was assessed to guideline OECD 205 (1984).

The authors verified the test substance was maintained in the test diet throughout the test at over 80 % (95 – 101 %) of the nominal concentration, as specified in the guidelines. The analytical method has been evaluated by HSE Chemistry specialists in Vol. 3 CA Part B5.1.2.6. The following was concluded for this method: “Acceptable method. LOQ: 120 ppm in avian diet”.

Overall the study had no major deviations and is considered to be valid, but the following minor points are noted for reference:

- **Duckling age:** The guideline recommends mallard ducklings should be aged 10-17 days. In this study, the birds were only five days old at the start of the test which generates uncertainty.
- **Acclimation:** The recommended acclimation period is a minimum of 7 days however since the chicks were younger than this at the start of the test this time period was not possible. Additionally, the survival of chicks in acclimation is not stated (the guidelines state that there should be < 5 % mortality in the batch used). However, the authors state healthy birds were used for the test, and the absence of mortality or abnormal behaviours in the controls shows this has not had an adverse effect on the study outcome.
- **Environmental conditions:** There is a minor difference in temperature (1.8 °C over recommended on day 1) and a lower humidity overall (29.8–67.7 %, mean 49.3 % measured; recommended 60-85 %) in the test compared to the recommended environmental conditions in the guideline. However, the performance of the controls shows this was not an issue with the study.
- **Food consumption:** It is noted that it was not possible to monitor feed consumption in one replicate at the 5620 ppm a.s. treatment level for the period of day 0-1 due to the food being wet, so the data for day 0-1 is based on one replicate. Data was successfully obtained for all other timepoints and so the absence of one replicate at one timepoint is not detrimental to the study outcome.

HSE has considered the additional report (██████████, 2016) and argumentation (shown in *italics* above) regarding the observed effects on body weight and notes the following points:

- **562 and 1780 ppm a.s. treatment levels:** Whilst there are significant differences in absolute body weight and % body weight change at the 562 and 1780 ppm a.s. treatment levels during the exposure period days 0-5 compared to control, this was also the case at study initiation generating some uncertainty (see table 9.1.1.2-5). The birds were adequately randomly assigned to pens at the start of the study, so these differences are likely due to natural variation. Additionally, there are not significant differences in absolute body weight change at these treatment levels (see table 9.1.1.2-5).
- **5620 ppm a.s. treatment level:** Whilst the absolute mean body weight is significantly less than control on day 5 and 8 in the 5620 ppm a.s. treatment level, the change and percentage change in weight is not significantly different from the control (see table 9.1.1.2-5).

- **3160 ppm a.s. treatment level:** The 3160 ppm a.s. treatment level does have a significantly less absolute weight for day 8, change and percentage change for days 5-8, and total weight change. However, significant differences in change or total change (absolute and percentage) are not seen in the higher treatment level of 5620 therefore this effect does not appear to be treatment related in a dose-response manner (see table 9.1.1.2-5).
- Ultimately, there were no mortalities in this study at any of the treatment levels.

The statistical analyses used in the study has also been considered and are deemed appropriate:

- A lack of mortality in the test means that statistical calculation of LC₅₀ was not conducted
- Comparison of body weight data was carried out using a Dunnett's t-test
- No statistical analysis was conducted for the food consumption data

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

- LC₅₀ > 2437 mg a.s./kg bw/day (nominal concentration)

B.9.1.1.3. Sub-chronic toxicity and reproduction to birds

Report: K-CA 8.1.1.31, [REDACTED], [REDACTED] S, [REDACTED], [REDACTED]. (2015). SYN545974 - A Reproduction Study with the Northern Bobwhite, Report Number 528-396. [REDACTED] (Syngenta File No. SYN545974_10130)

Report: K-CA 8.1.1.31, [REDACTED], [REDACTED] (2016) SYN545974: Response to ANSES comments regarding the reproduction study with the Northern Bobwhite ([REDACTED] *et al.*, 2015). (Syngenta File No. SYN545974_10449)

GUIDELINES

OECD Guidelines for Testing of Chemicals, Method 206: Avian Reproduction Test (1984)

US EPA Ecological Effects Test Guidelines, OPPTS 850.2300: Avian Reproduction Test (1996)

GLP: Yes

MATERIALS

Test Material SYN545974 tech.

Lot/Batch #: SMU2EP12007

Purity: 98.5%

Treatments

Test rates: Nominal dietary concentration: 200, 1000 and 5000 ppm alongside an untreated control

Food: Basal diet: Game bird food ration, contained at least 27 % protein, 2 % crude fat, and no more than 5 % crude fibre, approx. 1.12 % calcium. Additional 5 % limestone added to basal diet of adults to raise calcium content to 3 %. Offspring received basal diet without test substance and without addition of 5 % supplemental limestone. Provided *ad libitum* during acclimation and testing.

Water: [REDACTED] public water supply; provided *ad libitum* during acclimation and testing. Offspring received water-soluble vitamin and electrolyte mix in water.

Diet preparation:	SYN545974 was mixed into a premix which was used to prepare the final diet. Control and treatment diets were prepared at least weekly.
Analysis of test concentrations:	Yes, HPLC analysis
Test organisms	
Species:	Bobwhite quail (<i>Colinus virginianus</i>), 21 weeks old (at test start, i.e. 1 st day of exposure to test diet), pen-reared, phenotypically indistinguishable from wild type, from same hatch, approaching first breeding season, not used in previous testing.
Source:	
Acclimatisation period:	4 weeks, birds segregated by sex and placed in group housing to receive medicated water, then moved to pair housing. Birds that appeared unhealthy, injured, unable to acclimate or outside the weight range were excluded from the study.
Treatment for disease:	None reported, birds received medicated water during acclimation period.
Weight:	176 to 234 g at test start
Test design	
Test cage description:	Pens (25 x 51 cm). The pens had sloping floors that resulted in ceiling height ranging from 20 to 26 cm
Replication:	18 pens per group for treated and control birds
No. of birds/pen:	Two (1 male, 1 female)
Pen/treatment assignment:	Randomized
Observations:	Daily during acclimation. Daily during study for signs of toxicity or abnormal behaviour. Offspring observed daily from hatching until 14 days. All mortalities and clinical observations recorded.
Necropsy:	All dead/euthanised birds were subjected to gross necropsy.
Duration of test:	Study phases: Acclimation - 4 weeks. Pre-photostimulation - 8 weeks. Pre-egg laying (with photostimulation) - 3 weeks. Egg laying - 10 weeks. Post-adult termination (final incubation, hatching, and 14-day offspring rearing period) - 6 weeks.
Endpoints:	Adult birds: mortality, clinical observations, gross necropsy, adult body weight, adult feed consumption Reproductive parameters: eggs laid/hen/day, eggs cracked of eggs laid, viable embryos of eggs set, live 3-week embryos of viable embryos, hatchlings of 3-week embryos, 14-day old survivors of hatchlings, hatchlings of eggs set, 14-day old survivors of eggs set, egg shell thickness, offspring body weight
Environmental test conditions	
Temperature:	Adults: 16.1-22.7 °C Chicks: 38 °C
Humidity:	Adults: 20-76 % Chicks: 18 ± 6%
Photoperiod:	Adults: 8 hours light per day from test initiation to Week 9; thereafter increased to 17 hours of light per day at 269 lux Chicks: 16 hours of light. Illumination provided by fluorescent lights that closely approximated colour spectrum of noonday sunlight.

STUDY DESIGN AND MEHODS

Experimental dates: 23 September 2013 to 31 March 2014

Northern bobwhite (72 males and 72 females) were randomly distributed into one control group and three treatment groups. Each treatment and control group contained 18 pairs of birds with one male and one female per pen. The three treatment groups were fed diets containing 200, 1000 or 5000 ppm SYN545974 for 21 weeks. The control group was fed a diet comparable to the treatment groups, but without the addition of the test substance.

All adult birds were observed daily throughout the test for signs of toxicity or abnormal behaviour. 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 due to possible adverse effects of handling on egg production. Feed consumption was measured weekly throughout the test. The amount of wasted feed was not quantified, so feed consumption is presented as an estimate of total feed consumption. At the beginning of Week 9, the photoperiod was increased to induce egg production. Eggs were collected daily from all pens, when available, and stored in a cold room at $13.6 \pm 0.3^{\circ}\text{C}$, RH $69 \pm 8\%$. Eggs were set weekly for incubation. Weekly, eggs were selected by indiscriminate draw for egg-shell thickness measurement and all remaining eggs were candled (using Speed King Model No. 32 egg-candling lamp) prior to incubation to detect egg-shell cracks or abnormal eggs. Eggs were also candled twice during incubation to detect infertile eggs (Day 11-12) or embryo mortality (Day 21). On Day 21 of incubation, the eggs were placed in an incubator configured for hatching and allowed to hatch. Once hatching was completed, hatchlings were removed from the incubator and the group body weight of the hatchlings by pen was determined. At 14 days of age, the average body weight by parental pen of all surviving offspring was determined.

Statistical analysis:

Upon completion of the test, an analysis of variance (ANOVA) was performed to determine statistically significant differences between groups. Dunnett's multiple comparison procedure was used to compare the three 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.

RESULTS AND DISCUSSION

Analytical measurements:

Analysis of control samples did not show any indication of the presence of the test substance. Samples collected during the test to verify test substance concentrations for the 100, 500 and 1000 ppm SYN545974 diets were found to be 97 to 101% of nominal concentrations at test week 1, 98 to 110 % of nominal concentration at test week 12 and 107 to 108% of nominal concentrations at test week 20. Dosing concentrations were therefore within 20 % of nominal concentrations.

Biological results:

Parental generation:

No mortalities occurred in the control group. However, four incidental mortalities, occurred: two in the 200 ppm SYN545974 treatment group, and one each in the 1000 and 5000 ppm SYN545974 treatment groups. None of these birds displayed clinical signs prior to death, and due to the nature of the lesions observed at necropsy, the mortalities that occurred were not considered to be related to treatment.

No overt signs of toxicity were observed at any of the tested concentrations. Incidental observations such as lesions on the head or feet were observed during the test but are normally associated with injuries and penwear. Aside from incidental observations, birds were noted as normal in appearance and behaviour. Findings from gross necropsy were considered unrelated to treatment.

There were no apparent treatment-related effects upon adult body weight at any of the concentrations tested. No statistically significant differences between the control group and test concentrations was observed for males or females. There were no apparent treatment-related effects upon feed consumption at the 200, 1000 and 5000 ppm a.i. test concentrations. There were significant differences between the control and each treatment group, but the

differences were not concentration responsive and were limited to one weekly interval in each treatment group. At the 200 ppm treatment level the difference occurred in week 20 ($p \leq 0.01$), at the 1000 ppm the difference occurred in week 8 ($p \leq 0.05$) and at the 5000 ppm treatment group the difference occurred in week 1 ($p \leq 0.05$). Adult mortality, growth and feed consumption are summarised in Table 9.1.1.3-1.

Reproductive results:

There were no treatment related effects upon egg-shell thickness at any of the concentrations tested. There were no treatment-related effects upon reproductive performance at the 200 or 1000 ppm a.i. test concentrations. At the 5000 ppm a.i. level there were treatment-level effects on multiple reproductive parameters. There was a statistically significant difference from the control in live 3-week embryos as a percentage of viable embryos ($p < 0.05$); hatchlings as a percentage of live 3-week embryos ($p < 0.01$); 14-day old survivors as a percentage of hatchlings ($p < 0.01$); hatchlings as a percentage of eggs set ($p < 0.01$) and 14-day survivors as a percentage of eggs set ($p < 0.01$). There were no statistically significant differences in offspring body weights at the 200 and 1000 ppm a.i. treatment level. At 5000 ppm a.i. there were treatment-related reductions in hatchling and 14-day old survivor body weights ($p < 0.01$ and $p < 0.05$ respectively). Reproductive effects are summarised in Table 9.1.1.3-2 and Table 9.1.1.3-3.

Table 9.1.1.3-1: Summary of effects of SYN545974 on survival, growth and feed consumption on adult northern bobwhite (*Colinus virginianus*) following dietary exposure

Nominal dose (ppm a.s.)	Mortality after 21 weeks (n)	Mean body weight (g)			Mean feed consumption (g/bird/day)			Estimated Daily Dietary Dose (mg a.s./kg bw/ day)
		Pre-egg production	Egg production	Overall	Pre-egg production	Egg production	Overall	Overall
		(1-11 wks)	(12-21 wks)	(1-21 wks)	(1-11 wks)	(12-21 wks)	(1-21 wks)	
Control	0	209	226	213	16	22	19	0
200	2	209	226	214	16	22	19	17.8
1000	1	209	227	214	16	22	19	90.1
5000	1	207	226	212	16	23	19	454

No statistically significant differences were noted for mortality, mean body weight and feed consumption compared to the control.

Table 9.1.1.3-2: Summary of the reproductive performance from northern bobwhite (*Colinus virginianus*) following dietary exposure to SYN545974

Reproductive parameter	Nominal dose (ppm a.s.)			
	Control	200	1000	5000
Number surviving replicates	18	16	17	17
Total eggs laid	871	624	738	674
Eggs cracked	18	34	12	16
Eggs set	766	476	634	553
Mean egg-shell thickness (mm)	0.230	0.229	0.238	0.230
Viable embryos	731	441	598	513
Live 3-week embryos	730	434	596	501

Hatchlings	691	410	517	320
14-day old survivors	605	350	441	232
Eggs / Hen	48	39	43	40
Eggs laid / Hen / Day ^a	0.53	0.43	0.48	0.44
14-day old survivors / Hen	34	22	26	14

^a Based on 91 days of egg production

Table 9.1.1.3-3: Summary of effects of SYN545974 on reproductive parameters and hatchling growth on northern bobwhite (*Colinus virginianus*) following dietary exposure

Nominal dose (ppm a.s.)	Total eggs laid	Eggs cracked / Eggs laid (%)	Viable embryos / Eggs set (%)	Live 3-week embryos / Viable embryos (%)	Hatchlings / Live 3-week embryos (%)	14-day old survivors / Hatchlings (%)	Mean body weight (g)	
							Hatchlings	14-day old survivors
Control	871	2	95	100	95	87	6.1	25
200	624	6	92	98	91	84	5.7	25
1000	738	2	93	100	87	85	5.8	25
5000	674	2	93	98*	59**	55**	5.4**	23*

* Statistically significant difference ($p < 0.05$) from the control group (Dunnett's t-test)

** Statistically significant difference ($p < 0.01$) from the control group (Dunnett's t-test)

VALIDITY CRITERIA

The validity criteria outlined in OECD 206 (1984) were met:

Validity criterion	Required	Observed
Mortality in control group	$\leq 10 \%$	0 %
Average number of 14-day-old survivors per hen in control group	≥ 12	34
Average egg-shell thickness for control group	$\geq 0.19 \text{ mm}$	$0.23 \pm 0.017 \text{ mm}$
Concentration of test substance in diet	$\geq 80 \%$	200 ppm a.i.: 98-108% 1000 ppm a.i.: 99-110 % 5000 ppm a.i.: 101-104 %

CONCLUSIONS

There were no adult treatment-related mortalities, overt signs of toxicity or treatment-related effects upon adult 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 200 or 1000 ppm a.s. test concentrations. However, at the 5000 ppm a.s. test concentration there were treatment-related effects upon multiple reproductive parameters and offspring body weights. Based upon the effects observed in the 5000 ppm a.s. treatment group, the no-observed-effect concentration for northern bobwhite exposed to SYN545974 in the diet during the study was 1000 ppm a.s. (90.1 mg a.s./kg bw/day).

(█ et al., 2015)

HSE evaluator comments

This study has been conducted in accordance with GLP and the validity criteria outlined in OECD 206 (1984) have been satisfactorily met. It is noted that the environmental conditions differed somewhat from those specified in OECD 206 (1984). Adult birds should have been maintained at a temperature of $22 \pm 5 \text{ }^{\circ}\text{C}$, however the temperature range recorded in the study room was $16.1\text{--}22.7 \text{ }^{\circ}\text{C}$ (average $19.6 \pm 1.8 \text{ }^{\circ}\text{C}$). The relative humidity

level specified for hatchlings in their first and second week is 50-75 %, however the average relative humidity in brooding pens was just 18 ± 6 %. Since the validity criteria were met and control birds behaved as expected, these deviations are not thought to have affected the study outcome.

OECD 206 (1984) states that feed consumption of young should be observed in the first and second week after hatching, but this has not been reported in the study. Since the mean body weight of hatchlings was reported, this omission is not critical to the study outcome or validity.

All analytical measurements were within 20 % of nominal test substance concentrations, so it is considered appropriate to express the endpoint in terms of nominal values.

Commission regulation 283/2013 requires estimates of EC_x (e.g. EC_{10} , EC_{20}) alongside the NOEC for chronic studies. EC_x values have not been provided here, nor has any justification for this omission.

Results were analysed statistically using ANOVA and, following angular transformation, Dunnett's multiple comparison test. These methods are in line with those specified in OECD 206 (1984).

HSE has also considered the additional report ([REDACTED] and [REDACTED], 2016) in response to comments by ANSES. ANSES provided the following comment:

"This study is valid but its results cannot be used to determine a NOEC of 1000 mg a.s./kg bw/day as basically concluded. Some significant effects have been observed on the hatching success (41 % below the hatching success in control) and 14 days old survival of juvenile (44 % below survival in control) from population exposed to 200 mg a.s. The discussion in the study report argues that this significant difference is due to the death of adults. This argumentation is not sufficient."

The applicant notes that the endpoints showing significant effects (Hatchlings/Max set (%) and 14 day old survivors/Max set (%)) are non-standard and are not prescribed in USEPA OPPTS 850.2300 or OECD 206 guidelines. Additionally they provide the following argumentation: *"Normalization of hatchling and 14-day old survivor numbers based on the maximum number of eggs set by the most productive hen over all test levels has no valid basis because it does not account for the numbers of eggs set within each treatment, hence artificially inflating the numbers of eggs set in this statistic. The number of eggs set is the most variable endpoint in bird reproduction studies"*. HSE agrees that endpoints normalised based on the maximum number of eggs set are not requirements of either USEPA or OECD guideline. HSE notes that no significant effects were seen in the standard endpoints at 200 ppm a.s. or at the 1000 ppm a.s. level, whereas nearly all reproductive endpoints at the 5000 ppm a.s. level were significantly reduced compared to the control. HSE therefore considers it acceptable for the NOEC to be set at 1000 ppm SYN545974.

The endpoint suitable for use in risk assessment is:

- NOEC = 1000 ppm SYN545974 (90.1 mg SYN545974/kg bw/ day).

Report:	K-CA 8.1.1.3, [REDACTED], [REDACTED], [REDACTED], [REDACTED]. (2014). SYN545974 - A Reproduction Study with the Mallard, Report Number 528-397. [REDACTED] [REDACTED] (Syngenta File No. SYN545974_10134)
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Guidelines

OECD Guidelines for Testing of Chemicals, Method 206: Avian Reproduction Test (1984)

US EPA Ecological Effects Test Guidelines, OPPTS 850.2300: Avian Reproduction Test (1996)

GLP: Yes

Materials

Test Material	SYN545974 tech.
Lot/Batch #:	SMU2EP12007
Purity:	98.5 %

Description:	Off white powder
Stability of test compound:	Stable under test conditions
Reanalysis/Expiry date:	30 June 2016
Treatments	
Test rates:	Nominal dietary concentration: 200, 1,000 and 5,000 ppm alongside an untreated control
Food:	Basal diet: Game bird food ration
Water:	██████ public water supply
Analysis of test concentrations:	Yes
Test organisms	
Species:	Mallard (<i>Anas platyrhynchos</i>) 24 weeks old (at test start, i.e. 1 st day of exposure to test diet)
Source:	██
Acclimatisation period:	10 weeks
Treatment for disease:	None reported
Weight:	903 to 1286 g at test start
Test design	
Test cage description:	Vinyl-coated wire mesh pens (75 X 90 X 45 cm high)
Replication:	18 pens per group for treated and control birds
No. of birds/pen:	Two (1 male, 1 female)
Duration of test:	Study phases: Acclimation - 10 weeks. Pre-photostimulation - 9 weeks. Egg laying - 11 weeks. Post-adult termination (final incubation, hatching, and 14-day offspring rearing period) – 6 weeks.
Environmental test conditions	
Temperature:	Adults: 20.4 to 23.1 °C Chicks: 38 °C
Humidity:	Adults: 23-78 % Chicks: 18 ± 6%
Photoperiod:	Adults: 8 hours light per day from test initiation to Week 9; thereafter increased to 17 hours of light per day at 280 lux Chicks: 16 hours of light

Study Design and Methods

Experimental dates: 23 September 2013 to 27 March 2014

Mallard (72 males and 72 females) were randomly distributed into one control group and three treatment groups. Each treatment and control group contained 18 pairs of birds with one male and one female per pen.

Prior to the experiment, birds were acclimated to the test conditions for a period of 10 weeks. At the start of acclimation, all birds used in the test were apparently healthy and phenotypically indistinguishable from wild type. Immediately prior to test initiation, all potential study birds were examined for physical injuries and general health. Birds that did not appear healthy, either due to injury or inability to acclimate to laboratory conditions, or

were outside the weight range for the test, were excluded from the study. No mortality was reported during the acclimation period.

After the acclimation period, the three treatment groups were fed diets containing 200, 1,000 or 5,000 ppm a.s. of SYN545974 for 20 weeks. The control group was fed diet comparable to the treatment groups, but without the addition of the test substance.

All adult birds were observed daily throughout the test for visible signs of toxicity or abnormal behaviour. Adult body weights were measured at test initiation, at the end of Weeks 2, 4, 6, 8, and at adult termination and feed consumption was measured weekly throughout the test. At the beginning of Week 10, the photoperiod was increased to induce egg production.

Following the start of egg production, eggs were selected by indiscriminate draw for measurements of egg shell thickness. The average thickness of the dried shell plus the membrane was determined by measuring five points around the waist of the egg using a micrometer. All remaining eggs were candled prior to incubation to detect egg shell cracks or abnormal eggs. Eggs were also candled twice during incubation to detect infertile eggs or embryo mortality. On Day 24 of incubation, the eggs were placed in an incubator configured for hatching and allowed to hatch. Once hatching was completed, hatchlings were removed from the incubator and the group body weight of the hatchlings by pen was determined. At 14 days of age, the average body weight by parental pen of all surviving offspring was determined.

Upon completion of the test, an analysis of variance (ANOVA) was performed to determine statistically significant differences between groups. Dunnett's multiple comparison procedure was used to compare the three 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.

Concentrations of test substance in the avian diet were analytically determined using HPLC. The method limit of quantitation (LOQ) was set at 50.0 ppm a.s..

Results and Discussion

Validity criteria

The validity criteria were met according to OECD 206 (1984):

Table 9.1.1.3-4: Validity criteria

Validity criterion	Required	Obtained
Mortality in the controls	Mortality in the controls should not exceed 10 % at the end of the test.	0 %
Brood survival	The average number of 14-day old survivors per hen in the controls should be at least 14.	41
Shell thickness	The average shell thickness for the control group should be ≥ 0.34 mm.	0.395 mm
Test substance concentration	Test substance should be maintained within ± 20 % of the nominal concentrations throughout the test period.	96 – 102 %

Samples collected during the test to verify test substance concentrations for the 200, 1,000, and 5,000 ppm SYN545974 diets were found to be 93 to 101 % of nominal concentrations at test week 1, and 99 to 104 % of nominal concentrations at test week 20. As such, the analysis and reporting of data was based on the nominal concentrations of test substance.

Adult mortality, growth and feed consumption are summarised in Table 9.1.1.3-5 below.

Table 9.1.1.3-5: Summary of effects of SYN545974 on survival, growth and feed consumption on adult Mallard (*Anas platyrhynchos*) following dietary exposure

Nominal dose (ppm a.s.)	Mortality after 20 weeks (n)	Mean body weight (g)			Mean feed consumption (g /bird /day)			Estimated Daily Dietary Dose (mg a.s. /kg bw/ day)
		Pre-egg production (1-9 weeks)	Egg production (11-20 weeks)	Overall (1-20 weeks)	Pre-egg production (1-9 weeks)	Egg production (11-20 weeks)	Overall (1-20 weeks)	Overall (1-20 weeks)
Control	0	1116	1174	1134	122	194	162	0
200	0	1097	1152	1114	113	180	150	26.9
1,000	0	1079	1143	1099	120	184	155	141
5,000	0	1103	1155	1119	115	176	149	664

No statistically significant differences were noted for mortality, mean body weight and feed consumption compared to the control.

No adult mortalities occurred in the control group or in any of the treatment groups during the test.

Reproductive effects are summarised in Table 9.1.1.3-6 and Table 9.1.1.3-7 below:

Table 9.1.1.3-6: Summary of the reproductive performance from mallard ducks (*Anas platyrhynchos*) following dietary exposure to SYN545974.

Reproductive parameter	Nominal dose (ppm a.s.)			
	Control	200	1,000	5,000
Number surviving replicates	18	18	18	18
Total eggs laid	1018	949	940	645
Eggs cracked	0	0	1	7
Eggs set	925	860	855	550
Viable embryos	840	757	737	428
Live 3-week embryos	833	753	723	416
Hatchlings	742	644	602	320
14 day old survivors	733	640	593	311
Eggs / Hen	57	53	52	36
Eggs laid / Hen / Day ^a	0.73	0.68	0.68	0.47
14 day old survivors / Hen	41	36	33	17

^a Based on 77 days of egg production

In addition to the effects shown in the table below, mean shell thickness was statistically significantly ($p < 0.01$) reduced in the 5,000 ppm a.s. treatment group (0.364 mm) in comparison with the control group and the 200 and 1,000 ppm a.s. treatment levels (0.395, 0.386 and 0.390 mm, respectively).

Table 9.1.1.3-7: Summary of effects of SYN545974 on reproductive parameters and hatchling growth on Mallard (*Anas platyrhynchos*) following dietary exposure.

Nominal dose (ppm a.s.)	Total eggs laid	Mean egg shell thickness (mm) [SD]	Eggs cracked / Eggs laid (%)	Viable embryos / Eggs set (%)	Live 3-week embryos / Viable embryos (%)	Hatchlings / Live 3-week embryos (%)	14 day old survivors / Hatchlings (%)	Mean body weight (g)	
								Hatchlings	14-day old survivors
Control	1018	0.395 [0.023]	0	92	99	88	99	37	324
200	949	0.386 [0.027]	0	88	99	84	99	37	329
1,000	940	0.390 [0.020]	0	86	98	83	98	37	312
5,000	645	0.364** [0.026]	1**	80	96*	70**	93	32**	277**

* Statistically significant difference ($p < 0.05$) from the control group (Dunnett's t-test)

** Statistically significant difference ($p < 0.01$) from the control group (Dunnett's t-test)

Abnormal behaviours and symptoms were reported in all conditions including the control condition. But as these were not shown to be significantly different from the control in any condition, they were determined to be incidental, and not treatment-related. A summary of the clinical effects which were observed in each treatment group is shown in Table 9.1.1.3-8 below:

Table 9.1.1.3-8: Summary of observations of abnormal behaviours or symptoms in Mallard (*Anas platyrhynchos*) following dietary exposure to SYN545974.

	Experimental groups (ppm a.s.)			
	0	200	1000	5000
Total number of birds displaying any clinical signs	17	17	13	15
Considered treatment related	0	0	0	0
Considered incidental (unrelated to treatment)	Feather loss (2) Foot lesions (16) Unkempt appearance (1) Wing Lesion (1)	Foot lesions (17) Ocular injury (1)	Distended abdomen (2) Foot lesions (13)	Foot lesions (15) Ocular injury (1) Ruffled appearance (1)

Conclusions

There were no adult treatment-related mortalities, overt signs of toxicity or treatment-related effects upon adult 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 200 or 1,000 ppm a.s. test concentrations. However, at the 5,000 ppm a.s. test concentration there were treatment-related effects upon multiple reproductive parameters, egg shell thickness and offspring body weights. Based upon the effects observed in the 5,000 ppm a.s. treatment group, the no-observed-effect concentration for mallard exposed to SYN545974 in the diet during the study was 141 mg a.s. /kg bw /day.

(██████ et al., 2014)

HSE evaluator comments

This study was conducted according to GLP, and was also in line with OECD 206 (1984): Avian Reproduction Test. All validity criteria were met. The following deviations were noted:

The OECD 206 (1984) guidelines recommend that hatchlings should be kept at 65 - 80 % relative humidity in their first and second week. The hatchlings in this study were kept at 18 ± 6 % humidity. The low humidity levels did not appear to have any impact on the survival of the hatchlings, as a 99 % rate of hatchling survival was observed after 14 days. The guidelines also state that the eggs should be placed in an incubator configured for hatching, and allowed to hatch, on day 23 of the incubation period. In the current study, the eggs were moved to the hatching incubator on day 24 of the incubation period. This is only a minor deviation from the guidelines, and as all the validity criteria have been met, it is unlikely to impact upon the reliability of the data.

Also stipulated in the OECD 206 (1984) guidelines is a minimum floor area of 1 m² per pair of mallard ducks. The floor area used for each pair of mallard ducks in this study was 75 x 90 cm (equivalent to 0.675 m²). Although not ideal from an animal welfare perspective, this shouldn't impact the reliability of the data, as all of the validity criteria were met, and there was no adult mortality throughout the test.

There were also some gaps in the measurements taken. It was not possible to determine the weekly feed consumption for pen 971 in the 5,000 ppm a.s. treatment group in weeks 16, 17, and 18, as the feed was wet. The feed consumption in pen 971 in the weeks before and after this period, was in line with the average feed consumption for the experiment, and so it can be assumed that the feed consumption in weeks 16, 17, and 18 would not differ substantially from this average. Additionally, the temperatures of 5 brooder pens, housing offspring, were not recorded on one day of testing (3rd March 2014). This shouldn't affect the reliability of the data, as the temperature is unlikely to have deviated much from the ambient room temperature. The average temperature in the adult mallard study room was 22.0 °C, and the air handling system was designed to replace up to 15 room air volumes every hour, maintaining uniform environmental conditions throughout the study room.

There were no adult treatment-related mortalities, signs of toxicity, or other treatment-related effects on adult body weight or feed consumption at any of the concentrations tested. However, in the 5,000 ppm a.s. treatment group there were several reproductive parameters which were significantly different from the control condition when assessed with Dunnett's t-test. These differences were determined to be treatment-related. The significantly different parameters were: Live 3-week embryos / viable embryos ($p < 0.05$); eggs cracked / eggs laid ($p < 0.01$); hatchlings / live 3-week embryos ($p < 0.01$); mean body weight (hatchlings) ($p < 0.01$); mean body weight (14 day old survivors) ($p < 0.01$).

Abnormal behaviours and symptoms were reported in all conditions including the control condition. The occurrence of abnormal behaviours and symptoms were not shown to be significantly different from the control in any condition, and as such, these abnormal behaviours were determined to be incidental, and not to be treatment related.

Commission regulation 283/2013 requires estimates of EC_x (e.g. EC₁₀, EC₂₀) alongside the NOEC for chronic studies. EC_x values have not been provided here, nor has any justification for this omission.

The OECD 206 (1984) guidelines do not provide a clear explanation of the statistical methods that should be used. The statistics used in the study do seem appropriate for this study type, and are detailed in the methods section above.

Based on the nominal concentrations, the NOEC value for mallard (*Anas platyrhynchos*) exposed to dietary SYN545974 was 141 mg a.s. /kg b.w. /day.

B.9.1.2. Effects on terrestrial vertebrates other than birds**B.9.1.2.1. Acute oral toxicity to mammals**

The consideration of the acute effects on terrestrial vertebrates other than birds can be found in 3CA B6 (Toxicology).

B.9.1.2.2. Long-term and reproduction toxicity to mammals

The consideration of the long term and reproductive effects on terrestrial vertebrates other than birds can be found in 3CA B6 (Toxicology).

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

No data submitted or required.

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

No data submitted or required.

B.9.1.5. Potential for endocrine disruption

The scientific criteria for determining endocrine disrupting properties in the context of pesticide regulation² have been finalized 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 that the adverse effects observed are not relevant at the (sub) population level:

- (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 pydiflumetofen 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 1107/2009³.

The assessment for pydiflumetofen is detailed below. Firstly the submitted literature review has been considered followed by an ecotoxicology assessment for each of the non-target organism groups in relevant sections (B.9.1.5 and B.9.2.3) for terrestrial and aquatic organisms respectively.

² COMMISSION REGULATION (EU) 2018/605 of 19 April 2018 amending Annex II to Regulation (EC) No 1107/2009 by setting out scientific criteria for the determination of endocrine disrupting properties

³ EFSA Journal 2018;16(6):5311

Relevant Literature on Endocrine Disrupting Properties

The applicant provided a summary report that detailed review of available studies and open literature in terms of endocrine disruption (██████, 2016).

██████, 2016 considered all data included open scientific literature in order to investigate potential endocrine disruption for pydiflumetofen. No relevant publications were identified.

HSE evaluator comments:

The literature review is described in detail in section B9.10 of the CA dossier. HSE considered the literature review acceptable for the endocrine disruption ecotoxicology assessment.
No publications were identified.

Summary of studies submitted relevant to endocrine disruption

A summary of submitted ecotoxicology studies suitable for consideration of endocrine disruption for birds are shown below. The toxicology data is detailed in 3CA B6 (Toxicology) Part II.

Table 9.1.5-1: Studies for ED assessment of pydiflumetofen in non-target organisms other than mammals.

Study ID	Study type	Species	Guideline	Reference
20	Avian reproduction test	<i>Colinus virginianus</i>	OECD 206	██████ <i>et al.</i> (2014)
21	Avian reproduction test	<i>Anas platyrhynchos</i>	OECD 206	██████ <i>et al.</i> (2014)

Birds:

A summary of the results has been provided below. The format is in accordance with EFSA/ECHA guidance i.e. appendix E and considers the information provided by the applicant (██████ *et al.* 2020 and ██████ *et al.* 2020a)

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

Study ID Matrix	Effect classification	Effect target	Species	Exposure	Route	Lowest Effect dose	Doses	Dose unit	Assessment of each line of evidence
20	Sensitive to, but not diagnostic of, EATS	Body weight	<i>Colinus virginianus</i>	21 weeks	Oral	5000	0, 200, 1000, 5000	mg a.s./kg body weight	No effect on adult body weight at any test concentration, Significant reduction in hatchling body weight at highest tested concentration, Significant reduction in 14 d survivor body weight at highest tested concentration
21	Sensitive to, but not diagnostic of, EATS	Body weight	<i>Anas platyrhynchos</i>	20 weeks	Oral	5000	0, 200, 1000, 5000	mg a.s./kg body weight	No effect on adult body weight at any test concentration, Significant reduction in hatchling body weight at highest tested concentration, Significant reduction in 14 d survivor body weight at highest tested concentration
20	Sensitive to, but not diagnostic of, EATS	Cracked eggs	<i>Colinus virginianus</i>	21 weeks	Oral	> 5000	0, 200, 1000, 5000	mg a.s./kg body weight	No effect

Study ID Matrix	Effect classification	Effect target	Species	Exposure	Route	Lowest Effect dose	Doses	Dose unit	Assessment of each line of evidence
21	Sensitive to, but not diagnostic of, EATS	Cracked eggs	<i>Anas platyrhynchos</i>	20 weeks	Oral	5000	0, 200, 1000, 5000	mg a.s./kg body weight	Significant increase in eggs cracked at highest tested concentration
20	Sensitive to, but not diagnostic of, EATS	Egg Production	<i>Colinus virginianus</i>	21 weeks	Oral	> 5000	0, 200, 1000, 5000	mg a.s./kg body weight	No effect on egg production
21	Sensitive to, but not diagnostic of, EATS	Egg Production	<i>Anas platyrhynchos</i>	20 weeks	Oral	> 5000	0, 200, 1000, 5000	mg a.s./kg body weight	Significant reduction in egg production at highest tested concentration
20	Sensitive to, but not diagnostic of, EATS	Egg viability	<i>Colinus virginianus</i>	21 weeks	Oral	> 5000	0, 200, 1000, 5000	mg a.s./kg body weight	No effect on viable embryos
21	Sensitive to, but not diagnostic of, EATS	Egg viability	<i>Anas platyrhynchos</i>	20 weeks	Oral	> 5000	0, 200, 1000, 5000	mg a.s./kg body weight	No effect on viable embryos
20	Sensitive to, but not diagnostic of, EATS	Gross pathology	<i>Colinus virginianus</i>	21 weeks	Oral	> 5000	0, 200, 1000, 5000	mg a.s./kg body weight	No effect
21	Sensitive to, but not diagnostic of, EATS	Gross pathology	<i>Anas platyrhynchos</i>	20 weeks	Oral	> 5000	0, 200, 1000, 5000	mg a.s./kg body weight	No effect
20	Sensitive to, but not diagnostic of, EATS	Hatchability	<i>Colinus virginianus</i>	21 weeks	Oral	5000	0, 200, 1000, 5000	mg a.s./kg body weight	Significant reduction in hatchlings at highest tested concentration

Study ID Matrix	Effect classification	Effect target	Species	Exposure	Route	Lowest Effect dose	Doses	Dose unit	Assessment of each line of evidence
21	Sensitive to, but not diagnostic of, EATS	Hatchability	<i>Anas platyrhynchos</i>	20 weeks	Oral	5000	0, 200, 1000, 5000	mg a.s./kg body weight	Significant reduction in hatchlings at highest tested concentration
20	Systemic toxicity	Mortality	<i>Colinus virginianus</i>	21 weeks	Oral	> 5000	0, 200, 1000, 5000	mg a.s./kg body weight	No effect
21	Systemic toxicity	Mortality	<i>Anas platyrhynchos</i>	20 weeks	Oral	> 5000	0, 200, 1000, 5000	mg a.s./kg body weight	No effect
20	Sensitive to, but not diagnostic of EATS	No. of 14 day old survivors	<i>Colinus virginianus</i>	21 weeks	Oral	5000	0, 200, 1000, 5000	mg a.s./kg body weight	Significant reduction in 14 d survivors at highest tested concentration
21	Sensitive to, but not diagnostic of EATS	No. of 14 day old survivors	<i>Anas platyrhynchos</i>	20 weeks	Oral	5000	0, 200, 1000, 5000	mg a.s./kg body weight	No effect
20	Sensitive to, but not diagnostic of EATS	Viable embryos	<i>Colinus virginianus</i>	21 weeks	Oral	5000	0, 200, 1000, 5000	mg a.s./kg body weight	Significant reduction in live 3-week embryos at highest tested concentration
21	Sensitive to, but not diagnostic of EATS	Viable embryos	<i>Anas platyrhynchos</i>	20 weeks	Oral	5000	0, 200, 1000, 5000	mg a.s./kg body weight	Significant reduction in live 3-week embryos at highest tested concentration

HSE ecotoxicology conclusion for birds

When considering reproductive toxicity, treatment related effects were seen in tests which were sensitive to, but not diagnostic of EATS. The lowest effect dose was 5000 ppm, the highest tested dose for both the Bobwhite Quail and Mallard Duck. No treatment related effects were seen in the parameters for systemic toxicity.

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

Currently there are no further tests available for assessing endocrine activity in birds hence HSE agrees with the applicant that further testing is not required at this stage.

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

The wild mammal endocrine disruption assessment is shown below.

Ecotoxicology consideration 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.

A discussion of the endocrine disrupting properties for pydiflumetofen has been provided in the CA section 6 dossier. The relevance of this to non-target mammals, as opposed to humans, is considered below.

Overall conclusion for EAS modalities (toxicology):

The following conclusion was reached in the toxicology section (see in the volume 3, CA section 6 dossier part II (B.6.8.3)):

‘Overall, HSE agrees with the assessment of EFSA and the EU peer-review process that in the absence of other EAS-mediated endocrine effects, changes in other developmental landmarks, ano-genital distance and other reproductive parameters and organs, the delay in sexual maturation in F1 generation pups alone is not sufficient evidence to support a direct effect of the test substance on the endocrine system. As there was no clear pattern of EAS-mediated adversity and no other effects on reproductive organs in either repeat-dose toxicity or two-generation reproductive toxicity studies, no further investigations into EAS-mediated activity were warranted. Based on the overall weight of evidence, pydiflumetofen does not cause EAS-mediated adversity. The ED criteria are not met because there is no “EAS-mediated” adversity. In addition, this modality has been sufficiently investigated.’

HSE (toxicology) concludes that: ‘based on the overall weight of evidence, pydiflumetofen does not cause EAS-mediated adversity. In addition, this modality has been sufficiently investigated’.

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.

Overall conclusion for T modality (toxicology):

The following conclusion was reached in the toxicology section (see volume 3, CA section 6 dossier part II (B.6.8.3)):

‘Overall, HSE agrees with the assessment of EFSA and the EU peer-review process that pydiflumetofen does not show a consistent pattern indicative of thyroid adversity across short-term, long-term, carcinogenicity and reproductive toxicity studies. As there was no clear pattern of adversity, no further investigations into T-mediated activity were warranted.’

Based on the overall weight of evidence, pydiflumetofen does not cause T-mediated adversity and this modality has been sufficiently investigated.'

HSE (toxicology) concludes that: 'based on the overall weight of evidence, pydiflumetofen does not cause T-mediated adversity and this modality has been sufficiently investigated'.

HSE (ecotoxicology) considers that based on the toxicology conclusion the ED criteria are not met for mammals as non-target organisms when considering T modality and that this modality has been sufficiently investigated.

Reptiles:

No publications or studies assessing effects on reptiles were submitted for pydiflumetofen. 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 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 pydiflumetofen 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 regarding pydiflumetofen for birds or reptiles when considering endocrine disruption.

For non-target wild mammals HSE concludes pydiflumetofen does not meet the criteria of being an ED based on EATS modalities based on current EFSA/ECHA 2018 guidance.

B.9.2. EFFECT ON AQUATIC ORGANISMS

B.9.2.1. Acute toxicity to fish

Report: K-CA 8.2.1 [REDACTED] (2012), SYN545974 - Acute Toxicity to Rainbow Trout (*Oncorhynchus mykiss*) Under Flow-Through Conditions, Report Number 1781.6840, [REDACTED] [REDACTED]. (Syngenta File No. SYN545974_10014)

GUIDELINES

OECD Guidelines for Testing of Chemicals, Method 203: Fish, Acute Toxicity Test (1992)

EC Guideline L142/446 Method C.1 Acute Toxicity for fish (EC, 1998)

US EPA Ecological Effects Test Guidelines, OPPTS 850.1075: Fish Acute Toxicity Test, Freshwater and Marine (1996)

GLP: Yes

MATERIALS

Test material	SYN545974 tech.
Lot/Batch #:	2637-BA/110
Purity:	99.5% (Certificate of analysis confirmed)
Treatments	
Test concentrations:	Dilution water control, solvent control (0.10 mL DMF/L), nominal concentrations of 0.063, 0.13, 0.25, 0.50 and 1.0 mg a.s./L, mean measured concentrations of 0.052, 0.12, 0.22, 0.47 and 0.92 mg a.s./L

Solvent:	Dimethylformamide (DMF, CAS No. 68-12-2)
Analysis of test concentrations:	Yes, at 0, 48 and 96 hours (all treatment levels and the dilution water and solvent controls) based on analysis of SYN545974 using LC-MS/MS
Test organisms	
Species:	Rainbow trout <i>Oncorhynchus mykiss</i>
Source:	Commercial supplier - [REDACTED] ([REDACTED] Lot No. 12A42)
Acclimatisation period:	14 days, in the same dilution water used in testing, no mortality observed in 7 days prior to testing.
Treatment for disease:	None reported
Weight and length of dilution water control fish at start of exposure period:	Mean length: 43 mm (range: 35 – 51 mm) Mean weight: 1.3 g (range: 0.81 – 2.1 g) Loading concentration: 0.22g/L
Feeding:	None during test
Test design	
Test vessels:	Glass aquaria measuring 30 x 15 x 20 cm, test solution volumes maintained at 6.8 L
Test medium:	Well water, same as that used in acclimatisation and dilution.
Replication:	0
No of fish per tank:	7
Exposure regime:	Flow-through using an intermittent-flow proportional diluter (Mount and Brungs, 1967), 6 solution volume replacements per day to provide a 90% test solution replacement rate of ~ 9 hours
Duration:	96 hours
Environmental conditions	
Test temperature:	14 – 16 °C
pH:	6.7 – 7.4
Dissolved oxygen:	7.7 – 9.4 mg/L (60% of saturation is 6.2 mg/L at 14 °C, and 5.9 mg/L at 16 °C)
Hardness of dilution water:	66 mg/L as CaCO ₃
Lighting:	330 – 490 Lux 16 hours fluorescent light and 8 hours dark, with 30-minute transition periods

STUDY DESIGN AND METHODS

Experimental dates: 27 April to 1 May 2012

A flow-through test system was employed. A 10 mg/mL diluter stock solution was prepared by placing 0.9970 g of test substance in a volumetric flask and bringing it to a volume of 100 mL with dimethylformamide (DMF). This stock solution was delivered at 0.0790 mL/cycle into the diluter system's chemical mixing chamber which also received 0.790 L of dilution water per cycle. The mixing chamber, holding a stir bar, was positioned over a magnetic stirrer and was also partially submerged in an ultrasonic water bath to ensure continuous mixing. The concentration of SYN545974 in the solution contained within the mixing chamber was equivalent to that of the highest nominal test concentration (1.0 mg a.s./L) and was proportionally diluted (50%) to produce the remaining nominal test concentrations. The concentration of DMF in the solvent control vessels was equivalent to the

concentration of solvent present in the highest treatment level solution (0.10 mL/L). The remaining control consisted of dilution water only.

At the start of the test seven fish were randomly allocated to each of the test concentrations and the dilution water and solvent controls. The aquaria were maintained in a temperature-controlled room and water bath, designed to maintain temperatures at 14 ± 1 °C. Observations for mortalities and symptoms of toxicity were made at 0, 24, 48, 72 and 96 hours.

Daily measurements of the controls and the test solutions of nominal ≤ 0.25 mg a.s./L were undertaken throughout the 96-hour period for pH, temperature and dissolved oxygen concentration. In the two highest treatment levels of nominal 0.50 and 1.0 mg a.s./L, all fish were dead at the 24-hour observation interval and no further measurements were taken.

The test concentrations were verified by chemical analysis of SYN545974 at 0, 48 and 96 hours using an LC-MS/MS method.

RESULTS AND DISCUSSION

Mean measured concentrations of SYN545974 ranged from 82 to 94% of nominal values (see table below) and defined the treatment levels tested as 0.052, 0.12, 0.22, 0.47 and 0.92 mg a.s./L. Analysis of quality control samples resulted in measured concentrations in the range of 94.6 to 118% of the nominal fortified values confirming the appropriate precision and quality control was maintained. The limit of quantification in this study was 0.0044 – 0.0058 mg a.s./L. Measured concentrations were used for the calculation and reporting of results.

Table 9.2.1-1: Analytical results

Nominal concentration (mg a.s./L)	Measured concentration at 0 hours (mg a.s./L)	Measured concentration at 48 hours (mg a.s./L)	Measured concentration at 96 hours (mg a.s./L)	Mean measured concentration (mg a.s./L) ^a	Percent of nominal ^a (%)
Control	< LOQ ^b	< LOQ	< LOQ	NA	NA
Solvent control	< LOQ	< LOQ	< LOQ	NA	NA
0.063	0.059	0.054	0.042	0.052	82
0.13	0.13	0.13	0.11	0.12	94
0.25	0.26	0.21	0.20	0.22	89
0.50	0.52	0.49	0.40	0.47	95
1.0	1.0	0.99	0.78	0.92	92

^a Mean and percent of nominal are based on the original raw data and not the rounded results presented in this table ^b LOQ = Limit of Quantification. The LOQ for each analysis is dependent upon the linear regression, the area of the low standards and the dilution factor of the controls. At 0, 48 and 96 hours, the LOQ was 0.0058, 0.0044 and 0.0051 mg a.s./L, respectively.

NA = Not applicable

The median lethal concentration (LC₅₀) was defined as the concentration resulting in 50% mortality of the fish in the time period specified. If at least one concentration caused mortality of $\geq 50\%$, a computer programme (Ives, 2011) was used to calculate the LC₅₀ values and 95% confidence intervals. The 96-hour LC₅₀ was determined using Spearman-Kärber estimates. The NOEC (No Observed Effect Concentration) was defined as the highest tested concentration which did not produce toxic-related mortalities or physical and behavioural abnormalities, when compared to the control organisms, and was determined by visual inspection of the data. No mortality or symptoms of toxicity were observed in the controls.

The mortality data and estimated LC₅₀ values are shown in the table below:

Table 9.2.1-2: Effects of SYN545974 on *Oncorhynchus mykiss*

Nominal Concentration (mg a.s./L)	Mean Measured Concentration (mg a.s./L)	Cumulative Percent Mortality (Number of Dead Fish) ^a			
		24 hours	48 hours	72 hours	96 hours
Control	Control	0 (0)	0 (0)	0 (0)	0 (0)
Solvent Control	Solvent Control	0 (0)	0 (0)	0 (0)	0 (0)
0.063	0.052	0 (0)	0 (0)	0 (0)	0 (0)
0.13	0.12	0 (0)	0 (0)	0 (0)	0 (0)
0.25	0.22	14 ^{bc} (1)	29 ^{def} (2)	86 ^f (6)	86 ^g (6)
0.50	0.47	100 (7)	100 (7)	100 (7)	100 (7)
1.0	0.92	100 (7)	100 (7)	100 (7)	100 (7)
LC ₅₀ (mg a.s./L)		0.29	0.26	0.18	0.18
95 % confidence interval (mg a.s./L)		0.24 – 0.35	0.21 – 0.33	0.15 – 0.21	0.15 – 0.21
NOEC (mg a.s./L)		0.12	0.12	0.12	0.12

LC₅₀ values were determined using Spearman-Kärber Estimates. ^a The actual number of mortalities is presented in parentheses ^b Three surviving fish exhibited a partial loss of equilibrium. ^c Three surviving fish were observed to be on the bottom of the test vessel. ^d Two surviving fish were observed to be dark in colouration and exhibited a partial loss of equilibrium. ^e Two surviving fish were observed to be on the bottom of the test vessel. ^f One surviving fish was observed to be dark in colouration and exhibited a complete loss of equilibrium. ^g One surviving fish was observed to be dark in colouration and exhibited a partial loss of equilibrium.

The dose-response curve for the calculated 96-hour LD₅₀ is shown in the figure below:

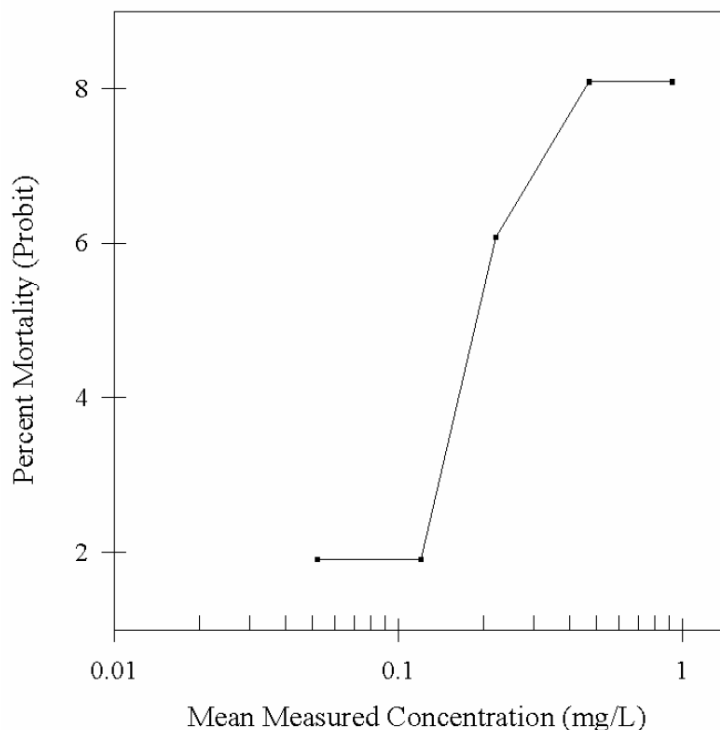


Figure 9.2.1-1: The 96-Hour Concentration-Response (Mortality) Curve for the Flow-Through Acute Exposure of Rainbow Trout (*Oncorhynchus mykiss*) to SYN545974

VALIDITY CRITERIA

The validity criteria for the study were met:

Table 9.2.1-3 Compliance with OECD 203 guidelines

Validity criterion	Required	Obtained
Mortality in the control(s)	$\leq 10 \%$	0 %
Dissolved oxygen concentration	At least 60 % of the air saturation value throughout the test	Observed 7.7 – 9.4 mg/L (60 % of saturation is 6.2 mg/L at 14 °C, and 5.9 mg/L at 16 °C)
Concentration of substance	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.	Mean measured concentrations ranged from 82 – 95 % of nominal. Results are based on mean measured concentrations.

CONCLUSIONS

Based on SYN545974 mean measured concentrations, the 96-hour LC_{50} was determined to be 0.18 mg a.s./L, with 95 % confidence intervals of 0.15 to 0.21 mg a.s./L. The 96-hour NOEC, based on mortality was determined to be 0.12 mg a.s./L.

(██████, 2012)

HSE evaluator comments

The study was carried out in accordance with GLP and follows OECD 203 (2019). Two deviations to the protocol were noted. Firstly, the temperature at test initiation and at the 24-hour-interval differed by $>1^{\circ}\text{C}$ from the target test temperature. Secondly, the fish were fed in the 48-hour period prior to test initiation. Since the temperature deviation was still within the accepted culture range, measured water quality parameters were acceptable, and control fish mortality was 0, these deviations were not found to have impacted the study outcome.

As per OECD 203 (2019), Guidance Document 23 should be consulted if the use of solvent is necessary. There was no observed mortality in the solvent control group, indicating that the solvent did not affect the study outcome. OECD 203 (2019) stipulates that dimethylformamide solvent should be avoided where possible on human health and safety grounds. Additionally, the use of silicone sealants in test vessels is not recommended under OECD 203 (2019). It is noted that the light intensity used was below that stipulated in OECD 203 (2019). These factors are not thought to have affected the study outcome since all validity criteria were met.

The analytical methods have been checked by HSE Chemistry specialists in Vol 3CA Part B5.1.2.6. The following was concluded: “Fit for regulatory purposes but the method is not fully validated in accordance with SANCO/3029/99 rev. 4. LOQ: 0.3 µg/L in aqueous matrices”.

The validity criteria in the OECD 203 (2019) guideline are fulfilled and the agreed endpoint for use in risk assessment is:

- **96-hour LC_{50} = 0.18 mg SYN545974/L (based on mean measured concentrations).**
- **96-hour NOEC = 0.12 mg SYN545974/L (based on mean measured concentrations).**

Report: K-CA 8.2.1 [REDACTED] (2013), SYN545974 - Acute Toxicity to Fathead Minnow (*Pimephales promelas*) Under Flow-Through Conditions, Report Number 1781.6883, [REDACTED] (Syngenta File No. SYN545974_10068)

GUIDELINES

OECD Guidelines for Testing of Chemicals, Method 203: Fish, Acute Toxicity Test (1992)

US EPA Ecological Effects Test Guidelines, OPPTS 850.1075: Fish Acute Toxicity Test, Freshwater and Marine (1996)

GLP: Yes

MATERIALS

Test material SYN545974
Lot/Batch #: SMU2EP12007
Purity: 98.5 %
Description: Off white powder
Stability of test compound: Stable under standard conditions
Reanalysis/expiry date: 30 June 2016
Density: Not applicable

Treatments

Test concentrations: Dilution water control, solvent control (0.10 mL DMF /L), nominal concentrations of 0.063, 0.13, 0.25, 0.50 and 1.0 mg SYN545974 /L, mean measured concentrations of 0.062, 0.11, 0.24, 0.50 and 0.94 mg SYN545974 /L
Solvent: Dimethylformamide (DMF, CAS No. 68-12-2)
Analysis of test concentrations: Yes, at 0, 48 and 96 hours (all treatment levels and the dilution water and solvent controls) based on analysis of SYN545974 using LC-MS/MS

Test organisms

Species: Fathead minnow (*Pimephales promelas*)
Source: Laboratory (testing facility) culture ([REDACTED] Lot No. 12A186)
Acclimatisation period: 14 days
Treatment for disease: None reported
Weight and length of a representative sample of fish (n = 30): Mean length: 29 mm (range: 24 – 32 mm)
 Mean weight: 0.57 g (range: 0.45 – 0.89 g)
Feeding: None during test

Test design

Test vessels: Glass aquaria measuring 30 x 15 x 20 cm, test solution volumes maintained at 6.8 L
Test medium: Well water
Replication: One replicate aquarium was established for each treatment level, control and solvent control
No of fish per tank: 7

Exposure regime:	Flow-through using an intermittent-flow proportional diluter (Mount and Brungs, 1967), 6 solution volume replacements per day to provide a 90 % test solution replacement rate of ~ 9 hours
Duration:	96 hours
Environmental conditions	
Test temperature:	21 – 23 °C
pH:	6.9 – 7.3
Dissolved oxygen:	7.8 – 9.9 mg /L (75 % of saturation is 6.7 mg /L at 21 °C, and 6.4 mg /L at 23 °C)
Hardness of dilution water:	52 - 56 mg /L as CaCO ₃
Lighting:	320 – 380 Lux 16 hours fluorescent light and 8 hours dark, with 30-minute transition periods

STUDY DESIGN AND METHODS

Experimental dates: 25 to 29 January 2013

Juvenile fathead minnow (*Pimephales promelas*) was selected as the test species since it is commonly used in freshwater acute toxicity tests. Prior to testing, the fish were acclimatised for 14 days under similar conditions as those used for the definitive test which were appropriate for the species. 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 7-day period prior to testing.

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 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.

A flow-through test system was employed. A 10 mg/mL diluter stock solution was prepared by placing 1.0014 g of test substance in a volumetric flask and bringing it to a volume of 100 mL with dimethylformamide (DMF). This stock solution was delivered at 0.0775 mL/cycle into the diluter system's chemical mixing chamber which also received 0.775 L of dilution water per cycle. The mixing chamber, holding a stir bar, was positioned over a magnetic stirrer and was also partially submerged in an ultrasonic water bath to ensure continuous mixing. The concentration of SYN545974 in the solution contained within the mixing chamber was equivalent to that of the highest nominal test concentration (1.0 mg a.s. /L) and was proportionally diluted (50 %) to produce the remaining nominal test concentrations. The concentration of DMF in the solvent control vessels was equivalent to the concentration of solvent present in the highest treatment level solution (0.10 mL/L). The remaining control consisted of dilution water only. The diluter system was calibrated prior to exposure initiation. At exposure termination, the calibration was checked by measuring delivery volumes of test substance and dilution water.

At the start of the test seven fish were randomly allocated to each of the test concentrations and the dilution water and solvent controls. The resulting test organism loading concentration was 0.10 g of biomass per liter of solution per aquarium per day. The aquaria were maintained in a temperature-controlled room and water bath, designed to maintain temperatures at 22 ± 1 °C.

Observations for mortalities and symptoms of toxicity were made at 0, 24, 48, 72 and 96 hours. Dead fish were recorded and removed, biological observations of adverse effects (e.g., loss of equilibrium, fish on the bottom of the vessel, lethargy) were also made and recorded at these times. Observations of the physical characteristics of the test solutions (e.g., presence of precipitate, film on the solution's surface) were made and recorded, if applicable. 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). Daily measurements of the controls and the test solutions of nominal ≤ 0.50 mg a.s. /L were undertaken throughout the 96-hour period for pH,

temperature and dissolved oxygen concentration. In the highest treatment level of nominal 1.0 mg a.s. /L, all fish were dead at the 24-hour observation interval and no further measurements were taken.

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 the 0.50 mg /L nominal treatment level using a VWR minimum/maximum thermometer.

At exposure initiation (0 hour), 48 hours and exposure termination (96 hours), two samples from each treatment level and control were collected. One sample was analysed for SYN545974 concentration while the duplicate was stored frozen as an archive backup sample. Each sample was collected from the approximate midpoint of the test vessel using a pipette. The test concentrations were verified by chemical analysis of SYN545974 at 0, 48 and 96 hours using an LC-MS/MS method. At 0, 48 and 96 hours, the LOQ was 0.0053, 0.0056 and 0.0047 mg a.s. /L, respectively.

The median lethal concentration (LC₅₀) was defined as the concentration resulting in 50 % mortality of the fish in the time period specified. If at least one concentration caused mortality of ≥ 50 %, a computer programme (Ives, 2011) was used to calculate the LC₅₀ values and 95 % confidence intervals. The 96-hour LC₅₀ was determined using Binomial/Graphical Estimates. The NOEC (No Observed Effect Concentration) was defined as the highest tested concentration which did not produce toxicant-related mortalities or physical and behavioural abnormalities, when compared to the control organisms, and was determined by visual inspection of the data.

RESULTS AND DISCUSSION

Analytical results

Mean measured concentrations of SYN545974 ranged from 88 to 100 % of nominal values (see table below) and defined the treatment levels tested as 0.062, 0.11, 0.24, 0.50 and 0.94 mg a.s. /L. Analysis of quality control samples resulted in measured concentrations in the range of 90.7 to 107 % of the nominal fortified values confirming the appropriate precision and quality control was maintained. The limit of quantification in this study was 0.0047 – 0.0056 mg a.s. /L. Measured concentrations were used for the calculation and reporting of results.

Table 9.2.1-4: Analytical results

Nominal concentration (mg a.s. /L)	Measured concentration at 0 hours (mg a.s. /L)	Measured concentration at 48 hours (mg a.s. /L)	Measured concentration at 96 hours (mg a.s. /L)	Mean measured concentration (mg a.s. /L) ^a	Percent of nominal ^a (%)
Control	< LOQ ^b	< LOQ	< LOQ	NA	NA
Solvent control	< LOQ	< LOQ	< LOQ	NA	NA
0.063	0.062	0.063	0.060	0.062	98
0.13	0.11	0.11	0.12	0.11	88
0.25	0.24	0.23	0.24	0.24	95
0.50	0.50	0.49	0.53	0.50	100
1.0	0.93	0.91	0.98	0.94	94

^a Mean and percent of nominal are based on the original raw data and not the rounded results presented in this table ^b LOQ = Limit of Quantification. The LOQ for each analysis is dependent upon the linear regression, the area of the low standards and the dilution factor of the controls. At 0, 48 and 96 hours, the LOQ was 0.0053, 0.0056 and 0.0047 mg a.s. /L, respectively.

NA = Not applicable

Biological results

No mortality was observed in the controls. Some signs of loss of equilibrium and lethargy were noted in the treatment concentrations (Table 9.2.1-5).

The mortality data along with the estimated LC₅₀ and NOEC values are shown in table 9.2.1-5: below, the 96 hour concentration/response curve is shown graphically in 9.2.1-2.

Table 9.2.1-5: Effects of SYN545974 on *Pimephales promelas*

Nominal Concentration (mg a.s. /L)	Mean Measured Concentration (mg a.s. /L)	Cumulative Percent Mortality (Number of Dead Fish) ^a			
		24 hours	48 hours	72 hours	96 hours
Control	Control	0 (0)	0 (0)	0 (0)	0 (0)
Solvent Control	Solvent Control	0 (0)	0 (0)	0 (0)	0 (0)
0.063	0.062	0 (0)	0 (0)	0 (0)	0 (0)
0.13	0.11	0 (0)	0 (0)	0 (0)	0 (0)
0.25	0.24	0 (0)	0 (0)	0 (0)	0 (0)
0.50	0.50	29 (2) ^{bc}	43 (3) ^{de}	86 (6) ^e	100 (7)
1.0	0.94	100 (7)	100 (7)	100 (7)	100 (7)
LC ₅₀ (mg a.s. /L)		0.56 ^{*1}	0.51 ^{*1}	0.38 ^{*1}	0.35 ^{*2}
95 % confidence interval (mg a.s. /L)		0.45 – 0.71	0.40 – 0.66	0.32 – 0.46	0.26 – 0.46
NOEC (mg a.s. /L)		-	-	-	0.24

^{*1} LC₅₀ value was determined using Spearman-Kärber Estimates

^{*2} LC₅₀ value was determined using Binomial/Graphical Estimates

^a The actual number of mortalities is presented in parentheses

^b One surviving fish exhibited a partial loss of equilibrium

^c Two surviving fish were observed to be lethargic

^d One surviving fish was observed to be on the bottom of the test vessel

^e One surviving fish exhibited a complete loss of equilibrium

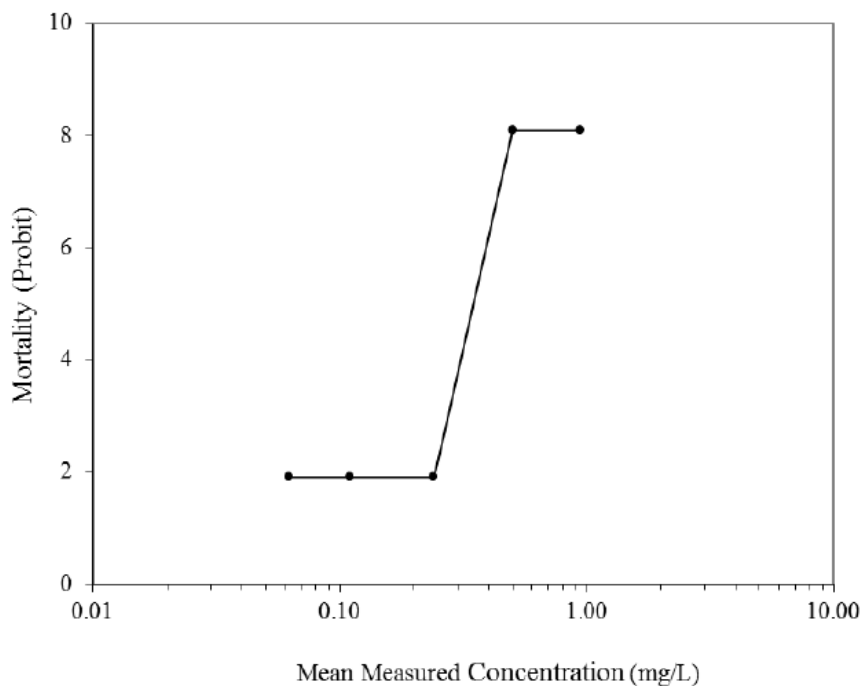


Figure 9.2.1-2: The 96h concentration response curve for mortality.

VALIDITY CRITERIA

The validity criteria for the study were met according to OECD 203 (1992) and OCSPP (Draft) Guideline 850.1075 (1996):

Table 9.2.1-6: Compliance with OECD 203 validity criteria

Validity criterion	Required	Obtained
Mortality in the control(s)	$\leq 10 \%$	0 %
Test conditions*	Constant conditions	A flow-through design was chosen, with 6 solution volume replacements per day. Constant conditions were maintained.
Dissolved oxygen concentration	At least 60 % of the air saturation value throughout the test	Dissolved oxygen concentration remained above 75 % 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 ranged from 88 - 100 % of nominal.

*Included in reference to OECD 203 (1992), this is no longer a requirement in OECD 203 (2019).

CONCLUSIONS

Based on SYN545974 mean measured concentrations, the 96 hour LC₅₀ was determined to be 0.35 mg a.s. /L, with 95 % confidence intervals of 0.26 to 0.46 mg a.s. /L. The 96-hour NOEC, based on mortality and visual abnormalities was determined to be 0.24 mg a.s. /L.

(██████████, 2013)

HSE evaluator comments

The study was carried out according to GLP and follows OECD 203 (1992), and OCSPP (Draft) Guideline 850.1075 (1996). The study was also evaluated against the most recent OECD 203 guideline (2019).

The following deviations were noted:

Observations for mortalities and symptoms of toxicity were made at 24, 48, 72 and 96 hours. This is in line with the OECD 203 (1992) guidelines that the study authors were subject to at the time of experimentation, which state that: “The fish are inspected at least after 24, 48, 72 and 96 hours. Observations at three and six hours after the start of the test are desirable.” However, the current OECD 203 (2019) guidelines state that where feasibly possible, a minimum of two observations should be conducted within the first 24 hours of the study (not including the measurement taken at 24h), and all vessels with living fish should be inspected twice daily from days 2-4 of the study. However, in the current study, only one observation was made per day. This may potentially mean that transient effects were missed. This will be considered further in the risk assessment.

The guideline specifies that during the acclimatisation period, fish should not be displaying visible signs of disease and stress and should be free of any apparent malformations. This detail isn’t included in the study report, however, the lack mortality/visible abnormalities in the control during the definitive test provides some reassurance that the fish were derived from a healthy stock population. This will be considered further in the risk assessment.

The statistical methods used to analyse the data are in-line with the guideline, however, Figure 9.2.1 indicates that the visual fit of the model to the data is not ideal. The three lower concentrations resulted in zero mortality, whilst there was a sharp increase at the two higher concentrations. This means that the accuracy of the predicted LD₅₀ could be uncertain. This will be considered further in the risk assessment.

The analytical methods have been checked by HSE Chemistry specialists in Vol 3CA Part B5.1.2.6. The following was concluded: “Fit for regulatory purposes but the method is not fully validated in accordance with SANCO/3029/99 rev. 4. LOQ: 0.3 µg/L in aqueous matrices”.

Based on the active substance mean measured concentration values, the 96-hour LC₅₀ for SYN545974 to the fathead minnow (*Pimephales promelas*) was 0.35 mg /L with 95 % confidence intervals of 0.26 to 0.46 mg a.s. /L. The 96-hour NOEC, based on mortality was determined to be 0.24 mg a.s. /L.

Report:	K-CA 8.2.1 ██████████ (2013a), SYN545974 - Acute Toxicity to Carp (<i>Cyprinus carpio</i>) Under Flow-Through Conditions, Report Number 1781.6882, ██████████ ██████████. (Syngenta File No. SYN545974_10066)
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GUIDELINES

OECD Guidelines for Testing of Chemicals, Method 203: Fish, Acute Toxicity Test (1992), US EPA Ecological Effects Test Guidelines, OCSPP 850.1075: Fish Acute Toxicity Test, Freshwater and Marine (1996)

GLP: Yes

MATERIALS

Test material	SYN545974 tech.
Lot/Batch #:	SMU2EP12007
Purity:	98.5 % w/w

Description:	Off white powder
Stability of test compound:	Stable under standard conditions
Reanalysis/expiry date:	30 June 2016
Treatments	
Test concentrations:	Dilution water control, solvent control (0.10 mL DMF/L) and nominal concentrations of 0.063, 0.13, 0.25, 0.50 and 1.0 mg a.s./L. Mean measured concentrations: 0.060, 0.13, 0.26, 0.51, 1.0 mg SYN545974/L
Solvent:	Dimethylformamide (DMF, CAS No. 68-12-2)
Analysis of test concentrations:	Yes from each treatment level and control, based on analysis of SYN545974 at initiation (0 hours), 48 hours and exposure termination (96 hours) using LC/MS/MS analysis
Test organisms	
Species:	Juvenile carp <i>Cyprinus carpio</i> (Lot No. 13A05)
Source:	Obtained from commercial supplier
Acclimatisation period:	13 days
Treatment for disease:	None reported
Weight and length of a representative sample of fish (n = 30):	Mean length: 32 mm (range: 26 to 39 mm) Mean weight: 0.60 g (range: 0.44 to 0.88 g)
Feeding:	None during test, or for 48 hours prior to exposure
Test design	
Test vessels:	Glass aquaria measuring 30 x 15 x 20 cm, test solution volumes maintained at 6.8 L
Test medium:	Well water
Replication:	None
No of fish per tank:	7
Exposure regime:	Flow-through using an intermittent-flow proportional diluter (Mount and Brungs, 1967), 6 solution volume replacements per day to provide a 90 % test solution replacement rate of ~ 9 hours
Duration:	96 hours
Environmental conditions	
Test temperature:	22 - 23° C
pH:	7.2 – 7.4
Dissolved oxygen:	7.6 – 9.4 mg/L (75 % of saturation is 6.5 mg/L at 22 °C, and 6.4 mg/L at 23 °C)
Hardness:	44 - 56 mg/L CaCO ₃
Lighting:	420 to 700 Lux 16 hours fluorescent light and 8 hours dark.

STUDY DESIGN AND METHODS

Experimental dates: 28 January to 01 February 2013.

A flow-through test system was employed. A 10 mg/mL primary stock solution was prepared by placing 2.0666 g of SYN545974 in a volumetric flask and bringing it to a volume of 200 mL with dimethylformamide (DMF). Appropriate volumes of the stock were then made up to 100 mL with DMF to produce secondary stocks with

concentrations of 0.63, 1.3, 2.5 and 5.0 mg a.s./mL. These secondary stock solutions were delivered at 0.0401 mL/cycle into the diluter system's chemical mixing chamber which also received 0.40 L of dilution water per cycle. The mixing chambers were positioned over a magnetic stirrer which continuously mixed the contents of the mixing chambers. The concentration of DMF was equal in each test concentration and was 0.10 mL/L, which is the highest concentration allowed by the OECD guideline. The control vessel contained the same dilution water and was maintained under the same conditions as the treatment level and solvent control vessels, but contained no SYN212974 or DMF.

At the start of the test, seven fish were randomly allocated to each of the test concentrations and the dilution water and solvent controls. The aquaria were maintained in a temperature-controlled room and water bath, designed to maintain temperatures at 22 ± 1 °C. Observations for mortalities and symptoms of toxicity were made at 0, 24, 48, 72 and 96 hours.

Daily measurements of the controls and the test solutions were undertaken throughout the 96 hour period for pH, temperature and dissolved oxygen concentration. Temperature was continuously monitored through-out the study in the 0.50 (day 0 to 3) and 0.25 mg a.s./L (day 3 to 4) nominal treatment levels.

The test concentrations were verified by chemical analysis of SYN545974 at 0, 48 and 96 hours using an LC-MS/MS method.

RESULTS AND DISCUSSION

Mean measured concentrations of SYN545974 ranged from 96 to 100 % of nominal values (see table below) and defined the treatment levels tested as 0.060, 0.13, 0.26, 0.51 and 1.0 mg a.s./L. Analysis of quality control samples resulted in measured concentrations in the range of 91 to 109 % of the nominal fortified values (0.0300, 0.200 and 1.00 mg a.s./L) confirming the appropriate precision and quality control was maintained. The limit of quantification in this study was 0.0047 – 0.0048 mg a.s./L.

Measured concentrations were used for the calculation and reporting of results.

Table 9.2.1-7: Analytical results

Nominal concentration (mg a.s./L)	MC at 0 hours (mg a.s./L)	% of nom ^a (%)	MC at 48 hours (mg a.s./L)	% of nom ^a (%)	MC at 96 hours (mg a.s./L)	% of nom ^a (%)	Mean measured concentration (mg a.s./L) ^a
Control	< LOQ ^b	NA	< LOQ	NA	< LOQ	NA	NA
Solvent Control	< LOQ	NA	< LOQ	NA	< LOQ	NA	NA
0.063	0.064	101.6	0.056	88.9	0.062	98.4	0.060
0.13	0.14	107.7	0.12	92.3	0.13	100.0	0.13
0.25	0.29	116.0	0.24	96.0	0.26	104.0	0.26
0.50	0.51	102.0	0.48	96.0	0.53	106.0	0.51
1.0	1.1	110.0	0.94	94.0	1.1	110.0	1.0

^a Mean and percent of nominal are based on the table values above rather than original raw data. ^b The limit of quantification (LOQ) for each analysis is dependent upon the linear regression, the area of the low standards and the dilution factor of the controls. At 0, 48 and 96 hours, the LOQ was 0.0047, 0.0048 and 0.0047 mg a.s./L, respectively.

NA : Not applicable, MC = Measured Concentration, % of nom = % of nominal

The median lethal concentration (LC₅₀) was defined as the concentration resulting in 50 % mortality of the fish in the time period specified. If at least one test concentration caused mortality of ≥ 50 % of the test population, then a computer program (Ives, 2011) was used to calculate LC₅₀ values and 95 % confidence intervals. The 96-hour LC₅₀ was determined using Binomial/Graphical Estimates. The NOEC (No Observed Effect Concentration) is defined as the highest tested concentration which did not produce an adverse effect when compared to the control and was determined by visual inspection of the data.

Treatment related mortalities were observed at mean measured concentrations of 0.26 mg a.s./L and above. Symptoms of toxicity observed included lethargy and were observed at concentrations of 0.26 mg a.s./L and above. No mortality or symptoms of toxicity were observed in the control or solvent control.

The mortality data and estimated LC₅₀ values are shown in the table below:

Table 9.2.1-8: Effects of SYN545974 on *Cyprinus carpio*

Nominal Concentration (mg a.s./L)	Mean Measured Concentration (mg a.s./L)	Cumulative Percent Mortality (Number of Dead Fish) ^a			
		24 hours	48 hours	72 hours	96 hours
Control	Control	0 (0)	0 (0)	0 (0)	0 (0)
Solvent Control	Solvent Control	0 (0)	0 (0)	0 (0)	0 (0)
0.063	0.060	0 ^b (0)	14 ^f (1)	14 (1)	14 (1)
0.13	0.13	0 (0)	0 (0)	0 (0)	0 (0)
0.25	0.26	0 (0)	0 ^g (0)	0 ^g (0)	14 (1)
0.50	0.51	0 ^c (0)	57 ^h (4)	100 (7)	100 (7)
1.0	1.0	28 ^{de} (2)	100 (7)	100 (7)	100 (7)
LC ₅₀ (mg a.s./L)		> 1.0	0.49 ^j	0.36 ^k	0.33 ^j
95 % confidence interval (mg a.s./L)		NA ⁱ	0.38 – 0.63	0.28 - 0.47	0.28 – 0.40
NOEC (mg a.s./L)		NC	NC	NC	0.13

a) The actual number of mortalities is presented in parentheses

b) One fish was observed to have a spinal deformity. Not considered to be toxicant related by study author

c) Several fish exhibited complete loss of equilibrium

d) Two fish exhibited a complete loss of equilibrium

e) Three fish were observed to be on the bottom of the test vessel

f) Fish was observed to have a spinal deformity likely resulting in a stressed fish. Mortality was not considered to be toxicant related

g) One fish exhibited a complete loss of equilibrium

h) All surviving fish exhibited a complete loss of equilibrium

i) NA = Not applicable. LC₅₀ value was empirically estimated therefore, confidence intervals could not be calculated.

j) LC₅₀ value was determined using Spearman-Kärber Estimates

k) LC₅₀ value was determined using Binomial/Graphical Estimates NC: Not calculated

VALIDITY CRITERIA

Validity criteria from OECD 203 (2019) have been satisfactorily met.

Table 9.2.1-9: Compliance with OECD 203 validity criteria

Validity criterion	Required	Obtained
Mortality in the control(s)	≤ 10 %	0 % in control 0 % in solvent control
Dissolved oxygen concentration	At least 60 % of the air saturation value throughout the test	94-98 % saturation

Validity criterion	Required	Obtained
Concentration of substance	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 ranged from 96 – 100 % of nominal.

CONCLUSIONS

Based on SYN545974 mean measured concentrations, the 96-hour LC_{50} for carp (*Cyprinus carpio*) was determined to be 0.33 mg a.s./L with 95 % confidence intervals of 0.28 – 0.40 mg a.s./L. The 96-hour NOEC, based on mortality was 0.13 mg a.s./L.

(██████████, 2013a)

HSE evaluator comments

The study was carried out according to GLP and follows OECD 203 (1992) with no significant deviations to the guideline. The study was also checked against the most recent OECD 203 guideline (2019) and no deviations were noted. One minor deviation noted was the acclimation period for the *Cyprinus carpi*;; this is stated as 13 days. OECD 203 (2019) section 13 dictates fish should be held for a minimum of 9 days, so this is within the most recent guidelines.

The fish loading of 0.1 g/L is stated in this study. This is in line with the guidance in OECD 203 (1992) but is marginally above the loading stated in the 2019 guideline. Given the validity criteria were met and there were no behavioural observations during the study this deviation from current guidelines is considered acceptable by HSE. This study uses the solvent dimethylformamide. OECD 203 (2019) states that if the use of solvent is needed Guidance Document No. 23 should be consulted. However, this requirement was part of the guideline published after study completion. The use of the solvent was noted, and a solvent control was used. The concentration of the solvent was 0.1 ml/L, not exceeding the maximum set out in the OECD 203 (2019) guidance. There was no observed mortality within the solvent control treatment group indicating the solvent had no effect on the outcome of the study.

The study used nylon and silicone seals within the holding tanks. This is not recommended in OECD 203 (2019), however the guidance is not present in OECD 203 (1992). It is not considered to have significantly influenced the study particularly as analytical measurements suggested relatively high recoveries and a flow through study design was used.

The statistical analysis was briefly reported and it was simply stated that the method used was Spearman-Kärber. Hence it was not possible to confirm whether data was transformed prior to analysis and not stated whether the analysis conducted was appropriate. However, the method used is detailed in OECD 203 and the endpoint derived is in-line with the experimental data. Therefore HSE considers the statistical analysis conducted by the study author appropriate.

The analytical methods have been checked by HSE Chemistry specialists in Vol 3CA Part B5.1.2.6. The following was concluded: “Fit for regulatory purposes but the method is not fully validated in accordance with SANCO/3029/99 rev. 4. LOQ: 0.3 µg/L in aqueous matrices”.

There were observed behaviours of one fish at 0.063 mg a.s./L but these were not considered to be due to toxicity. Therefore, the agreed endpoint suitable for use in the risk assessment is:

- **96-hour LC_{50} = 0.33 mg a.s./L (mean measured concentration)**
- **96-hour NOEC= 0.13 mg a.s./L (mean measured concentration)**

Report: K-CA 8.2.1 [REDACTED] (2013b). SYN545974 - Acute Toxicity to Sheepshead Minnow (*Cyprinodon variegatus*) Under Flow-Through Conditions, Report Number 1781.6884, [REDACTED]. (Syngenta File No. SYN545974_10067)

GUIDELINES

- OECD Guidelines for Testing of Chemicals, Section 2 - Effects on Biotic Systems, Method 203: Fish, Acute Toxicity Test (1992)
- US EPA Ecological Effects Test Guidelines, OPPTS 850.1075: Fish Acute Toxicity Test, Freshwater and Marine (1996)

GLP: Yes

MATERIALS

Test material SYN545974 tech.
Lot/Batch #: SMU2EP12007
Purity: 98.5 % w/w
Description: Off white powder
Stability of test compound: Stable under standard conditions
Reanalysis/expiry date: 30 June 2016
Density: Not applicable

Treatments

Test concentrations: Dilution water control, solvent control (0.10 mL DMF/L), nominal concentrations of 0.063, 0.13, 0.25, 0.50 and 1.0 mg a.s./L
Mean measured concentrations: 0.060, 0.11, 0.23, 0.48 and 0.90 mg a.s./L

Solvent: Dimethylformamide (DMF, CAS No. 68-12-2)

Analysis of test concentrations: Yes, at 0, 48 and 96 hours (all treatment levels and the dilution water and solvent controls) based on analysis of SYN545974 using LC-MS/MS

Test organisms

Species: Sheepshead minnow *Cyprinodon variegatus*
Source: Obtained from commercial supplier [REDACTED] (Lot No. 13A07)
Acclimatisation period: 14 days
Treatment for disease: None reported
Weight and length of a representative sample of fish (n = 30): Mean length: 15 mm (range: 13 – 17 mm)
Mean weight: 0.090 g (range: 0.050 – 0.13 g)
Feeding: None during test, or for 48 hours prior to exposure

Test design

Test vessels: Glass aquaria measuring 30 x 15 x 20 cm, test solution volumes maintained at 6.8 L

Test medium: Natural seawater from the Cape Cod Canal, Bourne, Massachusetts, USA, filtered and diluted to approximately 20 ‰.

Replication: 1 tank per control and per treatment condition.

No of fish per tank: 7

Exposure regime:	Flow-through using an intermittent-flow proportional diluter (Mount and Brungs, 1967), 6 solution volume replacements per day to provide a 90 % test solution replacement rate of ~ 9 hours
Duration:	96 hours
Environmental conditions	
Test temperature:	22 – 23 °C
Salinity:	20 – 21 ‰
pH:	7.7 – 7.8
Dissolved oxygen:	7.2 – 8.4 mg/L (75 % of saturation is 5.8 mg/L at 22 °C and 20 ‰, and 5.7 mg/L at 23 °C, and 20 and 21 ‰)
Lighting:	320 – 400 Lux 16 hours fluorescent light and 8 hours dark, with 30-minute transition periods

STUDY DESIGN AND METHODS

Experimental dates: 8 to 12 February 2013

A flow-through test system was employed. A 10 mg/mL diluter stock solution was prepared by placing 1.0226 g of test substance in a volumetric flask and bringing it to a volume of 100 mL with dimethylformamide (DMF). This stock solution was delivered at 0.0780 mL/cycle into the diluter system's chemical mixing chamber which also received 0.780 L of dilution water per cycle. The mixing chamber, holding a stir bar, was positioned over a magnetic stirrer and was also partially submerged in an ultrasonic water bath to ensure continuous mixing. The concentration of SYN545974 in the solution contained within the mixing chamber was equivalent to that of the highest nominal test concentration (1.0 mg a.s./L) and was proportionally diluted (50 %) to produce the remaining nominal test concentrations. The concentration of DMF in the solvent control vessels was equivalent to the concentration of solvent present in the highest treatment level solution (0.10 mL/L). The remaining control consisted of dilution water only.

At the start of the test seven fish were randomly allocated to each of the test concentrations and the dilution water and solvent controls. The aquaria were maintained in a temperature-controlled room and water bath, designed to maintain temperatures at 22 ± 1 °C. Observations for mortalities and symptoms of toxicity were made at 0, 24, 48, 72 and 96 hours.

Daily measurements of the controls and the test solutions were undertaken throughout the 96 hour period for pH, temperature, salinity and dissolved oxygen concentration, except in the highest treatment level of nominal 1.0 mg a.s./L. In this treatment all fish were dead at the 72-hour observation interval and no further measurements were taken. Temperature was continuously monitored through-out the study in the 0.50 mg a.s./L nominal treatment level.

The test concentrations were verified by chemical analysis of SYN545974 at 0, 48 and 96 hours using an LC-MS/MS method.

RESULTS AND DISCUSSION

Mean measured concentrations of SYN545974 ranged from 84 to 96 % of nominal values (see table below) and defined the treatment levels tested as 0.060, 0.11, 0.23, 0.48 and 0.90 mg a.s./L. Analysis of quality control samples resulted in measured concentrations in the range of 88.9 to 110 % of the nominal fortified values (0.0300, 0.200 and 1.00 mg a.s./L) confirming the appropriate precision and quality control was maintained. The limit of quantification in this study was 0.0048 – 0.0050 mg a.s./L. Measured concentrations were used for the calculation and reporting of results.

Table 9.2.1-10: Analytical results

NC (mg a.s./L)	MC at 0 hours (mg a.s./L)	% of NC at 0 hours	MC at 48 hours (mg a.s./L)	% of NC at 48 hours	MC at 96 hours (mg a.s./L)	% of NC at 96 hours	MMC ^a (mg a.s./L)
Control	< LOQ ^b	NA	< LOQ	NA	< LOQ	NA	NA
Solvent control	< LOQ	NA	< LOQ	NA	< LOQ	NA	NA
0.063	0.066	104.8	0.047	74.6	0.068	107.9	0.060
0.13	0.12	92.3	0.088	67.7	0.12	92.3	0.11
0.25	0.26	104.0	0.17	68.0	0.25	100.0	0.23
0.50	0.53	106.0	0.37	74.0	0.53	106.0	0.48
1.0	1.0	100.0	0.66	66.0	1.0	100.0	0.90

^a Mean based on the original raw data and not the rounded results presented in this table

^b LOQ = Limit of Quantification. The LOQ for each analysis is dependent upon the linear regression, the area of the low standards and the dilution factor of the controls. At 0, 48 and 96 hours, the LOQ was 0.0050, 0.0048 and 0.0049 mg a.s./L, respectively.

NA = Not Applicable, NC = Nominal Concentration, MC = Measured Concentration, % of NC = Percentage of Nominal Concentration, MMC = Mean Measured Concentration.

The median lethal concentration (LC₅₀) was defined as the concentration resulting in 50 % mortality of the fish in the time period specified. If at least one concentration caused mortality of ≥50 %, a computer programme (Ives, 2011) was used to calculate the LC₅₀ values and 95 % confidence intervals. The 96-hour LC₅₀ was determined using Binomial/Graphical Estimates. The NOEC (No Observed Effect Concentration) was defined as the highest tested concentration which did not produce an adverse effect when compared to the control, and was determined by visual inspection of the data.

No mortality or symptoms of toxicity were observed in the controls. The mortality data and estimated LC₅₀ values are shown in the table below:

Table 9.2.1-11: Effects of SYN545974 on *Cyprinodon variegatus*

Nominal concentration (mg a.s./L)	Mean measured concentration (mg a.s./L)	Cumulative Percent Mortality (Number of Dead Fish) ^a			
		24 hours	48 hours	72 hours	96 hours
Control	Control	0 (0)	0 (0)	0 (0)	0 (0)
Solvent Control	Solvent Control	0 (0)	0 (0)	0 (0)	0 (0)
0.063	0.060	0 (0)	0 (0)	0 (0)	0 (0)
0.13	0.11	0 (0)	0 (0)	0 (0)	0 (0)
0.25	0.23	0 (0)	0 (0)	0 (0)	0 (0)
0.50	0.48	0 (0)	0 (0)	0 (0)	0 (0)
1.0	0.90	0 (0)	57 ^b (4)	100 (7)	100 (7)
LC ₅₀ (mg a.s./L)		> 0.90	0.83* ¹	0.66* ²	0.66* ²
95% confidence interval (mg a.s./L)		NA	0.58 – 1.2	0.52 – 0.83	0.52 – 0.83
NOEC (mg a.s./L)		-	-	-	0.48

^a The actual number of mortalities is presented in parentheses

^b All surviving fish were observed to be lethargic

*¹ LC₅₀ value was determined using Trimmed Spearman-Kärber Estimates

*² LC₅₀ value was determined using Binomial/Graphical Estimates

NA = Not Applicable. LC₅₀ value was empirically estimated, therefore, 95 % confidence intervals could not be determined.

VALIDITY CRITERIA

The following validity criteria are set out in the guideline OECD 203 (2019):

Table 9.2.1-12: Compliance with OECD 203 validity criteria

Validity criterion	Required	Obtained
Mortality in the control(s)	≤ 10 %, or one fish if fewer than 10 control fish are tested.	No mortality in both controls.
Test conditions	Static, semi-static or flow-through	Flow-through design was used.
Dissolved oxygen concentration	At least 60 % of the air saturation value throughout the exposure in all vessels.	83-97 %
Concentration of test substance	<p>Analytical measurement of test concentrations (using a validated analytical method) is compulsory.</p> <p>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.</p>	<p>Analytical measurements of test substance concentration ranged from 66-107 % across the whole exposure. Therefore, the applicant based their results on the mean measured concentration across all timepoints.</p>

CONCLUSIONS

Based on mean measured concentrations, the 96-hour LC₅₀ for SYN545974 to sheephead minnow (*Cyprinodon variegatus*) was determined to be 0.66 mg a.s./L, with 95% confidence intervals of 0.52 to 0.83 mg a.s./L. The 96-hour NOEC, based on mortality, was determined to be 0.48 mg a.s./L.

(██████████, 2013b)

HSE evaluator comments

The study was carried out according to OECD GLP (1998) with the exception of routine water and food screening analyses which were outsourced to an external company. Since the external company used standard validated methods these exceptions had no impact on the study results. The study followed OECD 203 (1992) guidelines and OPPTS 850.1075 (1992). However, the most recent guideline is OECD 203 (2019) so the study was assessed against this more recent version.

There were minor deviations from the study guidelines however these were not deemed to affect the obtained results as explained below:

- Silicone sealant was used in the tank whereas the guidelines state to avoid this where possible due to its lipophilic properties. However, due to the adequate analytical measurement of the test chemical, this was not an issue.
- The pre-exposure holding temperature for the fish was reported as 21-22 °C, and the exposure temperature was 22-23 °C, whereas the guidelines for sheephead minnow state 23-27 °C. Additionally, the holding dissolved oxygen concentration was not recorded. This did not affect the overall results because no signs of stress or mortality were recorded in the holding period or exposure controls.

- The light intensity for the vessels was 320 to 400 lux whereas the guidelines recommend 540-100 lux. This did not affect the overall results because no signs of stress or mortality were recorded in the holding period or exposure controls.
- For the dilution water, the study uses natural filtered (20- and 5-micron pore size) seawater rather than reconstituted seawater. The precise elemental analysis of this water was not provided, but the water was sent for periodic testing to an external company and no contaminants were reported present according to ASTM 2002 standard practice. Additionally, the guidelines state that natural water should have TOC and nitrate content analysed. Although TOC was analysed and within acceptable range for the month of February (which was the month of exposure), nitrate was not reported. Additionally, water hardness was not reported at any point. However, since there was no mortality or negative physical observations recorded in both holding conditions and the test controls, it is concluded that these minor deviations in dilution water do not affect the overall result of the study.
- The guidelines state exposure tanks should be monitored twice daily whereas the study monitored them once a day. However, since meaningful data has been obtained for the 96-hour endpoint, this is not a significant issue.

The details of the statistical analysis were briefly reported. For example, it was unclear whether data was transformed or the exact method of analysis used. The report stated LC₅₀ values were calculated by 'Binomial/Graphical estimates.' Nonetheless, both methods are listed in OECD 203 guideline. The graphical method is stated as last resort in OECD 203 but given the lack of partial responses in experimental data this method could also be justified. Furthermore, the endpoint calculated is in-line with experimental data. Hence HSE considers the statistical analysis conducted by the study author appropriate.

The analytical methods have been checked by HSE Chemistry specialists in Vol 3CA Part B5.1.2.6. The following was concluded: "Fit for regulatory purposes but the method is not fully validated in accordance with SANCO/3029/99 rev. 4. LOQ: 0.3 µg/L in aqueous matrices".

Test solution concentration for SYN545974 were analysed at 0, 48 and 96 hours. The measured test concentrations for all levels at 48 hours were under the 80 % nominal threshold (ranging from 66-75 %), therefore the applicant used mean measured concentration across the three measured timepoints in their results and LC₅₀ calculations.

The substance SYN545974 was prepared using a solvent and therefore OECD Guidance Document 23 (2019) for testing of difficult substances has been considered. The solvent used (Dimethylformamide, DMF) is listed in the Guidance Document as effective for aquatic toxicity testing and is within the recommended concentration range of 0.10 mL DMF/L. Additionally, there was no observed mortality within the solvent control treatment group indicating the solvent had no effect on the outcome of the study.

Overall, the study had no major deviations from the OECD 203 (2019) guideline and the study fulfils all validity criteria of this guideline.

The agreed endpoints for SYN545974 suitable for use in the risk assessment are:

- **96-hour LC₅₀ = 0.66 mg a.s./L (mean measured concentrations)**
- **NOEC = 0.48 mg a.s./L (mean measured concentrations)**

Report:	K-CA 8.2.1 [REDACTED] (2014), SYN545974 - Acute Toxicity to Bluegill Sunfish (<i>Lepomis macrochirus</i>) Under Flow-Through Conditions, Report Number 1781.7025, [REDACTED], (Syngenta File No. SYN545974_10129)
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GUIDELINES

OECD Guidelines for Testing of Chemicals, Section 2 - Effects on Biotic Systems, Method 203: Fish, Acute Toxicity Test (1992)

US EPA Ecological Effects Test Guidelines, OPPTS 850.1075: Fish Acute Toxicity Test, Freshwater and Marine (1996)

GLP: Yes

MATERIALS

Test material	SYN545974
Lot/Batch #:	SMU2EP12007
Purity:	98.5%
Treatments	
Test concentrations:	Dilution water control and nominal concentrations of 0.063, 0.13, 0.25, 0.50 and 1.0 mg a.s./L (mean measured: 0.058, 0.091, 0.20, 0.42 and 0.82 mg a.s./L)
Solvent:	Dimethylformamide (DMF, CAS No. 68-12-2).
Analysis of test concentrations:	Yes, using LC-MS analysis
Test organisms	
Species:	Bluegill sunfish (<i>Lepomis macrochirus</i>)
Source:	██████████
Acclimatisation period:	14 days, no mortalities observed during the 48- hour period prior to testing
Treatment for disease:	None
Weight and length of dilution water control fish at end of exposure period:	Mean length: 23 mm Mean weight: 0.30 g Loading rate: 0.05g/L
Feeding:	None during test
Test design	
Test vessels:	Disposable glass vessels
Test medium:	Well water, used as dilution water and for acclimatisation
Replication:	None
No of fish per tank:	7
Exposure regime:	Flow-through
Duration:	96 hours
Environmental conditions	
Test temperature:	21 - 22° C
pH:	7.1 – 7.4
Dissolved oxygen:	7.2 – 8.9 mg /L
Hardness of dilution water:	64 to 66 mg/L CaCO ₃
Lighting:	16 hours fluorescent light and 8 hours dark with 30-minute dawn and dusk transition periods

STUDY DESIGN AND METHODS

Experimental dates: 22 to 26 September 2014

A nominal stock solution was prepared by dissolving 0.9989 g of the test item completely in 100 mL of dilution water by intense stirring. The control consisted of dilution water only. The concentration of DMF in the solvent control vessels was equivalent to the concentration of solvent present in the highest treatment level solution (0.10 mL/L).

At the start of the test seven fish were randomly allocated to each of the test concentrations and the dilution water control. The test was conducted in a temperature-controlled water-bath. Observations for mortalities and symptoms of toxicity were made at 6, 24, 48, 72 and 96 hours.

Daily measurements of the test solutions were undertaken throughout the 96-hour period for pH, temperature and dissolved oxygen concentration.

The test concentrations were verified by chemical analysis at 0 and 96 hours using an LC-MS/MS method.

RESULTS AND DISCUSSION

The measured concentrations are shown in the table below in terms of nominal concentrations. The test concentrations were maintained throughout the study. The limit of quantification in this study was 0.151 µg /L. Mean measured concentrations were used for the calculation and reporting of results.

Table 9.2.1-13: Analytical results

Nominal concentration (mg a.s./L)	0-Hour measured concentration (mg/L)	48-Hour measured concentration (mg/L)	96-Hour measured concentration (mg/L)	Mean measured concentration (mg a.s./L)	% of nominal
0.063	0.055	0.059	0.060	0.058	92
0.13	0.10	0.096	0.075	0.091	70
0.25	0.24	0.19	0.18	0.20	82
0.50	0.43	0.42	0.42	0.42	85
1.0	0.88	0.75	0.82	0.82	82

The LC₅₀ is defined as the concentration of the test substance in dilution water which caused mortality of 50% of the test organism population at the stated time interval. If at least one test concentration caused mortality of greater than or equal to 50% of the test population, then a computer program (Ives, 2013) was used to calculate the LC₅₀ values and 95% confidence intervals.

The No-Observed-Effect Concentration (NOEC) was also determined by visual inspection of the data. The NOEC is defined as the highest concentration tested at which there were no toxicant related mortalities or physical and behavioural abnormalities (e.g., lethargy, loss of equilibrium), with respect to the control organisms. The mortality data and estimated LC₅₀ values are shown in the table below:

Table 9.2.1-14: Effects of SYN545974 on the survival of *Lepomis macrochirus*

Mean measured concentration (mg a.s./L)	Mortality observed (Cumulative number of dead fish) (n = 7)				
	6 hours	24 hours	48 hours	72 hours	96 hours
Dilution water control	0	0	0	0	0
Solvent control	0	0	0	0	0
0.058	0	0	0	0	0
0.091	0	0	0	0	0
0.20	0	0	0	0	0
0.42	0	0	0	0 ^{ab}	2 ^{bc}
0.82	0	4 ^d	7	7	7

Mean measured concentration (mg a.s./L)	Mortality observed (Cumulative number of dead fish) (n = 7)				
	6 hours	24 hours	48 hours	72 hours	96 hours
LC ₅₀ mg/L	n.d.	0.75	0.59	0.59	0.48
95 % confidence interval	-	0.51 – 1.1	0.46 – 0.76	0.46 – 0.76	0.38 – 0.61
NOEC	0.82	0.42	0.42	0.42	0.20

^a One fish was observed to exhibit complete loss of equilibrium. ^b Several fish were observed to be lethargic. ^c One fish was observed to be on the bottom of the test vessel. ^d All surviving fish were observed to be on the bottom of the test vessel.

The dose-response curve for the calculated 96-hour LC₅₀ is shown in the figure below:

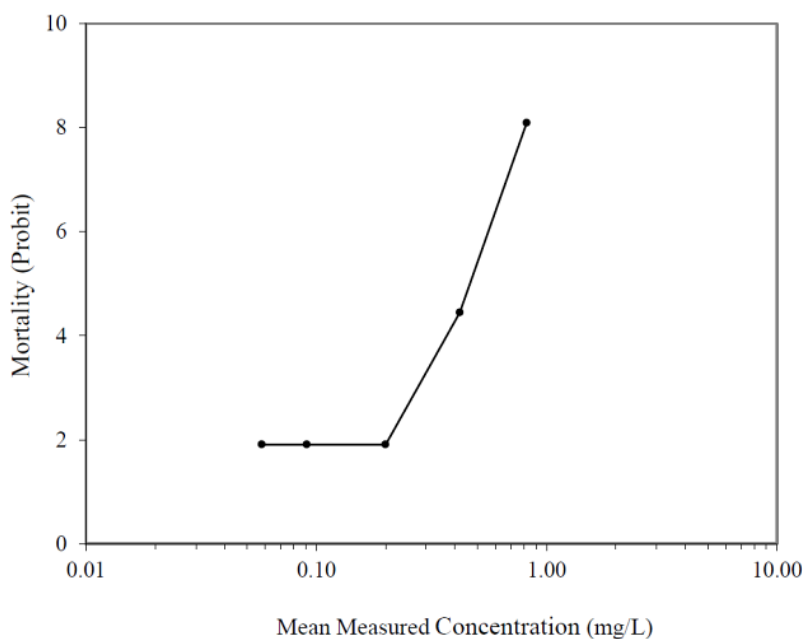


Figure 9.2.1-3: The 96-Hour Concentration-Response (Mortality) Curve for the Flow-Through Acute Exposure of Bluegill Sunfish (*Lepomis macrochirus*) to SYN545974

VALIDITY CRITERIA

The validity criteria for the study were met:

Table 9.2.1-15: Compliance with OECD 203 validity criteria

Validity criterion	Required	Obtained
Mortality in the control(s)	≤ 10%	0%
Dissolved oxygen concentration	At least 60 % of the air saturation value throughout the test	Observed 7.2 – 8.9 mg /L (75 % of saturation is 6.5 mg/L at 22 °C).
Concentration of substance	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.	Mean measured concentrations ranged from 70 – 92 % of nominal. Results are based on mean measured concentrations.

CONCLUSIONS

Based on mean measured concentrations, the 96-hour LC_{50} for SYN545974 to Bluegill sunfish (*Lepomis macrochirus*) was 0.48 mg a.s./L and the 96-hour NOEC was 0.2 mg a.s./L.

(██████████, 2014)

HSE evaluator comments

The study was carried out in accordance with GLP and follows OECD 203 (2019) with no significant deviations to the guidelines. A minor deviation noted was the use of light intensities below those stipulated in OECD 203 (2019). Light intensity was measured at 20- 43 footcandles, but OECD 203 states that light intensity at the water surface should be 30-100 footcandles. Since control survival exceeded the acceptable criteria this deviation is not thought to have affected the study outcome.

OECD 203 (2019) guidelines stipulate that where a solvent is used, Guidance Document 23 should be consulted. An appropriate solvent control was used and there was no observed mortality in the solvent control group. It is noted that OECD 203 (2019) guidance states that use of the solvent dimethylformamide should be avoided where possible, due to human health concerns. The use of silicone seals in test vessels is also not advised in OECD 203 (2019). These factors are not thought to have affected the study outcome.

The analytical methods have been checked by HSE Chemistry specialists in Vol 3CA Part B5.1.2.6. The following was concluded: “Fit for regulatory purposes but the method is not fully validated in accordance with SANCO/3029/99 rev. 4. LOQ: 0.3 µg/L in aqueous matrices”.

The validity criteria in OECD 203 (2019) are fulfilled and the agreed end point for use in risk assessment is:

- **96-hour LC_{50} = 0.48 mg SYN545974/L (based on mean measured concentrations).**

Report:	K-CA 8.2.1 ██████████, (2015), SYN545547 - Acute Toxicity Test with Rainbow Trout (<i>Oncorhynchus mykiss</i>) Under Static Conditions, Report Number 1781.7096, ██████████ ██████████, (Syngenta File No. SYN545547_10001)
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GUIDELINES

OECD Guidelines for Testing of Chemicals, Method 203: Fish, Acute Toxicity Test (1992)
US EPA Ecological Effects Test Guidelines, OCSPP 850.1075: Fish Acute Toxicity Test, Freshwater and Marine (1996)

GLP: Yes

MATERIALS

Test material	SYN545547
Lot/Batch #:	BPS 1510/1
Purity:	95 % w/w tested at 100 %
Description:	White powder
Stability of test compound:	Stable under test conditions
Reanalysis/expiry date:	End of May 2017

Treatments

Test concentrations:	Dilution water control, solvent control and nominal concentrations of 0.31, 0.63, 1.3, 2.5, 5.0 and 10 mg /L (0.28, 0.44, 0.97, 2.0, 4.2 and 7.9 mg /L geometric mean measured).
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Solvent:	Dimethylformamide (DMF)
Solvent control:	1.5 mL DMF added to 15 L of dilution water
Analysis of test concentrations:	Yes, based on analysis at 0 and 96 hours using HPLC/UV
Test organisms	
Species:	Rainbow trout <i>Oncorhynchus mykiss</i>
Source:	████████████████████
Acclimatisation period:	14 days
Treatment for disease:	None
Weight and length of dilution water control fish at end of exposure period:	Mean length: 49 mm (range 44 to 55 mm) Mean weight: 1.4 g (range 0.99 to 1.7 g)
Feeding:	None during test
Test design	
Test vessels:	39 x 20 x 25 cm (L x W x H) glass aquaria containing 15 L test medium
Test medium:	Dilution water (well water)
Replication:	One aquarium for the treatment and one for the control and solvent control
No of fish per tank:	7
Exposure regime:	Static
Duration:	96 hours
Environmental conditions	
Test temperature:	Continuous measurement: 13 to 15 °C Measurements taken at daily intervals: 14 to 15 °C
pH:	6.8 to 7.3
Dissolved oxygen:	7.3 to 9.5 mg /L
Hardness of dilution water:	66 mg /L CaCO ₃
Lighting:	840 to 1000 Lux. 16 hours fluorescent light and 8 hours dark

STUDY DESIGN AND METHODS

Experimental dates: 1 to 5 June 2015

Rainbow trout (*Oncorhynchus mykiss*) were chosen as the test organism for this study, as they are recommended by the U.S. EPA and OECD, and are commonly used in freshwater acute toxicity tests. Prior to experimentation, they were acclimatised to the test conditions for 14 days. Fish were fed with commercial trout feed during the acclimatisation period, but no feeding took place in the 48 hours prior to testing, or during the test. No mortality was observed among the test fish population in the 48 hours before testing.

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 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.

A stock solution with a nominal concentration of 100 mg /L was prepared by dissolving 2.5345 g of the test item in 25 mL volumetric flask and bringing it to volume with dimethylformamide. The primary (100 mg /L) and

secondary (50 mg /L) stock solutions were observed to be clear with a yellow tint, but no visible undissolved material. All further dilutions of this stock solution were observed to be clear and colourless, with no visible undissolved test substance. Appropriate volumes of the stock were made up to 15 L of dilution water in each test vessel to give the test concentrations. The control consisted of dilution water only.

At the start of the test seven fish were randomly allocated to each of the test concentrations and the dilution water control, with a mean organism loading of 0.63 g biomass /L of test solution. The test was conducted in a temperature-controlled water-bath. Observations for mortalities and symptoms of toxicity were made at 6, 24, 48, 72 and 96 hours. The following symptoms of toxicity were recorded: lethargy, residing on the bottom of the test vessel, loss of equilibrium, and discolouration.

Daily measurements of the test solutions were undertaken throughout the 96 hour period for pH, temperature and dissolved oxygen concentration. The test concentrations were verified by chemical analysis at 0 and 96 hours using a HPLC/UV method. Measured concentrations ranged from 71 % to 90 % of the nominal values, as a result, the geometric mean measured concentrations were used for the analysis and reporting of results. The limit of quantification in this study was 0.00606 mg /L.

The LC₅₀ is defined as the concentration of the test substance in dilution water which produces 50 % mortality of the test organism population at the stated time interval. As more than one test concentration caused mortality of greater than or equal to 50 % of the test population, a computer program (Ives, 2013) was used to calculate the LC₅₀ values and 95 % confidence intervals.

The No-Observed-Effect Concentration (NOEC) during the 96 hour exposure period was also determined. The NOEC is defined as the highest concentration tested at and below which there was no toxicant related mortality or physical and behavioural abnormalities (e.g., lethargy), with respect to the control organisms.

RESULTS AND DISCUSSION

Table 9.2.1-16: Analytical results

Nominal concentration (mg /L)	Measured concentration at 0 hours (mg /L)	Measured concentration at 96 hours (mg /L)	Geometric mean measured concentration (mg /L)	Percent of nominal ^a (%)
Control	<0.023	<0.026	NA	NA
Solvent control	<0.023	<0.026	NA	NA
0.31	0.35	0.22	0.28	90
0.63	0.48	0.41	0.44	71
1.3	1.1	0.84	0.97	75
2.5	2.3	1.8	2.0	82
5.0	4.6	3.8	4.2	84
10	9.1	6.9	7.9	79

Geometric mean measured concentrations are based on the original raw data and not the rounded results presented in this table. Concentrations expressed as less than values were below the limit of quantitation (LOQ). The LOQ for each analysis is dependent upon the linear regression, the area of the low standards and the dilution factor of the controls.

NA = Not Applicable

The geometric mean measured concentrations tested (from 0 and 96 hours) and the corresponding data derived from the definitive toxicity test were used to estimate the 24, 48, 72 and 96-hour median effective concentrations (LC₅₀).

The mortality data and estimated LC₅₀ values are shown in table 9.2.1-17 below. The 96-hour concentration/response relationship is displayed graphically in 9.2.1-4:

Table 9.2.1-17: Effects of SYN545547 on the survival of *Oncorhynchus mykiss*

Geometric mean measured concentration (mg /L)	Mortality observed (Cumulative number of dead fish) ^a				
	6 hour	24 hours	48 hours	72 hours	96 hours
Dilution water control	0	0	0	0	0
Solvent control	0	0	0	0	0
0.28	0	0	0	0	0
0.44	0	0	0	0 ^g	0 ^{ci}
0.97	0 ^{bc}	0 ^{bce}	0 ^{bce}	0 ^{bch}	0 ^{chj}
2.0	0 ^{bd}	6 ^f	7	7	7
4.2	7	7	7	7	7
7.9	7	7	7	7	7
LC ₅₀ mg /L	ND	1.5	1.4	1.4	1.4
95 % confidence interval	ND	1.3 – 1.9	1.1 – 1.8	1.1 – 1.8	1.1 – 1.8
NOEC	0.44	0.44	0.44	0.28	0.28

^a n = 7^b Two fish exhibited a complete loss of equilibrium.^c Several fish were observed to be lethargic.^d Several fish were observed to be on the bottom of the test vessel.^e One fish exhibited a partial loss of equilibrium.^f The surviving fish was observed to be on the bottom of the test vessel.^g Two fish were observed to be lethargic.^h One fish was observed to be on the bottom of the test vessel.ⁱ One fish was observed to be dark in color and lethargic.^j Several fish exhibited a complete loss of equilibrium.

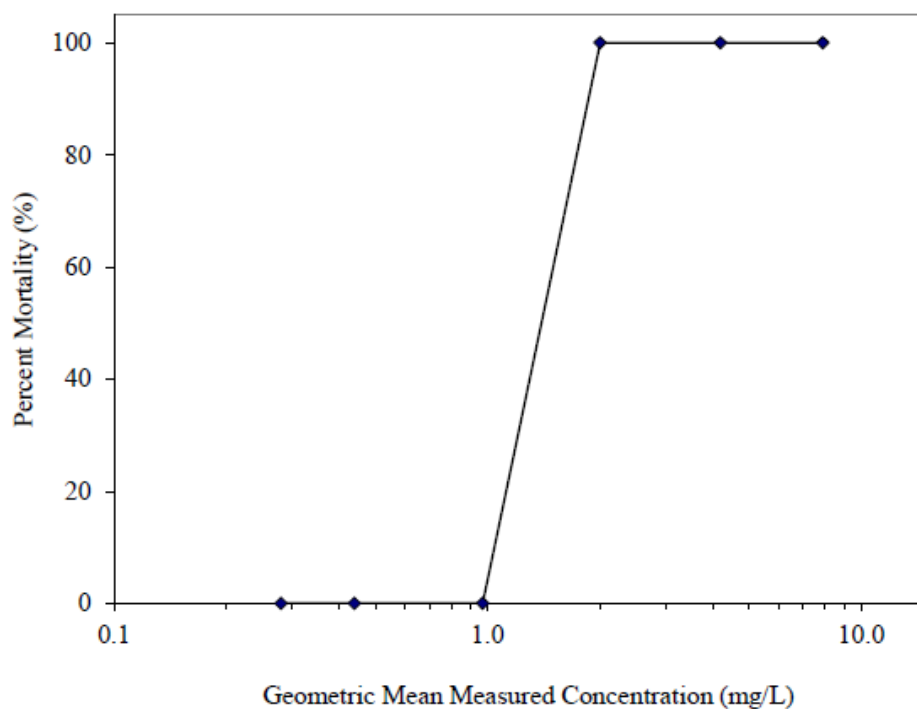


Figure 9.2.1-4. The 96 hour concentration-response curve for mortality

VALIDITY CRITERIA

The validity criteria for the study were met according to OECD 203 (1992) and OPPTS (1996):

Table 9.2.1-18 Compliance with validity criteria

Validity criterion	Required	Obtained
Mortality in the control(s)	≤ 10 %	0 %
Test conditions*	Constant conditions	To maintain constant conditions a static design was chosen.
Dissolved oxygen concentration	At least 60 % of the air saturation value throughout the test	71-94 %
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 ranged from 71-90 % of nominal. As a result, geometric mean measured concentrations were used for the calculation and reporting of results.

*Included in reference to OECD 203 (1992), this is no longer a requirement in OECD 203 (2019).

The measured concentrations are shown in the table below in terms of nominal concentrations. Geometric mean measured concentrations were used for the calculation and reporting of results

CONCLUSIONS

Based on geometric mean measured concentrations, the 96-hour LC_{50} for SYN545547 to rainbow trout (*Oncorhynchus mykiss*) was 1.4 mg /L, and the 96-hour based on mortality and visual/behavioural abnormalities NOEC was 0.28 mg /L.

HSE evaluator comments

This study was carried out according to GLP, and follows OECD 203 (1992), and OPPTS (1996) with no significant deviations from the guidelines. The study was also evaluated to the most recent version of OECD 203 (2019), and all validity criteria were met.

One minor point of note was that the measured temperature ranged from 14-15 °C, which was in line with the OECD 203 (1992) guidelines of 13-17 °C degrees, but was marginally outside of the since revised OECD 203 (2019) guidelines of 10-14 °C, which the study authors were not subject to at the time of experimentation. As the validity criteria were met, and there was no abnormal behaviour recorded in the control conditions, this would not be cause to invalidate the study.

Although the authors reported that there was no mortality in the 48h period prior to exposure, there was no mention of any observations for disease or other biological symptoms in the test organisms prior to testing. As there were no such observations reported in the control conditions throughout the test period, and the fish were kept under the same conditions in the acclimatisation period, it can be assumed that the fish were healthy at the point of exposure.

Observations for mortalities and symptoms of toxicity were made at 6, 24, 48, 72 and 96 hours. This is in line with the OECD 203 (1992) guidelines that the study authors were subject to at the time of experimentation, which state that: “The fish are inspected at least after 24, 48, 72 and 96 hours. Observations at three and six hours after the start of the test are desirable.” However, the current OECD 203 (2019) guidelines state that where feasibly possible, a minimum of two observations should be conducted within the first 24 hours of the study (not including the measurement taken at 24h), and all vessels with living fish should be inspected twice daily from days 2-4 of the study. However, in the current study after the first day, only one observation was made per day. This may potentially mean that transient effects were missed.

The authors state that “If at least one test concentration caused mortality of greater than or equal to 50 % of the test population, then a computer program (Ives, 2013) was used to calculate the LC_{50} values and 95 % confidence intervals.” As three of the tested concentrations resulted in 100 % mortality, this indicates that a computer program was used for the endpoint calculations as stated in the study report. However, no further information was provided about the statistical procedures which were used. OECD 203 (1992) (which the authors were subject to at the time of experimentation) states that “Where the data obtained are inadequate for the use of standard methods of calculating the LC_{50} , the highest concentration causing no mortality and the lowest concentration producing 100 percent mortality should be used as an approximation for the LC_{50} (this being considered the geometric mean of these two concentrations).” No tested concentration produced partial mortality. As such, in order to estimate the LC_{50} value, a geometric mean should have been taken of the highest concentration resulting in 0 % mortality, and the lowest concentration resulting in 100 % mortality. When calculated independently, this produces the same LC_{50} value as was obtained by the study authors. This provides some reassurance that the calculations were in accordance with the guidelines.

The current (2019) version of the OECD 203 guidelines states that “When an experiment results in no concentration with partial mortality, estimates of the LC_{50} can be made using various techniques such as the Spearman-Kärber method (Stephan, 1977), the binomial method (USEPA, 2002), the moving average method (ISO, 1996), or as a last resort, the graphical method (USEPA, 2002).” Minimal information was provided about the statistical procedures used, and as such, it was not possible to ascertain whether these were within the guidelines. Following a request for further information, the applicant has clarified that, “except for the 24 h LC_{50} value obtained by Spearman-Kärber estimation, the values for all other timepoints were binomial/graphical estimates”. Therefore the statistical methods used can be accepted.

Measured concentrations of test substance dipped below 80 % of the nominal concentration in some conditions, and so geometric mean measured concentrations were used for calculation and reporting of results. The analytical methods have been checked by HSE Chemistry specialists in Vol 3CA Part B5.1.2.6. The following was

concluded: “Fit for regulatory purposes but the method is not fully validated in accordance with SANCO/3029/99 rev. 4. LOQ: 0.015 mg/L in freshwater and AAP medium”.

Based on the geometric mean measured concentrations, the 96-hour LC₅₀ for SYN545547 to rainbow trout (*Oncorhynchus mykiss*) was 1.4 mg /L (with 95 % confidence intervals of 1.1-1.8 mg /L), and the 96-hour NOEC was 0.28 mg /L.

Report: K-CA 8.2.1 [REDACTED] and [REDACTED] (2016), SYN548261 - Acute Toxicity to *Oncorhynchus mykiss*, Report Number 3201085, [REDACTED] (Syngenta File No. SYN548261_10002).

GUIDELINES

OECD Guidelines 203: Fish, Acute Toxicity Test (1992)

GLP: Yes

MATERIALS

Test material SYN548261
Lot/Batch #: MES 333/2
Purity: 98 %
Description: White solid
Stability of test compound: Stable under test conditions
Reanalysis/expiry date: 30 April 2017

Treatments

Test concentrations: Dilution water control and a single nominal concentration of 100 mg a.s./L
Solvent: None
Analysis of test concentrations: Yes, at 0 and 96 hours using HPLC analysis

Test organisms

Species: Rainbow trout *Oncorhynchus mykiss*
Source: [REDACTED]
Acclimatisation period: 12 days
Treatment for disease: None
Weight and length of dilution water control fish at end of exposure period: Mean length: 5.20 cm
 Mean weight: 1.24 g
Feeding: None during test

Test design

Test vessels: 20 L Glass aquaria containing 15 L dilution water
Test medium: Dechlorinated water
Replication: None
No of fish per tank: 10
Exposure regime: Static
Duration: 96 hours

Environmental conditions

Test temperature: 14.6 – 16.6 ° C

pH:	6.3 to 7.54
Dissolved oxygen:	92 to 101 % gentle aeration provided
Lighting:	16 hours fluorescent light

STUDY DESIGN AND METHODS

Experimental dates: 16 July to 09 November 2015

At the start of the test, 1.5 g of the test substance was dissolved in a final volume of 15 L of treated mains water to give a test concentration of 100 mg/L. Dissolution was aided with 10 minutes sonication. The control consisted of dilution water only.

At the start of the test, ten fish were randomly allocated to each of the test concentrations and the dilution water control. The test was conducted in a temperature controlled room. Observations for mortalities and symptoms of toxicity were made at 3, 24, 48, 72 and 96 hours.

Daily measurements of the test solutions were undertaken throughout the 96 hour period for pH, temperature and dissolved oxygen concentration.

The test concentrations were verified by chemical analysis of SYN548261 at 0 and 96 hours using an HPLC analysis.

RESULTS AND DISCUSSION

The measured concentrations are shown in the table below in terms of nominal concentration. The test concentrations were maintained throughout the study. The limit of quantification in this study was 0.05 µg/mL. Nominal concentrations were used for the calculation and reporting of results.

Table 9.2.1-19: Analytical results

Nominal concentration (mg a.s./L)	Measured concentration (mg/L) 0 hours	% of nominal 0 hours	Measured concentration (mg/L) 96 hours	% of nominal 96 hours
100	98.3	98	99.5	100

No toxic effects were observed during the test; therefore formal statistical analysis was not performed. As statistical analysis was not performed all results were derived empirically. Toxicity results were expressed in terms of the lethal concentration that causes 50% mortality of the fish after 96 hours exposure with 95% confidence limits, where appropriate.

The highest test concentration causing no mortality and the lowest concentration causing 100% mortality, based on observation of the raw data, was reported, where appropriate. Throughout the results, numerical data may have been rounded for presentation purposes. Therefore, manual recalculation of the data may result in slightly different values to those shown.

The mortality data and estimated LC₅₀ values are shown in the table below:

Table 9.2.1-20: Effects of SYN548261 on the survival of *Oncorhynchus mykiss*

Nominal concentration (mg/L)	Mortality observed (Cumulative number of dead fish) (n = 10)			
	24 hours	48 hours	72 hours	96 hours
Dilution water control	0	0	0	0
100	0	0	0	1 *
LC ₅₀ mg/L	>100	>100	>100	>100

95% confidence interval	n.d.	n.d.	n.d.	n.d.
NOEC	100	100	100	100

n.d. – not determined, * Fish found dead on laboratory floor.

VALIDITY CRITERIA

Validity criteria from OECD 203 (2019) have been satisfactorily met.

Table 9.2.1-21: Compliance with OECD 203 validity criteria

Validity criterion	Required	Obtained
Mortality in the control(s)	≤ 10 %	0 %
Dissolved oxygen concentration	At least 60 % of the air saturation value throughout the test	92– 101 % ASV
Concentration of substance	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 ranged from 98 – 100 % of nominal. Results are based on nominal concentrations.

CONCLUSIONS

Based on nominal concentrations, the 96-hour LC₅₀ for SYN548261 to rainbow trout (*Oncorhynchus mykiss*) was >100 mg a.s./L and the 96-hour NOEC was 100 mg a.s./L.

(██████ and ██████, 2016)

HSE evaluator comments

The study was carried out according to GLP and follows OECD 203 (1992) guidance with no significant deviations to the guideline. The study was also checked against the most recent OECD 203 guideline (2019) with some deviations noted.

A fish loading of 0.83 g/L was reported, this is marginally above the maximum loading of 0.8 g/L in the OECD 203 (2019) guidance. However, the study was completed in 2016 and complies with the OECD 203 (1992) guidance of 1 g/L. Furthermore, the validity criteria were met and no behavioural observations noted. The study used dechlorinated mains water as the medium. The composition of this water is not in line with OECD 203 (2019), however this guidance was not present in OECD 203 (1992). As there were no mortalities or sub-lethal behavioural changes during this study the water source can be considered to have no effect. Additionally, the water hardness and water temperature were not in line with OECD 203 (2019) guidance for *Oncorhynchus mykiss* at 29.89 mg/L CaCO₃ and 14.6-16.6 °C respectively. In OECD 203 (1992) there is no preferred water hardness stated for *Oncorhynchus mykiss* and the temperature range is 13-17 °C.

The analytical methods have been checked by HSE Chemistry specialists in Vol 3CA Part B5.1.2.6. The following was concluded: “Acceptable method. LOQ: 0.05 µg/mL in treated mains water”.

A limit test was performed. There was one mortality in the duration of the study, this was not considered to be as a result of toxicity. Therefore, the agreed endpoint suitable for use in the risk assessment is:

- **96-hour LC₅₀ = > 100 mg a.s./L (nominal concentrations)**

Report:	K-CA 8.2.1 [REDACTED], (2009) M700F001 (metabolite of BAS 700 F): Acute Toxicity for Rainbow Trout, Report Number W/09/09, [REDACTED], (Syngenta File No. CA4312_10909)
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GUIDELINES

- OECD Guidelines for Testing of Chemicals, Section 2 - Effects on Biotic Systems, Method 203: Fish, Acute Toxicity Test (1992)
- Official Journal of the European Communities, Dir 92/69/EEC, O.J. L383A, Part C.1: Acute Toxicity For Fish (1992)

GLP: Yes**MATERIALS**

Test material M700F001 (Metabolite of BAS 700 F; synonym of NOA449410)
3-(difluoromethyl)-1-methyl-1H-pyrazole-4-carboxylic acid

Lot/Batch #: L80-68

Purity: 99.2 % (\pm 1.0 %)

Description: Pale pink powder

Stability of test compound: Stable under test conditions

Reanalysis/expiry date: 01 August 2010

Treatments

Test concentrations: Dilution water control and nominal concentration of 100 mg metabolite/L

Solvent: None

Analysis of test concentrations: Yes, based on analysis of M700F001 (NOA449410) at 0 and 96 hours using HPLC analysis with UV-VIS detection.

Test organisms

Species: Rainbow trout (*Oncorhynchus mykiss* Walb.)

Source: Obtained from [REDACTED]

Acclimatisation period: 2 weeks

Treatment for disease: Not reported

Weight and length of fish at end of exposure period: Mean length: 4.3 cm (standard deviation 0.44 cm)
Mean weight: 0.60 g (standard deviation 0.24 g)

Feeding: None during test

Test design

Test vessels: Glass aquaria containing 35 L water

Test medium: Natural dechlorinated tap water

Replication: 2 (4 tanks in total: 2 tanks each for control and test concentration)

No of fish per tank: 10

Exposure regime: Static

Duration: 96 hours

Environmental conditions

Test temperature:	13.6 – 14.4 °C
pH:	Test start: 6.10 – 7.54 Test end: 6.90 – 7.42
Dissolved oxygen:	Test start: 99.9 – 101.9 % Test end: 98.7 – 100.4 %, constant aeration provided
Hardness of dilution water:	150 mg/L CaCO ₃
Lighting:	16 hours natural light with additional fluorescent light and 8 hours dark.

STUDY DESIGN AND METHODS

Experimental dates: 20 to 24 April 2009

A stock solution was prepared by dissolving 8 g of M700F001 (NOA449410) completely in 80 mL of deionised water on the magnetic stirrer for 0.5 hours and 5 minutes at ultrasonic cleaner. Then 35 mL was pipetted to each replicate vessel and mixed thoroughly. The control consisted of dilution water only.

At the start of the test ten fish were allocated to each replicate of the test concentration and the dilution water control. Observations for mortalities and symptoms of toxicity were made at 3, 6, 24, 48, 72 and 96 hours. Daily measurements of the test solutions were undertaken throughout the 96 hour period for pH and dissolved oxygen concentration. The temperature was continuously monitored by thermo-logger.

The test concentrations were verified by chemical analysis of M700F001 (NOA449410) at 0 and 96 hours using an HPLC method with UV-VIS detection.

RESULTS AND DISCUSSION

At the start of the test, the analytically determined concentration of M700F001 (NOA449410) was 91.1 % of the nominal value and at the end of the test was 85.1 % (see table below). The limit of quantification in this study was 0.05 mg M700F001(NOA449410)/L. Nominal concentrations were used for the calculation and reporting of results.

Table 9.2.1-22: Analytical results

Nominal concentration (mg metabolite/L)	% of nominal 0 hours	% of nominal 96 hours
Control	n.d.*	n.d.*
100	91.1	85.1

* Not detected.

The median lethal concentration (LC₅₀) was defined as the concentration resulting in 50 % mortality of the fish in the time period specified and was estimated after 24, 48, 72 and 96 hours. The NOEC (No Observed Effect Concentration) is defined as the highest tested concentration which did not produce an adverse effect when compared to the control and was also determined. No mortalities were observed at the nominal concentration of 100 mg M700F001(NOA449410)/L. No symptoms of toxicity were observed at a concentration of 100 mg M700F001/L. No mortality or symptoms of toxicity were observed in the control.

The mortality data and estimated LC₅₀ values are shown in the table below:

Table 9.2.1-23: Effects of M700F001 (NOA449410) on the survival of *Oncorhynchus mykiss*

Nominal concentration (mg metabolite/L)	Mortality observed (n = 20)				
	3 hour	24 hours	48 hours	72 hours	96 hours
Dilution water control	0	0	0	0	0
100	0	0	0	0	0
LC ₅₀ mg/L	n.d.	> 100	> 100	> 100	> 100
NOEC mg/L	100				

VALIDITY CRITERIA**Table 9.2.1-24 : Compliance with OECD 203 validity criteria**

Validity criterion	Required	Obtained
Mortality in the control(s)	≤ 10 %, or one fish if fewer than 10 control fish are tested.	0 % mortality observed.
Test conditions	Static, semi-static or flow-through	Static test
Dissolved oxygen concentration	At least 60 % of the air saturation value throughout the exposure in all vessels.	Ranges from 98.7-102.8 % across all timepoints
Concentration of test substance	Analytical measurement of test concentrations using a validated analytical method is compulsory. If the deviation from the nominal concentration is ±20 % then results should be based on the measured concentration.	Test concentration was measured at t=0 hrs and t= 96 hrs using HPLC UV-VIS and was 91.1 % and 85.1 % of nominal concentration respectively. Therefore, nominal concentration can be used.

CONCLUSIONS

Based on nominal concentrations, the 96-hour LC₅₀ for M700F001 (NOA449410) to rainbow trout (*Oncorhynchus mykiss*) was > 100 mg metabolite/L and the 96-hour NOEC was 100 mg metabolite/L. (██████████, 2009)

HSE evaluator comments

The study followed OECD 203 (1992) guidelines. However, the most recent guideline is OECD 203 (2019) so the study was assessed against this more recent version.

Constant aeration was used, and the guideline requires evidence that this does not result in substantial loss of test chemical. The analytical measurements show a slight decrease from 91.9 % to 85.1 % of nominal concentrations however this is still within the guideline threshold, therefore results are reported using nominal concentration.

The analytical methods have been evaluated by HSE Chemistry specialists in Vol. 3 CA Part B5.1.2.6. The following was concluded for this method: “Acceptable method. LOQ: 0.05 µg/mL in water”.

There were only minor deviations from the study guidelines, which were not deemed to affect the obtained results as explained below:

- The solubility of the test substance in water is not stated in this report. The study authors record that the preparation of the concentrated stock solution of the test substance was pink in colour and turbid,

therefore it is unclear whether the test substance is completely dissolved in the stock. However, this should not have affected the outcome of the study because there was no record of visible precipitate in the test tanks at the test concentrations. Additionally, the tanks were continually aerated throughout the test which should help to prevent any localised accumulation of the test substance within the tank.

- The guideline states exposure tanks should be monitored twice daily whereas the study monitored them once a day. However, since meaningful data has been obtained for the 96-hour endpoint and there was no mortality or negative behavioural or physical effects observed, this is not an issue.
- Holding temperature and photoperiod were not recorded in the study but this is not a problem as the holding period mortality for the fish batch was within the acceptable range.
- The chemical analysis of the dilution water showed some minor differences where some elements exceeded the maximum levels shown in Annex 3 of the OECD 203 (2019) guideline. This includes COD and some metal ions. However, since validity criteria were met and there were no mortalities in the test and control vessels, this has not had a negative impact on the fish or on the study results.
- The intensity of the fluorescent lighting used during the test is not stated but the absence of any mortality, behavioural or physical problems in the control fish suggests that this was not an issue.

Due to the study results (no mortality at limit test concentration) statistical analysis was not conducted and it was not possible to calculate EC_{10/20} values.

Overall, the study had no major deviations from the OECD 203 (2019) guideline and the study fulfils all validity criteria of this guideline.

The agreed endpoints for M700F001 (NOA449410) suitable for use in risk assessments are:

- 96-hour LC₅₀ > 100 mg metabolite /L (nominal concentration)
- NOEC = 100 mg metabolite/L (nominal concentration)

B.9.2.2. Long-term and chronic toxicity to fish

B.9.2.2.1. Fish early life stage toxicity test

Report:	K-CA 8.2.2.1 [REDACTED] (2020), SYN545974 – Early Life-Stage Toxicity Test with Fathead Minnow (<i>Pimephales promelas</i>), Report Number 1781.6843, [REDACTED] [REDACTED]. (Syngenta File No. SYN545974_10080)
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GUIDELINES

OECD Guidelines for Testing of Chemicals, method 210 Fish, Early-life Stage Toxicity Test (1992)
 US EPA Ecological Effects Test Guidelines OPPTS 850.1400 Fish, Early-life Stage Toxicity Test (1996)
 EC Guideline L.142/603, Method C.15 Fish Short-Term Toxicity Test on Embryo and Sac-Fry Stages (2008)
GLP: Yes

MATERIALS

Test material	SYN545974 tech.
Lot/Batch #:	2637-BA/110
Purity:	99.5 % (tested as 100%)
Description:	Not reported
Stability of test compound:	Stable under standard conditions
Reanalysis/expiry date:	31 July 2013

Treatments

Test concentrations:	Nominal:	0.010, 0.026, 0.064, 0.16 and 0.40 mg a.s./L
	Mean measured:	0.0095, 0.025, 0.064, 0.15 and 0.38 mg a.s./L

	Dilution water control
Dilution water:	A mixture of unadulterated water from a 100-meter bedrock well and dechlorinated Town of Wareham well water.
Solvent:	Dimethylformamide (DMF), concentration: 0.0040 mL/L (equal to that of the highest test concentration)
Analysis of test concentrations:	Yes, based on analysis of SYN545974, on days 0, 4, 11, 17, 20, 27 and 32 using LC/MS/MS
Test organisms	
Species:	Fathead minnow (<i>Pimephales promelas</i>)
Source:	Embryos (~ 22 hours old) were obtained from brood stock maintained at the testing laboratory for > 30 years, and periodically added to from reputable commercial suppliers. The brood stock used for this exposure (Lot No. 12A09) was approximately 8 months old, and 0% mortality was observed in the 48 hours prior to testing. The stock originated from [REDACTED]. Water flowing culture unit was from the same source as the dilution water used in exposure,
Acclimatisation period:	None
Treatment for disease:	Not reported
Feeding:	Live brine shrimp nauplii (<i>Artemia salina</i>) three times daily from Day 4 (Day 0 post-hatch). No food was given during last 24 hours of the study.
Test design	
Exposure regime:	Flow-through, using a Mount and Brungs intermittent-flow proportional diluter system
Replication:	4
Test vessels:	Glass aquaria measuring 30 x 14.5 x 20 cm, with a 14.5 cm high side drain maintaining a solution volume of approximately 6.5 L. Embryo incubation cups: round glass jars with 40-mesh Nitex® screen bottoms. A rocker arm apparatus gently oscillated the incubation cups.
No of eggs per tank:	30
Duration:	28 days post-hatch (32 days exposure)
Environmental conditions	
Test temperature:	24 – 25 °C measured in all test vessels on Day 0 and in sequentially alternating replicates daily thereafter and continuously in one replicate of the control
pH:	7.1 – 7.8 measured in all test vessels on Day 0 and in sequentially alternating replicates daily thereafter
Dissolved oxygen:	6.92 – 8.73 mg/L (83.0 to 109 % saturation) measured in all test vessels on Day 0 and in sequentially alternating replicates daily thereafter
Hardness of dilution water:	64 – 72 mg/L as CaCO ₃ samples measured at exposure initiation and weekly thereafter in sequentially alternating replicates of the low and high concentration and the dilution water control
Conductivity of dilution water:	390 – 460 µS/cm samples measured at exposure initiation and weekly thereafter in sequentially alternating replicates of the low and high concentration and the dilution water control
Lighting:	16 hours fluorescent light and 8 hours dark. 50 to 120 foot candles (540 to 1300 lux). Sudden transitions from light to dark, and vice versa, were avoided.

STUDY DESIGN AND METHODS

Experimental dates: 10 August to 11 September 2012.

A flow-through test system was employed. At the start of the test 30 eggs, approximately 22 hours old, were randomly allocated to egg cups and one egg cup suspended in each of four replicate test vessels at each test and control treatment. Hence, 120 eggs were exposed at each treatment. The test was undertaken in a temperature controlled water-bath.

A 100 mg a.s./L diluter stock solution was prepared, prior to exposure initiation and as needed throughout the definitive exposure, by adding approximately 1.0 g of SYN545974 to 10 mL of dimethylformamide (DMF), mixed by inversion, and sonicated for less than one minute. A 28 µL/mL solvent stock solution was prepared by diluting 28 mL of DMF to a final volume of 1000 mL with reagent grade water.

The control, solvent control and test solutions were delivered to the exposure aquaria (50 L/ aquarium/day) using a Mount and Brungs intermittent-flow proportional diluter at a rate of approximately 7.7 aquarium volumes per 24-hour period, with a 90 % replacement time of approximately 7 hours.

The concentrations of SYN545974 in test solutions were measured at 0, 4, 11, 17, 27 and 32 days using LC/MS/MS.

On day 4, 15 surviving larvae from each egg cup was placed in a larval chamber. These were observed daily for behaviour, appearance and mortality. The loading rate did not exceed 0.041 g/L of flow through solution per day or 0.32 g/L of solution at any time.

Observations for time to hatch, hatching success, larval mortality and deformed larvae were made daily during the pre- and post-hatch phases, as appropriate. Day of hatch was considered to be day 4 when no more than 10 % unhatched viable embryos remained in any control or solvent control embryo incubation cup. At the end of the test, survival percentage was determined together with lengths and dry weights of the surviving fry.

RESULTS AND DISCUSSION

Analytical data

The mean measured concentrations ranged from 81 % to 120 % of their nominal concentrations. The limit of quantification (LOQ) for the method validation was 0.151 µg a.s./L. It was established that the concentrations of SYN545974 in the exposure solutions were generally consistent and that the delivery apparatus maintained the expected concentration. The mean measured concentrations were used for calculating and reporting the results.

Table 9.2.2.1-1: Analytical results

Nominal concentration (mg a.s./L)	Measured concentration mg a.s./L (% of nominal)						Mean measured concentration ^a (mg a.s./L)
	Day 0	Day 4	Day 11	Day 20	Day 27	Day 32	
Control	< LOQ ^b	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	NA
Solvent Control	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	NA
0.010	0.0089 (89.0)	0.011 (110.0)	0.0078 (78.0)	0.0077 (77.0)	0.012 (120.0)	0.011 (110.0)	0.0095
0.026	0.023 (88.5)	0.025 (96.2)	0.027 (103.8)	0.022 (84.6)	0.026 (100.0)	0.024 (92.3)	0.025
0.064	0.065 (101.6)	0.070 (109.4)	0.057 (89.1)	0.055 (85.9)	0.065 (101.6)	0.069 (107.8)	0.064
0.16	0.15 (93.8)	0.16 (100.0)	0.14 (87.5)	0.14 (87.5)	0.17 (106.3)	0.15 (93.8)	0.15
0.40	0.34	0.44	0.33	0.32	0.42	0.44	0.38

Nominal concentration (mg a.s./L)	Measured concentration mg a.s./L (% of nominal)						Mean measured concentration ^a (mg a.s./L)
	Day 0	Day 4	Day 11	Day 20	Day 27	Day 32	
	(85.0)	(110.0)	(82.5)	(80.0)	(105.0)	(110.0)	

^a Mean and percent of nominal are based on the original raw data, not the rounded values presented in this table. ^b The limit of quantification (LOQ) for each analysis is dependent upon the linear regression, the area of the low standards and the dilution factor of the controls. At the sampling days, the LOQ was in the range of 0.000883 and 0.00113 mg a.s./L.

NA = Not applicable

Biological data

The No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) were estimated from the data obtained as follows:

Quantal responses

Hatching success: determined on Day 4 and expressed as a percentage of the number of eggs at the start of the test (Day 0).

Survival at the end of the test: the number of surviving fry at the end of the test (Day 32) expressed as a percentage of the number of live larvae (30 per replicate) on the day they were transferred from the egg cups to the test vessels (Day 4).

Non-quantal responses

Length: mean total length of surviving fry per replicate at test end.

Dry weight: mean dry weight of surviving fry per replicate at test end.

Statistical analysis

Analyses were performed using the mean organism response in each replicate aquarium. Significant differences in the percentage hatching success, percent normal larvae at hatch, and percentage larval survival were evaluated after transformation (arcsine square-root percentage) of the data. The ShapiroWilk's Test was used to determine sample distribution normality; homogeneity of variance was evaluated using Bartlett's Test, except for percentage larval survival where data were analysed using a Modified Levene Equality of Variance Test and a Levene Equality of Variance Test; and Williams' Multiple Comparison Test and Dunnett's Test were used to establish treatment effects, except for percent live normal larvae at hatch, which did not meet the assumption of homogeneity of variance and was therefore evaluated using Fisher's Exact Test with Bonferroni-Holm's Adjustment. No significant difference was determined between the control and solvent control data (Equal Variance Two-Sample t-Test except Unequal Variance Two-Sample t-Test for fish length and percent live normal fry data) so these data were pooled for comparison to the exposure data. A computer program was used to perform the statistical computations.

The biological data are presented in the table below.

Table 9.2.2.1-2: Effects of SYN545974 on early life stages of *Pimephales promelas*

Mean measured concentration (mg a.s./L)	Mean embryo hatching success ^a (%)	Live, normal larvae at hatch (%)	28 Days Post-Hatch		
			Mean larval survival day 4 to end of test (%)	Mean total length (mm) ± SD ^b	Mean dry weight (mg) ± SD ^b
Dilution water	91	99	88	25.0 (0.0857)	29.1 (0.875)

Mean measured concentration (mg a.s./L)	Mean embryo hatching success ^a (%)	Live, normal larvae at hatch (%)	28 Days Post-Hatch		
			Mean larval survival day 4 to end of test (%)	Mean total length (mm) ± SD ^b	Mean dry weight (mg) ± SD ^b
Solvent control	94	100	88	25.3 (0.797)	30.4 (3.77)
Pooled control	92	100	88	25.2 (0.559)	29.8 (2.63)
0.0095	92	100	95	25.2 (0.411)	30.6 (2.81)
0.025	97	99	93	24.7 (0.483)	28.8 (1.76)
0.064	90	94 ^c	93	24.7 (0.442)	28.9 (1.69)
0.15	90	93 ^c	78 ^d	22.6 ^e (0.828)	26.3 ^d (2.08)
0.38 ^f	6	0 ^c	0 ^d	NA	NA

^a Values presented represent hatching success at the completion on hatch (day 4 for all treatments with the exception of the highest dose) ^b SD = Standard Deviation (presented in parentheses) ^c Significantly reduced compared to the pooled control, based on Fisher's Exact Test with Bonferroni-Holm's Adjustment ^d Significantly reduced compared to the pooled control, based on Dunnett's Multiple Comparison Test ^e Significantly reduced compared to the pooled control, based on William's Multiple Comparison Test

^f This treatment level was excluded by study author from statistical analysis on growth (length and dry weight) due to 0 % larval survival at termination NA = Not Applicable

Table 9.2.2.1-3: Summary of the effects of SYN545974 on early life stages of *Pimephales promelas*

Endpoint	NOEC (mg a.s./L)	LOEC (mg a.s./L)
Embryo Hatching Success	0.15	0.38
Live, Normal Larvae at Hatch	0.025 ^a	0.064
Larval Survival	0.064	0.15
Mean Total Length	0.064	0.15
Mean Dry Weight	0.064	0.15

^a Fisher's Exact Test (with Bonferroni-Holm's Adjustment) determined a significant difference at 0.064 and 0.15.

Statistical analysis determined a significant difference in percent of live, normal larvae among embryos exposed to the 0.064, 0.15 and 0.38 mg/L treatment levels, compared to the pooled control. The NOEC and LOEC for this endpoint were determined to be 0.025 and 0.064 mg/L, respectively. The study author considered that the effects observed at 0.064 and 0.15 mg/L (i.e. 94 and 93 % live and normal larvae post hatch) are minimal compared to the control response (100 % pooled control) and stated these values were within historical control limits for the laboratory.

VALIDITY CRITERIA

Table 9.2.2.1-4: Compliance with OECD 210 validity criteria

Validity criteria according to OECD 210 (2013)	Required	Obtained
Dissolved oxygen concentration	>60 % of air saturation	83 % to 109 %
Water temperature range	25 ± 1.5 °C Must not differ by > 1.5 °C between test chambers or successive days during the test	24 - 25 °C
Concentrations of test substance in solution	Within ± 20 % of the mean measured values	95 % of nominal
Survival of fertilised eggs in the controls	≥ 70 % hatching success ≥ 75 % post-hatch success	92 % mean hatching success 100 % live, normal at hatch 88 % mean larval survival

CONCLUSIONS

Based on the day 32 (day 28 post-hatch-completion) larval survival, mean length and mean dry weight and mean measured concentrations, the No-Observed-Effect-Concentration (NOEC) was determined by study author to be 0.025 mg a.s./L and the No-Observed-Adverse-Effect Concentration (NOAEC) was determined by study author to be 0.064 mg/L for SYN545974 and fathead minnow.

(██████, 2020)

Additional Statistical Analysis

In accordance with **Commission Regulation (EU) No 283/2013**, estimation of EC₁₀ and EC₂₀ values was conducted for ██████, 2020 (SYN545974_10080) in the following report:

Report:	K-CA 8.2.2.1 ██████ (2016) Pydiflumetofen – Statistical Reanalysis; SYN545974 – Early Life-Stage Toxicity Test with Fathead Minnow (<i>Pimephales promelas</i>), Report Number 1781.7192c, Smithers Viscient, 790 Main Street, Wareham, MA, USA (Syngenta File No: SYN545974_10469)
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Executive Summary

Report number 1781.6843 (██████, 2020; SYN545974_10080) did not provide EC₁₀ and EC₂₀ estimates for the response variables evaluated as part of the original study. Consequently, the data generated have been re-analysed in order to provide these values where they could be reliably determined.

Statistical Analysis

Mean embryo hatching success and percent live normal larvae at hatch were compared to the mean embryo hatching success and percent live normal larvae at hatch in the pooled control.

At exposure termination (28 days post-hatch), larval survival and growth (total length and dry weight) were compared to the mean larval survival and growth in the pooled control.

All statistical analyses were conducted using CETISM Version 1.8 (Ives, 2013). Several non-linear regression models were attempted to determine EC₁₀ and EC₂₀ values. For total length and dry weight 2P OECD exponential #2 was used to determine EC₁₀ and EC₂₀ values along with corresponding 95% confidence intervals.

Results and Conclusion

Statistical analyses of the available data at hatching and at 28 days post hatch (termination) revealed that the following EC₁₀ and EC₂₀ values were reliably calculated:

Table 9.2.2.1-5: Summary of reliably calculated EC₁₀ and EC₂₀ values from ██████, 2020 (Report number 1781.6843; effects of SYN545974 on *Pimephales promelas* after 32 days exposure)

Endpoint	Analysis	Estimate (mg/kg)	Lower CI (mg/kg)	Upper CI (mg/kg)
Total length	EC ₁₀	0.15	0.12	0.19

Endpoint	Analysis	Estimate (mg/kg)	Lower CI (mg/kg)	Upper CI (mg/kg)
Total length	EC ₂₀	0.32	0.24	0.4
Dry weight	EC ₁₀	0.13	0.056	0.22

CI = confidence intervals

(██████████, 2016)

HSE evaluator comments

As shown above the validity criteria were met.

This study was conducted according to OECD 210 Fish, Early-life Stage Toxicity Test (1992) and OPPTS 850.1400 Fish, Early-life Stage Toxicity Test (1996). The study was also checked against the most recent OECD 210 guideline (2013) and no deviations were noted. The study followed GLP.

The solvent DMF was used to dissolve SYN545974, a solvent control was used and the survival of the fertilised eggs was above 70%, therefore the solvent is not considered to have affected the outcome of the study. The study meets the validity criteria set out in OECD 210 (2013), the mean total length of the larvae in the pooled control were 25.2 mm, above the 18 mm required in OECD 210 (2013) at the end of the study.

There were some discrepancies within the study. The executive summary in the study report on page 10 stated lighting of 670-1100 lux, whereas page 15 which lists the test conditions stated lighting levels of 540-1300. This may be a typographical error in the report. Nonetheless, for this study it is unlikely light levels would significantly impact the endpoints, noting the photoperiod was identical to that recommended in OECD 210 (2013) for the chosen species. In addition, the validity criteria were met.

The study used an Equal Variance Two Sample t-Test to compare the solvent control and diluent control, resulting in the control data being pooled. This is not listed in OECD 210 (2013) but is within the U.S EPA (2002) guidance. The percentage hatching success data was transformed prior to statistical analysis, using arcsine square-root percentage transformation. Shapiro-Wilks test for normality was used to evaluate normal distribution, and Bartlett's test, Modified Levene Equality of variance Test, and Levene Equality of Variance Test to test homogeneity of variance. These are not listed in OECD 210 (2013) but are in U.S EPA (2002). Fisher's Exact Test with Bonferroni-Holm's Adjustment, Dunnett's Test and William's Multiple Comparison Test were used to evaluate the study endpoints and these methods are in line with OECD 210 (2013). Statistical reanalysis has been performed in ██████████ (2016) in order to calculate EC₁₀ and EC₂₀ values. HSE agrees with the approaches taken to calculate these values and accepts them for use in risk assessment.

The analytical methods have been checked by HSE Chemistry specialists in Vol 3CA Part B5.1.2.6. The following was concluded: "Fit for regulatory purposes but the method is not fully validated in accordance with SANCO/3029/99 rev. 4. LOQ: 0.3 µg/L in aqueous matrices".

After considering the biological data, HSE considers the appropriate NOEC to be 0.025 mg a.s./L when considering all parameters (including length, dry weight and larval survival). Whilst there were statistically significant effects on % of live healthy larvae at 0.064 mg a.s./L the difference is relatively low i.e. 94 % rate compared to 100 % in control.

The study is considered acceptable and suitable for risk assessment purposes.

The agreed endpoints suitable for use in the risk assessment are:

- NOEC (32 d) of 0.025 mg a.s./L (mean measured concentrations)
- EC₁₀ (32 d) based on body length 0.15 mg a.s./L (mean measured concentrations)
- EC₂₀ (32 d) based on body length 0.32 mg a.s./L (mean measured concentrations)
- EC₁₀ (32 d) based on body weight 0.13 mg a.s./L (mean measured concentrations)

Report:	K-CA 8.2.2.1 ██████████ (2015), SYN545974 – Early Life-Stage Toxicity Test with Sheepshead Minnow, <i>Cyprinodon variegatus</i> , Report Number 1781.6979, ██████████. (Syngenta File No. SYN545974_10293)
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GUIDELINES

- OECD Guidelines for Testing of Chemicals, method 210 Fish, Early-life Stage Toxicity Test (2013)
- US EPA Ecological Effects Test Guidelines OPPTS 850.1400 Fish, Early-life Stage Toxicity Test (1996)

GLP: Yes

MATERIALS

Test Material	SYN545974 tech
Description:	Off-white powder
Lot/Batch #:	SMU2EP12007
Purity:	98.5 %
Stability of test compound:	Stable under standard conditions
Reanalysis/expiry date:	30 June 2016

Treatments

Test concentrations:	Dilution water control, Nominal: 0.031, 0.063, 0.13, 0.25, and 0.50 mg a.s./L Mean measured: 0.024, 0.048, 0.090, 0.17, and 0.35 mg a.s./L
Control:	Dilution water is filtered, natural seawater
Solvent:	None
Analysis of test concentrations:	Yes, days 0, 6, 13, 20, 28 and 33

Test animals

Species:	Sheepshead minnow (<i>Cyprinodon variegatus</i>)
Source:	Sheepshead minnow embryos used during this testing were obtained from brood stock maintained at [REDACTED]
Acclimatisation period:	None
Treatment for disease:	None
Feeding:	From day 7 fed live brine shrimp nauplii (<i>Artemia salina</i>) three times daily. Not fed during the 24 hours prior to study termination.

Test design

Exposure regime:	Flow-through, using a Mount and Brungs intermittent-flow proportional diluter system using test substance stock from glass wool saturator columns.
Aeration:	Gentle
Replication:	At the start of the test there are 4 replicates containing 30 embryos, for each test concentration and control. This is reduced to 20 impartially selected surviving larvae for each test concentration and control in the 28-day post-hatch larval exposure.
Test vessels:	glass tanks (30 x 14.5 x 20 cm) with a working volume of 5.5 L
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:	25 to 26 °C (24.5 – 25.8 °C)
pH:	7.2 to 8.0 measured daily
Dissolved oxygen:	94 to 110 % ASV measured daily
Salinity of dilution water:	20 to 21 ‰

Lighting:

560 to 830 lux
16 hours fluorescent light and 8 hours dark

STUDY DESIGN AND METHODS

Experimental dates: 18 November 2014 to 30 January 2015.

A flow-through test system was employed. At the start of the test 30 eggs were randomly allocated to egg cups and one egg cup suspended in each of four replicate test vessels at each test and control treatment. Hence, 120 eggs were exposed at each treatment. At completion of hatching, 20 surviving larvae were impartially selected from each cup and placed into the respective exposure aquarium for the 28-day post-hatch larval exposure period. The test was undertaken in a temperature controlled water-bath.

For this exposure, glass wool saturator columns were used to deliver SYN545974 to the exposure system. Saturator column output and stability trials were performed prior to the exposure and demonstrated that the saturator column delivered a stable and consistent concentration of approximately 2.5 mg/L for approximately two weeks. This analytically confirmed output value was used to calculate the appropriate flow rate of stock solution into the diluter system. The glass columns were packed with glass wool, which was then coated with the test substance. The columns were designed to provide a constant flow of nearly saturated aqueous solutions (2.5 mg/L) of SYN545974 to the diluter system without the use of a carrier solvent. Columns were constructed entirely of chemically inert materials (glass and Teflon).

The concentrations of SYN545974 in test solutions were measured at 0, 7, 14, 21, 28 and 33 days using an LC-MS/MS method. Samples for analysis were taken from the centre of the test solutions.

Observations for time to hatch, hatching success, larval mortality, deformed larvae and other symptoms of toxicity were made daily during the pre and post-hatch phases, as appropriate. At the end of the test, lengths, dry weights and wet weights of the surviving fry were measured.

RESULTS AND DISCUSSION**Analytical data**

The concentrations of SYN545974 were determined in the test solutions. The measured concentrations ranged from 60 % to 90.3 % of their nominal concentrations. The limit of quantitation of 0.151 µg a.s./L. The flow-splitting accuracy of the dosing apparatus was within 5 % of the nominal delivery volume and the data demonstrates that the dosing apparatus operated satisfactorily throughout the test. The mean measured concentrations were used for calculating and reporting the results.

Table 9.2.2.1-6: Analytical results

NC (mg a.s./L)	Measured concentration (mg a.s./L)						MMC ^a (mg a.s./L)
	Day 0 MC (%NC)	Day 6 MC (%NC)	Day 13 MC (%NC)	Day 20 MC (%NC)	Day 28 MC (%NC)	Day 33 MC (%NC)	
Control	< 0.00093 ^b (NA)	< 0.00086 (NA)	< 0.00083 (NA)	< 0.0010 (NA)	< 0.00098 (NA)	< 0.00086 (NA)	NA
0.031	0.027 (87.1)	0.022 (71.0)	0.022 (71.0)	0.020 (64.5)	0.028 (90.3)	0.025 (80.6)	0.024
0.063	0.053 (84.1)	0.042 (66.7)	0.044 (69.8)	0.044 (69.8)	0.055 (87.3)	0.048 (76.2)	0.048
0.13	0.099 (76.2)	0.082 (63.1)	0.083 (63.8)	(0.081) 62.3	0.10 (76.9)	0.089 (68.5)	0.09
0.25	0.19 (76.0)	0.15 (60.0)	0.16 (64.0)	0.15 (60.0)	0.19 (76.0)	0.17 (68.0)	0.17
0.50	0.41 (82.0)	0.30 (60.0)	0.34 (68.0)	0.32 (64.0)	0.37 (74.0)	0.35 (70.0)	0.35

^a Mean is based on the original raw data, not the rounded values presented in this table.

^b The limit of quantification (LOQ) for each analysis is dependent upon the linear regression, the area of the low standards and the dilution factor of the controls. At the sampling days, the LOQ was in the range of 0.000883 and 0.00113 mg a.s./L.

Note: %NC on each day was calculated by HSE from the available data.

NA = Not applicable, NC = Nominal Concentration, %NC = % of Nominal Concentration, MC = Measured Concentration, MMC = Mean (arithmetic) Measured Concentration across all timepoints.

Biological data

Quantal responses

Egg survival: the number of eggs at the start of the test (day 0) minus the number of dead eggs identified on day 5, expressed as a percentage of the number of eggs at the start of the test.

Live normal larvae at hatch: the number of live normal larvae on the day they are transferred from the egg cups to the test vessels, expressed as a percentage of the number of surviving eggs.

Hatching success: the number of live normal larvae on the day they are transferred from the egg cups to the test vessels, expressed as a percentage of the number of eggs at the start of the test (day 0).

Survival at the end of the test: the number of surviving fry at the end of the test (day 34) expressed as a percentage of the number of live larvae (20 larvae) on the day they were transferred from the egg cups to the test vessels.

Non-quantal responses

Length: mean total length of surviving fry per replicate at test end.

Dry weight: mean dry weight of surviving fry per replicate at test end.

Wet weight: mean wet weight of surviving fry per replicate at test end.

Statistical analysis

At the termination of the early life-stage exposure, data obtained on hatch success, percent live, normal larvae at hatch, larval survival and larval growth (total length, wet and dry weight) were statistically analysed to establish exposure level effects. Analyses were performed using the mean organism response in each replicate aquarium. All statistical analyses were conducted at the 95 % level of certainty, except in the case of Shapiro-Wilk's Test and Bartlett's Test, in which the 99 % level of certainty was applied.

The highest mean measured concentration that did not elicit a statistically significant difference between the exposed organisms and the control (No-Observed-Effect Concentration, NOEC), the lowest mean measured concentration that did elicit a statistically significant effect on organism performance (Lowest Observed-Effect Concentration, LOEC), were also determined. Determination of these levels is based on the performance criteria evaluated (e.g., embryo hatching success, percent of embryos that produce live, normal larvae at hatch, organism survival at hatch, larval survival and growth (total length, wet and dry weight) at exposure termination).

The biological data are presented in the table below.

Table 9.2.2.1-7: Effects of SYN545974 on the growth of *Cyprinodon variegatus*

Mean measured concentration (mg a.s./L)	Hatching success from all loaded eggs (%) ^{a,b}	Live normal larvae at hatch from surviving eggs (%) ^b	28 Days Post-Hatch			
			Larval survival from day of transfer out of egg cups to test end ^b (%)	Mean length ^b (mm)	Mean wet weight ^b (g)	Mean dry weight ^b (g)
Control	87 (6)	98 (2.3)	94 (2.5)	19.91 (0.22)	0.1013 (0.0026)	0.0242 (0.0010)
0.024	86 (6.9)	99 (1.8)	95 (4.1)	20.14 (0.41)	0.1030 (0.0055)	0.0246 (0.0012)
0.048	93 (4.5)	100 (0.0)	89 (4.8)	20.23 (0.32)	0.1114 (0.0035)	0.0263 (0.0012)
0.090	86 (10)	100 (0.0)	91 (2.5)	20.52 (0.31)	0.1147 (0.0074)	0.0265 (0.0009)
0.17	89 (8.3)	99 (2.0)	88 (5.0)	19.95 (0.27)	0.1002 (0.0056)	0.0239 (0.0017)
0.35	73 (4.7)	94 (5.4)	0 (0.0)*	NA (NA)	NA (NA)	NA (NA)

^aValues presented represent hatching success at the completion of hatch (test day 5-7).

^bStandard deviation is presented in parentheses.

*Statistically significant difference from the control based on Fisher's Exact Test with Bonferroni-Holm's Adjustment.

NA = Not applicable. Treatment excluded from statistical analysis of growth (length and weight) due to a significant reduction in survival.

VALIDITY CRITERIA

The study meets the validity criteria of guideline OECD 210 (2013) as follows:

Table 9.2.2.1-8: Compliance with OECD 210 validity criteria:

Validity criterion	Required	Obtained
Dissolved oxygen concentration	Should remain >60 % of air saturation value throughout the test	94 to 110 % 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 <i>C. variegatus</i> .	24.5-25.8 °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 because the analytical results showed test concentrations varied from 60-90.3 % 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 <i>C. variegatus</i> .	Hatching success of controls 87 ± 7 % (SD = 6.0 %). Survival (28 days post-hatch success) 94 % (SD = 2.5 %).

CONCLUSIONS

Based on mean measured concentrations and larval survival, the NOEC was determined to be 0.17 mg a.s./L, and the LOEC was determined to be 0.35 mg a.s./L for sheephead minnow exposed to SYN545974.

(██████, 2015)

Additional Statistical Analysis

In accordance with **Commission Regulation (EU) No 283/2013**, estimation of EC₁₀ and EC₂₀ values was conducted for ██████, 2015 (SYN545974_10293) in the following report:

Report:	K-CA 8.2.2.1 ██████ (2016a) Pydiflumetofen – Statistical Reanalysis; SYN545974 – Early Life-Stage Toxicity Test with Sheepshead Minnow (<i>Cyprinodon variegatus</i>), Report Number 1781.7192d, ██████ (Syngenta File No: SYN545974_10467)
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Statistical Analysis

Mean embryo hatching success and percent live normal larvae at hatch were compared to the mean embryo hatching success and percent live normal larvae at hatch in the control. At exposure termination (28 days post-hatch), larval survival and growth (total length and dry weight) were compared to the mean larval survival and growth in the control.

All statistical analyses were conducted using CETIS™ Version 1.8 (Ives, 2013). Several non-linear regression models were attempted to determine EC₁₀ and EC₂₀ values. For embryo hatching success, 2P OECD exponential #2 was used to determine EC₁₀ values along with corresponding 95 % confidence intervals.

Results and Conclusion

Statistical analyses of the available data at hatching and at 28 days post hatch (termination) revealed that the following EC₁₀ value was reliably calculated:

Table 9.2.2.1-9: Summary of reliably calculated EC₁₀ values from ██████, 2015 (Report number 1781.6979; effects of SYN545974 on *Cyprinodon variegatus* after 32 days exposure)

Endpoint	Analysis	Estimate (mg/kg)	Lower CI (mg/kg)	Upper CI (mg/kg)
Embryo hatching success	EC ₁₀	0.34	0.12	0.58

CI=Confidence Intervals

Due to the data generated it was not possible to determine EC₁₀ values for the other parameters or EC₂₀ endpoints.

(██████, 2016a)

HSE evaluator comments

The above study (██████, 2015) was assessed using Guideline OECD 210 (2013). The study was carried out according to OECD GLP (1998) with the exception of routine water and food screening analyses which were outsourced to an external company. It is noted that this study on sheepshead minnow is an additional species but the applicant states that it was conducted to fulfil global registration regulatory requirements, therefore it has been adequately justified. The statistical reanalysis report (██████, 2016a) was also considered.

The authors used mean measured concentrations for reporting of results and statistical analyses due to the results of the analytical measurements of the test substance. HSE agrees with this approach given the flow through study design.

The analytical methods have been checked by HSE Chemistry specialists in Vol 3CA Part B5.1.2.6. The following was concluded: “Fit for regulatory purposes but the method is not fully validated in accordance with SANCO/3029/99 rev. 4. LOQ: 0.3 µg/L in aqueous matrices”.

There were no major deviations from the study guidelines, however a minor deviation was noted below, though this does not affect the overall results of the study:

- It is noted that the stage of embryonic development at the start of test was characterised at a mean average of 12 (range 11-13) where 11 is flat blastula, 12 is early gastrula and 13 is one-quarter epiboly. This is after cleavage of the blastodisc which is stated as preferable in the OECD 210 (2013) guideline, however this is not considered sufficient by HSE to invalidate the study.
- At completion of hatch, the authors do not take forward all surviving larvae. Instead they select twenty surviving larvae to take forward. This generates some uncertainty, although the authors do state that the larvae were selected impartially which should reduce this uncertainty.

The statistical analyses used by the authors has been examined in accordance with the OECD 210 (2013) guideline and are detailed below:

- In accordance with the guideline, the authors use appropriate statistical tests for the normality and homogeneity of the data:
 - The authors used Shapiro-Wilk-s Test for normality and Bartlett's Test for homoscedasticity to conclude that the data for growth endpoints ('length', 'wet weight' and 'dry weight') met the assumptions of normal distribution, homogeneity of variance and exhibited a non-monotonic response. Therefore, the parametric procedure Tamhane-Dunnett Step-Down Test was used to determine any significant effects of exposure level.
 - The authors state that the data of 'hatching success', 'live, normal larvae' and 'survival length' are binomial and were analysed using a Model III 2×2 contingency table with procedures of Fisher's Exact Test with Bonferroni-Holm Adjustment ('hatch success' and 'percent live, normal larvae at hatch') and Cochran-Armitage Trend Step Down Test ('percent survival') to determine any significant effects of exposure level.
- The authors reanalysed the study data at a later date to determine EC₁₀ and EC₂₀ values. Several nonlinear regression models were attempted and the authors report that EC₁₀ and EC₂₀ values could not be calculated for 'percent live, normal larvae at hatch', 'larval survival', 'total length', 'wet weight' and 'dry weight' since they do not meet the conditions set out in OECD Guideline 210 (2013). An appropriate EC₁₀ value was able to be calculated for 'hatching success'. The authors do not supply a graph of the fit of the model to the data, but the EC₁₀ value does meet the requirements set out in the OECD Guideline 210 (2013), as detailed below:
 - 95 % confidence interval does not contain 0. This criteria has been met.
 - 95 % confidence interval is not overly wide. This criteria is considered met by HSE.
 - 95 % confidence interval does not contain the control mean. This criteria has been met.
 - Test concentrations should bracket EC_x so that the EC_x comes from interpolation rather than extrapolation. Since the highest mean measured concentration is above the calculated EC₁₀, then this criteria has been met.

Overall, there are no major deviations from the guideline and the validity criteria has been met. However, there is one additional point which may be relevant for risk assessment:

- It is noted that the authors report that all treatment levels had hatch completion 2 days after control (exposure day 7 compared to exposure day 5).

There is a discrepancy in the reported time to hatch in the controls. In the study report methods section 3.10.1 completion of hatch is stated to be exposure day 6, whereas in results section 4.3 it is exposure day 5.

Following a request for further information, this discrepancy was clarified by the applicant in a 2022 amendment to the initial study report from 2015 as follows:

"Completion of hatch was considered to be exposure day 6, when all viable embryos in all control embryo incubation cups were hatched. Completion of hatch for the 0.024, 0.048, 0.090 and 0.17 mg/L mean measured treatment levels was also on day 6. Completion of hatch for the 0.35 mg/L treatment level was day 7".

The agreed endpoints suitable for use in risk assessment are:

- **NOEC = 0.17 mg a.s./L (mean measured concentration)**
- **EC₁₀ (note could only be calculated for embryo hatching success) = 0.34 (CI 0.12-0.58) mg a.s./L (mean measured concentration)**

B.9.2.2.2. Fish full life cycle test

None submitted.

B.9.2.2.3. Bioconcentration in fish

Report: K-CA 8.2.2.3 [REDACTED] (2017), SYN545974- Flow-Through Bioconcentration and Metabolism Study with Bluegill Sunfish (*Lepomis macrochirus*), Report Number 1781.6900, [REDACTED] [REDACTED]. (Syngenta File No. SYN545974_10093)

GUIDELINES

OECD Guidelines for Testing of Chemicals, Method 305: Bioaccumulation in Fish: Aqueous and Dietary Exposure (2012)

US EPA Ecological Effects Test Guidelines, OPPTS 850.1730: Fish BCF (1996)

GLP: Yes

MATERIALS

Radiolabelled Test Substance

Test Material: [Phenyl – U-¹⁴C]-SYN 545974
[Phenyl-U-¹⁴C]-CSCD678790

Description: Not reported

Lot/Batch #: RDR-XV-94

Purity: 97.5% (radiochemical purity 99.1%)

Specific activity 156.5 µCi/mg

Stability of test compound: Store in freezer

Expiry Date 31 May 2013

Non-Radiolabelled Test Substance

Test Material: SYN545974 Tech.
CSCD678790

Description: Off-white powder

Lot/Batch #: SMU2EP12007

Purity: 98.5 % w/w

Stability of test compound: Store at < 30 °C

Expiry Date 30 June 2016

Test concentrations: Vehicle control and nominal concentration of 4.9 µg [¹⁴C]SYN545974/L.
Mean measured concentration 4.77 µg [¹⁴C]SYN545974/L

Vehicle control: Acetone 0.025 mL/L / no positive control

Analysis of test concentration: Yes, on Days -2, -1 (prior to test initiation) 0, 1, 3, 7, 14 and 19 by HPLCRAM analysis

Test animals

Species: Bluegill sunfish (*Lepomis macrochirus*) (Lot No. 13A27)

Source: [REDACTED]

Acclimatisation period: 6 days

Treatment for disease: None reported

Weight and length of fish prior to exposure period: Mean weight: 0.85 g (range 0.72 to 0.98 g) (n = 30)
Mean length: 42 mm (range 40 to 44 mm) (n = 30)

Feeding:	Commercial pelletized food, at a rate of 1 - 2% of the total fish weight per day
Test design	
Test vessels:	Glass aquaria (75 x 39 x 30 cm)
Test medium:	Local well water, supplemented with dechlorinated water.
Replication:	None
No of fish per tank:	150
Exposure regime:	Continuous flow diluter
Duration:	Uptake phase: 19 days Depuration phase: 7 days
Environmental conditions	
Test temperature:	23 °C
pH range:	Exposure aquaria; 6.4 – 7.8 Depuration aquaria; 7.0 – 7.5
Dissolved oxygen:	Exposure aquaria; 72 – 92 % saturation Depuration aquaria; 77 – 97 % saturation
Total Organic Carbon	Exposure aquaria; 1.5 – 11 mg C/L Depuration aquaria; 0.82 – 14 mg C/L
Total hardness of dilution water:	Exposure aquaria; 32 mg/L as CaCO ₃
Lighting:	16 hours fluorescent light and 8 hours dark.
Length of test:	Uptake phase – 19 days Depuration phase – 7 days

STUDY DESIGN AND METHODS

Experimental dates: 25 March to 26 April 2013

Apparatus

A continuous flow-through system, similar to Benoit *et al.* (1982), was used to expose fish to a nominal [¹⁴C] SYN545974 concentration of 4.9 µg [¹⁴C]SYN545974/L for a 19-day exposure period, followed by a 7-day depuration phase. In addition, a solvent control was used. Clear glass aquaria with a working volume of 73 L were used to hold the test fish.

A primary radiolabelled stock solution of 1.20 mg/mL was prepared by adding the entire quantity of ¹⁴C-SYN545974 to 50 mL of acetone. A 2.016 mg/mL primary non-radiolabelled stock solution was prepared by placing 0.2047 g (0.2016 g as active ingredient) of SYN545974 into 100 mL of acetone.

A 0.195 mg SYN545974/mL diluter stock was prepared by combining 22.87 mL of the 1.20 mg/mL primary radiolabelled stock solution with 54.4 mL of the 2.016 mg/mL primary non-radiolabelled stock solution and diluted to a final volume of 700 mL with acetone. The mixed radiolabelled stock was sonicated for 5 minutes, and triplicate aliquots were assayed by LSC as well as immediately prior to use. The toxicant delivery system was calibrated to deliver 0.0105 mL/min of the diluter stock solution into the mixing cell. The mixing cell also received a flow of 420 mL/min of dilution water. The delivery rate provided a turnover rate equivalent to 8.3 aquarium volumes per 24 hours and 90% aquarium volume replacement every 7-hour period. Acetone was delivered at the same rate, resulting in a solvent concentration of 0.025 mL acetone/L in the solvent control.

Test procedure

The uptake phase was initiated by transferring 150 fish to each of the solvent control and treatment aquarium after steady-state concentration had been achieved. The initial total biomass per aquarium was 128 g (1.74 g/L per day). The population of fish in each tank were fed commercial pelletised food at a rate of 1 - 2% of the total fish weight per day. The end of the exposure phase was determined when a steady state tissue concentration was

established. This was shown at day 19 as there were three consecutive sampling intervals for which the measured [^{14}C] tissue concentrations did not statistically differ by more than $\pm 20\%$. At the end of the exposure phase (day 19), the remaining fish from the exposure aquarium (64) were transferred to the corresponding depuration aquaria. Dilution water flow rate was 420 mL/min. The depuration phase lasted 7 days. It was predicted that at day 7 there would be an appropriate reduction in the body burden of the test substance.

Analysis of fish tissues

Fish were sampled throughout the uptake and depuration period. Fish were taken from the exposure and control treatments for tissue analysis at 0, 3, 7, 14 and 19 days into the uptake phase, and on days 1, 3 and 7 during the depuration phase.

On each of the fish sampling occasions, four fish were randomly selected from each test vessel and dissected into edible (flesh) and non-edible (carcass and viscera) portions. The portions of each tissue type were then pooled and weighed, and the tissues solubilised with the solubilization reagent (1600 mL of 10% sodium hydroxide solution, 200 mL of methanol and 200 mL of Triton X-100). Samples were weighed and digested overnight on a shaker table with vigorous shaking (~ 200 rpm) at ~60°C. Three 1.0 mL subsamples were dispensed into scintillation vials and total weight recorded, 15 mL of scintillation cocktail was added to the samples and were then analysed by LSC after refrigeration for 30 minutes.

Determination was made using LSC to confirm total radioactivity residues (TRR) in the edible and nonedible tissues. The values obtained were added together to calculate the whole body total radioactive residue.

Four fish were collected on days 7 and 19 from each aquaria and cut into small pieces to determine the distribution of radioactivity. Samples were extracted with 25 mL dichloromethane and 25 mL methanol. Samples were analysed for total radioactivity by LSC and for concentration of [^{14}C]SYN545974 by HPLC/RAM.

The lipid content of the control fish was determined in control fish on days 0 and depuration day 7 and treated fish at steady state (day 19). Four fish from the exposure and solvent control tanks were removed, weighed before transfer to 500 mL plastic bottles and homogenized with chloroform and methanol, prior to filtration. Solids were then returned to the bottle, and suspended in chloroform and methanol, shaken on a shaker table, and filtered through the original filter paper. The two filtrates were combined. The filtrates were partitioned with saturated sodium chloride and allowed to stand for an hour to ensure complete separation. The organic (chloroform) layer was dried by gravity filtration through 30g anhydrous sodium sulphate into a round-bottom flask, and the organic fraction was concentrated by rotary evaporation, dissolved in hexane and sonicated, prior to filtration through a glass filter paper. The fraction was concentrated by rotary evaporation to remove hexane, and the flask containing the lipid residue was placed in a desiccator overnight.

Analysis of test water

Water samples were taken from the test concentration aquaria at -2, and -1 days and days 0, 1, 3, 7, 14 and 19 during the uptake phase. Following the start of the depuration phase water samples were taken after 0, 1, 3 and 7 days. Water samples were taken from the solvent control on days -2, 0, 1, 3, 7, 14 and 19.

The analytical methods employed to measure the concentrations of [^{14}C] test material in the test solutions were based on LSC to determine total [^{14}C] residues, and HPLC/RAM to characterise the [^{14}C] residues for water samples.

Physical and chemical parameters

Dissolved oxygen, pH, temperature and dilution water and stock solution flow measurements were made throughout the study. The, total hardness and total organic carbon in the dilution water were determined periodically. Representative samples of the laboratory freshwater supply were also analysed for heavy metals and pesticides on a periodic basis.

Calculation of Steady State Bioconcentration Factors (BCF_{ss})

The steady-state bioconcentration factor (BCF_{ss}) was calculated from the average steady-state fish and water concentrations (mean value of 7-, 14- and 19-day exposure) in the 4.9 μg [^{14}C] SYN520453/L test solution using the following equation:

Measured bioconcentration factor (BCF_{ss}) $BCF_{ss} = C_{tissue} / C_{water}$

BCF_{ss} based on lipid-normalized content were calculated using the following equation:

$$BCF_{ss, \text{ Lipid-Normalized}} (BCF_{ssl}) = BCF_{ss} \times 0.05 / Ln$$

Where Ln = Mean lipid content (based on wet weight).

Calculation of Kinetic Bioconcentration Factors (BCF_k)

The calculated bioconcentration factor, BCF_k , was calculated for edible, non-edible and whole fish tissues as follows:

Calculated bioconcentration factor (BCF_k) $BCF_k = K_u / K_d$

The uptake constant (K_u) was calculated as follows:

$$\text{Uptake constant } (K_u): C_t / C_w = (K_u / K_d) [1 - e^{(-K_d t)}]$$

Where:

C_t = tissue concentration at time t

C_w = mean water concentration during uptake phase

K_u = uptake constant

K_d = depuration constant from fish tissue

t_u = time at the end of the exposure phase the depuration constant

The depuration constant (K_d) was calculated as follows:

$$\text{Depuration constant } (K_d): C_t = C_{t,0} e^{(-K_d t)}$$

Where:

C_t = tissue concentration at time t ($\mu\text{g/g}$)

K_u = uptake constant (day^{-1})

$C_{t,0}$ = tissue concentration at the start of depuration period ($\mu\text{g/g}$)

The uptake and depuration constants were produced from a software curve-fitting program using the Marquardt-Levenberg algorithm.

The kinetic BCF (BCF_k) and lipid-normalized kinetic BCF (BCF_{kl}) were calculated as follows:

$$BCF_k = K_u / K_d$$

$$BCF_{kl} = BCF_k \times 0.05 / Ln$$

Calculation of depuration half-life

The depuration half-life value was calculated as follows:

$$\text{Half-life} = Ln(2) / \text{depuration rate constant } (K_d) = 0.693 / \text{depuration rate constant } (K_d)$$

RESULTS AND DISCUSSION

The measured total radioactivity during the exposure period was maintained between 97 and 104 % of the exposure mean of $4.9 \mu\text{g/L}$, representing 97 % of the nominal value as determined by LSC. The mean measured steady state concentration of [^{14}C]SYN545974 in the nominal $4.9 \mu\text{g/L}$ exposure water was $4.87 \mu\text{g}$ [^{14}C]SYN545974/L, calculated from the day 0, 3, 7, 14 and 19 aqueous samples.

Table 9.2.2.3-1 Analytical results for quality control water samples analysed by LSC and HPLC concurrently with exposure water samples during the 19-day exposure phase of the bioconcentration study with blue gill sunfish (*Lepomis macrochirus*) and [¹⁴C]SYN545974.

Exposure day	LSC			HPLC ^a		
	Fortified Concentration (µg/L)	Measured Concentration (µg/L)	Percent Recovered %	Fortified Concentration (µg/L)	Measured Concentration (µg/L)	Percent Recovered %
Day 0	4.80	4.95	93.8	4.80	4.95	93.8
Day 3	5.13	5.40	105	5.13	5.24	103
Day 7	5.46	5.51	101	5.46	5.51	101
Day 14	5.30	5.58	105	5.30	5.58	105
Day 19	6.59	6.70	102	6.59	6.70	102

^a HPLC results were calculated using the following equation: HPLC Results = LSC Results x % Parent. During this study, the percent parent remained at 100%. Therefore, the HPLC results, rounded to three significant figures were identical to the LSC results.

NOTE: calculations were performed using the actual unrounded analytical data.

The concentration of [¹⁴C] in the water samples is a result of the active substance (parent) as the HPLC and LSC results are the same. A representative HPLC/RAM analysis for the nominal 4.9 µg [¹⁴C]SYN545974/L exposure water demonstrates that the only residue detected was [¹⁴C]SYN545974.

The concentrations of [¹⁴C] SYN545974 in fish tissue during the 19-day uptake phase followed by the 7-day depuration phase for bluegill sunfish are given in the table below:

Table 9.2.2.3-2: Uptake and depuration of [¹⁴C] SYN545974 (TRR) in the bluegill sunfish (*Lepomis macrochirus*)

Day		Mean concentration of [¹⁴ C]SYN545974 equivalent (µg/kg)		
		Edible tissues	Non-edible tissues	Whole fish
Uptake phase	0	NA	NA	NA
	3	212.5	1543.5	900.9
	7	210.0	1342.9	767.9
	14	318.9	1301.7	824.8
	19	269.9	1167.0	738.8
Average Steady state		266.3	1270.5	777.2
Depuration phase	1	55.8	278.1	147.5
	3	34.3	88.3	64.6
	7	20.3	48.1	35.7
% of steady state on day 7		7.6	3.8	4.6

NA = not applicable.

TRR = [¹⁴C]Residue concentrations

Concentrations are reported as µg SYN545974 equivalent/kg fish tissue.

Calculations were performed using the actual unrounded analytical data and not the rounded values presented in this table.

The Average steady state was determined at day 19 of the uptake phase, using values from days 7 to 19. The limit of detection for LSC in water and fish tissue were 0.058 µg/L and 8.6 µg/L respectively. The PES detection limit in fish was 8.63 µg/L, and the limit of detection for fish tissue extract was 14.4 µg/L. The HPLC detection limit was 0.014 µg/L (14.3 µg/ml).

[¹⁴C]SYN545974 was rapidly eliminated from the fish during the depuration phase. At the end of the 7-day depuration period, the [¹⁴C] tissue concentration ranged from 20.3, 48.1, and 35.7 µg

[¹⁴C]SYN545974 kg for edible, non-edible, and whole fish tissue, respectively. The total tissue [¹⁴C]residue concentrations were 7.6, 3.8, and 4.6 % of the average steady-state concentration for the edible, non-edible, and whole fish tissue, respectively.

Steady State Bioconcentration Factor, TRR

The steady state bioconcentration factors (BCF_{ss}) were calculated from the average steady-state fish and water concentrations (mean value of day 7, 14 and 19 exposure) in the 4.9µg [^{14}C] SYN520453/L test solution.

$$(BCF_{ss}) = C_{\text{tissue}} / C_{\text{water}}.$$

Table 9.2.2.3-3: Measured bioconcentration factor from average steady state concentrations in water and fish tissue.

	Edible tissues	Non-edible tissues	Whole fish
Average steady state concentration in fish tissue (µg/kg)	266.3	1270.5	777.2
BCF_{ss} based on TRR	55.3	264	161

The measured BCF_{ss} based on ^{14}C -residues was 55.3, 264 and 161 in edible, non-edible and whole fish tissues, respectively.

The lipid-normalized bioconcentration factor BCF_{ss} for whole fish was 181.

Kinetic Bioconcentration Factor, TRR

The kinetic bioconcentration factor (BCF_k) was calculated using the uptake constant (K_u) and depuration constant (K_d). The kinetic BCF and lipid normalised BCF were calculated as follows:

$$BCF_k = K_u / K_d$$

$$BCF_{kl} = BCF_k \times 0.05/Ln$$

The depuration half life was calculated as:

$$\text{Half life} = Ln(2) / K_d = 0.693 / K_d$$

Table 9.2.2.3-4: Kinetic Bioconcentration factor calculated from the uptake constant and depuration constant.

	Edible tissues	Non-edible tissues	Whole fish
$K_u(\text{day}^{-1})$	70.8	443	284
$K_d(\text{day}^{-1})$	1.34	1.59	1.69
BCF_k	52.8	279	168
BCF_{kl}	82.3	253	189
Depuration half-life ($t_{1/2}$ (days))	0.52	0.44	0.41

The mean lipid content of whole fish on days 0 and 19 of exposure during steady state was 3.42 to 5.39 % for control fish and 3.34 to 5.58 % for the exposed fish tissue. The mean lipid content of whole fish on day 7 of depuration was consistent for control fish, 5.58 %, and slightly increased to 7.40 % in the exposed fish tissue.

The whole fish uptake rate constant (K_u) was calculated to be 284/day. The whole tissue depuration rate constant (K_d) was calculated to be 1.69/day. Based on these values the calculated bioconcentration factor (BCF_k) was 168. The BCF_k values for edible and non-edible tissues were 52.8 and 279, respectively. The lipid-normalised bioconcentration factors (BCF_{kl}) were 189, 82.3 and 253 for whole body, edible and non-edible fish tissue, respectively.

The depuration half-life of accumulation was 0.52, 0.44, and 0.41 days for edible, non-edible and whole fish respectively.

Steady State Bioconcentration Factor, SYN545974 (parent)

The day 7 and day 19 whole fish samples were extracted and analysed by HPLC/RAM for distribution of ^{14}C -residues. The radioactivity, expressed as [^{14}C]SYN545974 equivalents, was found to accumulate within the tissues and extractability declined with increasing exposure from 89.65 to 87.89 % total radioactive residue (TRR). The total ^{14}C -residues determined by extraction of the whole fish tissue at day 7 and day 19 was 0.856 and 1.113 µg/g respectively. From these results it is determined that the total radioactive residue was mainly associated with the metabolites of SYN545974 in fish tissues.

Table 9.2.2.3-5: Distribution of total radioactivity in whole body fish tissue by HPLC during the exposure phase for 4.9 µl of [¹⁴C]SYN545974

Sample day	% TRR	µg SYN545974/g tissue
7	6.95	0.0663
19	10.05	0.133
BCF_{ss} SYN545974	27.7	

The whole fish bioconcentration factor based on [¹⁴C]SYN545974 concentration (BCF_{ss}, SYN545974) was calculated using the mean measured steady state water concentration and the measured [¹⁴C]SYN545974 at Day 19 whole body fish tissue concentration (0.133 µg SYN545974/kg). BCF_{ss}, SYN545974 was calculated to be 27.7.

The lipid-normalized bioconcentration factor BCF_{ss} SYN545974 whole fish was 31.1.

Table 9.2.2.3-6: Summary of endpoints for whole fish

Endpoint	Whole fish
BCF _{ss} TRR	161
BCF _{ss} SYN545974	27.7
Lipid normalised BCF _{ss} TRR	181
Lipid normalised BCF _{ss} SYN545974	31.1
BCF _k TRR	168
BCF _{kl} TRR	189
Depuration half-life (t _{1/2} (days))	0.41

The physical and chemical data in both the solvent control and exposure tank showed little variation during the whole study period. Dissolved oxygen levels ranged from 72 to 97 % saturation, the pH values ranged from 6.4 to 7.8 and the temperature was 23 °C. The test solution flow rates of the stock solution and dilution water to the individual mixing cell was 0.0105 mL stock solution/min and 420 mL dilution water/min, respectively. No undissolved test substance was observed in the dilution system or test aquaria. There was one mortality during the study on day 5 of the depuration phase. Otherwise the fish appeared healthy and exhibited normal behaviour.

VALIDITY CRITERIA

Table 9.2.2.3-7: Compliance with OECD 305 validity criteria

Validity criteria	Required (OECD 305, 2012)	Obtained
Water temperature variation over the whole test period	± 2 °C	23 °C
Dissolved oxygen % saturation in all test vessels	> 60 %	72-97 %
Concentration of test substance in test chambers maintained within required range of the mean of the measured values during the uptake phase	± 20 %	97 %
The concentration of the test substance is below its limit of solubility in test water	Test concentration < water solubility of test item in test water	Yes
The mortality or other adverse effects/disease in both control and treated fish.	< 10% at test end	<1%

CONCLUSIONS

On basis of measured SYN545974 residues, the BCF_{ss} value for the whole fish tissues was 27.7 and the lipid normalized BCF_{ss} value for the whole fish tissues was 31.1. The depuration half-life of accumulated residues was 0.52, 0.44 and 0.41 days for edible, non-edible and whole fish, respectively.

(██████, 2017)

HSE evaluator comments

This study was conducted according to OECD 305 (2012) and OCSPP Guideline 850.1730. The study met the relevant validity criteria as shown in the table above.

According to OECD 305 (2012) the measurement of the total organic carbon (TOC) should be <2.0 mg/L in the dilution water. During the study the TOC concentration ranged from a mean value of 0.85 mg C/L to 13.5 mg C/L in exposure and solvent control water samples. The TOC levels in the source water were recorded as 1.5 mg C/L and 0.7 mg C/L in March and April respectively. It is stated that the high TOC concentration is due to contribution of the test substance and solvent. However, the levels of TOC during the study are inconsistent, raising some uncertainty. The applicants response for further information is shown below:

“During the study the dilution water had a TOC of less than 2 ppm C, and the higher TOC values were from the test substance and the solvent in the treated tank and the solvent in the control tank, and not from the dilution water. TOC measured weekly throughout the test material exposure phase ranged from 6.8 to 11 mg C/L. At depuration day 7, the TOC declined to a range of 0.82 to 1.5 mg C/L, indicating that nearly all the TOC was contributed by the test substance and solvent in the exposure phase. The TOC values were consistent with the treated tank, approximately 10 ppm C during equilibrium to day 14 of the study. The TOC value in the control tank started increasing from day 7, and remained constant by day 14. Higher organic carbon content would result in the sorption of the test substance and reduce the availability of the test substance in the water. But the test substance concentration in the water is within the range of 93 to 105 %, which confirms that the high TOC did not have any impact on the availability of the test substance.”

HSE agrees that despite the high level of TOC, the concentration of the test substance was maintained within 20 %, therefore this should not have had an impact on the calculated endpoints.

OECD 305 (2012) states that the natural particle content should not be greater than 5 mg/L. The natural particle content is not reported in the study report. The applicant has stated:

“No natural particle content was measured during the study. However, the analysed TC, DOC, and TOC of the dilution water were all under 2 ppm C, which confirms that the particle content in the water was less than 5 ppm.”

It is noted that pH deviated by >0.5 units during the test, however as all validity criteria were met, this is not considered to invalidate the test. The fish loading was 1.74 g/L per day during the study. This is marginally higher than the OECD 205 (2012) recommendations of 0.1 – 1 g/L per day. However, as the validity criteria were met, and there was only one mortality during the study it is not considered to have had an impact on the endpoints. The radiochemical purity of the test substance was listed as > 95 %, lower than > 98 % recommended in OECD 305 (2012). This is a minor difference and it is not considered to have affected the endpoints.

It is noted that the age of the fish was not reported. OECD 305 (2012) states that age of a fish may have a significant effect on BCF values, so all fish should be from the same year-class. According to the study report fish of the same batch and source were used and therefore effects on BCF values should have been minimised. However, as it is not clear whether fish were sexually mature adults, it cannot be confirmed whether the fish were in a spawning state or had recently spawned before or during the test, which may have affected the test results. The applicant has responded to a request for further information providing the following:

“Juvenile fish (i.e. not sexually mature) were used in the study and therefore the sex of the fish used were not determined. The age of the fish is unknown”

As the fish used were not sexually mature, HSE accepts that this would not have had an effect on the endpoints.

The sex of the fish was not reported; according to OECD 305 (2012) this should be stated and if both sexes are used, differences in growth and lipid content should be documented to be non-significant before exposure initiation. The fish were homogenised before lipid determination so any differences between males and females will not be recorded. Male and Female specific BCFs could deviate from the values calculated, making the endpoints uncertain. Additionally, pooling of data is not recommended in OECD 305 (2012) as it can lead to less robust statistics and reduce real variability. This can lead to uncertainty in the endpoints.

The individual lengths and weights of the fish were not measured before homogenisation. As such, it was not possible to consider growth dilution for the calculated BCFs. This is a potential limitation to the study and could lead to the endpoints being uncertain.

The fish were acclimatised for six days. OECD 305 (2012) recommends an acclimation period of two weeks. As the validity criteria are otherwise met this is not considered to invalidate the test.

The uptake phase lasted 19 days. Concentrations of total radioactive residues in whole fish are shown to be within 20 % in three consecutive analysis, in line with OECD 302 (2012) guidelines. The depuration phase lasted 7 days, the time predicted for an appropriate reduction (95 %) in the body burden of the test substance. At day 7 of the depuration phase 4.6 % of the average steady state concentration was detected. This is within OECD 305 (2012) guidelines.

This study is considered valid and the following endpoints are considered acceptable for use in the risk assessment:

Steady state bioconcentration factor (BCF_{ss}SYN545974) for whole fish = 27.7

Lipid normalized steady state bioconcentration factor (BCF_{SSL}SYN545974) for whole fish = 31.1

Depuration half-life of accumulated residues for whole fish = 0.41

B.9.2.3. Potential for endocrine disruption – SECTION SUBJECT TO SUBMISSION OF THE FINAL RADAR STUDY REPORT

B.9.2.3.1. Consideration of EAS modalities (aquatic organisms)

Three studies have been submitted, the Fish Short-Term Reproduction Assay (FSTRA) and two Early Life Stage (ELS) studies, noting that the latter measures parameters that are ‘sensitive to but not diagnostic of EATS’. Following advice from the Expert Committee on Pesticides (ECP) a RADAR assay was requested due to uncertainties with the results from the FSTRA. The studies that are valid have been summarised in the table below. The full study evaluations are shown in section B.9.2.2.1 (ELS) and B.9.2.3.3 (FSTRA and RADAR assay).

Table 9.2.3.1-1: Brief overview of aquatic studies relevant to assessment of EAS modalities:

Details	ELS (██████, 2020)	ELS (██████, 2015)	FSTRA	RADAR
Study ID ^a	22	23	25, 25.1, 25.2, 25.3	26
Species tested:	Fathead minnow (<i>Pimephales promelas</i>)	Sheepshead Minnow (<i>Cyprinodon variegatus</i>)	Fathead minnow (<i>Pimephales promelas</i>)	Japanese medaka
Exposure method and duration:	Flow-through, 32 days (28-days post hatch)	Flow-through, 34 days (28-days post hatch)	Continuous flow, 21 days exposure	<i>Oryzias latipes</i>
Test concentrations:	Test concentrations were maintained within $\pm 20\%$ of nominal concentrations (actual: 81 – 120 %), nevertheless authors chose to calculate and report results using mean measured concentrations: 0.0095, 0.025, 0.064, 0.15 and 0.38 mg a.s./L	Test concentrations were not maintained within $\pm 20\%$ of nominal concentrations (actual: 60 % to 90.3 %) hence results are based on mean measured concentrations: 0.024, 0.048, 0.090, 0.17, and 0.35 mg a.s./L	Test concentrations were not maintained within $\pm 20\%$ of nominal concentrations. Time-weighted average measured concentrations were used: 0.0013, 0.017, and 0.13 mg a.s./L	Unspiked mode: 130, 41, 13, 4.1 and 1.3 $\mu\text{g/L}$ MM1X + 0.2% DMSO (nominal) Spiked mode: 130, 41, 13, 4.1 and 1.3 $\mu\text{g/L}$ MM1X + 0.2% DMSO + 17-MT 3 $\mu\text{g/L}$ (nominal). Mean measured test concentrations: to be confirmed in final study report.
Guideline followed:	OECD 210 (1992), but assessed against OECD 210 (2013) and no deviations noted.	OECD 210 (2013)	OECD 229 (2012)	OECD 251 (2022)
Parameters measured:	Hatching success, larvae deformation, larvae survival, body length, body dry weight, behaviour/appearance	Egg survival, larvae deformations, hatching success, larvae survival, body length, body	Survival, behavioural/morphological abnormalities, fertilisation success, fecundity, SSC*, GSI*, gonad and liver histopathology, VTG* in blood plasma	Macroscopic observation of malformations and survival after 24, 48 and 72 hours.

		dry weight, body wet weight		Reading of fluorescence.
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*SSC = secondary sexual characteristics, GSI = gonadosomatic index, VTG = vitellogenin

^a Study ID used in table 9.2.3.1-6.

The study results have been discussed below for each study.

B.9.2.3.1.1. ELS study on Fathead minnow (*Pimephales promelas*) (██████, 2020):

A summary of the results from the ELS study on fathead minnow are shown in the table below.

Table 9.2.3.1-2: Chronic toxicity of pydiflumetofen to fathead minnow (*Pimephales promelas*) in a fish early life stage test (32 d)

Concentration corrected for purity (mean measured) [mg a.s./L]	Pooled Control (<LoQ)	0.0095	0.025	0.064	0.15	0.38
Mean embryo hatching success [%] ¹⁾	92	92	97	90	90	6***
Live, normal larvae at hatch ¹⁾ [%]	100	100	99	94*	93*	0*
Post-hatch larval fish survival (day 4 to 32) ¹⁾ [%]	88	95	93	93	78**	0**
Behaviour	None	None	None	None	None	NA ³⁾
Mean dry weight (32 d) [mg] (±SD ²⁾)	29.8 (2.63)	30.6 (2.81)	28.8 (1.76)	28.9 (1.69)	26.3** (2.08)	NA ³⁾
Mean length (32 d) [mm] (±SD ²⁾)	25.2 (0.559)	25.2 (0.411)	24.7 (0.483)	24.7 (0.442)	22.6*** (0.828)	NA ³⁾
Parameters	Endpoints [mg a.s./L]					
	Mean measured					
EC ₂₀ body length (32 d)	0.32 (95 % CI 0.24 – 0.4)					
EC ₁₀ body length (32 d)	0.15 (95 % CI 0.12 – 0.19)					
EC ₁₀ body dry weight (32 d)	0.13 (95 % CI 0.056 – 0.22)					
NOEC _{mortality, survival, growth} (32 d)	0.064					
NOEC _{normal larvae at hatch}	0.025					
NOEC _{hatching success}	0.15					
Overall NOEC (32 d) based on all parameters	0.025					

LoQ: Limit of quantification.

* Significantly reduced compared to the pooled control, based on Fisher's Exact Test with Bonferroni-Holm's Adjustment. The study author considered that the effects observed at 0.064 and 0.15 mg/L (i.e. 94 and 93 % live and normal larvae post hatch) are minimal compared to the control response (100 % pooled control) and stated these values were within historical control limits for the laboratory. After considering the biological data, it was concluded by HSE in section 3CA B9.2.2 that a NOEC of 0.025 mg a.s./L was the appropriate endpoint.

** Significantly reduced compared to the pooled control, based on Dunnett's Multiple Comparison Test

*** Significantly reduced compared to the pooled control, based on William's Multiple Comparison Test

¹⁾ data was transformed prior to statistical analysis, using arcsine square-root percentage transformation.

²⁾ SD = standard deviation

³⁾ This treatment level was excluded by study author from statistical analysis on growth (length and dry weight) due to 0 % larval survival at termination.

NA = Not Applicable – no surviving fish

B.9.2.3.1.1.1. Consideration of ELS results on fathead minnow (*Pimephales promelas*) (██████, 2020):

Results from ELS studies are considered sensitive to, but not diagnostic of EATS modalities.

Completion of hatch for all treatments, with the exception of the highest dose, was equivalent to the control (day 4). Statistically significant effects on embryo hatching were observed at the highest test concentration (0.38 mg a.s./L), with 6 % embryo hatching success, 0 % of which were 'live, normal larvae'. Systemic toxicity to post-hatch larvae was observed in the highest test concentration with 100 % mortality (0 % survival) of post-hatch larvae at 0.38 mg a.s./L and 22 % mortality (78 % survival) of post-hatch larvae at 0.15 mg a.s./L.

Statistically significant effects on growth (length and dry weight) were observed at a test concentration of 0.15 mg a.s./L (no data available for highest treatment level of 0.38 mg a.s./L due to 0 % larval survival).

Regarding the percentage of live, normal larvae at hatch (of the embryos that successfully hatched, those which did not have deformities or were dead at hatch), statistically significant effects were observed at the three highest test concentrations of 0.064, 0.15 and 0.38 mg a.s./L. For the test concentrations of 0.064 and 0.15 mg a.s./L the percentage of normal larvae was 94 % and 93 %, respectively, and is stated to be within the historical range for the laboratory, therefore the biological relevance of the statistical effect was noted to be questionable. The parameter of 'percentage live, normal fry at hatch' is the only parameter in which there is a statistically significant effect at the test concentration of 0.064 mg a.s./L. An EC₁₀ or EC₂₀ could not be reliably calculated for this parameter.

B.9.2.3.1.2. ELS study on sheepshead minnow (*Cyprinodon variegatus*) (■■■■■, 2015)

A summary of the results from the ELS study are shown in the table below.

Table 9.2.3.1-3: Chronic toxicity of pydiflumetofen to sheepshead minnow (*Cyprinodon variegatus*) in a fish early life stage test (34 d)

Concentration corrected for purity (mean measured) [mg a.s./L]	Control (<LoQ)	0.024	0.048	0.09	0.17	0.35
Mean embryo hatching success ^a (±SD) [%]	87 (6)	86 (6.9)	93 (4.5)	86 (10)	89 (8.3)	73 (4.7)
Live, normal larvae at hatch (±SD) [%]	98 (2.3)	99 (1.8)	100 (0.0)	100 (0.0)	99 (2.0)	94 (5.4)
Post-hatch larval fish survival (±SD) (day 6 to 34) [%]	94 (2.5)	95 (4.1)	89 (4.8)	91 (2.5)	88 (5.0)	0 (0.0)*
Behaviour	None	None	None	None	None	None
Mean dry weight (34 d) [mg] (±SD)	19.91 (0.22)	20.14 (0.41)	20.23 (0.32)	20.52 (0.31)	19.95 (0.27)	NA (NA) ^b
Mean length (34 d) [mm] (±SD)	0.0242 (0.0010)	0.0246 (0.0012)	0.0263 (0.0012)	0.0265 (0.0009)	0.0239 (0.0017)	NA (NA) ^b
Parameters	Endpoints [mg a.s./L]					
	Mean measured					
EC ₁₀ hatching success ^{**} (mg a.s./L)	0.34 (CI 0.12-0.58)					
Overall NOEC (34 d) based on all parameters (mg a.s./L)	0.17					

LoQ: Limit of quantification range = 0.000883 – 0.00113 mg a.s./L

SD=Standard deviation

*Statistically significant difference from the control based on Fisher's Exact Test with Bonferroni-Holm's Adjustment.

**Note, this is not the most sensitive endpoint, but it was not possible to derive EC₁₀ or EC₂₀ for any other parameter.

^a Values presented represent hatching success at the completion of hatch (test day 6 for control and treatment levels 0.024, 0.048, 0.09 and 0.17 mg a.s./L; test day 7 for the highest treatment level of 0.35 mg a.s./L).

^bNA=not applicable for growth (length and weight) analysis due to 0 % larval survival at this treatment level.

It was not possible to derive EC₁₀ or EC₂₀ values from the data for any endpoint apart from hatching success. The overall NOEC accounts for the most sensitive endpoint.

B.9.2.3.1.2.1. Consideration of ELS results on sheepshead minnow (*Cyprinodon variegatus*) (██████, 2015):

In the study, completion of hatch, defined as when all viable embryos in the control embryo incubation cups were hatched, was on day 6. Completion of hatch occurred on all treatment levels on day 6 as well with the exception of the highest treatment level of 0.35 mg a.s./L where the completion of hatch was delayed by 1 day, finishing on day 7 with a success rate of 73 % compared to 87 % in the control (not a statistically significant difference). Statistically significant effects on larval survival were observed at the highest test concentration (0.35 mg a.s./L) with 0 % survival. No other significant effects on any other parameters were observed.

B.9.2.3.1.3. FSTRA study on fathead minnow (*Pimephales promelas*) (██████, 2020a):

Request for Independent Scientific Advice from the Expert Committee on Pesticides

As there was some uncertainty regarding the interpretation of the results from the FSTRA, HSE sought Independent Scientific Advice (ISA) from the Expert Committee on Pesticides (ECP) in October 2022 in this area. The following provides a summary of the points discussed with the ECP, together with the response from the applicant and the agreed actions going forward. Text has subsequently been amended in the following sections to reflect the outcomes of the ISA:

HSE noted that there was some uncertainty regarding the interpretation of the female VTG results together with effects on oocyte atresia and fecundity. Specifically, whilst there was reduction in female VTG at all tested concentrations, only the middle dose tested was statistically significant in comparison to the control; in addition whilst there was severe oocyte atresia and a statistically significant reduction in fecundity, this was only observed at the highest tested concentration. There was also a large variability in male VTG results, making interpretation of the results difficult.

The ECP did not consider the variation in male VTG results to be of concern, however the statistically significant reduction in female VTG together with increased oocyte atresia and decreased fecundity was considered to be of potential concern. The ECP considered the results from the FSTRA to be unclear as to whether they were endocrine-mediated or due to reproductive toxicity, and hence recommended that a further mechanistic assay was conducted.

HSE considered the advice provided by the ECP alongside our initial consideration of the data from the FSTRA and concluded that there was significant uncertainty regarding the results and agreed with the ECP that further mechanistic data should be provided by the applicant and a Rapid Androgen Disruption Activity Reporter (RADAR) assay was requested. The applicant did not agree with this position and submitted a rebuttal paper to HSE in January 2023. HSE considered the information provided by the applicant in this rebuttal paper and again consulted the ECP in April 2023 to determine if this changed the ISA initially provided and to confirm the most appropriate mechanistic assay to assist in reaching a conclusion. The ECP confirmed that their initial advice had not changed in light of the applicant's rebuttal paper and that the RADAR assay was the most appropriate assay given the results from the FSTRA.

The ISA was relayed to the applicant and the applicant is currently in the process of conducting the RADAR assay. As such, it is not possible to conclude on the EAS modalities at present.

Summary of results from the FSTRA

Under this study, the maximum tolerated concentration (MTC) was 0.13 mg a.s./L. The study authors state that this was based on available chronic data from Wheeler et al., 2013⁴, in consultation with the study sponsor, and the early life-stage data from the 32-day pydiflumetofen study on fathead minnow (██████, 2020), which resulted in a 28-day post-hatch larval fish survival of 78 % compared to 88 % in the pooled control at the test concentration

⁴ Wheeler J.R., Panter G., Weltje L., Thorpe K.L., 2013. Organization for Economic Co-operation and Development; United States Environmental Protection Agency. Test concentration setting for fish in vivo endocrine screening assays. Chemosphere 92:1067-1076.

of 0.15 mg a.s./L (Table 9.2.3.1-2). Although it was not possible to derive an EC₁₀ or EC₂₀ for larval survival (see study summary in section B.9.2.2.1), the calculated 28-day post-hatch dry weight EC₁₀ was 0.13 mg a.s./L. The OECD 229 (2012) guideline defines the MTC as “the highest test concentration of the chemical which results in less than 10% mortality”. Based on the above, the highest test concentration of 0.13 mg a.s./L used in the FSTRA study meets this OECD definition.

The results from the FSTRA 21-day flow through GLP study are shown in the tables below for the parameters assessed. Liver and gonad histopathology findings are presented separately.

Table 9.2.3.1-4 Summary of biological results during the 21-day fish short-term reproduction assay (FSTRA) with fathead minnow.

Parameters measured		Nominal concentration pydiflumetofen [mg a.s./L]			
		Control	0.0013	0.013	0.13
		Time-weighted average concentration pydiflumetofen [mg a.s./L]			
		Control	0.0013	0.017	0.13
Male survival [%] ^A	Mean	88	88	100	88
	SD	25	25	0	25
Female survival [%] ^A	Mean	94	100	100	100
	SD	13	0	0	0
Egg fertilisation [%] ^B	Mean	98.3	98.9	99.1	98.2
	SD	1.0	0.42	0.43	1.2
Fecundity (mean no. eggs per surviving female per reproductive day) [n]	Mean	66	69	65	42 ^C
	SD	19	14	22	14
	%CV	29	20	34	33
Wet body weight males at study termination [g] ^D	Mean	3.7481	4.0247	4.1078	4.0927
	SD	0.3453	0.0901	0.3503	0.3614
Wet body weight females at study termination [g] ^D	Mean	2.8195	2.8417	2.8619	2.9492
	SD	0.2396	0.1369	0.2058	0.2871
Length males at study termination [mm] ^D	Mean	62.59	64.11	64.56	64.53
	SD	1.26	1.39	0.70	2.39
Length females at study termination [mm] ^D	Mean	58.61	59.49	59.53	58.69
	SD	1.35	0.75	1.00	2.17
VTG [ng/mL]; males at study termination	Mean	105	47.2 ^{F,G}	68.4	188
	SD	119	59.6	35.1	107
	% change from control	na	-55.0 %	-34.6 %	+79 %
VTG [ng/mL]; females at study termination	Mean	10.2 × 10 ⁶	6.21 × 10 ⁶	5.35 × 10⁶ ^{E,F,H}	6.99 × 10 ⁶
	SD	2.18 × 10 ⁶	2.50 × 10 ⁶	3.10 × 10 ⁶	2.60 × 10 ⁶
	% change from control	na	-39.3 %	-47.8 %	-31.8 %
Tubercle scores in males*	Mean	26	25	29	25
	SD	3.5	5.0	4.5	2.0
GSI in males [%]	Mean	1.8	1.5	1.9	1.8
	SD	0.28	0.26	0.26	0.17
GSI in females [%]	Mean	16	15	17	20
	SD	1.7	3.1	2.4	2.5

Values in bold are statistically significantly different compared to the control.

SD = Standard deviation; CV = Coefficient of Variation; VTG = Vitellogenin concentration in blood plasma;

GSI = Gonadosomatic Index (gonad weight/body weight × 100)

^A: No statistically significant difference between controls and treatments (one-tailed Fisher's Exact Test with Bonferroni-Holm's Adjustment, p<0.05)

^B: No statistically significant difference between controls and treatments (one-tailed Dunnett's Multiple Comparison Test, p>0.05)

^C: Statistically significant compared to the control (one-tailed Jonckheere-Terpstra's Step-Down Test, p<0.05)

^D: Length and body weight not statistically analysed for differences from the control as they are not endpoints in the study.

^E: Significantly reduced compared to the control (one-tailed Dunnett's Multiple Comparison Test, $p < 0.05$).

^F: An outlier was detected in the dataset during statistical analyses; however, the results of the statistical analysis of treatment results compared to the control were the same with and without the statistical outliers, so data including outliers is used as the basis for results and conclusions.

^G: For reference, mean value excluding outlier (see note ^F) is $16.4 \pm \text{SD } 14.8$ (-84.0 % compared to control)

^H: For reference, mean value excluding outlier (see note ^F) is $5.43 \times 10^6 \pm \text{SD } 2.94$ (-47.0 % compared to control)

* No tubercles were present in females

B.9.2.3.1.3.1. Consideration of egg production, wet weight and length results:

According to EFSA/ECHA guidance 2018, body weight and reproduction (fecundity, fertility) are considered sensitive to, but not diagnostic of EATS modalities.

Egg production was significantly reduced in the highest treatment level (0.13 mg a.s./L), with a mean of 42 eggs per female per reproductive day compared to 66 in the control (Table 9.2.3.1-4). However, there was no statistically significant differences in egg fertilisation success (across all treatments 98.2 - 99.1 % compared to 98.3 % in the control).

Terminal wet weight and length results were not evaluated statistically. The data (Table 9.2.3.1-4), shows male weight and length in all test concentrations are slightly higher than the control, but there is no obvious dose response effect. No notable differences in female weight or length are evident.

It is noted in the study summary (section B.9.2.3.3), that prior to exposure to the test item, the mean wet weight of both male and female fish was larger than that specified in Annex 2 of the OECD 229 (2012) guideline, as measured in a representative sample ($n=30$ per sex) of the laboratory fish culture population prior to study initiation: OECD 229 (2012) Annex 2 suggests $2.5 \text{ g} \pm 20 \%$ for male fish whereas the study measured 4.2 (range 3.4-5.0; 68 – 119 % of mean) g; and Annex 2 suggests $1.5 \text{ g} \pm 20 \%$ for female fish whereas the study measured 3.0 (range 2.4-3.6; 80 – 120 % of mean) g. The guideline does not provide any more information to indicate whether large fish are more or less sensitive in this type of assay. Therefore, this introduces some uncertainty.

Additionally, variation in the female fish population slightly exceeded guideline recommendations: for approximately 17 % of the female pre-exposure population were within $\pm 30 \%$ of the population mean, as opposed to $\pm 20 \%$ of the mean as recommended in the guideline. Despite this, it is noted that the pre-exposure population met the key validity requirement for being actively spawning at study initiation, were of recommended age, and the standard deviations in the female body weights at study termination do not indicate an inappropriate level of variation. Therefore, this is not expected to adversely impact the study sensitivity.

B.9.2.3.1.3.2. Consideration of vitellogenin results:

EFSA/ECHA Guidance Document (2018) states that vitellogenin (VTG) measurement has been developed as a biomarker for endocrine activity, with induction of VTG in males a biomarker for detecting estrogenic compounds, and a reduction of VTG in females being indicative of sexual steroid synthesis modulation. However, 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. Specifically, EFSA/ECHA (2018) mentions that “*a decrease in VTG, while generally considered EAS-mediated, needs to be interpreted with caution in combination with other observations*”.

In this study, pydiflumetofen-exposed males exhibited rising blood-plasma VTG levels across the three treatment groups, though this relationship is not consistent with the control data: only at the highest test concentration of 0.13 mg a.s./L is the mean VTG level higher than the control, and there are no statistical differences from control group at any treatment level. However, the large variation in the VTG dataset, as seen by a standard deviation which is larger than the mean in the control and lowest treatment level (Table 9.2.3.1-4), reduces the power of any comparisons of male VTG data between treatments and the control. This may be reducing the ability to detect statistical differences in these results, hence introducing some uncertainty.

In the study summary, seen in section B.9.2.3.3, the male VTG data is presented on a per-replicate basis, where it can be seen that the range of VTG level in the control covers at least one order of magnitude, from 7.6-270 ng/ μl , and that the datapoint responsible for the highest VTG level in the control is based on a single individual due to

the mortality of the other fish in the tank. In the lowest test concentration (0.0013 mg a.s./L), the range is 7-257 ng/μl, though the highest data point was identified as a statistical outlier.

According to the OECD guideline, VTG differences are:

‘considered positive if there is a statistically significant increase in VTG in males ($p < 0.05$), or a statistically significant decrease in females ($p < 0.05$) at least at the highest dose tested compared to the control group, and in the absence of signs of general toxicity’.

Following this definition from the guideline, the VTG results do not give a positive indication for endocrine activity for either males or females, noting though, that the large variation in the male control data introduces some uncertainty. However, variability of VTG is discussed in the OECD validation report for 21-day fish screening assay (2007), where 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’.

This suggests that the high variability is expected and should not compromise the detection of a true response using statistical analysis. However, it is noted that the sensitivity of male VTG levels for detecting effects in this study is very low.

For female fish, mean blood-plasma VTG levels are lower than the control at all three test concentrations, but this reduction was not dose-responsive and is only statistically significant in the middle treatment level (time-weighted average of 17 μg a.s./L). ~~Therefore, the VTG results do not give a positive indication for endocrine activity.~~ This raises some uncertainty regarding the interpretation of these results.

The applicant’s consideration regarding female VTG levels in the context of the gonad and liver histopathology (histopathology results are presented in Table 9.2.3.1-5) is stated below. It is noted that in the mammalian toxicology assessment (volume B6), liver toxicity is present in mammals.

“While VTG reduction in female fish can be caused by oestrogen antagonists or androgen agonist compounds, production of VTG in female fish can also be decreased by hepatotoxicity, stress, and other non-endocrine modes of toxicity (██████ et al., 2014).

Responses in the gonad and liver histopathological endpoints (i.e., oocyte atresia in gonad histopathology; cell necrosis/apoptosis in female liver histopathology), included above, suggest that the reduced VTG in female fish exposed to pydiflumetofen is likely the result of a general stress response and not indicative of an EAS-mediated response. Furthermore, it would be anticipated that decreased hepatic basophilia would occur in female fish when VTG concentration is reduced (██████ and ██████, 2018). However, the apparent decrease in plasma vitellogenin was not evident histopathologically as diminished liver basophilia in the pydiflumetofen-exposed females. The decreased VTG concentration in the 1.3 and 17 μg/L treatment levels did not have an adverse effect in the form of reproductive success since fecundity in both treatments were comparable to control.

Based on the histopathology effects, the reduced female plasma VTG concentration may have been a result of liver and ovarian damage rather than endocrine signalling interference. Therefore, based on the weight of evidence across the other endpoints in this assay, the apparent reduction in plasma VTG among pydiflumetofen-exposed females likely reflects liver toxicity, consistent with findings in mammalian toxicology studies.”

On the basis of the data and in the context of the guideline, HSE considers ~~there to be no clear treatment related~~ the effect of pydiflumetofen on female blood-plasma VTG levels ~~to be uncertain~~. For male fish, due to the large variation in the data, particularly in the control, the potential to identify biological effects of pydiflumetofen on male VTG levels from this dataset is limited and therefore no conclusion can be made for male fish.

It is further noted that there is a larger than expected difference in order of magnitude between control male and female VTG levels, which according to the OECD FSTRA study guideline (OECD 229) can compromise the responsiveness of the assay. The guideline states male and female VTG levels in control populations are expected to be separated by about 3 orders of magnitude for fathead minnow whereas in this study there is approximately 5 orders of magnitude difference. However, the large spread of the control data in the male VTG results is likely

partly responsible for this. As the male VTG results are inconclusive anyway this is not expected to affect the conclusions. For the female VTG results, this does introduce some uncertainty.

B.9.2.3.1.3.3. Consideration of secondary sexual characteristics (SSC) and gonadosomatic index (GSI)

No abnormal mating behaviour or changes in secondary sexual characteristics (SSC) were observed in either sex throughout the study. Gonadosomatic index (GSI), which is a percentage measure of gonad weight relative to total body weight, was not significantly different from control for either sex at any treatment level.

B.9.2.3.1.3.4. Consideration of histological analysis:

Histological analyses were carried out on gonads and livers of all surviving male and female fish. According to EFSA/ECHA 2018, gonad histopathology is considered to be EATS mediated and are considered for the definition of adversity. Liver histopathology is considered under the category of evidence of general toxicity. Selected results are shown in Table 9.2.3.1-5 and discussed below.

Table 9.2.3.1-5 Selected gonad and liver histopathology results from pydiflumetofen-exposed fish in a short-term reproduction test for 21 days

Term Reproduction test for 21 days

Findings in the Ovaries of Female Fathead Minnows																	
TWA Pydiflumetofen Treatment (µg/L):		Negative Control		1.3		17		130									
Total number of fish examined:		15		16		16		16									
Observation type		Score		Number of fish with observation													
Oocyte Atresia, Increased		Total		2		8		4		9							
		minimal		-		3		2		2							
		mild		1		1		-		-							
		moderate		1		4		2		3							
		severe		-		-		-		4							
Liver Findings by Sex																	
TWA Pydiflumetofen Treatment (µg/L):		Negative control		1.3		17		130		Negative control		1.3		17		130	
Total number of fish examined:		8		7		8		7 ^a		15		16		16		16	
Observation Type		Number of fish with observation															
		Males						Females									
Basophilia (minimal, mild and moderate severity; majority mild)		0		0		0		0		15		15		16		16	
Individual Cell Necrosis/Apoptosis (minimal to mild severity)		0		0		0		0		2		1		5		5	
Inflammation, Granulomatous (minimal to mild severity)		3		3		0		1		4		1		3		0	
Microsporidiosis (minimal severity)		3		3		1		0		0		0		0		0	
TWA: Time-weighted average																	
^a For one additional male in this group, liver tissue was not recovered, possibly due to autolysis																	

Male gonad histology

There were no treatment-related findings in the testes; any observations such as inflammation, mineralisation and spermatogenesis were in general at low prevalence and severity, and were in comparable numbers across control and treatments (see study summary for detailed results).

Female gonad histology

There was a treatment related effect in female gonads: there was increase in prevalence of oocyte atresia (breakdown) at all treatment levels compared to the control (Table 9.2.3.1-5), and an increase in severity of oocyte atresia at the highest test concentration (130 µg a.s./L). There were no treatment-related observations of granulomatous inflammation, microsporidiosis, or post-ovulatory follicle grades.

Ovarian stage scores were similar between treatments and controls, noting that for ovarian stage scores four fish in the highest treatment level (130 µg a.s./L) were unable to be characterised due to four ovaries with severe atresia as mentioned above. Additionally, one female in the lowest treatment level (1.3 µg a.s./L) had a relatively undeveloped immature Stage 1 ovary and a liver resembling that of a male fish with no hepatocyte basophilia, which taken together are consistent with decreased endogenous oestrogen activity in this individual fish.

Liver histopathology for both sexes

Livers were examined for autolysis, basophilia, cystic degeneration, hepatocellular vacuolation, individual cell necroses/apoptosis, granulomatous inflammation and microsporidiosis (infection).

A general observation of reduced prevalence of granulomatous inflammation in livers of both sexes and microsporidiosis in livers of males were generally decreased compared to the control. Consideration regarding this point from the applicant's report is provided below for reference:

“Given that pydiflumetofen is a fungicide, and microsporidia are most recently classified as fungi or fungi-like organisms (██████████, 2017), it is possible that this decreased prevalence represents an effect of pydiflumetofen treatment.”

As mentioned above, a single female in the lowest treatment level did not exhibit hepatocyte basophilia (Table 9.2.3.1-5) and this co-occurred with an immature ovary stage, indicating decreased endogenous oestrogen activity in this individual fish. However, due to the sporadic nature of this occurrence and no fish in higher treatment levels exhibiting similar symptoms, HSE considers this not to be treatment related.

A slight increase in individual cell necrosis/apoptosis was observed in females at the highest two treatment levels (17 and 130 µg/L) compared to the control (Table 9.1.3.1-5) but was not co-associated with other types of liver findings or ovarian changes.

Discussion of gonad and liver histopathology results in the context

In the study report, the applicant provided the following discussion regarding the gonad and liver histopathology results:

“Increased hepatocyte necrosis/apoptosis is prominently indicative of chronic cytotoxicity as opposed to hormonal perturbation (██████████ and ██████████, 2018), and can be induced by a variety of non-endocrine mechanisms, including oxidative stress (██████████ et al., 2013).”

“Apparent treatment-related increases in oocyte atresia (degeneration) hepatocyte apoptosis/necrosis in female fish can both be induced by direct cytotoxicity, stress, or other factors. Therefore, the histopathological responses in this study are not indicative of EAS-mediated endocrine activity.”

ECHA/EFSA guidance (2018) states that gonad histology can help interpret effects on reproduction and that oocyte atresia in female fish is of a primary diagnostic interest, but that this may also be influenced by non-endocrine-mediated methods of actions (MoAs). Therefore, it is probable that the observations of severe oocyte atresia and the significant reduction in fecundity at 0.13 mg a.s./L are linked. However, in the absence of significantly reduced VTG levels at 0.13 mg a.s./L (see below), and with the observed increase in liver necrosis/apoptosis (though this was noted in the results to not co-occur with any particular ovarian findings), there is the potential confounding influence of some general low-level toxicity. This also aligns with the OECD 150 conceptual framework, which regarding gonad histopathology results states that: *“Although these endpoints are indicative of endocrine activity, care should be taken in their interpretation because some (e.g. oocyte atresia) can also be caused by certain types of systemic toxicity”*. As such, it is considered uncertain as to whether the observed effects on oocyte atresia are indicative of endocrine activity or due to systemic toxicity.

B.9.2.3.1.4. RADAR assay (██████████-██████████ (2023))

Following independent scientific advice from the ECP, the applicant conducted a RADAR assay at the request of HSE due to uncertainties with results from the FSTRA regarding the interpretation of the female VTG results together with observed effects on oocyte atresia and fecundity. Specifically, whilst there was reduction in female VTG at all tested concentrations, only the middle dose tested (17 µg a.s./L) was statistically significant in comparison to the control and a clear dose-reponse was not evident. In addition, whilst there was severe oocyte atresia and a statistically significant reduction in fecundity, this was only observed at the highest tested concentration (130 µg a.s./L). Thus, it was unclear from the FSTRA results whether the observed effects were endocrine-mediated or attributable to systemic toxicity. The conduct of the RADAR assay together with the results is discussed below in the context of the observed effects from the FSTRA.

The RADAR assay was conducted at the following concentrations based on survival pre-tests:

Unspiked mode: 130, 41, 13, 4.1 and 1.3 µg a.s./L MM1X + 0.2% DMSO (nominal)

Spiked mode: 130, 41, 13, 4.1 and 1.3 µg a.s./L MM1X + 0.2% DMSO + 17-MT 3 µg/L (nominal).

The following controls were included in the test in line with OECD 251 guidelines:

Solvent control (MMIX + DMSO 0.2%); dilution water control (MMIX; 17-MT 3 and 10 µg/L MMIX + 0.2% DMSO (pro-androgenic standards); 17-MT 3 µg/L MMIX + Flutamide 167 and 500 µg/L + 0.2% DMSO (anti-androgenic standards).

The concentration range selected was in line with OECD 251 guidelines, with the MTC based on survival pre-tests, which demonstrated ≤10% mortality/sublethal effects at a concentration of 126 µg a.s./L; as such a concentration of 130 µg a.s./L was selected at the maximum concentration in the definitive test. The concentrations selected are also in the range used in the FSTRA (1.3, 17 and 130 µg a.s./L) discussed in section B9.2.3.1.4.

All validity criteria were met (see full study summary in section 9.2.3.3 for further details).

Results from the RADAR assay indicated that no significant increase or decrease in normalised mean fluorescence was observed in the spiked mode of the test in comparison to the 17MT 3 µg/L control. According to OECD 251, it can therefore be concluded that pydiflumetofen is inactive in the RADAR assay. However, it is noted that in the unspiked mode, a statistically significant decrease in normalised mean fluorescence was observed at the lowest test concentration (1.3 µg a.s./L) in comparison to the solvent control. GFP was not visible in the kidneys of the solvent control group or test groups. It is stated in the study report that this observation only occurred at run 2 of the test, however looking at the raw data, normalised mean fluorescence in all 3 runs of the test is below that of the solvent control, noting this has not been statistically determined.

Results are summarised in the tables below for spiked and unspiked mode for all 3 runs of the test:

Table B.9.2.3.1-6: Normalized fluorescence and statistical analysis for the unspiked mode (The results were normalised to the mean fluorescence of the 3 µg/L 17MT control group).

Treatment	Normalised mean fluorescence				SEM	CV	% of induction ¹	p-value ² (pooled results)
	Run 1	Run 2	Run 3	Pooled				
Dilution water control	0.0866	0.1344	0.0690	0.0977	0.0116	91	-	0.6200
Solvent control	0.0746	0.0705	0.0824	0.0758	0.0056	57	-	-
Pooled control ³	0.0804	0.1025	0.0757	0.0862	0.0064	82	-	Not calculated
Pydiflumetofen 1.3 µg/L	0.0666	0.0419	0.0464	0.0514	0.0036	53	-47.40	0.0006
Pydiflumetofen 4.1 µg/L	0.0664	0.0745	0.0671	0.0693	0.0037	41	-29.02	>0.9999
Pydiflumetofen 13 µg/L	0.0968	0.0479	0.0670	0.0706	0.0063	69	-27.76	>0.9999
Pydiflumetofen 41 µg/L	0.1133	0.0634	0.0443	0.0731	0.0062	65	-25.12	>0.9999
Pydiflumetofen 130 µg/L	0.1171	0.0486	0.0511	0.0734	0.0074	76	-24.85	0.5309

¹In comparison to the solvent control

²Based on a Kruskal-Wallis test; P <0.05 denotes statistical significance

³Pooled control calculated by HSE evaluator as per paragraph 51 of OECD 251 which states 'if there is no statistically significant difference between the test medium control and solvent control, the pooled test medium and solvent controls should be used'.

Table B.9.2.3.1-7: Normalized fluorescence and statistical analysis for the spiked mode (The results were normalised to the mean fluorescence of the 3 µg/L 17MT control group).

Treatment	Normalised mean fluorescence				SEM	CV	% of induction ¹	p-value ² (pooled results)
	Run 1	Run 2	Run 3	Pooled				
17MT 3 µg/L	1.0000	1.0000	0.8610	1.0000	0.1486	113	-	-
Pydiflumetofen 1.3 µg/L + 17MT 3 µg/L	0.9613	0.5437	0.7533	0.7523	0.1503	155	-24.77	0.3080
Pydiflumetofen 4.1 µg/L + 17MT 3 µg/L	2.2558	0.5975	0.9761	1.2647	0.2228	134	26.47	>0.9999
Pydiflumetofen 13 µg/L	1.5715	0.4375	1.2012	1.0464	0.1851	136	4.64	>0.9999
Pydiflumetofen 41 µg/L	1.1928	0.7168	0.8991	0.9362	0.1306	108	-6.38	>0.9999
Pydiflumetofen 130 µg/L	1.3959	0.7740	1.0653	1.0730	0.1929	138	7.30	>0.9999

¹In comparison to the 17MT 3 µg/L

²Based on a Kruskal-Wallis test; P <0.05 denotes statistical significance

Based on the above consideration, there is some uncertainty regarding the results from this study. Fluorescence decreases in unspiked mode are not expected as the eleutheroembryos do not synthesise detectable levels of androgen at this developmental stage. OECD 251 recommends where statistical significance is observed, the RADAR assay may not be appropriate for the chemical or a potential issue with the organisms or test conditions. As the controls performed appropriately and all validity criteria were met, it does not appear that there was an issue with the test conditions or test organisms. OECD 251 recommends statistical analysis of the individual runs of the test where statistical significance is observed for the pooled results, which has not been conducted. Regarding the concentration range selected, this appears to be appropriate in the context of setting the MTC and no mortality >10% or sublethal effects were observed at any of the tested concentrations, however a lower concentration range is suggested in OECD 251 where results of this nature are observed. OECD 251 also suggests that a different androgen axis activity test may be more appropriate in this case. Consideration of these uncertainties has been made in the following paragraph in the context of the results of the FSTRA assay:

The below table makes a comparison of the key results from the FSTRA and the RADAR assay as the concentrations span a similar range and thus a side by side comparison is useful when considering the observed effects on EAS modalities as a whole. Considering the results from the RADAR assay alongside those from the FSTRA, some of the uncertainty regarding the decrease in fluorescence at 1.3 µg a.s./L in the unspiked mode can potentially be mitigated. In the FSTRA, other than a moderate increase in oocyte atresia in comparison to the control at a concentration of 1.3 µg a.s./L, which may be related to systemic toxicity, there was no clear indication of endocrine-mediated effects at this concentration, with VTG and fecundity not statistically different to the control. Furthermore, the decrease in fluorescence in the RADAR assay was only observed at 1.3 µg a.s./L in the unspiked mode; there was no effect approaching statistical significance at the 4 other tested concentrations in the unspiked mode indicating that the concentration range tested was likely appropriate. The statistically significant decrease in female VTG observed at 17 µg a.s./L in the FSTRA does not correspond to any significant effect in the RADAR assay at the comparable concentration of 13 µg a.s./L. Whilst there remains some uncertainty with both the results of the FSTRA and the RADAR assay, taken together HSE considers the results support a negative conclusion for EAS modalities. In addition, the conclusion reached for toxicology was that based on the overall weight of evidence, pydiflumetofen does not cause EAS-mediated adversity and that this modality has been sufficiently investigated (see in the volume 3, CA section 6 dossier part II (B.6.8.3), adding further support to the overall conclusion that there is lack of adversity for EAS modalities.

Table B.9.2.3.1-8: Comparison of key results from the FSTRA and RADAR assay (results in **bold** indicate statistical significance in comparison to the control, (p <0.05))

Key results from FSTRA

Pydiflumetofen Treatment (µg/L):		Negative Control	1.3	17	130		
Total number of fish examined:		15	16	16	16		
Observation type	Score	Number of fish with observation					
Oocyte Atresia, Increased	Total	2	8	4	9		
	minimal	-	3	2	2		
	mild	1	1	-	-		
	moderate	1	4	2	3		
	severe	-	-	-	4		
Fecundity (mean no. eggs per surviving female per reproductive day) [n]	Mean	66	69	65	42		
	SD	19	14	22	14		
	%CV	29	20	34	33		
VTG [ng/mL]; females at study termination	Mean	10.2 × 10 ⁶	6.21× 10 ⁶	5.35 × 10 ⁶	6.99× 10 ⁶		
	SD	2.18× 10 ⁶	2.50× 10 ⁶	3.10× 10 ⁶	10 ⁶		
	% change from control	na	-39.3 %	-47.8 %	2.60× 10 ⁶ -31.8 %		
Key results from RADAR							
Pydiflumetofen Treatment (µg/L):		Control	1.3	4.1	13	41	130
Normalised mean fluorescence Unspiked mode	Mean	0.0758	0.0514	0.0693	0.0706	0.0731	0.0734
	% change from control	-	-47.40	-29.02	27.76	-25.12	-24.85
Normalised mean fluorescence Spiked mode	Mean	1.0000	0.7523	1.2647	1.0464	0.9362	1.0730
	% change from control	-	-24.77	26.47	4.64	-6.38	7.30

B.9.2.3.1.5. EAS summary of parameters

A summary of the results from both the FSTRA, RADAR and ELS studies has been provided in the table below. The format is in accordance with EFSA/ECHA guidance (2018) and was collated using the appendix E spreadsheet and considers the information provided by the applicant (██████████ *et al.* 2020 and ██████████ *et al.* 2020a). The results summarised are for the EAS modalities.

Table 9.2.3.1-69: Reporting and assessing the lines of evidence for adverse effects from fish studies (EAS modalities)

Integrated Line of Evidence	Evidence Group	Line of evidence	Study ID; Type	Species	Exposure duration (days) ¹	LOED (µg a.s./L)	Observed effect (positive and negative)	Assessment of each line of evidence	Assessment of integrated line of evidence	Modality
Endocrine activity	In vivo mechanistic	Vitellogenin (VTG) in females	25; FSTRA	<i>Pp</i>	21	17	Reduced VTG at all test concentrations but only statistically significantly at 17 µg a.s./L (other doses 1.3, 130 µg a.s./L). No consistent dose-response relationship - flat response.	Reduced VTG but not dose responsive, therefore this does not indicate evidence for endocrine activity.	Uncertain as to whether the reduction in female VTG is endocrine-mediated. Evidence does not indicate endocrine activity based on female data according to EFSA/ECHA 2018 (no dose/concentration response).	E,A,S
		Vitellogenin (VTG) in males	25.1; FSTRA	<i>Pp</i>	21	Inconclusive	Rising trend in mean VTG levels across test concentrations (1.3, 17 and 133 µg a.s./L) was observed, but only at 133 µg a.s./L was the mean VTG level higher than the control, and none were statistically significantly different from the control. However, large variation in control (mean 105 ng VTG/mL with standard deviation of 119) reduces the power of comparison.	Inconclusive VTG results in males due to large variation in control data.	Male data not considered reliable due to control variation.	E,A

Integrated Line of Evidence	Evidence Group	Line of evidence	Study ID; Type	Species	Exposure duration (days) ¹	LOED (µg a.s./L)	Observed effect (positive and negative)	Assessment of each line of evidence	Assessment of integrated line of evidence	Modality
		Fluorescence	26 RADAR assay	Ol	3	1.3	No statistically significant increase or decrease in fluorescence in the spiked mode of the assay. Statistically significant decrease in fluorescence in the unspiked mode of the assay at the lowest tested concentration (1.3 µg a.s./L)	Inactive in RADAR assay in spiked mode. Fluorescence decreases in unspiked mode are not expected as the eleutheroembryos do not synthesise detectable levels of androgen at this developmental stage.	Uncertainty with study results when considering lowest concentration (1.3 µg a.s./L) unspiked mode. At 4.1, 13, 41 and 130 µg a.s./L there were no significant differences in comparison to the controls in either spiked or unspiked mode.	E,A
Adversity	EATS-mediated parameters	Secondary sex characteristics*	25, 25.1; FSTRA	Pp	21	>130	No effect. No change in male secondary sex characteristics in females or males.	No indication.	Adverse effects on gonads in adult females at 130 µg a.s./L, corresponds with decreased fecundity. Decreased fry body weight	E, A
		Gonad histopathology – female ³	25; FSTRA	Pp	21	1.3	Increased prevalence of increased oocyte atresia at all tested concentrations (1.3, 17, 130 µg a.s./L) at minimum to moderate severity. Severe oocyte atresia observed at 130 µg a.s./L only ² .	The overall increase in atresia in the 130 µg/L treatment was consistent with the observation of decreased fecundity in this treatment level.		E, A, S
		Female ovarian stage scores ³	25.1; FSTRA	Pp	21	>130	No effect on ovarian stage scores at any tested dose. However, no feasible stage scoring for four ovaries with severe atresia at 130 µg a.s./L treatment level.			E, A, S
		Gonad histopathology – male ³	25.2; FSTRA	Pp	21	>130	No effect	No indication		E, A, S

Integrated Line of Evidence	Evidence Group	Line of evidence	Study ID; Type	Species	Exposure duration (days) ¹	LOED (µg a.s./L)	Observed effect (positive and negative)	Assessment of each line of evidence	Assessment of integrated line of evidence	Modality
		Male testicular score ³	25.3; FSTRA	<i>Pp</i>	21	>130	No effect		at length at 150 µg a.s./L (<i>P. promelas</i> only).	E, A, S
	Sensitive to, but not diagnostic of, EATS	Behaviour ³	25; FSTRA	<i>Pp</i>	21	>130	No effect. Injuries in two individual male fish (one in control and one in 1.3 µg a.s./L treatment) were a result of normal territorial aggression.	No indication		N
		Body weight	22; ELS	<i>Pp</i>	32	150	Significantly reduced weight of surviving fry at 150 µg a.s./L	Decrease (weight and length) for <i>P. promelas</i> fry only, from 150 µg a.s./L		N
			23; ELS	<i>Cv</i>	34	>170	No effect; noting measurement not possible at 350 µg a.s./L due to 0 % larval survival			N
			25; FSTRA	<i>Pp</i>	21	>130	No effect (adult) ³			N
		Body length	22; ELS	<i>Pp</i>	32	150	Significantly reduced length of surviving fry at 150 µg a.s./L			N
			23; ELS	<i>Cv</i>	34	>170	No effect; noting measurement not possible at 350 µg a.s./L due to 0 % larval survival			N
			25; FSTRA	<i>Pp</i>	21	>130	No effect (adult) ³			N
		Gonado-somatic index (male and female)	25; FSTRA	<i>Pp</i>	21	>130	No statistically significant effects on gonado-somatic index at any test concentration. Slight, non-significant increase in female GSI was observed at the 130 µg a.s./L treatment level (likely related to the decreased fecundity and increased oocyte atresia at this treatment level)	No indication.		N
		Hatching success and time-to-hatch	22; ELS	<i>Pp</i>	32	380	Hatching success significantly reduced at 380 µg a.s./L (also 0 % larval survival); this also corresponded to a delay in time-to-hatch.	No indication - effects on hatching success from 380 µg a.s./L and time-to-hatch from 350 µg a.s./L co-occur with systemic toxicity to larvae.		N
			23; ELS	<i>Cv</i>	34	350 ⁴	No effect on hatching success at any concentration (max. tested 350 µg a.s./L; 14 % reduction not statistically significant ⁴), but one day delay in time-to-hatch at 350 µg a.s./L (additionally, 0 % larval survival)			

Integrated Line of Evidence	Evidence Group	Line of evidence	Study ID; Type	Species	Exposure duration (days) ¹	LOED (µg a.s./L)	Observed effect (positive and negative)	Assessment of each line of evidence	Assessment of integrated line of evidence	Modality
		Morphological abnormalities ³	22; ELS	<i>Pp</i>	32	64	Significant reduction in percentage of normal live larvae at hatch at 64 and 150 µg/L. Reductions were marginal (6-7%) and within historic control range.	No indication		N
			23; ELS	<i>Cv</i>	34	>350	No effect of treatments on percentage of live, normal larvae at hatch			
			25; FSTRA	<i>Pp</i>	21	>130	No effect. No observations of morphological abnormalities			
		Reproduction: fecundity	25; FSTRA	<i>Pp</i>	21	130	Significant decrease in the mean number of eggs/female/reproductive day at 130 µg a.s./L.	Decrease in fecundity at 130 µg/L is consistent with increased oocyte atresia (see gonad histopathology)		N
		Reproduction: fertilisation success	25.1; FSTRA	<i>Pp</i>	21	>130	No effect on fertilisation success at any tested concentration			N
General toxicity	Systemic toxicity	Liver histopathology (male and female) ³	25; FSTRA	<i>Pp</i>	21	17	Reduced prevalence of minimal/mild granulomatous inflammation in livers of both sexes and reduction in minimal microsporidiosis in males at 17 and 130 µg a.s./L ⁵ Increased prevalence of individual cell necrosis/apoptosis (minimal to mild) in females at the 17 and 130 µg/L treatments (5/16 individuals at 17 and 130 µg a.s./L compared to 2/15 individuals in the control and 1/16 individuals at 1.3 µg a.s./L).	Increased hepatocyte necrosis/apoptosis is prominently indicative of chronic cytotoxicity as opposed to hormonal perturbation (can be induced by a variety of mechanisms such as oxidative stress)	Some possible signs of minimal toxicity/cellular stress on adults from 17 µg a.s./L based on liver histopathology.	-
			Survival	22; ELS	<i>Pp</i>	32	150	Decrease. Post hatch larval survival significantly reduced at 150 and 380 µg a.s./L		Post-hatch larval survival affected from 150 µg a.s./L.
		23; ELS		<i>Cv</i>	34	350	Decrease. Post hatch larval survival significantly reduced at 350 µg a.s./L.	Adult survival unaffected.	-	
		25; FSTRA		<i>Pp</i>	21	>130	No effect on adult survival at any test concentration		-	

Pp: *Pimephales promelas* (fathead minnow), *Cv*: *Cyprindon variegatus* (sheepshead minnow), *Ol*: *Oryzias latipes* LOED: Lowest Effect Dose, E = Estrogen, A = Androgen, S = Steroidogenesis, N = Not assignable to a specific modality, - = not applicable, stat sig = Statistically Significant

¹ Route of exposure for all parameters is uptake from water

² Oocyte atresia: 8/16, 4/16, 9/16 affected individuals at 1.3, 17 and 130 µg a.s./L respectively compared to 2/15 in the control; of those, 4/16 at 130 µg a.s./L had score of 'severe' which is the only test concentration resulting in this score. Further details in Table 9.2.3.1-5 and full details in FSTRA study summary section B.9.2.3.3.

³ No statistical testing performed on this parameter

⁴ Note that although the 14 % reduction in hatching success at 350 µg a.s./L was not statistically significant, due to the EC₁₀ for this parameter being determined as less than this at 340 µg a.s./L, and the observed delay in time-to-hatch at 350 µg a.s./L, then the LOED for hatching success has been conservatively set at 350 µg a.s./L as opposed to >350 µg a.s./L.

⁵ Male granulomatous inflammation: 3/7, 0/8, 1/7 affected individuals at 1.3, 17 and 130 µg a.s./L respectively compared to 3/8 in the control; male microsporidiosis: 3/7, 1/8, 0/7 affected individuals at 1.3, 17 and 130 µg a.s./L respectively compared to 3/8 in the control; female granulomatous inflammation 1/16, 3/16, 0/16 affected individuals at 1.3, 17 and 130 µg a.s./L respectively compared to 4/15 in the control;

B.9.2.3.1.6. Summary of HSE ecotoxicology consideration for aquatic organisms (EAS modalities):

The consideration has been carried out according to the EFSA/ECHA guidance document (2018) based on the available data summarised in Table 9.2.3.1-6 above.

Mechanistic endocrine activity of the EAS modalities has been sufficiently investigated through the FSTRA study. VTG results for males were inconclusive due to wide variation of the data, but there were no other indications of EAS-mediated adverse effects based on the other parameters measured, which supports the conclusion of no endocrine activity. For females, only the middling test concentration (0.017 mg a.s./L) had a significant decrease in VTG levels; although all treatments were reduced relative to the control, this results in uncertainty when interpreting the results from this assay, this was not dose responsive and therefore is not indicative of endocrine activity according to EFSA/ECHA guidance (2018).

Adversity based on EAS-mediated parameters has been sufficiently investigated through the FSTRA study which monitored Secondary Sex Characteristics (SSCs), gonad histopathology, ovarian stage scores and testicular stage scores. Evidence for parameters sensitive to, but not diagnostic of, EATS modalities, was also monitored in the FSTRA and two further ELS studies, namely: behaviour, body weight and length, reproduction and morphological abnormalities.

The 1-day delay in time-to-hatch of larvae was observed in both ELS studies, however, this was at the highest tested concentrations of 0.38 mg a.s./L and 0.35 mg a.s./L (for *P. promelas* and *C. variegatus*, respectively) where there was systemic toxicity demonstrated to larvae with 0 % post-hatch survival for both species tested and significant reductions in embryo hatching success (6 % success) and live, normal larvae at hatch (0 % live, normal) for *P. promelas*. Therefore, toxicity is the likely mechanism for these observations rather than EAS modalities.

For EAS-mediated effects in the FSTRA study, some adversity was observed in gonad histopathology, notably severe oocyte atresia at the highest test concentration (0.13 mg a.s./L) which correlates with a significant reduction in egg production (fecundity) at this treatment level. However, increased cell necrosis/apoptosis in the female liver histopathology in the highest two treatment levels indicates there may be some general toxicity co-occurring, which is a confounding factor according to the EFSA/ECHA guidance.

Regarding toxicity, there were no changes in fish survival during the FSTRA study, and HSE agrees with the study author statement regarding survival that: “These results, coupled with a lack of abnormal appearance or behaviour related to pydiflumetofen exposure, demonstrate that overt toxicity was not considered to be a mechanism of pydiflumetofen exposure at the concentrations in this assay” (██████, 2020a). Nevertheless, the liver histopathology is potentially indicative of some mild toxicity, which would cause a general stress response in the fish and could affect the gonad histopathology and fecundity. This observation of liver toxicity is consistent with the mammalian toxicology assessment (volume B6) which found that liver toxicity is present in mammals. Furthermore, in the ELS study, reduced post hatch survival and decreased body weight and length in surviving fry of fathead minnow (*P. promelas*), which is the same species as tested in the FSTRA, occurs at a LOED of 0.15 mg a.s./L. This LOED is a similar concentration to the highest tested dose of 0.13 mg a.s./L in the FSTRA, and therefore, although there is no effect on adult survival in the FSTRA study, based on the ELS observations of *P. promelas*, it is plausible that the adult fish are experiencing cellular stress from low level toxicity effects at this test concentration. Therefore, the weight of evidence does not suggest that the observations in EAS mediated parameters of the FSTRA study are due to hormonal changes.

The applicant consideration of the FSTRA study is provided below for reference:

“There were no effects of 21-day exposure to pydiflumetofen on in vivo mechanistic or EAS-mediated parameters in this study.

Parameters affected at the top test concentration (130 µg/L) in females (fecundity, increased oocyte atresia and hepatocyte apoptosis/necrosis) are sensitive to non-endocrine-mediated toxicities, and these effects are therefore likely to be inter-related responses to non-endocrine toxicity of the test substance, e.g. cytotoxicity. Increased oocyte atresia in females exposed to the top the test concentration corroborates the adverse apical response (decreased fecundity) at this concentration.

Plasma VTG concentrations were depressed in females at all three treatment levels, while this difference was only statistically significant at the middle level. Considering the absence of a consistent

concentration response relationship and the histopathological findings, these decreases in female plasma VTG concentrations likely reflect liver and ovarian damage resulting from direct cytotoxicity.

Based on the weight-of-evidence from complimentary endpoints in this study, there is no indication of endocrine activity of pydiflumetofen from this study.”

Considering the definition of a substance having endocrine disrupting properties in the EFSA/ECHA guidance, where all three parts of adversity in EAS-mediated parameters, EAS mechanistic activity, and a mode of action to link the two should be demonstrated, then HSE considers that based on the results from the FSTRA, it is uncertain as to whether the observed effects are endocrine-mediated or due to systemic toxicity. As such a **Rapid Androgen Disruption Activity Reporter (RADAR) assay was requested**, which the applicant is currently in the process of conducting and hence it is not possible to conclude on the EAS modalities at present. that there are no clear treatment related effects of pydiflumetofen on endocrine activity at the highest concentration tested in the FSTRA study (0.13 mg a.s./L) based on evidence from the submitted FSTRA study, and taking into consideration relevant observations from the two ELS studies.

The applicant conducted a RADAR assay and provided the draft study report to HSE. Results of the study demonstrated no statistically significant increases or decreases in fluorescence in spiked mode, indicating that pydiflumetofen is inactive in the RADAR assay. However, in the unspiked mode, a statistically significant decrease in fluorescence was observed relative to the solvent control when considering the pooled results from the 1.3 µg a.s./L test concentration. Decreases in fluorescence were observed for all 3 runs of the test at this concentration, noting that this was not statistically determined. As such, this gives some uncertainty to the interpretation of the results regarding whether the concentration range was appropriate or if the RADAR assay was the most appropriate androgen axis test for this test substance when following the decision logic in OECD 251, noting the reduction in fluorescence only applies to the lowest tested concentration.

Considering both the results from the RADAR assay and FSTRA together, overall HSE considers that EAS modalities have been sufficiently investigated. There is uncertainty regarding the lowest RADAR assay test concentration due to the significant decrease in fluorescence in the unspiked mode, however in the FSTRA, other than an increase in moderate oocyte atresia compared to the control at the same concentration, there were no clear endocrine mediated effects. No other significant effects were observed at the other tested concentrations in the RADAR assay in unspiked mode, suggesting the concentration range selected was broadly appropriate. As there were no statistically significant increases or decreases in fluorescence in spiked mode at any of the concentrations tested, this indicates that pydiflumetofen is inactive in the RADAR assay. The significant decrease in female VTG in the FSTRA at a concentration of 17 µg a.s./L does not correspond to any significant effects at a similar concentration in the RADAR assay, suggesting the observed decrease is not endocrine-mediated. Whilst there remains some uncertainty with both the results of the FSTRA and the RADAR assay, taken together HSE considers the results support a negative conclusion for EAS modalities. In addition, the conclusion reached for toxicology (noting uncertainty in ‘read across’ between vertebrates) was that based on the overall weight of evidence, pydiflumetofen does not cause EAS-mediated adversity and that this modality has been sufficiently investigated (see in the volume 3, CA section 6 dossier part II (B.6.8.3), adding further support to the overall conclusion that there is lack of adversity for EAS modalities.

B.9.2.3.1.7. Overall HSE ecotoxicology conclusion for EAS modality (aquatic organisms):

Overall, HSE considers that insufficient information has been submitted when considering EAS modalities and aquatic organisms. In accordance with current guidance (EFSA/ECHA, 2018)⁵ HSE concludes that further information is required and it has been requested that a RADAR assay is conducted by the applicant to provide further mechanistic data on this point. pydiflumetofen does not meet the endocrine disruption criteria for EAS modalities in aquatic organisms.

Overall, HSE considers that EAS modalities for aquatic organisms have been sufficiently investigated. Whilst there are some uncertainties with the results from the RADAR assay and FSTRA, the information taken together supports a negative conclusion for EAS modalities in aquatic organisms.

⁵ [Guidance for the identification of endocrine disruptors in the context of Regulations \(EU\) No 528/2012 and \(EC\) No 1107/2009 | EFSA \(europa.eu\)](#)

B.9.2.3.2. Consideration of T modality (aquatic organisms)

B.9.2.3.2.1. AMA Study

An Amphibian Metamorphosis Assay (AMA) was submitted and considered valid (██████, 2020). There were 4 replicates per treatment group, and the GLP study was conducted following OECD 231 (2009) - a flow through study of 21-days duration.

The endpoints evaluated during this AMA screening assay with pydiflumetofen and the African clawed frog (*Xenopus laevis*) were mortality, behaviour, gross morphology, sub-lethal observations, developmental stage, snout-vent length (SVL), hind limb length, hind limb length (normalized by SVL), whole body wet weight, and liver and thyroid histopathology.

A more detailed discussion of this study is provided at the end of the study summary later on in section B.9.2.3. The key points from this study have been considered below.

Analytical verification: Nominal test concentrations of 32, 100, and 320 µg a.s. /L were used for the test. Mean measured concentrations of the test substance were not maintained within 20 % of the nominal values (actual range was 71 – 92 %). Therefore, the results of the study were based on mean measured concentrations.

Maximum tolerated concentration (MTC): The MTC selected for the AMA study was 320 µg a.s./L. This concentration was chosen as during the preliminary exposure, approximately 50 % of the organisms at the 320 µg/L nominal treatment level briefly exhibited loss of equilibrium (i.e., for less than 24 hours). A certain level of uncertainty surrounds this MTC, as the maximum tolerated concentration (MTC) is defined in the OECD 231 (2009) guidelines as: *'The highest test concentration of the chemical which results in less than 10 % acute mortality.'* However, it is also stated that *'Using this approach assumes that there are existing empirical acute mortality data from which the MTC can be estimated.'* No tadpole mortality was observed in the preliminary exposure up to and including 1,000 µg a.s./L.

Another option proposed in the OECD 231 (2009) guidelines is the use of existing acute toxicity data to estimate an MTC: *'A useful approximation of the MTC can be derived from existing acute data by using 1/3 of the acute LC₅₀ value. However, acute toxicity data may be lacking for the species on test. If species specific acute toxicity data are not available, then a 96-hour LC₅₀ test can be completed with tadpoles that are representative (i.e., same stage) of those on test in the AMA.'* Additionally, *'if data from other aquatic species are available (e.g. LC₅₀ studies in fish or other amphibian species), then professional judgement may be used to estimate a likely MTC based on inter-species extrapolation.'* Toxicity data was available from both the accompanying FSTRA study, the fish acute toxicity studies, and early life stage (ELS) studies. Noting that whilst there is uncertainty when extrapolating between species, effects were seen in the fish acute toxicity and ELS studies at concentrations around the MTC chosen for use in the AMA study. However, the MTC selected for the current AMA study was more than double the selected MTC for the FSTRA study (130 µg a.s./L).

None of the suggested methods of MTC selection from OECD 231 (2009) were used, and no mention is made in the guidelines of the applicant's approach to MTC selection. The MTC for this study was chosen based upon the results from the preliminary exposure. The applicant states that the MTC of 320 µg a.s./L was selected as approximately 50% of the organisms at this treatment level briefly exhibited a loss of equilibrium (i.e. for less than 24 hours). It is not clear on what basis the applicant has chosen the threshold of 50 % immobilisation for MTC selection. This casts doubt on the suitability of the selected MTC, which is further compounded by the lack of any immobilisation or other behavioural observations in the highest tested concentration (MTC) condition in the definitive exposure.

There was, however, a statistically significant decline in mean whole body wet weight at 21 days in the 23 µg a.s./L and 300 µg a.s./L (mean measured concentrations) conditions ($p = 0.049$, and $p = 0.02$, respectively) (See Table 9.2.4-6 below). Whole body wet weight is considered sensitive to, but not diagnostic of, EATS modality for the amphibian metamorphosis assay, hence may have been due to general toxicity.

Overall, HSE concludes that the MTC for the AMA study has not been properly selected in line with any of the processes outlined in the MTC selection criteria from the OECD 231 (2009) guidelines. However, when considering the data from the FSTRA study and the other available fish toxicity studies, the selected MTC appears to be reasonable. HSE therefore does not consider the uncertainty surrounding the MTC selection process sufficient to invalidate the study.

Results

The results from this 21-day flow-through GLP AMA study are summarised below.

Table 9.2.3.2-1: Survival, growth and development of *Xenopus laevis* tadpoles after flow-through exposure to pydiflumetofen for 7 days

Nominal test concentration (µg a.s./L)	Mean measured test concentration (µg a.s./L)	Survival to day 7 ± SD (%)	Median developmental stage on day 7 (range)	Mean whole body wet weight ± SD (g)	Mean SVL ± SD (mm)	Mean hind limb length (normalised for SVL*) ± SD (mm)
Negative control	Negative control	100 ± 0.0	53 (53 – 54)	0.4299 ± 0.0466	17.86 ± 0.70	0.113 ± 0.005
32	23	100 ± 0.0	53 (53 – 54)	0.4252 ± 0.0898	17.92 ± 1.31	0.109 ± 0.003
100	90	100 ± 0.0	54 (53 – 54)	0.3897 ± 0.0322	17.39 ± 0.57	0.116 ± 0.002
320	300	100 ± 0.0	54 (54)**	0.3937 ± 0.0299	17.40 ± 0.46	0.112 ± 0.006

SD: standard deviation

*Normalised by dividing mean hind limb length by SVL

**Jonckheere-Terpstra's Step-Down Test determined a significant increase at day 7 (developmental stage) in highest concentration (300 µg a.s./L) compared to the control. The day 7 developmental stage distribution profile was analysed by applying the multi-quantal Jonckheere-Terpstra's Step-Down Test. The overall multi-quantal procedure determined no significant increase in day 7 percentile developmental stage at 300 µg a.s./L. It should be noted the multi-quantal analysis is the preferred statistical method for assessing developmental stage in the appropriate guidance (OECD, 2009 and U.S. EPA, 2009). HSE has also considered the raw data in terms of range and agrees there is no clear treatment related effect for development stage.

Table 9.2.3.2-2: Survival, growth and development of *Xenopus laevis* tadpoles after flow-through exposure to pydiflumetofen for 21 days

Nominal test concentration (µg a.s./L)	Mean measured test concentration (µg a.s./L)	Survival to day 21 ± SD (%)	Median developmental stage on day 21 (range)	Mean whole body wet weight ± SD (g)	Mean SVL ± SD (mm)	Mean hind limb length (normalised for SVL ^a) ± SD (mm)
Negative control	Negative control	100 ± 0.0	58 (57 – 59.8)	1.3796 ± 0.0654	25.50 ± 0.20	0.473 ± 0.026
32	23	100 ± 0.0	58 (56 – 59.4)	1.2132 ± 0.1154*	24.56 ± 0.61	0.447 ± 0.037
100	90	100 ± 0.0	58 (56.6 – 59.4)	1.3072 ± 0.0615	25.49 ± 0.53	0.432 ± 0.047
320	300	100 ± 0.0	58 (56 – 59)	1.1827 ± 0.0939**	24.70 ± 0.78	0.412 ± 0.035

SD: standard deviation

^a Normalised by dividing mean hind limb length by SVL

* Statistically significant reduction compared to the control (p = 0.049)

** Statistically significant reduction compared to the control (p = 0.02)

For the purposes of this assessment, based on the EFSA/ECHA 'Guidance for the identification of endocrine disruptors in the context of regulations (EU) No 528.2012 and (EC) No 1107.2009', the tested parameters from the AMA study have been divided into 'endpoints indicative of thyroid-mediated modality', and 'endpoints sensitive to, but not diagnostic of, the thyroid modality'.

Endpoints indicative of thyroid-mediated modality

Developmental stage: The median day 7 developmental stage for tadpoles in the control, 23, 90, and 300 µg /L treatment levels was 53, 53, 54, and 54, respectively. Jonckheere-Terpstra's Step-Down Test determined a significant increase in day 7 developmental stage among tadpoles exposed to the 300 µg /L treatment level compared to the control. The day 7 developmental stage distribution profile was analysed by applying the multi-quantal Jonckheere-Terpstra's Step-Down Test. The multi-quantal procedure determined no significant increase in day 7 percentile developmental stage at 300 µg/L. It should be noted the multi-quantal analysis is the preferred statistical method for assessing developmental stage in the appropriate guidance (OECD, 2009 and U.S. EPA, 2009). HSE has also considered the raw data in terms of range and agrees there is no clear treatment related effect for development stage. There was no significant reduction in developmental stage compared to the control at any treatment level, when assessed at 21 days.

Hindlimb length: There were no statistically significant differences in normalized hind-limb lengths on day 7 or day 21, in any treatment group in comparison to the control according to Dunnett's Multiple Comparison Test ($C \neq T$). There was a general trend observed for decreasing hind limb length in line with increasing test concentration at day 21, however, this was not statistically significant.

Thyroid gland histopathology: There were no clear treatment-related histopathologic findings involving the thyroid in this study. A proportion of control frogs exhibited baseline levels of thyroid follicular cell hypertrophy (mild) and/or follicular cell hyperplasia (mild), but the prevalence and severity of these findings in pydiflumetofen-treated frogs were generally comparable to those of the negative controls at highest tested concentration. There was a slight increase in follicular cell hyperplasia in the 23 µg /L condition, with one tadpole being graded as 'moderate', however, there was no clear dose-response relationship. The absence of treatment-related effects in the thyroid gland is consistent with the lack of treatment-related effects on developmental stage across the treatment levels. Thyroid histopathology results are displayed below in Table 9.2.3.2-3

Table 9.2.3.2-3: Prevalence and severity of thyroid histopathologic findings

Mean Measured Concentration (µg /L)			0.0 (Control)	23	90	300
Number of Tadpoles Examined			20	20	20	20
Thyroid	Follicular cell hyperplasia	Mild	6	4	8	6
		Moderate	-	1	-	-
		Severe	-	-	-	-
		Total	6	5	8	6
	Follicular cell hypertrophy	Mild	17	15	15	18
		Moderate	1	4	5	2
		Severe	-	-	-	-
		Total	18	19	20	20

Endpoints sensitive to, but not diagnostic of, the thyroid modality

Whole-body wet weight: Dunnett's Multiple Comparison Test ($C \neq T$) determined no significant reduction in day 7 whole body wet weight among tadpoles exposed to any of the treatment levels tested compared to the control. However, Dunnett's Multiple Comparison Test ($C \neq T$) determined a significant reduction in day 21 whole body wet weight among tadpoles exposed to the 23 and 300 µg /L treatment levels compared to the control ($p = 0.049$ and $p = 0.02$, respectively). Considering that there was no effect at 100 µg /L, and no effects on other parameters, the difference at 23 µg /L is unlikely to be treatment-related. Reductions in WBW at the 300 µg /L treatment level were likely to result from systemic toxicity, considering the liver histopathology findings at this treatment level and the behavioural symptoms of toxicity observed in the range-finder, and indicate that this treatment level approached the maximum tolerable concentration for pydiflumetofen.

Snout-vent length: There were no statistically significant differences in snout-to-vent lengths on day 7 or day 21 in any treatment group in comparison to the control according to Dunnett's Multiple Comparison Test ($C \neq T$) ($p < 0.05$).

Malformations (Spinal deformities): On day 7, spinal deformities (i.e. scoliosis, bent tail) were observed in 15 % of control animals, and in 20 %, 10 %, and 5 % of tadpoles exposed to the 23, 90, and 300 µg /L test conditions, respectively. On day 21, spinal deformities were observed in 56 % of control animals, and in 50 %, 43 %, and 38 % of tadpoles exposed to the 23, 90, and 300 µg /L nominal treatment levels, respectively. For the entire exposure spinal deformities were observed for 45 % of control animals and for 43, 35, and 30 % of tadpoles exposed to 23, 90, and 300 µg /L nominal treatment levels, respectively. The study authors have stated that the spinal deformities did not impact any endpoint collected for this assay or growth/survival of tadpoles and was not attributable to pydiflumetofen exposure. This is considered further in the 'Applicant response to CRD request for additional information' section below.

Mortality & behaviour: No tadpole mortality was observed in the control condition, or in any of the treatment groups, at test termination on day 21. Tadpoles in all treatment levels and the control condition exhibited normal behaviour throughout the exposure period. No tadpoles showed any abnormal behaviour, such as floating on the surface, lying on the bottom of the aquarium, inverted or irregular swimming, lack of surfacing activity, or non-responsiveness. No noticeable difference in food consumption between treatments was noticed. Also, no gross malformations or lesions were observed.

Liver histopathology: Liver histopathology revealed a relatively low-grade reduction in hepatocellular vacuolation (glycogen incorporation) in eight tadpoles at the 300 µg /L treatment level. Severity at this treatment level was graded as mild (grade 1) in six tadpoles and moderate (grade 2) in two tadpoles (See Table 9.2.3.2-4 below). The prevalence of this finding was also increased slightly in frogs of the 23 µg /L group, but this difference was less substantial and thus less likely to be treatment related. The relatively low-grade decrease in hepatocellular vacuolation in tadpoles of the 300 µg /L dose group is consistent with diminished hepatic glycogen/lipid storage (U.S. EPA, 2015). This non-specific finding suggests that the energy intake in those frogs was insufficient relative to physiological requirements for growth and activity.

Table 9.2.3.2-4: Prevalence and severity of liver histopathologic findings

Mean Measured Concentration (µg /L)			0.0 (Control)	23	90	300
Number of Tadpoles Examined			20	20	20	20
Liver	Hepatocellular vacuolation, decreased	Mild	2	6	2	6
		Moderate	-	-	-	2
		Severe	-	-	-	-
		Total	2	6	2	8*

* Findings were significantly different from the control

Performance and validity criteria

All the relevant validity criteria outlined in OECD 231 (2009) have been met. All performance criteria except for one were met. The performance criterion which was not met was as follows:

- pH should be maintained between 6.5 - 8.5. The inter-replicate/inter-treatment differentials should not exceed 0.5.

Inter-replicate differentials slightly exceeded 0.5 pH units on four separate days during the test. As these fluctuations were transient, and the performance of the control animals was within expectations, these deviations are not likely to have impacted the results or interpretation of the study. There is some uncertainty, as the raw pH data was not reported. However, HSE does not consider this deviation alone sufficient to invalidate this vertebrate study.

B.9.2.3.2.2. T summary of parameters

A summary of the results from the Amphibian Metamorphosis Assay (AMA) (on *Xenopus laevis*) has been provided in the table below. The format is in accordance with EFSA/ECHA guidance i.e. appendix E and considers the information provided by the applicant (██████ *et al.* 2020 and ██████ *et al.* 2020a). The results summarised are for the T modality.

Table 9.2.3.2-5: Lines of evidence for T modality of pydiflumetofen (aquatic organisms)

Effect	Effect target	Study ID	Duration of exposure	Exposure route	Dose based on tested concentrations*	Effect direction	Observed effect (positive and negative)	Assessment on the integrated line of evidence	Modality
EATS-mediated	Developmental stage	24	21	Uptake from water	300 µg a.s./L (m.m)	No effect	No evidence of asynchronous development at any tested dosed.	No indication of T-mediated endocrine activity.	T
	Developmental stage	24	7 days	Uptake from water	300 µg a.s./L (m.m)	No effect	No statistically significant effects based on recommended OECD 239 guideline approach i.e. multi-quantal analysis. Raw data did not indicate effects either as treatment and control ranges were comparable.		T

Effect	Effect target	Study ID	Duration of exposure	Exposure route	Dose based on tested concentrations*	Effect direction	Observed effect (positive and negative)	Assessment on the integrated line of evidence	Modality
	Hind limb length	24	7 days	Uptake from water	300 µg a.s./L (m.m.)	No effect	No statistically significant difference in hind-limb length at day 7 in any treatment group compared to the control.	No indication of T-mediated endocrine activity.	T
	Hind limb length	24	21 days	Uptake from water	300 µg a.s./L (m.m.)	No effect	There was a general trend observed for decreasing hind limb length in line with increasing test concentration at day 21, however, this was not statistically significant in any treatment group and there was no clear dose response or supporting histological observations to suggest thyroid activity.		T
	Hind limb length normalised by snout-vent length	24	7 days	Uptake from water	300 µg a.s./L (m.m.)	No effect	No significant effect on day 7 normalised HLL at any tested concentration.		T

Effect	Effect target	Study ID	Duration of exposure	Exposure route	Dose based on tested concentrations*	Effect direction	Observed effect (positive and negative)	Assessment on the integrated line of evidence	Modality
	Hind limb length normalised by snout-vent length	24	21 days	Uptake from water	300 µg a.s./L (m.m.)	No effect	There were no statistically significant effects on normalised HLL at d21 at any tested concentration, however, there was a general trend observed for decreasing normalised hind limb length in line with increasing test concentration.		T
	Thyroid histopathology (amphibian)	24	21 days	Uptake from water	300 µg a.s./L (m.m.)	No effect	No treatment-related histopathologic findings involving the thyroid.		T
Sensitive to, but not diagnostic of, EATS	Body weight (amphibian)	24	7 days	Uptake from water	300 µg a.s./L (m.m.)	No effect	No statistically significant difference in whole body wet weight on day 7, in any treatment group compared to the control.		N
	Body weight (amphibian)	24	21 days	Uptake from water	90 µg a.s./L (m.m.)	Decrease	Significant reduction in whole body wet weight for tadpoles exposed to the 23, and 300 µg /L treatment levels compared to the control.	Effect at 23 ug/L not regarded as treatment-related; effect at 300 ug/L potentially indicative of systemic toxicity.	N
	Snout-vent length/growth	24	7 days	Uptake from water	300 µg a.s./L (m.m.)	No effect	No statistically significant difference in the snout-to-vent length in any treatment group compared to the control, at day 7.		N
	Snout-vent length/growth	24	21 days	Uptake from water	300 µg a.s./L (m.m.)	No effect	No statistically significant difference in the snout-to-vent length in any treatment group compared to the control, at day 21.		N
	Malformations	24	21 days	Uptake from water	300 µg a.s./L (m.m.)	No effect	There was a high incidence of spinal deformity in all test conditions.		N

Effect	Effect target	Study ID	Duration of exposure	Exposure route	Dose based on tested concentrations*	Effect direction	Observed effect (positive and negative)	Assessment on the integrated line of evidence	Modality
	Mortality	24	21 days	Uptake from water	300 µg a.s./L (m.m.)	No effect	No statistically significant reduction in day 21 larval survival among tadpoles exposed to any of the treatment levels tested compared to the control.		N
	Behaviour	24	21 days	Uptake from water	300 µg a.s./L (m.m.)	No effect	No statistically significant or treatment-related effects on behaviour in any treatment group compared to the control at test termination		N

*This column displays the highest tested dose at which there were no observable effects.

(m.m.) = Mean measured concentrations; T = Thyroid; N = Not assignable to a specific modality

Applicant response to CRD request for additional information

HSE had noted the very high level of spinal deformities in the control and treatment groups in the provided AMA study. This was highest in the control (exposure total: 45%), followed by the 23 µg /L treatment group (exposure total 43 %), then the 90 µg /L nominal treatment group (exposure total 35 %), and then the 300 µg /L nominal treatment group (exposure total 30 %). It is acknowledged that these results do not suggest overt toxicity, in addition, there is no clear dose-response relationship present, and as such, the incidence of spinal deformities do not appear to be treatment related. However, the wide range and high general occurrence demonstrate the limited sensitivity of this parameter, casting doubt on its suitability as a measure of toxicity.

Additional consideration of this point was requested from the applicant in a request for additional information. The applicant response explained that *‘Scoliosis in larval Xenopus is an idiopathic phenomenon that is poorly understood among the scientific community that routinely culture this species. Genetic and nutritional aetiologies have been proposed [...] In all but the most severe manifestation the condition typically resolves through the process of metamorphosis.’* Additionally, the applicant highlighted that *‘the exposure window of the AMA coincides with the post-embryonic developmental period where key morphological changes are thyroid-regulated, rather than the embryogenesis period, where the fundamental elements of the body plan are formed along with major organs. It may therefore be considered unlikely that gross morphological deformities resulting from disruption of these processes would be manifest in the AMA as a response to general toxicity.’*

It was also pointed out by the applicant that *‘There is no evidence in the scientific literature (e.g. Coady et al., 2014) that this phenomenon [scoliosis] can be induced by overt (systemic) toxicity of an exogenous chemical.’* Nevertheless, it is important that it is reported, as *‘the potential for scoliosis to confound detection or interpretation of responses of core parameters to the test material should be considered. Specifically, in severe cases, scoliosis may interfere with measurement of snout-vent length (it makes it very hard to position the larvae appropriately to see the vent), which may then impact the derived parameter of hind-limb length normalised by snout-vent length. The reporting of this phenomenon contributes to a general assessment of whether the biological needs of the test organism have been met during the test, in the same way as the median stage of controls at test termination. Incidence of scoliosis is not, therefore, an indicator of toxicity per se, and the variability of its incidence alone should not be considered an indicator of test validity.’*

HSE accepts the applicant’s explanation of the high levels of scoliosis among the test organisms, and this point would not provide reason for the study to be repeated.

Justification for the reduction in feeding rates used in this test, compared to the feeding rates recommended by OECD, was also requested from the applicant. The applicant had initially stated that the reduced feeding rate was based on extensive experience with performing this study type, however, without further data or evidence to support this claim, it was not possible to rule out the contribution of the reduced feeding rate to the effects observed. In response, the applicant has stated that this reduced feeding rate has become common practice among contract research laboratories performing this test, who observe consistent benefits in doing so. The applicant cited Tobor-Kaplan (2020), which had demonstrated that reduced feeding provides the following benefits:

- Reduced build-up of waste
- Reduced microbial growth
- Better maintenance of dissolved oxygen concentrations
- Better maintenance of intended test concentrations
- Slower growth resulting in fewer ‘late-stage’ larvae at test termination (high incidence of which makes statistical evaluation of growth parameters more complicated)

Tobor-Kaplan (2020) also reported that incidence of scoliosis was lower when feeding rate is reduced relative to the OECD 231 test guideline recommendation.

HSE accepts the applicant’s response, but some uncertainty remains as this is still a deviation from the guideline. However, consideration of the justification provided by the applicant, and the fact that all validity criteria were met means that the study can be considered acceptable. References provided by applicant are detailed below:

Coady KK, Lehman CM, Currie RJ, Marino TA. Challenges and approaches to conducting and interpreting the amphibian metamorphosis assay and the fish short-term reproduction assay. *Birth Defects Research Part B - Developmental and Reproductive Toxicology*. 2014;101(1):80-89. Syngenta File No. VV-940734

Lambert FM, Malinvaud D, Glaunes J, Bergot C, Straka H, Vidal P-P (2009) Vestibular asymmetry as the cause of idiopathic scoliosis: a possible answer from *Xenopus*. *The Journal of Neuroscience* **29**(40): 12477-12483 Syngenta File No. VV-940733

Tobor-Kaplon MA (2020) Endocrine Testing in Aquatic Vertebrates, 11th International Akademie Fresenius Conference "Endocrine Disruptors", 24 Nov - 25 Nov 2020, Online Conference, Die Akademie Fresenius GmbH

B.9.2.3.2.3. HSE ecotoxicology conclusion for aquatic organisms (T modality):

Overall, there were no clear treatment related effects on T-mediated parameters in the submitted AMA study up to the MTC. There was a slight effect on the whole body wet weight parameter, which is sensitive to but not diagnostic of thyroid-mediated effects. This is likely due to general toxicity, meaning that overall, the case is strong enough to conclude that there is no evidence of treatment-related changes in thyroid activity.

There was a change to the OECD 231 study design, in that the feeding rate was reduced by 50 % from that recommended in the guidance. Clarification on this issue was sought from the applicant (the response is discussed above in detail), and HSE considers this justification to be sufficient. Furthermore, there was uncertainty regarding MTC but this was not considered by HSE sufficient to invalidate the study.

As such, pydiflumetofen is considered not to have endocrine disruption properties **as regards the T-modality**, in accordance with EFSA/ECHA 2018 guidance based on available information.

B.9.2.3.3. Endocrine disruption studies testing on fish and amphibians

Report:	K-CA 8.2.3 [REDACTED], 2020, Pydiflumetofen-Amphibian Metamorphosis Assay with African Clawed Frog (<i>Xenopus laevis</i>), Report Number 1781.7310, [REDACTED] (formerly [REDACTED]) [REDACTED] (Syngenta File No. VV-858948)
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Guidelines

OECD Guideline for the Testing of Chemicals. No. 231. Amphibian Metamorphosis Assay. OECD, 2009.
Endocrine Disruptor Screening Program Test Guidelines OPPTS 890.1100 Amphibian Metamorphosis (Frog). EPA 740-C-09-002. October 2009.

GLP: Yes

Materials

Test Material	SYN545974 technical (Pydiflumetofen)
Lot/Batch #:	SMU2EP12007
Purity:	98.5 %
Description:	Off-white powder
Stability of test compound:	Stable when stored < 30 °C
Reanalysis/Expiry date:	30 April 2020

Treatments

Test concentrations:	Nominal: 32, 100, and 320 µg Pydiflumetofen /L Mean measured: 23, 90, and 300 µg Pydiflumetofen /L
Control:	Dilution water (laboratory well water)

Vehicle and/or positive control:	No positive control
Analysis of test concentrations:	Yes, prior to start of exposure and during exposure weekly; on test days: 0, 7, 14 and 21, under a full validated LC-MS/MS method (LOQ = 0.0500 µg /L)
Test animals	
Species:	Stage 51 (Nieuwkoop and Faber, 1994) <i>Xenopus laevis</i> tadpoles
Source:	Tadpoles originated from adult brood stock maintained at test facility (Brood stock was originally obtained from [REDACTED], and maintained as in-house breeders for at least 3 months prior to use for generating tadpoles). Prior to selection, all healthy tadpoles from the rearing vessel were pooled in a holding vessel containing dilution water, which was maintained at approximately 22 °C.
Acclimatisation period:	ca. 13 days
Treatment for disease:	None reported
Feeding:	During the pre-exposure and in-life exposure periods, tadpoles were fed [REDACTED] tadpole food ([REDACTED]), a commercially available diet that is suitable for normal growth. During the pre-exposure period tadpoles were fed twice per day at approximately 2.7 to 16 mg /Tadpole. For the in-life portion, tadpoles were fed twice per day at rates of 16 to 43 mg /Tadpole.
Test design	
Exposure regime:	Flow-through using an intermittent flow proportional diluter (Mount & Brungs, 1967).
Aeration:	None reported
Replication:	4
Test vessels:	10 L exposure aquaria measuring 30 x 14.5 x 20 cm with a 12.5 cm high-side drain that maintained a constant exposure solution volume of approximately 5.5 L
No of tadpoles per tank:	20 tadpoles per replicate
Duration:	21 days
Environmental conditions	
Test temperature:	21 to 22 ° C continuously monitored in the control replicate A. Daily individual vessel temperature monitoring established a range of 20 to 22 °C.
pH:	6.7 to 7.9 - Inter-replicate differentials slightly exceeded 0.5 pH units on four separate days during the test.
Dissolved oxygen:	5.9 to 8.8 mg /L (66 to 99 % of saturation)
Dilution/culture water source:	Dilution/culture water used in the experiment was a mixture of unadulterated on-site well water (taken from 100 m bedrock well), and de-chlorinated [REDACTED] well water (dechlorinated with UV light and activated carbon filtration). The two sources of water were passed individually through 1-µm polypropylene bag filters, a degasser, and were then mixed.
Hardness of dilution water:	60 to 80 mg /L as CaCO ₃
Alkalinity of dilution water:	20 to 22 mg /L
Conductivity:	460 to 650 µS /cm.

Iodide concentration:	5.8 µg /L (measured at exposure day 13)
Lighting:	12 hours light: 12 hours dark (610 to 1200 lux)

Study Design and Methods

Experimental dates: 30 May 2019 to 15 November 2019

The amphibian metamorphosis assay (AMA) for pydiflumetofen was performed with the African clawed frog (*Xenopus laevis*) under flow-through conditions. For this exposure, glass wool saturator columns were used to deliver pydiflumetofen to the exposure system, similar to those described in Kahl et al., 1999 and OECD 23 (2019). The glass columns were packed with glass wool, and then coated with the test substance. The columns were designed to provide a constant flow of saturated aqueous solutions (2800 µg /L) of pydiflumetofen to the diluter system without the use of a carrier solvent. Columns were constructed entirely of chemically inert materials (glass and Teflon). Once the column preparation was complete, these fittings attached the column to the water source (laboratory well water) and the appropriate delivery pump.

To coat each column, approximately 11 grams of pydiflumetofen was diluted with 50 mL of acetone (CAS No. 67-64-1). This solution was slowly poured into the glass column. After all of the solution was added, the column was attached to a vacuum pump. After visual inspection indicated that all of the wool was coated and all the solution was evaporated, each column was detached from the vacuum pump and attached to a FMI pump, which continuously passed dilution water through the column.

Prior to exposure initiation, two FMI pumps were each calibrated to continuously deliver approximately 12.4 mL /min of the 2800 µg /L saturator column solution to the mixing chamber of the diluter system at each cycle. The highest nominal test concentration (320 µg /L) was proportionally diluted by a constant factor of 3.15 to produce the remaining nominal test concentrations (i.e., 100 and 32 µg /L). The exposure system was operating properly for 6 days prior to exposure initiation to allow equilibration of the test substance in the diluter apparatus and exposure aquaria.

Once all tadpoles were at feeding stage, larvae from the highest quality spawn, based on the number of embryos and hatching success, were transferred to nine 10-L rearing tanks. The spawn selected for use in the exposure yielded approximately 2000 embryos, and the embryo survival rate from this spawn was estimated at > 90 %. One of the nine rearing tanks was designated as a replacement tank. These tadpoles were used as replacements in the event that mortalities occurred in the rearing tanks during the next week of rearing. During this pre-exposure period, tadpoles were maintained under flow-through conditions similar to that of the actual exposure. On pre-exposure day 13 (day 13 post-fertilization), the majority of the tadpoles had reached stage 51, this was confirmed with the use of a binocular dissection microscope. Tadpoles were assessed and were randomly distributed to the test vessels in groups of five until each test vessel contained 20 tadpoles. The aquaria were impartially positioned in pairs within the exposure system, with two pairs per test treatment.

Tadpoles were exposed to nominal concentrations of 32, 100, and 320 µg Pydiflumetofen /L (mean measured concentrations of 23, 90 and 300 µg Pydiflumetofen /L), and a dilution water control. The highest nominal test concentration was set as the maximum tolerated concentration (320 µg /L). This concentration was chosen, as approximately 50% of the organisms at the 320 µg /L nominal treatment level briefly exhibited loss of equilibrium (i.e., for less than 24 hours) during the preliminary exposure. Based on the transient observation of overt toxicity, and in consultation with the Study Sponsor, the maximum tolerable concentration (MTC) was determined to be 320 µg /L.

The concentrations of pydiflumetofen in test solutions were measured in one replicate of each treatment at 0, 7, 14 and 21 days using an LC-MS/MS method. Dissolved oxygen concentration, pH, and temperature measurements were taken in all vessels on day 0 and in one replicate of each concentration and control each day thereafter; replicates were sequentially alternated each day (A, then B, then C, etc.). Total hardness, total alkalinity, and conductivity were measured in replicate A of the control, low, and high test concentrations on day 0 and in sequentially alternating replicates weekly thereafter. Test solution temperature was continuously monitored in replicate A of the control. Continuous monitoring of the control established a temperature range of 21 to 22 °C throughout the exposure period. A representative sample of dilution water was analysed for iodide concentration, on D13 of the definitive exposure. Iodide concentration was measured to be 5.8 µg /L, within the guideline range. On Day 7, five tadpoles were randomly selected from each test vessel and euthanized for growth metrics. Following euthanasia, each tadpole was submerged in Davidson's fixative for approximately 72 hours, and then rinsed with 70 % reagent grade ethanol, and stored under 10 % neutral buffered formalin. Digital images were

taken of each tadpole for snout-vent length and hind limb length measurements. Developmental stage (Nieuwkoop and Faber, 1994) was then determined for each tadpole using a binocular dissection microscope. Each tadpole was then blotted dry prior to body weight determination to the nearest 0.1 mg.

At test termination, the remaining tadpoles were removed from the test vessels, euthanized with buffered MS-222 and developmental stage was then determined, and each tadpole was weighed to the nearest 0.1 mg. Following these procedures, each tadpole was transferred to a container filled with of Davidson's fixative. Tadpoles remained submerged for approximately 96 hours, and then they were rinsed with 70 % reagent grade ethanol and stored in 10 % natural buffered formalin.

Where possible, individual larvae selected for histological analysis were matched to the median developmental stage of the control. If five stage 58 tadpoles were not available for a replicate, then tadpoles at the next stage down (stage 57), followed by the next stage up (stage 59) were impartially selected for analysis until a total of five tadpoles were selected.

Endpoints and Observations

Development Stage: Developmental stage was determined at days 7 and 21 using the staging criteria of Nieuwkoop and Faber (1994). Development stage data were used to determine if development was accelerated, asynchronous, delayed or unaffected.

Snout-Vent Length: Snout-vent length was determined at days 7 and 21. Snout-vent length was measured for each individual tadpole to the nearest 0.01 mm. For consistency, the cranial aspect of the vent was used as the caudal limit of the measurement.

Hind Limb Length: Hind limb length was determined at days 7 and 21. For consistency, the left hind limb was used for this measurement. On day 7, hind limb measurement is straight forward. On day 21, measurements of hind limb length originated at the body wall and followed the midline of the limb through any angular deviations. Hind limb length is expressed as the total length and as a normalised value. Hind limb length normalised by snout-vent length, was calculated by taking the ratio of the hind limb length to snout-vent of each individual.

Whole Body Wet Weight: Whole body wet weight was determined at days 7 and 21. Body weights were measured on an analytical balance to the nearest 0.1 mg for each individual tadpole.

Thyroid Gland and Liver Histology: For histological analyses, a total of five tadpoles were randomly selected from each replicate test concentration on day 21. Where possible, individual larvae selected for histological analysis were matched to the median developmental stage of the control.

Additional Observations: 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, differences in food consumption, visible gross malformations or lesions were also recorded.

Statistical analysis: At the termination of the metamorphosis assay, data obtained on developmental stage, snout-vent length, hind limb length, and whole body wet weight were analysed to identify significant differences between test treatments and the dilution water control. All statistical conclusions were made at the 95 % level of confidence except in the case of the basic assumption tests, i.e., Shapiro-Wilks' Test (normal distribution) and Bartlett's Test (homogeneity of variance) in which the 99 % level of confidence was applied. Statistical analyses were conducted using the software programme CETIS (2019) version 1.9. The limit of quantification (LOQ) was set at 0.05 µg /L.

Results and Discussion

Validity Criteria

The validity criteria in the following table were used to determine whether the test was sufficient to assess thyroid activity. The table illustrates the criteria, the acceptable limits listed in the test guideline, and the performance of this exposure.

Criterion	Acceptable Limits	Study Performance	Criterion Met (Yes/No)
Treatment/Control Mortality	For any given treatment (including controls), mortality should not exceed 10 %. For any given replicate, mortality should not exceed three tadpoles	All replicate mortality < 10 % and ≤ 1 tadpole	Yes
Treatment levels analysed	At least two treatment levels, with four uncompromised replicates, will be used for analysis	All replicates uncompromised; no abnormal behaviour or gross malformations in any replicate	Yes
Test concentrations (non-control) without overt toxicity	≥ 2	No test concentrations had overt toxicity	Yes

Analytical data

Analysis of the exposure solutions during the pre-test period showed recoveries ranging from 71 to 110 % of nominal concentration for the treatment levels. 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.

Table 9.2.3.3-1: Pydiflumetofen - Concentrations Measured in the Exposure Solutions

Nominal Concentration (µg /L)	Mean Measured Concentration (µg /L)				Time-Weighted Average Concentration (SD) ^a	Percent of Nominal (%) ^a	% CV ^a
	Day 0	Day 7	Day 14	Day 21			
Control	< LOQ	< LOQ	< LOQ	< LOQ	NA	NA	NA
32	23	19	21	27	23 (3.0)	71	14
100	84	78	82	110	90 (15)	90	17
320	280	250	270	390	300 (58)	92	20

Concentrations expressed as less than values were below the limit of quantitation (LOQ) for the analytical method for this testing (i.e., 0.0500 µg /L) (NA = Not applicable)

^a Time-weighted average values, percent of nominal, standard deviations (SD), and coefficient of variance (CV) were calculated using the actual (unrounded) analytical results and not the rounded values (two significant figures) presented in this table.

NA = Not Applicable

A summary of the water quality measurements taken during the 21-day definitive exposure period are shown in Table 9.2.3.3-2 below. Values are presented as ranges.

Table 9.2.3.3-2: Summary of water quality measurements

Nominal Concentration (mg /L)	Ranges						
	Dissolved Oxygen ^a		Temperature ^{abc} (°C)	pH ^{ac}	Total Hardness ^d (mg /L as CaCO ₃)	Total Alkalinity ^d (mg /L as CaCO ₃)	Conductivity ^c (µS /cm)
	(mg /L)	(% of Saturation)					
Control	5.9 – 8.8	66 – 99	20.4 – 21.5	6.65 – 7.93	60 – 76	20	460 – 650
0.032	6.1 – 8.7	68 – 99	20.4 – 21.5	6.69 – 7.62	64 – 80	20 – 22	460 – 650
0.10	6.1 – 8.8	68 – 99	20.4 – 21.5	6.68 – 7.51	NA ^e	NA	NA

0.32	6.2 – 8.7	69 – 99	20.4 – 21.5	6.70 – 7.43	64 – 76	20 – 22	460 – 650
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^a N = 25

^b Continuous temperature monitoring of replicate A of the control established a temperature range of 21 to 22 °C throughout the exposure period, however no further data was presented in the study report.

^c Inter-replicate and inter-treatment temperature differentials slightly exceeded 0.5 °C on exposure day 16 and inter-replicate differentials slightly exceeded 0.5 pH units on four separate days during the test.

^d N = 4

^e NA = Not Applicable, measurements were not required as indicated by the protocol.

Histopathology results: Based on the preliminary exposure data and the indications of overt toxicity at the 320 µg /L treatment level in the definitive exposure (i.e., decrease in growth and remarkable liver histopathology effects), the highest concentration in the exposure was considered to have met the requirements for this assay.

There were no treatment-related histopathologic findings involving the thyroid. A proportion of control frogs exhibited baseline levels of thyroid follicular cell hypertrophy (mild) and/or follicular cell hyperplasia (mild), but the prevalence and severity of these findings in pydiflumetofen-treated frogs were generally comparable to those of the negative controls.

The number of frogs displaying histopathological symptoms are shown in Table 9.2.3.3-3 below, along with the severity grading schemes used in the thyroid histopathological assessments (Table 9.2.3.3-4). No grading scale for hepatocellular vacuolation was provided in the study report.

Table 9.2.3.3-3: Prevalence and severity of thyroid and liver histopathologic findings

Mean Measured Concentration (µg /L)			0.0 (Control)	23	90	300
Number of Tadpoles Examined			20	20	20	20
Thyroid	Follicular cell hyperplasia	Mild	6	4	8	6
		Moderate	-	1	-	-
		Severe	-	-	-	-
		Total	6	5	8	6
	Follicular cell hypertrophy	Mild	17	15	15	18
		Moderate	1	4	5	2
		Severe	-	-	-	-
		Total	18	19	20	20
Liver	Hepatocellular vacuolation, decreased	Mild	2	6	2	6
		Moderate	-	-	-	2
		Severe	-	-	-	-
		Total	2	6	2	8*

* Findings were significantly different from the control

Table 9.2.3.3-4: Severity grading schemes used for histopathological assessments

Symptom assessed	Grade	Descriptor	Criteria
Follicular cell hyperplasia	1	Mild	Focal or diffuse crowding of follicular cells affecting less than 20 % of the tissue.
	2	Moderate	60 – 80 % of the follicles exhibit focal hyperplasia characterised by pseudostratified or stratified follicular epithelium. Papillary infolding may be present.
	3	Severe	Over 80 % of follicles exhibit extensive hyperplasia with stratification 2 – 3 cell layers thick. Papillary infolding may be present.
Follicular cell hypertrophy	1	Mild	30 – 50 % of follicular cells exhibit hypertrophy.
	2	Moderate	60 - 80 % of follicular cells exhibit hypertrophy.
	3	Severe	Over 80 % of follicular cells exhibit hypertrophy.

Biological Observations

For the entire exposure (N = 20 per replicate), spinal deformities were observed for 45 % of control animals and for 43, 35, and 30 % of tadpoles exposed to the 32, 100, and 320 µg /L treatment levels, respectively. Table 9.2.3.3-5 below shows the average percentage of animals with spinal deformities per replicate for each treatment level, at each assessment point, and for the total exposure period.

Table 9.2.3.3-5: 21-day exposure of African clawed frog tadpoles to Pydiflumetofen – Percentage of animals displaying spinal deformities (curved spine)

Mean Measured Concentration (µg /L)	Replicate	Day 7 % Deformed ^{ab}	Day 21 % Deformed ^c	Exposure Total % Deformed ^d
Control	A	0	43	32
	B	20	67	55
	C	20	33	30
	D	20	80	65
	Mean	15	56	45
23 (Nominal: 32)	A	20	67	55
	B	20	27	25
	C	20	53	45
	D	20	53	45
	Mean	20	50	43
90 (Nominal: 100)	A	20	33	30
	B	0	33	25
	C	0	40	30
	D	20	67	55
	Mean	10	43	35
300 (Nominal: 320)	A	0	47	35
	B	20	27	25
	C	0	33	25
	D	0	47	35
	Mean	5	38	30

^a Based on tadpoles that were terminated on Day 7.

^b Calculated by dividing the number of tadpoles exhibiting spinal deformity by the total number of tadpoles terminated (i.e., x/5).

^c Calculated by dividing the number of tadpoles exhibiting spinal deformity by the total number of tadpoles terminated (i.e., x/15).

^d Calculated by dividing the number of tadpoles exhibiting spinal deformity by the total number of tadpoles terminated (i.e., x/20)

Following 21 days of exposure, the percent survival averaged 100 % for control animals. Also, tadpoles in all treatment levels and the control exhibited what was characterized by the study author as normal behaviour throughout the exposure period: tadpoles were suspended in the water column with the tail elevated above the head, regular rhythmic tail fin beating, periodic surfacing, operculating, and responsive to stimulus. No tadpoles showed any abnormal behaviour: i.e., floating on the surface, lying on the bottom of the aquarium, inverted or irregular swimming, lack of surfacing activity, or being non-responsive. No noticeable differences in food consumption between treatments were stated to be observed. Also, no gross malformations or lesions were observed.

At termination of this exposure, 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 were censored and not used in calculations or statistical analysis of means/medians for the growth metrics presented below (i.e., snout-vent length, wet body weight, and hind limb length normalized by snout-vent length). One animal in control replicate B (NF 61) and 0.10 mg /L replicate B (NF 61) were characterized as late-stage and were censored.

Whole Body Wet Weight: The mean day 7 whole body wet weight for tadpoles in the control and 32, 100, and 320 µg/L treatment was 0.4299, 0.4252, 0.3897, and 0.3937 g, respectively. Dunnett's Multiple Comparison Test ($C \neq T$) determined no significant reduction in day 7 whole body wet weight among tadpoles exposed to any of the treatment levels tested compared to the control. The mean day 21 whole body wet weight for tadpoles in the control and 32, 100, and 320 µg/L treatment was 1.3796, 1.2132, 1.3072, and 1.1827 g, respectively. Dunnett's Multiple Comparison Test ($C \neq T$) determined a significant reduction in day 21 whole body wet weight among tadpoles exposed to the 32 and 320 µg/L treatment levels compared to the control ($p = 0.049$ and $p = 0.02$, respectively). The data ranges, along with the mean values and percentage difference from the control, are presented in Table 9.2.3.3-6 (Day 7 results), and Table 9.2.3.3-7 (Day 21 results).

Snout-Vent Length: The mean day 7 snout-vent length for tadpoles in the control and 32, 100, and 320 µg/L treatment was 17.86, 17.92, 17.39, and 17.40 mm, respectively. The mean day 21 snout-vent length for tadpoles in the control and 32, 100, and 320 µg/L treatment was 25.50, 24.56, 25.49, and 24.70 mm. Dunnett's Multiple Comparison Test ($C \neq T$) indicated no significant difference in day 7 or day 21 snout-vent length between the control and any of the test treatments. The data ranges, along with the mean values and percentage difference from the control, are presented in Table 9.2.3.3-6 (Day 7 results), and Table 9.2.3.3-7 (Day 21 results).

Hind Limb Length (HLL): The mean day 7 HLL for tadpoles in the control and 32, 100, and 320 µg/L treatment was 2.01, 1.95, 2.01, and 1.95 mm, respectively. The mean day 21 HLL for tadpoles in the control and 32, 100, and 320 µg/L treatment was 11.90, 10.90, 11.02, and 10.13 mm, respectively. Dunnett's Multiple Comparison Test ($C \neq T$) indicated no significant difference in day 7 or in day 21 HLL between the control and any of the test treatments. The data ranges, along with the mean values and percentage difference from the control, are presented in Table 9.2.3.3-6 (Day 7 results), and Table 9.2.3.3-7 (Day 21 results).

Hind Limb Length Normalized by Snout-Vent Length (nHLL): The mean day 7 nHLL, for tadpoles in the control 32, 100, and 320 µg/L treatment levels was 0.113, 0.109, 0.116, and 0.112 mm respectively. Dunnett's Multiple Comparison Test ($C \neq T$) indicated no significant difference in day 7 nHLL between the control and any of the test treatments.

The median day 21 nHLL for tadpoles in the control, 32, 100, and 320 µg/L nominal treatment levels was 0.478, 0.428, 0.435, and 0.414 mm respectively. Whilst there was a weak trend for decreasing nHLL as the concentration of test item increased, Jonckheere-Terpstra's Step Down Test determined no significant difference in day 21 nHLL between the control and any of the test treatments. The data ranges, along with the mean values and percentage difference from the control, are presented in Table 9.2.3.3-6 (Day 7 results), and Table 9.2.3.3-7 (Day 21 results).

Developmental Stage: The median day 7 developmental stage for tadpoles in the control, 23, 90, and 300 µg/L treatment levels was 53, 53, 54, and 54, respectively. Jonckheere-Terpstra's Step-Down Test determined a significant increase in day 7 developmental stage among tadpoles exposed to the 300 µg/L treatment level compared to the control. The day 7 developmental stage distribution profile was analysed by applying the multi-quantal Jonckheere-Terpstra's Step-Down Test. The overall multi-quantal procedure determined no significant increase in day 7 percentile developmental stage at 300 µg/L. It should be noted the multi-quantal analysis is the preferred statistical method for assessing developmental stage in the appropriate guidance (OECD, 2009 and U.S. EPA, 2009). HSE has also considered the raw data in terms of range and agrees there is no clear treatment related effect for development stage.

There was no significant reduction in developmental stage compared to the control at any treatment level, when assessed at 21 days.

The median day 21 developmental stage for tadpoles in the control, 32, 100, and 320 µg/L treatment levels was 58 for all. Jonckheere-Terpstra's Step-Down Test ($C > T$) determined no significant reduction in day 21 developmental stage among tadpoles exposed to any of the treatment levels tested compared to the control. The overall multi-quantal procedure also determined no significant reduction in day 21 percentile developmental stage at 320 µg/L and corroborated the standard comparison test result for day 21 developmental stage. The data ranges, along with the mean values and percentage difference from the control, are presented in Table 9.2.3.3-6 (Day 7 results), and Table 9.2.3.3-7 (Day 21 results).

Thyroid and Liver Histology: Tissues from the 32, 100, and 320 µg/L treatment levels were submitted for histological analysis. Haematoxylin and eosin-stained tissue sections of thyroid glands and livers were examined from control and pydiflumetofen-exposed tadpoles. Five tadpoles per replicate were examined.

There were no treatment-related histopathologic findings involving the thyroid in this study. As anticipated, a proportion of control frogs exhibited baseline levels of thyroid follicular cell hypertrophy (mild) and/or follicular cell hyperplasia (mild), but the prevalence and severity of these findings in pydiflumetofen-treated frogs were generally comparable to those of the negative controls. The absence of treatment-related effects in the thyroid gland is consistent with the lack treatment-related effects on developmental stage across the treatment levels.

Liver histopathology revealed a relatively low-grade reduction in hepatocellular vacuolation (glycogen incorporation) in eight tadpoles at the 320 µg/L treatment level. Severity at this treatment level was graded as mild (grade 1) in six tadpoles and moderate (grade 2) in two tadpoles. The prevalence of this finding was also increased slightly in frogs of the 32 µg/L group, but this difference was less substantial and thus less likely to be treatment related. The relatively low-grade decrease in hepatocellular vacuolation in tadpoles of the 320 µg/L dose group is consistent with diminished hepatic glycogen/lipid storage (U.S. EPA, 2015). This non-specific finding suggests that the energy intake in those frogs was insufficient relative to physiological requirements for growth and activity.

All endpoints and effects are also summarised in the tables below:

Table 9.2.3.3-6: Effects of pydiflumetofen on *Xenopus laevis* following 7 days of exposure

Mean measured concentration (µg pydiflumetofen /L)	D Stage		Snout-Vent Length (mm)			Hind-Limb Length (mm)		Hind Limb Length (Normalized by SVL)			Whole Body Wet Weight (g)			
	Range	Median	Range	Mean	Percent change from control (%)	Range	Mean	Range	Average		Percent change from control (%)	Range	Mean	Percent change from control (%)
									Mean	Median				
Control	53-54	53	16.85-18.45	17.86	-	1.88-2.16	2.01	0.109-0.119	0.113	0.112	-	0.3634-0.4701	0.4299	-
23 (Nominal: 32)	53-54	53	16.51-19.28	17.92	+ 0.34	1.84-2.06	1.95	0.105-0.111	0.109	0.110	- 3.54	0.3315-0.5229	0.4252	- 1.09
90 (Nominal: 100)	53-54	54	16.92-18.21	17.39	- 2.63	1.96-2.08	2.01	0.114-0.119	0.116	0.116	+ 2.65	0.3582-0.4294	0.3897	- 9.35
300 (Nominal: 320)	53-54	54*	16.83-17.91	17.40	- 2.58	1.82-2.06	1.95	0.105-0.118	0.112	0.114	- 0.89	0.3667-0.4326	0.3937	- 8.42

D stage = Developmental stage.

* Jonckheere-Terpstra's Step-Down Test determined a significant increase at day 7 (developmental stage) in highest concentration (300 µg a.s./L) compared to the control. The day 7 developmental stage distribution profile was analysed by applying the multi-quantal Jonckheere-Terpstra's Step-Down Test. The overall multi-quantal procedure determined no significant increase in day 7 percentile developmental stage at 300 µg/L. It should be noted the multi-quantal analysis is the preferred statistical method for assessing developmental stage in the appropriate guidance (OECD, 2009 and U.S. EPA, 2009). HSE has also considered the raw data in terms of range and agrees there is no clear treatment related effect for development stage.

Table 9.2.3.3-7: Effects of pydiflumetofen on *Xenopus laevis* following 21 days of exposure

Mean measured concentration (µg pydiflumetofen /L)	D Stage		Snout-Vent Length (mm)			Hind-Limb Length (mm)		Hind Limb Length (Normalized by SVL)				Whole Body Wet Weight (g)		
	Range	Median	Range	Mean	Percent change from control (%)	Range	Mean	Range	Average		Percent change from control (%)	Range	Mean	Percent change from control (%)
									Mean	Median				
Control	57-59.8	58	25.22-25.64	25.50	-	11.14-12.77	11.90	0.46-0.505	0.473	0.478	-	1.29-1.435	1.3796	-
23 (Nominal: 32)	56-59.4	58	23.69-25.13	24.56	- 3.69	10.12-12.17	10.90	0.366-0.487	0.447	0.428	- 5.50	1.066-1.337	1.2132*	- 12.06
90 (Nominal: 100)	56.6-59.4	58	24.94-25.98	25.49	- 0.04	9.91-12.15	11.02	0.357-0.497	0.432	0.435	- 8.67	1.237-1.373	1.3072	- 5.25
300 (Nominal: 320)	56-59	58	24.05-25.73	24.70	- 3.14	9.18-11.42	10.13	0.388-0.46	0.412	0.414	- 12.90	1.111-1.319	1.1827*	- 14.27

D stage = Developmental stage.

*Significantly reduced compared to the control based on one-tailed Dunnett's Multiple Comparison Test.

Table 9.2.3.3-8: Summary of Day 7 and Day 21 endpoints

Endpoints		Nominal Concentration (µg /L)		
		32	100	320
Day 7	Developmental Stage	-	-	-*
	Hind Limb Length	-	-	-
	Hind Limb Length (Normalized by Snout-Vent Length; SVL)	-	-	-
	Snout-Vent Length	-	-	-
	Whole Body Wet Weight	-	-	-
Day 21	Survival	-	-	-
	Developmental Stage	-	-	-
	Hind Limb Length	-	-	-
	Hind Limb Length (Normalized by SVL)	-	-	-
	Snout-Vent Length	-	-	-
	Whole Body Wet Weight	↓	-	↓
	Thyroid Gland Histology	NF	NF	NF
	Liver Histology	NF	NF	F

* Based on statistical analysis recommended in OECD 239.

- Endpoint not impacted by the concentration of pydiflumetofen compared to the control.

↑ Endpoint significantly increased based on a one-tailed Jonckheere-Terpstra's Step-Down Test compared to the control.

↓ Endpoint significantly reduced based on a one-tailed Dunnett's Multiple Comparison Test compared to the control.

NF – No histological findings

F – histological findings

As indicated in the table above, there were no statistically significant differences in any parameters measured on day 7 of the study. At completion of the study (21 days of exposure), statistically significant differences relative to the control were evident in whole body wet weight (WBW). For WBW, considering there was no effect at 100 µg /L, and no effects on other parameters, the difference at 32 µg /L is unlikely to be treatment-related.

Reductions in WBW at the 320 µg /L treatment level, were likely to result from systemic toxicity, considering the liver histopathology findings at this treatment level and the behavioural symptoms of toxicity observed in the range-finder, and indicate that this treatment level approached the maximum tolerable concentration for pydiflumetofen. The relatively low grade decrease in hepatocellular vacuolation in tadpoles of the 320 µg/L dose group is consistent with diminished hepatic glycogen/lipid storage (U.S. EPA, 2015), suggesting that the energy intake in those frogs was insufficient relative to physiological requirements for growth and activity.

Based on the results, neither advanced development nor asynchronous development (i.e., disruption of the relative timing of the morphogenesis or development of different tissues, and the inability to clearly establish the developmental stage of animals by morphological landmarks) occurred at any concentration. Moreover, there is no evidence of antagonistic/inhibitory effects of pydiflumetofen, with no retardation of development (i.e. reductions in HLL and or developmental stage) and no remarkable histological effects in the thyroid. Therefore, these results indicate that pydiflumetofen does not exhibit thyroid-mediated activity in *Xenopus laevis* tadpoles.

Performance Criteria

The performance criteria of the test and controls were acceptable, all were met except one:

Criterion	Acceptable Limits	Study Performance	Criterion Met (Yes/No)
Test concentrations	Maintained at ≤ 20 % coefficient of variation over the 21 day test	CV values ranged from 14 to 20 %	Yes

Criterion	Acceptable Limits	Study Performance	Criterion Met (Yes/No)
Control mortality	≤ 10 % mortality in any one replicate in the controls should not exceed 2 tadpoles	No mortality was observed in any of the control replicates	Yes
Minimum median developmental stage of controls at the end of test	57	At test termination, the median developmental stage in the control was 58	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	The difference between the 10th and 90th percentile of the developmental stage in the controls was 2.0 stages	Yes
Dissolved Oxygen	≥ 40 % air saturation	Dissolved oxygen was maintained between 66 to 69 % of air saturation	Yes
pH	6.5 to 8.5; inter-replicate/inter-treatment not to exceed a difference of 0.5	Inter-replicate and inter-treatment temperature differentials slightly exceeded 0.5 °C on exposure day 16 and inter-replicate differentials slightly exceeded 0.5 pH units on four separate days during the test.	No
Water temperature	22 ± 1 °C; inter-replicate/inter-treatment not to exceed a difference of 0.5 °C	Water temperature was maintained between 20 and 22 °C; inter-replicate/inter-treatment did not exceed a difference of 0.5 °C	Yes
Test concentrations (non-control) without overt toxicity	≥ 2	No test concentrations had overt toxicity	Yes
Replicate performance	Mortality cannot exceed two tadpoles in any treatment or control replicate	No mortality was observed in any of the treatments or control replicates	Yes

Conclusions

The reduced day 21 growth (whole body wet weight) at 320 µg/L was likely a result of systemic toxicity, considering the behavioural effects noted in the range-finder. Based on the absence of effects (no advance, delay, or asynchrony) on development, as well as the absence of thyroid histopathological findings, there is no evidence that pydiflumetofen has endocrine disrupting properties relative to the thyroid system.

(██████████, 2020)

HSE evaluator comments

This study was conducted according to GLP, and followed OECD 231 (2009): Amphibian Metamorphosis Assay. All validity criteria were met, and all performance criteria were met except for one:

The pH for all exposure vessels ranged from 6.7 to 7.9, which was within the guideline range, however, the inter-replicate differentials slightly exceeded 0.5 pH units on four separate days during the test. As these fluctuations were transient, and the performance of the control animals was within expectations, these deviations are not likely to have impacted the results or interpretation of the study. No raw pH data were provided in the study report, and as such, there is insufficient information available to demonstrate if any of the effects seen were related to deviations in the pH.

The following deviations from the OECD guideline were noted:

The OECD 231 (2009) guideline states that: ‘Test solutions from each replicate tank at each concentration should be sampled for analytical chemistry analyses at test initiation (Day 0), and weekly during the test for a minimum

of four samples.’ The frequency of the sampling was in line with the guideline, however, analytical verification was only carried out on two out of four replicates from each condition on each occasion, rather than from all replicates at each timepoint, as is required (replicates A&B were sampled for D0 and D14, and replicates C&D were sampled for D7 and D21). Although this does not give the same degree of confidence as sampling as per the guideline requirement, it wouldn’t provide reason to invalidate the study, as all four replicates were each sampled on at least two consecutive sampling days, so it was still possible to evaluate the test item concentrations over time. Additionally, the experiment uses a flow-through study design, and so should have relatively consistent dosing. The uncertainty will be considered further in the risk assessment.

The analytical methods have been evaluated by HSE Chemistry specialists in Vol. 3CA Part B5.1.2.6. The following was concluded for this method: “Acceptable method. LOQ: 0.05 µg/mL in well water”.

The test organism feeding rates were reduced from the recommended feeding regime of [REDACTED] in the Test Guideline (OECD 231, 2009). However, the applicant states that based on extensive experience with performing this study type, these rates employed with [REDACTED] Tadpole Food have consistently shown to support proper growth and development of *Xenopus laevis* tadpoles. However, no further data or evidence to support this claim was initially provided, it was not possible to rule out the contribution of the reduced feeding rate to the effects observed.

Justification for the reduction in feeding rates used in this test, compared to the feeding rates recommended by OECD, was therefore requested from the applicant. In response, the applicant has stated that this reduced feeding rate has become common practice among contract research laboratories performing this test, who observe consistent benefits in doing so. The applicant cited Tobor-Kaplan (2020), which had demonstrated that reduced feeding provides the following benefits:

- Reduced build-up of waste
- Reduced microbial growth
- Better maintenance of dissolved oxygen concentrations
- Better maintenance of intended test concentrations
- Slower growth resulting in fewer ‘late-stage’ larvae at test termination (high incidence of which makes statistical evaluation of growth parameters more complicated).

Tobor-Kaplan (2020) also reported that incidence of scoliosis was lower when feeding rate is reduced relative to the OECD 231 test guideline recommendation. HSE considers this response to be acceptable.

The statistical procedures used in this study were in line with those detailed in OECD 231 (2009).

The delivery of the test substance was achieved with the use of a glass wool saturator column. Acetone was used as the solvent carrier to coat the saturator column with pydiflumetofen, and a vacuum pump was then used to evaporate all the acetone, leaving only pydiflumetofen in the saturator column. This procedure was in line with that recommended by OECD 23 (2019): Guidance document on aqueous-phase aquatic toxicity testing of difficult test chemicals, and the total evaporation of acetone during this procedure negates the requirement for a solvent control.

There was a very high incidence of spinal deformity for the tadpoles in all conditions. The highest rate was observed in the control condition, with 45 % of control animals displaying spinal deformities. The study authors have justified this by saying: “Based on historical data from amphibian metamorphosis assays performed at [REDACTED] and other facilities, incidence of spinal deformities can range widely and is not typically associated with overt toxicity (Coady et al., 2014). The presence of spinal deformities did not affect the survival or growth of the tadpoles in any treatment or control for this study. Also, the ability to determine developmental stage and measure growth parameters were not compromised in any of the affected tadpoles. Therefore, the incidence of spinal deformities did not impact any endpoint collected for this assay.” As there are no performance or validity criteria concerning the rate of spinal deformity, the high levels observed throughout the experiment would not provide reason to invalidate the study, however, it demonstrates the limited sensitivity of this parameter, and casts doubt on the suitability of this parameter as a measure of toxicity.

Additional consideration of this point was requested from the applicant in a request for additional information. The applicant response explained that ‘Scoliosis in larval *Xenopus* is an idiopathic phenomenon that is poorly understood among the scientific community that routinely culture this species. Genetic and nutritional aetiologies have been proposed [...] In all but the most severe manifestation the condition typically resolves through the

process of metamorphosis.’. Additionally, the applicant highlighted that ‘the exposure window of the AMA coincides with the post-embryonic developmental period where key morphological changes are thyroid-regulated, rather than the embryogenesis period, where the fundamental elements of the body plan are formed along with major organs. It may therefore be considered unlikely that gross morphological deformities resulting from disruption of these processes would be manifest in the AMA as a response to general toxicity.’

It was also pointed out by the applicant that ‘There is no evidence in the scientific literature (e.g. Coady et al., 2014) that this phenomenon [scoliosis] can be induced by overt (systemic) toxicity of an exogenous chemical.’. Nevertheless, it is important that it is reported, as ‘the potential for scoliosis to confound detection or interpretation of responses of core parameters to the test material should be considered. Specifically, in severe cases, scoliosis may interfere with measurement of snout-vent length (it makes it very hard to position the larvae appropriately to see the vent), which may then impact the derived parameter of hind-limb length normalised by snout-vent length. The reporting of this phenomenon contributes to a general assessment of whether the biological needs of the test organism have been met during the test, in the same way as the median stage of controls at test termination. Incidence of scoliosis is not, therefore, an indicator of toxicity per se, and the variability of its incidence alone should not be considered an indicator of test validity.’.

Based on the mean measured test concentrations, there were no statistically significant differences from the control group in any parameters except developmental stage measured on Day 7 of the study. For developmental stage at day 7, the developmental stage distribution profile was analysed by applying the multi-quantal Jonckheere-Terpstra’s Step-Down Test, which identified a significant increase among tadpoles exposed to the 300 µg/L treatment level compared to the control. The overall multi-quantal procedure determined no significant increase in day 7 percentile developmental stage at 300 µg/L. It should be noted the multi-quantal analysis is the preferred statistical method for assessing developmental stage in the appropriate guidance (OECD, 2009 and U.S. EPA, 2009). HSE has also considered the raw data in terms of range and agrees there is no clear treatment related effect for development stage.

For the Day 21 measurements, there were no signs of increased mortality, or differences in developmental stage, hind limb length, or snout-vent length, relative to the control. However, the tadpoles in the 32 and 320 µg a.s./L conditions displayed a significant reduction in whole body wet weight when compared to the control. As there was no reduction at 100 µg a.s./L, and no effects on the other tested parameters, the difference at 32 µg a.s./L is unlikely to be treatment related. Based on the behavioural effects observed in the preliminary exposure, the study authors concluded that the reduced day 21 growth (whole body wet weight) at 320 µg a.s./L was likely a result of systemic toxicity, rather than the effects of the test-item.

Liver histopathology revealed mild to moderate vacuolation (glycogen) in some of the tadpoles at the 320 µg a.s./L treatment level on Day 21. This is consistent with diminished hepatic glycogen/lipid storage, suggesting that energy intake in those frogs was insufficient to their requirements for growth and activity.

This study has been considered further in section B.9.2.3.2.

Report:	K-CA 8.2.3 [REDACTED], (2020a), Pydiflumetofen – Fish Short-Term Reproduction Assay (FSTRA) with Fathead Minnow (<i>Pimephales promelas</i> , Report Number 1781.7303, [REDACTED] (formerly [REDACTED]) [REDACTED] (Syngenta File No.VV-857838)
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Guidelines

OECD, 2012a. Fish Short Term Reproduction Assay. OECD Guideline for the testing Chemicals: Test No. 229. Paris, France. 40 pp

U.S. EPA, 2009. Office of Prevention, Pesticides and Toxic Substances. Endocrine Disruptor Screening Program Test Guideline, OPPTS 890.1350. Fish Short-Term Reproduction Assay. EPA 740-C-09-007. October 2009.
U.S. Environmental Protection Agency. Washington, D.C.

GLP: Yes

MATERIALS

Test Material

Description: SYN545974 technical (Pydiflumetofen Technical)
Lot/Batch #: SMU2EP12007
Purity: 98.5% according to certificate of analysis from study sponsor
Stability of test compound: Stable when stored < 30 °C
Description: off-white powder
Reanalysis/Expiry date: 30 April 2020

Treatments

Test concentrations: *Nominal:* 1.3, 13, and 130 µg/L. Highest test concentration was based on the maximum tolerated concentration (MTC) via available data and in consultation with the Study Sponsor. See assessment of endocrine disruption for further details. *Time-Weighted Average (TWA) Concentrations:* 1.3, 17, and 130 µg/L
Control: A negative (dilution water) control
Solvent: None
Analysis of test concentrations: Yes, prior to start of exposure and during exposure weekly; on test days: 0, 7, 14 and 21, under a full validated LC-MS/MS method (LOQ = 0.0500 µg/L)

Test animals

Species: Fathead minnow (*Pimephales promelas*)
Source: Single population of [REDACTED] laboratory culture: [REDACTED] Lot 18A527
Age: approximately 20 weeks old at the initiation of the pre-exposure.
Pre-exposure period: 15 days prior to exposure
Treatment for disease: None
Weight of fish Representative sample of the laboratory fish culture population (n = 30) male (n=30) female, weighed prior to pre-exposure:

- Male mean wet weight: 4.2 g (range 3.4 – 5.0 g; 68 – 119 % of mean)
- Female mean wet weight: 3.0 g (range 2.4 – 3.6 g; 80 – 120 % of mean)

Of the fish used for pre-exposure assessment (112 females, 56 males):

- 100 % of male fish were \pm 20 % of the representative sample mean.
- 83 % of female fish were \pm 20 % of the representative sample mean.
- Remaining female fish were \pm 30 % of representative sample mean.

Note: weight is outside the approximate range stated for fathead minnow in Annex 2 of the OECD 229 (2012 guideline), which suggests male 2.5 g \pm 20 % and female 1.5 g \pm 20 %. See HSE comments below for further notes.

Feeding: Frozen brine shrimp twice daily (2.0 mL) and once daily with fish flake food (2.5 mL), exception at weekends, when there were two daily feedings (one of brine shrimp and one of flake fish food).

Test design

Exposure regime: Glass wool saturator column was used to deliver pydiflumetofen to the exposure system
Dilution water: Laboratory well water (same as culture water). Tested periodically for presence of pesticides, PCBs, toxic metals, and TOC. No compounds were detected at toxic levels. TOC: 0.60 and 0.76 mg/L for May and June 2019 (within the acceptable range of < 2mg/L specified in the OECD 229 (2012) guideline).
Replication: 4 tanks per test concentration
Test vessels: 18 L aquaria constructed of glass, silicone sealant and nylon, measuring 39 × 20 × 25 cm (L × W × H), filled with approximately 10 L of test water (13 cm depth). Spawning substrate provided in line with that described in guideline OECD 229 (2012).
No of fish per tank: 2 males and 4 females impartially assigned to pre-exposure vessels (spawning assessment) & 16 groups selected at exposure vessels
Duration: 15 Days Pre-Exposure;
 21 Day Exposure

Environmental conditions

Test temperature:	25 to 26 °C
pH:	7.3 to 8.1
Dissolved oxygen:	84.3 to 102 % of saturation
Hardness:	40 to 70 mg/L as CaCO ₃
Conductivity:	110 to 540 µS/cm
Alkalinity:	23 to 28 mg/L
TOC:	0.60 and 0.76 mg/L
Lighting:	16 hours light: 8 hours dark, with a 15- to 30-minute transition period (540 to 750 lux)

STUDY DESIGN AND METHODS

Experimental dates: 15 May to 5 June 2019

Pre-exposure phase description:

Prior to exposure initiation, the adult fish were housed in aquaria within a pre-exposure system to evaluate reproductive performance over a 15-day period. This 15-days is slightly over the one to two-week pre-exposure recommended in the guideline but is acceptable because the purpose of the pre-exposure period is to determine that fish are actively spawning, which was successfully demonstrated. The aquaria were the same construction as those used in the definitive exposure.

Prior to adding fish to the pre-exposure system, a subsample of 30 male and 30 female fish from the laboratory test population were weighed (see above). From the laboratory population, four females and two males were impartially assigned to each of the 28 pre-exposure vessels (total 112 females, 56 males), 15 days prior to exposure initiation. Each fish used in pre-exposure was weighed to determine whether their weight fell within the range of the laboratory subsample (see above). 16 spawn groups are required for the definitive exposure, but the additional pre-exposure chambers were set up to account for a potential lack of spawning in some chambers and/or mortality during this phase.

Each group was monitored daily for active spawning and fecundity data was collected. During this phase, suitability for testing was established when regular spawning occurred in each replicate chamber at least twice in the immediate 7-day period preceding exposure initiation or when an egg production rate of ≥ 15 eggs/female/day/replicate was achieved.

Exposure system and analytical details:

For this exposure, a glass wool saturator column was used to deliver pydiflumetofen to the exposure system, similar to that described in Kahl et al., 1999 and OECD, 2019. The glass column was packed with wool, and then coated with the test substance. The column was designed to provide a constant flow of saturated aqueous solutions (2.5 mg/L) of pydiflumetofen to the diluter system without the use of a carrier solvent.

To coat a column, approximately 11 grams of pydiflumetofen was diluted with 50 mL of acetone and the solution was ultrasonicated in a 100-mL glass beaker for ca. 1 minute until no undissolved material was visible. This solution was then slowly poured into the glass column. After all the solution was added, the column was attached to a vacuum pump. The vacuum pump was 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 it had visually appeared that all of the glass wool was coated and all the solution was evaporated, the column was detached from the vacuum pump and attached to an FMI pump, which delivered a flow of dilution water through the column at 13 mL/min to the diluter mixing chamber.

During the exposure phase, a saturator column was prepared weekly and replaced on the exposure system, after flushing to drain for approximately 48 hours. The column output concentration was used to calculate the appropriate flow rate of the stock solution into the diluter system.

The exposure system consisted of a 2-L intermittent-flow proportional diluter and a two-tiered water bath, consisting of an upper and lower-level water bath (one positioned over the other). The exposure system was designed to provide three concentrations of the test substance, and a control, to four replicate test vessels per treatment. The upper bath contained replicates A and B and the lower bath contained replicates C and D and each replicate was positioned impartially within the respective water baths. The exposure system was operating properly for 11 days prior to exposure initiation to allow equilibration of the test substance in the diluter apparatus and test vessels.

Prior to the start of the definitive exposure, samples from the exposure solution of each replicate of each treatment level and the control, as well as the saturator column stock solution were collected and analysed for pydiflumetofen concentration. During the definitive exposure test, two replicates of each exposure concentration were sampled on days 0, 7, 14, and 21 and four replicates of the 13 µg/L concentration were sampled on day 5 of the exposure for the analysis of pydiflumetofen concentration.

All exposure solutions and QC samples were analysed for pydiflumetofen (SYN545974) using a LC-MS/MS validated method. This method was validated by fortification of laboratory well water (LWW, an ecotoxicology testing matrix) with SYN545974 at concentrations of 0.0500 (LOQ) and 100,000 (High) µg/L.

Exposure phase and measurements:

Once successful spawning was established during the pre-exposure phase, 16 spawn groups of four females and two males were added to the exposure system using a random block distribution, which divided groups into blocks based on spawning activity and then randomized the blocks to exposure vessels. This randomised block design according to egg production output is recommended in the OECD 229 (2012) guideline.

During the exposure period, the survival, appearance of the fish (e.g., coloration patterns or bands, differences in body shape in head and pectoral region), behaviour, fecundity, and fertilisation success were assessed daily. 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). The fish were then euthanised by transfer to a buffered solution of MS-222 (tricaine methanesulfonate) and measured for standard length and wet weight.

Blood samples were then taken for plasma vitellogenin (VTG) analysis, which was carried out in duplicate using a commercially available enzyme-linked immunosorbent assay (ELISA) kit according to manufacturer instructions (BioSense Laboratories, Bergen, Norway). Duplicate QC samples and standard curve were also included, along with fortification of culture samples using a purchased VTG standard of a separate lot as recommended by OECD 229 (2012a). The QC sample was prepared by rehydrating lyophilised VTG standard with male plasma from a [REDACTED] culture population of fathead minnow and following the dilution scheme used for the test samples. A limit of quantification (LOQ) was calculated for each plate based on the standard curve. If a sample resulted in a value below detectable limits, then one-half the LOQ was used as the result for that sample in statistical analysis.

The gonads were then fixed *in situ*, removed, and placed into pre-labelled plastic tissue cassettes and preserved for histological analyses. Fish carcasses were also preserved for subsequent tubercle scoring. Liver histopathology was also performed. Excised fathead minnow gonads and the associated carcasses were submitted to [REDACTED], for histologic processing and pathologic evaluation.

Statistical analysis

At the termination of the assay, data obtained on survival, fecundity, fertilisation success, nuptial tubercle scores, and plasma vitellogenin concentration were analysed to identify significant differences in the treatment organisms compared to the control organisms. CETIS Version 1.9 (Ives, 2019) was used to perform all statistical computations. Histopathology results for liver and gonads were evaluated qualitatively.

Analyses were performed using the mean organism response in each replicate aquarium. 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. For this study, all continuous data were normally distributed and passed the qualifying test for homogeneity of variance.

Deviations from study plan: Minor study plan deviations relating to dilution water source and monitoring, causing no impact in the results or interpretation of the study:

1. A pump failure for laboratory well water on pre-exposure day 5 meant that the dilution water changed from a mixture of unadulterated on-site well water and de-chlorinated Town of Wareham well water to 100 % de-chlorinated Town of Wareham well water. This lasted until exposure day 5, when the laboratory well water supply was restored. All water quality parameters were still within protocol criteria during the water interruption, and fish survival and fecundity was unaffected.
2. On test day 14 water quality measurements were taken from the incorrect replicate (replicate A instead of replicate C). Additional replicates were measured on day 21 as a conservative measure. All replicates demonstrated water parameters within expectations, therefore this deviation did not impact the results or interpretation of the study.

RESULTS AND DISCUSSION

Analytical data:

Exposure solutions were clear, colourless, and free of visible precipitate.

The recoveries of the 13 µg/L samples on day 0 were approximately 140 % of nominal concentration and out of trend from the other two exposure concentrations. Hence recalibration was required and further analysis was conducted on day 5 showing recoveries closer to nominal (Table 9.2.3.3-9).

Due to the unequal spacing between analytical intervals, a time-weighted average was calculated for each treatment level. Across all treatment levels, time-weighted average concentrations ranged from 100 to 130 % of nominal concentration and defined the treatment levels tested as 1.3, 17, and 130 µg/L. The coefficient of variation for all measured concentrations ranged from 6.0 to 12 %. The limit of quantitation was 0.0500 µg pydiflumetofen/L. Time-weighted average concentrations were used for calculating and the reporting of results.

Table 9.2.3.3-9: Pydiflumetofen - Concentrations Measured in the Exposure Solutions

Nominal concentration (µg a.s./L)	Measurement Day ^c	Measured concentration of a.s. (µg/L)			TWA: Time weighted average concentration (µg a.s./L) ^a	Percent of nominal ^a
		Mean (% CV ^b)	Percent of nominal ^b	Percent of TWA ^b		
Control	0	< LoQ	NA	NA	NA	NA
	5	< LoQ	NA	NA		
	7	< LoQ	NA	NA		
	14	< LoQ	NA	NA		
	21	< LoQ	NA	NA		
1.3	0	1.5 (10)	112	112	1.3 SD: 0.16 % CV: 12	100
	5	NA	NA	NA		
	7	1.1 (0.0)	85	85		
	14	1.5 (6.7)	115	115		
	21	1.4 (3.4)	112	112		
13	0	18 (0.0)	138	106	17 SD: 1.1 % CV: 6.8	130
	5	16 (9.7)	119	91		
	7	15 (3.4)	112	85		
	14	18 (2.9)	135	103		
	21	18 (2.9)	135	103		
130	0	130 (3.7)	104	104	130 SD: 7.7 % CV: 6.0	100
	5	NA	NA	NA		
	7	120 (0.0)	92	92		
	14	130 (7.7)	100	100		
	21	140 (0.0)	108	108		

Concentrations expressed as less than values were below the limit of quantitation (LoQ) for the analytical method for this testing (i.e., 0.0500 µg/L) (NA = Not applicable)

^a Time-weighted average values, percent of nominal, standard deviations (SD) and coefficient of variation (% CV) were calculated by study authors using the actual (unrounded) analytical results and not the rounded values (two significant figures) presented in this table.

^b CV: coefficient of variation (%). Mean measured concentration as calculated by study author; % CV, Percent of nominal and percent of TWA calculated by HSE from available raw data.

^c Number of replicates measured is two on each day except Day 5 where four replicates were measured. Concentrations on days 0 and 14 are from samples of replicates C and D. Concentrations on days 7 and 21 are from replicates A and B. Concentrations on day 5 are from all four replicates A, B, C and D.

Biological data:

Biological data is described in sections according to the following endpoints:

- Survival
- Behavioural/morphological abnormalities
- Fertilisation success and fecundity
- Secondary sexual characteristics (SSC)
- GSI (gonadosomatic index)
- Gonad histopathology
- Liver histopathology
- VTG (Vitellogenin in blood plasma)

Survival:

Survival data is shown in Table 9.2.3.3-10. Following 21 days of exposure, mean percent survival of the control fish was 88 % for males (one single mortality in replicate B on exposure day 20) and 94 % for females (one single

mortality in replicate C on exposure day 5). Combined male and female percent survival in the control fish was 92 %. Statistical analysis determined no significant difference in mean percent survival among male, female or male and female combined fish exposed to any of the treatment levels tested compared to the control (one-tailed Fisher's Exact Test with Bonferroni-Holm's Adjustment; this statistical test was chosen because data resembled a monotonic concentration response and met the assumptions for normality and homogeneity of variance).

Behavioural/morphological abnormalities:

No observations of abnormal behaviour such as hyperventilation, loss of equilibrium, uncoordinated swimming, atypical quiescence, or feeding abstinence were noted in any of the treatment levels tested or the control during daily observations, with two exceptions: 1) a single male fish on day 16 in replicate A of the 1.3 µg/L treatment level, which had loss of equilibrium and injuries attributed to normal male territorial aggression and subsequently died; 2) a single male in replicate D of the control on exposure day 20 which was observed to be lethargic and on the bottom of the exposure vessel, which was also attributed to territorial aggression by the other male in the aquarium. Normal male territorial behaviour was observed daily in all replicates of all treatment levels and the control.

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. Detailed examination of ovaries and testes is discussed further in the gonad histopathology section.

Fertilisation success and fecundity:

Fecundity was expressed as the mean number of eggs produced by surviving females per reproductive day per replicate. The percent fertility rate was expressed as the number of viable embryos divided by the total number of eggs $\times 100$ for each replicate per reproductive day.

No significant differences were observed for fertilisation success at any treatment levels compared to control (one-tailed Dunnett's Multiple Comparison Test; data did not resemble a monotonic concentration response but met the assumptions for normality and homogeneity of variance) (Table 9.2.3.3-10).

For fecundity, there was a statistically significant reduction at the highest tested concentration compared to the control (one-tailed Jonckheere-Terpstra's Step-Down Test; test chosen because data resembled a monotonic concentration response and met the assumptions for normality and homogeneity of variance), with a mean of 42 eggs per female per reproductive day at test concentration of 130 µg/L compared to 66 in the control (Table 9.2.3.3-10).

Table 9.2.3.3-10: Effects of pydiflumetofen on survival, egg fertilisation and fecundity for fathead minnow in a short-term reproduction test for 21 days

Time-Weighted Average Concentration (µg/L)	Percent survival to Day 21 (Mean \pm SD)			Percent egg fertilisation (Mean \pm SD)	Fecundity ^b (Mean \pm SD)
	Male	Female	Combined Male/Female		
Negative Control	88 \pm 25	94 \pm 13	92 \pm 10	98.3 \pm 1.0	66 \pm 19; CV 29 % ^a
1.3	88 \pm 25	100 \pm 0	96 \pm 8	98.9 \pm 0.42	69 \pm 14; CV 20 % ^a
17	100 \pm 0	100 \pm 0	100 \pm 0	99.1 \pm 0.43	65 \pm 22; CV 34 % ^a
130	88 \pm 25	100 \pm 0	96 \pm 8	98.2 \pm 1.2	42 \pm 14*; CV 33 % ^a
SD = Standard Deviation					
* Significantly reduced compared to the control based on one- tailed Jonckheere-Terpstra's Step-Down Test					
^a CV: Coefficient of variation (%), calculated by HSE from available data					
^b mean number of eggs per surviving female per reproductive day					

Secondary sexual characteristics

Nuptial tubercles were counted, mapped and scored on preserved fish. Tubercles were mapped according to the template in the applicable guidelines (OECD, 2012a; U.S. EPA, 2009).

Nuptial 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. Median male tubercle scores are presented in the table below

(Table 9.2.3.3-11), and no significant differences were observed between the treatments and the control (one-tailed Jonckheere-Terpstra's Step-Down Test).

No abnormal mating behaviour or notable changes in secondary sex characteristics were observed in either sex throughout the 21-day study. Specifically, there were no observations of abnormalities in body colour (light or dark), coloration patterns, body shape, size of dorsal nape pad in males, or ovipositor size in females during the exposure period or at study termination in any of the treatment levels or the control.

GSI (gonadosomatic index)

Gonadosomatic index (GSI) is a percentage measure of gonad weight relative to total body weight and is defined as the (gonad weight / body weight) \times 100. GSI is not an endpoint in the OECD 229 (2012) guideline but is a recommended endpoint in the US EPA guideline OPPTS 890.1350 (2009).

Statistical analysis of GSI data was performed as follows: male GSI data did not resemble a monotonic concentration response but met the assumptions for normality and homogeneity of variance. Therefore, male GSI data was analysed using a two-tailed Dunnett's Multiple Comparison Test. Female GSI data resembled a monotonic concentration response and met the assumptions for normality and homogeneity of variance. Therefore, female GSI data was analysed using a one-tailed Jonckheere-Terpstra's Step-Down Test.

No significant difference in mean male or female GSI among fish exposed to any of the treatment levels tested were found compared to controls (Table 9.2.3.3-11). Male GSI in all treatment levels and control was in the expected male range of 1-2 % as stated in EPA guideline OPPTS 890.1350 (2009). Female GSI was slightly above expected female range of 8-13 % as stated in the EPA guideline in all treatments and control (16, 15, 17 and 20 % in the control, 1.3, 1.7 and 130 $\mu\text{g a.s./L}$ treatment levels respectively).

Table 9.2.3.3-11: Effects of pydiflumetofen on fathead minnow size, gonadosomatic index and tubercle score in a short-term reproduction test for 21 days

TWA pydiflumetofen concentration ($\mu\text{g a.s./L}$)	Male termination endpoints (mean \pm SD)				Female termination endpoints (mean \pm SD) ^b		
	Tubercle score (median \pm SD)	Length ^a (mm)	Wet body weight ^a (g)	GSI %	Length ^a (mm)	Wet body weight ^a (g)	GSI %
Negative Control	26 \pm 3.5	62.59 \pm 1.26	3.7481 \pm 0.3453	1.8 \pm 0.28	58.61 \pm 1.35	2.8195 \pm 0.2396	16 \pm 1.7
1.3	25 \pm 5.0	64.11 \pm 1.39	4.0247 \pm 0.0901	1.5 \pm 0.26	59.49 \pm 0.75	2.8417 \pm 0.1369	15 \pm 3.1
17	29 \pm 4.5	64.56 \pm 0.70	4.1078 \pm 0.3503	1.9 \pm 0.26	59.53 \pm 1.00	2.8619 \pm 0.2058	17 \pm 2.4
130	25 \pm 2.0	64.53 \pm 2.39	4.0927 \pm 0.3614	1.8 \pm 0.17	58.69 \pm 2.17	2.9492 \pm 0.2871	20 \pm 2.5

TWA = Time-weighted average

SD = Standard Deviation

GSI = gonadosomatic index: gonad weight/body weight \times 100

^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 Note: Nuptial tubercles were not observed on any female fish.

Values presented in this table have been rounded to two significant figures. All calculations were performed using the actual (unrounded) data.

Gonad Histopathology

Histological examination of the gonads in male and female fish was conducted to determine whether specific gonad histopathological effects occurred as a result of pydiflumetofen exposure. This histopathology was performed on all surviving fish at all test concentrations and control at the end of the test, apart from in the control where an additional single male which died one day prior to study termination was also submitted for analysis.

Male gonad histopathology

Testes were examined for autolysis, granulomatous inflammation, increased interstitial cells, mineralisation and decreased spermatids, spermatocytes or spermatogonia and were graded as severity scores of minimal, mild, moderate or severe. Testes stage was scored on a scale of 0.0 to 3.5 from entirely immature to predominantly mature sperm with minimal germinal epithelium. Testes were also examined for autolysis, inflammation, mineralisation and changes in prevalence of interstitial cells or developmental gametes.

Detailed male gonad histopathology results are presented in Table 9.2.3.3-12. Testicular stage scores were comparable among control and pydiflumetofen-treated males (mean testis stage score 2.1, 1.9, 1.9 and 2.1 in the control, 1.3, 17 and 130 µg a.s./L, respectively). There were occasional incidences of other observations such as inflammation, mineralisation and spermatogenesis stages as seen in Table 9.2.3.3-12, but these were in general at low prevalence and severity, and were in comparable numbers across control and treatment levels. Therefore, overall, there were no treatment-related findings in the testes.

Table 9.2.3.3-12: Effects of pydiflumetofen on male gonads in a short-term reproduction test for 21 days

Findings in the Testes of Male Fathead Minnows					
TWA Pydiflumetofen Treatment (µg/L):		Negative Control	1.3	17	130
Total number of fish examined:		8	7	8	8
Observation Type	Score	Number of fish			
Autolysis	minimal	-	-	-	1
Inflammation, granulomatous	mild	2	-	-	-
	moderate	1	-	-	-
Interstitial cells, increased	minimal	1	1	2	-
Mineralisation	minimal	-	1	-	-
Mineralisation, collecting ducts	minimal	1	-	1	-
Decreased spermatids and spermatocytes	moderate	1	-	-	-
Increased spermatogonia	minimal	1	-	-	-
Testicular Stage Scores	Stage 1.5	3	2	3	3
	Stage 2.0	2	4	4	2
	Stage 2.5	2	1	1	2
	Stage 3.0	-	-	-	1
	Stage 3.5	1	-	-	-
Mean Score		2.1	1.9	1.9	2.1
TWA: Time-weighted average					

Female gonad histopathology

Ovary stage was scored on a scale of 0.0 to 4.0 from immature oocytes to mature and spawning follicles with large yolk granules. Post-ovulatory follicles were also examined. Oocyte atresia (degradation) was graded with increasing severity from 1 to 4, where grade 1 is average 3-5 atretic follicles per ovary section and grade 4 is vast majority of oocytes are atretic. Ovaries were also examined for presence of granulomatous inflammation, microsporidiosis infection and ovarian cysts (Table 9.2.3.3-13).

Detailed female gonad histopathology results are presented in Table 9.2.3.3-13. There was an increased prevalence of minimal to moderate oocyte atresia in the 1.3 and 17 µg/L treatment relative to the control, with a total of 8 fish in the lowest treatment level and total 4 fish in the middling treatment level. There was increased prevalence and severity (minimal to severe) of oocyte atresia in the 130 µg/L treatment, with a total 9 fish, four of which exhibited severe atresia, and this was the only test concentration exhibiting severe atresia (Table 9.2.3.3-13). This is considered a treatment related effect. The overall increase in atresia in the 130 µg/L treatment was consistent with the observed statistical effect of decreased fecundity in this treatment level.

Ovarian stage scoring was not feasible for four ovaries in the highest treatment level with severe atresia, and those were listed as “unable to stage”. Otherwise, mean ovarian stage scores were comparable among control and pydiflumetofen-treated females.

Granulomatous inflammation (minimal to mild) was observed periodically in the ovaries of both control and pydiflumetofen-exposed females and was occasionally associated with the presence of microsporidian organisms. This minor inflammation was only rarely associated spatially with atresia and did not appear to affect the interpretation of other histopathological findings or outcome of the study, therefore, is not considered treatment related.

Table 9.2.3.3-13: Effects of pydiflumetofen on female gonads in a short-term reproduction test for 21 days

Findings in the Ovaries of Female Fathead Minnows					
TWA Pydiflumetofen Treatment (µg/L):		Negative Control	1.3	17	130
Total number of fish examined:		15	16	16	16
Observation type	Score	Number of fish			
Inflammation, granulomatous	Total	5	7	4	6
	minimal	3	6	4	6
	mild	2	1	-	-
Microsporidiosis	Total	1	2	-	-
	minimal	1	2	-	-
Oocyte Atresia, Increased	Total	2	8	4	9
	minimal	-	3	2	2
	mild	1	1	-	-
	moderate	1	4	2	3
	severe	-	-	-	4
Post-Ovulatory Follicles ^a	Total	7	3	4	6
	Grade 1	1	-	1	3
	Grade 2	1	2	-	1
	Grade 3	5	1	3	2
Ovarian Stage Scores	Stage 1.0	-	1	-	-
	Stage 2.0	2	-	-	-
	Stage 2.5	-	-	3	1
	Stage 3.0	3	4	3	5
	Stage 3.5	7	10	9	4
	Stage 4.0	3	1	1	2
	Mean Score	3.3	3.3	3.3	3.3
	Unable to Stage ^b	-	-	-	4
^a Because they are normal structures, post-ovulatory follicles displayed here as grades rather than severity scores. ^b Stage scoring was not feasible for the four ovaries with severe atresia TWA: Time-weighted average					

Liver histopathology

Liver histopathology was performed on all surviving fish at the end of the test, apart from in the control where an additional single male which died one day prior to study termination was also submitted for analysis. Livers were examined for autolysis, basophilia, cystic degeneration, hepatocellular vacuolation, individual cell necroses/apoptosis, granulomatous inflammation and microsporidiosis (infection).

Detailed liver histopathology observations for both sexes are presented in Table 9.2.3.3-14. A description of the findings:

- All female livers, including the control, exhibited some degree of hepatocyte basophilia, with one exception in the 1.3 µg/L (lowest) treatment level. That particular female, whose liver resembled that of a male fish, also had a relatively undeveloped immature Stage 1 ovary; taken together, both findings are consistent with decreased endogenous oestrogen activity in this individual fish.
- Cystic degeneration in females was generally low and occurred at similar prevalence and severity in all treatments and control, therefore is not considered a treatment related finding.

- Hepatocellular vacuolation was observed in males and females at all treatment levels and control at similar severities (mostly moderate for males; mostly mild for females) and is therefore not considered a treatment related finding.
- There was slight increase in the prevalence of individual cell necrosis/apoptosis (minimal to mild) in the livers of females exposed to 17 and 130 µg/L treatments compared to control females. Individual cell necrosis/apoptosis was not co-associated with other types of liver findings in females, nor did it appear to correspond with any particular ovarian changes.
- Prevalence of granulomatous inflammation (minimal to mild) in the livers of both sexes, and microsporidiosis (minimal) in the livers of males, were generally decreased in pydiflumetofen-exposed fish compared to the control.

Table 9.2.3.3-14: Effects of pydiflumetofen on male and female livers in a short-term reproduction test for 21 days

Liver Findings by Sex									
Sex:		Males				Females			
TWA Pydiflumetofen Treatment (µg/L):		Negative control	1.3	17	130	Negative control	1.3	17	130
Total number of fish examined:		8	7	8	7 ^a	15	16	16	16
Observation Type	Score	Number of fish							
Autolysis	Total	1	-	-	-	-	-	-	-
	Moderate	1	-	-	-	-	-	-	-
Basophilia	Total	0	0	0	0	15	15	16	16
	minimal	-	-	-	-	-	3	1	-
	mild	-	-	-	-	15	12	14	15
	moderate	-	-	-	-	-	-	1	1
Cystic Degeneration	Total	0	0	0	0	3	1	3	3
	minimal	-	-	-	-	2	1	3	2
	mild	-	-	-	-	1	-	-	1
Hepatocellular Vacuolation	Total	7	7	8	7	15	16	16	16
	minimal	1	-	-	-	1	3	2	4
	mild	-	1	1	-	13	11	14	12
	moderate	6	6	7	7	1	2	-	-
Individual Cell Necrosis/Apoptosis	Total	0	0	0	0	2	1	5	5
	minimal	-	-	-	-	2	1	4	5
	mild	-	-	-	-	-	-	1	-
Inflammation, Granulomatous	Total	3	3	0	1	4	1	3	0
	minimal	1	3	-	1	4	1	3	-
	mild	2	-	-	-	-	-	-	-
Microsporidiosis	Total	3	3	1	0	0	0	0	0
	minimal	3	3	1	-	-	-	-	-

TWA: Time-weighted average
^a For one additional male in this group, liver tissue was not recovered, possibly due to autolysis

VTG (Vitellogenin) in blood plasma.

Male and female blood plasma VTG concentrations were evaluated to determine if an induction or reduction of VTG occurred as a result of pydiflumetofen exposure.

Presence of potential outliers in the male and female plasma VTG data sets were statistically determined according to the US EPA guideline OPPTS 890.1350 (2009), where outliers were identified by values that exceed the median plus three times the interquartile range (the difference between the 75th and 25th percentiles). The female data was log-transformed prior to outlier analysis, whereas the male data was not transformed for outlier analysis.

Two data points (i.e., individual fish plasma concentrations) were identified as statistical outliers during analysis: one male in the 1.3 µg/L treatment level, replicate C; one female in the 17 µg/L treatment level, replicate D. The

multiple comparison analyses (using a one-tailed Dunnett's Multiple Comparison Test, chosen for its suitability for non-monotonic concentration response data) were conducted with and without calculated outliers. Since there was no difference in statistical significance between analyses with and without the potential statistical outliers, results presented for male and female VTG include all samples, including these two outliers.

A summary of vitellogenin results is presented in Table 9.2.3.3-15. In the controls, there are 5 orders of magnitude difference between the male mean and the female mean VTG levels (105 ng/ml and 10.2×10^6 ng/ml, respectively). This is larger than the expected 3 orders of magnitude as stated in the OECD 229 (2012) guideline.

Pydiflumetofen-exposed females at all treatment levels exhibited a non-concentration-dependent (flat) decrease in plasma VTG concentration relative to control; however, only the 17 µg/L treatment level was significantly reduced from the control (Table 9.2.3.3-15).

Pydiflumetofen-exposed males demonstrated an increase in blood-plasma VTG with increasing treatment concentration, although this was not relative to the control, as control VTG was higher than the lowest and middling treatment concentration. Statistical analysis determined no significant increase in mean male vitellogenin among fish exposed to any of the treatment levels tested compared to the control data (Table 9.2.3.3-15). However, the large spread of the data, as represented by the standard deviation, reduces the power of the statistical analysis. Therefore, detailed per-replicate male VTG results are presented in Table 9.2.3.3-16 for further consideration. For reference, female VTG results are presented in the same way in Table 9.2.3.3-17. Some points to note from this male VTG data breakdown include:

- The control VTG varies across at least one order of magnitude, from 7.6-270 ng/µl.
- In the control, replicate tank B is responsible for the highest VTG level in the control, and is based on a single individual due to the mortality of the other fish in this tank
- The lowest test concentration has VTG of 7-9.1 ng/µl in two replicates, though one of these replicates is based on a single individual due to mortality. The remaining replicates vary from the others by at least an order of magnitude at 38.5 and 134 ng/µl
- 3 out of 4 replicates at the middling treatment level have mean VTG at levels from 37.7-67.6 ng/µl. The final replicate has 118 ng/µl VTG.
- 3 out of 4 replicates at the highest treatment level have mean VTG levels from 201-264 ng/µl. The final replicate has 33.5 ng/µl VTG

Table 9.2.3.3-15: Effects of pydiflumetofen on male and female blood plasma VTG levels in a short-term reproduction test for 21 days

TWA pydiflumetofen concentration (µg a.s./L)	Male VTG concentration (ng/mL) (mean ± SD)	Male % change from control ^b	Female VTG concentration × 10 ⁶ (ng/mL) (mean ± SD)	Female % change from control ^b
Negative Control	105 ± 119	-	10.2 ± 2.18	-
1.3	47.2 ± 59.6 (excl. outlier ^a : 16.4 ± 14.8)	-55.0 % (excl. outlier ^a : -84.0 %)	6.21 ± 2.50	-39.3 %
17	68.4 ± 35.1	-34.6 %	5.35 ± 3.10* (excl. outlier ^a : 5.43 ± 2.94*)	-47.8 % (excl. outlier ^a : -47.0 %)
130	188 ± 107	+79.0 %	6.99 ± 2.60	-31.8 %

SD = Standard Deviation

TWA = Time-weighted average.

* Significantly reduced compared to the control, based on one-tailed Dunnett's Multiple Comparison Test

^a There was no difference in statistical significance between multiple comparison analyses with and without the statistical outliers (see text), so data including statistical outliers is used as the basis for results and conclusions. However, results excluding statistical outliers are additionally presented here for reference.

^b a negative value indicates a decrease compared to control, a positive percentage indicates an increase.

Table 9.2.3.3-16: Male blood plasma VTG concentration broken down by replicate tank in a short-term reproduction test for 21 days.

Replicate tank	VTG conc (ng/μl) in each TWA treatment level							
	Control		1.3 μg a.s./L		17 μg a.s./L		130 μg a.s./L	
	Raw data	Replicate mean	Raw data	Replicate mean	Raw data	Replicate mean	Raw data	Replicate mean
A	7.6	7.6	7	7	126	67.6	131	254
	7.6		NA		9.1		376	
B	270	270	9.1	9.1	94.5	50.8	22.6	33.5
	NA		9.1		7		44.4	
C	37.8	29.3	257*	134	52.5	118	325	264
	20.7		10.9		183		203	
D	6.95	112	35.9	38.5	6.95	37.7	201	201
	217		41.1		68.5		NA	
Treatment mean VTG concentration (ng/μl) ± SD	-	105 ± 119	-	47.1 ± 59.6	-	68.4 ± 35.1	-	188 ± 107
% change from control**	-	-	-	-55 %	-	-34.6 %	-	+79 %

*Identified as outlier (see text) but there were no differences in the results of the statistical analysis with or without outliers.
 **a negative value indicates a decrease compared to control, a positive percentage indicates an increase.
 NA – not applicable – fish died.
 SD = standard deviation
 TWA = Time-weighted average

Table 9.2.3.3-17: Female blood plasma VTG concentration broken down by replicate tank in a short-term reproduction test for 21 days.

Replicate tank	VTG conc (× 10 ⁶ ng/μl) in each TWA treatment level							
	Control		1.3 μg a.s./L		17 μg a.s./L		130 μg a.s./L	
	Raw data	Replicate mean	Raw data	Replicate mean	Raw data	Replicate mean	Raw data	Replicate mean
A	19.1	12.6	6.65	7.28	6.35	8.09	2.43	3.40
	17.7		4.65		6.70		5.70	
	11.1		10.2		3.39		1.46	
	2.38		7.6		15.9		4.01	
B	10.8	11.0	3.83	4.99	2.20	6.95	4.70	7.05
	16.7		9.00		14.3		14.6	
	12.5		2.66		3.54		5.50	
	3.99		4.45		7.75		3.38	
C	7.65	7.37	2.11	3.46	3.70	5.33	13.3	9.53
	11.3		4.63		7.25		11.8	
	3.17		3.44		4.83		5.45	
	NA		3.65		5.55		7.55	
D	22.1	10.0	1.90	9.13	1.29	1.01	5.00	7.98
	6.55		22.7		1.96		2.75	
	2.91		3.00		0.80		4.85	
	8.50		8.90		0.00237*		19.3	
Treatment mean VTG concentration ± SD (× 10⁶ ng/μl)	-	10.2 ± 2.18	-	6.21 ± 2.50	-	5.34 ± 3.10	-	6.99 ± 2.60

Replicate tank	VTG conc ($\times 10^6$ ng/ μ l) in each TWA treatment level							
	Control		1.3 μ g a.s./L		17 μ g a.s./L		130 μ g a.s./L	
	Raw data	Replicate mean	Raw data	Replicate mean	Raw data	Replicate mean	Raw data	Replicate mean
% change from control**	-	-	-	-39.3 %	-	-47.8 %		-31.8 %
<p>*Identified as outlier (see text) but there were no differences in the results of the statistical analysis with or without outliers. Female VTG data was log-transformed prior to outlier analysis.</p> <p>**a negative value indicates a decrease compared to control, a positive percentage indicates an increase.</p> <p>NA – not applicable – fish died.</p> <p>SD = standard deviation</p> <p>TWA = Time-weighted average</p>								

Validity Criteria

All validity criteria were met and therefore, the study was assessed as being acceptable. The table below illustrates the criteria, the acceptable limits listed in the test guideline, and the performance of this study:

Criterion	Study Results	Criterion Met (Yes/No)
Demonstrate that the concentrations of the test substance have been maintained within 20 % of the mean measured values per treatment level.	Concentrations of the test substance (i.e., total measured active substance) were maintained at ± 17 % of time-weighted average values.	Yes
Control mortality ≤ 10 % at end of exposure period.	Mean male/female combined mortality 8 %. For reference: Mean male control mortality 12 %, mean female control mortality 6 %.	Yes, for combined male/female mortality*
Evidence that fish are actively spawning in all replicates prior to initiating chemical exposure	Fecundity was monitored daily during pre-exposure phase; only fish from groups which spawned at least twice 7-days before exposure, or where egg production was of ≥ 15 eggs/female/day/replicate, were used in exposure.	Yes
Evidence that fish are actively spawning in control replicates during the test.	Control fecundity averaged 66 eggs/female/reproductive day (Table 9.2.3.3-10). Fertilisation success in the control averaged 98.3 %.	Yes
Dissolved oxygen ≥ 60 % air saturation for the duration of testing.	Dissolved oxygen was maintained at ≥ 84.3 % 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, and water temperature should be maintained at 25 ± 1 °C throughout the exposure.	Daily water temperature measurements and continuous temperature monitoring of the control (replicate A in upper water bath and replicate C of the lower water bath) established a temperature range of 25 to 26 °C throughout the exposure period. At no point in the exposure were test chambers measured to differ more than ± 1 °C or between successive days.	Yes

*Both the male and female mortality percentages reflect a single mortality in a single replicate for each sex. The exceedance of 10 % for the male mortality is due to there being fewer male fish than female in the test (2 male and 4 female per replicate). Therefore, the combined mortality is deemed most appropriate by HSE for acceptance of this validity criterion.

Conclusions

- Survival, behaviour and fertility:** There were no statistically significant or treatment-related effects on the fish mortality or fertility for either sex (Table 9.2.3.3-10). No treatment-related behavioural effects

were observed.

- **Fecundity:** A significant reduction in fecundity was observed among fish exposed to the highest treatment level tested of 130 µg a.s./L (Table 9.2.3.3-10).
- **SSC and GSI:** There were no effects on the GSI or secondary sexual characteristics (SSC) for either sex (Table 9.2.3.3-11). Although not treatment related, it is noted that the mean female GSI across treatment and control groups is larger than the expected value stated in the US EPA OPPTS 890.1350 (2009) guideline for females. Whereas, the male GSI is within the expected range.
- **Gonad histopathology:** There were no treatment-related effect on male gonad histopathology (Table 9.2.3.3-12), but for females there was increased prevalence of oocyte atresia in all test concentrations compared to control, which included increased severity of oocyte atresia at the highest test concentration 130 µg a.s./L (Table 9.2.3.3-13) (not statistically evaluated).
- **Liver histopathology:** There was slightly increased individual cell necrosis/apoptosis in the liver histopathology findings of female fish at the highest two treatment levels of 17 and 130 µg a.s./L (not statistically evaluated) (Table 9.2.3.3-14). General decreases in granulomatous liver inflammation in both sexes and decreased liver microsporidiosis in males (Table 9.2.3.3-14) (not statistically evaluated) were observed in pydiflumetofen-exposed fish compared to control. Any other observations were deemed sporadic background and not treatment related.
- **VTG concentration in blood plasma:** Female VTG levels were depressed at all three treatment levels in a non-concentration dependent manner and noting that this difference was only statistically significant at the middle treatment level of 17 µg a.s./L (Table 9.2.3.3-15). Male VTG levels increased with pydiflumetofen concentration, but only the VTG level at highest treatment concentration was increased compared to the control, and none of the male VTG levels were significantly different to the control. However, notably the wide range of the male data, particularly in the control where the standard deviation exceeds the mean, introduces uncertainty. The difference in male and female VTG levels in the controls (5 orders of magnitude) is larger than the 3 orders of magnitude which is expected according to the OECD 229 (2012) guideline.

(██████████ 2020a)

HSE evaluator comments

This study was conducted to GLP and was assessed according to the OECD 229 (2012) guideline. The study meets the acceptability criteria of this guideline.

The author monitored the concentration of the test item during exposure and results are based on time-weighted mean average concentration. The analytical methods have been evaluated by HSE Chemistry specialists in Vol. 3CA Part B5.1.2.6. The following was concluded for this method: “Acceptable method. LOQ: 0.05 µg/mL in water”. The guideline does not specify the use of time-weighted average but HSE deems the use of this as acceptable as the measured concentrations are within the guideline acceptance criteria of ± 20 % this mean concentration and had relatively low coefficient of variation over time and between replicates (Table 9.2.3.3-9).

The statistical procedures used are described in adequate detail. They are in line with the guideline: the applicant tested the data for assumptions of normality and homogeneity of variance, considered whether they were monotone dose-response, and adjusted their selection of suitable statistical tests accordingly.

Potential sources of uncertainty:

Three sources of uncertainty are noted below to be considered at hazard assessment:

1. Fish size
2. Spread of data in male VTG measurements

3. Difference in order of magnitude between male and female control VTG measurements.

1. Note on fish size

Regarding fish weight:

- a. The variation in individual female fish weight at the start of the pre-exposure with regards to mean weight is within $\pm 30\%$ of the mean for approximately 17 % of the pre-exposure population, as opposed to $\pm 20\%$ of the mean as recommended in the guideline.
- b. The mean wet weight of both male and female fish is larger than that specified in the guideline: OECD 229 (2012) Annex 2 suggests $2.5\text{ g} \pm 20\%$ for male fish whereas the study measured 4.2 (range 3.4-5.0) g; and Annex 2 suggests $1.5\text{ g} \pm 20\%$ for female fish whereas the study measured 3.0 (range 2.4-3.6) g.

a. Regarding weight variation, the OECD 229 (2012) states that “it is important to minimise variation in weight of the fish at the beginning of the assay” and that the range of weights “should be kept, if possible, within $\pm 20\%$ of the arithmetic mean weight of the same sex”. The applicant states the following justification for female fish being outside the weight range of $\pm 20\%$ of the mean: “Since the number of females outside the $\pm 20\%$ range was minimal relative to the entire population and all spawn groups in the pre-exposure system satisfied the minimum reproduction requirements, the presence of females slightly beyond $\pm 20\%$ of the arithmetic mean weight in the pre-exposure population is not expected to impact the results or interpretation of the study.” Therefore, on the bases that the weight range of $\pm 20\%$ of the mean for each sex is not a strict guideline requirement, and that the pre-exposure population met the requirement for being actively spawning, HSE agrees that the $\pm 30\%$ variation in female fish was acceptable.

b. Regarding the large weight of both male and female fish, the applicant does not provide any more information as to why this is the case, and the guideline does not provide any more information to indicate whether large fish are more or less sensitive in this type of assay. The larger fish size could explain the larger GSI observed for all female fish in this test compared to the expected GSI in the US EPA OPPPTS 890.1350 guideline. Although the fish are all of the recommended age and adequate spawning status at the start of the exposure, the larger fish size adds some potential uncertainty which will be considered further in the hazard assessment discussion.

2. Note on VTG variation in male data

The range of the data for male VTG levels, which in some cases the standard deviation exceeds the mean (Table 9.2.3.3-15), is a source of uncertainty for drawing conclusions with the data. Specifically, the large standard deviation in the male control, which exceeds the mean, reduces the ability to detect statistical differences between treatments and draw meaningful conclusions from the data. Variation in male VTG could partly be because of the nature of the test and low individual numbers (n=2 individuals per each of the 4 replicates per treatment). The additional raw data on an individual level is presented in Table 9.2.3.3-16 for further consideration. This uncertainty will be considered further at hazard assessment.

3. Larger than expected difference in order of magnitude between control male and female VTG levels

It is also noted that the VTG levels in the controls are approximately 5 orders of magnitude higher in female fish than male fish, whereas the guideline suggests males and female VTG levels are expected to be separated by about 3 orders of magnitude for fathead minnow. The guideline suggests that particularly low VTG in females or high VTG in males in the controls can compromise the responsiveness of the assay. Although for this study, the observed order of magnitude difference is potentially compounded by the large spread of the male control data as detailed above, it is nevertheless something to be considered at hazard assessment.

This study and results will be considered further in the endocrine disruption hazard assessment for pydiflumetofen (section B.9.2.3.1).

Report:	KCA 8.2.3
	██████████ (2023)
	Test Item: Pydiflumetofen
	Rapid Androgen Disruption Activity Reporter (RADAR) assay
	Report No.: ██████████
	DRAFT REPORT

Guidelines: OECD 251 (2022)
GLP: No

MATERIAL AND METHODS

Test item:	Pydiflumetofen Purity: 98.5% Batch No.: SMU2EP12007 CAS No.: 1228284-64-7 Expiry date: Aug 2026
Test species:	<i>Oryzias latipes</i> (Japanese medaka) Newly hatched eleutheroembryo (day post hatch zero, dph 0) from a stable transgenic line that harbours a genetic construct comprised of the promoter of the male stickleback (<i>Gasterosteus aculeatus</i>) spiggin 1 (Spg1) gene, coupled to the coding sequence for a fluorescent reporter gene (GFP). Source: Not reported
Breeding method:	Adult homozygous Japanese medaka of the Spg1-GFP line were placed in breeding tanks with a 14:10 light: dark cycle and allowed to breed freely. Eggs were collected and sorted at neurula stages to remove asynchronous, unfertilised or dead eggs. After collection, eggs were cleaned with a 0.5% liquid iodine-based virucidal disinfectant for fish egg (Buffodine®, Evans Vanodine, R074EEV2) diluted 50 times into dechlorinated animal facility water containing 1 mg/L of methylene blue (final concentration of Buffodine® = 2%). The eggs were then raised in crystallizing vessels containing dechlorinated animal facility water + 1 mg/L of methylene blue. The maximal density was 500 eggs/ crystallizing vessel. The medium was renewed once within the 10-day pre-hatching period.
Number of runs:	3 independent runs
Organisms per concentration:	60 (20 per run; 5 per well)
Exposure:	Duration: 72 hours Renewal frequency of test solutions: every 24 hours
Test vessels:	Plastic 6-well plates made of chemically inert material containing 8mL of test solution per well.
Loading rate:	0.871 mg/mL (mean eleutheroembryo fresh weight: 1.394 mg)
Test item solubility:	The test item proved to be fully soluble at 10 g/L dimethyl sulfoxide (DMSO). After dilution into the test medium at 10.0 mg/L, 5.0 mg/L and 2.5 mg/L (0.2% DMSO in the final mixture), solubilisation of the test item was not fully achieved: residual tiny suspended particles and/or tensioactivity were observed. After dilution into the test medium at 2.0 mg/L (0.2% DMSO in the final mixture), solubilisation of the test item was fully achieved.
Test concentrations:	Unspiked mode: 130, 41, 13, 4.1 and 1.3 µg a.s./L MM1X + 0.2% DMSO (nominal) Spiked mode: 130, 41, 13, 4.1 and 1.3 µg a.s./L MM1X + 0.2% DMSO + 17-MT 3 µg/L (nominal). Mean measured test concentrations: to be confirmed in final study report. Controls: Solvent control (MMIX + DMSO 0.2%); dilution water control (MMIX; 17-MT 3 and 10 µg/L MM1X + 0.2% DMSO (pro-androgenic standards); 17-MT

	<p>3 µg/L MM1X + Flutamide 167 and 500 µg/L + 0.2% DMSO (anti-androgenic standards).</p> <p>First lethality pre-test Maximum tolerated concentration (MTC) was based on a survival pre-test conducted with the following nominal test concentrations: 2.0 – 0.625 – 0.195 – 0.061 – 0.019 mg/L (spacing factor, SF = 3.2). Lethal and sub-lethal toxicity was observed at all tested concentrations.</p> <p>Second lethality pre-test In order to refine the MTC and define a range of concentrations for the definitive test a second lethality pre-test was performed at the following test concentrations: 1.260 - 0.400 – 0.126 – 0.040 – 0.013 – 0.004 mg/L (SF = 3.16). No toxicity was observed up to and including the concentration of 0.126 mg/L. Mortality exceeded 10% at the next test concentration (0.4 mg/L), therefore 0.126 mg/L was identified as the MTC in this test.</p>														
Test medium:	<p>The test medium was medaka medium MM1X, details shown below:</p> <table border="1"> <thead> <tr> <th colspan="2">Composition</th></tr> <tr> <th>Salt</th><th>Concentration</th></tr> </thead> <tbody> <tr> <td>NaCl</td><td>50 mg/L</td></tr> <tr> <td>CaCl₂</td><td>1.51 mg/L</td></tr> <tr> <td>MgSO₄</td><td>0.975 mg/L</td></tr> <tr> <td>KCl</td><td>1.5 mg/L</td></tr> <tr> <td>pH</td><td>[7.2 – 8.0]</td></tr> </tbody> </table>	Composition		Salt	Concentration	NaCl	50 mg/L	CaCl ₂	1.51 mg/L	MgSO ₄	0.975 mg/L	KCl	1.5 mg/L	pH	[7.2 – 8.0]
Composition															
Salt	Concentration														
NaCl	50 mg/L														
CaCl ₂	1.51 mg/L														
MgSO ₄	0.975 mg/L														
KCl	1.5 mg/L														
pH	[7.2 – 8.0]														
Endpoints (observation time points)	<p>Macroscopic observation of malformations and survival after 24, 48 and 72 hours. Reading of fluorescence using Olympus MVX10 microscope, Olympus DP74 camera, Cool LED pE-300 fluorescence source, CUBE U-F19002 GFP AT LP filters (excitation 475/40 emission 515LP dichroic 505). Any significant observations of the test medium (e.g., presence of undissolved material, change in colour) were also noted.</p>														
Test conditions	<p>Photoperiod/light intensity: None study conducted in the dark Water temperature: 26°C pH range: 7 – 7.80</p>														
Analytical verification:	<p>Stock solutions, and test solutions for dilution water control (MM1X), solvent control (MM1X + 0.2% DMSO), test groups containing 130, 41, 13, 4.1, 1.3 µg/L for both unspiked and 17-MT - spiked modes were sampled in order to perform chemical analysis of the test item.</p> <p>Samples were taken at 0 hours in fresh solutions, and then every 24 hours in aged and renewed solutions for each test group and for each run of the study.</p> <p>For the stock solution, prepared in 100% DMSO, 500 µL samples were taken and immediately frozen in amber glass vials. The test solution samples from each test group were split into two amber glass vials, with 5 mL added to each vial. All samples were then frozen at -20°C until sent for analysis (maximum of 29 days).</p>														
Statistical analyses:	<p>Data of the 3 runs were pooled and analysed. The results were normalised to the mean fluorescence of the 17MT 3 µg/L control group. The data were analysed following the recommendations of the OECD for the analysis of ecotoxicology experiments (Hypothesis Testing, in Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to Application”, OECD 2006). Each experimental group was analysed to determine whether there is a normal distribution of the values. If the values are normally distributed, the homogeneity</p>														

of variance between the groups was tested with a Levene's test. If the variance is homogeneous between the different groups, a variance test (ANOVA) was carried out followed by a parametric post-hoc test to compare the groups in a pairwise manner (Dunnett's post-hoc test). If the values of one or more experimental groups are not normally distributed or the variance is not homogenous, a variance test (Kruskal-Wallis) was carried out followed by a pairwise non-parametric post-hoc test (Dunn's post-hoc test) to compare the groups with each other.

For statistical analysis, unspiked test substance groups as well as the 17-MT (10 µg/L) control group were compared to the solvent control. For statistical analysis of the spiked groups, the spiked test substance groups were compared to the 17-MT (3 µg/L) control group. Likewise, the flutamide (167 µg/L) control group as well as the flutamide (500 µg/L) control group were compared with the 17-MT (3 µg/L) control group.

Control data of the 3 runs, including the solvent control, 17-MT controls, and 17-MT + flutamide controls, were analysed to ensure that the validity criteria were reached in each run. The data of the three runs were then pooled and analysed to obtain the final results of the test.

Calculations were carried out using Microsoft Excel® and Graphpad Prism® (version 10) software.

RESULTS

Analytical measurements

The analytical phase of the test was not available at the time of submission of the draft report. All results are therefore based on nominal test concentrations and will be revisited when the final report is received.

Measurement of pH

The initial pH of the test medium (MMIX) and the higher exposure solutions containing the test item was measured at the beginning of the test (day 0) and before every renewal (days 1 and 2) and was within the tolerated range (6.5 to 8).

Biological results:

Survival

The results are summarised in the table below for each test run.

Table B.9.2.3.3-17: Survival during test runs.

Concentration nominal	Survival % at 72 hours			Malformations
	Run 1	Run 2	Run 3	
Dilution water control	100	100	100	None
Solvent control (Medaka medium + DMSO 0.2 %)	95	100	100	None
17MT 3 µg/L	100	95	100	None
17MT 10 µg/L	100	95	100	None
17MT 3 µg/L + Flutamide 167 µg/L	95	100	100	None
17MT 3 µg/L + Flutamide 500 µg/L	100	100	100	None
Test item 1.3 µg a.s./L	100	100	100	None
Test item 4.1 µg a.s./L	100	100	100	None
Test item 13 µg a.s./L	100	100	100	None
Test item 41 µg a.s./L	100	100	100	None
Test item 130 µg a.s./L	100	100	100	None
Test item 1.3 µg a.s./L + 17MT 3 µg/L	100	100	100	None
Test item 4.1 µg a.s./L + 17MT 3 µg/L	100	100	95	None
Test item 13 µg a.s./L + 17MT 3 µg/L	100	100	95	None

Concentration nominal	Survival % at 72 hours			Malformations
	Run 1	Run 2	Run 3	
Test item 41 µg a.s./L + 17MT 3 µg/L	95	100	100	None
Test item 130 µg a.s./L + 17MT 3 µg/L	100	100	100	None

Androgenic hormone = 17α-MT (17α -Methyltestosterone).

No malformations were observed in any of the test or controls groups. There was a maximum of 5% mortality (1 eleutheroembryo) in any group in any run of the test.

Fluorescence measurements

The results of the three runs were pooled before data analysis to obtain a maximum of n = 60 fluorescence values in each group.

Controls

Results of the controls are reported in the summary table below:

Table B.9.2.3.3-18: Normalized fluorescence and statistical analysis for the control groups – Pool of the three runs (The results were normalised to the mean fluorescence of the 3 µg/L 17MT control group).

Group name	Dilution water control	Solvent control	17MT 3 µg/L	17MT 10 µg/L	17MT 3 µg/L + Flutamide 167 µg/L	17MT 3 µg/L + Flutamide 500 µg/L
Number of values	59	60	58	58	59	59
Normalised mean fluorescence	0.0977	0.0758	1.0000	1.1955	0.4018	0.1353
SEM	0.0116	0.0056	0.1486	0.1750	0.1002	0.0325
CV	91	57	113	111	192	185
% of induction	0.00	-22.36	923.89	1124.06	-59.82	-86.47
Normality test p- value	<0.001	<0.0001	0.0024	<0.0001	<0.0001	<0.0001
Result of normality test	No	No	No	No	No	No
Statistical test applied	Mann- Whitney	-	Kruskal- Wallis	Kruskal- Wallis	Kruskal-Wallis	Kruskal-Wallis
Statistical test p- value	0.6200	-	<0.0001	<0.0001	0.0023	<0.0001

All controls performed appropriately and met the validity criteria outlined in OECD 251 (see HSE evaluator comments for further details).

Unspiked mode:

In unspiked mode, a statistically significant inhibition of fluorescence was observed for the lowest test concentration only (P=0.0006). However no GFP fluorescence was observed in the images obtained for the solvent control or test groups. The mean fluorescence results from each run of the test (calculated by the HSE evaluator from the raw results in Appendix I – K of the study report), together with pooled results, SEM, CV, % of induction in comparison to the solvent control and consideration of statistical significance are presented in the table below:

Table B.9.2.3.3-19: Normalized fluorescence and statistical analysis for the unspiked mode (The results were normalised to the mean fluorescence of the 3 µg/L 17MT control group).

Treatment	Normalised mean fluorescence				SEM	CV	% of induction ¹	p-value ² (pooled results)
	Run 1	Run 2	Run 3	Pooled				

Dilution water control	0.0866	0.1344	0.0690	0.0977	0.0116	91	-	0.6200
Solvent control	0.0746	0.0705	0.0824	0.0758	0.0056	57	-	-
Pooled control ³	0.0804	0.1025	0.0757	0.0862	0.0064	82	-	Not calculated
Pydiflumetofen 1.3 µg/L	0.0666	0.0419	0.0464	0.0514	0.0036	53	-47.40	0.0006
Pydiflumetofen 4.1 µg/L	0.0664	0.0745	0.0671	0.0693	0.0037	41	-29.02	>0.9999
Pydiflumetofen 13 µg/L	0.0968	0.0479	0.0670	0.0706	0.0063	69	-27.76	>0.9999
Pydiflumetofen 41 µg/L	0.1133	0.0634	0.0443	0.0731	0.0062	65	-25.12	>0.9999
Pydiflumetofen 130 µg/L	0.1171	0.0486	0.0511	0.0734	0.0074	76	-24.85	0.5309

¹In comparison to the solvent control

²Based on a Kruskal-Wallis test; P <0.05 denotes statistical significance

³Pooled control calculated by HSE evaluator as per paragraph 51 of OECD 251 which states 'if there is no statistically significant difference between the test medium control and solvent control, the pooled test medium and solvent controls should be used'.

A graphical representation of the results is also provided below:

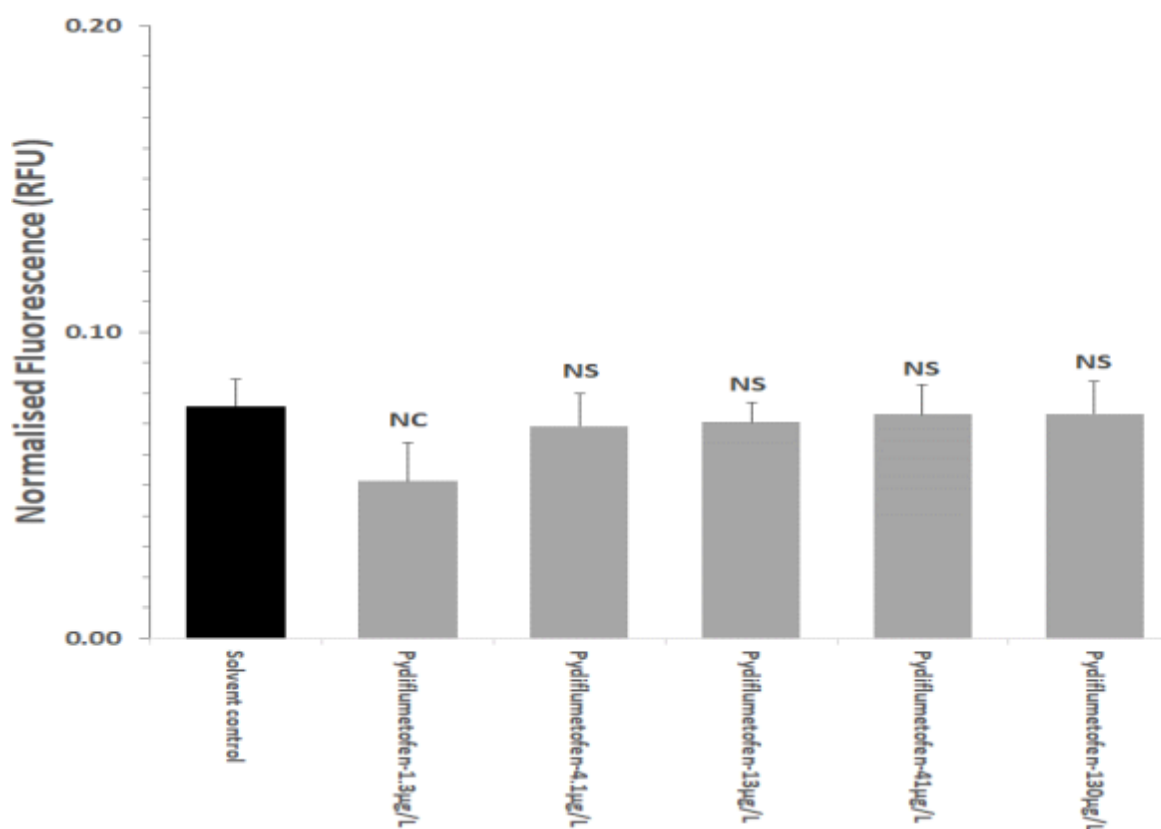


Figure B9.2.3.3-1 : Normalised mean fluorescence in unspiked mode. The fluorescence of each group was normalised to the mean fluorescence of the 3 µg/L 17-MT control group. Error bars represent 1 SEM. NS - Not significant; NC - Not considered (unspecific fluorescence inhibition, §56 of the OECD TG 251) by the applicant.

The following is stated in the study report regarding the statistically significant reduction in normalised mean fluorescence observed in the 1.3 µg a.s./L treatment group:

Examination of the three individual runs indicated that this test concentration gave a fluorescence value lower than the solvent control group uniquely in run 2. Due to the lack of reproducibility of this result between runs and the lack of biological plausibility for an inhibition of androgen signalling when androgen signalling in the solvent control was below measurable levels, in line with the decision logic of the RADAR assay, this result was not considered (NC).

Looking at the individual runs for the 1.3 µg a.s./L treatment group, as presented in the above table, there was a reduction in normalised mean fluorescence in comparison to the solvent control (and pooled control) for all runs of the test. This will be considered further in the HSE evaluator comments.

Spiked mode:

There were no statistically significant differences in fluorescence between spiked treatment groups (pydiflumetofen + 17-MT 3 µg/L) and 17-MT 3 µg/L control, at any of the tested concentrations. The results are presented in the table and figure below.

Table B.9.2.3.3-20: Normalized fluorescence and statistical analysis for the spiked mode (The results were normalised to the mean fluorescence of the 3 µg/L 17MT control group).

Treatment	Normalised mean fluorescence				SEM	CV	% of induction ¹	p-value ² (pooled results)
	Run 1	Run 2	Run 3	Pooled				
17MT 3 µg/L	1.0000	1.0000	0.8610	1.0000	0.1486	113	-	-
Pydiflumetofen 1.3 µg/L + 17MT 3 µg/L	0.9613	0.5437	0.7533	0.7523	0.1503	155	-24.77	0.3080
Pydiflumetofen 4.1 µg/L + 17MT 3 µg/L	2.2558	0.5975	0.9761	1.2647	0.2228	134	26.47	>0.9999
Pydiflumetofen 13 µg/L	1.5715	0.4375	1.2012	1.0464	0.1851	136	4.64	>0.9999
Pydiflumetofen 41 µg/L	1.1928	0.7168	0.8991	0.9362	0.1306	108	-6.38	>0.9999
Pydiflumetofen 130 µg/L	1.3959	0.7740	1.0653	1.0730	0.1929	138	7.30	>0.9999

¹In comparison to the 17MT 3 µg/L

²Based on a Kruskal-Wallis test; P <0.05 denotes statistical significance

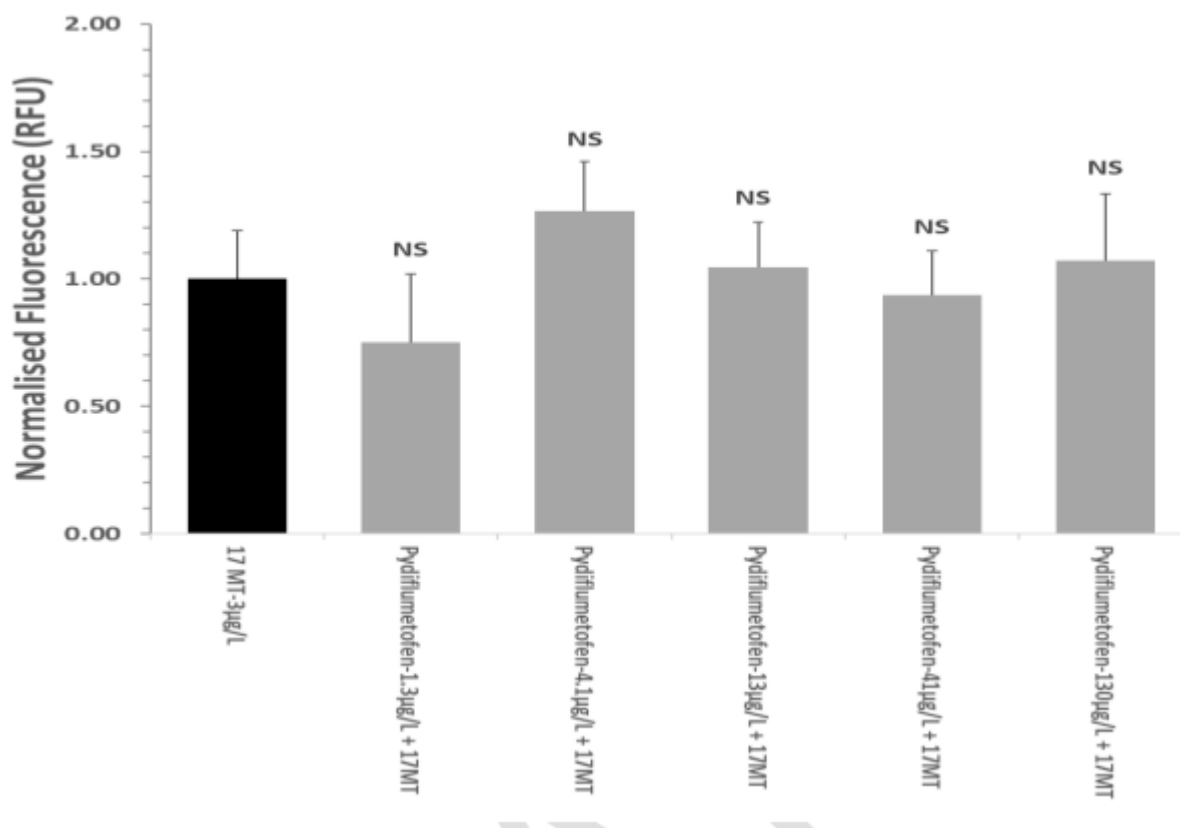


Figure B9.2.3.3-2: Normalised mean of fluorescence in spiked mode. The fluorescence of each group was normalised to the mean fluorescence of the 3 µg/L 17-MT control group. Error bars represent 1 SEM. NS - Not significant; NC - Not considered (unspecific fluorescence inhibition, §56 of the OECD TG 251) by the applicant.

Overall conclusion in test report (study author conclusion shown in *italics*):

Mortality did not exceed 10% in any group, in each of the three runs and in the pooled data, and all validity criteria were met according to OECD TG 251.

There were no effects of the test substance on GFP-fluorescence in spiked or unspiked mode.

At the nominal concentrations of 130, 41, 13, 4.1, 1.3 µg/L, pydiflumetofen did not exhibit endocrine activity in the Rapid Androgen Disruption Activity Reporter (RADAR) assay

HSE evaluator comments

The above study was **not** conducted in compliance with GLP, however the chemical analysis was conducted under GLP/officially recognised testing facilities.

At present it is not possible to comment on the analytical recovery as the test report is draft and does not contain these details. This will be considered when the final report is received.

The MTC was selected through use of survival pre-tests. The first survival pre-test was conducted with 5 concentrations across a range of 0.019 – 2.0 mg a.s./L and ≥10% mortality was observed at all concentrations except the lowest (0.019 mg a.s./L). Sublethal effects were also observed at all tested concentrations. A second survival pre-test was conducted at a lower concentration range of 0.004 mg a.s./L – 1.260 mg a.s./L again with 5 test concentrations. At this range, ≥10% mortality was observed at concentrations above 0.126 mg a.s./L with sublethal effects only observed in 1 embryo at 0.013 mg a.s./L. As such, the MTC was set as 0.126 mg a.s./L (120 µg a.s./L). The selection of the MTC for the definitive test (130 µg a.s./L) was therefore conducted in line with OECD 251.

The validity criteria and study performance are shown in the tables below.

Criterion	Acceptable Limits	Study Performance	Criterion Met (Yes/No)
Validity criteria according to OECD 251 The following criteria should be met for each run (three in total)			
Fluorescence	A statistically significant induction of fluorescence should be measured between the solvent control group and the 17MT 10 µg/L control group. The mean fluorescence of the 17MT 10 µg/L control group should be at least 300 % the mean of fluorescence of the solvent control group.	Run 1: 1591% Run 2: 762% Run 3: 1286% All runs demonstrated a statistically significant induction of fluorescence (p < 0.001)	Yes
Mortality	The combined mortality, sublethal effects such as malformation, changes in body colour or immobility and invalid data due to poorly positioned eleutheroembryos should not exceed 10 % in each control group.	Mortality was between 0 – 5% in all control groups	Yes
The following criteria should be met for the pool of the three runs:			
Fluorescence	The mean fluorescence of the 17MT 10 µg/L control group should be at least 10% higher than the mean of fluorescence of the 17MT 3 µg/L control group. This ensures that the mean fluorescence of the 17MT 10 µg/L group is higher than that of the 3 µg/L group, which historical data has shown to be important for ensuring that the assay performed correctly. Generally, the difference is much greater than 10%.	19.55%	Yes
Fluorescence	A statistically significant inhibition of fluorescence should be measured between the 17MT 3 µg/L control group and the 17MT 3	-86% (p < 0.001)	Yes

	µg/L + flutamide 500 µg/L control group.		
Mortality/invalid data	For the pool of the three runs, a test should have at least five uncompromised test concentrations. A treatment group (ideally 60 individuals) is considered uncompromised if in each of the three runs (ideally 20 individuals per run) it passes validity criteria (combined mortality, and/ or malformations and invalid data due to poorly positioned eleutheroembryos	All above criteria met for all 5 test concentrations	Yes
Note from OECD 251 guideline:	These validity criteria are applicable after image quality control if performed. If a minor deviation from the validity criteria is observed, the consequences should be considered in relation to the reliability of the test data and these considerations should be included in the report		

All validity criteria were met for the study.

Consideration of the results has been done using the decision scheme in OECD 251:

- It was not reported whether the fluorescence of the test item was checked as stated in OECD 251: 'A limitation of this test guideline is that it should not be used for test chemicals emitting fluorescence between 500 and 550 nm when excited at wavelengths between 450 and 500 nm and able to accumulate in the eleutheroembryos.' A protocol to assess this is detailed in the guideline. However, in the interpretation of the study results the study report does state that no GFP fluorescence was present in the images obtained for the solvent control or test groups, therefore it can be assumed this criteria is met.
- In the unspiked mode, a statistically significant decrease in fluorescence was observed in the 1.3 µg a.s./L treatment group in comparison to the control. GFP was not visible in the kidneys of the solvent control group. The three individual runs were checked for reliability of the response in the unspiked mode and it was noted by the HSE evaluator that there was a decrease in fluorescence for all runs of the test (no statistical analysis was conducted by the HSE evaluator or applicant for the individual runs of the test). According to the decision scheme, it should be considered that one or more runs of the RADAR assay is repeated, potentially over a different concentration range or that a different test should be used.
- The results from the spiked mode showed no significant increase or decrease in fluorescence at any of the test concentrations in comparison to the 3 µg/L 17MT control. As such, in accordance with the decision scheme in OECD 251, pydiflumetofen is inactive in the RADAR assay.

Based on the above consideration, there is some uncertainty regarding the results from this study. Fluorescence decreases in unspiked mode are not expected as the eleutheroembryos do not synthesise detectable levels of androgen at this developmental stage. OECD 251 recommends where statistical significance is observed, the RADAR assay may not be appropriate for the chemical or a potential issue with the organisms or test conditions. As the controls performed appropriately and all validity criteria were met, it does not appear that there was an issue with the test conditions or test organisms. OECD 251 recommends statistical analysis of the individual runs of the test where statistical significance is observed for the pooled results, which has not been conducted.. Regarding the concentration range selected, this appears to be appropriate in the context of setting the MTC and no mortality >10% or sublethal effects were observed at any of the tested concentrations, however a lower

concentration range is suggested in OECD 251 where results of this nature are observed. OECD 251 also suggests that a different androgen axis activity test may be more appropriate in this case.

B.9.2.4. Acute toxicity to aquatic invertebrates**B.9.2.4.1. Acute toxicity to *Daphnia magna***

Report: K-CA 8.2.4.1 [REDACTED] (2017). SYN545974 – Acute Toxicity to Water Fleas (*Daphnia magna*) Under Static Conditions, Report number 1781.6839, Smithers Viscient, 790 Main Street, Wareham, MA 02571-1037, USA. (Syngenta File No. SYN545974_10016)

GUIDELINES

OECD Guidelines for Testing of Chemicals, Method 202: *Daphnia* sp., Acute Immobilisation Test (2004)
 Official Journal of the European Communities, Commission Regulation (EC) No 761/2009, Method C.2: Acute Toxicity for *Daphnia*. L142/456 (2009)
 US EPA Ecological Effects Test Guidelines, OPPTS 850.1010: Aquatic Invertebrate Acute Toxicity Test, Freshwater Daphnids (1996)

GLP: Yes

MATERIALS

Test Material	SYN545974 tech.
Lot/Batch #:	2637-BA/110
Purity:	99.5 % (certificate of analysis confirmed).
Reference testing	A 24-hour reference test was conducted with daphnids (<24 hours old) from laboratory culture using potassium dichromate (K ₂ Cr ₂ O ₇). Tested from 27 to 28 February 2012
Treatments	
Test concentrations:	Dilution water control, solvent control, and nominal concentrations of 0.063, 0.13, 0.25, 0.50 and 1.0 mg SYN545974/L (mean measured: 0.057, 0.11, 0.22, 0.48 and 0.96 mg SYN545974/L)
Solvent:	Dimethylformamide (DMF, CAS No.: 68-12-2), 0.1 mL/L
Analysis of test concentrations:	Yes, analysis of SYN545974, at 0 and 48 hours using LC/MS/MS
Test organisms	
Species:	<i>Daphnia magna</i>
Age:	< 24 hours
Source:	Continuous laboratory cultures, Smithers Viscient Laboratory
Acclimatisation period:	Not necessary since test was performed in the same medium as used in culture.
Feeding:	None during test
Culture medium:	Fortified well water, based on formula for hard water U.S. EPA 1975
Test design	
Test vessels:	250-mL glass beakers containing 200 mL test medium
Test medium:	Fortified well water adjusted to hardness of approximately 170 - 190 mg/L as CaCO ₃ , and filtered prior to test initiation
Replication:	4 test vessels per control and test concentration
No. of organisms per tank:	5, randomly assigned to a tank
Exposure regime:	Static

Duration:	48 hours
Environmental conditions	
Test temperature:	20 – 21 °C
pH range:	8.0 to 8.3
Dissolved oxygen:	7.9 to 8.9 mg/L (no aeration)
Total hardness of dilution water:	170 mg/L CaCO ₃
Lighting:	16 hours light (79 – 87 footcandles) and 8 hours dark cycle with a 30 minute transition period

STUDY DESIGN AND METHODS

Experimental dates: 16 to 18 April 2012

A 10 mg/mL primary stock solution was prepared by placing 0.2502 g of SYN545974 in a 25-mL volumetric flask, bringing it to volume with dimethylformamide (DMF), and mixing by inversion for approximately one minute. Four secondary stock solutions were prepared from the primary stock solution, and the primary and secondary solutions were used to prepare the test solutions, which were observed to be clear and colourless with no visible undissolved test substance. The solvent control was prepared by adding 0.10 mL of DMF to 1.0 L of dilution water, and the water control was prepared with filtered, fortified well water containing no test substance or solvent. Appropriate volumes of the test solution were added to the test vessels and the *Daphnia* added without conscious bias. The study was performed under static conditions.

The immobility of the daphnids was determined by visual observations after 24 and 48 hours of exposure. Organisms unable to swim within 15 seconds after gentle agitation of the test beaker were considered to be immobile.

The pH, temperature and dissolved oxygen were measured at the start and end of the test in each test concentration and the control.

The test concentrations were verified by chemical analysis of SYN545974 at 0 and 48 hours using LC/MS/MS. The 48-hour samples were taken from pooled replicates.

RESULTS AND DISCUSSION

Mean measured concentrations ranged from 83 to 96% of the nominal values (see table below). Analysis of quality control samples resulted in measured concentrations in the range of 96.3 to 104% of the nominal fortified values confirming that the appropriate precision and quality control was maintained. The limit of quantification in this study was 0.151 µg a.s./L. Mean measured concentrations were used for the calculation and reporting of results.

Table 9.2.4.1-1: Analytical results

Nominal concentrations (mg a.s./L)	Concentration measured at 0 hours (mg a.s./L)	Concentration measured at 48 hours (mg a.s./L)	Mean measured concentration (mg a.s./L) ¹	Percent of Nominal (%) ¹
Control	<LOQ _{ana}	<LOQ _{ana}	NA	NA
Solvent control	<LOQ _{ana}	<LOQ _{ana}	NA	NA
0.063	0.057	0.058	0.057	91
0.13	0.11	0.11	0.11	83
0.25	0.23	0.22	0.22	89
0.50	0.47	0.49	0.48	96
1.0	0.98	0.94	0.96	96

¹mean measured concentrations and percent of nominal are based on the original raw data and not the rounded results presented in this table. LOQ_{ana} = 0.151 µg a.s./L. NA. = Not applicable

The median effective concentration (EC₅₀) was defined as the concentration resulting in 50% mortality of the *Daphnia* in the time period specified. If at least one test concentration caused immobilization of $\geq 50\%$ of the test population, then a computer programme (Ives, 2011) was used to calculate the EC₅₀ values and 95% confidence intervals. The NOEC (No Observed Effect Concentration) is defined as the highest tested concentration which did not produce an adverse effect when compared to the control and was determined directly from the raw data. There was no immobility observed in the controls. Immobility data and estimated EC₅₀ values are shown in the table below:

Table 9.2.4.1-2: Effects of SYN545974 on *Daphnia magna* following exposure for 48-hours in a static test

Mean measured concentrations (mg a.s./L)	Immobilised daphnids after 24 hours		Immobilised daphnids after 48 hours	
	Number	%	Number	%
Control	0	0	0	0
Solvent control	0	0	0	0
0.057	0	0	0	0
0.11	0	0	0	0 ^a
0.22	0	0 ^b	0	0 ^b
0.48	0	0 ^c	13	65 ^c
0.96	2	10 ^c	20	100
EC ₅₀ (mg a.s./L)	>0.96		0.42	
95% Confidence limits	ND		0.36 – 0.49	
NOEC (mg a.s./L)	-		0.057	

^a Two daphnids were observed to be lethargic ^b Several daphnids were observed to be lethargic ^c All remaining daphnids were observed to be lethargic. ND = not determined

The dose-response curve for the 48-hour EC₅₀ value is shown in the figure below:

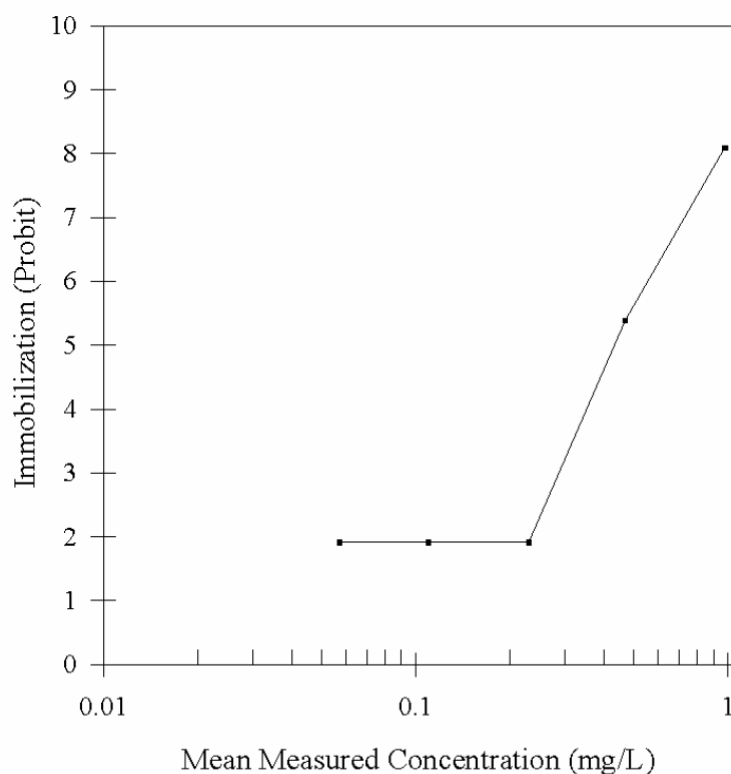


Figure 9.2.4.1-1: The 48-Hour Concentration-Response (Immobilization) Curve for the Static Acute Exposure of *Daphnia magna* to SYN545974

VALIDITY CRITERIA

The validity criteria for the test were met according to OECD 202 (2004):

Table 9.2.4.1-3: Compliance with OECD 202 validity criteria

Validity criterion	Required	Obtained
Immobilisation and sub-lethal effects in control during test	≤ 10 %	0 %
Dissolved oxygen concentration at the end of the test	≥ 3 mg/L	≥ 7.9 mg/L

CONCLUSIONS

Based on SYN545974 mean measured concentrations, the 48-hour EC₅₀ was 0.42 mg a.s./L with 95% confidence intervals of 0.36 to 0.49 mg a.s./L. The 48-hour NOEC was determined to be 0.057 mg a.s./L.

(██████, 2017)

HSE evaluator comments

The study was carried out in accordance with GLP and follows OECD 202 (2004) with no significant deviations from the protocol. All validity criteria outlined in OECD 202 (2004) have been satisfactorily met. The reference item test results (24-hour EC₅₀ = 1.9 mg/L) were within the range stipulated in OECD 202 (2004) (0.6-2.1 mg/L) and are, therefore, acceptable.

It is not specified whether test vessels were covered to minimise evaporation and entry of dust, as is recommended in OECD 202 (2004).

An appropriate solvent control was used, and no immobilisation was observed in this group, indicating that the solvent did not impact the study outcome.

The analytical methods have been checked by HSE Chemistry specialists in Vol 3CA Part B5.1.2.6. The following was concluded: "Fit for regulatory purposes but the method is not fully validated in accordance with SANCO/3029/99 rev. 4. LOQ: 0.3 µg/L in aqueous matrices".

EC₅₀ values and 95 % confidence intervals were calculated using Probit analysis as recommended in OECD 202 (2004). Therefore, the agreed endpoint suitable for use in risk assessment is:

- **48-hour EC₅₀ = 0.42 mg SYN545974/L (based on mean measured concentrations).**

Report: K-CA 8.2.4.1 [REDACTED] (2015a). SYN545547 - Acute Toxicity to Water Fleas (*Daphnia magna*) Under Static Conditions. Report number 1781.7095, Smithers Viscient 790 Main Street Wareham, MA 02571-1037 USA. (Syngenta File No. SYN545547_10000)

GUIDELINES

OECD Guidelines for Testing of Chemicals, Method 202: *Daphnia* sp., Acute Immobilisation Test (2004)

US EPA Ecological Effects Test Guidelines, OPPTS 850.1010: Aquatic Invertebrate Acute Toxicity Test, Freshwater Daphnids (1996)

GLP: Yes

MATERIALS

Test Material SYN545547

Lot/Batch #: BPS 1510/1

Purity: 95% w/w (Tested as 100 %)

Description: White powder

Stability of test compound: Stable under standard conditions.

Reanalysis/Expiry date: End of May 2017

Treatments

Test concentrations: Dilution water control and nominal concentrations of 0.31, 0.63, 1.3, 2.5, 5.0 and 10 mg /L (0.30, 0.61, 1.2, 2.5, 5.3 and 9.8 mg /L mean measured)

Solvent: Dimethylformamide (DMF, CAS No.: 68-12-2)

Solvent control: 1.5 mL DMF added to 1.5 L dilution water

Positive control: Potassium dichromate

Analysis of test concentrations: Yes, analysis at 0 and 48 hours using HPLC/UV analysis

Test organisms

Species: *Daphnia magna* Straus

Source: Continuous laboratory cultures

Feeding: None during test

Culture medium: Dilution water

Test design

Test vessels: 250 mL glass beakers containing 200 mL

Test medium: Dilution water

Replication: 4 replicates of 5 daphnids

Exposure regime:	Static
Duration:	48 hours
Environmental conditions	
Test temperature:	20 to 23 °C
pH range:	8.1 to 8.4.
Dissolved oxygen:	7.8 to 8.7 mg /L (no aeration).
Total hardness of dilution water:	210 mg /L CaCO ₃ .
Lighting:	410 to 860 Lux
Conditions:	16 hours light and 8 hours dark, with a 30-minute dawn/dusk period The test vessels were placed in a temperature-controlled water bath designed to maintain exposure solution temperatures

STUDY DESIGN AND METHODS

Experimental dates: 2 to 4 June 2015

Daphnids (*Daphnia magna*) were selected as the test organism for this study since they are recommended by U.S. EPA and OECD, and they are commonly used in freshwater acute invertebrate toxicity tests. Daphnids were cultured under conditions similar to those in the definitive test, culture daphnids showed no signs of stress or poor health. Prior to the experiment, the daphnid cultures were fed with unicellular green algae, in addition to a suspension of yeast, cereal leaves and flaked fish. No feeding took place during the test.

The dilution water used in this study was taken from the same fortified well water source as the *Daphnid* cultures, which conformed to the OECD 202 guideline on the composition of holding and dilution water. Representative samples of the dilution water source were analysed periodically for the presence of pesticides, PCBs and toxic metals, and analysed monthly for total organic carbon (TOC) concentration.

Four test vessels were used, each containing five *Daphnia* (< 24 hours old), which were added without conscious bias. A static test design was employed. A 100 mg /mL primary stock solution was prepared by placing 2.5345 g of SYN545547 in a 25 mL volumetric flask and bringing it to volume with dimethylformamide. Using this stock solution, the remaining nominal test concentrations as stated above were prepared by serial dilution. The control consisted of dilution water only. The solvent control was prepared by adding 0.15mL of DMF to 1.5 L of dilution water. A 24-hour reference test was conducted with daphnids (< 24 hours old) from the laboratory culture using potassium dichromate.

The pH, temperature and dissolved oxygen of the test solution was measured at the start and end of the test, in each test concentration and the control, continuous temperature monitoring was performed in a satellite vessel in the environmental chamber throughout the exposure period. The immobility of the daphnids was determined by visual observations after 24 and 48 hours of exposure. Organisms unable to swim within 15 seconds after gentle agitation of the test beaker were considered to be immobile. 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 test concentrations were verified by chemical analysis of SYN545547 at 0 and 48 hours using high performance liquid chromatography with ultra violet-visible detection. The limit of quantification (LOQ) in this study was 0.00606 mg /L.

The median effective concentration (EC₅₀) was defined as the concentration resulting in 50 % mortality of the *Daphnia* in the time period specified. If at least one test concentration caused immobilization of greater than or equal to 50 % of the test population, then a computer program (Ives, 2013) was used to calculate the EC₅₀ values and 95 % confidence intervals. Trimmed Spearman Kärber Estimates were used for determination of the EC₅₀ value and the 95 % confidence intervals. The No-Observed-Effect Concentration (NOEC) and Lowest-Observed-Effect Concentration (LOEC) during the 48 hour exposure period were determined by visual inspection of the data.

RESULTS AND DISCUSSION

Analytical results

At the start of the test, the measured concentrations were in the range 91 to 110 % of the nominal values and at the end of the test were in the range 89 to 108 %. Mean measured concentrations ranged from 94 to 110 % of the nominal values. The limit of quantification (LOQ) in this study was 0.00606 mg /L. Mean measured concentrations were used for the calculation and reporting of results.

Biological results

A 5 % immobility rate was observed in the solvent control condition after 48 hrs

Immobility data and estimated EC₅₀ values are shown in table 9.2.4.1-4 below:

Table 9.2.4.1-4: Effects of SYN545547 on *Daphnia magna* following exposure for 48 hours in a static test

Mean measured concentration (mg /L)	Immobilised daphnids after 24 hours		Immobilised daphnids after 48 hours	
	Number	%	Number	%
Control	0	0	0	0
Solvent control	0	0	1	5
0.30	0	0	0	0
0.61	0	0	0	0
1.2	0	0	0	0
2.5	0	0	0	0
5.3	2	10 ^a	8	40 ^a
9.8	8	40 ^a	12	60 ^a
48 hr EC₅₀ mg /L	7.3			
95% Confidence limits	4.5 –12			
48 hr NOEC	2.5			

^a All surviving daphnids were observed to be on the bottom of the test vessel.

The 24-hour reference test established that the EC₅₀ value for *Daphnia magna* and potassium dichromate was 0.78 mg/L. This result was within the expected range for *D. magna* exposed to potassium dichromate.

The percentage of immobilised daphnids in each condition is shown in Figure 9.2.4.2 below.

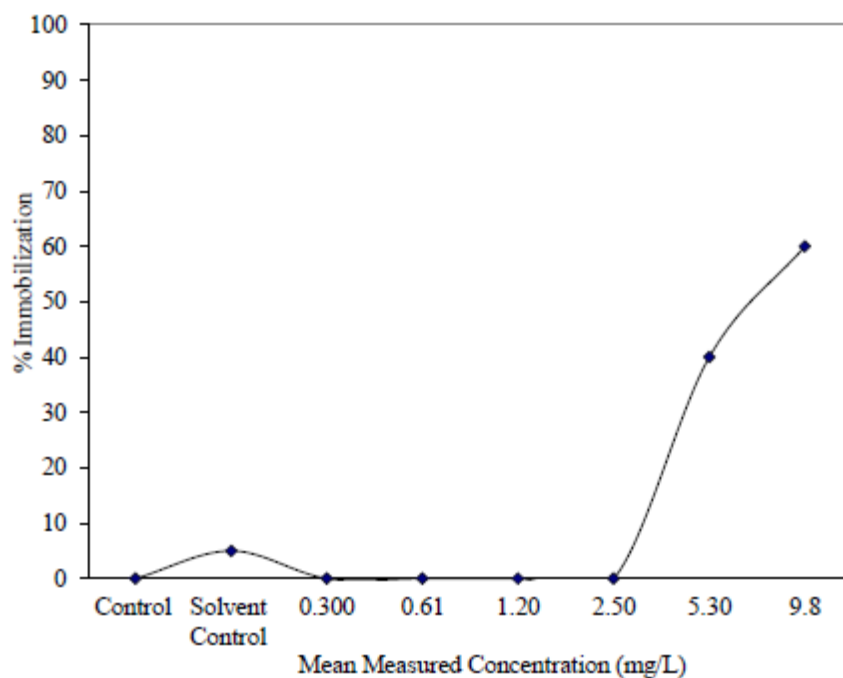


Figure 9.2.4.2-2: The 48-hour concentration-response (immobilisation) curve for the static acute exposure of daphnids (*Daphnia magna*) to SYN545547

VALIDITY CRITERIA

Table 9.2.4.1-5: Compliance with OECD 202 validity criteria

Validity criterion	Required	Obtained
Immobilisation in the controls	≤ 10 % immobilisation or other signs of disease or stress in the control(s) (dilution water control, solvent control)	Dilution water control: 0 % Solvent control: 5 %
Dissolved oxygen concentration	≥ 60 % of the air saturation value in all test vessels throughout the exposure	Dissolved oxygen concentration remained above 85 % of the air saturation throughout the test.

CONCLUSIONS

Based on mean measured concentrations, the 48 hour EC₅₀ for SYN545547 to *Daphnia magna* was 7.3 mg /L, with 95% confidence intervals of 4.5 – 12 mg /L. The 48-hour NOEC was 2.5 mg SYN545547 /L.

(██████, 2015a)

HSE evaluator comments

This study was carried out to GLP and conducted in accordance with OECD 202 (2004) and OPPTS (1996).

The following deviations to the guideline (OECD 202) were noted:

The guideline strongly recommends that the daphnids used in the definitive test are not first brood progeny to limit variability. It's not clear from the study report if this was the case, however, as the culture daphnids showed no signs of stress or poor health and control survival met the validity criterion of $\leq 10\%$, this would not be cause to invalidate the study.

The water temperature of the daphnid culture for the two weeks prior to exposure initiation ranged from 21 – 23 °C. The test solution temperatures at the 24 hr testing interval also ranged from 21 – 23 °C. OECD 202 (2004) recommends that Daphnids should be kept in media ranging from 18 - 22 °C. However, as the temperature did not vary more than ± 1 °C, and the test temperatures were very similar to the culture temperatures, where no signs of stress, poor health or increased mortality were noticed, this was deemed not likely to have had any negative impact on the results.

The statistical methods used to analyse the data are in line with the guidelines, and visual inspection of the data in Figure 9.2.4.1-2 supports the calculated endpoints.

The analytical methods have been checked by HSE Chemistry specialists in Vol 3CA Part B5.1.2.6. The following was concluded: "Fit for regulatory purposes but the method is not fully validated in accordance with SANCO/3029/99 rev. 4. LOQ: 0.015 mg/L in freshwater and AAP medium".

Based on mean measured concentrations, the 48 hour EC₅₀ for SYN545547 to *Daphnia magna* was 7.3 mg /L, with 95% confidence intervals of 4.5 - 12 mg /L. The 48-hour NOEC was 2.5 mg SYN545547 /L.

Report:	K-CA 8.2.4.1 [REDACTED] & [REDACTED] (2016a), SYN548261 - Acute Toxicity to Water Fleas, (<i>Daphnia magna</i>) under Static Conditions. Report number 3201086, Smithers Viscient (ESG) Ltd.108 Woodfield Drive Harrogate North Yorkshire, HG1 4LS, UK (Syngenta File No. SYN548261_10000).
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GUIDELINES

OECD Guideline 202: *Daphnia* sp., Acute Immobilisation Test (2004)

GLP: Yes

MATERIALS

Test Material	SYN548261
Lot/Batch #:	MES 333/2
Purity:	98 % w/w
Description:	White solid
Stability of test compound:	Stable under standard conditions.
Reanalysis/Expiry date:	30 April 2017

Treatments

Test concentrations:	Dilution water control and a single nominal concentration of 100 mg metabolite/L
Solvent:	None
Positive control:	Potassium dichromate
Analysis of test concentrations:	Yes, analysis at 0 and 48 hours using HPLC analysis with UV detection

Test organisms

Species:	<i>Daphnia magna</i> Straus
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Source:	Continuous laboratory cultures, originally obtained from Smithers Viscient, Shawbury
Feeding:	None during test
Culture medium:	Elendt M4
Test design	
Test vessels:	100 mL glass beakers covered to reduce evaporation
Test medium:	Elendt M4
Replication:	2 replicates of 5 daphnids
Exposure regime:	Semi-Static
Duration:	48 hours
Environmental conditions	
Test temperature:	21.0 – 21.9 °C
pH range:	6.01 - 7.57
Dissolved oxygen:	9.21 to 9.74 mg/L (no aeration).
Total hardness of dilution water:	208 - 224 mg/L CaCO ₃ .
Lighting:	16 hours light and 8 hours dark, with a 30 minute dawn/dusk period

STUDY DESIGN AND METHODS

Experimental dates: 20 July to 24 August 2015

At the start of the test, an amount of test substance (ca 50 mg) was dissolved in 500 mL of Elendt M4 medium to give the initial 100 mg/L test solution. Dissolution was aided by 10 minute stirring followed by 10 minutes of sonication. The control consisted of dilution water only.

The immobility of the daphnids was determined by visual observations after 24 and 48 hours of exposure. Organisms unable to swim within 15 seconds after gentle agitation of the test beaker were considered to be immobile.

The pH, temperature and dissolved oxygen were measured at the start and end of the test in each test concentration and the control.

The test concentrations were verified by chemical analysis of SYN548261 at 0 and 48 hours using high performance liquid chromatography with ultra violet-visible detection.

RESULTS AND DISCUSSION

The limit of quantification (LOQ) for SYN548261 in Elendt M4 medium using this method was 0.05 mg/L. Nominal concentrations were used for the calculation and reporting of results.

Table 9.2.4.1-6: Analytical results

Nominal concentrations (mg/L)	% of nominal measured at 0 hours	% of nominal measured at 24 hours (old)	% of nominal measured at 24 hours (new)	% of nominal measured at 48 hours (old)
100	101	101	101	101

No toxic effects were observed during the test; therefore formal statistical analysis was not performed. As statistical analysis was not performed all results were derived empirically.

The highest test substance concentration where no significant immobilisation ($\leq 10\%$ immobile *Daphnia magna*) i.e. the no observed effect concentration (NOEC), based on observation of the data was also reported.

Throughout the results, numerical data may have been rounded for presentation purposes. Therefore, manual recalculation of the data may result in slightly different values to those shown.

There was no immobility observed in the dilution water control. Immobility data and estimated EC_{50} values are shown in the table below:

Table 9.2.4.1-7: Effects of SYN548261 on *Daphnia magna* following exposure for 48-hours in a semi-static test

Nominal concentration (mg/L)	Immobilised daphnids after 24 hours		Immobilised daphnids after 48 hours	
	Number	%	Number	%
Dilution water control	0	0	0	0
100	0	0	0	0
EC_{50} mg/L	>100		>100	
95 % Confidence limits	n.d.		n.d.	
NOEC	100		100	

n.d. – not determined

VALIDITY CRITERIA

Table 9.2.4.1-8 : Compliance with OECD 202 validity criteria

Validity criterion	Required	Obtained
Immobilisation and sub-lethal effects in control during test	$\leq 10\%$	0 %
Dissolved oxygen concentration at the end of the test	≥ 3 mg/L	≥ 9.21 mg/L

CONCLUSIONS

Based on nominal concentrations, the 48-hour EC_{50} for SYN548261 to *Daphnia magna* was >100 mg metabolite/L. The 48-hour NOEC was 100 mg metabolite/L.

(██████ and ██████, 2016a)

HSE evaluator comments

The study was carried out according to GLP and follows OECD 202 (2004) with no significant deviations to the guideline or the study plan. All validity criteria outlined in OECD 202 (2004) have been satisfactorily met. The reference item test results showed a 24-hour EC_{50} of 0.79 mg $K_2Cr_2O_7$ /L. This shows appropriate sensitivity of the species (OECD 202 considers an appropriate range of 0.6 to 2.1 mg potassium dichromate/L after 24 hours). No acclimation period was present in the study, the test species were taken from laboratory cultures held at the test facility.

The analytical methods have been evaluated by HSE Chemistry specialists in Vol 3CA Part B5.1.2.6. The following was concluded: “Acceptable method. LOQ: 0.05 μ g/mL in Elendt M4 water”.

The mean measured concentrations of the test solutions were maintained within 20 % of the nominal, therefore nominal concentrations are used for reporting biological results.

No immobilisation was observed at any time point during the study for any test concentration or in control. Due to the absence of treatment-related effects at any tested concentration, statistical evaluation of EC_{10/20/50} values was not possible. Therefore, the agreed endpoint suitable for use in the risk assessment is:

- **48-hour EC₅₀ = > 100 mg metabolite/L (nominal concentrations)**
- **48-hour NOEC = 100 mg metabolite/L (nominal concentrations)**

Report: K-CA 8.2.4.1 [REDACTED], 2009a. M700F001 (metabolite of BAS 700 F) - *Daphnia magna*, acute immobilization test. Report number W/10/09, Institute of Industrial Organic Chemistry Branch Pszczyna Department of Ecotoxicology, Doświadczalna 27, 43-200 Pszczyna, Poland. (Syngenta File No. CA4312_10908)

GUIDELINES

- OECD Guidelines for Testing of Chemicals, Section 2 - Effects on Biotic Systems, Method 202: *Daphnia* sp., Acute Immobilisation Test (2004)
- Official Journal of the European Communities, Directive 67/548 EC, Annex No. V, Part C: C.2. Acute toxicity for *Daphnia* (1992)

GLP: Yes

MATERIALS

Test Material M700F001 (Metabolite of BAS 700F; synonym of NOA449410)
3-(difluoromethyl)-1-methyl-1H-pyrazole-4-carboxylic acid

Lot/Batch #: L80-68
Purity: 99.2 % (± 1 %)
Description: Pale pink powder
Stability of test compound: Stable under standard conditions
Reanalysis/Expiry date: 01 August 2010

Treatments

Test concentrations: Dilution water control and nominal concentrations of 10, 18, 32, 56 and 100 mg M700F001(NO449410)/L
Solvent: None
Positive control: Potassium dichromate on a regular basis.
Analysis of test concentrations: Yes, analysis of M700F001 (NOA449410) at 0 and 48 hours using HPLC analysis with UVVIS detection.

Test organisms

Species: *Daphnia magna* Straus
Source: Standard laboratory cultures maintained at the Institute of Industrial Organic Chemistry, Branch Pszczyna, Department of Ecotoxicology, Laboratory of Aquatic Toxicology
Feeding: None during test
Culture medium: Elendt M7 medium

Test design

Test vessels: 150 mL glass beakers containing 25 mL test solution
Test medium: Elendt M7 aerated for at least 48 hours prior to test initiation
Replication: 4 replicates of 5 daphnids corresponding to a total of 20 daphnids per test concentration and control.

Exposure regime: Static
Duration: 48 hours

Environmental conditions

Test temperature: 20.3 – 21.0 °C
pH range: Test start: 6.07 to 7.00
 Test end: 6.91 to 7.25
Dissolved oxygen: Test start: 7.02 to 8.48 mg/L
 Test end: 7.61 to 7.74 mg/L
Total hardness of dilution water: Not reported
Lighting: 16 hours light and 8 hours dark.

STUDY DESIGN AND METHODS

Experimental dates: 05 to 07 May 2009

A stock solution with a nominal concentration of 1.0 mg M700F001(NOAA449410)/mL was prepared by dissolving 53 mg of M700F001 (NOAA449410) item completely in 53 mL of dilution water by stirring for 0.5 hours on a multi-position magnetic stirrer and five minutes at ultrasonic cleaner. Using this stock solution, the remaining nominal test concentrations as stated above were prepared by dilution. The control consisted of dilution water only. Test solutions were added to the test vessels and the *Daphnia* added without conscious bias. The test animals used were less than 24 hours old, progeny of 21 – 25 days old parents.

The immobility of the daphnids was determined by visual observations after 24 and 48 hours of exposure. Organisms unable to swim within 15 seconds after gentle agitation of the test beaker were considered to be immobile.

The pH, temperature and dissolved oxygen were measured at the start and end of the test in each test concentration and the control.

The test concentrations were verified by chemical analysis of M700F001 (NOAA449410) at 0 and 48 hours using high performance liquid chromatography with ultraviolet-visible detection.

RESULTS AND DISCUSSION

At the start of the test, the measured concentrations were in the range 93.1 to 97.9 % of the nominal values and at the end of the test were in the range 94.3 to 98.6 % of the initial measured values (see table below). The limit of quantification in this study was 0.05 mg M700F001(NOAA449410)/L. Nominal concentrations were used for the calculation and reporting of results.

Table 9.2.4.1-9: Analytical results

Nominal concentrations (mg/L)	Mean (n=3) % of nominal measured at 0 hours	Mean (n=3) % of initial measured at 48 hours
Control	<LOQ	<LOQ
10	93.10	94.30
18	95.39	98.22
32	96.37	96.47
56	96.91	97.95
100	97.93	98.58

<LOQ – less than the limit of quantification, n = number of analytical measurements (range was not reported)

The median effect concentration (EC₅₀) was defined as the concentration resulting in 50 % immobilisation of the *Daphnia* in the time period specified and was calculated after 24 and 48 hours. The NOEC (No Observed Effect Concentration) is defined as the highest tested concentration which did not produce an adverse effect when compared to the control, according to the study report it was determined by Fisher's Exact Binomial Test with Bonferroni Correction. There was no immobility observed in the dilution water control. Immobility data and estimated EC₅₀ values are shown in the table below:

Table 9.2.4.1-10: Effects of M700F001 (NOA449410) on *Daphnia magna* (n=20) following exposure for 48-hours in a static test

Nominal concentration (mg/L)	Immobilised daphnids after 24 hours		Immobilised daphnids after 48 hours	
	Number	%	Number	%
Dilution water control	0	0	0	0
10	0	0	0	0
18	0	0	0	0
32	0	0	0	0
56	1*	5	1*	5
100	0	0	0	0
EC ₅₀ mg/L	> 100		> 100	
95% Confidence limits	Not reported		Not reported	
NOEC mg/L	100		100	

* Immobilized due to a handling problem

VALIDITY CRITERIA

The following validity criteria are set out in the guideline OECD 202 (2004):

Table 9.2.4.1-11: Compliance with OECD 202 validity criteria

Validity criterion	Required	Obtained
Immobilisation in the control(s)	≤ 10 % immobilisation or other signs of disease or stress (for example, discolouration or trapping at surface water)	0 % immobilisation. No abnormal signs or behavior were recorded.
Dissolved oxygen concentration	≥ 3 mg/L in control and test vessels at the end of the test	7.61-7.74 mg/L.

CONCLUSIONS

Based on nominal concentrations, the 48-hour EC₅₀ for M700F001 (NOA449410) to *Daphnia magna* was > 100 mg/L. The 48-hour NOEC was 100 mg/L, the highest concentration tested. (██████, 2009a)

HSE evaluator comments

This study was assessed using Guideline OECD 202 (2004). The study was conducted in compliance with Principles of GLP.

Data for a positive control acute toxicity test of *Daphnia magna* using reference substance potassium dichromate was supplied for the dates 23.04.2009-25.04.2009, approximately 2 weeks before the definitive test. This test showed an immobilisation 24-hour EC₅₀ of 1.05, which is within the range of 0.6-2.1 mg/L as specified in OECD 202 guideline.

There were some minor deviations from the OECD 202 (2004) guidelines, however these did not affect the outcome of the study as detailed below:

- The study did not specify whether they monitored for abnormal behaviour and signs of stress in addition to mortality/immobilisation, and none were reported.
- The study did not state whether they covered the test vessels, which is recommended to prevent water evaporation and dust entry. However, the stability of the test substance concentrations and the absence of immobilisation in the test vessels shows that neither of these things were a problem and did not affect the outcome of the study.
- It is noted that the single immobilisation occurring at the nominal concentration of 56 mg metabolite/L was due to a handling error. The absence of immobilisation in the highest nominal concentration supports this claim.

Analytical measurements of the test substance in the test solution were carried out at the start and end of the test. It was unclear at the end of the study exactly how samples for chemical analysis were collected. The report states: ‘the samples of each test concentration and the control for analysis at t48 were collected from the test vessels’. This suggests the samples were pooled prior to analysis, particularly since there were four replicates in the study and three analytical measurements taken per group. It is not specifically stated that any replicates were excluded and given the relatively high recoveries HSE has not considered this further. The mean measured concentrations of test substance ranged from 93.10 - 98.58 % of nominal, which is within the acceptable range of 80-120 % set out in the OECD 202 (2004) guideline, therefore the authors presented their results using nominal concentrations. The analytical methods have been evaluated by HSE Chemistry specialists in Vol. 3CA Part B5.1.2.6. The following was concluded for this method: “Acceptable method. LOQ: 0.05 µg/mL in water”.

Due to the study results it was not possible to statistically determine EC_{10/20/50} values.

There were no major deviations from the OECD 202 (2004) guideline and the study fulfils all validity criteria of this guideline.

Therefore, the agreed endpoint for M700F001 (NOA449410) suitable for use in risk assessment is:

- **48-hour EC₅₀ > 100 mg metabolite/L (nominal concentration)**

The agreed NOEC for M700F001 (NOA449410) is:

- **100 mg metabolite/L (nominal concentration)**

B.9.2.4.2. Acute toxicity to additional aquatic invertebrate species

Report:	K-CA 8.2.4.2 [REDACTED] (2016). SYN545974 – Acute Toxicity to Mysid (<i>Americamysis bahia</i>), Under Static Conditions, Report number 1781.6838, Smithers Viscient, 790 Main Street, Wareham, MA 02571-1037, USA. (Syngenta File No. SYN545974_10015; updated to included Amendment 1)
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GUIDELINES

US EPA Ecological Effects Test Guidelines, OPPTS 850.1035: Mysid Acute Toxicity Test (1996)

US EPA Ecological Effects Test Guidelines, OPPTS 850.1000: Special Considerations for Conducting Aquatic Laboratory Studies (1996)

GLP: Yes

MATERIALS

Test material SYN545974 tech.

Lot/Batch #: 2637-BA/110

Purity: 99.5%

Treatments

Test concentrations: Dilution water control, solvent control (0.10 mL DMF/L) and nominal concentrations of 0.063, 0.13, 0.25, 0.50 and 1.0 mg a.s./L (mean measured concentrations: 0.056, 0.11, 0.23, 0.46 and 0.99 mg a.s./L)

Dilution water: Filtered (5 µm) seawater collected from Cape Cod Canal, Bourne, Massachusetts

Solvent: Dimethylformamide (DMF, CAS No. 68-12-2)

Analysis of test concentrations: Yes, at 0 and 96 hours (all treatment levels and the dilution water and solvent controls) based on measurements of SYN545974 using LC-MS/MS analysis.

Test organisms

Species: Saltwater mysid, *Americamysis bahia*

Source: Test facility-maintained cultures, from brood stock originally obtained from MBL Aquaculture, Sarasota, Florida, U.S.A.

Acclimatisation period: Adults acclimated for 14 days prior to collection of juveniles

Mortality/observations during acclimation: None reported

Treatment for disease: None

Life stage of test organism: Juveniles <24 hours old

Feeding: Live brine shrimp nauplii (*Artemia salina*) daily during test

Test design

Test vessels: 1.0-L glass beakers containing 0.9 L of test solution

Test medium: Filtered natural seawater diluted with laboratory well water to a salinity of 20 ± 3‰. Total organic carbon: 1.4 mg/L

Introduction of test organisms: 10 mysids per control and test concentration, selected impartially and added two at a time to each vessel until each vessel contained 10 mysids

Replication: 2 replicates; 10 mysids per replicate

Exposure regime: Static

Duration: 96 hours

Environmental conditions

Test temperature: 24 - 25 °C

pH: 7.8 – 8.2

Dissolved oxygen: 5.1 – 7.3 mg/L (60% of saturation is 4.4 mg/L at 25°C)

Salinity of dilution water: 20‰

Lighting: 830 – 970 lux.

16 hours fluorescent light and 8 hours dark with 30-minute transition periods

STUDY DESIGN AND METHODS

Experimental dates: 20 to 24 April 2012

A 10 mg a.s./mL primary stock solution was prepared by placing 0.2502 g of SYN545974 in a 25-mL volumetric flask, bringing it to volume with dimethylformamide (DMF), and mixing by inversion for approximately one minute. Four additional stock solutions were prepared from the primary stock solution, and these were used to prepare the test solutions, which were observed to be clear and colourless with no visible undissolved test substance. The solvent control was prepared by adding 0.2 mL of DMF to 2.0 L of dilution water, and the water control was prepared with natural filtered seawater containing no test substance or solvent. The study was performed under static conditions.

At the start of the test mysids were randomly allocated, two at a time, to each test and control vessel until each vessel contained 10 organisms. There were 2 vessels per treatment and control. The test was conducted in a temperature-controlled water-bath set to maintain a temperature range of $25 \pm 2^\circ\text{C}$, and observations for mortalities and symptoms of toxicity were made at 0, 24, 48, 72 and 96 hours. Additionally, 3 quality control samples were prepared at each sampling interval.

Daily measurements of the test solutions were undertaken throughout the 96-hour period for pH, temperature, dissolved oxygen concentration and salinity.

The test concentrations were verified by chemical analysis of SYN545974 at 0 and 96 hours using an LCMS/MS method.

RESULTS AND DISCUSSION

Mean measured concentrations ranged from 85 to 99% of nominal values (see table below). Analysis of quality control samples resulted in measured concentrations in the range of 95 to 120% of the nominal fortified values confirming that the appropriate precision and quality control was maintained. The limit of quantification in this study was $0.151 \mu\text{g a.s./L}$. Mean measured concentrations were used for the calculation and reporting of results.

Table 9.2.4.2-1: Analytical results

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 hours	96 hours		
Dilution water control	< 0.0049 ^b	< 0.0045	NA	NA
Solvent control	< 0.0049	< 0.0045	NA	NA
0.063	0.056	0.057	0.056	90
0.13	0.11	0.11	0.11	85
0.25	0.23	0.23	0.23	92
0.50	0.48	0.43	0.46	91
1.0	1.0	0.97	0.99	99

^a Mean and percent of nominal are based on the original raw data and not the rounded results presented in this table. ^b Concentrations expressed as less than values were below the limit of quantification (LOQ). The LOQ for each analysis is dependent upon the linear regression, the area of the low standards and the dilution factor of the controls. NA = Not Applicable

The median lethal concentration (LC_{50}) was defined as the concentration resulting in 50% mortality of the test organism population in the time period specified. If at least one concentration caused mortality of $\geq 50\%$ of the test population then a computer programme (Ives, 2011) was used to calculate the LC_{50} values and 95% confidence intervals. The 96-hour LC_{50} was determined using trimmed Spearman-Kärber estimates. The NOEC (No Observed Effect Concentration) was defined as the highest concentration tested that showed no difference from the control organisms and was determined by visual inspection of the data.

Mortalities were observed in all test concentrations, with 100% mortality observed at the mean measured concentrations of $\geq 0.46 \text{ mg a.s./L}$ after 24 hours, and 90% and 95% mortality in the 0.23 mg a.s./L treatment

level after 72 and 96 hours, respectively. Mortality in the 0.23 mg a.s./L treatment level at 24 and 48 hours was observed to be 5% and 50%, respectively, and in the 0.11 mg a.s./L treatment level was 5% at 96 hours. Mortality of 50% was observed in one replicate of the 0.056 mg a.s./L treatment level after 72 hours, while in the other replicate no mortality was observed. Since the NOEC was considered to be the next higher concentration, the vessel was considered to be compromised and the observed mortality not to be toxicant related. Mortality was 10% in the solvent control and 5% in the dilution water control. ASTM (2002) allows a response $\leq 10\%$ in control populations.

The mortality data and estimated LC₅₀ values are shown in the table below:

Table 9.2.4.2-2: Effects of SYN545974 on saltwater mysids (*Americamysis bahia*) following exposure for 96 hours in a static test

Nominal concentration (mg a.s./L)	Mean measured concentration (mg a.s./L)	Mean cumulative mortality (%)			
		24 hours	48 hours	72 hours	96 hours
Dilution water control	-	0	0	5	5
Solvent control	-	0	0	10	10
0.063	0.056	0	0	25	25 ^b
0.13	0.11	0	0	5	5
0.25	0.23	5	50	90 ^a	95
0.50	0.46	100	100	100	100
1.0	0.99	100	100	100	100
LC ₅₀ (mg a.s./L)		0.31	0.23	0.16	0.16
95% confidence interval (mg a.s./L)		0.29 – 0.34	0.19 – 0.27	0.15 – 0.18	0.15 – 0.17
NOEC (mg a.s./L)		-	-	-	0.11

LC₅₀ values were determined by Spearman-Kärber estimates (24 and 48 hours) and by Trimmed Spearman-Kärber estimates (72 and 96 hours)

^a One surviving mysid was observed to be lethargic ^b Mortality is not consistent with associated replicate (replicate A 0% mortality; replicate B 50% mortality) and therefore vessel is considered to be compromised. Mortality is not considered to be toxicant related.

VALIDITY CRITERIA

The validity criteria outlined in OPPTS 850.1035 were satisfied:

- All test vessels (and retention chambers) were identical
- Treatments were randomly assigned to individual test vessel locations and test organisms were randomly assigned to test vessels
- A dilution water control and solvent (vehicle) control was included in the test
- No more than 10 % of organisms in the dilution water or vehicle control showed signs of disease, stress (e.g., discolouration, unusual behaviour, immobilization), and/or death
- No surfactant or dispersant was used in the preparation of a stock or test solution

CONCLUSIONS

Based on mean measured concentrations, the 96-hour LC₅₀ was 0.16 mg a.s./L, with 95% confidence intervals of 0.15 to 0.17 mg a.s./L. The 96-hour NOEC was determined to be 0.11 mg a.s./L.

(██████████, 2016)

HSE evaluator comments

This study has been conducted to GLP and follows OPPTS 850.1035 with no significant deviations from the protocol. The validity criteria have been fully satisfied.

Since a static design was used, endpoints should be expressed in terms of geometric mean measured values rather than arithmetic mean values. HSE calculated geometric mean measured concentrations but considered the values equivalent to the mean measured concentrations presented above. The use of mean measured values is therefore considered acceptable.

The NOEC was determined to be 0.11 mg a.s./L, despite the lower concentration (0.056 mg a.s./L) resulting in greater mortality. However, mortality was only observed in one of the two replicates and the vessel was considered to be compromised. The data was still considered in statistical evaluation of the LC₅₀, resulting in a more conservative estimation of the endpoint (0.16 mg a.s./L when included, 0.17 mg a.s./L when excluded). HSE has considered the additional information supplied by the applicant (KCA 8.2.4.2/02) and considers this approach acceptable.

The analytical methods have been checked by HSE Chemistry specialists in Vol 3CA Part B5.1.2.6. The following was concluded: “Fit for regulatory purposes but the method is not fully validated in accordance with SANCO/3029/99 rev. 4. LOQ: 0.3 µg/L in aqueous matrices”.

The 24- and 48-hour LC₅₀ values were determined using Spearman-Kärber estimates and the 96- and 72-hour LC₅₀ values were determined statistically using Trimmed Spearman-Kärber estimates. These methods are in line with those outlined in OPPTS 850.1000. The NOEC was determined by visual inspection of the data, so no evaluation of statistical methodology is required.

The endpoints suitable for use in risk assessment are:

- **96-hour LC₅₀ = 0.16 mg SYN545974/ L (based on mean measured concentrations)**

Additional information requested by the RMS is included in the report below.

Report:	K-CA 8.2.4.2, ██████████ (2016a) SYN545974: Response to ANSES comments regarding the acute toxicity test with mysids (<i>Americamysis bahia</i>) (██████████, 2016) (Syngenta File No. SYN545974_10451)
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Summary

The RMS requested the following comments to be addressed:

This study is valid but it is RMS opinion that the vessel B should not be excluded. Please provide three calculations of EC50: one with all vessels (including 0.056 mg a.s./L), one excluding 0.056 mg a.s./L and one excluding 0.11 mg a.s./L. The most conservative EC50 among EC50 values that have confidence intervals in a reasonable range should be used in the risk assessment.

Syngenta response

It is noted that mortality in the 0.056 mg a.s./L treatment level was observed in one replicate only, but as the next higher test concentration was considered to be the NOEC, this mortality was not considered to be toxicant related. The NOEC was determined by visual inspection of the data. The NOEC is defined as the highest concentration tested at which there were no toxicant-related mortalities or physical and behavioral abnormalities (e.g., lethargy, loss of equilibrium), with respect to the control organisms.

As the ‘B’ replicate of the 0.056 mg a.s./L treatment level was not consistent with the associated replicate, the vessel was considered to be compromised. However, the effects recorded in both test vessels were not excluded from the LC₅₀ calculation. The resulting 96 hr LC₅₀ was 0.16 mg a.s./L (95% CI: 0.15 – 0.17 mg a.s./L). This was not made clear in the study report; however the report has been amended.

The RMS requested that an LC₅₀ be calculated considering the exclusion of the 0.11 mg a.s./L treatment level. However, there is no scientific rationale for excluding this treatment level from the LC₅₀ calculation. The RMS

also requested that an LC₅₀ be calculated considering the exclusion of the 0.056 mg a.s./L treatment level. Considering the effects seen were considered not treatment related, this may be acceptable. The resulting 96 hr LC₅₀ was 0.17 mg a.s./L (95% CI: 0.15 – 0.18 mg a.s./L).

In summary, the mortality effects in the ‘B’ replicate of the 0.056 mg a.s./L treatment level was not consistent with the associated replicate and as such, not considered to be toxicant related. However, this treatment level and the effects recorded in both test vessels were not excluded from the LC₅₀ calculation in the report. The resulting 96 hr LC₅₀ was 0.16 mg a.s./L (95% CI: 0.15 – 0.17 mg a.s./L). Alternatively, the 0.056 mg a.s./L treatment level could conceivably be excluded from the LC₅₀ calculation due to inconsistency with the data between replicates and among treatment levels (i.e. non-monotonic response). The resulting 96 hr LC₅₀ is 0.17 mg a.s./L (95% CI: 0.15 – 0.18 mg a.s./L).

The originally reported 96 hr LC₅₀ value of 0.16 mg a.s./L will continue to be used in the risk assessment.

(██████████, 2016a)

Report: K-CA 8.2.4.2 ██████████ (2014a). SYN545974 – Toxicity to Eastern Oyster (*Crassostrea virginica*) Under Flow-Through Conditions, Report number 1781.6885, Smithers Viscient, 790 Main Street, Wareham, MA 02571-1037, USA. (Syngenta File No. SYN545974_10099)

GUIDELINES

US EPA Ecological Effects Test Guideline, OPPTS 850.1025: Oyster Acute Toxicity Test (Shell Deposition). (1996)

GLP: Yes

MATERIALS

Test material	SYN545974 tech.
Lot/Batch #:	SMU2EP12007
Purity:	98.5 % w/w (tested as 100 %)
Treatments	
Test concentrations:	Dilution water control, solvent control (0.010 mL DMF/L) and nominal concentrations of 0.063, 0.13, 0.25, 0.50 and 1.0 mg SYN545974/L (mean measured; 0.083, 0.15, 0.25, 0.41 and 0.95 mg SYN545974/L)
Dilution water:	Natural filtered seawater
Solvent:	Dimethylformamide (DMF, CAS No. 68-12-2)
Analysis of test concentrations:	Yes, at 0 and 96 hours (all treatment levels and the controls) based on measurements of SYN545974 using LC-MS/MS analysis
Test organisms	
Species:	Eastern oyster, <i>Crassostrea virginica</i>
Source:	Northside Shellfish, Barnstable, Massachusetts
Acclimatisation period:	10 days, < 1 % mortality observed during 7 days prior to test initiation
Treatment for disease:	None reported
Life stage of test organism:	Reproductively immature, mean valve height 37 ± 3.3 mm
Feeding:	Algae (<i>Tetraselmus maculata</i>) three times daily. Average concentration maintained at 104 cells/mL in the test solutions.
Test design	

Test vessels:	Glass aquaria measuring 49.5 x 25.5 x 29 cm, with an overflow drain at a height of 14 cm maintaining a test solution volume of approximately 18 L
Test medium:	Filtered natural seawater
Replication:	One replicate of 20 oysters, per treatment level and control
Exposure regime:	Flow-through using a constant-flow serial diluter (Benoit, et al., 1982). Flow rate was 75 mL/minute, providing approximately 6 solution volume replacements per day. Recirculation flow rate was 1.75 L/minute.
Duration:	96 hours
Environmental conditions	
Test temperature:	21 – 23 °C
pH:	7.5 – 8.1
Dissolved oxygen:	4.5 – 7.2 mg/L. Gentle aeration was initiated in all test vessels at the 24-hour observation interval.
Salinity of dilution water:	18 – 20 ‰
Lighting:	200 – 2200 lux. 16 hours fluorescent light and 8 hours dark with transition periods

STUDY DESIGN AND METHODS

Experimental dates: 21 to 25 June 2013

A 10 mg a.s./mL diluter stock solution was prepared by placing 3.0558 g of SYN545974 in a 300 mL volumetric flask and bringing it to volume with dimethylformamide (DMF). A 0.50 mL/mL solvent stock solution was prepared by bringing 125 mL of DMF to a final volume of 250 mL with deionised water. The diluter stock solution was delivered into the chemical mixing chamber of the constant-flow serial diluter at a rate of 0.015 mL/minute, together with 0.075 L/minute of dilution water, and the contents continuously stirred using a magnetic stirrer, stir bar, and water-driven magnetic stir plate partially submerged in an ultrasonic water bath. The concentration of the active ingredient in the mixing chamber was equivalent to 1.0 mg a.s./L (the highest nominal test concentration) and was serially diluted to produce the remaining nominal test concentrations.

At the start of the test 20 oysters were randomly allocated to each test aquarium. They were placed equidistant from each other with the left (convex) valve down, and with their valve inflow openings toward the flow from the circulator tube. Aquaria were placed in a temperature-controlled water bath designed to maintain a temperature of 20 ± 2 °C. Biological observations were made at 0, 24, 48, 72 and 96 hours. At the end of the exposure period, new shell growth was measured microscopically to the nearest 0.1 mm using a calibrated micrometer.

Daily measurements of the test solutions were undertaken throughout the 96-hour period for pH, temperature, dissolved oxygen concentration and salinity. The test concentrations were verified by chemical analysis of SYN545974 at 0 and 96 hours using an LC-MS/MS method. Additionally, three quality control samples were prepared at each sampling interval.

RESULTS AND DISCUSSION

Mean measured concentrations ranged from 82 to 130 % of nominal values. Analysis of quality control samples resulted in measured concentrations in the range of 86.4 to 101 % of the nominal fortified levels, confirming the appropriate precision and quality control was maintained. The limit of quantification in this study was 0.0045 and 0.0050 mg a.s./L, at 0 hours and 96 hours, respectively. Mean measured concentrations were used for calculation and reporting of results.

Table 9.2.4.2-3: Analytical results

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 hours	96 hours		
Dilution water control	< LOQ ^b	< LOQ	NA	NA
Solvent control	< LOQ	< LOQ	NA	NA
0.063	0.063	0.10	0.083	130
0.13	0.12	0.19	0.15	120
0.25	0.21	0.30	0.25	100
0.50	0.33	0.49	0.41	82
1.0	0.92	0.97	0.95	95

^a Mean measured and percent of nominal are based on the original raw data and not the rounded results presented in this table. ^b LOQ = limit of quantification. The LOQ for each analysis is dependent upon the linear regression, the area of the low standards and the dilution factor of the controls. LOQ at 0-hour and 96-hour were 0.0045 and 0.0050 mg a.s./L, respectively. NA = Not Applicable

The EC₅₀ is defined as the estimated concentration of test substance in seawater which reduced shell deposition (growth) of the exposed oysters by 50 %, as compared to control oysters. The mean measured exposure concentrations and the corresponding biological-response data derived from the definitive 96-hour test were used to statistically estimate an EC₅₀ (and corresponding 95 % confidence intervals) using a non-linear regression. After comparison using a t-test, control data were pooled for comparison of the treatment responses.

No mortality was observed among oysters exposed to any of the treatment levels, and no mortality or sublethal effects were observed among oysters in the control or solvent control. A summary of the results of shell growth analyses are presented in the table below.

Table 9.2.4.2-4: Effects of SYN545974 on the survival and shell deposition of the eastern oyster (*Crassostrea virginica*) following exposure for 96 hours under flow-through conditions

Mean measured concentration (mg a.s./L)	Mean mortality (%)	Shell deposition ^a (mm)		Mean reduction (%)
		Mean	Standard deviation	
Control	0	1.5	0.8	-
Solvent Control	0	1.4	0.7	-
Pooled Control	0	1.4	0.7	-
0.083	0	1.2	0.7	16
0.15	0	1.1	0.8	20
0.25	0	1.0	0.6	27
0.41	0	0.39	0.4	73
0.95	0	0.16	0.2	89
96-hour EC₅₀ Growth (mg a.s./L)	0.31			
95% confidence interval (mg a.s./L)	0.24 – 0.39			

^a mean shell deposition represents the measurements of 20 oysters per treatment

VALIDITY CRITERIA

The validity criteria outlined in OCSPP 850.1025 (2016) were partially fulfilled:

Table 9.2.4.2-5: Compliance with OCSPP 850.1025 validity criteria

Validity criterion	Required	Observed
Test vessels throughout test	Identical	Identical
Assignment of treatments and individuals to test vessels	Random	Random
Control groups	Dilution water control and (if vehicle (solvent) is used) vehicle control included.	Dilution water and solvent control used
Signs of disease, stress (<i>e.g.</i> , shell gaping, excessive mucus), and/or death in control during test	< 10 %	0
Shell growth in control groups	A mean of at least 2 mm of new shell growth observed in each control group	Dilution water control: 1.5 mm Solvent control: 1.4 mm
Preparation of stock/ test solutions	No use of surfactant or dispersant in preparation of stock/test solutions.	None used
Evidence of spawning	No evidence of spawning	None noted

CONCLUSIONS

Based on mean measured concentrations, the 96-hour EC₅₀ was determined to be 0.31 mg a.s./L, with 95% confidence intervals of 0.24 to 0.39 mg a.s./L.

(██████████, 2014a)

HSE evaluator comments

The study was carried out in accordance with GLP and follows OCSPP 850.1025 (2016). The full validity criteria for this test have not been met, since an overall mean of at least 2 mm of new shell growth was not observed in each control group, as stipulated in OCSPP 850.1025 (2016). The applicant provided historic data showing the mean shell growth of control oysters during previous 96-hour studies, arguing that the data presented here falls within the historic range (1.3-4.3 mm) and is therefore representative of the species. However, 77 % of the historic studies did record growth in the control oysters of ≥ 2 mm, and the overall mean was 2.5 ± 0.6 mm, which would indicate that the validity criterion of 2 mm is appropriate for this species. As such, this historic data does not adequately justify the failure to meet the validity requirements set out in OCSPP 850.1025 (2016).

Three additional deviations from the study guidelines are noted by the applicant. Firstly, the water temperature of test solutions ranged from 20 to 23 °C and was therefore not maintained at 20 ± 2 °C as stipulated in OCSPP 850.1025 (2016). Secondly, at the 96-hour interval the total dissolved oxygen concentration dropped below the 60 % saturation level stipulated in OCSPP 850.1025 (2016) and was corrected by aeration at the 24-hour interval. Thirdly, an acclimatisation period of ten days, rather than the suggested twelve to fifteen days, was implemented. The light intensity spanned a range of 200-2200 lux, including light intensities outside the range (540 to 1080 lux) stipulated in OCSPP 850.1025 (2016). The applicant did not provide a measure of total organic carbon in the dilution water.

The analytical methods have been checked by HSE Chemistry specialists in Vol 3CA Part B5.1.2.6. The following was concluded: "Fit for regulatory purposes but the method is not fully validated in accordance with SANCO/3029/99 rev. 4. LOQ: 0.3 µg/L in aqueous matrices".

At present, the EC₅₀ value is not suitable for use in risk assessment, since the validity criteria outlined in OCSPP 850.1025 (2016) have not been fulfilled.

Report:	K-CA 8.2.4.2 ██████████ (2015). SYN545974 – Acute Toxicity of SYN545974 to <i>Asellus aquaticus</i> , Report number CEA.1644, Cambridge Environmental Assessments, ADAS Boxworth, Battlegate Road, Boxworth, Cambridgeshire, CB23 4NN, UK. (Syngenta File No. SYN545974_10305)
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GUIDELINES:

The study was not conducted according to any specific regulatory guideline, but the following was consulted: OECD Guidelines 202: Daphnia sp., Acute Immobilisation Test (2004)

GLP: Yes

MATERIALS

Test material SYN545974 technical
Description: Off-white powder
Lot/Batch #: SMU2EP12007
Purity: 98.5 %
Stability of test compound: Stable under test conditions
Reanalysis/Expiry date: 30th June 2016

Treatments

Test concentrations: 0.427, 0.939, 2.07, 4.55 and 10 mg a.s. /L nominal
(0.328, 0.700, 1.21, 3.07, and 6.88 mg a.s. /L mean measured)
Dilution water: Filtered (30 µm) mesocosm water
Vehicle and/or positive control: dimethylformamide, DMF,
None
Solvent control: 0.1 mL /L DMF
Analysis of test concentrations: Yes at 0 and 48 hours

Test organisms

Species: *Asellus aquaticus*
Source: Test facility

Acclimatisation period: 7 days

Treatment for disease: None

Life stage of test organism: Juvenile

Feeding: *Elodea* sp. and alder leaves – no feeding during test

Test design

Test vessels: 120 mL glass beakers each containing 60 mL of the control medium
Replication: 20 replicates of 1 individual
Exposure regime: Static
Duration: 48 hours

Environmental conditions

Test temperature: 19.8 to 20.7 °C
pH range: 8.03 to 8.29
Dissolved oxygen: 90.3 to 98.6 %
Lighting: 16 hours fluorescent light and 8 hours dark daily
Light intensity: 601 lux

STUDY DESIGN AND METHODS

Experimental dates: 13 July to 6 August 2015

Juvenile (< 28 days old) *Asellus aquaticus* were used as the test organism. Prior to experimentation, adult *Asellus aquaticus* were acclimatised to the test conditions for 7 days, and any juveniles produced were isolated prior to use in the definitive test. No feeding took place during the test.

The filtered mesocosm water used as the dilution water in this study was from the same source as the water used during acclimation, which conforms to the characteristics of an acceptable dilution water listed in OECD 202 (2004). The temperature (°C), pH, and dissolved oxygen concentration (% ASV, Air Saturation Volume) were measured at the start and end of the test in each test concentration and the control groups. The concentrations of SYN545974 in the test solutions were measured using LC-MS/MS.

A static test design was implemented, using loosely covered glass beakers containing the test and control solutions. At the start of the test, a primary solvent stock solution (100 mg /mL) was prepared by dissolving 1 g of SYN545974 into 10 mL of DMF. Further solvent stock solutions were prepared by serial dilution of the primary stock in DMF to give dosing solutions of 4.27, 9.39, 20.7, and 45.5 mg a.s /mL. All stock solutions were mixed by inversion for approximately one minute, or until no undissolved test item was visible.

In addition to the primary stock of 100 mg /mL of SYN545947, the dosing solutions were used to provide the test media at 0.427, 0.939, 2.07, 4.55 and 10 mg /L, respectively, by the addition of 0.2 mL of the solvent stock solutions into individual 2 L volumetric flasks containing 2 L of filtered (30 µm) mesocosm water using a micro-syringe. All test media were homogenised by inversion and in addition, the 2.07, 4.55, 10 mg /L test media was treated with ultrasound for 0.5, 5 and 30 minutes respectively, until no test item or undissolved material was visible prior to use in the test. Similarly, the solvent control was prepared by the addition of 0.2 mL DMF to 2 L filtered (30 µm) mesocosm water using a microsyringe and was mixed by inversion.

The test organisms were observed daily at approximate 24 hr intervals for signs of immobility and, where possible, mortality. For the purposes of this study, immobility was defined as the absence of free movement within 30 seconds following stimuli, i.e. gentle swirling of the media. As mortality is difficult to confirm in invertebrates, this was only recorded where cessation of life was certain e.g. the clear absence of any response or by obvious sign of necrosis or decomposition. It is noted that the total number of immobile organisms included the number of dead. The test organisms were observed for any behavioural/morphological abnormalities (such as slow response or abnormal colouration), but none were reported.

The Fisher's Exact Binominal Test, used to perform a pair-wise comparison between the control and solvent control, showed there was no significant difference between control groups (mortality and immobility $p(i) = 1.513$). As a result, the data were analysed in comparison to the pooled control.

The LC_{50} and EC_{50} values were determined by interpolation (Spearman-Kärber, 0 % trim) in which the confidence limits were approximated by $\pm 2 \cdot se(\ln(EC_{50}))$ where se = the standard error. For all parameters and time points, the NOEC was determined using the step-down Cochran-Armitage test procedure.

RESULTS AND DISCUSSION

Analytical results

At the start of the test, the measured concentrations were in the range 53 to 79 % of the nominal values and at the end of the test ranged from 60 to 78 % (Table 9.2.4.2-6 below). Mean measured concentrations ranged from 58 to 77 % of the nominal values. The limit of quantification (LOQ) in this study was 0.05 µg /L. Mean measured concentrations were used for the calculation and reporting of results.

Table 9.2.4.2-6: Analytical results

Nominal concentration (mg a.s. /L)	% of nominal 0 hours	% of nominal 48 hours	Mean measured concentration (mg a.s. /L)
0.427	79	75	0.328
0.939	71	78	0.700
2.07	53	64	1.21
4.55	66	69	3.07
10	78	60	6.88

Biological results

A 5 % mortality rate was observed in both the dilution water and solvent control conditions. No LC₅₀ value could be reliably calculated for mortality due to the absence of a clear dose response to treatment. The mortality data from each condition is presented in Table 9.2.4.2-7 below.

Table 9.2.4.2-7: Effects of test material on mortality of *Asellus aquaticus* following exposure for 48 hours in a static test

Mean measured concentration (mg a.s. /L)	Cumulative mortality observed (%)	
	24 hour	48 hour
Dilution water control	0	5
Solvent control	0	5
Pooled control	0	5
0.328	0	5
0.700	0	0
1.21	0	0
3.07	0	10
6.88	10	30
LC ₅₀ (95 % confidence limits)	n.d.	n.d.
NOEC	6.88	6.88

n.d. = not determined

Note: No LC₁₀, LC₂₀ or LC₅₀ values could be reliably calculated due to the absence of a clear dose response to treatment.

A significant dose related immobility response was observed at 24 and 48 hrs following exposure, EC₅₀ values were calculated and are presented in Table 9.2.4.2-8 below.

Table 9.2.4.2-8: Effects of test material on immobility of *Asellus aquaticus* following exposure for 48 hours in a static test

Mean measured concentration (mg a.s. /L)	Cumulative immobility observed (%)	
	24 hour	48 hour
Dilution water control	5	5
Solvent control	0	5

Mean measured concentration (mg a.s. /L)	Cumulative immobility observed (%)	
	24 hour	48 hour
Pooled control	2.5	5
0.328	0	5
0.700	0	0
1.21	5	0
3.07	0	15
6.88	60*	90*
EC ₅₀ (95 % confidence limits)	6.041 (4.999 – 7.300)	4.209 (3.488-5.081)
NOEC	3.07	3.07**

* A significant difference ($p < 0.05$) was observed in comparison to the solvent control

** There was 15 % immobility at 3.07 mg a.s. /L after 48 hours, NOEC amended by CA to 1.21 mg a.s. /L (refer to evaluator comments).

Note: The number of immobile organisms includes dead

Note: Test organisms were observed for any behavioural/morphological abnormalities (such as slow response or abnormal colouration), but none were reported.

Note: No 24 hr EC₁₀ or EC₂₀ values could be reliably calculated.

VALIDITY CRITERIA

Table 9.2.4.2-9: Compliance with OECD 202 validity criteria

Validity criterion	Required	Obtained
Mortality in the controls	≤ 10 % mortality in the control(s) (dilution water control, solvent control)	Dilution water control: 5 % Solvent control: 5 %
Dissolved oxygen concentration	≥ 60 % of the air saturation value in all test vessels throughout the exposure	Dissolved oxygen concentration ranged from 90.3 to 98.6 % 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 ranged from 53-79 % of nominal.

CONCLUSIONS

Based on mean measured concentrations, the NOEC for SYN545974 on the mortality and immobility of *Asellus aquaticus* was determined to be 3.07 mg a.s. /L. The 48 hour EC₅₀ was 4.209 mg a.s. /L based on immobility, with 95 % confidence intervals of 3.488 - 5.081 mg a.s. /L. No LC₅₀ values could be determined.

HSE evaluator comments

This study was conducted according to GLP. It was not conducted according to any particular guideline, although OECD 202 (2004) was consulted. The work was checked against OECD 202, all validity criteria were met, but the following deviations were noted:

The minimum water temperature recorded at the end of the test was 17.8 °C. This was marginally outside of the OECD 202 (2004) recommended range of 18 - 22 °C, however, the temperature remains within the normal ecological range for *Asellus aquaticus* and only represented a slight deviation from the recommended range. The raw data showing this temperature was not available, however, the temperature ranges provided suggest that it remained within the guideline limits. Additionally, all OECD 202 validity criteria were met, as such, this issue was deemed unlikely to have had any negative impact on the results.

Culture conditions also transiently varied from those of the test conditions. The temperature of the culture media dropped to 17.8 °C at one point, and the dissolved oxygen levels dipped to 56.4 % ASV. Light intensity also fell outside the range stated in the protocol (500 - 1000 LUX) on four tested dates in the 7 day acclimation period. The measured light intensities were 401, 200, 170, and 490 LUX. These deviations were judged to have had a negligible impact on the results produced in the study, as they were transient, only marginally outside of the recommended ranges and also, low mortality and immobility were observed in the control groups, during the range finding and definitive tests.

The OECD 202 (2004) *Daphnid* guidelines which were consulted for this study strongly recommend that the daphnids used in the definitive test are not first brood progeny to limit variability. It's not clear from the study report if first brood progeny test organisms were used, the applicant states that "Periodically, adult cultures were checked for juveniles and, where present, these were isolated into separate cultures" suggesting that first brood progeny were not used. However, as the control survival rate met the validity criterion of $\leq 10\%$, this would not be cause to invalidate the study.

As the test species is non-standard, no positive control data was provided by the applicant. It may be necessary to ask the applicant if any data is available.

The study authors state that test organisms were observed for any behavioural/morphological abnormalities (such as slow response or abnormal colouration), however, no observations were reported. It may be necessary to check with the applicant if there is any data on this.

The analytical methods have been checked by HSE Chemistry specialists in Vol 3CA Part B5.1.2.6. The following was concluded: "Fit for regulatory purposes but the method is not fully validated in accordance with SANCO/3029/99 rev. 4. LOQ: 0.05 µg/L".

The statistical methods used were in line with the guidelines. However, the EC₅₀ value was calculated by interpolation, which is not ideal considering that only the highest tested concentration produced an immobilisation response which was significantly different to the control conditions after 48 hours. This means that the accuracy of the predicted EC₅₀ value could be uncertain. Additionally, the NOEC value was reported by the study authors as 3.07 mg a.s. /L, however, inspection of the data reveals a 15 % mortality rate after 48 hours at this concentration. Although this datapoint is not significantly different from the control, it does appear to be part of a dose-response relationship. As such, the 48 hour NOEC value has been amended to 1.21 mg a.s. /L.

Based on mean measured concentrations, the NOEC for SYN545974 value for immobility of *Asellus aquaticus* was determined to be 1.21 mg a.s. /L. The 48 hour EC₅₀ was 4.209 mg a.s. /L based on immobility, with 95 % confidence intervals of 3.488 - 5.081 mg a.s. /L.

Report:	K-CA 8.2.4.2 [REDACTED] (2015). SYN545974 – Acute Toxicity of SYN545974 to <i>Chaoborus crystallinus</i> , Report number CEA.1666, Cambridge Environmental Assessments, ADAS Boxworth, Battlegate Road, Boxworth, Cambridgeshire, CB23 4NN, UK. (Syngenta File No. SYN545974_10341)
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GUIDELINES

The study was not conducted according to any specific regulatory guideline, but the following was consulted:

OECD Guidelines 202: Daphnia sp., Acute Immobilisation Test (2004)

GLP: Yes

MATERIALS

Test Material SYN545974 technical

Lot/Batch #: SMU2EP12007

Purity: 98.5 %

Description: Off-white powder

Stability of test compound: Stable under standard conditions.

Reanalysis/Expiry date: 30 June 2016

Treatments

Test concentrations: Dilution water control and nominal concentrations of 0.04, 0.088, 0.194, 0.427, 0.939, 2.07, 4.55 and 10 mg/L (corresponding to 0.0313, 0.0688, 0.162, 0.333, 0.676, 1.59, 4.30 and 6.54 mg/L mean measured)

Solvent: dimethylformamide, DMF

Positive control: None

Analysis of test concentrations: Yes, analysis at 0 and 48 hours using method GRM061.01A at CEMAS, UK

Test organisms

Species: *Chaoborus crystallinus* larvae

Age: larvae

Source: Larvae collected from Cambridge Environmental Assessments mesocosm facility

Feeding: None during test

Culture medium: Filtered (30 µm) mesocosm water

Test design

Test vessels: 60 mL glass vessels each containing 60 mL of media

Test medium: Filtered (30 µm) mesocosm water

Replication: 2 replicates of 5 individuals

Exposure regime: Static

Duration: 48 hours

Environmental conditions

Test temperature: 18.4 and 20.4 °C

pH range: 7.70 to 8.38

Dissolved oxygen: 86.6 to 93.3 % ASV

Lighting: 550 Lux
16 hours light and 8 hours dark

STUDY DESIGN AND METHODS

Experimental dates: 15 July to 18 September 2015

At the start of the test, a primary solvent stock solution (100 mg a.s./mL) was prepared by dissolving 1 g of SYN545974 into 10 mL of DMF. Further solvent stock solutions were prepared by serial dilution of the primary stock in DMF to give dosing solutions of 0.4, 0.88, 1.94, 4.27, 9.39, 20.7, and 45.5 mg a.s./mL. All stock solutions were mixed by inversion for approximately one minute, or until no undissolved test item was visible.

In addition to the primary stock of 100 mg/mL of SYN545974, the dosing solutions were used to provide the test media at 0.04, 0.088, 0.194, 0.427, 0.939, 2.07, 4.55 and 10 mg a.s./L, respectively, by the addition of 0.1 mL of the solvent stock solutions into individual 1 L volumetric flasks containing 1 L of filtered (30 µm) mesocosm water using a micro-syringe. All test media were homogenised by inversion and in addition, the 2.07, 4.55, 10 mg a.s./L test media were treated with ultrasound for 0.5, 5 and 30 minutes respectively, until no test item or undissolved material was visible prior to use in the test. Similarly, the solvent control was prepared by the addition of 0.1 mL DMF to 1 L filtered (30 µm) mesocosm water using a microsyringe and was mixed by inversion.

The test organisms were observed daily at approximate 24-hr intervals for signs of immobility and, where possible, mortality. For the purposes of this study, immobility was defined as the absence of free movement within 30 seconds following stimuli, i.e. gentle swirling of the media. As mortality is difficult to confirm in invertebrates, this was only recorded where cessation of life was certain e.g. the clear absence of any response or by obvious sign of necrosis or decomposition. For the purposes of this study, where an organism was recorded as dead, it was also recorded as immobile. Any other notable observations (such as slow response or abnormal colouration) were also recorded.

The pH, temperature and dissolved oxygen were measured daily in each test concentration and the control. The concentrations of SYN545974 in the test solutions were measured using the validated method GRM061.01A at CEMAS, UK.

RESULTS AND DISCUSSION

At the start of the test, the measured concentrations were in the range 71 to 99 % of the nominal values and at the end of the test were in the range 52 to 90 % (see table below). Mean measured concentrations were used for the calculation and reporting of results.

Table 9.2.4.2-10 : Analytical results

Nominal concentrations (mg a.s./L)	% of nominal measured at 0 hours	% of nominal measured at 48 hours	Mean measured concentrations (mg a.s./L)	Geometric mean measured concentrations calculated by HSE* (mg a.s./L)
0.04	81	76	0.0313	0.0314
0.088	84	73	0.0688	0.0689
0.194	91	76	0.162	0.1613
0.427	78	78	0.333	0.3331
0.939	71	73	0.676	0.6760
2.07	75	78	1.59	1.5832
4.55	99	90	4.30	4.2949
10	78	52	6.54	6.3687

The Fisher's Exact Binominal Test used to perform a pair-wise comparison between the control and solvent control showed there was no significant difference between control groups (mortality and immobility $p(i) = 0.1$). In any case, the diluent control was excluded prior to analysis. * Given not all test concentrations were maintained within ± 20 % of initial measured concentrations, HSE calculated geometric mean concentrations. However, the difference is minor and therefore calculating endpoints based on mean measured concentrations is considered acceptable by HSE.

The EC₅₀ values at 24 and 48 hrs were determined by interpolation (Spearman-Kärber, 0 % trim) in which the confidence limits were approximated by $\pm 2 \times \text{se}(\text{Ln}(\text{EC}_{50}))$ where se = the standard error. For all parameters and time points, the NOEC was determined using the step-down Cochran-Armitage test procedure.

Mortality

There was no significant dose related response after 24 or 48 hrs; as a result no LC_x values could be reliably determined.

Table 9.2.4.2-11: Cumulative mortality for *Chaoborus crystallinus* treated with SYN545974

Mean measured concentration (mg a.s./L)	% mortality after 24 hours	% mortality after 48 hours
Control	0	10
Solvent control	0	0
Pooled control	0	5
0.0313	0	0
0.0688	0	5
0.162	5	5
0.333	0	10
0.676	0	0
1.59	0	0
4.30	0	30
6.54	5	15
LC₅₀ (95% confidence limits)	n.d.	n.d.
NOEC	6.54	6.54

Initial population treatment = 20 (19 individuals for 0.162 mg/L)

Pooled control = 40 n.d. – not determined

Immobility

A significant dose related response was observed at 24 and 48 hrs following exposure and as a result, the EC₅₀ values are presented in the table below.

Table 9.2.4.2-12: Cumulative immobility for *Chaoborus crystallinus* treated with SYN545974

Mean measured concentration (mg a.s./L)	% immobility after 24 hours	% immobility after 48 hours
Control	10	15
Solvent control	10	10
Pooled control	10	12.5
0.0313	10	5
0.0688	5	10
0.162	5	5

Mean measured concentration (mg a.s./L)	% immobility after 24 hours	% immobility after 48 hours
0.333	15	15
0.676	35*	25
1.59	15*	10
4.30	50*	60*
6.54	50*	60*
EC ₅₀ (95 % confidence limits)	2.496 (1.729 – 3.604)	2.489 (1.759 – 3.524)
NOEC	0.333	1.59

Note: the number of immobile organisms includes dead

Initial population treatment = 20 (19 individuals for 0.162 mg/L) Pooled control = 40

* Statistically different from pooled control (p < 0.05)

CONCLUSIONS

Statistical analyses of the available data for mortality revealed that no LC_x values could be reliably calculated. As a result, the NOEC was considered to be greater than 6.54 mg a.s./L.

The 48 hr NOEC for SYN545974 on the immobility of *Chaoborus crystallinus* was determined to be 1.59 mg a.s./L, and the 48 hour EC₅₀ was 2.489 mg a.s./L, based on mean measured concentrations.

(██████, 2015)

HSE evaluator comments

The study was carried out according to GLP.

There is no agreed OECD guideline for *Chaoborus crystallinus*. As such it was not possible to confirm validity criteria, generating some uncertainty. Instead, the study author based the test on OECD 202 (2004). The immobility of *Chaoborus* in the pooled control was 12.5 %, marginally higher (exceedance equivalent to one additional immobilised organism) than 10 % stated in OECD 202 (2004) guidelines for *Daphnia*. The consideration has been presented below. Given the relatively low immobilisation in the control, HSE considers it is unlikely to have significantly impacted the derived endpoint (EC₅₀).

Validity criteria for different species (<i>Daphnia</i> OECD 202, 2004)	Required	Obtained in <i>Chaoborus</i> sp study
Immobilisation and sub-lethal effects in control during test	≤ 10 %	12.5 %
Dissolved oxygen concentration at the end of the test	≥ 3 mg/L	≥ 7.01 mg/L

Chaoborus were acclimatised for a period of at least a day. This is considered acceptable by HSE as the *Chaoborus* were cultivated in culture conditions similar to those used in test conditions.

The identification of immobility of organisms used in this study was no movement 30 seconds after stimulus. OECD 202 (2004) defines immobility as no movement 15 seconds after stimulus. There are no validated guidelines for *Chaoborus*. It was noted this study's immobility definition is less protective than that for a *Daphnia* study (OECD 202) i.e. double the time is allowed before organism is considered immobile. Nonetheless this is unlikely to result in major differences in endpoints given the relatively short time of assessments (increase in duration of 15 seconds).

The solvent DMF was used to dissolve the active substance in this study. Preparation of the stock solutions followed the OECD 23 guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures (2000). Deviation from these guidelines involved assessing solubility by visibility of substance. This is not

recommended in OECD 23 (2000). However, as the study has included analytical information for the active substance, it can be considered that this had no impact on the derived endpoints.

There were some discrepancies in the report. In the body of the text the pooled control mortality was quoted as 15 %, however, in Table 3 (in study report) this was shown as 5 %. The raw data showed there were 2 mortalities out of a total of 40 individuals, corresponding with a figure of 5 %. Additionally, the nominal concentration in Table 2 (in study report) was listed as 0.004 mg a.s./L, but in other areas of the report it is listed as 0.04 mg a.s./L. After considering the methodology the correct concentration appears to be 0.04 mg a.s./L, which is also confirmed by supporting analytical data.

The analytical methods have been checked by HSE Chemistry specialists in Vol 3CA Part B5.1.2.6. The following was concluded: “Fit for regulatory purposes but the method is not fully validated in accordance with SANCO/3029/99 rev. 4. LOQ: 0.05 µg/L”.

A validated guideline for this species is not available. However, the statistical analysis method used is in-line with other comparable OECD guidelines. The reporting of the analysis was brief and it was unclear whether the data was transformed prior to analysis. Nonetheless, the resulting EC₅₀ is considered appropriate by HSE and supported by the experimental data.

The mean measured concentrations of the test solutions were not maintained within 20 % of the nominal concentrations or initial measured concentrations for all test concentrations. Therefore, ideally geometric mean measured concentrations would have been calculated. However, in this case the difference is minor therefore HSE considers endpoints based on mean measured concentrations acceptable.

There is an interrupted data response making the mortality NOEC unclear. It was also noted there was some recovery of the *Chaoborus* between 24 hours and 48 hours when measuring immobility raising some uncertainty. The agreed endpoints suitable for use in the risk assessment are:

- **48-hour NOEC = 1.59 mg a.s./L (mean measured concentration)**, noting interrupted dose response.
- **48-hour EC₅₀ = 2.489 mg a.s./L (mean measured concentration)**, noting uncertainties detailed above.

Report:	K-CA 8.2.4.2 [REDACTED], (2015a). SYN545974 – Acute Toxicity of SYN545974 to <i>Chironomus riparius</i> , Report number CEA.1667, Cambridge Environmental Assessments, ADAS Boxworth, Battlegate Road, Boxworth, Cambridgeshire, CB23 4NN, UK. (Syngenta File No. SYN545974_10316)
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GUIDELINES

The study was not conducted according to any specific regulatory guideline, but the following was consulted:

- OECD Guidelines 202: *Daphnia* sp., Acute Immobilisation Test (2004)

GLP: Yes

MATERIALS

Test Material:	SYN545974
Lot/Batch #:	SMU2EP12007
Purity:	98.5 %
Description:	Off-white powder
Stability of test compound:	Stable under standard conditions.
Reanalysis/ Expiry date:	30 June 2016
Density:	n/a

Treatments

Test concentrations:	Dilution water control, solvent control and nominal concentrations of 0.427, 0.939, 2.07, 4.55 and 10 mg a.s./L (equivalent to 0.351, 0.741, 1.55, 3.51 and 6.99 mg a.s./L mean measured)
Solvent:	dimethylformamide, DMF
Analysis of test concentrations:	Yes 0 and 48 hours analysed using GRM061.01A method at CEMAS, UK

Test organisms:

Species:	<i>Chironomus riparius</i>
Age:	First instar (< 24 hours old)
Source:	Not stated
Feeding:	None during test
Culture medium:	Filtered (30 µm) mesocosm water

Test design:

Test vessels:	60 mL glass beakers containing 60 mL of media
Test medium:	Filtered (30 µm) mesocosm water
Replication:	4 replicates of 5 chironomids
Exposure regime:	Static
Duration:	48 hours

Environmental conditions

Test temperature:	19.5 to 21.6 °C
pH range:	7.58 to 8.42
Dissolved oxygen:	85.8 to 89.3 % ASV
Total hardness of dilution water:	180 to 220 mg/L CaCO ₃
Lighting:	16 hours light (648 Lux) and 8 hours dark

STUDY DESIGN AND METHODS

Experimental dates: 12 to 21 August 2015

At the start of the test, a primary solvent stock solution (100 mg/mL) was prepared by dissolving 1 g of SYN545974 into 10 mL of DMF. Further solvent stock solutions were prepared by serial dilution of the primary stock in DMF to give dosing solutions of 4.27, 9.39, 20.7, and 45.5 mg/mL. All stock solutions were mixed by inversion for approximately one minute, or until no undissolved test item was visible.

In addition to the primary stock of 100 mg/mL of SYN545947, the dosing solutions were used to provide the test media at 0.427, 0.939, 2.07, 4.55 and 10 mg/L, respectively, by the addition of 0.1 mL of the solvent stock solutions into individual 1 L volumetric flasks containing 1 L of filtered (30 µm) mesocosm water using a micro-syringe. All test media were homogenised by inversion and in addition, the 2.07, 4.55, 10 mg/L test media were treated with ultrasound for 0.5, 5 and 30 minutes respectively, until no test item or undissolved material was visible prior to use in the test. Similarly, the solvent control was prepared by the addition of 0.1 mL DMF to 1 L filtered (30 µm) mesocosm water using a microsyringe and was mixed by inversion.

The immobility of the chironomids was determined by visual observations after 24 and 48 hours of exposure. For the purposes of this study, immobility was defined as the absence of free movement within 30 seconds following stimuli, i.e. gentle swirling of the media. As mortality is difficult to confirm in invertebrates, this was only recorded where cessation of life was certain e.g. the clear absence of any response or by obvious sign of necrosis or decomposition. For the purposes of this study, where an organism was recorded as dead, it was also recorded as immobile. Any other notable observations (such as slow response or abnormal colouration) were also recorded.

The pH, temperature and dissolved oxygen were measured at the start and end of the test in each test concentration and the control.

The test concentrations were verified by chemical analysis of SYN545974 at 0 and 48 hours using GRM061.01A method at CEMAS, UK.

RESULTS AND DISCUSSION

At the start of the test, the concentrations of SYN545974 were in the range 76 to 91 % of the nominal values and at the end of the test were in the range 57 to 77 % (see table below). The limit of quantification in this study was 0.05 µg/L. Mean measured concentrations were used for the calculation and reporting of results.

Table 9.2.4.2-13: Analytical results

Nominal concentrations (mg a.s./L)	Measured concentration 0 hours (mg a.s./L)	% of nominal measured at 0 hours	Measured concentration 48 hours (mg a.s./L)	% of nominal measured at 48 hours	Mean measured concentration (mg a.s./L)
0.427	0.39	91	0.32	74	0.351
0.939	0.76	81	0.72	77	0.741
2.07	1.57	76	1.53	74	1.55
4.55	3.69	81	3.32	73	3.51
10	8.30	83	5.70	57	6.99

Prior to the determination of concentration response functions, a pair-wise comparison between the control and solvent control was performed using Fisher's Exact Binominal Test, to determine if there was any significant difference between control groups. For both parameters, as the probability $p(i) = >0.05$, no differences were apparent and the diluent control data was excluded.

As the data at 24 and 48 hrs were inappropriate for regression analysis due to the lack of suitable responses to treatment, the EC_{50} and LC_{50} after were determined by interpolation (Spearman-Kaerber, 0 % trim) in which the confidence limits were approximated by $\pm 2 \cdot se(\ln(EC_{50}))$ where se = the standard error. For all parameters and time points, the NOEC was determined using the step-down Cochran-Armitage test procedure.

Mortality

A significant dose related response for mortality was observed at 24 and 48 hrs following exposure and as a result, the LC_{50} values are presented in the table below.

Table 9.2.4.2-14: Mortality of *Chironomus riparius* following exposure with SYN545974

Mean measured concentration (mg a.s./L)	Cumulative mortality observed (%)	
	24 hour	48 hour
Dilution water control	5	5
Solvent control	5	5
Pooled control	5	5
0.351	0	0
0.741	50*	70*
1.55	30*	55*

Mean measured concentration (mg a.s./L)	Cumulative mortality observed (%)	
	24 hour	48 hour
3.51	100*	100*
6.99	95*	100*
LC₅₀ (95% confidence limits)	1.317 (1.029 – 1.686)	0.902 (0.715- 1.138)
NOEC	0.351	0.351

Initial population = 20 (Pooled control = 40)

* Statistically different from pooled control (p =< 0.05)

Immobility

A significant dose related response for mortality was observed at 24 and 48 hrs following exposure and as a result, the EC₅₀ values are presented in the table below.

Table 9.2.4.2-15: Immobility of *Chironomus riparius* following exposure with SYN545974

Mean measured concentration (mg a.s./L)	Cumulative immobility observed (%)	
	24 hour	48 hour
Dilution water control	5	5
Solvent control	5	5
Pooled control	5	5
0.351	0	0
0.741	75*	80*
1.55	50*	80*
3.51	100*	100*
6.99	100*	100*
EC₅₀ (95 % confidence limits)	0.902 (0.715 – 1.138)	0.691 (0.570 – 0.838)
NOEC	0.351	0.351

Note: the number of immobile organisms includes dead.

Initial population = 20 (Pooled control = 40)

* Statistically different from pooled control (p =< 0.05)

VALIDITY CRITERIA

This test can be regarded as valid since:

- The control mortality did not exceed 15 % and there were no signs of disease or other stress (including trapping at the water surface)
- The concentration of Dissolved Oxygen (DO) was maintained at ≥3 mg/L, noting DO was measured in % ASV but was equivalent to ≥ 3 mg/L

CONCLUSIONS

Based on mean measured concentrations, the 48-hour EC₅₀ for SYN545974 to *Chironomus riparius* was 0.691 mg a.s./L, the 48 hour LC₅₀ was 0.902 mg a.s./L and the 48-hour NOEC was 0.351 mg a.s./L.

(■■■■, 2015a)

HSE evaluator comments

For this type of water-only acute 48-hour toxicity test for *Chironomus riparius* HSE has considered the validity criteria of OECD guideline 235 (2011) for *Chironomus* acute immobilisation water-only test. In this study the validity criteria were met.

Overall, the study is considered scientifically robust and reliable. The following points are noted for reference but do not have an impact on the outcome of the study:

- The substance SYN545974 was prepared using a solvent and therefore OECD Guidance Document 23 (2019) for testing of difficult substances has been considered. The solvent used (Dimethylformamide, DMF) is listed in the Guidance Document as effective for aquatic toxicity testing and is within the recommended concentration range of 0.10 mL DMF/L. Additionally, the absence of significant mortality in the solvent control treatment group compared to dilution water control indicates that the solvent had no effect on the outcome of the study.
- The authors note minor deviations from their protocol in the maintained ranges of temperature during the test, and temperature, light and dissolved oxygen in the maintenance cultures. These deviations were small, transient, and additionally due to adequate performance of controls HSE agrees that there was no impact on the validity and integrity of the study. In addition, the environmental conditions were within those recommended in OECD 235.
- The authors recorded immobility, mortality and any other abnormal responses of the test organisms. The authors defined immobility as “*absence of free movement within 30 seconds following stimuli*”. For reference, the definition of immobilisation in the OECD guideline 235 (2011) *Chironomus* acute immobilisation water-only study guideline has been considered. This guideline defines immobilisation as lack of response for 15 seconds following stimuli [“*Those animals that are not able to change their position (by crawling or swimming movements) within 15 seconds after mechanical stimulation*”)]. The longer time period to define immobilisation in this study is considered acceptable because the resulting observations are the same and the study authors also detail any additional abnormal responses of the test organisms. The agreed endpoints listed below are EC₅₀ from the immobilisation data rather than LC₅₀ from mortality data, as immobilisation is the typical endpoint detailed in OECD 235.
- The authors considered the immobility data “*inappropriate for regression*” therefore determined EC₅₀ from interpolation of data with Trimmed Spearman-Kärber analysis. NOEC was determined using the step-down Cochran-Armitage test procedure. The authors do not report applying any transformation procedures to the data. The 48 hour EC₅₀ appears to be in-line with experimental data and guideline therefore HSE considers the statistical analysis appropriate.

The analytical methods have been checked by HSE Chemistry specialists in Vol 3CA Part B5.1.2.6. The following was concluded: “Fit for regulatory purposes but the method is not fully validated in accordance with SANCO/3029/99 rev. 4. LOQ: 0.05 µg/L in aqueous matrices”.

The agreed endpoints for use in risk assessment are:

- 48-hour EC₅₀ 0.691 mg a.s./L (mean measured concentration).
- 48-hour NOEC 0.351 mg a.s./L (mean measured concentration).

Report:	K-CA 8.2.4.2 [REDACTED] (2015a). SYN545974 – Acute Toxicity of SYN545974 to <i>Cloeon dipterum</i> , Report number CEA.1664, Cambridge Environmental Assessments, ADAS Boxworth, Battlegate Road, Boxworth, Cambridgeshire, CB23 4NN, UK. (Syngenta File No. SYN545974_10315)
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GUIDELINES

The study was not conducted according to any specific regulatory guideline, but the following was consulted:

OECD Guidelines 202: *Daphnia* sp., Acute Immobilisation Test (2004)

GLP: Yes

MATERIALS

Test Material	SYN545974 tech.
Lot/Batch #:	CSCD678790 SMU2EP12007
Purity:	98.5% w/w [certificate of analysis confirmed]
Treatments	
Test concentrations:	Dilution water control, solvent control and nominal concentrations of 0.427, 0.939, 2.07, 4.55 and 10 mg SYN545974/L (mean measured 0.321, 0.762, 1.52, 3.24 and 5.01 mg SYN545974/L)
Solvent:	Dimethylformamide (DMF)
Positive control	None
Analysis of test concentrations:	Yes, analysis of SYN545974 at 0 and 48 hours by LC-MS/MS analysis
Test organisms	
Species:	<i>Cloeon dipterum</i> (larval stage 1 or 2)
Source:	Continuous laboratory cultures, originally obtained from the CEA mesocosm facility.
Acclimatisation period:	Acclimatised to test conditions for at least one day prior to use
Mortality/ observations during acclimatisation period:	None stated
Feeding:	None during test. Fed ad-hoc during acclimatisation using a periphytometer colonised by algae and bacteria
Culture medium:	30 µm filtered Mesocosm water, aerated to 97 % saturation during acclimatisation period
Test design	
Test vessels:	60 mL glass beakers containing 60 mL of test media, loosely covered with a lid
Test medium:	Filtered (30 µm) mesocosm water, collected from CEA mesocosm facility
Introduction of organisms:	20 individuals per control and test concentration, each individual randomly assigned to a test vessel via random number generator.
Replication:	20 replicate test vessels per control and test concentration
No. of organisms per tank:	1
Exposure regime:	Static
Duration:	48 hours
Environmental conditions	
Test temperature:	18.1 – 20.6 °C
pH range:	7.73 – 8.25
Dissolved oxygen:	88.9 – 95.5% ASV (no aeration during testing).

Total hardness of dilution water: 180 – 220 mg/L CaCO₃.

Lighting: 760 Lux
16 hours light and 8 hours dark

STUDY DESIGN AND METHODS

Experimental dates: 13 August 2015 to 09 September 2015

At the start of the test, a primary solvent stock solution (100 mg/mL) was prepared by dissolving 1 g of SYN545974 into 10 mL of DMF. Further solvent stock solutions were prepared by serial dilution of the primary stock in DMF to give dosing solutions of 4.27, 9.39, 20.7, and 45.5 mg/mL. All stock solutions were mixed by inversion for approximately one minute.

In addition to the primary stock of 100 mg/mL of SYN545947, the dosing solutions were used to provide the test media at 0.427, 0.939, 2.07, 4.55 and 10 mg/L, respectively, by the addition of 0.2 mL of the solvent stock solutions into individual 2 L volumetric flasks containing 2 L of filtered (30 µm) mesocosm water using a micro-syringe. All test media were homogenised by shaking and in addition, the 2.07, 4.55, and 10 mg/L test media was treated with ultrasound until no test item or undissolved material was visible prior to use in the test. Similarly, the solvent control was prepared by the addition of 0.2 mL DMF to 2 L filtered (30 µm) mesocosm water using a microsyringe and was mixed by inversion.

The immobility of the *Cloeon dipterum* was determined by visual observations after 24 and 48 hours of exposure. Organisms unable to swim within 30 seconds after gentle agitation of the test beaker were considered to be immobile. As mortality is difficult to confirm in invertebrates, this was only recorded where cessation of life was certain e.g. the clear absence of any response or by obvious sign of necrosis or decomposition. Any other notable observations (such as slow response or abnormal colouration) were also recorded.

The pH, temperature and dissolved oxygen were measured at the start and end of the test in each test concentration and the control.

The test concentrations were verified by chemical analysis of SYN545974 at 0 and 48 hours using LC-MS/MS.

RESULTS AND DISCUSSION

At the start of the test, the measured concentrations were in the range 49 to 81 % of the nominal values and at the end of the test were in the range 51 to 81% (see table below). The limit of quantification in this study was 0.05 µg SYN545974/L. Mean measured concentrations were used for the calculation and reporting of results.

Table 9.2.4.2-16: Analytical results

Nominal Concentration (mg/L)	Measured Concentration (mg/L)			
	0 hr (Fresh)	48 hr (Expired)	Proportion of Initial Measured	Mean
Diluent Control	<LOQ	0.000101	N/A	N/A
Solvent Control	0.000439	0.000722	N/A	0.000580
0.427	0.329 (77)	0.312 (73)*	95%	0.321 (75)
0.939	0.763 (81)	0.762 (81)*	100%	0.762 (81)
2.07	1.55 (75)	1.50 (72)	97%	1.52 (74)
4.55	3.13 (69)	3.36 (74)	107%	3.24 (71)
10	4.92 (49)	5.09 (51)	104%	5.01 (50)

LOQ: Limit of quantification (0.05 µg/L). Values expressed in brackets are percent of the nominal (%). *the original sample analysis showed these results were initially transposed however analysis of the reserve samples confirmed the correct exposure concentrations.

The Fisher's Exact Binominal Test used to perform a pair-wise comparison between the control and solvent control showed there was no significant difference between control groups (mortality and immobility $p(i) = 1.0$). As a result, the data were analysed in comparison to the pooled control.

After 48 hours no significant dose response was observed, therefore no ECx values could be reliably determined. The NOEC (No Observed Effect Concentration) was determined using the Bonferroni Fisher test procedure.

Table 9.2.4.2-17: Cumulative mortality for *Cloeon dipterum* treated with SYN545974

Mean measured concentration (mg a.s./L)	% mortality after 24 hours	% mortality after 48 hours
Control	0	5
Solvent control	0	0
Pooled control	0	2.5
0.321	0	0
0.762	0	0
1.52	0	0
3.24	0	0
5.01	0	5
LC₅₀ (95% confidence limits)	n.d.	n.d.
NOEC	5.01	5.01

n.d. – not determined

Table 9.2.4.2-18: Cumulative immobility for *Cloeon dipterum* treated with SYN545974

Mean measured concentration (mg a.s./L)	% immobility after 24 hours	% immobility after 48 hours
Control	0	5
Solvent control	0	0
Pooled control	0	2.5
0.321	0	0
0.762	0	0
1.52	0	0
3.24	0	0
5.01	0	10
EC₅₀ (95% confidence limits)	n.d.	n.d.
NOEC	5.01	5.01

n.d. – not determined

VALIDITY CRITERIA

It was not possible to compare this study to validity criteria since no validated guideline exists for this species. However, it is noted that the study conforms to the validity criteria outlined in OECD 202 (2004) for use in daphnids, the standard species for acute toxicity studies in aquatic invertebrates.

Table 9.2.4.2-19: Compliance with OECD 202 validity criteria

Validity criterion	Required	Obtained
Immobilisation and sub-lethal effects in control during test	$\leq 10 \%$	5 %
Dissolved oxygen concentration at the end of the test	$\geq 3 \text{ mg/L}$	$\geq 8.3 \text{ mg/L}$

CONCLUSIONS

The 48-hour EC_{50} and LC_{50} could not be calculated. Based on mean measured concentrations, the 48-hour NOEC was 5.01 mg a.s./L for *Cloeon dipterum*.

(██████████, 2015a)

HSE evaluator comments

This study was carried out in accordance with GLP. This study used a non-standard species, and as such no specific regulatory guideline was followed. As such it is not possible to confirm the validity of this study.

The applicant proposes their own validity criteria for use with non-daphnid species ($\leq 15 \%$ mortality in control groups and $> 60 \%$ dissolved oxygen), however the reasoning behind these criteria and suitability for the test species have not been discussed. The validity criteria stipulated in OECD 202 (2004) have been satisfactorily fulfilled, although there is uncertainty with regard to their applicability to non-standard species.

A reference test was not conducted as recommended in OECD 202 (2004). As a non-standard species was used, without a reference test there may be uncertainty as to whether the test species is sensitive enough to detect effects of the test item on this species.

A minor protocol deviation reported by the applicant was that the culture medium temperature was not constant within $\pm 1 \text{ }^{\circ}\text{C}$, ranging from 18.1 – 20.6 $^{\circ}\text{C}$. Since temperatures remained within the normal ecological range for *Cloeon dipterum*, and control immobilisation was within the validity requirements, this deviation is not thought to have impacted the study outcome.

Measured concentrations differed by $> 20 \%$ from the nominal values, so results were based on mean measured values. It is noted that the concentration of SYN545974 in the control groups was above the limit of quantification (0.05 $\mu\text{g/L}$), however, since control immobilisation was below the limits of the validity criteria ($<10 \%$), this is not thought to have impacted the study outcome. The analytical methods have been checked by HSE Chemistry specialists in Vol 3CA Part B5.1.2.6. The following was concluded: “Fit for regulatory purposes but the method is not fully validated in accordance with SANCO/3029/99 rev. 4. LOQ: 0.05 $\mu\text{g/L}$ in aqueous matrices”.

A Fisher’s Exact Binomial test determined that there was no statistical difference between the immobilisation seen in the control and treatment groups. As no significant treatment-related effects were observed at any test concentration, statistical evaluation of EC_{50} values was not suitable. The agreed endpoint for use in risk assessment is therefore:

- **48-hour $EC_{50} = > 5.01 \text{ mg SYN545974/L}$**

Due to the uncertainties surrounding the use of non-standard species, it is recommended that this study is considered further in the context of the risk assessment for standard species.

Report: K-CA 8.2.4.2 [REDACTED] (2015b). SYN545974 – Acute Toxicity of SYN545974 to *Crangonx pseudogracilis*, Report number CEA.1661, Cambridge Environmental Assessments, ADAS Boxworth, Battlegate Road, Boxworth, Cambridgeshire, CB23 4NN, UK. (Syngenta File No. SYN545974_10306)

GUIDELINES

The study was not conducted according to any specific regulatory guideline, but the following was consulted: OECD Guidelines 202: Daphnia sp., Acute Immobilisation Test (2004)

GLP: Yes

MATERIALS

Test material SYN545974 technical
Description: Off-white powder
Lot/Batch #: SMU2EP12007
Purity: 98.5 %
Stability of test compound: Expiry date: 30 June 2016

Treatments

Test concentrations: 0.427, 0.939, 2.07, 4.55 and 10 mg a.s. /L nominal (corresponding to 0.333, 0.852, 1.69, 3.73 and 7.38 mg a.s. /L mean measured)
Dilution water: Filtered (30 µm) mesocosm water
Vehicle and/or positive control: dimethylformamide (DMF) at 0.1 mL /L, none
Analysis of test concentrations: Yes using method GRM061.01A

Test organisms

Species: *Crangonx pseudogracilis* (< 28 days old)
Source: Test facility (collected from the CEA mesocosm facility and bred in the laboratory.)

Acclimatisation period: 7 days

Treatment for disease: None

Life stage of test organism: Juvenile

Feeding: None during test

Test design

Test vessels: 60 mL glass beakers containing 60 mL of the prepared treated or control media covered with a lid
Replication: 20 replicates containing 1 organism
Exposure regime: Static
Duration: 48 hours

Environmental conditions

Test temperature: 19.2 to 20.9 °C.
pH range: 7.04 to 8.45
Dissolved oxygen: 89.6 to 95.5 %

Total hardness of dilution 180-220 mg /L

water:

Lighting: 16 hours fluorescent light and 8 hours dark daily (591 lux).

STUDY DESIGN AND METHODS

Experimental dates: 28 July to 04 September 2015

Juvenile (< 28 days old) *Crangonyx pseudogracilis* were used as the test organism in this study. Adult cultures were periodically checked for juveniles, which were isolated for use in the test. Test organisms were acclimatised to the conditions of the experiment for at least 7 days prior to experimentation. No feeding took place during the test.

The filtered mesocosm water used as the dilution water in this study was from the same source as the water used during acclimatisation, which conforms to the characteristics of an acceptable dilution water listed in OECD 202. Representative samples of the mesocosm water source were analysed in the 3-month period prior to experimentation, for the presence of pesticides, PCBs, and toxic metals, and measurements were also taken of the total organic carbon (TOC) concentration.

A static test design was implemented, using loosely covered glass beakers containing the test and control solutions. At the start of the test, a primary solvent stock solution (100 mg /mL) was prepared by dissolving 1 g of SYN545974 into 10 mL of DMF. Further solvent stock solutions were prepared by serial dilution of the primary stock in DMF to give dosing solutions of 4.27, 9.39, 20.7, and 45.5 mg /mL. All stock solutions were mixed by inversion for approximately one minute and no undissolved test item was visible.

In addition to the primary stock of 100 mg /mL of SYN545947, the dosing solutions were used to provide the test media at 0.427, 0.939, 2.07, 4.55 and 10 mg /L, respectively, by the addition of 0.2 mL of the solvent stock solutions into individual 2 L volumetric flasks containing 2 L of filtered (30 µm) mesocosm water using a micro-syringe. All test media were homogenised by shaking and in addition, the 2.07, 4.55, 10 mg /L test media was treated with ultrasound for 5, 10 and 30 minutes respectively, until no undissolved material was visible prior to use in the test. Similarly, the solvent control was prepared by the addition of 0.2 mL DMF to 2 L filtered (30 µm) mesocosm water using a microsyringe and was mixed by inversion.

The temperature (°C), pH, and dissolved oxygen concentration (% ASV, Air Saturation Volume) were measured at the start and end of the test in each test concentration and the control groups. The concentrations of SYN545974 in the test solutions at 0 and 48 hours were measured using the LCMS/MS validated method GRM061.01A at CEMAS, UK.

The test organisms were observed daily at approximate 24-hr intervals for signs of immobility and, where possible, mortality. For the purposes of this study, immobility was defined as the absence of free movement within 30 seconds following stimuli, i.e. gentle swirling of the media. For the purposes of data reporting, the number of immobilised organisms included dead organisms. As mortality is difficult to confirm in invertebrates, this was only recorded where cessation of life was certain e.g. the clear absence of any response or by obvious sign of necrosis or decomposition. Any other notable observations (such as slow response or abnormal colouration) were also recorded.

The Fisher's Exact Binominal Test used to performed a pair-wise comparison between the control and solvent control showed there was no significant difference between control groups (mortality and immobility $p(i) = 1.0$ and 1.395, respectively). As a result, the data were analysed in comparison to the pooled control.

For all time points and parameters, probit analysis with linear maximum likelihood regression was used for the evaluation of the LC_x and EC_x values. The NOEC was determined using the step-down Cochran- Armitage test procedure.

RESULTS AND DISCUSSION

Analytical results

Measured concentrations of SYN545974 ranged from 78 to 96 % of the nominal values at 0 hours, and after 48 hours, ranged from 69 to 85 % of the nominal. The limit of quantification in this study was 0.05 µg a.s. /L. Mean measured concentrations were used for the calculation and reporting of results. The measured concentrations are shown in Table 9.2.4.2-20 below, in terms of nominal concentrations.

Table 9.2.4.2-20: Analytical results

Nominal concentration (mg a.s. /L)	% of nominal 0 hours	% of nominal 48 hours	Mean measured concentration (mg a.s. /L)
0.427	84	72	0.333
0.939	96	85	0.852
2.07	86	77	1.69
4.55	85	79	3.73
10	78	69	7.38

Biological results

No mortality was observed following 24 hr exposure therefore, it was not possible to calculate a LC₅₀ value. A 10 % mortality rate was observed after 48 hours in the dilution water control. Averaged with the solvent control mortality rate of 5 %, this gave a pooled control mortality rate of 7.5 %. At 48 hrs, a significant dose response was observed and the LC₅₀ value is presented in Table 9.2.4.2-21 below.

Table 9.2.4.2-21: Effects of test material on the mortality of *Crangonx pseudogracilis*

Mean measured concentration (mg a.s. /L)	Cumulative mortality observed (%)	
	24 hour	48 hour
Dilution water control	0	10
Solvent control	0	5
Pooled control	0	7.5
0.333	0	5
0.852	0	15
1.69	0	15
3.73	0	55*
7.38	0	60*
LC ₅₀ (95% confidence limits)	n.d.	4.532 (2.937 – 9.620)
NOEC	7.38	1.69

Initial population = 20

n.d. – not determined

* Statistically different from pooled control (p = < 0.05)

A 10% immobility rate was observed in both the dilution water control and the solvent control conditions. A significant dose response was observed at 24 and 48 hrs. EC₅₀ values were calculated and are presented in Table 9.2.4.2-22 below.

Table 9.2.4.2-22: Effects of test material on the immobilisation of *Crangonx pseudogracilis*

Mean measured concentration (mg a.s. /L)	Cumulative immobility observed (%)	
	24 hour	48 hour
Dilution water control	0	10
Solvent control	0	10
Pooled control	0	10
0.333	0	10
0.852	10*	40*
1.69	25*	50*
3.73	90*	90*
7.38	100*	100*
EC ₅₀ (95% confidence limits)	2.040 (1.640 – 2.540)	1.226 (0.888 – 1.641)
NOEC	0.333	0.333

Note: the number of immobile organisms includes dead

Initial population = 20

* Statistically different from pooled control ($p < 0.05$)

Values expressed in brackets are percent immobility (%)

The 48-hour concentration-response (immobility) data is displayed graphically in Figure 9.2.4.2-3.

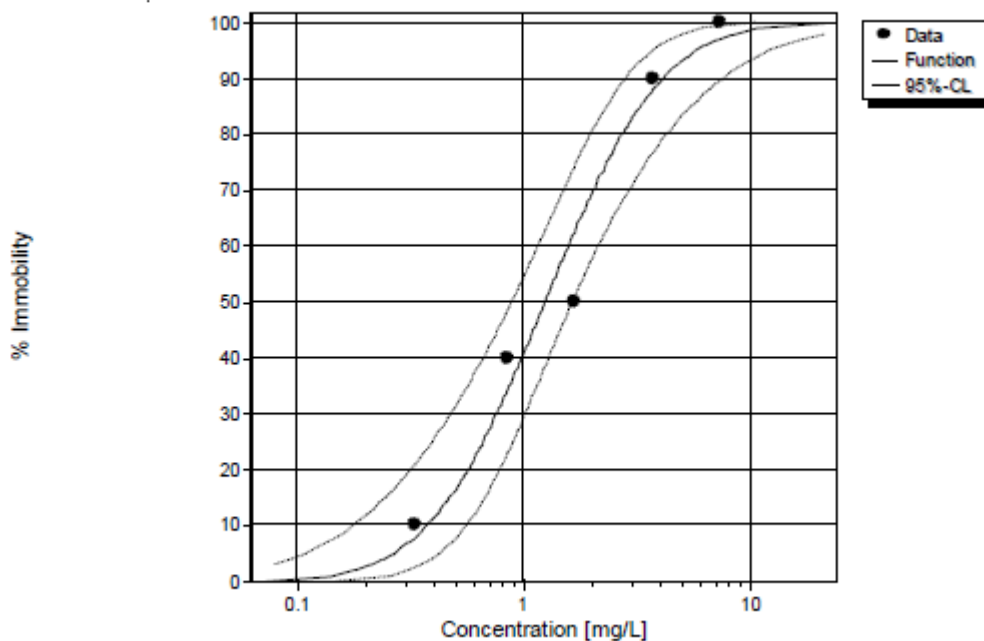


Figure 9.2.4.2-3: 48hr concentration-response (immobility) curve for the static acute exposure of *Crangonyx pseudogracilis* exposed to SYN545974.

VALIDITY CRITERIA

Table 9.2.4.2.-23: Compliance with OECD 202 validity criteria

Validity criterion	Required	Obtained
Mortality (immobility) in the controls	≤ 10 % mortality (immobility) in the control(s) (dilution water control, solvent control)	Dilution water control: 10 % Solvent control: 5 %
Dissolved oxygen concentration	≥ 60 % of the air saturation value in all test vessels throughout the exposure	Dissolved oxygen concentration remained above 89.6 % 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 ranged from 69 – 96 % of nominal.

CONCLUSIONS

The 48 hour LC₅₀ for *Crangonx pseudogracilis* exposed to SYN545974 was 4.532 mg a.s. /L based on mean measured concentrations. The 48-hour EC₅₀ was 1.226 mg /L a.s., with 95% confidence intervals of 0.888 - 1.641 mg /L. The NOEC of SYN545974 on the mortality and immobility of *Crangonx pseudogracilis* was determined to be 1.69 and 0.333 mg a.s. /L, respectively, based on mean measured concentrations.

HSE evaluator comments

The study was carried out according to GLP, but was not conducted according to any specific regulatory guideline, however, OECD 202 (2004) was consulted. When assessed against the guidelines, one deviation was noted.

During the 7 day acclimatisation period, the temperature of the culture media room dipped to 17.8, 17.9 and 17.9 °C on 3 separate dates, which is marginally below the required temperature of 20 °C ± 2 °C. This deviation was judged to have had a negligible impact on the results produced in the study, as these measurements were taken from the ambient room temperature and not the culture media itself, and also, the conditions remained within the normal ecological range for *Crangonx pseudogracilis*.

The definitive test was initially conducted and abandoned after 20 % immobility was observed after 24 hrs, exceeding the acceptable immobility threshold in the guideline. The definitive test was repeated and met the validity criteria. Immobility in the controls was measured at 10 % (the pooled control mortality rate was measured as 7.5 %). This is equal to the OECD 202 limit of 10 % (for *Daphnids*).

The authors state that in addition to observations of immobility and mortality, “any other notable observations (such as slow response or abnormal colouration) were also recorded” over the course of the experiment, although none were reported. Additionally, no mention was made of whether the test organisms came from a healthy stock which was free of disease. As the mortality levels in the controls were below the maximum permitted level, it can be assumed that the test organisms were healthy at the start of the experiment.

As *Crangonx pseudogracilis* is a non-standard testing organism, no positive control data was provided, it may be necessary to request this from the applicant.

For all time points and parameters, probit analysis with linear maximum likelihood regression was used for the evaluation of the LC_x and EC_x values. The NOEC was determined using the step-down Cochran- Armitage test procedure. The statistical methods used to analyse the data are in line with the guideline, and visual inspection of the data in Figure 9.2.4.2-3 supports the calculated end-point data.

The analytical methods have been checked by HSE Chemistry specialists in Vol 3CA Part B5.1.2.6. The following was concluded: “Fit for regulatory purposes but the method is not fully validated in accordance with SANCO/3029/99 rev. 4. LOQ: 0.05 µg/L in aqueous matrices”.

Based on mean measured concentrations, the immobility 48-hour EC₅₀ value for the effects of SYN545974 on *Crangonx pseudogracilis* was determined to be 1.226 mg /L a.s., with 95% confidence intervals of 0.888 – 1.641 mg /L. The NOEC values for the immobility were determined to be 0.333 mg a.s. /L, also based on mean measured concentrations.

Report: K-CA 8.2.4.2 (2015b). SYN545974 – Acute Toxicity of SYN545974 to *Cyclops agilis speratus*, Report number CEA.1662, Cambridge Environmental Assessments, ADAS Boxworth, Battlegate Road, Boxworth, Cambridgeshire, CB23 4NN, UK. (Syngenta File No. SYN545974_10347)

GUIDELINES

No specific guideline used, but the following was consulted:
OECD Guidelines for Testing of Chemicals, Section 2 - Effects on Biotic Systems, Method 202: *Daphnia* sp., Acute Immobilisation Test (2004)

GLP: Yes

MATERIALS

Test Material	SYN545974 technical CSCD678790
Lot/Batch #:	SMU2EP12007
Purity:	98.5 % w/w
Description:	Off white powder
Stability of test compound:	Stable under standard conditions
Reanalysis/Expiry date:	30 June 2016
Density:	Not applicable

Treatments

Test concentrations:	Dilution water control, solvent control and nominal concentrations of 0.427, 0.939, 2.07, 4.55 and 10 mg a.s./L (mean measured: 0.340, 0.822, 1.94, 3.68 and 7.76 mg a.s./L)
Solvent:	Dimethylformamide (DMF)
Positive control:	None
Analysis of test concentrations:	Yes, analysis of SYN545974 at 0 and 48 hours using LC-MS/MS analysis

Test organisms

Species:	<i>Cyclops agilis speratus</i> (adults)
Source:	Laboratory-maintained cultures, collected from Cambridge Environmental Assessments mesocosm facility as gravid adults and acclimatised to test conditions for 3 days prior to use
Feeding:	None during test
Culture medium:	2 mm filtered mesocosm water

Test design

Test vessels:	60 mL glass vessels containing 60 mL of test media
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Test medium:	Filtered (30 µm) mesocosm water collected from the laboratory mesocosm facility and acclimatised to test conditions for at least one day prior to use
Replication:	4 replicates of 5 organisms
Exposure regime:	Static
Duration:	48 hours
Environmental conditions	
Test temperature:	18.4 to 21.9 °C
pH range:	7.66 to 8.49
Dissolved oxygen:	86.5 to 94.4 % ASV (Air Saturation Value) (no aeration)
Total hardness of dilution water:	180 – 220 mg/L CaCO ₃
Lighting:	601 Lux 16 hours light and 8 hours dark

STUDY DESIGN AND METHODS

Experimental dates: 20 July to 4 August 2015

At the start of the test, a primary solvent stock solution (100 mg/mL) was prepared by dissolving 1 g of SYN545974 into 10 mL of DMF. Further solvent stock solutions were prepared by serial dilution of the primary stock in DMF to give dosing solutions of 4.27, 9.39, 20.7, and 45.5 mg/mL. All stock solutions were mixed by inversion for approximately one minute, or until no undissolved test item was visible.

In addition to the primary stock of 100 mg/mL of SYN545947, the dosing solutions were used to provide the test media at 0.427, 0.939, 2.07, 4.55 and 10 mg/L, respectively, by the addition of 0.1 mL of the solvent stock solutions into individual 1 L volumetric flasks containing 1 L of filtered (30 µm) mesocosm water using a micro-syringe. All test media were homogenised by inversion and in addition, the 2.07, 4.55, 10 mg/L test media were treated with ultrasound for 0.5, 5 and 30 minutes respectively, until no test item or undissolved material was visible prior to use in the test. Similarly, the solvent control was prepared by the addition of 0.1 mL DMF to 1 L filtered (30 µm) mesocosm water using a micro-syringe and was mixed by inversion.

The immobility of the organisms was determined by visual observations after 24 and 48 hours of exposure. Organisms unable to swim within 30 seconds after gentle agitation of the test media were considered to be immobile. As mortality is difficult to confirm in invertebrates, this was only recorded where cessation of life was certain e.g. the clear absence of any response or by obvious sign of necrosis or decomposition. For the purposes of this study, where an organism was recorded as dead, it was also recorded as immobile. Any other notable observations (such as slow response or abnormal colouration) were also recorded.

The pH, temperature and dissolved oxygen were measured at the start and end of the test in each test concentration and the controls.

The test concentrations were verified by chemical analysis of SYN545974 at 0 and 48 hours using LCMS/MS.

RESULTS AND DISCUSSION

At the start of the test, the measured concentrations were in the range 82 to 107 % of the nominal values and at the end of the test were in the range 73 to 80 % (see table below). The limit of quantification in this study was 0.05 µg a.s./L. Mean measured concentrations were used for the calculation and reporting of results.

Table 9.2.4.2-24: Analytical results

Nominal concentrations (mg a.s./L)	Measured concentration (mg a.s./L) at 0 hours	% of nominal measured at 0 hours	Measured concentration (mg a.s./L) at 48 hours	% of nominal measured at 48 hours	Mean measured concentrations (mg a.s./L)
0.427	0.361	85	0.340	75	0.340
0.939	0.944	101	0.822	75	0.822
2.07	2.22	107	1.94	80	1.94
4.55	3.86	85	3.68	77	3.68
10	8.25	82	7.76	73	7.76

LOQ: Limit of Quantification (0.05 µg SYN545974/L) n.a. = not applicable

The Fisher's Exact Binominal Test used to performed a pair-wise comparison between the control and solvent control showed there was no significant difference between control groups (mortality and immobility $p(i) = 1.0$). As a result, the control data were pooled prior to analysis.

The 48-hour EC_{50} and LC_{50} values were evaluated by Probit analysis using linear maximum likelihood regression. The 24-hour EC_{50} and LC_{50} values were evaluated using a Trimmed Spearman-Kärber method. The NOEC (No Observed Effect Concentration) was determined using the step-down Cochran Armitage test procedure.

Mortality

A significant dose related response was observed at 24 and 48 hrs and as a result, LC_{50} values were calculated and are presented in the table below.

Table 9.2.4.2-25: Mortality of *Cyclops agilis speratus* following exposure with SYN545974

Mean measured concentration (mg a.s./L)	Cumulative mortality observed (%)	
	24 hour	48 hour
Dilution water control	0	0
Solvent Control	0	0
Pooled control	0	0
0.340	0	0
0.822	0	0
1.94	0	0
3.68	0	20*
7.76	95*	95*
LC_{50}	5.552	4.744
(95 % confidence limits)	(5.154 - 5.981)	(4.035 - 5.672)
NOEC	3.68	1.94

* Significant statistical difference compared to the solvent control (Fisher's Exact Test, $p = < 0.05$)

Immobility

A significant dose related response was observed at 24 and 48 hrs and as a result, EC_{50} values were calculated and are presented in the table below.

Table 9.2.4.2-26: Immobiility of *Cyclops agilis speratus* following exposure with SYN545974

Mean measured concentration (mg a.s./L)	Cumulative immobility observed (%)	
	24 hour	48 hour
Dilution water control	0	0
Solvent Control	0	0
Pooled control	0	0
0.340	10	0
0.822	5	0
1.94	15*	5
3.68	30*	25*
7.76	100*	100*
EC₅₀ (95 % confidence limits)	3.414 (2.702 - 4.313)	4.168 (3.512 - 5.025)
NOEC	0.822	1.94

* Significant difference compared to the solvent control (Fisher's Exact Test, $p < 0.05$)

CONCLUSIONS

Based on mean measured concentrations, the 48-hour EC₅₀ for SYN545974 to *Cyclops agilis speratus* was calculated to be 4.168 mg a.s./L. The 48-hour LC₅₀ was calculated to be 4.744 mg a.s./L. The 48-hour NOEC was determined to be 1.94 mg a.s./L for both mortality and immobilisation. The 24 hour NOEC for immobilisation was 0.822 mg a.s./L and has been discussed below.

(██████, 2015b)

HSE evaluator comment

The study was carried out according to GLP. This study does not follow a set guideline but OECD 202 (2004) was consulted.

There is no agreed OECD guideline for *Cyclops agilis speratus*. As such it was impossible to confirm validity criteria. However, the immobility of *Cyclops* in the pooled control was 0 %, below the 10 % stated in OECD 202 (2004) guidelines for *Daphnia*. Consideration of OECD 202 validity criteria is shown below.

Validity criteria	Required	Obtained
Immobilisation and sub-lethal effects in control during test	≤ 10 %	0 %
Dissolved oxygen concentration at the end of the test	≥ 3 mg/L	≥ 7.15 mg/L

The identification of immobility of organisms used in this study was no movement 30 seconds after stimulus. OECD 202 (2004) defines immobility as no movement 15 seconds after stimulus. It was noted this study's immobility definition is less protective than that for a *Daphnia* study (OECD 202) i.e. double the time is allowed before organism is considered immobile. Nonetheless this is unlikely to result in major differences in endpoints given the relatively short time of assessments (increase in duration of 15 seconds).

A validated guideline for this species is not available. However, the statistical analysis method used is in-line with other comparable OECD guidelines. The reporting of the analysis was brief and it was unclear whether the data was transformed prior to analysis. Nonetheless, the resulting EC₅₀ is considered appropriate by HSE and supported by the experimental data (at 48 hours). It was noted the 24 hour EC₅₀ was not in-line with experimental data but calculated endpoint is more conservative than observed data.

The solvent DMF was used to dissolve the active substance in this study. Preparation of the stock solutions followed the OECD 23 guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures (2000). Deviation from these guidelines involved assessing solubility by visibility of substance. This is not recommended in OECD 23 (2000). However, as the study has included analytical information for the active substance, it can be considered that this had no impact on the derived endpoints.

The analytical methods have been checked by HSE Chemistry specialists in Vol 3CA Part B5.1.2.6. The following was concluded: “Fit for regulatory purposes but the method is not fully validated in accordance with SANCO/3029/99 rev. 4. LOQ: 0.05 µg/L in aqueous matrices”.

The mean measured concentrations of the test solutions were not maintained within 20 % of the nominal concentrations. However, they were within ± 20 % of initial mean measured. Hence mean measured concentrations were, therefore, used for reporting biological results.

There is some recovery of the organisms between 24 hours and 48 hours at 1.94 mg a.s./L and 3.68 mg a.s./L for immobility. This lends some uncertainty regarding observations of immobile organisms. The agreed endpoints suitable for use in the risk assessment are:

- **48-hour NOEC = 1.949 mg a.s./L (mean measured concentration), noting uncertainty (see above)**
- **48-hour EC₅₀ = 4.168 mg a.s./L (mean measured concentration), noting uncertainty (see above)**

Report: K-CA 8.2.4.2 [REDACTED] [REDACTED] [REDACTED] (2015). SYN545974 – A 48-Hour Static Acute Toxicity Test with the Freshwater Amphipod (*Hyalella azteca*), Report number 528A-287, Wildlife International, 8598 Commerce Drive, Easton, MD 21601 USA. (Syngenta File No. SYN545974_10354)

GUIDELINES

- OECD 202 (2004)
- OPPTS 850.1010 (1996)

GLP: Yes

MATERIALS

Test material	SYN545974 Technical
Description:	Off-white powder
Lot/Batch #:	SMU2EP12007
Purity:	98.5 % (w/w)
Stability of test compound:	Stable under test conditions

Treatments

Test concentrations:	Nominal concentrations of 0.0029, 0.0095, 0.031, 0.10, 0.31 and 1.0 mg a.s./L alongside dilution water control
Test water:	Laboratory well water
Solvent:	None
Analysis of test concentrations:	Yes at 0 and 48 hours using LC/MS/MS

Test organisms

Species:	Freshwater amphipod (<i>Hyalella azteca</i>)
Source:	Maintained at test facility
Treatment for disease:	None
Feeding:	None during test

Test design

Test vessels:	250 mL glass beakers containing 200 mL test water
Replication:	4 replicates with 5 amphipods for biological response
Exposure regime:	Static
Duration:	48 hours

Environmental conditions

Test temperature:	22.6 to 24.7 °C
pH range:	8.2 to 8.5
Dissolved oxygen:	≥7.0 mg/L (≥81 % of saturation),
Lighting:	16 hours fluorescent light and 8 hours dark with a 30 minute transition period (695 lux)

STUDY DESIGN AND METHODS

Experimental dates: 21 to 28 August 2015

Test chambers were 250 mL glass beakers filled with approximately 200 mL of test water. The depth of the test water in a representative chamber was 6.8 cm. Two approximately 2x2 cm squares of nylon mesh screen were placed on the bottom of each test compartment prior to test initiation to serve as a substrate for the organisms. The chambers were indiscriminately positioned by treatment group in a temperature controlled environmental chamber.

A primary stock solution was prepared by mixing a calculated amount of test substance (0.00406 g) in 4000 mL of UV sterilized well water at a nominal concentration of 1.0 mg a.s./L, the highest concentration tested. Aliquots of the primary stock solution were proportionally diluted with UV sterilized well water to prepare five additional test solutions at nominal concentrations of 0.0029, 0.0095, 0.031, 0.10 and 0.31 mg a.s./L. The solutions were stirred for 15 minutes and approximately 250 mL of solution was placed in each of four replicate test chambers per treatment group. The negative control solution was dilution water only.

The test concentrations were verified by analysis of SYN545974. The method used for the analysis of SYN545974 in freshwater consisted of diluting the samples with a ratio of 20 : 80 (v/v) methanol : freshwater. Samples were analysed by high performance liquid chromatography with tandem mass spectrometric detection (LC/MS/MS).

All organisms were observed periodically to determine the number of mortalities in each treatment group. Mortality was defined as a lack of reaction by the test organism to application of a gentle stimulus. The numbers of individuals exhibiting signs of toxicity or abnormal behaviour also were evaluated. Observations were made approximately 5, 24 and 48 hours after test initiation.

RESULTS AND DISCUSSION

The measured concentrations in test solution are shown in the table below. Nominal concentrations were used for the calculation and reporting of results.

Table 9.2.4.2-27: Measured Concentrations of SYN545974 in Test Solution Samples

Nominal concentration (mg a.s./L)	Measured SYN545974 Concentration at 0 hours (%)	Measured SYN545974 Concentration at 48 hours (%)	Mean measured concentration (mg a.s./L)
0.029	98.5	99.1	0.0028
0.095	96.3	93.7	0.0090
0.031	103	91.0	0.030
0.10	94.6	90.3	0.093
0.31	106	86.8	0.29
1.0	90.9	84.8	0.89

Estimates of LC_{50} , slopes of the concentration-response curves, and confidence intervals for both 24 and 48-hour data responses were determined using probit analysis. The protocol stated that the LC_{50} and 95 % confidence interval would be calculated by probit analysis, the moving average method, or by binomial probability with nonlinear interpolation using the computer program of [REDACTED]. However, there was one mortality in the negative control group, and it was noted that algorithm used by [REDACTED] to calculate maximum likelihood estimates of the LD_{50} ignores mortality in the control group. Therefore, the mortality data were analysed using the CETIS computer program of Tidepool Scientific instead. This program is designed to calculate the LC_{50} value and the 95 % confidence interval by probit analysis, and does incorporate control mortality into the maximum likelihood estimate of the LC_{50} and 95 % confidence interval. The no-observed-effect concentration (NOEC) was determined using the Jonckheere-Terpstra Step-Down Test.

Table 9.2.4.2-28: Mortality of *Hyalella azteca* treated with SYN545974 in a 48 hour test (n=20)

Mean measured concentration (mg a.s./L)	Number showing trapping at ~5 hours*	Cumulative mortality at ~24 hours	Number showing trapping at ~24 hours*	Cumulative mortality at ~48 hours	Number showing trapping at ~48-hours*	Cumulative Percentage mortality (%)
Negative control	2 (10 %)	0	3 (15 %)	1	2 (10.5 %)	5
0.0028	3 (15 %)	0	3 (15%)	1	2 (10.5 %)	5
0.0090	2 (10 %)	0	5 (25 %)	4	3 (18.8 %)	20
0.030	1 (5 %)	0	5 (25 %)	6	4 (28.6 %)	30
0.093	2 (10 %)	2	2 (11 %)	7	0 (0 %)	35
0.29	1 (5 %)	6	0 (0 %)	13	0 (0 %)	65
0.89	4 (20 %)	11	1 (11 %)	20	N/A	100
LC₅₀ (mg a.s./L)	-	0.68	-	0.12	-	-
95 % confidence limits	-	0.41 – 1.7	-	0.057 – 0.21	-	-

n.d. – not determined

*Data added by HSE from the study report Table 3. This number reflects the individuals showing the observed effects of both ‘Q,AN = floating and trapped at water surface but appear normal after gentle submersion and ‘Q,C’ = floating and trapped at water surface and appear lethargic after gentle submersion’. Other behavioural observations are not included. Percentage calculated from surviving organisms.

N/A: not applicable.

VALIDITY CRITERIA

The following criteria were used to judge the validity of the test and were met:

- Mortality of the amphipods in the negative control group will not exceed 10 % by the end of the test. Mortality in the control was 5 %.
- The dissolved oxygen concentration will be at least 60 % of the air-saturation value throughout the test. Dissolved oxygen concentrations remained ≥ 99 % saturation (8.5 mg/L) during the test.

CONCLUSIONS

The freshwater amphipod, *Hyalella azteca*, was exposed for 48 hours under static conditions to six mean measured concentrations of SYN545974 ranging from 0.0028 to 0.89 mg a.s./L. Based on mean measured concentrations, the 48-hour LC₅₀ value was 0.12 mg a.s./L, with a 95 % confidence interval of 0.057 to 0.21 mg a.s./L. The NOEC was 0.009 mg a.s./L.

(██████████ et al, 2015)

HSE evaluator comments

For this type of water-only acute 48-hour toxicity test for *Hyalella azteca* there is no agreed guideline and validity criteria. The authors state that the study protocol was conducted with consultation of the OECD guideline 202 (2004) *Daphnia* acute immobilisation test. HSE notes there is also another OECD 235 (2011) guideline for *Chironomus* sp. acute water only 48 hour test. This study met the validity criteria of both guidelines, with the exception of trapping at water surface (detailed below). However, it is not possible to fully conclude on the validity of the study due to lack of specific guidelines for this species.

Overall, the study is considered scientifically robust and reliable. The following points are noted for reference but do not have an impact on the outcome of the study:

- The authors confirmed the concentration of the test substance using samples from 2 out of 4 test vessels and these were within ± 20 % of nominal, therefore the authors could have used nominal concentrations in the calculation and reporting of results. The authors chose to use the mean measured concentrations instead which is also acceptable. The analytical methods have been checked by HSE Chemistry specialists in Vol 3CA Part B5.1.2.6. The following was concluded: “Acceptable method. LOQ: 0.0025 mg/L in water”.
- The non-lethal effect of organisms becoming trapped at the water surface but returning to normal upon re-immersion was observed in all treatment levels and the controls, including at 24 hours in the control where this was seen in 15 % of test organisms, though this reduced to 10.5 % at 48 hours (Table 9.2.4.2-28). If this were a *Daphnia* sp. study this would violate the validity criteria of the study (OECD 202), but not that of a chironomid study (OECD 235). However, the results show this water trapping is a transient effect that is sporadic between replicates, therefore HSE agrees this effect does not have an impact on the endpoints of the study.
- It is noted that there are single individuals exhibiting lethargy at the 0.0028, 0.030 and above treatment levels but not the control or the 0.0090 treatment level. For the 0.0028 treatment level the authors justify the decision to consider the single mortality and single lethargic organism as “incidental and not treatment related, since there was a comparable mortality noted in the negative control”, which is in line with the data.
- It was noted there was 20 % mortality at 0.009 mg a.s./L (proposed NOEC). The level of mortality at NOEC is not considered suitably protective by HSE. Nonetheless as a NOEC value is not required from this study for the risk assessment further consideration is not required.
- The authors define mortality as “as a lack of reaction by the test organism to application of a gentle stimulus.” Since this definition is similar to definitions of immobility in the OECD guideline 202 (2004), and immobility is the standard endpoint for most acute aquatic invertebrate studies, then the endpoint listed below is considered as ‘immobility’ rather than ‘mortality’ and is therefore presented as an EC₅₀ instead of LC₅₀.

The agreed endpoint for use in risk assessment is:

- **48-hour EC₅₀: 0.12 mg a.s./L (mean measured concentration)**

Report:	K-CA 8.2.4.2 [REDACTED] (2015c). SYN545974 – Acute Toxicity of SYN545974 to <i>Lumbriculus variegatus</i> , Report number CEA.1642, Cambridge Environmental Assessments, ADAS Boxworth, Battlegate Road, Boxworth, Cambridgeshire, CB23 4NN, UK. (Syngenta File No. SYN545974_10304)
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GUIDELINES

The study was not conducted according to any specific regulatory guideline, but the following was consulted: OECD Guidelines 202: *Daphnia* sp., Acute Immobilisation Test (2004)

GLP: Yes

MATERIALS

Test Material	SYN545974 technical
Lot/Batch #:	SMU2EP12007
Purity:	98.5%
Description:	Off white powder
Stability of test compound:	Stable under standard conditions.

Reanalysis/Expiry date:	30 June 2016
Density:	n/a
Treatments	
Test rates:	0.427, 0.939, 2.07, 4.55 and 10 mg a.s./L nominal (0.317, 0.822, 1.08, 3.14, and 6.87 mg a.s./L mean measured)
Control:	Filtered pond water
Toxic standard:	None
Test organisms	
Species:	<i>Lumbriculus variegatus</i>
Age at test start:	Juvenile (2-4 cm long)
Source:	Maintained at test facility (originally: Smithers Viscient (Harrogate, UK))
Feeding:	2 g of dried flakes fish food (Neptune Goldfish Flakes, Bn: 12634914)
Test design	
Vessels:	60 mL glass beakers each containing 60 mL of the prepared treated or control media
Test medium:	Filtered (30 µm) mesocosm water
Replication:	20
No. of worms/arena:	1
Duration of test:	48 hours
Environmental test conditions	
Temperature:	Continuous media temperature: 16.2 to 23.3 °C Measurements taken at 24 h & 48 h: 19.1 to 21.2 °C (not more than +/- 1 °C in each vessel)
pH:	7.93 to 8.57
Dissolved oxygen:	88.8 to 91.2 %
Photoperiod:	16 hours light: 8 hours dark (510 lux)
Hardness as CaCO₃	180 – 220 mg /L

STUDY DESIGN AND METHODS

Experimental dates: 15 July to 07 August 2015

Juvenile (2-4 cm long) *Lumbriculus variegatus* were used as the test organism for this study. Prior to use in the experiment, juvenile worms were acclimated to the test conditions for 7 days. During this time, the test organisms were fed with dried fish flakes, but no food was provided during the test period. No mention was made of any observations of mortality or behavioural abnormalities during this period. Organisms used in the test were selected randomly from the stock cultures, and randomly added to the test vessels.

A static test design was used for this experiment. At the start of the test, a primary solvent stock solution (100 mg /mL) was prepared by dissolving 1 g of SYN545974 into 10 mL of DMF. Further solvent stock solutions were prepared by serial dilution of the primary stock in DMF to give dosing solutions of 4.27, 9.39, 20.7, and 45.5 mg /mL. All stock solutions were mixed by inversion for approximately one minute, or until no undissolved test item was visible. In addition to the primary stock of 100 mg /mL of SYN545947, the dosing solutions were used to provide the test media at 0.427, 0.939, 2.07, 4.55 and 10 mg /L, respectively, by the addition of 0.2 mL of the solvent stock solutions into individual 2 L volumetric flasks containing 2 L of filtered (30 µm) mesocosm water using a micro-syringe. All test media were homogenised by shaking and in addition, the 2.07, 4.55, 10

mg /L test media was treated with ultrasound for 0.5, 5 and 30 minutes respectively, until no test item or undissolved was visible prior to use in the test. Similarly, the solvent control was prepared by the addition of 0.2 mL DMF to 2 L filtered (30 µm) mesocosm water using a microsyringe and was mixed by inversion.

The filtered (30 µm) mesocosm water used as the dilution water during this study was from the same source as the water used during acclimation. The dilution water conformed to the chemical characteristics defined as acceptable in OECD 202.

The pH, temperature and dissolved oxygen were measured at the start and end of the test in each test concentration and the controls. The test concentrations were verified by chemical analysis of SYN545974 at 0 and 48 hours using LCMS/MS. The limit of quantification (LOQ) in this study was 0.05 µg a.s. /L.

The test organisms were observed daily at approximate 24-hr intervals for signs of immobility and, where possible, mortality. For the purposes of this study, immobility was defined as the absence of free movement within 30 seconds following stimuli, i.e. gentle swirling of the media. This tally of immobilised organisms included dead organisms. As mortality is difficult to confirm in invertebrates, this was only recorded where cessation of life was certain e.g. the clear absence of any response or by obvious sign of necrosis or decomposition. The test organisms were observed for any behavioural/morphological abnormalities (such as slow response or abnormal colouration), but none were reported.

Prior to the determination of concentration response functions, a pair-wise comparison between the control and solvent control was performed using Fisher's Exact Binominal Test, to determine if there was any significant difference between control groups. For both parameters, as the probability $p(i) = 1.0$ was greater than 0.05, no differences were apparent, and the control data were pooled. Probit analysis with linear maximum likelihood regression was used for the evaluation of the 24 and 48 hrs LC_x values and 24 hr EC_x values, whereas for the evaluation of the EC_x values at 48 hrs, interpolation (trimmed Spearman- Kärber) was used. The NOEC was determined using the step-down Cochran- Armitage test procedure and Bonferroni Fisher test procedure, respectively.

RESULTS AND DISCUSSION

Analytical results

The measured concentrations are shown in Table 9.2.4.2-29 below in terms of the nominal concentrations. Measured concentrations ranged from 53 – 93 % of the nominal concentration at 0 hours, and ranged from 51 – 82 % of the nominal concentration at 48 hours. The limit of quantification (LOQ) in this study was 0.05 µg a.s. /L. Mean measured concentrations were used for the calculation and reporting of results.

Table 9.2.4.2-29: Analytical results

Nominal concentration (mg a.s. /L)	% of nominal 0 hours	% of nominal 48 hours	Mean measured concentration (mg a.s. /L)
0.427	77	72	0.317
0.939	93	82	0.822
2.07	53	51	1.08
4.55	78	60	3.14
10	71	66	6.87

Biological results

No mortality was definitively observed at 24 hrs following exposure and as a result, it was not possible to calculate a LC₅₀ value. At 48 hrs, a significant dose response was observed and the LC₅₀ value is presented in Table 9.2.4.2-30 below. The 48 hour concentration-response (mortality) data is displayed graphically in Figure 9.2.4.2-4

Table 9.2.4.2-30: Effects of SYN545974 on mortality of *Lumbriculus variegatus*

Mean measured concentration (mg a.s. /L)	Cumulative mortality observed (%)	
	24 hour	48 hour
Dilution water control	0	0
Solvent control	0	0
Pooled control	0	0
0.317	0	0
0.822	0	0
1.08	0	0
3.14	0	0
6.87	0	75*
LC ₅₀ (95% confidence limits)	-	5.535 (4.764 – 6.659)
NOEC	6.87	3.14

* A significant difference ($p < 0.05$) was observed in comparison to the pooled control

Note: No 24 hr LC₅₀ values could be reliably calculated due to the absence of a clear dose response to treatment.

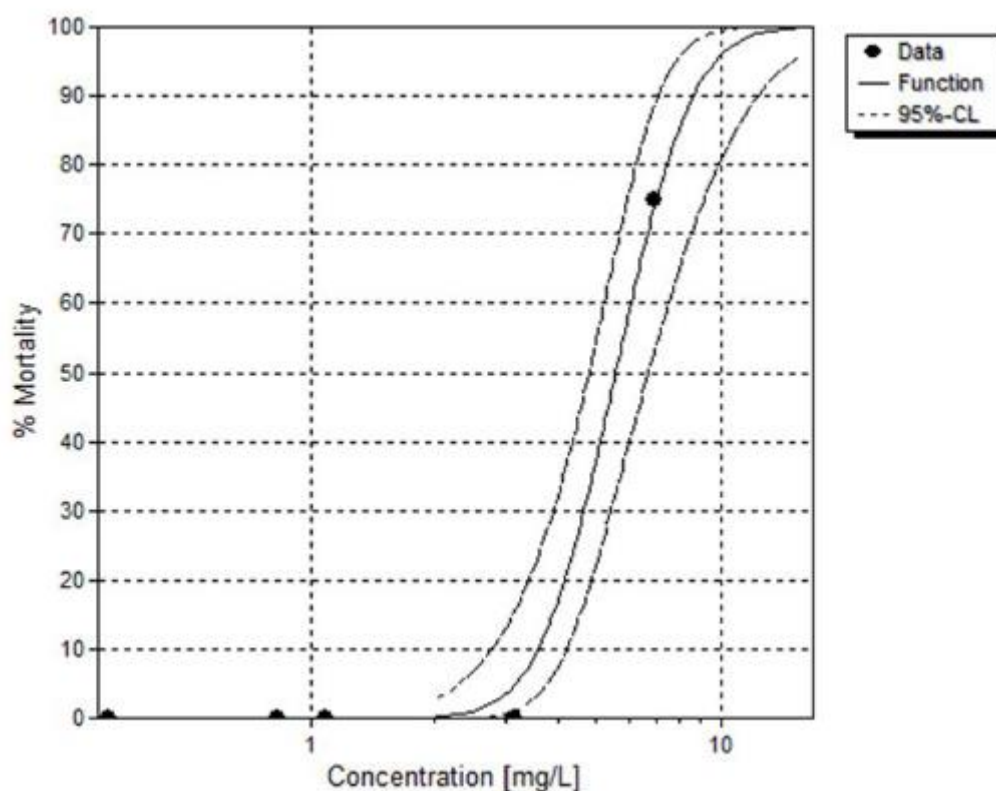


Figure 9.2.4.2-4: 48 hr concentration-response (mortality) curve for the static acute exposure of *Lumbriculus variegatus* to SYN545974.

A 2.5 % immobility rate was observed in the pooled control after 48 hours. A significant dose related immobility response was observed at 24 and 48hrs in the test conditions following exposure. EC₅₀ values were calculated and are presented in Table 9.2.4.2-31 below. The 24 hour concentration-response (immobility) data is displayed graphically in Figure 9.2.4.2-5.

Table 9.2.4.2-31: Effects of SYN545974 on immobility of *Lumbriculus variegatus*

Mean measured concentration (mg a.s. /L)	Cumulative immobility observed (%)	
	24 hour	48 hour
Dilution water control	5	5
Solvent control	0	0
Pooled control	2.5	2.5
0.317	0	5
0.822	0	5
1.08	0	5
3.14	0	0
6.87	90*	85*
EC ₅₀ (95% confidence limits)	4.780 (4.049 – 5.808)	4.651 (3.880 – 5.575)
NOEC	3.14	3.14

* A significant difference ($p < 0.05$) was observed in comparison to the pooled control

Note: The number of immobilised organisms was defined as the total number of organisms with absence of free movement within 30 seconds following stimuli (including dead organisms). No observations of behavioural abnormalities were taken.

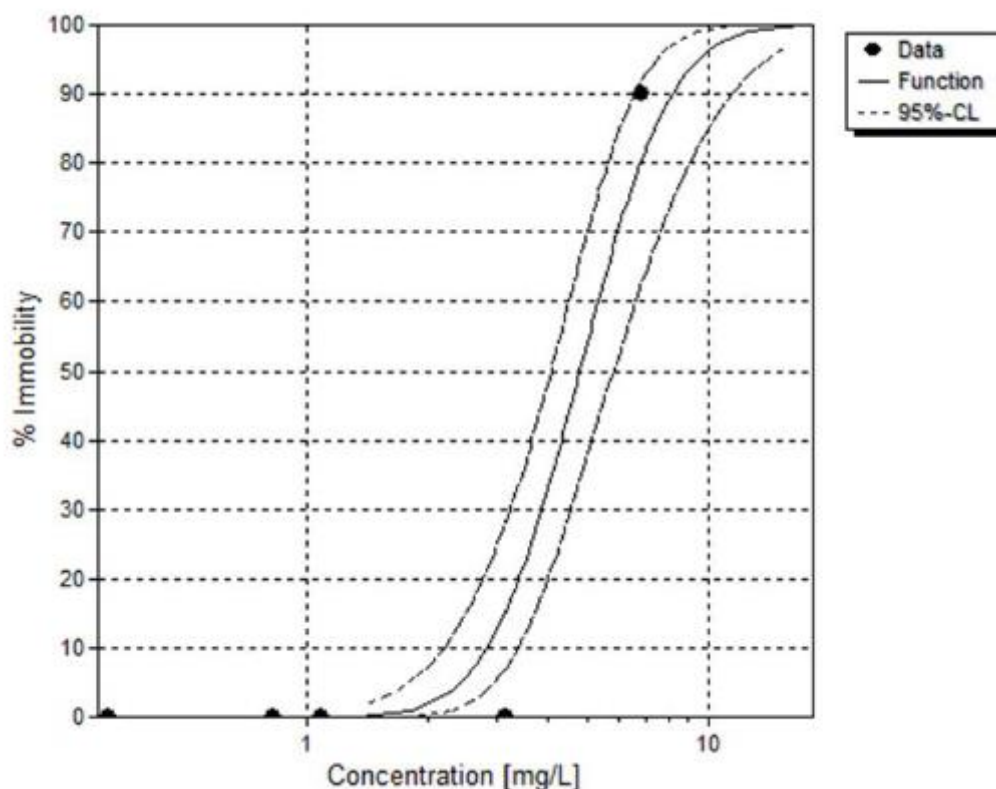


Figure 9.2.4.2-5: 24 hr concentration-response (immobility) curve for the static acute exposure of *Lumbriculus variegatus* to SYN545974

VALIDITY CRITERIA

Table 9.2.4.2-32: Validity criteria

Validity criterion	Required	Obtained
Mortality in the controls	≤ 10 % mortality in the control(s) (dilution water control, solvent control)	0 %
Dissolved oxygen concentration	≥ 60 % of the air saturation value in all test vessels throughout the exposure	Dissolved oxygen concentration remained above 88.8 % 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 ranged from 51-93 % of nominal.

CONCLUSIONS

Based on mean measured concentrations, the NOEC of SYN545974 on the mortality and immobility of *Lumbriculus variegatus* was determined to be 3.14 mg a.s. /L. The 48 hour LC₅₀ was determined to be 5.535 mg a.s. /L and the 48 hour EC₅₀, based on immobilisation, was 4.651 mg a.s. /L.

HSE evaluator comments

The study was conducted according to GLP. It was not conducted according to any specific regulatory guideline, but OECD 202 (2004) was consulted. When assessed against the guidelines, the following deviations were noted:

Although the temperature of the test media remained within the OECD 202 recommended range at all times, the minimum recorded temperature in the culture room was 17.8 °C, and during the definitive test the minimum temperature recorded in the continuously monitored representative sample of media was 16.2 °C. This is unlikely to have had any negative impact on the data, as the temperature in the culture room was only transiently 0.2 °C below the recommended temperature. Additionally, the low temperatures measurements taken from the representative sample do not correspond to the acceptable temperatures observed in the test media.

Light intensity fell outside the range stated in the protocol (500-1000 LUX) on four occasions during the 7-day acclimation period. The measured light intensities were 200, 170, 480 and 490 LUX. These deviations were judged to have had a negligible impact on the results produced in the study, as the conditions remained within the normal ecological range for *Lumbriculus variegatus*, and there were low levels of mortality and immobility observed in the control conditions during the test.

As the test species is non-standard, no positive control data was provided by the applicant. It may be necessary to ask the applicant if any data is available. Additionally, no 48 h immobility graph was provided as part of the study report. The graphical display of the 24 h immobility data (Figure) shows that the majority of the data points are clustered at the bottom, with a sharp increase in response at the highest tested concentration. The visual fit of the curve to the data isn't ideal, meaning that the predicted EC₅₀ value could be unreliable. The 48 h immobility curve will need to be requested from the applicant, and the uncertainty surrounding the endpoint may need to be considered further in the risk assessment.

The 48-hour EC₅₀ value was estimated by interpolation (trimmed Spearman- Kärber). The NOEC was determined using the step-down Cochran- Armitage test procedure and Bonferroni Fisher test procedure, respectively. These statistical methods were in line with the guidelines. However, the use of interpolation may not be ideal, considering that only the highest tested concentration produced an immobilisation response which was significantly different

to the control conditions after 48 hours. This means that the accuracy of the predicted EC₅₀ value could be uncertain.

The analytical methods have been checked by HSE Chemistry specialists in Vol 3CA Part B5.1.2.6. The following was concluded: “Fit for regulatory purposes but the method is not fully validated in accordance with SANCO/3029/99 rev. 4. LOQ: 0.05 µg/L in aqueous matrices”.

Based on mean measured concentrations, the NOEC of SYN545974 on *Lumbriculus variegatus* was determined to be 3.14 mg a.s./L. The 48 hour EC₅₀, based on immobilisation, was 4.651 mg a.s./L, with 95% confidence intervals of 3.880 – 5.575 mg /L.

Report: K-CA 8.2.4.2 [REDACTED] (2015d). SYN545974 – Acute Toxicity of SYN545974 to *Lymnaea stagnalis*, Report number CEA.1645, Cambridge Environmental Assessments, ADAS Boxworth, Battlegate Road, Boxworth, Cambridgeshire, CB23 4NN, UK. (Syngenta File No. SYN545974_10303)

GUIDELINES

The study was not conducted according to any specific regulatory guideline, but the following was consulted: OECD Guidelines 202: *Daphnia* sp., Acute Immobilisation Test (2004)

GLP: Yes

MATERIALS

Test Material	SYN545974 technical
Lot/Batch #:	SMU2EP12007
Purity:	98.5 %
Description:	Off white powder
Stability of test compound:	Stable under standard conditions.
Reanalysis/Expiry date:	30 June 2016

Treatments

Test concentrations:	Dilution water control and a single nominal concentration of 10 mg/L (7.30 mg a.s./L mean measured)
Solvent:	Dimethylformamide (DMF)
Positive control:	None
Analysis of test concentrations:	Yes, analysis at 0 and 48 hours

Test organisms

Species:	Juvenile (<21 days old) <i>Lymnaea stagnalis</i>
Source:	Collected from CEA mesocosms facility
Feeding:	0.2 g of dried flake fish food (Neptune Goldfish Flakes, Bn: 12634914) and 6 g fresh cucumber approximately three times a week
Culture medium:	Filtered (30 µm) mesocosm water

Test design

Test vessels:	120 mL glass beakers each containing 100 mL of test medium
Test medium:	Filtered (30 µm) mesocosm water
Replication:	4 replicates of 5 <i>Lymnaea</i>

Exposure regime:	Static
Duration:	48 hours
Environmental conditions	
Test temperature:	19.0 and 20.8 °C
pH range:	7.89 to 8.68
Dissolved oxygen:	76.5 to 102.3 %
Total hardness of dilution water:	180 to 220 mg/L CaCO ₃
Lighting:	589 Lux 16 hours light and 8 hours dark

STUDY DESIGN AND METHODS

Experimental dates: 18 to 27 August 2015

The definitive test concentrations and test media preparation were selected based on the results of the range finding test. Due to the lack of clear biological response at 10 mg a.s./L the definitive test was conducted as a limit test. The definitive limit test was comprised of one exposure concentration (nominally 10 mg a.s./L), a diluent control (containing 30 µm filtered pond water only) and a solvent control (containing diluent with 0.1 mL/L DMF).

At the start of the test, a primary solvent stock solution (100 mg/mL) was prepared by dissolving 1 g of SYN545974 into 10 mL of DMF. The solvent stock solution was mixed by inversion for approximately one minute, until no undissolved test item was visible.

The test media was prepared by the addition of 0.1 mL of the solvent stock (100 mg/mL) into a 1 litre volumetric flask containing 1 L of filtered (30 µm) mesocosm water using a micro-syringe. The test media was homogenised by shaking by hand for 5 minutes and was treated with ultrasound for 30 minutes, until no test item or undissolved test item was visible prior to use in the test. Similarly, the solvent control was prepared by the addition of DMF at a rate of 0.1 mL to 1 L filtered (30 µm) mesocosm water using a microsyringe and mixed by inversion.

The test organisms were observed daily at approximate 24-hr intervals for signs of immobility and, where possible, mortality. For the purposes of this study, immobility was defined as the absence of free movement within 30 seconds following stimuli, i.e. gentle swirling of the media. A 0.25 cm² grid was placed beneath the test vessel to aid detection of movement. As mortality is difficult to confirm in invertebrates, this was only recorded where cessation of life was certain e.g. the clear absence of any response or by obvious sign of necrosis or decomposition. Any other notable observations (such as slow response or abnormal colouration) were also recorded.

The pH, temperature and dissolved oxygen were measured at the start and end of the test in each test concentration and the controls.

The concentrations of SYN545974 in the test solutions were measured using the validated method GRM061.01A at CEMAS, UK.

RESULTS AND DISCUSSION

The measured concentrations are shown in the table below in terms of nominal concentrations. The limit of quantification in this study was 0.05 µg a.s./L. Mean measured concentrations were used for the calculation and reporting of results.

Table 9.2.4.2-33: Analytical results

Nominal concentrations (mg a.s./L)	% of nominal measured at 0 hours	% of nominal measured at 48 hours	Mean measured concentrations (mg a.s./L)
10	76	70	7.30

Prior to the determination of concentration response functions, a pair-wise comparison between the control and solvent control was performed using Fisher's Exact Binominal Test, to determine if there was any significant difference between control groups. For both parameters, as the probability $p(i) = 1.0$ was greater than 0.05, no differences were apparent and the control data were pooled.

As a limit test was conducted, the data were not suitable for concentration response analysis and it was not possible to calculate LCx and ECx values.

To determine the NOEC for immobility, the Fisher's Exact Binominal Test was used to performed a pairwise comparison between the number immobile organisms within the pooled control and treatment groups at 24 and 48 hrs.

Mortality

For the duration of the test, no mortality was observed in the 7.30 mg a.s./L treatment group or the pooled control. Therefore the NOEC is considered to be 7.30 mg a.s./L.

Table 9.2.4.2-34: Mortality of SYN545974 on *Lymnaea stagnalis*

Mean measured concentration (mg a.s./L)	Cumulative mortality observed (%)	
	24 hours	48 hours
Dilution water control	0	0
Solvent Control	0	0
Pooled control	0	0
7.30	0	0
LC50 (95% confidence limits)	n.d.	n.d.
NOEC	7.30	7.30

n.d. – not determined

Immobility

After 48 hrs of exposure, immobility at 7.30 mg a.s./L was 10 %, whereas no immobility was observed in the pooled control. The NOEC was determined to be 7.30 mg a.s./L.

Table 9.2.4.2-35: Immobility of SYN545974 on *Lymnaea stagnalis*

Mean measured concentration (mg a.s./L)	Cumulative immobility observed (%)	
	24 hours	48 hours
Dilution water control	5	0
Solvent Control	0	0
Pooled control	2.5	0
7.30	5	10

Mean measured concentration (mg a.s./L)	Cumulative immobility observed (%)	
	24 hours	48 hours
EC ₅₀ (95% confidence limits)	n.d.	n.d.
NOEC	7.30	7.30

Note: the number of immobile organisms includes dead; Initial population = 20 n.d. – not determined

CONCLUSIONS

Based on mean measured concentration, the 48-hour NOEC for SYN545974 to *Lymnaea stagnalis* was 7.30 mg a.s./L. The 48 hour EC₅₀ and LC₅₀ could not be calculated.

(██████████, 2015d)

HSE evaluator comments

The study was carried out according to GLP. This study does not follow a set guideline but OECD 202 (2004) was consulted.

There is no agreed OECD guideline for *Lymnaea stagnalis*. As such it was impossible to confirm validity criteria. However, the immobility of *Lymnaea stagnalis* in the pooled control was 2.5 %, lower than 10 % stated in OECD 202 (2004) guidelines for *Daphnia*. Consideration of OECD 202 validity criteria is shown below.

Validity criteria	Required	Obtained
Immobilisation and sub-lethal effects in control during test	≤ 10 %	2.5 %
Dissolved oxygen concentration at the end of the test	≥ 3 mg/L	≥ 6.28 mg/L

The identification of immobility of organisms used in this study was no movement 30 seconds after stimulus. OECD 202 (2004) defines immobility as no movement 15 seconds after stimulus. It was noted this study's immobility definition is less protective than that for a *Daphnia* study (OECD 202) i.e. double the time is allowed before organism is considered immobile. Nonetheless this is unlikely to result in major differences in endpoints given the relatively short time of assessments (increase in duration of 15 seconds).

HSE notes there is a draft OECD guideline for *Lymnaea stagnalis* reproduction test. Whilst this was an acute study the temperature and light levels are in-line with draft guideline i.e. lux between 250 – 500 and temperature of 20 ± 1.0 °C, noting temperature was not continuously monitored for the study (measurements at initiation and termination).

The solvent DMF was used to dissolve the active substance in this study. Preparation of the stock solutions followed the OECD 23 guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures (2000). Deviation from these guidelines involved assessing solubility by visibility of substance. This is not recommended in OECD 23 (2000). As the study has included analytical information for the active substance, it can be considered that this had no impact on the validity of the results. A solvent control was included in the study, there were no recorded mortalities or immobilisations in the solvent control.

The analytical methods have been checked by HSE Chemistry specialists in Vol 3CA Part B5.1.2.6. The following was concluded: "Fit for regulatory purposes but the method is not fully validated in accordance with SANCO/3029/99 rev. 4. LOQ: 0.05 µg/L in aqueous matrices".

The mean measured concentrations of the test solutions were not maintained within 20 % of the nominal concentrations. However, they were within ± 20 % of initial mean measured. Hence mean measured concentrations were, therefore, used for reporting biological results. Very low levels of the active substance were found in both the diluent and solvent controls. As there was 0 % mortality in the pooled controls it is not considered to have had an effect.

Fisher's exact binomial test was used to show no significant difference between the diluent control and solvent control. As a result, these were pooled prior to analysis. As this study was a limit test, it was not suitable for concentration result analysis i.e. to determine EC_{10/20/50} values. However, Fisher's exact binomial test was used to compare the pooled control with the treatment concentrations and no significant difference was recorded.

Therefore, the agreed endpoint suitable for use in the risk assessment is:

- 48-hour NOEC = 7.30 mg a.s./L (mean measured concentration)
- 48-hour EC₅₀ = >7.30 mg a.s./L (mean measured concentration)

B.9.2.5. Long-term and chronic toxicity to aquatic invertebrates

B.9.2.5.1. Reproductive and development toxicity to *Daphnia magna*

Report: K-CA 8.2.5.1 [REDACTED] (2016a). SYN545974 – Full Life-Cycle Toxicity Test with Water Fleas, *Daphnia magna*, Under Static-Renewal Conditions, Report Number 1781.6842, Smithers Viscient. 790 Main Street, Wareham, MA 02571-1037, USA. (Syngenta File No. SYN545974_10017; updated with Amendments 5 and 6)

GUIDELINES

- OECD Guidelines for Testing of Chemicals, Method 211: *Daphnia magna* Reproduction test (1998)
- US EPA Ecological Effects Test Guidelines, OPPTS 850.1300: *Daphnia* Chronic Toxicity Test (1996)
- Official Journal of the European Communities, Dir 92/69/EEC, L142/674, Part C.20: *Daphnia magna* Reproduction Test (2009)

GLP: Yes

MATERIALS

Test material	SYN545974 tech.
Lot/Batch #:	2637-BA/110
Purity:	99.5 %
Description:	White powder
Stability of test compound:	Stable under standard conditions
Reanalysis date:	31 July 2013
Treatments	
Test concentrations:	Dilution water control, solvent control and nominal concentrations of 0.0048, 0.012, 0.024, 0.048, 0.12 and 0.30 mg SYN545974/L Mean measured concentrations: 0.0045, 0.011, 0.023, 0.042, 0.12 and 0.31 mg SYN545974/L
Solvent:	Dimethylformamide (DMF), 0.1 mL/L
Positive control:	None
Analysis of test concentrations:	Yes, on days 0, 2, 16 and 19 (new solutions) and days 2, 5, 19 and 21 (aged solutions), using LC/MS/MS
Test animals	
Species:	<i>Daphnia magna</i>
Source:	Continuous laboratory cultures, Smithers Viscient Laboratory
Feeding:	Daily, with alga (<i>Ankistrodesmus falcatus</i>) and YCT (a mixture of yeast, cereal leaves and flaked fish food), equivalent to approximately 0.2 mg carbon/daphnid/day
Culture medium:	Fortified well water, meeting U.S. EPA specifications

Test design

Test vessels:	100-mL glass beakers containing 80 mL medium
Test medium:	Fortified well water adjusted to hardness of approximately 160 - 180 mg/L as CaCO ₃ and filtered prior to test initiation.
Replication:	Ten replicate vessels for each control and test concentration (one organism <24 hours old per vessel)
Exposure regime:	Static-renewal
Duration:	21 days

Environmental conditions

Test temperature:	20 – 21 °C
pH range:	7.8 – 9.0
Dissolved oxygen:	7.0 – 13 mg/L (60 % of dissolved oxygen saturation = 5.4 mg/L at 20 °C)
Water hardness:	180 – 190 mg/L as CaCO ₃
Lighting:	Fluorescent bulbs, intensity range 10 – 13 µE.m ⁻² s ⁻¹ 16 hours light and 8 hours dark, with 15-minute transition periods

STUDY DESIGN AND METHODS

Experimental dates: 6 to 27 June 2012

Prior to test initiation, a 3.0 mg a.s./mL primary stock solution was prepared by placing 0.0752 g of SYN545974 in a 25 mL volumetric flask and bringing it to volume with dimethylformamide (DMF). From this primary solution five additional stock solutions were prepared and these were used to prepare the test solutions at test initiation, and on alternate days thereafter. Exposure solutions were mixed using a glass rod for approximately one minute. The solvent control was prepared by adding 0.15 mL of DMF to 1.5 L of dilution water (the same ratio of stock volume to dilution water volume as for the exposure solutions) and the remaining control consisted of dilution water only.

The test was initiated by impartially adding one animal (< 24 hours old) to each replicate vessel. The test vessels were held in a temperature-controlled water bath at 20 °C ± 1 °C. The test medium was renewed every other day and the daphnids carefully transferred to the fresh medium along with food solutions.

The *Daphnia* were fed daily on a mixture of 200 µL of algal suspension and 50 µL of YCT suspension, so that the quantity of algal suspension supplied to each daphnid was approximately equivalent to 0.2 mg carbon/daphnid/day.

Observations of immobilisation and abnormal behaviour among adult daphnids were recorded daily. Numbers of offspring were determined at first brood release, and daily thereafter. The time to first brood and the number of immobilised offspring were recorded and at each observation interval offspring were removed, counted and discarded. At the end of the test the length of all surviving adult daphnids was measured to the nearest 0.05 mm, and their dry weight measured to the nearest 0.01 mg.

The concentrations of SYN545974 in the test solutions were measured in freshly prepared solutions on days 0, 2, 16 and 19 and in the reciprocal old solutions on days 2, 5, 19 and 21. Duplicate samples were removed from each treatment level with one being analysed for SYN545974 and the other being stored frozen as backup. Three quality control (QC) samples were also prepared at each sampling interval. All solutions and QC samples were analysed using LC/MS/MS.

Dissolved oxygen, pH and temperature were measured in all test concentrations and controls at the beginning (new solutions) and end (aged solutions) of each renewal period. Aged solutions were removed from a composite of all available replicate vessels. Water bath temperature was continuously monitored, and the appearance of the test medium was visually recorded at each test organism observation.

RESULTS AND DISCUSSION

The measured concentrations of SYN545974 in fresh solutions were in the range 82 to 110 % of the nominal values and the measured concentrations in aged solutions were in the range 86 to 110 % (see table below). Mean measured concentrations ranged from 88 to 100 % of nominal concentrations and were used for the calculation and reporting of the results. The limit of quantification in this study was 0.151 mg a.s./L.

Table 9.2.5.1-1: Analytical results

Sample	Nominal concentrations (mg a.s./L)						
	Control and solvent control	0.0048	0.012	0.024	0.048	0.12	0.30
	Measured concentration (mg a.s./L) / % of nominal ^a						
Day 0 new media	<LOQ ^b	0.0053 / 110	0.011 / 94	0.025 / 100	0.043 / 89	0.12 / 100	0.31 / 100
Day 2 aged media	<LOQ	0.0043 / 89	0.011 / 91	0.022 / 90	0.041 / 86	0.12 / 97	0.29 / 98
Day 2 new media	<LOQ	0.0043 / 90	0.012 / 96	0.023 / 96	0.041 / 87	0.12 / 100	0.31 / 100
Day 5 aged media	<LOQ	0.0044 / 92	0.012 / 98	0.022 / 93	0.042 / 87	0.12 / 99	0.29 / 97
Day 16 new media	<LOQ	0.0042 / 88	0.011 / 90	0.021 / 87	0.039 / 82	0.12 / 97	0.29 / 98
Day 19 aged media	<LOQ	0.0046 / 95	0.011 / 94	0.023 / 95	0.044 / 91	0.12 / 98	0.31 / 100
Day 19 new media	<LOQ	0.0046 / 95	0.012 / 100	0.024 / 100	0.045 / 93	0.12 / 100	0.31 / 100
Day 21 aged media	<LOQ	0.0045 / 94	0.012 / 97	0.023 / 95	0.043 / 90	0.12 / 100	0.32 / 110
Mean (% nominal)^a	NA	0.0045 (94)	0.011 (95)	0.023 (95)	0.042 (88)	0.12 (100)	0.31 (100)

^aPercent of nominal was calculated using unrounded analytical results. The values presented in this table are rounded.

^bLOQ = Limit of Quantification. The LOQ for each analysis is dependent upon the regression, the area of the low standards and the dilution factor of the controls. For the different samplings LOQ varied between 0.000122 and 0.000163 mg a.s./L.

NA = not applicable

Survival of the adult animals was 100 % in the solvent control, and 80 % in the water control (pooled control = 90 %). In the 0.0045, 0.011, 0.023, 0.042, 0.12 and 0.31 mg a.s./L treatment levels, survival was 90, 90, 100, 100, 80 and 80 %, respectively.

The first brood juveniles were observed on Day 7 in the controls and all test concentrations up to and including 0.042 mg a.s./L. Hence, time to first brood was unaffected at these concentrations. At 0.12 mg a.s./L first brood release occurred on Day 10 and at 0.31 mg a.s./L no juveniles were produced throughout the test.

The mean number of juveniles per surviving adult showed a statistically significant inhibitory effect on the reproduction of *D. magna* over 21 days at concentrations of 0.12 and 0.31 mg a.s./L (see table below).

Table 9.2.5.1-2: Effects of SYN545974 on Daphnia adult survival, reproduction and growth

Nominal concentrations (mg a.s./L)	Mean measured concentrations (mg a.s./L)	Mean adult survival (%)	Mean number of offspring released (SD)	Mean Growth	
				Length (mm) (SD)	Dry Weight (mg) (SD)
Control	ND	80	171 (14)	4.80 (0.08)	0.98 (0.11)
Solvent control	ND	100	177 (11)	4.78 (0.07)	1.00 (0.07)
Pooled control	ND	90	174 (12)	4.79 (0.07)	0.99 (0.09)
0.0048	0.0045	90	171 (6)	4.74 (0.05)	0.96 (0.07)
0.012	0.011	90	163 (11)	4.69 (0.08) ^a	0.98 (0.09)
0.024	0.023	100	172 (13)	4.77 (0.09)	0.91 (0.11)
0.048	0.042	100	179 (11)	4.77 (0.09)	0.96 (0.08)
0.12	0.12	80	146 (21) ^{b,d}	4.74 (0.05)	1.14 (0.05)
0.30	0.31	80	0 (0) ^b	3.84 (0.15) ^c	0.55 (0.11) ^b

SD = Standard deviation

ND = Not detected. The limit of quantification for SYN545974 was 0.151 mg/L

^a Significantly reduced compared to the pooled control, based on Wilcoxon's Test with Bonferroni's Adjustment; however, this effect was not considered to be treatment related^b Significantly reduced compared to the pooled control, based on Bonferroni's Adjusted t-Test^c Significantly reduced compared to the pooled control, based on Wilcoxon's Test with Bonferroni's Adjustment^d Brood release was also delayed by three days compared to the controls and lower test concentrations.

The NOEC (No Observed Effect Concentration) was defined as the highest tested concentration that elicited no statistically significant difference between the exposed organisms and the pooled control, the LOEC was defined as the lowest test concentration that elicited a statistically significant effect on organism performance, and the EC₅₀ was defined as the concentration in dilution water resulting in a 50 % immobility or reduction in survival or reproductive output of the test organism population at the stated time interval. Effects on survival were established using Fisher's Exact Test with Bonferroni-Holm's Adjustment, effects on reproduction and dry weight were established using Bonferroni's Adjusted t-Test, and Wilcoxon's Test with Bonferroni's Adjustment was used to determine effects for total body length. The statistical analysis computations were performed using CETIS™ Version 1.8.4.20.

The results are summarised in the table below.

Table 9.2.5.1-3: Summary of the effects of SYN545974 on Daphnia magna after 21 days exposure

Endpoint	EC ₁₀ (mg a.s./L) (95 % CI)	EC ₂₀ (mg a.s./L) (95 % CI)	EC ₅₀ (mg a.s./L) (95 % CI)	NOEC (mg a.s./L)	LOEC (mg a.s./L)
Survival	0.094 (0.054 – NA)	> 0.31 (ND)	> 0.31 (ND)	0.31	> 0.31
Reproduction	0.085 (0.063 – 0.12)	0.13 (0.11 – 0.14)	0.19 (0.18 – 0.20)	0.042	0.12
Growth	Body length = 0.21 (0.20 – 0.22) Dry weight = 0.16 (0.14 – 0.16)	Body length > 0.31 (ND) Dry weight = 0.20 (0.18 – 0.21)	Body length and dry weight > 0.31 (ND)	Body length and dry weight = 0.12	Body length and dry weight = 0.31

CI: Confidence interval

NA: could not be determined.

ND: not determined. EC₅₀ value was empirically estimated to be greater than the highest mean measured concentration tested; therefore, corresponding 95% confidence intervals could not be calculated.

VALIDITY CRITERIA

The validity criteria for the test were met:

- Parent mortality in the control $\leq 20\%$ (measured 20 % and 0%, control and solvent control, respectively)
- Mean number of living offspring per surviving parent in the control was ≥ 60 (measured 171 and 177, control and solvent control, respectively)
- The coefficient of variation in the mean number of living offspring per surviving parent in the control was $\leq 25\%$ (measured 7.9 % and 6.1 %, control and solvent control, respectively)

CONCLUSIONS

Based on SYN545974 mean measured concentrations, the 21-day EC_{50} s for survival and reproduction were determined to be >0.31 and 0.19 mg a.s./L, respectively. Based on reproduction (the most sensitive indicator of toxicity), the 21-day NOEC was determined to be 0.042 mg a.s./L and the 21-day LOEC was determined to be 0.12 mg a.s./L.

(██████████, 2016a)

HSE evaluator comments

This study was conducted to GLP. This study was conducted according to OECD Guideline 211 (1998), but the most recent version is OECD 211 (2012). Therefore, this study was assessed according to the more recent version of the guideline.

The substance SYN545974 was prepared using a solvent and therefore OECD Guidance Document 23 (2019) for testing of difficult substances has been considered. The solvent used (Dimethylformamide, DMF) is listed in the Guidance Document as effective for aquatic toxicity testing and is within the recommended concentration range of 0.10 mL DMF/L. Additionally, there were no significant differences in results between the solvent control and the dilution water control, indicating the solvent had no effect on the outcome of the study.

Analytical measurements showed test substance remained within 80-120 % of nominal concentrations, therefore authors may use nominal concentrations to report results. However, authors chose to report results and conduct statistical analysis using mean measured concentrations, which is also acceptable.

The analytical methods have been evaluated by HSE Chemistry specialists in Vol. 3CA Part B51.2.6. The following was concluded for this method: "Fit for regulatory purposes but the method is not fully validated in accordance with SANCO/3029/99 rev. 4. LOQ: 0.3 µg/L in aqueous matrices".

It is noted that the daphnids were fed using the green algae *Ankistrodesmus falcatus*, which is not one of the listed recommended feed species in the guideline. However, the performance of controls shows that this has not affected the overall results of the study and is an adequate food source.

The statistical analyses used by the authors has been examined in accordance with the OECD 211 (2012) guideline. There were no major issues though some points have been noted below for reference:

- The authors confirmed homogeneity and normality of the data using Bartlett's Test and Shapiro-Wilk's Test respectively. All data passed this test except for body length, which failed normality. Therefore, for body length the authors used non-parametric method Wilcoxon's Test with Bonferroni's Adjustment to analyse the data, in accordance with OECD guidelines. For the remaining data, the parametric Bonferroni's Adjusted T-test was used to determine significant difference, in accordance with OECD guidelines.
- The authors used an equal variance two-sample t-test to determine there was no significant difference between the dilution water control and the solvent control, therefore the two controls were pooled for subsequent comparisons with test concentration data.
- Some mortality of parents did occur throughout the test, however this was not statistically significant in test concentrations compared to the pooled control, as determined by the authors using Fisher's Exact Test with Bonferroni-Holm's Adjustment. Visual inspection of the data also shows this mortality was not

in a dose-response relationship with the test concentrations. Therefore, the authors excluded the offspring from adults which died during the test from the results, as recommended in the OECD 211 (2012) guideline when there is no dose-response relationship for parental mortality.

- It is noted that the authors observed a statistically significant difference in body length at the treatment level of 0.012 mg a.s./L compared to the control, but they used the higher treatment concentration of 0.12 mg a.s./L as the NOEC. They justify that the effect in the 0.012 mg a.s./L treatment level “*was not considered to be treatment related*”. Due to the lack of significant effects in any measured parameters for at least another two higher treatment levels, and the relatively low difference in means (4.69 mm for the 0.012 mg a.s./L treatment level compared to 4.79 mm in the pooled control), it is agreed that the significant result obtained for length at 0.012 mg a.s./L treatment level is anomalous and the higher NOEC is acceptable.
- The authors present EC₅₀ values, with EC₁₀ and EC₂₀ analysis having been added at a later date. Guideline 211 (2012) states that regression on the whole dataset is required for calculations of EC_x. For determination of EC_x, the authors do not explain their exact statistical methods or supply figures showing model fit but state that “*If at least one test concentration caused immobilization or reduction in reproduction of greater than or equal to 50 % of the test population, then CETIS™ Version 1.8.4.20 (Ives, 2011) was used to calculate the EC₅₀ values and 95 % confidence intervals.*” There is no reported transformation of the data. Due to the use of an appropriate software and the reported EC_x appearing in line with the observed results, the use of these EC_x is deemed appropriate despite lack of full model details supplied.

Overall, the study had no major deviations from the OECD 211 (2012) guideline and the study fulfils all validity criteria of this guideline.

The agreed endpoints for use in risk assessment are:

- 21-day reproductive EC₁₀ = 0.085 mg a.s./L (mean measured concentrations)
- 21-day reproductive NOEC = 0.042 mg a.s./L (mean measured concentrations)

B.9.2.5.2. Reproductive and development toxicity to an additional aquatic invertebrate species

Report:	K-CA 8.2.5.2 [REDACTED] (2015a), SYN545974 – Life-Cycle Toxicity Test with Mysids (<i>Americamysis bahia</i>). Report Number 1781.6886, Smithers Viscient, 790 Main Street Wareham, MA 02571-1037 USA (Syngenta File No. SYN545974_10167)
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GUIDELINES

US EPA Ecological Effects Test Guideline, OCSPP 850.1350: Mysid Chronic Toxicity Test (1996)

GLP: Yes

MATERIALS

Test material	SYN545974 tech.
Lot/Batch #:	SMU2EP12007
Purity:	98.5 % [certificate of analysis confirmed]
Treatments	
Test concentrations:	Dilution water control and nominal SYN545974 concentrations of 0.0025, 0.005, 0.010, 0.020, 0.040, 0.080 mg a.s./L
Dilution water:	Dilute, filtered, natural seawater collected from Cape Cod Canal, Massachusetts, adjusted to salinity of 20 ± 3 ‰ with laboratory well water and filtered (20, 5 and 1 µm filters)

Analysis of test concentrations:	Yes, at day 0, 7, 14, 21 and 28 days (alternating replicate solutions at each treatment and the control) using LC/MS/MS analysis
Solvent:	None used
Test organisms	
Species:	Mysid (<i>Americamysis bahia</i>)
Source:	In-house cultures. Brood stock originally obtained from MBL Aquaculture, Sarasota, Florida and maintained for 27 months prior to use.
Life stage of test organism:	Juvenile (≤ 24 hours old)
Acclimatisation period:	Not necessary since the test was performed in the same medium as the culturing
Feeding:	Live brine shrimp nauplii (<i>Artemia salina</i>) twice daily during test. At least one feed was enriched with Selco®
Test design	
Test vessels:	28 exposure aquaria were set up. Each glass aquarium was 30 x 20 x 25 cm with a 10 cm-high side drain that maintained a constant exposure solution 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- μ m mesh collar), partially submerged. Pairing chambers were 6 cm diameter petri dishes, with a 14 cm high 350 μ m mesh size opening attached. During reproductive phase of the exposure, each exposure aquarium contained one retention chamber and a maximum of 5 pairing chambers.
Replication:	4 replicates per treatment and control
No. of organisms per replicate:	20, yielding 80 organisms for each treatment level and the control.
Exposure regime:	Flow-through
Duration:	28 days
Environmental conditions	
Test temperature:	25 \pm 2 °C
pH range:	7.6 to 8.1
Dissolved oxygen:	5.08 to 6.73 mg/L (71.0 to 93.0 % of saturation)
Salinity of dilution water:	19 – 22 ‰
Lighting:	16 hours fluorescent light and 8 hours dark daily, with 30-minute dawn and dusk transition periods. Light intensity 230 to 340 lux.

STUDY DESIGN AND METHODS

Experimental dates: 7 March to 4 April 2014

The life-cycle toxicity test was conducted using an exposure system consisting of an intermittent-flow proportional diluter, and a set of 28 exposure aquaria, each containing a retention chamber or during the reproductive phase one retention chamber and a maximum of 5 pairing chambers.

A glass wool saturator column was used to deliver SYN545974 to the exposure system. To prepare a column (which was done at test initiation and then biweekly throughout the exposure), approximately 6 g of test material was diluted with 35 mL of acetone. This solution was poured in the glass column. The column was then attached to a vacuum pump which was used to draw the solution through the column and coat the wool with the test

substance and evaporate the acetone. The vacuum pump was detached once it appeared that all of the wool was uniformly coated, and the column was attached to an FMI pump.

The FMI pump was calibrated to deliver a flow of water of 6 mL/min or 0.062 L/cycle to the diluter system's mixing chamber. The chamber also received approximately 1.94 L of dilution water at each cycle. The solution in the mixing chamber constituted the highest nominal test concentration (0.080 mg/L) and was diluted (50%) to provide the remaining nominal test concentrations (0.040, 0.020, 0.010, 0.0050, 0.0025 mg/L).

To initiate the test, mysids ≤ 24 hours old were randomly distributed between 28 beakers maintained at 25 °C until each beaker contained 20 mysids. Each group of 20 mysids was then randomly assigned to an exposure aquarium. For the first 12 days of exposure, each aquarium contained one retention chamber to retain sexually immature mysids. Upon maturation (day 13), male and female pairs were transferred to separate pairing chambers, unpaired mysids were pooled and maintained in the retention chamber. Following this distribution, each aquarium contained one retention chamber and a maximum of five pairing chambers.

Observations of survival, number of offspring and abnormal appearance or behaviour were recorded daily throughout the study. Throughout the test, mysids were fed live brine shrimp nauplii, twice daily.

During the reproductive phase, groups of offspring ($n = 10$, if possible) were removed from pairing chambers in each replicate vessel and placed in a separate pairing chamber in that replicate. These F_1 mysids were monitored for survival 96 hours post-release.

At test termination all mysids were euthanized and separated into male and female groups for each replicate exposure. Individual body length was measured and mysids were then dried in an oven at 91 to 99 °C for 23 hours and placed in a desiccator. Individual body lengths and dry weights were measured to the nearest 0.01 mm and 0.01 mg, respectively.

The concentrations of test material in the test solutions were measured at test initiation and at test day 0, 7, 14, 21 and 28 using LC/MS/MS.

At test termination, data were statistically analysed to establish treatment level effects. Data were assessed for normal distribution and homogeneity using Shapiro-Wilks and Bartlett's tests before using parametric analyses. Non-parametric analyses were used where data were not normally distributed. Survival data were analysed and evaluated using Fisher's Exact Test with Bonferroni-Holm's adjustment and Dunnett's Multiple Comparison test. Data were analysed using CETIS (Ives, 2013).

RESULTS AND DISCUSSION

The measured concentrations of SYN545974 ranged from 76 to 110% of nominal values. Mean measured concentrations were used for the calculation and reporting of results.

Table 9.2.5.2-1: Analytical results

Nominal concentration (mg a.s./L)	Measured concentration (mg a.s./L)					Mean measured concentration ^a	Percent of nominal ^a
	Day 0	Day 7	Day 14	Day 21	Day 28	(mg a.s./L)	(%)
Control	< 0.00028 ^b	< 0.00024	< 0.00025	< 0.00023	< 0.00023	NA	NA
0.0025	0.0024	0.0021	0.0018	0.0024	0.0023	0.0022	87
0.0050	0.0055	0.0051	0.0049	0.0051	0.0053	0.0052	100
0.010	0.012	0.0078	0.010	0.011	0.011	0.010	100
0.020	0.020	0.018	0.018	0.020	0.020	0.019	97
0.040	0.041	0.033	0.036	0.034	0.042	0.037	93
0.080	0.083	0.061	0.073	0.080	0.085	0.076	95

^a Mean measured concentrations and percent of nominal were calculated using actual analytical data (not rounded data)

^b Concentrations expressed as less than values were below the LOQ (which can vary somewhat among different runs) NA = Not applicable

Table 9.2.5.2-2: Effects of SYN545974 on *Americamysis bahia* reproduction, growth and survival of the adult (F0) generation

Mean Measured Concentrations (mg a.s./L)	Mean F0-survival at 28 days (SD) (%)	Females producing young (SD) (%)	Mean number offspring / female (SD)	Mean F1-survival at 96 h post-release (SD) (%)	Mean Dry Weight (SD) (mg)		Mean Body Length (SD) (mm)	
					Males	Females	Males	Females
Control	80 (10)	100 (0)	14.7 (2.1)	100 (0)	0.79 (0.04)	1.07 (0.08)	7.25 (0.20)	7.25 (0.13)
0.0022	67 (16)	100 (0)	11.9 (3.3)	98 (5)	0.82 (0.03)	1.07 (0.14)	6.99 ^b (0.10)	7.15 (0.39)
0.0052	81 (7)	85 (19)	14.7 (4.7)	95 (10)	0.80 (0.04)	1.17 (0.11)	7.14 (0.11)	7.30 (0.07)
0.010	59 ^a (25)	85 (19)	14.8 (2.7)	96 (7)	0.93 (0.09)	1.16 (0.17)	7.10 (0.11)	7.40 (0.19)
0.019	70 (7)	100 (0)	16.4 (2.3)	98 (5)	0.76 (0.03)	1.11 (0.06)	7.08 (0.15)	7.36 (0.11)
0.037	79 ^a (12)	95 (10)	14.8 (4.9)	95 (6)	0.80 (0.05)	1.08 (0.08)	7.00 (0.88)	7.28 (0.14)
0.076	75 (12)	95 (10)	10.4 (1.8)	100 (0)	0.81 (0.06)	1.09 (0.05)	7.06 (0.11)	7.20 (0.12)

^a Significantly reduced compared to the control, based on Fisher's Exact test with Bonferroni-Holms adjustment

^b Significantly reduced compared to the control, based on Dunnett's multiple comparison test SD= standard deviation

Statistical analyses of the study results did not determine significant differences between any of the SYN545974 treatment levels compared to the control data for mean number of offspring per female, for growth measured as average total body length and average dry body weight for all surviving F0 mysids and F1 mysid survival.

With regard to mean F0-survival at test end, Fisher's Exact Test with Bonferroni-Holm's Adjustment determined a significant difference in survival among organisms exposed to the 0.010 and 0.037 mg a.s./L treatment levels compared to the control data. Conducting gender specific analyses, no significant difference is determined in male survival of any treatment level compared to the control and also for females a statistically significant difference can be determined only among females exposed to the 0.010 mg a.s./L treatment level compared to the control. Taking into account both the gender specific results and the lack of a clearly defined dose response for the mean F0-survival data, the effect observed at the 0.010 and 0.037 mg a.s./L treatment levels was not considered to be toxicant related.

Likewise, the significant difference determined in the total body length of male mysids exposed to the 0.0022 mg a.s./L treatment level compared to the control data was not considered to be toxicant related due to the lack of a clearly defined dose response and a lack of matching effects in the weight endpoints.

VALIDITY CRITERIA

The validity criteria for the test were met;

Table 9.2.5.2-3: 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	11.8 - 16.4
Post-pairing survival*	>70 %	83-100 %

* Criterion not specified in OCSPP 850.1350 (1996)

CONCLUSIONS

The chronic toxicity of SYN545974 to the mysid (*Americamysis bahia*) was determined under flow-through conditions. Mysids were exposed to nominal concentrations of 0.0025, 0.0050, 0.010, 0.020, 0.040 and 0.080 mg a.s./L, together with a dilution water control. Results are based on the mean measured concentrations of 0.0022, 0.0052, 0.010, 0.019, 0.037 and 0.076 mg a.s./L.

The 28-day NOEC was determined to be 0.076 mg a.s./L, and the 28 day LOEC was determined to be > 0.076 mg a.s./L. As no concentration resulted in ≥ 50 % mortality the LC₅₀ was estimated to be > 0.076 mg a.s./L.

(██████, 2015a)

ADDENDUM:

In accordance with **Commission Regulation (EU) No 283/2013**, estimation of EC₁₀ and EC₂₀ values was conducted for ██████, 2015a (SYN545974_10167) in the following report:

Report:	K-CA 8.2.5.2 ██████ (2016b) Pydiflumetofen – Statistical Reanalysis; SYN545974 – Life-Cycle Toxicity Test with Mysids (<i>Americamysis bahia</i>), Report Number 1781.7192e, Smithers Viscient, 790 Main Street, Wareham, MA, USA (Syngenta File No: SYN545974_10465)
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Executive Summary

Report number 1781.6886 (██████, 2015a; SYN545974_10167) did not provide EC₁₀ and EC₂₀ estimates for the response variables evaluated as part of the original study. Consequently, the data generated have been re-analysed in order to provide these values.

Statistical analysis of the available data revealed that EC₁₀ and EC₂₀ values could not be reliably determined.

Statistical Analysis

Mean 28-day survival, male survival and female survival at termination were compared to the mean 28- day survival, male survival and female survival at termination in the control.

At exposure termination, male and female growth (total dry body weight and total body length) were compared to the mean male and female growth in the control.

At exposure termination, the number of young released per female was compared to the number of young released per female in the control.

At exposure termination, F1 survival 96 hours post-release was compared to the F1 survival 96 hours post-release in the control.

All statistical analyses were conducted using CETIS™ Version 1.8 (Ives, 2013). Several statistical models were attempted to determine EC₁₀ and EC₂₀ values.

Results and Conclusion

Statistical analysis of the available data revealed no reliable LC/EC₁₀ and LC/EC₂₀ values for any of the endpoints monitored due to lack of fit with the available models.

(██████, 2016b)

Additional information requested by the RMS is included in the report below.

Report:	K-CA 8.2.5.2, ██████ and ██████ (2016a) SYN545974: Response to ANSES comments regarding the life-cycle toxicity test with mysids (<i>Americamysis bahia</i>) (██████, 2015a) (Syngenta File No. SYN545974_10471)
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Summary

The RMS requested the following comment to be addressed:

This study is valid but RMS considers that the effects observed should not be excluded as confounding effect occurs on mysid. It is RMS opinion that no NOEC may be defined based on this study. At least, as requested above, please provide EC₁₀ and EC₂₀ values.

Syngenta response:

A study was conducted by Syngenta and submitted to the RMS to evaluate the potential toxic effects of SYN545974 technical on the survival, reproduction and growth of mysids (*Americamysis bahia*). First generation mysids were exposed for 28 days to concentrations of 2.5, 5.0, 10, 20, 40 and 80 µg a.s./L, nominal; 2.2, 5.2, 10, 19, 37 and 76 µg a.s./L, mean measured. First generation (F0) survival, reproduction and growth and second generation (F1) survival were used as the indicators of toxicity. This study followed USEPA OPPTS 850.1035 and ASTM E 1191-03a standard testing guidelines. This study was conducted to fulfil global registration requirements only and was included in the submission for completeness.

It is noted that a significant reduction in male body length occurred at the lowest treatment level. Additionally, a significant reduction in mean F0 survival at 28 days was noted in the 10 and 37 µg a.s./L test concentrations.

Effects on male body length

With regard to the significant reduction in male length at the lowest test concentration, the study report highlights that ‘...due to the lack of a clearly defined dose response and a lack of matching effects in the weight endpoints, the effect observed at the 2.2 µg/L treatment level was not considered to be toxicant related.’ It is important to underscore that, per OECD guidance, all test concentrations above the LOEC must have a harmful effect equal to or greater than those observed at the LOEC. As such, the statistically significant effect noted for male length at the lowest treatment level does not demonstrate a true dose-response relationship, nor should it be considered related to exposure to SYN545974.

Table 9.2.5.2-4: Mysid life-cycle exposure to SYN545974 – first generation (f0) male and female total body length

Mean Measured Concentration (µg a.s./L)	Replicate	Mean Total Body Length (mm)	
		Males	Females
Control	A	7.23	7.11
	B	7.46	7.43
	C	6.99	7.24

Mean Measured Concentration (µg a.s./L)	Replicate	Mean Total Body Length (mm)	
		Males	Females
	D	7.31	7.21
	Mean (SD) ^a	7.25 (0.20)	7.25 (0.13)
	A	6.95	7.14
	B	6.95	6.65
	C	6.93	7.22
2.2	D	7.15	7.6
	Mean (SD)	6.99 (0.10) *	7.15 (0.39)
	A	7.27	7.28
	B	7.02	7.26
	C	7.09	7.4
5.2	D	7.18	7.25
	Mean (SD)	7.14 (0.11)	7.30 (0.07)
	A	7.01	7.2
	B	7.24	7.6
	C	7.03	7.52
10	D	7.15	7.27
	Mean (SD)	7.10 (0.11)	7.40 (0.19)
	A	6.98	7.33
	B	7.11	7.44
	C	7.28	7.45
19	D	6.95	7.21
	Mean (SD)	7.08 (0.15)	7.36 (0.11)
	A	6.97	7.08
	B	7.11	7.4
	C	7.01	7.3
37	D	6.92	7.32
	Mean (SD)	7.00 (0.08)	7.28 (0.14)
	A	6.94	7.2
	B	7.12	7.33
	C	7.18	7.04
76	D	7.01	7.23
	Mean (SD)	7.06 (0.11)	7.20 (0.12)

^a Mean values are presented with standard deviations (SD) in parentheses.

* Significantly reduced compared to the control, based on Dunnett's Multiple Comparison Test. However, due to the lack of a clearly defined dose response, the effect observed at this treatment level was not considered to be toxicant related.

NOTE: Values presented have been rounded; however, statistical analysis was performed using unrounded values.

The historical control data for mean male total body length for mysid life cycle studies conducted at the testing laboratory (n = 9) between 2013 and 2014, demonstrate that the control growth of mature male mysids during this study (6.99 mm) fell within the normal expected range (6.76 to 7.53 mm).

Furthermore, the overall range of minimum and maximum values among the nine studies represents a 10% difference, which should be considered the naturally occurring variability amongst control mysids.

Table 9.2.5.2-5: Historical Control Data for Mean Total Body Length during Mysid Life-Cycle Studies Conducted at the Testing Facility

Study ID	Control Replicate	Male Total Body Length (mm)	Mean (mm)	Standard Deviation	Coefficient of Variation (%)
1	A	6.97	7.31	0.270	3.69
	B	7.57			
	C	7.48			

Study ID	Control Replicate	Male Total Body Length (mm)	Mean (mm)	Standard Deviation	Coefficient of Variation (%)
	D	7.23			
2	A	7.29	7.34	0.163	2.22
	B	7.58			
	C	7.22			
	D	7.27			
3	A	7.22	7.29	0.088	1.20
	B	7.30			
	C	7.23			
	D	7.41			
4	A	7.59	7.38	0.173	2.35
	B	7.44			
	C	7.28			
	D	7.20			
5	A	7.23	7.25	0.196	2.71
	B	7.46			
	C	6.99			
	D	7.31			
6	A	7.06	7.20	0.135	1.87
	B	7.15			
	C	7.38			
	D	7.19			
7	A	7.88	7.53	0.376	5.00
	B	7.80			
	C	7.10			
	D	7.32			
8	A	6.70	6.76	0.299	4.42
	B	7.20			
	C	6.54			
	D	6.61			
9	A	6.92	6.95	0.081	1.17
	B	7.06			
	C	6.93			
	D	6.87			
Minimum			6.76		4.42
Maximum			7.53		5.00

The percent reduction for each treatment level observed during this exposure ($\leq 3.6\%$) fell well within the normal control variability of adult male mysids (10%). Furthermore, apical growth endpoints, particularly for invertebrates, are frequently used for corroboration of true biological responses. For this study, the dry weight data for F0 male mysids exposed to all treatment levels were all very consistent with the control data and no statistically significant effects were noted, further supporting that the effect noted for the male length endpoint was not biologically relevant.

In the study report, Dunnett's Multiple Comparison Test was used. This test is one of a number of standard approaches and is considered appropriate for this data set. Consideration could also be given to alternative statistical analysis. Dunn's Test with Bonferroni-Holm's adjustment is an alternative method of multiple comparison test. Using Bonferroni-Holm's adjustment minimises the likelihood of false positives. When this test is applied, there is no significant difference at the lowest treatment level, implying that the significant difference detected by the Dunnett's test was potentially a false positive.

Therefore, the NOEC for male body length is considered to be 76 µg a.s./L.

Effects on F0 survival at 28 days

A significant reduction in mean F0 survival at 28 days was noted in the 10 and 37 µg a.s./L test concentrations. However, in the study report, the author highlighted that '*...due to the lack of a clearly defined dose response, the effect observed at the 10 and 37 µg/L treatment levels was not considered to be toxicant related*'.

It is important to underscore that, per OECD guidance, all test concentrations above the LOEC must have a harmful effect equal to or greater than those observed at the LOEC. As such, the statistically significant effect noted for F0 survival at the 10 and 37 µg a.s./L treatment levels do not demonstrate a true doseresponse relationship, nor should they be considered related to exposure to SYN545794. Therefore, due to the lack of a clearly defined dose response, the NOEC is considered to be 76 µg a.s./L for 28d survival.

Table 9.2.5.2-6: Mysid life-cycle exposure to SYN545794 – first generation (f0) survival

Mean Measured Concentration (µg a.s./L)	Replicate	Male Survival ^a (%)	Female Survival ^a (%)	Post-Pairing Survival (%)	28-Day Survival (%)
Control	A	86	100	93	82
	B	100	100	100	67
	C	80	88	83	83
	D	86	100	94	89
	Mean (SD) ^b	88 (9)	97 (6)	93 (7)	80 (10)
2.2	A	67	90	79	75
	B	86	67	82	78
	C	80	80	80	71
	D	38	67	50	44
	Mean (SD)	67 (22)	76 (11)	73 (15)	67 (16)
5.2	A	89	100	93	76
	B	82	100	86	75
	C	91	100	95	90
	D	100	89	93	82
	Mean (SD)	90 (9)	97 (6)	92 (4)	81 (7)
10	A	100	100	100	85
	B	67	67	67	53
	C	29	33	31	28
	D	40	92	76	72
	Mean (SD)	59 (32)	73 (30) *	69 (29)	59 (25) *
19	A	100	75	82	70
	B	73	57	67	60
	C	100	90	93	76

Mean Measured Concentration ($\mu\text{g a.s./L}$)	Replicate	Male Survival ^a (%)	Female Survival ^a (%)	Post-Pairing Survival (%)	28-Day Survival (%)
	D	63	89	76	72
	Mean (SD)	84 (19)	78 (15)	80 (11)	70 (7)
37	A	100	78	88	82
	B	86	100	95	95
	C	100	100	100	71
	D	83	78	80	67
	Mean (SD)	92 (9)	89 (13)	91 (9)	79 (12) *
76	A	89	90	89	89
	B	73	67	71	63
	C	100	100	100	79
	D	86	100	76	68
	Mean (SD)	87 (11)	89 (16)	84 (13)	75 (12)

^a Calculations of male and female survival began after pairing.

^b Mean values are presented with standard deviations (SD) in parentheses.

* Significantly reduced compared to the control, based on Fisher's Exact Test with Bonferroni-Holm's Adjustment. However due to the lack of a clearly defined dose response, the effect observed at this treatment level was not considered to be toxicant related.

NOTE: Values presented have been rounded; however, statistical analysis was performed using unrounded values.

28-day survival in the 2.2, 5.2, 10, 19, 37 and 79 $\mu\text{g a.s./L}$ treatment levels differed from the control by 16, 0, 26, 12, 1 and 6%, respectively. The statistically significant effect on 28-day survival in the 37 $\mu\text{g a.s./L}$ treatment is questionable, as survival is 99% of the control value (nearly identical to the control mean survival value and standard deviation).

The significance of the statistical differences in survival in the 10 $\mu\text{g a.s./L}$ treatment is also questionable, as no statistically significant treatment related effects were noted in male survival or in post-pairing survival. As can be seen in the table above, one of the four replicates (replicate C) was consistently lower than the others across all survival endpoints. Effects at this level were not noted in any other replicate of the other treatment levels. Additionally, it should be noted that despite potential effects on mean survival, no statistically significant treatment related effects were noted in the reproduction and growth endpoints (females producing young, number of offspring per female, F1 survival, dry weight and body length).

In the study report, Fisher's Exact Test with Bonferroni-Holm's Adjustment was used. This test is one of a number of standard approaches and was considered by the study director as the most appropriate. William's test is the US EPA's preferred method of analysis for these data. William's test should be used where a dose response is expected and observed. In this case, a dose response is expected, but not observed, so the appropriateness of this method is questionable. However, use of the William's test reveals no significant differences from control for survival (female and 28-day).

In addition, data obtained during the preliminary test have been statistically analysed and the results support the conclusion that there is no treatment related effect on survival at concentrations around 10 $\mu\text{g a.s./L}$.

Therefore, the NOEC for 28-d survival is considered to be 76 $\mu\text{g a.s./L}$.

EC10 and EC20 values

EC10 and EC20 values could not be determined since the data did not meet the criteria for ECx determination. This is not unexpected due to the lack of effects observed in the study. In addition, the study is primarily designed to achieve a robust and reliable NOEC that is the required endpoint for the risk assessment. There are no ECx values suitable for use in the risk assessment.

Conclusion

Based on the points described above, the concern for the lack of a NOEC is unfounded. The clear lack of a dose-response for all endpoints evaluated during this exposure, consideration of alternative statistical analysis and the historical growth performance of mysids in numerous chronic studies, demonstrates that the current reported NOEC of 76 µg a.s./L should be considered robust and useful for assessment of risk. EC10 and EC20 values could not be determined since the data did not meet the criteria for ECx determination. This is not unexpected due to the lack of effects observed in the study. Therefore, there are no ECx values suitable for use in the risk assessment.

HSE evaluator comments

This study was carried out in accordance with GLP and follows OCSPP 850.1350 (1996) with no significant deviations to the protocol. The validity criteria outlined in OCSPP 850.1350 (1996) were satisfactorily fulfilled. The applicant included an additional validity criterion (post-pairing survival), which was not specified within the OCSPP 850.1350 (1996) guideline. This criterion was met, since post-pairing survival exceeded 70 %. OCSPP 850.1350 (1996) guidelines recommend a photoperiod of 14 h light and 10 h darkness, however, a photoperiod of 16 h light and 8 h darkness has been used here. This deviation is not thought to have affected the study outcome, since the validity criteria were fulfilled.

Measured concentrations of SYN545974 ranged from 76-110 % of nominal, so results are based on mean measured concentrations. OCSPP 850.1350 (1996) guidelines require the calculation of a maximum-acceptable-toxicant-concentration (MATC) value, which is defined as the geometric mean of the LOEC and NOEC for the most sensitive test criterion. Since no dose-response relationship was established, the 28-day NOEC was determined to be 0.076 mg a.s./L, and the 28-day LOEC was determined to be > 0.076 mg a.s./L. Therefore, the MATC could not be calculated.

It is noted that for the 0.076 mg a.s./L group, the number of offspring produced was reduced compared to the lower concentrations. Although not statistically significant, this may be biologically relevant, since it is uncertain if this reduction is due to natural variation, and no other parameters were noticeably reduced in proportion. It is possible that the reduction in offspring could be due to action of the test item. A more conservative NOEC estimate may therefore be appropriate i.e. 0.037 mg a.s./L.

EC_{20/10} values were not calculated as part of the original study report since no dose-response relationship was established for any test criteria, as discussed in the addendum supplied by the applicant above. HSE accepts that calculation of EC_{20/10} values was not possible due to lack of treatment-related effects. The LC₅₀ value was not determined statistically, so no consideration of statistical methodology is required.

The analytical methods have been evaluated by HSE Chemistry specialists in Vol. 3CA Part B5.1.2.6. The following was concluded for this method: “Fit for regulatory purposes but the method is not fully validated in accordance with SANCO/3029/99 rev. 4. LOQ: 0.3 µg/L in aqueous matrices”.

The agreed endpoint for use in risk assessment is therefore:

- 28-day LC₅₀ > 0.076 mg SYN545974 /L
- 28-day NOEC (based on number of offspring) = 0.037 mg SYN545974 /L

(██████████ and ██████████, 2016a)

B.9.2.5.3. Development and emergence in *Chironomus riparius*

Report:	K-CA 8.2.5.3 ██████████, ██████████, ██████████ & ██████████ (2015), SYN545547 - A Prolonged Sediment Toxicity Test with the Midge (<i>Chironomus riparius</i>) Using Spiked Sediment, Report Number 528A-286, Wildlife International, 8598 Commerce Drive, Easton, MD 21601 USA. (Syngenta File No. SYN545547_10004).
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GUIDELINES

OECD Guideline 218 Sediment-Water Chironomid Toxicity Test using Spiked Sediment (2004)

GLP: Yes

MATERIALS

Test Material SYN545547
Description: White powder
Lot/Batch #: BPS 1510/1
Purity: 95 % w/w
Stability of test compound: Stable under test conditions

Reanalysis/Expiry date: 30th May 2017

Treatments

Test concentrations: Dilution water control, solvent control and nominal concentrations of 8.1, 27, 90, 300 and 1000 mg a.s. /kg of sediment (corresponding to 7.2, 21, 80, 285 and 1044 mg a.s. /kg mean measured)
Solvent: Acetone
Analysis of test concentrations: Yes (0 and 28 days)

Test organism

Species: *Chironomus riparius*, first instar (3 days post hatch)
Source: Wildlife International, Easton, Maryland, original culture supplied by: Environmental Consulting and Testing, Superior, Wisconsin 54880
Feeding: 20 – 30 mg ground TetraMin® flake food approximately three times per week, beginning on Day 0

Test design

Test vessels: 1-quart (~950 mL) glass jars containing approximately 2 cm (approximately 150 mL) of sediment and approximately 600 mL of overlying water
Test medium: Filtered well water
Artificial Sediment: 5 % sphagnum peat (air dried and finely ground)
 20 % silt and clay (kaolin clay)
 75 % industrial quartz sand
 The organic carbon content of the final sediment mixture was 1.7 %
 Sediment pH = 7.0 (± 0.5)
 pH of 1:1 ratio of sediment:water = pH 7.7
Sediment moisture content: 68.6 %
Replication: Eight replicate test vessels, four vessels for use in analytical sampling, and four test vessels with 20 larvae per vessel.
Duration: 28 days

Environmental conditions

Test temperature: 20.4 – 20.7 °C (in test vessels).
pH range of overlying water: 8.0 – 8.6

Dissolved oxygen of overlying water:	8.0 – 9.1 mg /L (≥ 88 % of saturation)
Total hardness:	158 mg /L CaCO ₃ for media batch used at start of test. 164 mg /L CaCO ₃ for control and 158 mg /L CaCO ₃ for highest test concentration.
Lighting:	16 hours fluorescent light (552 lux at water surface) and 8 hours dark with 30 minute dawn and dusk transition periods

STUDY DESIGN AND METHODS

Experimental dates: 4 September to 21 October 2015

This study examined the effects of SYN545547 on the emergence and development of *Chironomus riparius*. A 30 mL primary stock solution was prepared by mixing a calculated amount of test substance into HPLC-grade acetone at a nominal concentration of 100 mg a.s./mL. Four secondary stock solutions (30 mL each) were prepared in acetone at nominal concentrations of 0.81, 2.7, 9.0 and 30.0 mg a.s./mL by serial dilution of the primary or previous stock.

Eight replicate test chambers were prepared for each treatment and control group. Four replicates per group were used for biological observations. The additional four replicates per group were prepared for use in analytical confirmation of the test item concentrations. At the beginning of the sediment preparation process, an 18 mL volume of the appropriate stock solution was added to 90 g of sand, which was then placed into a fume hood, and the solvent was allowed to evaporate for one hour. This mixture was then combined with a further 810 g of untreated formulated sediment, and was mixed for one hour. This mixture was then combined with the remaining 900 g of untreated formulated sediment, to reach a final volume of 1,800 g. This final mixture was then mixed for approximately 67 hours prior to use in the experiment.

After mixing the batch sediments, approximately 2 cm (approximately 150 mL) of the appropriate dosed sediment was placed in the bottom of each test chamber (one quart glass jars) on a top-loading balance, and the weight of the sediment was recorded. Approximately 600 mL of overlying water was slowly added to each test chamber, while avoiding disturbance of the sediment, and each test chamber was loosely covered. This provided a sediment : water ratio of 1 : 4 parts. After preparation, the test chambers were impartially positioned in a temperature-controlled environmental chamber, and gentle aeration was applied to each test chamber. The sediment/water mixtures were allowed to acclimate under static conditions for approximately 48 hours prior to introduction of the organisms.

To initiate the test, one to two first-instar larvae (3 days old) were added to a test chamber until it contained 20 individuals; this was repeated until all chambers contained 20 larvae. The test chambers prepared for analytical sampling on Day 0 did not contain midges. All transfers were made below the water surface using wide-bore pipettes.

The test chambers were observed daily during the test to make visual assessments of any abnormal behaviour (e.g., leaving the sediment, unusual swimming). During the period of expected emergence, the sex and number of fully emerged midges were recorded daily, this information was also used to record the duration of the development time. After identification, the midges were removed from the test chambers. When the total number of adults emerged in each replicate at the end of the test (Day 28) was less than the number initially placed in each replicate, then those individuals not accounted for were considered dead.

At the start and at the end of the test and on a weekly basis the pH and dissolved oxygen were measured in each test vessel. Water temperature was recorded continuously by means of a data logger. The hardness was measured of the medium batches used at the start of the test and of the overlying water in the control and the highest test concentration at the end of the test.

The concentrations of test material were determined at Day 0, Day 7, and Day 28 in the overlying water, pore water, and sediment, using high performance liquid chromatography with ultraviolet absorbance detection (HPLC/UV). The test chambers prepared for analytical sampling on day 0 did not contain any test organisms.

The limit of quantification (LOQ) for sediment analysis was 2.49 mg a.s. /kg and the LOQ for water analysis was 2.00 mg a.s. /kg.

A preliminary analysis was performed to determine if there were any differences in the sensitivity of sexes to the test substance. This analysis revealed a concentration-related trend in the proportion of emerged males and females in each treatment group, and also a significant interaction between sex and treatment was demonstrated with an ANOVA performed on development rate. Since both tests were significant at the 0.05 probability level for development rate, the sexes were evaluated separately in subsequent analyses.

The emergence ratio, development time, and development rate data were evaluated for normality and homogeneity of variance ($p = 0.01$) using the Chi-Square test and Levene's test, respectively.

As the male development rate data passed the assumptions of normality and homogeneity, the data in the treatment groups were compared to the pooled control data using a Bonferroni t-test to identify any significant differences ($p = 0.05$).

The assumption of homogeneity was not met in the emergence ratio, development time and female development rate data. An unsuccessful attempt was made to correct the condition by log transformation of the data. As such, the data in the treatment groups were compared to the negative control data (development time and female development rate) or pooled control data (emergence ratio), using a Kruskal-Wallis non-parametric test to identify any significant differences ($p = 0.05$). The LC_{50} value and the 95 % confidence intervals were calculated by probit analysis, using the moving average method. Non-linear interpolation was used to calculate the 28-day LC_{50} value and binominal probability was used to calculate the 95 % confidence interval. Due to the method used to calculate the LC_{50} value, the slope of the concentration-response curve could not be calculated. The EC_{10} and EC_{20} values based on emergence ratios and development rates at the end of the test period were also calculated using the Versteeg method.

RESULTS AND DISCUSSION

Analytical Results

The initial measured concentrations of test material in the sediment were in the range 77 to 115 % of nominal and were 1.0 to 4.4 % in the overlying water. After 28 days of the test, concentrations of test material measured in the sediment were in the range 65.2 to 78.5 % of the nominal concentrations, and were between 1.5 to 9.6 % of the nominal concentrations in the overlying water (see table 9.2.5.3-1 below). Biological results are therefore based on mean measured concentrations of 7.2, 21, 80, 285 and 1044 mg a.s. /kg.

Table 9.2.5.3-1: Analytical results

Nominal concentration (mg a.s. /kg)	% of nominal measured in the overlying water		% of nominal measured in pore water		% of nominal measured in sediment		Mean measured concentration (mg a.s. /kg)
	Day 0	Day 28	Day 0	Day 28	Day 0	Day 28	
8.1	4.4	4.3	1.1	0.5	115	67.8	7.2
27	4.4	9.6	1.1	0.7	77.0	65.2	21
90	3.5	8.7	1.0	0.6	96.0	72.9	80
300	2.7	4.8	0.4	0.3	100	71.0	285
1000	1.0	1.5	0.1	0.1	101	78.5	1044

Biological Results

The effects of SYN545547 on *C. riparius* emergence and development, based on mean measured concentrations are given in the table below:

Table 9.2.5.3-2: Effects of SYN545547 on emergence and development of *Chironomus riparius*

Mean measured Sediment Concentration (mg a.s. /kg)	Number emerged ^a			Mean emergence ratio ^{b, d}	Mean development time (Days) ^d	Mean development rate ^{c, d}	
	Males	Females	Total			Males	Females
Control	51	28	79	0.99	14.6	0.0732	0.0679
Solvent Control	41	36	77	0.96	15.5	0.0732	0.0626
Pooled Control	92	64	156	0.98	--	0.0732	--
7.2	48	31	79	0.99	15.2	0.0742	0.0617
21	29	51	80	1.00	16.5	0.0712**	0.0591
80	30	42	72	0.90	18.6***	0.0663**	0.0503***
285	0	2	2	0.03*	18.5	--	0.0559***
1044	0	0	0	--	--	--	--

^a Each replicate contained 20 midge larvae at test initiation, for a total of 80 larvae per control and treatment group.

^b Emergence ratio is calculated as the number of emerged midges divided by the initial number exposed, and corresponds to percent emergence.

^c The development rate represents that portion of larval development which takes place per day.

^d Calculated using SAS or Excel 2010. Manual calculations may differ slightly.

* Indicates a statistically significant difference in comparison to the pooled control ($p \leq 0.05$) using a non-parametric Kruskal-Wallis test.

** Indicates a statistically significant difference in comparison to the pooled control ($p \leq 0.05$) using a Bonferroni t-test.

*** Indicates a statistically significant difference in comparison to the negative control ($p \leq 0.05$) using a non-parametric Kruskal-Wallis test.

-- = Not calculated.

Table 9.2.5.3-3: Summary of SYN545547 endpoints for emergence and development of *Chironomus riparius*

Endpoint	Emergence (mg a.s. /kg)	Male development (mg a.s. /kg)	Female development (mg a.s. /kg)
NOEC	80	7.2	21
LOEC	285	21	80
EC ₁₀ (Confidence interval)	82.7 (66.5 - 103)	81.1 (64.2 - 102)	35.4 (< 7.2 - 324)
EC ₂₀ (Confidence interval)	97.8 (81 - 118)	192 (116 - 317)	67.3 (14.1 - 323)
EC ₅₀ (Confidence interval)	122 (80 - 285)	-	-

- Not calculated

VALIDITY CRITERIA

The validity criteria were met according to OECD 218 (2004):

Table 9.2.5.3-4: Validity criteria

Validity criterion	Required	Obtained
Emergence in the controls	Emergence in the controls should be ≥ 70 % at the end of the test.	Emergence in the control and solvent control conditions was 99 and 96 %, respectively.
	Emergence to adults in the control vessels should occur between 12 and 23 days after their insertion into the vessels.	Control emergence occurred between 13 and 21 days after initiation.
pH and dissolved O ₂ concentration	At the end of the test, O ₂ concentration should be at least 60 % of the air saturation value, pH of overlying water should be in the range of 6-9 in all test vessels.	At the end of the test, pH of overlying water ranged from 8.0 to 8.6. Dissolved O ₂ concentration ranged from 88 to 100 %
Water temperature	Water temperature should not vary by more than ± 1.0 °C.	Temperature ranged from 20.4 to 20.7 °C

CONCLUSIONS

Midge larvae (*Chironomus riparius*) were exposed to SYN545547 at nominal test concentrations of 8.1, 27, 90, 300 and 1000 mg a.s. /kg for 28 days under static conditions. Mean measured concentrations in the sediment were 7.2, 21, 80, 285 and 1044 mg a.s. /kg. There were treatment-related effects observed on both emergence and development. Based on the mean measured concentrations in sediment, the 28-day EC₅₀ value for emergence was 122 mg a.s. /kg, with a 95 % confidence interval of 80 to 285 mg a.s. /kg. Based on the effects observed on male development rate, the LOEC for the study was 21 mg a.s. /kg and the NOEC was 7.2 mg a.s. /kg.

(██████ et al., 2015)

HSE evaluator comments

This study was conducted according to GLP, and was also conducted in accordance with OECD 218 (2004), all validity criteria were met. No deviations were noted. The statistics used were in line with the OECD 218 (2004) guidelines.

Although the male development rate was the most sensitive NOEC endpoint, it is noted that both the EC₁₀ and EC₂₀ values were lower for female development than for male development. The 95 % confidence intervals for both male and female development EC_x values were large, most notably so for the female development rate. No probit curve was provided by the applicant, and so the fit of the data to the model cannot be visually inspected. Following a request for additional information, probit curves were provided by the applicant. The data appeared to fit the model, despite large confidence intervals for female development.

The study authors did not report the use of a positive control reference item. Although not explicitly required, the OECD 218 (2004) guidelines do recommend that one is used periodically to verify the sensitivity of the test system.

The analytical methods have been checked by HSE Chemistry specialists in Vol 3CA Part B5.1.2.6. The following was concluded: “Acceptable method. LOQ: 0.2 mg/Lin water samples and 2.49 mg/kg dry sediment”.

Based on mean measured concentrations, the NOEC for male development was 7.2 mg SYN5455547 /kg sediment.

B.9.2.5.4. Sediment dwelling organisms

Report: K-CA 8.2.5.4 [REDACTED] (2015), SYN545974 – Life-Cycle Toxicity Test Exposing Midges (*Chironomus dilutus*) to Spiked Sediment, Report Number 1781.6889, Smithers Viscient, 790 Main Street, Wareham, MA 02571-1037 USA. (Syngenta File No. SYN545974_10095)

GUIDELINES

U.S. EPA, Office of Water, Methods for Measuring the Toxicity and Bioaccumulation of Sediment associated Contaminants with Freshwater Invertebrates. Test method 100.4 (2000)

US EPA Ecological Effects Test Guidelines 850.1760. Whole Sediment Life Cycle Toxicity Test with *Chironomus* spp. (1996)

GLP: Yes

MATERIALS

Test Material

Description: SYN545974 tech.
Lot/Batch #: SMU2EP12007
Purity: 98.5 %
Stability of test compound: Stable under standard conditions
Reanalysis/Expiry date: 30 June 2016

Treatments

Test concentrations: Negative control, solvent control and nominal concentrations of 2.6, 6.4, 16, 40, 100 mg a.s./kg dry weight of sediment

Solvent: Stocks were prepared in acetone. 10 ml of acetone (containing no test substance for solvent control or the appropriate amount of test substance for respective treatments) was added to 0.050 kg of fine silica sand and then the solvent was allowed to completely evaporate off.

Analysis of test concentrations: Yes (0, 20 and 59 days) – based on measurements of SYN545974 in the overlying water, pore water, and sediment.

Test organism

Species: *Chironomus dilutus*, < 24 hours old at the start of the exposure

Source: Continuous laboratory cultures from Smithers Viscient culture facility

Feeding: Fish food (Tetramin) suspension (4 mg/mL). During the exposure food was introduced at 1.5 mL flaked fish food per vessel per day

Test design

Test vessels: 300 mL glass vessels with two slots cut on the top edge of the beaker covered with 40-mesh Nitex® screen for drainage and containing 72.7 g of dry sediment and 175 mL of overlying water (laboratory well)

Test medium: Laboratory well water, with a total hardness of 38 to 52 mg/L as CaCO₃, and a pH range of 7.3 to 7.7

Artificial Sediment:	Smithers Viscient artificial sediment batch number 012913B prepared to OECD guideline 218 (2004) 6 % sphagnum peat (air dried and finely ground) 20 % kaolin clay (kaolinite content >30 %) 74 % fine sand 110 g Calcium carbonate (to adjust the pH) The organic carbon content of the final sediment mixture was 2.2 % (Organic carbon was characterised by Agvise Laboratories, North Dakota, USA)
Sediment moisture content:	18.6%
Replication:	Twenty replicates, each containing 12 individuals, were established for each control and treatment level.
Duration:	59 days
Environmental conditions	
Test temperature:	21 to 26°C (in test vessels).
pH range of overlying water:	7.3 to 7.7
Dissolved oxygen of overlying water:	Maintained above 2.5 mg/L throughout the exposure.
Total hardness :	38 to 52 mg/L CaCO ₃
Lighting:	16 hours fluorescent light (370 – 810 lux) and 8 hours dark

STUDY DESIGN AND METHODS

Experimental dates: 27 December 2012 to 26 July 2013

A 25 mg/mL stock solution was prepared by dissolving 1.2573 mg of SYN545974 in 50 mL volumetric flask and bringing to volume with acetone. Stock solution was diluted with acetone to give the dosing solutions with the concentrations 19.5, 7.8, 3.12, 1.25 and 0.510 mg a.s./mL. 10 mL of each dosing stock solution was applied to 0.05 kg of fine silica. The solvent was allowed to evaporate off for 60 minutes. The dry sand containing the test substance was then added to 3.5 kg of wet sediment in individual glass jars to produce the required test concentrations. Jars were sealed and rolled for two hours at room temperature, and then left to equilibrate for 30 days in a refrigerator. Once a week and prior to distribution of the sediments into replicate test vessels, jars were rolled for two hours.

100 mL of sediment (approximately 4 cm layer) was transferred per test chamber (300 mL glass jars) and overlaid carefully with 175 mL water. Larvae of *Chironomus dilutus* were exposed to the test item in glass jars filled with sediment and overlying water until emergence. All vessels were terminated on day 59, regardless if all individuals loaded had emerged. Treated and control sediments were allocated to test vessels one day prior to exposure. The larvae were randomly distributed amongst the test vessels. Throughout the test the larvae were fed daily and from day one the overlying water was renewed in a calibrated water renewal system providing 350 mL per vessel every 24 hours (i.e. 2 volume additions) until day 11, from which overlying water renewal was increased to 4 volume additions per day.

Daily observations of mortality (larvae or pupae) on the sediment surface and abnormal behaviour were made and the physical characteristics of the test solutions were recorded.

Twenty replicates, each containing 12 individuals, were established for each control and treatment level. Twelve replicates were used to evaluate biological response of the test organisms; four of these were used for survival and growth (ash-free dry weight) measurements on test day 20, and the remaining eight replicates were used for assessment of emergence and reproduction. Four additional replicates were established on test day 10 for production of auxiliary males during the emergence and reproduction phase of the test. The final four replicates were maintained for chemical analysis. Starting on test day 18 and daily thereafter, male and female adult midges emerged from each replicate test vessel were recorded and were placed in reproductive/oviposit chambers. Egg

masses were collected and survival of individual midges (male and female) was recorded daily until death. The number of eggs produced in each primary egg mass laid by female midges in each treatment level and control by replicate were counted the day the egg mass was laid.

Prior to test day 20, four replicate vessels were randomly selected for Midge Larval Survival and Growth determination. The sediment was sieved to remove all surviving midges, before pooling and drying at $60 \pm 1^\circ\text{C}$ for 24 hours. The dry larvae were weighted, then ashed at $550 \pm 50^\circ\text{C}$ for two hours. The ashed larvae were weighed.

Dissolved oxygen, pH and temperature of each test vessel were measured on days 0, 10, 20 and 59. On remaining days, dissolved oxygen and temperature were measured daily in one alternating replicate of each treatment and control. Water temperature was recorded continuously by means of a data logger in an auxiliary vessel. Total hardness, alkalinity, conductivity and ammonia concentration of the overlying water were measured at exposure initiation, day 10, day 20 and test termination in each treatment level and control solution.

The concentrations of test material were determined on days 0, 20 and 59 in the sediment, pore water and overlying water using an LC/MS/MS method.

RESULTS AND DISCUSSION

The initial measured concentrations of test material in the sediment were in the range 97 – 110% of nominal. After 59 days of the test, concentrations of test material measured in the sediment were in the range 75 and 85% of nominal (see table below). The Limit of Quantification (LOQ) for sediment analysis was 0.210 mg a.s./kg.

Table 9.2.5.4-1: Analytical results

Nominal concentrations in sediment (mg a.s./kg)	Measured overlying water concentration mg a.s./L (% of nominals)			Measured pore water concentration mg a.s./L (% of nominals)			Measured sediment concentration mg a.s./kg (% of nominals)			Mean measured conc. in sediment (mg a.s./kg)	Geometric mean conc. In sediment (mg a.s./kg) ^a
	Day 0	Day 20	Day 59	Day 0	Day 20	Day 59	Day 0	Day 20	Day 63		
Control	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	NA	NA
Solvent control	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	NA	NA
2.6	0.0015 (0.057)	0.001 (0.038)	0.001 (0.038)	0.022 (0.85)	0.014 (0.54)	0.017 (0.65)	2.5 (96.2)	2.5 (96.2)	2.2 (84.6)	2.4 (92.3)	2.4 (92.3)
6.4	0.016 (0.25)	0.0023 (0.036)	0.0013 (0.02)	0.082 (1028)	0.019 (0.3)	0.045 (0.70)	6.2 (96.9)	6.0 (93.8)	5.0 (78.1)	5.8 (90.6)	5.7 (89.1)
16	0.079 (0.49)	0.01 (0.062)	0.0055 (0.034)	0.21 (0.31)	0.21 (0.31)	0.13 (0.81)	17 (106.3)	15 (93.8)	12 (75)	15 (93.8)	14.5 (90.6)
40	0.55 (1.38)	0.019 (0.048)	0.02 (0.05)	0.57 (1.43)	0.55 (1.38)	0.43 (1.08)	40 (100)	38 (95)	31 (77.5)	36 (90)	36.1 (90.25)
100	0.24 (0.24)	0.041 (0.041)	0.077 (0.077)	0.91 (0.91)	1.3 (1.3)	0.12 (0.12)	110 (110)	95 (95)	79 (79)	93 (93)	93.8 (93.8)

LOQ = Limit of Quantification. The LOQ for each analysis is dependent upon the regression, the area of the low standards and the dilution factors of the controls. NA = Not Applicable

^aGeometric mean calculated by HSE

The control and solvent control endpoints were compared using an Equal Variance t Two-Sample Test or Wilcoxon's Rank Sum Two-Sample Test to compare the performance. A significant difference was observed between control and solvent control data for day 59 male emergence rate. Therefore, these data were compared to the solvent control data to determine treatment-related effects. All remaining statistical analyses were performed comparing treatment data to the pooled control data, since no significant differences were observed between control and solvent control data.

The LOEC was defined as the lowest tested concentration at which the test substance was observed to have a statistically significant effect for a given endpoint when compared with the control. However, all test

concentrations above the LOEC should have an effect equal to or greater than that observed at the LOEC. The NOEC was defined as the test concentration immediately below the LOEC, which when compared to the selected control, had no statistically significant effect. These were calculated using Wilcoxon's Test with Bonferroni's Adjustment to establish treatment effects for time to oviposition and egg masses per female, and Bonferroni's Adjusted t-Test or Dunnett's Multiple Comparison Test were used to establish treatment effects for all other endpoints. The EC₅₀ is defined as the estimated test concentration that results in 50% reduction in the specified endpoint, and the LC₅₀ is defined as the estimated test concentration that results in 50% mortality. Since no concentration tested resulted in $\geq 50\%$ reduction or mortality, these endpoints were empirically estimated to be greater than the highest mean measured sediment concentration tested.

Day 20 Midge Larvae survival and Growth

Following 20 days of exposure, larval survival in the control and solvent control averaged 96 % and 88 % respectively. Larval growth in the control and solvent control averaged 1.07 and 1.30 mg ash-free dry weight respectively.

On day 20, the larvae exposed to the 2.4, 5.8, 15, 36 and 93 mg/kg treatment levels had average survivals of 92, 83, 85, 67, and 50 % respectively. Those larvae exposed to the 36 and 96 mg/kg treatment levels showed significant difference in survival compared to the pooled control (Bonferroni's Adjusted t Test).

On day 20, ash-free dry weight of the larvae exposed to the 2.4, 5.8, 15, 36 and 93 mg/kg treatment levels averaged 1.32, 1.19, 1.22, 1.93, and 1.56 respectively. There was no significant difference in any of the treatment levels compared to the pooled control (Bonferroni's Adjusted t Test).

Table 9.2.5.4-2: Effects of SYN545974 on survival and ash-free dry weight of *Chironomus dilutus* after 20 days exposure

Mean measured sediment concentration (mg a.s./kg)	Day 20	
	Mean percent survival (%)	Mean ash-free dry weight per larvae (mg)
Control	96	1.07
Solvent control	88	1.30
Pooled control	92	1.19
2.4	92	1.32
5.8	83	1.19
15	85	1.22
36	67 ^a	1.93
93	50 ^a	1.56

^a Significantly reduced compared to the pooled control, based on Bonferroni's adjusted t-test

Day 59 Midge Emergence, Emergence Rate and Days to Death

The mean emergence among adult midges in the control and solvent control was 65 and 75 % respectively. The emergence for adult midges exposed to the 2.4, 5.8, 15, 36 and 93 mg/kg treatment levels averaged 64, 67, 67, 44, and 38 % respectively. There was a statistically significant difference in percent emergence for midges exposed to the 36 and 93 mg/kg treatment levels compared to the pooled control (Bonferroni's Adjusted t Test).

Male mean emergence rate in the control and solvent control was 0.0435 and 0.0385 respectively. There was a significant difference between the control and solvent control emergence rate for male midges, so the treatment results were compared to the solvent control. Mean emergence for the male midges exposed to the 2.4, 5.8, 15, 36 and 93 mg/kg treatment levels was 0.0354, 0.0430, 0.0352, 0.0398, and 0.0401 respectively. There was no

significant difference in mean emergence rate among male midges in any of the treatment levels compared to the solvent control (Bonferroni's Adjusted t Test).

Mean Female emergence rate in the control and solvent control was 0.0357 and 0.0343 respectively. Mean emergence rate for female midges exposed to 2.4, 5.8, 15, 36 and 93 mg/kg treatment levels was 0.0301, 0.0341, 0.0329, 0.0382, and 0.0391 respectively. There was a significant difference in the mean emergence of female midges in the 2.4 mg/kg treatment level (Bonferroni's Adjusted t Test). However, there were no statistically significant reductions at the higher treatment levels, this reduction was determined to be a result of biological variability and not a result of test substance toxicity.

Mean number of days to death for male midges in the control and solvent control was 3.8 and 3.6 days respectively. The mean number of days to death in the male midges exposed to the 2.4, 5.8, 15, 36 and 93 mg/kg treatment levels was 4.8, 3.6, 3.8, 5.5, and 3.8 days respectively. There was no significant difference in mean number of days to death in any of the treatment levels when compared to the pooled control (Bonferroni's Adjusted t Test).

Mean number of days to death for female midges in the control and solvent control was 3.7 and 4.4 days respectively. The mean number of days to death in the female midges exposed to the 2.4, 5.8, 15, 36 and 93 mg/kg treatment levels was 4.1, 4.8, 4.0, 4.8, and 5.2 days respectively. There was no significant difference in mean number of days to death in any of the treatment levels when compared to the pooled control (Bonferroni's Adjusted t Test).

Table 9.2.5.4-3: Effects of SYN545974 on emergence rate after 59 days exposure

Mean measured sediment concentration (mg a.s./kg)	Day 59				
	Mean percent emergence	Mean male emergence rate (equivalent to developmental rate)	Mean female emergence rate (equivalent to developmental rate)	Mean male days to death	Mean female days to death
Control	65	0.0435	0.0357	3.84	3.74
Solvent control	75	0.0385	0.0343	3.64	4.37
Pooled control	70	NA ^c	0.0350	3.73	4.03
2.4	64	0.0354	0.0301 ^b	4.83	4.14
5.8	67	0.0430	0.0341	3.56	4.78
15	67	0.0352	0.0329	3.81	3.98
36	44 ^a	0.0398	0.0382	5.46	4.81
93	38 ^a	0.0401	0.0391	3.80	5.16

^a Significantly reduced compared to the pooled control, based on Bonferroni's adjusted t-test

^b Significantly reduced compared to the pooled control, based on Bonferroni's adjusted t-test. However, due to the lack of a clear dose response at the higher treatment levels the effect observed at this treatment was not considered to be related to SYN545974 exposure

^c The control and solvent control were not statistically similar and therefore the solvent control was used for treatment comparisons.

59 Day Reproduction

The mean number of egg masses from mated females in the control and solvent control was 0.74 and 0.77 respectively. The mean number of egg masses per mated female exposed to the 2.4, 5.8, 15, 36 and 93 mg/kg treatment levels was 0.68, 0.67, 0.62, 0.46, and 0.39 respectively. There was a significant difference in the mean number of eggs per mated female in the 93 mg/kg treatment level compared to the pooled control (Wilcoxon's Test with Bonferroni's Adjustment).

The mean number of eggs per egg mass in the control and solvent control was 777 and 693 respectively. The mean percent hatch per replicate of the egg masses in the control and solvent control was 96 and 94 % respectively. The mean number of eggs per mass in midges exposed to the 2.4, 5.8, 15, 36 and 93 mg/kg treatment levels were 630, 885, 860, 661, and 762 respectively. There was no significant difference in mean number of eggs per mass in any of the treatment levels compared to the pooled control (Bonferroni's Adjusted t Test).

The mean number of eggs per mated female among midges in the control and solvent control was 575 and 561 respectively. The mean number of eggs per mated female exposed to the 2.4, 5.8, 15, 36 and 93 mg/kg treatment levels were 443, 603, 531, 296, and 253 respectively. There was significant difference in mean number of eggs per mated female in the midges exposed to the 36 and 93 mg/kg treatment levels compared to the pooled controls (Bonferroni's Adjusted t Test).

The mean percent hatch in the control and solvent control were 94 and 96 % respectively. The mean percent hatch in midges exposed to the 2.4, 5.8, 15, 36 and 93 mg/kg treatment levels were 95, 94, 95, 82, and 65 % respectively. There was a significant difference in mean hatch in midges exposed to the 36 and 93 mg/kg treatment levels compared to the pooled control (Bonferroni's Adjusted t Test).

The mean number of days to oviposition was 1.3 and 1.8 in the control and solvent control respectively. The mean number of days to oviposition in midges exposed to the 2.4, 5.8, 15, 36 and 93 mg/kg treatment levels were 1.8, 1.6, 1.5, 1.3, and 1.6 respectively. There was no significant difference in mean days to oviposition in any of the treatment levels (Wilcoxon's Test with Bonferroni's Adjustment).

Table 9.2.5.4-4: Effects of SYN545974 on *Chironomus dilutus* reproduction

Mean measured sediment concentration (mg a.s./kg)	Day 59				
	Mean egg masses per mated female	Mean eggs per egg mass	Mean number of eggs per mated female	Mean percent hatch (%)	Mean days to oviposition
Control	0.74	777	575	96	1.3
Solvent control	0.77	693	561	94	1.8
Pooled control	0.76	732	568	95	1.5
2.4	0.68	630	443	95	1.8
5.8	0.67	885	603	94	1.6
15	0.62	860	531	95	1.5
36	0.46	660	296 ^a	82 ^a	1.3
93	0.39 ^a	762	253 ^a	65 ^a	1.6

^a Significantly reduced compared to the pooled control based on the Wilcoxon's test with Bonferroni's adjustment. The NOEC, LOEC and EC₅₀ data are tabulated in Table 9.2.5.4-5.

Below is a summary of the endpoints:

Table 9.2.5.4-5: Summary of the effects of SYN545974 on *Chironomus dilutus* after 20 and 59 days exposure

Endpoint	NOEC (mg a.s./kg) (mean measured concentration)
Day 20 midge survival	15
Day 20 midge growth	93
Day 59 emergence percent	15

Endpoint	NOEC (mg a.s./kg) (mean measured concentration)
Day 59 Male emergence rate ^a	93
Day 59 Female emergence rate ^a	93
Day 59 Egg masses per mated female	36
Day 59 Eggs per egg mass	93
Day 59 Eggs per mated female	15
Day 59 Percent hatch	15
Day 59 Days to oviposition	93

ND = Not determined

^a emergence rate is identical to developmental rate as described in OECD 218.

CONCLUSIONS

For Day-20 midge larval survival and growth, the EC₅₀ was >93 mg a.s./kg of dry sediment. The NOEC for midge survival was 15 mg a.s./kg of dry sediment and for midge growth was 93 mg a.s./kg of dry sediment.

For Day-59 emergence and reproduction endpoints, the lowest EC₅₀ was 47 mg a.s./kg of dry sediment, calculated for eggs per mated female. The lowest NOEC was 15 mg a.s./kg of dry sediment, obtained for emergence, eggs per mated female and percent hatch. The corresponding LOEC was 36 mg a.s./kg of dry sediment.

(██████████, 2015)

Additional statistical analysis

In accordance with **Commission Regulation (EU) No 283/2013**, estimation of EC₁₀ and EC₂₀ values was conducted for ██████████, 2015 (SYN545974_10095) in the following report:

Report:	K-CA 8.2.5.4 ██████████ (2016), Pydiflumetofen - Statistical Reanalysis; SYN545974 - Life-Cycle Toxicity Test Exposing Midges (<i>Chironomus dilutus</i>) to Spiked Sediment, Report Number 1781.7192a, Smithers Viscient 790 Main Street, Wareham, MA 02571-1037 USA. (Syngenta File No: SYN545974_10457)
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Executive Summary

Report number 1781.6889 (██████████, 2015) did not provide EC₁₀ and EC₂₀ estimates for the response variables evaluated as part of the original study. Consequently, the data generated have been re-analysed in order to provide these values where they could be reliably determined.

Statistical Analysis

Mean larval survival and growth (ash-free dry weight) at day 20 were compared to the mean larval survival and growth in the pooled control. Mean percent emergence, female emergence rate, male days to death, female days to death, eggs per egg mass, egg masses per female, eggs per female and percent hatch at termination (day 59) in each of the treatment levels were compared to the pooled control.

Mean male emergence rate at termination (day 59) was compared to the solvent control due to a statistical difference between the negative control and solvent control.

All statistical analyses were conducted using CETISM Version 1.8 (Ives, 2013). Linear interpolation was used to determine EC₁₀ and EC₂₀ values along with corresponding 95% confidence intervals.

Results and Conclusion

Statistical analyses of the available data after days 20 and day 59 (termination) revealed that the following EC₁₀ and EC₂₀ values were reliably calculated:

Table 9.2.5.4-6: Summary of reliably calculated EC10 and EC20 values from [REDACTED], 2015 (Report number 1781.6889; effects of SYN545974 on *Chironomus dilutus* after 20 and 59 days exposure)

Endpoint	Analysis	Estimate (mg/kg)	Lower CI (mg/kg)	Upper CI (mg/kg)	Model
20-Day Growth	EC ₁₀	> 93	N/A	N/A	Linear Interpolation
	EC ₂₀	> 93	N/A	N/A	
Percent emergence	EC ₂₀	22	16	27	
Male emergence rate	EC ₁₀	> 93	N/A	N/A	
	EC ₂₀	> 93	N/A	N/A	
Female emergence rate	EC ₁₀	> 93	N/A	N/A	
	EC ₂₀	> 93	N/A	N/A	
Male days to death	EC ₂₀	> 93	N/A	N/A	
Female days to death	EC ₁₀	> 93	N/A	N/A	
	EC ₂₀	> 93	N/A	N/A	
Eggs per egg mass	EC ₁₀	> 93	N/A	N/A	
	EC ₂₀	> 93	N/A	N/A	
Percent hatch	EC ₁₀	30	20	41	
	EC ₂₀	49	30	68	

CI = confidence interval. NA = Not Applicable; EC value was empirically estimated, so 95 % CI could not be calculated.

([REDACTED], 2016)

HSE evaluator comments

Validity Criteria (OECD 218 (2004) and OECD 233 (2010))	Required	Obtained
Emergence in the controls	≥ 70 %	70 % (pooled control)
Days for emergence to adults	Day 18 onwards	20- 65 days for <i>C. dilutus</i>
pH of overlying water	6-9	7.3 – 7.7
Oxygen concentration in overlying water	≥ 60 % ASV	30.26 % (2.5 mg/L)
Water temperature	Should not differ by more than ± 1°C	21 – 26

The study was carried out according to GLP. This study follows U.S. EPA, Office of Water, Methods for Measuring the Toxicity and Bioaccumulation of Sediment associated Contaminants with Freshwater Invertebrates. Test method 100.4 (2000) and US EPA Ecological Effects Test Guidelines 850.1760. Whole Sediment Life Cycle Toxicity Test with *Chironomus* spp. (1996). The study was checked against OECD 218 (2004) and OECD 233 (2010). Some deviations were noted.

The table above shows how the study meets the validity criteria of OECD 218 (2004) and OECD 233 (2010).

The emergence for the water and solvent control were pooled to meet the validity criteria of 70 %. It was noted however that the emergence in the water control was 65 %, lower than specified in the validity criteria. As the emergence in the solvent control was 75 %, HSE considers that this criterion has been met.

During the study the temperature recorded reached a maximum of 26 °C, this was not within 20°C ± 1°C. The study report states that outside of this deviation the temperature was within the acceptable range. As 26 °C is within the tolerance of the test organism, it is not considered that this deviation had an adverse effect on the endpoints. Additionally, the oxygen in the overlying water was recorded as 2.5 mg/L. OECD 233 states that 60 % ASV is equivalent to 5.46 mg/L, therefore, the concentration of oxygen in the overlying water was significantly below the recommended level in OECD 218 (2004) and OECD 233 (2010). Due to this, it is not considered that the validity criteria have been met in this study.

There was a statistically significant difference to the control for mean female emergence rate at 2.4 mg a.s./kg. However, there was no clear dose response at this test concentration, and the difference only occurred at the lowest concentration.

The measured concentrations of active substance in the sediment were not maintained within ± 20 of initial measured concentrations. Therefore, ideally geometric mean measured concentrations would have been used. HSE calculated geometric mean concentrations and they were broadly comparable to mean measured concentrations. Hence the use of mean measured concentrations in calculation of endpoints is considered acceptable by HSE. The analytical methods have been evaluated by HSE Chemistry specialists in Vol. 3CA Part B5.1.2.6. The following was concluded for this method: “Acceptable method. LOQ: 0.151 $\mu\text{g/Lin}$ water samples and 0.021 mg/kg dry sediment”.

EC_{10/20} values were provided in [REDACTED] (2016) and were calculated by linear interpolation. Reliable estimates could only be made for percent emergence and percent hatch, and values for other parameters were empirically estimated to be greater than the highest treatment level. The values are summarized below.

- 20-day growth, male emergence rate, female emergence rate, male days to death, female days to death, eggs per egg mass: EC₁₀ and EC₂₀ > 93 mg SYN545974/kg.
- Percent emergence EC₂₀ = 22 mg SYN545974/kg
- Percent hatch EC₁₀ = 30 mg SYN545974/kg; EC₂₀ = 49 mg SYN545974/kg.

The agreed endpoints are as follows:

- Day-59 NOEC_{percentemergence} = 15 mg a.s./kg (mean measured concentration)

This endpoint will not be relied upon in risk assessment as the validity criteria were not fully met.

Report:	K-CA 8.2.5.4 [REDACTED] (2015b). SYN545974 - 10-Day Toxicity Test Exposing Estuarine Amphipods (<i>Leptocheirus plumulosus</i>) to a Test Substance Applied to Sediment under Static Conditions, Report Number 1781.7069, Smithers Viscient 790 Main Street, Wareham, MA 02571-1037 USA. (SYN545974_50120)
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GUIDELINE

U.S. EPA, Ecological Effects Test Draft Guidelines: OPPTS 850.1740 Whole Sediment Acute Toxicity Invertebrates, Freshwater (1996)

GLP: Yes

MATERIALS

Test Material SYN545974 tech.

Lot/Batch #: SMU2EP12007

Purity: 98.5 % w/w

Treatments

Test concentrations: 0.78, 1.6, 3.1, 6.3, 13, 25, 50 and 100 mg/kg sediment dry weight (0.61, 1.2, 2.3, 5.7, 13, 21, 46 and 92 mg/kg sediment dry weight mean measured)

Solvent: Acetone

Analysis of test concentrations: Yes at 0 and 10 days based on measurements of SYN545974 in the overlying water, pore water and sediment by LC/MS/MS from replicates established for chemical analysis

Test organism

Species: *Leptocheirus plumulosus*, immature, ranging from 2.0-4.0 mm in length, able to pass through a 1.0 mm sieve but retained in a 0.5 mm sieve.

Source: Obtained from Chesapeake Cultures, Hayes, Virginia

Acclimation:	96 h acclimation period using culture water and sediment from same source as used during exposure period. No mortality observed prior to exposure initiation.
Feeding:	Pre-test: ~200 mg of flaked fish food; provided on first and fourth day of holding following renewal of overlying water. During test: None
Test design	
Test vessels:	Chemically cleaned 1000-mL glass beakers containing 175 mL (approximately 2 cm) of sediment and 725 mL of overlying water.
Test medium:	Filtered seawater (salinity of 32‰ and pH of 7.7 to 7.8); diluted with laboratory well water to salinity of 20-21 ‰
Sediment:	Sediment collected from Sequim Bay, Sequim, Washington; wet pressed through 0.25 mm sieve to remove large particles and indigenous organisms. 37 % sand 37 % silt 26 % clay The organic carbon content was 3.2 % and the pH was 7.8
Sediment moisture content:	69.8%
Replication:	5 replicate vessels of 20 amphipods (100 amphipods per treatment/control group)
Duration:	10 days
Exposure regime:	Static
Environmental conditions	
Test temperature:	24 to 26°C
pH range of overlying water:	7.8 to 8.4
Dissolved oxygen of overlying water:	5.5 to 7.0 mg/L
Lighting:	Continuously illuminated (620 to 750 lux)

STUDY DESIGN AND METHODS

Experimental dates: 17 to 27 February 2015

A 20 mg/mL primary stock solution was prepared by dissolving 0.5002 g of SYN545974 with 25 mL of acetone. Eight individual dosing stock solutions were prepared in acetone for application of the test substance to the sediment. A jar-rolling technique was used to apply the test substance to the sediment. A 5.0-mL volume of each dosing stock solution was applied to 0.050 kg of fine silica sand placed in glass petri dishes. The acetone solvent was then allowed to evaporate from the sand for 30 minutes, leaving the material adhered to the sand. Following evaporation, the entire sand/test substance mixture was added to 2.75 kg of wet sediment (0.9399 kg total dry weight based on a percent solids value of 32.36% and including the 0.050 kg of sand) in a jar. The test substance was applied to the sediment and each jar was then rolled for four hours at room temperature at approximately 15 rpm. The spiked sediments were then allowed to equilibrate for a 27-day period in a dark refrigerator.

The negative control sediment group was prepared as described above using only untreated sediment (no test substance or added 0.050 kg of sand). A solvent control sample was prepared in the same manner as the treated sediment by adding 5.0 mL of acetone to 0.050 kg of fine silica sand and the solvent was allowed to evaporate.

One day prior to exposure initiation (day -1), the treated and control sediments and overlying water were allocated to each treatment or control vessel. Overlying water was gently added to each vessel to avoid suspension of the sediment layer. Each vessel was then placed in the water bath. Each test vessel was covered with a plastic plate and aeration was supplied with a constant trickle flow of bubbles from a 1 mL glass pipette.

Five replicate vessels were used to evaluate the biological response of the test organisms. Four replicates were also established and designated for chemical analysis of the test substance and pore water quality measurements. Each vessel contained 20 amphipods, a total of 100 amphipods per treatment or control. Amphipods were added impartially to each replicate, five at a time.

All vessels were examined at exposure initiation (day 0) and daily thereafter, until test termination (day 10). Observations of mortality and abnormal behaviour were made, and the physical characteristics of the test samples were recorded. At test termination (day 10), the total number of surviving amphipods was determined in each test vessel. Missing animals or all observed animals failing to respond to gentle prodding (i.e., neuromuscular twitch of pleopods or antennae) were recorded as dead.

The concentrations of test material were determined on days 0, and 10 in the sediment, pore water and overlying water using an LC/MS/MS method.

The LC_{50} is the estimated sediment concentration of the test substance which produces 50% mortality in the test population of amphipods at test termination compared to the appropriate control data. If $\geq 50\%$ mortality was observed, then an appropriate statistical model within CETIS™ Version 1.8 was used to determine the LC_{50} value for survival. If no treatment level tested resulted in $\geq 50\%$ mortality, the LC_{50} value was empirically estimated to be greater than the highest mean measured sediment concentration tested.

Determination of adverse effects on percent survival for determination of a NOEC and LOEC was made after angular transformation (arcsine square-root) of the data, using multiple comparison procedures.

RESULTS AND DISCUSSION

Analytical results:

Analysis of the dosing stock solutions resulted in measured concentrations ranging from 84 to 100% of the nominal concentrations. Analysis of the dosed sediment samples after application and mixing, prior to allocation into the test vessels, resulted in recoveries ranging from 76 to 87% of nominal concentrations. These results indicated that an appropriate amount of test substance was applied to the sediment for each treatment level. Analytical results are shown in Table 9.2.5.4-7.

Table 9.2.5.4-7: Analytical results in sediment samples

Nominal concentration s in sediment (mg a.s./kg)	Measured overlying water concentration (mg a.s./L)		Measured pore water concentration (mg a.s./L)		Measured sediment concentration (mg a.s./kg)		Mean measured conc. in sediment (mg a.s./kg)	Mean Percent Recovery in the sediment (%)
	Day 0	Day 10	Day 0	Day 10	Day 0	Day 10		
Control	< 0.00016 ^a	< 0.00016	< 0.00015	< 0.00016	< 0.056	< 0.065	NA	NA
Solvent control	< 0.00016	< 0.00016	< 0.00015	< 0.00016	< 0.056	< 0.065	NA	NA
0.78	0.0014	0.0030	0.0057	0.0049	0.64	0.58	0.61	78
1.6	0.0026	0.0062	0.014	0.010	1.3	1.0	1.2	75
3.1	0.0060	0.014	0.033	0.025	2.6	2.0	2.3	74
6.3	0.011	0.026	0.073	0.053	6.6	4.8	5.7	90
13	0.032	0.064	0.15	0.10	14	11	13	98
25	0.056	0.15	0.30	0.23	25	17	21	83
50	0.11	0.30	0.58	0.46	55	37	46	91
100	0.16	0.50	0.99	1.1	100	79	92	92

^a Concentrations expressed as less than values were below the limit of quantitation (LOQ). The LOQ for each analysis is dependent upon the regression, the area of the low standards and the dilution factor of the controls. NA = Not Applicable.

Biological results:

There were no significant differences detected between control and solvent control organism survival (Wilcoxon's Rank Sum Two-Sample t-Test). There was a significant difference in survival among amphipods exposed to the 92 mg/kg treatment level compared to the negative control (Dunnett's Multiple Comparison Test). No sublethal or behavioural effects were noted at any of the treatment levels. No concentration tested resulted in $\geq 50\%$ mortality, so the 10-day LC₅₀ value for survival was determined empirically to be > 92 mg/kg sediment dry weight (the highest mean measured concentration tested). The effects of SYN545974 on *Leptocheirus plumulosus* after 10-day exposure, based on mean measured sediment concentrations are given in Table 9.2.5.4-8 below:

Table 9.2.5.4-8: Effects of SYN545974 on survival of *Leptocheirus plumulosus* after 10 days exposure

Mean measured sediment concentration (mg a.s./kg sediment d.w.)	Mean Percent Survival (%)
Control	98
Solvent Control	98
0.61	97
1.2	94
2.3	96
5.7	98
13	94
21	93
46	90
92	74*
10-day LC ₅₀ (95% confidence limits)	>92 (NA)
LOEC	92
NOEC	46

NA = Not Applicable. LC₅₀ value was empirically estimated; therefore, corresponding 95% confidence intervals could not be determined * Significant difference compared to the control, based on Dunnett's Multiple Comparison Test.

VALIDITY CRITERIA

Validity criteria specified in OPPTS 850.1740 were met:

- All test vessels were identical and contained the same amount of sediment and overlying water.
- Test organisms were randomly assigned to test vessels.
- A negative sediment control and solvent sediment control were included.
- Average survival of amphipods in the negative sediment control and solvent sediment control was $\geq 80\%$ at test termination (98 % observed).

CONCLUSIONS

Based on mean measured sediment concentrations, the 10-day LC₅₀ for SYN545974 on survival of *Leptocheirus plumulosus* was determined to be >92 mg a.s./kg sediment dry weight. The 10-day NOEC was 46 mg a.s./kg sediment dry weight.

([REDACTED], 2015b)

HSE evaluator comments

This study was conducted to GLP and meets the validity criteria outlined in OPPTS 850.1740 (2016).

It is noted that the test area was continuously illuminated throughout the study duration, despite the guideline recommending a lighting regime of either 12 h light 12 h dark or 16 h light 8 h dark for *L. plumulosus*. However, since the behaviour of the control groups was as expected, and the validity criteria were met, this deviation from the guideline is not thought to have affected the study outcome.

The guideline recommends the use of 7-8-day old *L. plumulosus*. The age of the test organisms used was not reported, however they were reported as being immature juveniles, and their size (2.0-4.0 mm) allowed them to pass through a 1.0 mm sieve but be retained in a 0.5 mm sieve, as described in the guideline. Therefore, the use of this test organism is deemed acceptable.

The results presented were based upon arithmetic mean-measured concentrations of the test item. Since the test design was static, OECD 23 (2000) guidance stipulates that results must be based on geometric mean concentrations where nominal values are not maintained. HSE has calculated geometric mean measured concentrations in sediment in the table below, noting that they are calculated using the rounded (to 2 sig. fig.) measured sediment concentration values, rather than the raw analytical results.

Measured sediment concentration (mg a.s./kg)		Geometric mean measured conc. in sediment (mg a.s./kg)
Day 0	Day 10	
<0.056	<0.065	NA
< 0.056	< 0.065	NA
0.64	0.58	0.61
1.3	1.0	1.1
2.6	2.0	2.3
6.6	4.8	5.6
14	11	12
25	17	21
55	37	45
100	79	89

The 10-day LC₅₀ value was determined empirically and so no consideration of statistical analysis is required for this endpoint. The NOEC and LOEC values were determined statistically using Dunnett's Multiple Comparison Test, following angular transformation of the survival data. The assumptions of normality were met (Shapiro-Wilk's Test and Bartlett's Equality of Variance Test) and these statistical methods are in accordance with the guidance in OTTPS 850.1740.

The analytical methods have been evaluated by HSE Chemistry specialists in Vol. 3 KCA Part B5.1.2.6. The following was concluded for this method: "Acceptable method. LOQ: 0.151 µg/L in water samples and 0.021 mg/kg dry sediment".

The following endpoints are suitable for use in risk assessment:

- 10-day LC₅₀ > 89 mg SYN545974/ kg sediment d.w. (based on geometric mean measured concentrations)
- 10-day LOEC = 89 mg SYN545974/ kg sediment d.w. (based on geometric mean measured concentrations)
- 10-day NOEC = 45 mg SYN545974/ kg sediment d.w. (based on geometric mean measured concentrations)

Report: K-CA 8.2.5.4 [REDACTED] (2015a), SYN545974 – 42-Day Toxicity Test Exposing Freshwater Amphipods (*Hyalella azteca*) to Spiked Sediment, Report Number 1781.6890, Smithers Viscient 790 Main Street, Wareham, MA 02571-1037 USA. (Syngenta File No. SYN545974_10094)

GUIDELINES

- U.S. EPA, Office of Water, Methods for Measuring the Toxicity and Bioaccumulation of Sediment associated Contaminants with Freshwater Invertebrates. Test method 100.4 (2000)
- US EPA Ecological Effects Test Guidelines 850.1770; Whole Sediment Life Cycle Toxicity Test with *Hyalella azteca*. (2009)

GLP: Yes

MATERIALS

Test Material SYN545974 tech.
Lot/Batch #: SMU2EP12007
Purity: 98.5 % w/w
Description: Off-white powder
Stability of test compound: Stable under standard conditions
Reanalysis/Expiry date: 30 June 2016

Treatments

Test concentrations: 4.1, 9.1, 20, 45 and 100 mg a.s./kg sediment, alongside a solvent control and negative control (Mean measured concentrations: 3.3, 7.6, 16, 36 and 88 mg a.s./kg sediment)

Solvent: Stocks were prepared in acetone. 10 ml of acetone (containing no test substance for solvent control or the appropriate amount of test substance for respective treatments) was added to 0.050 kg of fine silica sand and then the solvent was allowed to completely evaporate off.

Analysis of test concentrations: Yes at 0, 14 and 28 days based on measurements of SYN545974 in the overlying water, pore water and sediment by LC/MS/MS from replicates established for chemical analysis.

Test organism

Species: *Hyalella azteca*

Source: Obtained from laboratory cultures maintained at Smithers Viscient

Feeding: Pre-test: Combination of yeast, Cerophyl and flaked fish food suspension (YCT) once daily, and unicellular green algae *Ankistrodesmus falcatus* as supplemental.
During test: 1.5 mL YCT daily

Test design

Test vessels: Chemically cleaned 300 mL glass beakers containing 100 mL (approximately 4 cm) of sediment and 175 mL of overlying water.

Test medium: Laboratory well water, with a hardness of 36 to 44 mg/L as CaCO₃, and a pH range of 6.9 to 7.8

Sediment:	Artificial sediment prepared according to OECD Guideline No. 218 and characterised as having: 6 % sphagnum peat (air dried and finely ground) 20 % kaolin clay (kaolinite content > 30 %) 74 % fine sand The organic carbon content was 2.3 % and the pH was 6.6
Replication:	15 replicate test vessels (12 replicates to evaluate the biological response [A to L] and three replicates for chemical analyses and pore water quality), 10 amphipods per vessel
Duration:	42 days
Environmental conditions	
Test temperature:	Range 22 – 25 °C (mean 23.5-23.7 °C)
pH range of overlying water:	7.0 – 7.4
Dissolved oxygen of overlying water:	4.9 – 8.2 mg/L
Lighting:	16 hours fluorescent light (240 – 790 Lux) and 8 hours dark

STUDY DESIGN AND METHODS

Experimental dates: 28 June to 16 August 2013

A 20 mg a.s./mL primary stock solution was prepared by placing 0.9988 g of SYN545974 in a 50-mL volumetric flask and bringing it to volume with acetone. Dosing stock solutions were prepared by adding acetone to appropriate amounts of the primary stock solution, to achieve final dosing stock volumes of 25 mL. To apply the dosing solution to the sediment, 10 mL of each dosing stock was applied to 0.05 kg of fine silica sand placed in glass petri dishes and the solvent allowed to completely evaporate. The dry sand was then added to 2.75 kg of wet sediment (1.6706 kg d.w. based on 58.93 % solids) in individual glass jars. The jars were sealed and positioned horizontally on a rolling mill. Each jar was then rolled for four hours at approximately 15 rpm, before being allowed to equilibrate vertically in a refrigerator for 28 days. Weekly during the equilibration period and prior to addition into the test vessels, the jars were mixed on the rolling mill for an additional two hours at room temperature. A solvent control and negative control were prepared in a similar manner, without SYN545974. The negative control also did not have the addition of the 0.05 kg of fine silica sand.

One day prior to test initiation (day - 1), the treated and control sediments were allocated to the fifteen replicate vessels per treatment or control (100 mL sediment per vessel). Overlying water (175 mL) was added to each test vessel and then each vessel was randomly placed in the water bath. A turbulence reducer was used to minimise disruption of the sediment layer during the introduction of the overlying water, and was removed after the addition of the water. During the study, the overlying water was renewed by adding two volume additions of water (350 mL) per test vessel per day using an intermittent delivery system in combination with a calibrated water-distribution system. The water delivery system cycled approximately 7 times per day, providing approximately 350 mL per vessel every 24 hours.

At test initiation, juvenile amphipods (8 days old) were added to each test vessel. During the exposure, 1.5 mL of a combination of yeast, Cerophyl and flaked fish food suspension (YCT) was added to each vessel daily.

Dissolved oxygen concentration, temperature and pH was measured in the overlying water of each replicate used for biological monitoring during the 42 day exposure. The ammonia concentration (as nitrogen) of the overlying water was monitored at test initiation (day 0), day 28, day 29 and test termination (day 42) from a composite sample of each treatment.

Observations of abnormal behaviour, and the physical characteristics of the test solutions, were recorded daily. Prior to test day 28, four of the 12 replicates maintained for biological observations were selected, and amphipod survival and growth in these vessels was assessed on day 28 by sieving the sediment to remove all surviving amphipods. Adults were preserved for up to two weeks in a sugar formalin solution prior to determining growth

by measuring body length from the base of the first antenna to the tip of the third uropod along the curve of the dorsal surface to the nearest 0.01mm using an image analyser.

Amphipods in the remaining replicates were removed on day 28 by sieving, and survival assessed. Surviving amphipods were placed in 300-mL water-only exposure vessels and reproduction and survival was assessed on days 35 and 42 by removing and counting the adults and offspring in each replicate beaker. Any offspring observed at the end of the sediment exposure phase (day 28) were also counted and recorded, and on day 35, after counting adults and offspring, and assessing reproduction, amphipods were returned to their respective test vessels.

At test termination (day 42), the total number of surviving adults and young was determined in each test vessel. Adult amphipods were preserved in a sugar formalin solution for up to 2 weeks prior to taking images for length determination, after which gender was determined, including the number of gravid females (identified by the presence or absence of eggs in the brood pouch). Mature males were identified by the enlarged second gnathopod, and those amphipods not identified as males were recorded as females.

The concentrations of test material were determined on days 0, 14 and 28 in the sediment, pore water and overlying water using an LC/MS/MS method.

RESULTS AND DISCUSSION

Analysis of the dosing stock solutions resulted in recoveries ranging from 96 to 110 % of nominal concentrations. Analysis of the dosed sediment samples taken during the equilibration period resulted in recoveries ranging from 74 to 86 % of nominal concentrations. During the definitive exposure, mean measured sediment concentrations ranged from 80 to 88 % of nominal (see table below). Biological results are based on mean measured concentrations of SYN545974.

Table 9.2.5.4-9: Analytical results for overlying water and pore water concentration

Nominal concentrations in sediment (mg a.s./kg)	Measured overlying water concentration (mg a.s./L)			Measured pore water concentration (mg a.s./L)			Measured sediment concentration (mg a.s./kg)			Mean measured conc. in sediment (mg a.s./kg)
	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28	
Control	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	NA
Solvent control	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	NA
4.1	0.0084	0.0022	0.00096	1.9	0.032	0.031	3.6	3.3	2.9	3.3
9.1	0.015	0.0055	0.0017	0.82	0.10	0.12	7.5	7.7	7.6	7.6
20	0.044	0.027	0.011	1.2	0.22	0.24	17	16	17	16
45	0.33	0.057	0.057	2.8	0.61	0.67	38	35	36	36
100	0.22	0.13	0.064	4.4	1.5	1.6	93	91	80	88

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.

LOQ = Limit of Quantification. The LOQ for each analysis is dependent upon the regression, the area of the low standards and the dilution factors of the controls.

NA = Not applicable

Table 9.2.5.4-10: Analytical results for sediment concentration

Nominal concentrations in sediment (mg a.s./kg)	Measured sediment concentration (mg a.s./kg) [%*]			Mean measured conc. in sediment (mg a.s./kg)	Mean measured conc. in sediment (% nominal)
	Day 0	Day 14	Day 28		
Control	< LOQ	< LOQ	< LOQ	NA	NA
Solvent control	< LOQ	< LOQ	< LOQ	NA	NA
4.1	3.6 [88]	3.3 [81]	2.9 [71]	3.3	80
9.1	7.5 [82]	7.7 [85]	7.6 [84]	7.6	84
20	17 [85]	16 [80]	17 [85]	16	82
45	38 [84]	35 [78]	36 [80]	36	81
100	93 [93]	91 [91]	80 [80]	88	88

*this percentage calculated by HSE using the data in this table

An Equal Variance t Two-Sample Test or Wilcoxon's Rank Sum Two-Sample Test was conducted on all endpoints to compare the performance of control organisms with that of solvent control organisms. A significant difference was observed between control and solvent control data for day 35 survival and day 42 survival and reproduction endpoints, therefore these data were compared to the solvent control data to determine treatment-related effects. All remaining statistical analyses were performed comparing treatment data to the pooled control data, since no significant differences were observed between control and solvent control data.

The LOEC was defined as the lowest tested concentration at which the test substance was observed to have a statistically significant effect for a given endpoint when compared with the control. However, all test concentrations above the LOEC should have an effect equal to or greater than that observed at the LOEC. The NOEC was defined as the test concentration immediately below the LOEC, which when compared to the selected control, had no statistically significant effect.

These were calculated using Wilcoxon's Test with Bonferroni's Adjustment to establish treatment effects for 28- and 42-day survival and 42-day male:female ratio, and Bonferroni's Adjusted t-Test to establish treatment effects for all other endpoints. The EC₅₀ is defined as the estimated test concentration that results in 50% reduction in the specified endpoint, and the LC₅₀ is defined as the estimated test concentration that results in 50 % mortality. Since no concentration tested resulted in ≥ 50 % reduction or mortality, these endpoints were empirically estimated to be greater than the highest mean measured sediment concentration tested.

The effects of SYN545974 on *Hyalella azteca* after 28-day exposure, based on mean measured sediment concentrations are given in the tables below:

Table 9.2.5.4-11: Effects of SYN545974 on survival and growth of *Hyalella azteca* after 28 days exposure

Mean measured sediment concentration (mg a.s./kg sediment)	Mean Percent Survival (SD)	Mean Length/Amphipod in mm (SD)
Control	93 (7)	4.54 (0.04)
Solvent Control	95 (9)	4.66 (0.21)
Pooled Control	94 (8)	4.60 (0.15)
3.3	95 (8)	4.49 (0.11)
7.6	96 (5)	4.42 (0.13)
16	90 (9)	4.51 (0.10)
36	89 (29)	4.53 (0.09)
88	79 ^a (17)	4.16 ^a (0.19)

^a Statistically significant reduction compared to pooled control data.

SD=Standard Deviation.

The effects of SYN545974 on *Hyalella azteca* after 35-day exposure, based on mean measured sediment concentrations are given in the tables below:

Table 9.2.5.4-12: Effects of SYN545974 on survival and reproduction of *Hyalella azteca* after 35 days exposure

Mean measured sediment concentration (mg a.s./kg sediment)	Mean Percent Survival (SD)	Mean number of Offspring per Surviving Female Amphipod (SD)
Control	91 (6)	1.5 (1.1)
Solvent Control	98 (5)	2.7 (1.2)
Pooled Control	NA ^c	2.1 (1.3)
3.3	94 (9)	2.0 (1.0)
7.6	94 (5)	2.7 (1.5)
16	85 ^a (9)	1.9 (0.99)
36	96 (8)	2.4 (1.4)
88	78 ^b (17)	0.90 (0.94)

^a Statistically significant reduction compared to applicable control data; not toxicant related due to performance at higher concentrations

^b Statistically significant reduction compared to applicable control data.

^c NA = Not applicable due to statistically significant difference between control groups.

SD = Standard Deviation.

The effects of SYN545974 on *Hyalella azteca* after 42-day exposure, based on mean measured sediment concentrations are given in the tables below:

Table 9.2.5.4-13: Effects of SYN545974 on survival, growth reproduction and male:female ratio of *Hyalella azteca* after 42 days exposure

Mean measured sediment concentration (mg a.s./kg)	Mean Percent Survival (SD)	Mean Length per Amphipod in mm (SD)	Mean number of Offspring per Surviving Female Amphipod (SD)	Mean Male:Female Ratio (SD)
Control	89 (8)	5.30 (0.29)	2.4 (1.6)	0.68 (0.54)
Solvent Control	96 (5)	5.32 (0.29)	4.4 (1.7)	0.49 (0.25)
Pooled Control	NA ^c	5.30 (0.28)	NA ^c	0.58 (0.42)
3.3	93 (9)	5.30 (0.23)	3.5 (0.69)	0.34 (0.20)
7.6	91 (6)	5.38 (0.17)	4.1 (2.2)	0.42 (0.24)
16	83 ^a (12)	5.40 (0.20)	2.9 (1.7)	0.22 ^d (0.08)
36	96 (8)	5.27 (0.17)	4.2 (1.3)	0.40 (0.30)
88	74 ^b (18)	5.13 (0.54)	2.9 (2.8)	0.35 (0.23)

^a Statistically significant reduction compared to applicable control data; however, considered not test substance related due to performance at higher concentrations

^b Statistically significant reduction compared to applicable control data.

^c NA = Not applicable due to statistically significant difference between control groups.

^d Statistically significant reduction compared to pooled control data; not toxicant related due to lack of a significant reduction at higher concentrations.

SD = Standard Deviation.

The NOEC, LOEC and EC₅₀ data for exposure of *Hyalella azteca* to SYN545974 applied to sediment are tabulated below:

Table 9.2.5.4-14: Summary of the effects of SYN545974 on *Hyalella azteca* after 28, 35 and 42 days exposure

Endpoint	Test Day	LC/EC ₅₀ (mg a.s./kg)	95 % Confidence limits (mg a.s./kg)	NOEC (mg a.s./kg)	LOEC (mg a.s./kg)
Survival	28	>88	NA	36	88
Growth		>88	NA	36	88
Survival	35	>88	NA	36	88
Reproduction		76 ^a	52, NA	88	ND
Survival	42	>88	NA	36	88
Growth		>88	NA	88	ND
Reproduction		>88	NA	88	ND
Male:Female Ratio		NA ^b	NA	88	ND

NA = Not Applicable

ND = Not Determined

^a An EC₅₀ was attainable, however, this point estimate may not be considered reliable as bracketing confidence intervals could not be determined. In addition, the percent minimum significant difference (PMSD) value for this endpoint was 62 %, which resulted in the inability to statistically define an LOEC, and has subsequently resulted in the NOEC being greater than the EC₅₀. As the PMSD is greater than 50 %, estimation of an EC₅₀ may not be considered appropriate for this endpoint.

^b Given the nature of this endpoint and the data set, an EC₅₀ assessment was not applicable.

VALIDITY CRITERIA

Validity criteria for the test were met;

- Amphipod survival in the pooled control was 94 % at day 28 (must be ≥ 80 %)
- Mean amphipod length in the pooled control was 4.60 mm at day 28 (must be ≥ 3.2 mm).
- Mean number of offspring was 2.4 per female in the control and 4.4 per female in the solvent control by day 42 (must be ≥ 2 offspring per control female between test days 28 and 42).

CONCLUSIONS

Based on mean measured sediment concentrations, the 28-, 35- and 42-day LC_{50} values for survival were >88 mg a.s./kg. The 28-, 35- and 42-day NOECs were 36 mg a.s./kg.

No concentration tested with surviving adult amphipods resulted in ≥ 50 % reduction of growth, so the 28- and 42-day EC_{50} values for growth were empirically estimated to be > 88 mg a.s./kg. The corresponding NOECs were 36 mg a.s./kg and 88 mg a.s./kg, respectively.

The 35- and 42-day EC_{50} for reproduction were 76 and >88 mg a.s./kg, respectively. The corresponding NOECs were 88 mg a.s./kg.

The 42-day NOEC for male:female ratio was 88 mg a.s./kg.

(████████, 2015a)

In accordance with **Commission Regulation (EU) No 283/2013**, estimation of EC_{10} and EC_{20} values was conducted for ██████████, 2015a (SYN545974_10094) in the following report:

Report:	K-CA 8.2.5.4 ██████████ (2016a), Pydiflumetofen - Statistical Reanalysis; SYN545974 - 42-Day Toxicity Test Exposing Freshwater Amphipods (<i>Hyaella azteca</i>) to Spiked Sediment, Report Number 1781.7192b, Smithers Viscient 790 Main Street, Wareham, MA 02571-1037 USA. (Syngenta File No: SYN545974_10455)
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Executive Summary

Report number 1781.6890 (████████, 2015) did not provide EC_{10} and EC_{20} estimates for the response variables evaluated as part of the original study. Consequently, the data generated have been re-analysed in order to provide these values where they could be reliably determined.

Statistical Analysis

Mean survival and growth (length) at day 28 were compared to the mean survival and growth in the pooled control.

Mean survival at day 35 in each of the treatment levels was compared to the mean survival in the solvent control due to a statistical difference between the negative control and solvent control. Mean reproduction at day 35 was compared to mean reproduction in the pooled control.

Mean survival and reproduction at termination (day 42) in each of the treatment levels was compared to the solvent control due to a statistical difference between the negative control and solvent control. Mean growth (length) at termination (day 42) was compared to the mean length in the pooled control.

All statistical analyses were conducted using CETISM Version 1.8 (Ives, 2013). Linear interpolation was used to determine EC_{10} and EC_{20} values along with corresponding 95 % confidence intervals.

Results and Conclusion

The following endpoints were proposed by the study author:

Table 9.2.5.4-15: Summary of EC₁₀ and EC₂₀ values from [REDACTED], 2015a (Report number 1781.6890; effects of SYN545974 on *Hyalella azteca* after 28, 35 and 42 days exposure)

Endpoint	Analysis	Estimate (mg/kg)	Lower CI (mg/kg)	Upper CI (mg/kg)	Model
28-Day Survival	LC ₂₀	> 88	NA	NA	Linear Interpolation
28-Day Length	EC ₁₀	> 88	NA	NA	Linear Interpolation
	EC ₂₀	> 88	NA	NA	
42-Day Length	EC ₁₀	> 88	NA	NA	Linear Interpolation
	EC ₂₀	> 88	NA	NA	

CI = Confidence Intervals

NA = Not Applicable. EC value was empirically estimated; therefore, corresponding 95 % confidence intervals could not be calculated.

([REDACTED], 2016a)

HSE evaluator comments

The study was conducted to GLP, but the statistical reanalysis was not. The study report states the methods are designed to meet the testing requirements of the U.S. EPA Test method 100.4 (2000) and a draft guideline of OCSPP 850.1770 (U.S. EPA 2009, document 712-C-08-069). The draft guideline is not publicly available hence HSE has assessed the study using the U.S. EPA Test method 100.4 (in document EPA/600/R-99/064, March 2000).

Overall there are no major deviations from the EPA 100.4 (2000) guideline and the study fulfils the acceptability requirements. The following minor point is noted for reference:

- The water quality measurements of the overlying water show that ammonia drops in all test concentrations on days 28 and 29 to ≤ 0.10 - 0.12 mg/L, whereas at the other timepoints of 0 and 42 days it is in the range of 0.25 - 0.51 mg/L. The guideline notes in the acceptability requirements that water quality parameters should not differ by more than 50 % during the sediment exposure. This drop is not explained by the authors and although the variation is over 50 % the guideline also states that in protocol evaluations, there were no significant correlations observed between the biological endpoints and water-quality characteristics such as ammonia. Therefore, this variation in ammonia is not a reason to invalidate the study. Water hardness, alkalinity and conductivity do not exhibit such variation. For other water quality measurements such as the daily measured dissolved oxygen and pH, it is unclear from the study report how often these measurements were made, but the values presented were acceptable throughout the test.

Analytical measurements of the test substance were conducted in the overlying water, the pore water and in the sediment for each treatment throughout the 28 day sediment exposure period. The analytical methods have been evaluated by HSE Chemistry specialists in Vol. 3CA Part B5.1.2.6. The following was concluded for this method “Acceptable method. LOQ: 0.151 µg/Lin water samples and 0.021 mg/kg dry sediment”. The sediment measurements shows a mean concentration of 80-88 % of nominals across the whole test with ranges on individual timepoints from 71-93 % of nominals. HSE notes that the concentrations were maintained within ± 20 % of initial measured concentrations (range 81 – 103 %) suggesting initial values could be used i.e. 3.6, 7.5, 17, 38 and 93 mg a.s./kg sediment. Instead the authors used mean measured concentrations of 3.3, 7.6, 16, 36 and 88 mg a.s./kg sediment. These values are broadly comparable or more conservative, hence the approach is considered acceptable by HSE.

The determination of endpoints has also been considered:

- Statistical differences between the control and the solvent control for endpoints at day 42 (reproduction) or day 35 and 42 (survival) meant that the authors used the solvent control rather than the pooled control in their analyses for these endpoints. Both controls do separately meet the acceptability requirements listed in the EPA 100.4 (2000) guideline, so this statistical difference in performance does not indicate a problem with the study and is likely due to inherent biological variation. The use of the solvent control

data in cases where it is significantly different from the control is deemed appropriate, as this produces more conservative comparisons due to the performance of the solvent control being higher than the control.

- The NOEC was determined using statistical tests. It is noted that at 35 days (survival) and 42 days (survival and male:female ratio) a statistically significant effect was observed at the 16 mg a.s./kg treatment level, but HSE agrees with the author's explanation that this is not likely to be treatment related due to the performance at the higher test concentrations. Additionally, the endpoints of growth and reproduction do not have statistical differences at this level, and the male:female ratio is not an assessed endpoint. Therefore, the NOEC is at a higher treatment level above 16 mg a.s./kg and is considered acceptable.
- There is a statistically significant decrease in survival for all timepoints at the highest treatment level of 88 mg a.s./kg. Although this is also reflected in growth at the 28 day timepoint, no other endpoints at this treatment level have a significant differences therefore the proposed NOECs are at this concentration. However, the standard deviations for the reproduction endpoint at this treatment level are relatively high (mean offspring per female 35-day: 0.90 (SD=0.94); 42-day: 2.9 (SD=2.8)) and therefore create some uncertainty over having the NOEC at the highest treatment level in this case, especially as there is a significant effect for mortality.
- The additional report for statistical reanalysis was also considered. In the reanalysis the authors attempt to apply a linear interpolation model to the data to determine LC/EC₁₀ and LC/EC₂₀. However, the LC₁₀ and LC₂₀ values (survival) or EC₁₀ and EC₂₀ values (reproduction) obtained using this method were not considered reliable due to either overly wide confidence intervals or lack of upper confidence limits. The values presented were instead empirically estimated. For EC values of growth, authors state that it was not possible to calculate them and so they were also empirically estimated.

The following endpoints are agreed for consideration in risk assessment:

- NOEC 42-day reproduction: 88 mg a.s./kg sediment (mean measured concentration)
- NOEC survival (28, 35 and 42 days): 36 mg a.s./kg sediment (mean measured concentration)
- EC₂₀ survival (28 days): > 88 mg a.s./kg sediment (mean measured concentration)
- EC₁₀ growth (28 and 42 days): > 88 mg a.s./kg sediment (mean measured concentration)
- EC₂₀ growth (28 and 42 days): > 88 mg a.s./kg sediment (mean measured concentration)

B.9.2.6. Effects on algal growth

B.9.2.6.1. Effects on growth of green algae

Report:	K-CA 8.2.6.1 [REDACTED] (2013). SYN545974 – 96-Hour Toxicity Test with the Freshwater Green Alga, <i>Pseudokirchneriella subcapitata</i> , Report Number 1781.6841, Smithers Viscient. 790 Main Street, Wareham, MA 02571-1037, USA. (Syngenta File No. SYN545974_10013)
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GUIDELINES

OECD Guidelines for Testing of Chemicals, Method 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test (2006)

US EPA Ecological Effects Test Guidelines, OPPTS 850.5400: Algal Toxicity, Tiers I and II, (1996)

Official Journal of the European Communities, Commission Regulation (EC) No 761/2009, Part C.3: Algal inhibition test (2009)

GLP: Yes

MATERIALS

Test Material SYN545974 tech.

Batch #:	2637-BA/110
Purity:	99.5 %
Description:	White powder
Stability of test compound:	Stable under standard conditions
Reanalysis/expiry date:	31 July 2013

Treatments

Test concentrations:	Culture medium control and nominal concentrations of 0.0093, 0.03, 0.095, 0.31, 0.98, 3.1 and 10 mg SYN545974 /L. (Mean measured: 0.0079, 0.026, 0.093, 0.28, 0.90, 2.9 and 5.9 mg SYN545974 /L, respectively)
Solvent:	Dimethylformamide (DMF)
Solvent control:	Yes, DMF at 0.1 mL /L
Positive control:	None
Analysis of test concentrations:	Yes, analysis of SYN545974 at 0 and 96 hours using LC/MS/MS

Test organism

Species:	<i>Pseudokirchneriella subcapitata</i> , strain 1648
Source:	Continuous laboratory cultures, originally obtained from University of Texas, Austin, Texas, USA

Test design

Test vessels:	250 mL glass flasks containing 100 mL of medium and covered with stainless steel caps permitting gas exchange
Test medium:	AAP medium, according to guideline (OECD 201)
Replication:	Six vessels for the solvent control and three for each of the test concentrations and the dilution water control
Starting cell density:	Approx. 1.0×10^4 cells /mL (actual: 0.99×10^4 cells /mL)
Exposure regime:	Static
Aeration:	No
Duration:	96 hours, followed by a 96-hour recovery period using an aliquot of the 10 mg a.s. /L treatment level

Environmental conditions

Test temperature:	23 – 24 °C
pH:	Test start: 7.3 – 7.5 Test end: 7.9 – 9.6
Lighting:	Continuous illumination at 3900 to 4700 Lux

STUDY DESIGN AND METHODS

Experimental dates: 9 to 17 April 2012

Pseudokirchneriella subcapitata was the freshwater green alga chosen as the test organism for this study, as it is easy to maintain in various culture media, and cell density measurements can be easily performed.

The test dilution water was prepared in the same way as the culture medium. Algal Assay Procedure (AAP) medium was used for both purposes, and was prepared using sterile, deionised water. The composition of the AAP medium is in accordance with the growth media requirements from OECD 201 (2011).

A static test design was employed, using six vessels for each of the controls, and three vessels for each test condition. A primary stock solution with a nominal concentration of 100 mg /mL was prepared by placing 5.000 g of SYN545974 in a 50 mL volumetric flask, bringing it to volume with dimethylformamide (DMF), and mixing for 30 minutes with a Teflon®-coated stir bar and magnetic stir plate followed by 10 minutes of sonication. A 31 mg /mL primary stock solution was prepared by placing 0.775 g of SYN545974 in a 25 mL volumetric flask, bringing it to volume with DMF and mixing by multiple shakes and inversions. The 100 mg /mL primary stock solution was used to prepare highest test concentration of 10.0 mg a.s. /L, and the 31 mg /mL stock solution was used to prepare the lower test concentrations. The controls consisted of culture medium only, and a solvent control (DMF).

The 0.98, 3.1 and 10 mg a.s. /L exposure solutions were observed to contain increasing amounts of undissolved test substance and were subsequently mixed for approximately two hours with a Teflon®-coated stir bar and magnetic stir plate and multiple manual shakes and inversions. The 10 mg a.s. /L exposure solution was sonicated for a further 20 minutes and observed to contain undissolved test material. The soluble portion was removed and observed to be slightly cloudy.

An aliquot of test solution was placed into each test vessel and the test was started by inoculation of approximately 9,900 algal cells per mL of test medium. The inoculum culture used to initiate the toxicity test was taken from a stock culture that had been transferred to fresh medium three days before testing. Test solutions were continuously stirred by magnetic stirrers and held in a temperature-controlled water bath under continuous illumination.

Small volumes of all test concentrations and controls were taken from all test flasks after 24, 48, 72 and 96 hours of exposure. The algal cell densities in these samples were determined using a hemacytometer and a compound microscope. Cell density was calculated for each daily interval by dividing the number of cells counted by the number of fields examined for each cell count and multiplying by 10,000 to yield cells /mL. In addition, after 96 hours exposure, a sample was taken from the control and from the test concentration of nominal 0.31 mg a.s. /L. The shape of the algal cells was examined microscopically in these samples.

At the end of the 96-hour exposure period, a recovery test was conducted over a 4-day period, using a sample from a composite of the three replicates of the nominal 10 mg a.s. /L treatment level. The sample was diluted with freshly-prepared AAP medium to prepare a subculture with a nominal concentration of 0.095 mg a.s. /L, and an estimated cell density of 0.53×10^4 cells /mL. The subculture was incubated under the same conditions as the definitive exposure and was examined microscopically every other day for resumption of cell growth.

The pH was measured at the start and at the end of the exposure phase and of the recovery period. The water temperature was measured daily in a flask incubated under the same conditions as the test flasks.

The test concentrations were verified by chemical analysis of SYN545974 at 0 and 96 hours, using LC/MS/MS. The limit of quantification (LOQ) was determined as 0.151 µg /L for SYN545974 in filtered seawater. In order to estimate the impact that the presence of algal biomass had on the test substance concentration, an additional replicate flask (D) of the 0.31 mg /L (nominal) treatment level was prepared. This flask, which was not inoculated with algae, was analysed at 96-hours of exposure for SYN545974 concentration.

An equal variance t-test was conducted to compare the dilution water control data and the solvent control data. Since no significant differences were observed for any of the endpoints analysed, the treatment data were compared to the pooled control data for all endpoints.

The algal cell densities were measured at 24, 48, 72 and 96 hours and the mean biomass, growth rate and yield calculated. The 72-hour and 96-hour E_bC_{50} , E_yC_{50} and E_rC_{50} values (defined as the concentration resulting in 50 % reduction of each parameter) and their 95 % confidence intervals were determined by linear interpolation of response versus the mean measured concentration. Where response was < 50 %, the EC_{50} value was empirically estimated. The NOEC (defined as the highest concentration which demonstrated no statistically adverse effect ($p \leq 0.05$) when compared to the pooled control) was determined using Bonferroni's Adjusted t-test. Recovery of growth was determined by microscopic examination.

RESULTS AND DISCUSSION

Mean measured concentrations ranged from 85 to 98 % of nominal values for treatment levels

≤ 3.1 mg a.s. /L (see table below). The low recovery (59 %) at the highest treatment level was expected, since the nominal concentration (10 mg a.s. /L) exceeded the functional limit of solubility of SYN545974 (i.e. approximately 5 mg /L). Analysis of quality control samples resulted in measured concentrations in the range of 93.9 to 106 % of the nominal fortified values confirming that the appropriate precision and quality control was maintained. Mean measured concentrations were used for the calculation and reporting of results. The measured concentrations of test substance, the combined mean concentrations for each condition, and the corresponding percentages of the nominal values are displayed in Table 9.2.6.1-1 below.

Table 9.2.6.1-1: Analytical results

Nominal concentrations (mg a.s. /L)	Measured concentration (mg a.s. /L)			Percent of nominal ^a (%)
	0 hours	96 hours	Mean ^a	
Control	< 0.00025 ^b	< 0.00019 ^b	NA	-
Solvent control	< 0.00025	< 0.00019	NA	-
0.0093	0.0079	0.0080	0.0079	85
0.030	0.026	0.025	0.026	85
0.095	0.098	0.089	0.093	98
0.31	0.28 (0.28 ^c)	0.28 (0.31 ^c)	0.28	91
0.98*	0.87	0.93	0.90	92
3.1*	2.8	3.0	2.9	94
10*	6.8	5.0	5.9	59

^a Mean measured concentrations and percent nominal were calculated using actual analytical data and not the rounded data presented in this table.

^b Concentrations expressed as less than values were below the limit of quantification (LOQ). The LOQ for each analysis is dependent upon the linear regression, the area of the low standards and the dilution factor of the controls.

^c Result of the additional sample without algae present to determine biological uptake/degradation.

* The 0.98, 3.1 and 10 mg a.s. /L solutions were observed to contain increasing amounts of undissolved test substance, and were subsequently mixed for a further two hours. The 10 mg a.s. /L exposure solution was sonicated for a further 20 minutes and was still observed to contain undissolved test material. The soluble portion was removed and observed to be slightly cloudy.

NA = Not applicable

There were no abnormalities, observed microscopically, in the controls or the mean measured concentrations ≤ 2.9 mg a.s. /L at 96 hours. Cells exposed to the 5.9 mg a.s. /L treatment level were observed to be bloated.

Biological results

Growth rates

The growth rate 0 to 72 hours and 0 to 96 hours were calculated for each replicate culture and the means are shown in Table 9.2.6.1-2 below, alongside the estimated EC₅₀ values.

Table 9.2.6.1-2: Mean values at each concentration of SYN545974 for the growth rate at 72 and 96 hours for *Pseudokirchneriella subcapitata* and relevant endpoints

Mean measured concentrations (mg a.s. /L)	Mean growth rate (1 /day) 0 – 72 hrs	Percentage inhibition ^{1,2}	Mean growth rate (1 /day) 0 – 96 hrs *	Percentage inhibition ^{1,2}
Control	1.48	-	1.30	-
Solvent control	1.45	-	1.31	-
Pooled control	1.46	-	1.30	-
0.0079	1.51	-3	1.36	-5
0.026	1.44	1	1.35	-4
0.093	1.5	-3	1.33	-2
0.28	1.48	-1	1.21**	7
0.90	1.47	-1	1.22**	6
2.9	1.28**	12	1.11**	15
5.9	1.18**	19	1.02**	22

¹ Calculated using the exact raw data. The tabulated results represent rounded values.

² Percent inhibition was calculated relative to the pooled control. Negative values indicate an increase relative to the control mean.

* The 96 h endpoints are superfluous to the UK requirements (see evaluator comments)

** Mean value statistically significantly lower than in the control (Bonferroni's Adjusted t-Test, $p \leq 0.05$)

The natural log cell densities in each condition over time is shown graphically in Figure 9.2.6.1-1.

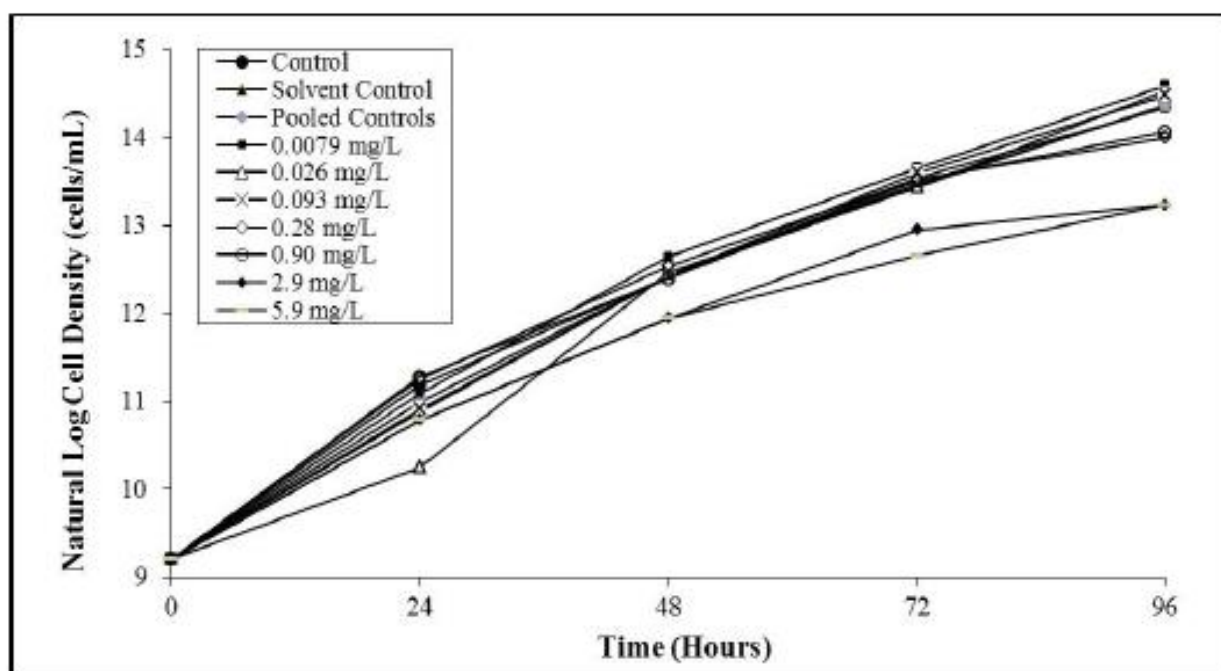


Figure 9.2.6.1-1: Algal growth curves (cell density x time) for *Pseudokirchneriella subcapitata* exposed to SYN545974

Yield

The yield 0 to 72 hours and 0 to 96 hours were calculated for each replicate culture and the means are shown in Table 9.2.6.1-3 below, alongside the estimated EC₅₀ values.

Table 9.2.6.1-3: Mean values at each concentration of SYN545974 for the yield at 72 and 96 hours for *Pseudokirchneriella subcapitata* and relevant endpoints

Mean measured concentrations (mg a.s. /L)	Mean yield (x 10 ⁴ cells /mL) 0 – 72 hrs	Percentage inhibition ^{1,2}	Mean yield (x 10 ⁴ cells /mL) 0 – 96 hrs *	Percentage inhibition ^{1,2}
Control	74.92	-	169.22	-
Solvent control	68.08	-	173.28	-
Pooled control	70.36	-	171.93	-
Mean measured concentrations (mg a.s. /L)	Mean yield (x 10 ⁴ cells /mL) 0 – 72 hrs	Percentage inhibition ^{1,2}	Mean yield (x 10 ⁴ cells /mL) 0 – 96 hrs *	Percentage inhibition ^{1,2}
0.0079	84.75	-20	217.50	-27
0.026	67.50	4	205.50	-20
0.093	80.00	-14	195.33	-14
0.28	74.00	-5	119.97**	30
0.90	73.00	-4	127.39**	26
2.9	41.25	41	78.25**	54
5.9	30.50**	57	55.08**	68

¹ Calculated using the exact raw data. The tabulated results represent rounded values.

² Percent inhibition was calculated relative to the pooled control. Negative values indicate an increase relative to the control mean.

* The 96 h endpoints are superfluous to the UK requirements (see evaluator comments)

** Mean value statistically significantly lower than in the control (Bonferroni's Adjusted t-Test, $p \leq 0.05$)

Biomass (Area under the growth curve)

Biomass (expressed as area under the growth curve) was determined at 72 and 96 hours for each replicate culture, and the means are shown in Table 9.2.6.1-4 below, alongside the estimated EC₅₀ values.

Table 9.2.6.1-4: Mean values at each concentration of SYN545974 for cell biomass at 72 and 96 hours for *Pseudokirchneriella subcapitata* and relevant endpoints

Mean measured concentrations (mg a.s. /L)	Mean biomass integral (area, 10 ⁴ x day) 0 – 72 hrs	Percentage inhibition ^{1,2}	Mean biomass integral (area, 10 ⁴ x day) 0 – 96 hrs *	Percentage inhibition ^{1,2}
Control	65.71	-	192.44	-
Solvent control	60.85	-	186.14	-
Pooled control	62.47	-	188.24	-
0.0079	76.18	-22	233.08	-24
0.026	59.25	5	200.96	-7
0.093	66.7	-7	209.62	-11
0.28	69.08	-11	169.77	10

Mean measured concentrations (mg a.s. /L)	Mean biomass integral (area, 10 ⁴ x day) 0 – 72 hrs	Percentage inhibition ^{1,2}	Mean biomass integral (area, 10 ⁴ x day) 0 – 96 hrs *	Percentage inhibition ^{1,2}
0.90	64.94	-4	168.96	10
2.9	36.96**	41	98.99**	47
5.9	32.77**	48	77.2**	59

¹ Calculated using the exact raw data. The tabulated results represent rounded values.

² Percent inhibition was calculated relative to the pooled control. Negative values indicate an increase relative to the control mean.

* The 96 h endpoints are superfluous to the UK requirements (see evaluator comments)

** Mean value statistically significantly lower than in the control (Bonferroni's Adjusted t-Test, $p \leq 0.05$)

The calculated endpoints are presented in Table 9.2.6.1-5 below:

Table 9.2.6.1-5: Summary of results for the toxicity of SYN545974 to *Pseudokirchneriella subcapitata* after 72 and 96 hours

Parameter	After 72 h (mg a.s. /L)			After 96 h (mg a.s. /L)*		
	AUC	Growth rate	Yield	AUC	Growth rate	Yield
EC ₅₀ (95% CI)	4.3 (0.78 – ND)	>5.9 (ND)	3.6 (1.4 – 6.0)	2.7 (1.5 – 3.5)	>5.9 (ND)	1.8 (ND – 2.4)
EC ₂₀ (95% CI)	1.6 (ND - 2.2)	5.7 (2.1 – ND)	1.6 (ND - 2.2)	ND	ND	ND
EC ₁₀ (95% CI)	1.0 (ND - 1.5)	2.3 (0.48 - 3.3)	1.1 (ND - 1.7)	ND	ND	ND
Parameter	After 72 h (mg a.s. /L)			After 96 h (mg a.s. /L)*		
	AUC	Growth rate	Yield	AUC	Growth rate	Yield
NOEC	0.90	0.90	0.90 ^a	0.90	0.093	0.093
LOEC	2.9	2.9	2.9	2.9	0.28	0.28

* The 96 h endpoints are superfluous to the UK requirements (see evaluator comments)

^a Due to > 20 % effect at the 2.9 mg /L treatment level, 0.90 mg /L will reported as the conservative NOEC value.

AUC = area under the growth curve

ND = not/could not be determined

Recovery

After a 4-day post-exposure recovery period, a cell density of 66 x 10⁴ cells /mL was observed in the subculture of the 5.9 mg a.s. /L treatment level. This represented an approximately 125 x increase from initiation of the recovery phase, indicating that the test substance had an algistatic, rather than algicidal, effect on the growth of *Pseudokirchneriella subcapitata*.

VALIDITY CRITERIA

The validity criteria for the study were met according to OECD 201 (2011):

Table 9.2.6.1-6: Compliance with OECD 201 validity criteria

Validity criterion	Required	Obtained
Biomass	Biomass in control cultures should have increased exponentially by a factor of at least 16 within 72 hours.	Exponential increase of a factor of 71 after 72 hours.
Coefficient of variation	Mean coefficient of variation for section by-section specific growth rates in the control cultures must not exceed 35 % after 72 hours.	The 72 h and 96 h values were 34 % and 40 %, respectively.
	Coefficient of variation of average specific growth rates in replicate control cultures must not exceed the value stated in the guideline for that species (7 % for <i>P. subcapitata</i>).	The 72 h and 96 h values were 5.5 % and 1.2 %, respectively.

CONCLUSIONS

Based on SYN545974 mean measured concentrations, the 72-hour E_rC_{50} was > 5.9 mg a.s. /L, the E_yC_{50} was 3.6 mg a.s. /L and the E_bC_{50} was 4.3 mg a.s. /L. The 96-hour E_rC_{50} was > 5.9 mg a.s. /L, the E_yC_{50} was 1.8 mg a.s. /L and the E_bC_{50} was 2.7 mg a.s. /L.

The No Observed Effect Concentration (NOEC) at 72 hours based on growth rate, yield and biomass was 0.90 mg a.s. /L. The NOEC at 96 hours based on growth rate and yield was 0.093 mg a.s. /L and based on biomass was 0.90 mg a.s. /L.

Following a 4-day recovery period using a subculture of the 5.9 mg a.s. /L treatment level, there was an approximately 125 x increase in cell density from initiation of the recovery phase, indicating that SYN545974 had an algistatic, rather than algicidal, effect on the growth of *Pseudokirchneriella subcapitata*.

HSE evaluator comments

This study was carried out according to GLP and follows guidance document OECD 201 (2011). The following deviations were noted:

All three validity criteria were met after 72 hours. It is noted that the coefficient of variation for section-by-section growth rate after 96 hours was 40 % (over the OECD 201 recommended maximum of 35 %). However, this does not invalidate the test, as OECD 201 (2011) only covers a test period of 72 hours.

Measured concentrations of active substance in the test conditions (excluding the highest nominal concentration of 10 mg a.s. /L) varied from 93.9 to 106 % of the nominal values; the mean measured concentrations in these conditions varied from 85 to 98 % of the nominal. The mean measured concentration of the active substance in the nominal condition of 10 mg a.s. /L was only 59 %. The study authors state that this was because the nominal concentration exceeded functional limit of solubility. Due to the fact that the mean measured concentrations were used for the analysis and reporting of data, this is unlikely to have had any impact on the reliability of the results. The analytical methods have been checked by HSE Chemistry specialists in Vol 3CA Part B5.1.2.6. The following was concluded: "Fit for regulatory purposes but the method is not fully validated in accordance with SANCO/3029/99 rev. 4. LOQ: 0.3 µg/L in aqueous matrices. It should be noted unacceptable recoveries were determined at a test concentration of 10,000 µg/L. The applicant has justified this as the solubility limit of the test substance in AAP medium has been reached."

The use of a positive control/toxic reference item was not reported. The OECD (201) guidelines state that 'Potassium dichromate can also be used as a reference substance for green algae. It is desirable to test a reference substance at least twice a year'. As the use of a positive control is not explicitly required by the guidelines, this wouldn't be cause to invalidate the study.

During the calculation of cell density in each test flask, the number of cells counted was divided by the number of fields examined for each cell count. This was then multiplied by the rounded starting cell density of 10,000 cells /mL. It is unclear why the study authors rounded this value up, as they also provided an actual starting density of 9,900 cells /mL. This is unlikely to impact the overall results, as the same value was used for the correction of all treatments, and so the relative differences between treatments will remain the same.

Raw temperature data from the experiment is not provided in the study report, the authors have only provided the temperature range. As the provided range is within the guideline limits, and the validity criteria are met, this wouldn't be cause to invalidate the study.

The upper 95 % confidence limit for the 72-hour E_rC_{20} was indeterminable, as were both the upper and lower 95 % confidence limits for the 72-hour E_rC_{50} . This could cast doubt on the precision of the provided endpoints. This will be considered further in the risk assessment.

Based on mean measured concentrations, the 72-hour E_rC_{10} value was calculated to be 2.3 mg /L (with 95 % confidence intervals of 0.48 - 3.3 mg /L); the 72-hour E_rC_{20} value was calculated as 5.7 mg /L (with 95 % confidence intervals of 2.1 – ND); the 72-hour E_rC_{50} value was calculated as > 5.9 mg /L (with indeterminable 95 % confidence intervals), and the 72-hour NOEC value was calculated to be 0.90 mg /L.

Report: K-CA 8.2.6.1 [REDACTED] (2015). SYN545547 – 96-Hour Toxicity Test with the Freshwater Green Alga, *Pseudokirchneriella subcapitata*. Report number 1781.7094, Smithers Viscient 790 Main Street Wareham, Massachusetts 02571-1037 (Syngenta File No. SYN545547_10002)

GUIDELINES

OECD Guidelines for Testing of Chemicals, Method 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test (2006)

US EPA Ecological Effects Test Guideline, OCSPP 850.4550: Cyanobacteria (*Anabaena flos-aquae*) Toxicity (2012)

GLP: Yes

MATERIALS

Test Material SYN545547

Lot/Batch #: BPS 1510/1

Purity: 95 % w/w

Description: White powder

Stability of test compound: Stable under standard conditions

Reanalysis/expiry date: End of May 2017

Treatments

Test concentrations: Culture medium control, solvent control and nominal concentrations of 0.63, 1.3, 2.5, 5.0 and 10 mg metabolite/L, (0.51, 1.0, 2.0, 4.0 and 7.0 mg metabolite/L, mean measured)

Solvent: Dimethylformamide (DMF)

Positive control: None referenced

Analysis of test concentrations: Yes, analysis at 0 and 96 hours

Test organism

Species: *Pseudokirchneriella subcapitata*, Strain No. 1648

Source:	Continuous laboratory cultures, originally obtained from UTEX, The Culture Collection of Algae at the University of Texas, Austin, Texas
Test design	
Test vessels:	250 mL glass Erlenmeyer flasks containing 100 mL of media covered with steel caps allowing for gas exchange
Test medium:	AAP algal medium
Replication:	Treatment and culture medium control: 4 Solvent control: 8 Treatment concentrations: 4
Starting cell density:	1.0×10^4 cells/mL
Exposure regime:	Static
Aeration:	None referenced
Duration:	96 hours
Environmental conditions	
Test temperature:	23 to 25 °C
pH:	test start: 7.1 to 7.3 test end: 7.4 to 9.7
Lighting:	Continuous illumination at 4400 to 8880 Lux

STUDY DESIGN AND METHODS

Experimental dates: 1 to 13 June 2015

A stock solution with a nominal concentration of 100 mg/L was prepared by dissolving 1 g of the test item completely in 10 mL of dimethylformamide (DMF). Appropriate volumes of the stock solution were diluted to give the test concentration series. The test concentrations and the solvent control contained 100 µ/L DMF, the upper limit recommended in OECD (2006). The control consisted of culture medium only.

An aliquot of test solution was placed into each test vessel and the test was started by inoculation of 10,000 algal cells per mL of test medium. Test solutions were constantly shaken and were held in a temperature controlled incubator under continuous illumination.

Small volumes of all test concentrations and controls were taken from all test flasks after 24, 48, 72 and 96 hours of exposure. The algal cell densities in these samples were determined by counting with a hemacytometer and a compound microscope. In addition, after 96 hours exposure, a sample was taken from the control and from a test concentration with reduced algal growth. The shape of the algal cells was examined microscopically in these samples.

The pH was measured at the start and at the end of the test. The water temperature was measured daily in a flask incubated under the same conditions as the test flasks.

The test concentrations were verified by chemical analysis of SYN545547 at 0 and 96 hours, using high performance liquid chromatography with ultraviolet detection.

RESULTS AND DISCUSSION

The limit of quantification in this study was 0.00606 mg/L. Mean measured concentrations were used for the calculation and reporting of results.

Table 9.2.6.1-7: Analytical results

NC (mg/L)	MC at 0 hours (mg/L)	% of NC at 0 hours	MC at 96 hours (mg/L)	% of NC at 96 hours	MM (mg/L)	Geometric mean concentration*
Control	<0.046	-	<0.050	-	NA	NA
Solvent control	<0.046	-	<0.050	-	NA	NA
0.63	0.53	84.1	0.50	79.4	0.51	0.5
1.3	1.1	84.6	0.99	76.2	1.0	1.0
2.5	2.1	84.0	1.8	72.0	2.0	1.9
5.0	4.2	84.0	3.8	76.0	4.0	4.0
10	8.2	82.0	5.8	58.0	7.0	6.9

NM = Nominal Concentration, MC = Measured Concentration, MM = Mean Measured concentration, GM = Geometric Mean measured concentration, calculated by HSE based on reported data. Based on recoveries, in accordance with OECD 201 it is appropriate to calculate geometric mean measured concentrations. However, for this study the difference between mean measured and geometric mean measured concentrations is relatively minor. Hence HSE accepts the use of mean measured concentrations in calculating endpoints.

Mean measured concentrations calculated using actual analytical data and not the rounded (2 significant figures) data presented in this table. Concentrations expressed as less than values were below the limit of quantitation (LOQ). The LOQ for each analysis is dependent upon the linear regression, the area of the low standards and the dilution factor of the controls NA = Not Applicable

The algal cell densities were measured at 24, 48, 72 and 96 hours and the mean biomass, growth rate and yield calculated. 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 for all endpoints. The 72-hour and 96-hour E_bC_{50} , E_yC_{50} and E_rC_{50} values (defined as the concentration resulting in 50 % reduction of each parameter) were determined by linear interpolation of response (percent inhibition of endpoint as compared with the appropriate control) using the ICP method (Norberg-King, 1993).

For determination of the LOEC (Lowest Observed Effect Concentration) and NOEC (No Observed Effect Concentration) values, the data were first checked for normality using Shapiro-Wilks' Test (U.S. EPA, 2002) and for homogeneity of variance using Bartlett's Test (U.S. EPA, 2002). If the data sets passed the tests for homogeneity of variance and normality, then Williams' Multiple Comparison Test (U.S. EPA, 2002), a parametric procedure, was used to determine the NOEC and LOEC values. If the data sets failed the tests for normality or homogeneity of variance, NOEC and LOEC values were determined using Jonckheere-Terpstra's Step-Down Test, a non-parametric statistical procedure.

Following 96 hours of exposure, cell fragments were observed in the 4.0 mg/L treatment level.

Beginning at the 48-hour observation interval and throughout the remainder of the exposure period, cells exposed to the 7.0 mg/L treatment level were observed to be chlorotic. White clumps of material were observed in the 7.0 mg/L treatment level, which was otherwise clear, at both 72 and 96 hours. Following agitation at these intervals, the solution was observed to be clear with fine particulates. Additionally, the particulate matter in the 7.0 mg/L treatment level was observed as crystals when observed microscopically. Cells exposed to the remaining treatment levels tested (0.51, 1.0 and 2.0 mg/L) and the controls were observed to be normal throughout the exposure period.

Growth rates

The growth rate 0 to 72 hours and 0 to 96 hours were calculated for each replicate culture and the means are shown below, alongside the estimated EC_{50} values.

Table 9.2.6.1-8: Mean values at each concentration of SYN545547 for the growth rate at 72 and 96 hours for *Pseudokirchneriella subcapitata* and relevant endpoints

Mean measured concentrations (mg/L)	Mean growth rate (1/day) 0 – 72 hrs	Percentage inhibition (relative to pooled control)	Mean growth rate (1/day) 0 – 96 hrs	Percentage inhibition (relative to pooled control)
Control	1.61	-	1.46	-
Solvent control	1.62	-	1.45	-
Pooled control	1.61	-	1.45	-
0.51	1.63	-1	1.44	1
1.0	1.62	0	1.42	2
2.0	1.58	2	1.38#	5
4.0	0.83*	48	0.71#	51
7.0	0.15*	90	0.05#	97

* Statistically significant inhibition compared to the pooled control, based on Williams' Multiple Comparison Test. # Statistically significant inhibition compared to the pooled control, based on Jonckheere-Terpstra's Step-Down Test. Negative values indicate an increase relative to the control

Yield

The yield 0 to 72 hours and 0 to 96 hours were calculated for each replicate culture and the means are shown below, alongside the estimated EC₅₀ values.

Table 9.2.6.1-9: Mean values at each concentration of SYN545547 for the yield at 72 and 96 hours for *Pseudokirchneriella subcapitata* and relevant endpoints

Mean measured concentrations (mg/L)	Mean yield (x 10 ⁴ cells/mL) 0 – 72 hrs	Percentage inhibition (relative to pooled control)	Mean yield (x 10 ⁴ cells/mL) 0 – 96 hrs	Percentage inhibition
Control	113.83	-	288.00	-
Solvent control	116.57	-	281.63	-
Pooled control	115.66	-	283.75	-
0.51	120.33	-4	271.75	4
1.0	116.25	-1	250.13	12
2.0	105.38	9	215.38*	24
4.0	10.75*	91	15.88*	94
7.0	0.63*	99	0.25*	100

* Statistically significant inhibition compared to the pooled control, based on Jonckheere-Terpstra's Step-Down Test.

Negative values indicate an increase relative to the control

Biomass (area under the growth curve)

The areas under the growth curve for 0 to 72 hours and 0 to 96 hours were calculated for each replicate culture and the means are shown below, alongside the estimated EC₅₀ values.

Table 9.2.6.1-10: Mean values at each concentration of SYN545547 for the biomass integral (area under the growth curve) at 72 and 96 hours for *Pseudokirchneriella subcapitata* and relevant endpoints

Mean measured concentrations (mg/L)	Mean biomass integral (x 10 ⁴) 0 – 72 hrs	Percentage inhibition (relative to pooled control)	Mean biomass integral (x 10 ⁴) 0 – 96 hrs	Percentage inhibition (relative to pooled control)
Control	75.90	-	265.80	-
Solvent control	78.71	-	266.89	-
Pooled control	77.77	-	266.52	-
0.51	78.46	-1	263.75	1
1.0	76.38	2	249.51	6
2.0	71.39	8	222.96#	16
4.0	11.59*	85	24.17#	91
7.0	0.46*	99	0.88#	100

* Statistically significant inhibition compared to the pooled control, based on Williams' Multiple Comparison Test # Significant difference compared to the pooled control, based on Jonckheere-Terpstra's Step-Down Test. Negative values indicate an increase relative to the control

Table 9.2.6.1-11: Summary of biological results for toxicity of SYN545547 to *Pseudokirchneriella subcapitata*, at 72 and 96 hours

Parameter	After 72 h (mg metabolite/L)			After 96 h (mg metabolite/L)		
	AUC	Growth rate	Yield	AUC	Growth rate	Yield
EC ₅₀ (95 % CI)	3.0 (2.7 – 3.1)	4.1 (3.8 to 4.4)	2.9 (2.6 - 3.0)	2.8 (2.5 – 2.9)	4.0 (3.5 to 4.3)	2.6 (2.4 – 2.8)
EC ₂₀ (95 % CI)	2.2 (1.8 – 2.5)	2.6 (2.5 to 2.8)	2.2 (1.5 – 2.4)	2.1 (1.2 – 2.3)	2.6 (2.4 to 2.7)	1.6 (0.46 – 2.3)
EC ₁₀ (95 % CI)	2.0 (0.082 – 2.3)	2.3 (2.1 to 2.4)	1.9 (0.059 – 2.2)	1.3 (ND – 2.4)	2.2 (2.1 to 2.3)	0.87 (0.13 - 1.7)
NOEC	2.0	2.0	2.0	1.0	1.0	1.0
LOEC	4.0	4.0	4.0	2.0	2.0	2.0

ND = not determined

VALIDITY CRITERIA

Table 9.26.1-12 : Compliance iwth OECD 201 validity criteria

Validity criteria OECD 201 (2011)	Required	Obtained
Biomass in the control(s)	Increased by a factor or ≥ 16 within 72 hours	Cell growth increased by a factor of 117 after 72 hours.
Mean coefficient of variation for section by section specific growth rates in the control(s)	Must not exceed 35 %	Pooled control was 25 % based on study report.
Coefficient of variation of average specific growth rates in the control(s)	Must not exceed 7 % in tests with <i>Pseudokirchneriella subcapitata</i> .	3.2 % after 72 hours.

CONCLUSIONS

Based on mean measured concentrations, the 72-hour E_rC_{50} for SYN545547 to *Pseudokirchneriella subcapitata* was 4.1 mg metabolite/L, the E_yC_{50} was 2.9 mg metabolite/L and the E_bC_{50} was 3.0 mg metabolite/L. The 96-hour E_rC_{50} was 4.0 mg metabolite/L, the E_yC_{50} was 2.6 mg/L and the E_bC_{50} was 2.8 mg metabolite/L.

The Lowest Observed Effect Concentration at 96 hours, based on growth rate, yield and biomass integral, was 2.0 mg metabolite/L, and the No Observed Effect Concentration was 1.0 mg metabolite/L.

(████████, 2015)

HSE evaluator comments

As shown above the validity criteria were met for this study.

The study was carried out to GLP and follows guidance document OECD 201 (2006) and OCSPP 850.4550 (2012). The study was reviewed according to OECD 201 (2011). There were no deviations to the guidelines. During the preparation of the test solution white, visible, undissolved test substance was observed which was filtered out. As the test concentrations were analysed, HSE deems that this did not affect the outcome of the study.

Results of a reference substance test have not been reported therefore there is uncertainty with the sensitivity of the test system. However, as the use of a reference item is not an essential part of the test under OECD 201 the lack of results does not invalidate the endpoints derived.

Equal Variance Two-Sample t-Test was used to compare the control and the solvent control. No significant difference was observed, and the control tests were pooled. The results from the test concentrations were compared to the pooled control. The data was checked for normality using Shapiro-Wilk's Test and tested for homogeneity of variance using Bartlett's Test. For 72 hour data these tests were passed but not for 96 hour data hence different methods were used for NOEC/LOEC determination either William's Multiple Comparison Test or Jonckheere-Terpstra's Step-Down Test. The EC values were calculated by linear interpolation of endpoint. Reporting of the statistical analysis was brief and it was unclear whether the data were transformed prior to analysis. The analysis used is mentioned in OECD 201 and the endpoints calculated appeared to be in-line with the experimental data. Therefore, HSE considers the statistical analysis conducted appropriate.

HSE considers the validity criteria from OECD 201 (2011) have been met for this study, however, there were some minor discrepancies. HSE calculated some minor differences in values (mean growth rate and section by section growth rate for control) but considers these are unlikely to impact study endpoints (% inhibition values almost identical) and notes validity criteria for section by section growth rate was still met. HSE calculated the mean coefficient of variation for section by section specific growth rates in the control as 28.5 % rather than 25 %. This is still below 35 %, meeting the validity criteria.

The analytical methods have been checked by HSE Chemistry specialists in Vol 3CA Part B5.1.2.6. The following was concluded: "Fit for regulatory purposes but the method is not fully validated in accordance with SANCO/3029/99 rev. 4. LOQ: 0.015 mg/L in freshwater and AAP medium".

The measured concentrations were not within $\pm 20\%$. Not all test concentrations were maintained within $\pm 20\%$ of initial measured concentrations either. Therefore, ideally geometric mean measured concentrations would have been used. HSE calculated geometric mean concentrations and they were broadly comparable to mean measured concentrations. Hence the use of mean measured concentrations in calculation of endpoints is considered acceptable by HSE. It should be noted that analytical data was only measured at 96 hours rather than 72 hours. Hence the 72 hour endpoints below are based on analytical data over a 96 hour period.

The agreed endpoints are:

- 72-hour E_rC_{50} for SYN545547 = 4.1 mg metabolite/L (mean measured concentrations)
- 72-hour E_rC_{20} for SYN545547 = 2.6 mg metabolite/L (mean measured concentrations)
- 72-hour E_rC_{10} for SYN545547 = 2.3 mg metabolite/L (mean measured concentrations)
- 72-hour E_yC_{50} for SYN545547 = 2.9 mg metabolite/L (mean measured concentrations)
- 72-hour E_bC_{50} for SYN545547 = 3.0 mg metabolite/L (mean measured concentrations)

- 96-hour E_rC_{50} for SYN545547 = 4.0 mg metabolite/L (mean measured concentrations)
- 96-hour E_yC_{50} for SYN545547 = 2.6 mg metabolite/L (mean measured concentrations)
- 96-hour E_bC_{50} for SYN545547 = 2.8 mg metabolite/L (mean measured concentrations)

- 72-hour NOEC for SYN545547 = 2.0 mg metabolite/L (mean measured concentrations)
- 96-hour NOEC for SYN545547 = 1.0 mg metabolite/L (mean measured concentrations)

Report: K-CA 8.2.6.1/03 [REDACTED] and [REDACTED] 2016b), SYN548261 - Inhibition of Growth to the Alga *Pseudokirchneriella subcapitata* in a 96-hour test, Report Number 3201084, Smithers Viscient (ESG) Ltd. 108 Woodfield Drive, Harrogate, North Yorkshire, HG1 4LS, UK, (Syngenta File No. SYN548261_10001).

GUIDELINES

OECD Guideline 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test (2011)

GLP: Yes

MATERIALS

Test Material SYN548261
Lot/Batch #: MES 333/2
Purity: 98 % w/w
Description: White solid
Stability of test compound: Stable under standard conditions
Reanalysis/expiry date: End of April 2017

Treatments

Test concentrations: Culture medium control and a single nominal concentration of 100 mg metabolite/L
Solvent: None
Positive control: Potassium dichromate
Analysis of test concentrations: Yes, analysis at 0 and 96 hours

Test organism

Species: *Pseudokirchneriella subcapitata*, Strain No. 278/4
Source: Continuous laboratory cultures, originally obtained from Culture Collection of Algae and Protozoa (CCAP)

Test design

Test vessels: 250 mL glass Erlenmeyer flasks containing 100 mL of media plugged with foam bungs

Test medium:	EC medium
Replication:	Six vessels for the control and three vessels for each test concentration
Starting cell density:	1.0×10^4 cells/mL
Exposure regime:	Static
Aeration:	No
Duration:	96 hours
Environmental conditions	
Test temperature:	22.5 – 23.1 °C
pH:	test start: 7.04 to 7.89 test end: 8.62 to 10.16
Lighting:	Continuous illumination at 6900 – 7110 Lux

STUDY DESIGN AND METHODS

Experimental dates: 15 July to 25 August 2015.

At the start of the test, ca 50 mg of SYN548261 was dissolved in 500 mL of EC medium to give the 100 mg/L test concentrations. Dissolution was aided by 10 minutes of stirring followed by 10 minutes of sonication. A control treatment was prepared by adding EC medium only to the control vessels.

An aliquot of test solution was placed into each test vessel and the test was started by inoculation of 10,000 algal cells per mL of test medium. Test solutions were constantly shaken and were held in a temperature-controlled incubator under continuous illumination.

Small volumes of all test concentrations and controls were taken from all test flasks after 24, 48, 72 and 96 hours of exposure. The algal cell densities in these samples were determined by counting with a particle counter. In addition, after 96 hours exposure, a sample was taken from the control and from a test concentration with reduced algal growth. The shape of the algal cells was examined microscopically in these samples.

The pH was measured at the start and at the end of the test. The water temperature was measured daily in a flask incubated under the same conditions as the test flasks.

The test concentrations were verified by chemical analysis of SYN548261 at 0 and 96 hours, using high performance liquid chromatography with UV detection.

RESULTS AND DISCUSSION

The limit of quantification (LOQ) for SYN548261 in EC medium using this method was 0.05 mg/L. Nominal concentrations were used for the calculation and reporting of results.

Table 9.2.6.1-13: Analytical results

Nominal concentration (mg metabolite/L)	Measured Concentration 0 Hours in New Media (mg metabolite/L)	% of nominal measured at 0 hours	Measured Concentration 96 Hours in Old Media (mg metabolite/L)	% of nominal measured at 96 hours
Control	0.00257*	-	0.00287*	-
100	100.4	100	109.3	109

*Possible contamination was not thought to have impacted the test as the values were below the limit of quantification (0.05 mg/L).

To distinguish between EC_x values determined using areas under the growth curve, final yield and growth rates, the symbols E_bC_x, E_yC_x and E_rC_x were used, respectively. Section-by-section percentage inhibition in growth rate and section-by-section growth rates for control vessels were also calculated and reported.

At 0 hours the test preparations were observed and recorded as colourless solutions for both the control group and 100 mg metabolite/L test group.

At 96 hours both the control and 100 mg metabolite/L test group were observed and recorded as green homogenous hazy dispersions of algal cells.

Growth rates

The growth rate 0 to 72 hours and 0 to 96 hours were calculated for each replicate culture and the means are shown below, alongside the estimated EC₅₀ values. In this study, 96 hr EC₁₀ and EC₂₀ values were not able to be calculated, as the definitive study consisted of a single test concentration.

Table 9.2.6.1-14: Mean values at each concentration of SYN548261 for the growth rate at 72 and 96 hours for *Pseudokirchneriella subcapitata* and relevant endpoints

Nominal concentrations (mg/L)	Mean growth rate (cells × 10 ⁻² /mL) 0 – 72 hrs	Percentage inhibition	Mean growth rate (cells × 10 ⁻² /mL) 0 – 96 hrs	Percentage inhibition
Control	6.485	-	5.775	-
100	6.598	-1.74 %*	5.699	1.316*
E_rC₅₀ mg /L	>100		>100	
NOEC	100		100	

Negative values indicate an increase in growth compared to control.

*Calculated by HSE based on available data.

Yield

The yield 0 to 72 hours and 0 to 96 hours were calculated for each replicate culture and the means are shown below, alongside the estimated EC₅₀ values.

Table 9.2.6.1-15: Mean values at each concentration of SYN548261 for the yield at 96 hours for *Pseudokirchneriella subcapitata* and relevant endpoints

Nominal concentrations (mg/L)	Mean yield (x 10 ⁴ cells/mL) 0 – 72 hrs ¹	Percentage inhibition ¹	Mean yield (x 10 ⁴ cells/mL) 0 – 96 hrs	Percentage inhibition
Control	105.820 ^a	-	256.101	-
100	114.900 ^a	-5.747 ^a	239.177	6.608 ^a
E_yC₅₀ mg/L	>100 ^b		>100	
NOEC	100 ^b		100	

Negative values indicate an increase in growth compared to control.

^a Added and/or calculated by HSE.

^b Was not reported, added by HSE based on available data.

Biomass (area under the growth curve)

The areas under the growth curve for 0 to 72 hours and 0 to 96 hours were calculated for each replicate culture and the means are shown below, alongside the estimated EC₅₀ values.

Table 9.2.6.1-16: Mean values at each concentration of SYN548261 for the biomass integral (area under the growth curve) at 72 and 96 hours for *Pseudokirchneriella subcapitata* and relevant endpoints

Nominal concentrations (mg/L)	Mean biomass integral (x 10 ⁴) 0 – 72 hrs	Percentage inhibition	Mean biomass integral (x 10 ⁴) 0 – 96 hrs	Percentage inhibition
Control	1916.948	-	6260.000	-
100	2107.188	-9.924	6356.108	-1.535
EbC₅₀ mg/L	>100		>100	
NOEC	100		100	

VALIDITY CRITERIA

The validity criteria for the OECD 201 (2011) Guideline were met as detailed below:

Table 9.2.6.1-17: Compliance with OECD 201 validity criteria

Validity criterion	Required	Obtained
Increase in biomass within the test period in the control cultures	Increase exponentially by a factor of at least 16 over 72-hours (specific growth rate of 0.92 day ⁻¹).	106 over 72 hours* 256 over 96 hours
Mean value of the coefficients of variation for section-by-section specific growth rates in the control cultures	Must not exceed 35 %	7.45 across 72 hours* 25.6 across 96 hours*
Coefficient of variation of average specific growth rates during the whole test period	≤ 7 % for <i>Pseudokirchneriella subcapitata</i>	1.43 over 72 hours* 2.14 over 96 hours
Analytical measurements of test concentration	Must be carried out using validated method at regular intervals. If deviation from nominal or measured initial concentration is more than ± 20 % then results should be expressed as geometric mean or using model describing concentration change.	Analytical measurements were taken at the start and end of the test. They were within the acceptable range therefore nominal concentrations were used for reporting results.

*calculated by HSE based on available data.

CONCLUSIONS

The objective of the study was to determine the effects of SYN548261 on the growth of the green alga, *Pseudokirchneriella subcapitata*, during a 96 hour growth inhibition toxicity test. No significant inhibition of growth was observed at the highest concentration tested, 100 mg/L.

Based on nominal concentrations, the 96-hour E_yC₅₀ and the 0-96 hour E_bC₅₀ and E_rC₅₀ values were calculated to be greater than 100 mg/L, respectively. The corresponding NOEC values for yield, biomass and specific growth rate after 96 hours were 100 mg/L, respectively.

(██████ and ██████, 2016b)

HSE evaluator comments

This study was conducted to GLP. The study was assessed against OECD Guideline 201 (2011).

Analytical measurements were taken at the start and end of the test (0 and 96 hours). They were within the acceptable range ($\pm 20\%$ of nominal) therefore nominal concentrations were used for reporting results.

The analytical methods have been evaluated by HSE Chemistry specialists in Vol. 3CA Part B5.1.2.6. The following was concluded for this method: “Acceptable method. LOQ: 0.05 µg/mL in EC medium”.

It is noted that there are some minor discrepancies in pH. The starting pH of the control cultures was higher than the test cultures at pH 7.98 compared to pH 7.04. The 96-hour pH of both the control and test cultures also rose by more than the recommended maximum of 1.5 units during the test (control cultures rising by 2.1 units to a mean of pH 10.1, test cultures rising by 1.8 units to mean of pH 8.84). However, since the control cultures meet the growth validity criteria, this is not deemed to be an issue.

It is also noted that the maintenance liquid cultures of *P. subcapitata* used to inoculate the test are listed as ‘semi-axenic’ rather than a pure culture. The presence of other micro-organisms could affect the sensitivity of the culture to the test substance. However, the authors do report the results of a 72-hour positive control test with potassium dichromate which was performed 8-months prior, and which demonstrates expected toxicity results for their laboratory with an E_rC_{50} of 1.667 and E_bC_{50} of 0.8173. This is outside the range referenced in ISO 8692 (1993), which states an E_rC_{50} (0-72h) of 0.60-1.03 and E_bC_{50} (0-72h) of 0.20-0.75. Therefore, although there is some indication of lower sensitivity, the differences are relatively minor and are not sufficient to invalidate the study.

It is noted that the authors state that formal statistical analysis was not conducted because “*SYN548261 did not inhibit the growth of Pseudokirchneriella subcapitata under the conditions of the test*”, and that toxicity values were derived empirically from the data. The absence of inhibition is true for the growth rate and yield data at 72 hours, but not for 96 hours where there is a small amount of inhibition. Since the desired end point for assessment is 72 hours, then this is not a significant issue with the study.

The 96-hour section-by-section coefficient of variation was reported by the study authors as part of the validity criteria. Recalculation by HSE based on the raw data available in the study report obtained a value higher than that reported by the study authors. The higher value was still within the validity criteria, therefore this discrepancy is not a further issue. EC_{10} and EC_{20} values were not able to be calculated, as the definitive study consisted of a single test concentration.

Overall, there were no major deviations from the study guidelines and the study meets all validity criteria.

The agreed end-points for use in risk assessments are:

- 72 hour $E_rC_{50} > 100$ mg metabolite/L (nominal concentration)
- 72 hour $E_bC_{50} > 100$ mg metabolite/L (nominal concentration)
- 72-hour NOEC for growth rate and yield > 100 mg metabolite/L (nominal concentration, not statistically determined)

Report:	K-CA 8.2.6.1 [REDACTED], 2009b, M700F001 (Metabolite of BAS 700 F) - <i>Pseudokirchneriella subcapitata</i> SAG.61.81 Growth Inhibition Test, Report Number W/11/09, Institute of Industrial Organic Chemistry, Branch Pszczyna, Department of Ecotoxicology, Doświadczalna 27, 43-200 Pszczyna, Poland. (Syngenta File No. CA4312_10907)
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GUIDELINES

OECD Guidelines for Testing of Chemicals, Section 2 - Effects on Biotic Systems, Method 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test (2006)

Official Journal of the European Communities, Dir 92/69/EEC, O.J. L383A, Part C.3: Algal inhibition test (1992)

GLP: Yes

MATERIALS

Test Material	M700F001 (Metabolite of BAS 700 F; synonym of NOA449410) 3-(difluoromethyl-1-methyl-1H-pyrazole-4-carboxylic acid)
Lot/Batch #:	L80-68
Purity:	99.2 % (\pm 1.0%)
Treatments	
Test concentrations:	Culture medium control and nominal concentrations of 10, 18, 32, 56 and 100 mg M700F001(NOA449410)/L
Solvent:	None
Positive control:	3,5 dichlorophenol (97 % purity) at 5 concentrations in the range 0.03 to 3.2 mg/L was tested in similar conditions between 5 May and 8 May 2009
Analysis of test concentrations:	Yes, analysis of M700F001 (NOA449410) at 0 and 72 hours by HPLC with UV-VIS detection
Test organism	
Species:	<i>Pseudokirchneriella subcapitata</i> , Strain No. 61.81 SAG (Reinsch) Korshikov (syn. <i>Selenastrum capricornutum</i> Prinz)
Source:	Laboratory culture, originally obtained from The Algae Collection of the Göttingen University, Germany
Test design	
Test vessels:	250 mL glass Erlenmeyer flasks containing 80 mL of media
Test medium:	AAP nutrient solution prepared according to OECD 201 (2011).
Replication:	Six vessels for the control and three vessels for each test concentration
Starting cell density:	1.0×10^4 cells/mL
Exposure regime:	Static
Aeration:	No
Duration:	72 hours
Environmental conditions	
Test temperature:	21.7 – 22.7 °C
pH:	test start: 4.21 to 7.14 test end: 3.98 to 7.49
Lighting:	Continuous illumination at 9096 to 9960 lux

STUDY DESIGN AND METHODS

Experimental dates: 5 May 2009 to 8 May 2009.

A stock solution with was prepared by dissolving 254.4 mg of M700F001 (NOA449410) completely in 25.44 mL of test medium. It was placed on a magnetic stirrer for 0.5 hour and then placed at ultrasonic cleaner for 5 minutes. Appropriate volumes of the stock solution were diluted to give the test concentration series. The control consisted of culture medium only.

An aliquot of test solution was placed into each test vessel and the test was started by inoculation of 10,000 algal cells per mL of test medium. Test solutions were constantly shaken on a mechanical shaker and were held in a temperature-controlled incubator under continuous illumination.

Small volumes of all test concentrations and controls were taken from all test flasks after 24, 48 and 72 of exposure. The algal cell densities in these samples were determined by spectrophotometric absorbance. In addition, after 72 hours exposure, the algal cells were examined for morphological changes.

The pH was measured at the start and at the end of the test.

The test concentrations were verified by chemical analysis of M700F001 (NOA449410) at 0 and 72 hours, using high performance liquid chromatography with UV-VIS detection.

RESULTS AND DISCUSSION

At the start of the test, the measured concentrations were in the range 87.1 to 96.9% of the nominal values and at the end of the test were in the range 84.5 to 102.3% (see table below). The limit of quantification in this study was 0.05 mg M700F001(NO449410)/L. Nominal concentrations were used for the calculation and reporting of results.

Table 9.2.6.1-18: Analytical results

Nominal concentrations (mg/L)	% of nominal measured at 0 hours	% of nominal measured at 72 hours
Control	<LOQ	<LOQ
10	87.20	84.50
18	95.94	102.28
32	92.41	92.00
56	96.86	96.75
100	87.06	89.51

<LOQ – less than the limit of quantification

The algal cell densities were measured at 24, 48 and 72 hours and the mean growth rate and yield calculated. The 72-hour E_yC_{50} and E_rC_{50} values (defined as the concentration resulting in 50% reduction of each parameter) were calculated using Probit analysis.

There were no abnormalities observed, in the control or 18 mg/L test culture at 72 hours. At 32, 56 and 100 mg/L the algal cells were swollen compared to the control.

Growth rates

The growth rate 0 to 72 hours were calculated for each replicate culture and the means are shown below, alongside the estimated EC_{50} values.

Table 9.2.6.1-19: Mean values at each concentration of M700F001 (NOA449410) for the growth rate at 72 hours for *Pseudokirchneriella subcapitata* and relevant endpoints

Nominal concentrations (mg/L)	Mean growth rate (1/day) 0 – 72 hrs	Percentage inhibition (%)
Control	1.831	0.0
10	1.787	2.4
18	1.813	1.0
32	1.088	40.6
56	0.291	84.1
100	0.216	88.2
E_rC_{50} mg/L (95% confidence limits)	36.31 (30.77 – 42.87)	
E_rC_{20} mg/L (95% confidence limits)	25.03 (16.98 – 29.76)	

ErC10 mg/L (95% confidence limits)	20.61 (11.93 – 25.66)
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Yield

The yield 0 to 72 hours were calculated for each replicate culture and the means are shown below, alongside the estimated EC₅₀ values.

Table 9.2.6.1-20: Mean values at each concentration of M700F001 (NOA449410) for the yield at 72 hours for *Pseudokirchneriella subcapitata* and relevant endpoints

Nominal concentrations (mg/L)	Mean yield 0-72 hrs	Percentage inhibition
Control	2.427	0.0
10	2.136	12.0
18	2.295	5.4
32	0.515	78.8
56	0.017	99.3
100	0.009	99.6
E _y C ₅₀ mg/L (95% confidence limits)	26.42 (18.30 – 30.26)	
E _y C ₂₀ mg/L (95% confidence limits)	21.60 (9.72 – 25.62)	
E _y C ₁₀ mg/L (95% confidence limits)	19.43 (6.87 – 23.87)	

The dose-response curve for growth rate and yield is shown in the figure below:

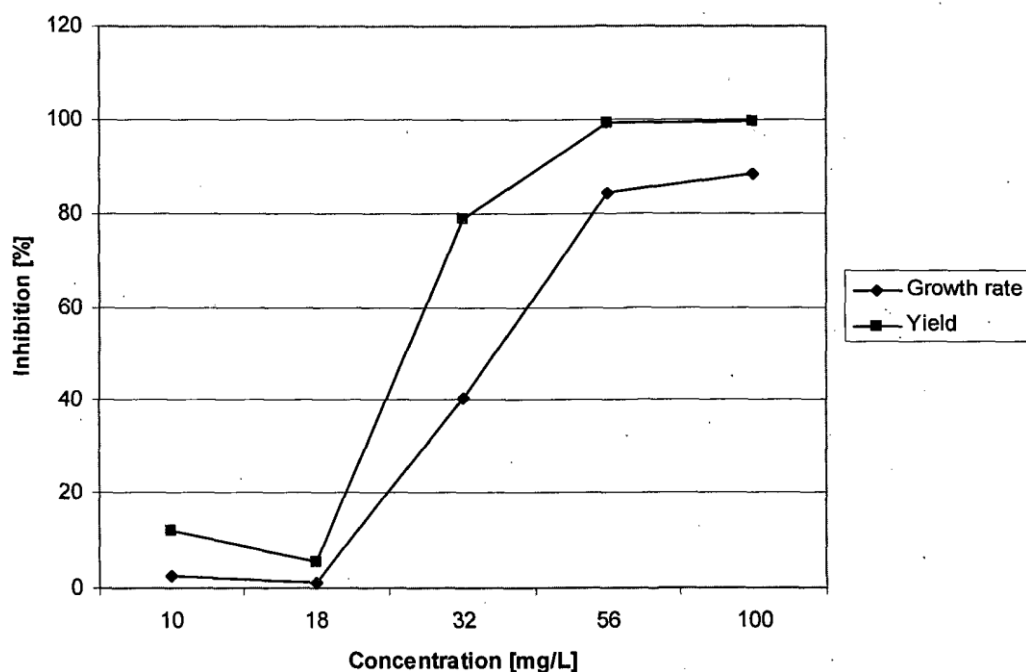


Figure 9.2.6.1-2: Dose-response curve for growth rate and yield**VALIDITY CRITERIA**

The validity criteria outlined in OECD 201 (2011) were satisfactorily met:

Table 9.2.6.1-21: Compliance with OECD 201 validity criteria

Validity criterion	Required	Obtained
Increase in biomass in control	Increase by a factor of 16 within 72 h	Increased by a factor of 244 within 72 h
Coefficient of variation for daily growth rates in control	Must be ≤ 35 % over 72 h	20.9 % over 72 h
Coefficient of variation for average specific growth rates in replicate control cultures	Must be < 7 % during whole test period	1.3 %

CONCLUSIONS

Based on nominal concentrations, the 72-hour E_rC_{50} for M700F001 (NOA449410) to *Pseudokirchneriella subcapitata* was 36.31 mg/L and the E_yC_{50} was 26.42 mg/L.

(██████████, 2009b)

HSE COMMENTS

This study was performed in accordance with GLP and follows OECD 201 (2011). The validity criteria have been satisfactorily met. The result of the reference test with 3,5-dichlorophenol (72 h E_rC_{50} = 2.36 mg/L) was within the range (3.38 ± 1.30 mg/L) stipulated in ISO 8692 (2012) and is therefore acceptable. Measured concentrations of M700F001 ranged from 87.06-96.86 % of nominal values at test initiation and from 84.50-102.28 % of nominal at 72 h. Since measured concentrations remained within ± 20 % of nominal values, presented results are based on nominal concentrations. The analytical methods have been evaluated by HSE Chemistry specialists in Vol. 3 CA Part B5.1.2.6. The following was concluded for this method: “Acceptable method. LOQ: 0.05 µg/mL in water”.

It is noted that the light intensity used (9096 to 9960 lux) exceed the maximum intensity advised in OECD 201 (2011) (8880 lux). This deviation from the protocol is minor and not thought to have affected the study outcome.

The acidity of the test item resulted in very low pH at higher test concentrations. At 56 mg/L and 100 mg/L, the pH at t0 measured 4.96 and 4.21 respectively, dropping to 4.47 and 3.98 respectively at t72. The applicant states: ‘The test solutions have not been buffered to reach standard conditions and definitely a part of the observed growth effects was caused by the acidification and thus not optimal condition for algal growth.’. Since the pH of the control medium remained within ± 1.5 units, as stipulated in OECD 201 (2011), the low pH seen at high concentrations is thought to be an action of the test item and is not cause for concern.

$EC_{50/20/10}$ values and 95 % confidence intervals were calculated using probit analysis, as recommended in OECD 201 (2011). No NOEC values were determined. The endpoints suitable for use in risk assessment are therefore:

- 72 h E_rC_{50} = 36.31 mg M700F001(NO449410)/L
- 72 h E_rC_{20} = 25.03 mg M700F001(NO449410)/L
- 72 h E_rC_{10} = 20.61 mg M700F001(NO449410)/L
- 72 h E_yC_{50} = 26.42 mg M700F001(NO449410)/L
- 72 h E_yC_{20} = 21.60 mg M700F001(NO449410)/L
- 72 h E_yC_{10} = 19.43 mg M700F001(NO449410)/L

B.9.2.6.2. Effects on growth of an additional algal species

Report: K-CA 8.2.6.2 [REDACTED] (2013). SYN545974 - Toxicity Test to the Freshwater Blue-Green Alga, *Anabaena flos-aquae*, Report Number 1781.6881, Smithers Viscient, 790 Main Street, Wareham, Massachusetts 02571-1037, USA, (Syngenta File No. SYN545974_10091)

GUIDELINES

OECD Guidelines for Testing of Chemicals, Method 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test (2006)

US EPA Ecological Effects Test Guideline, OCSPP 850.4550: Cyanobacteria (*Anabaena flos-aquae*) Toxicity. (2012)

GLP: Yes

MATERIALS

Test Material	SYN545974 tech.
Lot/Batch #:	SMU2EP12007
Purity:	98.5 %
Description:	Off white powder
Stability of test compound:	Stable under standard conditions
Reanalysis/expiry date:	30 June 2016
Density:	Not applicable

Treatments

Test concentrations:	Culture medium control, solvent control, and nominal concentrations of 0.1, 0.33, 1.0, 3.2 and 10 mg a.s./L (0.087, 0.28, 0.82, 2.7 and 4.9 mg a.s./L mean measured, respectively)
Solvent:	Dimethylformamide (DMF), 0.1 mL/L
Positive control:	None
Analysis of test concentrations:	Yes, 0 and 96 hours (based on measurements of SYN545974 by LC/MS/MS)

Test organism

Species:	<i>Anabaena flos-aquae</i> , Strain 1444, class Cyanophyceae
Source:	Laboratory cultures, originally obtained from the University of Texas, Austin, Texas, USA

Test design

Test vessels:	250 mL glass flasks containing 100 mL of test solution, covered with glass dish
Test medium:	AAP algal medium prepared according to OECD guideline 201
Replication:	Eight vessels for the solvent control and four vessels for each test concentration and dilution water control
Starting cell density:	5.0×10^4 cells/mL
Exposure regime:	Static
Aeration:	None reported
Duration:	96 hours

Environmental conditions

Test temperature:	23 - 25 °C
pH:	test start: 7.0 to 7.2 test end: 7.5 to 9.6
Lighting:	Continuous illumination at 180 to 230 foot candles (1900 to 2500 Lux)

STUDY DESIGN AND METHODS

Experimental dates: 17 to 25 June 2013.

A 100 mg a.s./mL primary stock solution was prepared prior to test initiation by placing 2.5002 g of SYN545974 in a 25-mL volumetric flask and bringing it to volume with DMF. Appropriate volumes of this solution were diluted with DMF to prepare the secondary stock solutions and 0.10 mL of each secondary stock solution was diluted with 1000 mL of AAP medium to prepare the test concentrations. These were mixed for approximately 1.5 hours using a magnetic stir plate with stir bar, after which the 3.2 and 10 mg a.s./L test concentrations were observed to contain a large amount of undissolved test substance. These were sonicated for approximately 30 minutes, and the 10 mg a.s./L test concentration was then filtered, and the filtrate used for testing. After these preparations all test solutions appeared to be clear and colourless with no visible undissolved test substance. A solvent control solution was prepared by adding 0.1 mL of DMF to a 1000 mL volumetric flask and bringing it to volume with AAP media, and the blank controls contained culture medium only.

In order to estimate the impact of the presence of algal biomass, an additional replicate flask of the 1.0 mg a.s./L (nominal) treatment level was prepared. This was not inoculated with algae and was analysed at 96 hours of exposure. The results of this analysis were compared with the results for the 1.0 mg a.s./L solution containing algae.

An aliquot of test solution was placed into each test vessel and the test was started by inoculation of 50,000 algal cells per mL of test medium. The solutions were hand shaken at least once a day and were incubated in an environmental chamber under continuous illumination.

Small volumes of all test concentrations and the control were taken from all test flasks after 24, 48, 72 and 96 hours of exposure. The algal cell densities in these samples were determined by haemocytometer and a compound microscope. The pH was measured at the start and at the end of the test in each test concentration and the control. The water temperature and light intensity was measured daily. The appearance of the test media was also recorded daily. Additionally, the water temperature was continuously recorded with a data logger. The test concentrations were verified by chemical analysis of SYN545974 at 0 and 96 hours, using LC-MS/MS.

RESULTS AND DISCUSSION

The mean measured concentrations of SYN545974 ranged from 49 to 87 % of nominal concentrations (see table below). The limit of quantification in this study was reported as 0.151 µg a.s./L (0.000151 mg/L). Mean measured concentrations were used for the calculation and reporting of results.

Table 9.2.6.2-1: Analytical results

Nominal concentrations (mg a.s./L)	Measured concentration (mg a.s./L)			
	0 hour	96 hour	Mean	Geometric mean concentration *
Control	<0.0086 ^b	<0.0082	NA	NA
Solvent Control	<0.0086	<0.0082	NA	NA
0.10	0.092	0.083	0.087	0.087
0.33	0.29	0.27	0.28	0.28

Nominal concentrations (mg a.s./L)	Measured concentration (mg a.s./L)			
	0 hour	96 hour	Mean	Geometric mean concentration *
1.0	0.88	0.76 (0.85 ^c)	0.82	0.88
3.2	3.0	2.5	2.7	2.74
10	5.7	4.0	4.9	4.77

^a Mean measured concentrations and percent nominal were calculated using actual analytical data and not the rounded (two significant figures) data presented in this table ^b Concentrations expressed as less than values were below the limit of quantitation (LOQ). The LOQ for each analysis is dependent upon the regression, the area of the low standards and the dilution factor of the controls. ^c Result of the additional sample without algae present to determine the effect of the presence of algae.

^d Due to filtration of the test solutions at 0 hour, the measured concentrations of the 10 mg SYN545974/L treatment level were expected to be below the nominal test concentration. NA = Not applicable

* Geometric Mean measured concentration, calculated by HSE based on reported data. Based on recoveries, in accordance with OECD 201 it is appropriate to calculate geometric mean measured concentrations. However, for this study the difference between mean measured and geometric mean measured concentrations is relatively minor. Hence HSE accepts the use of mean measured concentrations in calculating endpoints.

The analytical result of the sample taken at 96 hours from the 1.0 mg a.s./L nominal treatment level, with algae present, was 0.76 mg a.s./L. The equivalent 1.0 mg a.s./L test solution without algae present resulted in a recovery of 0.85 mg a.s./L after 96 hours. These results indicate that the presence of algae had a slight impact on the concentration of SYN545974 in the test solution.

The algal biomass was measured at 24, 48, 72 and 96 hours and the biomass integral, growth rate and yield were calculated. No significant difference (Equal Variance Two-Sample Test, $p \leq 0.05$) was determined between the negative and solvent controls, therefore the biological results were compared to the pooled control data. The data were checked for normality using Shapiro-Wilks' Test and for homogeneity of variance using Levene's Equality of Variance or Bartlett's Tests. If the data set passed tests for homogeneity and normality, then Dunnett's Multiple Comparison Test was used to determine the NOEC and LOEC. If the data set did not pass tests for homogeneity and normality, then Dunnett's T3 Multiple Comparison or Dunnett's Multiple Comparison Test with Bonferroni-Holm Adjustment were used to determine the NOEC and LOEC. The 72- and 96-hour E_bC_{50} , E_yC_{50} and E_rC_{50} values (defined as the concentration resulting in 50 % reduction of each parameter) and their 95 % confidence intervals were determined by linear interpolation of response.

Cells from all treatment levels and controls were observed to be normal throughout the exposure period.

Growth rates

The growth rate 0 to 72 hours and 0 to 96 hours were calculated for each replicate culture and the means are shown below.

Table 9.2.6.2-2: Mean growth rate values at each concentration of SYN545974 at 72 and 96 hours for *Anabaena flos-aquae*

Mean measured concentration (mg a.s./L)	Mean growth rate (1/day) 0 – 72 hrs	Percentage inhibition ^{a,b}	Mean growth rate (1/day) 0 – 96 hrs	Percentage inhibition ^{a,b}
Control	1.36	-	1.18	-
Solvent Control	1.35	-	1.14	-
Pooled Control	1.35	-	1.15	-
0.087	1.29	4	1.12	3
0.28	1.27	6	1.15	0
0.82	1.29	4	1.06 ^c	8
2.7	1.34	1	1.04 ^c	10
4.9	0.00 ^d	100	-0.18 ^c	116

^a Percent inhibition relative to the pooled control ^b Mean and percent inhibition were calculated from original raw data, not from the rounded values presented in this table ^c Significantly reduced compared to the pooled control, based on Dunnett's Multiple Comparison Test with Bonferroni-Holm Adjustment ^d Significantly reduced compared to the pooled control, based on Dunnett's Multiple Comparison Test

Yield

The yield (based on biomass) from 0 to 72 hours and 0 to 96 hours were calculated for each replicate culture and the means are shown below.

Table 9.2.6.2-3: Mean yield (based on biomass) values at each concentration of SYN545974 at 72 and 96 hours for *Anabaena flos-aquae*

Mean measured concentration (mg a.s./L)	Mean yield (x 10 ⁴ cells/mL) 0 – 72 hrs	Percentage inhibition ^{ab}	Mean yield (x 10 ⁴ cells/mL) 0 – 96 hrs	Percentage inhibition ^{ab}
Control	251.06	-	483.63	-
Solvent Control	237.48	-	421.69	-
Pooled Control	242.01	-	442.33	-
0.087	204.96	15	381.94	14
0.28	187.56	22	433.06	2
0.82	205.07	15	295.69 ^c	33
2.7	231.59	4	278.31 ^c	37
4.9	-5.00 ^c	102	-0.28 ^d	100

^a Percent inhibition relative to the pooled control ^b Mean and percent inhibition were calculated from original raw data, not from the rounded values presented in this table ^c Significantly reduced compared to the pooled control, based on Dunnett's Multiple Comparison Test with Bonferroni-Holm Adjustment

^d Significantly reduced compared to the pooled control, based on Dunnett's Multiple Comparison Test

Biomass (area under the growth curve)

The areas under the growth curve for 0 to 72 hours and 0 to 96 hours were calculated for each replicate culture and the means are shown below.

Table 9.2.6.2-4: Mean biomass integral values (area under the growth curve) at each concentration of SYN545974 at 72 and 96 hours for *Anabaena flos-aquae*

Mean measured concentration (mg a.s./L)	Mean biomass integral (10 ⁴ days/mL) 0 – 72 hrs	Percentage inhibition ^{ab}	Mean biomass integral (10 ⁴ days/mL) 0 – 96 hrs	Percentage inhibition ^{ab}
Control	204.14	-	572.25	-
Solvent Control	203.28	-	533.55	-
Pooled Control	203.56	-	546.45	-
0.087	175.49	14	469.55	14
0.28	190.77	6	501.73	8
0.82	209.94	-3	460.84	16
2.7	203.90	0	459.38	16
4.9	-12.07 ^c	106	-14.72 ^d	103

^a Percent inhibition relative to the pooled control ^b Mean and percent inhibition were calculated from original raw data, not from the rounded values presented in this table ^c Significantly reduced compared to the pooled control, based on Dunnett's Multiple Comparison Test ^d Significantly reduced compared to the pooled control, based on Dunnett's T3 Multiple Comparison Test

Table 9.2.6.2-5: Summary of results for the toxicity of SYN545974 to *Anabaena flos-aquae* after 72 and 96 hours

Parameter	after 72 h (mg a.s./L)			after 96 h (mg a.s./L)		
	AUC	Growth rate	Yield	AUC	Growth rate	Yield
EC ₅₀ (9 5% CI)	3.6 (3.3 – 3.6)	3.6 (3.6 – 3.7)	3.5 (3.2 – 3.7)	3.4 (3.2 - 3.6)	3.4 (3.2 – 3.9)	3.1 (2.7 – 3.3)
EC ₂₀ (95 % CI)	3.0 (2.7 – 3.1)	3.0 (2.9 – 3.1)	2.8 (ND – 3.1)	NA	NA	NA
EC ₁₀ (95 % CI)	2.8 (ND – 2.9)	2.8 (2.7 – 2.9)	ND	NA	NA	NA
NOEC	2.7	2.7	2.7	2.7	0.28	0.28
LOEC	4.9	4.9	4.9	4.9	0.82	0.82

AUC = area under the growth curve

ND = not/could not be determined

NA = not applicable

VALIDITY CRITERIA

As shown below, this study did not meet the validity criteria in OECD 201.

Table 9.2.6.2-6: Compliance with OECD 201 validity criteria

Validity criteria OECD 201 (2011)	Required	Obtained
Biomass in the control(s)	Increased by a factor of ≥ 16 within 72 hours	Cell growth increased by a factor of 49.4 after 72 hours in pooled control.

Validity criteria OECD 201 (2011)	Required	Obtained
Mean coefficient of variation for section by section specific growth rates in the control(s)	Must not exceed 35 %	Pooled control was 40 %
Coefficient of variation of average specific growth rates in the control(s)	For less frequently used species (not <i>Pseudokirchneriella subcapitata</i> or <i>Desmodesmus subspicatus</i> the value should not exceed 10 %.	5.2 % after 72 hours in pooled control.

CONCLUSIONS

Based on mean measured concentrations, the 72-hour E_rC_{50} was 3.6 mg a.s./L, the E_yC_{50} was 3.5 mg a.s./L and the E_bC_{50} was 3.6 mg a.s./L. The 96-hour E_rC_{50} was 3.4 mg a.s./L, the E_yC_{50} was 3.1 mg a.s./L and the E_bC_{50} was 3.4 mg a.s./L.

The NOEC and LOEC at 72 hours, based on all parameters, were 2.7 and 4.9 mg a.s./L, respectively. The NOEC and LOEC at 96 hours, based on growth rate and yield, were 0.28 and 0.82 mg a.s./L, respectively, and based on biomass integral were 2.7 and 4.9 mg a.s./L, respectively.
(██████, 2013)

HSE evaluator comments

The study was carried out to GLP and follows guidance document OECD 201 (2006) and OCSPP 850.4550 (2012). The study was reviewed according to OECD 201 (2011). There were some deviations to the guidelines. The applicant stated that this additional algal study was conducted to meet data requirements for another regulatory system.

The AAP algal medium prepared had different amounts of K_2HPO_4 , NH_4Cl , NH_4Cl , and KH_2PO_4 than advised in OECD 201 (2006). However, the deviations were minor and are not considered to have had an effect on the endpoints. It was noted that the pH increased by 2.2 units during the study. This is not within OECD 201 (2006) guidance, which states pH should not increase by more than 1.5 units. Again, this is a marginal difference and it not considered to have affected the study endpoints. The inoculation contained 50,000 algal cells per mL of test medium. This is five times higher than the amount suggested in OECD 201 (2012), which recommends 10,000. During the preparation of the test solution white, visible, undissolved test substance was observed which was filtered out. Whilst this is not ideal, as the test concentrations were analysed, and the mean concentrations used for endpoint generation, HSE deems that this did not affect the outcome of the study.

Results of a reference substance test have not been reported therefore there is uncertainty with the sensitivity of the test system. However, as the use of a reference item is not an essential part of the test under OECD 201 the lack of results does not invalidate the endpoints derived.

It is unclear if the data was transformed before statistical analysis. Equal Variance Two-Sample t-Test was used to compare the control and the solvent control. No significant difference was observed, and the control tests were pooled. The results from the test concentrations were compared to the pooled control. The 72-hour data was checked for normality using Shapiro-Wilk's Test and tested for homogeneity of variance using Levene's Equality of Variance Test. Based on these results, Dunnett's Multiple Comparison Test was used to compare the test concentration to the pooled control to determine the 72-hour treatment-related effects. The 96-hour data was checked for normality using Shapiro-Wilk's Test and tested for homogeneity of variance using Bartlett's Test. The data was subsequently compared to the pooled control using Dunnett's T3 Multiple Comparison Test to determine the 96-hour treatment-related effects. These are in line with OECD 201 (2011). Statistical tests were performed on CETIS™ Version 1.8.

The analytical methods have been checked by HSE Chemistry specialists in Vol 3CA Part B5.1.2.6. The following was concluded: "Fit for regulatory purposes but the method is not fully validated in accordance with SANCO/3029/99 rev. 4. LOQ: 0.3 µg/L in aqueous matrices. It should be noted unacceptable recoveries were

determined at a test concentration of 10,000 µg/L. The applicant has justified this as the solubility limit of the test substance in AAP medium has been reached.”

The validity criteria from OECD 201 (2011) have not been met in this study. After 72-hours the mean coefficient of variation for section by section growth rates in the control was 40 %. This is above 35 % stipulated for in OECD 201 (2011). *Anabaena flos-aquae* has more variable growth due to its structure, it is more filamentous than green algae which may have caused the difference in section by section growth rate. Whilst this adds uncertainty, the other validity criteria are met for this study (including overall CV). Therefore, given endpoints are based on 0 – 72 or 0 – 96 hours. HSE considers the study valid.

The measured concentrations were not within ± 20 %. Not all test concentrations were maintained within ± 20 % of initial measured concentrations either. Therefore, ideally geometric mean measured concentrations would have been used. HSE calculated geometric mean concentrations and they were broadly comparable to mean measured concentrations. Hence the use of mean measured concentrations in calculation of endpoints is considered acceptable by HSE. It should be noted that analytical data was only measured at 96 hours rather than 72 hours. Hence the 72 hour endpoints below are based on analytical data over a 96 hour period.

The measured concentrations were not within ± 20 % so mean measured concentrations were used. The agreed endpoints are:

- 72-hour E_rC_{50} for SYN545974 = 3.6^a mg a.s./L (mean measured concentrations)
- 72-hour E_rC_{20} for SYN545974 = 3.0^a mg a.s./L (mean measured concentrations)
- 72-hour E_rC_{10} for SYN545974 = 2.8^a mg a.s./L (mean measured concentrations)

- 72-hour E_yC_{50} for SYN545974 = 3.5^a mg a.s./L (mean measured concentrations)
- 72-hour E_bC_{50} for SYN545974 = 3.6^a mg a.s./L (mean measured concentrations)
- 72-hour NOEC for SYN545974 = 2.7^a mg a.s./L (mean measured concentrations)

- 96-hour E_rC_{50} for SYN545974 = 3.4^a mg a.s./L (mean measured concentrations)
- 96-hour E_yC_{50} for SYN545974 = 3.1^a mg a.s./L (mean measured concentrations)
- 96-hour E_bC_{50} for SYN545974 = 3.4^a mg a.s./L (mean measured concentrations)

- 96-hour NOEC (growth rate) for SYN545974 = 0.28^a mg a.s./L (mean measured concentrations)

^a = These endpoints are not considered suitable for use in risk assessment as not all validity criteria were met, specifically the section by section growth rate CV exceeded the required amount by 5 %.

Report: K-CA 8.2.6.2 [REDACTED] (2015), SYN545974 - 96-Hour Toxicity Test with the Freshwater Diatom (*Navicula pelliculosa*), Report Number 1781.6879, Smithers Viscient, 790 Main Street, Wareham, Massachusetts 02571-1037, USA, (Syngenta File No. SYN545974_10097)

GUIDELINES

- OECD Guidelines for Testing of Chemicals, Method 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test (2006)
- US EPA Ecological Effects Test Guideline, OCSPP 850.4550: Cyanobacteria (*Anabaena flos-aquae*) Toxicity (2012)

GLP: Yes

MATERIALS

Test Material	SYN545974 tech.
Lot/Batch #:	SMU2EP12007
Purity:	98.5 % (w/w)
Description:	Off white powder

Stability of test compound:	Stable under standard conditions
Reanalysis/expiry date:	30 June 2016
Treatments	
Test concentrations:	Culture medium control, solvent control and nominal concentrations of 0.034, 0.10, 0.33, 1.0, 3.2 and 10 mg a.s./L (0.031, 0.095, 0.31, 0.89, 2.7 and 5.6 mg a.s./L, mean measured)
Solvent:	Dimethylformamide (DMF), 0.1 mL/L
Positive control:	None
Analysis of test concentrations:	Yes, analysis of SYN545974 at 0 and 96 hours, using LC/MS/MS
Test organism	
Species:	Freshwater diatom (<i>Navicula pelliculosa</i>), strain 661
Source:	Laboratory cultures, originally obtained from UTEX, The Culture Collection of Algae at the University of Texas at Austin
Test design	
Test vessels:	250 mL Erlenmeyer flasks containing 100 mL of media fitted with stainless steel caps which permitted gas exchange
Test medium:	AAP medium
Replication:	Four vessels for each treatment and culture medium control group, eight replicates for the solvent control
Starting cell density:	1.0×10^4 cells/mL
Exposure regime:	Static
Aeration:	No
Duration:	96 hours
Environmental conditions	
Test temperature:	24.0 - 26.0 °C
pH:	Test start: 7.3 - 7.6 Test end: 7.3 - 8.7
Lighting:	Constant illumination (range: 4700-5900 Lux)

STUDY DESIGN AND METHODS

Experimental dates: 9 to 22 September 2013

A primary stock solution with a nominal concentration of 100 mg a.s./mL was prepared by placing 1.0003 g of SYN545974 in a volumetric flask, bringing it to volume with dimethyl formamide (DMF) and sonicating for two minutes. Secondary stock solutions at nominal concentrations of 0.34, 1.0, 3.3, 10 and 32 mg a.s./mL were prepared in dimethyl formamide by dilution of the primary stock solution. Appropriate volumes of the secondary stock solutions were diluted with culture medium to give the test concentration series. Solutions were then mixed with a magnetic stir plate and Teflon-coated stir bar for two hours. The 3.2 and 10 mg a.s./L solutions contained visible undissolved test substance so were sonicated for 20 minutes after which they still contained visible undissolved test substance. They were therefore passed through polyester filter floss and the filtrate was used for testing. The concentration of the solvent in the solvent control was 0.1 mL/L and the blank control consisted of culture medium only.

An aliquot of test solution was placed into each test vessel and the test was started by inoculation of 10,000 algal cells per mL of test medium. Test solutions were shaken by hand at least once daily during the exposure period and were held in a temperature controlled chamber with continuous illumination.

Small volumes of all test concentrations and controls were taken from all test flasks after 24, 48, 72 and 96 hours of exposure. The algal cell densities in these samples were determined using a haemocytometer and a microscope. Observations of the health of the algal cells were made at each 24-hour interval.

The pH was measured at the start and at the end of the test. The water temperature was measured continuously in a flask incubated under the same conditions as the test flasks.

The test concentrations were verified by chemical analysis of SYN545974 at 0 and 96 hours, using liquid chromatography/mass spectrometry (LC/MS/MS).

RESULTS AND DISCUSSION

The mean measured concentrations ranged from 33.0 to 100 % of nominal concentrations. The limit of quantification (LOQ) for each analysis was dependent upon the linear regression, the area of the low standards and the dilution factor of the controls. The LOQ values for the 0 and 96 hour sampling intervals were 0.0024 and 0.0025 mg/L, respectively. Mean measured concentrations were used for the calculation and reporting of results by study author.

Table 9.2.6.2-7: Analytical results

Nominal concentrations (mg a.s./L)	Measured concentration (mg a.s./L)					
	0 hours	% of nominal (0 hours)	96 hours	% of nominal (96 hours)	Mean Measured (mg a.s./L) ^a	Geometric mean measured ^e (mg a.s./L)
Control	<LOQ ^b	-	<LOQ	-	NA	NA
Solvent control	<LOQ	-	<LOQ	-	NA	NA
0.034	0.034	100.0	0.028	82.4	0.031	0.031
0.10	0.10	100.0	0.086	86.0	0.095	0.093
0.33	0.35	106.1	0.28 (0.29 ^c)	84.8	0.31	0.313
1.0	1.0	100.0	0.77	77.0	0.89	0.877
3.2	2.8	87.5	2.7	84.4	2.7	2.750
10	8.0	80.0	3.3	33.0 ^d	5.6	5.138

^a Mean measured concentrations and percent nominal were calculated using actual analytical data and not the rounded (two significant figures) data presented in this table.

^b Concentrations measured were below the limit of quantitation (LOQ). The LOQ values for the 0 and 96 hour sampling intervals were 0.0024 and 0.0025 mg a.s./L, respectively. The LOQ for each analysis is dependent upon the regression, the area of the low standards and the dilution factor of the controls.

^c Result of the additional sample without algae present to determine the effect of the presence of algae.

^d Due to filtration of the test solution at 0 hour, the measured concentrations of the 10 mg a.s./L treatment level were expected to be below the nominal test concentration.

^e Geometric mean measured concentration determined by HSE as nominal concentrations were not maintained at ± 20 % of nominals at all time points/concentrations in accordance with OED 201 (2011). However, the difference between geometric mean and mean measured concentrations is relatively low. Therefore, HSE accepts the use of mean measured concentrations by the study author.

The algal cell densities were measured at 24, 48, 72 and 96 hours and the mean biomass, growth rate and cell density calculated. The 72-hour EC₁₀, EC₂₀ and EC₅₀ values (defined as the concentration resulting in 10, 20 and 50 % reduction, respectively, of biomass, growth rate and yield) were determined by linear interpolation of response using the ICp method (Norberg-King, 1993). For determination of the LOEC (Lowest Observed Effect Concentration) and NOEC (No Observed Effect Concentration) values, a Dunnett's test was used to identify significant differences in the calculated mean biomass, growth rate and yield of test item treatments compared to the pooled control data.

Growth rates

The growth rate 0 to 72 hours and 0 to 96 hours were calculated for each replicate culture and the means are shown below.

Table 9.2.6.2-8: Mean growth rate values at each concentration of SYN545974 at 72 and 96 hours for *Navicula pelliculosa*

Mean measured concentrations (mg a.s./L)	Mean growth rate 0 – 72 hrs (1/day)	Percentage inhibition ^{ab}	Mean growth rate 0 – 96 hrs (1/day)	Percentage inhibition ^{ab}
Control	1.20	-	1.24	-
Solvent control	1.22	-	1.24	-
Pooled control	1.21	-	1.24	-
0.031	1.28	-6	1.26	-2
0.095	1.32	-9	1.22	2
0.31	1.34	-10	1.20	4
0.89	1.24	-2	1.13 ^c	9
2.7 ^d	0.00	100	0.00	100
5.6 ^d	0.00	100	0.00	100

^a Percent inhibition relative to the pooled control. Negative values indicate stimulation compared to control.

^b Calculated from original raw data.

^c Significantly different compared to the pooled control, based on Dunnett's Multiple Comparison Test.

^d Due to the zero cell density observed in the 2.7 and 5.6 mg a.s./L treatment levels, these treatment levels were excluded from growth rate statistical analysis.

Yield

The yield (based on biomass) at 72 hours and 96 hours were calculated for each replicate culture and the means are shown below.

Table 9.2.6.2-9: Mean yield (based on biomass) values at each concentration of SYN545974 at 72 and 96 hours for *Navicula pelliculosa*

Mean measured concentrations (mg a.s./L)	Mean cell density (x 10 ⁴ cells/mL) 72 hrs	Percentage inhibition ^{ab}	Mean cell density (x 10 ⁴ cells/mL) 96 hrs	Percentage inhibition ^{ab}
Control	35.13	-	133.27	-
Solvent control	36.63	-	134.71	-
Pooled control	36.13	-	134.23	-
0.031	44.25	-22	145.21	-8
0.095	49.69	-38	122.35	9
0.31	51.38	-42	112.17	16
0.89	38.63	-7	85.94	36
2.7	-1.00 ^c	103	-1.00 ^d	101
5.6	-1.00 ^c	103	-1.00 ^d	101

^a Percent inhibition relative to the pooled control. Negative values indicate stimulation compared to control.

^b Calculated from original raw data.

^c Significantly different compared to the pooled control, based on Dunnett's Multiple Comparison Test.

^d Significantly different compared to the pooled control, based on Dunnett's T3 Multiple Comparison Test.

Biomass (area under the growth curve)

The areas under the growth curve for 0 to 72 hours and 0 to 96 hours were calculated for each replicate culture and the means are shown below.

Table 9.2.6.2-10: Mean biomass integral (area under the growth curve) values at each concentration of SYN545974 at 72 and 96 hours for *Navicula pelliculosa*

Mean measured concentrations (mg a.s./L)	Biomass (x 10 ⁴ cells·days/mL) 0 - 72 hrs	Percentage inhibition ^{ab}	Biomass (x 10 ⁴ cells·days/mL) 0- 96 hrs	Percentage inhibition ^{ab}
Control	33.40	-	116.14	-
Solvent control	33.96	-	118.14	-
Pooled control	33.77	-	117.47	-
0.031	37.95	-12	131.03	-12
0.095	42.38	-25	126.91	-8
0.31	45.06	-33	125.41	-7
0.89	34.33	-2	95.53	19
2.7	-2.08 ^c	106	-3.06 ^d	103
5.6	-2.38 ^c	107	-3.36 ^d	103

^a Percent inhibition relative to the pooled control. Negative values indicate stimulation compared to control.

^b Calculated from original raw data.

^c Significantly reduced compared to the pooled control, based on Dunnett's Multiple Comparison Test.

^d Significantly reduced compared to the pooled control, based on Dunnett's T3 Multiple Comparison Test.

Table 9.2.6.2-11: Summary of biological results for toxicity of SYN545974 to *Navicula pelliculosa*, at 72 and 96 hours

Parameter	after 72 h (mg a.s./L)			after 96 h (mg a.s./L)		
	AUC	Growth rate	Yield	AUC	Growth rate	Yield
EC ₅₀ (95% CI)	1.5 (1.2 – 1.6)	1.6 (1.5 – 1.7)	1.5 (1.2 – 1.7)	1.4 (1.1 – 1.6)	1.5 (1.5 – 1.6)	1.1 (0.89 – 1.4)
EC ₂₀ (95% CI)	0.98 (0.56 – 1.2)	1.1 (1.0 – 1.2)	0.97 (0.51 – 1.3)	ND	ND	ND
EC ₁₀ (95% CI)	0.71 (0.37 – 1.2)	0.97 (0.86 – 1.1)	0.68 (0.37 – 1.2)	ND	ND	ND
NOEC	0.89	0.89	0.89	0.89	0.31	0.31
LOEC	2.7	2.7	2.7	2.7	0.89	0.89

ND = not determined

VALIDITY CRITERIA

The test was considered valid:

- The cell growth in the pooled control increased by a factor of 37.13 after 72 hours (must be at least a factor of 16)

- The mean coefficient of variation of the daily growth rates in the pooled control replicates was 28 and 26% over 72 and 96 hours, respectively (must be ≤ 35 %)
- The average specific growth rates of the pooled control replicates was 6.8 and 1.9 % after 72 and 96 hours, respectively (must be ≤ 10 %).
- The 96-hour pooled control coefficient of variation for mean yield and growth rate was 9.5 and 1.6 %, respectively (should be < 15 %).

CONCLUSIONS

Based on mean measured concentrations, the 72-hour E_rC_{50} for SYN545974 to *Navicula pelliculosa* was 1.6 mg a.s./L, the E_yC_{50} was 1.5 mg a.s./L and the E_bC_{50} was 1.5 mg a.s./L. The 96-hour E_rC_{50} was 1.5 mg a.s./L, the E_yC_{50} was 1.1 mg a.s./L and the E_bC_{50} was 1.4 mg a.s./L. The 72-hour NOECs for growth rate, yield and biomass were 0.89 mg a.s./L. The 96-hour NOECs for growth rate and yield were 0.31 mg a.s./L, and for biomass was 0.89 mg a.s./L.

(██████, 2015)

HSE evaluator comments

This study was conducted to GLP and followed OECD guideline 206 (2006) and OCSP 850.4550. However, the most recent guideline is OECD 206 (2011) so the study was assessed against this more recent version.

The authors note that the study deviates from their protocol at the end of the test in the method used to determine whether the test substance had algistatic or algicidal effects using a sample from one of the test cultures. Since this data is not required in the OECD 206 (2011) guideline and has no effect on the relevant endpoints listed below, then this part of the study was not considered.

The test substance SYN545974 was prepared using a solvent and therefore OECD Guidance Document 23 (2019) for testing of difficult substances has been considered. The solvent used (Dimethylformamide, DMF) is listed in the Guidance Document as effective for aquatic toxicity testing and is within the recommended concentration range of 0.10 mL DMF/L. Additionally, there was no significant difference between control and solvent control indicating the solvent had no effect on the outcome of the study.

The authors note that the nominal test solutions with test substance concentrations of 3.2 and 10 mg a.s./L contained visible undissolved test substance. The highest test concentration was filtered prior to testing. The analytical measurements show that as expected, this resulted in a lower measured concentration than nominal at 10 mg a.s./L.

Ideally given the measured concentrations were not within ± 20 % of nominal concentrations, for all test concentrations and sampling points, geometric mean measured concentrations should have been determined in-line with OECD 206 (2011) guideline. However, the study author derived mean measured concentrations. Nonetheless, the geometric mean concentrations calculated by HSE (table 9.2.6.2-7) are considered sufficiently comparable to the mean measured concentrations. Therefore, HSE accepts the use of mean measured concentrations to determine endpoints.

The analytical methods have been checked by HSE Chemistry specialists in Vol 3CA Part B5.1.2.6. The following was concluded: "Fit for regulatory purposes but the method is not fully validated in accordance with SANCO/3029/99 rev. 4. LOQ: 0.3 μ g/L in aqueous matrices. It should be noted unacceptable recoveries were determined at a test concentration of 10,000 μ g/L. The applicant has justified this as the solubility limit of the test substance in AAP medium has been reached."

It is noted that there is no reference test performed however the demonstration of growth inhibition in the study shows that the test system is effective.

The temperature of the cultures was maintained at 24-26 °C rather than 21-24 °C as recommended in the guideline, however adequate performance of the control cultures shows that this does not affect the outcome of the study.

The following statistical analysis was performed and is in line with the guidelines:

- As recommended in the guidelines, the authors tested the data for normality and homogeneity. The tests they used for this were Shapiro-Wilks' Test for normality and Bartlett's Test (biomass AUGC data and growth rates data) or Levene's test (biomass yield data) for equality of variance.
- For data which passed the normality and variance tests, NOEC and LOEC were determined using Dunnett's multiple comparison test (72 and 96-hour growth rate data, 72-hour biomass as yield data, 72-hour biomass as AUGC data). For data which failed the homogeneity of variance test, NOEC and LOEC were determined using Dunnett's T3 Multiple Comparison Test (96-hour biomass as yield data and 96-hour biomass as AUGC data). The authors excluded the treatment levels of 2.7 and 5.6 mg/L from statistical analysis of NOEC and LOEC due to zero cell density.
- EC_x values were calculated by "linear interpolation of response using the ICp method" and did include zero cell density treatment levels in the calculation.

It should be noted the mean measured concentrations are based on 96 hour duration i.e. samples taken at 0 and 96 hours. EC_{10/20} values at 96 hours were not determined by study author. The agreed end-points for use in risk assessments are:

- 72 hour ErC₅₀ : 1.6 mg a.s./L (mean measured concentration)
- 72 hour ErC₂₀ : 1.1 mg a.s./L (mean measured concentration)
- 72 hour ErC₁₀ : 0.97 mg a.s./L (mean measured concentration)
- 72 hour EyC₅₀ : 1.5 mg a.s./L (mean measured concentration)
- 72 hour EyC₂₀ : 0.97 mg a.s./L (mean measured concentration)
- 72 hour EyC₁₀ : 0.68 mg a.s./L (mean measured concentration)
- 72 hour NOEC for growth rate and yield : 0.89 mg a.s./L (mean measured concentration)
- 96 hour ErC₅₀ : 1.5 mg a.s./L (mean measured concentration)
- 96 hour EyC₅₀ : 1.1 mg a.s./L (mean measured concentration)
- 96 hour NOEC for growth rate and yield : 0.31 mg a.s./L (mean measured concentration)

Report:	K-CA 8.2.6.2 [REDACTED], (2014), SYN545974 – 96-Hour Toxicity Test with the Marine Diatom, <i>Skeletonema costatum</i> , Report Number 1781.6880 Smithers Viscient, 790 Main Street, Wareham, MA 02571-1037 USA. (Syngenta File No. SYN545974_10105)
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GUIDELINES

OECD Guidelines for Testing of Chemicals, Method 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test (2006)

US EPA Ecological Effects Test Guidelines, OCSPP 850.5400: Algal Toxicity, Tiers I and II, (2012)

GLP: Yes

MATERIALS

Test Material	SYN545974 tech.
Lot/Batch #:	SMU2EP12007
Purity:	98.5% (tested as 100%)
Treatments	
Test concentrations:	Culture medium control, solvent control (DMF 0.1 mL/L) and nominal concentrations of 0.10, 0.33, 1.0, 3.2 and 10 mg a.s./L
Solvent:	Dimethyl formamide (DMF, CAS No. 68-12-2)
Analysis of test concentrations:	Yes, analysis of SYN545974 at 0 and 96 hours
Test organism	

Species:	<i>Skeletonema costatum</i> Strain CCMP 1332		
Source:	Continuous laboratory cultures, originally obtained from Bigelow Laboratories, West Boothbay Harbor, Maine USA		
Test design			
Test vessels:	250 mL glass Erlenmeyer flasks containing 100 mL of media covered with stainless steel dishes		
Test medium:	Artificially enriched seawater (AES) on the basis of filtered, natural seawater; salinity adjusted to 30 ±2 g/L, pH 8.0 ±0.1		
Replication:	Four vessels for the control and for each test concentration, eight replicate flasks for the solvent control		
Starting cell density:	1.0 × 10 ⁴ cells/mL		
Exposure regime:	Static		
Aeration:	No		
Duration:	96 hours		
Environmental conditions			
Test temperature:	20-22 °C,		
pH:	test start:	7.8 to 8.1	
	test end:	7.7 to 8.5	
Conductivity:	48 to 50 mS/cm		
Lighting:	14: 10 hours light/darkness cycles; light intensity range of 3700 to 4900 Lux		

STUDY DESIGN AND METHODS

Experimental dates: 2 to 14 December 2013

A primary stock solution with a nominal concentration of 100 mg a.s./mL was prepared by placing 1.0004 g of SYN545974 in a 10-mL volumetric flask, bringing it to volume with dimethyl formamide (DMF) followed by multiple shakes and inversions. Secondary stock solutions at nominal concentrations of 1.0, 3.3, 10 and 32 mg a.s./mL were prepared in dimethyl formamide by dilution of the primary stock solution. Appropriate volumes of the secondary stock solutions were diluted with culture medium to give the test concentration series. Following mixing with a Teflon-coated stir bar and stir plate for approximately 3 hours, all solutions were observed to be clear and yellow in colour with no visible undissolved test substance with the exception of the 3.2 and 10 mg a.s./L test solutions. The 3.2 and 10 mg/L test solutions were sonicated for approximately 30 minutes. After sonication, the 3.2 mg a.s./L solution was observed to be clear and colourless with no visible undissolved test substance while the 10 mg a.s./L solution still contained visible undissolved test substance. The 10 mg a.s./L solution was then filtered with polyester filter floss to remove undissolved material that remained after sonication and the filtrate was used for testing. After complete preparation, all test solutions were observed to be clear and yellow in colour with no visible undissolved test substance.

A 100-mL aliquot of test solution was placed into each test vessel and the test was started by inoculation of 10,000 algal cells per mL of test medium. Test solutions were shaken daily by hand and were held in a temperature-controlled incubator under a photoperiod of 14 hours of light and 10 hours of darkness.

Small volumes of all test concentrations and controls were taken from all test flasks after 24, 48, 72 and 96 hours of exposure. The algal cell densities in these samples were determined by counting using a hemacytometer and a compound microscope. In addition, observations of the health of the algal cells were made at each sampling interval. After 96 hours exposure, a sample was taken from the composite of the four replicates of the maximum test concentration level and diluted with fresh AES medium to prepare a subculture with a nominal concentration of 0.10 mg a.s./L. The performance of the sub-culture was used to determine if the effects of the test substance on the algae were algistatic or algicidal. Due to the nature of *Skeletonema* to aggregate, each test solution was vigorously pipetted multiple times prior to each observation.

The pH was measured at the start and at the end of the test and conductivity was measured at test start. The water temperature was measured continuously in a flask incubated under the same conditions as the test flasks.

The test concentrations were verified by chemical analysis of for SYN545974 at 0 and 96 hours, using liquid chromatography/mass spectrometry.

RESULTS AND DISCUSSION

At the start of the test, the measured concentrations were in the range 87 to 99% of the nominal values and at the end of the test were in the range 10 to 88% (see table below). Mean measured concentrations were used for the calculation and reporting of results.

Table 9.2.6.2-12: Analytical results

Nominal concentrations (mg a.s./L)	Measured concentration (mg a.s./L)			Percent of nominal ^a (%)
	0 hours	96 hours	Mean	
Control	<LOQ ^b	<LOQ	-	-
Solvent control	<LOQ	<LOQ	-	-
0.10	0.099	0.060	0.077	77
0.33	0.32	0.21	0.26	79
1.0	0.93	0.67 (0.88 ^c)	0.79	79
3.2	3.0	2.0	2.4	76
10	8.7	1.0	3.0	30

^a Mean measured concentrations and percent nominal were calculated using actual analytical data and not the rounded (two significant figures) data presented in this table. ^b Concentrations measured were below the limit of quantitation (LOQ). The LOQ values for the 0 and 96-hour sampling intervals were 0.0085 and 0.0084 mg a.s./L, respectively. The LOQ for each analysis is dependent upon the regression, the area of the low standards and the dilution factor of the controls. ^c Result of the additional sample without algae indicates a slight impact of the presence of algae on a.s. concentration levels.

The algal cell densities were measured at 24, 48, 72 and 96 hours and the mean biomass, growth rate and yield calculated. The 72-hour and 96-hour E_bC_{50} , E_yC_{50} and E_rC_{50} values (defined as the concentration resulting in 50% reduction of each parameter) were calculated using Dunnett's test. For determination of the LOEC (Lowest Observed Effect Concentration) and NOEC (No Observed Effect Concentration) values, a Dunnett's test was used to identify significant differences in the calculated mean biomass, growth rate and yield of test item treatments compared to the control.

Growth rates

The growth rate 0 to 72 hours and 0 to 96 hours were calculated for each replicate culture and the means are shown below, alongside the estimated EC_{50} values.

Table 9.2.6.2-13: Mean growth rate values at each concentration of SYN545974 at 72 and 96 hours for *S. costatum* and relevant endpoints

Mean measured concentrations (mg a.s./L)	Mean Growth rate 0 – 72 hrs ^a (1/day)	Percentage inhibition ^{a,b}	Mean Growth rate 0 – 96 hrs ^a (1/day)	Percentage inhibition ^{a,b}
control	1.03	-	1.05	-
Solvent control	1.02	-	1.10	-
Pooled control	1.03	-	1.08 ^c	-

Mean measured concentrations (mg a.s./L)	Mean Growth rate 0 – 72 hrs ^a (1/day)	Percentage inhibition _{a,b}	Mean Growth rate 0 – 96 hrs ^a (1/day)	Percentage inhibition _{a,b}
0.077	1.05	-2	1.11	-2
0.26	1.05	-2	1.10	-1
0.79	1.10	-7	1.12	-3
2.4	1.06	-4	1.09	0
3.0	0.0	100 ^d	0.00	100 ^d

^a Calculated using the exact raw data. The tabulated results represent rounded values. ^b Percent inhibition relative to the pooled control; negative values indicate an increase relative to the pooled control mean ^c Control and solvent control are significantly different but arbitrarily pooled because all other statistical comparisons are based on pooled control data ^d Excluded from statistical analysis, due to zero growth rate and empirically estimated to be different from the pooled control.

Yield

The yield 0 to 72 hours and 0 to 96 hours were calculated for each replicate culture and the means are shown below, alongside the estimated EC₅₀ values.

Table 9.2.6.2-14: Mean yield values at each concentration of SYN545974 at 72 and 96 hours for *S. costatum* and relevant endpoints

Nominal concentrations (mg a.s./L)	Mean Yield 0 – 72 hrs ^a (x 10 ⁴ cells/mL)	Percentage inhibition _{a,b}	Mean Yield 0 – 96 hrs ^a (x 10 ⁴ cells/mL)	Percentage inhibition _{a,b}
Control	19.38	-	60.06	-
Solvent control	19.44	-	74.66	-
Pooled control	19.42	-	69.79	-
0.077	20.81	-7	74.88	-7
0.26	20.31	-5	73.94	-6
0.79	23.75	-22	78.63	-13
2.4	21.75	-12	70.00	0
3.0	-1.00 ^c	105	-1.00 ^d	101

^a Calculated using the exact raw data. The tabulated results represent rounded values. ^b Percent inhibition relative to the pooled control; negative values indicate an increase relative to the pooled control mean ^c Significantly different compared to pooled control (Dunnett's Multiple Comparison test, $p \leq 0.05$) ^d Significantly different compared to pooled control (Dunnett's T3 Multiple Comparison test, $p \leq 0.05$)

Biomass (area under the growth curve)

The areas under the growth curve for 0 to 72 hours and 0 to 96 hours were calculated for each replicate culture and the means are shown below, alongside the estimated EC₅₀ values.

Table 9.2.6.2-15: Mean biomass integral (area under the growth curve) values at each concentration of SYN545974 at 72 and 96 hours for *S. costatum* and relevant endpoints

Nominal concentrations (mg a.s./L)	Mean Biomass integral 0 – 72 hrs ^a (x 10 ⁴ * day)	Percentage inhibition a,b	Mean Biomass integral 0 – 96 hrs ^a (x 10 ⁴ * day)	Percentage inhibition _{a,b}
Control	14.48	-	53.93	-
Solvent control	15.51	-	62.23	-
Pooled control	15.17	-	59.46	-
0.077	15.91	-5	63.43	-7
0.26	15.78	-4	62.58	-5
0.79	18.92	-25	69.76	-17
2.4	17.46	-15	63.01	-6
3.0	-1.75 ^c	112	-2.74 ^c	105

^a Calculated using the exact raw data. The tabulated results represent rounded values. ^b Percent inhibition relative to the pooled control; negative values indicate an increase relative to the pooled control mean ^c Significantly different compared to pooled control (Dunnett's Multiple Comparison test, $p \leq 0.05$)

Table 9.2.6.2-16: Summary of biological results for toxicity of SYN545974 to *S. costatum*, at 72 and 96 hours

Parameter	after 72 h (mg a.s./L)			after 96 h (mg a.s./L)		
	AUC	Growth rate	Yield	AUC	Growth rate	Yield
EC ₅₀ (95% CI)	2.7 (2.6-2.7)	2.7 (2.7-2.7)	2.7 (2.6 – 2.7)	2.7 (2.6-2.7)	2.7 (2.7-2.7)	2.7 (2.6-2.7)
EC ₂₀ (95% CI)	2.5 (2.5 - 2.5)	2.5 (2.5 - 2.5)	2.5 (2.4 - 2.5)	NA	NA	NA
EC ₁₀ (95% CI)	2.5 (2.4 - 2.5)	2.5 (2.4 - 2.5)	2.5 (1.2 – 2.5)	NA	NA	NA
NOEC	2.4	2.4	2.4	2.4	2.4	2.4
LOEC	3.0	3.0	3.0	3.0	3.0	3.0

VALIDITY CRITERIA

In OCSPP 850. 5400 (2012) if one or more of the following criteria are met the study is considered *invalid*:

1. All test vessels and closures were not identical.
2. Treatments were not randomly assigned to test vessels, and test vessels were not randomly assigned to positions in the growth chamber.
3. A medium (untreated) control [and solvent (vehicle) control, when a solvent was used] was not included in the test.
4. The concentration of solvent in the range used affected growth of the test species.
5. During the 96- hour test period, cell counts in the controls did not increase by a factor of at least 100 times for *P. subcapitata* and a factor of at least 30 times for *S. costatum* (i.e., logarithmic growth in the controls was not reached during the test).
6. A minimum of five test concentrations were not used in the definitive test.
7. Controls were contaminated with the test substance.
8. The lowest test concentration level was not less than the 96-hour yield, average specific growth rate, and area under the growth curve IC50 values based on cell density.

9. For testing with industrial chemicals, a surfactant or dispersant was used in the preparation of a stock or test solution.
10. Temperature and light intensity were not measured as specified during the test.

These criteria were not met, and **the study is considered valid.**

CONCLUSIONS

Based on mean measured concentrations, the 72-hour E_rC_{50} was 2.7 mg a.s./L, the E_yC_{50} was 2.7 mg a.s./L and the E_bC_{50} was 2.7 mg a.s./L. The 96-hour E_rC_{50} was 2.7 mg a.s./L, the E_yC_{50} was 2.7 mg a.s./L and the E_bC_{50} was 2.7 mg a.s./L.

The Lowest Observed Effect Concentration at 72 and 96 hours, based on growth rate, yield and biomass integral, was 3.0 mg a.s./L, and the No Observed Effect Concentration was 2.4 mg a.s./L.

(██████, 2014)

HSE evaluator comments

This study has been performed in accordance with GLP and follows OCSPP 850.5400 (2012) guidelines. The validity criteria outlined in OCSPP 850.5400 (2012) have been met. Test concentrations were not maintained within ± 20 % of nominal values, so results are based on geometric mean measured concentrations.

OCSPP 850.5400 (2012) guidelines state that the mean coefficient of variation for mean yield in the pooled control cultures should be ≤ 15 % yet was observed to be 18% over 96 hours. This is thought to be a relatively small deviation, and since the validity criteria were met, the study outcome is not thought to have been affected. It is noted that the pH was not maintained at 8 ± 0.1 as stipulated and instead ranged from 7.8 to 8.1 at start, and from 7.7 to 8.5 at test end. This is also thought to be a minor deviation and the study outcome is not thought to have been affected.

Statistical analysis was reported briefly, and $EC_{50/20/10}$ values and 95 % confidence intervals were determined statistically using liner interpolation. NOEC and LOEC values were determined statistically using Dunnet's multiple comparison test or Dunnet's T3 multiple comparison test for non-parametric data. These methods are in line with those recommended in OCSPP 850.5400 (2012). For average specific growth rate, the control and solvent control were pooled together, despite a significant difference being detected between them at 96 hours. Although significant differences between control and solvent control may indicate a confounding effect of the solvent on growth, since no significant difference was detected at 72 hours for this response variable, or for any other response variable at 72 or 96 hours, the solvent is unlikely to be a confounding factor and pooling this data is deemed acceptable. At the highest test concentration (3 mg/L) there was 100 % inhibition in growth rate. This data was excluded from statistical analysis and instead empirically considered a significant reduction compared to the control. It was not clear how this empirical estimation was conducted or what values were used in deriving the E_rC_x values.

Following a request for additional information, the applicant provided the following clarification on the statistical methods: "The data was excluded because in each replicate a zero value was observed. Inclusion of this group would violate the assumption of normal distribution and homogeneity of variances (in case of this group the variance was zero) and therefore, a parametric test was not possible. Paragraph 3.9 provides information about the information about estimation of EC_x values. As no notion was made that data was excluded, the assumption is that all data was included in this analysis."

Since it has been confirmed that all data was included for calculation of E_rC_x values, they can be considered reliable and suitable for use in risk assessment.

The analytical methods have been checked by HSE Chemistry specialists in Vol 3CA Part B5.1.2.6. The following was concluded: "Fit for regulatory purposes but the method is not fully validated in accordance with SANCO/3029/99 rev. 4. LOQ: 0.3 $\mu\text{g/L}$ in aqueous matrices. It should be noted unacceptable recoveries were determined at a test concentration of 10,000 $\mu\text{g/L}$. The applicant has justified this as the solubility limit of the test substance in AAP medium has been reached."

The following endpoints are suitable for use in risk assessment and are based on geometric mean measured concentrations:

- 72 h E_rC₅₀ 2.7 mg SYN545974/L
- 72 h E_yC₅₀ = 2.7 mg SYN545974/L
- 72 h E_bC₅₀ = 2.7 mg SYN545974/L
- 72 h E_yC₁₀ = 2.5 mg SYN545974/LNOEC (72/96 h growth rate/yield) = 2.4 mg SYN545974/L

B.9.2.7. Effects on aquatic macrophytes

Report: K-CA 8.2.7 [REDACTED] (2015a), SYN545974 – 7-Day Toxicity Test with Duckweed (*Lemna gibba*). Report Number 1781.6878. Smithers Viscient, 790 MainStreet, Wareham, Massachusetts 02571-1037, USA. (Syngenta File No. SYN545974_10088)

GUIDELINES

OECD Guidelines for Testing of Chemicals, Method 221: *Lemna* sp. Growth Inhibition Test (2006)

US EPA Ecological Effects Test Guidelines, OPPTS 850.4400: Aquatic Plant Toxicity using *Lemna* spp., Tiers I and II, (1996)

GLP: Yes

MATERIALS

Test Material	SYN564974 tech.
Lot/Batch #:	SMU2EP12007
Purity:	98.5 % w/w
Description:	Off white powder
Stability of test compound:	Stable under standard conditions
Reanalysis/expiry date:	30 June 2016

Treatments

Test concentrations:	Dilution water control; solvent control; nominal concentration of 0.10, 0.33, 1.0, 3.2 and 10 mg a.s./L (mean measured concentrations; 0.099, 0.33, 0.97, 3.1 and 6.3 mg a.s./L)
Solvent:	Dimethylformamide (DMF), 0.1 mL/L
Solvent control:	Yes, DMF 0.1 mL/L
Analysis of test concentrations:	Yes, analysis of SYN545974 from freshly prepared and aged test media on days 0 and 3 using LC/MS/MS analysis.

Test organisms

Species:	<i>Lemna gibba</i>
Source:	In-house cultures, originally obtained from the Canadian Phycological Culture Centre (CPCC) at the University of Waterloo (Waterloo, Ontario, Canada). The fronds used to initiate the test were taken from a stock culture that had been transferred to fresh medium nine days prior to testing.

Test design

Test vessels:	270-mL Pyrex glass crystallising dishes containing 100 mL of test medium
Test medium:	20X AAP-Growth Medium according to OECD test guideline

Replication:	Four replicates per treatment level and control, and eight replicates for the solvent control
Initial frond number:	3-4 fronds per plant, total 12 fronds per replicate
Exposure regime:	Semi-static, media were replaced with freshly-prepared media on days 3 & 5
Duration:	7 days
Environmental conditions	
Temperature:	24 - 25 °C
pH:	7.8 – 8.2 new solutions; 8.4 – 9.0 aged solutions
Lighting:	Continuous illumination, range: 4900 - 6400 Lux

STUDY DESIGN AND METHODS

Experimental dates: 1 to 12 March 2013

A 7-day preliminary static-renewal exposure was conducted from 1 to 8 February 2013 at nominal SYN545974 concentrations of 0.0010, 0.010, 0.10, 1.0 and 10 mg/L, a control and a solvent (DMF) control.

For the definitive test the toxicity of SYN545974 to the aquatic plant *Lemna gibba* was determined in a 7-day semi-static test. 20x AAP medium was used in preparation of the pre-exposure culture media, and the test media. The composition was in accordance with the OECD 221 guidelines. A primary stock solution of 100 mg a.s./L was prepared at test initiation by placing 2.5000 g of SYN545974 in a 25 mL volumetric flask and bringing it to volume with DMF. This solution was mixed by multiple shakes and inversions of the flask and sonicating for two minutes. Secondary stock solutions were prepared by diluting appropriate volumes of the primary solution with DMF. Appropriate volumes of primary or secondary stock solutions were mixed with 20X AAP medium to prepare the test media which were mixed for approximately 2 hours using a magnetic stir plate and stir bar. Additionally, the 3.2 and 10 mg a.s./L solutions were sonicated for approximately 30 minutes and then filtered, and the filtrate used for testing.

The 0.10, 0.33 and 1.0 mg/L solutions were observed to be clear and colorless with no visible undissolved test substance following preparation. The 3.2 and 10 mg/L solutions were sonicated for approximately 30 minutes and were observed to be clear and colorless with large aggregates of undissolved test substance floating throughout the water column. The 3.2 and 10 mg/L solutions were additionally filtered through polyester filter floss to remove undissolved material that remained present after sonication and the filtrate was used for testing. After complete preparation, all test solutions were observed to be clear and colorless with no visible undissolved test substance.

At the start of the test, *Lemna* colonies were transferred aseptically from the pre-culture into the different test vessels in a randomized order. The test was started with three randomly selected colonies per vessel (12 fronds/3 colonies). At the test medium renewal dates, the test plants were transferred under aseptic conditions to clean test vessels with freshly prepared test medium of the corresponding concentration. Assessments of frond number were made on days 3, 5 and 7. Fronds were harvested and dried for measurement of dry weight after frond density determinations were complete.

Water temperature was measured continuously in the temperature-controlled water bath and was measured in a vessel filled with water (incubated under the same conditions as the test vessels) daily. The light intensity was recorded once at test start and at each subsequent 24-hour interval, and the pH of the exposure solutions was measured at test initiation (new solutions), in each aged and new solution at the renewal periods, and at test termination (aged solution). Each of the test concentrations were verified by chemical analysis of SYN545974 at the start of the test (new) and on Day 3 (aged), using LC/MS/MS analysis. The limit of quantification in this study was 0.151 µg a.s./L.

Data for frond number and dry weight were used to calculate growth rates and yield for the control and each exposure concentration. Linear interpolation of response was then used to calculate the 7-day E_rC_{50} and E_yC_{50} ,

based on percent inhibition relative to the pooled control. For the No Observed Effect Concentration and Lowest Observed Effect Concentration, Dunnett's Multiple Comparison Test was used to determine values significantly different to the pooled control. Mean measured concentrations were used for the calculation and reporting of results.

RESULTS AND DISCUSSION

The analytically determined concentrations of SYN545974 were between 63 to 100 % of the nominal values averaging the initial and final measured exposure concentrations for each treatment level (see Table 9.2.7-1 below). The limit of quantification in this study was 0.151 µg a.s./L. Mean measured concentrations were used for the calculation and reporting of results.

Table 9.2.7-1: Analytical results

Nominal concentrations (mg a.s./L)	Measured concentration ^a (mg a.s./L)			Percent of nominal ^a
	Day 0 (new)	Day 3 (aged)	Mean measured ^a	
Control	<0.0084 ^b	<0.0082	NA	NA
Solvent control	<0.0084	<0.0082	NA	NA
0.10	0.10	0.095	0.099	100
0.33	0.35	0.32	0.33	100
1.0	1.0	0.93	0.97	97
3.2	3.2	2.9	3.1	96
10	8.5	4.1	6.3	63

^a Measured concentrations and percent of nominal values were calculated using the actual analytical data and not the rounded data presented in this table. ^b Concentrations expressed as less than values were below the limit of quantitation (LOQ). The LOQ for each analysis is dependent upon the regression, the area of the low standards and the dilution factor of the controls. NA = Not applicable

Mean frond numbers are presented below along with the growth rate, yield and respective inhibition values, alongside estimated EC₅₀ values:

Table 9.2.7-2: Effect of SYN545974 on growth rate and yield of *Lemna gibba* (based on frond number)

Mean measured concentration (mg a.s./L)	Mean No. fronds/replicate (Day 7)	Based on Frond Number (0-7 days)			
		Growth rate	Inhibition of Growth rate (%)	Yield (Mean No. fronds/replicate)	Inhibition of Yield (%)
Control	267	0.44	NA	255	NA
Solvent Control	257	0.44	NA	245	NA
Pooled Control	260	0.44	NA	248	NA
0.099	249	0.43	2	237	5
0.33	244	0.43	2	232	7
0.97	256	0.44	0	244	2
3.1	211	0.41*	7	199*	20
6.3	277	0.45	-2	265	-7
EC₅₀ (mg a.s./L)		>6.3		>6.3	
95% confidence limits^a		ND		ND	
EC₂₀ (mg a.s./L)		>6.3		>6.3	
95% confidence limits^a		ND		ND	
EC₁₀ (mg a.s./L)		>6.3		>6.3	
95% confidence limits^a		ND		ND	
NOEC (mg a.s./L)		6.3		6.3	
LOEC (mg a.s./L)		ND		ND	

*Significantly reduced compared to pooled control, based on Dunnett's Multiple Comparison Test. However, since growth was not significantly reduced at the higher treatment level 6.3 mg a.s./L compared to the pooled control data, the reduction in the 3.1 mg a.s./L treatment level was not considered to be test substance related.

^a EC value was empirically estimated, therefore corresponding 95% confidence limit(s) could not be determined

(-) = increase in growth relative to that of control

NA = Not applicable

ND = Not determined

Mean dry weights are presented in Table 9.2.7-3 below along with the growth rate, yield and respective inhibition values, alongside estimated EC₅₀ values:

Table 9.2.7-3: Effect of SYN545974 on growth rate and yield of *Lemna gibba* (based on dry weight)

Mean measured concentration (mg a.s./L)	Mean Dry Weight (Day 7) (mg)	Based on Dry Weight (0-7 days)			
		Growth rate	Inhibition of Growth rate (%)	Yield	Inhibition of Yield (%)
Control	27.2	0.57	NA	26.7	NA
Solvent Control	26.1	0.57	NA	25.6	NA
Pooled Control	26.5	0.57	NA	26.0	NA
0.099	24.3	0.56	2	23.8	9
0.33	24.8	0.56	2	24.3	7
0.97	23.7	0.55	4	23.2	11
3.1	21.0	0.54*	5	20.5*	21
6.3	25.9	0.57	0	25.4	2
EC ₅₀ (mg a.s./L)		> 6.3		> 6.3	
95% confidence limits ^a		ND		ND	
EC ₂₀ (mg a.s./L)		> 6.3		> 6.3	
95% confidence limits ^a		ND		ND	
EC ₁₀ (mg a.s./L)		> 6.3		> 6.3	
95% confidence limits ^a		ND		ND	
NOEC (mg a.s./L)		6.3		6.3	
LOEC (mg a.s./L)		ND		ND	

*Significantly reduced compared to pooled control, based on Dunnett's Multiple Comparison Test. However, since growth was not significantly reduced at the higher treatment level 6.3 mg a.s./L compared to the pooled control data, the reduction in the 3.1 mg a.s./L treatment level was not considered to be test substance related. ^a EC value was empirically estimated, therefore corresponding 95% confidence limit(s) could not be determined

(-) = increase in growth relative to that of control

NA = Not applicable

ND = Not determined

No abnormalities in appearance of the test plants were recorded in the control, solvent control or any test concentrations during the 7-day exposure to SYN545974.

VALIDITY CRITERIA

The validity criterion was met according to OECD Guidelines for Testing of Chemicals, Method 221: *Lemna sp.* Growth Inhibition Test (2006).

Table 9.2.7-4: Compliance with OECD 221 validity criterion

Validity criterion	Required	Obtained
Frond number	Doubling time of frond number in the	Doubling time for frond density was 1.6 days.

Validity criterion	Required	Obtained
	control must be less than 2.5 days (60h), corresponding to approximately a seven-fold increase in seven days and an average specific growth rate of 0.275 d ⁻¹ .	

CONCLUSIONS

For *Lemna gibba*, the 7-day frond number EC₅₀ for yield (E_yC₅₀) and growth rate (E_rC₅₀) for SYN545974 were > 6.3 mg a.s./L, the highest concentration tested, based on mean measured concentrations.

The 7-day dry weight EC₅₀ for yield (E_yC₅₀) and growth rate (E_rC₅₀) were > 6.3 mg a.s./L, the highest concentration tested, based on mean measured concentrations.

The 7-day frond number NOEC, based on growth rate and yield, was determined to be 6.3 mg a.s./L, and the 7-day LOEC was not determined.

The 7-day dry weight NOEC, based on growth rate and yield, was determined to be 6.3 mg a.s./L, and the 7-day LOEC was not determined.

(██████, 2015a)

HSE evaluator comments

This study was conducted according to GLP, and to OECD 221 (2006), all validity criteria were met. The following deviations were noted:

The composition of the 20x AAP medium used was in line with the guidelines, as were the temperature and the pH ranges throughout the experiment. However, the light intensity was below the recommended range of 6,500 to 10,000 LUX. The measured light intensity throughout the experiment ranged from 4,900 to 6,400 LUX. As the validity criteria were met, this is unlikely to affect the reliability of the experiment.

No mention was made in the study of the use of a reference substance as a positive control. OECD 221 (2006) recommends that one is used at least twice per year, but does not require it. Therefore, this would not be cause to invalidate the study.

Although it was determined that SYN564974 has no effect on growth inhibition of *Lemna gibba* at any of the tested concentrations, a certain degree of uncertainty surrounds the results from the two highest tested concentrations:

Firstly, the 3.1 mg /L condition showed a significantly increased level of growth inhibition when compared to the controls, whereas the highest tested concentration of 6.3 mg /L did not. The study authors stated that “Since all other treatment levels tested were not significantly reduced compared to the pooled control data, the reduction observed in the 3.1 mg/L treatment level was not considered to be biologically relevant.” This conclusion seems slightly more tenuous when observing the data for inhibition of yield based on dry weight (shown in Table 9.2.7-3). Although not statistically significant, there appears to be a trend for increasing inhibition of yield in the 0.33 mg /L, 0.97 mg /L, and then 3.1 mg /L conditions. It may be the case that if more replicates were carried out, that this trend would become significant, meaning that the 6.3 mg /L results rather than the 3.1 mg /L results may have been anomalous, as concluded by the study authors.

Secondly, there were issues with the solubility of the test substance in the two highest tested conditions. All test solutions were mixed for approximately two hours after preparation, but the 3.2 mg /L and 10 mg /L solutions were observed to be clear and colourless with large aggregates of undissolved test substance. The remainder of the test solutions had no visible undissolved test substance following preparation. The 3.2 mg /L and 10 mg /L

solutions were sonicated for 30 minutes, and then filtered through polyester filter floss to remove any remaining undissolved material. These filtrates were used as the experimental media.

The OECD 221 (2006) guidelines state that “In semi-static tests where the concentration of the test substance is not expected to remain within $\pm 20\%$ of the nominal concentration, it is necessary to analyse all freshly prepared test solutions and the same solutions at each renewal”. Samples of the initially prepared test media were analysed for concentration of test substance at day 0 (new), and day 3 (aged). The results showed that the mean measured concentration of test substance was not maintained within $\pm 20\%$ of the nominal value in the 10 mg /L condition, and as such, analytical measurements should have additionally been taken of all new and aged solutions at each subsequent media renewal. This casts some uncertainty on the reliability of the reported concentrations of test substance in each medium. Following a request for additional information, the applicant provided the following justification for the lack of additional analytical samples: “A decrease of exposure concentrations was not foreseen and therefore, no additional sampling dates were planned. Explanation regarding the possible cause of the decrease is given in the report. No negative effects were observed in this study. The endpoints are given based on mean measured concentrations providing the worst-case.”. HSE does not consider this adequate justification to omit analytical measurements and the endpoints cannot be considered reliable as the concentration of test item for the duration of the study cannot be confirmed.

Although it was not mentioned in the study report whether the fronds were taken from healthy monocultures which were free from contamination, the study authors reported that no abnormalities in appearance of the test plants were recorded in the control, solvent control or any test concentrations during the 7-day exposure to SYN545974. No further information was provided on which parameters were measured for the frond appearance. These issues are unlikely to affect the reliability of the results, as the validity criterion was met.

Linear interpolation of response was used to calculate the 7-day E_rC_{50} and E_yC_{50} values, based on percent inhibition relative to the pooled control. As no concentration resulted in a 50% reduction in growth rate, the EC values were empirically estimated to be greater than the highest mean measured concentration tested. For the NOEC and LOEC values, Dunnett’s Multiple Comparison Test was used to determine values significantly different from the pooled control. The statistics used were in line with the OECD 221 (2006) guidelines.

The analytical methods have been checked by HSE Chemistry specialists in Vol 3CA Part B5.1.2.6. The following was concluded: “Fit for regulatory purposes but the method is not fully validated in accordance with SANCO/3029/99 rev. 4. LOQ: 0.3 $\mu\text{g/L}$ in aqueous matrices. It should be noted unacceptable recoveries were determined at a test concentration of 10,000 $\mu\text{g/L}$. The applicant has justified this as the solubility limit of the test substance in AAP medium has been reached.”

Overall, HSE does not consider the endpoints suitable for use in risk assessment, since the concentration of the test item cannot be confirmed through the duration of the study, due to lack of analytical measurements. This is discussed further in the risk assessment.

B.9.2.8. Further testing on aquatic organisms

None submitted.

B.9.3. EFFECTS ON ARTHROPODS

B.9.3.1. Effects on bees

Report:	K-CA 8.3.1.1.1, [REDACTED], (2012), SYN545974 – Acute Oral and Contact Toxicity to the Honey bee <i>Apis mellifera</i> L. in the Laboratory, Report Number S11-03873. Eurofins Agroscience Services EcoChem GmbH, Eutinger Str. 24, 75223 Niefern-Öschelbronn, Germany. (Syngenta File No. SYN545974_10010)
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Guidelines

OECD Guidelines for Testing of Chemicals, Method 213: Honey bees, acute oral toxicity test (1998) OECD Guidelines for Testing of Chemicals, Method 214: Honey bees, acute contact toxicity test (1998)

GLP: Yes.

Materials

Test Material	SYN545974 tech.
Lot/Batch #:	2637-BA/110
Purity:	99.5 % w/w
Description:	White powder
Stability of test compound:	Stable under standard conditions.
Reanalysis/Expiry date:	31 July 2013
Density:	Not applicable

Treatments

Test rates:	Oral: nominal 100 µg SYN545974/bee (actual consumed dose: 116 µg SYN545974/bee) Contact: 100 µg SYN545974/bee
Controls:	Oral: 50 % (w/v) aqueous sucrose solution; one additional group treated with a mixture of 50 % (w/v) aqueous sucrose solution and acetone (ratio 10:1) Contact: mineral water; one additional group treated with pure acetone
Toxic standard:	Perfekthion /BAS 152 11 I (nominally 400 g dimethoate/L; measured 411.7 g dimethoate/L) Oral: Nominal: 0.08, 0.11, 0.15 and 0.20 µg a.s./bee Contact: Nominal: 0.10, 0.13, 0.17 and 0.26 µg a.s./bee
Administration:	Oral: ingestion in aqueous sucrose solution Contact: cuticular absorption following the application of droplets dorsally to the thorax of each bee

Test organisms

Species:	<i>Apis mellifera</i> L. (Hymenoptera,:Apidae)
Source:	Healthy colony of young adult worker bees descended from a breeding line of a beekeeper in Ayora, Spain (responsible beekeeper: Carlos Feuerriegel, Ciudad Jardin 54, S-44620 Ayora, Spain)
Food:	50 % w/v aqueous sucrose solution

Test design

Test cage description:	Stainless steel chambers (approximately 8.2 x 4.0 x 6 cm) with a transparent window and a perforated bottom plate which allows sufficient air supply into the vessel. The test cages were lined with filter paper.
Replication:	5
No. of bees/arena :	10
Duration of test:	Oral: 48 hours Contact: 48 hours

Environmental test conditions

Temperature:	25.0 – 26.0 °C
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Humidity:	50 – 65 %, with two brief periods of 48 % (RH)
Photoperiod:	Constant darkness

Study Design and Methods

Experimental dates: 31 January to 2 February 2012

Honey bees (*Apis mellifera*) were collected randomly the day before the start of the test. They were held under laboratory conditions.

Honey bees (*Apis mellifera*) were exposed to SYN545974 dissolved in acetone via two routes of administration: (1) oral ingestion in aqueous sucrose solution; and (2) contact, i.e. cuticular absorption following the application of a droplet dorsally to the thorax of each bee; after each application the applicator needle was cleaned with a mixture of water and water-wetting agent. To immobilise the bees during the course of treatment, they were anaesthetised using short bursts of CO₂.

Oral test procedures: Bees were starved for 2 hours prior to treatment. Each group of bees was offered 250 µL (equivalent to 25 µL/bee) of the test material, controls, or toxic standard dispersed in aqueous sucrose solution. Treatments were calculated so that the target dose per bee was contained in 20 µL, however 25 µL was actually provided per bee. This was to ensure sufficient consumption of the test material so that the target dose was achieved. The doses were measured into the eppendorf cups and the weights of these were recorded before the doses were made available to the bees. The bees were allowed to consume the test solutions up to a maximum of six hours after which the eppendorf cups were replaced and 50 % w/v aqueous sucrose solution provided *ad libitum*. All cups with test solutions were weighed after feeding in order to calculate actual mean consumption per bee for each treatment.

Contact test procedures: Bees were treated with a 2 µL droplet of the test solution, the controls or the toxic standard, applied to the dorsal surface of the thorax using a micro applicator. Droplets of 2 µL were chosen in deviation to the guideline recommendation of 1 µL, since a higher volume was considered to ensure a more reliable dispersion of the test item. No adverse effects on the outcome of the study were expected. The bees were returned to the test unit, allowed to recover and kept in the CE room with a continuous supply of 50 % w/v aqueous sucrose solution.

In both the oral and contact tests there were five replicates per treatment. Mortality and sub-lethal effects were assessed at 4, 24 and 48 hours for the test material, controls and toxic standard for both oral and contact tests. The mortality per treatment was calculated from the number of dead bees and the total number of introduced bees per treatment group. Since 2% mortality occurred in the mineral water control group of the contact toxicity test, the reference item mortality was corrected according to the formula of Abbott (1925), modified by Schneider-Orelli (1947):

Corrected mortality

$$M = \frac{(t - c)}{(100 - c)} \times 100 \%$$

M = Corrected mortality (%)

t = Mortality in the treated group (%)

c = Mortality in the control group (%)

The LD₅₀ values with 95% confidence limits of the reference and test item treatments were calculated by means of a probit analysis. The oral LD₅₀ values for the reference and test item treatments were calculated with the single consumption values per replicate.

Results and Discussion

Mortality data for the test material and toxic standard are summarised in the tables below.

Table 9.3.1-1 Summary of acute oral toxicity of SYN545974 to the Honey bee

Target Dose SYN545974 (µg a.s./bee)	Consumed Dose SYN545974 (µg a.s./bee)	Mortality 24 hours (%)	Mortality 48 hours (%)
Control	-	0	0
Solvent control	-	0	0
100	116	2	2
LD₅₀ (µg a.s./bee)		>116	>116
95 % confidence interval (µg a.s./bee)		NA	NA

The 48-hour oral LD₅₀ for the reference item Perfekthion was 0.10 µg a.i./Bee, the 24-hour oral LD₅₀ for the reference item Perfekthion was 0.12 µg a.i./Bee.

Table 9.3.1-2: Summary of acute contact toxicity of SYN545974 to the Honey bee

Dose SYN545974 (µg a.s./bee)	Mortality 24 hours (%)	Mortality 48 hours (%)
Control	0	2
Solvent control	0	0
100	0	0
LD₅₀ (µg a.s./bee)	>100	>100
95% confidence interval (µg a.s./bee)	NA	NA

NA. = not applicable

The corrected 48-hour contact LD₅₀ for the reference item Perfekthion was 0.15 µg a.i./Bee, the corrected 24-hour contact LD₅₀ for the reference item Perfekthion was 0.20 µg a.i./Bee.

No remarkable behavioural abnormalities were observed throughout the whole 48 hours observation period in any treatment group from either test.

Conclusions

The 48-hour oral LD₅₀ for the test material was >116 µg a.s./bee, the only concentration tested.

The 48-hour contact LD₅₀ for the test material was >100 µg a.s./bee, the only concentration tested.

No sublethal effects were observed throughout the 48-hour observation period in either test.

(██████, 2012)

HSE evaluator comments

Validity criteria	Recommended	Obtained
The average mortality for the total number of controls must not exceed 10 per cent at the end of the test:		
Oral test:		
Water control	≤ 10 %	0.0 %
Solvent control	≤ 10 %	0.0 %
Contact test:		
Water control	≤ 10 %	2.0 %
Solvent control	≤ 10 %	0.0 %
The 24-hour LD₅₀ of the toxic standard meets the specified range:		
Oral test Perfekthion (Dimethoate):	0.10 – 0.30 µg a.s./bee	0.12 µg a.s./bee)
Contact test Perfekthion	0.10 – 0.35 µg a.s./bee	0.20 µg a.s./bee)

Validity criteria	Recommended	Obtained
(Dimethoate):		

The study was carried out to GLP and follows guidance documents OECD 213 & OECD 214 (1998). All the validity criteria set out in OECD 213 (1998) and OECD 214 (1998) were met.

There were some deviations to the guidelines. The volume of test substance applied to each bee in the contact test was 2 µl. In OECD 214 (1998) a 1 µl droplet is recommended. As there were no mortalities or sublethal effects observed during the contact test it is not considered to have affected the endpoints.

Secondly, in OECD 213 it states that when the test item has low solubility, acetone may be used as a vehicle when preparing test item concentrations. According to OECD 213 the concentration of the vehicle (in this case, acetone) considered appropriate is 1 % and should not be exceeded. In the oral test, test item feeding solutions were prepared and contained 10 % acetone which clearly deviates from what is recommended in the OECD guideline. However, no toxicity was observed in both the solvent control and the test item groups. From this, this increase in solvent percentage is not considered to have had an effect on the outcome and integrity of the study and can be accepted.

The agreed endpoints suitable for use in the risk assessment are:

- **Acute oral 48-hour LD₅₀ = > 116 µg a.s./bee.**
- **Acute contact 48-hour LD₅₀ = > 100 µg a.s./bee.**

Report:	K-CA 8.3.1.3 [REDACTED] (2015) SYN545974 - A laboratory study to determine the chronic effects on the brood of the honey bee <i>Apis mellifera</i> L. (Hymenoptera: Apidae), Report Number 037SRFR15C06, SynTech Research France SAS 613 route du Bois de Loyse 71570 La Chapelle de Guinchay, France (Syngenta file No. SYN545974_10279)
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Guidelines

OECD Guidelines for Testing of Chemicals, DRAFT method: Honey bee (*Apis mellifera*) larval toxicity test, repeated exposure (2014)

GLP: Yes

Materials

Test Material	SYN545974 tech.
Lot/Batch #:	SMU2EP12007
Actual content of active ingredients:	98.5 % w/w
Description:	Off-white powder
Stability of test compound:	Stable under test conditions
Reanalysis/Expiry date:	End of June 2016

Treatments

Test rates:	Nominal does: 0.021 µg a.s./larva (equivalent to 0.15 mg a.s./L diet) Measured dose: 0.014 µg a.s./larva (equivalent to 0.1 mg a.s./L diet)
Control:	Untreated
Toxic standard:	ROGOR PLUS (Dimethoate (400 g/L, equivalent to 37.9 % w/w))
Application method:	Oral application via artificial diet

Test organisms

Species:	honey bee <i>Apis mellifera</i> L. (Hymenoptera: Apidae)
Age:	First instar (L1) during grafting

Source:	Maintained at test facility
Food:	Artificial diet (containing 50 % royal jelly and 50 % aqueous sugar [sugar: equal parts D-glucose & D-fructose] + yeast extract solution) supplemented with SYN545974.
Test Design	
Test cage description:	1 individual cell (queen starter). 48 cells per culture plate. Each well of the culture plate was half filled with a piece of dental roll.
Replication:	3 (one colony per replicate was used)
No. of larvae/replicate:	12
Environmental test conditions	
Temperature:	34.2 °C to 35.0 °C during incubation. When dosing (days 3, 4, 5 and 6 of larval rearing period) temperature ranged between 23.2 to 25.6 °C
Humidity:	The actual hygrometry was stated as 45 to 99 %. Raw data was not reported. In the method section the test conditions were stated as: 90 – 100 % RH (day 1 to 7), 75 – 85 % RH (day 8 to 14) and 55 % RH (day 15 onwards). When dosing (days 3, 4, 5 and 6 of larval rearing period) humidity ranged between 48 and 72 %.
Photoperiod:	Constant darkness
Duration of test:	22 days

Study Design and Methods

Experimental dates: 8 to 29 June 2015

The study comprised an untreated control, a toxic reference item and limit dose of the test item treatment (0.014 µg a.s./larva, actual measured dose). Exposure to the treatments occurred via the diet during the larval rearing period.

Honey bee larvae *Apis mellifera* L. were exposed to a repeated oral application of 0.021 µg a.s./larva (equivalent to 0.15 mg a.s./kg diet) (measured 0.014 µg a.s./larva (equivalent to 0.1 mg a.s./L diet)) in an *in vitro* limit test. One control group and a reference item group were included in the test. The larvae of the control treatment were fed with untreated artificial diet, which served as a vehicle for the test item and reference item.

Treatments were applied on days 3, 4, 5 and 6 of the larval rearing period (chronic exposure); using a calibrated micropipette.

The number of dead larvae was recorded on Day 4, Day 5, Day 6, Day 8, (plus uneaten food) and, Day 15. On Day 22 the number of emerged adult bees was counted (pupal mortality), behaviour and development were recorded.

Results (except toxic reference results) were analysed with the statistical software Minitab® Release 14 (Fisher test with Bonferroni correction) to determine any significant differences. Behavioural observations were not evaluated for statistical significance due to the non-quantitative nature of the observations. Mortality results were corrected for control mortality using an adaptation of Abbott's formula (1925).

Results and Discussion

Mortality data and other observations for the test material are summarised in the table below.

Table 9.3.1-3: Summary of chronic toxicity of test material to Honey bee larvae

Test item	SYN545974
Test organism / Exposure	Honey bee larvae / Repeated exposure (Chronic)

Application rate		8-day cumulative mean larval mortality (%) ^a	Pupal mortality (%) 22 days ^a	22-day cumulative effects (%)
(µg a.s./larva)	(µg a.s./larva/day) ^b			
Control		8.333	6.061	NA
0.014	0.0035	21.21 ^a (*)	22.21 ^a	38.71 ^a (*)
LD ₅₀ / day (µg a.s./larva/day)		> 0.0035	> 0.0035	> 0.0035
NOED / day (µg a.s./larva/day)		< 0.0035	0.0035	< 0.0035

The pupal mortality is the inverse of the emergence effect from the day 8 to day 22. ^a value corrected from untreated control, according to

Abbott (1925). ^b Mean value of daily consumed dose.

* Treatment groups significantly different from the control (Fisher test with Bonferroni correction, after Log transformation).f.p. NA = Not Applicable

Analytical Verification

The actual analysed concentrations 0.0010 and 0.0006 g a.s./L, were not within the required range of 80-120% of the nominal concentration (actual values: -33.3 and -57.3 %, therefore the actual measured concentration was taken into account.

Conclusions

The objective of the study was to determine the lethal and sublethal effects of SYN545974 on the brood of the honey bee *Apis mellifera* L. (Hymenoptera: Apidae), when mixed with artificial diet and fed to larvae.

There was a significant difference in larval mortality between the control and the test item dose. Thus, the NOED during larval development was < 0.0035 µg a.s./larva/day and the LD₅₀ during larval development was estimated to be > 0.0035 µg a.s./larva/day.

There was no significant difference in pupal mortality between the control and the test item dose. Thus, the NOED during pupation was 0.0035 µg a.s./larva/day and the LD₅₀ during pupation was estimated to be > 0.0035 µg a.s./larva/day.

There was a significant difference in emergence between the control and the test item dose. Thus the NOED for the entire development period was < 0.0035 µg a.s./larva/day and the LD₅₀ for the entire development period was estimated to be > 0.0035 µg a.s./larva/day.

(██████████, 2015)

HSE evaluator comments

Validity criteria	Recommended	Obtained
Cumulative mortality from D3-D8	≤ 15 % across all replicates	8.33 %
Adult emergence rate on D22	≥ 70 % across all replicates	86.11 %
Positive control: Dimethoate larval mortality on D8	≥ 50 % across all replicates	54.55 %

The study was reported and conducted in line with ENV/JM/MONO(2016)34, Series on testing & Assessment, No. 239 and follows GLP. The validity criteria as shown above have all been met. A limit test design was used and the report stated the chosen concentration was based on the maximum achievable solubility of the active substance in water. In-line with the aforementioned guidance if statistically significant effects occur compared to control, as is the case above, a full study should be conducted.

Raw data was not provided for humidity during different days, instead the range of values were reported and the values during dosing. Nonetheless, the intended test conditions detailed in the study report are in-line with

ENV/JM/MONO(2016)34. The actual humidity values during dosing and over the study deviated but given validity criteria were met it is unlikely these deviations significantly impacted the study results.

There were deviations to the guideline in the study. The analysed concentrations of the stock solution were not within the required range of 80-120 % of the nominal concentration. The actual measured concentration of the first analysed concentration was taken into account and it is not considered to have had an effect on the endpoints. The analytical method has been considered by HSE Chemistry specialists in Vol. 3 CA Part B5.1.2.6. The method was considered to be acceptable.

The mortality results were corrected for control mortality using Abbot's formula, in line with OECD 239 (2016). The data was transformed prior to analysis. Fisher test with Bonferroni correction was used to determine if the treatment groups were significantly different from the control group, in line with OECD 54 (2006).

The agreed endpoints to consider are as follows:

- **NOED 8 and 22-day = Not possible to determine (< 0.0035 µg a.s./larva/day)**
- **LD₅₀ 8 and 22-day = > 0.0035 µg a.s./larva/day**

Report:	K-CA 8.3.1.3. [REDACTED], (2018), Pydiflumetofen – Effects on the honeybee brood of <i>Apis mellifera</i> L. following chronic oral exposure under field conditions. Report Number 17 48 BFB 0001. BioChem agrar, Labor für biologische und chemische Analytik GmbH, Kupferstr. 6, 04827 Machern OT Gerichshain, Germany. (Syngenta file no SYN545974_10619)
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GUIDELINES

Oomen P.A., De Ruijter A. and van der Steen J., OEPP/EPPO Bulletin, 22 613-616 (1992)

GLP: Yes.

EXECUTIVE SUMMARY

The purpose of this study was to investigate potential effects of pydiflumetofen on free-flying honeybee (*Apis mellifera* L.) brood following chronic oral exposure via in-hive application of treated sucrose solution, according to the method described by Oomen et al. (1992) under field conditions. Honeybees were exposed to five different concentrations of the test item (ranging from 2.50 mg pydiflumetofen/kg food (test item I) to 32.0 mg pydiflumetofen/kg food (test item V)) in 50 % w/v sucrose solution containing 2 % v/v acetone. An untreated control and reference item treatment were also included. Following exposure, no adverse effects on adult bee mortality and behaviour were observed between any of the test item treatments and the control. An increase in pupal mortality occurred on single days but not in all replicates in all treatments. No differences in colony strength and general brood development were observed between any of the test item treatments and the control during the entire course of the study. The detailed bee brood development assessment of initially labelled eggs revealed no impact of the test item on brood development during the entire trial. Overall, based on the results of this study, oral exposure of honeybees to pydiflumetofen, up to 32 mg a.s./kg food, does not adversely affect colony development and survival.

MATERIALS

Test Material	SYN545974
Lot/Batch #:	SMU2EP12007
Purity:	98.5% w/w (certificate of analysis confirmed)

Treatments

Test rates:	2.5, 5.0, 10.5, 20.0 and 32.0 mg a.s./kg
Control:	Untreated sucrose solution containing 2% acetone
Toxic standard:	Insegar (fenoxycarb) – 1.28 g a.s./kg (0.748 g a.s./colony/day) Batch no. SMO2K434 Active ingredient 250 g/kg (nominal)
Analysis of test concentrations:	Analysis of daily feeding solutions to confirm exposure using HPLC MS-MS. Method; SYN545974 - Analytical Method ECO_066_03A and Validation for the Determination of SYN545974 in pollinator matrices (pollen, nectar, foliage and flowers) and in feeding solutions (sucrose) from honey bee oral laboratory studies” (BioChem Projext No.: 17 35 CRB 0148). LOQ for Pydiflumetofen = 0.005 mg/kg

Test organisms

Species:	Honeybee, <i>Apis mellifera</i> L. (Buckfast) from well-fed queen-right colonies.
Colony health:	Health examination for <i>Nosemia apis</i> (absence or low level infestation, 4 colonies with level 1 infestation i.e. 1-7 spores counted), <i>Varroa destructor</i> (absent) and diverse viruses (2 colonies with BQCV). Health examinations were non-GLP.
Age:	Whole colonies with adults and juveniles (all brood stages)
Source:	Apiary at BioChem agrar GmbH, Kupferstr. 6, 04287 Machern OT Gerichshain, Germany
Food:	Treated/untreated sucrose solution via feeding together with natural food resources in surrounding area (bee colonies free flying)
Colony strength:	10238-12825 on DAT -2 (mean of 11612 bees/colony)
Colony brood status:	Mean 9923 cm ² /colony; comprising eggs of mean 1081 cm ² /colony; larvae of mean 2284 cm ² /colony; pupae of mean 6558 cm ² /colony.

Test Design

Monitoring site:	Borsdorf near Leipzig in Saxony, Germany. Latitude: 51°20'58.07"N; longitude: 12°33'48.47"E. Height above sea level: 127 m. No agricultural or mass flowering crops were present in the surrounding area.
Number of treatments:	Seven – control, five test item treatments and a reference item treatment
Replication:	Four colonies per treatment
Application:	The application was carried with an in-hive feeding method by providing 0.5 L treated or untreated 50 % (w/v) sucrose solution daily over a 9-day period.
Duration of test:	Three bee brood cycles (61 days following start of exposure).
Exposure scenario:	Pre-application/pre-exposure phase: DAT -4 to DAT 0. Application/exposure phase: DAT 1 to DAT 9. Post-application/post-exposure phase: DAT 10 to DAT 61.
Beehive set-up:	Colonies set up 5 days before start of applications. Each colony was equipped with a dead bee trap at hive entrance. Colonies arranged in a block scheme to prevent drifting or robbing.

Environmental test conditions

Field conditions with open access to natural food sources (without intensive agriculture in the surrounding area). Temperature, relative humidity and precipitation were recorded daily by a data logger at the monitoring site close to the colonies (GLP-conditions) and additionally by a weather station located 1.8 km from the monitoring site (non-GLP-conditions).

STUDY DESIGN AND METHODS

Experimental dates: 23rd June 2017 – 28th August 2017

The aim of the study was to determine the potential effects of pydiflumetofen (SYN545974) on honeybee (*Apis mellifera*) brood following chronic oral exposure via an in-hive application of treated sucrose solution according to the method described by Oomen *et al.* (1992) under field conditions where bees are free flying. Detailed brood development was assessed via photo documentation of initially marked eggs which represented the main endpoint of the study (brood termination rate – BTR). In addition, bee mortality, behaviour and colony development were monitored for three bee brood cycles. The chronological test schedule is shown in the table below:

Time schedule

Assessment day	Date	Activity / Assessment
DAT -5	23.06.2017	- Bee hive set-up at the monitoring site
DAT -4 to DAT 0	24.06.2017 to 28.06.2017	- Mortality - Behaviour
DAT -2 (BFD 0)	26.06.2017	- General brood/colony development - Detailed brood development of cells containing eggs (n=300)
DAT 0 to DAT 8	28.06.2017 to 06.07.2017	- Application of the control, test item I-V, reference item solutions - Sampling of feeding solution for analysis (control and test item I-V)
DAT 1 to DAT 9	29.06.2017 to 07.07.2017	- Determination of actual food consumption for the control, test item I-V and reference item
DAT 1 to DAT 26	29.06.2017 to 24.07.2017	- Mortality - Behaviour
DAT 2 (BFD 4), DAT 7 (BFD 9), DAT 14 (BFD 16), DAT 20 (BFD 22)	30.06.2017, 05.07.2017, 12.07.2017, 18.07.2017	- General brood/colony development - Detailed brood development of initially eggs (n=300)
DAT 26 (BFD 28) DAT 33 (BFD 35) DAT 41 (BFD 43) DAT 48 (BFD 50) DAT 55 (BFD 57) DAT 61 (BFD 63)	24.07.2017, 31.07.2017, 08.08.2017, 15.08.2017, 22.08.2017, 28.08.2017	- General brood/colony development
DAT 61	28.08.2017	- Transporting colonies back to the apiary (in the evening)

DAT: Days after treatment; BFD: Brood area fixing day

The test item was dissolved in 2% acetone before being added to 50% (w/v) sucrose solution. Feeding solutions were fed daily for a period of nine days; each day 0.5 L of sucrose solution containing the appropriate amount of the test item were fed to colonies via a feeder placed on top of each hive. Bees had open access to the feeding solution from within the hive. The solutions were provided for one day before replacement. To determine actual consumption the amount of sucrose solution was determined daily by weighing before and after feeding. Analysis of SYN545974 (pydiflumetofen) were determined in the daily feeding solutions for each test item treatment

group/control in order to confirm exposure. The control group was fed with untreated 50 % sucrose solution containing the same solvent level (2 % v/v acetone) as used for the test item. The reference item Insegar 25 WG was dissolved directly in the 50 % sucrose solution. Prior to and during feeding applications, assessments were made on the mortality of adults and pupae; behaviour of bees; colony strength; presence of healthy egg-laying queen and the comb area covered with brood and food. Bee behaviour was assessed in bees returning to the hive entrance during the evaluation period. Abnormal behaviours such as aggressiveness, intensive cleaning or accumulation of bees at the hive entrance were noted. Detailed examination of the bee brood development was conducted for the first brood cycle until DAT 20. Observations and assessments were not performed when environmental conditions were considered likely to have a negative influence on those assessments or bee development.

Brood termination rate was calculated based on the failure of individual eggs to develop. Failure to develop was indicated by the bee brood in a cell not reaching the expected brood stage at the specified assessment day or food being stored in the cell during BFD 4 to BFD 16. The brood index and brood compensation index were also calculated. The endpoints for statistical evaluation were mortality [number of dead bees], brood termination rate [% BTR], brood-index and brood compensation index. The arithmetic mean and the standard deviation per replicate and treatment were calculated for these endpoints, as well as for colony strength, brood and food area.

Pre-treatment data were statistically evaluated using a multiple testing method; comparing the treatment means (control, test item and reference item) against each other. Therefore, the evaluation was done using the Tukey-test to reveal statistically significant differences between the treatment groups. The post-treatment data were evaluated using pair-wise statistical testing methods comparing treatments (test item or reference item) separately against the control. The Student t-test (for homogeneous variance data) or the Welch t-test (for inhomogeneous variance data) was used for pair-wise comparison of treatments (test item, reference) against the control (one-sided greater: mortality, brood termination rate; one-sided less: brood index and brood compensation index).

For all statistical tests, a significance level of $\alpha = 0.05$ was used. The statistical analysis was performed with the software Easy Assay 4.0 (■■■■■■, 1998) and ToxRat Professional 3.2.1 (■■■■■■, 2015).

RESULTS AND DISCUSSION

Table 9.3.1-4: Overview of main endpoints for control, test item treatment I and test item treatment II

Evaluation / Assessment		Treatment group					
		Control (untreated)		Test item I (2.50 mg a.i./kg)		Test item II (5.00 mg a.i./kg)	
		mean ¹	SD	mean ¹	SD	mean ¹	SD
Adult mortality² [bees/colony/day]	Pre-exposure phase (DAT -4 to DAT 0)	8.3	2.8	9.6	3.1	10.4	3.1
	Exposure phase (DAT 1 to DAT 9)	9.8	6.7	11.3	7.7	8.1	4.7
	Post-exposure phase (DAT 10 to DAT 26)	8.0	5.5	12.5*	7.2	10.4*	7.7
	Overall after start of exposure (DAT 1 to DAT 26)	8.6	6.1	12.1*	7.3	9.6	6.9
Pupal mortality²	Pre-exposure phase (DAT -4 to DAT 0)	0.0	0.0	0.0	0.0	0.0	0.0

Evaluation / Assessment		Treatment group					
		Control (untreated)		Test item I (2.50 mg a.i./kg)		Test item II (5.00 mg a.i./kg)	
		mean ¹	SD	mean ¹	SD	mean ¹	SD
[bees/colony/day]	Exposure phase (DAT 1 to DAT 9)	0.0	0.0	0.0	0.0	0.0	0.0
	Post-exposure phase (DAT 10 to DAT 26)	0.0	0.0	0.1	0.6	0.1	0.5
	Overall after start of exposure (DAT 1 to DAT 26)	0.0	0.0	0.1	0.5	0.1	0.4
Brood termination rate [%]	Eggs (BFD 22) ³	10.3	6.5	18.1	13.5	10.8	8.6
Brood–index	Eggs (BFD 22) ³	4.5	0.3	4.1	0.7	4.5	0.4
Brood compensation index	Eggs (BFD 22) ³	4.5	0.3	4.3	0.4	4.6	0.3

¹⁾ mean of 4 replicates; ²⁾ dead honeybees found in dead bee trap; ³⁾ at the last relevant assessment when development is expected to be completed, i.e. BFD 22 for marked eggs; * = statistically significant different when comparing treatment against control via Student or Welch t-test at post-application phase; one-sided greater: mortality, brood termination rate; one-sided smaller: brood index and brood compensation index. The percent-values of the brood termination rate were arcsine- transformed to ensure the homogeneity of the data before conducting the t-test procedure.

Table 9.3.1-5: Overview of main endpoints for test item treatments III, IV and V

Evaluation / Assessment		Treatment group					
		Test item III (10.5 mg a.i./kg)		Test item IV (20.0 mg a.i./kg)		Test item V (32.0 mg a.i./kg)	
		mean ¹	SD	mean ¹	SD	mean ¹	SD
Adult mortality ² [bees/colony/day]	Pre-exposure phase (DAT -4 to DAT 0)	8.1	2.5	9.2	2.5	9.5	2.6
	Exposure phase (DAT 1 to DAT 9)	8.4	3.8	9.7	8.9	8.9	6.7
	Post-exposure phase (DAT 10 to DAT 26)	9.9	6.5	11.9*	9.2	12.2*	7.7
	Overall after start of exposure (DAT 1 to DAT 26)	9.4	5.7	11.1*	9.1	11.1*	7.5
Pupal mortality ²	Pre-exposure phase (DAT -4 to DAT 0)	0.0	0.0	0.0	0.0	0.0	0.0

[bees/colony/day]	Exposure phase (DAT 1 to DAT 9)	0.0	0.0	0.0	0.0	0.0	0.0
	Post-exposure phase (DAT 10 to DAT 26)	0.9	3.1	0.3	1.4	1.3	5.2
	Overall after start of exposure (DAT 1 to DAT 26)	0.6	2.6	0.2	1.1	0.8	4.2
Brood termination rate [%]²	Eggs (BFD 22) ³	14.2	3.3	11.1	5.8	19.2	10.7
Brood-index²	Eggs (BFD 22) ³	4.3	0.2	4.4	0.3	4.0	0.5
Brood compensation index²	Eggs (BFD 22) ³	4.4	0.2	4.6	0.2	4.2	0.3

¹⁾ mean of 4 replicates; ²⁾ dead honeybees found in dead bee trap; ³⁾ at the last relevant assessment when development is expected to be completed, i.e. BFD 22 for marked eggs; * = statistically significant different when comparing treatment against control via Student or Welch t-test at post-application phase; one-sided greater: mortality, brood termination rate; one-sided smaller, brood index and brood compensation index. The percent-values of the brood termination rate were arcsine- transformed to ensure the homogeneity of the data before conducting the t-test procedure.

Table 9.3.1-6: Overview of main endpoints for the reference item

Evaluation / Assessment		Treatment group	
		Reference item (1.28 g a.i./kg)	
		mean ¹	SD
Adult mortality² [bees/colony/day]	Pre-exposure phase (DAT -4 to DAT 0)	8.5	2.6
	Exposure phase (DAT 1 to DAT 9)	8.8	6.4
	Post-exposure phase (DAT 10 to DAT 26)	10.8*	7.4
	Overall after start of exposure (DAT 1 to DAT 26)	10.1	7.1
Pupal mortality² [bees/colony/day]	Pre-exposure phase (DAT -4 to DAT 0)	0.0	0.0
	Exposure phase (DAT 1 to DAT 9)	0.0	0.0
	Post-exposure phase (DAT 10 to DAT 26)	16.2	14.7
	Overall after start of exposure (DAT 1 to DAT 26)	10.8	14.1
Brood termination rate [%]²	Eggs (BFD 22) ³	85.1*	11.0
Brood-index²	Eggs (BFD 22) ³	0.8*	0.6
Brood compensation index²	Eggs (BFD 22) ³	0.8*	0.6

¹) mean of 4 replicates; ²) dead honeybees found in dead bee trap; ³) at the last relevant assessment when development is expected to be completed, i.e. BFD 22 for marked eggs; * = statistically significant different when comparing treatment against control via Student or Welch t-test at post-application phase; one-sided greater: mortality, brood termination rate; one-sided smaller, brood index and brood compensation index. The percent-values of the brood termination rate were arcsine- transformed to ensure the homogeneity of the data before conducting the t-test procedure.

Bee Behaviour

The exposure of bee colonies to the test item pydiflumetofen did not result in any honeybee behavioural abnormalities or signs of intoxication up to DAT 26 in the control or any of the test item treatment groups.

Food consumption

There was no evidence of unpalatability of the test diet. A summary of nominal dose to consumed dose is shown in the table below.

Summary actual intake	Nominal dose ¹		Daily mean consumed dose	Cumulative consumed dose
	Daily	Cumulative		
Nominal concentration	[mg a.i./colony]	[mg a.i./colony]	[mg a.i./colony]	[mg a.i./colony]
Control untreated	-	-	-	-
Test item I 2.50 mg a.i./kg (2.94 mg a.i./L)	1.471	13.239	1.458	13.125
Test item II 5.00 mg a.i./kg (5.88 mg a.i./L)	2.942	26.478	2.923	26.311
Test item III 10.5 mg a.i./kg (12.4 mg a.i./L)	6.178	55.604	6.091	54.816
Test item IV 20.0 mg a.i./kg (23.5 mg a.i./L)	11.768	105.912	11.616	104.542
Test item V 32.0 mg a.i./kg (37.7 mg a.i./L)	18.829	169.459	18.676	168.084
Reference item 1274 mg a.i./kg (1500 mg a.i./L)	750.0	6750	747.532	6727.8

¹ 0.5 L test diet was applied each colony daily over 9 days.

Colony strength

Before the start of exposure, the mean estimated colony strength on BFD 0 (DAT -2) was comparable and there were no statistically significant differences (Student t-test, one-sided (less), $p > 0.05$) between the control and any treatment including the reference item.

During the first brood cycle through BFD 22 (DAT 20) the colonies developed in a similar way, in terms of colony strength, in the test item treatment groups I-V compared to the control; no statistically significant differences (Student t-test, one-sided (less), $p > 0.05$) were determined. At BFD 22, the means were 14288 (+28 %), 15131 (+23 %), 14738 (+25 %), 13978 (+17 %), 14963 (+33 %), 15863 (+36 %) in the control, test item I, II, III, IV, V, respectively.

During the following two brood cycles through BFD 63 (DAT 61) colony strength continued to develop in a comparable way between the control and all test item treatment groups without any significant differences (Student t-test, one-sided (less), $p > 0.05$). The final means were 14456 (+30 %), 15919 (+30 %), 15441 (+31 %), 15806 (+33 %), 16622 (+47 %) and 15694 (+34 %) in the control and test item treatment groups I, II, III, IV, V, respectively.

In the reference item group, the mean estimated colony strength after the first brood cycle was 11784 bees/colony (+5 %); a similar level compared to BFD 0. In the following two brood cycles through to BFD 63 (DAT 61) the colony strength decreased by -67 % to 3741 bees/colony and demonstrated a statistically significant decrease (Student t-test, one-sided (less), $p < 0.05$) in colony strength.

With respect to the test item concentration, no dose response relationship was observed in the test item treatment groups I-V. Therefore, it is assumed the test item had no adverse effects on the colony strength after chronic oral treatment with ≤ 32.0 mg a.i./kg.

General brood/colony development

The mean brood area (total area occupied by eggs, larvae and pupae) before application (BFD 0/DAT -2) was on a comparable level across all treatment groups and no statistical differences were determined between the control and any of the treatment groups including the reference item. The entire brood area was 10043, 10159, 9592, 9991, 10224, 9927 and 9527 cm²/colony for the control, test item I, II, III, IV, V and reference item, respectively.

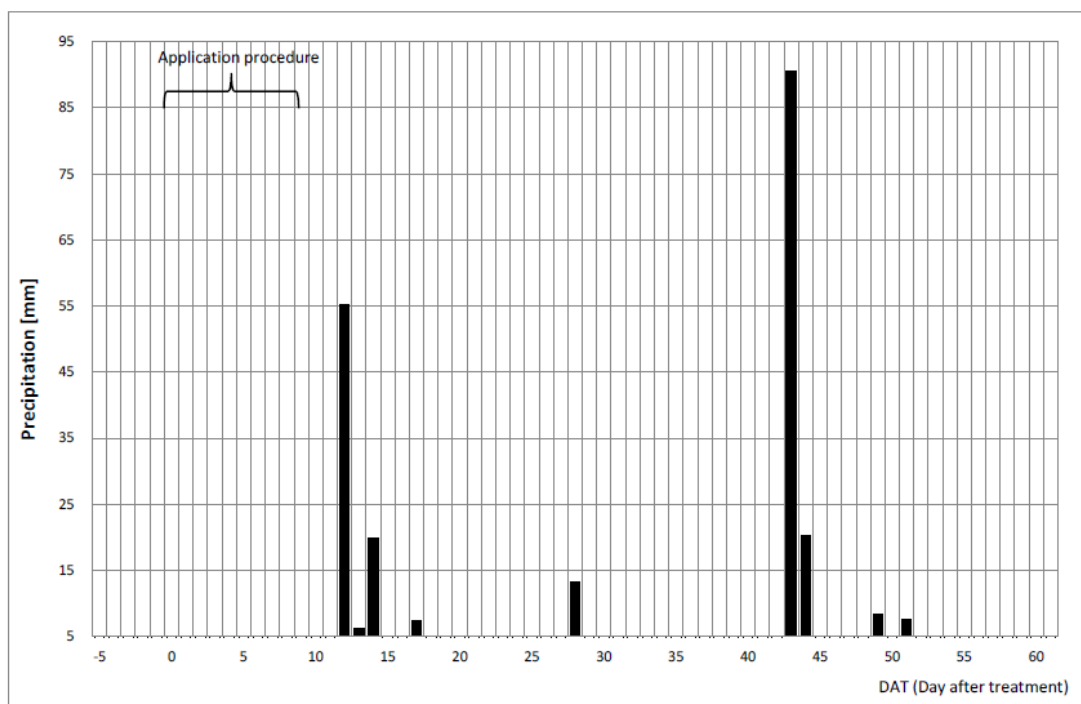
The estimated mean brood area for the test item groups I-V remained on a similar level during the first brood cycle compared to BFD 0 and showed no statistically significant differences compared to the control (Student t-test, one-sided (less), $p > 0.05$). The mean values for the test item I, II, III, IV and V were 10701 (+5 %), 9037 (-6 %), 9282 (-7 %), 10120 (-1 %) and 9579 cm²/colony (-4 %), respectively, and similar to the control 9179 cm²/colony (-9 %). This development and slight decrease in the brood nest occurred until BFD 63 (DAT 61) and was 8212 (-18 %), 8921 (-12), 6678 (-30), 8689 (-13 %), 6910 cm²/colony (-30 %) for the control, test item I, II, III, IV and V, respectively, when compared with the values at BFD 0. There was no statistically significant difference (Student t-test, one-sided (less), $p > 0.05$) between control and test item treatment groups I-V. Therefore, this slight decrease in brood nest is due to the seasonal shift of brood activity at this time of year (July/August).

In contrast the reference item treatment revealed distinctly reduced levels of the estimated mean brood nest area within the first brood cycle until BFD 22 (by -79 % to 2037 cm²/colony) and was maintained at a similar level low level of 1624 (-83 %) by BFD 63. Therefore, statistically significant reduction (Student t-test, one-sided (less), $p < 0.05$) compared to the control was observed from BFD 9 until the last assessment at BFD 63.

The mean estimated food stores (pollen and nectar) on DAT -2/BFD 0 were similar and a sufficient supply (without starvation) was available in all colonies. The following assessments determined sufficient food supplies and no limitation during the course of the experimental phase through to the end of the study (DAT 61).

Climate conditions

During pre-exposure from DAT -4 to DAT 0, it was warm in the daytime and precipitation occurred on single day at a low level. Thus, the climate conditions were good for bee colonies (with respect to foraging activity) to get familiar with the environment at the monitoring site. During application/exposure phase from DAT1 to DAT 9 it was warm and there were few days of rainfall on a low level, which occurred during the early exposure phase. During the post-exposure phase from DAT 10 to DAT 61, it was warm and some precipitation was observed which was on a more intensive level on DAT 12, DAT 14, DAT 43 and DAT 44. The precipitation data recorded by the climate station over the test duration is displayed in the figure below.

Figure 9.3.1-1: Climate data- precipitation (climate station, non-GLP):

The environmental conditions during the test recorded by the climate station and data logger are summarised in the Tables below:

Table 9.3.1-7: Environmental conditions during the entire test (DAT-5 to DAT 61)- Climate station (non-GLP)

	minimum	maximum	average/total
Air temperature	7.2 °C	32.7 °C	19.1 °C
Precipitation	0.0 mm	90.6 mm	287.3 mm
Relative air humidity (daily mean)	29.0 %	96.0 %	67.4 %

Table 9.3.1-8: Environmental conditions during the entire test (DAT -5 to DAT 61)- Data logger (GLP)

	minimum	maximum	average/total
Air temperature	6.8 °C	33.9 °C	19.1 °C
Relative air humidity (daily mean)	33.9 %	99.9 %	78.6 %

Analytical verification of feeding solutions

Recoveries of SYN545974 in the feeding solutions were in the range of 81%-95%. No active ingredient was detected in any control samples. The analytical results are summarised in the table below:

Table 9.3.1-9: Analytical results

Treatment group	Nominal concentration (mg a.s./kg)	Mean recovery (%)
Control	0.00	< LOQ
Test item I	2.39	87
Test item II	4.77	91
Test item III	10.34	83
Test item IV	19.70	87
Test item V	31.52	83

VALIDITY CRITERIA

The study is considered to be valid:

- Mean brood termination of initially marked eggs of the reference item treatment was 85.1 % at the end of the first brood cycle (day 22) and was therefore significantly higher compared to the control which was 10.3 %
- Pupal mortality in the reference item treatment was increased from day 9 until the last assessment at day 61. In contrast, no dead pupae were found in the control during the entire course of the study.

CONCLUSIONS

The purpose of this study was to investigate potential effects of pydiflumetofen on honeybee (*Apis mellifera* L.) brood following chronic oral exposure via in-hive application of treated sucrose solution according to the method described by Oomen *et al.* (1992) under field conditions where bees are free-flying.

Honeybees were exposed to five different concentrations of the test item in 50 % w/v sucrose solution containing 2 % v/v acetone. The bee colonies were exposed to concentrations ranging from 2.50 mg pydiflumetofen/kg food (test item I) to 32.0 mg pydiflumetofen/kg food (test item V). An untreated control and reference item treatment were also included.

Following exposure, no adverse effects on adult bee mortality and behaviour were observed between any of the test item treatments and the control. An increase of pupal mortality occurred on single days but not in all replicates in all treatments. No differences in colony strength and general brood development were observed between any of the test item treatments and the control during the entire course of the study. The detailed bee brood development assessment of initially labelled eggs revealed no impact of the test item on brood development during the entire trial.

Therefore, it can be concluded that the test item provided to honeybees at a rate of ≤ 32.0 mg pydiflumetofen/kg food has no effects on the above mentioned endpoints in this field study. Overall, based on the results of this study, pydiflumetofen via oral exposure to honeybees does not adversely affect colony development and survival.

(██████, 2018)

HSE evaluator comments

This study was conducted in accordance with GLP and was based broadly on ‘The Oomen method’ EPPO 22 (1992)⁶ guidelines and the modified ‘Oomen method’ from the 2013 EFSA guidance document on the risk assessment of plant protection products on bees⁷. Some of the brood assessment measurements (e.g. brood index and brood compensation index) are requirements of OECD 75 (2007)⁸. No validity criteria are specified in these guidelines, however the applicant has cited the significantly higher brood termination rate of the reference item treatment (85.1 %) compared to the control (10.3 %) and increased pupal mortality from day 9 to 61 in the reference treatment compared to the control (1123 dead pupae in reference vs 0 dead pupae in control) as criteria to confirm the study validity. The reference item Insegar was used at a rate of 1.28 g a.i./kg, but no justification was provided as to why this rate was used. It is noted that the OECD 75 (2007) honeybee brood test guidance recommends the use of Insegar ‘at a rate of at least 600 g/ha corresponding to 150 g Fenoxycarb/ha’, however the units g a.s./kg and g a.s./ha are not strictly comparable and it is possible that less reference item would be required to achieve a similar effect when being applied as a feed solution as compared to a sprayed crop. As the suitability of the reference item dose cannot be confirmed and no specific validity criteria are provided for the Oomen method, it is not possible to verify the validity of this study.

The following guideline deviations are noted. Firstly EPPO (1992) states the application of the test item should be in 1 L of 50 % sucrose solution, whereas in this study 0.5 L of 50 % sucrose solution has been used. No justification is provided for this deviation. Secondly, in EPPO (1992), the test item is provided as a single application, whereas in this study the test item has been provided daily for 9 days. However, the 2013 EFSA guidance document on the risk assessment of plant protection products on bees contains information on a modified version of the ‘Oomen method’, in which daily feeding is recommended to ensure all larval stages are exposed. Therefore, the mode of treatment use in this study is considered to be acceptable. Finally, although acetone is considered to be a suitable solvent, and its volume has been kept to 2 %, which is in line with recommendations for other larval bee studies (such as OECD 239/237), no solvent control group has been tested. This means it is not possible to differentiate potential effects of the solvent from effects of the test item and raises uncertainty over the reliability of the endpoints derived.

Additionally, the following omissions from the study report are noted. It is stated that no agricultural or flowering crops were present in the surrounding area, but it is not stated how large the measured surrounding area is. This is particularly pertinent considering the foraging range of honeybees can reach several kilometres (██████████ & ██████████ (1982). Furthermore, the endpoints presented are expressed as mg Pydiflumetofen/ kg food, rather than as a daily dietary dose. Although details of the consumed dose are available (mg a.s./colony/day), they do not account for potential evaporative losses of the test solution and therefore may not accurately reflect the consumption of the test item by the colonies. Although it is acknowledged that evaporative losses may be difficult to measure under field conditions, there did not appear to be any attempt to minimise loss from evaporation throughout the test either.

No dose response was observed for any of the parameters measured, however significant effects on adult mortality compared to the control were determined in treatment groups I, III IV and V for the post-exposure phase (DAT 10-26) in overall and daily means. The mortalities were classed as ‘low and on a comparable level in all test item groups compared to the control’. Significant effects on single days were attributed to the natural variation of adult bee mortality between colonies. It is noted that during the post-exposure period on DAT 12 and DAT 14 there were days of more intense rainfall (55.2 mm and 19.8 mm, respectively), and DAT 15 recorded a cooler temperature than the rest of the period (14.8 °C, mean DAT 9-61 was 19.2 °C). When considering daily mortality by replicate, there did not appear to be much variation in mean mortalities per replicate within each treatment group. There was a slight decrease in brood nest area across all treatment levels and the control until DAT 61. This decrease was attributed to a seasonal shift in brood activity at the time of year (July/August), however no

⁶ Oomen P.A., De Ruliter A. and van der Steen J. (1992) Method for honey bee brood feeding tests with insect growth-regulating insecticides. Bulletin OEPP/EPPO Bulletin, 22 613–616 (1992).

⁷ EFSA Journal 2013;11(7):3295 pp 211-212

⁸ OECD 75: ENV/JM/MONO(2007)22 OECD Environment, Health and Safety Publications Series on Testing and Assessment No. 75 Guidance Document on the honey bee (*Apis mellifera* L.) brood test under semi-field conditions. Environment Directorate Organisation for Economic Co-operation and Development.

supporting information has been provided to corroborate this claim. Overall, these effects are not considered to be dose related.

Statistical analysis was conducted for mortality, brood termination rate (%; arcsine-transformed), brood index and brood compensation index. Pre-treatment data were evaluated using multiple testing methods (Tukey test) and post-treatment data were evaluated using pair-wise comparisons (Student T-test for homogenous variances and Welch t-test for non-homogenous variances). There are no specified statistical methods in any of the guidelines used, however the procedures used are in line with the OECD 54 (2006)⁹ guidance document. No statistical analysis was conducted on pupal mortality with the study report stating, “Due to no pupal mortality occurring in the control group on all days, no statistical comparison was performed”. However, statistical comparison does seem to have been conducted between the control and reference treatment pupal mortality, and the study report states, “The sum mortality per colony between DAT 1 to DAT 26 ranged from 195-375 dead pupae (10.8 dead pupae/colony/day), which was significantly increased in comparison to the control (0 dead pupae/colony/day)”. Furthermore, dead pupae were observed in all treatment groups in the post-exposure period in single replicates for treatments I, II and III and in two replicates for treatment groups IV and V, and the study report states there is “presumed an effect of higher concentrations on the pupal mortality even though not all replicates were affected”. It is therefore unclear why treatment groups I-V were not statistically analysed for pupal mortality, and the lack of mortality in the control group is not considered adequate justification.

As there was > 80 % recovery of the test item in the analytical phase, it is considered acceptable to base endpoints on nominal test concentrations. The analytical method has been evaluated by HSE Chemistry specialists in Vol. 3 CA Part B5.1.2.6. The following was concluded for this method: “Acceptable method. LOQ : 0.005 mg/kg”. The endpoint below is suitable for use in risk assessment, noting uncertainties discussed above:

- No effect on colony development or survival up to 32 mg SYN545974/ kg food (18.7 mg SYN545974/colony/day).

B.9.3.2. Effects on non-target arthropods other than bees

Not relevant. Studies for non-target arthropods other than bees have been conducted on the formulated product A21857B, please see the 3CP-B9 section for further details.

B.9.4. EFFECTS ON NON-TARGET SOIL MESO- AND MACROFAUNA

B.9.4.1. Earthworm – sub-lethal effects

No sub-lethal study with the active substance was submitted for earthworms. The following acute toxicity study was submitted, however this was not evaluated as it is not a data requirement under 283/2013 and was not used in the risk assessment.

Report:	K-CA 8.4. [REDACTED] (2012), SYN545974 – Acute toxicity to the earthworm <i>Eisenia fetida</i> Report No. 12 10 48 076 S Document No. VV-401989 , SYN545974_10008
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B.9.4.2. Effects on non-target soil meso- and macrofauna (other than earthworms)

No studies with the active substance were submitted for -target soil meso- and macrofauna (other than earthworms).

⁹ OECD 54 ENV/JM/MONO(2006)18 OECD Environment Health and Safety Publications Series on Testing and Assessment No. 54 Current Approaches In The Statistical Analysis Of Ecotoxicity Data: A Guidance To Application

B.9.5. EFFECTS ON SOIL NITROGEN TRANSFORMATION

Report: K-CA 8.5, [REDACTED] (2015) SYN545974 - Effects on the Activity of Soil Microflora (Nitrogen and Carbon Transformation Tests), Report Number 15 10 48 111 C/N. BioChem agrar Labor für biologische und chemische Analytik GmbH Kupferstraße 6 04827 Gerichshain, Germany. (Syngenta file No. SYN545974_10275)

Guidelines

- OECD Guideline for Testing of Chemicals Method 216 Soil Microorganisms: Nitrogen Transformation Test (2000)
- OECD Guideline for Testing of Chemicals Method 217 Soil Microorganisms: Carbon Transformation Test (2000)

GLP: Yes

Materials

Test Material SYN545974 tech.
Lot/Batch #: SMU2EP12007
Purity: 98.5 % w/w
Description: White powder
Stability of test compound: Stable under test conditions
Reanalysis/Expiry date: End of June 2016
Density: NA

Treatments

Test rates: 0.54 and 2.71 mg a.s./kg dry soil (corresponding to 0.41 and 2.03 kg a.s./ha respectively)
Control: Untreated
Toxic standard: Dinoterb

Test design

Soil type: Loamy sand
Test units: Nitrogen transformation test: 500 mL wide mouth glass flask containing 200 g soil d.w.
Carbon transformation test: 4 L steel test vessels containing 1000 g soil dry weight.
Replication: 3
Sampling intervals: 3 hours, 7, 14, and 28 days after application
Duration of test: 28 days

Environmental test conditions

Temperature: 19.8 - 21.2 °C
pH of soil: 6.4
Soil moisture content: Nitrogen transformation test: 16.28 - 17.24 g/100 g soil d.w. (equivalent to 45.75 - 48.44 % of WHC)
Carbon transformation test: 16.99 - 17.78 g/100 g soil d.w. (equivalent to 47.75 - 49.95 % of WHC)
Photoperiod: Constant darkness

Study Design and Methods

Experimental dates: 31 July to 28 August 2015

Soil samples were treated with SYN545974 at two doses, 0.54 and 2.71 mg /kg dry soil. Test concentrations related to a soil depth of 5 cm and a soil density of 1.5 g/cm³.

The test item was mixed with deionised water and the test solution was subsequently mixed with the soil in the laboratory mixer. Water was added to the soil to achieve a water content of approximately 45 % of WHC. The water content of the soil in each test vessel was determined at test start (after application) and adjusted once a week to the required range of 40 - 50 % of WHC.

Three replicate soil samples were prepared for each treatment rate and the control for the nitrogen transformation test and carbon transformation test.

Mean nitrogen content (mg NO₃/kg soil d.w.), standard deviation and coefficient of variation as well as the mean nitrogen content/day (mg NO₃/kg soil d.w./day) were calculated for each treatment group and sampling date.

For the evaluation of the results the relative deviations (%) of the test item treatment groups from the control were calculated (based on the mean nitrogen content/day) for each sampling date.

The cumulative O₂-consumption after 12 hours was calculated (using regression analysis; the goodness of fit (R²) was > 0.99 in all replicates and on all days).

Furthermore, standard deviation and coefficient of variation were calculated for each treatment group and sampling dates. For evaluation of the results the relative deviations (%) of the test item treatment groups from the control were calculated for each sampling date. Statistical evaluation of the test results (2-sided Student-t-test at 5 % significance level) was performed.

Results and Discussion

Results from the Nitrogen transformation test and the Carbon transformation test are summarised in the tables below.

Table 9.5-1: Effects on Nitrogen Transformation in Soil after Treatment with the Test Item

Time Interval (days)	Control		0.54 mg a.s./kg soil dry weight			2.71 mg a.s./kg soil dry weight		
	NO ₃ -N (mg/kg soil d.w.)	NO ₃ -N (mg/kg soil d.w./day)	NO ₃ -N (mg/kg soil d.w.)	NO ₃ -N (mg/kg soil d.w./day)	Deviation from control (%) ¹⁾	NO ₃ -N (mg/kg soil d.w.)	NO ₃ -N (mg/kg soil d.w./day)	Deviation from control (%) ¹⁾
0 - 7	43.6	3.97	45.9	4.30	+8.4	47.0	4.50	+13.4
0 - 14	62.4	3.33	60.1	3.16	-4.9	62.6	3.37	+1.2
0 - 28	78.6	2.24	77.8	2.22	-1.3	75.5	2.15	-4.4
7 – 14*	-	2.69	-	2.02	-24.65	-	2.23	-16.84
14 – 28*	-	1.16	-	1.27	+9.24	-	0.92	-20.33

The calculations were performed with non-rounded values

1) based on NO₃-nitrogen-production; - = inhibition; + = stimulation

No statistically significant differences between the control and the test item treatments were calculated.

* Values in these rows have been calculated by HSE.

Table 9.5-2: Effects on Carbon Transformation in Soil after Treatment with the Test Item

Days after application	Control		0.54 mg a.s./kg soil dry weight			2.71 mg a.s./kg soil dry weight		
	O2-consumption (mg/kg soil d.w./h)	CV (%)	O2-consumption (mg/kg soil d.w./h)	CV (%)	Deviation from control (%) ¹	O2-consumption (mg/kg soil d.w./h)	CV (%)	Deviation from control (%) ¹
0	17.96	1.5	17.50	2.2	-2.5	16.38*	0.8	-8.8
7	15.91	1.2	15.07*	2.1	-5.3	14.49*	0.9	-8.9
14	15.27	0.9	14.48	3.8	-5.2	13.95*	0.3	-8.6
28	14.28	1.1	14.08	1.3	-1.4	13.56*	2.5	-5.1

The calculations were performed with non-rounded values.

CV [%] = Coefficient of Variation

¹ Based on O₂-consumption; - = inhibition; + = stimulation

* = statistically significantly different to control (Student-t-test for homogeneous variances, 2-sided, p ≤ 0.05)

Validity Criteria

The validity criteria were met:

- The coefficient of variation in the Nitrogen and Carbon transformation tests were 4.5 and 1.5 % respectively (must be ≤ 15 %)
- The toxic standard caused effects of +33.2 % and +46.9 % at concentrations 16.00 and 27.00 mg /kg soil d.w. in the Nitrogen transformation test, demonstrating the sensitivity of the test system (must be ≥ 25 %)
- The toxic standard caused effects of -30.1 % and -39.6 % at concentrations 16.00 and 27.00 mg /kg soil d.w. in the Carbon transformation test, demonstrating the sensitivity of the test system (must be ≥ 25 %)

Conclusions

The test item SYN545974 (tested at 0.54 mg a.s./kg dry soil corresponding to 0.41 kg a.s./ha and 2.71 mg a.s./kg dry soil corresponding to 2.03 kg a.s./ha) caused no adverse effects on soil nitrogen transformation (measured as NO₃-N-production) and on soil carbon transformation (measured as O₂-consumption) at the end of the 28-day incubation period.

(██████, 2015)

HSE evaluator comments

This study was conducted to GLP and follows guidelines OECD 216 (2000). The study has been assessed against this guideline. The study includes data on carbon transformation in addition to nitrogen transformation but since carbon transformation data is no longer a data requirement this part of the study has not been considered.

The following points are noted:

- A test with a reference item successfully demonstrates the sensitivity of the test system. This reference test was done on different, earlier batch of soil but taken from the same test site as the test soil so is deemed to be adequately representative.
- The time periods for calculation of nitrogen transformation rates were expressed as days 0-7, 0-14 and 0-28, whereas the necessary time periods are 0-7, 7-14 and 14-28. HSE calculated the nitrogen transformation rates for these time periods and added them to the summary report. These rates still fall within the guidelines of conducting the study to no more than 28 days as they are ≤ 25 % effects compared to control in the last time period.

Therefore, the agreed end-point for use in risk assessment is no effects on nitrogen transformation rate, greater than or equal to ± 25 %, were observed by day 28 at up to 2.71 mg a.s./kg dry soil.

Report: K-CA 8.5 [REDACTED], (2017) SYN545974 – Effects on the Activity of Soil Microflora (Nitrogen and Carbon Transformation Tests), Report Number 17 48 SMO 0015. BioChem agrar, Labor für biologische und chemische, Analytik GmbH, Kupferstraße 6, 04827 Gerichshain, Germany.
(Syngenta file No. SYN 545974_10535).

Guidelines

OECD guidelines 216, Soil Microorganisms: Nitrogen Transformation Test (2000)

OECD guidelines 217, Soil Microorganisms: Carbon Transformation Test (2000)

GLP: Yes

Materials

Test Material Pydiflumetofen - SYN545974
Lot/Batch #: SMU2EP12007
Purity: 98.5 % w/w
Description: off-white powder
Stability of test compound: Stable under test conditions
Reanalysis/Expiry date: End of April 2020

Treatments

Test rates: 5.4 mg test item/kg soil dry weight and 13.5 mg test item/kg soil dry weight
Control: untreated
Toxic standard: Dinoterb

Test design

Soil type: loamy sand , pH 6.6, 1.48 % C_{org}, Microbial biomass 3.96 % of C_{org}
 WHC: 38 g/100 g dry soil
Test units: Nitrogen: 500 mL wide mouth glass flask
 Carbon: steel test vessels (4 L)
Replication: 3
Sampling intervals : 3 hours, 7, 14, and 28 days after application
Duration of test: 28 days

Environmental test conditions

Temperature: 19.4 - 21.4 °C
pH of soil: 6.6
Soil moisture content: Nitrogen transformation test 17.38 - 18.29 g/100 g soil d.w.
 (equivalent to 45.75 - 48.12 % of WHC)
 Carbon transformation test 17.93 - 18.58 g/100 g soil d.w.
 (equivalent to 47.18 - 48.89 % of WHC)

Photoperiod: Constant darkness

Experimental dates: 10 May 2017 to 07 June 2017

Study Design and Methods

Soil samples were treated with the SYN545974 at two doses, 5.4 and 13.5 mg SYN545974/kg dry soil. Test concentrations related to a soil depth of 5 cm and a soil density of 1.5 g/cm³.

The test item was mixed with quartz sand, which was subsequently added to the soil and mixed with a hand stirrer. The test item quartz sand mixture was applied at a ratio of about 10g per kg soil dry weight. Water was added to

the soil to achieve a water content of approximately 45 % of WHC. The water content of the soil in each test vessel was determined at test start (after application) and adjusted once a week to the required range of 40 - 50 % of WHC.

Three replicate soil samples were prepared for each treatment rate and the control for the nitrogen transformation test and carbon transformation test. 200g of soil dry weight were weighed into each vessel and then placed into a mixing vessel. Lucerne meal (5g/kg soil dry weight) was then added to each 200g soil sample.

Soil samples (10g soil d.w. per replicate) were taken at intervals of 3 hours, 7, 14, and 28 days after application. The $\text{NH}_4\text{-N}$ -, $\text{NO}_3\text{-N}$ -, and $\text{NO}_2\text{-N}$ - contents were determined by adding 50mL 1M KCL to 10g soil d.w. and mixed in a rotator at 150 rpm for 60 minutes. The mixtures were centrifuged and stored deep-frozen prior to analysis at $-20^\circ\text{C} \pm 5^\circ\text{C}$.

Samples were analysed within one week after day 28, using an Autoanalyzer. The LOQ for $\text{NO}_3\text{-N}$ -, $\text{NH}_4\text{-N}$ -, and $\text{NO}_2\text{-N}$ were 0.84 mg/100 g soil d.w., 0.09 mg/100 g soil d.w., and 0.14 mg/100 g soil d.w. respectively.

Mean nitrate content (mg NO_3 /kg soil d.w.), standard deviation and coefficient of variation as well as the mean nitrate content/day (mg NO_3 /kg soil d.w./day) were calculated for each treatment group and sampling date.

For the evaluation of the results the relative deviations (%) of the test item treatment groups from the control were calculated (based on the mean nitrate content/day) for each sampling date.

Statistical evaluation of the test results (2-sided Student-t-test at 5 % significance level) was Performed.

The cumulative O_2 -consumption after 12 hours was calculated (using regression analysis; the goodness of fit (R^2) was > 0.97 in all replicates and on all days).

Furthermore, standard deviation and coefficient of variation were calculated for each treatment group and sampling date.

For evaluation of the results the relative deviations (%) of the test item treatment groups from the control were calculated for each sampling date.

Statistical evaluation of the test results (2-sided Student-t-test at 5 % significance level) was Performed.

Results and discussion

Results from the Nitrogen transformation test and the Carbon transformation test are summarised in the tables below.

No differences of greater than 25 % were found for the tested concentrations of the test item at the end of the 28-day incubation period in comparison to the respective controls.

Table 9.5-3: Effects on Nitrogen Transformation in Soil after Treatment with the Test Item

Interval (days)	Control		5.4 mg test item/kg soil dry weight			13.5 mg test item/kg soil dry weight		
	$\text{NO}_3\text{-N}$ [mg/kg soil d.w.]	$\text{NO}_3\text{-N}$ [mg/kg soil d.w./day]	$\text{NO}_3\text{-N}$ [mg/kg soil d.w.]	$\text{NO}_3\text{-N}$ [mg/kg soil d.w./day]	Deviation from control [%] ¹⁾	$\text{NO}_3\text{-N}$ [mg/kg soil d.w.]	$\text{NO}_3\text{-N}$ [mg/kg soil d.w./day]	Deviation from control [%] ¹⁾
0-7	62.4	5.04	65.1	5.54	+10	68.4	5.90*	+17
0-14	74	3.35	74.6	3.45	+3.1	76.1	3.5	+4.3
0-28	101.6	2.66	103.2	2.75	+3.3	103.4	2.72	+2.4

The calculations were performed with non-rounded values.

¹⁾ based on $\text{NO}_3\text{-N}$ products; - = inhibition, + = stimulation

* = statistically significant differences between the control and the test item treatments were calculated (student-t test for homogenous variances, 2-sided, $p \leq 0.05$).

Table 9.5-4: Effects on Carbon Transformation in Soil after Treatment with the Test Item

Days after application	Control		5.4 mg test item/kg soil dry weight			13.5 mg test item/kg soil dry weight		
	O ₂ -consumption (mg/kg soil d.w./h)	CV (%)	O ₂ -consumption (mg/kg soil d.w./h)	CV (%)	Deviation from control (%)	O ₂ -consumption (mg/kg soil d.w./h)	CV (%)	Deviation from control (%)
0	26.15	0.8	25.75	0.6	-1.5	24.97*	1.9	-4.5
7	23.53	2	22.84	1.5	-2.9	21.95*	1	-6.7
14	20.57	0.9	20.47	1.3	-0.5	20.06*	0.5	-2.5
28	16.58	1.4	15.77*	1.4	-4.9	15.51*	1.7	-6.4

The calculations were performed with non-rounded values.

- = inhibition; + = stimulation

* = statistically significant differences between the control and the test item treatments were calculated (Student-t-test for homogeneous variances, 2-sided, $p \leq 0.05$).

Validity criteria

The validity criteria are listed below:

- The coefficient of variation in the Nitrogen and Carbon transformation tests were 7.5% and 2 % respectively (must be less than ± 15 %)
- The toxic standard caused effects of +38.6 % and +51.8 % at concentrations 13.60 mg and 27.20 mg /kg soil d.w. in the Nitrogen transformation test, demonstrating the sensitivity of the test system (must be ± 25 %)
- The toxic standard caused effects of -34.8 % and -43.8 % at concentrations 13.60 mg and 27.20 mg /kg soil d.w. in the Carbon transformation test, demonstrating the sensitivity of the test system (must be ± 25 %)

Conclusions

The test item SYN545974 (tested at 5.4 mg/kg soil dry weight and 13.5 mg/kg soil dry weight) caused < 25 % effects compared to control on soil nitrogen transformation (measured as NO₃-N-production) and on soil carbon transformation (measured as O₂-consumption) at the end of the 28-day incubation period.

(████████, 2017)

HSE evaluator comments

HSE has evaluated the Nitrogen Transformation test according to OECD 216 (2000), however, as the Carbon Transformation test is no longer a requirement, this has not been evaluated.

Validity criteria according to OECD 216 (2000)	Obtained in this study
In the control(s), the coefficients of variation for NO ₃ must be <15%	7.5 %

The study was carried out according to GLP and follows OECD 216 (2000) with no significant deviations to the guideline or the study plan. All validity criteria outlined in OECD 216 (2000) have been satisfactorily met.

A toxic reference item test is not required in OECD 216 (2000) but Dinoterb was tested in a separate study and showed an effect of + 38.6 % and 51.8 % on the nitrogen transformation when tested at concentrations of 13.6 mg and 27.2 mg Dinoterb/kg soil dry weight.

Mean nitrogen content, standard deviation and coefficient of variation were calculated for each treatment group and sampling date. This is in line with recommendations in OECD 216 (2000).

The agreed endpoint suitable for use in the risk assessment is:

- < 25 % effects on nitrogen transformation rate compared to control at 13.5 mg active substance/kg dry soil

B.9.6. EFFECTS ON TERRESTRIAL NON-TARGET HIGHER PLANTS

B.9.6.1. Summary of screening data

No active substance data was submitted testing non-target plants. Please refer to 3CP Part B9.11.

B.9.6.2. Testing on non-target plants

No active substance data was submitted testing non-target plants. Please refer to 3CP Part B9.11.

B.9.7. EFFECTS ON OTHER TERRESTRIAL ORGANISMS (FLORA AND FAUNA)

No data submitted or required.

B.9.8. EFFECTS ON BIOLOGICAL METHODS FOR SEWAGE TREATMENT

Report: K-CA 8.8 [REDACTED] (2013), SYN545974 - Toxicity to Activated Sludge in a Respiration Inhibition Test, Report Number D64647, Harlan Laboratories Ltd., Zelgliweg 1, 4452 Itingen, Switzerland. (Syngenta File No. SYN545974_10061)

GUIDELINES

OECD Guidelines for Testing of Chemicals, Method 209: Activated Sludge, Respiration Inhibition Test (Carbon and Ammonium Oxidation) (2010).

MATERIALS

Test Material	SYN545974 technical
Lot/Batch #:	SMU2EP12007
Purity:	98.5% (w/w)
Treatments	
Test concentrations:	Blank and positive controls; nominal concentrations of 10, 100 and 1000 mg a.s./L
Solvent:	None
Blank control:	Equal volume of activated sludge and synthetic medium, no test or reference item.
Positive control:	3,5-dichlorophenol at nominal concentrations of 3.2, 10 and 32 mg a.s./L; based on the findings the 3-hour EC ₅₀ for total respiration was calculated to be 8 mg a.s./L.
Analysis of test concentrations:	No
Test organism	
Organisms:	Aerobic activated sludge microorganisms, nominally 3 g/L dry weight, equivalent to 1.5 g/L in the final incubation mixture

Source:	A wastewater treatment plant (ARA Ergolz II, Füllinsdorf, Switzerland) treating predominantly domestic wastewater, and held at the test facility for two days at room temperature under continuous aeration prior to use
Test design	
Test vessels:	2000-mL glass beakers containing 500 mL of incubation mixture BOD-flasks for O ₂ measurements
Replication:	One replicate for each of the 10 and 100 mg a.s./L test concentrations and the positive controls, 3 replicates for the 1000 mg a.s./L test concentration, and 4 replicates for the blank control.
Exposure regime:	Static
Environmental conditions	
Test temperature:	Test start: 19 °C Test end: 20 °C
pH:	Test start: 7.2 - 7.8 Test end: 7.6 - 8.0
Aeration:	Continuous aeration provided by intense stirring on magnetic stirrers
Oxygen:	Test start: 7.9 - 8.4 mg O ₂ /L Test end: 7.8 - 8.5 mg O ₂ /L

STUDY DESIGN AND METHODS

Experimental dates: 21 to 23 January 2013

Due to the low solubility of the test material no stock solution could be prepared. Instead, weighed amounts of the test item (5.09, 50.02, 500.24, 500.74 and 500.24 mg SYN545974) were transferred to the test vessels and mixed into 234 mL of test water (deionized water) by intense stirring for 2 hours at room temperature. No emulsifiers or solvents were used. The appearance of the test medium was evaluated after the 2-hour stirring period and all the test concentrations were clearly above the SYN545974 water solubility limit under test conditions. At time 0, a control flask consisting of 16 mL of synthetic sewage feed, 234 mL of test water and 250 mL of the activated sludge inoculum (3 g solids/L d.w.) was prepared. The sludge was added in time intervals of 15 minutes first to a second control, secondly to the test solutions of the reference item, thirdly to the test suspension of the test item and finally to a further two controls.

The test flasks were continuously aerated throughout the 3-hour incubation period as a result of the intense stirring procedure. After 3 hours, well-mixed samples of test media were poured into BOD-flasks and were not aerated further. The samples were continuously stirred on a magnetic stirrer and the respiration rate measured using an oxygen electrode. The dissolved oxygen concentration was continuously recorded, and the respiration rates calculated. The inhibitory effect of the test item was expressed as percentage of the mean respiration rate of the controls.

RESULTS AND DISCUSSION

The concentration of dissolved oxygen did not drop below 7.8 mg/L during the incubation period. The results from the controls (Coefficient of Variation of the oxygen consumption rates of the four controls: 2.25 %), and the reference item 3-hour EC₅₀ value (8 mg a.s./L) demonstrated the suitability of the sludge used. SYN545974 had no significant inhibitory effect (Student t-test) on the respiration rate of activated sludge after the 3-hour incubation period at any of the tested loading rates. Concentrations of ≥ 10 mg a.s./L were above the water solubility limit of SYN545974 under test conditions as the test item was not completely dissolved in the test medium.

The nominal concentrations were used for reporting of results which are presented in the table below:

Table 9.8-1: Influence of SYN545974 on oxygen consumption of activated sludge in a 3-hour respiration inhibition test

Nominal concentration of test chemical (mg/L)	Oxygen consumption rate		Inhibition ^a (compared to mean control) (%)	pH values		Oxygen concentration (mg O ₂ /L)	
	Respiration rate (R) (mg O ₂ /L*h)	Specific respiration rate (R _s) (mg O ₂ /g*h)		Start	End	Start	End
Control #1	80.91	53.94	NA	7.6	7.6	8.1	7.8
Control #2	81.20	54.13	NA	7.6	7.6	7.9	8.1
Control #3	78.69	52.46	NA	7.2	7.7	8.0	8.0
Control #4	77.49	51.66	NA	7.2	7.7	8.0	8.3
Mean control (SD)	79.57 (1.79)	53.05 (1.19)	-	-	-	-	-
SYN545974: 10	76.11	50.74	4.3	7.3	7.7	8.0	8.0
SYN545974: 100	77.83	51.89	2.2	7.3	7.7	8.0	8.3
SYN545974: 1000	84.51	56.34	-6.2	7.3	7.7	8.0	8.1
SYN545974: 1000	76.00	50.67	4.5	7.3	7.7	8.0	8.1
SYN545974: 1000	76.20	50.80	4.2	7.3	7.7	8.0	8.2
3,5-dichlorophenol: 3.2	53.89	35.93	32.3	7.7	7.9	8.1	8.5
3,5-dichlorophenol: 10	38.85	25.90	51.2	7.8	8.0	8.3	8.3
3,5-dichlorophenol: 32	8.73	5.82	89.0	7.6	8.0	8.4	8.3

^a Negative value = increased oxygen consumption rate relative to mean control value NA = not applicable SD = standard deviation

VALIDITY CRITERIA

The validity criteria outlined in OECD 209 (2010) were fulfilled:

Table 9.8-2: Compliance with OECD 209 validity criteria

Validity criterion	Required	Observed
Respiration rate of blank controls	20 mg O ₂ /gh	52-54 mg O ₂ /gh
Coefficient of variation of oxygen uptake rates in control	< 30 %	2.25 %
Reference item EC ₅₀	2 to 25 mg a.s./L	8 mg a.s./L

CONCLUSIONS

After the incubation period of three hours, SYN545974 had no significant inhibitory effect (<15%) on the respiration rate of activated sludge at concentrations up to and including 1000 mg a.s./L, the highest concentration tested.

The 3-hour EC₂₀, EC₅₀ and EC₈₀ could not be calculated but were clearly > 1000 mg a.s./L, the highest concentration tested.

The 3-hour NOEC was 1000 mg a.s./L.

(■■■■■, 2013)

HSE evaluator comments

This study was a range-finding test, conducted in accordance with GLP and OECD 209 (2010). No statistically significant effects of the test item on respiration rate were detected at the highest tested concentration; 1000 mg SYN545974/L. The EC₅₀ of the reference item 3,5-dichlorophenol for total respiration was found to be 8 mg a.s./L and was therefore within the range of the guideline (2 to 25 mg a.s./L), demonstrating the sensitivity of the test system. The validity criteria outlined in OECD 209 (2010) have been satisfied.

There is some uncertainty since all test concentrations were above the limit of solubility for SYN545974, however the study is considered acceptable, especially considering the lack of effects compared to the control at all test concentrations. According to Vol 3CA Part B2.5, the solubility limit of Pydiflumetofen in water is 1.5 mg/L at 25 °C, pH 6.6.

Determination of the NOEC was conducted statistically using a Student t-test, which is in line with the guidance provided in OECD 209 (2010). EC_x values were not determined statistically due to lack of treatment-related effects.

Given the solubility issues experienced in the study, HSE considers it most appropriate for the endpoints to be defined as greater than the limit of solubility as follows:

- 3-hour NOEC = > 1.5 mg SYN545974/L
- 3-hour EC₅₀ > 1.5 mg SYN545974/L

B.9.9. MONITORING DATA

No data submitted or required.

B.9.10. BIOLOGICAL ACTIVITY OF METABOLITES POTENTIALLY OCCURRING IN GROUNDWATER**Scientific peer-reviewed open literature report**

This document is a Literature Review Report for SYN545974 and metabolites SYN545547, SYN548261, and NOA449410.

Literature reviews have been carried out for the active substance pydiflumetofen (SYN545974) and its metabolites; SYN545547, SYN548261, NOA449410. The reviews have been conducted in accordance with Article 8(5) of Regulation No. 1107/2009 and are based on the EFSA guidance document as published in EFSA Journal 2011; 9(2):2092.

The key objective of the submitted literature review was to establish whether any scientific peer-reviewed open literature published within the last ten years before the date of submission of the dossier would be relevant for the risk assessment of pydiflumetofen and its metabolites. The dates of the searches are shown in table 9.10-1. All available CAS numbers of stereoisomers of pydiflumetofen and metabolites have been included in the search profile.

Top up searches for ecotoxicology and ED (non-target organisms) were conducted by the applicant in August 2022 at the request of HSE to cover until the date of submission and are provided in green highlight.

Table 9.10-1: Dates of the conducted literature searches

Date of initial SYN545974 search	16 April 2015
Date of most recent update to SYN545974 search	9 November 2015
Date of most recent update to SYN545974 search (ecotox and ecotox ED)	02 August 2022 (time window 01 November 2015 – 31 July 2020)
Date of SYN545974 specific metabolites search	16 November 2015
Date of SYN545974 metabolites that are common SDHI metabolites search	17 November 2015
Date span of the search	50-45 years

A summary of the process of selection of relevant scientific peer reviewed open literature was done in the following steps.

- A very broad search was conducted in 18 scientific source databases (detailed in Table 9.5-2) for SYN545974 and its metabolites using the search terms listed in CA 9.5.1.
- Duplicate titles from between the data bases were automatically removed from the output.
- A rapid assessment of the titles was conducted to remove any additional duplicates and any obviously irrelevant titles (where enough information was available from the title alone).
- A further rapid assessment was conducted using summary abstracts and any clearly irrelevant titles were removed.

Databases searched

A total of 18 databases were searched, covering the minimum requirement of 10 years prior to the date of the search **plus a subsequent top up search covering up to the date of submission for 13 databases**. A web search, search for journal table of contents, and a search for reference lists were not conducted as the reported database search was considered to provide an adequately comprehensive search of the quality peer reviewed literature.

Table 9.10–2: Databases searched as part of the pydiflumetofen literature review (ecotoxicology)

Provider	Database	Justification
Host STN	MEDLINE	Contains information on every area of medicine providing comprehensive coverage from 1948 to present. Sources include journals and chapters in books or symposia. The database is updated 5 times each week with an annual reload and therefore stays very current in its cover.
	EMBASE	The database, covers worldwide literature in the biomedical and pharmaceutical fields, including biological science, biochemistry, human medicine, forensic science, pediatrics, pharmacy, pharmacology and drug therapy, pharmacoeconomics, psychiatry, public health, biomedical engineering and instrumentation, and environmental science. Sources include more than 4,000 journals from approximately 70 countries, monographs, conference proceedings, dissertations, and reports. The databases covers data from 1974present and is updated daily.
	ESBIOBASE	A database providing comprehensive coverage of the entire spectrum of biological research worldwide. Coverage includes the following areas: applied microbiology, biotechnology, cancer research, cell & developmental biology, clinical chemistry, ecological & environmental sciences, endocrinology, genetics, immunology, infectious diseases, metabolism, molecular biology, neuroscience, plant and crop science, protein biochemistry, and toxicology. Records are selected from over 1,700 international scientific journals, books, and conference proceedings. The database covers the period 1994 - present and is updated weekly.
	AGRICOLA	A bibliographic database containing selected worldwide literature of agriculture and related fields. Coverage of the database includes agricultural economics and rural sociology, agricultural production, animal sciences, chemistry, entomology, food and

		human nutrition, forestry, natural resources, pesticides, plant science, soils and fertilizers, and water resources. Also covered are related areas such as biology and biotechnology, botany, ecology, and natural history. The database draws on bibliographies, serial articles, book chapters, monographs, computer files, serials, maps, audiovisuals, and reports. It covers the period 1970-present and is updated monthly.
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Provider	Database	Justification
Host STN	BIOSIS	A large and comprehensive worldwide life science database covers original research reports, reviews, and selected U.S. patents in biological and biomedical areas, with subject coverage ranging from aerospace biology to zoology. Sources include periodicals, journals, conference proceedings, reviews, reports, patents, and short communications. Nearly 6,000 life source journals, 1,500 international meetings as well as review articles, books, and monographs are reviewed for inclusion. It covers the period 1926 – present and is updated weekly.
	CABA	Covers worldwide literature from all areas of agriculture and related sciences including biotechnology, forestry, and veterinary medicine. Sources include journals, books, reports, published theses, conference proceedings, and patents. It covers the period 1973-present and is updated weekly.
	HCAPLUS	Covers worldwide literature from all areas of chemistry, biochemistry, chemical engineering, and related sciences including applied, macromolecular, organic, physical, inorganic, and analytical chemistry. Current sources include over 8,000 journals, patents, technical reports, books, conference proceedings, dissertations, product reviews, bibliographic items, book reviews, and meeting abstracts. Electronic-only journals and Web preprints are also covered. Cited references are included for journals, conference proceedings and basic patents from the U.S., EPO, WIPO, and German patent offices added to the CAS databases from 1999 to the present. Also provides early access to the bibliographic information, abstracts and CAS Registry Numbers for documents in the process of being indexed by CAS. Covers the period 1907 – present and is updated daily
	FSTA	The database provides worldwide coverage of all scientific and technological aspects of the processing and manufacture of human food products including basic food sciences, biotechnology, hygiene and toxicology, engineering, packaging, and all individual foods and food products. Sources include more than 2,200 journals, books, reviews, conference proceedings, patents, standards, and legislation. It covers the period 1969 – present and is updated weekly.

Provider	Database	Justification
Host STN	FROSTI	The database contains citations to the worldwide literature on food science and technology including food and beverages, analytical methods, quality control, manufacturing, microbiology, food processing, health and nutrition, recipes, and additives. Sources include approximately 800 scientific and technical journals, bulletins, technical reports, conference proceedings, grey literature, and British, European (EP), U.S., Japanese, and international (PCT) patent applications. Covers the period 1972 – present and is updated twice weekly.

	GEOREF	Covers international literature on geology and geosciences. Sources include the Bibliography of North American Geology, Bibliography and Index of Geology Exclusive of North America, Geophysical Abstracts, Bibliography of Fossil Vertebrates, selected records from Geoline and from geology sections of PASCAL and state and national geological surveys. Covers the period 1669 – present and is updated twice a month.
	TOXCENTER	Covers the pharmacological, biochemical, physiological, and toxicological effects of drugs and other chemicals. It is composed of the following subfiles: BIOSIS, CAlplus, IPA and MEDLINE and sources include abstracts, books and book chapters, bulletins, conference proceedings, journal articles, letters, meetings, monographs, notes, papers, patents, presentations, research and project summaries, reviews, technical reports, theses, translations, unpublished material, web reprints. Covers the period 1907 – present and is updated weekly
	PQSCITECH	Is a huge resource in all areas of science and technology from engineering to lifescience. The file is a merge of 25 STN databases formerly known as CSA databases (Cambridge Scientific Abstracts): AEROSPACE, ALUMINIUM, ANTE, AQUALINE, AQUASCI, BIOENG, CERAB, CIVILENG, COMPUAB, CONFSCI, COPPERLIT, CORROSION, ELCOM, EMA, ENVIROENG, HEALSAFE, LIFESCI, LISA, MATBUS, MECHENG, METADEX, OCEAN, POLLUAB, SOLIDSTATE, and WATER. Sources are journals, patents, books, reports, and conference proceedings spanning the period 1962 – present and it is updated monthly.

Provider	Database	Justification
Host STN	PASCAL	The database provides access to the world's scientific and technical literature including physics and chemistry, life sciences (biology, medicine, and psychology), applied sciences and technology, earth sciences, and information sciences. French and European literature is particularly well represented. Approximately 5,000 journal titles are indexed. References to theses and to conference proceedings are also included. Spans the period 1977 to present and is updated weekly
	SCISEARCH	Is an international index to the literature covering virtually every subject area within the broad fields of science, technology, and biomedicine. SciSearch contains all the records published in Science Citation Index Expanded™ and additional records from the Current Contents series of publications. Bibliographic information and cited references from over 5,600 scientific, technical, and medical journals are contained in the database. Spans the period 1974 to present and is updated weekly.
	ANABST	Covers worldwide literature on analytical chemistry. The ANABSTR file contains bibliographic records with abstracts (since 1984) for documents reported in printed Analytical Abstracts. Sources for ANABSTR include journals, books, conference proceedings, reports, and standards. Spans the period 1980 to present and is updated weekly.
	HCHEMLIST	The database identifies the regulatory requirements for a specific substance from many of the world's most significant regulated substances lists. Includes, substance identity information, inventory status, source of information, and summaries of regulatory activity, reports, and other compliance information.
	CROPU	The Derwent Crop Protection File covers all aspects of pesticides, including their use in crop protection and pest control. Information on plant and insect growth regulators, attractants, repellents and biological control is also included. The database draws on 1,100 scientific journals, conference proceedings, and patents beginning in 1996. Records contain bibliographic information, titles, abstracts, in-depth indexing, and Enzyme Commission Numbers.

Host STN	CROPB	The Crop Protection Backfile is the companion backfile to the current Crop Protection File, CROPU, covering all aspects of pesticides, including their use in crop protection and pest control. Information on plant and insect growth regulators, attractants, repellents and biological control is also included. Bibliographic information and indexing terms are searchable.
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* Total number of summary records retrieved after removing duplicates

Table 9.10-3: Bibliographical databases used in the top-up literature search

Databases	Frequency of updates
MEDLINE	1948 to present; Updated 6 times each week with an annual reload
EMBASE	1974 to present; Updated daily
ESBIOBASE	1994 to present; Updated weekly
AGRICOLA	1970 to present; Updated monthly
BIOSIS	1926 to present; Updated weekly
CABA	1973 to present; Updated weekly
HCAPLUS	1907 to present; Updated daily
FSTA	1969 to present; Updated weekly
GEOREF	1669 to present; Updated twice a month
TOXCENTER	1907 to present; Updated weekly
PQSCITECH	1962 to present; Updated monthly
SCISEARCH	1974 to present; Updated weekly
ANABSTR	1980 to present; Updated weekly

Search parameters

The CAS numbers searched were extensive, covering the active substance and metabolites of the active substance. The search parameters are shown in the table below. HSE considers the search terms and parameters to be acceptable.

Table 9.10-34: Detailed Search Parameters for Ecotoxicological studies of pydiflumetofen and metabolites (CA 8.1 to 8.15)

Search Strategy	
Initial SYN545974 search :	
L1	QUE (1228284-64-7 OR 1639015-49-8 OR 1639015-48-7)
L2	QUE (1485419-47-3 OR 1485419-44-0 OR (FUSHA(10A)FUNGICID?))
L3	QUE (SYN545974 OR (SYN(W)545974))
L4	QUE L1-3 FUSHA PLUS STEREOISOMERS
L5	QUE (1658468-84-8 OR 1561039-73-3 OR 1336797-48-8)
L6	QUE (1335518-65-4 OR 1245827-93-3 OR 1228286-43-8)
L7	QUE (1228284-63-6 OR 1204298-65-6 OR 1192017-82-5)
L8	QUE (1105713-22-1 OR 1004285-82-8 OR 960053-63-8)
L9	QUE (925689-10-7 OR 176969-34-9 OR 151734-02-0)
L10	QUE (SYN545547 OR SYN547894 OR SYN547892 OR SYN547893)
L11	QUE (NOA449410 OR SYN547895 OR SYN547890 OR SYN545720)
L12	QUE (SYN508272 OR SYN545357 OR SYN547896 OR SYN547897)
L13	QUE (SYN547891 OR SYN548263 OR SYN548264 OR SYN548265)
L14	QUE (SYN548279 OR (SYN(W)548279) OR (NOA(W)449410))
L15	QUE (SYN(W)(545547 OR 547894 OR 547892 OR 547893))
L16	QUE (SYN(W)(547895 OR 547890 OR 545720))
L17	QUE (SYN(W)(508272 OR 545357 OR 547896 OR 547897))
L18	QUE (SYN(W)(547891 OR 548263 OR 548264 OR 548265))
Top-up SYN545974 search :	

L1	QUE SPE=ON ABB=ON PLU=ON (1639015-49-8 OR 1639015-48-7 OR 1485419-47-3 OR 1485419-44-0 OR 1228284-64-7)
L2	QUE SPE=ON ABB=ON PLU=ON (DIFLUOROMETHYL(1W)METHOXY(1W)METHYL(2W)METHYL(4W) TRICHLOROPHENYL (W) ETHYL(1W)PYRAZOL?(1W)CARBOXAMID? OR DIFLUOROMETHYL (1W) METHYL(1W)PYRAZOL?(1W)CARBOXYLIC(W)ACID(W)METHOXY(1W)METHYL(4W)TRICHLORO(W) PHENYL(W)ETHYL(W)AMID?)
L3	QUE SPE=ON ABB=ON PLU=ON (ADEPIDYN OR FUSHA OR PYDIFLUMETOFEN# OR SYN545974 OR SYN(W)545974)
L4	QUE SPE=ON ABB=ON PLU=ON (L1 OR L2 OR L3)
L5	QUE SPE=ON ABB=ON PLU=ON (1658468-84-8 OR 1561039-73-3 OR 1336797-48-8 OR 1335518-65-4 OR 1245827-93-3 OR 1228286-43-8 OR 1228284-63-6 OR 1204298-65-6 OR 1192017-82-5 OR 1105713-22-1 OR 1004285-82-8 OR 960053-63-8 OR 925689-10-7 OR 176969-34-9 OR 151734-02-0)
L6	QUE SPE=ON ABB=ON PLU=ON (SYN545547 OR SYN547894 OR SYN547892 OR SYN547893 OR NOA449410 OR SYN547895 OR SYN547890 OR SYN545720 OR SYN508272 OR SYN545357 OR SYN547896 OR SYN547897 OR SYN547891 OR SYN548263 OR SYN548264 OR SYN548265)
L7	QUE SPE=ON ABB=ON PLU=ON (SYN548279 OR (SYN(W)548279) OR (NOA(W)449410) OR SYN(W) (545547 OR 547894 OR 547892 OR 547893) OR SYN(W)(547895 OR 547890 OR 545720) OR SYN(W)(508272 OR 545357 OR 547896 OR 547897) OR SYN(W)(547891 OR 548263 OR 548264 OR 548265))
L8	QUE SPE=ON ABB=ON PLU=ON (DICHLOROPHENYL(1W)METHYLETHYL(1W)DIFLUOROMETHYL (1W)METHOXY(1W)METHYL(1W)PYRAZOLE(1W)CARBOXAMIDE OR DIFLUOROMETHYL(1W) HYDROXY(1W)METHYL(2W)METHYL(4W)TRICHLOROPHENYL(W)ETHYL(1W)PYRAZOLE (1W) CARBOXAMIDE OR DIFLUOROMETHYL(1W)METHYL(1W)PYRAZOL#(1W)CARBOXAMID#)
L9	QUE SPE=ON ABB=ON PLU=ON (DIFLUOROMETHYL(1W)METHYL(1W)PYRAZOL#(1W)CARBOXYLIC (W)ACID OR DIFLUOROMETHYL(1W)METHYL(1W)PYRAZOL# (1W)CARBOXYLIC(W)ACID(2W) HYDROXY(1W)METHYLETHYL(W)AMIDE OR DIFLUOROMETHYL(1W) METHYL(1W)PYRAZOL # (1W) L(W)CARBONYL(1W)ALANINE)
L10	QUE SPE=ON ABB=ON PLU=ON (DIFLUOROMETHYL(1W)METHYL(2W)METHYL(4W) TRICHLOROPHENYL (W) ETHYL (1W) PYRAZOL#(1W)CARBOXAMIDE ORDIFLUOROMETHYL(1W) METHYLPYRAZOL#(1W)CARBOXAMIDE OR DIFLUOROMETHYL(1W)METHYLPYRAZOL # (1W) CARBOXYLIC(W)ACID)
L11	QUE SPE=ON ABB=ON PLU=ON (DIFLUOROMETHYL(1W)PYRAZOL#(1W)CARBOXYLIC(W)ACID OR DIFLUOROMETHYL(2W)HYDROXY(1W)METHYLETHYL(1W)METHOXY(1W)METHYL(1W) PYRAZOL# (1W)CARBOXAMIDE OR DIFLUOROMETHYL(3W)HYDROXYL(1W)METHYLETHYL(1W)METHYL (1W) PYRAZOL#(1W)CARBOXAMID#)
L12	QUE SPE=ON ABB=ON PLU=ON (METHYL(1W)DIFLUOROMETHYL(W)PYRAZOL#(1W) CARBOXYLIC (W)ACID OR TRICHLORO(1W)METHYL(1W)BENZENEETHANAMINE OR TRICHLORO(1W)METHYL(W) BENZENE (W) ETHANAMIN#)

Search Strategy

SYN545974 specific metabolites search :

L1 QUE SPE=ON ABB=ON PLU=ON (960053-63-8 OR DIFLUOROMETHYL(1W)METHYL(2W)METHYL(4W) TRICHLOROPHENYL (W) ETHYL(2W)PYRAZOL?(1W)CARBOXAMID# OR SYN545547 OR SYN(W)545547)

L2 QUE SPE=ON ABB=ON PLU=ON (3784-03-0 OR 2591-21-1 OR 95-95-4 OR 88-06-2 OR 89465-86-1 OR 77001-45-7)

L3 QUE SPE=ON ABB=ON PLU=ON (TRICHLOROPHENOL OR TRICHLOROPHENATE OR TRICHLOROPHENOLATE OR TRICHLOROPHENOXIDE OR TRICHLORO(W)PHENOL OR TRICHLOROPHENOXY OR TRICHLOROPHENIC(W)ACID OR TRICHLORO(1W)HYDROXYBENZENE)

L4 QUE SPE=ON ABB=ON PLU=ON (DOWICIDE OR NSC(W)2266 OR NSC2266 OR PREVENTOL OR TCP OR 2(W)4(W)6(W)TCP OR BTS(W)45186 OR BTS45186 OR NSC(W)2165 OR NSC2165 OR OMAL OR PHENACHLOR)

L5 QUE SPE=ON ABB=ON PLU=ON (FUNGI!ID? OR MOLDICID? OR PESTI!ID? OR MICROBIO!ID? OR MICROBI!ID? OR BIO!ID? OR BI!ID? OR ANTIFUNG? OR ANTI(W)FUNG?)

L6 QUE SPE=ON ABB=ON PLU=ON L4(10A)L5

L7 QUE SPE=ON ABB=ON PLU=ON (1192017-82-5 OR SYN548264 OR SYN(W)548264)

L8 QUE SPE=ON ABB=ON PLU=ON (DIFLUOROMETHYL(1W)METHYL(1W)PYRAZOL#(1W)CARBONYL (W) AMINO (W) PROPANOIC(W)ACID OR DIFLUOROMETHYL(1W)METHYL(1W)PYRAZOL#(1W) CARBONYL (W) AMINO(W)PROPANOAT# OR DIFLUOROMETHYL(1W)METHYL(1W)PYRAZOL#(1W) YL(W)CARBONYL(1W)ALANIN#)

L9 QUE SPE=ON ABB=ON PLU=ON (SYN547891 OR SYN(W)547891 OR DIFLUOROMETHYL(1W) METHOXY (2W) METHYL(4W)TRICHLOROPHENYL(W)ETHYL(1W)PYRAZOL?(1W)CARBOXAMID?)

L10 QUE SPE=ON ABB=ON PLU=ON (SYN547897 OR SYN(W)547897 OR DIFLUOROMETHYL(1W) METHYL (1W)PYRAZOL?(1W)CARBOXYLIC(W)ACID(W)METHOXY(1W)METHYL(4W)TRICHLORO(1W) HYDROXYL (W)PHENYL(W)ETHYL(W)AMID?)

L11 QUE SPE=ON ABB=ON PLU=ON (SYN548263 OR SYN(W)548263 OR DIFLUOROMETHYL(1W)METHYL (W) PYRAZOL?(1W)CARBONYL(W)METHOXY(W)AMINO(W)PROPANOIC(W)ACID OR DIFLUOROMETHYL (1W) METHYL(W)PYRAZOL?(1W)CARBONYL(W)METHOXY(W) AMINO(W) PROPANOAT? OR DIFLUOROMETHYL(1W)METHYL(W)PYRAZOL?(1W)CARBONYL(1W) METHOXY (W)ALANIN#)

L12 QUE SPE=ON ABB=ON PLU=ON (SYN548261 OR SYN(W)548261 OR DIFLUOROMETHYL(1W)METHYL (W)PYRAZOL?(1W) CARBONYL(W)METHOXY(W)AMINO(W)(BUTANOIC OR BUTYRIC OR PROPANECARBOXYLIC)(W)ACID OR DIFLUOROMETHYL(1W)METHYL(W)PYRAZOL?(1W)CARBONYL (W)METHOXY(W)AMINO(W)(BUTANOAT? OR BUTYRATE? OR PROPANECARBOXYLAT?))

L13 QUE SPE=ON ABB=ON PLU=ON (SYN547948 OR SYN(W)547948 OR DIFLUOROMETHYL(4W) HYDROXY (1W) METHYL(4W)TRICHLOROPHENYL(W)ETHYL(1W)METHOXY(1W)METHYL(W) PYRAZOL? (1W)CARBOXAMID?)

L14 QUE SPE=ON ABB=ON PLU=ON (1639015-49-8 OR 1639015-48-7 OR 1485419-47-3 OR 1485419-44-0 OR 1228284-64-7)

L15 QUE SPE=ON ABB=ON PLU=ON (DIFLUOROMETHYL(1W)METHOXY(1W)METHYL(2W)METHYL(4W)

	TRICHLOROPHENYL	(W)ETHYL(1W)PYRAZOL?(1W)CARBOXAMID?	OR
	DIFLUOROMETHYL(1W)		
	METHYL (1W) PYRAZOL? (1W)CARBOXYLIC(W)ACID (W)METHOXY(1W)METHYL(4W)		
	TRICHLORO	(W) PHENYL(W)ETHYL(W)AMID?)	
L16	QUE SPE=ON ABB=ON PLU=ON (ADEPIDYN OR FUSHA OR PYDIFLUMETOFEN# OR SYN545974 OR		
	SYN(W)545974)		
L17	QUE SPE=ON ABB=ON PLU=ON (HYDROXY OR OXY?)(3W)(L14 OR L15 OR L16)		
L18	QUE SPE=ON ABB=ON PLU=ON ((L1 OR L2 OR L3) OR (L6 OR L7 OR L8 OR L9 OR L10 OR L11		
	OR L12		
	OR L13) OR L17)		
SYN545974 metabolites that are common SDHI metabolites search :			
L1	QUE SPE=ON ABB=ON PLU=ON (176969-34-9 OR 1334398-13-8 OR NOA (W)449410 OR NOA449410 OR		
	R(W)648993	OR	R648993
	DIFLUOROMETHYL(1W)METHYL(1W)PYRAZOL?(1W)CARBOXYLIC(W)		OR
	ACID OR (PYRAZOL?(1W)CARBOXYLIC(W)ACID)(1A)(DIFLUOROMETHYL(1W)METHYL))		
L2	QUE SPE=ON ABB=ON PLU=ON (METHYL(1W)DIFLUOROMETHYL(1W)PYRAZOL?(1W)CARBOXYLIC		
	(W)ACID OR DIFLUOROMETHYL(1W)METHYL(1W)PYRAZOL? (1W)CARBOXYLAT? OR		
	DIFLUOROMETHYL (1W)METHYLPYRAZOL?(1W)CARBOXYLIC(W)ACID)		
L3	QUE SPE=ON ABB=ON PLU=ON (925689-10-7 OR SYN(W)508272 OR SYN508272 OR R(W)423363		
OR		R423363	OR
	DIFLUOROMETHYL(1W)METHYL(1W)PYRAZOL?(1W)CARBOXYLIC(W)ACID(W)AMID?		
	OR DIFLUOROMETHYL(1W)METHYL(1W)PYRAZOL?(1W)CARBOXAMID##)		
L4	QUE SPE=ON ABB=ON PLU=ON ((PYRAZOL?(1W)CARBOXAMIDE)(1A)(DIFLUOROMETHYL		
(1W)	METHYL) OR DIFLUOROMETHYL(1W)METHYLPYRAZOL?(1W)CARBOXAMIDE##)		

Search Strategy

L5 QUE SPE=ON ABB=ON PLU=ON (L1 OR L2 OR L3 OR L4)

Plus ecotoxicology areas

- L1 QUE (RIPARIAN? OR REPTILE? OR SNAKE? OR LIZARD?)
- L2 QUE (TORTOISE? OR TURTLE? OR TERRAPIN? OR CROCODIL?)
- L3 QUE (ALLIGATOR? OR CAIMAN? OR GHARIAL? OR HOVERFLIES)
- L4 QUE ((MEADOW#(W)VOLE#) OR PSEUDOKIRSCHNERIELLA)
- L5 QUE (RHAPHIDOCCELIS OR NITZSCHIA OR CYCLOTELLA OR MICROCYSTIS)
- L6 QUE (OSCILLATORIA OR APHANIZOMENON OR ANKISTRODESMUS)
- L7 QUE (TEILINGRIA OR MONORAPHIDIUM OR RADIOCOCCACAE OR TETRASPORALES)
- L8 QUE (TETRAEDRON OR TREUBARIA OR WILLEA OR COSMOCLADIUM)
- L9 QUE (HYPOASPIS OR (SOIL(3A)MICROORGAN?) OR ECHINOCHLOA OR SPARTINA)
- L10 QUE (SALVINIA OR NAJAS OR CALLITRICHE OR MYOSOTIS OR STRATIOTES)
- L11 QUE (HIPPIRUS OR PERSICARIA OR CLOEON? OR CORBICULA?)
- L12 QUE (NEOCARIDINIA? OR NEOCARIDINA? OR MYSID? OR CICHLIDAE)
- L13 QUE (CICHLID# OR LEPOMIS? OR SERRANIDAE OR PERCIFORMES)
- L14 QUE (ICTALURUS? OR POECILIA? OR ORYZIAS? OR GASTEROSTEUS?)
- L15 QUE (GASTEROSTEIDAE OR SALVELINUS OR BRACHYDANIO? OR CARASSIUS?)
- L16 QUE (MISGUMUS? OR CYPRINODON? OR FUNDULUS? OR MISGURNUS?)
- L17 QUE (BREAM OR ROTIFER# OR GAMMARUS OR GAMMARID? OR MAYFLY?)
- L18 QUE (BIVALVE# OR MUSSEL# OR MOLLUSK# OR MOLLUSC# OR BUFO)
- L19 QUE (NEWT# OR SCALLOP# OR CLAM# OR GAMBUSIA OR OREOCHROMIS)
- L20 QUE (OSTRAC? OR TUBIFEX? OR TURBELLARIA OR COPEPODA)
- L21 QUE (PREDACE? OR PREDACI? OR PARASITOID? OR APIS OR APIDAE)
- L22 QUE (BOMBUS OR BOMBINAE OR WORM# OR LUMBRICIDAE OR LUMBRICUS)

L23 QUE (ALLOBOPHORA? OR DENDROBAENA? OR APORRECTODEA? OR DENDRODRILUS?)
 L24 QUE (EISENIA? OR OCTOLASION? OR (LACE(W)WING#) OR NEUROPTER?)
 L25 QUE (CARABID? OR CARBUS OR STAPHYLINID? OR COCCINEL? OR ADALIA?)
 L26 QUE (STETHORUS? OR SCYMNUS? OR WASP# OR VESPIDAE OR SPHECOIDEA)
 L27 QUE (SPHECIDAE OR STIZIDAE OR OPIUS OR (ICHNEUMON(W)FL?))
 L28 QUE (ICHNEUMONID? OR BRACONID? OR CHALCID? OR CYNIP? OR APHIDI?)
 L29 QUE (EUCOILID? OR IBALIID? OR FIGITID? OR EURYTOM? OR TORYM?)
 L30 QUE (ORYM? OR EUCHARIT? OR PERILAMP? OR PTEROMAL? OR CHRYSOLAMP?)
 L31 QUE (EUPELM? OR ENCYRT? OR SIGNIPHOR? OR APHELIN? OR ELASMID?)
 L32 QUE (ELASMUS OR TETRACAMP? OR MYMAR? OR HELOR? OR PROCTOTRUP?)
 L33 QUE (DIAPRI? OR SCELION? OR PLATYGASTR? OR PLATYGASTER?)
 L34 QUE (CERAPHRON? OR MEGASPIL? OR ARANE? OR OPILION? OR PHALANG?)
 L35 QUE (ARACHNID? OR HARVESTM? OR DADDYLONGLEG? OR (DADDY(W)LONG (W)LEG?))
 L36 QUE ((DADDY(W)LONGLEG?) OR COLLEMB? OR (SPRING(W)TAIL?) OR CYDNODROMUS?)
 L37 QUE (PARDOSA? OR ORIUS? OR TYPHLODROM? OR PHYTOSEIULUS? OR SYRPHID?)
 L38 QUE (METASYRPHUS? OR SYRPHUS? OR EUPEODES? OR EPISYRPHUS? OR SYRPHIAN?)
 L39 QUE (EPISTROPHE? OR AMBLYSEIUS? OR POECILUS? OR TRECHUS? OR BEMBIIDION?)
 L40 QUE (NEBRIA? OR PTEROSTICHUS? OR CALOSOMA? OR TACHYPORUS? OR NABIDAE?)
 L41 QUE (GEOCORIS? OR HYMENOPT? OR HAEMATOLOECHA? OR CHRYSOPID? OR SYMPHYTA?)
 L42 QUE (OULEMA? OR APHYTIS? OR BATHYPLECTES? OR LINPHIIDAE? OR LYNPHIIDAE?)
 L43 QUE (LINYPHIIDAE? OR ERIGONE? OR BATHYPHANTES? OR MEIONETA? OR OEDOTHORAX?)
 L44 QUE (LEPTYPHANTES? OR LYCOSID? OR LYCOSA? OR CHRYSOPA? OR DACNUSA?)
 L45 QUE (CYRTORHINUS? OR CRYPTOLAEMUS? OR ZETZELLIA? OR LEPTOMASTIX?)
 L46 QUE (TRICHOGRAMMA? OR ENCARSIA? OR MACROLOPHUS? OR CHRYSOPERLA?) L47 QUE
 (ALEOCHARA? OR CHRYSOPID# OR CHRYSOPIDAE OR DIABROTICA)
 L48 QUE (PALEXORISTA? OR MAMMAL## OR ANIMAL? OR RABBIT? OR RODENT#)
 L49 QUE (BLACKBIRD# OR (BLACK(W)BIRD#) OR ((TURDUS OR T)(W)MERULA))
 L50 QUE (CHAFFINCH? OR ((FRINGILLA OR F)(W)COELEBS) OR GREENFINCH?)
 L51 QUE (((CARDUELIS OR C)(W)CHLORIS) OR SONGTHRUSH?)
 L52 QUE ((SONG(W)THRUSH?) OR ((TURDUS OR T)(W)PHILOMELOS) OR WREN#)
 L53 QUE (((TROGLODYTES OR T)(W)TROGLODYTES) OR (WILLOW(W)WARBLER#))
 L54 QUE (((PHYLLOSCOPUS OR P)(W)TROCHILUS) OR (GREAT(W)TIT#))
 L55 QUE (((PARUS OR P)(W)MAJOR) OR ROBIN# OR GOLDFINCH?)
 L56 QUE (((ERITHACUS OR E)(W)RUBECULA) OR DUNNOCK#)
 L57 QUE (((CARDUELIS OR C)(W)CARDUELIS) OR LINNET#)
 L58 QUE (((PRUNELLA OR P)(W)MODULARIS) OR SKYLARK# OR (SKY(W)LARK#))

Search Strategy

L59	QUE ((HEDGE(W)(SPARROW# OR ACCENTOR#)))
L60	QUE (((CARDUELIS OR C)(W)CANNABINA) OR ((ALAUDA OR A)(W)ARVENSIS))
L61	QUE ((RED(W)LEGGED(W)PARTRIDGE#) OR ((ALECTORIS OR A)(W)RUFA))
L62	QUE ((MEADOW(W)PIPIT#) OR MEADOWPIPIT# OR ((ANTHUS OR A)(W)PRATENSIS))
L63	QUE (LAPWING# OR ((VANELLUS OR V)(W)VANELLUS) OR PEEWIT#)
L64	QUE (STARLING# OR ((STURNUS OR S)(W)VULGARIS))
L65	QUE ((TURTLE(W)DOVE#) OR ((STREPTOPELIA OR S)(W)TURTUR))
L66	QUE (YELLOWHAMMER# OR (YELLOW(W)HAMMER#) OR (YELLOW(W)WAGTAIL#))
L67	QUE (((EMBERIZA OR E)(W)CITRINELLA) OR (YELLOW(W)WAG(W)TAIL#))
L68	QUE (((MOTACILLA OR M)(W)FLAVA) OR (FAN(W)TAILED(W)WARBLER#))
L69	QUE ((GREY(W)LAG(W)G!!SE) OR ((ANSER OR A)(W)ANSER))
L70	QUE (REEDBUNTING# OR (REED(W)BUNTING#) OR ((EMBERIZA OR E)(W)SCHOENICLUS))
L71	QUE (CHAFFINCH? OR BLUETIT? OR (BLUE(W)TIT?))
L72	QUE (((PARUS OR P)(W)CAERULEUS) OR (SYLVIA(W)COMMUNIS))
L73	QUE (((GALERIDA OR G)(W)CRISTATA) OR (TREE(W)SPARROW#))
L74	QUE (((COTURNIX OR C)(W)COTURNIX) OR (GREY(W)PARTRIDGE#))
L75	QUE (((PERDIX OR P)(W)PERDIX) OR ((PHASIANUS OR P)(W)COLCHICUS))
L76	QUE (((MILIARIA OR M)(W)CALANDRA?) OR GREYLAGG!!SE)
L77	QUE ((GREYLAG(W)G!!SE) OR ((COLUMBA OR C)(W)PALUMBUS?))
L78	QUE (((STREPTOPELIA OR S)(W)(ORIENTALIS? OR RISORIA?)))
L79	QUE (((MOTACILLA OR M)(W)ALBA?) OR (CRESTED(W)LARK#))
L80	QUE ((WHITE(W)WAGTAIL#) OR (WOOD(W)PIGEON#) OR (BIRD(W)LIFE))
L81	QUE ((SONG(W)BIRD#) OR VANELLUS? OR (PEE(W)WIT#))
L82	QUE (AVIFAUNA? OR (AVI(W)FAUNA?) OR SONGBIRD?)
L83	QUE (ORNITHOLOG? OR PASSERINE? OR WOODPIGEON#)
L84	QUE (((PASSER OR P)(W)MONTANUS) OR QUAIL# OR (CALANDRA(W)LARK#))
L85	QUE (CISTICOLA? OR (Z(W)CISTICOLA?) OR BIRDLIFE)
L86	QUE (GEESE OR GOOSE OR SPARROWS OR PIGEONS OR LARK#)
L87	QUE (WARBLER# OR PARTRIDGE# OR BUNTING# OR WAGTAIL#)
L88	QUE (WHITETHROAT# OR PIED# OR (WHITE(W)THROAT#))
L89	QUE ((FORAGING OR FARMLAND OR GRASSLAND)(3A)BIRD#)
L90	QUE (BLUEBIRD# OR (ROCK(W)PTARMIGAN#) OR (BLACK(W)REDSTART#))
L91	QUE ((PREDATOR? OR NONTARGET? OR (NON(W)TARGET?))(3A)BIRD#)
L92	QUE ((CORN(W)BUNTING#) OR SERINS OR SERINUS)
L93	QUE (L49 OR L50 OR L51 OR L52 OR L53 OR L54 OR L55 OR L56 OR L57 OR L58 OR L59 OR L60 OR L61 OR L62 OR L63 OR L64 OR L65 OR L66 OR L67 OR L68 OR L69 OR L70 OR L71 OR L72 OR L73 OR L74 OR L75 OR L76 OR L77 OR L78 OR L79 OR L80 OR L81 OR L82 OR L83 OR L84 OR L85 OR L86 OR L87 OR L88 OR L89 OR L90 OR L91 OR L92)
L94	QUE L93 NOT (JAPANESE? OR JAPONICA?)
L95	QUE (((SMALL OR WILD)(3A)MAMMAL#) OR (WILD(3A)ANIMAL?))
L96	QUE (VOLE# OR GLIS OR DORMOUSE OR DORMICE OR ELIOMY#)
L97	QUE (LEROT# OR LAGOMORPH# OR LEPORID? OR LEPUS OR ORYCTOLAGUS?)
L98	QUE (HARE# OR SORICIDAE? OR SOREX? OR NEOMY# OR CROCIDURA?)
L99	QUE (SHREW# OR WOODMOUSE OR WOODMICE OR APODEMUS? OR MICROTUS?)
L100	QUE (CLETHRIONOMYS? OR CRICETIDAE? OR MICROTIN?)
L101	QUE (RAPTOR# OR MARMOSET# OR GOPHER# OR GRASSCUTTER#)
L102	QUE ((PREDATOR? OR NONTARGET? OR (NON(W)TARGET?))(3A)MAMMAL#)
L103	QUE ((WOOD(W)(MOUSE OR MICE)) OR ARVICOLA?)
L104	QUE (MEADOW#(W)VOLE#)
L105	QUE (L95 OR L96 OR L97 OR L98 OR L99 OR L100 OR L101 OR L102 OR L103 OR L104)
L106	QUE (ECOTOX? OR LC50 OR ((LC OR EC OR LR)(W)50) OR EC50 OR LR50)
L107	QUE (ECO OR ECOL OR ECOLOG? OR ENV OR ENVIRONM? OR AQUATIC?)
L108	QUE (L107(5A)(TOX? OR RISK? OR IMPACT? OR EFFECT?))
L109	QUE (AQUATIC? OR FRESHWATER? OR (FRESH(W)WATER?))
L110	QUE (FLORA OR FAUNA OR BIOTA OR ORGANISM? OR INSECT?)
L111	QUE (ENVIRONM? OR LIFE OR INVERTEB? OR CRUSTACE? OR SPECIES)
L112	QUE (ENTOMOFAUNA OR (ENTOMO(W)FAUNA))
L113	QUE (L109(5A)(L110 OR L111 OR L112))

L114	QUE (MAGNA? OR (D(W)MAGNA?) OR CHIRONOM? OR BRACHIONUS?)
L115	QUE (LIMNEA? OR CRASSOSTREA? OR ALGA# OR FISH OR FISHES)
L116	QUE (ONCORHYNCHUS? OR SALMONIDAE? OR CYPRINUS? OR CYPRINID?)
L117	QUE (PIMEPHALES? OR PISCES OR TROUT OR SUNFISH? OR CARP)

Search Strategy

L118	QUE (MINNOW? OR (F(W)MINNOW?) OR CATFISH? OR ZEBRAFISH?)
L119	QUE (GOLDFISH? OR (ZEBRA(W)DANIO#) OR GUPPY OR GUPPIES)

L120 QUE (KILLFISH? OR FATHEAD? OR BLUEGILL? OR SALMON#)
 L121 QUE (THUNDERFISH? OR (WATER(W)(FLY OR FLEA?)) OR WATERFLEA?)
 L122 QUE (FROG# OR AMPHIBIA? OR SHRIMP# OR PRAWN# OR CRAB# OR TOAD#)
 L123 QUE (TADPOLE# OR CRAYFISH? OR SHELLFISH? OR LOBSTER#)
 L124 QUE (OYSTER# OR SNAIL# OR RANA OR RANIDAE? OR PLANKTON?) L125 QUE L106 OR L108
 L126 QUE ((NONTARGET? OR (NON(W)TARGET?))(5A)(PLANT? OR FLORA?))
 L127 QUE ((AQUATIC(3A)(PLANT? OR (PHYTO(W)TOX?) OR PHYTOTOX?))
 L128 QUE (SEDIMENT? OR HYDROSOIL? OR DUCKWEED? OR PONDWEED?)
 L129 QUE (((DUCK OR POND)(W)WEED#) OR MACROPHYT? OR PERIPHYTON?)
 L130 QUE (POTAMOGETON? OR CHAROPHYTA? OR ELODEA? OR HYDROCHARITA?)
 L131 QUE (CERATOPHYL? OR CHLAMYDOMON? OR SELENASTRUM? OR CHLORELLA?)
 L132 QUE (SCENEDESMUS? OR SKELETONEMA? OR NAVICULA? OR ANABAENA?)
 L133 QUE (MYRIOPHYLLUM? OR GLYCERIA?)
 L134 QUE (NONTARGET? OR (NON(W)TARGET?) OR BENEFICIAL?)
 L135 QUE (EFFECT? OR INVERTEB? OR ORGANISM? OR ARTHROPOD? OR INSECT?)
 L136 QUE (FAUNA OR SPECIES OR (ENTOMO(W)FAUNA?) OR ENTOMOFAUNA?) L137 QUE
 ((L134(5A)(L135 OR L136)))
 L138 QUE (PREDAT? OR (NATURAL(W)ENEM?) OR BEE OR BEES OR HONEYBEE#)
 L139 QUE (BUMBLEBEE# OR ((HONEY OR BUMBLE)(W)BEE#) OR EARTHWORM?)
 L140 QUE ((EARTH(W)WORM?) OR LADYBUG# OR LADYBEETLE# OR LADYBIRD#)
 L141 QUE ((LADY(W)(BUG# OR BEETLE# OR BIRD#)) OR HOVERFLY)
 L142 QUE (HOOVERFLIES OR SAWFLY OR SAWFLIES OR DRONEFLY)
 L143 QUE (DRONEFLIES OR FLOWERFLY OR FLOWERFLIES OR LACEWING?)
 L144 QUE (((HOVER OR DRONE OR FLOWER OR SAW)(W)(FLY OR FLIES)))
 L145 QUE (SPIDER# OR SPRINGTAIL? OR (ROOT(W)WORM#) OR ROOTWORM#)
 L146 QUE (L137 OR L138 OR L139 OR L140 OR L141 OR L142 OR L143 OR L144 OR L145)
 L147 QUE (BIRD? OR AVES OR AVIAN? OR (AVI(W)FAUNA?) OR AVIFAUNA?)
 L148 QUE (SONGBIRD? OR (SONG(W)BIRD?) OR ORNITHOLOG?)
 L149 QUE (L147 OR L148)
 L150 QUE ((WILD(3A)(LIFE OR ANIMAL#)) OR WILDLIFE OR SQUIRREL?)
 L151 QUE (VOLE# OR SCIURUS OR GLIRID? OR GLIS OR DORMOUSE)
 L152 QUE (DORMICE OR ELIOMYS OR LEROT# OR MUSTELID? OR MINK#)
 L153 QUE (MUSTELINE# OR WEASEL? OR STOAT? OR MUSTEL? OR BADGER?)
 L154 QUE (MELES OR MELINAE OR OTTER# OR LUTRA OR LUTRINAE)
 L155 QUE (LAGOMORPH# OR LEPORID? OR LEPUS OR ORYCTOLAGUS OR HARE#)
 L156 QUE (TALPA OR MOLE OR MOLES OR HEDGEHOG? OR (HEDGE(W)HOG?))
 L157 QUE (CROCIDURA? OR SHREW# OR WOODMOUSE OR WOODMICE OR APODEMUS)
 L158 QUE (MICROTUS OR ARVICOLA OR CLETHRIONOMYS? OR CRICETIDAE?)
 L159 QUE (ERINACEUS OR ERINACEIDAE? OR SORICIDAE? OR SOREX)
 L160 QUE (ENDOCRIN? OR HORMON?)
 L161 QUE (DISRUPT? OR MIMIC? OR MODULAT? OR DISORDER? OR DISEASE?) L162 QUE
 (L160(5A)L161)
 L163 QUE (DAPHNI? OR CERIODAPHNI? OR HYALELLA? OR ASSELLUS)
 L164 QUE L113 OR (L114 OR L115 OR L116 OR L117 OR L118 OR L119 OR L120 OR L121 OR L122 OR
 L123 OR L124) OR L163
 L165 QUE (PHYTOPLANKTON? OR AUFWUCH# OR LEMNA? OR ARALES OR CHARA)
 L166 QUE (L126 OR L127 OR L128 OR L129) OR (L130 OR L131 OR L132 OR L133) OR L165
 L167 QUE (NEOMYS OR MICROTINAE?)
 L168 QUE (L150 OR L151 OR L152 OR L153 OR L154 OR L155 OR L156 OR L157 OR L158 OR L159) OR
 L167 L169 QUE (LOACH? OR STICKLEBACK? OR MUMMICHOG# OR TILAPIA? OR ASELLUS) L170
 QUE L164 OR L169
 L171 QUE L125 OR L170 OR L166 OR L146 OR L149 OR L168 OR L162
 L172 QUE (L1 OR L2 OR L3 OR L4 OR L5 OR L6 OR L7 OR L8 OR L9 OR L10 OR L11 OR L12 OR L13 OR
 L14 OR L15
 OR L16 OR L17 OR L18 OR L19 OR L20 OR L21 OR L22 OR L23 OR L24 OR L25 OR L26 OR L27 OR L28 OR
 L29 OR
 L30 OR L31 OR L32 OR L33 OR L34 OR L35 OR L36 OR L37 OR L38 OR L39 OR L40 OR L41 OR L42 OR L43
 OR L44 OR L45 OR L46 OR L47 OR L48)
 L173 QUE (L171 OR L172 OR L94 OR L105)

Top-up search conducted August 2022

Endpoint specific search terms were used to filter for results relevant to ecotoxicology endpoints

Patent literature was not considered to be relevant to the results of this search. Since some of the databases used also include patent information, the search was filtered to remove patent document types.

The search was filtered to restrict the publication date.

STN was used to automatically remove duplicate results.

Table 9.10-5: Top-up literature search for ecotoxicology

Search Strategy top-up – Ecotoxicology

- L1 QUE ((RIPARIAN? OR REPTILE? OR SNAKE? OR LIZARD?) OR (TORTOISE? OR TURTLE? OR TERRAPIN? OR CROCODIL?) OR (ALLIGATOR? OR CAIMAN? OR GHARIAL? OR HOVERFLIES) OR ((MEADOW#(W)VOLE#) OR PSEUDOKIRSCHNERIELLA))
- L2 QUE ((RHAPHIDOCELIS OR NITZSCHIA OR CYCLOTELLA OR MICROCYSTIS) OR (OSCILLATORIA OR APHANIZOMENON OR ANKISTRODESMUS) OR (TEILINGRIA OR MONORAPHIDIUM OR RADIOCOCCACAE OR TETRASPORALES))
- L3 QUE ((TETRAEDRON OR TREUBARIA OR WILLEA OR COSMOCLADIUM) OR (HYPOASPIS OR (SOIL(3A)MICROORGAN?) OR ECHINOCHLOA OR SPARTINA) OR (SALVINIA OR NAJAS OR CALLITRICHE OR MYOSOTIS OR STRATIOTES))
- L4 QUE ((HIPURUS OR PERSICARIA OR CLOEON? OR CORBICULA?) OR (NEOCARIDINIA? OR NEOCARIDINA? OR MYSID? OR CICHLIDAE) OR (CICHLID# OR LEPOMIS? OR SERRANIDAE OR PERCIFORMES))
- L5 QUE ((ICTALURUS? OR POECILIA? OR ORYZIAS? OR GASTEROSTEUS?) OR (GASTEROSTEIDAE OR SALVELINUS OR BRACHYDANIO? OR CARASSIUS?) OR (MISGUMUS? OR CYPRINODON? OR FUNDULUS? OR MISGURNUS?))
- L6 QUE ((BREAM OR ROTIFER# OR GAMMARUS OR GAMMARID? OR MAYFLY?) OR (BIVALVE# OR MUSSEL# OR MOLLUSK# OR MOLLUSC# OR BUFO) OR (NEWT# OR SCALLOP# OR CLAM# OR GAMBUSIA OR OREOCHROMIS))
- L7 QUE ((OSTRAC? OR TUBIFEX? OR TURBELLARIA OR COPEPODA) OR (PREDACE? OR PREDACI? OR PARASITOID? OR APIS OR APIDAE) OR (BOMBUS OR BOMBINAE OR WORM# OR LUMBRICIDAE OR LUMBRICUS))
- L8 QUE ((ALLOBOPHORA? OR DENDROBAENA? OR APORRECTODEA? OR DENDRODRILUS?) OR (EISENIA? OR OCTOLASION? OR (LACE(W)WING#) OR NEUROPTER?) OR (CARABID? OR CARBUS OR STAPHYLINID? OR COCCINEL? OR ADALIA?))
- L9 QUE ((STETHORUS? OR SCYMNUS? OR WASP# OR VESPIDAE OR SPHECOIDEA) OR (SPHECIDAE OR STIZIDAE OR OPIUS OR (ICHNEUMON(W)FL?)) OR (ICHNEUMONID? OR BRACONID? OR CHALCID? OR CYNIP? OR APHIDI?))
- L10 QUE ((EUCOILID? OR IBALIID? OR FIGITID? OR EURYTOM? OR TORYM?) OR (ORYM? OR EUCHARIT? OR PERILAMP? OR PTEROMAL? OR CHRYSOLAMP?) OR (EUPELM? OR ENCYRT? OR SIGNIPHOR? OR APHELIN? OR ELASMID?))
- L11 QUE ((ELASMUS OR TETRACAMP? OR MYMAR? OR HELOR? OR PROCTOTRUP?) OR (DIAPRI? OR SCELION? OR PLATYGASTR? OR PLATYGASTER?) OR (CERAPHRON? OR MEGASPIL? OR ARANE? OR OPILION? OR PHALANG?))
- L12 QUE ((ARACHNID? OR HARVESTM? OR DADDYLONGLEG? OR (DADDY(W)LONG(W)LEG?) OR ((DADDY(W)LONGLEG?) OR COLLEMB? OR (SPRING(W)TAIL?) OR CYDNODROMUS?) OR (PARDOSA? OR ORIUS? OR TYPHLODROM? OR PHYTOSEIULUS? OR SYRPHID?))
- L13 QUE ((METASYRPHUS? OR SYRPHUS? OR EUPEODES? OR EPISYRPHUS? OR SYRPHIAN?) OR (EPISTROPHE? OR AMBLYSEIUS? OR POECILUS? OR TRECHUS? OR BEMBIIDION?) OR (NEBRIA? OR PTEROSTICHUS? OR CALOSOMA? OR TACHYPORUS? OR NABIDAE?))
- L14 QUE ((GEOCORIS? OR HYMENOPT? OR HAEMATOLECHA? OR CHRYSOPID? OR SYMPHYTA?) OR (OULEMA? OR APHYTIS? OR BATHYPLECTES? OR LYNPHIIDAE? OR LYNPHIIDAE?) OR (LYNPHIIDAE? OR ERIGONE? OR BATHYPHANTES? OR MEIONETA? OR OEDOTHORAX?))
- L15 QUE ((LEPHTYPHANTES? OR LYCOSID? OR LYCOSA? OR CHRYSOPA? OR DACNUSA?) OR (CYRTORHINUS? OR CRYPTOLAEMUS? OR ZETZELLIA? OR

	LEPTOMASTIX?) OR (TRICHOGRAMMA? OR ENCARSIA? OR MACROLOPHUS? OR CHRYSOPERLA?))
L16	QUE ((ALEOCHARA? OR CHRYSOPID# OR CHRYSOPIDAE OR DIABROTICA) OR (PALEXORISTA? OR MAMMAL## OR ANIMAL? OR RABBIT? OR RODENT#))
L17	QUE ((BLACKBIRD# OR (BLACK(W)BIRD#) OR ((TURDUS OR T)(W)MERULA)) OR (CHAFFINCH? OR ((FRINGILLA OR F)(W)COELEBS) OR GREENFINCH?) OR (((CARDUELIS OR C)(W)CHLORIS) OR SONGTHRUSH?) OR (SONG(W)THRUSH?))
L18	QUE (((TURDUS OR T)(W)PHILOMELOS) OR WREN#) OR ((TROGLODYTES OR T)(W)TROGLODYTES) OR (WILLOW(W)WARBLER#) OR (((PHYLLOSCOPUS OR P)(W)TROCHILUS) OR (GREAT(W)TIT#)) OR (((PARUS OR P)(W)MAJOR) OR ROBIN# OR GOLDFINCH?)
L19	QUE (((ERITHACUS OR E)(W)RUBECULA) OR DUNNOCK#) OR (((CARDUELIS OR C)(W)CARDUELIS) OR LINNET#) OR (((PRUNELLA OR P)(W)MODULARIS) OR SKYLARK# OR (SKY(W)LARK#))
L20	QUE ((HEDGE(W)(SPARROW# OR ACCENTOR#)) OR (((CARDUELIS OR C)(W)CANNABINA) OR ((ALAUDA OR A)(W)ARVENSIS)) OR ((RED(W)LEGGED(W)PARTRIDGE#) OR ((ALECTORIS OR A)(W)RUFA))
L21	QUE ((MEADOW(W)PIPIT#) OR MEADOWPIPIT# OR ((ANTHUS OR A)(W)PRATENSIS)) OR (LAPWING# OR ((VANELLUS OR V)(W)VANELLUS) OR PEEWIT#) OR (STARLING# OR ((STURNUS OR S)(W)VULGARIS))
L22	QUE ((TURTLE(W)DOVE#) OR ((STREPTOPELIA OR S)(W)TURTUR)) OR (YELLOWHAMMER# OR (YELLOW(W)HAMMER#) OR (YELLOW(W)WAGTAIL#)) OR (((EMBERIZA OR E)(W)CITRINELLA) OR (YELLOW(W)WAG(W)TAIL#))
L23	QUE (((MOTACILLA OR M)(W)FLAVA) OR (FAN(W)TAILED(W)WARBLER#)) OR ((GREY(W)LAG(W)G!!SE) OR ((ANSER OR A)(W)ANSER)) OR (REEDBUNTING# OR (REED(W)BUNTING#) OR ((EMBERIZA OR E)(W)SCHOENICLUS))
L24	QUE (CHAFFINCH? OR BLUETIT? OR (BLUE(W)TIT?)) OR (((PARUS OR P)(W)CAERULEUS) OR (SYLVIA(W)COMMUNIS)) OR (((GALERIDA OR G)(W)CRISTATA) OR (TREE(W)SPARROW#))
L25	QUE (((COTURNIX OR C)(W)COTURNIX) OR (GREY(W)PARTRIDGE#)) OR (((PERDIX OR P)(W)PERDIX) OR ((PHASIANUS OR P)(W)COLCHICUS)) OR (((MILIARIA OR M)(W)CALANDRA?) OR GREYLAGG!!SE)
L26	QUE ((GREYLAG(W)G!!SE) OR ((COLUMBA OR C)(W)PALUMBUS?)) OR (((STREPTOPELIA OR S)(W)(ORIENTALIS? OR RISORIA?))) OR (((MOTACILLA OR M)(W)ALBA?) OR (CRESTED(W)LARK#))
L27	QUE ((WHITE(W)WAGTAIL#) OR (WOOD(W)PIGEON#) OR (BIRD(W)LIFE)) OR ((SONG(W)BIRD#) OR VANELLUS? OR (PEE(W)WIT#)) OR (AVIFAUNA? OR (AVI(W)FAUNA?) OR SONGBIRD?)
L28	QUE (ORNITHOLOG? OR PASSERINE? OR WOODPIGEON#) OR (((PASSER OR P)(W)MONTANUS) OR QUAIL# OR (CALANDRA(W)LARK#)) OR (CISTICOLA? OR (Z(W)CISTICOLA?) OR BIRDLIFE)
L29	QUE (GEESE OR GOOSE OR SPARROWS OR PIGEONS OR LARK#) OR (WARBLER# OR PARTRIDGE# OR BUNTING# OR WAGTAIL#) OR (WHITETHROAT# OR PIED# OR (WHITE(W)THROAT#) OR MALLARD OR DUCK OR BOBWHITE OR ANAS? OR COLINUS?)
L30	QUE ((FORAGING OR FARMLAND OR GRASSLAND)(3A)BIRD#) OR (BLUEBIRD# OR (ROCK(W)PTARMIGAN#) OR (BLACK(W)REDSTART#)) OR ((PREDATOR? OR

	NONTARGET? OR (NON(W)TARGET?)(3A)BIRD#) OR ((CORN(W)BUNTING# OR SERINS OR SERINUS)
L31	QUE (L17 OR L18 OR L19 OR L20 OR L21 OR L22 OR L23 OR L24 OR L25 OR L26 OR L27 OR L28 OR L29 OR L30)
L32	QUE (L31 NOT (JAPANESE? OR JAPONICA?))
L33	QUE (((SMALL OR WILD)(3A)MAMMAL#) OR (WILD(3A)ANIMAL?)) OR (VOLE# OR GLIS OR DORMOUSE OR DORMICE OR ELIOMY#) OR (LEROT# OR LAGOMORPH# OR LEPORID? OR LEPUS OR ORYCTOLAGUS? OR VERTEBRAT? OR RAT)
L34	QUE (HARE# OR SORICIDAE? OR SOREX? OR NEOMY# OR CROCIDURA?) OR (SHREW# OR WOODMOUSE OR WOODMICE OR APODEMUS? OR MICROTUS?) OR (CLETHRIONOMYS? OR CRICETIDAE? OR MICROTIN?)
L35	QUE (RAPTOR# OR MARMOSET# OR GOPHER# OR GRASSCUTTER#) OR ((PREDATOR? OR NONTARGET? OR (NON(W)TARGET?))(3A)MAMMAL#) OR ((WOOD(W)(MOUSE OR MICE)) OR ARVICOLA?) OR (MEADOW#(W)VOLE#)
L36	QUE (L33 OR L34 OR L35)
L37	QUE (ECOTOX? OR LC50 OR ((LC OR EC OR LR)(W)50) OR EC50 OR LR50)
L38	QUE ((ECO OR ECOL OR ECOLOG? OR ENV OR ENVIRONM? OR AQUATIC?) (5A)(TOX? OR RISK? OR IMPACT? OR EFFECT?))
L39	QUE (AQUATIC? OR FRESHWATER? OR (FRESH(W)WATER?))
L40	QUE (FLORA OR FAUNA OR BIOTA OR ORGANISM? OR INSECT?) OR (ENVIRONM? OR LIFE OR INVERTEB? OR CRUSTA? OR SPECIES) OR (ENTOMOFAUNA OR (ENTOMO(W)FAUNA))
L41	QUE (L39(5A)L40)
L42	QUE (MAGNA? OR (D(W)MAGNA?) OR CHIRON? OR BRACHIONUS?) OR (LIMNEA? OR CRASSOSTREA? OR ALG? OR FISH OR FISHES) OR (ONCORHYNCHUS? OR SALMONIDAE? OR CYPRINUS? OR CYPRINID?)
L43	QUE (PIMEPHALES? OR PISCES OR TROUT OR SUNFISH? OR CARP) OR (MINNOW? OR (F(W)MINNOW?) OR CATFISH? OR ZEBRAFISH?) OR (GOLDFISH? OR (ZEBRA(W)DANIO#) OR GUPPY OR GUPPIES)
L44	QUE (KILLFISH? OR FATHEAD? OR BLUEGILL? OR SALMON#) OR (THUNDERFISH? OR (WATER(W)(FLY OR FLEA?)) OR WATERFLEA?) OR (FROG# OR AMPHIB? OR SHRIMP# OR PRAWN# OR CRAB# OR TOAD#)
L45	QUE (TADPOLE# OR CRAYFISH? OR SHELLFISH? OR LOBSTER#) OR (OYSTER# OR SNAIL# OR RANA OR RANIDAE? OR PLANKTON? OR crusta? OR ESTUARINE OR GASTROPOD?)
L46	QUE (L37 OR L38)
L47	QUE ((NONTARGET? OR (NON(W)TARGET?))(5A)(PLANT? OR FLORA?)) OR ((AQUATIC(3A)PLANT? OR (PHYTO(W)TOX?) OR PHYTOTOX?)) OR (SEDIMENT? OR HYDROSOIL? OR DUCKWEED? OR PONDWEED?)
L48	QUE (((DUCK OR POND)(W)WEED#) OR MACROPHYT? OR PERIPHYTON?) OR (POTAMOGETON? OR CHAROPHYTA? OR ELODEA? OR HYDROCHARITA?) OR (CERATOPHYL? OR CHLAMYDOMON? OR SELENASTRUM? OR CHLORELLA?)
L49	QUE (SCENEDESMUS? OR SKELETONEMA? OR NAVICULA? OR ANABAENA?) OR (MYRIOPHYLLUM? OR GLYCERIA?)
L50	QUE (NONTARGET? OR (NON(W)TARGET?) OR BENEFICIAL?)
L51	QUE (EFFECT? OR INVERTEB? OR ORGANISM? OR ARTHROPOD? OR INSECT?) OR (FAUNA OR SPECIES OR (ENTOMO(W)FAUNA?) OR ENTOMOFAUNA?)

L52	QUE (L50 (5A)L51)
L53	QUE (PREDAT? OR (NATURAL(W)ENEM?) OR BEE OR BEES OR API? OR HONEYBEE#) OR (BUMBLEBEE# OR ((HONEY OR BUMBLE)(W)BEE#) OR EARTHWORM?) OR ((EARTH(W)WORM?) OR LADYBUG# OR LADYBEETLE# OR LADYBIRD#)
L54	QUE ((LADY(W)(BUG# OR BEETLE# OR BIRD#)) OR HOVERFLY) OR (HOOVERFLIES OR SAWFLY OR SAWFLIES OR DRONEFLY) OR (DRONEFLIES OR FLOWERFLY OR FLOWERFLIES OR LACEWING?)
L55	QUE (((HOVER OR DRONE OR FLOWER OR SAW)(W)(FLY OR FLIES))) OR (SPIDER# OR SPRINGTAIL? OR (ROOT(W)WORM#) OR ROOTWORM# OR MACRO(W)ORGANISM)
L56	QUE (L52 OR L53 OR L54 OR L55)
L57	QUE (BIRD? OR AVES OR AVIAN? OR (AVI(W)FAUNA?) OR AVIFAUNA?) OR (SONGBIRD? OR (SONG(W)BIRD?) OR ORNITHOLOG?)
L58	QUE ((WILD(3A)(LIFE OR ANIMAL#)) OR WILDLIFE OR SQUIRREL?) OR (VOLE# OR SCIURUS OR GLIRID? OR GLIS OR DORMOUSE) OR (DORMICE OR ELIOMYS OR LEROT# OR MUSTELID? OR MINK#)
L59	QUE (MUSTELINE# OR WEASEL? OR STOAT? OR MUSTEL? OR BADGER?) OR (MELES OR MELINAE OR OTTER# OR LUTRA OR LUTRINAE) OR (LAGOMORPH# OR LEPORID? OR LEPUS OR ORYCTOLAGUS OR HARE#)
L60	QUE (TALPA OR MOLE OR MOLES OR HEDGEHOG? OR (HEDGE(W)HOG?)) OR (CROCIDURA? OR SHREW# OR WOODMOUSE OR WOODMICE OR APODEMUS) OR (MICROTUS OR ARVICOLA OR CLETHRIONOMYS? OR CRICETIDAE?) OR (ERINACEUS OR ERINACEIDAE? OR SORICIDAE? OR SOREX)
L61	QUE ((ENDOCRIN? OR HORMON?)(5A)(DISRUPT? OR MIMIC? OR MODULAT? OR DISORDER? OR DISEASE?))
L62	QUE (DAPHNI? OR CERIODAPHNI? OR HYALELLA? OR ASSELLUS)
L63	QUE (L41 OR L42 OR L43 OR L44 OR L45 OR L62)
L64	QUE (PHYTOPLANKTON? OR AUFWUCH# OR LEMNA? OR ARALES OR CHARA OR SEDIMENT(W)DWELL OR HAZARD OR ADVERSE OR BIOACCUMULAT? OR BIOMAGNIFICAT? OR BIOCONCENTRAT? OR POISON OR SEWAGE OR ACTIVATED(W)SLUDGE)
L65	QUE (L47 OR L48 OR L49 OR L64)
L66	QUE (NEOMYS OR MICROTINAE? OR MICROBIAL OR VEGETATIVE(W)VIGO? OR SEEDLING OR GERMINAT? OR MONOCOT? OR DICOT?)
L67	QUE (L58 OR L59 OR L60 OR L66)
L68	QUE (LOACH? OR STICKLEBACK? OR MUMMICHOG# OR TILAPIA? OR ASELLUS)
L69	QUE (L63 OR L68)
L70	QUE (L46 OR L69 OR L65 OR L56 OR L57 OR L67 OR L61)
L71	QUE (L1 OR L2 OR L3 OR L4 OR L5 OR L6 OR L7 OR L8 OR L9 OR L10 OR L11 OR L12 OR L13 OR L14 OR L15 OR L16)
L72	QUE (L70 OR L71 OR L32 OR L36)
	SAVE L72 ECOTOX/Q
	STN Search (conducted 02 August 2022):
	FILE MEDLINE EMBASE ESBIOBASE AGRICOLA BIOSIS CABA HCAPLUS FSTA GEOREF TOXCENTER PQSCITECH SCISEARCH ANABSTR

SET DUPORDER FILE

L1 s PYDIPM/Q AND ECOTOX/Q

L2 s L1 NOT PY<2015

L3 s L2 NOT ED<20151101

L4 s L3 NOT ED>20200731

L5 s L4 NOT PATENT/DT

L6 DUP REM L5

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L1 QUE ((RIPARIAN? OR REPTILE? OR SNAKE? OR LIZARD?) OR (TORTOISE? OR TURTLE? OR TERRAPIN? OR CROCODIL?) OR (ALLIGATOR? OR CAIMAN? OR GHARIAL?) OR ((MEADOW#(W)VOLE#)))

L2 QUE ((HIPPURUS) OR (CICHLIDAE) OR (CICHLID# OR LEPOMIS? OR SERRANIDAE OR PERCIFORMES))

L3 QUE ((ICTALURUS? OR POECILIA? OR ORYZIAS? OR GASTEROSTEUS?) OR (GASTEROSTEIDAE OR SALVELINUS OR BRACHYDANIO? OR CARASSIUS?) OR (MISGUMUS? OR CYPRINODON? OR FUNDULUS? OR MISGURNUS?))

L4 QUE ((BREAM) OR (BUFO) OR (NEWT# OR GAMBUSIA OR OREOCHROMIS))

L5 QUE (MAMMAL## OR RABBIT? OR RODENT#)

L6 QUE ((BLACKBIRD# OR (BLACK(W)BIRD#) OR ((TURDUS OR T)(W)MERULA)) OR (CHAFFINCH? OR ((FRINGILLA OR F)(W)COELEBS) OR GREENFINCH?) OR (((CARDUELIS OR C)(W)CHLORIS) OR SONGTHRUSH?) OR (SONG(W)THRUSH?))

L7 QUE (((TURDUS OR T)(W)PHILOMELOS) OR WREN#) OR ((TROGLODYTES OR T)(W)TROGLODYTES) OR (WILLOW(W)WARBLER#) OR (((PHYLLOSCOPUS OR P)(W)TROCHILUS) OR (GREAT(W)TIT#)) OR (((PARUS OR P)(W)MAJOR) OR ROBIN# OR GOLDFINCH?)

L8 QUE (((ERITHACUS OR E)(W)RUBECULA) OR DUNNOCK#) OR (((CARDUELIS OR C)(W)CARDUELIS) OR LINNET#) OR (((PRUNELLA OR P)(W)MODULARIS) OR SKYLARK# OR (SKY(W)LARK#))

L9 QUE ((HEDGE(W)(SPARROW# OR ACCENTOR#))) OR (((CARDUELIS OR C)(W)CANNABINA) OR ((ALAUDA OR A)(W)ARVENSIS)) OR ((RED(W)LEGGED(W)PARTRIDGE#) OR ((ALECTORIS OR A)(W)RUFA))

L10 QUE ((MEADOW(W)PIBIT#) OR MEADOWPIBIT# OR ((ANTHUS OR A)(W)PRATENSIS)) OR (LAPWING# OR ((VANELLUS OR V)(W)VANELLUS) OR PEEWIT#) OR (STARLING# OR ((STURNUS OR S)(W)VULGARIS))

L11 QUE ((TURTLE(W)DOVE#) OR ((STREPTOPELIA OR S)(W)TURTUR)) OR (YELLOWHAMMER# OR (YELLOW(W)HAMMER#) OR (YELLOW(W)WAGTAIL#)) OR ((EMBERIZA OR E)(W)CITRINELLA) OR (YELLOW(W)WAG(W)TAIL#)

L12 QUE (((MOTACILLA OR M)(W)FLAVA) OR (FAN(W)TAILED(W)WARBLER#)) OR ((GREY(W)LAG(W)G!!SE) OR ((ANSER OR A)(W)ANSER)) OR (REEDBUNTING# OR (REED(W)BUNTING#) OR ((EMBERIZA OR E)(W)SCHOENICLUS))

L13 QUE (CHAFFINCH? OR BLUETIT? OR (BLUE(W)TIT?)) OR (((PARUS OR P)(W)CAERULEUS) OR (SYLVIA(W)COMMUNIS)) OR (((GALERIDA OR G)(W)CRISTATA) OR (TREE(W)SPARROW#))

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- L14 QUE (((COTURNIX OR C)(W)COTURNIX) OR (GREY(W)PARTRIDGE#)) OR (((PERDIX OR P)(W)PERDIX) OR ((PHASIANUS OR P)(W)COLCHICUS)) OR (((MILIARIA OR M)(W)CALANDRA?) OR GREYLAGG!!SE)
- L15 QUE ((GREYLAG(W)G!!SE) OR ((COLUMBA OR C)(W)PALUMBUS?)) OR (((STREPTOPELIA OR S)(W)(ORIENTALIS? OR RISORIA?))) OR (((MOTACILLA OR M)(W)ALBA?) OR (CRESTED(W)LARK#))
- L16 QUE ((WHITE(W)WAGTAIL#) OR (WOOD(W)PIGEON#) OR (BIRD(W)LIFE)) OR ((SONG(W)BIRD#) OR VANELLUS? OR (PEE(W)WIT#)) OR (AVIFAUNA? OR (AVI(W)FAUNA?) OR SONGBIRD?)
- L17 QUE (ORNITHOLOG? OR PASSERINE? OR WOODPIGEON#) OR (((PASSER OR P)(W)MONTANUS) OR QUAIL# OR (CALANDRA(W)LARK#)) OR (CISTICOLA? OR (Z(W)CISTICOLA?) OR BIRDLIFE)
- L18 QUE (GEESE OR GOOSE OR SPARROWS OR PIGEONS OR LARK#) OR (WARBLER# OR PARTRIDGE# OR BUNTING# OR WAGTAIL#) OR (WHITETHROAT# OR PIED# OR (WHITE(W)THROAT#) OR MALLARD OR DUCK OR BOBWHITE OR ANAS? OR COLINUS?)
- L19 QUE ((FORAGING OR FARMLAND OR GRASSLAND)(3A)BIRD#) OR (BLUEBIRD# OR (ROCK(W)PTARMIGAN#) OR (BLACK(W)REDSTART#)) OR ((PREDATOR? OR NONTARGET? OR (NON(W)TARGET))(3A)BIRD#) OR ((CORN(W)BUNTING#) OR SERINS OR SERINUS)
- L20 QUE (L6 OR L7 OR L8 OR L9 OR L10 OR L11 OR L12 OR L13 OR L14 OR L15 OR L16 OR L17 OR L18 OR L19)
- L21 QUE (L20 NOT (JAPANESE? OR JAPONICA?))
- L22 QUE (((SMALL OR WILD)(3A)MAMMAL#)) OR (VOLE# OR GLIS OR DORMOUSE OR DORMICE OR ELIOMY#) OR (LEROT# OR LAGOMORPH# OR LEPORID? OR LEPUS OR ORYCTOLAGUS? OR VERTEBRAT? OR RAT)
- L23 QUE (HARE# OR SORICIDAE? OR SOREX? OR NEOMY# OR CROCIDURA?) OR (SHREW# OR WOODMOUSE OR WOODMICE OR APODEMUS? OR MICROTUS?) OR (CLETHRIONOMYS? OR CRICETIDAE? OR MICROTIN?)
- L24 QUE (RAPTOR# OR MARMOSET# OR GOPHER# OR GRASSCUTTER#) OR ((PREDATOR? OR NONTARGET? OR (NON(W)TARGET?))(3A)MAMMAL#) OR ((WOOD(W)(MOUSE OR MICE)) OR ARVICOLA?) OR (MEADOW#(W)VOLE#)
- L25 QUE (L22 OR L23 OR L24)
- L26 QUE (ECOTOX? OR LC50 OR ((LC OR EC OR LR)(W)50) OR EC50 OR LR50)
- L27 QUE ((ECO OR ECOL OR ECOLOG? OR ENV OR ENVIRONM? OR AQUATIC?) (5A)(TOX? OR RISK? OR IMPACT? OR EFFECT?))
- L28 QUE (AQUATIC? OR FRESHWATER? OR (FRESH(W)WATER?))
- L29 QUE (FAUNA OR BIOTA OR ORGANISM?) OR (ENVIRONM? OR LIFE OR SPECIES)
- L30 QUE (L28(5A)L29)
- L31 QUE (FISH OR FISHES) OR (ONCORHYNCHUS? OR SALMONIDAE? OR CYPRINUS? OR CYPRINID?)
- L32 QUE (PIMEPHALES? OR PISCES OR TROUT OR SUNFISH? OR CARP) OR (MINNOW? OR (F(W)MINNOW?) OR CATFISH? OR ZEBRAFISH?) OR (GOLDFISH? OR (ZEBRA(W)DANIO#) OR GUPPY OR GUPPIES)
- L33 QUE (KILLFISH? OR FATHEAD? OR BLUEGILL? OR SALMON#) OR (THUNDERFISH?) OR (FROG# OR AMPHIB? OR TOAD#)
- L34 QUE (TADPOLE#) OR (RANA OR RANIDAE?)

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L35 QUE (L26 OR L27)

L36 QUE (NONTARGET? OR (NON(W)TARGET?) OR BENEFICIAL?)

L37 QUE (EFFECT? OR ORGANISM?) OR (FAUNA OR SPECIES)

L38 QUE (L36 (5A)L37)

L39 QUE (BIRD? OR AVES OR AVIAN? OR (AVI(W)FAUNA?) OR AVIFAUNA?) OR (SONGBIRD? OR (SONG(W)BIRD?) OR ORNITHOLOG?)

L40 QUE ((WILD(3A)(LIFE OR ANIMAL#)) OR WILDLIFE OR SQUIRREL?) OR (VOLE# OR SCIURUS OR GLIRID? OR GLIS OR DORMOUSE) OR (DORMICE OR ELIOMYS OR LEROT# OR MUSTELID? OR MINK#)

L41 QUE (MUSTELINE# OR WEASEL? OR STOAT? OR MUSTEL? OR BADGER?) OR (MELES OR MELINAE OR OTTER# OR LUTRA OR LUTRINAE) OR (LAGOMORPH# OR LEPORID? OR LEPUS OR ORYCTOLAGUS OR HARE#)

L42 QUE (TALPA OR MOLE OR MOLES OR HEDGEHOG? OR (HEDGE(W)HOG?)) OR (CROCIDURA? OR SHREW# OR WOODMOUSE OR WOODMICE OR APODEMUS) OR (MICROTUS OR ARVICOLA OR CLETHRIONOMYS? OR CRICETIDAE?) OR (ERINACEUS OR ERINACEIDAE? OR SORICIDAE? OR SOREX)

L43 QUE ((ENDOCRIN? OR HORMON?)(5A)(DISRUPT? OR MIMIC? OR MODULAT? OR DISORDER? OR DISEASE?))

L44 QUE (L30 OR L31 OR L32 OR L33 OR L34)

L45 QUE (HAZARD OR ADVERSE)

L46 QUE (NEOMYS OR MICROTINAE?)

L47 QUE (L40 OR L41 OR L42 OR L46)

L48 QUE (LOACH? OR STICKLEBACK? OR MUMMICHOG# OR TILAPIA?)

L49 QUE (L44 OR L48)

L50 QUE (L35 OR L49 OR L45 OR L39 OR L47 OR L43)

L51 QUE (L1 OR L2 OR L3 OR L4 OR L5)

L52 QUE (L50 OR L51 OR L21 OR L25)

L53 QUE (ADRENAL? OR GONAD? OR SPERM OR METAMORPH? OR VAGINA OR ANDROGEN? OR GNRH OR STEROID? OR SECONDARY(W)SEX? OR PITUITARY OR AROMATASE OR GLUCOCORTICOID? OR TESTIS OR SILURANA OR OVARIAN(W)FOLLICLE OR CORTICOTROP? OR HORMONE?)

L54 QUE (?TESTOSTERONE OR SPIGGIN? OR OVIDUCT OR OVARY OR ENDOCRINE(W)DISRUPT? OR HYPOTHALAM? OR THYROID? OR TUBERCLES OR ?ESTRADIOL OR EPIDIDYM? OR LUTEINI!ING(W)HORMONE OR LH)

L55 QUE (THYROXINE OR VITELLOGEN? OR ?ESTRUS OR ?ESTROUS OR ?ESTROGEN? OR NEURODEVELOPMENT OR TRIIODOTHYRONINE OR XENOPUS OR TSH OR FOLLICLE(W)STIMULATING(W)HORMONE OR FSH OR VAGINAL(W)OPENING)

L56 QUE (L52 OR L53 OR L54 OR L55)

SAVE L56 ECOTOXEDSYN/Q

Relevance and reliability

The relevance of literature studies is defined as the extent to which a test is appropriate for a particular hazard or risk assessment, the way a study can be used and the framework used for evaluation, hence a study, may be relevant

in one framework but not in another. The criteria for assessing the relevance of the literature data are shown in the table below.

Table 9.10-4: Proposed List of Criteria for relevance for each data requirement

Data requirements(s) (indicated by the correspondent CA data point (s))	Criteria for relevance
Ecotoxicological studies (CA 8.1 to 8.15)	<p><u>Laboratory Studies</u></p> <p>Well defined test material (including purity/content)</p> <p>Number of organisms per group sufficient to establish a statistical significance</p> <p>Applicable test species</p> <p>Test organisms are not previously exposed to the test material or other contaminants</p> <p>Several dose levels tested, at least 3, including a negative control, to establish a dose-response, unless the study design is specifically a limit test. Control must be run concurrently with treatments and mortality to be within test validity criteria.</p> <p>Exposure route is clearly defined, is environmentally relevant and, if appropriate, suitably quantified.</p> <p>If conducted, analytical confirmation of dosing or sufficient information provided to determine concentrations were within acceptable range (e.g. 80/120%) of nominal targets.</p> <p>Effects are related to single test item, and a quantitative relationship exists between the reported endpoint and risk assessment endpoints of growth, mortality, behaviour and/or reproduction.</p> <p>Sufficient experimental information provided to substantiate and evaluate whether the study conclusions and endpoints are robust.</p> <p>Study conditions should not differ significantly from recommended protocols.</p> <p>Study conditions should not interfere with the interpretation of the study results.</p> <p><u>Field Studies</u></p> <p>Appropriate and relevant geoclimatic conditions (setting), appropriate application method and rates (exposure) and observation data (biological relevance) to derive endpoints.</p> <p>Well defined test material (including purity/content)</p> <p>Applicable test species</p> <p>Exposure route is clearly defined, is environmentally relevant and, if appropriate, suitably quantified.</p> <p>Sufficient experimental information provided to substantiate and evaluate whether the study conclusions and endpoints are robust (e.g. pre-treatment details, characterisation of physico-chemical parameters, replication, statistical methods and appropriate sampling regime).</p> <p>Study conditions should not differ significantly from recommended protocols, if available for field study.</p> <p>Study conditions should not interfere with the interpretation of the study results</p>

* Recommended protocols under each data point include but are not limited to those listed in the Commission Communications 2013/C 95/01 and 2013/C 95/02

HSE agrees with the relevance criteria based on guidance in EFSA journal, 2011. HSE agrees with the reliability of the search based on the table above, the inherent quality of the study is based on methodology, conduct, reporting, and reproducibility.

Results

The results of the search method employed by the applicant is presented in table 9.1-5 below.

Table 9.10-56: Results of study selection process

Data requirement(s) captured in the search	Number SYN545974 Initial Search	Number SYN545974 Top-Up Search	Number SYN545974 Specific Metabolites Search	Number Common SDHI Metabolites Search	Total
Total number of <i>summary records</i> retrieved after <i>all*</i> searches of peer-reviewed literature (excluding duplicates)	2	31	3465	4	3502
Number of <i>summary records</i> excluded from the search results after rapid assessment for relevance**	2	31	3465	4	3502
Total number of <i>full-text</i> documents assessed in detail*	0	0	0	0	0
Number of <i>studies</i> excluded from further consideration after detailed assessment for relevance	0	0	0	0	0
Number of <i>studies</i> not excluded for relevance after detailed assessment (i.e. relevant studies and studies of unclear relevance)	0	0	0	0	0

*both from bibliographic databases and other sources of peer-reviewed literature

** aligned with EFSA Journal 2011; 9(2) 2092: rapid assessment means exclusion of “obviously irrelevant records” based on titles.

All of the references were excluded from the rapid assessment, as no external research has been published on the parent molecule SYN545974, the SYN545974 specific environmental metabolites (SYN545547 and SYN548261) and the SYN545974 common SDHI environmental metabolite (NOA449410). The SYN545974 specific metabolite search which returned many thousands of hits did not contain any of the SYN545974 metabolites found in the environment, rather many hits for common classes for chemistry e.g. trichlorophenols. Therefore no full text was assessed and no studies were identified as potentially relevant for this submission of SYN545974.

Top-up search

Following an update to the literature review requested by HSE to include publications up until the date of submission, the applicant provided the below consideration.

Table 9.10-7: Rational for exclusion of papers during rapid assessment - Ecotoxicology

Criteria for exclusion	Number of publications excluded at rapid assessment of title or abstract
	Search (August 2022)
Biochemistry	12
ED	5
Efficacy	60
Exposure scenario not environmentally relevant	1
Fate	4
Fate in non-environmentally relevant scenario	27
Fate, non relevant geoclimatic area	10
Human health	41
In silico	18
Metabolism	6
Methods of analysis	53
Mixture of test substances	18
No data	1
Not representative for chemical under evaluation	6

No representative species for risk assessment	3
Not test substance	9
Physical properties	1
Remediation	109
TOTAL EXCLUDED	384

Table 9.10-8: Rational for exclusion of papers during rapid assessment – Ecotoxicology ED

Criteria for exclusion	Number of publications excluded at rapid assessment of title or abstract
	Search: August 2022
	Pydiflumetofen ED search - ecotoxicology
Publication relevant to proceedings from conferences/meetings	1
Publication relevant to environmental fate	2
Publication relevant to efficacy, resistance, or pest control	19
Publication concerned with cost effectiveness of disease control	1
Publication not on the a.i. of interest	1
Publication concerned with methods to select top dose in toxicity testing	1
Publication relevant to toxicology	1
TOTAL EXCLUDED	26

HSE evaluator comments:

Overall, the literature review is considered acceptable. HSE agrees with the exclusion of literature based on guidance in EFSA Journal 2011. The search terms used were acceptable to HSE and a range of databases were utilised.

Following an update to the literature review requested by HSE to include publications up until the date of submission, no publications relevant to ecotoxicology were found and no further consideration is required.

As this is a new active substance the literature search did not provide any results.

HSE notes that an updated literature has been submitted by the applicant. An initial assessment suggests there are no relevant studies from the updated literature review. However, this will need further consideration and HSE will update accordingly.

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
KCA1 8.1.1.1	██████ ████ ██████	2013	SYN545974 – An Acute Oral Toxicity Study with the Northern Bobwhite Using a Sequential Testing Procedure Report No. 528-393 Document No. VV-404460 , SYN545974_10062 Test Facility ██████████ GLP Unpublished	Y	Y	Data for first approval	SYN	N
KCA1 8.1.1.1	██████ ████ ██████	2013a	SYN545974 – An Acute Oral Toxicity Study with the Canary using a Sequential Testing Procedure Report No. 528-394 Document No. VV-404576 , SYN545974_10065 Test Facility ██████████ GLP Unpublished	Y	Y	Data for first approval	SYN	N
KCA1 8.1.1.2	██████ ████ ██████ ████ ██████	2013	SYN545974 – A Dietary LC50 Study with the Northern Bobwhite Report No. 528-391 Document No. VV-404461 , SYN545974_10063 Test Facility ██████████ GLP Unpublished	Y	Y	Data for first approval <u>Not used in the risk assessment</u>	SYN	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
KCA1 8.1.1.2	[REDACTED]	2013	SYN545974 – A Dietary LC50 Study with the Mallard Report No. 528-392 Document No. VV-404462 , SYN545974_10064 Test Facility [REDACTED] GLP Unpublished	Y	Y	Data for first approval <u>Not used in the risk assessment</u>	SYN	N
KCA1 8.1.1.2	[REDACTED]	2016	SYN545974 - Response to ANSES comments regarding the bird dietary toxicity studies Report No. N/A Document No. VV-137213 , SYN545974_10459 Test Facility N/A Not GLP Unpublished	N/A	N	N/A	SYN	N
KCA1 8.1.1.3	[REDACTED]	2014	SYN545974 – A Reproduction Study with the Mallard Report No. 528-397 Document No. VV-411097 , SYN545974_10134 Test Facility [REDACTED] GLP Unpublished	Y	Y	Data for first approval	SYN	N
KCA1 8.1.1.3	[REDACTED]	2015	SYN545974 – A Reproduction Study with the Northern Bobwhite Report No. 528-396 Document No. VV-410869 , SYN545974_10130 Test Facility [REDACTED] GLP Unpublished	Y	Y	Data for first approval	SYN	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
KCA1 8.1.1.3	[REDACTED]	2016	SYN545974 - Response to ANSES comments regarding the reproduction study with the Northern Bobwhite ([REDACTED] et al., 2015) Report No. N/A Document No. VV-137209 , SYN545974_10449 Test Facility N/A Not GLP Unpublished	N/A	N	N/A	SYN	N
KCA1 8.1.5 Also under data point 8.2.3	[REDACTED]	2020a	Pydiflumetofen - Review for Potential Endocrine Disruption Report No. TK0259197 Document No. VV-639581 , SYN545974_10638 Test Facility Syngenta - Jealott's Hill Not GLP Unpublished	N	N	N/A	SYN	N
KCA1 8.1.5 Also under data point 8.2.3	[REDACTED]	2020	Pydiflumetofen - Endocrine Disruption - Appendix E Update July 2020 Report No. N/A Document No. VV-866644 Test Facility Syngenta - Jealott's Hill Not GLP Unpublished	N	N	N/A	SYN	N
KCA1 8.1.5 Also under data point 8.2.3	[REDACTED]	2016	SYN545974 – Review for Potential for Endocrine Disruption in Ecotoxicological Species Report No. N/A Document No. VV-415537 , SYN545974_10363 Test Facility Syngenta - Jealott's Hill Not GLP Unpublished This is CONFIDENTIAL INFORMATION	N	N	N/A	SYN	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
KCA1 8.2.1	██████████ ██████████	2016	SYN548261 - Acute Toxicity to Oncorhynchus mykiss Report No. 3201085 Document No. VV-414937 , SYN548261_10002 Test Facility ██████████ GLP Unpublished	Y	Y	Data for first approval	SYN	N
KCA1 8.2.1	██████████	2012	SYN545974 – Acute Toxicity to Rainbow Trout (Oncorhynchus mykiss) Under Flow-Through Conditions Report No. 1781.6840 Document No. VV-402859 , SYN545974_10014 Test Facility ██████████ ██████████ GLP Unpublished	Y	Y	Data for first approval	SYN	N
KCA1 8.2.1	██████████	2013a	SYN545974 – Acute Toxicity to Carp (Cyprinus carpio) Under Flow–Through Conditions Report No. 1781.6882 Document No. VV-404432 , SYN545974_10066 Test Facility ██████████ ██████████ GLP Unpublished	Y	Y	Data for first approval	SYN	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
KCA1 8.2.1	████████	2013b	SYN545974 – Acute Toxicity to Sheepshead Minnow (Cyprinodon variegatus) Under Flow-Through Conditions Report No. 1781.6884 Document No. VV-404433 , SYN545974_10067 Test Facility ██████████ GLP Unpublished	Y	Y	Data for first approval	SYN	N
KCA1 8.2.1	████████	2013	SYN545974 – Acute Toxicity to Fathead Minnow (Pimephales promelas) Under Flow-Through Conditions Report No. 1781.6883 Document No. VV-404422 , SYN545974_10068 Test Facility ██████████ GLP Unpublished	Y	Y	Data for first approval	SYN	N
KCA1 8.2.1	████████	2014	SYN545974 – Acute Toxicity to Bluegill Sunfish (Lepomis macrochirus) Under Flow – Through Conditions Report No. 1781.7025 Document No. VV-410863 , SYN545974_10129 Test Facility ██████████ GLP Unpublished	Y	Y	Data for first approval	SYN	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
KCA1 8.2.1	██████████	2009	M700F001 (metabolite of BAS 700 F) - Acute toxicity for rainbow trout Report No. W/09/09 2009/1021591 Document No. VV-401998 , CA4312_10909 CA4312_50005 Test Facility ██████████ GLP Unpublished	Y	Y	Data for first approval	SYN	N
KCA1 8.2.1	██████████	2015	SYN545547 - Acute Toxicity Test with Rainbow Trout (Oncorhynchus mykiss) under static conditions Report No. 1781.7096 Document No. VV-414084 , SYN545547_10001 Test Facility ██████████ GLP Unpublished	Y	Y	Data for first approval	SYN	N
KCA1 8.2.2.1	██████████	2015	SYN545974 - Early Life-Stage Toxicity Test with Sheepshead Minnow, Cyprinodon variegatus Report No. 1781.6979 Document No. VV-414304 , SYN545974_10293 Test Facility ██████████ GLP Unpublished	Y	Y	Data for first approval	SYN	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
KCA1 8.2.2.1	██████	2016	Pydiflumetofen - Statistical Reanalysis; SYN545974 - Early Life-Stage Toxicity Test with Fathead Minnow (Pimephales promelas) Report No. 1781.7192c Document No. VV-134508 , SYN545974_10469 Test Facility ██████ ██████ Not GLP Unpublished This is CONFIDENTIAL INFORMATION	Y	N	N/A	SYN	N
KCA1 8.2.2.1	██████	2016a	Pydiflumetofen - Statistical Reanalysis; SYN545974 - Early Life-Stage Toxicity Test with Sheepshead Minnow (Cyprinodon variegatus) Report No. 1781.7192d Document No. VV-134507 , SYN545974_10467 Test Facility ██████ ██████ Not GLP Unpublished This is CONFIDENTIAL INFORMATION	Y	N	N/A	SYN	N
KCA1 8.2.2.1	██████	2020	SYN545974 - Early Life-Stage Toxicity Test with Fathead Minnow (Pimephales promelas) Report No. 1781.6843 incl. amendments Document No. VV-405320 , SYN545974_10080 Test Facility ██████ ██████ GLP Unpublished	Y	Y	Data for first approval	SYN	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
KCA1 8.2.2.3	██████	2017	SYN545974 – Flow-through Bioconcentration and Metabolism Study with Bluegill Sunfish (Lepomis macrochirus) Report No. 1781.6900 incl. amendments Document No. VV-406411 , SYN545974_10093 Test Facility ██████ ██████ GLP Unpublished	Y	Y	Data for first approval	SYN	N
KCA1 8.2.3	██████	2020a	Pydiflumetofen – Fish Short-Term Reproduction Assay with Fathead Minnow (Pimephales promelas) Report No. 1781.7303 Document No. VV-857838 Test Facility ██████ GLP Unpublished	Y	Y	Data for first approval	SYN	N
KCA1 8.2.3	██████	2020	Pydiflumetofen - Amphibian Metamorphosis Assay with African Clawed Frog (Xenopus laevis) Report No. 1781.7310 Document No. VV-858948 Test Facility ██████ GLP Unpublished	Y	Y	Data for first approval	SYN	N
KCA1 8.2.3	██████ ██████████ ██████	2023	Test Item: Pydiflumetofen Rapid Androgen Disruption Activity Reporter (RADAR) assay Report No. : ██████████ DRAFT REPORT Test facility ████████████████████ Unpublished	Y	Y	Data for first approval	SYN	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
KCA1 8.2.4.1	██████████ ██████████	2016a	SYN548261 - Acute Toxicity to Water Fleas, (Daphnia magna) under Static Conditions Report No. 3201086 Document No. VV-414931 , SYN548261_10000 Test Facility Smithers Viscient (ESG) Ltd GLP Unpublished	N	Y	Data for first approval	SYN	N
KCA1 8.2.4.1	██████████	2017	SYN545974 – Acute Toxicity to Water Fleas (Daphnia magna) Under Static Conditions Report No. 1781.6839 incl. amendment Document No. VV-402832 , SYN545974_10016 Test Facility Smithers Viscient GLP Unpublished	N	Y	Data for first approval	SYN	N
KCA1 8.2.4.1	██████████	2009a	M700F001 (metabolite of BAS 700 F) - Daphnia magna, acute immobilization test Report No. 2009/1021592 W/10/09 Document No. VV-401997 , CA4312_50006 CA4312_10908 Test Facility Institute of Industrial Organic Chemistry GLP Unpublished	N	Y	Data for first approval	SYN	N
KCA1 8.2.4.1	██████████	2015a	SYN545547 - Acute Toxicity to Water Fleas (Daphnia magna) Under Static Conditions Report No. 1781.7095 Document No. VV-413198 , SYN545547_10000 Test Facility Smithers Viscient GLP Unpublished	N	Y	Data for first approval	SYN	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
KCA1 8.2.4.2	██████	2015	SYN545974 – A 48-hour static acute toxicity test with the freshwater amphipod (<i>Hyalella azteca</i>) Report No. 528A-287 Document No. VV-415217 , SYN545974_10354 Test Facility Wildlife International Ltd. GLP Unpublished	N	Y	Data for first approval	SYN	N
KCA1 8.2.4.2	██████.	2016a	SYN545974 - Response to ANSES comments regarding the acute toxicity test with mysids (<i>Americamysis bahia</i>) (██████, 2016) Report No. N/A Document No. VV-137210 , SYN545974_10451 Test Facility N/A Not GLP Unpublished	N/A	N	N/A	SYN	N
KCA1 8.2.4.2	██████	2014a	SYN545974 – Toxicity to Eastern Oyster (<i>Crassostrea virginica</i>) Under Flow-Through Conditions Report No. 1781.6885 Document No. VV-407528 , SYN545974_10099 Test Facility Smithers Viscient GLP Unpublished	N	Y	Data for first approval <u>Not used in risk assessment</u>	SYN	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
KCA1 8.2.4.2		2016	SYN545974 – Acute toxicity to Mysid (Americamysis bahia), under static conditions Report No. 1781.6838 (Including Amendment 1) Document No. VV-402952 , SYN545974_10015 Test Facility Smithers Viscient GLP Unpublished	N	Y	Data for first approval	SYN	N
KCA1 8.2.4.2		2015	SYN545974 – Acute toxicity of SYN545974 to Chaoborus crystallinus Report No. CEA.1666 Document No. VV-414780 , SYN545974_10341 Test Facility Cambridge Environmental Assessments GLP Unpublished	N	Y	Data for first approval	SYN	N
KCA1 8.2.4.2		2015b	SYN545974 – Acute toxicity of SYN545974 to Cyclops agilis speratus Report No. CEA.1662 Document No. VV-414891 , SYN545974_10347 Test Facility Cambridge Environmental Assessments GLP Unpublished	N	Y	Data for first approval	SYN	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
KCA1 8.2.4.2	██████	2015a	SYN545974 – Acute toxicity of SYN545974 to Chironomus riparius Report No. CEA.1667 Document No. VV-414602 , SYN545974_10316 Test Facility Cambridge Environmental Assessments GLP Unpublished	N	Y	Data for first approval	SYN	N
KCA1 8.2.4.2	██████	2015	SYN545974 – Acute toxicity of SYN545974 to Asellus aquaticus Report No. CEA.1644 Document No. VV-414265 , SYN545974_10305 Test Facility Cambridge Environmental Assessments GLP Unpublished	N	Y	Data for first approval	SYN	N
KCA1 8.2.4.2	██████	2015a	SYN545974 – Acute toxicity of SYN545974 to Cloeon dipterum Report No. CEA.1664 Document No. VV-414583 , SYN545974_10315 Test Facility Cambridge Environmental Assessments GLP Unpublished	N	Y	Data for first approval	SYN	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
KCA1 8.2.4.2		2015b	SYN545974 – Acute Toxicity of SYN545974 to Crangonx pseudogracilis Report No. CEA.1661 Document No. VV-414266 , SYN545974_10306 Test Facility Cambridge Environmental Assessments GLP Unpublished	N	Y	Data for first approval	SYN	N
KCA1 8.2.4.2		2015c	SYN545974 – Acute toxicity of SYN545974 to Lumbriculus variegatus Report No. CEA.1642 Document No. VV-414260 , SYN545974_10304 Test Facility Cambridge Environmental Assessments GLP Unpublished	N	Y	Data for first approval	SYN	N
KCA1 8.2.4.2		2015d	SYN545974 – Acute toxicity of SYN545974 to Lymnaea stagnalis Report No. CEA.1645 Document No. VV-414259 , SYN545974_10303 Test Facility Cambridge Environmental Assessments GLP Unpublished	N	Y	Data for first approval	SYN	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
KCA1 8.2.5.1	██████████	2016a	SYN545974 – Full Life-Cycle Toxicity Test with Water Fleas, Daphnia magna, Under Static Renewal Conditions Report No. 1781.6842 (Including Amendments 5 and 6) Document No. VV-402673 , SYN545974_10017 Test Facility Smithers Viscient GLP Unpublished	N	Y	Data for first approval	SYN	N
KCA1 8.2.5.2	██████████ ██████████.	2016a	SYN545974 - Response to ANSES comments regarding the life-cycle toxicity test with mysids (Americamysis bahia) (██████████, 2015a) Report No. N/A Document No. VV-137220 , SYN545974_10471 Test Facility N/A Not GLP Unpublished	N/A	N	N/A	SYN	N
KCA1 8.2.5.2	██████████	2015a	SYN545974 - Life-Cycle Toxicity Test with Mysids (Americamysis bahia) Report No. 1781.6886 Document No. VV-411300 , SYN545974_10167 Test Facility Smithers Viscient GLP Unpublished	N	Y	Data for first approval	SYN	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
KCA1 8.2.5.2	[REDACTED]	2016b	Pydiflumetofen - Statistical Reanalysis; SYN545974 - Life-Cycle Toxicity Test with Mysids (Americamysis bahia) Report No. 1781.7192e Document No. VV-134506 , SYN545974_10465 Test Facility Smithers Viscient Not GLP Unpublished This is CONFIDENTIAL INFORMATION	N	N	N/A	SYN	N
KCA1 8.2.5.3	[REDACTED]	2015	SYN545547 - A Prolonged Sediment Toxicity Test with the Midge (Chironomus riparius) Using Spiked Sediment Report No. 528A-286 Document No. VV-414378 , SYN545547_10004 Test Facility Wildlife International Ltd. GLP Unpublished	N	Y	Data for first approval	SYN	N
KCA1 8.2.5.4	[REDACTED]	2015b	SYN545974 - 10-Day Toxicity Test Exposing Estuarine Amphipods (Leptocheirus plumulosus) to a Test Substance Applied to Sediment under Static Conditions Report No. 1781.7069 Document No. VV-413425 , SYN545974_50120 Test Facility Smithers Viscient GLP Unpublished	N	Y	Data for first approval	SYN	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
KCA1 8.2.5.4		2015a	SYN545974 – 42-Day Toxicity Test Exposing Freshwater Amphipods (Hyalella Azteca) to Spiked Sediment Report No. 1781.6890 Document No. VV-407470 , SYN545974_10094 Test Facility Smithers Viscient GLP Unpublished	N	Y	Data for first approval	SYN	N
KCA1 8.2.5.4		2015	SYN545974 – Life-cycle Toxicity Test Exposing Midges (Chironomus dilutes) to Spiked Sediment Report No. 1781.6889 Document No. VV-407472 , SYN545974_10095 Test Facility Smithers Viscient GLP Unpublished	N	Y	Data for first approval <u>Not used in risk assessment</u>	SYN	N
KCA1 8.2.5.4		2016	Pydiflumetofen - Statistical Reanalysis; SYN545974 - Life-Cycle Toxicity Test Exposing Midges (Chironomus dilutus) to Spiked Sediment Report No. 1781.7192a Document No. VV-134290 , SYN545974_10457 Test Facility Smithers Viscient Not GLP Unpublished This is CONFIDENTIAL INFORMATION	N	N	N/A	SYN	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
KCA1 8.2.5.4	████████	2016a	Pydiflumetofen - Statistical Reanalysis; SYN545974 - 42-Day Toxicity Test Exposing Freshwater Amphipods (<i>Hyalella azteca</i>) to Spiked Sediment Report No. 1781.7192b Document No. VV-134288 , SYN545974_10455 Test Facility Smithers Viscient Not GLP Unpublished This is CONFIDENTIAL INFORMATION	N	N	N/A	SYN	N
KCA1 8.2.6.1	████████ ████████	2016b	SYN548261 - Inhibition of Growth to the Alga <i>Pseudokirchneriella subcapitata</i> in a 96-hour test Report No. 3201084 Document No. VV-414932 , SYN548261_10001 Test Facility Smithers Viscient (ESG) Ltd GLP Unpublished	N	Y	Data for first approval	SYN	N
KCA1 8.2.6.1	████████	2013	SYN545974 – 96-hour Toxicity Test with the Freshwater Green Alga, <i>Pseudokirchneriella</i> <i>subcapitata</i> Report No. 1781.6841 Document No. VV-402845 , SYN545974_10013 Test Facility Smithers Viscient GLP Unpublished	N	Y	Data for first approval	SYN	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
KCA1 8.2.6.1		2009b	M700F001 (metabolite of BAS 700 F): Pseudokirchneriella subcapitata SAG.61.81 growth inhibition test Report No. 2009/1021593 W/11/09 2009/1102103 Document No. VV-401996 , CA4312_10907 Test Facility Institute of Industrial Organic Chemistry GLP Unpublished	N	Y	Data for first approval	SYN	N
KCA1 8.2.6.1		2015	SYN545547 – 96-Hour Toxicity Test with the Freshwater Green Alga, Pseudokirchneriella subcapitata Report No. 1781.7094 Document No. VV-413967 , SYN545547_10002 Test Facility Smithers Viscient GLP Unpublished	N	Y	Data for first approval	SYN	N
KCA1 8.2.6.2		2013	SYN545974 – Toxicity Test to the Freshwater Blue-Green Alga, Anabaena flos-aquae Report No. 1781.6881 Document No. VV-406480 , SYN545974_10091 Test Facility Smithers Viscient GLP Unpublished	N	Y	Data for first approval	SYN	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
KCA1 8.2.6.2	██████	2014	SYN545974 – 96-Hour Toxicity Test with the Marine Diatom, Skeletonema costatum Report No. 1781.6880 Document No. VV-409188 , SYN545974_10105 Test Facility Smithers Viscient GLP Unpublished	N	Y	Data for first approval	SYN	N
KCA1 8.2.6.2	██████	2015	SYN545974 – 96-Hour Toxicity Test with the Freshwater Diatom, Navicula pelliculosa Report No. 1781.6879 Document No. VV-407284 , SYN545974_10097 Test Facility Smithers Viscient GLP Unpublished	N	Y	Data for first approval	SYN	N
KCA1 8.2.7	██████	2015a	SYN545974 – 7-Day Toxicity Test with Duckweed (Lemna gibba) Report No. 1781.6878 Document No. VV-406021 , SYN545974_10088 Test Facility Smithers Viscient GLP Unpublished	N	Y	Data for first approval <u>Not used in risk assessment</u>	SYN	N
KCA1 8.3.1.1.1	██████	2012	SYN545974 – Acute Oral and Contact Toxicity to the Honeybee Apis mellifera L. in the Laboratory Report No. S11-03873 Document No. VV-402698 , SYN545974_10010 Test Facility Eurofins Agroscience Services EcoChem GmbH GLP Unpublished	N	Y	Data for first approval	SYN	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
KCA1 8.3.1.3		2015	SYN545974 - A laboratory study to determine the chronic effects on the brood of the honey bee <i>Apis mellifera</i> L. (Hymenoptera: Apidae). Report No. 037SRFR15C06 Document No. VV-414128 , SYN545974_10279 Test Facility SynTech Research France SAS GLP Unpublished	N	Y	Data for first approval	SYN	N
KCA1 8.3.1.3		2018	Pydiflumetofen - Effects on the honeybee brood <i>Apis mellifera</i> L. following chronic oral exposure under field conditions Report No. 17 48 BFB 0001 Document No. VV-469686 , SYN545974_10619 Test Facility BioChem agrar GLP Unpublished	N	Y	Data for first approval	SYN	N
KCA1 8.4		2012	SYN545974 – Acute toxicity to the earthworm <i>Eisenia fetida</i> Report No. 12 10 48 076 S Document No. VV-401989 , SYN545974_10008 Test Facility BioChem agrar GLP Unpublished	N	Y	Data for first approval <u>Not evaluated or used in the risk assessment</u>	SYN	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
KCA1 8.5	██████	2015	SYN545974 - Effects on the Activity of Soil Microflora (Nitrogen and Carbon Transformation Tests) Report No. 15 10 48 111 C/N Document No. VV-413949 , SYN545974_10275 Test Facility BioChem agrar GLP Unpublished	N	Y	Data for first approval	SYN	N
KCA1 8.5	██████	2017	SYN545974 – Effects on the Activity of Soil Microflora (Nitrogen and Carbon Transformation Tests) Report No. 17 48 SMO 0015 Document No. VV-468002 , SYN545974_10535 Test Facility BioChem agrar GLP Unpublished	N	Y	Data for first approval	SYN	N
KCA1 8.8	██████	2013	SYN545974 – Toxicity to Activated Sludge in a Respiration Inhibition Test Report No. D64647 Document No. VV-404118 , SYN545974_10061 Test Facility Harlan Laboratories Ltd. GLP Unpublished	N	Y	Data for first approval	SYN	N