

Draft Assessment Report

Evaluation of Active Substances

Plant Protection Products

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

**Aqueous extract from the germinated seeds
of sweet *Lupinus albus***

Volume 3 – B.9 (AS)

Ecotoxicology Data

Great Britain

February 2025

Version History

When	What
June 2024	Initial DAR
February 2025	Updates made after ECP
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	[Updates made after any additional steps not covered by the above]

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B.9. Ecotoxicology data

Background information

The active substance aqueous extract from the germinated seeds of sweet *Lupinus albus*, is a plant extract with fungicidal properties that can be used on food and non-food crops. It is extracted from the germinated seeds of sweet *Lupinus albus* and manufactured into PROBLAD PLUS. In general, botanical active substances are complex mixtures comprising of numerous components, therefore, the whole technical grade is regarded as the active substance which is described as a UVCB substance (Substance of Unknown or Variable composition, Complex reaction product or of Biological material).

A major component of the aqueous extract from the germinated seeds of sweet *Lupinus albus* is the BLAD protein. BLAD is a naturally occurring seed storage protein present in germinated sweet lupins. It is a 20 kDa¹ polypeptide that is comprised of 173 amino acid residues and is a stable intermediate of the catabolism of β -conglutin², or characterised as a fragment of the amino acid sequence of β -conglutin, therefore, BLAD has no specific molecular or structural formula. BLAD is present in the aqueous extract from the germinated seeds of sweet *Lupinus albus* as a 210 kDa glyco-oligomer which is mainly composed of the 20 kDa polypeptide. The published literature (Monteiro et al. 2015; see Volume 3 CA B8 for summary) indicates that the 210 kDa protein present in PROBLAD PLUS is a glyco-oligomer composed of several polypeptides, the major ones exhibiting molecular masses of 14, 17, 20 (BLAD) 32, 36, 48 and 50 kDa. It is noted that BLAD contains a high proportion of the nitrogen-rich amino acids, which is consistent with its role as a seed storage protein.

BLAD forms 20% w/w of the PROBLAD PLUS formulation and is not isolated during the preparation of the product. The ecotoxicology data has been submitted with PROBLAD PLUS (sometimes also named PROBLAD). The active substance aqueous extract from the germinated seeds of sweet *Lupinus albus* and the plant protection product 'PROBLAD PLUS' are identical in composition and are derived from the same continuous manufacturing process. Therefore all toxicity data has been submitted and evaluated in this dossier. Additionally, a range of published literature studies have been provided, which are summarised and evaluated here.

This section summarises the available toxicity data and relevant literature studies.

¹ Unit of molecular mass, 1 Dalton (Da) = 1 g/mol

² Major *Lupinus* seed storage protein

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

No acute avian oral toxicity studies in accordance with OECD 223 (2016) guidelines have been submitted. However, several published literature studies have been submitted which investigate the inclusion of lupin seeds in bird diets. These studies are summarised below with the inclusion of HSE comments on their relevance and reliability.

Reference:	K-CA 8.1.1.1/01
Report Title:	The effects of processed white lupin seeds (<i>Lupinus albus</i> L.) on growth performance of Japanese quail
Author(s) & Year:	Arslan, C., Seker, E. (2002).
Document No:	Revue Méd.Vét., 2002, 153, 10, 643-646.
Substance used:	White lupin seed (<i>Lupinus albus</i> L.)
Method of analysis:	No analysis conducted
Guideline(s):	No, study from published literature
Deviations:	N/A, study from published literature
GLP or GEP:	No, study from published literature
Acceptability:	Supplementary
Study relied upon:	Yes, however data protection not applicable to published literature studies

I. MATERIALS AND METHODS

A. MATERIALS

Test material: White lupin seed (*Lupinus albus* L.)
Lot/batch no.: NA
Purity: NA
Treatment groups: Control (C); 15% raw (R); 15% processed via Turkish Traditional Method (TTM); 15% extruded lupin (E).

Test animals:

Species:	Japanese quail (<i>Coturnix japonica</i>)
Age:	One day
Weight at dosing:	7.00 g mean
Source of test species:	Not stated
Housing conditions:	Not stated
Climatic conditions:	Not stated

B. STUDY DESIGN AND METHODS

Study Design

A total of 360 one day old chicks were randomly assigned to each of the four groups (three lupin diet groups and control group), with each group divided into six sub-groups with fifteen birds per group. The diets of the three treatment groups (consisting of mainly corn, soybean meal and fish meal, formulated to meet NRC requirements for quail) were amended with lupin seeds. The treatment groups were as follows: 15% raw lupin seed, 15% lupin seeds processed according to Turkish method and 15% extruded lupin. A control group was also set up which received the same diet, without lupin. The Turkish method for processing the lupin seeds involves soaking in water for 12 h, filtering then boiling in water for 1 h, soaking in water for three days then drying under sunlight. The diets were given in mash form and the birds had free access to water and feed throughout the 42-day exposure period. The extrusion method involves exposing the seeds to 145°C for 5 seconds. Live weight and feed consumption were recorded weekly, live weight gain and feed efficiency (kg feed:kg gain) were calculated at the same time. Data obtained were subjected to statistical analysis with one-way ANOVA procedures. Where significant values were obtained, Duncan multiple range test was performed.

II. RESULTS AND DISCUSSION

The results of feed consumption, live weight and feed conversion efficiency for each of the treatments are summarised in the following table.

Table 9.1.1.1-1: Effect of lupin diets on the feeding, weight gain and feed conversion efficiency in Japanese quail

Treatments	Week relative to hatching					
	1	2	3	4	5	6
Feed consumption, g						
C	3.56	8.78	14.86	19.62 ^a	21.30	21.10
R	4.14	9.53	14.92	19.92 ^a	22.36	20.13
TTM	3.83	9.40	14.92	19.11 ^a	22.30	22.23
E	3.36	7.39	12.76	16.15 ^b	22.48	22.12
SEM	0.29	0.77	0.71	0.59	0.97	0.91
Live weight gain, g						
C	20.82 ^a	46.78 ^a	84.78 ^a	114.79 ^a	140.76	166.79
R	17.62 ^b	36.65 ^b	72.69 ^c	101.54 ^c	133.00	160.99
TTM	20.08 ^a	45.46 ^a	81.43 ^b	111.31 ^{ab}	141.16	166.53
E	18.83 ^{ab}	42.73 ^{ab}	75.47 ^c	106.83 ^b	136.04	161.58
SEM	0.63	1.25	1.09	1.54	3.12	3.22
Feed conversion efficiency						
C	1.82 ^b	2.37 ^b	2.74	4.67 ^a	5.80	5.81
R	2.71 ^a	3.06 ^a	3.16	4.85 ^a	5.00	5.19
TTM	2.17 ^b	2.43 ^{ab}	2.76	4.62 ^a	5.42	6.19
E	2.00 ^b	2.15 ^b	2.75	3.63 ^b	5.48	6.25
SEM	0.17	0.20	0.15	0.22	0.24	0.44

a,b,c: Different superscripts within columns differ significantly ($P < 0.05$)

SEM: standard error of the mean

During the initial three weeks, there was no effect of the lupin diets on feed consumption. In week 4, only consumption of the extrusion seed diet was lower than the other groups. There was however a reduced growth rate of the individuals with the raw lupin diet compared to the control and the other lupin treatment groups. The lower weight could be attributed to greater alkaloid content of the raw lupin seeds, which may adversely affect utilisation of diet and as such reduce the feed to weight gain efficiency. However later in the study, when chicks were over three weeks old there was no difference in weight shown between any of the treatments or control group. This could indicate that the younger chicks are not able to tolerate the higher amounts of alkaloid in the raw lupin. By the end of the study there were no differences in feed conversion efficiency between any of the groups and so it was concluded that inclusion of lupin seeds up to 15% of the diet of Japanese quails did not compromise the performance or growth of the birds.

III. CONCLUSIONS

According to the results of the study, lupin addition at 15% level in quail diet had no adverse effect on growth or performance of the birds.

HSE COMMENTS

This study is from the scientific literature and does not conform to standardised test guidelines, nor is it GLP compliant. The study's methodology, reporting and analysis have been considered with regard to how well they meet common standards of scientific practice, based on 'expert judgement'.

The study appears to be well conducted in accordance with scientific principles, although details on the source of test organism and their housing conditions before and during the test were lacking. There is also some uncertainty regarding the test item, for which no batch number or purity is reported, although the same species of lupin has been used as the active substance is derived from. Japanese quail is the standard test species used in avian toxicity tests and the route of exposure was dietary, so the study is deemed relevant to the risk assessment. The comparative assessment of the effects of different processing methods is not considered to be relevant to the risk assessment. However, the study does indicate that inclusion of raw lupin seeds at up to 15% in the diet, over a six week period, does not negatively affect feed consumption, live weight gain or feed conversion efficiency in Japanese quail. The results may be considered as supporting evidence in the risk assessment.

Reliability: Reliable with reservations

(Studies are graded as one of the following categories: 1) Reliable, 2) Reliable with reservations, 3) Unreliable. Categorisation is based on the evaluator's subjective assessment of the quality of the methods, reporting and analysis as presented in the study.)

Reference:	K-CA 8.1.1.1/02
Report Title:	Effects of feeding growing broiler chickens with practical diets containing sweet lupin (<i>Lupinus angustifolius</i>) seed meal.
Author(s) & Year:	Rubio, L.A., Brenes, A. & Centeno, C. (2003)
Document No	British Poultry Science, 2003, 44, 3, 391-397. DOI: 10.1080/0007166031000085553
Substance used:	Raw whole or dehulled sweet lupin seed meal (species <i>Lupinus angustifolius</i>)
Method of analysis:	No analysis conducted
Guideline(s):	No, study from published literature

Deviations:	N/A, study from published literature
GLP or GEP:	No, study from published literature
Acceptability:	Supplementary
Study relied upon:	Yes, however data protection not applicable to published literature studies

I. MATERIALS AND METHODS

A. MATERIALS

Test material:	Sweet Lupin (<i>Lupinus angustifolius</i>) seed meal: Raw (not heat treated) whole sweet lupin seed meal (400 g/kg diet), or, dehulled sweet lupin seed meal (320 g/kg diet)
Control:	commercial type feed based on wheat and heat-treated soybean (440 g/kg protein) meals.
Lot/batch no.:	NA
Purity:	NA

Test animals

Species:	Male cobb broiler chicken (<i>Gallus gallus domesticus</i>)
Age:	One day old
Weight at dosing:	40 ± 2 g mean
Source:	Not reported

B. STUDY DESIGN AND METHODS

For each experimental treatment, 32 birds were allocated to 4 replicates of 8 birds each in electrically heated battery brooders, and maintained on the control or lupin diets for 21 days. Feed and water were available at all times. Room temperature was maintained at 24°C and lighting was constant. Diets were formulated to contain the same amounts of metabolisable energy (12.55 kJ/g) and protein (210 g/kg). The control diet was a commercial type of feed based on wheat and heat treated soybean (440 g/kg protein) meals. Raw (not heat treated) sweet (low in alkaloids) whole lupin (400 g/kg) or dehulled

lupin meal (320 g/kg) were used to formulate lupin-based diets. Diets were completed with either defatted soybean or casein to reach the required amount of protein without altering the final composition. In experiment 1 the effects of using dehulled or whole lupin seed meal were studied. Experiment 2 was designed to investigate the effect of the inclusion of a commercial protease on apparent ileal digestibility of amino acids and liver composition.

II. RESULTS AND DISCUSSION

A summary of the feed intake, weight gain and gain: feed ratio over the 21 day exposure period is presented in the following table.

Table 9.1.1.1-2: Productive parameters and selected liver parameters in growing broiler chickens fed whole or dehulled lupin-based diets for 21 days.

	Final body weight (g)	Feed intake (g/chick)	Gain:feed ratio	Fresh relative liver weight (g per 100 g of total weight)	Liver RNA composition (mg/g dry weight)
Experiment 1					
Control	471 ^a	639 ^a	0.68	Not assessed	Not assessed
Lupin/soy	434 ^b	577 ^b	0.68	Not assessed	Not assessed
Dehulled lupin/soy	497 ^a	669 ^a	0.69	Not assessed	Not assessed
Pooled SD	60	32	0.03	n/a	n/a
Experiment 2					
Control	515 ^a	724 ^a	0.65	2.45 ^a	53.5 ^a
Lupin/soy	462 ^{ab}	658 ^b	0.62	2.97 ^b	11.8 ^b
Lupin/casein	445 ^b	635 ^b	0.65	3.03 ^b	22.7 ^b
Lupin/soy + enz	509 ^a	728 ^b	0.62	2.68 ^{ab}	21.5 ^b

	Final body weight (g)	Feed intake (g/chick)	Gain:feed ratio	Fresh relative liver weight (g per 100 g of total weight)	Liver RNA composition (mg/g dry weight)
Lupin/casein +enz	501 ^a	687 ^{ab}	0.67	2.71 ^{ab}	13.5 ^b
Pooled SD	87	33	0.04	0.24	10.4

a,b means in each column and experiment not sharing a common letter differ significantly (P < 0.05).

enz = commercial protease enzyme included in diet.

Final body weight and feed intake of chicks fed diets containing whole lupin seed meal (400 g/kg) were significantly lower than those of the control but grain:feed ratios were not different to control. The birds given the dehulled (320 g/kg) instead of whole lupin seed meal had similar body weight, feed intake and grain:feed values to the control. The lower weight gain in the whole lupin seed meal fed birds than in controls was most likely due to the depressed feed intake in chickens given this diet as the gain:feed values were not affected. Also as the weight gain of the dehulled lupin seed meal was not affected which suggests that the large amounts of insoluble lupin fibre in the hulls affected feed intake.

Plasmic uric acid concentration was higher (p < 0.05) while cholesterol and triglycerides were lower (p < 0.05) than controls in birds fed on whole lupin meal. Plasma amino acid concentrations of arginine were higher (p < 0.05) than controls in birds fed dehulled lupin/soy diets, while glycine, methionine and phenylalanine concentrations were lower (p < 0.05). No differences were found in apparent ileal digestibility of amino acids for the different lupin diets compared to control.

The fresh weight of the liver was higher (p < 0.05) in birds fed lupin/soy or lupin/casein diets than in the control but this difference was eliminated by protease supplementation. With respect to liver composition, protein values were not affected by dietary treatment, while glycogen values tended to drop and DNA values tended to rise, even though only values in birds fed lupin/casein + enzyme were significantly different (p < 0.05), and RNA values were significantly lower (p < 0.05) in lupin fed birds than in controls.

III. CONCLUSIONS

Final body weight and feed intake of chickens fed diets consisting of whole lupin seed meal (400 g/kg) were lower than control weight and intake but the gain in weight to feed ratio were not different, which would suggest that the high insoluble fibre content of the lupin

seed hull affected digestibility. Birds fed dehulled (320 g/kg) seed meal were comparable in weight and feed intake as the control.

HSE COMMENTS

This study was submitted as evidence that a reduction in chicken growth rate from lupin based diets is as a result of lower palatability.

The study from the scientific literature was designed in two parts: to assess the productive and physiological effects of 1) including whole or dehulled sweet lupin seed meal in chicken diets, and 2) including whole sweet lupin seed meal diet with and without protease supplementation, both for the purpose of further understanding the digestibility and protein utilisation of legume-based diets in chicken feed. Given the purpose of this study, it does not conform to a standard ecotoxicological test guideline, nor is it GLP compliant. Therefore, the study's methodology, reporting and analysis have been considered with regard to how well they meet common standards of scientific practice, based on 'expert judgement'.

The methodology, study design and statistical analysis are well described and are in accordance with general scientific principles, although details on the source of the chickens is not provided. The chickens were all males. Detailed composition of the test diets is provided. Since the test item is not a single chemical, analytical verification is not applicable, though the authors do provide a detailed analysis of the amino acid composition of the diets which are all broadly comparable, as they were designed to be. The seed meal tested is from the species *Lupinus angustifolius*, which is different to the species being evaluated in the current dossier (*L. albus*).

The main finding is that after 21-days, chickens fed whole sweet lupin (*Lupinus angustifolius*) seed meal (400 g/kg diet) had lower final body weight compared to dehulled sweet lupin seed meal (320 g/kg diet) or the control. The authors suggest that "this was most likely due to depressed feed intake in chickens given whole lupin seed meal, as gain:feed values were not affected" and further expand on possible reasons behind this, stating: "feed intake and body weight were not affected in chickens fed on dehulled lupin seed meal, which suggests that the presence of large amounts of insoluble lupin fibre in lupin hulls affected feed intake." The results may be considered as supporting evidence in the risk assessment.

Additional 21-day findings relating to blood plasma diagnostics (uric acid, cholesterol, triglycerides, some amino acids), liver weight and liver composition were discussed by the author who suggested that "liver metabolism was probably affected by lupin inclusion in the diet" and discuss the findings in the context of potential effects on general N metabolism relating to differences in absorbed peptide structure from different proteins in the diet, including the potential for some absorbed peptides having an unidentified biological effect or acting as an 'ANF' (antinutritional factor). However, much of this discussion highlighted theories requiring further investigation, and the authors note further ongoing work into characterising amino acid absorption from different protein structures. Therefore, the results

of this physiological diagnostic part of the study may be less useful for risk assessment as mechanistic conclusions from the data are limited.

Overall, the results from the chicken body weight part of the study may be considered as supporting evidence in the risk assessment, noting the tested sweet lupin seed meal is from species *Lupinus angustifolius*. Furthermore, the relevance of testing of the large amount of seed meal in the diet in this study compared with the amount of active substance under assessment, which is the aqueous extract from the germinated seeds of sweet *Lupinus albus*, needs consideration at risk assessment. The results from additional diagnostics (plasma, liver) may be less useful in for risk assessment as mechanistic conclusions from the data are limited.

Reliability: Reliable with reservations.

(Studies are graded as one of the following categories: 1) Reliable, 2) Reliable with reservations, 3) Unreliable. Categorisation is based on the evaluator's subjective assessment of the quality of the methods, reporting and analysis as presented in the study.)

Reference:	K-CA 8.1.1.1/03
Report Title:	Cholesterol-lowering effects of dietary lupin (<i>Lupinus albus</i> var <i>Multolupa</i>) in chicken diets
Author(s) & Year:	Viveros, A., Centeno, C., Arijia, I., and Brenes, A. (2007)
Document No	Poultry Science, 86:2631-2638 DOI: 10.3382/ps.2007-00128
Substance used:	Lupin (<i>Lupinus albus</i> var <i>Multolupa</i>) seed meal
Method of analysis:	No analysis conducted
Guideline(s):	No, study from published literature
Deviations:	N/A, study from published literature
GLP or GEP:	No, study from published literature
Acceptability:	Supplementary

Study relied upon:	Yes, however data protection not applicable to published literature studies
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I. MATERIALS AND METHODS

A. MATERIALS

Treatments:

Test item:	Lupin (<i>Lupinus albus</i> var Multolupa) seed meal	Cholesterol
Source:	Grown in Badajoz, Spain	Sigma-Aldrich Química SA, Spain
Test diets:	0, 200 and 400 g/kg	0 and 10 g/kg

Test animals:

Animal:	Male broiler chickens, one day old
Replicates:	Three pens per treatment
Animals/replicate:	Six chicks per pen
Test duration:	21 days

B. STUDY DESIGN AND METHODS

This study was conducted in order to investigate the effect of different concentrations of lupin and cholesterol on performance, relative liver weight, liver fat, intestinal pH and viscosity, and different blood serum parameters (glucose, cholesterol, triglycerides, total biliary salts, amylase, total protein and albumin, and globulin fractions).

The lupin cultivar used in this experiment was grown in Badajoz, Spain. The experimental diets were formulated to meet or exceed the minimum NRC (1994) requirements for broiler chickens. A total of 108 one-day-old male broiler chicks were housed in heated battery brooders in an environmentally controlled room. The chicks were allocated to 18 pens, each pen containing 6 chicks, to receive 6 dietary treatments with 3 replicates of each treatment. Diets in mash form and water were provided ad libitum. Celite, a source of acid insoluble ash, was added at 10 g/kg to all diets as an indigestible marker. The starter broiler diets with different inclusion levels of lupin (0, 200 and 400 g/kg) with and without cholesterol added (10 g/kg) were fed for three weeks. For the diets containing lupin meal, the diet was adjusted to have a corresponding reduction in soybean meal. At the end of the experimental period, birds were weighed, and feed consumption was recorded for feed efficiency computation.

At 21 days of age, 9 birds (3 per pen) were randomly selected from each treatment, and blood samples were obtained to determine different biochemical parameters. After the birds were killed by cervical dislocation, the liver, jejunum, and the caeca were removed for liver lipid, viscosity, and pH determination, respectively, using 9 randomly selected chicks per treatment.

Blood samples were obtained by cardiac puncture for determination of glucose, cholesterol, triglyceride, biliary salt, total protein, amylase, and albumin and globulin fractions and albumin:globulin ratio in serum.

After the chicks were killed, the liver was removed, cleaned from adhering tissue, and weighed using 9 randomly selected chicks per treatment. Digesta contents were also collected from 9 birds per treatment for the viscosity determination (jejunum) and pH determination was directly measured on caecal digesta (9 chicks per treatment).

Chemical analysis demonstrated that the lupin seed was a low-alkaloid cultivar and had a high content of protein (388 g/kg), fat (99.8 g/kg), and fibre (219.1 g/kg neutral detergent fibre).

Table 9.1.1.1-3: Ingredients and nutrient composition of experimental diets

Item	Lupin seed (g/kg as fed)		
	0	200	400
Ingredients			
Corn	476.3	439.0	403.4
Soybean meal (48% CP)	421.0	256.0	87.9
Lupin meal ¹	-	200.0	400.0
Sunflower oil	62.7	63.3	64.0
Dicalcium phosphate	20.8	23.0	25.1
Calcium carbonate	9.8	8.7	7.6
NaCl	3.0	3.0	3.0
DL-Met	1.4	2.0	2.8

Item	Lupin seed (g/kg as fed)		
	0	200	400
L-Lys	-	-	1.2
Vitamin and mineral premix ²	5.0	5.0	5.0
Cholesterol	+/-	+/-	+/-
Analysed composition			
Protein	228	229	
Calculated analysis³			
AME (kcal/kg)	3,050	3,050	3,050
Lys	13.7	12.4	12.4
Met+Cys	9.0	9.0	9.0
Ca	10.0	10.0	10.0
Available P	4.5	4.5	4.5

¹ Protein content of lupin meal was 38.8%.

² Vitamin and mineral premix supplied the following per kilogram of diet: vitamin A, 8,250 IU; cholecalciferol, 1,000 IU; vitamin E, 11 IU; vitamin K, 1.1mg; vitamin B12, 11.5 µg; riboflavin, 5.5 mg; Ca pantothenate, 11 mg; niacin, 53.3 mg; choline chloride, 1,020 mg; folic acid, 0.75 mg; biotin, 0.25 mg; delquin, 125 mg; DL-Met, 500 mg; amprol, 1 g; Mn, 55 mg; Zn, 50 mg; Fe, 80 mg; Cu, 5 mg; Se, 0.1 mg; I, 0.18 mg; and NaCl, 2,500 mg.

³ Estimated using FEDNA (2003) tables.

Data were analysed as a completely randomized design and subjected to ANOVA using the GLM procedure of SAS software. The experiments were analysed by ANOVA in 3 (lupin concentration) × 2 (cholesterol concentration) factorial arrangements of treatments. Significant differences among treatment means were determined at $p < 0.05$ by Duncan's multiple range test.

II. RESULTS AND DISCUSSION

The effects of dietary lupin concentration and cholesterol supplementation on growth performance, relative liver weight, liver fat, caecal pH, and jejunum viscosity are summarised in the below table.

The data indicate that increasing amounts of lupin seed in the diet depressed body weight (up to 12%; $p < 0.001$) feed-to-gain ratio (up to 7%; $p < 0.001$), liver fat (17%; $p < 0.001$; in the higher-lupin concentration), and caecal pH (up to 6%; $p < 0.01$) and increased intestinal viscosity (up to 14%; $p < 0.001$). Cholesterol concentration in the diet increased relative liver weight (10%; $p < 0.001$), liver fat (46%; $p < 0.001$), and intestinal viscosity (10%; $p < 0.001$). A significant interaction was only observed in liver fat ($p < 0.05$) and intestinal viscosity ($p < 0.001$).

Table 9.1.1.1-4: Effects of dietary lupin meal concentration with and without cholesterol

Item	Lupin (g/kg)	Cholesterol (g/kg)	BW ¹ (g)	FC ¹ (g)	FGR1	RLW ¹ (g/100g BW)	LF ² (%)	pH ²	IV ² (cP)
Treatments									
1	0	0	624 ^a	826	1.30 ^c	2.90 ^{bc}	5.34 ^c	6.25 ^a	2.19 ^b
2	200	0	580 ^{bc}	748	1.37 ^{ab}	3.08 ^{abc}	6.56 ^b	6.25 ^a	2.33 ^b
3	400	0	543 ^d	761	1.40 ^a	2.83 ^c	4.57 ^c	6.07 ^a	2.32 ^b
4	0	10	621 ^a	813	1.33 ^{ab}	3.13 ^{abc}	8.74 ^a	6.15 ^a	2.20 ^b
5	200	10	607 ^{ab}	810	1.33 ^{bc}	3.17 ^{ab}	8.09 ^a	6.28 ^a	2.31 ^b
6	400	10	555 ^{cd}	782	1.41 ^a	3.36 ^a	7.09 ^b	5.61 ^b	2.68 ^a
Pooled SEM			48.0	37.1	0.02	0.16	0.54	0.21	0.08
Main effects									
Lupin (L)									
0			622 ^a	819	1.31 ^c	3.12	7.04 ^a	6.20 ^a	2.19 ^c

Item	Lupin (g/kg)	Cholesterol (g/kg)	BW ¹ (g)	FC ¹ (g)	FGR1	RLW ¹ (g/100g BW)	LF ² (%)	pH ²	IV ² (cP)
200			594 ^b	779	1.35 ^b	3.11	7.32 ^a	6.26 ^a	2.32 ^b
400			549 ^c	771	1.40 ^a	3.01	5.83 ^b	5.84 ^b	2.50 ^a
Cholesterol (CH)									
0			582	802	1.35	2.94 ^a	5.49 ^b	6.19	2.28 ^b
10			595	778	1.35	3.22 ^a	8.03 ^a	6.01	2.50 ^a
Source of variation			-- Probabilities --						
L effect			0.001	NS	0.001	NS	0.001	0.01	0.001
CH effect			NS	NS	NS	0.001	0.001	NS	0.001
LxCH effect			NS	NS	NS	NS	0.05	NS	0.001

Body weight (BW), feed consumption (FC), feed-to-gain ratio (FGR), relative liver weight (RLW), liver fat (LF), caecal pH (pH), and intestinal viscosity (IV).

^{a-d} Means in columns with no common superscript differ significantly ($p < 0.05$).

¹ Data are means of 3 pens of 6 chicks.

² Data are means of 3 pens of 3 chicks

Serum glucose, cholesterol, triglyceride, total biliary salts, and total protein contents were reported in the below table. Increasing dietary lupin concentration reduced serum glucose (up to 20%; $p < 0.001$), cholesterol (up to 27%; $p < 0.01$), total biliary salts (up to 47%; $p < 0.01$), and total protein (up to 14%; $p < 0.001$) compared with those of chicks fed the control diet. The addition of 10g/kg of cholesterol reduced serum glucose (14%; $p < 0.001$) and increased plasma cholesterol (60%; $p < 0.001$), total biliary salts (60%; $p < 0.001$), and total protein (39%; $p < 0.001$). A significant interaction was observed for glucose ($p < 0.01$), cholesterol ($p < 0.05$), and triglycerides ($p < 0.001$).

Table 9.1.1.1-5: Effects of dietary lupin meal concentration with and without cholesterol.

Item	Lupin (g/kg)	Cholesterol (g/kg)	G (mg/dL)	CH (mg/dL)	T (mg/dL)	BS (nmol/dL)	P (mg/dL)
Treatments							
1	0	0	254 ^a	103 ^c	33 ^b	12 ^{bc}	2.66 ^{cd}
2	200	0	249 ^a	109 ^{bc}	26 ^{bc}	9 ^{bc}	2.87 ^c
3	400	0	199 ^c	74 ^d	15 ^d	7 ^c	2.44 ^d
4	0	10	220 ^b	182 ^a	23 ^c	22 ^a	3.90 ^a
5	200	10	207 ^c	134 ^b	20 ^{cd}	15 ^{ab}	3.87 ^a
6	400	10	183 ^d	137 ^b	42 ^a	11 ^{bc}	3.28 ^b
Pooled SEM			6.17	15.30	3.68	3.68	0.18
Main effects							
Lupin (L)							
0			239 ^a	142 ^a	28	17 ^a	3.28 ^a
200			228 ^b	122 ^b	23	12 ^b	3.34 ^a
400			191 ^c	104 ^b	29	9 ^b	2.83 ^b
Cholesterol (CH)							
0			236 ^a	95 ^b	25	10 ^b	2.66 ^b
10			203 ^b	152 ^a	28	16 ^a	3.69 ^a
Source of variation			-- Probabilities --				

L effect	0.001	0.01	NS	0.01	0.001
CH effect	0.001	0.001	NS	0.001	0.001
L x CH effect	0.01	0.05	0.001	NS	NS

Serum glucose (G), cholesterol (CH), triglyceride (T), total biliary salts (BS), and total protein (P).

^{a-d} Means in columns with no common superscript differ significantly ($p < 0.05$).

Data are means of 3 pens of 3 chicks

Serum albumin, α -1 globulin, α -2 globulin, β -globulin, albumin:globulin ratio, and amylase concentrations are shown in the below table. The main effects data indicated that increasing concentration of lupin seed in the diets reduced serum albumin (up to 13%; $p < 0.05$), β -globulin (up to 24%; $p < 0.01$), γ -globulin (up to 32%; $p < 0.001$), and albumin:globulin ratio (up to 13%; $p < 0.01$). The addition of 10g/kg of cholesterol increased serum albumin (36%; $p < 0.001$), α -1 globulin (125%; $p < 0.001$), α -2 globulin (39%; $p < 0.001$), γ -globulin ($p < 0.01$), and β -globulin (45%; $p < 0.001$) and reduced albumin:globulin ratio (6%; $p < 0.05$) and amylase (74%; $p < 0.001$). A significant interaction was observed between lupin seed concentration and cholesterol for serum α -1 globulin ($p < 0.05$), β -globulin ($p < 0.05$), albumin:globulin ratio ($p < 0.01$), and amylase ($p < 0.05$), indicating that the response of cholesterol was more effective, in the case of β -globulin, albumin:globulin ratio, and amylase, with 400g/kg of lupin seed concentration, and, in the case of serum α -1 globulin, with the lowest lupin seed concentration.

Table 9.1.1.1-6: Effects of dietary lupin meal concentration with and without cholesterol (3)

Item	Lupin (g/kg)	Chol. (g/kg)	ALB (%)	α-1 GLB (%)	α-2 GLB (%)	β- GLB (%)	γ- GLB (%)	ALB:GLB ratio	Amylase (U/L)
Treatments									
1	0	0	1.43 ^b	0.16 ^d	0.29 ^b	0.32 ^{cd}	0.20 ^{bc}	1.33 ^a	40.75 ^a
2	200	0	13.8 ^b	0.20 ^{cd}	0.37 ^{ab}	0.39 ^{bcd}	0.25 ^{ab}	1.06 ^b	36.00 ^b
3	400	0	1.25 ^b	0.26 ^c	0.27 ^b	0.29 ^d	0.17 ^c	1.07 ^b	40.62 ^a
4	0	10	1.94 ^a	0.51 ^a	0.47 ^a	0.60 ^a	0.30 ^a	1.07 ^b	10.25 ^c
5	200	10	1.85 ^a	0.46 ^{ab}	0.42 ^a	0.44 ^b	0.27 ^a	1.05 ^b	10.71 ^c
6	400	10	1.70 ^a	0.38 ^b	0.40 ^{ab}	0.41 ^{bc}	0.17 ^c	1.14 ^b	9.87 ^c
Pooled SEM			0.14	0.04	0.07	0.05	0.04	0.06	1.65
Main effects									
Lupin (L)									
0			1.69 ^a	0.33	0.38	0.46 ^a	0.25 ^a	1.20 ^a	25.5
200			1.61 ^{ab}	0.33	0.40	0.42 ^a	0.26 ^a	1.05 ^b	24.2
400			1.47 ^a	0.32	0.34	0.35 ^b	0.17 ^b	1.11 ^b	25.5
Cholesterol (CH)									
0			1.35 ^b	0.20 ^b	0.31 ^b	0.33 ^b	0.21 ^b	1.16 ^a	39.12 ^a
10			1.83 ^a	0.45 ^a	0.43 ^a	0.48 ^a	0.25 ^a	1.09 ^b	10.26 ^b

Source of variation	-- Probabilities --						
	0.05	NS	NS	0.01	0.001	0.01	NS
L effect	0.05	NS	NS	0.01	0.001	0.01	NS
CH effect	0.001	0.001	0.001	0.001	0.01	0.05	0.001
LxCH effect	NS	0.001	NS	0.01	NS	0.01	0.05

Cholesterol (Chol), Serum albumin (ALB), α 1 globulin (α -1 GLB), α -2 globulin (α -2 GLB), β -globulin (β GLB), albumin:globulin ratio (ALB:GLB). NS: not significant

^{a-d} Means in columns with no common superscript differ significantly ($p < 0.05$).

Data are means of 3 pens of 3 chicks.

III. CONCLUSION:

This study demonstrated that the inclusion of raw low-alkaloid lupin seeds up to 20% in the diets for broiler chickens depressed growth rate. One of the most prominent adverse effects observed in animals fed lupin was feed refusal, likely associated with poor palatability. Part of this effect may also have been related to the higher fibre content found in the hulls, or the concentration of non-starch polysaccharides (NSP) within the cotyledon. The results obtained in the experiment, with an increase in the intestinal viscosity and a reduction in the caecal pH, suggest that considerable amounts of NSP become soluble when ingested by the chicken.

The results of the study also demonstrated that dietary lupin seeds caused blood parameter changes in chicks, with a decrease in serum cholesterol, glucose, total biliary salts, and total protein concentration as well as a reduction in liver fat were found after legume diet intake. The reduction in serum cholesterol level observed with lupin diets in this study was also accompanied by a significant lower serum albumin, β -globulin, γ -globulin, and albumin:globulin ratio. The decrease observed in serum protein as well as the concentration of serum albumin and γ -globulin in response to increasing amount of lupin can be caused by the reduction of feed intake and growth rate observed in this experiment.

The increase in total serum protein and α - and β -globulin and the decrease in albumin:globulin ratio observed in response to cholesterol added could indicate inflammatory disease in the liver. Inflammatory disease state frequently results in increasing protein globulin fraction. On the other hand, significant reductions of serum α -amylase (more than 3 times) in response to cholesterol have also been observed.

In summary, the addition of lupin seed in chicken diets caused a negative effect on performance. The observed low concentrations of serum glucose, cholesterol, total biliary

salts, and total protein and the modification in the electrophoretic bands of serum protein caused by lupin feeding may have some physiological importance and may be extrapolated to humans. The overall results suggest that lupin seed intake may be effective in lowering the cholesterol absorption as well as the serum glucose level in chickens and that this seed may have a potential application as a cholesterol-reducing agent.

HSE COMMENTS

This study was submitted as evidence that feed avoidance is one of the causes of growth depression observed in chickens fed lupine-based diets.

This study is from the scientific literature with the purpose of investigating the effect of including lupin seed meal, with and without cholesterol, in chicken diets on growth and other physiological parameters. Given the purpose of this study, it does not conform to a standard ecotoxicological test guideline, nor is it GLP compliant. Therefore, the study's methodology, reporting and analysis have been considered with regard to how well they meet common standards of scientific practice, based on 'expert judgement'.

The methodology, study design and statistical analysis are well described and are in accordance with general scientific principles, although details on the source of the chickens and the housing environmental conditions were not provided. The chickens were all males. Detailed composition of the test diets is provided in the study. Since the test item is not a single chemical, analytical verification to confirm exposure is not applicable. The lupin seed species (*L. albus* var *Multolupa*) was shown to be a low-alkaloid cultivar. *L. albus* is the same species that is being evaluated in the current dossier.

The comparison of dietary cholesterol supplementation is not relevant for risk assessment, however the comparison of lupin-containing and non-lupin-containing diets does yield relevant information: the inclusion of lupin seed meal at 200 and 400 g/kg into chicken diet fed ad libitum for 21 days did not result in overt toxic effects or mortality, but did result in a significant reduction in body weight. Changes in blood parameters were discussed by the authors but mechanistic conclusions were limited and no direct association with body weight was inferred.

The authors suggest: "Feed refusal, likely associated with poor palatability, is probably 1 of the most prominent adverse effects observed in animals fed lupins". However, it can be seen that although food consumption was reduced in the lupin-containing diets, this was not statistically significant. Whereas, feed-to-gain ratio was significantly increased at both test concentrations in a dose-responsive manner. This suggests that feed refusal does not wholly explain the body-weight reduction. The authors do also discuss the impact of the lupin diets being high in fibre including non-starch polysaccharides (NSPs), on causing potential changes to intestinal digestion and nutrient absorption.

The results may be considered as supporting evidence in the risk assessment. It is noted that the relevance of the tested concentrations of seed meal in the diet to the concentration

of the active substance under assessment, which is an aqueous extract of lupin seeds, needs further consideration at risk assessment.

Reliability: Reliable with reservations.

(Studies are graded as one of the following categories: 1) Reliable, 2) Reliable with reservations, 3) Unreliable. Categorisation is based on the evaluator's subjective assessment of the quality of the methods, reporting and analysis as presented in the study.)

Reference:	K-CA 8.1.1.1/04
Report Title:	Influence of graded inclusion of white lupin (<i>Lupinus albus</i>) meal on performance, nutrients digestibility and intestinal morphology of broiler chickens.
Author(s) & Year:	Kaczmarek, S.A., Mejdysz, M., Kubiz, M., & Rutkowski, A. (2016)
Document No	British Poultry Science, 57, 3, 364 – 374. DOI: 10.1080/00071668.2016.1171295
Substance used:	White Lupin Meal (WLM) made from ground seeds of white lupin <i>Lupinus albus</i> , a low-alkaloid cultivar
Method of analysis:	No analysis conducted
Guideline(s):	No, study from published literature
Deviations:	N/A, study from published literature
GLP or GEP:	No, study from published literature
Acceptability:	Supplementary
Study relied upon:	Yes, however data protection not applicable to published literature studies

I. MATERIALS AND METHODS

A. MATERIALS

Treatments

Test item:	White lupin (<i>Lupinus albus</i>)
Test diets:	Control diet with only maize, or with addition of 100, 150, 200, 250, and 300 g/kg white lupin
Source:	Seeds were obtained from the Plant Breeding Stations in Przebędowo and Wiatrowo, Poland.

Test animals

Animal:	480 male broiler (Ross 308) chicks
Body weight:	Initial: 40 ± 2 g
Replicates:	10 replicates per treatment
Animals/replicate:	Eight birds per replicate
Test duration:	35 days

Test conditions

Temperature:	32°C during the first week and gradually decreased to approx. 23°C by the end of the third week
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B. STUDY DESIGN

The aim of this study was to investigate the effect of white lupin (*Lupinus albus*) meal (WLM) addition on the intestinal viscosity, bird performance, nutrient utilisation, and villi morphology of growing broiler chicks.

White lupin seeds were obtained from the Plant Breeding Stations in Przebędowo and Wiatrowo, Poland. To obtain white lupin meal, seeds were ground using a hammer mill with a screen size of 2.0 mm.

The experiment was conducted with 480 male broiler (Ross 308) chicks with an initial weight of 40 ± 2 g. Six dietary treatments were assessed including a control diet with only maize, and five experimental diets containing 100, 150, 200, 250, and 300 g/kg white lupin seeds. Each treatment had 10 replicates, to each of which were added eight birds.

Replicates were maintained at 32°C during the first week, which was gradually decreased to approximately 23°C by the end of the third week. Test diets were offered ad libitum from day 1 to day 35, and water was freely available throughout the trial.

Collection trays were installed in the floor of the pens on day 34 to allow for excreta collection, with 10 samples collected per pen of each treatment.

At the end of the experiment, chickens were killed and the ileum removed. Digesta were flushed from the terminal ileum and pooled by pen for analysis. Intestinal morphology measurements included the height and width of intestinal villi and intestinal crypt depth in intestines.

Body weight and feed intake were monitored weekly by pen. Birds were fasted for four hours prior to weighing. Mean weight gain, feed intake, and feed-to-gain ratio were used to determine growth performance.

II. RESULTS AND DISCUSSION

The overall mortality was approximately 4%, and was not related to any specific dietary treatments. An increase in dietary levels of WLM from 0 to 300 g/kg (until day 14 of the experiment) resulted in a quadratic decrease in body weight gain (BWG) ($p < 0.001$), a tendency to a linear decrease in feed intake, and a quadratically increased ($p < 0.001$) feed conversion ratio (FCR). Birds fed 200 g/kg WLM had a lower BWG and higher FCR than control birds ($p < 0.05$) in the 14-day growing period. From day 14 to 35, birds fed 100 g/kg WLM or more had a lower BWG ($p < 0.05$) than control birds. Birds fed 250 or 300 g/kg of WLM had a higher feed intake than control animals ($p < 0.05$). Body weight gain linearly decreased ($p < 0.05$) as WLM increased from 0 to 300 g/kg. WLM inclusion linearly increased ($p < 0.05$) FCR and quadratically reduced feed intake ($p < 0.05$). In the period from day 0 to day 35, birds fed with 200, 250, or 300 g/kg WLM were characterised by lower BWG than control chickens ($p < 0.05$). Feed intake was reduced in birds fed on diets containing 200, 250, or 300 g/kg compared to the control birds ($p < 0.05$). The use of 100g/kg did not affect FCR, but higher dosages of WLM increased FCR compared to the control treatment ($p < 0.05$).

Table 9.1.1.1-7: Bird performance by diet (1)

WLM (g/kg)	Day 1-14			Day 14-35			Day 1-35		
	BWG	FI	FCR	BWG	FI	FCR	BWG	FI	FCR
Control	368	497	1.21	1733	2556	1.48	2100	3053	1.46
100	367	488	1.20	1630*	2488	1.53	1996	2976	1.49
150	370	502	1.22	1646*	2554	1.55*	2016	3056	1.52*
200	342*	474	1.24	1575*	2460	1.56*	1917*	2934	1.53*
250	319*	497	1.38*	1504*	2358*	1.57*	1823*	2855*	1.57*
300	260*	460*	1.53*	1408*	2238*	1.59*	1668*	2698*	1.62*
Pooled SEM	0.614	0.51	0.188	190	227	0.0852	238	253	0.009

Values are means of 10 replicates.

* Means in the same column differ from control treatment at $p < 0.05$.

WLM: White lupin meal; BWG: Body weight gain (g/bird); FI: Feed intake (g/bird);

FCR: Feed conversion ratio (g/g)

SEM: Standard error of the mean.

Apparent metabolisable energy and apparent ileal digestibility of dry matter, ether extract, crude protein and starch linearly decreased ($p < 0.05$) as WLM increased from 0 to 300 g/kg.

Apparent ileal P digestibility decreased quadratically ($p < 0.05$) as WLM inclusion increased. Apparent ileal dry matter digestibility and diet AME_N depression was noted at 100 g/kg WLM inclusion ($p < 0.05$). Use of 300 g/kg of WLM reduced the AME_N of diet by 2.4 MJ/kg compared to a control diet ($p < 0.05$). Ether extract and crude protein digestibility was decreased when 250 and 300 g/kg of WLM was incorporated into the diet ($p < 0.05$). There was a tendency ($p < 0.1$) to decrease apparent ileal P digestibility when a 150 g/kg or higher dose of WLM was used. Ileal starch digestibility was high in all treatments and ranged from 0.90 (300 and 250 g/kg of WLM) to 0.99 in the control treatment. Birds fed on diets with 250 and 300 g/kg of WLM digested starch less efficiently than control chickens ($p < 0.05$).

Table 9.1.1.1-8: Bird performance by diet (2)

WLM (g/kg)	AMEN	Apparent ileal digestibility					Ileal digestibility
		Dry matter	Ether extract	Crude protein	P	Na	Starch
Control	12.8	0.70	0.74	0.74	0.40	0.62	0.99
100	11.5*	0.62*	0.68	0.74	0.36	0.58*	0.94
150	11.6*	0.63*	0.68	0.74	0.27*	0.58*	0.96
200	11.2*	0.58*	0.68	0.73	0.24*	0.57*	0.96
250	10.9*	0.48*	0.56*	0.70*	0.24*	0.57*	0.90*
300	10.4*	0.48*	0.56*	0.68*	0.21*	0.55*	0.90*
Pooled SEM	0.187	0.0121	0.0197	0.0074	0.0230	0.0261	0.0089

Values are means of 6 replicates.

* Means in the same column differ from control treatment at $p < 0.05$.

WLM: White lupin meal; AMEN: Apparent metabolisable energy corrected to N equilibrium (MJ/kg); P: Phosphorous; Na: Sodium

SEM: Standard error of the mean.

The inclusion of WLM generally had an adverse effect on sialic acid excretion compared to the control diet ($p < 0.05$). There was a quadratic effect ($p < 0.05$) of WLM dose on sialic acid excretion. A strong negative linear correlation was found between the excretion of sialic acid and AMEN ($p < 0.05$, $r^2 = 0.62$) (Figures 1 to 3). The regression analysis revealed that for every 100 $\mu\text{mol/g}$ titanium dioxide increase in the excretion of sialic acids, there was a concomitant reduction of 18.6% AMEN.

The viscosity of ileal digesta was linearly increased ($p < 0.05$) as WLM increased. There was a tendency ($p < 0.1$) to increased ileal viscosity after 100 g/kg WLM and a dramatic increase in ileal viscosity ($p < 0.05$) when higher dosages of WLM were used compared to the control treatment. The difference between the control and 300 g/kg treatment was over 110% higher (% increase relative to the control treatment, $p < 0.05$).

Histomorphological evaluations showed that ileum villus height (VH) and ileum villus area

(VA) were affected after the WLM supplementation ($p < 0.05$). There was a linear effect ($p < 0.05$) of WLM dose on VH and a quadratic one on VA ($p < 0.05$). Both parameters decreased as WLM increased from 0 to 300 g/kg. Compared to the control treatment, 150 g/kg WLM inclusion negatively affected VH and VA ($p < 0.05$). VH tended to decrease quadratically ($p < 0.074$) after WL supplementation from 0 to 300 g/kg. WLM dose had no effect on intestinal crypt depth.

Table 9.1.1.1-9: Bird performance by diet (3)

WLM (g/kg)	Sialic acid excreted ($\mu\text{moles/g}$ TiO_2)	Viscosity (cP)	Ileum villus (mm)			Crypt depth (mm)
			Height	Width	Area	
Control	219	1.76	0.861	0.155	416	0.140
100	282*	2.18 [†]	0.935	0.121	351	0.136
150	314*	2.41*	0.703*	0.127	301*	0.156
200	308*	3.06*	0.698*	0.122	266*	0.142
250	310*	3.08*	0.721*	0.139	286*	0.141
300	316*	3.74*	0.704*	0.126	281*	0.142
Pooled SEM	9.91	0.162	0.0224	0.0037	12.0	0.002

Values are means of 10 replicates.

* Means in the same column differ from control treatment at $p < 0.05$.

[†] Means in the same column differ from control treatment at $p < 0.01$. WLM: White lupin meal; SEM: Standard error of the mean.

III. CONCLUSION:

The use of over 150 g/kg of white lupin meal (WLM) in the diets of broiler diets depressed the performance results. Depression of nutrient utilisation was only observed when 250 or 300 g/kg WLM was used. An important observation was that the increase in digesta viscosity and phytic acid concentration associated with WLM inclusion into a diet may account for effects of endogenous losses from birds rather than by only a decrease in the nutritional value of the diets. The extent to which the effect of WLM addition on nutrient

digestion is associated with endogenous losses or phytic acid concentration requires further research.

HSE COMMENTS

This study was submitted as evidence that birds may show avoidance of lupin-based diets.

This study is from the scientific literature with the purpose of investigating the effect of including lupin seed meal in chicken diets on growth and intestinal physiology. Given the purpose of this study, it does not conform to a standard ecotoxicological test guideline, nor is it GLP compliant. Therefore, the study's methodology, reporting and analysis have been considered with regard to how well they meet common standards of scientific practice, based on 'expert judgement'.

The methodology, study design and statistical analysis are well described and are in accordance with general scientific principles. The chickens were all males. Furthermore, although the described mortality is low ("overall mortality of approximately 4%" over 35 days), this data is not provided. Detailed composition of the test diets is provided in the study. Since the test item is not a single chemical, analytical verification to confirm exposure is not applicable. The lupin seed species (white lupin *L. albus*) was shown to be a low-alkaloid cultivar. The species *L. albus* is the same as the species being evaluated in the current dossier.

The results show inclusion of 200 g/kg or more (up to 300 g/kg) WLM (white lupin meal, made from ground seeds) for 35 days in chicken diet had a negative effect on body weight and feed conversion ratio. The authors suggest "This could be the effect of quadratic decreases in feed intake when WLM was introduced into the diet". Food intake rate decrease was significantly reduced for 250 and 300 g/kg treatment rates (0-35 d) whereas feed conversion ratio was significantly increased from 150 g/kg (0-35 d), suggesting intake rate is not wholly responsible for body weight reduction. The authors suggest: "decreases in feed intake after WLM addition could be partially explained by the expected high insoluble NSP content" and that "Observed increases in ileal viscosity may also explain some of the depression in performance of birds observed in study", discussing the potential of general changes to gut digestion and nutrient absorption in response to high levels of viscosity and NSPs (non-starch polysaccharides) in the lupin diet, incorporating the results from ileal digestibility and apparent metabolisable energy.

The results may be considered as supporting evidence in the risk assessment. The relevance of the test dose concentrations will need to be considered further at risk assessment. In a standard avian reproductive toxicity test, the maximum recommended concentration of test item in the diet is 1000 mg/kg (1 g/kg), and this literature study has a lowest test concentration 100 times higher than this (100 g WLM/kg), noting that the test item in this study is whole seed meal whereas the active substance under assessment is an aqueous extract of germinated lupin seeds.

Reliability: Reliable with reservations.

(Studies are graded as one of the following categories: 1) Reliable, 2) Reliable with reservations, 3) Unreliable. Categorisation is based on the evaluator’s subjective assessment of the quality of the methods, reporting and analysis as presented in the study.)

Reference:	K-CA 8.1.1.1/05
Report Title:	Total and ileal digestible tryptophan contents of feedstuffs for broiler chickens
Author(s) & Year:	Ravindran, G., Ravindran, V., and Bryden, W.L. (2006)
Document No	J Sci Food Agric, 2006, 86:1132-1137
Substance used:	Multiple feedstuffs assessed. The lupin species assessed were: <i>L. albus</i> and <i>L. angustifolius</i> .
Method of analysis:	No analysis conducted
Guideline(s):	No, study from published literature
Deviations:	N/A, study from published literature
GLP or GEP:	No, study from published literature
Acceptability:	Supplementary
Study relied upon:	Yes, however data protection not applicable to published literature studies

I. MATERIALS AND METHODS

A. MATERIALS

Test organism	Male broiler chickens from 35-42 days old
Replicates:	Three replicates
Animals/replicate:	Four birds per replicate
Test duration:	Seven days

B. STUDY DESIGN AND METHODS

This study presents the variability in contents and apparent ileal digestibility for 6-week-old broiler chickens of tryptophan in 74 samples representing 24 feedstuffs.

The samples from a previous survey of amino acid digestibility of 24 feedstuffs were used in this study. The feedstuffs included five cereals (barley, maize, sorghum, triticale, wheat), two cereal by-products (rice polishings and wheat middlings), full-fat canola, four oilseed meals (canola, cottonseed, soybean, sunflower), five grain legumes (chickpea, faba bean, field pea, narrow-leaf (blue) lupin, white lupin), five animal protein meals (blood, feather, fish, meat, meat-and-bone), and two purified proteins (casein and gelatin). In addition, a soybean meal sample was purchased and included as a reference ingredient in seven of the 14 digestibility assays to measure assay-to-assay variations.

The proportions of dextrose and the test feedstuff were varied in each diet to obtain 200 g/kg crude protein. For lupins the test ingredient was present at 666 g/kg (air dry basis). As a source of acid-insoluble ash (AIA), 20 g/kg was added to all diets. This functioned as an indigestible marker in the calculation of digestibility coefficients.

Each assay diet was offered ad libitum to three pens (four birds per pen) of male broilers from 35 to 42 days old. On day 42, all birds were euthanised and the contents of the lower half of the ileum were obtained. Ileal digesta of birds within a pen were pooled and freeze dried. Dried samples were ground to pass through a 0.5 mm sieve and stored in airtight containers at -4°C for chemical analyses.

Determination of tryptophan was done using a chromatographic method. The precision of the method was 1 to 4% relative standard deviation.

Nitrogen determinations were performed in duplicate using an FP-428 nitrogen determinator. Crude protein contents of the ingredients were calculated as N x 6.25.

The apparent ileal tryptophan digestibility coefficients were calculated from the dietary ratio of tryptophan to AIA relative to the corresponding ratio in the ileal digesta.

II. RESULTS AND DISCUSSION

Results of grain legumes including the two lupin species are presented in the below table. For feedstuffs with three or more samples, the mean, standard deviation (SD), and coefficient of variation (CV) were calculated for the content and digestibility coefficient of tryptophan to provide information on variability.

Table 9.1.1.1-10: Content (g/kg as received) and ileal digestibility coefficient of tryptophan in grain legumes

Feedstuff	Sample	Dry matter	Crude protein¹	Tryptophan content	Ileal Digestibility coefficient of tryptophan²
Chickpea	1	930	213	1.80	0.71
Faba bean	1	923	238	1.90	0.63
Field pea	1	906	229	1.80	0.75
	2	911	216	2.00	0.63
Lupin (<i>L. angustifolius</i>) ³	1	920	304	2.60	0.77
	2	926	340	2.70	0.78
	3	929	299	2.50	0.82
	4	907	288	2.80	0.79
	Mean	921	308	2.65	0.79
	SD	8.4	19.5	0.11	0.02
	CV (%)	0.9	6.3	4.2	2.4
Lupin (<i>L. albus</i>) ⁴	1	935	347	2.80	0.83

¹ Nitrogen x 6.25² Each value represents the mean of three pens (four 6-week-old broilers per pen)³ Narrow-leaf (or blue) lupin⁴ White lupin

To ensure that the digestibility assay was reproducible and that the digestibility values of the ingredients from the different assays could be compared, a sample of soybean (crude protein 485 g/kg, tryptophan 6.60 g/kg) was included in seven assays as a reference ingredient. Determined tryptophan digestibility coefficients ranged from 0.82 to 0.86, and statistical analysis showed no significant assay effects on digestibility. The CV for the digestibility of tryptophan in the reference soybean sample across different assays was

less than 3%. These findings indicate that the assay-to-assay variation was small and that the digestibility values obtained in different assays can be compared.

The average ileal tryptophan digestibility coefficient was high in wheat (0.83), intermediate in sorghum (0.75) and triticale (0.75) and lowest in maize (0.71). Although considerable variability was observed in the tryptophan concentrations of sorghum and wheat, the variability in digestibility coefficients was lower and grain protein level had no influence on tryptophan digestibility. Tryptophan digestibility in wheat middlings (0.76) was lower than in wheat and considerably higher than in rice polishings (0.65).

Among the oilseed meals, average tryptophan digestibility coefficients in soybean meal (0.84) and sunflower meal (0.81) were higher than in canola meal (0.78) and cottonseed meal (0.75). It is of interest that the digestibility estimate for the full-fat canola sample (0.86) was higher than those determined for canola meal samples (0.73 to 0.80). This observation may indicate possible adverse effects of processing conditions on tryptophan digestibility.

Among the grain legumes, tryptophan in both species of lupin (*L. angustifolius* and *L. albus*) was better digested than that in chickpeas, faba beans, and field peas.

The tryptophan digestibility coefficient in fish meal (0.77) and blood meal (0.84) was substantially higher than in meat meal (0.62), meat-and-bone meal (0.63) and feather meal (0.52). Marked variations in tryptophan digestibility were observed among samples of fish meal, meat-and-bone meal and meat meal, highlighting significant batch-to-batch differences.

The paper notes that the methodology used to determine the tryptophan digestibility in cereals, cereal-by-products, and grain legumes suffered from inherent limitations which may have caused an underestimation of the apparent digestibility coefficients.

III. CONCLUSION:

This study presents the variability in contents and apparent ileal digestibility for 6-week-old broiler chickens of tryptophan in 74 samples representing 24 feedstuffs. The average ileal tryptophan digestibility coefficient in wheat was 0.83, in sorghum and triticale was 0.75, in maize 0.71, soybean meal 0.84, sunflower meal 0.81, canola meal 0.78, and cottonseed meal 0.75. Among the grain legumes, tryptophan in lupins was better digested than that in chickpeas, faba beans, and field peas. Among the animal protein meals, the tryptophan digestibility coefficients in fish meal (0.77) and blood meal (0.84) were substantially higher than those in meat meal (0.62), meat-and-bone meal (0.63), and feather meal (0.52). Marked variations in tryptophan digestibility were also observed among samples of fish meal, meat-and-bone meal, and meat meal, highlighting significant batch to-batch differences. For most feedstuffs, considerable variability was observed in the tryptophan concentrations, but such variations were not reflected in digestibility coefficients.

HSE COMMENTS

This study was submitted as evidence that digestibility of lupin-based diets is a potential cause of lower weight gain in chickens fed lupin-based diets.

This study is from the scientific literature with the purpose of investigating the digestibility of the amino acid tryptophan from multiple different feedstuffs, including two lupin varieties.

Given the purpose of this study, it does not conform to a standard ecotoxicological test guideline, nor is it GLP compliant. Therefore, the study's methodology, reporting and analysis have been considered with regard to how well they meet common standards of scientific practice, based on 'expert judgement'.

The methodology and study design are well described and are in accordance with general scientific principles, although details on the source of the chickens and environmental housing conditions throughout the test were not provided. The chickens were adults and were all males. Detailed composition of the test diets is provided in the study. Since the test item is not a single chemical, analytical verification to confirm exposure is not applicable. The results of the other feedstuffs apart from the lupins are not relevant for risk assessment. Of the two lupin species tested, *L. albus* is the most relevant species since *L. albus* is the species being evaluated in the current dossier.

The study does not report any signs of toxicity or mortalities, but it was not stated whether this was explicitly monitored in the study, and results of this are not presented.

The study outputs are related to the digestibility of tryptophan from samples of the contents of the lower half of the ileum from adult male chickens fed test diets (for lupin diet: 666 g lupin/kg diet) for 7 days. The score for digestibility of tryptophan in *L. albus* was higher than the other grain legumes including the other tested lupin *L. angustifolius*, higher than most cereals apart from wheat which was a similar score, higher than oilseed meals apart from sunflower and soybean meal which had a similar score, and higher than animal protein meals. No statistical analysis was performed. No other performance related parameters of the chickens were presented.

The results are of **limited** supporting evidence in the risk assessment.

Reliability: Reliable with reservations.

(Studies are graded as one of the following categories: 1) Reliable, 2) Reliable with reservations, 3) Unreliable. Categorisation is based on the evaluator's subjective assessment of the quality of the methods, reporting and analysis as presented in the study.)

Reference:	K-CA 8.1.1.1/06
Report Title:	Feeding High Levels of Lupine Seeds to Broiler Chickens: Plasma Micronutrient Status in the Context of Digesta Viscosity and Morphometric and Ultrastructural Changes in the Gastrointestinal Tract
Author(s) & Year:	Olkowski, B. I. et al. (2005)
Document No	Poultry Science 84:1707–1715
Substance used:	Lupin seeds (<i>Lupinus angustifolius</i> , <i>Lupinus luteus</i> and <i>Lupinus albus</i>) raw or dehulled
Method of analysis:	No analysis conducted
Guideline(s):	No, study from published literature
Deviations:	N/A, study from published literature
GLP or GEP:	No, study from published literature
Acceptability:	Supplementary
Study relied upon:	Yes, however data protection not applicable to published literature studies

I. MATERIALS AND METHODS

A. MATERIALS

Test Material	Lupin seeds (<i>Lupinus angustifolius</i> , <i>Lupinus luteus</i> and <i>Lupinus albus</i>) raw or dehulled
Treatments	
Test rates:	400 g/kg diet lupine seeds raw (equivalent to 40% of diet) 350 g/kg diet lupine seeds dehulled (equivalent to 35% of diet) Offered ad libitum
Control:	350 g/kg soybean meal 48% CP (Crude Protein) (equivalent to 35% of diet). Offered ad libitum

Analysis of test concentrations:	NA
Test organisms	
Species:	Broiler chicks, male
Age:	1 day
Source:	Not reported
Health:	Only apparently normal chickens were used in the study – the lack of subclinical tissue and organ injury was confirmed by biochemical tests.
Feeding:	Soybean meal (SBM) or meal from whole or dehulled <i>Lupinus albus</i> , <i>Lupinus luteus</i> , and <i>Lupinus angustifolius</i> seeds was used as the primary protein source. The level of protein in the diets was equilibrated using corn gluten, and energy was balanced with canola oil and supplemented respectively with synthetic amino acids, dicalcium phosphate, limestone, choline chloride, mineral-vitamin premix, and salt.
Test design	
Test vessel:	84 x 46 cm metal cages
Replication:	4 replicates
No. animals/vessel:	4 males per cage (total 16 individuals per treatment group)
Duration of test:	21 days
Environmental test conditions	Not reported
Test facility:	University of Saskatchewan, Saskatoon, Saskatchewan S7N 5A8

B. STUDY DESIGN AND METHODS

All diets were isocaloric (~13 MJ of AME) and isonitrogenous (~23% crude protein). Soybean meal (SBM) or meal from whole or dehulled *Lupinus albus*, *Lupinus luteus*, and *Lupinus angustifolius* seeds was used as the primary protein source. The level of protein in the diets was equilibrated using corn gluten, and energy was balanced with canola oil and supplemented respectively with synthetic amino acids, dicalcium phosphate, limestone, choline chloride, mineral-vitamin premix, and salt.

Each diet was offered ad libitum to a group of 16 (4 replicate cages of four 1-d-old male broiler chicks per cage) for 21 d. The chickens were housed at 4 per cage in 84 x 46 cm metal cages (Jamesway Manufacturing Co., Ft. Atkinson, WI) with raised metal wire flooring. Water was provided ad libitum.

Body weight gain and feed consumption were recorded weekly. The birds were monitored 3 times per day for overt signs of toxicity.

At the completion of the experiment (d 21) blood samples from 8 normal broilers from each dietary treatment were collected for measurement of riboflavin, zinc, and LDH. After this, the birds were euthanized using T-61 Euthanasia Solution (Intervet Canada Ltd., Whitby, ON, Canada) and subjected to postmortem examination. Body weights were recorded immediately after death. Measurements of NSP and monosugars content were analysed using a gas chromatography-mass spectrometry method. Ileal digesta from all birds from each treatment (pooled by replication) were analysed for viscosity using a Brookfield DVII viscometer (Brookfield Engineering Labs, Inc., Stoughton, MA).

Statistical analyses were carried out by 1-way ANOVA from the microcomputer package Number Cruncher Statistical System (Hintze, 1995). The means were compared using Fisher's LSD test (Snedecor and Cochran, 1989). Statistical significance was assumed to exist when the probability of making a type I error was less than 0.05.

II. RESULTS AND DISCUSSION

Broilers fed lupine-based diets showed markedly decreased feed intake and growth rate (see table below). These effects of dietary lupine were the main adverse effects observed in the birds during the first week and persisted throughout the trial. Dehulling significantly increased body weight gain only in birds fed diets based *L. angustifolius* and *L. albus* ($P < 0.05$), but their performance was still lower ($P < 0.05$) than that of birds fed SBM-based diet, when comparing the body weight gains. The changes in feed consumption and feed conversion associated with dehulling were not statistically significant.

No birds showed any overt signs of toxicity during the course of the study, and all appeared normal upon clinical examination at sample collection. There were no changes after gross pathological examination, and blood plasma LDH levels did not indicate that there was any subclinical tissue or organ injury. Blood plasma LDH activity did not differ between birds fed lupine- and SBM-based diets, and mean values for birds fed SBM, raw lupine, and dehulled lupine based diets were 0.35, 0.34, and 0.28 nmol/mL per min, respectively. Broilers fed diet based on *L. angustifolius* had the highest viscosity of digesta. Dehulling decreased digesta viscosity in these birds.

Table 9.1.1.1-11: Total non-starch polysaccharide content in whole and dehulled lupine seeds, and the effects of lupine species and dehulling of seeds on different variables in broilers

Variable	Treatment group						
	SBM	Lupinus angustifolius		Lupinus luteus		Lupinus albus	
		Whole	Dehulled	Whole	Dehulled	Whole	Dehulled
Total non-starch polysaccharide (%)	21.7	33.5	25.3	24.2	10.8	27.0	17.6
Viscosity (cP)	1.8 ^{a*}	32.7 ^b	4.0 ^a	3.5 ^a	2.9 ^a	4.2 ^a	2.0 ^a
Body weight gain (g)	667.5 ^c	442.0 ^a	507.7 ^b	427.0 ^a	431.6 ^a	437.9 ^a	511.9 ^b
Feed consumption (g)	891.7 ^c	636.4 ^{ab}	693.5 ^b	611.6 ^a	650.3 ^{ab}	631.1 ^{ab}	665.6 ^{ab}
Feed conversion ratio (g:g)	1.33	1.45	1.37	1.43	1.51	1.44	1.31

^{a-c} Means with different superscript letters indicate significant differences at $\alpha = 0.05$ as a result of a protected Fishers least significant difference test. Absence of superscript letters indicates no statistical significance was found. Values of SEM (standard error of the mean) for viscosity, body weight gain, feed consumption and feed consumption ratio were 2.6, 18, 28, 0.05, respectively.

SBM: soybean meal

All sections of the intestinal tract were significantly enlarged ($P < 0.01$) in all groups fed lupine-based diets in comparison with broilers fed a SBM diet (see table below). The weight and length of each section differed significantly ($P < 0.001$) between broilers fed lupine-based diets and those fed a SBM-based diet.

There were no differences in the overall gross architecture of villi from duodenum, jejunum, or ileum. However, in comparison to birds fed a SBM-based diet, the mucosa of the ceca in broilers fed a lupine-based diet appeared thickened with grossly enlarged plicae and rounded villi tips. Cross-sectional examination of duodenum, jejunum, and ileum did not reveal any significant differences in the architecture of mucosa, submucosa, muscularis interna and externa, or serosa between broilers fed a lupine-based diet and those fed a SBM-based diet.

Table 9.1.1.1-12: Effects of lupine species and dehulling (D) of lupine seeds on morphometric parameters of intestinal tract¹

Treatment group	Duodenum		Jejunum		Ileum		Ceca	
	(cm)	(g)	(cm)	(g)	(cm)	(g)	(cm)	(g)
SBM	3.54 ^a	1.19 ^a	7.54 ^a	1.80 ^a	7.05 ^a	1.25 ^a	3.54 ^a	0.49 ^a
<i>Lupinus angustifolius</i>	4.90 ^{bc}	1.56 ^c	10.94 ^c	2.26 ^c	10.14 ^{bc}	1.61 ^c	4.46 ^c	0.59 ^b
<i>Lupinus luteus</i>	5.07 ^{bc}	1.52 ^{bc}	10.52 ^c	2.14 ^{bc}	9.75 ^{bc}	1.47 ^{bc}	4.57 ^c	0.56 ^{ab}
<i>Lupinus albus</i>	5.11 ^c	1.47 ^{bc}	10.51 ^c	2.17 ^{bc}	9.88 ^{bc}	1.45 ^{abc}	4.45 ^c	0.56 ^{ab}
<i>L. angustifolius</i> D	4.49 ^b	1.46 ^{bc}	10.07 ^c	2.03 ^{ab}	9.64 ^{bc}	1.45 ^{abc}	3.94 ^b	0.54 ^{ab}
<i>L. luteus</i> D.	4.22 ^b	1.30 ^{ab}	9.13 ^b	1.82 ^a	8.63 ^b	1.30 ^a	3.99 ^b	0.57 ^{ab}
<i>L. albus</i> D	4.44 ^b	1.26 ^a	9.34 ^{bc}	1.84 ^a	8.30 ^b	1.26 ^a	4.02 ^b	0.52 ^{ab}
SEM	0.21	0.08	0.32	0.08	0.32	0.07	0.13	0.02

^{a-c} Means with different superscript letters indicate significant differences at $\alpha = 0.05$ as a result of a protected Fishers least significant difference test.

¹ Values are normalised for body weight (g or cm/100 g)

SBM: soybean meal. SEM: standard error of the mean. D= dehulled

Blood plasma zinc concentration did not differ ($P = 0.45$) between broilers fed lupine- and SBM-based diets, but broilers fed diets based on lupine seeds had a significantly higher ($P < 0.001$) plasma riboflavin level in comparison to those fed a SBM diet.

III. CONCLUSION

This paper was conducted to examine the effects of lupine-based diets on the status of the host's riboflavin and zinc.

Overall, broilers fed lupine-based diets had more viscous digesta than those fed the soybean meal diet, but the differences were statistically not significant. Blood plasma Zn concentration did not differ between broilers fed lupine-based diets and those fed soybean-meal-based diets, and all broilers fed lupine-based diets had significantly higher ($P < 0.001$) riboflavin concentrations. In this context, it is apparent that the bioavailability of these micronutrients from lupine diets is not compromised. Intestinal tissue hyperplasia may be interpreted as physiological adaptation to increase absorptive capacity and thus maximize absorption of essential nutrients in the face of antinutritional factors in the diet.

Assessment and conclusion by applicant:

This paper has not been previously evaluated for EU regulatory purposes.

The study was not performed according to a regulatory guideline, but some consideration against a corresponding regulatory study guideline is possible:

The test meets the current OECD 205 (1984) validity criteria relating to control group performance:

- Control mortality was 0% (< 10%)

More generally, the paper is deficient in several key details usually required for a valid and reliable study. The source and purity of the test substance are not given, and there is little information on the test organism, including species name. Also, methodological detail is not provided on how some parameter assessments were made.

Conclusion:

For regulatory ecotoxicology purposes the study must be limited to supportive information only, due to a lack of test item, test organism and methodological details.

Overall, broilers fed lupine-based diets had more viscous digesta than those fed the soybean meal diet, but the differences were statistically not significant. Blood plasma Zn concentration did not differ between broilers fed lupine-based diets and those fed soybean-meal-based diets, and all broilers fed lupine-based diets had significantly higher ($P < 0.001$) riboflavin concentrations. In this context, it is apparent that the bioavailability of these micronutrients from lupine diets is not compromised. Intestinal tissue hyperplasia may be interpreted as physiological adaptation to increase absorptive capacity and thus maximize absorption of essential nutrients in the face of antinutritional factors in the diet.

HSE COMMENTS

This study was submitted as evidence that a reduction in body weight observed in chickens fed lupin-based diets is not a result of toxicological effects but due to high fibre content and changes in digestibility.

This study is from the scientific literature with the purpose of investigating the effect of lupine-based diets on micronutrient availability, using plasma riboflavin and zinc as examples, and considering this in the context of intestinal morphology and digesta (food undergoing digestion) viscosity. Given the purpose of this study, it does not conform to a standard ecotoxicological test guideline, nor is it GLP compliant. Therefore, the study's methodology, reporting and analysis have been considered with regard to how well they meet common standards of scientific practice, based on 'expert judgement'.

The methodology, study design and statistical analysis are well described and are in accordance with general scientific principles, although details on the source of the chickens and lupine seeds are not provided and neither are the housing environmental conditions throughout the test. The chickens were all males. Detailed composition of the test diets is provided in the study. Since the test item is not a single chemical, analytical verification to confirm exposure is not applicable. Three species of lupin seed were tested, both in whole and dehulled form. The findings for the lupin species *L. albus* are most relevant for risk assessment since this is the species being evaluated in the current dossier.

The main findings after the chickens were fed lupine seed meal diets (at 350-400 g/kg diet) for 21 days were enlargement of the intestinal tract and reduced feed intake and growth rate compared to the control. Regarding the intestinal findings, the authors discuss that

these were due to hyperplasia (increased cell number) rather than hypertrophy (increased cell size) and suggest that “Based on these features it can be inferred that the remodelling of intestinal morphology in lupine-fed birds is indicative of adaptive responses rather than pathological changes” and that “Intestinal tissue hyperplasia may be interpreted as physiological adaptation to increase absorptive capacity”.

Body weight and feed consumption were recorded weekly but quantitative results were only presented for test termination (21 days), however it is noted that adverse effects on these parameters were observed from the first week onwards. The authors could not conclude on a reason for growth reduction but did note that “Growth depression associated with high levels of dietary lupines in otherwise normal broilers may be in part explained by reduced feed intake” and emphasise the lack of any overt signs of toxicity or biochemical evidence of organ damage in the study.

In summary, chickens (male, 1 day old at start of test) fed with lupin seed meal up to 400 g/kg diet whole or 350 g/kg dehulled for 21 days, did not result in toxic effects but did result in adverse effects on body weight and feed consumption from 1 week onwards. Increased intestinal size was also observed for lupine diets but was attributed by the authors to a physiological adaptation to increase absorptive capacity rather than pathological response to the diet.

The results may be considered as supporting evidence in the risk assessment. The relevance of the doses in this study needs further consideration at risk assessment, given that this study tests with whole or dehulled seed meal diets, at a relatively high concentration in the diet (35-40% of diet), whereas the active substance under assessment is an aqueous extract of lupin seeds.

Reliability: Reliable with reservations.

(Studies are graded as one of the following categories: 1) Reliable, 2) Reliable with reservations, 3) Unreliable. Categorisation is based on the evaluator’s subjective assessment of the quality of the methods, reporting and analysis as presented in the study.)

B.9.1.1.2. Short-term dietary toxicity to birds

No data submitted or required.

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

No toxicity data is available addressing the long-term and reproductive risks to birds. The main components of aqueous extract from the germinated seeds of sweet *Lupinus albus* (PROBLAD PLUS) are water, proteins and carbohydrates (see Volume 4) which are naturally occurring and will already be present in avian diets. PROBLAD PLUS and its lead component BLAD have been demonstrated to be readily biodegradable in soil and water, so it is unlikely that chronic exposure to birds would occur. As such, it is considered that no chronic studies with birds are required. The published literature investigating inclusion of lupin seeds in bird diets (summarised in B9.1.1.1) will also be considered as supporting information in the risk assessment.

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 Volume 3 CA B6 (Toxicology).

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

No toxicity data is available addressing the long-term and reproductive risks to mammals. The main components of aqueous extract from the germinated seeds of sweet *Lupinus albus* (PROBLAD PLUS) are water, proteins and carbohydrates (see Volume 4) which are naturally occurring and will already be present in mammalian diets. PROBLAD PLUS and its lead component BLAD have been demonstrated to be readily biodegradable in soil and water, so it is unlikely that chronic exposure to mammals would occur. Additionally, as stated in Volume 3CA B6.1.1, the lead component, BLAD, will be broken down under enzymatic processes in the gastrointestinal tract, enter the amino acid pool and be consumed into normal metabolic processes. BLAD is known to be susceptible to proteolytic degradation. Furthermore, there were no indications in the 90 day oral study in the rat, of adverse effects on reproductive organs (see Volume 3 CA B6.2.1). Therefore consideration of the chronic risks to mammals is not considered to be necessary.

Lupin seeds are a commonly used as a feedstuff for livestock. Several published literature studies have been submitted which investigate the inclusion of lupin seeds in livestock diets, which may be used as supporting information in the risk assessment. These literature studies are summarised below with the inclusion of HSE comments on their relevance and reliability.

Reference:	K-CA 8.1.2.2/01
Report Title:	Nutrient intake, digestibility and growth performance of Washera lambs supplemented with graded levels of sweet blue lupin (<i>Lupinus angustifolius</i> L.) seed.
Author(s) & Year:	Ephrem, N., Tegegne, F., Mekuriaw, Y., & Yeheyis, L. (2015)
Document No	Small Ruminant Research, 130 (2015) 101-107.
Substance used:	Sweet blue lupin (<i>Lupinus angustifolius</i> L.) seed, cultivar 'Sanabor'
Method of analysis:	No analysis conducted

Guideline(s):	No, study from published literature
Deviations:	N/A, study from published literature
GLP or GEP:	No, study from published literature
Acceptability:	Supplementary
Study relied upon:	Yes, however data protection not applicable to published literature studies

I. MATERIALS AND METHODS

A. MATERIALS

Treatments

Test feeds: 150 g wheat bran (WB) (T1), 150 g WB + 195 g sweet lupin seed (SLS) (T2), 150 g WB + 245 g SLS (T3) and 150 g WB and 295 g SLS (T4).

Test animals

Animal: Intact Washera lambs with an average age of four months.
Body weight: Initial: 16.9 ± 0.32 kg (mean ± SD)
Source: Purchased from Adet livestock market
Treatment for disease: De-wormed and vaccinated against endo- and ecto-parasites and diseases
Acclimation : Adapted for 15 days to the experimental diets

Test conditions

Test site: Zenzelima campus, Bahir Dar University (11°37'N, 379'E, 1912 m)
Environmental conditions: Long-term average daily minimum and maximum temperatures 7 to 29°C, respectively. Average annual rainfall calculated for the same period was 1445 mm.
Replicates: Five replicates per group
Animals/group: Four lambs

B. STUDY DESIGN AND METHODS

Twenty intact Washera lambs with average age of four months and initial body weight of 16.9 ± 0.32 kg (mean ± SD) were purchased from Adet livestock market, 45 km southeast of Bahir Dar. The lambs were de-wormed and vaccinated against endo- and ecto-parasites and diseases and they were adapted for 15 days to the experimental diets and individual pens. The experimental animals were kept under experimental houses constructed according to ILRI (International Livestock Research Institute) standard design.

The design of the experiment was randomized complete block design (RCBD). Experimental animals were grouped into five blocks of four animals each based on their

initial body weight. Natural pasture hay was provided as a basal diet and offered ad libitum with 20% refusal. The hay used in this study was local mixed grass hay which was harvested from natural pasture during November 2013 (matured) and purchased from surrounding farmers during March 2014. The four experimental supplement feeds were 150 g wheat bran (WB) (T1), 150 g WB + 195 g sweet lupin seed (SLS) (T2), 150 g WB + 245 g SLS (T3) and 150 g WB and 295 g SLS (T4).

The SLS used for this study was purchased from local growers in Arebgebeya (50 km East of Bahir Dar) and Durbete (60 km southwest of Bahir Dar). The cultivar of sweet blue lupin seed used in this study was 'Sanabor'. From the three treatments (T2, T3 and T4), T3 was assumed to provide 127 g/day crude protein (CP), while the other two treatments were formulated to provide 110 and 144 g/day CP, respectively. Mineral block (common salt) and water were provided ad libitum. The supplemental feeds were divided into two halves and offered twice a day.

Daily feed intake was calculated as a difference of daily offer and daily left over from each animal. Body weight measurements were taken by Salter scale (50 kg capacity with 100 g precision) weekly after overnight fasting. Average daily gain (ADG) was determined as a difference between final and initial body weight divided over the feeding days. Feed conversion efficiency was calculated as the proportion of daily weight gain to daily dry matter intake. Feed conversion ratio was calculated as the proportion of daily dry matter intake to daily weight gain.

The digestibility trial was conducted at the end of the growth trial. Lambs were acclimatised to faecal collection bags for 3 days followed by 7 days of total faecal collection for each lamb. Feed refusals and faeces voided by each lamb within 24-h period were collected and weighed. After weighing the daily total faecal output of each animal, the faeces was thoroughly mixed and sub sample of 10% was taken to make up a single weekly composite for each lamb. Apparent digestibility of nutrients was calculated as the proportion of the difference between nutrient consumed and nutrient in faeces to nutrient consumed.

II. RESULTS AND DISCUSSION

The daily feed and nutrient intake of Washera sheep fed the basal diet of natural pasture hay supplemented with graded levels of sweet lupin seed is presented in the below table. No significant difference was observed on hay dry matter (DM) intake among treatment groups. Supplementation with SLS did not improve basal feed (hay) DM intake, nor was there a substitution effect. A significant difference ($p < 0.01$) in total DM intake among treatments was found, and total DM intake increased as the level of SLS supplementation increased. Supplementation of SLS increased ($p < 0.01$) CP intake and there was an increasing trend as the level of supplementation increased. There was also a significant difference in organic matter (OM) intake among treatment groups. Organic matter intake increased with the level of SLS supplementation, as did the intake of the fibre fraction (NDF, ADF, and ADL).

Table 9.1.2.2-1: Daily feed and nutrient intake of Washera sheep by diet

Source (g/day)	Treatment (mean \pm SD)				SL
	T1	T2	T3	T4	
Hay DMI	426.9 \pm 23.5	424.6 \pm 19.8	411.9 \pm 13.9	416.1 \pm 16.3	NS
Supplement DMI	150	345	395	445	**
Total DMI	576.9 \pm 23.5 ^c	769.6 \pm 19.8 ^b	806.9 \pm 13.9 ^{ab}	861.1 \pm 16.3 ^a	NS
Total DMI (%BW)	3.14 \pm 0.04	2.97 \pm 0.08	3.04 \pm 0.07	2.94 \pm 0.08	**
CPI	40.2 \pm 1.01 ^d	100.3 \pm 0.85 ^c	115.2 \pm 0.59 ^b	130.8 \pm 0.7 ^a	**
NDFI	379.2 \pm 17.8 ^b	428.3 \pm 14.9 ^{ab}	431.7 \pm 10.6 ^{ab}	447.9 \pm 12.3 ^a	*
ADFI	303.9 \pm 15.7 ^b	337.0 \pm 13.2 ^{ab}	337.4 \pm 9.3 ^{ab}	349.1 \pm 10.9 ^a	*
ADLI	93.2 \pm 4.57 ^b	105.4 \pm 3.86 ^a	106.1 \pm 6.08 ^a	110.2 \pm 7.11 ^a	**
OMI	535.0 \pm 22.1 ^c	704.8 \pm 18.6 ^b	736.9 \pm 13.1 ^{ab}	785 \pm 15.3 ^a	

^{abcd} Mean values in the same row with different superscript differ significantly; SE = Standard Error; DMI = Dry matter intake; CPI = Crude protein intake; NDFI = Neutral detergent fibre; ADFI = Acid detergent fibre intake; ADLI = Acid detergent lignin intake; OMI = Organic matter intake; %BW = Percentage body weight; SL = Significance level; NS = Not significant; T1 (control) = Hay + 150 g wheat bran (WB); T2 = Hay + 150 g WB + 195 g Sweet lupin seed (SLS); T3 = Hay + 150 g WB + 245 g SLS; T4 = Hay + 150 g WB + 295 g SLS. * $p < 0.05$. ** $p < 0.01$

Apparent digestibility of DM and OM was higher ($p < 0.05$) for sheep supplemented with SLS than for the control group, but there was no significant difference within the supplemented sheep. The NDF and ADF digestibility in the study was also not affected by supplementation. The apparent digestibility of CP was higher ($p < 0.001$) for supplemented sheep, increasing as the level of SLS supplementation increased.

Table 9.1.2.2-2: Apparent digestibility of nutrients in Washera sheep by diet

Digestibility (%)	Treatment (mean \pm SD)				SL
	T1	T2	T3	T4	
DM	60.4 \pm 0.9 ^b	71.5 \pm 2.2 ^a	68.2 \pm 2.7 ^a	69.5 \pm 1.8 ^a	*
CP	40.1 \pm 1.9 ^b	70.4 \pm 2.01 ^a	70.6 \pm 2.06 ^a	74.2 \pm 1.03 ^a	***
NDP	46.2 \pm 0.78	50.8 \pm 2.75	44.5 \pm 3.53	44.6 \pm 2.71	NS
ADF	55.9 \pm 0.57	60.9 \pm 2.17	57.8 \pm 2.66	57.7 \pm 2.15	NS
OM	63.9 \pm 0.85 ^b	73.1 \pm 2.16 ^a	76.7 \pm 2.57 ^a	73.5 \pm 1.78 ^a	*

^{ab} Mean values in the same row with different superscript differ significantly SE = Standard Error; DM = Dry Matter; CP = Crude Protein; NDF = Neutral Detergent Fibre; ADF = Acid Detergent Fibre; OM = Organic Matter; SL = Significance level; NS = Not significant; T1 (control) = Hay + 150 g wheat bran (WB); T2 = Hay + 150 g WB + 195 g sweet lupin seed

(SLS); T3 = Hay + 150 g WB + 245 g SLS; T4 = Hay + 150 g WB + 295 g SLS. * $p < 0.05$. *** $p < 0.001$.

Significantly higher body weight was obtained in the supplemented groups from the control group ($p < 0.05$). Significantly higher average daily gain (ADG), body weight change, and feed conversion efficiency ($p < 0.01$) was recorded for the supplemented groups.

Table 9.1.2.2-3: Body weight change parameters Washera sheep by diet

Body weight (BW) parameter	Treatment (mean ± SD)				SL
	T1	T2	T3	T4	
Initial BW (kg)	16.2 ± 0.38	17.2 ± 0.54	16.9 ± 0.96	17.2 ± 0.61	NS
Final BW (kg)	18.1 ± 0.58 ^c	22.8 ± 0.8 ^b	24.4 ± 0.97 ^{ab}	25.2 ± 0.56 ^a	*
Body weight change (kg)	1.88 ± 0.3 ^c	5.56 ± 0.49 ^b	7.54 ± 0.07 ^a	8.02 ± 0.09 ^a	**
ADG (g)	20.9 ± 3.35 ^c	61.8 ± 5.47 ^b	83.8 ± 0.84 ^a	89.1 ± 0.95 ^a	***
FCR	29.6 ± 3.37 ^a	12.8 ± 0.9 ^b	9.62 ± 0.17 ^b	9.7 ± 0.2 ^a	**
FCE	0.03 ± 0.005 ^c	0.08 ± 0.005 ^b	0.1 ± 0.003 ^a	0.1 ± 0.002 ^a	**

^{abc}Mean values in the same row with different superscript differ significantly; ADG = Average Daily Gain; SE = Standard Error; FCR = Feed conversion ratio; FCE = Feed conversion efficiency; SL = Significance level; NS = Not significant; T1 (control) = Hay + 150 g wheat bran (WB); T2 = Hay + 150 g WB + 195 g Sweet lupin seed (SLS); T3 = Hay + 150 g WB + 245 g SLS; T4 = Hay + 150 g WB + 295 g SLS. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$.

Average daily gain was strongly and positively correlated ($p < 0.01$) with Dry matter intake was strongly and positively correlated ($p < 0.01$) with total DM, CP and OM intake and CPD.

Table 9.1.2.2-4: Correlation between nutrient intake, apparent digestibility of nutrients, and average daily gain

	TDMI	CPI	NDFI	ADFI	OMI	DMD	CPD	NDFD	ADFD	OMD	ADG
TDMI	1										
CPI	0.96**	1									
NDFI	0.87**	0.71**	1								
ADFI	0.79**	0.52*	0.99**	1							
OMI	0.99**	0.95**	0.89**	0.81**	1						
DMD	0.54*	0.61**	0.32	0.24	0.53*	1					
CPD	0.84**	0.93**	0.53*	0.41	0.83**	0.82**	1				
NDFD	-0.09	-0.09	-0.06	-0.05	-0.09	0.71**	0.19	1			
ADFD	0.19	0.18	0.15	0.14	0.19	0.86**	0.43	0.96**	1		
OMD	0.46*	0.52*	0.26	0.18	0.45*	0.99**	0.76**	0.77**	0.90**	1	
ADG	0.95**	0.97**	0.54*	0.47*	0.95**	0.53*	0.87**	-0.15	0.14	0.46*	1

ADG = Average Daily Gain; SE = Standard Error; FCR = Feed conversion ratio; FCE = Feed conversion efficiency; SL = Significance level; NS = Not significant; T1 (control) = Hay + 150 g wheat bran (WB); T2 = Hay + 150 g WB + 195 g Sweet lupin seed (SLS); T3 = Hay + 150 g WB + 245 g SLS; T4 = Hay + 150 g WB + 295 g SLS. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$.

III. CONCLUSION:

Supplementation of Washera lamb feed with sweet lupin seed (SLS) significantly increased total dry matter, crude protein, and organic matter intakes. Supplementation of SLS improved the digestibility of dry matter and organic matter ($p < 0.05$) and crude protein ($p < 0.001$). However, the digestibility of neutral detergent fibre and acid detergent fibre were not affected by supplementation ($p < 0.05$). Average daily gain (ADG) was higher ($p < 0.001$) for the SLS supplemented groups (61.8 to 89.1 g/day).

HSE COMMENTS

This study is from the scientific literature and does not conform to standardised test guidelines, nor is it GLP compliant. The study's methodology, reporting and analysis have been considered with regard to how well they meet common standards of scientific practice, based on 'expert judgement'.

Whilst the study has been well conducted in accordance with scientific principles, it is performed with seeds of *Lupinus angustifolius*, rather than *Lupinus albus*, from which the active substance is derived. This raises some uncertainty as to the relevance of this study for the assessment of aqueous extract from the germinated seeds of sweet *Lupinus albus*. Additionally the study has been conducted with lambs, which are not a standard test organism and the assessment of nutrient digestibility is not of relevance to the mammalian risk assessment. Body weight change has been measured and average daily gain was higher in groups supplemented with sweet lupin seeds. This indicates that seeds of *Lupinus angustifolius* do not adversely affect growth in Washera lambs but there is uncertainty applying this conclusion to seeds of *Lupinus albus*.

Reliability: Reliable with reservations

(Studies are graded as one of the following categories: 1) Reliable, 2) Reliable with reservations, 3) Unreliable. Categorisation is based on the evaluator's subjective assessment of the quality of the methods, reporting and analysis as presented in the study.)

Reference:	K-CA 8.1.2.2/02
Report Title:	Performance and intestinal responses to dehulling and inclusion level of Australian sweet lupins (<i>Lupinus angustifolius</i> L.) in diets for weaner pigs.
Author(s) & Year:	Kim, J.C (2012)
Document No	Animal feed science and technology. 201-209 172.
Substance used:	<i>Lupinus angustifolius</i> (whole and dehulled seeds), cultivar 'Coromup'
Method of analysis:	No analysis conducted
Guideline(s):	No, study from published literature
Deviations:	N/A, study from published literature
GLP or GEP:	No, study from published literature
Acceptability:	Supplementary
Study relied upon:	Yes, however data protection not applicable to published literature studies

I. MATERIALS AND METHODS

A. MATERIALS

B. STUDY DESIGN

Test Material	Lupinus angustifolius (whole and dehulled seeds)
Purity:	319 g protein, 346 g insoluble non-starch polysaccharides (NSP) and 24 g soluble NSP
Treatments	
Test feed:	Control: a wheat-based diet containing 240 g/kg of milk products, whole or de-hulled lupins at 60, 120, 180 and 240 g/kg of diet (replace 25%, 50%, 75%, 100% of the milk product).
Test organisms	
Organism:	180 entire male pigs weaned at 21 days of age and weighing 6.4 ± 0.1 kg
Species:	Not stated
Source:	A high health status commercial farm

Test design

Experimental unit: Housed in pens as pairs (space allowance 0.4 m² per pig)

Replication: 10 pigs per treatment (two replicate studies, 90 pigs each, total of 180 pigs)

High protein *Lupinus angustifolius*, cv. Coromup, was selected for the study. The lupin was collected from the northern agricultural region of Western Australia (Geraldton, WA) which has a reasonably generic soil type (sand over loam). The lupin contained 319 g protein, 346 g insoluble non-starch polysaccharides (NSP) and 24 g soluble NSP (Kim et al., 2009a).

A total of 180 entire male pigs weaned at 21 days of age and weighing 6.4 ± 0.1 kg (mean \pm standard error of mean (SEM)) was acquired from a high health status commercial farm and was transported to the Medina Research Station. Two replicate studies using 90 pigs per replicate were conducted, with an interval of a month between each. Upon arrival, pigs were weighed, ear tagged, housed as pairs (space allowance 0.4 m² per pig) and were allocated to 9 dietary treatments based on weaning weight.

The experiment was a completely randomised block design with 9 dietary treatments, as follows: (1) a wheat-based control diet containing 240 g/kg of whey and skim milk powder, and (2) 2 x 4 diets containing whole or dehulled lupins (cv. Coromup) that substituted the milk products at 60, 120, 180 and 240 g/kg of diet (replace 25%, 50%, 75%, or 100%). The whole and dehulled lupins were hammer milled to a mean particle size of 700 μ m and directly dumped into the mixer. Digestible energy and ileal digestible indispensable amino-acid contents were equalised using soy protein concentrate, canola oil, full fat soya and meat meal. The diets were isoenergetic and were formulated to contain the same ileal standardised digestible lysine content (Sauvant et al., 2004) and ideal patterns of other indispensable amino-acid. All diets contained 2 g/kg titanium dioxide as a digestibility marker (Short et al., 1996) to estimate the total tract apparent digestibility (TTAD) of dry

matter (DM). Nutrient composition of dehulled lupins used in this study was not chemically determined. Tabulated values reported in the previous publication (Kim et al., 2007) were used for diet formulation, as nutrient composition of lupin kernels shows a low variation.

Experimental procedure and measurements

Pigs were fed their respective diets as a mash form and on an ad libitum basis for 3 weeks. Fresh water was available throughout the experiment. Pigs were weighed weekly and feed intake was measured on a daily basis.

Data for faecal consistency were expressed as the mean faecal consistency value of pigs within a diet having the score 1 (25%), score 2 (50%), score 3 (75%), and score 4 (100%). A faecal consistency score of 4 represented pigs with diarrhoea, and the incidence of post-weaning diarrhoea (PWD) was expressed as the mean proportion of days pigs had faecal score 4 with respect to days (14 d) (after Mateos et al., 2006).

Faecal swabs from individual pigs were taken on days 0, 3, 5, 7 and 9 to examine the degree of -haemolytic *E. coli* proliferation (Kim et al., 2008b). Swabs were cultured onto sheep blood (50 mL/L) agar plates (Columbia base, Oxoid, London, UK) and then assessed for -haemolytic colonies displaying the characteristic morphology of *E. coli*, after overnight incubation at 37°C in air (McDonald et al., 2001). The sheep blood agar plates were given a swab score according to the number of streaked sections that contained viable haemolytic *E. coli*, where: 0 = no growth, 1 = haemolytic *E. coli* in 1st section, 2 = haemolytic *E. coli* in 2nd section, 3 = haemolytic *E. coli* in 3rd section, 4 = haemolytic *E. coli* in 4th section, 5 = haemolytic *E. coli* present right out to the 5th section of the plate.

Faecal samples were collected from the pen floor as voided by the pigs for 3 consecutive days at the end of weeks 1 and 3 to determine faecal moisture content and the TTAD of DM. Samples were pooled per pen at the end of each collection and subsamples were stored at -20°C until analysed.

Blood samples were collected from individual pigs on days 7 and 21. Samples were collected from anterior vena cava into lithium heparin coated vacutainer. The blood samples were immediately placed on ice and then centrifuged at 2000 × g for 10 min at 5°C. Plasma was stored at -20°C until analysed for plasma urea nitrogen (PUN).

Chemical analyses

The PUN level was determined using an enzymatic (urease) kinetic method (Randox, Crumlin, Co. Antrim, UK). Dry matter was measured using the AOAC official method 930.15 (AOAC, 1997). Titanium dioxide was measured spectrophotometrically at 410 nm after acid hydrolysis (7.4 M-H₂SO₄; Short et al., 1996). The nitrogen (N) content was determined using combustion method 990.03 (AOAC, 1997). Crude protein content was calculated as N content × 6.25. Crude fat content was determined using AOAC official method 2003.06 (AOAC, 1997). The neutral detergent fibre (NDF), acid detergent fibre (ADF) and lignin were determined using the AOAC official method 925.10 (AOAC, 1997). Gross energy

content was determined using a ballistic bomb calorimeter (SANYO Gallenkamp, Loughborough, UK). Total phosphorus (P) was determined using inductively coupled atomic emission spectroscopy as described by McQuaker et al. (1979). Phytate-P content was determined spectrophotometrically using the principle that phytate forms stable complexes with ferric ions in dilute acid solution (Xu et al., 1992). The insoluble and soluble NSP content of the lupin samples was determined as alditol acetates by gas-liquid chromatography (GLC) using the method of Theander and Westerlund (1993). Total NSP content was calculated by adding the insoluble and soluble NSP contents. The sum of insoluble and soluble NSP was calculated using the following polymerisation factors:

Sum of total, insoluble and soluble NSP = (Rha + Fuc + Rib)×0.89 + (Ara + Xyl)×0.88 + (Man + Gal + Glu)×0.90, where Rha = rhamnose; Fuc = fucose; Rib = ribose; Ara = arabinose; Xyl = Xylose; Man = mannose; Gal = galactose; Glu = glucose.

Polymerisation factors were used to correct for differences in total molecular weights due to dehydration during the polymerisation process. For example, each glucosidic linkage for a glucose (hexose) molecule loses one molecule of water during polymerisation. Therefore, a factor of 0.9 was used in the calculation to account for differences in molecular weights between glucose (180) and water (18) [i.e., $(180 - 18)/180 = 0.9$]. The same calculation was applied for deoxy-sugars (molecular weight 164 and hence a factor of 0.89) and pentoses (molecular weight 150 and hence a factor of 0.88).

Statistics

The treatment effects were assessed by one-way analysis of variance (ANOVA) and faecal score was analysed using repeated measure ANOVA as it was recorded daily for 14 days. When significant diet effect was found in the ANOVA test, then the variables were tested for Fisher's-protected least significant difference analysis to separate means where significant main effect occurred under the ANOVA analysis. Statistically significant difference between treatments was accepted at $P < 0.05$.

II. RESULTS AND DISCUSSION

Post-weaning performance

Pigs fed diets containing whole lupins up to 240 g/kg and dehulled lupins up to 180 g/kg ate comparable amounts of feed and had similar feed conversion ratio (FCR) and daily gains compared to pigs fed the milk-powder-based control diet. Although feed conversion ratio (FCR) was comparable, pigs receiving 240 g/kg of dehulled lupins grew slower ($P < 0.05$) than pigs fed the other diets, predominantly due to decreased feed intake.

Indices of gastrointestinal tract function

Faecal consistency, the number of antibiotic treatments, and the incidence of PWD were generally low and unaffected by up to 240 g/kg inclusion of whole or dehulled lupins in the diet. However, pigs fed diets containing 180 g/kg and 240 g/kg of dehulled lupins had

greater faecal β -haemolytic *E. coli* scores on day 3 after weaning ($P < 0.05$). Faecal β -haemolytic *E. coli* scores were not different in the other days.

Plasma urea nitrogen

Inclusion of 240 g/kg of whole lupin or more than 180 g/kg of dehulled lupins increased ($P < 0.001$) the PUN level. Increased dispensable amino acid levels in the diets with greater lupin concentrations showed a positive relationship ($P < 0.001$) to the PUN level.

Table 9.1.2.2-5: Effect of dehulling and concentration of lupins in the diet for weaner pigs on performance of weaner pigs measured for 21 days after weaning

Item	Control	Whole lupin, g/kg				Dehulled lupin, g/kg				SEM ^a	P-value
		60	120	180	240	60	120	180	240		
Average daily gain, g/day	324 ^Y	351 ^Y	342 ^Y	364 ^Y _Z	344 ^Y	362 ^Y _Z	359 ^Y _Z	326 ^Y	305 ^X	6.4	0.040
Average daily feed intake, g/day	509	523	524	519	498	516	502	507	424	10.6	0.202
Feed conversion ratio	1.56	1.46	1.55	1.43	1.43	1.44	1.36	1.52	1.49	0.029	0.734

^{XYZ} Means in the same row with different superscripts differ ($P < 0.05$) Control diet contained none of either whole or dehulled lupins. ^a Pooled standard error of mean.

Table 9.1.2.2-6: Effect of dehulling and concentration of lupins in the diet for weaner pigs on faecal consistency, faecal dry matter (DM), incidence of post-weaning diarrhoea (PWD) and number of antibiotic treatment

Item	Control	Whole lupin, g/kg				Dehulled lupin, g/kg				SE M ^c	P-value
		60	120	180	240	60	120	180	240		
n	20	20	20	20	20	20	20	20	20		
Faecal consistency ^d (%)											
D 1-7	22.1	22.6	22.0	21.6	24.4	24.6	22.6	24.1	22.3	0.410	NS
D 8-14	22.4	23.7	21.6	20.6	21.9	22.9	22.6	22.9	22.6	0.355	NS
Faecal DM ^e (%)											
D 7	31.8	37.1	34.0	30.6	35.3	31.3	34.4	32.9	35.2	0.69	NS
D 21	29.5	28.4	28.2	29.2	30.9	28.4	27.5	29.3	30.1	0.26	NS
Incidence of PWD ^f (%)											
D 1-7	2.1	0.7	0.7	0.7	2.9	2.9	2.9	2.1	0.7	0.42	NS
D 8-14	0.1	0.7	0.0	0.0	0.7	0.7	0.0	0.0	1.4	0.21	NS
Number of antibiotic treatments ^g											
D 1-7	0.1	0.1	0.1	0.0	0.3	0.2	0.2	0.2	0.1	0.03	NS
D 8-14	0.0	0.1	0.0	0.0	0.0	0.1	0.0	0.0	0.1	0.01	NS

^c Standard error of the mean.^d Faecal consistency was expressed as % cumulative score per day of pigs having more liquid faeces; higher values are associated with more liquid faeces.^e The pen was experimental unit for faecal DM analysis (n = 10).^f A faecal consistency score of 4 represented pigs with PWD, observed during the first 14 days after weaning, is expressed as the mean percentage of days with diarrhoea relative to the total 14 days after weaning. Data are mean values per treatment combination assessed between days 1 and 14.^g Mean for total number of pigs injected with antibiotics with respect to the number of pigs in each feeding group error of mean.

Total tract apparent digestibility of dry matter

The TTAD of DM decreased ($P < 0.001$) as inclusion of lupins was increased, and the extent of the reduction was greater in the whole lupin diet than in the dehulled lupin diet. The TTAD of DM was negatively correlated to the NDF and ADF concentration of the diets ($P < 0.001$).

III. CONCLUSION:

The purpose of this study was to examine the performance and intestinal responses to whole and dehulled *Lupinus angustifolius* as alternatives to more expensive animal protein sources.

Pigs receiving 240 g/kg of dehulled lupins grew slower ($P < 0.05$) than pigs fed the other diets mainly due to decreased feed intake. Pigs fed diets containing more than 180 g/kg of dehulled lupins had a higher faecal -haemolytic *Escherichia coli* score on day 3 after weaning ($P < 0.05$). However, the significant increase in the faecal -haemolytic *E. coli* score in pigs fed more than 180 g dehulled lupins/kg diets (Fig. 1) suggests that fermentable lupin fibre may have negative effects on gastrointestinal tract function, although this did not result in more PWD. It is likely that poly-galacturonans (major polysaccharides in lupin kernel) increased water holding capacity and increased digesting retention time in the gastrointestinal tract, which is evident in the decreased feed intake in 240 g/kg dehulled lupin-fed pigs. In turn, increased retention time and availability of fermentable fibre contributed to higher proliferation of -haemolytic *E. coli*. Moreover, inclusion of 240 g/kg of whole lupin or more than 180 g/kg of dehulled lupins increased ($P < 0.001$) plasma urea nitrogen (PUN) levels. Total tract apparent digestibility (TTAD) of dry matter decreased ($P < 0.001$) in all lupin diets compared with the control diet.

HSE COMMENTS

This study is from the scientific literature and does not conform to standardised test guidelines, nor is it GLP compliant. The study's methodology, reporting and analysis have been considered with regard to how well they meet common standards of scientific practice, based on 'expert judgement'.

Whilst the study has been well conducted in accordance with scientific principles, it is performed with seeds of *Lupinus angustifolius*, rather than *Lupinus albus*, from which the active substance is derived. This raises some uncertainty as to the applicability of this study to the assessment of aqueous extract from the germinated seeds of sweet *Lupinus albus*. Additionally there is some uncertainty with the test organism: the study has been conducted with pigs, which are not a standard test organism, there are no details of the species tested and all of the test organisms were male. The digestibility parameters measured are not of particular relevance to the standard mammalian risk assessment. However, growth rate was measured and the inclusion of 240 g/kg dehulled lupin seeds resulted in slower growth than the control, possibly due to reduced feed intake, although there is uncertainty applying this conclusion to seeds of *Lupinus albus*.

Reliability: Reliable with reservations

(Studies are graded as one of the following categories: 1) Reliable, 2) Reliable with reservations, 3) Unreliable. Categorisation is based on the evaluator’s subjective assessment of the quality of the methods, reporting and analysis as presented in the study.)

Reference:	K-CA 8.1.2.2/03
Report Title:	Whole white lupin (<i>Lupinus albus</i> cv. Amiga) seeds as a source of protein for growing-fattening rabbits.
Author(s) & Year:	Z. Volek, M. Marounek (2009)
Document No	Animal Feed Science and Technology 152 (2009) 322–329
Substance used:	Whole white lupin seeds (<i>Lupinus albus</i>), cultivar ‘Amiga’
Method of analysis:	No analysis conducted
Guideline(s):	Recommendations and guidelines for applied nutrition experiments in rabbits. World Rabbit Sci. 13, 209–228 (Fernandez, 2005) (Non-standard test guideline)
Deviations:	N/A, study from published literature
GLP or GEP:	No, study from published literature
Acceptability:	Supplementary
Study relied upon:	Yes, however data protection not applicable to published literature studies

I. MATERIALS AND METHODS

A. MATERIALS

Test Material	Whole white lupin seeds
Purity:	NA
Treatments	
Test rates:	150 g/kg <i>Lupinus albus</i> , 100 g/kg soybean meal, 170 g/kg sunflower meal,
Test organisms	Growing-fattening rabbits
Species:	Hyplus rabbits (<i>Oryctolagus cuniculus</i>)

Source:	Not stated
Feeding:	Fed ad libitum,
Test design	
Experimental unit:	Individually housed in cages (50 cm × 40 cm × 42.5 cm) and assigned at random to one of the three experimental diets
Replication:	13 per treatment

B. STUDY DESIGN

The objective of this study was to compare the effect of including three protein sources (white lupin, soybean meal and sunflower meal) in rabbit diets on the digestibility of nutrients, caecal traits, performance and carcass traits.

Experimental procedure and measurements

There were three experimental diets that included 100 g soybean meal, 170 g sunflower meal or 150 g white lupin /kg as the main protein source. The experimental diets had similar crude protein (CP), starch, ether extract (EE) and amino-acids (AA) content. The main additional components were alfalfa meal, wheat bran, sugar beet pulp, oats and barley. Synthetic AA were added to the vitamin premix in substitution of the premix carrier (wheat flour), and included in the diets. The soybean meal and white lupin diets contained more wheat bran than the sunflower meal diet. Thus, the sunflower meal diet had more cellulose and lignin and less digestible energy than the other diets. Diets were offered as 3 mm pellets with a length of 5-10 mm. No medication was included in the feed or in the drinking water.

For the performance trial, 90 Hyplus rabbits were individually housed, randomly allocated to three groups and fed one of the three experimental diets from 37 to 79 days of age. Animals were housed in all-wire cages (40 cm × 40 cm × 42.5 cm) in an environmentally controlled building. The temperature was maintained at $16 \pm 2^{\circ}\text{C}$. Feeds and water were offered to all rabbits for ad libitum consumption. Rabbits were weighed every 7 days and feed intake was measured in this period. Health status of the digestive tract was controlled through individual observation of clinical signs of digestive troubles such as diarrhoea, mucus in faeces and abnormal caecotrophy.

At the end of the experiment, 18 rabbits per treatment were randomly selected, weighed, humanely killed without previous fasting, and used for the evaluation of carcass traits. The individual rabbit was considered as the experimental unit for all of the parameters studied. In addition, 39 additional Hyplus rabbits (950 ± 92 g) weaned at 33 days of age were used in a digestibility trial to determine the coefficient of total tract apparent digestibility (CTTAD) of organic matter (OM), CP, gross energy (GE), EE, starch, neutral detergent fibre, and acid detergent fibre of the diets, following the European reference method (Perez et al., 1995).

The rabbits were individually housed in digestibility cages (50 cm × 40 cm × 42.5 cm) and assigned at random to one of the three experimental diets (13 per treatment). Diets were

offered to rabbits *ad libitum*. Following an adaptation period of 15 days, daily feed intake and total faecal output were recorded from 48 to 52 days of age for each rabbit and then, all the rabbits were humanely killed. After laparotomy, the caeca were excised, weighted, emptied by gentle squeezing, and then the digesta pH was measured. For analyses of caecal volatile fatty acids (VFA), lactic acid and ammonia concentration, portions of caecal digesta were diluted with distilled water (1:2), and mercury (II) chloride was added to inactivate microbial growth. Three rabbits were excluded from the evaluation due to transitory diarrhoea.

Chemical analyses

The components, listed in the table below were determined using the appropriate methods in accordance with the methods published by the Association of Official Agricultural Chemists (AOAC).

Table 9.1.2.2-7: Chemical composition of protein sources (g/kg as-fed basis unless otherwise stated).

	Soybean meal	Sunflower meal	White lupin seeds
Determined values			
Dry matter	868	887	883
Crude protein	438	275	297
aNDFom ^a	108	386	330
ADFom ^b	67	280	230
Lignin	25	95	59
RSO ^c	8.7	1.9	11.2
Ether extract	23	28	114
Calculated values			
Water-insoluble pectins	82	71	104
Lysine	26.2	9.9	15.8
Methionine + cysteine	12.1	11.8	7.3
Threonine	16.2	10.2	11.5

^a Neutral detergent fibre expressed exclusive of residual ash

^b Acid detergent fibre expressed exclusive of residual ash

^c Oligosaccharides of the raffinose series expressed in mmol/100 g

Statistics

Data on growth performance, caecal and carcass traits and the CTTAD of the diets were examined by one-way analysis of variance using the GLM procedure of the Statistical Analysis Systems Institute (2001). Scheffe's test was used for mean comparison where appropriate. Morbidity was analysed using the chi-square test. Differences among

treatment means with $P < 0.05$ were accepted as representing statistically significant differences. Differences within the range 0.05-0.10 were considered a trend.

II. RESULTS AND DISCUSSION

In the present study, the weight of the caecum and its contents was highest in rabbits fed the soybean meal based diet. There was a significantly higher level of lactic acid in the caecal contents of rabbits fed the white lupin diet than in rabbits fed the other diets, probably due to the higher oligosaccharides of the raffinose series content of white lupin.

No adverse effect of feeding white lupin on rabbit performance was observed. In the present study, we observed a higher feed intake in rabbits fed the sunflower meal diet than in those fed the soybean meal or white lupin diets. The highest incidence of diarrhoea was observed in rabbits fed the soybean meal diet. The dressing-out percentage was higher in rabbits fed the white lupin diet in comparison with those fed the sunflower meal or soybean meal diet.

Table 9.1.2.2-8: Growth performance of rabbits fed diets based on soybean meal (SBM), sunflower meal (SFM) or whole white lupin seed (WL).

	Soybean meal	Sunflower meal	White lupin seeds
Live weight (g)			
At 37 days	1055	1052	1057
At 79 days	2630	2699	2683
37–58 days of age			
Weight gain (g/d)	43.8	45.5	43.7
Feed intake (g/d)	113.3	121.4	115.2
Feed conversion	2.61	2.71	2.63
Morbidity	0	1	1
58–79 days of age			
Weight gain (g/d)	32.7	34.2	35.4
Feed intake (g/d)	133.0	149.2	142.9
Feed conversion	4.17	4.40	4.04
Morbidity	8	3	1
37–79 days of age			
Weight gain (g/d)	38.4	40.1	40.0
Feed intake (g/d)	122.9	135.0	128.7
Feed conversion	3.22	3.40	3.24
Morbidity	8	4	2

III. CONCLUSION:

The objective of this study was to compare the effect of including three protein sources (white lupin, soybean meal and sunflower meal) in rabbit diets on the digestibility of nutrients, caecal traits, performance and carcass traits.

White lupin (*Lupinus albus* cv. Amiga) is a suitable dietary protein source for growing-fattening rabbits that can fully replace traditionally used protein sources without adverse effects on the digestibility of nutrients, caecal traits, performance or carcass parameters.

HSE COMMENTS:

This study is from the scientific literature and does not conform to standardised test guidelines, nor is it GLP compliant. The study's methodology, reporting and analysis have been considered with regard to how well they meet common standards of scientific practice, based on 'expert judgement'.

The study appears to be well conducted and in accordance with scientific principles. There is some uncertainty regarding the test item as no batch number or purity is reported, however the same species of lupin (*L. albus*) has been used as the active substance is derived from. No mortality was seen in the study and there was no effect on weight gain or feed conversion ratio in the sweet lupin treatment. The results indicate that there is no adverse effect of feeding white lupin seeds to rabbits over a period of 79 days at a concentration of 150 g/kg in the diet.

Reliability: Reliable with reservations

(Studies are graded as one of the following categories: 1) Reliable, 2) Reliable with reservations, 3) Unreliable. Categorisation is based on the evaluator's subjective assessment of the quality of the methods, reporting and analysis as presented in the study.)

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

No data submitted or required. See Volume 3 CP B9 for discussion.

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

No endocrine disruptor studies were conducted or were considered necessary in the toxicology assessment (Volume 3CA B6), nor are they considered necessary for the ecotoxicology assessment. The main components of aqueous extract from the germinated seeds of sweet *Lupinus albus* (PROBLAD PLUS) have been identified as water, proteins, carbohydrates and lipids (see Volume 4). These components are naturally occurring and will already contribute a large portion of the diet of terrestrial vertebrates. Additionally,

seeds of lupins, including *Lupinus albus*, are widely used as a food source in agriculture and aquaculture. A variety of published literature has been submitted for assessment, which investigate the effects of including lupin seeds in bird and mammal diets. No indications of toxicity or sub-lethal effects were demonstrated in these studies. Additionally, there were no indications in the 90 day oral study in the rat, of adverse effects on the endocrine system (see Volume 3CA B6.2.1 and 6.8.2). As stated in Volume 3CA B6.1.1, the lead component, BLAD, is a polypeptide which, under normal mammalian metabolism, will be broken down under enzymatic processes in the gastrointestinal tract, enter the amino acid pool and be consumed into normal metabolic processes.

HSE considered the literature review acceptable for the endocrine disruption ecotoxicology assessment. The literature review is described in detail in section Volume 3CA B9.11. As such, the potential effects on endocrine disruption are not considered to be relevant and no further consideration is required.

B.9.2. Effect on aquatic organisms

B.9.2.1. Acute toxicity to fish

Reference:	K-CA 8.2.1/01
Report Title:	Assessment of toxic effects of PROBLAD on Rainbow Trout (<i>Oncorhynchus mykiss</i>) (Teleostei, Salmonidae).
Author(s) & Year:	<div></div> (2011)
Document No	CEV SA, Unpublished report No.: S10-02621
Substance used:	PROBLAD (Batch: 201009, 20% BLAD, 1.30 g/cm ³) CoA: 01/09/2010
Method of analysis:	No analysis conducted
Guideline(s):	OECD Guideline No. 203 (1992)
Deviations:	Yes, see HSE comments
GLP or GEP:	Yes
Acceptability:	Supplementary

Study relied upon:	Yes, used in a reasoned case to support the aquatic risk assessment.
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I. MATERIALS AND METHODS

A. MATERIALS

Test Substance

Test item:	PROBLAD (Batch: 201009, 20% BLAD, 1.30 g/cm ³) CoA: 01/09/2010 (checked by HSE)
Test concentrations:	The test was a range-finding study performed with 0.01, 0.1, 1, 10 and 100 mg PROBLAD/L.

The test item solutions were prepared using test medium. The necessary amount of PROBLAD for preparing the stock solutions was weighed on weighing scoops and transferred to volumetric flasks. Test medium was added up to the mark and solution was homogenised by shaking.

Test item (required) (mg)	Final Volume (mL)	Test Solution (L)	Application (mL)	Nom Conc. test vessel (mg/L)
2500	500	20	400	100
200	500	20	500	10
40	200	20	100	1
	-	20	10	0.1
	-	20	1	0.01
Control -	-	20	0	-

Control:	Untreated test medium
Reference item:	None
Test Organism	Oncorhynchus mykiss (Walbaum) 4-6 cm in size from [REDACTED]

Fish for the test were acclimatised for > 12 days and held under testing conditions in 300 L containers with continuous renewal of water (5-10% per day) and permanent aeration of water. The fish were held under identical conditions for the entire pre-experimental period. Loading of 1 g fish/L.

Water composed of dechlorinated drinking water and deionised water was used as the holding water. The pH value of the aerated water was between 6.0-8.5. The dissolved oxygen was above 60% saturation and the total hardness was between 140-250 mg/L (as CaCO₃). There was a 12-16 h photoperiod daily and the water temperature was 15-17°C. The fish were fed each day with granular rearing food with approx. 2% of their body-weight. 24 h before initiation of the test feeding was stopped. Following a 48-hour settling-in period, mortality was recorded. No mortality above 5% was observed throughout the acclimatisation period.

Test Procedure

Experimental design:	7 animals were tested per concentration with no replicates.
Exposure unit:	25 L stainless steel tanks, filled with 20 L test medium.

Test medium: Dechlorinated drinking water and deionised water with a conductivity of 455 $\mu\text{S}/\text{cm}$.

B. STUDY DESIGN AND METHODS

Study dates: 27/09/2010- 01/10/2010

Environmental Conditions:

Semi-static 96 hour study. Test media was renewed every 24 hours.

Temperature:	15.7°C-16.6°C (required: 10-14°C)
pH:	7.87-8.43 (required: 6.0-8.5)
Lighting:	12-16 h daily
Feeding:	none
Oxygen concentration:	$\geq 89\%$ of air saturation (required: $\geq 60\%$)
Aeration:	Continuous aeration of the test tanks with a membrane pump using a Pasteur pipette

Observations:

The pH value, temperature and O_2 concentration were measured at 24 hour intervals from fresh and/or aged solutions. One control with untreated test medium was used. The test was performed without replication and without a reference item.

Fish were observed at 3, 6, 24, 48 and 96 hours after test start. Fish were considered dead if there was no visible movement (e.g. gill movement), and if touching of the caudal peduncle produced no reaction. Records were made on visible abnormalities as: loss of equilibrium, swimming behaviour, respiratory function, pigmentation and all other observed events. Dead fish were removed if observed and mortality recorded. At termination of the test, all dead and surviving animals were weighed and measured.

Analytics

None performed.

Statistics:

Since no mortality occurred during the test, the estimation of the LC₅₀ value did not require statistical evaluation.

II. RESULTS AND DISCUSSION:

Mortality

No mortality was observed in the control and the concentrations of the test item up to and including 100mg/L in the range-finding test. Therefore no main test was performed.

Table 9.2.1-1: % mortality of fish in the range-finding test

Time (h)	Test concentration (mg/L)					
	Control	0.01	0.1	1	10	100
	Mortality (%)					
3	0	0	0	0	0	0
6	0	0	0	0	0	0
24	0	0	0	0	0	0
48	0	0	0	0	0	0
72	0	0	0	0	0	0
96	0	0	0	0	0	0

Observations

No sub-lethal or lethal effects were observed during the test in the control and up to and including 100 mg/L. At termination of the test, all animals were weighed and measured. The average weight of the test organisms was 0.99 ± 0.18 g; the average length was 49 ± 3 mm. The test organisms were killed immediately at test termination and weighed and measured individually.

Table 9.2.1-2: Size and body weight of the fish at the end of the test

	Nominal concentration of PROBLAD (mg/L)					
	Control	0.01	0.1	1	10	100
	Length (mm)					
Mean	49	49	47	51	50	48
Std Dev	2	1	4	2	2	3
Mean ± SD	49 ± 3					
	Weight (g)					
Mean	0.96	1.03	0.89	1.10	1.03	0.95
Std Dev	0.12	0.15	0.23	0.14	0.17	0.21
Mean ± SD	0.99 ± 0.18					

Evaluation

Since no mortality was observed up to the highest test item concentration of 100 mg/L, the evaluation of the toxicological data did not require the application of statistical methods. The LC₅₀ (96 h) was determined to be above 100 mg/L (nominal).

III. CONCLUSION

According to OECD 203 this study is regarded as valid since: the mortality in the control did not exceed one fish at the end of the test and the dissolved oxygen concentration was at least 60% of the air saturation value throughout the test (Minimum: 89%).

HSE COMMENTS

The submitted study has been produced using the OECD 203 (1992) guidelines. This version is now out of date and HSE has therefore assessed the study against the most recent version of the guidelines, OECD 203 (2019).

Validity criteria:	Achieved:
In the control, the mortality should not exceed 10% at the end of exposure	Yes 0%
The dissolved oxygen should be $\geq 60\%$ of the air saturation value in all test vessels throughout the exposure	Yes (95, 98, 97, 96, 94, 93%)
Analytical measurement of test concentrations is compulsory	No

There is no indication of the age of the fish used in the study. OECD 203 (2019) states that the fish should be juveniles, and be of the same age. The holding period is recommended to be 9 days long with 48 hours settling-in period and 7 days acclimatisation whereas in this study it was reported as > 12 days. The guidelines also states that for a semi-static renewal test, maximum fish loading should be 0.8 g wet weight fish/L, whereas this study has used 1 g wet weight fish/L. The temperature exceeded the required range outlined in the guidelines and there is no indication of the light intensity. It is acknowledged that many of these deviations arise because the study followed the 1992 guideline, which does not have any specific requirements for fish age or light intensity, and has different requirements for acclimatisation, fish loading and temperature.

No analytical assessments were conducted, so the stability of the test item could not be assessed and the exposure of the test organism cannot be confirmed. This means that the OECD 203 (2019) validity criteria have not been met and the reliability of the results is questionable. **As a result, this study will only be used as supporting evidence in the risk assessment.**

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

No toxicity data is available addressing the long-term and chronic risks to fish. However, several published literature studies have been submitted which investigate the inclusion of lupin seeds as a feed supplement in aquaculture, which may be used as supporting evidence in the risk assessment. These literature studies are summarised below with the inclusion of HSE comments on their relevance and reliability.

Reference:	K-CA 8.2.2/01
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Report Title :	Optimal inclusion of lupin and pea protein concentrates in extruded diets for rainbow trout (<i>Oncorhynchus mykiss</i>)
Author(s) & Year :	Zhang et al. (2012a)
Document No :	Aquaculture (2012) 344-349 100–113
Substance used:	Lupin protein concentrates (LPC)- White lupine (<i>Lupinus albus</i>)
Guideline(s):	No, study from published literature
Deviations:	N/A, study from published literature
GLP or GEP:	No, study from published literature
Acceptability:	Supplementary
Study relied upon :	Yes, however data protection not applicable to published literature studies

I. MATERIALS AND METHODS

A. MATERIALS

Test Material	Lupin protein concentrates (LPC) and pea protein concentrates (PPC)
Description:	White lupine (<i>Lupinus albus</i>), produced by dehulling, milling, aqueous extraction of lupine seeds to remove sugars and soluble non-starch polysaccharides, heating and spray drying.
Purity:	100%
Treatments	
Test rates:	Lupin protein concentrates 300 or 500 g plant protein/kg dietary protein mixed with pea protein concentrates(PPC) L/P ratio at 3:0,2:1,1:2 and 0:3
Test animals	
Species:	Rainbow trout (<i>Oncorhynchus mykiss</i>)

Source:	In house culture
Feeding:	LT®Fishmeal
Weight:	58 g trout (mean)

Test Design

Test vessel:	Cylindrical 200-L fibreglass tanks
Test substrate:	Freshwater at a flow rate of 6-7 L/min
Replicates:	Duplicate tanks
Animals/vessel:	40
Test duration:	62 days feeding test, followed by a 20-day digestibility experiment.
Temperature:	8.2°C

B. STUDY DESIGN AND METHODS

This study consisted of a 62-day feeding and subsequent 20-day digestibility experiment.

Ingredients and diets

The Lupin protein concentrates were derived from white lupine (*Lupinus albus*), produced by dehulling, milling, aqueous extraction of lupine seeds to remove sugars and soluble non-starch polysaccharides, heating and spray-drying. Pea protein concentrate was produced from yellow field pea (*Pisum sativum* L.) by dehulling, fine grinding and air classification. A 2x4 inclusion levels of plant protein concentrate were prepared (300 or 500 g plant protein/kg dietary protein) at 4 mixing ratios between EAA-supplemented Lupin protein concentrates and PPC in diets (L/P ratio at 3:0, 2:1, 1:2 and 0:3). The diet with LT® Fishmeal as the sole source of protein (LT fish meal diet) was produced with 538 g crude protein (CP) and 228 g crude lipid (CL)/kg, formulated to keep the same ratio between protein and lipid as the diets with plant protein sources. Yttrium oxide was used as a marker for digestibility measurement.

Fish feeding and sampling

The 62-day feeding experiment was carried out using an indoor recirculation system. Before the feeding experiment started, the fish were deprived of feed for 48 h. A total of 720 juvenile rainbow trout with an average weight of 58 g were randomly assigned to 18 cylindrical 200-L fibreglass tanks, forty fish per tank. Each tank was supplied with freshwater at a flow rate of 6-7 L/min, and constant light was maintained. During the feeding

experiment, water temperature ranged from 7.9 to 8.8°C, with a mean of 8.2°C. Dissolved oxygen levels were above 6.0 mg/L in the outlet water, based on daily measurements. Each type of diet was fed to fish in duplicate tanks, and the trout were fed three times per day.

Fish were fed 10% in excess, based on average feed intake over the previous 3-day period. Before the start of the experiment, 2 × 5 fish from the holding tank were euthanized with an overdose of MS-222, and stored at -20°C for whole body analysis. Fish were anaesthetised with MS-222 (90 mg/L) and batch-weighed in the beginning (Day 0) and the middle (Day 31) of the experiment. At the end of feeding experiment, five fish were randomly sampled from each tank for blood and tissue samples for histology. The fish were weighed individually, and blood was collected from the caudal vein with heparinised vacutainers, kept on ice until centrifuged at 3000g for 10 min then frozen in liquid N₂ and kept at -80°C until analysis.

The intact gastrointestinal (GI) tracts of the same fish were removed. All the intestinal sections were opened longitudinally, and tissue samples of 1 cm² were fixed in phosphate buffered formalin (4%; pH 7.4) for 24 h, and then transferred to 70% ethanol for storage until processing. The liver was also removed from the same fish, weighed, and 1 cm² × 0.5 cm histological samples were taken following the same procedure. Another 10 fish were taken from each tank, weighed individually, dissected, and eviscerated. Surface fat and connective tissue were carefully removed. The contents (digesta) from the PR, MI and DI sections were collected in Eppendorf tubes separately, frozen in liquid N₂ and kept at -80°C for bile acid and trypsin activity analysis, brush border leucine amino peptidase and maltase activities. Three fish were taken from each tank, weighed individually, killed by a blow to the head, cut open to remove the intestinal contents, and stored at -20°C for whole body analysis. The remaining 22 fish in each tank were batch-weighed and kept for 3 lots of faeces stripping. At each time point, fish were fed using the same procedure as in the feeding experiment for 6 d, then anaesthetized by MS-222, and stripped for faeces by the method of Austreng (1978). All the faecal samples from the same tank were pooled and stored at -20°C prior to analysis. Only the samples from the fish fed the LT fish meal diet and the diets with 500 g plant protein/kg inclusion were measured for trypsin activity and bile acid concentration in the intestinal contents and brush border enzyme activities. Only the samples from the fish fed the LT fish meal diet and the diets with the highest inclusion of Lupin protein concentrates were used for histological evaluation.

Calculations and statistical analysis

The results were analysed using the GLM procedure of SAS statistical software (SAS, 1990). One-way analysis of variance (ANOVA) was used to compare effects of the LT fish meal diet with those of the plant protein diets. Factorial ANOVA used to analyse the effects of plant protein inclusion level and L/P ratio. Significant ($P < 0.05$) interactions between inclusion level and L/P ratio were rationalized by regression analysis of $L/(L+P)$ within inclusion level, if at least one of the main effects were significant (Snedecor and Cochran, 1967). The results were expressed as the means and pooled standard errors of means

(S.E.M). Duncan's multiple-range test was used to rank significant differences among diets in the one way ANOVA and main effects in the factorial ANOVA.

Analytical method

The initial and final whole body samples were homogenised with dry ice (CO₂) in a food processor and freeze-dried. Pooled faeces samples were freeze-dried and ground with a pestle and mortar. Fish scales were removed prior to analysis. Feed ingredients, feeds, and freeze dried faeces samples were analysed for dry matter (EU 71/393), Kjeldahl nitrogen (N) (EU 93/28), lipid (HCl hydrolysis and diethylether extraction (EU 98/64)), ash (EU 71/250) and minerals (ICP-AES/ICP-MS) (Nordic Committee on Food Analysis (NMKL) method 161) and starch (AOAC enzymatic method 996.11).

Freeze-dried whole body samples were analysed for proximate composition using the same methods, except that no HCl hydrolysis was employed. Gross energy was measured by bomb calorimetry (Parr 1271 Bomb calorimeter, Parr, Moline, IL, USA). Amino acid (except tryptophan) analysis of all samples was according to EC (1998) on a Biochrom 30 amino acid analyser (Biochrom Ltd., Cambridge, UK). Tryptophan was analysed according to EC (2000) on a Dionex Summit HPLC system, with a Shimadzu RF-535 fluorescence detector. Yttrium oxide concentration in feed and faeces was determined by inductively coupled plasma mass spectroscopy (ICPMS) after complete digestion of the homogenised and dried samples in HNO₃ after cooking in a microwave oven for 1 h.

Plasma cholesterol and tri-acylglycerols were analysed by Cobas® Integra enzyme kits and automatic analyser equipment (Cobas Mira, Hoffman-La Roche & Co., Basel, Switzerland). Bile acids in the digesta samples were analysed by enzymatic assay (Bio-Stat Ltd, Stockport, UK). Activities of brush border membrane bound leucine amino peptidase (LAP) and maltase were analysed as described by Krogdahl et al. (2003). Trypsin activity of digesta samples was determined colorimetrically according to methods described by Kakade et al. (1973) and Krogdahl et al. (2003), using the substrate Na-benzoyl-dl-arginine-p-nitroanilide (BAPNA) (Sigma no. B-4875; Sigma Chemical Co., St. Louis, MO, USA), and a curve generated from a standardized bovine trypsin solution.

Histological evaluation

Processing of the histological tissues was done at the Section for Anatomy and Pathology of the Norwegian School of Veterinary Science (Oslo, Norway) using standard histological techniques. The MI was sectioned transversely, whereas the DI sample was sectioned longitudinally (i.e. perpendicular to the macroscopically complex folds; approximately 5µm thick) and stained with haematoxylin and eosin (HE). Blind histological examination was performed using light microscopy. Two independent evaluations were performed. Tissue morphology was evaluated according to the descriptions of Amin et al. (1992) and Baeverfjord and Krogdahl (1996).

II. RESULTS AND DISCUSSION

Extrusion parameters and physical pellet quality

Mainly, feeding rate and water addition to the preconditioner were the two adjustable parameters used to optimise bulk density. Consequently, torque and specific mechanical energy (SME) varied among the diets. Overall more water was needed for the diets with 500 g plant protein/kg dietary protein compared to 300 g/kg, and the water addition was slightly increased with the proportion of Lupin protein concentrates in diet. Least water was added to the process when the LT fish meal diet was extruded and this diet also generated lower SME and torque compared to the diets with high content of PPC. For the diets with 300 g plant protein/kg dietary protein inclusion, a linear increase of durability with the increasing PPC was observed, while for the diets with 500 g protein/kg dietary protein inclusion, such effects on durability was not as pronounced. Durability of the pellets was mainly associated with torque. The effects of inclusion level and L/P ratio on the breaking point showed the same trend as the durability, but less distinct. Expansion ratio ranged between 39 and 52% among the diets and was affected by ingredient composition and extruder parameters.

Growth and feed utilisation

No mortality occurred, and all of the fish had high feed intake and grew well. Average weight gain was 210% and the average feed conversion ratio was 0.72 g DM ingested (g gain)⁻¹. Both during the first 30 days and over the whole period, FI and weight gain did not differ significantly among diets. Neither inclusion level of plant protein sources nor the ratio between Lupin protein concentrates and PPC (L/P ratio) caused significant differences in FI or weight gain. The feed conversion ratio of trout fed the LT fish meal diet was not significantly different from that of the fish fed the LLP3, LLP4, HLP2, or HLP 4 diets during the first 30 days and the LLP4 diet for the whole experiment. All other diets resulted in significantly higher feed conversion ratio than the LT fish meal diet.

Trout fed the diets with 500 g plant protein/kg dietary protein inclusion had significantly higher feed conversion ratio than those fed the diets with 300 g/kg inclusion. The main effects of L/P ratio were significant, indicating higher feed conversion ratio for the diets with the most Lupin protein concentrates (L/P ratio = 3:0). Significant interaction between plant protein inclusion level and L/P ratio for feed conversion ratio was seen for the first 30 days of feeding. The interaction is rationalised by a quadratic response at the inclusion level of 300 g plant protein/kg dietary protein, where the feed conversion ratio levelled off at higher L/P ratios. The response at the inclusion level of 500 g/kg was quadratic also, but the feed conversion ratio value was significantly increased for the highest L/P ratio.

Digestibility

The apparent digestibility coefficient (ADC) of crude protein of fish fed the FM diet was lower ($P < 0.001$) than that of fish fed the LLP4, HLP3 and HLP4 diets, but not significantly different from that of fish fed the other diets. The ADC of lipid of fish fed the FM diet was significantly lower than that of fish fed the LLP4, HLP1 and HLP2 diets. The ADC of starch and energy of fish fed the FM diet were not significantly different from that of fish fed the

LLP4 and HLP4 diets, but significantly higher than that of fish fed the other diets. The ADC of crude protein and lipid were significantly higher for the 500 g plant protein/kg dietary protein inclusion level than for the 300 g/kg. The ADC of energy was not affected by inclusion level. The diets with most PPC (L/P ratio = 0:3) resulted in significantly higher ADC of crude protein and energy than the diets with less PPC. The diets with most Lupin protein concentrates (L/P ratio = 3:0) gave a significantly higher ADC of lipid than that of the diets with L/P ratio of 1:2, but did not differ from the other treatments. A significant interaction between plant protein inclusion level and L/P ratio for ADC of lipid was seen. At high plant protein inclusion level, the ADC of lipid increased with the increasing L/P ratio, while this was not significant at the low inclusion level.

The ADC of starch was significantly higher at 300 g plant protein/kg dietary protein inclusion than at 500 g/kg. A significant interaction between inclusion level and L/P ratio for ADC of starch was seen where the ADC of starch declined at a greater rate at 500 g plant protein/kg dietary protein inclusion than at 300 g/kg. The ADC of starch decreased in a linear manner from more than 97% to 86% with increasing levels of Lupin protein concentrates in the diets. There was a significant negative relationship between the analytical residue and ADC of starch for both the 300 and 500 g plant protein/kg dietary protein inclusion levels.

Compared to the FM diet, the EAA Arg, Lys, Met and Phe were significantly more efficiently digested in the plant protein diets. Also Trp was more efficiently digested in the plant protein diets, except for the LLP2 and LLP3 diets. The same was found for Thr, except for the LLP3 and HLP3 diets and for Val, except for the LLP3, HLP2 and HLP3 diets. Leu was more efficiently digested in the LLP1, LLP4, HLP1 and HLP4 diets, Ile in the LLP1, LLP4 and HLP4 diets, and His in the LLP4 and HLP4 diets. All plant protein diets had similar or higher EAA (including Cys) digestibility than the FM diet.

Higher inclusion of plant protein in the diets resulted in significantly lower ADC of His, but higher ADC of Met and Trp. The ratio between Lupin protein concentrates and PPC significantly affected the ADC of His, Ile, Lys, Met, Phe, Trp, Thr, and Cys. The diets with most PPC (L/P ratio=0:3) resulted in significantly higher ADC of His than the diets with less PPC, and significantly higher ADC of Ile, Lys, Phe, Trp, and Thr than the diet with L/P ratio of 1:2. The diets with most Lupin protein concentrates (L/P ratio=3:0) resulted in significantly higher ADC of Met, Phe and Trp than treatments with L/P ratio of 1:2. The diets with L/P ratio of 1:2 resulted in significantly lower ADC of Cys than other diets. No interaction between inclusion level and L/P ratio was found for EAA and Cys digestibility. Regression analysis did not reveal any consistent relationships between ADC of Cys and any of the extrusion or physical quality parameters.

Body composition, nutrient retention and metabolic loss

No significant differences were found in whole body composition among diets. Neither the inclusion level of plant protein nor the L/P ratio resulted in significant differences in whole body composition except for ash, which was significantly lower in the fish fed the diets with 500 g plant protein/kg crude protein than with 300 g/kg.

Retention of both ingested and digested nitrogen of the trout fed the FM diet were significantly lower than for those fed the plant protein diets, except for the ingested nitrogen retention of those fed the LLP3 and HLP1 diets and the digested nitrogen retention of those fed the LLP3, HLP1, and HLP4 diets. Retentions of ingested and digested energy did not significantly differ among diets. The inclusion level of plant protein sources and the L/P ratio did not significantly affect the retention of ingested or digested nitrogen or energy. The FM diet resulted in higher metabolic loss of nitrogen. The metabolic loss of nitrogen was higher in trout fed the FM diet than any of the dietary treatments with 300 g plant protein/kg dietary protein, and the fish fed the HLP2 and HLP3 diets. The metabolic loss of nitrogen was higher for the 500 g plant protein/kg dietary protein inclusion level than for the 300 g/kg. A significant interaction between inclusion level and L/P ratio for metabolic nitrogen loss was found. This was rationalized by a parabolic effect of L/P ratio at the high inclusion, while no significant effect was seen at low inclusion. The EAA profile of whole body samples were evaluated only in fish fed the HLP1, HLP4, and FM diets. The whole body EAA profile of the initial and final samples were similar, and also close to the profile of LT fish meal, except for the level of His which was lower in LT fish meal. In each treatment, the retentions of digestible Arg and Trp were lowest and His was highest among all EAAs. The retentions of digestible Arg and Phe in trout fed the FM diet were significantly higher than that of trout fed HLP1 and HLP4 diets.

Plasma triacylglycerol level did not differ significantly among dietary treatments. Plasma cholesterol level was significantly higher in trout fed the FM diet than in trout fed the plant protein diets. Inclusion of 500 g plant protein/kg dietary protein resulted in significantly higher triacylglycerols level, but lower cholesterol level than inclusion of 300 g/kg. The dietary treatments with L/P ratio = 3:0 resulted in significantly lower plasma cholesterol level than L/P = 2:1 and L/P = 0:3, while L/P = 1:2 was intermediate.

Generally, both trypsin activity and bile acid concentration in intestinal content decreased throughout the intestine. Only the trypsin activity in the content of mid-intestine was significantly affected by L/P ratio, and fish fed the HLP1 diet had the lowest and those fed the HLP3 diet had the highest value. Digestibility of tryptophan decreased with increasing trypsin activity (TA) in the mid-intestine up to a TA of 250 U (mg DM)⁻¹, and levelled out at higher TA levels. The bile acid concentration in the contents of different intestinal sections was not affected by the L/P ratio. Leucine aminopeptidase (LAP) activity in the intestinal tissue was not significantly affected by the L/P ratio. No significant differences were seen for maltase activity (MA) in MI by ANOVA. A significant quadratic decrease in MA with increasing L/P ratio (LPR, expressed by L/(L+P)) ($MA = 4 \times 10^{-4} LPR^2 + 7.94 \times 10^{-2} LPR + 15.675$, $R^2 = 0.58$) was seen. Significant differences were seen for MA in DI, where the FM diet resulted in a lower value than the HLP2 diet. In contrast to what was found in MI, MA in DI increased with increasing L/P ratio ($MA = -1 \times 10^{-3} LPR^2 + 1.08 \times 10^{-2} LPR + 31.916$, $R^2 = 0.76$).

Histology of liver and intestine

No obvious histological differences were found in the MI and DI in trout fed the FM or the Lupin protein concentrates diet. No inflammation was observed in trout fed the PPC diet, but a slight decrease in mucosal fold height and a slight increase in fold fusion were seen. Hepatosomatic Index (HSI) was significantly higher in trout the FM diet than in trout fed the HLP1 and HLP4 diets. Only the trout fed the HLP1 diet, however, had lower hepatocyte vacuolization compared to those fed the FM and HLP 4 diets.

III. CONCLUSION

The present experiment has demonstrated the potentials of lupin protein concentrates and pea protein concentrates as major protein sources in extruded diets for rainbow trout. This growth rate of rainbow trout achieved in this study is comparable to the growth in similar previous studies. The rapid growth achieved could be attributed to both high feed intake and efficient feed conversion. The high intake and absence of significant differences among diets in the present experiment are consistent with previous work with lupin protein concentrates (LPC) or pea protein concentrates (PPC) used individually in diets for rainbow trout.

The high feed intake in the present experiment demonstrated that neither the level of saponins in pea protein concentrates (PPC) nor alkaloids in lupin proteins concentrates (LPC) negatively affected feed intake of rainbow trout, even at inclusion levels as high as 407 g PPC or 439 g LPC/kg diet.

Both lupin and pea protein concentrates were shown to be useful dietary protein sources for rainbow trout. Pea protein concentrates had a higher nutritional value than lupin protein concentrates, mainly due to the lower non-starch polysaccharide content. The results suggested that any combination of LPC and PPC with EAA supplementation can be efficiently used when total plant protein inclusion is limited to 300 g/kg crude protein in extruded diets for rainbow trout. At higher inclusion level, PPC seemed to be the preferable source of protein. This indicates that the benefit of mixing PPC and LPC may be limited at high inclusion level.

HSE COMMENTS

This study is from the scientific literature and does not conform to standardised test guidelines, but is GLP compliant. The study's methodology, reporting and analysis have been considered with regard to how well they meet common standards of scientific practice, based on 'expert judgement'.

The study appears to be well conducted in accordance with scientific principles, although details on the batch of test item and its stability during the test were lacking. *O. mykiss* is a standard species for ecotoxicity testing. The measurements on pellet composition, feed utilisation, digestibility, nutrient retention, and histology are not relevant for use in the risk assessment. Additionally, exposure was via the diet so the results are of limited relevance in the aquatic risk assessment, which considers exposure via a concentration in water. However, the study does indicate that inclusion of white lupin protein concentrates at up to

439 g LPC/kg diet, over a 82 day period, does not negatively affect growth of rainbow trout. The results may be considered as supporting evidence in the risk assessment.

Relevance: Not relevant (dietary route of exposure)

Reliability: Reliable with reservations

(Studies are graded as one of the following categories: 1) Reliable, 2) Reliable with reservations, 3) Unreliable. Categorisation is based on the evaluator's subjective assessment of the quality of the methods, reporting and analysis as presented in the study.)

Reference :	K-CA 8.2.2/02.
Report Title :	Criteria for safe use of plant ingredients in diets for aqua-cultured fish (Opinion of the Panel on Animal Feed of the Norwegian Scientific Committee for Food Safety) Note that the present summary includes details on lupins. The paper also analyses the effect of the use of soybean, pea, canola, sunflower, cotton seed, wheat gluten, potato protein
Author(s) & Year :	Hemre et al., (2009)
Document No :	1365-2109 in Aquaculture Research, 2010, 1-12
Substance used :	Proteins in kernel meals of dehulled white (<i>Lupinus albus</i>), sweet (<i>L. angustifolius</i>) and yellow (<i>L. luteus</i>) lupines
Method of analysis :	No analysis conducted
Guideline(s) :	No, study from published literature
Deviations :	N/A, study from published literature
GLP or GEP :	No, study from published literature
Acceptability :	Supplementary
Study relied upon :	Yes, however data protection not applicable to published literature studies

Utilisation of plant protein resources by fish.

As the use of fishmeal in the aquaculture industry decreases for various reasons, alternative, more cost-effective feedstuffs are being increasingly used as protein sources in formulated feeds for farmed fish. Various sources have been attempted from plant,

microbial, and other animal sources. However, some of these alternative feed ingredients have been reported to have negative consequences on growth and feed utilization of farmed fish, depending on fish species and inclusion level in their diets. The long-term implications of alternative protein source may have on fish production, health, and product quality are questionable, and this topic deserves continued, substantial investments in further research to preserve and enhance the sustainability of the aquaculture industry.

The main challenges in using plant protein sources in diets for carnivorous fish lie in their often lower levels of protein and high levels of starch, unfavourable amino acid and mineral profiles, high levels of fibre and the presence of anti-nutritional factors (ANFs) and/or antigens. Especially the long-term implications they may have on fish production, health, and product quality are largely unknown.

Today various plant-derived ingredients are used in fish feed. Wheat is the most widely used ingredient, and is used as a carbohydrate source with the currently most utilised feed technology (extrusion). As a protein ingredient, among others, the following have been used: soybean (with varying degrees of processing), maize, peas, lupine, and broad beans.

As demonstrated in studies on several fish species, the proteins in kernel meals of dehulled white (*Lupinus albus*), sweet (*L. angustifolius*) and yellow (*L. luteus*) lupines are highly digestible. It has been estimated that sweet lupine kernel meal may be included in diets for rainbow trout up to 30 or 40% of total diet without significantly influencing growth and nutrient utilisation. No histological changes were observed in the pyloric caeca or distal intestine of rainbow trout fed up to 50% yellow lupine kernel meal (of total diet), although hepatocytes appeared to have a lower level of lipid droplets in the fish fed the 50% inclusion level. Nor were histological changes observed in the distal intestine of Atlantic salmon fed 24% of total diet of dehulled, low-alkaloid white lupine meal, or 30% of total diet of kernel meals or protein concentrates from yellow lupine or sweet lupine. In the stomachs of these fish, however, higher severity of ulcer-like lesions connected with the lupine-containing diets was reported.

Table 9.2.2-1: Important anti-nutrients present in some commonly used alternative fish feed ingredients (Francis et al., 2001a)

Plant feed-stuff	Anti-nutrient present
Soybean meal	Proteinase inhibitors, lectins, phytic acid, saponins, phytoestrogens, antivitamins, phytosterols, allergens
Kidney beans	Proteinase, amylase and lipase inhibitors, lectins, phytic acid, saponins, phytoestrogens, antivitamins, phytosterols, allergens
Rapeseed meal	Proteinase inhibitors, glucosinolates, phytic acid, tannins
Lupin seed meal	Proteinase inhibitors, saponins, phytoestrogens, alkaloids
Pea seed meal	Proteinase inhibitors, lectins, tannins, cyanogens, phytic acid, saponins, antivitamins
Sunflower oil cake	Proteinase inhibitors, saponins, arginase inhibitor

Cotton seed meal	Phytic acid, phytoestrogens, gossypol, antivitamin, cyclopropenoid acid
Alfalfa leaf meal	Proteinase inhibitors, saponins, phytoestrogens, antivitamin
Mustard oil cake	Glucosinolates, tannins
Sesame meal	Phytic acid, proteinase inhibitors

The following components can be found in lupin seed meals:

Proteinase inhibitors

In the intestine, proteinase inhibitors first form a rather stable complex with trypsin, thereby reducing trypsin activity. This in turn stimulates secretion of PZ-CCK (Pancreozymin Cholecystokinin) from the gut wall. This hormone stimulates secretion of trypsin from pancreatic tissue and stimulates the gall bladder to empty its content into the intestine. In some animals, proteinase inhibitors cause pancreatic hypertrophy. Whether this also takes place in fish is not clear.

In studies with salmonids, proteinase inhibitors have been found to reduce apparent digestibility not only of protein but also of lipid (Berg-Lea et al., 1989; Krogh et al., 1994; Olli et al., 1994). The effects on digestibility correspond to a decrease in trypsin activity and presumably chymotrypsin, which is also inhibited by soybean proteinase inhibitors. Saturated fatty acids appear more severely affected than unsaturated.

The results indicate that the proteinase inhibitors stimulate pancreatic enzyme secretion, causing the enzyme level of the intestinal content (trypsin protein) to increase. However, the activity in the intestinal content is not increased. The enzyme activity seems unaffected when fed diets with the lower inhibitor levels and short-term feeding, but higher levels decrease the activity. After longer-term feeding it may seem that the pancreas no longer manages to compensate for decreased enzyme activity by increasing secretion. Thus enzyme production does not appear to keep up with the increased demand.

Saponins

Saponins are amphipathic glycosides that disrupt cell membranes and can have antimicrobial, immune-stimulatory, glucocorticoid and antioxidant activities. They inhibit protein and lipid digestion, vitamin absorption, and cholesterol metabolism. The involvement of saponins from soybeans in an inflammatory response in the distal intestine of salmon has recently been indicated, albeit not alone but in combination with unidentified components found in lupin.

Phytoestrogens/-sterols

The research on effects of phytoestrogens/-sterols in farmed fish has focused on reproductive parameters. Little is known about other physiological responses following dietary intake of phytoestrogens in any fish. Glucosinolates/goitrogens are a group of substances that disrupt thyroid hormone production, partially by interfering with iodine

availability. They also cause reduced palatability and thus reduced growth, as well as affect liver and kidney functions.

Quinolizidine alkaloids

Quinolizidine alkaloids, such as lupinine, gramine and sparteine in lupin meals, are toxins that inhibit motor coordination and muscular control in mammals. Data regarding this or other anti-nutritional activities are lacking in fish, but the bitter taste may be responsible for reduced feed intake in rainbow trout and Atlantic salmon. Tolerance levels for these fish maybe between 100 and 500 mg/kg. Tolerance levels for cod and halibut are unknown as are long term-effects for any fish species.

Antinutritional factors(ANFs)

Combined effects of antinutritional factors (ANFs) have not been extensively studied in any animals. Tannins in combination with lectins, cyanogenic glycosides and saponins appear to reduce the deleterious effects of the individual ANFs. However, additive interactions with deleterious effects on intestinal function or structure have been reported for saponin and lectin on rabbit tissue in vitro, soybean lectin and protease inhibitor on Atlantic salmon intestinal tissue in vitro, and saponin and unidentified component(s) of lupin meal in Atlantic salmon distal intestine in vivo. Thus there is a need to test combinations of plant ingredients and ANFs on a case-by-case basis to assess any potential consequences to fish health.

III. CONCLUSION

With the exception of full-fat and extracted soybean meal for salmonids, substituting at least part of the fish meal fraction of aqua-feeds with individual plant ingredients, such as lupin, seems promising, at least in the short to medium term. Indeed in some cases, diets containing up to 20% of high quality plant protein sources resulted in better nutrient digestibility and growth parameters than the fishmeal-based control diets. This reveals that not all fish meals are of optimal nutritional quality. When substituting fishmeal with plant ingredients, however, it may be necessary to adjust the diets regarding limiting amino acids and minerals.

Information regarding anti-nutritional factors in the plant ingredients and feeds containing them is rarely given in publications reporting data from feeding trials. Combining various plant ingredients in feeds, thereby limiting the concentration of anti-nutritional factors/antigens inherent in single ingredients, has promise regarding complete substitution of fishmeal.

However, each blend of plant ingredients needs to be thoroughly tested in order to rule out adverse effects caused by combinations of anti-nutritional factors and antigens. Long term studies of metabolic and health aspects of individual plant ingredients as well as various mixes in aqua-feeds are needed for all species. Also, little knowledge exists regarding the consequences of adding various plant ingredients to diets of juveniles for the various fish species and effects on brood-stock and subsequent reproductive parameters.

Knowledge regarding responses to various qualities/processing methods of soybean meal in feeds for salmonids as well as to various extracts of and/or purified anti-nutritional ERM factors in soybeans has resulted in some insights in anti-nutritional factors effects and their possible role in the SBM-induced enteritis.

Saponins, non-starch poly-/oligosaccharides, phytoestrogens, phytosterols and/or antigenic peptides may potentially have a role in inducing the inflammation. However, it cannot be ruled out that as-yet unidentified components as well as the gut microbiota may be involved.

Various processing measures presently employed in feed manufacturing may decrease the activity or concentration of individual anti-nutritional factors. However, recent findings suggest combinations of various anti-nutritional factors, may have particular significance in causing adverse effects to intestinal structure, function and defence mechanisms at lower levels than what the individual anti-nutritional factors would elicit.

HSE COMMENTS

This summary does not conform to standardised test guidelines and is not GLP compliant. The summary’s discussion has been considered with regard to how well it meet common standards of scientific practice, based on ‘expert judgement’.

The above summary is not a supporting data study and instead comprises a summary of alternative plant ingredients for use in diets of aqua-cultured fish. The summaries provided for all other alternative plant ingredients except lupines are not relevant for use in the risk assessment. The summary for dehulled white, sweet and yellow lupines cannot be used in the risk assessment as no data is presented, but may be used as supportive information. It is shown that meals containing lupines can positively influence growth of aqua-cultured fish such as Atlantic salmon (*Salmo salar*), rainbow trout (*Onchorhyncus mykiss*), Atlantic halibut (*Hippoglossus hippoglossus*) and Atlantic cod (*Gadus morhua*).

Relevance : Not relevant (dietary route of exposure)

Reliability : Reliable with reservations

(Studies are graded as one of the following categories: 1) Reliable, 2) Reliable with reservations, 3) Unreliable. Categorisation is based on the evaluator’s subjective assessment of the quality of the methods, reporting and analysis as presented in the study.)

Reference:	K-CA 8.2.2/03
Report Title:	Effect of lupin kernel meal as plant protein source in diets of Red hybrid tilapia (<i>Oreochromis niloticus</i> x <i>O. mossambicus</i>) on growth performance and nutrients utilisation

Author(s) & Year:	Yones, A. M. (2010)
Document No :	African J. Biol. Sci., 6 (1): 1-16 (2010) ISSN 1687-4870
Substance used:	<i>Lupinus albus</i> seeds ‘lupin kernel meal’ 100% purity- no batch number provided
Method of analysis :	No analysis conducted
Guideline(s):	No, study from published literature
Deviations:	N/A, study from published literature
GLP or GEP:	No, study from published literature
Acceptability:	Supplementary
Study relied upon :	Yes, however data protection not applicable to published literature studies

I. MATERIALS AND METHODS

A. MATERIALS

Test Material	<i>Lupinus albus</i>
Description:	Seeds “lupin kernel meal”
Purity:	100%
Test rates:	0, 25, 50 or 60% lupin seed meal

Test animals

Species:	Red hybrid tilapia (<i>Oreochromis</i> sp.)
Source:	Kelo 21 hatchery, General Authority for fish resource development, Alexandria road, Egypt

B. STUDY DESIGN AND METHODS

The study was conducted using the research facilities of Shakshouk fish Research Station, Fayoum (NIOF) Governorate. Red hybrid tilapia (*Oreochromis* sp.) fingerlings were

obtained from the Kelo 21 hatchery belonging to General Authority for fish resource development, Alexandria road, Egypt.

The experimental treatments were triplicated and fingerlings were randomly distributed and stocked at 50 fingerling per each fibre glass tank (1 m^3) with initial average weight of $2.62 \pm 0.08 \text{ g/fingerling}$. Water was supplied in a flow through filter system. Before starting the experiment fish were fed the experimental diets during an acclimatisation period for two weeks. The experimental period lasted 120 days. Physicochemical characteristics of water tanks were examined every week.

Four iso-nitrogenous diets were formulated to contain an average of 30.12 ± 0.06 crude protein percentage. The diets were given at a rate of 3% of live body weight (BW) twice daily at 10.00 am and 16.00 pm. Four experimental diets were formulated; the control diet (C) was formulated without lupin meal, where only animal source protein was used. Diets L25, L50 and L60 were formulated by partial inclusion of 25, 50 and 60 g per 100 g extruded lupin meal to replace fish meal, respectively. All diets were processed into dry sinking pellet, using California pelleting machine with 3 mm diameter.

At the start and end of the experiment, a number of 10 fish were killed, decapitated, homogenised in a blender, stored in polyethylene bags, frozen and then dried for subsequent protein (Kjeldahl), ether extract (Soxhlette) and moisture analysis. The following parameters were analysed/calculated in the Lupin kernel meal: protein levels, amino acid composition, Nitrogen free extract, gross energy, Tryptophan content, fatty acids, total alkaloids using (after appropriate extractions) HPLC, hydrolysis, GC, gravimetry. Fish were analysed for growth performance, feed utilization in terms of final individual fish weight (g), final weight gain (g), average daily gain (g/fish/day), specific growth rate (SGR%), feed conversion ratio (FCR), protein efficiency ratio (PER) and protein productive value (PPV) using the appropriate analytical methods and calculations.

Fish were fed Chromium(III)-oxide (Cr_2O_3) (starting 10 days before the end of the experimental phase) in order to study the apparent digestibility coefficient (ADC %) of nutrients. Chromium(III)-oxide levels were determined by atomic absorption spectrometry from faecal samples collected daily.

Blood samples were withdrawn from the caudal vein with heparinised syringes. Haematocrit percentage and haemoglobin, plasma protein, glucose, triglyceride and cholesterol were determined.

III. RESULTS AND DISCUSSION

Water physicochemical characteristics revealed that temperature, pH, dissolved oxygen, salinity and unionised ammonia are within the optimum ranges for *Oreochromis* sp. in all tanks.

The lupin kernel meal showed good protein content (40.0%), high fat content (10%), high fibre content (11.1%) and allowable ash content 5.48%, while nitrogen free extract recorded

moderate value (33.2%). However, total alkaloid determined on dry matter basis in the present study was 0.22 (g/kg dry matter). The amino acid experimental diet were almost similar in protein content (30.03-30.22%), while fat, nitrogen free extract, fibre and ash differed slightly in the experimental diet.

Table 9.2.2-2: Lupin kernel meal composition (g/100 g)

Chemical composition	Lupin kernel meal (% on dry matter basis)
Crude protein	40
Crude fat	10
Nitrogen free extract	33.2
Crude fibre	11.1
Ash	5.48
Total alkaloid (g/kg)	0.22

Table 9.2.2-3: Experimental diets composition (g/100 g)

Ingredients (g/100 g)	Control	L25	L50	L60
Crude protein	30.1	30.03	30.14	30.22
Crude fat (ether extract)	11.22	11.95	10.58	10.51
Nitrogen free extract	44.26	44.42	45.34	44.12
Crude fibre	6.5	6.79	7.95	9.56
Ash	7.92	6.83	8.99	5.59

Growth performance

The highest performances in growth were obtained with fish fed L50 diet followed by that fed control and L25 diets, respectively. However, the lowest performance parameters were recorded with fish fed L60 diet. It was observed that feed consumed was increased with increasing lupin kernel meal level in the diet.

Table 9.2.2-4: Average growth performance parameters of *Oreochromis* sp. fed the experimental diets.

Ingredients (g/100 g)	Control	L25	L50	L60
Initial weight	2.5	2.7	2.4	2.6
Final weight	45.8	42.6	47.5	36.2
Gain	43.3	39.9	45.1	33.6
Feed consumed	58	58	59	63
Average daily gain	0.36	0.33	0.37	0.28
Specific growth rate	2.42	2.30	2.49	2.19
Feed conversion ratio	1.33	1.45	1.30	1.87
Protein efficiency ratio	2.42	2.29	2.53	1.76
Protein productive value	39.17	36.19	39.53	26.90

Furthermore, protein productive value (PPV) revealed significant ($P < 0.05$) differences between treatments, where fish fed L50 involved diet showed the highest PPV followed with the fed control and L25 involved diets, respectively and the lowest PPV was recorded with fish fed L60 diet.

Apparent digestibility coefficient

No significant differences ($P < 0.05$) among dietary treatments could be attributed to the partial inclusion of extruded Lupin up to 50% in fish diets.

Table 9.2.2-5: Average growth performance parameters of *Oreochromis* sp. fed the experimental diets.

Nutrient	Control	L25	L50	L60
Dry matter	86.5	85.4	86.2	81.0
Protein	95.6	95.2	95.2	90.0
Fat	96.0	95.8	96.6	95.0
Nitrogen free extract	77.0	70.2	73.6	68.3

Blood contents

The blood contents, (haematocrit and haemoglobin) and plasma contents of Protein; Glucose, Triglyceride and Cholesterol levels were not significantly ($P < 0.05$) different with lupin kernel meal inclusion compared to the control groups.

Table 9.2.2-6: Haematocrit, haemoglobin and some plasma contents of *Oreochromis* sp. fed the experimental diets

Parameters	Control	L25	L50	L60
Haematocrit (%)	30.0	29.2	29.4	29.2
Haemoglobin (103 mg/L)	83	81	82	81
Protein (g/L)	43.4	44.2	44.3	44.6
Glucose (mmol/L)	4.3	4.4	4.5	4.6
Triglyceride (mmol/L)	4.6	4.5	4.4	4.2
Cholesterol (mL mol/L)	7.2	7.3	7.3	7.4

III. CONCLUSION:

The results of the present trial indicated that the 50% replacement level of extruded lupin kernel meal is promising in feeding of *Oreochromis* sp. It was demonstrated that extruded lupin kernel meal can be used without adversely effects on growth performance, digestibility coefficient and blood characteristics of *Oreochromis* sp. fingerlings.

HSE COMMENTS:

This study is from the scientific literature and does not conform to standardised test guidelines and is not GLP compliant. The study's methodology, reporting and analysis have been considered with regard to how well they meet common standards of scientific practice, based on 'expert judgement'.

The study appears to be well conducted in accordance with scientific principles, although details on the batch of test item and its stability during the test were lacking. The test species also did not conform to the standardised test guidelines. The measurements on meal composition and blood contents are not relevant for use in the risk assessment. Additionally, exposure was via the diet so the results are of limited relevance to the aquatic risk assessment, which considers exposure via a concentration in water. However, the study does indicate that inclusion of white lupin seeds at up to 50% in the diet, over a 120 day period, does not negatively affect growth of *Oreochromis* sp. fingerlings. The results may be considered as supporting evidence in the risk assessment.

Relevance: Not relevant (dietary route of exposure)

Reliability: Reliable with reservations

(Studies are graded as one of the following categories: 1) Reliable, 2) Reliable with reservations, 3) Unreliable. Categorisation is based on the evaluator’s subjective assessment of the quality of the methods, reporting and analysis as presented in the study.)

Reference :	K-CA 8.2.2/04.
Report Title :	Evaluation of the potential of Andean lupin meal (<i>Lupinus mutabilis</i> Sweet) as an alternative to fish meal in juvenile <i>Litopenaeus vannamei</i> diets.
Author(s) & Year :	Molina-Poveda, C., Lucas, M., and Jover, M. (2013)
Document No :	Aquaculture 410-411 (2013) 148-156
Substance used :	Andean lupin meal (<i>Lupinus mutabilis</i> Sweet)
Method of analysis :	No analysis conducted
Guideline(s) :	No, study from published literature
Deviations :	N/A, study from published literature
GLP or GEP :	No, study from published literature
Acceptability :	Supplementary
Study relied upon :	Yes, however data protection not applicable to published literature studies

I. MATERIALS AND METHODS

A. MATERIALS

Test organism	Whiteleg shrimp (<i>Litopenaeus vannamei</i>) juveniles	
Source :	National Aquaculture and Marine Research Centre (CENAIM), Guayaquil, Ecuador	
Study :	Indoor study	Outdoor study
Body weight at initiation :	1.23 ± 0.18 g (mean ± SD)	5.84 ± 0.25 g (mean ± SD)
Test design		
Test vessels :	60 x 30 x 36 cm 50 L 30 mm polyethylene aquaria covered with 2 mm mesh netting to prevent shrimp from escaping. Water depth was 30 cm.	Bottomless polyethylene mesh cage of 1 m ² surface area and 1.5 m height, in a 1000 m ² greenhouse.
Replicates :	Six replicates per treatment	Five replicates per treatment
Animals/replicate :	Eight shrimp per aquarium	30 shrimp per cage
Test duration :	57 days	45 days
Test conditions		
Temperature :	25.3-29.3°C (mean 27.3°C)	33 ± 1°C
Salinity :	35 – 36 g/L (mean 35 g/L)	Not reported
Dissolved oxygen :	3.19-7.00 mg/L (mean 5.83 mg/L)	8.7 ± 3 mg/L
Photoperiod :	12 h light : 12 h dark at 1427 ± 103 lux	Greenhouse, 25,474 ± 19,287 lux

B. STUDY DESIGN AND METHODS

This study was conducted in order to assess the suitability of substituting fish meal protein (FM) with lupin kernel meal (LKM) when feeding juvenile Whiteleg shrimp (*Litopenaeus vannamei*).

Six replicate flow-through aquaria were assigned to each treatment, and each received eight shrimp. Aquaria were 60 x 30 x 36 cm 30 mm polyethylene aquaria with a capacity of 50 L. At test initiation, shrimp had a mean \pm SD body weight of 1.23 ± 0.18 g. During the first two days, dead shrimp were replaced with shrimp of a similar weight.

Seawater entered the aquaria through a sand filter and a 20 μ m cartridge, and exchanged at a rate of 1000% per day. The temperature and dissolved oxygen concentration were monitored using a handheld oxygen meter twice a week, and a refractometer was used to track salinity. Over the experimental period, the temperature ranged from 25.3 to 29.3°C (mean 27.3°C), the salinity ranged from 35 to 36 g/L (mean 35 g/L), and the dissolved oxygen content ranged from 3.19 to 7.00 mg/L (mean 5.83 mg/L). Test vessels were under a 12 hour light to 12 hours dark photoperiod, with daylight fluorescent tubes providing 1427 ± 103 lux.

Once all of the dry ingredients were mixed by hand, soy lecithin and fish oil were added. Finally, 400 to 500 mL/kg water was gradually added until the resulting dough could be easily extruded. The moist mixture was pelleted in a 2-mm diameter meat mincer. Dried strands were broken into pellets of about 1 cm in length prior to use.

Experimental diets providing nominally 35% protein and 11% lipid were prepared, within which 0, 25, 50, 75 or 100% of fish meal protein (FM) was substituted by lupin kernel meal (LKM) on a protein basis. All diets contained 10% squid meal as an attractant. Only the corn starch and fish oil contents of the diets were varied (31.0 to 36.4% and 5.2 to 7.1%, respectively) in order to keep the protein and lipid content of the diets constant across treatments. Proximate analysis of the diets showed levels that closely mirrored the calculated values, with the actual crude protein content ranging from 35.79 to 37.64% and lipid from 10.70 to 12.07%.

Shrimp were fed ad libitum four times a day during the first five weeks, reduced to twice a day for the following three. In order to estimate the acceptability based on the ingestion rate over 13 consecutive days, the uneaten feed was collected 2 hours after each feeding and weighed, taking into account feed losses due to water movement, aeration, siphoning, and rinsing.

Seven days before the end of the growth trial, the shrimp were fed on their assigned diets supplemented with 0.5% chromic oxide in order to acclimatise them to the new feed. The supplied feeds were left in the water for an hour and then uneaten feed and faeces were removed. Dried faeces were dried and ground, and assessed for content of protein and chromic oxide.

Every 15 days the aquaria were thoroughly cleaned, and the shrimp were weighed and counted in order to determine weight gain and survival rate.

Table 9.2.2-7: Ingredients and proximate composition of the diets

Ingredients (%)¹	LKM0	LKM25	LKM50	LKM75	LKM100
Fish meal	32.91	24.68	16.46	8.23	0.00
Lupin meal	0.00	9.12	18.25	27.37	36.50
Corn starch	36.44	35.12	33.75	32.40	31.05
Fish oil	5.28	5.71	6.17	6.63	7.08
Proximate composition (% dry matter)					
Moisture (at mixing)	8.44	8.13	8.13	9.52	6.01
Crude protein (N x 6.25)	35.21	35.79	36.85	36.92	37.64
Crude lipid	11.23	12.07	11.08	11.51	10.70
Carbohydrate	39.29	35.20	36.20	35.18	36.02
Ash	5.83	8.81	7.74	6.87	9.63
Energy (kJ/g dry matter)	17.9	17.7	17.8	17.8	17.8

¹ Diets also included the following (%) : Squid meal (10.00), wheat gluten (5.00), lecithin liquid (2.00), cholesterol (0.50), vitamin premix (2.00), mineral premix (2.00), antioxidant (0.02), mould inhibitor (0.10), carboxymethyl cellulose (3.00), astaxanthine (0.25), and chromic oxide (0.50).

In order to confirm the results of the laboratory bioassay, and determine the contribution of natural productivity and experimental feeds to the overall nutritional budget of shrimp, an experiment under commercial conditions was carried out. This growth trial was conducted in bottomless cages of 1 m² surface area and 1.5 m height in a single 1000 m² greenhouse. Cages were made with polyethylene mesh (15 x 20 mm aperture), and were buried approximately 20 cm and spaced 40 cm apart.

Thirty juvenile shrimp were installed in each cage. Feed was offered on a tray at 6.5% (5.5 to 9 g), 6% (9 to 11.5 g) and 5.5% (11.6 g to harvest) of the biomass twice a day for 45

days. Five replicates were performed for each treatment. Additionally, five cages with shrimp, but without feed supply (NF), were placed in the pond. The five diets and the unfed control, as well as the animals, were randomly allocated to the 30 cages.

Dissolved oxygen concentration (8.7 ± 3 mg/L) and temperature ($33 \pm 1^\circ\text{C}$) in the water were monitored daily. Light intensity in the greenhouse was also monitored daily, and was $25,474.9 \pm 19,287$ lux. Cages were sampled weekly to estimate the mean weight and adjust the amount of feed. At the end of the experimental period all shrimp were counted and weighed to estimate the feed conversion ratio, survival, biomass gain, and final mean weight. Other parameters assessed included specific growth rate (SGR), daily feed intake (DFI), feed conversion ratio (FCR), and contribution of natural productivity (CNP).

The feed ingredients and diets were milled to fine powder (300 μm) and their proximate compositions were analysed using standard laboratory procedures. Dry matter was calculated from weight loss after drying in an oven at 105°C for two hours. Crude protein ($\%N \times 6.25$) was measured using Kjeldahl method after acid digestion. Crude fat was calculated after extraction with diethyl ether extraction following the Soxhlet technique. Ash was determined after ignition of the samples at 550°C for four hours in a muffle furnace. Amino acids were determined by high-performance liquid chromatography after hydrolysis of samples in 6 N HCl for 24 hours at 110°C . Then, samples were derivatised with o-phthaldialdehyde (OPA) according to Antoine et al. (1999).

II. RESULTS AND DISCUSSION

Pellet water stability was positively related ($p < 0.05$) to the level of dietary LKM. The diet with the highest lupine inclusion level, LKM100, was the most stable ($p < 0.05$) after two hours of immersion at 95%, and the diet without lupine, LKM0, presented the lowest value, at 82%. The rest of the diets had intermediate values of stability, between 85 and 90%. The palatability results in the laboratory trial revealed a better acceptance of the LKM0 diet (8.4%/d) compared with the rest of the test diets. Feed intake for diets LKM75 and LKM100 were by far ($p < 0.05$) the lowest among all the other diets (3.4%/d).

Table 9.2.2-8: Pellet water stability (PWS) and palatability of diets containing different replacement levels of fishmeal by lupin

Diet	LKM0	LKM25	LKM50	LKM75	LKM100	SEM
Pellet water stability (%)	82.2 ^a	85.5 ^b	86.7 ^b	90.3 ^c	95.2 ^d	0.84
Palatability (%/d)	8.40 ^a	7.15 ^b	4.47 ^c	3.37 ^d	3.47 ^d	0.27

Mean of three replicates for PWS and thirteen replicates for palatability.

SEM : Standard Error of the Mean

Values in the same row with different superscripts are significantly different from one another ($p < 0.05$).

The apparent dry matter digestibility (ADMD) decreased from 78.5 to 66.5% with the increase of LKM in the diet. The three highest replacement diets (50, 75 and 100%) had significantly ($p < 0.05$) lower ADMD than the two lowest replacement diets (0 and 25%). The apparent protein digestibility (APD) did not differ among the diets with 25, 50, 75 and 100% replacement, but was significantly ($p < 0.05$) lower with the control diet.

No difference in initial individual weight using ANOVA indicated that the shrimp were homogeneously distributed between the treatments and replicates at stocking. There were no differences found on survival, but there were statistical differences in SGR, DFI, and FCR between the treatments. Shrimp fed diets LKM0, LKM25, and LKM50 exhibited similar results for SGR and FCR, with higher values than those fed on LKM75 and LKM100. In terms of daily feeding intake, this was highest for LKM0 and lowest for LKM100.

At the conclusion of the cage experiment in the field, no significant differences were recorded in final weight, SGR, survival, DFI, or FCR between the experimental shrimp diets. In contrast, the control cages without feed supply presented a lower growth and survival rate than the average of the fed cages ($p < 0.05$).

The estimation of percentage contribution of natural productivity to the nutritional requirements of juvenile shrimp fed on the different diets varied between 26 and 31%, and there were no statistical differences between them.

The apparent dry matter (ADMD) results indicate that the lupin seed-based diets are not as well digested by juvenile *L. vannamei* as diets with no LKM. A significant decrease in apparent digestibility of dietary protein was observed when fish meal was replaced by any increment of LKM when compared to the control diet (80.5%). The APD values ranged from 75.5 to 77.6% in diets containing 9 to 36% dietary lupin, with no differences between the diets.

Table 9.2.2-9: Apparent dry matter (ADMD) and protein (APD) digestibility of diets containing different replacement levels of fishmeal by lupin

Diet	LKM0	LKM25	LKM50	LKM75	LKM100	SEM
ADMD (%)	78.5 ^a	72.7 ^b	67.7 ^c	66.0 ^c	66.5 ^c	1.48
APD (%)	80.5 ^a	77.6 ^b	75.5 ^b	76.8 ^b	76.0 ^b	1.01

Mean of six replicates. SEM : Standard Error of the Mean Values in the same row with different superscripts are significantly different from one another ($p < 0.05$).

The body weight gain in the shrimp reared in aquaria followed the trend of feed intake and decreased gradually with the increase in concentration of LKM in the feed. In the present study, *L. vannamei* displayed good growth with a 50% substitution of fish meal by de-fatted and dehulled *L. mutabilis* seed, but a significant decrease in weight gain and SGR when levels of 75% and above of the dietary fishmeal was replaced by lupin, meaning that 45% of the total dietary protein was from the LKM (27.4% inclusion level).

The low levels of methionine in lupin protein did not appear to limit the nutritional value of the feed when the dietary lupin inclusion level was 18.25% (LKM50), equivalent to about 32% of the total dietary protein. Despite the lower methionine level (1.6% of protein) in LKM50 diet, the average weight gain and SGR were not significantly different from those of the control diet.

No differences in mortality were found, and the main cause for the decrease in survival rate was the shrimp jumping out of the aquarium, despite it being covered with 2 mm mesh.

Table 9.2.2-10: Shrimp growth performance when reared for 57 days in 50 L indoor aquaria

Mean of six replicates using initial weight as covariate. SEM : Standard Error of the Mean
Values in the same row with different superscripts are significantly different from one another (p < 0.05).

Diet	LKM0	LKM25	LKM50	LKM75	LKM100	SEM
Initial weight (g/shrimp)	1.19	1.18	1.24	1.20	1.27	0.06
Final weight(g/shrimp)	7.02 ^a	6.68 ^a	6.70 ^a	5.22 ^b	4.76 ^b	0.31
SGR (5/day)	3.05 ^a	3.00 ^a	2.99 ^a	2.52 ^b	2.42 ^b	0.09
Survival (%)	82.3	81.8	96.8	90.4	86.1	5.68
DFI(%BW/day)	6.48 ^a	5.31 ^b	5.99 ^{ab}	4.87 ^b	3.51 ^c	0.32
FCR	2.71 ^a	2.17 ^a	2.48 ^a	2.24 ^a	1.66 ^b	0.15

The growth experiments conducted in cages in earthen ponds showed that, unlike the study conducted in aquaria, the gradual increase of LKM in diets did not produce a significant decrease in shrimp growth even when all the FM in the experimental feed was replaced by LKM. This may be because the bottomless cages allowed the shrimp free access to the substrate and the flora and fauna found there.

Table 9.2.2-11: Shrimp growth performance when reared for 45 days in 1 m² bottomless cages

Diet	LKM0	LKM25	LKM50	LKM75	LKM100	SEM
Initial weight (g/shrimp)	5.87	5.98	5.63	5.85	5.79	0.11
Final weight (g/shrimp)	11.67	11.68	11.89	11.06	12.21	0.49
SGR (5/day)	1.53	1.54	1.57	1.43	1.63	0.09
Survival (%)	77.1	80.4	70.8	78.6	69.2	5.1
DFI (%BW/day)	2.87	2.73	3.12	2.90	2.77	0.17
FCR	2.17	1.99	2.35	2.32	2.04	0.20
Harvest biomass (g/m)	268	281	250	261	249	12.6
CNP (%)	31.0	31.3	37.0	25.9	37.8	5.7

Mean of five replicates using initial weight as covariate. SEM : Standard Error of the Mean Values in the same row with different superscripts are significantly different from one another ($p < 0.05$).

Table 9.2.2-12: Comparison of growth performance of shrimp reared for 45 days in 1 m² bottomless cages and fed experimental diets and fed natural productivity

Diet	Experimental diet	Natural productivity
Initial weight (g/shrimp)	5.82 ± 0.05	5.92 ± 0.11
Final weight (g/shrimp)	11.68 ^a ± 0.23	9.84 ^b ± 0.52
SGR (5/day)	1.53 ^a ± 0.05	1.13 ^b ± 0.10
Survival (%)	75.5 ^a ± 2.2	56.2 ^b ± 4.9
Harvest biomass (g/m)	262 ^a ± 5.3	161 ^b ± 11.9

Mean of twenty five replicates for diet and five for natural using initial weight as covariate. Values in the same row with different superscripts are significantly different from one another ($p < 0.05$).

III. CONCLUSION :

Two growth trials were conducted with juvenile *Litopenaeus vannamei* using experimental diets providing 35% protein and 11% lipid, where 0, 25, 50, 75 and 100% of fish meal protein (FM) was substituted by lupin kernel meal (LKM). At the end of the 57-day feeding trial, the average survival of the shrimp was > 80% and there was no variation ($p > 0.05$) when FM was replaced partially nor totally with LKM. The results of this study showed that LKM can replace 50% of FM protein without significantly discouraging growth (6.7 to 7.0 g final weight) ($p > 0.05$), but the substitution of 75 and 100% resulted in lower growth (4.8 to 5.2 g final weight). The inclusion of LKM at any of the tested levels resulted in a statistical reduction ($p < 0.05$) of the apparent dry matter digestibility (ADMD) and apparent protein digestibility (APD) of the feed. The gradual increases of LKM in diets produced a significant decrease ($p < 0.05$) in ingestion rate.

To demonstrate the inherent effects of water quality and natural food sources found in shrimp ponds, a growth trial was conducted in 1 m² bottomless cages in a single 1000 m² pond greenhouse. Juveniles weighing 5.84±0.25 g (mean ±SD) were stocked in the cages at a density of 30 individuals per m². The feed was offered on a tray twice a day for 45 days. Five replicates were performed for each treatment. At the end of the 45-day field evaluation, no significant differences ($p > 0.05$) in final weight (11.1 to 12.2 g), specific growth rate (1.4 to 1.6%/day), survival (69 to 79%) nor feed conversion ratio (2.0 to 2.3) were found in any of the experimental shrimp diets. These findings show that *Lupinus mutabilis* Sweet has very good potential as

an alternative protein source replacing at least 50% of protein from FM, equivalent to one third of the total protein in the diet for growth-out phase of *L. vannamei*.

HSE COMMENTS

This study is from the scientific literature and does not conform to standardised test guidelines and is not GLP compliant. The study’s methodology, reporting and analysis have been considered with regard to how well they meet common standards of scientific practice, based on ‘expert judgement’.

The study appears to be well conducted in accordance with scientific principles, although details on the batch of test item and its stability during the test were lacking. The test species also did not conform to the standardised guidelines and was not a species of fish. This study would therefore not be considered in the fish risk assessment, but may be considered as supporting evidence for the aquatic invertebrates or general aquatic risk assessment. The measurements on dry matter and protein digestibility and the effects of different test vessels are not relevant for use in the risk assessment. Additionally, exposure was via diet so the results are of limited relevance to the aquatic risk assessment which considers exposure via a concentration in water. However, the study does indicate that inclusion of lupin kernel meal as a potential alternative protein source replacing at least 50% of protein from fish meal, over a 45 day period, does not negatively affect growth or survival of Whiteleg shrimp.

Relevance : Not relevant (dietary route of exposure)

Reliability : Reliable with reservations

(Studies are graded as one of the following categories : 1) Reliable, 2) Reliable with reservations, 3) Unreliable. Categorisation is based on the evaluator’s subjective assessment of the quality of the methods, reporting and analysis as presented in the study.)

Reference:	K-CA 8.2.2/05
Report Title:	Feeding high inclusion of whole grain white lupin (<i>Lupinus albus</i>) to rainbow trout (<i>Oncorhynchus mykiss</i>): effects on growth, nutrient digestibility, liver and intestine histology and muscle fatty acid composition.
Author(s) & Year:	Borquez et al. (2010)

Document No :	Aquaculture Research, 2010, 1-12.
Method of analysis :	No analysis conducted
Substance used:	<i>Lupinus albus</i> var. Hamburg
Guideline(s):	No, study from published literature
Deviations :	N/A, study from published literature
GLP or GEP:	Yes
Acceptability:	Supplementary
Study relied upon :	Yes, however data protection not applicable to published literature studies

I. MATERIALS AND METHODS

A. MATERIALS

Test Material	<i>Lupinus albus</i> var. Hamburg
Lot/Batch #:	Not stated
Description:	Whole white Lupin grain
Purity:	Not stated
Stability:	Not determined in the test system
Treatments	
Test rates:	Control diet and three diets containing 30%, 40% and 50% of whole white lupin grain (<i>Lupinus albus</i> var. Hamburg) (L0, L30, L40 and L50 respectively). These diets were formulated to replace 0%, 25%, 33% and 42% of the total crude protein of the diet with lupin protein.
Analysis of test concentrations:	None
Acclimation:	10 days
Test animals	
Species:	<i>Oncorhynchus mykiss</i>
Source:	
Feeding:	Formulated fish food twice a day
Weight:	54.0 ± 6.2 and 181.9 ±3.4 g
Test Design	
Test vessel:	500 L fibreglass tanks
Test substrate:	Well water
Replicates:	Three replicates per treatment group

Animals/vessel:	30 fish
Test duration:	11-week (growth trial) and 4-week (digestibility trial)
Test conditions	
Temperature:	14.0 ± 2°C
pH:	Not stated
Photoperiod:	Not stated

B. STUDY DESIGN AND METHODS

The aim of this study was to evaluate the effect of high inclusion levels of whole grain lupin in rainbow trout extruded diets on growth, nutrient utilisation and fatty acids in the muscle.

Four experimental extruded diets were evaluated: a control diet (L0) and three diets containing 30%, 40% and 50% of whole white lupin grain (*Lupinus albus* var. Hamburg) (L30, L40 and L50 respectively). These diets were formulated to replace 0%, 25%, 33% and 42% of the total crude protein of the diet with lupin protein. Chromium-oxide (Cr₂O₃) was used as an inert marker. The compositions of all of the ingredients used in the diet preparations, and the formulation and proximate composition of the diets are presented in the below tables.

Table 9.2.2-13: Composition of the ingredients used in this study

Chemical composition (g/kg)	LT fish meal	Whole grain <i>Lupinus albus</i> var. Hamburg	Wheat	Sunflower defatted meal	Feather meal
Dry matter	908	912	878	890	900
Crude protein	680	340	100	370	820
Crude lipid	106	95	20	22	70
Ash	140	36	14	79	29
Carbohydrates*	74	529	866	529	81
Gross energy (MJ/kg)	215.1	208.8	180.5	187.0	235.1
Arginine	39	39	6	32	55
Histidine	25	8	3	9	10
Isoleucine	28	17	4	16	39
Leucine	49	26	8	24	68
Lysine	52	18	3	13	22
Methionine	19	3	2	8	6
Phenylalanine	27	14	6	18	41
Threonine	28	13	3	14	38
Valine	33	15	5	19	59

* Calculated as the remainder of crude protein + crude lipid + ash

Table 9.2.2-14: Composition of the ingredients used in this study

Ingredient composition (g/kg)	Diets			
	L00	L30	L40	L50
LT fish meal	400.0	350.0	300.0	250.0
Fish oil	145.2	136.2	134.0	131.6
Whole grain white lupin	0	300.0	400.0	500.0
Wheat flour	150.0	63.5	63.5	63.4
Sunflower defatted meal	138.1	49.0	0.00	0.00
Feather meal	122.6	85.4	107.5	102.5
Vitamin and mineral premix	5.0	5.0	5.0	5.0
Mono-calcium phosphate	0.3	5.7	10.5	14.3
L-Lysine	0.2	0.5	1.9	3.1
DL-methionine	0.0	1.4	2.4	3.1
Chromium oxide	10.0	10.0	10.0	10.0
Chemical composition (g/kg DM)				
Dry matter	918.9	927.7	919.0	911.2
Crude protein	491.3	527.7	527.0	484.4
Crude lipid	258.1	254.1	243.7	223.0
Ash	85.3	77.6	72.4	71.0
Carbohydrates	165.3	140.7	156.9	221.6
Gross energy (kJ kg ⁻¹)	24.09	24.49	24.30	23.05

Growth experiment

Juvenile rainbow trout (*Oncorhynchus mykiss*) (initial mean weight of 54.0 ± 6.2 g) were randomly distributed into twelve 500 L circular fibreglass tanks (supplied with freshwater ($14.0 \pm 2.7^\circ\text{C}$; flow rate 12 L/min). Before the start of the growth experiment the fish were acclimated for 10 days and fed a control diet. Subsequently, triplicate groups of fish were fed the experimental diets by hand, ad libitum twice a day, for 11 weeks.

At the beginning of the growth experiment, 15 fish were randomly sampled in order to determine the fatty acid profile, body composition and histology. At the end of the experiment, the fish were fasted for a day, and then weighed individually. Two fish from each tank (six per treatment) were randomly taken for whole body composition and stored at -20°C for proximate analysis. An additional three fish were removed from each tank (nine per treatment) and samples of dorsal muscle were dissected,

skinned, deboned, homogenized and stored at -20°C for fatty acid analysis. These same fish were later sampled for liver weight determination, and both liver and intestine samples were collected for histological examination. Fish were killed by a blow to the head after an overdose of benzocaine.

Digestibility experiment

Apparent digestibility coefficients (ADC) were determined using the modified Guelph method, using Cr₂O₃ as an inert indicator (Cho, Cowey & Watanabe 1985). Thirty Juvenile rainbow trout (*O. mykiss*) (initial mean weight of 181.9 ± 3.4 g) were randomly allocated into twelve 500 L cylindro-conical fibreglass tanks equipped with faecal settling columns connected to the outlet of each tank and supplied with well water (14.0 ± 2°C; flow rate 12 L/min). Fish were fed the experimental diets in triplicate by hand, to apparent visual satiety twice a day, for 4 weeks. After an adaptation period to the dietary treatments of 1 week, faeces were collected daily in each tank from settling columns, centrifuged at 1235g for 15 min and frozen at -20°C until analysis.

Calculations

Growth was assessed using the thermal growth coefficient (TGC) and weight gain (G).

Feed conversion ratio (FCR) was calculated as: $FCR = F \times G^{-1}$, and protein efficiency ratio (PER) was determined as: $(PER) = G \times PI^{-1}$, where F is consumption of dry matter from feed, G is the weight gain and PI is the protein intake. The Hepato-somatic index (HSI) was calculated as: $HSI = 100 \times (LW \times FW^{-1})$, where LW and FW represent wet liver weight and wet body weight respectively.

Chemical analyses

Proximate compositions (crude protein, crude lipid, ash and moisture) of diets and carcass were determined according to the methods of AOAC (1998). Carbohydrates were calculated by difference (as the remainder of crude protein + crude lipid + ash). Gross energy was estimated using the following coefficients: 23.4 kJg⁻¹ for crude protein, 39.8 kJg⁻¹ for crude lipid and 17.2 kJg⁻¹ for carbohydrates (Cho, Slinger & Bayley 1982).

The extraction of total lipids from diets and muscle tissues was carried out according to the method of Folch, Lees and Sloane-Stanley (1957) and analysed by gas chromatography. Helium was used as a carrier gas. Fatty acids were identified by comparison with fatty acid standards and expressed as the percentage of total fatty acids identified.

The total amino acid contents of the experimental diets were determined using near-infrared reflectance spectroscopy. Samples were ground to a 300 µm particle size before analyses.

Histological analysis

Liver and intestine samples were fixed in Bouin solution for 24 h, and stored in 70% ethanol at 4°C. The tissues were subsequently dehydrated according to standard histological techniques in a graded ethanol series, and embedded in paraffin. Sections (4-6 µm) were cut and stained with haematoxylin and eosin and then blindly examined under a light microscope (Leica Microsystems model DM750, Leica, Bannockburn, IL, USA).

Statistical analyses

Second-order polynomial regression models were used to describe the effects of whole lupin grain dietary inclusion on different parameters studied. The significance level was set to $P < 0.05$. All statistical analyses were conducted using SAS for Windows version 8.01 (SAS Institute, Cary, NC, USA), and data are presented as mean standard error of mean.

II. RESULTS AND DISCUSSION

Growth performance and feed utilization

During the growth trial, all diets were well accepted by fish and the survival rate was 98%. The mean treatment final body weights varied from 173.6 (L50) to 183.2 g (L40); fish fed the diet containing 500 g/kg of whole lupin grain (L50) achieved the lowest weight gain (119.63 g) and the highest FCR (1.38), although there were no significant correlations with the dietary inclusion of whole lupin grain. Correspondingly, no significant correlations were observed between the increase in the level of whole lupin grain in the feeds and feed intake, TGC and PER ($R^2 = 0.16$, 0.34 and 0.04 respectively).

Whole body composition and HSI

In general, the inclusion level of lupin in the diet did not change the chemical composition of the fish. Hepato-somatic index, however, showed a significant decreasing trend ($R^2 = 0.75$, $P < 0.05$). The HSI values ranged from 1.19 (L50) to 1.45 (L30).

Histology examination

The cellular morphology of enterocytes showed a displacement of the nucleus in the direction of the distal cell pole. Simultaneously, a reduction in the number of basophils and abundant number of lipid drops were also observed in the fish fed the

diet containing 40% whole grain lupin meal (L40). Hepatocytes, on the other hand, showed a slight lipid infiltration in the fish fed the control and L40.

Surprisingly, the above effect was observed to a lesser degree in the treatment L50.

Digestibility coefficients

The ADC values for dry matter, crude protein, lipid and carbohydrates showed slight variations among dietary treatments, but significant effects of increasing dietary whole lupin grain were not observed. Phosphorus digestibility was also not affected by the dietary inclusion of whole lupin grain.

Muscle fatty acid composition

The incorporation of high levels of whole grain lupin into diets did not lead to significant changes in the total content of saturated fatty acids. The most abundant fatty acid was palmitic acid, which showed a slight decrease in the diets containing lupin; however, this trend was not significant.

Histological analysis of the fish fed diets containing 40% and 50% of whole grain lupin exhibited morphological changes in the mid intestine that included a decrease in the number of basophil granulocytes, distal displacement of enterocyte nucleus and an increment in lipid drops.

III. CONCLUSION:

The findings concerning growth performance and nutrient digestibility obtained in the present study have demonstrated the potential use of whole grain sweet white lupin (*L. albus*) in commercial extruded diets for rainbow trout (*O. mykiss*).

The growth performances and feed intake of fish fed diets containing up to 50% of whole lupin grain were comparable to those of fish fed the control diet. Marginal effects were observed on fish nutrient digestibility and muscle fatty acid compositions. This indicates that white lupin seed may be utilized successfully as a feed ingredient in rainbow trout diets without removing the seed coat, avoiding the cost of a preliminary dehulling process.

HSE COMMENTS

This study is from the scientific literature and does not conform to standardised test guidelines, but it is GLP compliant. The study's methodology, reporting and analysis have been considered with regard to how well they meet common standards of scientific practice, based on 'expert judgement'.

The study appears to be well conducted in accordance with scientific principles, although details on the batch of test item and its stability during the test were lacking. The same lupin species has been used as the active substance under assessment is derived from and *O. mykiss* is a standard species for ecotoxicity testing. The measurements on diet content, digestibility and histology are not relevant for use in the risk assessment. Additionally, as exposure was via diet, the results are of limited use in the aquatic risk assessment which considers exposure via a concentration in water. However, the study does indicate that inclusion of whole white lupin grain at up to 50% in the diet (500 g/kg), over a fifteen week period, does not negatively affect mortality, feed consumption or growth of Rainbow trout. The results may be considered as supporting evidence in the risk assessment.

Relevance: Not relevant (dietary route of exposure)

Reliability: Reliable with reservations

(Studies are graded as one of the following categories: 1) Reliable, 2) Reliable with reservations, 3) Unreliable. Categorisation is based on the evaluator's subjective assessment of the quality of the methods, reporting and analysis as presented in the study.)

Reference:	K-CA 8.2.2/06.
Report Title:	Mixtures of lupin and pea protein concentrates can efficiently replace high-quality fishmeal in extruded diets for juvenile black sea bream (<i>Acanthopagrus schlegeli</i>)
Author(s) & Year:	Zhang, Y. et al. (2012b)
Document No :	Aquaculture 354-355 (2012) 68-74
Substance used :	Lupin protein concentrates (LPC)- White lupine (<i>Lupinus albus</i>)
Method of analysis :	No analysis conducted
Guideline(s) :	No, study from published literature
Deviations :	N/A, study from published literature
GLP or GEP :	No, study from published literature

Acceptability :	Supplementary
Study relied upon :	Yes, however data protection not applicable to published literature studies

I. MATERIALS AND METHODS

A. MATERIALS

Test Material

Lupin protein concentrates (LPC) and pea protein concentrates (PPC)
 White lupine (*Lupinus albus*), produced by dehulling, milling, aqueous extraction of lupine seeds to remove sugars and soluble non-starch polysaccharides, heating and spray drying
 Purity: 100%

Treatments

Lupin protein concentrates 300 or 500 g plant protein/kg dietary protein mixed with pea protein concentrates (PPC)
 L/P ratio at 3:0, 2:1, 1:2 and 0:3

Test animals

Species: Black sea bream (*Acanthopagrus schlegeli*)
 Source: In house culture
 Feeding: LT®Fishmeal
 Weight: 13 g (mean)

Test Design

Test vessel: Cylindrical 500 L tanks
 Test substrate: with sand-filtered seawater at a flow rate of 1.5 L/min
 Replicates: Duplicate tanks
 Animals/vessel: 50
 Test duration: 60 days
 Temperature: 25.0°C
 Salinity: 29‰ (g/L)

B. STUDY DESIGN AND METHODS

Ingredients and diets

The LPC was derived from white lupine (*Lupinus albus*), produced by dehulling, milling, aqueous extraction of lupine seeds to remove sugars and soluble non-starch polysaccharides (NSP), heating and spray-drying. The PPC was produced from yellow field pea (*Pisum sativum* L.) by dehulling, fine grinding and air-classification. The LPC and PPC were each supplemented with the first-three limiting essential amino acids to balance the essential amino acid profile to that of LT fish meal. A 2 × 4 factorial design was used in the present experiment, where the factors were

inclusion level of plant protein concentrate (300 or 500 g protein/kg dietary protein), and ratio between essential amino acid-supplemented LPC and PPC in the diets (L/P ratio at 3:0, 2:1, 1:2 and 0:3). The diets were isonitrogenous (530 g crude protein (CP)/kg) and isolipidic (160 g crude lipid (CL)/kg). In addition, a diet with LT fish meal as the sole source of protein (FM diet) was produced with 570 g CP and 180 g CL/kg, and formulated to keep the same ratio between protein and lipid ratio as the 8 diets with plant protein sources. Yttrium oxide was used as a marker for digestibility measurement (Austreng et al., 2000). Feed processing and equipment are described in detail by Zhang et al. (2012). All dry ingredients were ground, mixed, preconditioned and extruded in a twin screw extruder with 2.0 mm dies and the pellets were dried and coated with fish oil in a vacuum coater. Yttrium oxide was added to the diets for determination of nutrient digestibility, but faecal collection by stripping was not successful, and digestibility results are not presented.

The experiment was conducted at the Joint Laboratory of Nutrition and Feed for Marine Fish, Marine Fisheries Research Institute of Zhejiang Province (Putuo, Zhoushan, China). The black sea bream juveniles were obtained from a hatchery in Fodu (Putuo), acclimated in an indoor concrete pond for three weeks, and fed a commercial diet (52% CP, 8% fat). Before the start of the experiment, 900 bream with an initial weigh of 13 g were depleted of feed for 24 h, anaesthetised with MS-222 (90 mg.L⁻¹), batch-weighed, then randomly assigned to 18 circular 500 L tanks, fifty fish per tank. Each tank was supplied with sand-filtered seawater at a flow rate of 1.5 L.min⁻¹ and additional aeration via air stone. A natural photoperiod (13 h light, 11 h dark) was applied throughout the feeding period. The average water temperature and salinity were 25.0°C and 29 g.L⁻¹, respectively. Each diet was fed to fish in duplicate tanks and all the fish were fed three times per day, at 06:30, 11:30, and 17:30. Before each feeding, the water flow was stopped, while continuous aeration was maintained. The fish were fed by hand for 1 h. After each feeding, all uneaten feed particles remained intact, and the number of uneaten pellets in the bottom of each tank was counted and siphoned out immediately. The amount of uneaten feed was set by multiplying the number of uneaten pellets with the average pellet weight for each feed (counting 4 × 100 pellets). The daily feeding rate was tentatively set 10% in excess based on the average feed intake over the last 3-day feeding, but the fish received more feed if they showed signs of feed intake at the end of the one hour meals. The feeding experiment lasted for 60 days.

Sampling

Before the start of the experiment, 2×8 fish (depleted of feed for 24 h) from the acclimation pond were killed by overdose of MS-222, and kept at -20°C for whole body analysis. Fish were anaesthetised with MS-222 (90 mg.L⁻¹) and batch-weighed in the beginning (Day 0) of the experiment. At the end of the feeding experiment, five fish were randomly sampled from each tank for blood samples. The fish were weighed individually, blood was collected from the caudal vein by a 1 mL disposable syringe with a 27-gauge needle, and kept on ice until centrifuged at 3000×g for 10

min. The plasma was aliquoted into two Eppendorf (EP) tubes, frozen in liquid N₂, and kept at -80°C until analysis. Another three fish were taken from each tank, individually measured for weight and length, and then killed by a blow to the head. The sea breams were cut open to remove the intestinal contents, the whole viscera, liver and carcass were weighed separately, and then put together and stored at -20°C for whole body analysis. Ten fish were taken from each tank, weighed individually, then the intact gastrointestinal tracts were gently removed and divided into 3 regions as follows : stomach, mid intestine (MI, from distal side of the stomach region to distal intestine) and distal intestine (DI, from the start of the last fold of intestine until the anus). Surface fat and connective tissue were carefully removed. The intestinal tissue walls of MI and DI were placed in pre-weighed EP tubes, frozen in liquid N₂ and kept at -80°C for the determination of brush border maltase activity. The remaining fish in each tank were weighed.

Chemical analyses

The initial and final fish samples were autoclaved at 120°C for 20 min, homogenised and oven-dried at 70°C. The dried whole fish samples and feed samples were analysed for dry matter, crude protein, lipid, ash, and energy. Dry matter was determined by drying at 105°C to constant weight. Crude protein content was measured using a 2300 Kjeltac Analyser Unit (Foss, Tecator, Sweden). Lipid was determined by petroleum ether extraction using a Soxtec system (Soxtec 2055, Foss Analytical, Denmark), ash by combustion at 550°C, and gross energy by bomb calorimetry (Phillipson Microbomb Calorimeter ; Gentry Instruments Inc., Aiken, SC, USA). Minerals in feed samples were determined by inductively coupled plasma mass spectroscopy (ICP-MS) after complete digestion of the homogenised and dried samples in HNO₃ after cooking in a microwave oven for 1 h. For each measurement, duplicate samples were analysed. Phytic acid was determined according to the method described by Carlsson et al. (2001). Plasma cholesterol and triacylglycerols were analysed by RSBIO® kits (Shanghai Rongshen Biotech Co., Ltd. Shanghai, China) and spectrophotometer micro-plate reader (PowerWave XS, BioTek Instruments Inc., Winooski, VT, USA). Activities of brushborder membrane bound maltase in MI and DI were analysed as described by Krogdahl et al. (2003). Only the samples from the fish fed the FM diet and the diets with 500 g plant protein/kg crude protein inclusion were measured for maltase activity.

Calculations and statistical analysis

Feed intake (FI) was calculated by subtracting uneaten feed from feed fed on a dry matter basis. Specific growth rate (SGR) was calculated as : $100 \times (\ln(\text{FBW}) - \ln(\text{IBW})) \times d^{-1}$, where $\ln(\text{IBW})$ and $\ln(\text{FBW})$ are the natural logarithms of initial and final body weight of individual fish (tank mean), and d is feeding days. Feed conversion ratio (FCR) was calculated as: $\text{FI} \times (\text{FBW} - \text{IBW})^{-1}$. Nitrogen or energy retentions (%) were calculated as: $100 \times (\text{N}_1 \times \text{FBW} - \text{N}_0 \times \text{IBW}) \times (\text{N}_d \times \text{FI})^{-1}$, where N_0 and N_1 represent the nitrogen or energy content in the initial and final

whole fish samples (pooled samples of 3 fish per tank), respectively. Hepatosomatic index (HSI, %) or viscerosomatic index was calculated as: $100 \times (\text{weight of organ}) / (\text{total fish weight})$. Condition factor (CF) was calculated as: $100 \times (\text{fish weight}) / (\text{body length})^3$, where weight is expressed in g and length is in cm.

Each tank was considered an experimental unit. The results were analysed using the GLM procedure of SAS statistical software (SAS, 1990). One-way analysis of variance (ANOVA) was used to compare effects of the FM diet with those of the diets with plant protein. Factorial ANOVA was used to analyse the effects of plant protein inclusion level and L/P ratio. Significant ($P < 0.05$) interactions between inclusion level and L/P ratio were rationalised by regression analysis of $L/(L+P)$ within inclusion level, provided that at least one of the main effects were significant (Snedecor and Cochran, 1967). The results were expressed as means and pooled standard errors of means (S.E.M). Duncan's multiple-range test was used to rank significant differences among diets in the one way ANOVA and main effects in the factorial ANOVA.

II. RESULTS AND DISCUSSION

Growth and feed utilisation

All fish had good appetite and grew well on all diets. The fish weights were more than tripled after the 60-day feeding period. Only one fish died during the experimental period ; one fed the HLP3 diet on day 14. An average feed conversion ratio (FCR) of 1.13 g DM ingested (g gain)⁻¹ was achieved. The feed intake (FI) of the fish fed the FM diet was significantly lower than that of the fish fed the HLP1 diet, and did not differ significantly from those fed the other diets. No significant difference was found among diets for growth (WG). The FCR for the LLP1, LLP3, LLP4 and HLP4 diets did not differ from that of FM diet, while the others were significantly higher.

The diets with most LPC (L/P ratio=3 :0) resulted in significantly higher FI than the diets with less LPC. Diets with 500 g plant protein/kg resulted in significantly lower WG than the diet with 300 g/kg. No significant effect of L/P ratio on WG was found. Higher dietary inclusion of plant protein resulted in significantly higher FCR during both feeding periods, and diets with the highest LPC ratio (L/P ratio=3:0) resulted in significantly higher FCR compared to diets with less LPC during both feeding periods. The FCR for the diets with L/P ratios of 2:1, 1:2 and 0:3 did not differ significantly from each other. Significant interaction was seen between inclusion level and L/P ratio for FCR. This was due to a steep and significant increase in FCR with increasing dietary LPC at 500 g plant protein/kg inclusion, while, this effect was not significant for 300 g/kg.

Body composition and nutrient retention

No significant difference was found in whole body composition among diets except for ash. The ash content in the fish fed the FM diet was significantly higher than those fed the dietary plant protein based diets, except for the LLP3 and LLP4 diets. Neither inclusion level nor the L/P ratio resulted in significant differences in whole body composition, except for ash, which was significantly lower in fish fed the diets with 500 g/kg plant protein inclusion than with 300 g/kg inclusion. The nitrogen (N) retention of fish fed the FM diet was significantly higher than those fed the HLP1 and HLP2 diets, but did not differ from those fed the other diets. Both N and energy retentions of fish fed diets with inclusion of 500 g plant protein/kg crude protein were significantly lower than those fed diets with 300 g/kg inclusion. The diets with lower LPC levels (L/P ratio = 1:2 or 0:3) resulted in significantly higher N retention than diets with the highest LPC level (L/P ratio = 3:0). No significant difference was found for the somatic indices, HIS, VSI, and CF, or the plasma parameters, total plasma cholesterol and triacylglycerol levels among the different diets. Also, none of these parameters was significantly affected by plant protein inclusion level or L/P ratio. No significant differences were seen for maltase activity in MI, but the activity in DI was significantly lower in fish fed the HLP4 diet. The growth rates obtained in the present experiment, corresponding to SGR ranging from 1.4 to 1.7) were consistent with previous findings in this species with comparable fish size and rearing conditions (Shao et al., 2008, SGR ranging from 1.8 to 2.3 for fish with 13 g start weight; Peng et al., 2009, SGR ranging from 1.7 to 2.1 for fish with 18–19 g start weight). The similar growth rates among fish fed the FM diet and those fed the other plant protein based diets, showed that using multiple amino acid supplemented diets with LPC or PPC alone or in combination could provide 50% of dietary protein for black sea bream without impairing growth.

Adequate feed intake is a precondition to guarantee a precise nutritional evaluation of plant proteins in fish feed. A feed intake reduction has been observed when including high levels of plant protein concentrates in the diet for gilthead sea bream (Kissil et al., 2000). This may be related to the dilution or removal of palatable constituents derived from FM and the presence of detractive compounds in plant-derived ingredients. The high feed intake in the present experiment was consistent with previous findings with LPC and PPC in rainbow trout fed the same diets, but with higher content of lipid (Zhang et al., 2012). The current results, thus, show that the concentration of detractive components, such as alkaloids from lupin and saponins from pea were not sufficient to impair feed intake, even at a dietary inclusion level of 467 and 433 g/kg LPC and PPC respectively.

The FCR values in the present experiment suggest efficient utilization of the feed by black sea bream. One main explanation is the removal of indigestible material from the plant protein ingredients, since the aqueous extraction and air- classification mainly remove the soluble NSP from the lupin seed meal and carbohydrates from the pea meal. However, other ANF may still exist. Extrusion is known both to improve protein utilisation by inactivating heat labile ANF, and by unfolding globular

storage proteins to facilitate access of the digestive enzymes. In addition, extrusion results in gelatinisation of the starch, which is necessary for efficient digestion (Sørensen, 2003). Thus, the high performance of sea bream obtained in the present experiment can both be related to the nutritional qualities of the LPC and PPC (Carter and Hauler, 2000 ; Refstie et al., 2006; Øverland et al., 2009) and the use of extrusion to produce the diets.

The main advantage of PPC over LPC as a dietary protein source seems to be the lower content of NSP. This is in keeping with previous observations (Carter and Hauler, 2000 ; Zhang et al., 2012). NSP are almost indigestible for fish due to the absence of α -galactosidase and β -xylanase in the digestive tract (Kuz'mina, 1996; Bansleben et al., 2008). In addition, the undigested NSP in digesta can negatively affect the digestion and absorption of other nutrients (Sinha et al., 2011). Soluble NSP have a viscous nature and can bind to the intestinal brush border and form a thick unstirred water layer adjacent to the mucosa to block the access of substrates to brush border enzymes, and reduce nutrient digestibility by increasing the intestinal viscosity. A major reason for processing lupin into LPC was to remove the soluble carbohydrate fraction, thus soluble NSP was not the major reason for the preference of PPC over LPC at high inclusion rate.

The total phosphorus concentration of all the diets exceeded the levels sufficient for maximum for growth of black sea bream (Shao et al., 2008). The lack of successful collection of faeces made it impossible to estimate phosphorus availabilities in the current experiment. The whole body composition of the black sea bream was similar to that reported by Peng et al. (2009) for fish fed a fish oil control diet. Whole-body ash content (WBA) significantly increased with increasing dietary total P concentration (DPc) ($WBA = -0.10 DPc^2 + 4.27 DPc + 11.2$; $R^2 = 0.66$, $P = 0.0001$). Whole-body ash concentration was not significantly related to dietary phytate concentration. This indicates that the total dietary P concentration was limited for bone mineralisation. The growth rates of the sea bream were, however, not significantly affected by dietary P-concentration. This is in keeping with the results obtained by Shao et al. (2008) who found that black sea bream of similar size had a requirement of available dietary P for growth at 0.55%, while the estimated requirement for vertebral mineralisation was higher (0.87%). The whole-body ash concentrations in the sea bream fed diets with the lowest inclusion of plant protein concentrates or the fish meal diet, were also similar to or higher than the values obtained in fish fed diets with adequate P-supply by Shao et al. (2008). Thus, the results indicate that diets not supplemented with P and containing 500 g plant protein concentrates/kg resulted in mild deficiency of this element. No indications of short term phosphorus deficiency were detected in the fish fed diets with 300 g plant protein concentrates/kg. Other essential elements like Ca and Mg are highly abundant in and taken up by the fish from saltwater, while Cu, I, Mn and Zn were supplemented by the micromineral premix (Zhang et al., 2012). There is no published information available on N and energy retentions for black sea bream. The

significantly lower N retention in fish fed HLP1 and HLP2 compared with the other diets may have been related to the high inclusion of LPC. The design of the experiment does not, however, provide explanations to whether this was caused by the high NSP content, differences in nutrient digestibility, or other differences between the two plant protein concentrates. The protein retention efficiencies indicate that essential amino acids were provided in excess by all diets. Thus, inadequate amino acid supply was not a plausible explanation to the differences in N retention.

Hughes (1991) and Lairon (1996) reported that NSP of legume seeds was an effective cholesterol-reducing agent. A clear hypocholesterolemic effect was also observed in our previous experiment with rainbow trout (Zhang et al., 2012). The absence of hypocholesterolemic effect in the present experiment indicates that such effect may be species specific.

The brush border enzymes are responsible for the final stages of hydrolysis of protein and starch. Their activities do not only indicate the capacity of digestion but also the integrity of intestinal structure, especially in the distal part. The reduction of maltase activity in DI is in keeping with our previous finding with rainbow trout (Zhang et al., 2012). The trout also had a slight decrease in mucosal fold height and a slight increase in fold fusion in DI of fish fed diet with the highest level of PPC. This may indicate mild changes associated with the mechanism that resulted in inflammation in DI at higher dietary PPC levels (Penn et al., 2011). Histological studies are, however, needed to find out if the reduced maltase activity is related to changes in the integrity of the distal intestine.

III. CONCLUSIONS

To conclude, both LPC and PPC are promising dietary protein sources for black sea bream. Any combination of LPC and PPC in essential amino acid-supplemented extruded diets, accounting for up to half of dietary protein, can be used without impairing fish growth. At higher inclusion, combinations with more PPC are preferred, while high inclusion of LPC resulted in less efficient feed conversion.

HSE COMMENTS

This study is from the scientific literature and does not conform to standardised test guidelines, but is GLP compliant. The study's methodology, reporting and analysis have been considered with regard to how well they meet common standards of scientific practice, based on 'expert judgement'.

The study appears to be well conducted in accordance with scientific principles, although details on the batch of test item and its stability during the test were lacking. The test species also did not conform to the standardised guidelines. The

measurements on feed utilisation and nutrient retention are not relevant for use in the risk assessment. Additionally, as exposure was via the diet, the results are of limited relevance to the aquatic risk assessment which considers exposure via a concentration in water. However, the study does indicate that inclusion of white lupin protein concentrates at up to 500 g LPC/kg diet, over a 60-day period, does not negatively affect growth of black sea bream. The results may be considered as supporting evidence in the risk assessment.

Relevance: Not relevant (dietary route of exposure)

Reliability: Reliable with reservations

(Studies are graded as one of the following categories: 1) Reliable, 2) Reliable with reservations, 3) Unreliable. Categorisation is based on the evaluator's subjective assessment of the quality of the methods, reporting and analysis as presented in the study.)

Reference:	K-CA 8.2.2/07.
Report Title:	Growth, physiological and immunological responses of rainbow trout (<i>Oncorhynchus mykiss</i>) to different dietary inclusion levels of dehulled lupin (<i>Lupinus angustifolius</i>)
Author(s) & Year:	Farhangi, M. and Carter, C. G. (2001)
Document No	Aquaculture Research, 2001, 32 (Suppl. 1), 329-340
Substance used:	Commercial dehulled lupin (<i>Lupinus angustifolius</i>)
Method of analysis:	No analysis conducted
Guideline(s):	No, study from published literature
Deviations:	N/A, study from published literature
GLP or GEP:	No, study from published literature
Acceptability:	Supplementary
Study relied upon:	Yes, however data protection not applicable to published literature studies

I. MATERIALS AND METHODS

A. MATERIALS

Test Material	Commercial dehulled lupin (<i>Lupinus angustifolius</i>)
Lot/Batch #:	Not reported
Purity:	Not reported
Reanalysis/Expiry date:	Not reported
Density:	Not reported
Treatments	
Test rates:	Diets with different inclusion levels of dehulled lupin (10%, 20%, 30%, 40% and 50%)
Control:	Diet with fish meal as the sole protein source
Reference item:	None
Solvent/vehicle:	None
Analysis of test concentrations:	None
Test organisms	
Species:	Rainbow trout (<i>Oncorhynchus mykiss</i>)
Age:	Juvenile
Source:	School of Aquaculture, University of Tasmania
Acclimatisation:	2 weeks
Weight at test start:	47.1 ± 0.2 g
Feeding:	Experimental diet
Test design	
Test vessel:	300-L fibre glass tanks covered with black plastic mesh
Aeration:	Continuous (11 L/min)
Replication:	2 replicates
No. animals/vessel:	38 fish per group
Duration of test:	8 weeks

Environmental test conditions

Temperature:	15 ± 0.5°C
Dissolved oxygen:	Not reported
pH:	Not reported
Photoperiod:	16:8 h light: dark

B. STUDY DESIGN AND METHODS

Prior to the experiment, fish were fed with a commercial diet for a 2-week adaptation period. At the end of this period, fish were re-weighed as 47.1 ± 0.2 (SE)g prior to the start of the experiment.

Juvenile trout were weighed and randomly stocked into 300-L fibre glass tanks in duplicate groups of 38 fish. Tanks were covered with black plastic mesh to minimize disturbance and prevent escape, and supplied with continuously aerated fresh water (11 L/min) at a temperature of $15 \pm 0.5^\circ\text{C}$.

Diets with different inclusion levels of dehulled lupin (10%, 20%, 30%, 40% and 50%) were compared with a diet in which fish meal was the sole protein source (0% diet). Experimental diets were isonitrogenous and isoenergetic. Protein from dehulled lupin replaced 9.88%, 19.76%, 29.64%, 39.53% and 49.41% of protein from fish meal in the diets.

Commercial dehulled lupin (*L. angustifolius*) was sourced through Milne Feeds Company (Western Australia). The dehulled lupin was finely ground to a homogenized flour. All ingredients were mixed and were cold pelleted (3 mm pellet size) after adding 10% water. The moisture content of the diets was lowered using a drier (30°C for 24h).

The experiment lasted for 8 weeks and experimental diets were fed twice a day at 9:00am and 4:30pm at 2% body weight. Feed was distributed by hand to control hierarchy and observe feeding behaviour. Fish weight was recorded every fortnight and rations adjusted accordingly. Mortality was recorded daily, and the weight and number of dead fish noted to adjust feed offered to each tank.

Temperature, dissolved oxygen, total ammonia, nitrite and pH were measured three times weekly and did not exceed values recommended for rainbow trout. A constant photoperiod of 16:8h light: dark was used.

Before starting the experiment random samples of diets were collected for chemical analysis. Ten fish at the start of the experiment and three fish per tank (six fish per treatment) at the end of the experiment were randomly sampled for carcass analysis. At the end of the experiment, four fish were killed, individually weighed and liver and

pyloric caeca weights recorded. Samples of pyloric caeca were frozen in liquid nitrogen and stored at -80°C for digestive enzyme extraction. Samples of proximal intestine were taken, flushed with cold (4°C) phosphate-buffered saline (pH 7.2) to remove the faeces and then fixed in 10% buffered formalin at room temperature for histology and image analysis.

After about 17h fasting, another four fish per tank were randomly sampled, anesthetized and their blood collected from the caudal vein. Blood samples were stored at -80°C until required.

All statistical tests were performed using the SPSS Statistical Analysis Software Program (version 9.0.1 for Windows, 1999). Kolmogorov-Smirnov and Levene's tests were applied to test normality and homogeneity of variance, respectively. One-way analysis of variance was also conducted to assess the effect of dietary treatments on different responses and, when appropriate, the differences between means were tested using Tukey's multiple range test. Probability values of less than 0.05 were considered as significant.

II. RESULTS AND DISCUSSION

The amino acid concentration of both dehulled and whole lupin were lower than that of fish meal. However, this value improved for dehulled lupin compared with whole lupin. The amino acid profiles for both lupin used were well above the fish requirement, with the exception of methionine and cysteine.

Table 9.2.2-15: Comparative amino acid profile of whole lupin, dehulled lupin and fish meal

	Lupin (g/kg)	Dehulled lupin (g/kg)	Fish meal (g/kg)
Arg	33.11	48.70	52.74
His	7.70	11.40	23.91
Iso	12.60	17.60	30.90
Leu	20.00	27.65	50.74
Lys	13.20	16.25	63.33
Met + Cys	6.20	8.40	27.26
Phe + Tyr	23.30	33.35	50.92
Val	12.00	16.55	35.89
Essential amino acid index	1.14	1.58	3.24

Rainbow trout requirements for all essential amino acids were exceeded by all experimental diets.

Table 9.2.2-16: Comparison of the amino acid profile of experimental diets and rainbow trout requirement

	Percentage of dehulled lupin in diet (g/kg)						Requirement (NRC, 1993)
	0%	10%	20%	30%	40%	50%	
Arg	32.3	33.9	35.6	37.3	38.9	40.6	15
His	14.6	14.3	14	13.7	13.4	13.1	7
Iso	18.9	18.8	18.7	18.5	18.4	18.3	9
Leu	31.1	30.7	30.4	30.1	29.8	29.4	14
Lys	38.8	36.5	34.3	32.1	29.8	27.6	18
Met + Cys	16.7	15.9	15.2	14.2	13.4	12.6	10
Phe + Tyr	31.2	31.4	31.6	31.9	32.1	32.3	18
Val	22	21.4	20.9	20.4	19.8	19.3	12
Essential amino acid index	32.3	33.9	35.6	37.3	38.9	40.6	15

All experimental diets were well accepted and survival was nearly 100% for all treatments. Final weight ranged between 125.9 and 139.1 g and there was a significant difference between the control diet (0% dehulled lupin) and 50% dehulled lupin diet. However, there was no significant difference between the control and the other treatments. Feed conversion ratio (FCR) ranged from 0.91 to 1.00 and showed significant differences between the control and 50% dehulled lupin diet. However, FCR remained similar for all other groups. Although protein efficiency ratio (PER) and protein productive value (PPV) were lower at higher inclusion levels of dehulled lupin, there were no significant differences between all treatments. The obvious differences occurred in the energy efficiency ratio (EER), which decreased at higher inclusion levels of dehulled lupin. This value was reasonably similar between the control diet and diets with up to 30% dehulled lupin.

Table 9.2.2-17: Growth response and feed utilisation of rainbow trout fed different experimental diets

Parameter	Percentage of dehulled lupin in diet (g/kg)						P
	0%	10%	20%	30%	40%	50%	
Initial weight (g)	46.9	47.5	46.85	47.75	47.05	46.5	ns
	0.3	0.6	0.15	0.25	0.25	0.4	
Final weight (g)	139.05 ^b	136.5 ^{ab}	134.2 ^{ab}	133.65 ^a _b	131.1 ^{ab}	125.85 ^a	<0.038
	0.45	3.4	1.1	0.75	2.8	1.95	
Weight gain (g)	93.95 ^b	88.99 ^{ab}	87.31 ^{ab}	85.89 ^{ab}	84.06 ^{ab}	79.36 ^a	<0.016
	0.985	2.76	0.975	1.13	2.57	1.6	
Condition factor	1.62	1.58	1.56	1.64	1.52	1.55	ns
	0.05	0.02	0.05	0.1	0.01	0.02	
FCR	0.91 ^a	0.96 ^{ab}	0.98 ^b	0.985 ^b	0.975 ^{ab}	1 ^b	<0.017
	0.01	0.01	0.01	0.01	0.01	0.01	
PER	2.49	2.39	2.37	2.34	2.37	2.31	ns
	0.02	0.02	0.03	0.04	0.04	0.02	
PPV	41.37	41.54	38.3	39.74	39.51	38.94	ns
	0.41	0.27	1.24	1.04	0.81	0.76	
LER	4.94	4.85	4.84	4.9	4.98	4.71	ns
	0.03	0.04	0.06	0.08	0.08	0.05	
LPV	76.46	73.48	74.16	71	67.83	68.24	ns
	4.33	1.93	0.72	0.26	2.22	3.21	
EER	47.76 ^c	45.88 ^{bc}	46.12 ^{bc}	45.09 ^{bc}	44.46 ^{ab}	41.81 ^a	<0.003
	0.04	0.35	0.62	0.73	0.71	0.42	
Mortality (%)	1.4	0	0	0	0	0	ns
	1.4	0	0	0	0	0	

Each value is the mean (SEM) of two replicates.

^{a-c} Means with the same letter are not significantly different (Tukey multiple range test).

Condition factor = $100 \times [\text{whole wet body weight (g)}/\text{fork length (cm)}^3]$

FCR = feed intake (g)/weight gain (g)

PER = weight gain (g)/crude protein intake (g)

PPV (%) = $100 \times [\text{protein retained (g)}/\text{protein intake (g)}]$

LER (lipid efficiency ratio) = weight gain (g)/lipid intake (g)

LPV (lipid productive value = $100 \times [\text{lipid retained (g)}/\text{lipid intake (g)}]$)

EER (g MJ^{-1}) = weight gain (g)/energy intake (MJ)

Carcass composition generally showed similar values for all treatments. However, crude protein composition was significantly higher (16.81%) for the 10% dehulled lupin diet compared with the 20% dehulled lupin (16%).

Table 9.2.2-18: Proximate composition (% wet weight) of rainbow trout fed different experimental diets

Parameter	Percentage of dehulled lupin in diet (g/kg)						P
	0%	10%	20%	30%	40%	50%	
Dry matter	34.46	34.63	34.31	34.05	33.45	33.86	ns
	0.41	0.31	0.24	0.5	0.37	0.54	
Crude protein	16.31 ^{ab}	16.81 ^b	16 ^a	16.53 ^{ab}	16.3 ^{ab}	16.43 ^{ab}	<0.03
	0.23	0.18	0.11	0.2	0.06	0.14	
Crude fat	15.18	14.96	15.1	14.55	14	14.55	ns
	0.49	0.36	0.27	0.58	0.32	0.64	
Ash	2.18	2.23	2.17	2.25	2.18	2.25	ns
	0.03	0.03	0.04	0.02	0.07	0.04	

Each value is the mean (SEM) of two replicates.

^{a,b} Means with the same letter are not significantly different (Tukey multiple range test).

There were no significant differences between any of the digestive tract responses and enzyme activities. Although villus height for the proximal intestine gradually decreased at higher inclusion levels of dehulled lupin, this trend was not significant.

Table 9.2.2-19: Digestive enzyme activity and digestive tract characteristics of rainbow trout fed different experimental diets

Parameter	Percentage of dehulled lupin in diet (g/kg)						P
	0%	10%	20%	30%	40%	50%	
Trypsin activity (mmol pNA g ⁻¹ tissue min ⁻¹)	0.075	0.086	0.08	0.072	0.077	0.076	ns
	0.003	0.002	0.001	0.003	0.004	0.002	
Amylase activity (mmol maltose mL ⁻¹ min ⁻¹)	164.15	157.1	160.55	155.95	162.83	156.85	ns
	3.76	5.06	5.6	2.81	4.05	5.73	
Pyloric caeca index (%)	5.49	4.82	5.46	5.03	4.61	4.8	ns
	0.12	0.14	0.35	0.28	0.07	0.04	
Hepatosomatic index (%)	1.62	1.58	1.56	1.64	1.52	1.55	ns
	0.05	0.02	0.05	0.09	0.01	0.02	
Villus height (Proximal intestine) (µm)	0.075	0.086	0.08	0.072	0.077	0.076	ns
	0.003	0.002	0.001	0.003	0.004	0.002	

Each value is the mean (SEM) of two replicates.

Hematocrit and total Ig were similar for all treatments. However, total plasma protein was significantly lower in the 30%, 40% and 50% dehulled lupin diets compared with the 10% dehulled lupin diet. The white blood cell count also showed similar values for lymphocytes and monocytes. However, the neutrophils count was significantly higher in the 20% dehulled lupin diet compared with the 30% group.

Table 9.2.2-20: Non-specific immune responses of rainbow trout to different experimental diets

Parameter	Percentage of dehulled lupin in diet (g/kg)						P
	0%	10%	20%	30%	40%	50%	
Hematocrit (%)	43.9	43.52	43.18	42.27	4.22	41.31	ns
	1.43	1.5	1.52	1.4	1.68	1.72	
Total protein (mg.mL ⁻¹)	46.3 ^{ab}	48.49 ^b	45.63 ^{ab}	39.38 ^a	38.49 ^a	38.32 ^a	0.001
	1.73	1.13	1.69	1.86	1.76	3.01	
Total Ig (mg.mL ⁻¹)	15.65	15.79	15.39	15.37	15.02	14.66	ns
	0.73	0.73	0.8	0.54	0.71	0.66	
White blood cell count lymphocyte (%)	91.87	90.75	90.25	93.62	91.37	90.87	ns
	1.31	1.39	0.70	0.86	1.42	1.39	
Monocyte (%)	3.12	3.62	2.25	2.5	2.75	2.87	ns
	0.69	0.75	0.55	0.42	0.88	0.51	
Neutrophils (%)	5 ^{ab}	5.62 ^{ab}	7.5 ^b	3.37 ^a	4.87 ^{ab}	6.25 ^{ab}	0.02
	0.77	0.86	0.42	0.59	0.95	1.03	

Each value is the mean (SEM) of two replicates.

^{a,b} Means with the same letter are not significantly different (Tukey multiple range test).

Significant relationships between dietary inclusion level of dehulled lupin and measurement of growth and feed efficiency were described by regression analysis. There was a strong negative linear relationship between higher inclusion levels of dehulled lupin and the growth rate ($R^2 = 0.81$, $P < 0.0001$). A similar negative relationship was observed between the inclusion level of dehulled lupin and the EER ($R^2 = 0.81$, $P < 0.0001$). This relationship between different inclusion levels of lupin and PER was plasma was not significantly different between treatments, total protein concentration of plasma generally decreased at higher inclusion levels of lupin. This parameter was significantly lower in 30%, 40% and 50% dehulled lupin diets compared with the 10% dehulled lupin diet.

III. CONCLUSION

This paper was conducted to examine the effects of dehulled lupin as a partial replacement for fish meal in the diets of rainbow trout.

Results show that dehulled lupin can be included at up to 40% of a rainbow trout diet without a significant effect on growth performance or nutrient utilization. Rainbow

trout utilise dehulled lupin protein as efficiently as fish meal protein but have a lower ability to utilize the energy content of dehulled lupin at higher inclusion levels.

HSE COMMENTS

This study is from the scientific literature and does not conform to standardised test guidelines and is not GLP compliant. The study’s methodology, reporting and analysis have been considered with regard to how well they meet common standards of scientific practice, based on ‘expert judgement’.

The study appears to be well conducted in accordance with scientific principles, although details on the batch of test item and its stability during the test were lacking. *O. mykiss* is a standard species for ecotoxicity testing. The measurements on amino acid profile, feed utilisation, enzyme activity and immune responses are not relevant for use in the risk assessment. The test item was the whole/dehulled seeds of *Lupinus angustifolius*, so the results may be of limited relevance to the assessment of this active substance, which is an aqueous extract from *Lupinus albus* seeds. Additionally, exposure was via diet, so the study results are of limited relevance to the aquatic risk assessment, which considers exposure via concentration in water. However, the study does indicate that inclusion of commercial dehulled lupin (*Lupinus angustifolius*) at up to 40% in the diet, over an 8-week period, does not negatively affect growth of *Oncorhynchus mykiss* juveniles. The results may be considered as supporting evidence in the risk assessment.

Relevance: Not relevant (dietary route of exposure)

Reliability: Reliable with reservations

(Studies are graded as one of the following categories: 1) Reliable, 2) Reliable with reservations, 3) Unreliable. Categorisation is based on the evaluator’s subjective assessment of the quality of the methods, reporting and analysis as presented in the study.)

Reference :	K-CA 8.2.2/08.
Report Title :	Feeding lupins to fish: A review of the nutritional and biological value of lupins in aquaculture feeds
Author(s) & Year :	Glencross, (2001)
Document No :	p. 126. Department of Fisheries–Research Division, Government of Western Australia

Substance used :	N/A, literature review paper
Method of analysis :	No analysis conducted
Guideline(s) :	No, study from published literature
Deviations :	N/A, study from published literature
GLP or GEP :	No, study from published literature
Acceptability :	Supplementary
Study relied upon :	Yes, however data protection not applicable to published literature studies

I. MATERIALS AND METHODS

A. MATERIALS

This report is a review paper; therefore materials vary between papers referenced.

B. STUDY DESIGN

This review considered a number of published studies to detail several facets of the use of lupins in diets for aquaculture species.

Studies on lupin composition as well as the effects of the use of feed containing lupin on a number of aquaculture species were considered.

Full references are not provided in this summary, however are available in the full-text report.

II. RESULTS AND DISCUSSION

The table below details the nutritional value of lupin to a number of species using data from a number of studies referenced in the full paper.

Table 9.2.2-21: Summary of the nutrient value (digestibility %) and available nutrient content of lupin whole-seed and kernel meals and defatted soybean meals in various aquaculture species as determined on an ingredient specific basis

Species and ingredient	Digestibility (%)				Available nutrients		
	Dry matter	Nitrogen	Energy	Phosphorus	Protein (g/kg)	Energy (MJ/kg)	Phosphorus (g/kg)
Rainbow trout (<i>Oncorhynchus mykiss</i>)							
L. angustifolius (whole seed meal)	-	85.5	61	-	275	11.1	-
L. albus (kernel meal)	69.7	96.2	77	62	385	15.7	2
Defatted soyabean meal	71.2	90.1	56	22	439	9.9	2
Silver Perch (<i>Bidyanus bidyanus</i>)							
L. angustifolius (whole seed meal)	57	91.8	51	-	296	9.2	-
L. angustifolius (kernel meal)	68	93.3	62	-	364	11.7	-
L. albus (whole seed meal)	64.7	96.1	73	78	344	14.2	3
L. albus (kernel meal)	77.8	101.4	85	74	406	17.4	4
Defatted soyabean meal	73	95	82	-	463	14.4	-

Species and ingredient	Digestibility (%)				Available nutrients		
	Dry matter	Nitrogen	Energy	Phosphorus	Protein (g/kg)	Energy (MJ/kg)	Phosphorus (g/kg)
Tiger Prawn (<i>Penaeus monodon</i>)							
L. angustifolius (whole seed meal)	67	94	68	-	303	12.3	-
Defatted soyabean meal	67	92	71	-	448	12.5	-
Greenlip Abalone (<i>Haliotis laevis</i>)							
L. angustifolius (whole seed meal)	-	91	80	-	293	14.5	-
L. luteus (whole-seed meal)	61	91	83	84	349	16.3	4
Defatted soyabean meal	57	87	84	86	424	14.8	6

Values derived from a number of studies referenced in the full text of the paper.

III. CONCLUSION

This paper presented a review of literature on the nutritional value of lupins on a number of aquatic species.

In all aquaculture species for which a nutritional assessment was made, they were shown to be a well-accepted and nutritionally useful ingredient. The extent of the nutritional value varied between species and also between studies.

Assessment of the biological value of a range of species and processing forms of lupins, across a range of aquaculture species, also showed a good capacity by fish and crustaceans to use this ingredient in compound feeds.

HSE COMMENTS

This summary does not conform to standardised test guidelines and is not GLP compliant. The summary's discussion has been considered with regard to how well it meet common standards of scientific practice, based on 'expert judgement'.

The above summary is not a supporting data study and instead comprises a summary of the nutritional value of a range of species and processing forms of lupins. The summary for *L. angustifolius*, *L. luteus* and defatted soyabean meal cannot be used in the risk assessment, but may be used as supportive information. It is shown that meals containing lupines can positively influence growth of aqua-cultured fish such as rainbow trout (*Oncorhynchus mykiss*) and Silver Perch (*Bidyanus bidyanus*).

Relevance : Not relevant (dietary route of exposure)

Reliability : Reliable with reservations

(Studies are graded as one of the following categories: 1) Reliable, 2) Reliable with reservations, 3) Unreliable. Categorisation is based on the evaluator's subjective assessment of the quality of the methods, reporting and analysis as presented in the study.)

B.9.2.2.1. Fish early life stage toxicity test

No data submitted or considered necessary.

B.9.2.2.2. Fish full life cycle test

No data submitted or considered necessary.

B.9.2.2.3. Bioconcentration in fish

No data submitted or considered necessary.

B.9.2.3. Potential for endocrine disruption

The main components of aqueous extract from the germinated seeds of sweet *Lupinus albus* (PROBLAD PLUS) have been identified as water, proteins, carbohydrates and lipids (see Volume 4). These components are naturally occurring and will already contribute a large portion of the diet of fish. Additionally, seeds of lupins, including *Lupinus albus*, are widely used as a food source in aquaculture. A variety of published literature has been submitted for assessment, which investigate the effects of including lupin seeds in the diets of aquatic organisms. No indications of toxicity or sub-lethal effects were demonstrated in these studies. As such, the potential effects on endocrine disruption are not considered to be relevant. However, two studies were identified as potentially relevant to endocrine disruption as a result

of the literature search for quinolizidine alkaloids (CEV/02/01-LRR3, 2019). These studies have been summarised and evaluated below. The literature review is described in detail in section Volume 3 CA B9.11 and HSE considered the literature review acceptable for the endocrine disruption ecotoxicology assessment.

Reference:	K-CA 8.2.3/01
Report Title:	Histology and growth performance in rainbow trout (<i>Oncorhynchus mykiss</i>) in response to increasing dietary concentration of sparteine, a common alkaloid in lupins
Author(s) & Year:	Serrano, E. et al. (2011)
Document No	Aquaculture Nutrition, doi: 10.1111/j.1365-2095.2011.00899.x
Substance used:	Sparteine, Purity: 99%
Method of analysis:	No analysis conducted
Guideline(s):	No, study from published literature
Deviations:	Not conducted under GLP/Officially recognised testing facilities
GLP or GEP:	No, study from published literature
Acceptability:	Supplementary
Study relied upon:	Yes, however data protection not applicable to published literature studies

I. MATERIALS AND METHODS

A. MATERIALS

Test Material	Sparteine
Purity:	99%
Source:	Alfa Aesar GmbH & Co., Karlsruhe, Germany
Treatments	
Test rates:	0, 50, 100, 250, 500, 1000, 2500 and 5000 mg sparteine/kg diet

Solvent/vehicle: 30 mL ethanol, fish oil

Test organisms

Species: Rainbow trout (*Oncorhynchus mykiss*)

Acclimatisation period: Ten days

Initial weight: 61 ± 7 g (mean ± SEM)

Feeding: Fish meal-based experimental diet, twice daily to satiety

Test design

Test vessel: Fibreglass tanks of 0.3 m³

No. of animals/vessel : Sixteen fish per tank

Replication: Three replicates per condition

Duration of test: 62 days

Environmental test conditions

Temperature: 13.7 ± 1.8°C

B. STUDY DESIGN

This study was conducted to evaluate the effects of sparteine, a lupin alkaloid, on growth and organ histology in the rainbow trout (*Oncorhynchus mykiss*) after feeding with sparteine-incorporated diets for 62 days.

Fish meal-based diet was manufactured at the Centre for Feed Technology, Norwegian University of Life Sciences, Ås, Norway. Varying concentrations of the quinolidizine alkaloid sparteine (99% purity) were dissolved in 30 mL ethanol, mixed with fish oil and added to batches of the fish meal-based diet to obtain dietary sparteine concentrations of 0, 50, 100, 250, 500, 1000, 2500 and 5000 mg/kg. All experimental diets were formulated to be isonitrogenous and isolipidic (50% crude protein and 17% lipid).

Sixteen juvenile rainbow trout with an initial mean body weight of 61 g were randomly distributed into 24 0.3 m³ fibreglass tanks, which were supplied with freshwater at a flow rate of 7.5 L/min and were held at 13.7 ± 1.8°C.

Prior to the start of the experiment, fish were fed a control diet without sparteine for a period of ten days. Following this period, the eight test diets were hand-fed to the fish to apparent satiety twice daily for 62 days. Satiety was determined visually by a loss in the feeding activity, and was usually reached within a one-hour period.

All fish were individually weighed at the start of the experiment and at the end on day 62.

At the end of the feeding trial, three fish from each tank were assessed for chemical analysis of body composition, and another three were dissected for histological examination of the liver, spleen, kidney and mid and distal intestines. The weights of liver, spleen and intestine in each fish were also measured.

Tissue processing for histology was performed at the Section for Pathology at the Norwegian School of Veterinary Science, Oslo, Norway. Tissues were fixed in 10% phosphate-buffered formalin for 24 hours, dehydrated in ethanol, cleared in xylene and embedded in paraffin. Samples were sectioned (4 to 6 µm thick), stained with haematoxylin and eosin and evaluated by light microscopy.

Thermal growth coefficients (TGC) were calculated as: $TGC = (BW_1 - BW_0) / \Sigma D^\circ$, where BW_1 and BW_0 are final and initial body weights (tank means), respectively, and ΣD° is the thermal sum (feeding days \times mean daily temperature). Weight gain (G) was calculated as: $G = BW_1 - BW_0$. Feed efficiency ratio (FER) was calculated as: $FER = G \times F/1$, where F is the feed intake. Hepato-somatic index (I) was calculated as $I = 100 \times (LW \times BW/1)$, where LW and BW are weight of liver and weight of body, respectively. Intestine- and spleen-somatic indices (ISI and SSI) were calculated in the same manner.

Regression analysis was used to determine the relationships between the response variables and sparteine concentrations in the diet using SAS 8.01. The coefficient of determination (R^2) was used to estimate the proportion of variability explained by the model, at $P < 0.05$.

II. RESULTS AND DISCUSSION

Feed intake, fish growth rate and feeding parameters

Fish survival was not affected by sparteine inclusion, as there was only one mortality over the course of the test, in the 100 mg/kg treatment group. Feed intake was significantly influenced by the dietary inclusion of sparteine. A reduction in feed intake was observed at levels of sparteine above 100 mg/kg diet ($P < 0.0001$). Feed intake decreased from 135 g/fish in the 100 g/kg feed group to 13 g/fish in the 5000 mg/kg feed group.

Growth, assessed as weight gain, final body weight and TGC, was largely consistent with the feed intake. Weight gain decreased quadratically ($P < 0.0001$) with increasing dietary level of sparteine, however the weight gain was similar in all groups receiving diets containing up to 100 mg sparteine/kg. These groups achieved

a weight gain four times higher than those fed the 5000 mg sparteine/kg diet. A similar quadratic trend was also observed for both final body weight and TGC.

Feed efficiency ratio was affected by the dietary inclusion of sparteine. A decline in FER was noticed with inclusion of dietary sparteine above 1000 mg/kg ($P < 0.0001$).

Whole-body composition and organ somatic indices

ISI and I decreased and SSI increased in a quadratic manner with increasing dietary intake of sparteine.

Dry matter content of fish decreased quadratically ($P < 0.0001$) with increasing dietary sparteine. Fish fed diets containing sparteine below 250 mg/kg had higher dry matter content than those fed the higher concentrations. Similarly, the crude lipid content decreased quadratically ($P < 0.0001$), showing a maximum lipid concentration of 120 g/kg in the fish fed 100 mg/kg sparteine. Crude protein and ash contents of fish were not significantly affected by dietary intake of sparteine.

Table 9.2.3-1: Growth performance and organ somatic indices of fish fed experimental diets with increasing doses of sparteine

	Sparteine inclusion (mg/kg)								SEM	Regression	
	0	50	100	250	500	1000	2500	5000		r2	P-value
IW (g)	61.0	60.7	61.3	60.7	60.7	61.0	61.1	61.2	0.45	0.07	0.4479
FW (g)	222.5	232.2	237.3	211.2	196.9	157.2	99.2	65.2	11.90	0.97	<0.0001
TGC	0.23	0.24	0.24	0.22	0.20	0.16	0.07	0.01	0.01	0.98	<0.0001
I	1.28	1.39	1.32	1.17	1.15	1.12	0.86	0.71	0.10	0.83	<0.0001
SSI	0.07	0.07	0.07	0.07	0.08	0.07	0.09	0.15	0.01	0.80	<0.0001
ISI	6.83	6.34	6.83	6.27	6.36	6.40	5.68	4.01	0.43	0.81	<0.0001

IW: initial weight; FW: final weight; SEM: pooled standard error of the mean; TGC: thermal growth coefficient; HIS: hepato-somatic index; SSI: spleen-somatic index; ISI: intestine-somatic index; S: dietary sparteine inclusion.

Each value is the mean of thee replicates.

Histology

Dietary inclusion of sparteine did not cause observable histopathological effects on kidney, spleen, liver or mid and distal intestines. Differences in hepatocyte vacuolation were observed in the liver; fish fed sparteine inclusions up to 1000 mg/kg showed moderate amounts of vacuole within hepatocytes, however, after exceeding this limit less hepatocyte vacuolation was observed.

The mid intestine displayed a decrease in the size of absorptive vacuoles with inclusions up to 100 mg/kg. Sparteine inclusions above 1000 mg/kg resulted in a decrease in size and number of folds in the mid intestine. No differences were observed in any other tissues.

III. CONCLUSION

Assessment and conclusion by applicant

This literature study is supportive only as it was not conducted to GLP or to recognised test guidelines, however the test material was well defined and a relevant species used.

Conclusion:

The findings of this study show that sparteine alkaloid exerts an anti-palatability effect when fed to rainbow trout. A sparteine concentration of 100 mg/kg diet appeared to be the maximum tolerance level with regard to feed intake and growth.

The diet containing 5000 mg/kg resulted in poor growth performance due to insufficient feed consumption to cover the maintenance energy requirement.

In the present experiment, histological examination of the liver, kidney, spleen and mid and distal intestines found no evidence of any tissue alteration attributable to sparteine alkaloid exposure. Intestinal tissue enterocytes did however show a slight decrease in size in dietary treatments over 1000 mg/kg, consistent with the lowered ISI, due to the very low feed intake in these groups.

No hepatotoxic effects were observed in rainbow trout at any dietary concentration.

Histological examination of liver tissue indicated no differences in glycogen and/or lipid storage in hepatocytes from fish fed sparteine concentrations up to 1000 mg/kg. Feed consumption was, however, largely reduced with dietary sparteine levels above 100 mg/kg, which was also reflected in the I values.

Chemical changes in whole-body composition observed in this experiment showed a depletion of the lipid and water content of the fish carcass. These changes are thought to be a result of reduced feed intake.

HSE COMMENTS

This study was submitted as part of the literature search.

This study is from the scientific literature and does not conform to a standard ecotoxicological test guideline, nor is it GLP compliant. Therefore, the study's methodology, reporting and analysis have been considered with regard to how well they meet common standards of scientific practice, based on 'expert judgement'.

The methodology, study design and statistical analysis are well described and are in accordance with general scientific principles. Detailed composition and preparation of the test diets is provided in the study, as is the source of the test chemical. Analytical verification of exposure is not relevant as exposure is through diet rather than via the water.

The purpose of the study was to determine the effects of the alkaloid 'sparteine' on the health and growth of rainbow trout and investigate the toxicity through histological analysis. The alkaloid 'sparteine' is stated to be one of the main alkaloids in yellow lupin (*Lupinus luteus*). This is a different species to the lupin under assessment in this dossier, *L. albus*, which is a 'sweet' lupin (low in alkaloids).

The study does indicate that the inclusion of 'sparteine' at up to 100 mg/kg in rainbow trout diet did not have a negative effect on growth parameters, whereas from 250 mg/kg onwards adverse effects on growth were observed. The adverse effect on growth was attributed to reduced palatability of the test diet causing

reduced feed intake, rather than short-term toxic effects, with any histopathological or body composition changes (mainly occurring from 1000 mg/kg onwards) being discussed by the authors in the context of general starvation responses.

However, the results of this study may be of limited relevance in the risk assessment since the test item ‘sparteine’ is a single compound, rather than a whole extract, and furthermore is from a different lupin species than that currently under assessment. Furthermore, since this study exposed fish through diet, it may be of limited use in risk assessment because it is a different exposure route to the risk assessment, which is via water exposure.

Reliability: Reliable with reservations.

(Studies are graded as one of the following categories: 1) Reliable, 2) Reliable with reservations, 3) Unreliable. Categorisation is based on the evaluator’s subjective assessment of the quality of the methods, reporting and analysis as presented in the study.)

Reference:	K-CA 8.2.3/02
Report Title:	Responses in rainbow trout (<i>Oncorhynchus mykiss</i>) to increasing dietary dose of lupinine alkaloid
Author(s) & Year:	Serrano, E. et al. (2008)
Document No	J.A. Palta and J.B. Berger (eds). 2008. ‘Lupins for Health and Wealth’ Proceedings of the 12 th International Lupin Conference ISBN 0-86476-153-8
Substance used:	Lupinine, Purity: 97%
Method of analysis:	No analysis conducted
Guideline(s):	No, study from published literature
Deviations:	Not conducted under GLP/Officially recognised testing facilities
GLP or GEP:	No, study from published literature
Acceptability:	Supplementary
Study relied upon:	Yes, however data protection not applicable to published literature studies

I. MATERIALS AND METHODS

A. MATERIALS

Test Material	Lupinine
Purity:	97%
Source:	Alfa Aesar GmbH & Co., Karlsruhe, Germany

Treatments

Test rates:	0, 50, 75, 100, 250, 500, 1000 and 5000 mg lupinine/kg feed
Solvent/vehicle:	Ethanol and fish oil

Test organisms

Species:	Rainbow trout (<i>Oncorhynchus mykiss</i>)
Initial weight:	330 g
Feeding:	Low temperature fish meal (LT-fish meal) with varying levels of lupinine

Test design

Test vessel:	300-L circular fibreglass tanks
No. of animals/vessel :	16
Replication:	Two replicates per condition
Duration of test:	60 days

Environmental test conditions

Temperature:	14°C
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B. STUDY DESIGN

This study was conducted to evaluate the effects of lupinine, a lupin alkaloid, on growth, tissue histology and nutritional composition in the rainbow trout (*Oncorhynchus mykiss*) after feeding with lupinine-incorporated diets for 60 days.

The diets contained low temperature fish meal (LT-fish meal) as a main protein source and were formulated to contain 45% crude protein and 20% lipids. The diets were extruded and pelleted prior to the addition of lipids and lupinine. Lupinine powder (97% purity) was dissolved in a small volume of ethanol and mixed with fish oil. The solutions containing different levels of lupinine were added to uncoated feed

by vacuum-coating at concentrations of 0, 50, 75, 100, 250, 500, 1000 and 5000 mg lupinine/kg feed, respectively. All feed was manufactured at the Centre for Feed Technology, Norwegian University of Life Sciences, Ås, Norway.

Twelve rainbow trout with an average initial body weight of 330 g were randomly allocated in 16 300-L circular fibreglass tanks, which were supplied with freshwater at a flow rate of 7.5 L/min and were held at an average temperature of 14°C. Each test diet was given to duplicate tanks over a period of 60 days, with fish being fed twice daily using automatic feeders set to 20% overfeeding based on estimated intake.

All individual fish were weighed at test start, and days 20, 40 and 60.

At the end of the trial, three fish from each tank were assessed for chemical analysis of whole body composition, and another three had kidneys, spleens, brains, hearts, livers and middle and distal intestines dissected.

Tissue samples for histology were fixed in 4% phosphate buffered formalin, dehydrated in ethanol, embedded in paraffin, pieced (4 to 6 µm) and stained with haematoxylin and eosin. Sample sections were evaluated for lesions under a light microscope.

All data were analysed by linear regression with lupinine levels as the classification variable, using SAS at $P \leq 0.05$. A one-way ANOVA was also performed and significant difference between means was determined by Duncan's test.

II. RESULTS AND DISCUSSION

Mortality was low throughout the entire experimental period, only disturbed by a biofilter fault in the middle of the experiment. Mortalities were not related to dietary treatments. Fish receiving the negative control (5000 mg lupinine/kg) were terminated for welfare reasons after 40 days due to poor feed intake and growth performance. Feed intake and growth rates declined in a linear manner in response to dietary inclusion of lupinine. Additional analysis of variance confirmed a minimal effects on growth and feeding parameters by the inclusion of lupinine levels below 250 mg/kg.

Histological examination of liver tissue showed a slight reduction of fat droplets and glycogen stores in the hepatocytes with increasing levels of lupinine.

There were no significant differences in the fish body composition that could be related to lupinine inclusion or starvation.

Rainbow trout in this study displayed no evident lesion in the middle and distal intestine, kidney, heart or liver when fed lupinine alkaloid.

These results suggest that the rainbow trout may be able to tolerate lupin alkaloids to a higher extent than other vertebrates. Histopathological conditions such as parenchymatous and vacuolar degeneration of hepatocytes, inflammatory lesions in

hepatocytes, focal necrosis of epithelium of renal tubules and focal hyaline degeneration of heart muscle fibres were not observed at any lupinine inclusion level with rainbow trout.

III. CONCLUSION

Assessment and conclusion by applicant

This literature study is supportive only as it was not conducted to GLP or to recognised test guidelines, however the test material was well defined and a relevant species used.

Conclusion:

The findings of this study show that the lupinine alkaloid had a clear palatability reducing effect on rainbow trout at inclusion levels above 100 mg/kg diet. When incorporated into the diet at levels below 250 mg/kg, only minimal effects on growth and feeding parameters were observed. No significant differences in the fish body composition could be related to lupinine inclusion or starvation.

Inclusion of the lupinine alkaloid in diet had no effect on the mortality of fish.

Histological examination of liver tissue showed a slight reduction of fat droplets and glycogen stores in the hepatocytes with increasing levels of lupinine. No other histopathological effects were observed

HSE COMMENTS

This study was submitted as part of the literature search.

This study is from the scientific literature and does not conform to a standard ecotoxicological test guideline, nor is it GLP compliant. Therefore, the study's methodology, reporting and analysis have been considered with regard to how well they meet common standards of scientific practice, based on 'expert judgement'.

Detailed composition of the test diets is provided in the study, as is the source of the test chemical and place of feed manufacture. Analytical verification is not relevant as exposure is through diet rather than via the water. The methodology, study design and statistical analysis are well described and are accordance with general scientific principles, although the raw data on mortality is not provided, nor is the tabular data for body weight, feed intake or feed conversion ratio (only figures are provided). This may be because the paper was prepared as a summary for conference proceedings rather than journal publication.

The purpose of the study was to determine the effects of the alkaloid 'lupinine' on the health of rainbow trout and determine the acute toxicity concentration. The alkaloid 'lupinine' is stated to be the main quinolizidine alkaloid from a species of yellow lupin (*Lupinus luteus*). This is a different species to the lupin under assessment in this dossier, *L. albus*, which is a 'sweet' lupin (low in alkaloids).

The study does indicate that the inclusion of 'lupinine' at up to 100 mg/kg in rainbow trout diet did not have a negative effect on growth parameters, whereas from

250 mg/kg onwards adverse effects were observed. These were attributed by the authors to reduced palatability of the test diet causing reduced feed intake rather than short-term toxic effects, as evidenced by the absence of histopathological effects in all organs apart from liver, for which the authors suggest is a common starvation response.

However the results of this study may be of limited relevance in the risk assessment since the test item ‘lupinine’ is a single compound, rather than a whole lupin extract, and furthermore is from a different lupin species than that currently under assessment. Also, due to the exposure in this study being through diet, the relevance of this will need to be considered in risk assessment, where exposure is estimated via water, therefore it may be difficult to compare exposure routes.

Reliability: Reliable with reservations.

(Studies are graded as one of the following categories: 1) Reliable, 2) Reliable with reservations, 3) Unreliable. Categorisation is based on the evaluator’s subjective assessment of the quality of the methods, reporting and analysis as presented in the study.)

Summary and conclusions for aquatic organism endocrine disruption:

Two literature studies were identified as potentially relevant to endocrine disruption as a result of literature search CEV/02/01-LRR3, 2019 (see section 9.1.1 for summary). Having reviewed the studies, HSE considers them both to be reliable with reservations, but of limited relevance for the assessment of aqueous extract from the germinated seeds of sweet *Lupinus albus*. Both studies focussed on the effects of specific alkaloids found in lupins, rather than a whole lupin extract. Additionally, the exposure was via diet, rather than via water. Histopathological examinations were made of liver tissues but the measured parameters would not be sufficient for investigation into EATS-mediated activity.

As previously stated, the main components of aqueous extract from the germinated seeds of sweet *Lupinus albus* are water, proteins and carbohydrates which are already likely to form part of the diet of aquatic organisms. Although there is some uncertainty in relying on dietary exposure studies to assess the risk via aquatic exposure, the published literature does not indicate any adverse effects from inclusion of lupin seeds in fish diets. Therefore HSE considers that no further consideration of endocrine disruption is required.

B.9.2.4. Acute toxicity to aquatic invertebrates

Reference:	KCA 8.2.4.1/01
Report Title:	Assessment of Toxic Effects of PROBLAD on <i>Daphnia magna</i> using the 48 h Acute Immobilisation Test.

Author(s) & Year:	Weber, K. (2011)
Document No	CEV SA, Unpublished report No. : S10-02622
Substance used:	PROBLAD, (Batch: 201009, 20% BLAD, 1.30 g/cm ³)
Method of analysis:	No analysis conducted
Guideline(s):	OECD Guidelines for Testing of Chemicals, Method 202: <i>Daphnia</i> sp., Acute Immobilisation Test (2004)
Deviations:	Yes, see HSE comments
GLP or GEP:	Yes
Acceptability:	Supplementary
Study relied upon:	Yes, used in a reasoned case to support the aquatic risk assessment.

I. MATERIALS AND METHODS

A. MATERIALS

Test Material

PROBLAD PLUS,

Lot/Batch #:

Batch no. 201009

Purity:

BLAD content 20.0% w/w, according to certificate of analysis.

Reference testing

Potassium dichromate tested at 1.0 mg/L and 2.0 mg/L.

Treatments

Range-finding test:

0 (dilution water control), 0.01, 0.10, 1.00, 10.0, 100 mg/L.

Test concentrations:

0 (dilution water control), 7.81, 15.6, 31.3, 62.5, 125 and 250 mg/L.

Solvent:

N/A

Analysis of test concentrations:

No analytical verification performed.

Test organisms

Species:	<i>Daphnia magna</i> STRAUS, Clone V.
Age:	6–24 hours.
Source:	Continuous laboratory cultures, originally purchased from Umweltbundesamt in Berlin, Germany.
Breeding conditions :	One daphnid per 100ml. Water composed of dechlorinated drinking water and deionised water. pH 6-9. Dissolved oxygen > 60% saturation. Hardness 140-268 mg/L. Temperature $20 \pm 2^{\circ}\text{C}$. 16h illumination and 8h darkness. Medium changed 3 x per week.
Feeding :	During culturing: Single cell green algae (<i>Desmodesmus subspicatus</i>) at least 3 x per week. During test: none.
Acclimatisation period:	Not necessary since test was performed in the same medium as used in culture.

Test design

Test vessels:	100 ml glass beakers, filled with 50 ml test medium.
Test medium:	Dechlorinated drinking water and deionised water. pH at initiation: 8.08. Dissolved oxygen : 97% saturation. Total hardness : 196.33 mg/L as CaCO_3 .
Replication:	4 test vessels per control and test concentration
No. of organisms per tank:	5 (total 20 daphnids per concentration)
Exposure regime:	Semi-static, media renewal at 24 hours.
Duration:	48 hours

Environmental conditions

Test temperature:	20.2–21.0°C
pH range:	7.87–8.47

Dissolved oxygen:	27–103%
Total hardness of dilution water:	196.33 mg/L CaCO ₃ at test initiation
Lighting:	16 hours light and 8 hours dark, 1100 lux

B. STUDY DESIGN AND METHODS

Daphnids were exposed over a period of 48 hours to PROBLAD PLUS at nominal concentrations corresponding to 0 (dilution water control), 7.81, 15.6, 31.3, 62.5, 125 and 250 mg/L. Two concentrations of the reference item, potassium dichromate (1.0 mg/L, 2.0 mg/L) were also tested. To prepare the stock solution, 125 mg of test item was weighed on weighing scoops and transferred to a volumetric flask. Test medium was added up to the bench mark and the solution was homogenised by shaking. The lower test solutions were prepared by dilution of the stock solution. Four tests vessels per treatment level and for the control were prepared. Daphnids < 24 hours old were selected and distributed until each vessel contained 20 daphnids. The test was initiated when 5 daphnids were introduced to each replicate exposure vessel. The daphnids were not fed during the 48 hour exposure period.

The number of immobilized daphnids observed in each replicate test vessel was recorded at test initiation and after 24 and 48 hours of exposure. All daphnids not able to swim within 15 seconds after gentle agitation of the test vessel were considered to be immobilised.

Measurements of temperature, pH and dissolved oxygen concentrations were made at 0, 24 and 48 hours in one replicate of each treatment level and the control. The water temperature was 20.2–21°C. Dissolved oxygen was 27–103%. The pH of the test media was 7.87–8.47.

The 48 hour EC₅₀ was determined using Spearman-Kaerber estimator (Anellis and Werkowsky, 1968).

II. RESULTS AND DISCUSSION

Range-finding test:

After 24 hours of exposure no immobilisation was observed in the control and up to the highest test item concentration of 100 mg/L. After 48 hours no immobilisation was observed in the control and up to 10.0 mg/L. At the highest test item concentration of 100 mg/L 30% immobilisation was observed. The results are presented in the table below.

Table 9.2.4-1: Corresponding immobilization and observations made during the 48-hour range-finding exposure of daphnids (D. magna) to PROBLAD PLUS

Nominal Concentration (mg PROBLAD PLUS/L)	Cumulative Percent of Immobilized Organisms										
	24-Hour					48-Hour					
	1	2	3	4	Total	1	2	3	4	Total	%
Control	0	0	0	0	0	0	0	0	0	0	0
0.01	0	0	0	0	0	0	0	0	0	0	0
0.10	0	0	0	0	0	0	0	0	0	0	0
1.00	0	0	0	0	0	0	0	0	0	0	0
10.0	0	0	0	0	0	0	0	0	0	0	0
100	0	0	0	0	0	0	2	3	1	6	30

Main test:

Details are provided in Table 9.2.4-2. Following 48 hours of exposure, no immobilisation was observed in the control and up to 62.5 mg/L. At 125 mg/L 10% immobilisation was observed. At the highest test item concentration of 250 mg/L 15% immobilisation was observed. After 48 hours no immobilisation was observed in the control and up to 15.6 mg/L. At 31.3 mg/L one immobile daphnid was observed, 15% immobilisation was observed at 62.5 mg/L and 20% immobilisation at 125 mg/L. At the highest test item concentration of 250 mg/L 75% of the daphnids were immobile.

Table 9.2.4-2: Corresponding immobilization and observations made during the 48-hour exposure of daphnids (*D. magna*) to PROBLAD PLUS

Nominal Concentration (mg PROBLAD PLUS/L)	Cumulative Percent of Immobilized Organisms											
	24-Hour						48-Hour					
Replicate:	1	2	3	4	Total	%	1	2	3	4	Total	%
Control	0	0	0	0	0	0	0	0	0	0	0	0
7.81	0	0	0	0	0	0	0	0	0	0	0	0
15.6	0	0	0	0	0	0	0	0	0	0	0	0
31.3	0	0	0	0	0	0	0	0	0	1	1	5
62.5	0	0	0	0	0	0	1	0	1	1	3	15
125	1	1	0	0	2	10	1	3	0	0	4	20
250	1	1	0	1	3	15	5	5	0	5	15	75

Environmental conditions:

The oxygen concentration at the highest test item concentration of 250 mg/L decreased at t = 24 hours aged to 27% and at t = 48 hours aged to 29%. This deviation from the study plan was due to the influence of the test item and it is considered to have no impact on the study since the control is valid. The oxygen content in the control test vessels and each of the test item vessels with the exception of the highest test concentration of 250 mg/L remained within the required criteria of > 3 mg/L (approximately 33%) for the duration of the study. The control group consistently remained above 95% oxygen saturation and so indicated the test design was valid. For the highest concentration of 250 mg/L in the 24h and 48h aged samples only the oxygen saturation dropped marginally below the required criteria of 3 mg/L or equivalent of 33% dissolved oxygen (27 and 29% respectively). The fresh media at the renewals were shown to have high oxygen saturation of 98 and 91% and the mean saturation was 61% (approximately 6 mg/L) which exceeds the required oxygen concentration. The drop in oxygen in the one test item group is not considered to have affected the study.

Toxic reference item:

The results of the toxic reference item study with potassium dichromate are summarised in the table below.

Table 9.2.4-3: Corresponding immobilization and observations made during the 48-hour exposure of daphnids (*D. magna*) to potassium dichromate

K₂Cr₂O₇	24 h		48 h	
	1.0mg/L	2.0mg/L	1.0mg/L	2.0mg/L
Replicate	Immobilised daphnids			
1	0	5	2	5
2	0	4	2	5
3	0	4	1	5
4	0	5	1	5
Σ	0	18	6	20
%	0	90	30	100

An EC₅₀ value has not been calculated for the toxic reference item but the results indicate it is between 1.0 and 2.0 mg/L.

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 in all test vessels	≥ 3 mg/L (33%)	29% in 48h aged media in 250 mg/L test concentration.

III. CONCLUSION

Based on nominal concentrations, the 48-hour EC₅₀ of PROBLAD PLUS to *Daphnia magna* was determined to be 159.32 mg/L. The 48-hour NOEC was determined to be 31.3 mg/L (nominal).

HSE COMMENTS

This study has been conducted in accordance with GLP and follows OECD 202 (2004).

The reference item potassium dichromate has been tested at concentrations of 1.0 and 2.0 mg/L. An EC₅₀ has not been calculated however the results indicate that it likely lies between 1.0 and 2.0 mg/L. This is in line with the range from ring-tests referenced in OECD 202 of 0.6-2.1 mg/L and therefore demonstrates the sensitivity of the test system.

There is some uncertainty as to whether the validity criterion for dissolved oxygen concentration has been met. The validity criterion requires a dissolved oxygen concentration of 3 mg/L, which is roughly equivalent to 33%. This was achieved in the control test vessels and all concentrations of the test item, excluding the 250 mg/L treatment, at which 27% dissolved oxygen was reported in the aged test media after 24h and 29% dissolved oxygen was reported after 48h. This is stated to have been an effect of the test item but no further detail has been provided. It is not stated whether or not the test medium was aerated prior to exposure in order to reach O₂ saturation, as is recommended in the guideline, however the fresh media oxygen saturation ranged from 91-98%.

No analytical verification has been conducted in this study. The guideline stipulates that, at a minimum, samples should be taken in the highest and lowest concentrations at the beginning and end of the test. As such it is not possible to verify that the nominal test item concentrations were achieved or maintained over the course of the study. This raises uncertainty in the reliability of the endpoint derived.

The EC₅₀ value has been determined using Spearman-Kaerber procedure. No dose-response curve has been provided so it is not possible to check the visual fit of the data. Additionally, no 95% confidence intervals are provided, which raises further uncertainty in the endpoint determined.

Additionally, the following minor guideline deviations are noted:

- Abnormal behaviour or appearance has not been measured or reported as recommended.
- Temperature has been reported at 0, 24 and 48h, rather than continuously as recommended in the guideline. However since the temperature did not vary by > 0.3°C across all time points and test vessels, this is not a critical deviation.

Overall there are a number of uncertainties with the study design and the endpoints derived. The validity criteria do not appear to have been met, there have been no

analytical measurements taken and no 95% confidence intervals have been provided. **As such the endpoint is not considered suitable for use in risk assessment, but could be used as supporting information.**

Reference:	K-CA 8.2.4.1/02
Report Title:	PROBLAD PLUS: A 48-hour static-renewal acute toxicity test with the cladoceran (<i>Daphnia magna</i>).
Author(s) & Year:	Gerke, A.K. and Schneider, S.Z. (2019)
Document No	CEV SA, Unpublished report No.: 896A-101
Substance used:	PROBLAD PLUS, (Batch: F0-1218, 21% BLAD, 1.21 g/cm ³)
Method of analysis:	Enzyme-Linked Immunosorbent Assay (ELISA). Method reference S18-08251.
Guideline(s):	OECD Guidelines for Testing of Chemicals, Method 202: <i>Daphnia</i> sp., Acute Immobilisation Test (2004) U.S. EPA OCSPP 850.1010
Deviations:	Yes, see HSE comments
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

I. MATERIALS AND METHODS

A. MATERIALS

Test Material	PROBLAD PLUS, ,
Lot/Batch #:	Batch no. F0-1218
Purity:	100% w/w , 21.0% BLAD
Reference testing	Potassium dichromate tested at 1.0 mg/L and 2.0 mg/L.

Treatments

Rang-finding study:	0.39, 1.6, 6.2, 25 and 100 mg/L.
Nominal:	0 (dilution water control), 7.5, 15, 30, 60, 120 mg/L.
Mean measured:	<LOQ, 2.2, 5.0, 12, 31 and 75 mg/L.
Solvent:	N/A
Analysis of test concentrations:	Yes, sampling at day 0 and 24 of two replicates per test concentration. Analysis via Enzyme-Linked Immunosorbent Assay (ELISA)

Test organisms

Species:	Cladoceran (<i>Daphnia magna</i>) neonates
Age:	< 24 hours old.
Source:	In-house culture
Acclimatisation period:	Cultured in water from the same source and at approximately the same temperature as used during the test
Feeding:	None during test

Test design

Test vessels:	250-mL glass beakers containing approx. 200 mL test solution, with a depth of 6.1 cm
Test medium:	Fresh well water from a 40-m deep on-site well
Replication:	Four chambers per test concentration
No. of organisms per tank:	Five daphnids per chamber
Exposure regime:	Semi-static, media renewal at 24 hours.
Duration:	48 hours

Environmental conditions

Test temperature:	19.9–20.6°C
pH range:	8.3–8.6
Dissolved oxygen:	5.1–9.0 mg/L, no aeration
Total hardness of dilution water:	128 mg/L CaCO ₃ on D0
Lighting:	16 hours light and 8 hours dark, with 30 minute transition period at 584 lux

B. STUDY DESIGN

Study design

This study was conducted to determine the effects of PROBLAD PLUS exposure on the cladoceran *Daphnia magna* during a 48-hour static-renewal exposure. Test concentrations were selected based on the results of exploratory range-finding toxicity data.

Daphnids were exposed to a geometric series of five test concentrations and a negative control (dilution water) for 48 hours under static-renewal conditions. The daphnids were transferred to newly-prepared test solutions at approximately 24 hours. Four replicate test chambers were maintained in each treatment and control group, with five daphnids in each test chamber, for a total of 20 daphnids per concentration. Nominal test concentrations selected were 7.5, 15, 30, 60 and 120 mg test item/L. Test concentrations were measured in samples of test water collected from each treatment and control group at the beginning of the test, before and after renewal at approximately 24 hours, and at the end of the test.

Neonate daphnids < 24-hours old were impartially assigned to exposure chambers at test initiation. Observations of immobility and other signs of toxicity were made approximately 3, 24 and 48 hours after test initiation. Cumulative percent immobility observed in the treatment groups was used to determine EC₅₀ values at 24 and 48-hour intervals. The no-immobility concentration and the 100% immobility concentration were determined by visual interpretation of the immobility data. The NOEC was empirically estimated from the immobility data.

Adult daphnids were fed a mixture of yeast, cereal grass media, and trout chow, supplemented with a vitamin stock solution and a suspension of green alga during the 24-hour period prior to test initiation, however neonates were not fed during the course of the test.

Water used for culturing and testing was freshwater obtained from a well approximately 40 meters deep located on the Eurofins-Easton site. The well water

was passed through a sand filter to remove particles greater than approximately 25 µm, aerated, filtered to 0.45 µm, and passed through a UV steriliser. Test chambers were 250 mL glass beakers containing approximately 200 mL test solution, the depth of solution in a representative test chamber was 6.1 cm. Each test chamber was loosely covered with a plastic petri dish and placed in an environmental chamber to maintain the target water temperature throughout the test period.

A primary stock solution was prepared by mixing a calculated amount of test substance in 2 L of dilution water at a nominal concentration of 120 mg/L, the highest concentration tested. The stock solution concentration was not adjusted for the purity of the test substance during preparation, and all concentrations are based on the test substance as received. The stock solution was brought to volume and stirred on a magnetic stir plate for one minute. The stock solution appeared clear and pale yellow with no visible particulates. Aliquots of the primary stock solution were proportionally diluted with dilution water to a final volume of 1 L to prepare four additional test solutions at nominal concentrations of 7.5, 15, 30 and 60 mg/L. The solutions were stirred on a magnetic stir plate for one minute, and approximately 200 mL of solution was placed in each of four replicate test chambers per treatment group. The negative control solution was dilution water only. New test solutions were prepared on Day 1 and surviving daphnids were transferred to the new test solutions at approximately 24 hours.

Test systems were illuminated following a 16-hour light to eight-hour dark photoperiod, with a 30-minute transition period in between. Light intensity at test initiation was 584 lux at the surface of the water of one representative test chamber. Temperature was measured in each test chamber at the beginning of the test, at approximately 24 hours during the test (both pre- and post-renewal), and at the end of the test using a digital thermometer. Temperature was also monitored continuously in a representative vessel adjacent to the test system.

Dissolved oxygen and pH were measured in each test chamber at the beginning of the test, at approximately 24 hours during the test (both pre- and post-renewal), and at the end of the test. Water temperatures ranged from 19.9 to 20.6°C over the course of the test. Dissolved oxygen concentrations remained $\geq 56\%$ of the air saturation (5.1 to 9.0 mg/L), and measurements of pH ranged from 8.3 to 8.7.

Analytical method

Samples of the control and treated media were analysed using the validated analytical method report reference S18-08251.

Triplicate water samples were collected from each treatment and control group at the beginning and end of each renewal period during the test to determine concentrations of the test substance. Newly prepared batch solutions were sampled

on Day 0 and at 24 hours (± 1 hour), and the 24-hour old solutions in two of the four replicate test chambers in each group were sampled at 24 and 48 hours (± 1 hour). Samples (20 mL) were collected from mid-depth and placed in polypropylene centrifuge tubes. Two sets of samples collected at each sampling interval were stored frozen (i.e., $\leq -18^{\circ}\text{C}$) until shipment for analysis. The other set of samples was stored frozen as back-up samples for possible analysis. Samples were analysed by Enzyme-Linked Immunosorbent Assay (ELISA).

II. RESULTS AND DISCUSSION

Range-finding test:

Percent immobility in the 0.39, 1.6, 6.2, 25 and 100 mg/L treatment groups at test termination was 0, 0, 0, 0 and 0%, respectively. No sub-lethal effects were observed at test termination, but daphnids in the 25 and 100 mg/L treatment groups appeared larger than the daphnids in the negative control group.

Definitive study:

All daphnids in the negative control and all the treatment groups appeared normal throughout the test.

At test termination, survival in the negative control and each of the 2.2, 5.0, 12, 31, and 75 mg/L treatment groups was 100%, as shown in Table 9.2.4-1. No statistical analyses were conducted on account of the lack of effect.

The resulting NOEC and LOEC for survival were determined to be 75 and > 75 mg/L, respectively. EC_{10} , EC_{20} , and EC_{50} values were empirically estimated to be greater than the highest concentration tested (> 75 mg/L).

Table 9.2.4-4: Cumulative immobility of Daphnia magna exposed to PROBLAD PLUS for 48 hours

Mean measured concentration (mg/L)	Number exposed	Cumulative immobility (%)		
		3 hours	24 hours	48 hours
Negative control	20	0.0	0.0	0.0
2.2	20	0.0	0.0	0.0
5.0	20	0.0	0.0	0.0
12	20	0.0	0.0	0.0
31	20	0.0	0.0	0.0
75	20	0.0	0.0	0.0

A summary of the resulting NOEC, LOEC, and ECx values is presented in the table below:

Table 9.2.4-5: Summary of endpoints

Parameter	NOEC (mg/L)	LOEC (mg/L)	EC ₁₀ (mg/L)	EC ₂₀ (mg/L)	EC ₅₀ (mg/L)
Immobility	75	> 75	> 75	> 75	> 75

95% confidence limits could not be calculated

Analytical results:

Measured concentrations of the test item in fresh and spent solutions are reported in the table below and expressed as a percentage of the nominal value. Study endpoints were based on mean measured concentrations.

Table 9.2.4-6: Summary of analytical results

Nominal concentrations (mg a.s./L)	Measured 0 h (fresh) (mg a.s./L)	% of nominal	Measured 24 h (spent) (mg a.s./L)	% of nominal	Measured 24 h (fresh) (mg a.s./L)	% of nominal	Measured 48 h (spent) (mg a.s./L)	% of nominal
Control	<LOQ	N/A	<LOQ	N/A	<LOQ	N/A	<LOQ	N/A
7.5	3.38	45.1	1.96	26.1	1.28	17.1	2.02	26.9
15	8.16	54.4	5.17	34.5	3.66	24.4	3.02	20.1
30	14.52	48.4	14.20	47.3	10.72	35.7	7.34	24.5
60	40.75	67.9	32.54	54.2	23.01	38.4	27.62	46.0
120	64.87	54.1	103.77	86.5	62.84	52.4	68.58	57.2

LOQ = 0.03 mg/L with LOD of 0.01 mg/L.

Table 9.2.4-7: Mean measured concentrations and percentage of nominal

Nominal concentrations (mg a.s./L)	Mean measured concentration ¹ (mg a.s./L)	Percent of Nominal ² (%)
Control	<LOQ	NA
7.5	2.2	29
15	5.0	33
30	11.7	39
60	31.0	52
120	75.0	63

¹Based on four measured values. ²Mean measured concentration as a % of nominal concentration

Validity Criteria:

The validity criteria in accordance with OECD 202 (2004) were met:

Validity criterion	Required	Obtained
Immobilisation and sub-lethal effects in control during test	≤ 10%	0%
Dissolved oxygen concentration at the end of the test in all test vessels	≥ 3 mg/L (33%)	≥ 5.1 mg/L

III. CONCLUSION:

The cladoceran, *Daphnia magna*, was exposed for 48 hours under static-renewal conditions to five mean measured concentrations of PROBLAD PLUS ranging from 2.2 to 75 mg/L. Based on the mean measured concentrations, the 48-hour EC₅₀ value was > 75 mg/L, the highest concentration tested. The no-immobility concentration was 75 mg/L, the 100% immobility concentration was > 75 mg/L and the NOEC was 75 mg/L.

HSE COMMENTS:

This study has been conducted in accordance with GLP and follows OECD 202 (2004) guidelines. The validity criteria have been satisfied.

The OECD 202 guideline states “A reference substance may be tested for EC₅₀ as a means of assuring that the test conditions are reliable”. A reference test has not been conducted in this study, nor are the results of previous reference tests reported. This calls in to question the sensitivity of the test system but since a reference test is not an explicit requirement or part of the validity criteria, the omission is not cause to invalidate the study.

A minor deviation noted is that the total hardness of the test medium was lower than recommended in OECD 202. The reported hardness was 128 mg/L, while the guideline recommends a hardness of 140-250 mg/L. However, since the validity criteria were met, this deviation is not thought to have impacted the study outcome.

The validity of the analytical method has been considered in Volume 3 CA B5. The following conclusion was reached: “The analytical method for the determination of PROBLAD PLUS in W4 can be considered fully validated in accordance with SANTE/2020/12830 rev. 1”. The analytical recoveries indicated that the test item was not within ± 20% of nominal concentration in either the fresh or aged solutions, which would suggest that the correct dosing of test item was not achieved or maintained. It is also noted that higher concentrations appeared to be recovered in the spent solutions at 48 h than were present at 24 h. HSE considers that these

results reflect the difficulties in detecting and quantifying UVCB substances and considers that there may be limitations in the ability of the ELISA method to provide quantitative results, despite the method being validated. It is noted that there was some uncertainty in the quantification of the ELISA method used in the Residues assessment (method validation in Volume 3 CA B5).

Considering the method of preparation of the test solutions, HSE has some degree of confidence that the nominal concentrations would have been achieved in the fresh solutions, despite the low recoveries. The method of preparation is relatively simple, with a defined amount of test item being added to a fixed volume of dilution medium to produce a stock solution, which is then serially diluted. The precise preparation methods are available from the raw data and could be recreated. HSE considers that, while there is some uncertainty in the measured test concentrations, the nominal dosing was likely to have been achieved in the fresh solutions and the mean measured concentrations over the course of the test will provide a conservative estimate of the toxicity endpoint.

As no immobility was observed in any of the treatment levels, statistical analyses could not be conducted for determination of the EC₅₀ or NOEC values. The EC₅₀ was instead estimated to be > 75 mg/L, the highest test item concentration tested.

Overall, the endpoint suitable for use in risk assessment is:

- 48 h EC₅₀ > 75 mg PROBLAD PLUS/L (mean measured).

B.9.2.5. Long-term and chronic toxicity to aquatic invertebrates

B.9.2.5.1. Reproductive and development toxicity to *Daphnia magna*

Reference:	K-CA 8.2.5.1/01
Report Title:	PROBLAD PLUS: A semi-static life-cycle toxicity test with the cladoceran (<i>Daphnia magna</i>)
Author(s) & Year:	Gerke, A.K. and Schneider, S.Z. (2019)
Document No	CEV SA, Unpublished report No.: 896A-102
Substance used:	PROBLAD PLUS (Batch: F0-1218, 21% BLAD, 1.21 g/cm ³)
Method of analysis:	Enzyme-Linked Immunosorbent Assay (ELISA). Method reference S18-08251.

Guideline(s):	OECD Guidelines for Testing of Chemicals, Method 211: <i>Daphnia magna</i> Reproduction test (1998) US EPA Ecological Effects Test Guidelines, OPPTS 850.1300: <i>Daphnia</i> Chronic Toxicity Test (1996)
Deviations:	Yes, see HSE comments
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

I. MATERIALS AND METHODS

A. MATERIALS

Test material	PROBLAD PLUS
Lot/Batch #:	F0-1218
Purity:	100% w/w, 21.0% BLAD, as per certificate of analysis checked by HSE
Treatments	
Test concentrations:	Nominal concentrations: 0, 0.75, 1.5, 3.0, 6.0 and 12 mg/L Mean measured concentrations: < LOQ, 0.08, 0.20, 0.38, 1.0 and 2.7 mg/L
Solvent:	None
Positive control:	None
Analysis of test concentrations:	Yes, via ELISA, mean measured concentrations 10–22% of nominal

Test animals

Species:	Cladoceran (<i>Daphnia magna</i>) neonates (< 24 hours old at test initiation)
Source:	In-house culture
Feeding:	Fed daily a mixture of 0.5 mL trout chow (YCT), 1.0 mL freshwater green alga <i>Raphidocelis subcapitata</i> , and 0.40 mL vitamin solution. Approximately 0.7 mg C/daphnid/day.
Acclimatisation period:	Cultured in water from the same source and at approximately the same temperature as used during the test (19.9-20.9°C).
Test design	
Test vessels:	250-mL glass beakers containing approx. 200 mL test solution, with a depth of 5.8 cm
Test medium:	Fresh well water from a 40-m deep on-site well
Replication:	Ten replicates per treatment group, 20 for the control
Exposure regime:	Static-renewal
Duration:	21 days
Environmental conditions	
Test temperature:	19–20.8°C
pH range:	8.3–8.7
Dissolved oxygen:	7.4–9.1 mg/L (≥ 81% of air saturation)
Water hardness:	127 mg/L as CaCO ₃
Lighting:	16 h light:8 h dark with a 30-min transition period at 868 lux
Statistical analysis:	Fisher’s Exact test/ Jonckheere-Terpstra test. SAS software used.

B. STUDY DESIGN AND METHODS

Study design:

This study was conducted to determine the effects of PROBLAD PLUS exposure on the survival, growth, and reproduction of the cladoceran *Daphnia magna* during a 21-

day static-renewal exposure. Test concentrations were selected based on the results of exploratory range-finding toxicity data.

Daphnids were exposed to a geometric series of five test concentrations and a negative (dilution water) control. Ten replicate test chambers containing one daphnid each were tested for each treatment group and 20 replicate test chambers were tested for the negative control group. Nominal test concentrations were 0.75, 1.5, 3.0, 6.0, and 12 mg/L. Test solutions were renewed daily throughout the test in maintain test concentrations as consistently as possible. Mean measured test concentrations were determined from samples of test solution collected from each treatment and control group at the beginning and end of the first and last renewals of the test, and at the beginning and the end of at least one renewal cycle each week during the test.

Daphnids were impartially assigned to exposure chambers at test initiation. First-generation daphnids were observed daily during the test for immobility, the onset of reproduction, and clinical signs of toxicity. Following the onset of reproduction, the numbers of second-generation daphnids were counted daily until test termination on Day 21. Total body lengths and dry weights of the surviving first-generation daphnids were measured at the end of the exposure period. Observations of the effects of PROBLAD PLUS on survival, reproduction, and growth were used to determine the NOEC and LOEC. EC₁₀, EC₂₀, and EC₅₀ values were estimated based on survival and immobility, reproduction, and growth at test termination, where possible.

Test organism

During culturing and testing, *Daphnia* were fed daily a mixture of 0.5 mL trout chow (YCT), 1.0 mL freshwater green alga *Raphidocelis subcapitata*, and 0.40 mL vitamin solution.

The four adult daphnids used to supply neonates for the test were held for 27 days prior to collection of the neonates for testing and had each produced at least five previous broods. Adult daphnids in the culture had produced an average of at least three young per adult per day over the 7-day period prior to the test. Adults showed no signs of disease or stress and no ephippia were produced during the holding period. Juvenile daphnids were collected from the cultures and indiscriminately transferred one or two at a time to each test chamber to initiate the test.

Dilution water

Water used for culturing and testing was freshwater obtained from a well approximately 40 meters deep located on the Eurofins-Easton site. The well water was passed through a sand filter to remove particles greater than approximately 25 µm. Aerated, filtered to 0.45 µm, and passed through a UV steriliser.

Test apparatus

Test chambers were 250 mL glass beakers containing approximately 200 mL test solution, the depth of solution in a representative test chamber was 5.8 cm. Each test chamber was loosely covered with a plastic petri dish and placed in an environmental chamber to maintain the target water temperature throughout the test period.

Preparation of test concentrations

At each preparation, a primary stock solution was prepared by mixing a calculated amount of test substance in 4 L dilution water at a nominal concentration of 12 mg/L, the highest concentration tested. Aliquots of the primary stock solution were proportionally diluted with dilution water to a final volume of 2 L to prepare four additional test solutions at nominal concentrations of 0.75, 1.5, 3.0, and 6.0 mg/L.

Environmental conditions

Test systems were illuminated following a 16-hour light to eight-hour dark photoperiod, with a 30-minute transition period in between. Light intensity at test initiation was 868 lux at the surface of the water of one representative test chamber. Temperature was measured in two replicate test chambers in each treatment and control group at test initiation, in the old and new solutions daily, and at test termination using a digital thermometer. Temperature was also monitored continuously in a representative vessel adjacent to the test system.

Dissolved oxygen and pH were measured in the newly prepared batch solution for each treatment and control group at test initiation and daily on renewal days throughout the test, and in the old solutions from two replicate test chambers in each treatment and control group daily on renewal days and at test termination. Water temperatures ranged from 19.0 to 20.8°C over the course of the test. Dissolved oxygen concentrations remained $\geq 81\%$ of the air saturation (7.4 to 9.1 mg/L), and measurements of pH ranged from 8.3 to 8.7.

Biological observations

Observations of each first-generation daphnid were made daily during the test. At these times, the numbers of immobile daphnids were recorded along with any clinical signs of toxicity (e.g. inability to maintain position in the water column, uncoordinated swimming, lethargy, opaque colour, cessation of feeding). Daphnids not able to swim within 15 seconds after gentle agitation of the test vessel were considered immobilised. The presence of eggs in the brood pouch, aborted eggs, males, or ephippia was recorded daily. With the onset of reproduction, neonates produced by the first generation daphnids were counted and then discarded daily during the test. The total body length and dry weight of each surviving first generation daphnid were measured at the end of the test.

Analytical method

Samples of the control and treated media were analysed using the validated analytical method report no.: S18-08251.

Freshwater samples of PROBLAD PLUS were analysed by Enzyme-Linked Immunosorbent Assay (ELISA) at Eurofins Agrosience Services Ltd.

Triplicate water samples were collected from each treatment and control group at test initiation, at the beginning and the end of a renewal cycle each week, and at test termination. Samples of “new” solutions were collected from the batch test solutions at test initiation and at the beginning of the renewal cycle. Samples of “old” solutions were collected from two alternating replicates of each treatment and control group at the end of the renewal cycle and at test termination. Samples (20 mL) were collected from mid-depth and placed in polypropylene centrifuge tubes. One set of samples collected at each sampling interval was stored frozen until shipment for analysis. One set of samples was stored frozen until shipment in case re-analysis was needed. The other set was stored frozen as back-up samples for possible analysis.

II. RESULTS AND DISCUSSION

Validity criteria:

The validity criteria outlined in OECD 211 (2012) have been met:

Validity criterion	Required	Obtained
Mortality of parent animals in the control(s)	≤ 20% mortality at the end of the test	5%
Mean number of living offspring produced per parent animal surviving at the end of the test	≥ 60	244 ± 12.4
Analytical measurements of test concentration	Must be carried out using validated method at regular intervals. If deviation from nominal or measured initial concentration is ± 20% then results should be expressed as time-weighted	Results based on mean-measured test concentrations

	mean.	
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Analytical results:

Measured test concentrations in the new test solutions on day 0, 11 and 20 ranged from 5–21%, 11–44% and 37–39% of the nominal concentration, respectively. Concentrations in the 24-hour old test solutions collected on days 1, 12, and 21 ranged from < LOQ–5%, < LOQ–5.6% and < LOQ–13% of nominal concentration, respectively. Recoveries for each treatment level are presented in Table 9.2.5-2 below. When the measured concentrations of the samples collected during the test were averaged, the mean measured test concentrations were determined to be 0.08, 0.20, 0.38, 1.0, and 2.7 mg/L, corresponding to 10, 13, 13, 17, and 22% of nominal, respectively, as shown in Table 9.2.5-1 below. Results of the study were therefore based on the mean measured test concentrations.

Table 9.2.5-1: Summary of mean measured concentrations and percentage recovery of test item

Nominal concentrations (mg a.s./L)	Mean measured concentration ¹ (mg a.s./L)	Percent of Nominal ² (%)
Control	< LOQ	NA
0.75	0.08	10
1.5	0.20	13
3.0	0.38	13
6.0	1.00	17
12.0	2.7	22

LOQ = 0.03 mg/L, LOD = 0.01 mg/L. ¹Based on 6 measured time points. ²Mean measured concentration as a % of nominal concentration.

Table 9.2.5-2: Analytical recoveries of the test item in fresh and spent solutions

Nominal concentrations (mg a.s./L)	Measured d 0 (fresh) (mg a.s./L)	% of nominal	Measured d 1 (spent) (mg a.s./L)	% of nominal	Measured d 11 (fresh) (mg a.s./L)	% of nominal	Measured d 12 (spent) (mg a.s./L)	% of nominal	Measured d 20 (fresh) (mg a.s./L)	% of nominal	Measured d 21 (spent) (mg a.s./L)	% of nominal
Control	<LOQ	N/A	<LOQ	N/A	<LOQ	N/A	<LOQ	N/A	<LOQ	N/A	<LOQ	N/A
0.75	0.04	5	<LOQ	N/A	0.09	11	<LOQ	N/A	0.28	37	<LOQ	N/A
1.5	0.10	7	<LOQ	N/A	0.40	27	<LOQ	N/A	0.62	41	0.03	2
3.0	0.42	14	<LOQ	N/A	0.36	12	<LOQ	N/A	1.46	49	<LOQ	N/A
6.0	1.24	21	0.10	1.5	1.54	26	0.04	1	2.73	46	0.33	5
12.0	2.54	21	0.60	5	5.29	44	0.67	5.6	5.28	44	1.5	13

LOQ = 0.03 mg/L, LOD = 0.01 mg/L.

Survival and Clinical Observations

Daphnids in the negative control and all treatments groups that survived to test termination generally appeared normal. One daphnid in both the 0.08 and 0.38 mg/L treatment groups appeared weak or discoloured (pale), however these observations were infrequent and did not follow a dose-response pattern and were therefore not considered to be treatment-related. No parental-generation daphnids were observed to be male during the test.

At test termination, survival in the negative control and the 0.08, 0.20, 0.38, 1.0, and 2.7 mg/L treatment groups was 95.0, 100, 100, 100, 90.0, and 90.0%, respectively. No statistically significant decreases in survival were observed in any treatment group in comparison to the negative control.

The resulting NOEC and LOEC for survival were determined to be 2.7 and > 2.7 mg/L, respectively. EC₁₀, EC₂₀, and EC₅₀ values were empirically estimated to be greater than the highest concentration tested (> 2.7 mg/L).

Reproduction

With the exception of one daphnid in the 2.7 mg/L treatment group which produced a brood on day 7 of the study, the first day of brood production in all control and treatment replicates was day 8 of the test, indicating that there was no apparent delay in the onset of production at any PROBLAD PLUS concentration tested. In the negative control, two immobile neonates were produced, however no immobile neonates were produced in any of the test item groups. In the negative control and the 0.20 and 0.38 mg/L treatment groups, 37, one, and nine aborted eggs were produced during the test, respectively. No ephippia were produced by first-generation daphnids during the test.

The mean production rate of first brood for all treatment groups ranged from 0.1333 to 0.1354. No statistically significant decreasing trend was evident in the treatment group data.

The resulting NOEC and LOEC for reproduction were determined to be 2.7 and > 2.7 mg/L, respectively. EC₁₀, EC₂₀, and EC₅₀ values were empirically estimated to be greater than the highest concentration tested (> 2.7 mg/L). Adult daphnids in the negative control and the 0.08, 0.20, 0.38, 1.0, and 2.7 mg/L treatment groups produced an average of 244, 238, 265, 262, 271, and 274 live young per parent animal, respectively. No statistically significant decreasing trend was evident in the treatment group data.

Growth

Daphnids in the negative control and the 0.08, 0.20, 0.38, 1.0, and 2.7 mg/L treatment groups had mean total lengths of 4.9, 5.0, 5.0, 5.1, 5.1, and 5.1 mm,

respectively, and mean dry weights of 1.20, 1.23, 1.21, 1.24, 1.36, and 1.45 mg, respectively. No statistically significant decreasing trend was evident in the treatment group data.

The resulting NOEC and LOEC for growth were determined to be 2.7 and > 2.7 mg/L, respectively. EC₁₀, EC₂₀, and EC₅₀ values were empirically estimated to be greater than the highest concentration tested (> 2.7 mg/L).

Table 9.2.5-3: Summary of survival, reproduction and growth of *Daphnia magna* exposed to PROBLAD PLUS for 21 days

Mean measured concentration (mg/L)	Percent Adult survival	Production rate of first brood	Mean no. neonates per adult at test initiation	Mean no. neonates per adult at test termination	Mean total length	Mean dry weight
Negative control	95.0	0.1333	244 ± 12.4	244 ± 12.4	4.9 ± 0.077	1.20 ± 0.105
0.08	100	0.1333	238 ± 37.9	238 ± 37.9	5.0 ± 0.15	1.23 ± 0.187
0.20	100	0.1333	265 ± 10.4	265 ± 10.4	5.0 ± 0.074	1.21 ± 0.237
0.38	100	0.1333	262 ± 13.6	262 ± 13.6	5.1 ± 0.11	1.24 ± 0.229
1.0	90.0	0.1333	271 ± 12.6	271 ± 12.6	5.1 ± 0.050	1.36 ± 0.154
2.7	90.0	0.1354	274 ± 29.9	274 ± 29.9	5.1 ± 0.093	1.45 ± 0.0714

Table 9.2.5-4: Percent inhibition of endpoints compared to the negative control

Mean measured concentration (mg/L)	Adult survival	Production rate of first brood	Mean no. neonates per adult at test initiation ¹	Mean no. neonates per adult at test termination	Mean total length	Mean dry weight
0.08	-5.3	0.00	2.2	2.2	-0.4	-2.6
0.20	-5.3	0.00	-8.8	-8.8	-1.4	-1.3
0.38	-5.3	0.00	-7.7	-7.7	-3.0	-3.5
1.0	5.3	0.00	-11.3	-11.3	-3.9	-13.4
2.7	5.3	-1.6	-12.7	-12.7	-3.0	-21.5
Negative values indicate percentage increase. ¹ Excluding live neonates produced by first generation daphnids which accidentally and/or inadvertently died during the test.						

A summary of the resulting NOEC, LOEC, and ECx values is presented in the table below:

Table 9.2.5-5: Summary of endpoints

Parameter	NOEC (mg/L)	LOEC (mg/L)	EC₁₀ (mg/L)	EC₂₀ (mg/L)	EC₅₀ (mg/L)
Survival	2.7	> 2.7	> 2.7	> 2.7	> 2.7
Production rate of first brood	2.7	> 2.7	> 2.7	> 2.7	> 2.7
Live young produced per parent animal alive at test initiation	2.7	> 2.7	> 2.7	> 2.7	> 2.7
Live young produced per parent animal alive at test termination	2.7	> 2.7	> 2.7	> 2.7	> 2.7
Total length	2.7	> 2.7	> 2.7	> 2.7	> 2.7
Dry weight	2.7	> 2.7	> 2.7	> 2.7	> 2.7

III. CONCLUSION

The cladoceran (*Daphnia magna*) was exposed to PROBLAD PLUS at mean measured concentrations of 0.08, 0.20, 0.38, 1.0, and 2.7 mg/L under static-renewal conditions for 21 days. There were no statistically significant treatment-related effects on survival, reproduction, or growth at concentrations ≤ 2.7 mg/L. Consequently, the overall NOEC and LOEC for the study were determined to be 2.7 and > 2.7 mg/L, respectively. The EC₁₀, EC₂₀, and EC₅₀ values based on immobility, reproduction, and growth were all > 2.7 mg/L.

HSE COMMENTS

This study has been conducted in accordance with GLP and follows OECD 211 (2012) guidelines. The validity criteria have been met. The only noted deviation to the guidelines was in the feeding rate. The guideline recommends that daphnids should be fed at a rate of 0.1-0.2 mg carbon/Daphnia/day, however the feeding in this study equated to 0.7 mg carbon/Daphnia/day. The study report states that feeding was in excess in order to maintain sufficient feed to support reproductive output. Since the validity criteria for reproductive output and survival were met in the control, and no significant effects of the test item were noted, this deviation is not thought to have affected the study outcome.

The validity of the analytical method has been considered in Volume 3 CA B5. The following conclusion was reached: “The analytical method for the determination of PROBLAD PLUS in W4 can be considered fully validated in accordance with SANTE/2020/12830 rev. 1”. The analytical recoveries indicated that the test item was not within $\pm 20\%$ of nominal concentration in either the fresh or aged solutions, which would suggest that the correct dosing of test item was not achieved or maintained. HSE considers that these results reflect the difficulties in detecting and quantifying UVCB substances and considers that there may be limitations in the ability of the ELISA method to provide quantitative results, despite the method being validated. It is noted that there was some uncertainty in the quantification of the ELISA method used in the Residues assessment (method validation in Volume 3 CA B5).

Considering the method of preparation of the test solutions, HSE has some degree of confidence that the nominal concentrations would have been achieved in the fresh solutions, despite the low recoveries. The method of preparation is relatively simple, with a defined amount of test item being added to a fixed volume of dilution medium to produce a stock solution, which is then serially diluted. The precise preparation methods are available from the raw data and could be recreated. HSE considers that, while there is some uncertainty in the measured test concentrations, the nominal dosing was likely to have been achieved in the fresh solutions and the mean measured concentrations over the course of the test will provide a conservative estimate of the toxicity endpoint.

Since no effect of the test item was seen at any of the tested concentrations, the EC_x values could not be determined statistically. They were instead estimated to be > than the highest test concentration (2.7 mg/L).

The endpoint suitable for use in risk assessment is therefore:

- $EC_{50/20/10}$ (growth, reproduction, immobility) > 2.7 mg/L
- NOEC (growth, reproduction, immobility) = 2.7 mg/L

B.9.2.5.2. Reproductive and development toxicity to an additional aquatic invertebrate species

No data submitted or required.

B.9.2.5.3. Development and emergence in *Chironomus* species

No data submitted or required.

B.9.2.5.4. Sediment swelling organisms

No studies with sediment-dwelling organisms have been conducted. PROBLAD PLUS is readily biodegradable and any residues in water bodies will be rapidly degraded. However, given the high Koc (see Volume 3 CA B8), partitioning into sediment is likely to occur. In the absence of specific data with sediment-dwelling organisms, the margin of safety in the chronic aquatic invertebrate risk assessment will be considered.

B.9.2.6. Effects on algal growth**B.9.2.6.1. Effects on growth of green algae**

Reference:	K-CA 8.2.6.1/01
Report Title:	PROBLAD: Testing of Effects to the single cell green alga <i>Desmodesmus subspicatus</i> in a 72 h static test
Author(s) & Year:	Falk, S. (2011).
Document No	CEV SA, Unpublished report No.: S10-02623.
Substance used:	PROBLAD, (Batch: 201009, 20% BLAD, 1.30 g/cm ³)
Method of analysis:	No analysis conducted
Guideline(s):	OECD Guidelines for Testing of Chemicals, Method 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test (2006)
Deviations:	Yes, see HSE comments
GLP or GEP:	Yes
Acceptability:	No

Study relied upon:	No, due to lack of analytical measurements
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I. MATERIALS AND METHODS

A. MATERIALS

Test Substance

Test item : PROBLAD (Batch: 201009, 20% BLAD, 1.30 g/cm³) CoA: 20/09/2010 (checked by HSE assessor)

Test concentrations : The main test was performed with six replicates for the control and three for 0.137, 0.412, 1.23, 3.70, 11.1, 33.3 and 100 mg/L.

Control : Untreated test medium

Reference item : None

Test Organism *Desmodesmus subspicatus*. SAG strain no. 86.81, from the Georg-August-Universität Göttingen, Wilhelmsplatz 1, 37073 Göttingen, Germany.

The algae are grown semi-continuously in the laboratory in aerated liquid cultures under permanent illumination. Old medium is periodically replaced by fresh mineral solution in order to keep the algae in an exponential growth state. Stock cultures are also kept on slanted agar tubes and are ordered regularly from the culture collection. Culture conditions were as follows:

- Illumination: from the top by light tubes, approx. 4400 – 8000 lux at cell culture level
- Temperature: 21–24°C, controlled at $\pm 2^\circ\text{C}$
- Culture flasks: 1000 mL screw cap bottles

- CO₂ supply by aeration. The air is sterilised by membrane filters

- Visually healthy cells were used for the test

Cells from this semi-continuous liquid stock culture were used for test

B. STUDY DESIGN AND METHODS

Experimental design : 72h static test. Homogeneity of test solutions was obtained by continuous shaking during the entire test period. The cell density was adjusted to an initial concentration of 0.5×10^4 cells/mL in each test vessel.

Exposure unit : 500 mL Erlenmeyer flasks with aluminium caps and two baffles. CO₂ supply by shaking on a rotating shaker, 110 rpm

Test medium : AAP medium (checked by HSE assessor, in-line with guidelines)

Test procedures : A range finding test was performed before the definitive study.

The algae were exposed to different concentrations of the test item under defined conditions in a synthetic growth medium during several generations. The initial cell density was adjusted to 0.5×10^4 cells/mL.

Each test item dilution was prepared with 3 replicates, the controls were prepared with 6 replicates.

By comparing the cell division under test conditions with and without the influence of the test item, an inhibition of the cell multiplication was calculated. The cell numbers were determined by fluorescence detection. At defined dates (1, 2 and 3 days), the number of cells in each replicate was evaluated. Thereby the concentration which did not yet cause any inhibition (NOEC) and the lowest observed effect concentration (LOEC) were determined for day 3. The other response variable tested was yield, which is defined as the cell numbers at the end of the exposure period minus the cell numbers at the start of the exposure period, was evaluated.

Study dates : 19/10/2010 – 29/07/2011

Environmental Conditions:

Static 72 hour study.

Temperature :	22.5°C and 24.0°C (required: 21–24 ± 2°C)
pH :	6.72-8.85 (should not +/- > 1.5 unit)
Lighting :	Continuously from the top by light tubes, 4400–5600 lux at cell culture level (required: 4440–8880 lux)

Observations:

Measurements of pH-value were performed at t = 0d and t = 3d. The temperature was measured at day 0, 1, 2, and 3. No analytical evaluation was performed. Daily, fluorescence measurement with Fuji FLA-3000 laser emitter at 633 nm (Fuji Photo Film Co., LTD), Filter R674, 16 bits per pixel, pixel size: 100 µm 100 µm, 10 x sensitivity.

Determination of cell number

The cell numbers were determined with a calibration curve, where cell numbers (x axis) were plotted versus fluorescence signals (y axis). Calibration data acquisition was done within a sensitivity range of 10 x. To establish a calibration curve, the cell numbers were counted with a Neubauer chamber after preparation of a dilution series of a logarithmic growing *Desmodium* culture.

Statistics:

The cell numbers in the test cultures and controls were tabulated together with the concentration of the test item and the times of measurement. The mean value of the cell numbers for each test item concentration and the control was plotted versus time to produce growth curves.

The percentage inhibition of growth rates (% I_μ) was calculated as the difference between the growth rates of the control (μ_c) and the growth rates in the treatment (μ_t).

The yield is calculated as the cell numbers at the end of the test minus the starting cell numbers for each single vessel of controls and treatments. For each test concentration and control, a mean value for yield is calculated, along with variance estimates. The percent inhibition in yield (% I_y) is calculated for each treatment replicate.

The statistical evaluation for day 3 was performed for cell number, yield and growth rate using SAS® (2002 – 2008). The dataset was transformed via Boxcox transformation and NOEC and LOEC were determined by using the Dunnetts-t-Test. A test for normality of the data was performed by calculating the Shapiro-Wilk's statistic. A test for homogeneity of variance for the data was performed using the Levene-test.

The E_yC₅₀ (for yield) –values were determined using Moving average analysis (Thompson, 1947). Due to statistical reasons, values of the concentrations 0.137, 0.412, 1.23 and 3.7 were not taken into account. The EC₅₀ of growth rate was not calculable due to statistical reasons (see HSE comments).

II. RESULTS AND DISCUSSION

Statistically significant differences to the control were observed on day 3 at 33.3 and 100 mg/L.

Table 9.2.6-1: Average cell number and yield for each sampling time and concentration in the main test

Conc. [mg/L]	Average cell numbers/mL ¹⁾			
	0d	1d	2d	3d
Control	0.5	1.70	9.98	42.49
0.137	0.5	1.43	10.08	45.78
0.412	0.5	1.48	10.59	49.25
1.23	0.5	1.29	10.78	47.08
3.70	0.5	1.55	12.43	55.42
11.1	0.5	1.42	11.40	55.99
33.30	0.5	1.54	3.30	4.47
100	0.5	2.08	5.21	7.84

¹⁾ Algae counts are divided by 10000. At the start, the cell density was adjusted to 0.5×10^4 cells/mL.

Table 9.2.6-2: Percentage inhibition of growth rate and yield in the main test

Conc. [mg/L]	% inhibition of growth rate			% inhibition of yield		
	0d-1d	0d-2d	0d-3d	0d-1d	0d-2d	0d-3d
Control	0.0	0.0	0.0	0.0	0.0	0.0
0.137	13.1	-0.6	-1.8	22.5	-1.1	-7.8
0.412	9.0	-2.4	-3.6	18.3	-6.4	-16.1
1.23	35.5	-2.8	-2.5	34.2	-8.4	-10.9
3.70	21.6	-7.6	-6.2	12.5	-25.8	-30.8
11.1	18.4	-4.7	-6.4	23.3	-15.0	-32.2
33.30	9.6	36.8	51.4	13.3	70.5	90.5
100	-19.3	21.7	40.5	-31.7	50.3	82.5

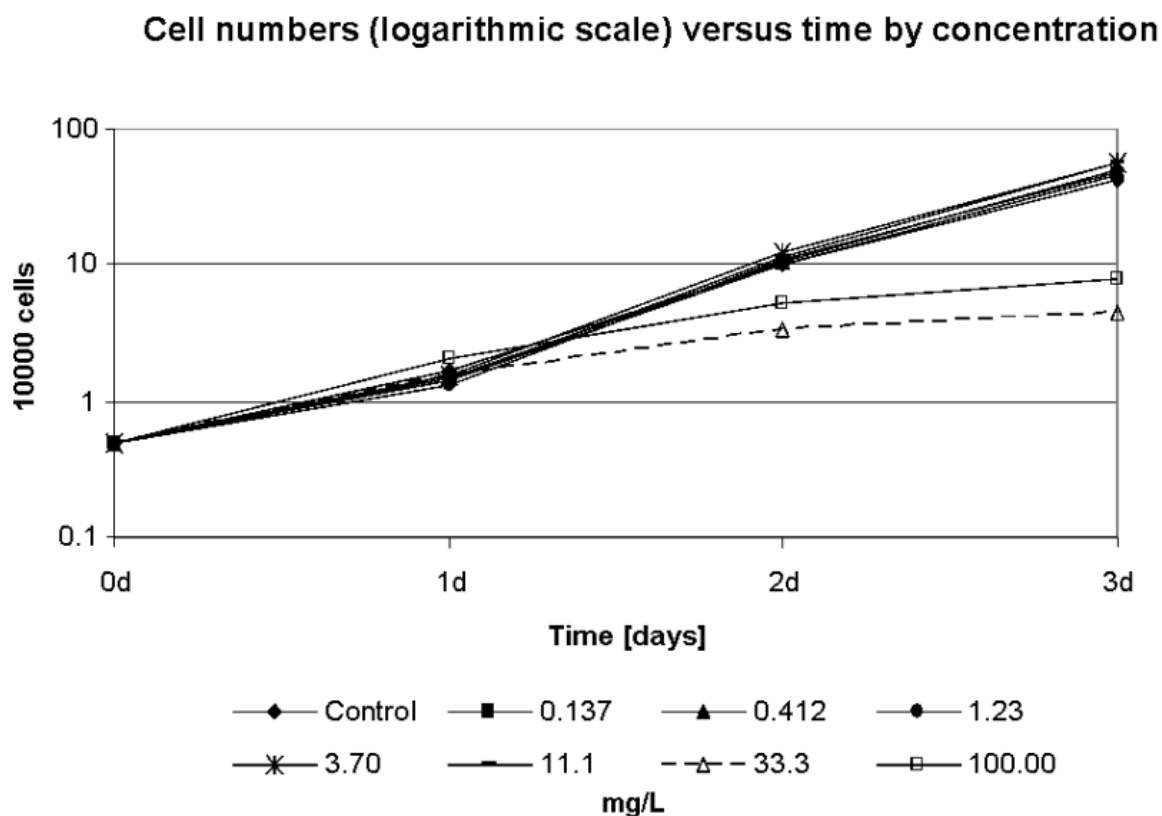


Figure 9.2.6.1-01/1. Growth curve

III. CONCLUSION

Statistically significant differences to the control were determined at 33.3 and 100 mg/L on day 3. Therefore, the LOEC is determined to be 33.3 mg/L and the NOEC is estimated to be 11.1 mg/L. The EC₅₀ was estimated to be 28.7 mg/L for yield.

HSE COMMENTS

The submitted study has been produced using the OECD 201 (2006) guidelines, this version is now out of date and HSE has therefore assessed the study against the most recent version of the guidelines OECD 201 (2011).

The HSE assessor has re-calculated the CV to be 22.2 for section by section and 3.7 for growth rate.

Validity criteria :	Achieved :
The biomass in the control cultures should have increased exponentially by a factor of at least 16 within the 72-hour test period. This corresponds to a specific growth rate of 0.92 day ⁻¹	Yes 85 fold
The mean coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2 and 2-3, for 72-hour tests) in the control cultures must not exceed 35%.	Yes 22.2%
The coefficient of variation of average specific growth rates during the whole test period in replicate control cultures must not exceed 7% in tests with <i>Desmodesmus subspicatus</i> .	Yes 3.7%

No analytical assessments were conducted so it cannot be confirmed that the concentration of test item was maintained at the nominal rate. No reference item was tested to check the sensitivity of the test species. There was also no assessment of the appearance of the algal cells during the study. The pH also increased by more than 1.5 units during the test. As a result of these deviations, the reliability of the endpoints derived is uncertain.

The statistical methods used in the study were in line with the guidelines. The study report states that the ErC_{50} was not able to be reliably calculated 'due to statistical reasons' however it is not made clear what the nature of the statistical reason was. It is presumed that the issue was a lack of clear dose response. It is noted that the second highest test concentration gave rise to approximately 50% effects on growth rate, whereas in the highest test concentration, only 40% effect was observed. Further consideration of the statistical analysis and the ErC_{50} endpoint would be required were the study to be used to support the risk assessment. Currently the only suitable endpoint is the $EyC_{50} = 28.7$ mg PROBLAD/L. Given that no analytical measurements were made to confirm the correct dosing of the test system, along with

other deviations from the guideline described above, **this study is not considered to be suitable for use in the risk assessment.**

Reference:	K-CA 8.2.6.1/02
Report Title:	PROBLAD PLUS: A 72-hour toxicity test with the freshwater alga (<i>Raphidocelis subcapitata</i>).
Author(s) & Year:	Arnie et al. (2019).
Document No	CEV SA, Unpublished report No.: 896P-101.
Substance used:	PROBLAD (Batch: F0-1218, 21% BLAD, 1.21 g/cm ³)
Method of analysis:	Enzyme-Linked Immunosorbent Assay (ELISA). Method reference S18-08251.
Guideline(s):	OECD Guideline No. 201 (2006) (assessed against OECD 201 (2011))
Deviations:	Yes, see HSE comments
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

I. MATERIALS AND METHODS

A. MATERIALS

Test Substance

Test item : PROBLAD (Batch: F0-1218, 100% w/w sweet *Lupinus albus* L. germ. Extract, 21% BLAD protein. Density: 1.21 g/cm³) CoA: 20/12/2018 (checked by HSE assessor)

Test concentrations : 7.5, 15, 30, 60, and 120 mg/L (based on range finding study results)

Geometric means: 3.30, 6.61, 13.77, 12.65, 30.69, 75.65 mg/L

A primary stock solution was prepared by mixing 0.1200 g of PROBLAD PLUS in 1000 mL of freshwater AAP medium, to achieve a nominal concentration of 120 mg/L. The primary stock was inverted at least 20 times and stirred for thirty-one minutes to mix. No surface slicks or particulates were observed in the primary stock. Four additional test solutions were prepared at nominal concentrations of 7.5, 15, 30, and 60 mg/L by diluting aliquots of the 120 mg/L primary stock with freshwater AAP medium. The 7.5, 15 and 30 mg/L test solutions appeared clear and colourless. The 60 mg/L test solution appeared slightly cloudy with a yellow tint.

Control : Untreated test medium

Reference item : None tested

Test Organism Raphidocelis subcapitata

Algal cells used in this test were obtained from Eurofins – Easton cultures that had been actively growing in culture medium under similar environmental conditions as used in this test for at least two weeks prior to test initiation. Algal cells for this study were taken from a culture that had been transferred to fresh medium four days prior to test initiation. The negative control organisms were expected to exhibit exponential growth over the 72-hour exposure period.

B. STUDY DESIGN AND METHODS

Study dates : 22/02/2019 – 25/02/2019

Experimental design : 72 hour static study. Three replicate test chambers were maintained in each treatment group and six replicate test chambers were maintained in the negative control group throughout the exposure period. An additional abiotic

replicate was included in the nominal 30mg/L treatment group to evaluate stability of the test substance under test conditions without the test organism present.

Prior to test initiation, the concentration of algal cells in the stock culture was determined using a hemacytometer and microscope, and was 4.19×10^6 cells/mL. In order to achieve the desired initial cell density of approximately 10,000 cells/mL, 0.239 mL of stock culture was added to each replicate test chamber at test initiation with an Eppendorf pipette.

Exposure unit :	Test chambers were sterile, 250-mL glass Erlenmeyer flasks plugged with sterile foam stoppers, and contained 100 mL of test or control medium. Test chambers were indiscriminately positioned on a mechanical shaker table in an environmental chamber and were shaken continuously at approximately 100 rpm.
Test medium :	AAP medium (checked by HSE assessor, in-line with guidelines)
Test procedures :	Samples were collected from each replicate test chamber at approximately 24-hour intervals during the test to determine cell densities. Cell densities were used to determine growth rates and yields which were subsequently used to calculate percent inhibition values relative to the negative control over the 72-hour exposure period. EC50 and ErC50 values (i.e., the theoretical concentrations that would produce an 50% reduction in cell density and growth rate, respectively) and their 95% confidence intervals were determined, when possible, at 24, 48, and 72 hours of exposure.

Environmental Conditions :

Temperature :	22.70–23.01°C (required: 21–24 ± 2°C)
pH :	Start: 7.4–7.7, end: 7.5–9.6 (should not +/- > 1.5 unit)
Lighting :	5810–6310 lux (mean 6010 lux) (required: 4440–8880 lux)

Observations:

At test initiation, pH was measured in the individual batches of test solution prepared for each treatment and control group. At exposure termination, pH was measured in pooled samples of test solution collected from each of the biotic replicates of each treatment and control group and from the single abiotic replicate included in the nominal 30 mg/L treatment group.

One test medium sample was collected from each replicate of the treatment and control groups at each sampling interval for the determination of algal cell densities. Samples were collected at approximately 24-hour intervals during the 72-hour exposure and were held in the dark for a maximum of three days under refrigerated conditions sufficient to inhibit growth until cell counts could be performed. Cell counts were performed using a hemacytometer and microscope. Samples were sonicated and swirled to disperse algal cells evenly in the samples prior to dilution. Each sample was diluted using an electrolyte solution (Isoton®), as needed, to maintain counting accuracy. A small amount of each sample was loaded onto a hemacytometer and the total number of cells was counted in 10 grids to calculate the cell density of the sample. At the end of the exposure period, samples of test solution were collected from each of the replicates per treatment and control group, pooled within their respective treatments, and subsamples were removed and examined microscopically for atypical cell morphology (e.g., changes in cell shape, size or colour). Cells in the replicate test chambers also were assessed for aggregation or flocculation of cells, and adherence of the cells to the test chamber.

Statistics:

The calculation of cell densities, yield and growth rates and percent inhibition values, as well as all statistical analyses, were conducted using “The SAS System for Windows, Version 9.4”. Growth rate was calculated for each replicate of the control and treatment groups at each 24-hour interval of exposure. Yield was calculated for each replicate of the control and treatment groups as the final biomass (cell density) in the exposure period minus the initial biomass (cell density; 10,000 cells/mL). Inhibition values were calculated for each treatment group as the percent reduction in cell density, growth rate, and yield relative to the negative control replicates.

EC_x, E_rC_x, and E_yC_x values and their corresponding 95% confidence intervals were calculated, when possible, using replicate data (cell density, growth rate, and yield, respectively) and exposure concentration data (nominal or geometric mean, measured concentrations).

The 72-hour cell density, growth rate, and yield data were evaluated for normality and homogeneity of variance ($\alpha = 0.01$) using Shapiro-Wilk's and Levene's tests, respectively. All data met assumptions of normality and homogeneity of variance. The responses of the treatment groups were compared to the negative control response using Dunnett's test ($\alpha = 0.05$). The results of the statistical analyses of the cell density, growth rate, and yield data, as well as an evaluation of the concentration-response pattern, were used to determine the NOEC relative to each parameter at 72 hours.

Analytical verification:

Samples of the test solutions were collected to measure concentrations of the test substance at 0 (test initiation, Day 0) and 72 hours (test termination, Day 3). Duplicate samples were collected at approximately 0 and 72 hours. Samples collected at test initiation (0 hour) were collected from the individual batches of test solution prepared for each treatment and control group prior to distribution into the test chambers. At exposure termination (72 hours), samples were collected from the pooled replicates from each treatment and control group. At each sampling interval, approximately 20 mL of test solution was added to 50 mL glass plastic centrifuged tubes. Quantification was performed using ELISA with measurement of optical density. LOQ: 0.03 mg/L, LOD: 0.01 mg/L.

II. RESULTS AND DISCUSSION:

Temperatures remained within the $24 \pm 2^\circ\text{C}$ range established for the test. The pH of the test solutions at test initiation ranged from 7.4 to 7.7. At test termination, pH in the pooled replicates of each respective treatment and control group ranged from 7.5 to 9.6. The observed increase in pH is typical for tests conducted with *R. subcapitata* and is attributed to the photosynthetic activity of the algae. The light intensity ranged from 5,810 to 6,310 lux, which was within the desired range of 6,000 lux $\pm 10\%$.

Table 9.2.6-3: Analytical results

Nominal concentration (mg/L)	Measured concentration 0 h (mg/L)	Measured concentration 72 h (mg/L)	Geometric mean measured concentration (mg/L)	Percent of nominal (%)
Negative control	<LOQ	<LOQ	<LOQ	-
7.5	3.21	3.39	3.3	42.9
15	5.10	8.57	6.6	44.0
30	13.37	14.19	14	46.7
60	36.95	25.49	31	51.7
120	94.84	60.35	76	63.3

After 72 hours of exposure, inhibition of cell density in the nominal 7.5, 15, 30, 60, and 120 mg/L treatment groups was 3, 11, 55, 91, and 96%, respectively, relative to the negative controls. Inhibition of yield in the nominal 7.5, 15, 30, 60, and 120 mg/L treatment groups was 3, 11, 55, 92, and 96%, respectively, relative to the negative control. Inhibition of growth rate in the nominal 7.5, 15, 30, 60, and 120 mg/L treatment groups was 1, 2, 14, 44, and 57%, respectively, relative to the negative control. Mean cell density, mean yield, and mean growth rate were significantly reduced (Dunnett's test; $p > 0.05$) in the 30, 60, and 120 mg/L treatment groups at 72 hours, when compared to the negative control. Consequently, the 72-hour NOEC was determined to be 15 mg/L.

After 72 hours of exposure, aggregation or flocculation was not observed in the controls or in any treatment groups. Adherence of the cells to the test chambers was observed in the controls and all treatment groups. This was not considered to be a treatment related effect since it was also documented in the control group. There were no noticeable changes in cell morphology in any of the treatment groups when compared to the control replicates during the microscopic examinations of the cells.

Table 9.2.6-4: Mean cell density, mean yield and % inhibition

Geometric mean measured (mg/L)	24 hours		48 hours		72 hours		0-72 hours	
	Mean cell density (cells/mL)	% inhib.	Mean cell density (cells/mL)	% inhib.	Mean cell density (cells/mL)	% inhib.	Mean yield (cells/mL)	% inhib.
Negative control	54667	-	430000	-	2658333	-	2648333	-
3.3	53000	3	403333	6	2570000	3	2560000	3
6.6	61667	-13	368333	14	2370000	11	2360000	11
14	47000	14	223333	48	1203333*	55	1193333*	55
31	45333	17	98333	77	233000*	91	223000*	92
76	36000	34	56000	87	109000*	96	99000*	96

*: statistically significant reduction when compared to negative control mean.

Table 9.2.6-5: Mean growth rate per hour and percent inhibition after 72-hour exposure to PROBLAD PLUS

Geometric mean measured conc. (mg/L)	0-24 h		0-48 h		0-72 h	
	Mean growth rate	% inhib.¹	Mean growth rate	% inhib.¹	Mean growth rate	% inhib.¹
Negative control	0.0706	-	0.0708	-	0.0775	-
3.3	0.0692	2	0.0770	1	0.0770	1
6.6	0.0756	-7	0.0751	4	0.0759	2
14	0.0641	9	0.0647	17	0.0665*	14
31	0.0628	11	0.0448	43	0.0437*	44
76	0.0533	25	0.0353	55	0.0331*	57

¹. Percent inhibition relative to the mean negative control response. Negative values indicate an increase.

*: statistically significant reduction when compared to negative control mean.

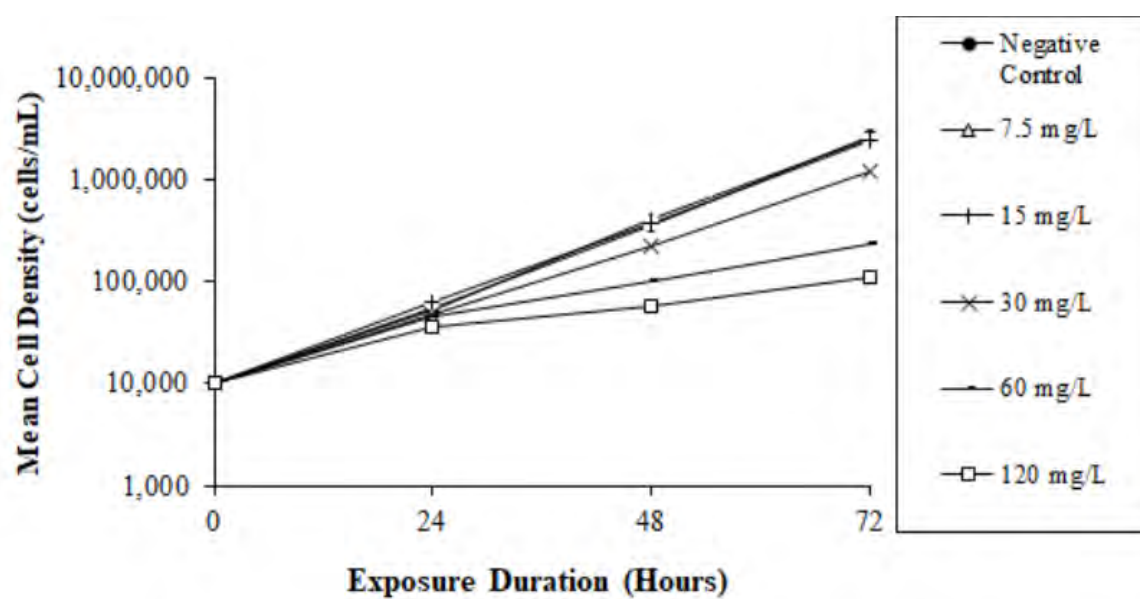


Figure 9.2.6.1/02-1. Growth of *R. subcapitata* in all experimental groups over the 72-hour exposure period

Table 9.2.6-6: NOEC, EC_x, E_rC_x and E_yC_x values at 72 hours of exposure

Endpoint	Nominal concentration (mg/L)	Geometric mean measured concentrations (mg/L)
Cell density		
NOEC ¹	15	6.6
EC ₁₀ ²	11	4.2
95% confidence interval ²	8.0–15	<3.3–6.2
EC ₂₀ ²	15	6.1
95% confidence interval ²	12–19	4.4–8.5
EC ₅₀ ²	27	12
95% confidence interval ²	23–23	10–15
Growth rate		
NOEC ¹	15	6.6
EC ₁₀ ²	18	7.5
95% confidence interval ²	14–25	5.0–11
EC ₂₀ ²	31	14
95% confidence interval ²	25–39	11–19
EC ₅₀ ²	87	51
95% confidence interval ²	78–97	44–59
Yield		
NOEC ¹	15	6.6
EC ₁₀ ²	11	4.3
95% confidence interval ²	8.2–15	2.9–6.3

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EC ₂₀ ²	15	6.2
95% confidence interval ²	12–19	4.5–8.5
EC ₅₀ ²	27	12
95% confidence interval ²	23–32	10–15

¹NOEC was determined based on the results of statistical analyses and evaluation of the dose response.

²EC_x values and their corresponding 95% confidence intervals were determined using non-linear regression with replicate data and exposure concentration data.

III. CONCLUSION:

The freshwater alga, *Raphidocelis subcapitata*, was exposed to a geometric series of five nominal concentrations of PROBLAD PLUS ranging from 7.5 to 120 mg/L. Effects were evaluated based on cell density, yield and growth rate using nominal and geometric mean, measured concentrations. The 72-hour EC₅₀, EyC₅₀, and ErC₅₀ values were determined to be 27, 27, and 87 mg/L, respectively, based on nominal concentrations (12, 12, and 51 mg/L based on geometric mean, measured concentrations). The 72-hour NOEC was determined to be 15 mg/L (6.6 mg/L based on geometric mean, measured concentrations).

HSE COMMENTS

The submitted study has been produced using the most up-to-date OECD 201 (2011) guidelines and is GLP compliant. The statistical analysis was in line with the guidelines.

The HSE assessor has re-calculated the CV to be 11.9% for section by section and 1.7% for growth rate. The CV the HSE assessor calculated is different to that the applicant submitted (16.6%) however, the validity criteria is still achieved.

Validity criteria :	Achieved :
The biomass in the control cultures should have increased exponentially by a factor of at least 16 within the 72-hour test period. This corresponds to a specific growth rate of 0.92 day ⁻¹	Yes 266
The mean coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2 and 2-3, for 72-hour tests) in the control cultures must not exceed 35%.	Yes 11.9%
The coefficient of variation of average specific growth rates during the whole test period in replicate control cultures must not exceed 7%	Yes 1.7%

No reference item was tested in the study and therefore creates uncertainty about the sensitivity of the test species. However, as the validity criteria have been met, this has been deemed to be a minor deviation.

The guideline recommends that the inoculum culture is prepared 2-4 days before the start of the test, however it is not stated when the inoculum culture is prepared in the study. The pH also increased more than 1.5 units during the study but this is not critical as the validity criteria were met.

The recovery of the active did not remain within 80-120% throughout the study, therefore the endpoints have been based on geometric mean measured concentrations. The validity of the analytical method has been considered in Volume 3 CA B5. The method was considered to be validated in accordance with SANTE/2020/12830 rev. 1 for the lowest fortification level of 0.03 mg/L, but was not validated for the higher fortification levels of 4 and 130 mg/L, due to recoveries outside of the acceptable range (recoveries 111–117%, acceptable range 70–110%). Further consideration was made considering further validation data presented in this study report. The following text is taken from Volume 3 CA B5:

“The fresh procedural recoveries were reported as presented in Table B.5.1.2-2.

Table B.5.1.2-2: Summary of procedural recoveries

Matrix	Analyte	Fortification level (mg/L)	Recovery (%) (mean)
Algae test water referred to as W2 in raw data	BLAD (in PROBLAD PLUS)	0.03	63, 87 (75)
		10	93, 108, 116 (106)
		120	80, 106 (92)

Considering the recovery determinations at 10 mg/L and 120 mg/L, although there is a limited number of samples tested (2 at each level), these provide extra reassurance that the method was working at the time of use. The validation data alone suggested that the method may not be fully validated and may be slightly over-estimating the amount present at these higher levels, however, these procedural recoveries provide sufficient reassurance that the method was working at the time of analysis. The method is considered sufficient for regulatory purposes”.

Overall the analytical method is not fully validated but can be considered sufficient for regulatory purposes.

The accepted endpoints for use in the risk assessment are:

- **E_rC₅₀ (growth rate geometric mean) = 51 mg PROBLAD PLUS/L (CI = 44-59 mg/L)**
- **NOEC (growth rate geometric mean) = 6.6 mg PROBLAD PLUS/L**

B.9.2.6.2. Effects on growth of an additional algal species

No data submitted or required. In accordance with assimilated Regulation No 283/2013, effects on additional algal species are only required for substances with herbicidal mode of action.

B.9.2.7. Effects on aquatic macrophytes

No data submitted or required. In accordance with assimilated Regulation No 283/2013, effects on aquatic macrophytes are only required for substances with herbicidal mode of action.

B.9.2.8. Further testing on aquatic organisms

No data submitted or required.

B.9.3. Effects on arthropods**B.9.3.1. Effects on bees****B.9.3.1.1. Acute toxicity to bees**

Reference:	K-CA 8.3.1.1.1/01
Report Title:	PROBLAD: Acute oral and contact toxicity to the Honey bee, <i>Apis mellifera</i> L., in the laboratory.
Author(s) & Year:	Kling (2010)
Document No	CEV SA, Unpublished report No.: S10-02558.
Substance used:	PROBLAD (Batch: 201009, 20% BLAD, 1.30 g/cm ³)
Method of analysis:	No analysis conducted or required
Guideline(s):	OECD Guideline No. 213 and No. 214 (1998)
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

I. MATERIALS AND METHODS**A. MATERIALS****Test Substance**

Test item : PROBLAD (Batch: #201009, 20% BLAD) CoA:
01/09/2010 (checked by HSE assessor)

Test concentrations: µg a.s./bee Oral : 100 (109.42 actual dose consumed)

	Contact : 100
Control :	Oral : aqueous sucrose solution with a final concentration of 500 g/L (50% w/v) Contact : tap water
Reference item :	Perfekthion/ BAS 152 11 I (Batch: 90924-06, 414.8 g/L) Oral: 0.06, 0.08, 0.11, 0.15 µg a.s./bee Contact: 0.10, 0.15, 0.23, 0.34 µg a.s./bee
Test Organism	<p>The honey bee <i>Apis mellifera</i> L., (Hymenoptera, Apidae) young adult workers derived from a healthy colony.</p> <p>One day before the start of the test, the bees were collected randomly from the outer combs of the colony for the oral and contact toxicity test.</p> <p>The hive used was adequately fed, healthy and as far as possible disease-free and queen-right.</p> <p>The hives were monitored regularly by an experienced apiarist and an authorised bee specialist.</p>

B. STUDY DESIGN AND METHODS

Study dates : 21/09/2010- 23/09/2010

Experimental design :	The studies consisted of one dose of the test item, one control for the oral toxicity (50% w/w sugar solution) and one control for the contact toxicity test (tap water), and four doses of the reference item. For each dose and treatment, there were 5 replicate groups of 10 bees.
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Exposure unit : The bees were kept in cages made of stainless steel (8.2 cm x 4.0 cm x 6.0 cm). The front side was equipped with a transparent window so the bees could be observed. The bottom of the cages consisted of perforated steel to guarantee sufficient air supply for the test organisms. The test cages were lined all round with filter paper.

Test procedures : **Oral:**

PROBLAD was dissolved in tap water to produce a stock solution. The final test dose was achieved by mixing the stock solution with an appropriate amount of 50% (w/v) aqueous sucrose solution, so that 20 µL contained the required nominal amount of active ingredient per bee (20% a.s.) even though 25 µL were provided.

The reference item was dissolved in tap water to produce a stock solution.

The test bees were starved for 2 hours before they were fed with solution. A quantity of 250 µL of test item or reference item was offered to each cage of 10 bees to ensure sufficient consumption of test or reference item. Bees within a cage share the test solution (via trophallaxis) and therefore are assumed to have received a similar dose. The amount of test solution consumed was determined by weighing the feeders (Eppendorf cups) before and after feeding. After a period of 6 hours, the feeding solution was totally consumed by the bees and feeders were exchanged. During the observation period, the bees were supplied ad libitum with untreated 50% aqueous sucrose solution. In the control group, the bees were fed with 50% (w/v) aqueous sucrose solution for up to 6 hours after the 2 hours starvation period.

Contact:

PROBLAD was directly dissolved in tap water so that 2 µL contained the required nominal amount of active

ingredient per bee. The reference item was dissolved in tap water in order to get a stock solution at the correct concentration for the highest dose so that 2 µL contained the required nominal amount of reference item per bee. The lower doses were prepared by mixing an appropriate amount of stock solution with an appropriate amount of tap water.

After the bees had been anaesthetised with carbon dioxide they were treated individually by topical application with a micro applicator. 2 µL of the control, test and reference item solutions were applied dorsally to the thorax of each bee. Between every application, the outside of the micro applicator was cleaned with a mixture of water and a water-wetting agent. This reduced the surface tension of the applied solution and ensured that the drop of the test item solution spread out immediately after application on each bee. After application, the bees were returned to the test cages and fed with 50% (w/v) aqueous sucrose solution ad libitum.

Environmental Conditions:

Conditions were measured continuously with a calibrated thermohygrograph.

Temperature :	25°C and 25.5°C (required: 25 ± 2°C)
Humidity :	61 and 66% (required 50-70%)
Lighting :	Constant darkness except at start of experimental phase in the oral toxicity test (feeding of the bees) and during the three assessments (4, 24 and 48 hours after test start). Feeding, application and assessments were made under neon light.

Observations :

In the oral and contact toxicity tests the number of dead bees in the individual test cages was recorded 4, 24 and 48 hours after the start of feeding and after application, respectively. In case of symptoms of poisoning, the behavioural

differences between the bees of the control group and those of the test item treatment were noted at each observation interval.

Statistics:

The mortality (%) per treatment was calculated from the number of dead bees and the total number of introduced bees per treatment group. There was no dead bees in any of the control groups. Therefore a correction of the mortality according to the formula of ABBOTT was not necessary.

The LD₅₀ values with 95% confidence limits of the reference item treatment were calculated by means of a probit analysis using the statistical program SAS Proprietary Software 9.2 (2002-2008). The oral LD₅₀ values for the reference item treatment were calculated with the consumption values per replicate.

II. RESULTS AND DISCUSSION:

Oral:

The actual consumption per bee in the oral test was 109.42 µg a.s./bee. The reference item was tested with the nominal dose levels of 0.06, 0.08, 0.11, 0.15 µg a.s./bee.

The mortality in the oral toxicity test is given as a function of the target dose and actual dose of PROBLAD or reference item and test solution consumed, respectively.

Table 9.3.1-1: Mortality and total consumption in the oral toxicity test in the control, the test item PROBLAD and reference item groups

Treatment (Target dose)	Test item consumed	Mortality (%)	
		24 h	48 h
Control (sugar solution)	--	0.0	0.0
Test item: PROBLAD (µg a.s./bee)			
100	109.42	0.0	0.0
Reference item: Perfekthion (µg a.s./bee)			
0.06	0.07	0.0	2.0
0.08	0.09	8.0	18.0
0.11	0.12	62.0	68.0
0.15	0.18	92.0	94.0

In the control group fed with sugar solution, no mortality occurred during the 48 hour observation period.

Table 9.3.1-2: LD₅₀ values in the oral toxicity test with the test item PROBLAD and the reference item

Treatment	24 h		48 h	
	LD ₅₀	Limits*	LD ₅₀	Limits*
	(µg a.s./bee)			
PROBLAD	> 109.42	-	> 109.42	-
Reference item	0.12	0.10 to 0.14	0.11	0.10 to 0.12

*lower and upper confidence limits; $p \leq 0.05$

No behavioural effects were observed in the oral toxicity test over the whole 48-hour test period.

Contact:

The reference item was tested with the nominal dose levels of 0.10, 0.15, 0.23, and 0.34 µg a.s./bee.

Table 9.3.1-3: Mortality in the contact toxicity test in the control, the test item PROBLAD and the reference item groups

Treatment	Mortality (%)	
	24 h	48 h
Control (tap water)	0.0	0.0
Test item: PROBLAD (µg a.s./bee)		
100	0.0	0.0
Reference item: Perfekthion (µg a.s./bee)		
0.10	0.0	0.0
0.15	16.0	26.0
0.23	68.0	72.0
0.34	66.0	66.0

In the control group treated with tap water, no mortality occurred during the 48 hour observation period.

Table 9.3.1-4: LD₅₀ values in the contact toxicity test with the test item PROBLAD and the reference item

Treatment	24 h		48 h	
	LD ₅₀	Limits*	LD ₅₀	Limits*
	(µg a.s./bee)			
PROBLAD	> 100	-	> 100	-

Treatment	24 h		48 h	
	LD ₅₀	Limits*	LD ₅₀	Limits*
	(µg a.s./bee)			
Reference item	0.23	0.20 to 0.26	0.21	0.17 to 0.24

*lower and upper confidence limits; $p \leq 0.05$

No behavioural effects were observed in the contact toxicity test over the whole 48-hour test period.

Validity criteria

	Oral	Contact
Average mortality must be $\leq 10\%$ in the control at the end of the test	0%	0%
24 h LD ₅₀ of the reference item must be within the appropriate range	0.12 (required: 0.10-0.35 µg a.s./bee)	0.23 (required: 0.10-0.30 µg a.s./bee)

III. CONCLUSION:

No mortality occurred in the control groups of the oral and contact toxicity tests during the 48-hour observation period.

In the oral toxicity and the contact toxicity tests, no mortality occurred and no behavioural effects were observed over the whole 48-hour test period.

The 24-hour oral and contact LD₅₀ values for the reference item are 0.12 and 0.23 µg dimethoate/bee. Consequently, validity criteria were met for both control and reference item mortality and the test was deemed valid.

HSE COMMENTS :

The studies were carried out according to GLP and follow OECD No. 213 and 214 with no significant deviations to the guidelines. The reference item was tested at a suitable concentration and provided results within the expected range according to the guidelines (oral: 0.10–0.35 µg a.s./bee; contact: 0.10–0.30 µg a.s./bee).

The final test dose in the oral study was achieved by mixing the stock solution with an appropriate amount of 50% (w/v) aqueous sucrose solution, so that 20 µL contained the required nominal amount of active ingredient per bee (20% a.s.) even though 25 µL were provided. For the contact test, a droplet of 2 µL was chosen in deviation to the guideline recommendation of 1 µL, since the higher volume ensures a more reliable dispersion of the test item. Experience has proven that higher volumes are suitable and no adverse effects on the outcome of the study are to be expected. The HSE assessor has deemed these to be minor deviations.

There was no mortality in the oral or contact toxicity study, therefore the endpoint is > highest dose tested.

HSE has agreed that the following endpoints are suitable for the risk assessment:

- **Oral LD₅₀: > 109.42 µg PROBLAD /bee**
- **Contact LD₅₀: > 100 µg PROBLAD /bee**

Reference:	K-CA 8.3.1.1.1/02
Report Title:	PROBLAD PLUS: Acute oral toxicity to the Honey bee, <i>Apis mellifera</i> L., under laboratory conditions
Author(s) & Year:	Aguilar-Alberola (2019)
Document No	CEV SA, Unpublished report No.: S19-21875.
Substance used:	PROBLAD PLUS, (Batch: CF01907-001, 21.2% BLAD, 1.20 g/cm ³)
Method of analysis:	No analysis conducted or required
Guideline(s):	OECD Guideline No. 213 (1998)
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

I. MATERIALS AND METHODS

A. MATERIALS

Test Substance

Test item : PROBLAD PLUS (Batch: CF01907-001, 21.2% w/w BLAD protein) CoA: 11/07/2019 (checked by HSE assessor)

Test concentrations: 96.5, 115.7, 138.9, 166.7 and 200.0 µg test item/bee

Actually consumed: 95.3, 112.7, 137.1, 162.8 and 196.8 µg test item/bee

Control : Aqueous sucrose solution with a final concentration of 500 g/L (50% w/v)

Reference item : Perfekthion/ BAS 152 11 I (Batch: FRE-001578, 429.0 g/L)

0.060, 0.090, 0.140, 0.210 µg a.s./bee

Actual consumed: 0.059, 0.089, 0.113, 0.175 µg a.s./bee

Test Organism

The honey bee *Apis mellifera* L., (Hymenoptera, Apidae) young adult workers derived from a healthy colony.

One day before the start of the test, the bees were collected randomly from the outer combs of the colony and kept under test conditions in the test units.

The hive used was adequately fed, healthy and as far as possible disease-free and queen-right. No chemical substances (such as antibiotics, anti-Varroa treatments, pesticides, etc.) were used in the hive for at least one month prior to this test.

The hives were monitored regularly by an experienced apiarist and an authorised bee special.

B. STUDY DESIGN AND METHODS

Study dates : 24/09/2019- 26/09/2019

- Experimental design :** The oral toxicity test was carried out as a dose-response test with duration of 48 hours. The test comprised one control group (C: pure 50% (w/v) aqueous sucrose solution), five test item treatment groups and four reference item treatment groups. Each treatment group consisted of 50 test organisms (divided in 5 replicates, containing 10 test organisms each) except the reference item group which had 40 test organisms (divided in 4 replicates, each containing 10 test organisms).
- Exposure unit :** Honey bees were kept in cages made of stainless steel (approx. base: 8.5 cm x 4.5 cm; height: 6.5 cm). The front side of the cages was equipped with a transparent pane to enable observation. The bottom of the cages consisted of perforated steel, which guaranteed sufficient air supply. The cages were lined with filter paper
- Preparation of application solutions :** Test and reference item were measured using a calibrated balance and/or micropipettes. For the preparation of the test item stock solution and the dilutions for the test item treated groups, 50% (w/v) aqueous sucrose solution was used as solvent. For the preparation of the reference item stock solution and the dilutions for the reference item treated groups, 50% (w/v) aqueous sucrose solution was used as solvent. All the treatment solutions were prepared immediately before the application.
- Test procedures :** For application, 2 mL syringes (feeders) containing the corresponding application solutions were used. The tips of the syringes were cut off to enable the bees to reach the

application solution. The application volume was 200 µL/replicate (corresponding to 20 µL/bee). The bees in one replicate share the application solution and thus receive similar doses (trophallaxis). The bees were starved approx. 2 hours prior to application start. Each test unit was provided with the application solution for up to 6 hours, to ensure a sufficient intake. The feeders were then removed and the bees were provided ad libitum with a 50% (w/v) aqueous sucrose solution. For dose verification the amount of application solution(s) consumed was determined by weighing the feeders before and after feeding using calibrated equipment.

Environmental Conditions:

Conditions were measured continuously with a calibrated data logger.

Temperature :	25.2°C–26.9°C (required : $25 \pm 2^\circ\text{C}$)
Humidity :	51.6 and 57.2% (required : 50–70%)
Lighting :	Constant darkness except at start of experimental phase in the oral toxicity test (feeding of the bees) and during the three assessments (4, 24 and 48 hours after test start).

Statistics:

The percentage of mortality was calculated for 24 and 48 hours for each treatment group from the number of dead individuals in relation to the number of introduced test organisms. In case of control mortality occurred, the cumulative mortalities for each test and reference item groups were expressed as percentage of the control populations after an adjustment according to the formula of Abbott.

The consumption of application solution per replicate was calculated from the initial and final weight of the syringes. For each treatment group, the mean consumption of application solution per replicate was calculated by averaging the replicate values.

The qualitative trend analysis by contrasts (monotonicity of concentration/response, $\alpha = 0.05$) did not reveal a linear trend between doses and mortality at the different study periods. Because of this, a Chi^2 2x2 table test with Bonferroni correction (one-sided greater, $\alpha = 0.05$) was used to evaluate whether there was a significant difference between the mortality data of the test item groups and the control group in

order to determine the NOED. Since consumptions were very similar between the test item groups (between 97.39 and 98.74% of the offered food) and the control group (97.98%), no statistical analysis was performed for this parameter. Because the mortality in the treatments no exceeds 50% in any case, the LD₅₀ cannot be calculated, but it was empirically estimated from the results. The 24h-LD₅₀ for the reference item was calculated using Probit analysis. For the statistical evaluation, the MS Excel Version 14.0 and the statistics program ToxRatPro® Version 3.2.1 was used.

Observations:

Mortality was recorded 4 hours after application (after start of feeding) and thereafter at 24 hours and 48 hours (\pm 30 min). Behavioural abnormalities such as symptoms of poisoning in comparison to the control were recorded at each observation interval. In the reference item group, behavioural abnormalities assessments were not conducted as it can be assumed that moribund and affected bees died by the end of the test.

Behavioural abnormalities were recorded according to the following categories:

- a = affected (bees still upright and attempting to walk but showing signs of reduced coordination).
- ap = apathy (bees show only low or delayed reactions to stimulation, e.g. light or blowing; bees are sitting motionless in the unit or are able to walk but not correctly).
- c = cramps (bees contracting abdomen or entire body).
- m = moribund (bees cannot walk and show only very feeble movements of legs and antennae, only weak response to stimulation; e.g. light or blowing; bees may recover but usually die).

II RESULTS AND DISCUSSION:

Bees of the control group consumed, on average, 97.98% of the offered food. Bees dosed with the test item consumed on average from a minimum of 97.39% (at treatment T2) to a maximum of 98.74% (at treatments T1 and T3) of the offered treated food. Bees dosed with the reference product consumed on average from a minimum of 80.67% to a maximum of 99.26% of the offered food. Since these consumptions were very similar, no statistical analysis was performed for this parameter.

By the end of the test, 48 hours after the application, one dead individual was observed in the control group. In the test item consumed doses of 95.3, 112.7, 137.1, 162.8 and 196.8 µg test item/bee, the cumulative mean corrected mortality at the end of the test, 48 h after dosing, was 4.08, 4.08, 4.08, 2.04 and 0.00%, respectively. No statistically significant differences were obtained for the mortality of the test item treated groups compared with the control group. According to the results of the study, the NOED value at 24 and 48 hours for the test was determined to be 196.8 µg test item/bee (actual value taking into account the consumed solution). Because the

mortality did not reach the 50% at the end of the test in any test item treatment, the median Lethal Dose (LD₅₀) value was empirically estimated from the results to be greater than 196.8 µg test item/bee. No sub-lethal effects were observed during the whole test period at all the test groups tested.

Table 9.3.1-5: Mortality in the oral toxicity test in the control and test product groups

Target doses (treatment group) [µg test item/bee]		Mortality (%)			Corrected mortality* [%]		
		4 hr	24 hr	48 hr	4 hr	24 hr	48 hr
0.00	I	0.00	0.00	2.00	--	--	--
96.5	(T1)	0.00	4.00	6.00	0.00	4.00	4.08
115.7	(T2)	0.00	6.00	6.00	0.00	6.00	4.08
138.9	(T3)	0.00	2.00	6.00	0.00	2.00	4.08
166.7	(T4)	0.00	4.00	4.00	0.00	4.00	2.04
200.0	(T5)	0.00	2.00	2.00	0.00	2.00	0.00

*Corrected by the control group according to the formula of Schneider-Orelli (1947)

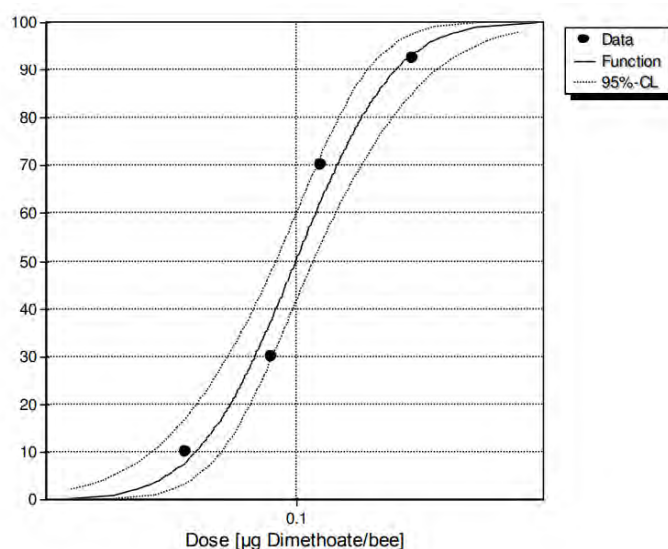


Figure 9.3.1/02-1. Dose-effect curve showing the influence of the test item on mortality of the introduced *Apis mellifera* as observed after 24 h

Validity criteria

Required :	Observed :
Average mortality must be $\leq 10\%$ in the control at the end of the test	2.0%
24 h LD ₅₀ of the reference item must be within the appropriate range	0.10 (required: 0.10–0.35 µg a.s./bee)

III. CONCLUSION:

The oral acute toxicity test of PROBLAD PLUS was tested under laboratory conditions over a period of 48 hours. The mortality in the control group was low (maximum 2.0%). The 24 hour LD₅₀ value for the reference item was 0.100 µg Dimethoate/bee. Consequently, validity criteria for both, control and reference item mortality, were met and the test was considered valid. According to the results of the study, the NOED value at 24 and 48 hours was determined to be 196.8 µg test item/bee. Because the mortality did not reach the 50% at the end of the test in any test item treatment, the median lethal dose (LD₅₀) values were empirically estimated from the results to be greater than the consumed dose of 196.8 µg test item/bee.

HSE COMMENTS

The study was carried out according to GLP and follows OECD No. 213 with no significant deviations to the guidelines. The reference item was tested at a suitable concentration and provided results within the expected range according to the guidelines (0.10- 0.35 µg a.s./bee). There was less than 50% mortality in the study, the endpoint was not determined statistically and is instead estimated to be > highest dose tested.

HSE has agreed that the following endpoint is suitable for the risk assessment:

Oral LD₅₀: > 196.8 µg PROBLAD PLUS/bee

Reference:	K-CA 8.3.1.1.2/02
Report Title:	PROBLAD PLUS: Acute oral and contact Toxicity to the Bumblebee <i>Bombus terrestris</i> L., under Laboratory Conditions.”
Author(s) & Year:	Aguilar-Alberola (2020)
Document No	CEV SA, Unpublished report No.: S20-00599
Substance used:	PROBLAD PLUS (Batch: CF01907-001, 21.2% BLAD, 1.20 g/cm ³)
Method of analysis:	Enzyme-Linked Immunosorbent Assay (ELISA). Method ref. S19-21256
Guideline(s):	OECD Guideline No. 213 (1998)
Deviations:	Yes, see HSE comments
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

I. MATERIALS AND METHODS

A. MATERIALS

Test Substance

Test item : PROBLAD (Batch : CF01907-001, 20% w/w BLAD) CoA : 11/07/2019 confirmed by HSE assessor

Test concentrations : Oral : 162.5, 325.0, 650.0, 1300.0, 2600.0 (4.06, 8.13, 16.25, 32.50, 65.0 g test item/L)

Contact : 75.0, 150.0, 300.0, 600.0, 1200.0 (18.75, 37.50, 75.0, 150.0, 300.0 g test item/L)

Test item preparation : Oral : 50% (w/v) aqueous sucrose solution : 500.00 g sucrose filled up with deionised water to 1000 mL

Oral toxicity test [μg test item/bee]

T1 ; 162.5 : 0.0407 g test item filled with 50% (w/v) aqueous sucrose solution to 10 mL

T2 ; 325.0 : 0.0815 g test item filled with 50% (w/v) aqueous sucrose solution to 10 mL

T3 ; 650.0 : 0.1623 g test item filled with 50% (w/v) aqueous sucrose solution to 10 mL

T4 ; 1300.0 : 0.3252 g test item filled with 50% (w/v) aqueous sucrose solution to 10 mL

T5 ; 2600.0 : 0.6502 g test item filled with 50% (w/v) aqueous sucrose solution to 10 mL

Contact : 0.1% Triton X solution : 0.107 g Triton X filled up with deionised water to 100 mL

Contact toxicity test [μg test item/bee]

T1 ; 75.0 : 0.1875 g test item filled up with 0.1% Triton X solution to 10 mL

T2 ; 150.0 : 0.3749 g test item filled up with 0.1% Triton X solution to 10 mL

T3 ; 300.0 : 0.7500 g test item filled up with 0.1% Triton X solution to 10 mL

T4 ; 600.0 : 1.5002 g test item filled up with 0.1% Triton X solution to 10 mL

T5 ; 1200.0 : 3.0000 g test item filled up with 0.1% Triton X solution to 10 mL

Control :

Oral : pure 50% (w/v) aqueous sucrose solution

Contact : 0.1% Triton X solution

Reference item :	Dimethoate (Batch : 10248664A, 414.0 g/L, 1.062 g/cm ³) CoA : 04/03/2019 confirmed by HSE assessor Oral toxicity test [µg dimethoate/bee] R ; 4.0 : 2.42 µL of BAS 152 65 I filled up with 50% (w/v) aqueous sucrose solution to 10 mL Contact toxicity test [µg dimethoate/bee] R ; 10.0 : 30.2 µL BAS 152 65 I filled up with 0.1% Triton X solution to 5 mL
Test Organism	<p><i>Bombus terrestris</i> L. (Hymenoptera : Apidae) Young adult worker Bumblebees obtained from commercial queen-right colonies free from disease and not treated within the last month were used as test organisms.</p> <p>Medium size workers were selected one day before application. Bumblebees were obtained directly from the hives and they were randomly allocated to test cages. Only medium size workers were used in the test. Overly small and overly big bumblebees as well as recently emerged individuals were excluded from the test group.</p> <p>Acclimatised : 02/02/2020- 03/03/2020</p>

B. STUDY DESIGN AND METHODS

Study dates : 03/03/2020- 05/03/2020

Experimental design :	<p>The oral toxicity test was carried out as a 48 hour dose-response test. The test comprised one control group, five test item treatment groups and one reference item treatment group. Each treatment group consisted of 35 test organisms (divided in 35 replicates, containing 1 test organism each).</p> <p>The contact toxicity test was carried out as a 48 hour dose-response test. The test comprised one control group, five test item treatment groups and one reference item treatment group. Each treatment group consisted of</p>
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30 test organisms (divided in 30 replicates, containing 1 test organism each).

Exposure unit : Bumblebees were kept individually in Nicot® cages and fed via 1 mL syringes deprived of the tip. Syringes were kept in position putting them in a piece of hose and by means of a plastic socket to prevent the syringes from sticking into the Nicot® cage. These test units were slightly inclined to ensure that the sugar solutions flowed to the opening of the syringes. Individual cages were placed next to each other.

Test procedures :

Oral : For application, 1 mL syringes (feeders) containing the corresponding application solutions were used. The tips of the syringes were cut off to enable the bees to reach the application solution. The application volume was 40 µL/replicate (corresponding to 40 µL/bee). The bees were starved 2 hours and 40 minutes prior to application start. Each unit was provided with the application solution for up to 4 hours, to ensure a sufficient intake. The feeders were then removed and the bees were provided ad libitum with a 50% (w/v) aqueous sucrose solution for the remainder of the test. For dose verification the amount of application solution(s) consumed was determined by weighing the feeders before and after feeding using calibrated equipment. Individuals that did not consume at least 80% of the mean consumption of the respective treatment group within the four hours of exposure were discarded from the test.

Contact : After being chilled with dry ice, a 4 µL droplet of the corresponding test solution was applied individually to the dorsal side of the thorax of each bumblebee. The application amount of 4 µL, instead of a 2 µL droplet ensures a more reliable dispersion of the application solution. Test Facility experience has proven that higher volumes are suitable and no adverse effects on the

outcome of the study are to be expected. Hand operated micro-applicators were used for application. After the application, the bees were returned to the test units. All the individuals recovered adequately from the anaesthesia (i.e., showed inconspicuous behaviour) in a short time.

Environmental Conditions :

Conditions were measured continuously with a calibrated thermohygrograph.

Temperature : Oral and contact : 24.1–25.1°C. (required : $25 \pm 2^\circ\text{C}$)

Humidity : Oral and contact : 57.9–80.6%. (required 40–80%)

Lighting : The collection of the bumblebees from the hive was performed under red light in a refrigerated chamber (temperature of approximately 15.4°C), applications and assessments were conducted under artificial light. The rest of the study was conducted under constant darkness.

Food : 50% (w/v) aqueous sucrose solution. Feeding was done ad libitum during acclimatisation and the test period for both the oral and contact tests, except during starvation and feeding of application solutions for the oral test.

Observations :

Mortality was recorded 4 hours after application (after start of feeding in the oral toxicity test and after application in the contact toxicity test) and thereafter at 24 hours and 48 hours (± 30 min).

Behavioural abnormalities such as symptoms of poisoning in comparison to the control were recorded at each observation interval. In the reference item group,

behavioural abnormalities assessments were not conducted as it can be assumed that moribund and affected bees died by the end of the test. Behavioural abnormalities were recorded according to the following categories :

- a = affected (bumblebees still upright and attempting to walk but showing signs of reduced coordination).
- m = moribund (bumblebees cannot walk and show only very feeble movements of legs and antennae, only weak response to stimulation ; e.g. light or blowing ; bumblebees may recover but usually die).

Analytics

Samples of the test item treated solutions from the lowest and highest concentrations were taken directly after its preparation for both, oral and contact solutions. Tested using ELISA. LOQ : 1.6 mg PROBLAD PLUS/L in 50% (w/v) aqueous sucrose solution. 0.2 mg PROBLAD PLUS/L in aqueous solution with 0.1% of Triton X. LOD : 0.02 mg PROBLAD PLUS/L ($\leq 30\%$ of the LOQ) in 50% (w/v) aqueous sucrose solution. 0.0625 mg PROBLAD PLUS/L ($\leq 30\%$ of the LOQ) in aqueous solution with 0.1% of Triton X.

Statistics :

Descriptive statistics for mortality and food consumption. Since no mortality occurred for the control group and the treatments with the test item in the oral test, no statistical analysis was performed for this parameter. In order to determine the NOED in the contact test at 24 and 48 hours, because the qualitative trend analysis by contrasts (monotonicity of dose/response, $\alpha = 0.05$) did not revealed a linear trend, a multiple sequentially-rejective Fisher test after Bonferroni-Holm (one-sided greater, $\alpha = 0.05$) was used. Since none of the treatments showed mortality higher than 50%, the LD₅₀ could not be calculated statistically. Statistical calculations were made with MS Excel 2010 v.14.0 and the statistical program ToxRatPro® Version 3.2.1.

II. RESULTS AND DISCUSSION :**Table 9.3.1-6: Analytical recoveries for PROBLAD PLUS**

Nominal PROBLAD PLUS concentration (mg/L)	Analysed PROBLAD PLUS concentration (mg/L)	Recovery (%)
4060	3361.68	83
65000	56030.0	86
18750	18712.50	100
300000	276000.0	92

Oral

The number of non-feeder bumblebees ranged from a minimum of no individuals in the T2 treatment group to a maximum of 15 individuals in the T5 treatment group. Thus, all the treatments had a minimum of 20 feeder individuals. The feeder bumblebees of the control groups consumed, on average, 97.6% of the offered food. The feeder bumblebees dosed with the test item consumed, on average, from a minimum of 89.3% (at treatment T5) to a maximum of 98.2% (at treatment T3) of the offered treated food. Feeder bumblebees dosed with the reference item consumed, on average, 92.6% of the offered food, equivalent to 3.7 µg Dimethoate/bumblebee.

By the end of the test (48 hours after dosing) and between the feeder individuals, no bumblebees were observed as dead. For this reason no statistical analysis was performed for this parameter. Therefore, under the conditions of this study, the NOED corresponded to the dose of 2320.9 µg test item/bumblebee (actual intake value).

Because the mortality did not reach the 50% at the end of the test in any treatment group, the LD₅₀ could not be calculated statistically, but was empirically estimated from the results as higher than 2320.9 µg test item/bumblebee (actual intake value) for all the periods. No individuals were recorded as having behavioural abnormalities (i.e. bumblebees affected or moribund) during the whole test.

Table 9.3.1-7: Mortality and mean consumption in the oral toxicity test

Nominal dose [μg test item/bee]	Consumed dose/bumblebee [μg PROBLAD PLUS/bee]	Mortality (%)			Corrected mortality (%)		
		4 h	24 h	48 h	4 h	24 h	48 h
0.0	-	0	0	0	0	0	0
162.5	158.5	0	0	0	0	0	0
325.0	313.3	0	0	0	0	0	0
650.0	638.5	0	0	0	0	0	0
1300.0	1263.7	0	0	0	0	0	0
2600.0	2320.9	0	0	0	0	0	0

Contact

Compared to the control group, none of the test item treated groups showed statistically significant increase in mortality at the 24 and 48 hour assessment. Therefore the NOED for mortality, under the conditions of this study, corresponded to the dose of 1200.0 μg test item/bumblebee. As no treatment showed mortality equal to or higher than 50%, the LD₅₀ could not be calculated statistically, but was empirically estimated from the results as higher than the applied dose of 1200.0 μg test item/bumblebee.

Symptoms of intoxication (affected bumblebees) were observed in one individual of the treatments T3, T4 and T5, 4 hours after the exposure. These individuals were observed as dead from the 24 hour assessment. In the 24 hour assessment and by the end of the test (48 hours after exposure), no individuals were observed as being affected.

Table 9.3.1-8: Mortality in the contact toxicity test

Nominal dose [μg test item/bee]	Mortality (%)			Corrected mortality (%)		
	4 h	24 h	48 h	4 h	24 h	48 h
0.0	0	0	0	-	-	-
75.0	0	0	0	0	0	0
150.0	0	0	0	0	0	0
300.0	0	3.33	6.67	0	3.33	6.67
600.0	0	3.33	3.33	0	3.33	3.33
1200.0	0	6.67	10.0	0	6.67	10.0

Validity criteria

Criterion	Oral	Contact
Average control mortality \leq 10%	0.00%	0.00%
Average reference item mortality \geq 50%	100%	86.67%

III. CONCLUSION :

All validity criteria were met and sensitivity of the test organisms could be confirmed. The measured concentration in the samples was within 20% of nominal test concentration used. Thus the concentrations of the test item were confirmed and the endpoints are based on nominal concentrations. According to the results of the study, the NOED value at 24 and 48 hours for the oral test was determined to be 2320.9 μg test item/bumblebee (actual intake value). Because the mortality did not reach the 50% at the end of the test in any treatment, the LD₅₀ value was empirically estimated from the results as higher than 2320.9 μg test item/bumblebee (actual intake value). According to the results of the study, the NOED value at 24 and 48 hours for the contact test was determined to be 1200.0 μg test item/bumblebee. Because the

mortality did not reach the 50% at the end of the test in any treatment, the LD₅₀ was empirically estimated from the results as higher than 1200.0 µg test item/bumblebee.

HSE COMMENTS :

The study was carried out according to GLP and follows OECD No. 246 and 247 (2017) with no significant deviations to the guidelines. The climatic conditions were suitable and the results of the study were statistically analysed according to the methods outlined in the guidelines. The reference item tested was within both guideline requirements. The validity criteria were met during the study.

The humidity rises above the specified range in the guidelines, however the HSE assessor accepts this as a minor deviation as it was only for a short period of time, and the validity criteria were met.

The guidelines state that a minimum of 2 mL of feeding solution be provided to each replicate, whereas in this study only 1 mL of feeding solution was provided. The applicant states that this volume was selected as it exceeds the expected daily intake amount for bees. HSE accepts this justification and considers the deviation to be minor.

The applicant states in the contact toxicity study 'The application amount of 4 µL, instead of a 2 µL droplet ensures a more reliable dispersion of the application solution. Test Facility experience has proven that higher volumes are suitable and no adverse effects on the outcome of the study are to be expected.' HSE accepts this justification and considers the choice of application volume a minor deviation.

HSE Chemistry have considered the validity of the analytical method in Volume 3 CA B5. The analytical method S19-21256 for the determination of PROBLAD PLUS in sucrose solution and deionised water is considered fully validated in accordance with SANTE/2020/12830 rev. 1.

The analytical recovery values were within the range of 80–120%, therefore the endpoint was based on nominal values. HSE considers that the study endpoints should be used as supporting evidence only for this application. Given the current lack of noted guidance for assessing the risk to bumblebees, the study is not required for use in risk assessment for this application.

HSE has agreed that the following endpoint is suitable for the risk assessment:

Oral LD₅₀: > 2320.9 µg test item/bumblebee

Contact LD₅₀: > 1200.0 µg test item/bumblebee

B.9.3.1.2. Chronic toxicity to bees

Reference:	K-CA 8.3.1.2/01
Report Title:	PROBLAD PLUS: Chronic toxicity test for adult honeybees (<i>Apis mellifera</i> L.)
Author(s) & Year:	Harkin (2015a)
Document No	CEV SA, Unpublished report No.: B2CF1000.
Substance used:	PROBLAD PLUS (Batch: D32-280414, 20.1% BLAD protein; CoA not available to confirm purity)
Method of analysis:	No analysis conducted or required
Guideline(s):	Method No. 230: Commission des Essais Biologiques Method for the Evaluation of Plant Protection Products on Honey Bees <i>Apis mellifera</i> L. EFSA Guidance Document on the risk assessment of plant protection products on bees (<i>Apis mellifera</i> , <i>Bombus</i> spp. And solitary bees) Current recommendations of the ring test group (2014) for a proposal for a new OECD Guideline for 10 day chronic adult honey bee toxicity test
Deviations:	Yes, see HSE comments
GLP or GEP:	Yes
Acceptability:	Supplementary
Study relied upon:	No, there are multiple uncertainties with the study conduct, and a more up to date study is available to address this data requirement.

I. MATERIALS AND METHODS**A. MATERIALS****Test Substance**

Test item : PROBLAD PLUS (Batch: D32-280414, 20.1% w/w BLAD protein; CoA not available to confirm purity))

Test concentrations : A range-finding test was run before the start of the main test.

1000 mg a.s./kg 50% (w/v) aqueous sucrose solution was decided as the test concentration for the limit test due to the results of the range-finding test

Control : Aqueous sucrose solution with a final concentration of 500 g/L (50% w/v)

Reference item : Danadim Progress- Dimethoate (Batch: 0001064092)
1.5 mg a.s./kg sucrose solution

Test Organism

The honey bee *Apis mellifera* L., (Hymenoptera, Apidae) young adult workers.

Newly emerged honeybees (less than 48 hours old) were used in the chronic test. They were allowed continual access to a 50% aqueous sucrose solution either with or without the test item through the form of a feeder inserted into the side of the plastic housing cage. This feeder was changed and weighed in and out every day, allowing the amount and dose consumed to be calculated.

The colony selected to provide brood for the test was queen-right, had an absence of statutory notifiable diseases, a low incidence of adult diseases and had not been treated with a varroacide within the 4 weeks before use in the study. At the end of the tests, bees used in the study were disposed of by freezing then incineration. The labelled frame was placed into a plastic 'bee emergence' cage and then placed into an environmental chamber held at $33 \pm 2^{\circ}\text{C}$ and $65 \pm 5\%$ RH. The cage was then left for bees to emerge.

The following day (day 0), all bees that had emerged overnight were taken directly from the comb into the test units (10 bees per pot) and returned to the incubator. They were given free access to untreated 50% (w/v) aqueous sucrose solution and pollen patty (organic pollen mixed with deionised

water to form a paste) overnight until the first day of dosing on day 1.

B. STUDY DESIGN AND METHODS

Study dates : 25/06/2014- 23/08/2014

Experimental design : The main test consisted of 5 cages of 10 bees per treatment group. The toxicity endpoint was mortality rate after 10 days of exposure

Exposure unit : The test units used to assess bee mortality consisted of an inverted 8 oz plastic pot lined with a filter paper. A hole was made in the side of the pot to allow a sucrose feeder to be inserted and held in place horizontally allowing the bees access to the sucrose solution while preventing its leakage. The sucrose feeders were replaced every day and were weighed in and out to allow consumption of sucrose to be calculated.

Treatment application : A stock solution of the test item was made up in water on Day 0, this was then used to make up the test feed solution which was stored at 0–10°C for the duration of the test. The toxic reference stock was made up in water on Day 0 and stored at 0–10°C until required for dosing, the toxic reference feed solutions were made up daily. All feed solutions were made up in 50% (w/v) aqueous sucrose solution. For dosing, a 1.5 mL micro-centrifuge tube was used, modified by making a small hole (approximately 1–1.5 mm diameter) at the narrowest end to act as a feeder pushed through a hole made in the side of the test unit. The feeders were labelled and filled with approximately 1.5 mL of dosing solution dilution (or control solution) using a syringe. Once filled, the feeder and contents were weighed before being placed in the appropriate test cage. Feeders were exchanged for new full feeders every day. Once removed, the feeders were re-weighed to allow calculation of the feed uptake

Environmental Conditions:

The test units were housed in a temperature and humidity controlled incubator for the duration of the test. Conditions were measured continuously with a calibrated data logger.

Temperature : $33^{\circ}\text{C} \pm 2^{\circ}\text{C}$ (required : $33 \pm 2^{\circ}\text{C}$)

Humidity : $65 \pm 5\%$ (required : 50–70%)

Lighting : Constant darkness

Statistics :

As no dose related toxicity was observed (there was no difference in mortality between the test item and control groups) no statistics were carried out.

Observations:

Observations of mortality and behaviour were recorded at 24 hour intervals after set up for 10 days (± 60 minutes). The number of live, knocked down, stumbling and dead bees were recorded. The condition of the bees was defined as follows:

Live – alive and apparently unaffected

Stumbling – moving but in a poorly co-ordinated manner

Knocked down – alive but immobile, i.e. twitching

Dead – no longer moving

If the bees were obviously agitated or affected in any other way (e.g., vomiting) this was also recorded.

II. RESULTS AND DISCUSSION:

Table 9.3.1-9: Summary of feed uptake

Treatment group	Nominal conc.	Mean uptake feeding solution (mg) per bee over the course of the test ^a											
		D0-1	D1-2	D2-3	D3-4	D4-5	D5-6	D6-7	D7-8	D8-9	D9-10	Sum ^b	Mean uptake (mg/bee/day) ^c
Control	0	28.0	16.3	34.6	32.7	35.8	37.6	36.2	52.0	35.4	64.7	373.3	37.3
PROBLAD PLUS	1000 (mg/kg)	25.9	22.5	32.0	34.9	46.9	40.2	52.3	51.8	48.3	66.3	421.1	42.1
Toxic Reference	1.5 (mg a.s./kg)	21.6	23.4	29.9	29.8	27.7	27.7	34.9	30.5	59.0	-	284.5	31.6

^a calculated average per living bee (rounded results, calculated from the exact data)^b sum of mean uptake feed/bee at test end over the course of the 10 days feeding period^c mean of mean uptake feed/bee per day at test end over the course of the 10 days feeding period

- all bees dead

The feed uptake in the treated group resulted in a mean daily dose of 42.10 µg test item/bee per day.

Table 9.3.1-10: Summary of dose consumed

Treatment group	Nominal concentration	Mean uptake feeding solution (mg) per bee over the course of the test ^a											
		D 0-1	D 1-2	D 2-3	D 3-4	D 4-5	D 5-6	D 6-7	D 7-8	D 8-9	D 9-10	Sum ^b	Mean (µg/bee/day) ^c
PROBLAD PLUS	1000 (mg/kg)	25.91	22.46	32.02	34.91	46.86	40.16	52.27	51.81	48.31	66.26	420.97	42.10
Toxic reference	1.5 (mg a.s./kg)	0.032	0.035	0.045	0.045	0.041	0.042	0.052	0.046	0.089	-	0.427	0.047

^a mean uptake per bee/day calculated by: total sucrose uptake of all replicates in group x concentration / no. live bees.

^bsum of mean uptake feed/bee at test end over the course of the 10 days feeding period

^cmean of mean uptake feed/bee per day at test end over the course of the 10 days feeding period

- all bees dead

Mortality:

The mortality observed within the control group was 2% meeting the acceptance criterion of < 10% mortality. In the test item group, mortality was also observed to be 2% (1 bee dead). In the toxic reference group, 100% mortality was observed, meeting the acceptance criterion of > 50% mortality at day 10.

Table 9.3.1-11: Percentage cumulative mortality of adult bees over the 10 day exposure period

Treatment group	Nominal concentration	Mean daily dose	Mean percentage cumulative mortality (%)									
			D 1	D 2	D 3	D 4	D 5	D 6	D 7	D 8	D 9	D 10
Control	0	-	0.0	0.0	0.0	0.0	2.0	2.0	2.0	2.0	2.0	2.0
PROBLAD PLUS	1000 (mg/kg)	42.10 (µg/bee/day)	0.0	0.0	0.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Toxic reference	1.5 (mg a.s./kg)	0.047 (µg a.s./bee/day)	0.0	0.0	0.0	2.0	12.0	32.0	60.0	96.0	100	100

Validity criteria:

Required:	Observed:
The average mortality across replicates for the untreated control and solvent control groups is \leq 15% at the end of the test (10 days following start of exposure)	2.0%
The average mortality in the reference substance treated group is \geq 50% at the end of the test (10 days following start of exposure).	100%

III. CONCLUSION

There was minimal difference in mean feed uptake per bee observed between the control and treated groups, with the treated bees consuming slightly more feed than the control group, indicating no antifeedant effects of the test item. Under the conditions of this study, and when compared to the results for the control treatment, PROBLAD PLUS was not found to cause an effect on mortality when fed to adult honey bees over 10 days at a concentration of 1000 mg test item/kg sucrose solution. This was equivalent to a mean daily dose of 42.10 µg test item/ bee per day. No adverse effects on the behaviour of the bees were observed.

HSE COMMENTS

The study was conducted before the latest guidelines were published, therefore the HSE assessor has reviewed this study against the most recent guidelines; OECD 245 (2017) guidelines: Honey bee *Apis mellifera* L. chronic oral toxicity test (10-day feeding). The study has met the validity criteria outlined in the guidelines. It is acknowledged that many of the deviations discussed below are a result of the finalised guideline not being available at study initiation.

The main test was carried out twice, but the first run did not pass the validity criteria. The second run of the test was successful and it is the data generated from this test that are reported.

There has been no analytical verification carried out on the stock solution used in the study. The guidelines recommends that for a stock solution not produced daily, a sample should be taken after preparation and at the end of the storage period for the stock solution and the test concentration. There has also not been any adjustment for evaporation of test solution from feeders using additional test cages. This has caused uncertainty, because the dose of the test item cannot be confirmed, but as the study was produced before release of the final guidelines, it does not invalidate the study.

It is recommended in the guidelines that for the reference item, between 0.5–1.0 mg a.s./kg feeding solution be tested to demonstrate > 50% mortality. In this study, 1.5 mg a.s./kg sucrose solution has been tested and demonstrated 100%

mortality. This has caused uncertainty in the sensitivity of the test system, but as the study was produced before release of the final guidelines, it does not invalidate the study. The guidelines also state that a minimum of 2 mL of feeding solution should be provided to each replicate, whereas in the study only 1.5 mL of feeding solution was provided. Since the validity criteria were met, this deviation can be considered as minor.

There is also no reporting of the number of bees with behavioural abnormalities, which is presumed to be due to none being identified. Additionally, the behavioural abnormality categories in this study are more vague than those specified in the guidelines. This study uses live, stumbling and dead to categorise the bees, whereas the guidelines suggest the use of: m = moribund (bees cannot walk and show only very feeble movements of legs and antennae, only weak response to stimulation; e.g. light or blowing; bees may recover but usually die); a = affected (bees still upright and attempting to walk but showing signs of reduced coordination; hyperactivity; aggressiveness; increased self-cleaning behaviour; rotations; shivering); c = cramps (bees contracting abdomen or entire body); ap = apathy (bees show only low or delayed reactions to stimulation e.g. light or puff of air; bees are sitting motionless in the unit); v = vomiting. As the study was produced before release of the final guidelines, this is deemed to be a minor deviation.

As less than 50% mortality occurred in the study, the endpoint was not determined statistically and is instead estimated as > highest dose tested.

Overall, there are a number of uncertainties with the study conduct. Additionally it is noted that there is no certificate of analysis provided in the study PDF. This means that the purity and batch number of the test item cannot be confirmed. Typically this would need to be requested for the study to be considered reliable for use in risk assessment. However, given that there is a newer study conducted to the latest OECD 245 (2017) guideline (K-CA 8.3.1.2-02), it is considered that this study is not necessary to meet the data requirement, so a request for the certificate of analysis is not necessary.

The following endpoint may be used as supporting information only for the risk assessment:

Chronic LDD₅₀: > 1000 mg test item/kg sucrose solution (42.10 µg test item/bee/day)

Reference:	K-CA 8.3.1.2/02
Report Title:	PROBLAD PLUS: Chronic Oral toxicity test (10-day feeding) to the Honey bee (<i>Apis mellifera</i> L.) under laboratory conditions.
Author(s) & Year:	Aguilar-Alberola (2019)
Document No	CEV SA, Unpublished report No.: S19-21016.
Substance used:	PROBLAD PLUS (Batch: CF01907-001, 21.2% BLAD, 1.20 g/cm ³)
Method of analysis:	Enzyme-Linked Immunosorbent Assay (ELISA). Method ref. S19-21256
Guideline(s):	OECD 245 (2017)
Deviations:	Yes, see HSE comments
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

I. MATERIALS AND METHODS

A. MATERIALS

Test Substance

Test item : PROBLAD PLUS (Batch: CF01907-001), 21.2% w/w BLAD protein) CoA: 11/07/2010 (checked by the HSE assessor)

Test concentrations : 1828, 4570, 11424, 28560, 71400 mg PROBLAD PLUS/ L diet
27.4, 68.5, 171.4, 428.4, 1071.0 µg PROBLAD PLUS/bee/day

Control : Aqueous sucrose solution with a final concentration of 500 g/L (50% w/v)

Reference item : BAS 152 11 I (Batch : FRE-001578)

0.90 mg dimethoate/ kg diet

Test Organism

Young adult worker honey bees, *Apis mellifera* L. (Hymenoptera, Apidae), not older than 48 hours, were used as test organisms. The colonies were examined for reportable bee epidemics by an authorised bee specialist and are inspected periodically according to the standard bee-keeping practices by an experienced apiarist. The hives used for honey bee collection were adequately fed, healthy, as far as possible parasite-free and queen-right. No chemical substances (such as antibiotics, anti-Varroa treatments, pesticides, etc.) were used in the hive within one month preceding the start of the test. Two days before the beginning of the test, frames with capped cells were transferred from the hive and put into a bioclimatic chamber. One day prior to test start, the bees were randomly collected directly from the frames, introduced into the test units and kept under test conditions until start of the test. Acclimatisation period lasted from collection to the start of the test. During this period bees were fed ad libitum with 50% w/v sucrose solution. Dead and moribund bees were rejected and replaced by healthy bees before starting the test.

B. STUDY DESIGN AND METHODS

Study dates : 04/09/2019- 14/09/2019

Experimental design : The study was conducted as a dose-response test. It comprised 1 control group C; 5 test item groups (T1 – T5) and 1 reference item group R. Test organisms were continuously exposed to the feeding solutions over a period of 10 days. Each treatment group consisted of 50 test organisms, divided in 5 parallel replicates, each containing 10 bees. Additionally, 5 test units without bees but with syringes containing pure 50% (w/v) aqueous

sucrose solution were placed in the climatic chamber for evaluation of evaporation.

Exposure unit :

Bees were kept in stainless steel cages (approx. base: 8.5 cm x 4.5 cm; height: 6.0 cm). The bottom of the cage consisted of perforated steel, which guarantees sufficient air supply. The cages were lined with filter paper. Two holes at the top of the cage allowed the use of feeders.

Treatment application :

A fresh stock solution was prepared daily by mixing a defined amount of test item with a defined amount of 50% (w/v) aqueous sucrose solution. The amount of test item needed for the daily preparation of the test item stock solution was measured using a calibrated balance. The test feeding solutions (diet) were freshly prepared every day by mixing aliquots of the stock solution with 50% (w/v) aqueous sucrose solution.

For the reference item treatment, a stock solution was prepared daily using 50% (w/v) aqueous sucrose solution as solvent. The definitive feeding solution was prepared every day from the stock solution with 50% (w/v) aqueous sucrose solution.

The feeding solutions were offered to the test organisms of each test unit using 5 mL syringes containing 1 mL of corresponding application solution (i.e., 0.1 mL/bee), injected with a micropipette into the syringes. This amount is in excess of the expected daily consumption by the bees. The tip of each feeder was removed to give the bees access to the feeding solution. Syringes with the feeding solutions were weighed before presentation to the bees, and then inserted in one of the holes in the upper surface of the cages. The bees in one replicate share the feeding solution and thus receive similar doses (trophallaxis). The application solutions remained in the cages for 24 h and the syringes were then weighed again in order to calculate actual food consumption. A fresh 1 mL of solution was then supplied to each cage. This was repeated daily.

Five additional cages with syringes with the feeding solution but no bees were maintained in the climatic chamber. Syringes of these additional cages were changed daily in concomitance with the test syringes and were weighed before and after each replacement for the calculation of sucrose solution evaporation. Daily consumption of the test solutions (the control and treatments with the test and the reference items) were adjusted taking into account the measured daily evaporation.

Environmental Conditions:

Conditions were measured continuously with a calibrated data logger.

Temperature :	31.9°C–34.2°C (required : $33 \pm 2^\circ\text{C}$)
Humidity :	57.0–69.0% (required : 50–70%)
Lighting :	Constant darkness

Statistics :

Cumulative mortality [%] for each treatment group across all replicates was calculated from the number of dead bees at the end of the assay (D10) in relation to the number of introduced test organisms per treatment group. The cumulative mortality of the test product treatments was corrected for corresponding control mortality according to the formula of Abbott.

Concerning consumption, Shapiro-Wilk's test ($\alpha = 0.01$) confirmed correspondence with the normal distribution and the hypothesis of homoscedasticity was accepted by Levene's test ($\alpha = 0.01$). A Williams multiple sequential t-test procedure ($\alpha = 0.05$, one-sided smaller) was used to compare consumption data of the control and each test item treatment group to determine if rejection of the test item solutions occurred. As signs of extra binomial variance in the data were found (Tarone's procedure, $\alpha = 0.01$), the step-down Rao-Scott-Cochran-Armitage test procedure ($\alpha = 0.05$, one-sided greater) was used to evaluate whether there was a significant difference

between the mortality data of the test item groups and the control group in order to determine the NOEC (No Observed Effect Concentration) and the corresponding No Observed Effect Dietary Dose (NOEDD). A moving average computation after Thompson was used to determine the LDD₅₀/LC₅₀ values. For the statistical evaluation, the MS Excel Version 14.0 and the statistics program ToxRatPro® Version 3.2.1 was used.

Observations:

Mortality:

Mortality was recorded on a daily basis starting 24 hours (\pm 2 hours) after the first treatment presentation and for the whole duration (10 days) of the test. At each assessment time dead bees were removed for sanitary reasons. Behavioural abnormalities such as symptoms of poisoning in comparison to the control group were recorded at each observation interval. Behavioural abnormalities were recorded according to the following categories:

- a = affected (bees still upright and attempting to walk but showing signs of reduced coordination).
- ap = apathy (bees show only low or delayed reactions to stimulation, e.g. light or blowing; bees are sitting motionless in the unit or are able to walk but not correctly).
- c = cramps (bees contracting abdomen or entire body).
- m = moribund (bees cannot walk and show only very feeble movements of legs and antennae; only weak response to stimulation; e.g. light or blowing; bees may recover but usually die).
- v = vomiting

Solution consumption

Consumption of application solution per bee was calculated by dividing the total consumption per replicate by the number of living bees at start of feeding. For each treatment group, the mean consumption of application solution/bee was calculated by averaging the replicate values. Data on food consumption was calculated for each treatment group and displayed as:

- Mean consumption of application solution per bee per day [mg/bee/day]; the number of living bees at the beginning of each feeding interval is taken for this calculation.

- Mean consumption of application solution per day over the 10 day test period [mg/bee/day].
- Mean intake of test/reference item per bee per day [μg or ng/bee/day].
- Mean intake of test/reference item per bee over the 10 day test period [μg or ng/bee/day].
- Accumulated intake of test/reference item per bee over the test period [μg or ng/bee].

The evaporation out of the food syringes was determined by daily weighing the syringes of the additional test cages which did not contain bees. Over the whole test period, the mean value of evaporation per day was determined and the daily food consumption of the control, test and reference items treatments was corrected by the corresponding mean value of evaporation on the corresponding day. If this correction led to a negative value, i.e. food consumption was lower than the mean daily evaporation, the food consumption of the respective replicate(s) was considered to be “0” (no food consumption).

Analytical verification:

Analytical data were required by the guideline to verify the actual concentration of the test item (representative sample) and its solubility in the solvent. The analysis of the samples was carried out in the analytical laboratory with an ELISA detection method.

Samples of the control, the highest and lowest concentration of the test item feeding solutions as well as of the stock solution were taken directly after their preparation on day 0. The samples were taken by quintuplicate, three samples for shipping and two for retention, with a volume of approximately 1.0 mL each. Additionally, samples of a fortified solution with a concentration corresponding to the treatment T3 were taken. The fortified samples also were taken by quintuplicate as the main samples.

II. RESULTS AND DISCUSSION

Analytical results:

The measured concentrations in the specimens were within 20% of nominal concentrations. Thus, the concentrations of the test item were sufficiently confirmed and the endpoints are based on nominal concentrations. LOD: 0.02 mg/L, LOQ: 1.6 mg PROBLAD PLUS/L.

Table 9.3.1-12: Analytical recoveries for PROBLAD PLUS

Sample description	Measured PROBLAD PLUS concentration (mg/L)	Nominal PROBLAD PLUS concentration (mg/L)	Recovery (%)
S19-21016-D0-C-A1	<LOQ	0	N/A
S19-21016-D0-T1-A1	2056	1828	112
S19-21016-D0-T5-A1	76469	71400	107
S19-21016-D0-St-A1	134232	142800	94

Test item consumption:

A statistically significant trend was obtained when comparing the consumption of the sucrose solution and the concentration of the test item (monotonicity of concentration/response, $\alpha = 0.05$). Statistically significant differences were obtained from the concentration of 11424 mg a.s./L diet and all the higher concentrations when comparing with the control group (Williams multiple sequential t-test procedure, $\alpha = 0.05$, one-sided smaller). This could indicate a possible rejection effect of bees towards the test item.

Table 9.3.1-13: Consumption of feeding solution per day over the 10 day test period

TRT	Concentration (mg t.i./L diet)	Mean amount of test solution consumed per bee (mg)									
		D1	D2	D3	D4	D5	D6	D7	D8	D9	D10
C	--	19.88	20.04	11.12	20.62	20.58	18.52	21.96	19.58	21.0	25.84
T1	1828	20.26	18.16	15.68	21.92	18.01	27.25	19.94	24.22	14.54	20.57
T2	4570	17.38	20.18	16.00	15.82	20.82	20.66	31.61	17.00	18.56	22.03
T3	11424	15.54	12.62	15.40	14.96	16.74	27.71	15.53	24.53	19.64	11.15
T4	28560	12.66	14.44	11.76	14.62	12.39	16.20	12.04	23.18	12.54	18.22
T5	71400	14.18	8.87	12.35	10.32	11.62	5.94	2.93	n.s.	n.s.	n.s.
R	1.07 ^a	16.92	13.72	15.08	15.65	3.45	10.10	n.s.	n.s.	n.s.	n.s.

TRT: Treatment; t.i.: test item; n.s.: no surviving individuals.

^amg dimethoate/L diet.

Table 9.3.1-14: Cumulative mean intake of solution, test item and reference item groups

TRT	Concentration (mg t.i./L diet)	Mean consumed solution			Average consumed (µg t.i./bee)	
		(mg/bee/d)	SD	SE	Daily	Cumulative (10 d)
C	--	19.91	7.39	1.045	--	--
T1	1828	20.06	7.60	1.075	30.8	308.2
T2	4570	20.01	8.12	1.148	76.8	768.3
T3	11424	17.38*	9.09	1.285	166.9	1668.8
T4	28560	14.81*	6.63	0.938	355.3	3553.3
T5	71400	9.98*	4.70	0.830	598.5	3830.4
R ^a	1.07	12.75	6.53	1.257	0.011	0.062

TRT: Treatment; SD: standard deviation; SE: standard error; t.i.: test item.

^afor the reference item, t.i. refers to the active substance dimethoate.

*Statistically significant difference compared to the control group (Williams multiple sequential t-test procedure (on-sided smaller, $\alpha = 0.05$)).

Mortality and behavioural abnormalities:

Compared to the control group, the two treatments with the highest test item concentrations (28560 and 71400 mg a.s./L diet) showed a statistically significant increase in mortality after 10 days of exposure using the step-down Rao-Scott-Cochran-Armitage test procedure ($\alpha = 0.05$, one-sided greater). Therefore, under the conditions of this study, the D10 NOEC/NOEDD were determined as 11424 mg test item/L diet (corresponding to 9600 mg a.s./kg diet) and 166.9 µg test item/bee/day (measured), respectively. The LDD50 value was determined to be 361.9 [308.8 – 424.2] µg test item/bee/day, and the LC₅₀ as to be 29720 [24425 – 36163] mg test item/L diet (corresponding to 24975 [20525 – 30389] mg test item/kg diet).

Table 9.3.1-15: Cumulative and corrected cumulative mortality

TRT	Concentration (mg t.i./L diet)	Total number of bees dosed	Total number of dead bees	Cumulative mortality (%)	SE (%)	Corrected mortality (%) ^a
C	--	50	1	2.00	2.00	--
T1	1828	50	4	8.00	8.00	6.12
T2	4570	50	4	8.00	5.83	6.12
T3	11424	50	4	8.00	8.00	6.12
T4	28560	50	22	44.00*	16.00	42.86
T5	71400	50	50	100.00*	0.00	100.00
R	1.07 ^b	50	50	100.00	0.00	100.00

t.i.: test item; SE: standard error.

^aCorrected mortality with the control group based on Abbott modified by Schneider-Orelli.

^bmg dimethoate/L diet.

*Statistically significant difference compared to the control group (step down Rao-Scott- Cochran-Armitage test (one-sided greater, $\alpha = 0.05$)).

Symptoms of intoxication (mainly affected bees) were observed sporadically for a few of the bees exposed to the test item. These symptoms were registered mainly in the two highest concentration treatments.

Table 9.3.1-16: Behavioural abnormalities

TRT	Concentration (mg t.i./L diet)	Affected bees (%) [*]									
		D1	D2	D3	D4	D5	D6	D7	D8	D9	D10
C	--	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
T1	1828	0.00	4.00	2.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00
T2	4570	0.00	2.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
T3	11424	0.00	0.00	0.00	0.00	0.00	6.12	0.00	0.00	0.00	0.00
T4	28560	0.00	2.04	2.22	4.55	2.63	0.00	0.00	3.03	0.00	7.14
T5	71400	0.00	2.04	0.00	0.00	0.00	40.0	-	-	-	-

t.i.: test item

*Percentage of affected individuals with respect to the remaining living ones.

Validity criteria:

Required:	Observed:
Mean mortality at the end of the test was $\leq 15\%$	2.0%
Mean mortality was $\geq 50\%$ at the end of the test	100.0%

III. CONCLUSION:

All validity criteria were met and the study was deemed valid. Statistically significant differences were observed in mean daily consumption between the bees exposed to a concentration of 11424 mg a.s./L diet and the higher concentrations compared to the control group. Symptoms of intoxication (mainly affected bees) were observed sporadically for a few of the bees exposed to the test item.

HSE COMMENTS

The study was carried out according to GLP and follows OECD No. 245 with no significant deviations to the guidelines. The climatic conditions were suitable and the results of the study were statistically analysed according to the methods outlined in the guidelines. The validity criteria were met during the study.

There is uncertainty caused by the guidelines stating that a minimum of 2 mL of feeding solution be provided to each replicate, whereas in the study only 1 mL of feeding solution was provided. The applicant states that this volume was selected as it exceeds the expected daily intake amount for bees. HSE accepts this as a minor deviation.

HSE Chemistry have considered the validity of the analytical method in Volume 3 CA B5. The analytical method S19-21256 for the determination of PROBLAD PLUS in sucrose solution and deionised water is considered fully validated in accordance with SANTE/2020/12830 rev. 1.

The analytical recovery values were within the range of 80–120%, therefore the correct dosing of the test system was achieved and the endpoint was based on nominal values.

There was a statistically significant reduction in the amount of consumed feeding solution at test concentrations T3 – T5, compared to the control. This could indicate a potential repellent effect of the test item.

The statistical analyses conducted are considered to be appropriate and in line with guideline recommendations.

The endpoints are confirmed as:

10 day LC₅₀: 29720 mg PROBLAD PLUS/L diet [95% CI: 24425–36163 mg/L diet]; (24975 mg PROBLAD PLUS/kg diet [95% CI: 20525–30389])

10 day LDD₅₀: 361.9 µg PROBLAD PLUS/ bee/day [95% CI: 308.8–424.2 µg/bee/day]

Reference:	K-CA 8.3.1.3/01
Report Title:	PROBLAD PLUS: In vitro chronic toxicity to larval stage honey bee (<i>Apis mellifera</i> L.)
Author(s) & Year:	Harkin (2015b)
Document No	CEV SA, Unpublished report No.: B2CF2000.
Substance used:	PROBLAD PLUS (Batch: D32-280414, 20.1 % BLAD protein; CoA not available to confirm purity)
Method of analysis:	No analysis conducted
Guideline(s):	Draft OECD Guidance document (2014): Honey Bee (<i>Apis mellifera</i>) Larval Toxicity Test, Repeated Exposure
Deviations:	Yes, see HSE comments
GLP or GEP:	Yes
Acceptability:	Supplementary
Study relied upon:	No, there are multiple uncertainties with the study conduct, and a more up to date study is available to address this data requirement.

I. MATERIALS AND METHODS

A. MATERIALS Test Substance

Test item : PROBLAD PLUS (Batch: D32-280414), 20.1% w/w BLAD protein; CoA not available to confirm purity)

Test concentrations : Based on the results of a range finder, the main test was performed using 6 doses of 10.24, 25.6, 64, 160, 400 and 1000 mg test item/kg diet.

Control : Untreated larval diet

Reference item : Danadim Progress (Dimethoate) (Batch: 0001064092)

10 mg dimethoate/ kg

Test Organism

Larval honey bees (*Apis mellifera* L.) were obtained from colonies belonging to the FERA National Bee Unit. The colonies used to provide the larvae were queen-right and showed no signs of American foul brood (AFB) or European foul brood (EFB). The colonies were not treated with a varroacide within the 4 weeks prior to collection. At the end of the tests all larvae used in the study were disposed of by incineration.

Collection of larvae :

Each queen was confined to a brood frame containing empty cells and emerging worker brood in a queen frame cage within their own colony. In order to ensure the production of larvae from at least 3 colonies in the main test, queens from a number of colonies were caged at one time. When eggs were laid, the queen was removed from the cage and the cage containing the frame replaced near the brood until hatching on day 0. On test day 0, the combs were taken to the laboratory to allow grafting of the young larvae into the test cells.

Larvae were grafted and reared using grafting cells with a 9 mm internal diameter housed in a 48 well tissue culture plate. The cells were first immersed for 30 minutes in absolute ethanol and then allowed to dry in a fume cupboard. Each cell was then placed into one well in the tissue culture plate. The wells had been previously half-filled with a piece of dental roll wetted with 15.5% (w/v) glycerol in 0.4% Methyl Benethonium Chloride (MBC) solution as a sterilant. The plates were warmed in the incubator prior to the grafting of the larvae.

The larvae were grafted onto 5 µL of pre-warmed untreated Diet A (50% fresh royal jelly, 50% aqueous solution containing 2% yeast extract, 12% glucose and 12% fructose (all w/w)) and placed in the bottom of each cell by delicately transferring from the comb to the cell on the surface of the diet substance using a grafting tool. When the plates were complete they were placed into a Plexiglas container in the incubator. Additional larvae to the number required were also grafted so that on day 1 of the study any damaged or dead larvae could be replaced before treatment.

The 48 well plates were placed into a hermetic Plexiglas desiccator with a dish filled with saturated K₂SO₄ solution in order to provide a water-saturated atmosphere. The desiccator was placed in an incubator set at 33 ± 2°C.

B. STUDY DESIGN AND METHODS

Study dates : 25/06/2014- 18/09/2014

Experimental design : In the main test, 3 colonies were used to supply the larvae for each of 6 test item dose rates. A single plate was used per dose rate, using 14 larvae from 1 hive and 16 from each of the 2 other hives giving a total of 46 larvae per plate. A record of the position of the larvae on the plates (and any transfers that occurred) and the source colonies were maintained to allow any differences between colonies to be identified if necessary. A single plate was also set up for the controls and toxic reference item using larvae from the 3 colonies in the same manner as for the test item.

Treatment preparation : The larval diet consisted of two components: i) Diet solution (either A, B or C dependant on developmental stage of the larvae) ii) Royal jelly.

To make the completed diet (A, B or C), the 2 components were mixed 50/50. The compositions of the diet solutions that were used were as follows:

- Diet Solution A: an aqueous solution containing 2% yeast extract, 12% glucose and 12% fructose

- Diet Solution B: an aqueous solution containing 3% yeast extract, 15% glucose and 15% fructose
- Diet Solution C: an aqueous solution containing 4% yeast extract, 18% glucose and 18% fructose.
(All figures expressed in % w/w)

Feeding :

During the test, the bees were fed with the diet treated with the appropriate amount of the test item.

- On days 1 and 2, the larvae were fed 5 and 10 µL of diet A
- On Day 3 they were fed 20 µL of diet B
- The larvae were fed diet C on Days 4 (30 µL), 5 (40 µL) and 6 (50 µL).

For each diet, the sugar solution was filtered through a 0.22 µm syringe filter before being mixed with the royal jelly. The diets were warmed to $33 \pm 2^\circ\text{C}$ prior to each feed.

Treatment application :

The diets were made up and weighed out into vials for each treatment group for each day and stored in the fridge at 0-10°C until required for dosing. The test item stock solutions were made up on Day 0 in deionised water. When the test item solution was added to the relevant aliquot of feed, it was mixed thoroughly to ensure homogeneity. It was then placed in the incubator to warm for approximately 30 minutes before being mixed again immediately before dosing the larvae. Different concentrations of the same substance were applied in order of increasing concentration. The toxic reference item stock was prepared in water on day 0 and the appropriate amount added to the diets daily as described for the test item. The larvae were dosed using an air displacement pipette to deliver the required volume of feed.

Environmental Conditions:

Conditions were measured continuously with a calibrated data logger.

Temperature :	33 ± 2°C (required: 34.5 ± 0.5°C, deviations allowed but must not drop below 23°C or rise above 40°C)
Humidity :	~ 95%
Lighting :	Constant darkness

Observations :

Treatment-related mortality checks were made each day at feeding. An immobile larva or one which did not respond when touched by a paintbrush, was recorded as dead and removed.

Statistics:

The toxicity endpoints were the mortality rates on day 7. The mortality rate was calculated as the number of dead larvae/number of larvae dosed on day 1. As there was no dose related mortality and no difference in mortality between the highest dose tested and the control group in the main test no statistics were carried out.

II. RESULTS AND DISCUSSION:

Mortality:

The mortality observed within the control group was below the 15% validity criterion at 13.0%. The mortality in the toxic reference treated group exceeded the 50% required to pass the validity criterion at 95.7% clearly demonstrating that the test system was sensitive to detecting toxic effects. Mortality within the test item treated groups was consistently equal to or below that of the control group (4.4%-13.0%) with no dose response seen.

Table 9.3.1-17: Percent cumulative mortality of larvae at each treatment dose over the 7 day exposure period

Treatment	Dose (mg t.i./kg)	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
PROBLAD PLUS	10	0.0	4.4	4.4	4.4	6.5	6.5	6.5
	25	0.0	4.4	4.4	4.4	4.4	4.4	4.4
	64	0.0	6.5	8.7	10.9	10.9	13.0	13.0
	160	0.0	0.0	2.2	2.2	4.4	4.4	6.5
	400	0.0	2.2	4.4	4.4	8.7	8.7	13.0
	1000	0.0	0.0	4.4	6.5	8.7	8.7	13.0
Control		0.0	4.4	8.7	8.7	10.9	10.9	13.0
Toxic reference		0.0	0.0	39.1	52.2	87.0	91.3	95.7

Validity criteria :

Required :	Observed :
Pupal mortality is $\leq 15\%$ in the control group	13%
Pupal mortality is $\geq 50\%$ in the reference item group	95.7%

III. CONCLUSION:

Under the conditions of this study, PROBLAD PLUS was found to cause no effect on larval mortality at day 7 following repeated exposure at a concentration of 1000 mg PROBLAD PLUS/kg when compared to the control group. It was not possible to generate an EC value, therefore the EC₅₀ can be stated as being > 1000 mg PROBLD PLUS/kg diet, based on the non-dose related mortality seen at this dose

rate, the highest dose offered in the main test. From the results obtained, the NOEC can be described by observation as at least 1000 mg PROBLAD PLUS/kg diet, equivalent to 155 µg PROBLAD PLUS per larva (based on an assumed uptake of the complete offered dose within 155 µl of treated diet and an assumed mean weight of 1 µL of larval diet of 1 mg). It was not possible to calculate a LOEC value.

HSE COMMENTS

The study was conducted to the 2014 draft guidance document. HSE have reviewed this study against the most recent OECD 239 (2021) guidelines: Honey bee *Apis mellifera* L. larval toxicity test, repeated exposure. There are a number of deviations from the OECD 239 guideline, as detailed below, but it is accepted that this is because the finalised guideline was not available at the study initiation.

In the main test the temperature dipped below the expected guideline range (34–35°C) to 33°C. However the guidelines does state: “Temporary deviations are allowed, however temperature should not drop below 23°C or go above 40°C, and these deviations should not last, as far as possible, more than 30 minutes once every 24 hour”. As the deviation was not for extended periods and the temperature did not drop below 23°C, this was deemed to be a minor deviation.

The guidelines say that during rearing of the larvae, the queen should be removed on D-3 to an exclusion cage and then returned on D-2 (maximum 30 hours after encaging) to reduce the variability in egg ages. In this study, it does not mention when the queen is removed to the exclusion cage, and is only returned on D0 at the start of exposure.

It states in the guidelines that the larvae should be fed every day except for day 2, however in the study the larvae are fed diet A on day 2. The guidelines also recommend that the larvae are fed 20 µl on day 1, but the larvae are only fed 5 µl.

The guidelines recommends that the reference item is tested at 48 mg dimethoate/kg diet, whereas this study only tests 10 mg dimethoate/kg diet. At this concentration, 95.7% mortality was observed which indicates a higher degree of sensitivity than would be expected.

Additionally, no analytical measurements were taken of the stock solutions. Therefore it is not possible to confirm that the correct dosing of the test diet was achieved. As the study was produced before release of the final guidelines, these deviations do not invalidate the study.

This study has been performed over 7 days, while the guidelines recommends it takes place over 22 days and assess adult emergence. This causes uncertainty as this means the study cannot meet the validity criteria specified in the guidelines. The

validity criteria according to the guidelines include: larval mortality in the control from D3 to D8 should be $\leq 15\%$, adult emergence on D22 should be $\geq 70\%$, and reference item mortality should be $\geq 50\%$. As the study did not proceed past day 7, this means that pupal mortalities and adult emergence were not measured during the study, however as the study has been conducted using the validity criteria from the single exposure study (OECD 237), which runs over 8 days, and these were met, this is deemed acceptable. Uneaten food and the behaviour of the larvae during the study were also not recorded or presented in the results.

Additionally it is noted that there is no certificate of analysis provided in the study PDF. It is therefore not possible to confirm the purity or batch number of the test item.

Given that a newer study conducted to the finalised OECD 239 guidance document is available (K-CA 8.3.1.3-02), this study is not required to meet the data requirement. It was therefore not considered necessary to request the certificate of analysis.

Reference:	K-CA 8.3.1.3/02
Report Title:	PROBLAD PLUS: Honey Bee (<i>Apis mellifera</i> L.) larval toxicity test following repeated exposure under laboratory conditions
Author(s) & Year:	Aguilar-Alberola (2019)
Document No	CEV SA, Unpublished report No.: S19-21015.
Substance used:	PROBLAD PLUS (Batch: CF01907-001, 21.2% BLAD, 1.20 g/cm ³)
Method of analysis:	Enzyme-Linked Immunosorbent Assay (ELISA). Method ref. S19-21256
Guideline(s):	OECD 239 (2016)
Deviations:	Yes, see HSE comments
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

I. MATERIALS AND METHODS

A. MATERIALS

Test Substance

Test item :	PROBLAD PLUS (Batch: CF01907-001), 21.2% w/w BLAD protein) CoA: 11/07/2019 (checked by HSE assessor)
Test concentrations :	28.16, 70.40, 176.00, 440.00 and 1100.00 mg PROBLAD PLUS/L diet (corresponding to 25.60, 64.00, 160.00, 400.00 and 1000.00 mg PROBLAD PLUS/kg diet), equivalent to cumulative doses of 3.94, 9.86, 24.64, 61.60 and 154.00 µg PROBLAD PLUS/larva
Control :	Untreated larval diet
Reference item :	BAS 152 I (Batch: COD-002332) 48 mg dimethoate/kg

Test Organism

First instar larvae (L1) of the honey bee, *Apis mellifera* L. (Hymenoptera, Apidae), were used as test organisms. The larvae originated from three different bee hives sited in a commercial apiary. Bees were foraging on wild flowers.

The colonies were examined for reportable bee epidemics by an authorised bee specialist and are inspected periodically according to the standard bee-keeping practices by an experienced apiarist. The hives used for honey bee larvae collection were adequately fed, healthy, and as far as possible parasite-free and queen-right. No chemical substances (such as antibiotics, anti-Varroa treatments, pesticides, etc.) were used in the hives within 4 weeks preceding the start of the test.

Four days prior to the grafting of larvae (D-3), in order to ensure the production of larvae, queens of several colonies were confined in their own colony within an excluder cage containing a comb with empty cells. Three days prior to the grafting (D-2), maximum 30 hours after caging, queens were released from the cages. The combs containing eggs were left in the excluder cages during the incubation stage until

hatching on day 1 (D1). On D1, the combs were transferred to the laboratory using an insulated container in order to avoid temperature variation. In the laboratory, the three combs were used for grafting. On D1 the test was initiated with larvae in excess. Therefore, reserve plates were prepared containing larvae of the same replicate hives. Before first application of the test product on day 3, it was assured that all larvae used were of similar size and alive. Thus, non-suitable larvae per replicate were replaced across all treatment groups by individuals from the reserve plates.

Grafting of larvae : On day 1 (D1), 20 µL of diet A were dropped into each grafting cell of the well plate. Using a grafting tool, one larva was delicately transferred from the comb to each cell on the surface of the diet.

B. STUDY DESIGN AND METHODS

Study dates : 26/08/2019-16/09/2019

Experimental design : The study was conducted as a dose response test with a duration of 22 days from grafting on day 1 (D1) to the final assessment on day 22 (D22). It comprised 1 control group C, 5 test item groups (T1 – T5) with five different concentrations of 28.16, 70.40, 176.00, 440.00 and 1100.00 mg PROBLAD PLUS/L diet (corresponding to 25.60, 64.00, 160.00, 400.00 and 1000.00 mg PROBLAD PLUS/kg diet), equivalent to cumulative doses of 3.94, 9.86, 24.64, 61.60 and 154.00 µg PROBLAD PLUS/larva; and 1 reference item group R with 52.80 mg Dimethoate/L diet (corresponding to 48.00 mg Dimethoate/kg diet), equivalent to a cumulative dose of 7.39 µg Dimethoate/larva. For each treatment group, 3 replicates of 16 individuals (48 larvae in total) were tested over 22 days. Each replicate came from different hives. All the individuals used in the study came from the same three hives.

Treatment preparation : The larval diet was freshly prepared prior the applications and stored in a fridge at ≤ 5°C (actual values between 1.0 and

4.0°C). Each larva was fed once a day (except on day 2) with a standardised amount of artificial diet until day 6. For feeding, a multi stepper pipette was used. Care was taken to avoid touching and drowning the larvae when feeding them. Food was dropped next to the larva, along the wall of the grafting cell. The diet was prepared with deionised water using the following ingredients:

- Diet A (D1, volume administered: 20 µL/larva): 50% weight of royal jelly + 50% weight of an aqueous solution containing 2% weight of yeast extract, 12% weight of glucose and 12% weight of fructose.
- Diet B (D3, volume administered: 20 µL/larva): 50% weight of royal jelly + 50% weight of an aqueous solution containing 3% weight of yeast extract, 15% weight of glucose and 15% weight of fructose.
- Diet C (from D4 to D6, volume administered: 30 µL/larva; 40 µL/larva and 50 µL/larva respectively): 50% weight of royal jelly + 50% weight of an aqueous solution containing 4% weight of yeast extract, 18% weight of glucose and 18% weight of fructose.

For the preparation of the larval diet, a commercial royal jelly was used. The absence of antibiotics, pesticides and heavy metals in the royal jelly was confirmed by a non-GLP multi-residues analysis.

Test unit :

Larvae were transferred into crystal polystyrene grafting cells having a diameter of 9 mm. Cells were initially sterilised by submerging them for 30 min in ethanol 70% (v/v), and then dried. Each cell was placed into a well of a sterile 48-well cellular culture plate, and then sterilised by exposure to Ultra-violet light during 15 minutes. The open plates of the control group, all test item treated groups and the reference item group were placed into hermetically sealed Plexiglas desiccators, containing a dish filled with a saturated potassium sulphate (K_2SO_4) solution in order to keep a water saturated atmosphere from day 1 until day 7. On day 7, the well plates were transferred to another Plexiglas desiccator, containing a dish with a saturated sodium chloride (NaCl)

solution in order to maintain a slightly lower relative humidity until day 15. On day 15, each plate was transferred into an emergence box in an incubator. Bees that emerged in the emergence box had access to aqueous sucrose solution *ad libitum*.

Treatment application : Preparation of Application Solutions

Test and reference item were measured using a calibrated balance. For the preparation of the test item stock solution and the test item dilutions, deionised water was used as solvent. For the preparation of the reference item stock solution and the preparation of the application solution of the reference item, deionised water was used as solvent. A unique stock solution of the reference item was prepared on D3 and it was stored in a refrigerator to be used until D6. The same volume of deionised water was added to the diet of the control group C from day 3 (D3) until day 6 (D6).

Preparation of Larval Diet from Day 3 until Day 6

Just before feeding, from day 3 until day 6, the test solutions were added to the diet using a micropipette. The volume of application solution in the diet did not exceed 10% of the final diet volume. The diet was homogenised using a vortex mixer.

Environmental Conditions :

Air temperature and relative air humidity were recorded at intervals of fifteen minutes with calibrated data loggers placed into each desiccator from day 1 until the end of the test on day 22. The bees were kept in constant darkness.

Climatic condition :	D1-D7	D7-D15	D15-D22
Temperature :	33.5–34.3°C	33.4–34.4°C	29.1–35.0°C
Humidity :	60.1–99.5%	53.2–99.5%	57.1–69.8%

Observations :

Assessment of larval mortality was conducted before feeding on D4, D5 and D6, also on D7 and D8. With assistance of a stereo microscope, larvae were recorded as dead if no respiration (movement of spiracles) was observed. On D8, during the assessment of mortality, the presence of uneaten food was qualitatively recorded. Assessment of mortality during pupation phase was conducted on day D15 and assessment of emergence on D22. Other observations (larval appearance and size) were recorded to aid in the interpretation of mortality in comparison to the control group. At each assessment time, dead larvae and pupae were removed for sanitary reasons.

Analytical verification:

Analysis of the samples was carried out in the analytical laboratory with ELISA and measurement of absorbance. Samples of the highest and lowest concentration of the test item solutions used to treat the diet were taken directly after their preparation from D3 to D6. The samples were taken by quintuplicate, three samples for shipping and two for retention, with a volume of approximately 1.0 mL each. Additionally, a fortified solution with a concentration corresponding to the solution used to treat the diet of the treatment T3 was prepared. The fortified samples also were taken by quintuplicate as the main samples.

Data evaluation:

For each test item treated group, the concentration correspondences were calculated based on the density of the diet (1.1 g/mL). The calculation of the equivalent doses per larva [μg test item/larva] was based on the given test item concentration [mg test item/L diet] and the feeding volume per larva (both for the daily volumes and the cumulative 140 μL per developmental period).

The cumulative larval mortality [%] for each treatment group was calculated from the number of dead larvae on day 8 (D8) in relation to the total number of larvae per treatment group across all replicates after selection on day 3 (D3). Mortality during the pupation phase was evaluated on day 15 (D15). The cumulative pupae mortality [%] for each treatment group was calculated from the number of larvae that had not transformed into pupae on D15 in relation to the total number of individuals after selection on D3.

The adult emergence rate [%] for each treatment group was evaluated on day 22 (D22) and was calculated from the number of adult emerged bees on D22 in relation to the total number of larvae per treatment group after selection on D3. In case control mortality occurred, the cumulative mortalities for each test and reference item groups were expressed as percentage of the control populations after an adjustment according to the formula of Abbott.

Chi² 2x2 table test with Bonferroni correction (one-sided greater, $\alpha = 0.05$) was used to evaluate whether there was a significant difference between the mortality data of the test item groups and the control group in order to determine the NOEC (No Observed Effect Concentration) and the corresponding NOED (No Observed Effect Dose) by taking into account the cumulative feeding volume per larva (140 μ L). Since no statistical significant dose-response was obtained, the ECx values were empirically estimated from the results. For the statistical evaluation, the MS Excel Version 14.0 and the statistics program ToxRatPro® Version 3.2.1 was used.

II. RESULTS AND DISCUSSION:

Analytical results

The measured concentration in the samples was within 20% of nominal test concentration used. Thus the concentrations of the test item were confirmed and the endpoints are based on nominal concentrations. LOD: 0.02 mg/L (\leq 30% of the LOQ), LOQ: 0.2 mg PROBLAD PLUS/L.

Table 9.3.1-18: Analytical results

Sample description	Measured PROBLAD PLUS concentration (mg/L)	Nominal PROBLAD PLUS concentration (mg/L)	Recovery (%)
D3-T1	288.36	281.60	102
D3-T5	10604.00	11000.00	96
D4-T1	238.94	281.60	85
D4-T5	10862.50	11000.00	99
D5-T1	248.37	281.60	88
D5-T5	10373.00	11000.00	94
D6-T1	261.18	281.60	93
D6-T5	11027.50	11000.00	100

Biological results

On day 8, the cumulative larval mortality was 4.17% in the control group C. On day 22, the adult emergence rate of the initial grafted larvae was 89.58% in the control group. Therefore the validity criteria for the control group were met for both test periods; the D8 mortality was under 15.00% and the D22 emergence rate was greater than 70.00%, across all replicates.

Mean corrected cumulative larval mortality on day 8 (D8) of the test item treated groups was -4.35, 4.35, 0.00, 0.00 and 0.00% in 28.16, 70.40, 176.00, 440.00 and 1100.00 mg PROBLAD PLUS/L diet, respectively. Mean corrected pupal mortality on day 15 (D15) of the test item treated groups was 2.17, 13.04, 6.52, 4.35 and 6.52% in 28.16, 70.40, 176.00, 440.00 and 1100.00 mg PROBLAD PLUS/L diet, respectively. Mean corrected mortality at the end of the test (D22) of the test item treated groups was -4.65, 9.30, 2.33, 6.98 and 2.33% in 28.16, 70.40, 176.00, 440.00 and 1100.00 mg PROBLAD PLUS/L diet, respectively.

On day 8, no individuals were observed with uneaten food or other affections. At the end of the test, in the final assessment of the emergence on day 22, no emerged bees were recorded as being affected. No statistically significant differences were found in the mortality of any of the test item groups compared to the control group at day 8 (D8), day 15 (D15) and at the end of the test on day 22 (D22). Accordingly, under the conditions of this study, the LOEC/LOED could not be determined and the No Observed Effect Concentration (NOEC) for these periods was determined as 1100.00 mg PROBLAD PLUS/L diet (corresponding to 1000.00 mg PROBLAD PLUS/kg diet). The corresponding No Observed Effect Dose (NOED) was determined as 154.00 µg PROBLAD PLUS/larva.

Since no statistical significant dose-response was obtained and the corrected mortality did not reach the 10% at any treatment, the EC_x values could not have been calculated, but were empirically estimated from the results. The EC_{10,20,50} were determined to be higher than 1100.00 mg PROBLAD PLUS/L diet (corresponding to 1000.00 mg PROBLAD PLUS/kg diet). The corresponding ED_{10,20,50} were determined to be higher than 154.00 µg PROBLAD PLUS/larva. Mean corrected cumulative mortality in the Reference Item group was 76.09% at day 8 and 100.00% at day 22 across all replicates.

Table 9.3.1-19: Effects of PROBLAD PLUS on Honeybee (*Apis mellifera* L.) larvae from repeated exposure

Treatment group	Concentration (mg t.i./L diet)	Cumulative Mortality (%)						
		D4	D5	D6	D7	D8	D15	D22
Control	--	0.00	2.08	2.08	4.17	4.17	4.17	10.42
Test item PROBLAD PLUS	28.16	0.00	0.00	0.00	0.00	0.00	6.25	6.25
	70.40	2.08	4.17	4.17	8.33	8.33	16.67	18.75
	176.0	4.17	4.17	4.17	4.17	4.17	10.42	12.50
	440.0	0.00	2.08	2.08	4.17	4.17	8.33	16.67
	1100.00	2.08	2.08	4.17	4.17	4.17	10.42	12.50
Reference item (Dimethoate) ^a	52.80	25.00	52.08	62.50	77.08	77.08	100.00	100.00

^aFor the reference item, the values indicate the amount of active ingredient (dimethoate).

Treatment group	Concentration (mg t.i./L diet)	Corrected Mortality (%) ^a						
		D4	D5	D6	D7	D8	D15	D22
Test item PROBLAD PLUS	28.16	0.00	-2.13	-2.13	-4.35	-4.35	2.17	-4.65
	70.40	2.08	2.13	2.13	4.35	4.35	13.04	9.30
	176.0	4.17	2.13	2.13	0.00	0.00	6.52	2.33
	440.0	0.00	0.00	0.00	0.00	0.00	4.35	6.98
	1100.00	2.08	0.00	2.13	0.00	0.00	6.52	2.33
Reference item (Dimethoate) ^b	52.80	25.00	52.08	62.50	77.08	77.08	100.00	100.00

^aCorrected for control mortality according to Abbott modified by Schneider-Orelli.

^bFor the reference item, the values indicate the amount of active ingredient (dimethoate).

Table 9.3.1-20: Emergence rate after 22 days

Treatment group	Concentration (mg t.i./L diet)	Emergence (%)
Control (untreated diet)	--	89.58
Test item PROBLAD PLUS	28.16	93.75
	70.40	81.25
	176.0	87.50
	440.00	83.33
	1100.00	87.50
Reference item (Dimethoate) ^a	52.80	0.00

^aFor the reference item, the values indicate the concentration in terms of active ingredient (Dimethoate).

Validity criteria:

Required:	Observed:
Cumulative larval mortality from day 3 (D3) until day 8 (D8) was $\leq 15\%$ across all replicates	4.17%
On day 22 (D22) the adult emergence rate was $\geq 70\%$ across all replicates	89.58%
The cumulative larval mortality was $\geq 50\%$ across all replicates on Day 8 (D8)	77.08%

III. CONCLUSION:

All validity criteria were met and sensitivity of the test organisms was confirmed. Accordingly, the study was deemed valid.

In a repeated exposure larval toxicity test with PROBLAD PLUS, the NOEC/NOED for D8, D15 and D22 was determined to be 1100.00 mg PROBLAD PLUS/L diet (corresponding to 1000.00 mg PROBLAD PLUS/kg diet) and 154.00 µg PROBLAD PLUS/larva, respectively based on the Chi² 2x2 table test with Bonferroni correction ($\alpha = 0.05$, one-sided greater).

Since no statistical significant dose-response was obtained, the ECx values could not be calculated, but were empirically estimated from the results. The EC_{10,20,50} were determined to be higher than 1100.00 mg PROBLAD PLUS/L diet (corresponding to 1000.00 mg PROBLAD PLUS/kg diet) and 154.00 µg PROBLAD PLUS/larva.

HSE COMMENTS

The HSE assessor has reviewed this study against the OECD 239 (2021) guidelines: Honey bee *Apis mellifera* L. larval toxicity test, repeated exposure. The study was conducted to GLP and met the validity criteria.

There are small, temporary deviations in the temperature during the study. The temperature drops lower than the specified range in the guidelines of 34–35°C, however the guidelines does state: ‘Temporary deviations are allowed, however temperature should not drop below 23°C or go above 40°C, and these deviations should not last, as far as possible, more than 30 minutes once every 24 hour.’ As the temperature does not drop below this range, the deviation is deemed to be minor. There is also no mention of warming the diet before use, or whether the grafting occurred on a warming plate. This is also deemed to be minor and not likely to have affected the results, since the validity criteria were met.

The analytical recovery of the test item was between 80–120%, therefore the correct dosing of the test diet is confirmed and the endpoints are based on nominal values.

HSE Chemistry have considered the validity of the analytical method in Volume 3 CA B5. The analytical method S19-21256 for the determination of PROBLAD PLUS in sucrose solution and deionised water is considered fully validated in accordance with SANTE/2020/12830 rev. 1.

As less than 50% effects were observed, the study endpoint is estimated to be > highest dose tested and 95% confidence intervals were not calculable.

The agreed endpoint for use in the risk assessment is:

- **EC₅₀ (D3-D22): > 1100 mg PROBLAD PLUS/L diet (> 1000 mg PROBLAD PLUS/kg diet)**
- **ED₅₀: > 154.0 µg PROBLAD PLUS/larva**
- **NOED = 154 µg PROBLAD PLUS/larva**
- **NOEC = 1100 mg PROBLAD PLUS/L diet (> 1000 mg PROBLAD PLUS/kg diet)**

B.9.3.2. Effects on non-target arthropods other than bees

Reference:	K-CA 8.3.2.1/01
Report Title:	Toxicity to the Aphid Parasitoid, <i>Aphidius rhopalosiphii</i> De Stefani Perez (Hymenoptera, Braconidae), in the Laboratory – Rate Response Test
Author(s) & Year:	Klug (2010a).
Document No	CEV SA, Unpublished report No.: S10-02555.
Substance used:	PROBLAD (Batch: 201009, 20% BLAD, 1.30 g/cm ³)
Method of analysis:	No analysis conducted or required
Guideline(s):	ESCORT I Guidance Document (BARRETT et al., 1994), ESCORT II Guidance Document (CANDOLFI et al., 2001) and IOBC (MEAD-BRIGGS et al., 2000)
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

I. MATERIALS AND METHODS**A. MATERIALS****Test Substance**

Test item : PROBLAD (Batch: #201009, 20% BLAD (plant protein extract from the genus *Lupinus*)) CoA: 01/09/2010 (checked by HSE assessor)

Test concentrations: mL product/ha (g a.s./ha) 268.8 (69.9), 672.0 (174.7), 1680.0 (436.8), 4200.0 (1092.0), 10500.0 (2730.0)

Applied in 200 L/ha water

Control : Deionised water

Reference item : Perfekthion/ BAS 152 11 I (Batch: 90924-06, 414.8 g/L)
applied in 200 L/ha water

Tested at 0.3 mL product/ha (0.12 g a.s./ha)

All spray solutions were prepared on the day of application as close as possible to the time of application. The spray solutions were prepared with deionised water. Required quantities of PROBLAD and Perfekthion were weighed into glass bottles and filled up with deionised water.

Test Organism

The aphid parasitoid, *Aphidius rhopalosiphi* De Stefani Perez (Hymenoptera, Braconidae).

Obtained from supplier 'Katz Biotech AG'

Feeding : Honey water solution (100 g honey, 50 g Aqua dest. (distilled water), 1.5 g gelatine) and sugar water solution was added ad libitum.

Preparation of the test organism: Adults less than 48 h old were used for the test. Mummies were exposed to $25 \pm 2^\circ\text{C}$ under long day conditions (16:8 hr light, intensity 1200 lux). Emerging adults were collected in a glass vessel and fed honey solution. They also had access to a cotton wool pad, soaked in water.

Five males and five females were placed in the exposure cage for each replicate using an aspirator.

After 48 hours of exposure to the treated glass plates, the surviving test organisms were removed from the cages. Up to a maximum of 17 of the survived and unaffected females per treatment were released individually into each fertility cage to parasitise aphids for a period of 24 h. After 24 h, the females were removed, and their condition (alive or dead) was assessed.

The plants bearing the aphids were maintained at test conditions and the number of parasitised aphids was counted after 12 days.

B. STUDY DESIGN AND METHODS

Study dates : 27/09/2010- 12/10/2010

Experimental design : Each treatment had 4 replicates containing 10 adults (5 males and 5 females)

The fertility test was conducted for the control group and all test item groups. Up to 17 randomly chosen surviving females were tested in each treatment group.

Exposure unit : Square glass plates (length of edges: 13 cm), assembled to cages with an aluminium frame (13 cm x 1.5 cm x 1 cm) after the spray residue had dried (~1 hour after application). Three sides of the metal frame contained six screened ventilation holes (diameter: 1 cm). The contact surfaces of the aluminium frame were taped with foam material.

The treated glass plates served as upper and lower covers (treated surface inwards). For ventilation of the exposure cages, an aquarium aeration pump was connected by means of a flexible tube to one ventilation hole in the frame.

The test organisms were introduced approximately 1 hour after the application of the test items, but not before the treated glass plates had dried.

Aphids (*Rhopalosiphum padi*) were prepared to test the parasitic capacity of *Aphidius rhopalosiphii*. A pot containing

10–15 barley seedlings (10–15 cm tall) infested with > 100 aphids was placed on a seed tray. Plexiglass tubes were then placed on top (10 cm x 25 cm). The tops of the tubes were covered with gauze.

Application of spray solutions :

The application was performed with an automatic laboratory track-sprayer. The track sprayer (1.8 bar, 2/3 replicate runs) was calibrated with deionised water before application by adjusting the spraying pressure, application speed and type of nozzle to provide an output of 200 L \pm 10% per ha. (min : 197 L/ha, max : 201 L/ha)

Environmental Conditions :

Conditions were measured continuously with a calibrated thermohygrograph.

Temperature : 20 \pm 2°C (actual : 18.5–21.5°C)

Humidity : 60–90% (actual : 60–77%)

Lighting : 16 h light:8 h dark

400–3000 lux for the exposure and the 24 h parasitisation phase (actual: 800–1400 lux); > 4000–20000 lux for development of the mummies (actual: 5000–7500 lux)

Observations:

Mortality:

The condition of the test organisms was observed 1 h, 2 h, 24 h and 48 h after introduction and they were classified as:

- Alive- alive and apparently unaffected
- Not found- wasp not recovered
- Affected- still upright and attempting to walk, but showing signs of reduced coordination
- Moribund- still twitching, unable to stand or walk
- Dead- not moving

Wasps not seen in the first 3 assessments were recorded as dead at the last assessment. The percentage mortality after 48 h was calculated for each replicate from the number of moribund and dead parasitoids and the number of released parasitoids. The mean value in the treated group was compared to the mean mortality of the control using the formula of ABBOTT (1925), modified by SCHNEIDER-ORELLI (1947).

Reproduction:

The mean number of offspring per female was determined for the treated groups and control group by averaging the replicate values. The effect on the fecundity was determined by calculating the reduction in reproduction rate (ABBOTT, 1925). Only results for wasps found alive when females were being removed after 24 h parasitisation period were used for calculation of reproduction rate.

Statistics:

Due to the results of the mortality and reproduction (< 50% effects occurred), the LR₅₀ and ER₅₀ could not be determined.

Fisher's exact test was used to detect significant differences between Bonferroni-Holm corrected mortality data of each test item treatment group and the control group.

Reproduction data were tested with Shapiro-Wilk's test and residual analysis. The data met the normality and homoscedasticity criteria. Thus, the Dunnett's t-test was conducted to detect significant differences between reproduction data of the test item treatment groups and the control group. SAS release 9.2 was used for the statistical analysis.

II. RESULTS AND DISCUSSION**Mortality:****Table 9.3.2-1: Mortality of *Aphidius rhopalosiphi* adults after 48 h exposure to treated glass plates**

Insects	PROBLAD (mL product/ha)					Control	Reference item
	268.8	672.0	1680.0	4200.0	10500.0		
#released	40	40	40	40	40	40	40
#alive	38	39	39	37	38	40	0
#affected	0	0	0	0	0	0	0
#moribund	0	0	0	0	0	0	0
#dead	2	1	1	3	2	0	40
M (%)	5.0	2.5	2.5	7.5	5.0	0.0	100.0*
Mcorr. (%)	5.0	2.5	2.5	7.5	5.0	-	100.0

M: Mortality, calculated as mean value of 4 replicates of each treatment, based on the number of dead and moribund organisms

Mcorr: Corrected mortality according to Schneider-Orelli (1947)

*: Statistically significant effects compared to the control (Fisher's Exact Test, Bonferroni-Holms corrected, one-tailed, $p \leq 0.05$)

No statistically significant effects of PROBLAD on mortality compared with the control were found at any test item rate.

Reproduction:**Table 9.3.2-2: Reproduction rate of *Aphidius rhopalosiphi***

	PROBLAD (mL product/ha)					Control
	268.8	672.0	168.0	4200.0	10500.0	
# females	16	17	16	17	16	17
# mummies	394	372	335	354	315	421
Mean no. mummies per female \pm SD	24.6 \pm 10.7	21.9 \pm 10.5	20.9 \pm 12.4	20.8 \pm 9.7	19.7 \pm 10.0	24.8 \pm 9.5
Reduction in reproduction rate (%)	0.8	11.7	15.7	16.1	20.6	-

SD: Standard Deviation

PROBLAD caused no significant effects on reproduction of *A. rhopalosiphi* at any test item rate.

Validity Criteria

Expected	Actual
Mean mortality in the control is \leq 13%	0.0%
Mean mortality in the reference group is \geq 75%	100.0%
Mean control parasitisation rate was $>$ 5 aphid mummies per surviving female	24.8
No more than two females of the control group failed to produce mummies	0 failed to produce mummies

III. CONCLUSION

It can be concluded that PROBLAD caused no statistically significant adverse effects on mortality or reproduction of *Aphidius rhopalosiphi* up to and including an application rate of 10500.0 mL product/ha. Therefore, the LR50 and ER50 of PROBLAD can be assumed to be above 10500.0 mL product/ha, the highest rate tested.

HSE COMMENTS

The study was carried out according to GLP and follows Mead-Briggs (2000) with no significant deviations to the guidelines. The reference item was tested at a suitable concentration and provided results within the expected range according to the guidelines (75–100%). All of the validity criteria were met.

After examining the raw data, it can be confirmed that no treatment rate caused more than 50% effects, therefore the LR₅₀ could not be statistically calculated. As this endpoint was not determined statistically, confidence intervals and goodness of fit graphs are not required. The LR₅₀ has been estimated to be > highest test concentration.

HSE has agreed that the following endpoint is suitable for the risk assessment:

LR₅₀: >10500 mL/ha PROBLAD in 200 L water /ha

Reference:	K-CA 8.3.2.1/02
Report Title:	PROBLAD PLUS – A rate-response extended laboratory bioassay of the effects of fresh residues on the Parasitic Wasp <i>Aphidius rhopalosiphi</i> (Hymenoptera, Braconidae)
Author(s) & Year:	Stevens (2014)
Document No	CEV SA, Unpublished report No.: CEV-14-1.
Substance used:	PROBLAD PLUS, (Batch: D32-280414, 20.1% BLAD, 1.22 g/cm ³)
Method of analysis:	No analysis conducted or required
Guideline(s):	ESCORT I Guidance Document (BARRETT et al., 1994), ESCORT II Guidance Document (CANDOLFI et al., 2001) and IOBC (MEAD-BRIGGS et al., 2009)
Deviations:	No
GLP or GEP:	Yes

Acceptability:	Yes
Study relied upon:	Yes

I. MATERIALS AND METHODS

A. MATERIALS

Test Substance

Test item : PROBLAD PLUS (Batch: D32-280414, measured: 20.10% w/w) CoA: 21/04/2014 (checked by HSE assessor)

Test concentrations: mL product/ha (g a.s./ha) 8000 (1600), 4000 (800), 2000 (400), 1000 (200), and 500 (100)

Control: Purified water

Reference item: Perfekthion/ BAS 152 11 I (Batch: FRE-000926, 400.9 g/L)

Tested at 10 mL product/ha in 400 L water/ha

Test Organism

For the bioassay, the test insects (*Aphidius rhopalosiphi*) were obtained from an in-house culture.

The wasps were reared on a mixed-species culture of cereal aphids containing the bird cherry-oat aphid *Rhopalosiphum padi* (L.) and the rose-grain aphid, *Metopolophium dirhodum* (Walk.). Parasitised aphid mummies of a uniform age were placed under upturned glass funnels and the emerging adults were collected in removable plastic pots fitted at the apex. The emergent adult wasps were provided with a 1:3 v/v solution of honey in water and female adults were used in bioassays within 48 h of their emergence.

During the period of wasp emergence, the culture was stored in a controlled- environment room maintained at 21–22°C and 67–74% relative humidity (RH), under a 16 h photoperiod of approximately 2111 lux.

B. STUDY DESIGN AND METHODS

Study dates : 08/07/2014- 21/07/2014

Experimental design: For the mortality assessments, there were six replicate units per treatment and five wasps were placed in each replicate arena, i.e., a total of 30 wasps per treatment. For the subsequent reproduction assessments, the reproductive performance of 15 individually-confined female wasps was evaluated for each treatment tested.

Exposure unit:

Mortality:

The adult parasitoids were exposed to fresh treatment residues applied to barley plants. The test plants were barley (*Hordeum vulgare* var. Westminster) grown in 11-cm-diameter pots of Levington F2S compost. The plants used in the test were 8 days old. They had two leaves (BBCH Growth Stage 12) and were approximately 10 cm in height (ca. 10 seedlings per pot). Around 1 h before product application, the seedlings were lightly sprayed with a 10% w/v solution of fructose in purified water. The spray was made using a hand-held atomiser and the plants were then left to dry. The soil in the pots was then covered with dry silver sand to create a uniform surface before the plants were treated. The treated plants were enclosed within clear acrylic cylinders (8 cm diameter, 20 cm high), the tops of which were covered with nylon netting (0.5 mm x 0.5 mm mesh). The netting contained an access hole for the introduction of the parasitoids and was sealed with a cotton wool bung.

Reproduction:

To evaluate their reproduction, female wasps were confined individually over pots containing 15 barley seedlings. These

were untreated and had been infested with host aphids (> 100 adults and nymphs of a mixed cereal aphid culture containing *M. dirhodum* and *R. padi*) seven days previously. The wasps were confined over the pots of plants using clear acrylic cylinders (9 cm diameter, 20 cm high), the tops of which were covered with nylon netting (0.5 mm x 0.5 mm mesh) for ventilation.

Application of spray solutions:

Spray applications were made at a volume rate equivalent to 400 L spray solution/ha. All dilutions were prepared in purified water shortly before their application and the spray solutions were thoroughly agitated to ensure their homogeneity. Treatments were applied in the order of control, test item (in ascending rate order) and finally the toxic reference item.

The diluted products were applied using a laboratory track-sprayer. The spray pressure selected was 3 bar and the moving spray boom was fitted with a single 80° flat-fan nozzle (Teejet 8003EVS).

The sprayer was calibrated in advance of applications using purified water, to confirm a deposition rate at target level equivalent to 400 L/ha. Calibration procedures involved spraying and weighing the deposits delivered onto glass plates (10 cm x 10 cm) positioned on 11 cm-high supports along the spray platform to simulate the mid-height of the plants to be sprayed. Three, pre-weighed plates were sprayed using purified water. The plates were then re-weighed, and the rate of deposition determined. (Min : 383 L/ha, Max : 400 L/ha)

Environmental Conditions :

Temperature and humidity was measured hourly, Light intensities were recorded at the start of assessments.

Temperature :	20 ± 2°C (actual : 21–22°C)
Humidity :	60–90% (actual 69–73%)
Lighting :	16 h light/ 8 h darkness 1828 lux mortality, 5601 lux reproduction

Observations:**Mortality and repellence:**

The treated plants were left to dry on the laboratory bench for approximately 35 minutes after treatment, before the arenas were set up. Using an aspirator, five wasps from the culture were then transferred to each test arena through a port in the netting, which was then secured with a cotton wool plug. The pots were stored in a controlled environment room.

The condition of the wasps was recorded at approximately 2, 24 and 48 h after their introduction and the insects were classed as being:

Live – alive and apparently unaffected

Affected – upright and attempting to walk but with reduced coordination

Moribund – on their back or side, twitching slightly

Dead – not moving

Not seen—wasp not observed (if not found at 48 h, missing wasps are grouped with the numbers of dead individuals)

To determine whether fresh residues of the test product were repellent, observations on the position of the individual wasps were made. Observations commenced 30 minutes after the wasps were released. Initially, five separate observations were made at 30-minute intervals, with each wasp being recorded as being on the:

Plant – on the treated plants

Cylinder – on the walls or ceiling of the test arena

Sand – on the sand below the plants

Wasps that were moribund, dead or unseen were omitted from this assessment. In the definitive bioassay there were a total of up to 150 observations for each treatment (i.e., 30 wasps on 5 occasions) with no repellence observed.

Reproduction:

Reproduction assessments were initiated at 48 h using surviving females ($n = 15$ per treatment) from the control treatment and from the three highest rates of the test item, each of which had resulted in $< 60\%$ corrected mortality rate. The female wasps categorised as being 'alive' were transferred to reproduction arenas using an aspirator. Female wasps were chosen with impartiality from the insects in each treatment and these were individually confined. The pots of seedlings and parasitoids were placed in a controlled environment room. The adult wasps were removed after 24 h, with a note made of where females were not found or were found dead, and the aphid-infested plants were then kept under similar conditions for a further 10 days, before the number of mummies that developed on each plant was recorded.

Statistics:

All statistical analyses were performed using validated computer software (SPSS, 2012).

Mortality

The percentage mortality of the test insects over 48 h was calculated. Mortality was defined as the numbers of moribund and dead insects combined. The data were not considered suitable for Probit analysis. Where there was treatment mortality at 48 h, this was compared to the control treatment using Fisher's Exact Test ($\alpha = 0.05$).

Repellence assessments

As a measurement of repellence of insects from the treated foliage during the initial 3 h of the bioassay, the percentages of wasps settled on the plants in each replicate was calculated for each of the five assessment occasions. A mean value was then obtained for each replicate. These values were angularly transformed (square root arcsine) and the normality of the data in individual treatments was assessed (Shapiro-Wilk test, $\alpha = 0.05$). As the control treatment did not conform to normality ($P < 0.05$), all results were compared to the control by non-parametric methods (Mann-Whitney U-test, 2-sided, $\alpha = 0.05$).

Reproduction

The data from the reproduction assessments (i.e., the numbers of mummies produced per female wasp found alive after the 24-h parasitism period) were square-root transformed prior to further analysis. Having checked the data sets for normality (Shapiro-Wilk test, $\alpha = 0.05$) and homogeneity of variance (Levene's test, $\alpha = 0.05$), the test-item treatments were compared to the control by one-way analysis of variance and Dunnett's two-sided t-test ($\alpha = 0.05$).

II. RESULTS AND DISCUSSION

Mortality:

At 48 h there was 0% mortality in the control treatment, compared with 13.3%, 6.7%, 0.0%, 6.7% and 0.0% mortality in the 8000, 4000, 2000, 1000 and 500 mL product/ha treatment rates of PROBLAD PLUS, respectively. In the toxic reference treatment, 86.7% mortality was observed.

Table 9.3.2-3: The percentage mortality of wasps (n = 30 per treatment) at 48 h

	Rate (mL product/ha)	% mortality ^a	Corrected % mortality ^b
Control	-	0.0	-
PROBLAD PLUS	8000	13.3	13.3
	4000	6.7	6.7
	2000	0.0	0.0
	1000	6.7	6.7
	500	0.0	0.0
Toxic reference	-	86.7 *	86.7

^a) The results for individual treatments were compared to the control using Fisher's Exact Test ($\alpha = 0.05$). Values that differed significantly are marked with an asterisk (*)

^b) Derived using Abbott's formula.

Wasp repellence:

During the initial 3 h of the bioassay, the percentage of observations where wasps were settled on the treated plants was 43.3% in the control, compared with 36.7%, 32.7%, 37.3%, 36.7% and 34.7% in the 8000, 4000, 2000, 1000 and 500 mL product/ha treatment rates of PROBLAD PLUS, respectively, and 26.0% in the toxic-reference treatment. Relative to the control, the settling rate during the initial 3 h was significantly reduced for the 4000 mL/ha treatment rate of the test item only (Mann-Whitney U-test, $\alpha = 0.05$).

Table 9.3.2-4: Summary of wasp repellence assessments

Treatment	Rate (mL product/ha)	% observations where wasps recorded to be settled on the treated plants during initial 3 h^{a)}
Control	-	43.3
PROBLAD PLUS	8000	36.7
	4000	32.7 *
	2000	37.3
	1000	36.7
	500	34.7
Toxic reference	-	26.0 *

^{a)} Treatments were compared to the control by Mann-Whitney U-test ($\alpha = 0.05$). An asterisk indicated where the results were significant.

Reproduction:

The mean number of mummies produced per surviving female was 39.3 in the control, compared with 39.5, 40.5 and 37.9 in the 8000, 4000 and 2000 mL product/ha treatment rates of PROBLAD PLUS, respectively. Therefore, relative to the control, there was an increase in reproduction of 0.6% and 2.9% in the 8000 and 4000 mL product/ha treatment rates, respectively, and a decrease of 3.7% in the 2000 mL product/ha treatment rate. Based on statistical comparisons with the

control, the reproductive performance of surviving wasps was not adversely affected by treatment rates up to and including 8000 mL product/ha ($\alpha = 0.05$).

Table 9.3.2-5: The mean number of mummies obtained per surviving female (n) wasp following a 24 h oviposition period

	Rate (mL product/ha)	N ^{a)}	Mean number of mummies per female ^{b)}	Standard deviation	Effect on reproduction (%) ^{c)}
Control	-	13	39.3	10.0	-
PROBLAD PLUS	8000	13	39.5	9.4	-0.6
	4000	13	40.5	12.5	-2.9
	2000	14	37.9	12.7	3.7

a) n = number of female wasps successfully assessed for reproductive capacity

b) The results for the test-item treatment and control were compared by one-way ANOVA and Dunnett's t-test ($\alpha = 0.05$), but results did not differ significantly.

c) Percentage effect on reproduction, relative to the control. A negative value indicated an increase, relative to the control.

Validity criteria

Required	Observed
Mortality in the control should be < 10%	0%
Mortality in the reference item at 48 h must be 50–100%	86.7%
The mean number of mummies in the control should be > 5.0 per female	39.3
There should be no more than two zero values in the control treatment	No zero values in control

Although not a criterion stated in the test guideline of Mead-Briggs et al. (2009), an additional validity criterion was set such that there should be a minimum of 30% of observations of wasps settled on the treated plants in the control treatment during the initial 3 h of the behavioural assessments. This was met during the study.

III. CONCLUSION

In an extended laboratory test to determine the effects of PROBLAD PLUS on the parasitic wasp *Aphidius rhopalosiphii*, the 48-h median lethal rate (LR₅₀) was > 8000 mL product/ha, the highest tested. Based on statistical comparisons with the control, the NOER (no-observed-effects rate) for mortality was 8000 mL product/ha. In terms of effects on the reproductive performance of surviving wasps, the median effect rate (ER₅₀) for PROBLAD PLUS was > 8000 mL product/ha, the highest rate tested. Based on statistical comparisons with the control, the NOER for reproduction was 8000 mL product/ha.

HSE COMMENTS

The study was carried out according to GLP and follows Mead-Briggs (2009) with no significant deviations to the guidelines. The reference item was tested at a suitable concentration and provided results within the expected range according to the guidelines (75–100%). All of the validity criteria were met.

After examining the raw data, it can be confirmed that no treatment rate caused more than 50% effects for the LR₅₀, therefore the LR₅₀ could not be statistically calculated. As this endpoint was not determined statistically, confidence intervals and goodness of fit graphs are not required. The LR₅₀ has been estimated to be > highest test concentration.

HSE has agreed that the following endpoint is suitable for the risk assessment:

- **LR₅₀: > 8000 mL/ha PROBLAD PLUS in 400 L water/ha**
- **Rate that caused < 50% effects on reproduction: > 8000 mL/ha PROBLAD PLUS in 400 L water/ha**

Reference:	K-CA 8.3.2.2/01
Report Title:	Toxicity to the Predatory Mite <i>Typhlodromus pyri</i> Scheuten (Acari, Phytoseiidae), in the Laboratory – Rate Response Test.
Author(s) & Year:	Klug (2010b)
Document No	CEV SA, Unpublished report No.: S10-02556.

Substance used:	PROBLAD (Batch: 201009, 20% BLAD, 1.30 g/cm ³)
Method of analysis:	No analysis conducted or required
Guideline(s):	ESCORT I Guidance Document (BARRETT et al., 1994), ESCORT II Guidance Document (CANDOLFI et al., 2001) and IOBC (MEAD-BRIGGS et al., 2009)
Deviations:	Yes, see HSE comments
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

I. MATERIALS AND METHODS

A. MATERIALS

Test Substance

Test item : PROBLAD (Batch: #201009, 20% BLAD (plant protein extract from the genus *Lupinus*)) CoA: 01/09/2010 (checked by HSE assessor)

Test concentrations: mL product/ha (g a.s./ha) 268.8 (69.9), 672.0 (174.7), 1680.0 (436.8), 4200.0 (1092.0), 10500.0 (2730.0)

Control : Deionised water

Reference item : Perfekthion/ BAS 152 11 I (Batch: 90924-06, 414.8 g/L)

Tested at 12.0 mL product/ha (4.8 g a.s./ha)

Test Organism

The predatory mite, *Typhlodromus pyri* Scheuten (Acari, Phytoseiidae) was used as the test organism. The mites were

obtained from a laboratory culture. Protonymphs not older than 24 hours were used in the study.

Feeding : The test organisms were provided with pollen of bean (*Vicia faba*) and birch (*Betula pendula*) as a food source. Each type of pollen was supplied separately at test initiation and replenished three times per week. The test organisms had continuous access to fresh water.

Preparation of the test organism: Three days before initiation of the nymph exposure, eggs of *T. pyri* (oviposited in a period of 24 hours) were collected from the culture. The eggs were transferred with a fine brush to a rearing arena (plastic tiles placed on foam rubber in small trays filled with water; the edges were covered with wet paper towels serving as a barrier) and incubated under conditions like the test conditions. Individuals from one protonymph cohort were used for the test, not later than 24 hours after moulting of the larvae. Protonymphs which already emerged one day before the start of the test were removed from the test cohort.

B. STUDY DESIGN AND METHODS

Study dates : 27/09/2010- 11/10/2010

Experimental design : Each treatment had 4 replicates containing 20 impartially selected protonymphs.

Exposure unit : Each test unit consisted of two glass cover slides with a size of 24 mm x 50 mm which were placed tightly together with their longitudinal sides. They were fixed together by means of two glass bars which were glued on them in the horizontal direction, both on the upper surface. To prevent mites escaping, a non-drying glue gel was applied on the centre of the glass cover slides. The glue barrier was formed as a

square arena which resulted in an exposure area of approximately 10–13 cm².

Following spray application and the drying of residues, the test units were placed treated surface upwards on wet filter paper on a plastic plate which laid on the top of a water-saturated foam, in such a manner that the filter paper was constantly provided with water. The thin gap between the two cover slides was filled with water by capillary forces and this served as drinking water source for the mites. The filter paper was kept permanently wet by placing each unit in a plastic tray with suitable water (mixture of tap water and deionised water).

Application of spray solutions :

All spray solutions were prepared on the day of application as close as possible to the time of application. The spray solutions were prepared with deionised water. Required quantities of PROBLAD and Perfekthion were weighed into glass bottles and filled up with deionised water.

The application was performed with an automatic laboratory track-sprayer. The track sprayer (1.8 bar, 2/3 replicate runs) was calibrated with deionised water before application by adjusting the spraying pressure, application speed and type of nozzle to provide an output of 200 L \pm 10% per ha. (Min : 197 L/ha, max : 201 L/ha)

Introduction of organisms :

After the test units had been set up, the protonymphs were transferred onto the glass plate with a fine-bristled brush under a stereo-microscope (not later than 1.5 hours after treatment). Healthy protonymphs of the correct age were chosen from the synchronised culture without bias. Immediately after transfer, mites which seemed to be injured due to the transfer and/or missing mites were replaced by healthy protonymphs from the culture. Following mite introduction, pollen was supplied as food and the units were incubated under the test conditions for the duration of the test.

Environmental Conditions:

Conditions were measured continuously with a calibrated thermohygrograph.

Temperature :	25 ± 2°C (actual : 23.5–26.0°C)
Humidity :	75 ± 15% (actual 66–76%)
Lighting :	16 h light/ 8 h darkness
	2000-4000 lux (actual: ~4200 lux)

Observations:

Mortality:

The condition of the test organisms was observed on day 3 and day 7 of exposure. Dead and surviving mites were counted. The number of escaped mites was determined. Dead mites were removed after counting. The mites were classified as:

- Alive- apparently unaffected
- Dead- not moving
- Escaped- Not recovered, stuck in the glue, or drowned in the water supply

The percentage of mortality after 3 and 7 days was calculated for each replicate from the number of dead plus escaped mites in relation to the number of introduced protonymphs.

Reproduction:

On day 7 of exposure the sex of the test organisms was determined by the shape and size of the body. Due to a delay in development of some individuals of treatment group 5, sex determination was completed 2 days later. A sex ratio of at least 1 male to 5 females was reached in all test item treatment groups, therefore a redistribution of males between replicates of the test item treatment group was not necessary. The number of offspring per female was determined by counting the number of females and eggs/juveniles on day 9, 11, and 14 of exposure. Eggs laid up to and including day 7 were removed from the test arena and were not counted. At each assessment, males and females were counted and the number of eggs and juveniles were determined. Dead animals, eggs and juveniles were removed before counting.

Statistical analysis:

The LR₅₀ could not be calculated as no mortality effects were $\geq 50\%$. Probit analysis was used to calculate the ER₅₀.

Fisher's exact test was used to detect significant differences between Bonferroni-Holms corrected mortality data of each item treatment group and control group.

Reproduction data was tested for normality and homoscedasticity using Shapiro-Wilk's test and residual analysis.

Dunnett's t-test was conducted to detect significant differences between reproduction data of the test groups and control groups.

The statistical program SAS release 9.2 was used.

Mortality:

At day 3 and 7 of the test, percentage of mortality was calculated for each replicate from the number of dead plus escaped mites in relation to the number of introduced protonymphs. A mean value and standard deviation of the replicates was calculated. For the escapees also the mean value and standard deviation were calculated for day 3 and 7. The corrected mortality and corrected escaping rate were obtained by comparing the mean values expressed as percentages observed in the treated series with those in the control series, according to the formula of ABBOTT (1925) modified by SCHNEIDER-ORELLI (1947).

Reproduction:

The number of offspring per female was determined by counting the number of females and offspring (number of eggs + number of larvae) at the 3 assessment days from day 7 on. For each replicate, the cumulative number of offspring per female was determined. For each treatment group, the mean and standard deviation was calculated.

II. RESULTS AND DISCUSSION**Mortality****Table 9.3.2-6: Mortality results and rate of escaping**

Treatment	Rate (mL/ha)	Mortality¹⁾ (%)	± SD	Mcorr (%)	Escapees²⁾ (%)	± SD	Ecorr (%)
Control	-	1.3	2.5	-	1.3	2.5	-
PROBLAD	268.8	1.3	2.5	0.0	1.3	2.5	0.0
	672.0	8.8	4.8	7.6	5.0	5.8	3.7
	1680.0	7.5	5.0	6.3	7.5	5.0	6.3
	4200.0	11.3*	4.8	10.1	8.8	6.3	7.6
	10500.0	23.8*	8.5	22.8	15.0*	4.1	13.9
Reference item	12.0	65.0*	21.2	64.5	26.3*	6.3	25.3

SD: Standard deviation

¹⁾ Mean mortality, based on number of missing and dead mites²⁾ Escaping rate, based on the number of missing mites

Mcorr: Corrected mortality according to Schneider-Orelli (1947)

Ecorr: Corrected rate of escaping according to Schneider-Orelli (1947)

*: Statistically significantly increased compared to the control (Fisher's Exact Test, one-tailed, $p \leq 0.05$)

The mean 7-day mortality was 1.3%, 8.8%, 7.5%, 11.3%, and 23.8% compared with 1.3% in the control. In the reference item group, there was 65.0% mortality. Therefore, corrected mortality was 0.0%, 7.6%, 6.3%, 10.1%, and 22.8% respectively. In the reference item group, there was 64.5% corrected mortality. Statistically significant effects were identified for test item groups 4200.0 and 10500.0 mL product/ha and for the reference item group, compared with the control (Fisher's exact test, Bonferroni-Holms corrected, one-tailed, $p \leq 0.05$). The number of escaped mites was statistically increased for the highest test item rate, applied with

10500 mL product/ha, compared with the control (Fisher's exact test, Bonferroni-Holms corrected, one-tailed, $p \leq 0.05$).

Reproduction :

Table 9.3.2-7: Reproduction results

Treatment	Rate (mL product/ha)	Mean no. Eggs/female	± SD	Reduction in reproduction (%)
Control	-	11.5	1.1	-
PROBLAD	268.8	9.1	2.5	20.9
	672.0	6.8*	4.0	40.9
	1680.0	5.9*	0.6	48.7
	4200.0	3.4*	2.0	70.4
	10500.0	1.9*	1.1	83.5

SD: Standard deviation

*: Significantly reduced compared to the control (Dunnett's t-test, one-tailed, $p \leq 0.05$)

After 3 assessments from day 7 (day 9, 11 and 14) the cumulative mean number of eggs per female in the PROBLAD treatments was 9.1, 6.8, 5.9, 3.4 and 1.9 compared with 11.5 in the control group. The reduction in reproduction was calculated as 20.9%, 40.9%, 48.7%, 70.4%, and 83.5% respectively. Significant effects on reproduction were found for the test item treatments, applied with 672.0 mL/ha, 1680.0 mL/ha, 4200.0 mL/ha and 10500 mL/ha, compared with the control group (Dunnett's t-test, one-tailed, $p \leq 0.05$). A developmental delay was recorded on day 7 of exposure for some individuals in the highest test item treatment group, applied with 10500.0 mL/ha.

Validity Criteria

Expected	Actual
Mean mortality in the control is $\leq 20\%$	1.3 %
Mean mortality in the reference group is between 50–100%	64.5%
Cumulative number of eggs per female in the control ≥ 4 eggs/female	11.5 eggs/female

III. CONCLUSION

The ER_{50} was calculated to be 1398 mL/ha with the 95% confidential limits of 1075 mL/ha to 1796 mL/ha.

HSE COMMENTS :

The study was carried out to the guidelines of Blümel et al., (2000). The study was conducted to GLP. All the validity criteria were satisfactorily met. There were no significant deviations from the guidelines.

After examining the raw data, it can be confirmed that no treatment rate caused more than 50% effects on mortality. Therefore the LR_{50} was not determined statistically, and confidence intervals and goodness of fit graphs are not required.

The applicant has proposed the endpoint to be the ER_{50} of 1398 mL/ha. However as this is a first tier *T. pyri* glass plate study, the endpoint for use in the risk assessment is the LR_{50} . The effects on reproduction will be investigated in the extended laboratory *T.pyri* study to determine a rate at which $< 50\%$ effects on reproduction occur.

This study has used fewer replicates (4) than recommended in the guidelines (5). The guidelines does say however that fewer replicates are required for dose response testing and there should be a minimum of three replicates analysed, so therefore this is deemed to be a minor deviation.

In the guidelines, sex determination is assessed at day 7 but in this instance the sex determination was completed on day 9. Due to a delay in development of some individuals of treatment group 5, sex determination was completed 2 days later. As the ratio of males: females was acceptable, this deviation is deemed to be minor.

HSE has agreed that the following endpoint is suitable for the risk assessment:

- **LR₅₀: > 10500.0 mL/ha PROBLAD in 200 L water/ha**

Reference:	K-CA 8.3.2.2/02
Report Title:	PROBLAD PLUS – A rate-response extended laboratory bioassay of the effects of fresh residues on the Predatory Mite <i>Typhlodromus pyri</i> Scheuten (Acari, Phytoseiidae)
Author(s) & Year:	Fallowfield (2014)
Document No	Study Code CEV-14-2.
Substance used:	PROBLAD PLUS, (Batch: D32-280414, 20.1% BLAD, 1.22 g/cm ³)
Method of analysis:	No analysis conducted or required
Guideline(s):	ESCORT I Guidance Document (BARRETT et al., 1994), ESCORT II Guidance Document (CANDOLFI et al., 2001) and IOBC (BLÜMEL et al., 2000)
Deviations:	Yes, see HSE comments
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

I. MATERIALS AND METHODS

A. MATERIALS

Test Substance

Test item : PROBLAD PLUS (Batch: D32-280414, measured: 20.10% w/w) CoA: 21/04/2014 (checked by HSE assessor)

Test concentrations: mL product/ha (g a.s./ha) 8000 (1600), 4000 (800), 2000 (400), 1000 (200), and 500 (100)

Control : Purified water

Reference item : Perfekthion/ BAS 152 11 I (Batch : FRE-000926, 400.9 g/L)

Tested at 37.5 mL product/ha (15 g a.s./ha)

Test Organism

The predatory mite, *Typhlodromus pyri* Scheuten (Acari, Phytoseiidae) was used as the test organism. The mites were obtained from a laboratory culture.

The mites were reared on arenas to a design described by Overmeer et al. (1982) consisting of a rectangular tile (10 cm x 20 cm in area and 1.5 mm thick) made from black PVC, with a layer of clear PVC film stretched over it. The mites were placed on a tile, which was rested on the upturned modified lid of a Perspex box, the base of which was filled with purified water. A strip of chromatography paper (10 cm x 30 cm), with two square 'windows' (7 cm x 7 cm) cut in it, was laid over the tile. The ends of the paper were submerged in the water so that it became saturated by wicking action. This wet paper surrounding the windows acted as both a barrier to mite dispersal and as a source of drinking water. To provide a further barrier against the escape of mites, a line of a non-drying sticky gel was laid on the paper, approximately 1 cm back from the edge of each window.

The temperature recorded in the cabinet in the 5-day period prior to the bioassay was 25–26°C. The culture was maintained under a 16 h photoperiod (750–1500 lux).

For the bioassays, eggs laid over a period of approximately 24 h were collected from the culture using a fine brush and were placed onto a fresh arena. Protonymphs less than 24 h old were used for the bioassay (i.e., mites collected 1-2 days after egg hatch).

Feeding : Pollen was provided as food for the mites. This was a 1:1 v/v mixture of almond (*Prunus* sp. Var. Butte) and apple (*Malus* sp. Var. Red Delicious) pollen.

B. STUDY DESIGN AND METHODS

Study dates : 21/07/2014- 04/08/2014

Experimental design : Each treatment had 3 replicate arenas per treatment, (60 mites per treatment)

Exposure unit : The test substrate comprised leaves of dwarf French bean plants (*Phaseolus vulgaris* L. var. The Prince). The plants were grown in pots of Levington F2 compost in a glasshouse until they reached the second trifoliate leaf stage (Growth Stage 14 on the BBCH scale). The first true leaves were taken from the bean plants shortly before spraying and 5-cm-diameter discs were cut from flattened sections of the leaves. The adaxial (upper) surfaces of the leaf discs were then sprayed.

Mortality:

The units comprised plastic Petri dish bases (9 cm in diameter) lined with water-saturated cotton wool. A 5-cm-high clear plastic collar was placed around each arena. The treated leaf discs were laid on the cotton wool, with their sprayed surface facing upwards. One end of a 5-mm-wide strip of 'Benchkote' was laid onto the leaf (5 mm distance) and the other end was extended onto the cotton wool (approx. 20 mm distance), to provide the mites with a source of drinking water. A line of the sticky gel barrier was drawn around the edge of the leaf, as a barrier to the dispersal of the mites. This ring was approximately 4 cm in diameter, enclosing an area of ca. 12.5 cm².

Reproduction:

The mites were left in situ on the test units.

Application of spray solutions :

Spray applications were made at a volume rate equivalent to 200 L spray solution/ha. All dilutions were prepared in purified water shortly before their application and the spray solutions were thoroughly agitated to ensure their homogeneity.

The diluted products were applied using a laboratory track-sprayer. The spray pressure selected was 3 bar and the moving spray boom was fitted with a single 80° flat-fan nozzle (Teejet 8003EVS).

The sprayer was calibrated in advance of applications using purified water, to confirm a deposition rate at target level equivalent to 200 L/ha. Calibration procedures involved spraying and weighing the deposits delivered onto glass plates (10 cm x 10 cm) positioned along the spray platform. Three, pre-weighed plates were sprayed using purified water. The plates were then re-weighed, and the rate of deposition determined. Treatments were applied in the order of control, test item (in ascending rate order) and finally the toxic reference item. (Min : 195 L/ha, max : 215 L/ha)

Environmental Conditions :

Temperature was measured hourly, Light intensities were recorded at the start of assessments.

Temperature :	25 ± 2°C (actual : 24–27°C)
Humidity :	75 ± 15% (actual 63–80%)
Lighting :	16 h light/ 8 h darkness
	2000–4000 lux (actual: 620–1300 lux)

Observations:

Mortality:

The bioassay was initiated within 1 h of treatments being applied. Using a fine brush, twenty protonymphal mites were placed into each of the arenas within 1.5 h of treatment application.

The condition of the mites was assessed with the aid of a binocular microscope at 1 and 7 days after treatment (DAT). They were recorded as being:

alive – still moving

dead – no sign of movement

stuck – embedded in the sticky barrier

drowned – dead on the water source

missing – not visible

Any dead, drowned, or stuck mites were removed at the time of each assessment.

Reproduction:

The numbers of male and female mites in each replicate were recorded at 7 DAT, this was to ensure a female to male ratio of at least 5:1. Any eggs that were produced prior to 7 DAT were removed and discarded. Untreated pollen was added as food and this was replenished daily during the following week. For 7 days, the total egg production (numbers of eggs plus live and dead juvenile stages) was recorded for each unit. Assessments of oviposition activities were carried out at 9, 11 and 14 DAT. Any eggs and nymphs present were recorded and then removed. In addition, the condition of the adult female and male mites in each arena was recorded on each date.

Statistics:

All statistical analyses were performed using validated computer software (SPSS, 2012).

The numbers of any drowned, stuck, or missing mites were added to the number of dead mites found in each treatment to derive the overall “mortality”. The mean percentage mortality after 7 days was calculated for the individual treatments and then corrected for any losses in the control treatment using Abbott’s formula. The percentage mortality in each treatment was compared to the control using Fisher’s Exact Test ($\alpha = 0.05$). The highest treatment rate at which no statistically significant

effect was observed was taken to be the no-observed-effect rate (NOER) with respect to mite survival. A Probit regression analysis was not deemed appropriate due to the outcome of the study.

For the reproduction data, the mean cumulative number of eggs per female was determined for the period 7-14 DAT. To calculate this value, the total number of eggs laid in each replicate between each assessment date was divided by half of the sum of the numbers of female mites recorded as alive at the start and end of each assessment period. Any progeny recorded as larvae/nymphs (i.e., eggs that had presumably been missed in a previous assessment and had subsequently hatched) were added to the egg totals from the previous assessment period, as per Blümel et al. (2000). The individual values were totalled for each replicate and the average for the three replicates was calculated.

The data sets were checked for normality (Shapiro-Wilk test, $\alpha = 0.05$) and equality of variance (Levene's test, $\alpha = 0.05$), prior to comparison of individual treatments with the control by one-way ANOVA and Dunnett's t-test. The highest treatment rate for which no statistically significant effect was observed was taken to be the NOER with respect to reproduction.

II. RESULTS AND DISCUSSION

Mortality:

At 7 days there was 3% (2 missing) mortality in the control treatment, compared with 8% (3 dead, 4 missing), 10% (4 dead, 2 stuck, 5 missing), 0%, 0% and 0% mortality in the 8000, 4000, 2000, 1000 and 500 mL product/ha treatment rates of PROBLAD PLUS, respectively. When adjusted for the deaths in the control, the corrected mortalities in the respective test-item treatments were 5%, 7%, 0%, 0% and 0%. Statistically, none of the test-item treatments differed significantly from the control (Fisher's Exact Test, $\alpha = 0.05$). The NOER for mortality at 7 days was therefore taken to be 8000 mL product/ha. In the toxic reference treatment, 100% mortality (100% corrected) was recorded at 7 days.

Table 9.3.2-8: Mean percentage mortality of mites at 7 days

Treatment	Application rate (mL product/ha)	Mean % mortality at 7 DAT^{a)}	Corrected % mortality at 7 DAT^{b)}
Control	-	3	-
PROBLAD PLUS	8000	8	5
	4000	10	7
	2000	0	0
	1000	0	0
	500	0	0
Toxic reference	-	100 *	100

^{a)} The results for mortality were compared using Fisher's Exact Test ($\alpha = 0.05$). Treatment means marked with an asterisk differed significantly from the control.

^{b)} Calculated using Abbott's formula.

Reproduction:

The mean number of eggs produced per female was 8.7 in the control treatment, compared with 6.0, 8.5, 6.8, 7.5 and 7.1 in the 8000, 4000, 2000, 1000 and 500 mL product/ha treatment rates of PROBLAD PLUS, respectively. These values indicated that the ER_{50} for PROBLAD PLUS was > 8000 mL product/ha, the highest rate tested. Statistically, none of the test-item treatments differed significantly from the control (ANOVA and Dunnett's t-test, $\alpha = 0.05$). The NOER with respect to reproduction was therefore taken to be 8000 mL product/ha.

Table 9.3.2-9: A summary of the reproduction of mites. The mean number of eggs laid per female 7-14 DAT and the percentage change in the mean reproduction in each treatment, relative to the control

Treatment	Application rate (mL product/ha)	Mean number of eggs per female a)	% change in reproduction relative to control b)
Control	-	8.7	-
PROBLAD PLUS	8000	6.0	30.7
	4000	8.5	2.9
	2000	6.8	22.0
	1000	7.5	13.7
	500	7.1	18.6

a) Results for reproduction over the assessment period were compared by one-way ANOVA and Dunnett's t-test ($\alpha = 0.05$), but there were no significant differences.

b) Percentage change in egg production, relative to the control. A positive value indicates a decrease.

Validity criteria:

All of the validity criteria were met:

Required :	Observed :
Mortality in the control should not exceed 20%	3%
Mortality in the toxic reference should be 50–100%	100%
The mean cumulative number of eggs produced should be ≥ 4 per female in the control	8.7

III. CONCLUSION :

In an extended laboratory test in which the predatory mite *Typhlodromus pyri* was exposed to freshly-dried foliar residues of PROBLAD PLUS, the 7-day median lethal rate (LR₅₀) was found to be > 8000 mL product/ha, the highest rate tested. Based on statistical comparison with the control, the no-observed-effect rate (NOER) with respect to mite survival was 8000 mL product/ha.

In terms of the reproductive performance of surviving mites, the ER₅₀ was > 8000 mL product/ha. Based on statistical comparison with the control, the NOER with respect to reproduction was 8000 mL product/ha.

HSE COMMENTS:

The study was carried out to GLP and the guidelines set out in Blümel et al., (2000). All the validity criteria were satisfactorily met.

Only three replicates of each treatment group was conducted, whereas the guidelines recommends five replicates per treatment group. According to the guidelines, only three replicates are required for analysis so this is deemed to be a minor deviation.

After examining the raw data, it can be confirmed that no treatment rate caused more than 50% effects on mortality. The LR₅₀ was therefore not determined statistically, so confidence intervals and goodness of fit graphs are not required.

The reference item was tested at a rate of 37.5 mL product/ha, which is more than double the expected dose according to the guidelines (9–15 mL product/ha). The rate of 9–15 mL prod/ha is chosen based on application to glass plates, which are worst-case compared to the leaf substrate used in this test. Although there is no ring-test data for reference items on leaf substrate, it can be assumed that a higher rate would be required to get the same level of effect as seen on glass plates. So whilst there is some uncertainty with the test system sensitivity, the results are not considered to be invalid.

The unit set-up for mortality and for reproduction do not match that which is outlined in the guidelines for any of the methods presented. It is assumed that the unit set-up is a modified form of the 'open method' due to the use of leaf substrate instead of glass plates. No further information is required on this deviation.

HSE has agreed that the following endpoints are suitable for the risk assessment:

- **LR₅₀: > 8000 mL/ha PROBLAD PLUS in 200 L water/ha**

- Rate that caused < 50% effects on reproduction: > 8000 mL/ha PROBLAD PLUS in 200 L water/ha

Reference:	K-CP 8.3.2.2/01
Report Title:	PROBLAD PLUS – A rate-response extended laboratory test to determine effects on the green lacewing <i>Chrysoperla carnea</i> (Neuroptera, Chrysopidae)
Author(s) & Year:	Vaughan, R. (2017)
Document No:	Unpublished report No.: CEV-17-1.
Substance used:	PROBLAD PLUS (Batch: C1609-002, 20.50% BLAD, 1.233 g/cm ³)
Method of analysis:	No analysis conducted or required
Guideline(s):	ESCORT I Guidance Document (BARRETT et al., 1994), ESCORT II Guidance Document (CANDOLFI et al., 2001) and IOBC (VOGT et al., 2000)
Deviations:	Yes, see HSE comments
GLP or GEP:	Yes
Acceptability:	Acceptable
Study relied upon:	Yes

I. MATERIALS AND METHODS

A. MATERIALS

Test Substance

Test item: PROBLAD PLUS (measured: 20.50% w/w) CoA: 12/12/2016 (checked by HSE assessor)

Lot/Batch # : Batch: C1609-002

Treatments

Test concentrations: 10.24 and 3.2 L product/ha. Corresponding to 12.8 and 3.2 mL test item diluted with purified water.

Control: Purified water

Reference item: Perfekthion/ BAS 152 11 I (Batch: FRE-001126, 420.3 g/L)

Tested at 80 mL product/ha

Test Organism

Rearing : Eggs of the green lacewing, *Chrysoperla carnea* Steph (Neuroptera, Chrysopidae), were obtained from a stock culture maintained at the test facility. For the bioassay, a cohort of eggs laid over a 24-h period was used. The emerging larvae were fed with UV light-killed eggs of the Angoumois grain moth, *Sitotroga cerealella* (Oliver). These moth eggs were stored at 0.3–9.3°C.

The lacewing eggs (on egg-laying substrate sheets) were stored in boxes at 23.8–25.8°C with a 16 h photoperiod (2700–3400 lux). Lacewing larvae emerging over a 24h period were used for the bioassay and were taken when they were 2–3 days old.

Test Plants: Leaves were taken from dwarf French bean plants (*Phaseolus vulgaris* var. The Prince). The beans were grown in pots of Levington F2+S compost in a glasshouse until their first true leaves were fully expanded (BBCH Growth Stage 12). The growing tips were removed from the plants to prevent further leaves from developing and the first true leaves were collected from the plants before being treated.

Feeding: Larvae and Adult food:

Larvae were fed every 1–3 days with UV light-killed eggs of *S. cerealella*. Three times per week adult lacewings were provided with the following food and water during the bioassays:

- a. An artificial diet consisting of 15 mL condensed milk, one hen's egg and one additional egg yolk, 30 g honey, 20 g fructose, 30 g brewer's yeast, 50 g wheatgerm and approximately 45 mL purified water. This was placed in a 3 cm diameter petri dish that was then inverted so that the lacewings could feed on the diet that flowed into the space between the lid and base, without getting stuck.
- b. A 1:2-1:3 honey/water solution on a cotton wool pad, placed in an inverted petri dish
- c. Fresh water on a cotton wool pad, placed in an inverted petri dish

Test Procedure

Experimental design: In the bioassay there were 40 individually-confined larvae per treatment. For the reproduction assessments, insects from the control were grouped in 2 boxes, while those from test item treatments were grouped in 1 box per treatment, but these were not considered as replicates for statistical purposes.

Exposure unit:

Mortality:

When residues had dried, the treated leaves were used to line the floor of simple test arenas. Each comprised a square glass plate (7.5 cm x 7.5 cm), a Perspex supporting plate of similar size, with a 5 cm diameter hole cut through it, and an acrylic cylinder (44 mm internal diameter, ca. 2.5 cm tall). The treated leaf was laid on the glass plate with its adaxial (upper) treated surface facing upwards and the Perspex sheet was placed on top, held together with elastic bands. The petiole of the leaf was wrapped in wet cotton wool, which was draped into a water trough. The acrylic cylinder was treated with an aqueous solution of polytetrafluoroethylene to prevent larvae from climbing

away from the treated leaf. A ventilated lid, covered with 0.5 mm x 0.5 mm mesh nylon netting, was placed over each cylinder to ensure that larger larvae could not climb out.

Pupal development

As the lacewing pupae developed they were transferred to large plastic storage boxes (27 cm x 27 cm x 14 cm). Treatments were kept in separate boxes and food and water was provided for the emerging adult lacewings.

Reproduction:

As the adult lacewings emerged, they were transferred to polystyrene boxes (15 cm x 27 cm x 10 cm) with close-fitting lids. A sheet of fibrous tissue was placed under the lid of each box to serve as an oviposition site.

Egg viability assessments:

The egg-bearing fibrous tissue was transferred to additional boxes of the same dimensions. The emerging larvae were provided with UV-killed eggs of *S. cerealella*, to limit any cannibalism of the eggs of *C. carnea*.

Application of spray solutions:

The treatments were applied from directly above the target leaves using a laboratory track-sprayer. The spray pressure selected was 3 bar and the moving spray boom was fitted with a single 80° flat-fan nozzle (Teejet 8003EVS).

The sprayer was calibrated in advance of applications using purified water, to confirm a deposition rate at target level equivalent to 200 L/ha. Calibration procedures involved spraying and weighing the deposits on glass plates. Three pre-weighed plates were sprayed with purified water. Once three consecutive applications had delivered the correct deposition rate (Min: 197 L/ha, Max: 209 L/ha), treatments were applied. The test item and reference item were diluted immediately before use. The

solutions were thoroughly agitated to ensure homogeneity of the spray solution.

Environmental conditions

Temperature and humidity was measured hourly, light intensity was recorded for each phase of the bioassay.

Temperature

25 ± 2°C (actual: 23.1–25.7°C)

Humidity:

75 ± 15% (actual 55–79%. Although the relative humidity fell below the intended minimum threshold on five occasions, each was for a period of less than 2 hours and so was not considered to be a deviation.)

Lighting:

16 h light/ 8 h darkness

≥ 1000 lux (actual: 3100–4500 lux)

B. STUDY DESIGN AND METHODS

Study dates: 07/02/2017- 16/03/2017

Bioassay observations:

Pre-imaginal mortality assessments

The freshly treated leaves were left to dry and the test arenas were then assembled with one larva introduced to each unit. Only larvae with healthy appearance were used. The condition of the larvae was assessed every 1–3 days until they pupated. They were categorised as follows:

- Alive – apparently healthy and unaffected
- Abnormal pupa – Larvae pupating without spinning a cocoon or appearing different from the norm
- Dead – no longer moving
- Pupated – larvae having pupated

Any larvae that escaped or were accidentally killed were noted and excluded from any data analysis.

As pupae developed, they were collected before emergence of adults, but were not removed from the surface to which they were attached. The number of lacewings that had emerged successfully was recorded every 2–3 days.

Reproduction assessments

Any insects emerging in the second week after the first adult lacewings had emerged were not included in the reproduction assessments, there being less than 3 females per treatment falling into this category. The sex of the adult lacewings was determined by eye. When the oviposition assessments were complete, the adults were killed in the freezer and then examined using a binocular microscope to confirm their sex, this allowed an accurate estimation of the mean number of eggs produced per female per day in each treatment.

Assessments commenced 7 days after the majority of the adult lacewings had emerged, which was also 7 days after egg laying had first been noted in the individual boxes. Eggs were sampled from the reproduction boxes by removing and replacing the fibrous sheet used to line the lids. The assessments made were as follows:

- a. The number of eggs laid in each box were recorded for two 24-h periods within one week.
- b. The variability of the eggs laid on the fibrous tissue was determined. Having first counted the number of eggs on the sheets, they were laid in individual boxes and UV-killed eggs of *S. cerealella* were provided for the emerging larvae. Once the larvae started to hatch, the sheets were removed once each day and shaken to remove those larvae. After at least 6 days, the number of unhatched eggs remaining was recorded so that the percentage viability could be calculated.

Statistics:

Statistical analyses were performed using validated computer software (SPSS, 2013).

The results have been expressed in terms of:

- Percentage pre-imaginal mortality of insects, both before and after correction of the data for any control losses using Abbott's formula.

- The mean number of eggs produced per female per day.
- The mean percentage viability of eggs on the fibrous sheet lining the box lid.

The pre-imaginal mortality in each treatment was compared to that in the control using Fisher's Exact test.

The percentage effect of the test item treatment on lacewing reproduction, relative to the control was also calculated.

II. RESULTS AND DISCUSSION

Pre-imaginal mortality assessments

Overall, there was 17.5% mortality in the control treatment, compared with 32.5% and 35.0% mortality (18.2% and 21.2% corrected mortality) in the 10.24 and 3.2 L product/ha treatments, respectively. There was no observed dose-response relationship for mortality amongst the individual treatment rates. The test item treatments did not differ significantly from the control (Fisher's Exact Test, $\alpha = 0.05$). There was 100% mortality in the toxic reference treatment.

Table 9.3.2-10: Mortality recorded during development of the test insects

Treatment	Rate (L product/ha)	% pre-imaginal mortality ^{a)}	Corrected % pre-imaginal mortality ^{b)}
Control	-	17.5	-
PROBLAD PLUS	10.24	32.5	18.2
	3.2	35.0	21.2
Toxic reference	-	100 *	100

^{a)} Data from individual treatments were compared to the control using Fisher's Exact Test ($\alpha = 0.05$)

^{b)} Corrected for any control treatment deaths using Abbott's formula (Abbott, 1925).

Reproduction assessments

The mean number of viable eggs produced per female per day was 26.8 in the control compared with 26.3 and 24.9 in the 10.24 and 3.2 L product/ha treatments, respectively. The reproductive performance in the control and test item treatment exceeded the validity thresholds of ≥ 15 eggs/female/day and $\geq 70\%$ hatching rate.

Table 9.3.2-11: Reproductive assessments

Treatment	Rate (L product/ha)	Mean number eggs/female/day^{a)}	Mean percentage egg viability^{b)}	Mean viable eggs/female/day	Effects on reproduction^{c)} [%]
Control	-	29.3	91.3	26.8	-
PROBLAD PLUS	10.24	29.6	88.8	26.3	1.9
	3.2	27.5	90.4	24.9	7.1

a) Based on two 24 h long assessments made for each oviposition box in each treatment

b) Based on all eggs laid on the fibrous tissue sheet lining the lid of each oviposition box

c) Percentage change in mean number of viable eggs per female: a positive value indicates a decrease relative to control.

Validity Criteria

Required:	Observed:
Pre-imaginal mortality should be $\leq 20\%$ in control	17.5%
Mean egg production in the control should be ≥ 15 eggs per female per day	29.3
Mean viability of eggs should be $\geq 70\%$	91.3%
Mortality should be $\geq 50\%$ in the toxic reference treatment	100%

III. CONCLUSIONS

In an extended laboratory test in which the foliar-active predator *Chrysoperla carnea* was exposed to freshly-dried foliar residues of PROBLAD PLUS, there were no significant effects on either the survival or reproductive capacity of the lacewings at treatment rates up to and including 10.24 L product/ha.

HSE COMMENTS:

The study was carried out to GLP and the guidelines set out in Vogt et al., (2000). All the validity criteria were satisfactorily met.

After examining the raw data, it can be confirmed that no treatment rate caused more than 50% effects for the LR₅₀. As this endpoint was not determined statistically, confidence intervals and goodness of fit graphs are not required.

The reference item was tested at a rate of 80 mL product/ha, which is more than the expected dose according to the guidelines (30–45 mL prod/ha). The rate of 30–45 mL prod/ha is chosen for glass plate studies, which are worst-case compared to the leaf substrate used in this test. Although there is no ring-test data for reference items on leaf substrate, it can be assumed that a higher rate would be required to get the same level of effect as would be seen on glass plates. So whilst there is some uncertainty with the test system sensitivity, the results are not considered to be invalid.

HSE has agreed that the following endpoints are suitable for the risk assessment:

- **LR₅₀: > 10.24 L/ha PROBLAD PLUS in 250 mL water/ha**
- **Rate that caused < 50% effects on reproduction: > 10.24 L/ha PROBLAD PLUS in 250 mL water/ha**

B.9.4. Effects on non-target soil meso- and macrofauna

The following acute toxicity study was submitted, however this was not evaluated as it does not form part of the data requirements under 283/2013 and is not required for the risk assessment.

Reference:	K-CA 8.4.1/01
Report Title:	PROBLAD – Acute Toxicity on Earthworms, <i>Eisenia fetida</i> using an Artificial Soil Test. CEV SA
Author(s) & Year:	Ganssmann (2010a)
Document No	CEV SA, Unpublished report No.: S10-02557
Substance used:	PROBLAD (Batch: 201009, 20% BLAD, 1.30 g/cm ³)

B.9.4.1. Earthworm – sub-lethal effects

Reference:	K-CA 8.4.1/02
Report Title:	Effect of PROBLAD PLUS on the earthworm <i>Eisenia andrei</i> in artificial soil. BioChem agrar, Gerichshain, Germany. CEV SA
Author(s) & Year:	S. Friedrich (2017)
Document No	CEV SA, Unpublished report No.: 17 48 TEC 0013.
Substance used:	PROBLAD PLUS (Batch: C1609-002, 20.50% BLAD, 1.233 g/cm ³)
Method of analysis:	No analysis conducted or required
Guideline(s):	OECD 222 (2016)
Deviations:	No

GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

I. MATERIALS AND METHODS

A. MATERIALS

Test Material

PROBLAD PLUS

Lot/Batch #: Batch no. C1609-002

Actual content of active substance: Protein (BLAD) content 20.0% w/w nominal; 20.50% w/w analysed, density $1.233 \pm 0.06 \text{ g/cm}^3$, according to the certificate of analysis.

Treatments

Test rates: 100, 55.56, 30.86, 17.15 and 9.53 mg PROBLAD PLUS/kg soil and untreated control (spacing factor: 1.8).

Control: Water only

Toxic reference test: A test with the reference item Maypon Flow (Carbendazim, SC 500) at 5 and 10 mg product/kg soil dry weight was carried out in January 2017 as part of routine testing. The number of juveniles was reduced by 57 and 100% at concentrations of 5 and 10 mg product/kg soil dry weight (mean number of juveniles = 46 and 0) after 8 weeks of test duration when compared to control (mean number of juveniles = 107).

The test confirms the sensitivity of the earthworms against a compound with known effects under the test conditions. The OECD 222 (2016) guideline states significant effects are expected between 1–5 mg a.s./kg soil dry weight, and in the reference test the effect over 57% at 5 mg a.s./kg soil dry weight shows the sensitivity of the test in this range.

Test animals

Species: *Eisenia andrei* (Bouché, 1972)

Source: Reared under ambient laboratory conditions in the test facility (original breeding of animals was purchased from a commercial supplier)

Age at start of test: Adult worms (approximately 4 months old with clitellum)

Acclimatisation:	At least 24 hours in artificial substrate (with food – horse manure)
Weight of animals used in the test:	403–596 mg/worm (this is in line with the 250–600 mg stated in the OECD 222 (2016) guideline).
Test design	
Test vessels:	Plastic vessel of Bellaplast (inside dimensions: about 16.5 cm x 12 cm x 6 cm) with a lid pervious to air and light.
Soil type and composition:	<p>Artificial soil, according to OECD 222 (2016) guideline:</p> <ul style="list-style-type: none"> • 10% sphagnum peat (close to pH 5.5–6.0; no visible plant remains, finely ground, dried to measured moisture content); • 20% kaolin clay (kaolinite content above 30%); • 0.5% calcium carbonate • 69.5% industrial quartz sand; type: Millisil W3 (fine sand is dominant with more than 50% of the particles between 50 and 200 microns) • Deionised water <p>Soil was mixed at 2 x 2 minutes for each replicate at 240 rpm.</p>
Soil storage and handling:	Dry artificial soil was pre-moistened by adding deionised water to obtain approximately half of the final water content one day before test start.
Application of test item:	<p>On the day of the test start, the test item was introduced by mixing the quantity of test item required to obtain the desired test concentration with the volume of water required to hydrate the soil to 40–60% of its WHC.</p> <p>The test item was prepared first by making a stock solution of the test item in deionised water of 1.000 g/L. The stock solution was then used to prepare four further test solutions by adding the required amount of the stock solution (23.81, 42.87, 77.16 and 138.89 mL) to 250 mL deionised water. 60 mL of each test solution was added to 750 g wet weight artificial soil, yielding 810 g wet artificial soil corresponding to 600 g dry weight (water content at confirmed to be 55.4–55.6% of WHC which is within the 40–60% of WHC in line with OECD 222 (2016) guideline requirements).</p>

Amount of soil per vessel:	810 g wet weight corresponding to 600 g dry weight
Replication:	Control: eight replicates
	Test rates: four replicates
	Each with ten individual worms
Observation intervals:	Weekly: observation of behavioural and pathological symptoms (including feeding activity
	After 4 weeks exposure: number of surviving adult worms per replicate, behaviour, pathology and morphological symptoms, fresh weight of surviving adult worms per replicate
	After 8 weeks exposure: number of juveniles per replicate, behaviour, pathology and morphological symptoms.
Duration of test:	8 weeks (4 weeks adult mortality and biomass; 4 weeks juvenile development)
Environmental test conditions	
Temperature:	18.0–21.3°C
pH of soil:	Guideline requirement: 6.0 +/- 0.5
	Measured (pooled replicates per treatment group):
	test start: 5.95–6.05
	test end: 5.70–5.75

Soil moisture content:	<p>Max. water holding capacity (WHC): 63.0 g/100 g dry soil.</p> <p>Guideline recommendations for moisture content: 40–60% of WHC</p> <p>Measured (pooled replicates per treatment group):</p> <p>Start of Test: 55.4–55.6% of WHC</p> <p>End of Test: 54.9–55.6% of WHC</p> <p>This is within the guideline variation of 10% variation from the start and end of the test.</p>
Photoperiod and lighting:	<p>Artificial light. Intensity: 590 lux.</p> <p>Duration: light : dark = 16 h:8 h</p>
Experimental Dates:	20 Dec 2016–14 Feb 2017
Statistical Analysis:	<p>The arithmetic mean and the standard deviation per treatment for each endpoint were calculated. The statistical analysis was performed with the software ToxRat Professional 3.2.1 (Ratte 2015). For identifying the NOEC values the Multiple Sequentially-rejective Fisher Test after Bonferroni-Holm was used for mortality data to compare the control and test groups.</p> <p>For biomass and number of juveniles, data were first tested for normality and homogeneity of variance using Shapiro-Wilk's Test and Levene's test, which found the data were normally distributed with homogenous variance, hence the parametric multiple comparison test of William's t-test was used to compare the control with the independent test item groups.</p> <p>For statistical evaluation of the biomass change, the changed mean fresh weight of surviving worms per replicate was used.</p> <p>No models for EC/LC_x values were used due to lack of effects seen in the study, these values are unbounded beyond the highest test concentration.</p>

B. STUDY DESIGN AND METHODS

The study was conducted in artificial soil in accordance with the OECD 222 guideline. The maximum water holding capacity (WHC) was 63.0 g/100 g dry soil. Adult earthworms used in the study were acclimatised to this soil with added horse manure for at least one day before the test start.

Five test concentrations were assessed, 100, 55.56, 30.86, 17.15 and 9.53 mg PROBLAD PLUS/kg soil. On the day of the test initiation, the required test item concentrations were mixed with an appropriate volume of water to hydrate the dried test soil to 40–60% of its water holding capacity. For control replicates water only was added to the soil. Approximately 500 g soil was then added to each test vessel. Adult earthworms were individually weighed weight of animals used in the test: 403–596 mg/worm and assigned randomly in groups of ten to each test vessel soil surface. After approximately 15 minutes any worms remaining on the soil surface was replaced to ensure only healthy individuals are included in the study. The test vessels were then sealed with a perforated lid to allow gas exchange but prevent earthworms escaping.

The test vessels were placed at random in a controlled environment test room (temperature $20 \pm 2^\circ\text{C}$, light 400–800 lux, light:dark cycle 16 h:8 h). For the control, eight replicate test vessels with ten earthworms were set up with four replicate vessels for each of the test item treatments. One day after application 5 g of air dried ground horse manure was scattered on the soil surface for food, which was sprinkled with 5 mL deionised water. The feeding interval was weekly during the first four weeks of the test, and the amount of manure (5 g) to be added depended on the feeding activity, which was assessed by visual estimation of the food remaining on the surface before addition of new food.

After four weeks the adult worms were removed from the test vessels. The number of surviving worms and fresh weight of the worms per vessel was then determined. With the adult worms removed the soil of each test vessel was mixed with 5 g manure and returned to the test vessels and left for another 4 weeks. After this time the number of juveniles per test vessel was determined by hand sorting. The number of surviving adults and total number of juveniles counted in each of the test item vessels was assessed compared to the control. In addition to these measurements, observation of behavioural and pathological symptoms, including feeding activity, was carried out weekly from the start to the end of the test.

II. RESULTS AND DISCUSSION

No statistically significant effects on mortality were observed at any of the concentrations tested (Multiple Sequentially-rejective Fisher Test after Bonferroni-Holm, $\alpha = 0.05$, one-sided greater) compared to the control. The test item caused no

statistically significant change in biomass (change in fresh weight after 4 weeks relative to initial fresh weight) and no statistically significant effects on reproduction compared to the control group at any concentration tested (Williams-t-test, $\alpha = 0.05$, one-sided smaller). The NOEC was therefore taken to be the highest concentration tested, 100 mg test item/kg soil d.w. and the LC_{50} and EC_x values to be > 100 mg test item/kg soil d.w. Results can be seen in Table 9.4.1-1.

Feeding activity was 100% in all test vessels at all test concentrations.

Table 9.4.1-1: Effects of PROBLAD PLUS on *Eisenia andrei* in a 56-day reproduction study

Parameter	Treatment group (mg test item/kg soil d.w.)					
	control	9.53	17.15	30.86	55.56	100
Mortality of adult worms after 4 weeks (%)	1.3	2.5	2.5	0.0	0.0	2.5
Mean biomass change (%)	15.9	14.9	13.5	17.3	15.4	14.7
Mean number of juveniles after 8 weeks	152.0	151.3	156.3	149.8	161.3	150.8
Reduction of reproduction compared to control (%)	-	0.5	-2.8	1.5	-6.1	0.8
Endpoints (mg test item/kg soil d.w.)						
NOEC (mortality)	100					
NOEC (biomass)	100					
NOEC (reproduction)	100					
LC₅₀ (mortality)¹	> 100					
EC₁₀ (reproduction)¹	> 100					
EC₂₀ (reproduction)¹	> 100					
EC₅₀ (reproduction)¹	> 100					

Not statistically significantly different compared to the control for mortality (Multiple Sequentially-rejective Fisher Test after Bonferroni-Holm, $\alpha = 0.05$, one-sided greater) and for biomass and reproduction (Williams-t-test, $\alpha = 0.05$, one-sided smaller)

Negative values = increase, relative to control

¹based on estimation of the data

Validity Criteria:

The validity criteria for the control group as per OECD 222 (2016) were met:

- Adult mortality: $\leq 10\%$ (being 1.3% after 4 weeks)
- Number of juveniles per replicate: ≥ 30 (being 146, 122, 139, 168, 154, 118, 176 and 193)
- Coefficient of variation of reproduction: $\leq 30\%$ (being 17.2%)

III. CONCLUSION

In a 56-day earthworm reproduction study with PROBLAD PLUS, no statistically significant adverse effects on mortality, biomass and reproduction of the earthworm *Eisenia andrei* in artificial soil were determined up to and including 100 mg test item/kg soil dry weight, i.e. the highest concentration tested.

The NOEC for mortality, biomass and reproduction was determined to be 100 mg test item/kg soil dry weight. The LC_{50} and the EC_{10} , EC_{20} and EC_{50} values for reproduction were estimated to be > 100 mg test item/kg soil dry weight.

HSE COMMENTS

This study was conducted to GLP. The study was conducted to OECD 222 (2016) and has been assessed to this guideline. HSE has added detail to the original summary report as required from the study PDF and corrected typos in the weight of the worms used in the test. The test soil contained 10% peat. There were no deviations from the study guideline and the validity criteria were met. The reference item test confirmed the sensitivity of the test system. Due to a lack of significant effect on reproduction, the $EC_{10/20/50}$ were not determined statistically and are instead estimated to be greater than the highest concentration tested. The NOEC for reproduction has been determined using Williams test, which is considered appropriate in accordance with the guideline.

The agreed endpoints from this study are:

- **NOEC (mortality, biomass, reproduction): 100 mg PROBLAD PLUS/kg soil d.w.**
- **LC_{50} : > 100 mg PROBLAD PLUS/kg soil d.w.**
- **$EC_{10,20,50}$ (reproduction): > 100 mg PROBLAD PLUS/kg soil d.w.**

Reference:	K-CA 8.4.1/03
Report Title:	PROBLAD PLUS: Sublethal Toxicity to the Earthworm <i>Eisenia andrei</i> (Oligochaeta, Lumbricidae) in Artificial Soil with 10% Peat.
Author(s) & Year:	Antón, B. (2020).
Document No	CEV SA, Unpublished report No.: S20-00600.
Substance used:	PROBLAD PLUS (Batch: CF01907-001, 21.2% BLAD, 1.20 g/cm ³)
Method of analysis:	No analysis conducted or required.
Guideline(s):	OECD 222 (2016)
Deviations:	No
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

I. MATERIALS AND METHODS

A. MATERIALS

Materials

Test Material	PROBLAD PLUS
Lot/Batch #:	CF01907-001
Purity:	Lead component BLAD protein present at 21.2% w/w, according to certificate of analysis.

Description:	Brown liquid; Soluble concentrate (SL)
Stability of test compound:	Stable at normal handling and storage conditions
Reanalysis/Expiry date:	July 2022, according to certificate of analysis
Density:	1.20 g/mL, according to certificate of analysis

Treatments

Test rates:	Concentration in soil (mg/kg dry weight of artificial soil): 0, 62.5, 125, 250, 500 and 1000 mg/kg (spacing factor 2)
Solvent/vehicle:	Deionised water
Analysis of test concentrations:	No
Reference item:	Carbendazim (formulation 'Sigma-Aldrich 97%') was tested in a separate study. The last reference test was performed between Feb–Apr 2019 (which is within a year of this experiment).

Test organisms

Species:	<i>Eisenia andrei</i> (Bouché, 1972) adult with clitellum, between 2 and 12 months old, with an age synchronised to a range of 4 weeks. Weights ranged between 250–600 mg, average weight 401 mg, and were observed to be reproductively mature.
Source:	Commercial supplier
Acclimatisation period:	2 days under experimental conditions
Feeding:	Horse manure, dried and finely ground
Treatment for disease:	None reported Treatment for disease is not mentioned in the OECD 222 (2016) guideline.

Test design

Test vessel:	Glass containers with a capacity of approximately 1.5 litres, filled with approximately 500 g (dry weight) test medium, covered with plastic lids with small openings to allow gaseous exchange
Test medium:	<p>Artificial soil: 70% quartz sand, 20% kaolin clay (no less than 30% kaolin content), 10% sphagnum peat, CaCO₃ added to adjust pH to 6 ± 0.5; this is in line with the OECD 222 (2016) study guideline.</p> <p>Two days prior to addition of test item, soil was pre-moistened to 25% water holding capacity (WHC) with deionised water. Upon application of test item, soil moisture was 50% of WHCmax.</p>
Application of test item	<p>For the application solutions preparation, the required amount of test item was weighed out and then deionised water was added up to a final volume proportional to 25% of the soil WHCmax. Each dilution was thoroughly mixed into the corresponding pre-moistened soil in order to achieve a final moisture content of 50% of the WHCmax. For the control, the adequate proportion of deionised water without test item was mixed into the corresponding premoistened soil.</p> <p>Thereby the desired water content of 50% of the WHCmax was achieved. Immediately after thoroughly mixing the test soil using an electric mixer, approximately 611 g of the test soil (corresponding to 500 g soil dry weight) was placed into the test units.</p>
Replication:	Four replicates per test item group; eight for control group
No. animals/vessel:	Ten worms per vessel. Prior to allocation to test vessels worms were rinsed in water, carefully dried and weighed as a total per replicate.
Duration of test:	56 days

Environmental test conditions

Temperature:	19.7°C to 20.4°C
pH:	Test start: 5.99–6.23; test end: 5.99–6.06
Photoperiod:	16:8 light cycle, intensity: 414.6 to 575.5 lux
Moisture content:	Test start: 26.17 to 26.33% of the dry weight Test end: 39.78 to 44.97% of the dry weight
Dates of work:	30 Jan 2020 to 27 Mar 2020

B. STUDY DESIGN AND METHODS

The objective of the study was to assess the effects of PROBLAD PLUS on mortality, reproduction and weight variation of *Eisenia andrei* in an artificial soil test over 56 days.

Dried and finely ground horse manure was provided on day one of the test and weekly thereafter until day 28 of the test, with any remaining food from the previous occasion being removed. 5 g manure was added to the surface of each replicate and was wetted with deionised water. After removal of adults on day 28 a further 5 g of food per replicate was mixed into the test soil, with no further food added for the remainder of the test.

On day 28 the test units were emptied onto a clean stainless steel tray. The adult test organisms were sorted from the soil and their reaction to a gentle mechanical stimulus at the anterior end was tested. Individuals showing no reaction or missing were counted as dead. Any observed behavioural or pathological symptoms were reported. After the assessment, the artificial soil containing all cocoons that had been produced was returned to their respective test container. On day 56 the total number offspring per test container was counted. Extraction of the juveniles from the soil was performed by heating the containers in water at 40–60°C as recommended in OECD 222 (2016). After approximately 30 min, the juveniles having surfaced were collected and counted.

Statistical analysis

Mortality data (arcsine-transformed) were analysed with the Shapiro-Wilk's test for normality of data distribution and with the Levene's test for homoscedasticity. Multiple Sequentially-rejective Welch-t-test After Bonferroni-Holm ($\alpha = 0.05$, one-sided) was used for hypothesis testing.

Adult body weight variation data were analysed with the Shapiro-Wilk's test for normality of data distribution and with the Levene's test for homoscedasticity. Multiple

Sequentially-rejective U-test After Bonferroni-Holm ($\alpha = 0.05$, two-sided) was used for hypothesis testing.

Reproduction data were analysed with the Shapiro-Wilk's test for normality of data distribution and with the Levene's test for homoscedasticity. Williams' Multiple Sequential t-test procedure ($\alpha = 0.05$, one-sided) was used for hypothesis testing.

The $EC_{10, 20, 50}$ were determined by Probit using maximum likelihood regression, together with their corresponding 95% confidence limits. Additionally, the normalised width (NW) of the confidence limits was calculated for quantifying the reliability of the determined EC_x values. To implement this classification, the EC_x values with $NW < 2$ were considered as acceptable.

All statistical analysis was conducted with ToxRat Professional software version 3.2.1.

II. RESULTS AND DISCUSSION

Sensitivity of the earthworm population was demonstrated in a positive control study (separate study conducted within a year of the current study) with carbendazim, where significant reduction in the number of juveniles was determined at 1.03 mg carbendazim/kg soil dry weight. This result is within the range expected from the OECD guideline 222 (2016) of 1–5 mg carbendazim/kg soil dry weight.

During the 28-day adult mortality and bodyweight observation period no mortalities occurred in the control group at treatments up to and including 125 mg/kg. At 250, 500 and 1000 mg/kg adult mortality was 7.5, 45 and 80%, respectively.

Adult mean bodyweights increased in all treatment and control groups, with a statistically significant increase in bodyweight change seen at 1000 mg/kg. These results are summarised in table 9.4.1-2.

Table 9.4.1-2: Mortality and bodyweight changes of adult earthworms (*Eisenia andrei*) exposed to PROBLAD PLUS

Concentration of PROBLAD PLUS (mg/kg)	Day 28 mortality (%)	Mean adult bodyweight (mg)		Mean % bodyweight increase
		Day 0	Day 28	
Control	0	427.49	622.24	45.55
62.5	0	430.08	642.43	49.40
125	0	424.10	646.50	52.45
250	7.5	423.33	626.97	48.11
500	45 ^a	425.55	524.82	23.35
1000	80 ^a	422.78	821.17	94.21 ^b

^a statistically significantly different compared to the control (Welsh-t-test After Bonferroni-Holm ($\alpha = 0.05$, one-sided)).

^b statistically significantly different compared to the control (U-test After Bonferroni-Holm ($\alpha = 0.05$, two-sided)).

At day 56 the reproductive output of the organisms exposed to the test item was not affected at concentrations up to and including 125 mg test item/kg soil dry weight. Statistically significant differences were found between the control and the test item concentration 250 mg/kg soil dry weight, up to and including 1000 mg/kg soil dry weight. Juvenile number results are summarised in Table 9.4.1-3.

Table 9.4.1-3: Juvenile numbers of earthworms (*Eisenia andrei*) present after 56 days

Concentration of PROBLAD PLUS (mg/kg)	Mean number juveniles per replicate	± Standard Deviation	Coefficient of Variation (%)	% Change in reproduction versus control group*
Control	233.88	22.66	9.69	-
62.5	228.75	35.72	15.62	-2.19
125	222.75	30.63	13.75	-4.76
250	192.75 ^a	22.54	11.70	-17.58 ^b
500	62.50 ^a	29.98	47.97	-73.28 ^b
1000	46.75 ^a	57.02	121.97	-80.01 ^b

^a statistically significantly different compared to the control (Williams Multiple Sequential t-test Procedure ($\alpha = 0.05$, one-sided)).

^b corresponds to a concentration where there was a statistically significant reduction in the mean number of juveniles per replicate compared to the control.

*negative values indicate lower reproduction compared to the control, positive values higher reproduction.

The resulting NOEC based on mortality, bodyweight gain and juvenile production following exposure to PROBLAD PLUS are 250, 500 and 125 mg/kg, respectively. The EC₁₀ for reproductive was determined to be 161.30 mg test item/kg soil dry weight, with a 95% confidence interval of 75.64–224.22 mg test item/kg soil dry weight. The Normalised Width (NW) was calculated to be 0.92 (See Table 9.4.1-4).

The EC₂₀ for reproductive was determined to be 219.83 mg test item/kg soil dry weight, with a 95% confidence interval: 128.18–284.88 mg test item/kg soil dry weight. NW as calculated to be 0.71 (See Table 9.4.1-4).

Table 9.4.1-4: Endpoints for reproductive (56 day) study on *Eisenia andrei*.

Endpoints	[mg test item/kg soil dry weight]	[mg BLAD lead component/kg dw]^a	95% confidence interval normalised width
LOEC mortality	500	106.00	--
NOEC mortality	250	53.00	--
LOEC body weight change	1000	212.00	--
NOEC body weight change	500	106.00	--
LOEC reproduction	250	53.00	--
NOEC reproduction	125	26.50	--
EC₁₀ reproduction (lower / upper 95% confidence limit)	161.30 (75.64 / 224.22)	34.20 (16.04 / 47.53)	0.92
EC₂₀ reproduction (lower / upper 95% confidence limit)	219.83 (128.18 / 284.88)	46.60 (27.17 / 52.76)	0.71
EC₅₀ reproduction (lower / upper 95% confidence limit)	397.49 (313.10 / 505.71)	84.27 (66.38 / 107.22)	0.48

sdw: soil dry weight; ^aConcentrations in terms of lead component are based on content of Blad protein (21.2% w/w) from the certificate of analysis.

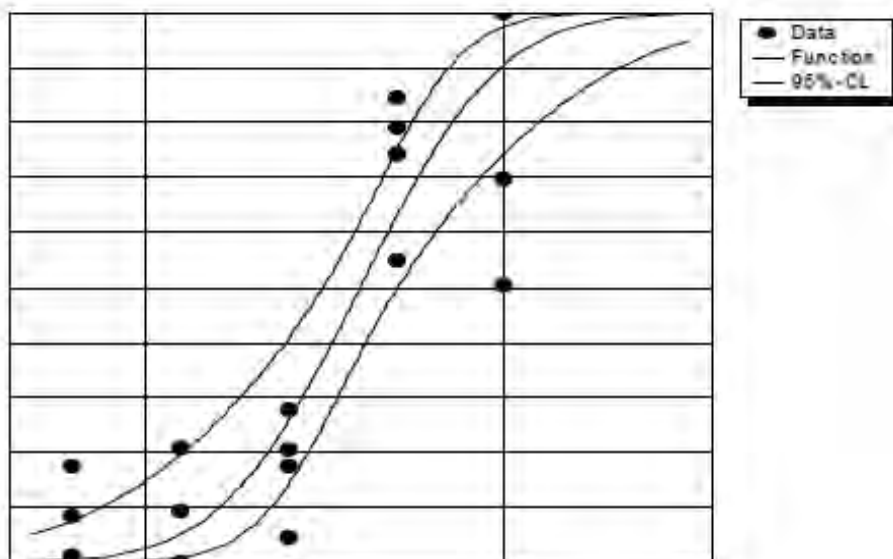


Figure 9.4.1/03-1 Concentration effect curve showing the influence of the test item on the number of offspring of the introduced *E. andrei* as observed after 56 d.

Validity Criteria:

Validity criteria according to OECD 222, 2016 were met:

- Earthworm mortality in the controls to be $\leq 10\%$ at test end (actual: 0.0%)
- Each control replicate to have produced ≥ 30 juveniles (actual: 208–270)
- Coefficient of Variation for control reproduction to be $\leq 30\%$ (actual: 9.69%)

III. CONCLUSION

The results demonstrated the NOEC of PROBLAD PLUS to the earthworm *Eisenia andrei*, to be 125 mg/kg, based on the critical parameter juvenile production. The corresponding EC_{10} and EC_{20} values are 161.3 and 219.8 mg/kg, respectively.

HSE COMMENTS

This study was conducted to GLP. The study was conducted to OECD 222 (2016) and has been assessed to this guideline. HSE has added details to the applicant's original summary report as required from the pdf study report. The test soil contained 10% peat.

There were no major deviations from the study guideline and the validity criteria were met.

The statistical analysis was adequately described and the procedures used were in line with the guideline, however it is noted that the OECD 222 (2016) guideline states that for a study with the aim of calculating both ECx and NOEC, a spacing factor not exceeding 1.8 is recommended, and 8 treatment concentrations should be used, whereas in the current study the spacing factor used is 2.0 and the number of treatment concentrations is 6. Therefore, according to the guideline only a NOEC is reliable to determine from this study. Additionally there is some uncertainty in the EC₁₀ value as the confidence intervals are very large and the upper CI overlaps with the EC₂₀ value. There is therefore uncertainty over the protectiveness of the EC₁₀. Given that the NOEC is the most conservative endpoint, no further consideration is required. The NOEC will be considered as reliable for use in risk assessment.

The agreed endpoints from this study are:

- **NOEC reproduction: 125 mg PROBLAD PLUS/kg soil d.w.**
- **NOEC mortality: 250 mg PROBLAD PLUS/kg soil d.w.**
- **EC₁₀ (reproduction) = 161.3 mg PROBLAD PLUS/kg soil d.w (95% C.I. 75.64–224.22)**
- **EC₂₀ (reproduction) = 219.8 mg PROBLAD PLUS/kg soil d.w (95% C.I. 128.18–284.88)**
- **EC₅₀ (reproduction) = 397.5 mg PROBLAD PLUS/kg soil d.w (95% C.I. 313.1 – 505.7)**

B.9.4.2. Effects on non-target soil meso- and macrofauna (other than earthworms)

No standard laboratory studies have been submitted for the soil macro-organisms *Hypoaspis aculeifer* or *Folsomia candida*. Instead, a case has been presented on the basis of published literature. In accordance with assimilated Regulation No 283/2013, for plant protection products applied as a foliar spray, “if data are available on both *Aphidius rhopalosiphi* and *Typhlodromus pyri* these may be used in an initial risk assessment” for assessing the risks to soil organisms other than earthworms. Given that an acceptable risk to both *A. rhopalosiphi* and *T. pyri* was demonstrated using standard Tier I glass plate studies, it is considered that no data is required on the soil macro-organisms *F. candida* and *H. aculeifer*. In addition, a study from published literature (K-CP 10.4.2/02) has been submitted and has been summarised and evaluated below, with the inclusion of HSE comments on the relevance and reliability.

Reference:	K-CP 10.4.2/02
Report Title:	Soil biota and crop residue decomposition during summer and autumn in south-western Australia
Author(s) & Year:	van Vliet, P.C.J., Gupta, V.V.S.R., and Abbott, L.K. (2000)
Document No	Applied Soil Ecology 14 (2000) 111-124
Substance used:	Narrow-leafed lupin (<i>Lupinus angustifolus</i> Myallie) standing dead residues from field crop, processed by cutting up into 5 cm pieces and oven drying, consisting of small sticks and stem.
Method of analysis:	No analysis conducted
Guideline(s):	No, study from published literature
Deviations:	N/A, study from published literature
GLP or GEP:	No, study from published literature
Acceptability:	Supplementary
Study relied upon:	Yes, however data protection not applicable to published literature studies

I. MATERIALS AND METHODS

A. MATERIALS

Test item	Paddock 1	Paddock 2
Litterbag:	16 x 16 cm fibreglass-nylon bags with a 1.0 x 1.5 mm mesh	
Litterbag contents:	10 g narrow-leaved lupin (Lupinus angustifolus Myallie) residues	5 g wheat (Triticum aestivum Spear) residues
Test site	East Beverly, Western Australia	
Soil properties:	95–98% sand ca. 1% clay	93–96% sand 1–2.5% clay
Test duration:	Six months	
Test start date	Litter bags were put in place on 23 and 24 December 1996 (Summer)	
Test conditions		
Soil temperature:	During the first 50 days: 23°C to 42°C	
Rainfall:	No rain in the first 50 days of the study First major event: 22.2 mm in six days towards the end of February Second major event: 86.2 mm in two days towards the end of March	

B. STUDY DESIGN AND METHODS

This study was conducted to determine the impact of the presence of lupin and wheat residues on decomposer fauna, and to assess the decomposition rate of these residues during summer and autumn in paddocks previously cropped with either wheat or lupin. Populations of various groups of decomposer soil biota and nitrogen dynamics (immobilisation and mineralisation) were measured using litterbags.

Two paddocks at East Beverly in Western Australia on a duplex soil (Alfisol) with a yellow-grey clayey subsoil, located next to each other were selected for this

experiment. Both paddocks were managed in a wheat-lupin rotation. Soil from Paddock 1, with lupin residues, consisted of 95 to 98% sand and ca. 1% clay, and soil from Paddock 2, with wheat residues, consisted of 93 to 96% sand and 1 to 2.5% clay.

On 29 April 1997, four months into the study, lupin was seeded directly in Paddock 2, and wheat was direct-drilled in Paddock 1 on 19 May 1997. Annual rainfall in East Beverly is about 420 mm, of which 340 mm is received during the growing season (May to October). Average yearly minimum and maximum temperatures were 10 and 24°C, respectively. The highest and lowest soil temperatures in the period of January to July 1997 were 43°C (January) and 8°C (June). A list of chemicals sprayed onto the two paddocks is presented in the table below.

Table 9.4.2-1: Dates and application rates of chemicals in the lupin and wheat paddocks

Date	Paddock 1 (lupin 1996, wheat 1997)		Paddock 2 (wheat 1996, lupin 1997)	
	Chemical	Rate (/ha)	Chemical	Rate (/ha)
24/04/1997	Sprayseed®	1000 mL		
24/04/1997	Simazine	1100 g		
24/04/1997	Atrazine	300 g		
01/05/1997			Roundup®	900 mL
01/05/1997			Diuron	300 mL
13/05/1997			Sprayseed®	1300 mL
13/05/1997			Logran®	25 g
17/06/1997	Sertin®	150mL		
17/06/1997	Simazine	400g		

Standing dead residues of narrow-leaved lupin (*Lupinus angustifolius* Myallie) were collected from Paddock 1 on December 13, 1996. Wheat residues (*Triticum aestivum* var. Spear) were collected from Paddock 2 on December 17, 1996. Both crop residues were cut into 5 cm pieces and dried in an oven at 60°C. Litterbags (16 x 16 cm) were prepared from fibreglass-nylon material with a 1.0 x 1.5 mm mesh. Lupin bags were filled with 10 g oven dried material, consisting of small sticks and one thicker part of the stem. Wheat bags were filled with 5 g oven-dried material. The carbon percentages of the lupin and wheat residues at the start of the experiment were 47.7 and 47.9%, respectively. The nitrogen percentage of the lupin residues was 3.5 times higher than that of the wheat residues (0.7% to 0.2%).

Each paddock was divided into four blocks to which x and y coordinates were assigned. The bags were randomly assigned to points in the Paddocks (Litterbags

with lupin residues were placed on soil after a lupin crop while litterbags with wheat residues were placed on soil that had grown wheat in the previous growing season.) On 23 and 24 December 1996, 208 bags were placed on the soil surface in each paddock. Bags were kept in place with a spike (20 cm long), with a metal frame protecting the bag from sheep and parrots.

Ten lupin and ten wheat bags were collected immediately after all the bags were placed in the field to determine any mass loss during transport and handling of the bags.

During the period January to June 1997, four litterbags were retrieved from each block in each paddock at regular intervals. One bag was used for the extraction of nematodes and one for the extraction of microarthropods. Litterbags from which microarthropods had been extracted were later used for the determination of litter mass loss and carbon and nitrogen content. The residues from the two other bags collected at each sampling time were cut into small pieces and combined; subsamples were taken for moisture, protozoa abundance and microbial biomass. On the first two sampling dates, only litter moisture, mass loss and percent carbon and nitrogen were determined. Substrate-induced respiration (SIR) measurements did not commence until the beginning of March.

Microbial biomass carbon was measured using a modified method of the SIR technique. The collected residues were chopped finely using scissors and weighed into six Erlenmeyer flasks (0.5 g residue/flask). A small amount of deionised water was added to moisten the residue pieces, after which the flasks were sealed and stored overnight at 5°C. The next morning, the flasks were left to equilibrate to room temperature for about 30 min. To half of the flasks, a sufficient volume of glucose solution (final concentration 20 mg glucose/g residue) was added to sustain the moist environment, to the other half deionised water was added in the same amount. Flasks were left to equilibrate on the bench for one hour, then sealed. After an incubation period of four hours at 20°C, the generated CO₂ was measured using an infrared gas analyser (Series 225 Gas Analyser). Microbial biomass C was estimated from the measured respiration rate using the formula: mg microbial C = 22.1 × $\mu\text{L CO}_2/\text{g/h}$, a formula especially developed for the conversion of SIR to microbial biomass carbon in Australian soils.

Protozoa were enumerated using the most probable number (MPN) method, with a five-fold dilution series in phosphate buffer and *Rhizobium* sp. as a food source. To 1 g of litter (wet weight), 20 mL of phosphate buffer was added. This mixture was shaken for 30 min, after which five five-fold dilutions were pipetted into 24 well plates and incubated at 20°C. Flagellates and ciliates were scored after 3 days of incubation, amoebae after 8 days. The numbers of ciliates, flagellates and amoebae

originally present in a gram of dry litter were estimated from the MPN data using the program MPNES v1.0.

Nematodes were extracted from litterbags using the Whitehead extraction technique. After 48h, the extracted nematodes were fixed with a mixture of hot and cold 5% formalin. The complete sample was counted using a compound microscope. For one sampling date in each month, 100 to 200 randomly selected nematodes were classified into four different functional groups: bacterivores, fungivores, omnivores/predators, and phytophages using oesophageal morphology.

Microarthropods were extracted using modified Tullgren extractors into a mixture of 70% ethanol and 5% glycerol. All animals were counted and classified into five groups:

Prostigmata, Oribatida (Cryptostigmata), Mesostigmata, Astigmata, and Collembola.

To determine mass loss, contents of the microarthropod-extracted litterbags were oven-dried at 60°C for 48h and the resulting mass recorded. To determine carbon and nitrogen content, residues were ground into a powder, dried at 60°C and analysed for total carbon and nitrogen using a combustion analyser. Sub-samples of each litterbag were ashed at 500°C for four hours to determine the sand content of the lupin and wheat residues. All carbon, nitrogen, and mass loss data were corrected for the presence of sand. Daily decay constants were calculated from the soil-corrected mass data using the single negative exponential decay function.

All data presented as percentages were arcsine transformed for statistical analysis. All soil biota data (including microbial biomass carbon) were log-transformed before analysis. Effects of residue type and sampling date were determined with the General Linear Model using SAS. Tukey's studentized range test (HSD) was used to determine significant differences between main effects. Comparisons by date were performed using t-tests.

II. RESULTS AND DISCUSSION

No rain was recorded during the first 50 days of the experiment. During this time, surface soil temperatures ranged from 23°C to 42°C. The first major rainfall, 22.2 mm in six days, occurred at the end of February, just before the fourth sampling date. The second major rainfall (86.2 mm in 2 days) occurred at the end of March. At the end of April (day 120) enough rain had fallen for seeding to commence. These climatic conditions are typical of a Mediterranean climate, with dry summers in which sporadic rainfall occurs. Moisture levels in the residue samples did not increase until more frequent rainfall events occurred in May and June.

Both lupin and wheat litterbags retained about 80 to 85% of their initial litter mass after 6 months exposure in the field. A significant mass loss of 15 to 20% of wheat and lupin residues occurred during the period between days 55 and 188. Because no significant mass loss occurred until after day 55, decomposition rates were calculated for the period between day 55 and 188. Decomposition rates for the lupin and wheat residues were different (0.0013 and 0.0011/day for lupin and wheat, respectively; $p < 0.005$). No mass loss occurred during the first 55 days of the experiment, due to the absence of rainfall and the high temperatures resulting in low residue moisture. The largest drop in residue mass (10%) occurred after the first major rainfall (22.2 mm). Successive rainfall events did not correspond to a similar decrease in mass. Between days 60 and 130 (March to the beginning of May), mass loss of both types of residue was more gradual; this coincided with large populations of microfauna. Mass losses of the residues were minimal during the period between 126 and 188 days when large populations of mesofauna were observed.

Lupin and wheat residues were not different in carbon concentration at the start of the experiment (47.7 and 47.9%, respectively). However, due to a difference in nitrogen concentration in the two residues, the C/N ratio of the wheat residue (211.4) was much higher than the C/N ratio of the lupin residue (66.7) at that time. Loss of carbon occurred from both residues during the 6-month period; a similar pattern as for mass-loss occurred for both residue types

An initial immobilisation of nitrogen was observed in both residue types. A decline in the amount of nitrogen in the residues occurred following the rainfall event at the end of February. However, no correlation was found between the amount of rainfall and the percentage of nitrogen lost from either type of residue during the whole sampling period. After the first major rainfall, nitrogen immobilisation continued, especially in association with the wheat residues. Significant losses of nitrogen (up to 46%) occurred from the lupin residues after the middle of March. At the end of the sampling period (June) the nitrogen concentration in the wheat residues was similar to that at the start of the sampling period.

The level of microbial biomass was greater on the lupin residues than on the wheat residues at the beginning of the study (March to April). Microbial biomass carbon on the wheat residues slowly increased over time until a maximum of 220 mg microbial biomass C/g residue was reached (comparing March to June: $p < 0.0001$). In June, microbial biomass levels on the lupin and wheat residues were similar.

Numbers of protozoa (flagellates, amoebae, and ciliates) were higher on the lupin residues at the first sampling date in March than on the wheat residues. Densities of ciliates and amoebae remained high during the entire sampling period. During the summer, abundances of amoebae and ciliates remained lower on the wheat than on the lupin residues (85 and 67% lower, respectively).

A sharp increase in nematode abundance occurred at the beginning of April on the lupin residues. Total numbers of free-living nematodes on the wheat residues were significantly lower than on the lupin residues ($p < 0.001$). Bacterial-feeding nematodes were the most abundant group on both the lupin and the wheat residues at all sampling dates.

Mites were not observed until the beginning of April. Microarthropods increased in number in May and their community structures associated with the lupin and wheat residues were different. Prostigmatic mites were the first mites to colonize both lupin and wheat residues. Numbers of Prostigmata on the wheat residues were significantly higher at the end of May and at the beginning of June than on the lupin residues. During this period more astigmatic mites and Collembola were present on the lupin than on the wheat residues. In addition, the number of mesostigmatic mites on the lupin residues during this period was greater than on the wheat residues. The abundance of microarthropods decreased at the end of June, corresponding with a period of low rainfall.

Colonization of lupin residues by microorganisms (estimated from levels of substrate-induced respiration) was more rapid than the colonisation of the wheat residues. Communities of microorganisms were larger on the lupin residues, especially at the beginning of the study.

Earlier colonisation of the lupin residues by microorganisms promoted earlier colonisation by predators such as protozoa and nematodes. Greater nematode abundance on the lupin than on the wheat residues could be attributed to greater abundance of microorganisms at the beginning of the study. The increase in nematode abundance might have prevented a further increase in the microbial biomass on the lupin residues.

III. CONCLUSION

During the summer period in Western Australia decomposition rates were low for lupin and wheat residues, with mass losses being only about 15–20%. Rainfall events had a large influence on the decomposition of both types of residue, causing leaching of nitrogen. About 30% of the initial nitrogen content was mineralized from the lupin residues in the first 5 months. The concentration of nitrogen in the wheat residues remained similar all through the summer; mineralization and immobilization occurred at similar rates.

Soil fauna colonized the lupin earlier than the wheat residues and in greater numbers. Food quality and predatory pressures may have affected the succession of different soil biota communities on the lupin and wheat residues. Different microarthropod communities were found on the residues at the end of May and at the

beginning of June. Prostigmatic mites were found in high numbers on the wheat, while Collembola were the most abundant microarthropods on the lupin residues.

HSE COMMENTS

This study was submitted as evidence that lupin residues do not demonstrate detrimental effects on soil biota or functioning.

This study is from the scientific literature and is a litterbag study with the purpose of investigating the impact of lupin and wheat residues on decomposer fauna and decomposition rate, including nitrogen dynamics. Given the purpose of this study, it does not conform to a standard ecotoxicological test guideline, nor is it GLP compliant. Therefore, the study's methodology, reporting and analysis have been considered with regard to how well they meet common standards of scientific practice, based on 'expert judgement'.

The methodology, study design and statistical analysis are well detailed and are in accordance with general scientific principles. Soil details and climatic data were provided. The study was carried out in south-western Australia and the authors note that in the early stages of the study the high temperature and low moisture was "typical of a Mediterranean climate". The location of this study is of limited relevance to UK climate as it was too dry (50 days no rain at start of the study) and the maximum soil temperature of 42°C is too high to be relevant to the UK. Commercial chemical treatments were applied to the sites after four months and details of this were provided. The chemicals and timings differed between the wheat and lupin paddocks. The tested lupin species is *Lupinus angustifolius* var. Myallie which is a different species to the *L. albus* that is being evaluated in the current dossier.

The data from the lupin and wheat litterbags were compared to each other. The results demonstrate that soil decomposition and colonisation by soil biota (protozoa, nematodes, microbial biomass, mites and Collembola) was able to occur on both the lupin residues and wheat residues. During lupin degradation, the mineralisation and immobilisation of nitrogen was not equal and more nitrogen was lost from lupin residues than wheat. However it is noted that nitrogen percentage of lupin residues was 3.5 times higher than wheat with higher C/N ratio.

Differences in biota community structure between lupin and wheat residues were expected due to the different food quality. Additionally, the authors suggest that this and predatory pressures "may have affected the succession of different soil biota communities".

Overall, the successful colonisation and decomposition of the lupin residues over 6 months suggests no negative impacts on the microbial and micro-arthropod soil community from the lupin residues.

The results may be considered as supporting evidence in the risk assessment but may be of limited relevance, since relating the amount of lupin residues per litterbag (10 g oven dried material, consisting of small sticks and one thicker part of the stem) to the amount of active substance under assessment, which is an aqueous extract of lupin seeds, might not be possible, so it may be difficult to relate the exposure from this study to the predicted exposure from the proposed use. Furthermore, the temperature and precipitation in study is not representative of typical UK climatic conditions.

Reliability: Reliable with reservations.

(Studies are graded as one of the following categories: 1) Reliable, 2) Reliable with reservations, 3) Unreliable. Categorisation is based on the evaluator's subjective assessment of the quality of the methods, reporting and analysis as presented in the study.)

B.9.5. Effects on soil nitrogen transformation

In addition to the standard laboratory study (K-CA 10.5/01), several published literature studies have been provided to support the assessment of risks to soil micro-organisms. These studies are summarised below with the inclusion of HSE comments on their relevance and reliability.

Reference:	K-CA 10.5/01
Report Title:	PROBLAD- Assessment of the Side Effects on the Activity of the Soil Microflora.
Author(s) & Year:	Ganssmann, M. (2010b)
Document No	CEV SA, Unpublished report No.: S10-02559
Substance used:	PROBLAD PLUS (Batch: 201009, 20% BLAD, 1.30 g/cm ³)
Method of analysis:	No analysis conducted or required
Guideline(s):	OECD guidelines 216, Soil Microorganisms : Nitrogen Transformation Test (2000)
Deviations:	No
GLP or GEP:	Yes

Acceptability:	Yes
Study relied upon:	Yes

I. MATERIALS AND METHODS

A. MATERIALS

Test Material PROBLAD PLUS

Lot/Batch #: Batch no. 201009

Actual content of active ingredients: BLAD content 20.0% w/w

Treatments

Test rates: 5.2 mg PROBLAD PLUS/kg dry soil and 52 mg PROBLAD PLUS/kg dry soil

Control: Untreated soil

Toxic standard: Dinoterb, tested once per year in a separate study at rate of 13.3 mg a.s./kg soil dry weight in June/July 2009.

Test design

Soil type: Agricultural sandy loam soil, sampled from a depth of 0-20 cm from a known location in Rhineland-Palatinate, Germany. Organic carbon content: 0.913% ; microbial biomass of total organic carbon: 2.37%; soil texture: sandy loam; 64.3% sand. Sample site had not been cultivated since 2006 and no plant protection products had been applied in previous five years. No organic fertiliser applied in past six months and no mineral fertiliser in past four years.

Soil storage and handling: Soil was sieved to a particle size of 2 mm and stored for approx. 6 weeks at $4 \pm 2^{\circ}\text{C}$. Soil was then conditioned prior to test start for four days at $20 \pm 2^{\circ}\text{C}$. Soil was amended with finely ground lucerne meal (C/N ratio: 15:1).

Test units: Glass bottles, closed loosely with screw caps.

Replication: Three replicates per treatment and control.

Sampling intervals: 0, 7, 14 and 28 days after application

Duration of test: 28 days

Environmental test conditions

Temperature: 19.0–21.5°C

pH of soil: 6.23 at test initiation

Soil moisture content: Test initiation: 42.0–44.9% of maximum water holding capacity

Test termination: 41.6–46.5% of maximum water holding capacity

Readjusted once per week according to weight of test vessels.

Photoperiod: Constant darkness

B. STUDY DESIGN AND METHODS

The objective of the study was the assessment of the effect of PROBLAD PLUS on nitrogen turnover. Two test concentrations were used, 5.2 mg PROBLAD PLUS/kg soil dry weight and 52 mg PROBLAD PLUS/kg soil dry weight and compared with soil nitrogen turnover in control samples. Nitrogen turnover was determined based on changes in the content of nitrate in the test soil over 28 days.

Test solutions were prepared by dissolving the necessary amount of test item in deionised water and were homogenised by shaking. The test item was applied to the soil, thoroughly mixed, added to glass bottles and then incubated at $20 \pm 2^\circ\text{C}$ in the dark. Three replicates for each concentrations and the control were used. In order to stimulate nitrogen transformation, the soil was amended with lucerne meal (0.5% of soil dry weight). Water content was adjusted to 45% WHC_{max}. The temperature was 19.0 to 21.5°C and pH 6.3. The soil nitrogen content was determined on 0, 7, 14 and 28 days after application of the test item. Soil nitrification was determined by measuring NO₃⁻ contents of aqueous soil extracts by ion sensitive electrode and the Orion expandable Ionanalyser. The concentrations of NO₃-N in the soil were

calculated from the measured values. The rate of nitrogen formation and percent deviation from the control was calculated for each time interval and treatment.

The results of the nitrification were tested for normal–y using Shapiro-Wilk's test and residual analysis (Zar, 1999). Dunnett's t-Test was used to analyse the data for significance. SAS version 9.2 service pack 4 (Ed. 2002-2008) was used for the statistical analysis.

II. RESULTS AND DISCUSSION

Results from the nitrogen transformation test are summarised in the tables below.

Table 9.5-1: Mean nitrate concentration after treatment with the test item

Time Interval (days)	Control NO₃-N [mg/kg soil d.w.]	5.2 mg/kg dry soil NO₃-N [mg/kg soil d.w.]	52 mg/kg dry soil NO₃-N [mg/kg soil d.w.]
0	10.77	10.87	10.87
7	12.90	12.03	12.67
14	26.20	22.43	23.60
28	37.30	34.17*	34.10*

* Statistically significantly different compared to the control (Dunnett's t-test, two sided, $p < 0.05$).

Table 9.5-2: Effects on nitrate formation rate after treatment with the test item:

Time Interval (days)	Control	5.2 mg test item/kg soil dry weight		52 mg test item/kg soil dry weight	
	NO ₃ -N [mg/kg soil d.w./day]	NO ₃ -N [mg/kg soil d.w./day]	Deviation from control [%]	NO ₃ -N [mg/kg soil d.w./day]	Deviation from control [%] ¹⁾
0 – 7	0.305	0.167	-45.31	0.257	-15.63
7 – 14	1.90	1.49	-21.80	1.56	-17.79
14- 28	0.79	0.84	+5.71	0.75	-5.41

+ = stimulation; - = inhibition

Reference test:

The reference item dinoterb resulted in statistically significant effects on nitrate formation after 28 days. For the 14–28 day period, the deviation from the control in the 13.3 mg a.s./kg dry soil treatment was + 178%. This demonstrates the sensitivity of the test system.

Validity Criteria:

The validity criteria outlined in OECD 216 were met:

Criterion	Required	Observed
Coefficient of variation in the control	Must be ≤ 15%	Nitrogen transformation test: max 4.5%

III. CONCLUSIONS

Based on the results of the study and OECD guideline 216 and 217, PROBLAD PLUS had no adverse effect on nitrogen turnover as there was < 25% deviation between treatments and control over 28 days.

HSE COMMENTS

This study has been conducted in accordance with GLP and follows OECD 216 (2000) guidelines. The validity criteria have been met.

The guideline recommends use of 0.1 M KCl for nitrate extraction, while in this study aluminium-potassium-sulphate has been used. However, the use of KCl is only a

recommendation and the guideline does indicate that other suitable extraction solvents may be used. Therefore, the use of an alternative extraction solvent may be accepted.

At the lower test rate there was a reduction in nitrogen transformation of 45.3% compared to the control in the 0–7d time period. However, no deviations > 25% were observed in the 14–28d time period at either test rate.

The endpoint suitable for us in risk assessment is:

- No effect on soil nitrogen transformation (< 25% deviation from control) at concentrations up to 52 mg PROBLAD PLUS/kg dry soil.

Reference :	K-CA 8.5/02
Report Title :	Microbial activity and biomass and N and P availability in a saline sandy loam amended with inorganic N and lupin residues.
Author(s) & Year :	Elgharably, A. and Marschner, P. (2011)
Document No	European Journal of Soil Biology 47 310 – 315.
Substance used :	Residues of lupin <i>Lupinus albus</i> L.
Method of analysis :	No analysis conducted
Guideline(s) :	No, study from published literature
Deviations :	N/A, study from published literature
GLP or GEP :	No, study from published literature
Acceptability :	Supplementary
Study relied upon :	Yes, however data protection not applicable to published literature studies

I. MATERIALS AND METHODS

A. MATERIALS

Test Soil

Source:	Monarto, South Australia (35°05'S, 139°06'E, 166 m)
Characteristics:	75% sand, 5% silt, and 20% clay
pH:	7.0 (prior to addition of saline solutions and lupin residues)
pHsoil:water suspension:	6.05–6.29 (with saline solution, prior to addition of nitrogen and residues)
Carbon:	Total C : 0.7%, microbial C : 223.1 µg/g soil
Water holding capacity (WHC):	19.8%

Treatments

Salinity:	S1: Control (untreated soil) S2: 117 mg NaCl, 588 mg CaCl ₂ .2H ₂ O/kg soil S3: 170 mg NaCl, 1255 mg CaCl ₂ .2H ₂ O/kg soil
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Test design

Test vessel:	Small containers with mesh at the bottom, placed into Mason jars containing a small container with 8 mL de-ionised water.
Temperature:	28 ± 3°C
Replicates:	Four replicates
Test duration:	45 days

B. STUDY DESIGN AND METHODS

This study was conducted in order to investigate the effects of NH₄-N and NO₃-N on microbial activity and biomass and N and P availability in a saline sandy loam.

Soil salination:

Several sub-samples of test soil (0 to 15 cm in depth) were bulked to give a composite sample. Soils were air-dried and sieved to ≤ 2 mm.

Three levels of salinity, denoted S1, S2, and S3 were induced in soil by addition of 0, 117, and 170 mg NaCl and 0, 588, and 1255 mg CaCl₂.2H₂O/kg soil by level, respectively. Salt solutions were thoroughly mixed with the soil using a cement mixer.

Soils were incubated in the dark at 25°C at 50% of the soil WHC to allow the microbial activity to equilibrate after re-wetting of air-dried soil. After incubation, soil pH (1:5 soil:water suspension) was 6.29, 6.11, and 6.05.

Nitrogen and residue addition:

After incubation, solutions of KNO₃ or (NH₄)₂SO₄ were added at 50 µg N/g soil to the saline sandy loam. A control treatment without N was also included.

Residues of lupin (*Lupinus albus* L.), ground to ≤ 1.5 mm were added to soil at 2% (w/w). A control soil without added residues was also included. Lupin residues contained 40.6% total C, 2.64% total N, and 0.11% total P, with a C/N ratio of 15.4 and a C/P ratio of 369. Residues contained 10.8, 4.0, and 75.3% total lipids, lignin, and cellulose, respectively. Test vessels were small containers with a mesh at the bottom for continuous measurement of soil respiration. To each of these were added 20 g dry weight equivalent soil. Also, 250 g dry weight equivalent soil with and without lupin residues were filled into pots for destructive sampling and determination of nutrients and microbial biomass. Throughout the experiment, the moisture of the soils in the pots and the small containers was maintained at 65% of WHC by adjusting the container weight with de-ionised water regularly.

Measurement of soil respiration:

The small containers were placed individually in Mason jars containing a small container with 8 mL de-ionised water. Jars were closed with an air-tight lid with a septum and incubated in the dark at $28 \pm 3^\circ\text{C}$. Released $\text{CO}_2\text{-C}$ was measured using a gas analyser by withdrawing air from the headspace with a needle through the lid's septum. After each measurement, the jars were opened to replace the CO_2 -enriched air inside the jars with fresh air. The baseline of $\text{CO}_2\text{-C}$ concentration was measured over 45 days and cumulative respiration is expressed as $\mu\text{g CO}_2\text{-C/g soil}$.

Measurement of microbial biomass and N and P availability :

In the uncovered pots, soil sub-samples were taken on days 15, 30 and 45 for determination of microbial biomass C, N and P and available P, and on days 20 and 40 for the measurement of available N (NH_4^+ and NO_3^-).

Statistical analysis:

The experiment was a completely randomised design with four replicates per condition. Two- and three-way analyses of variance and Tukey test were carried out using GenStat for Windows 8.0.

II. RESULTS AND DISCUSSION

Soil respiration

In the unamended soil with no salt or lupin residues, the respiration rate was low with an average of 0.65 $\mu\text{g CO}_2\text{-C/g soil/h}$ over the 45 days of incubation. With lupin residues incorporated, soil respiration was maximal with approximately 8 $\mu\text{g CO}_2\text{-C/g soil/h}$ on day 3. With no residue added, addition of inorganic N had no significant effect on soil respiration. Addition of inorganic N with residue significantly increased soil respiration with greater rates with $\text{NO}_3\text{-N}$ compared to $\text{NH}_4\text{-N}$.

Salinity significantly decreased soil respiration with or without amendments. Compared to S1, soil respiration was lower by approximately 46 and 65% with no amendments, 26 and 45% with lupin residues only, 20 and 33% with lupin and $\text{NH}_4\text{-N}$, and 15 and 23% with lupin residues and $\text{NO}_3\text{-N}$ in S2 and S3, respectively. Under all treatments, soil respiration decreased over time.

Cumulative respiration

Compared to unamended soil, with lupin residues incorporated cumulative respiration was three-fold at S1 and 2.5-fold higher at S3. Compared to soil with lupin residue, cumulative respiration was approximately 12% higher with addition of lupin residues and $\text{NH}_4\text{-N}$, and 22% higher with addition of lupin residues and $\text{NO}_3\text{-N}$. Relative to S1 in lupin residue-amended soil, cumulative respiration was 25 and 47% lower in S2 and S3, respectively. Salinity decreased cumulative respiration even with addition of lupin residues and inorganic N. Relative to S1, cumulative respiration was lower by approximately 20 and 36% with lupin residues and $\text{NH}_4\text{-N}$, and 13 and 28% with lupin residues and $\text{NO}_3\text{-N}$ in S2 and S3, respectively.

In soil with no Na^+ salt added, available P was less than that with NaCl. With no amendments (salinity, residue, inorganic N) the concentration of available N and P remained unchanged in 45 days, but with lupin residues added, P availability was two to three times higher and N availability was four to six times higher than that in unamended soil even under saline conditions.

Soil salinity had no direct effect on N availability. Residue and inorganic N addition increased N availability with significant differences between salinity treatments. In the unamended control soil, the concentration of available NH_4^+ was lower on day 40 than on day 20, however with addition of $\text{NH}_4\text{-N}$ the concentration of available NO_3^- did not change from day 20 to day 40, and microbial biomass N was lower with $\text{NH}_4\text{-N}$ than with $\text{NO}_3\text{-N}$.

Microbial biomass C, N, and P increased in the first 15 days with residue addition. This resulted in increased soil respiration, indicating that lupin residues enhanced

microbial activity in the sandy loam. Nitrifying bacteria were not completely inhibited even at EC_e 11.8 dS/m. However, soil respiration and microbial biomass C, N, and P decreased with increasing salinity.

In the sandy loam, with increasing salinity the reduction in rate of cumulative respiration and microbial biomass C, N, and P were far higher with no residue added than in the presence of lupin residues. This indicates that the salt tolerance of microorganisms was improved with residue addition. Increased microbial activity could have increased decomposition of lupin residues and, in addition to microbial turnover, resulted in release of N and P with a lower availability in soil with Na^+ and Ca^{2+} salts.

In soil amended with lupin residues, the higher soil respiration and microbial biomass with addition of inorganic N indicates that mineralised N from residues was inadequate. Furthermore, in presence of lupin residues with NH_4-N or NO_3-N , the concentration of available NH_4^+ was the same, and the NH_4/NO_3 ratio was lower with NO_3-N than with NH_4-N , suggesting a higher activity of the nitrifying bacteria with a constant rate of ammonification. Compared to NH_4-N , addition of NO_3-N had a stronger effect on microbial biomass N and P under saline conditions.

Addition of inorganic N further increased N availability greatly in soil with lupin residues but N availability did not change in the saline sandy loam from day 20 to day 40. Added N was in the soluble form and the higher cumulative respiration and microbial biomass in NO_3-N compared to NH_4-N treated soil resulted in greater microbial turnover and this was reflected on N and P availability.

Table 9.5-3: Cumulative respiration of soil over 45 days and available $\text{NH}_4^+\text{-N}$ and $\text{NO}_3\text{-N}$ on days 20 and 40 in sandy loam soil with or without 2% (w/w) lupin residues and 50 $\mu\text{g N/g}$ soil added.

Nitrogen	Residue	45 days			Day 20					
		Cumulative respiration (µg CO ₂ -C/g soil)			NH ₄ + -N (µg/g soil)			NO ₃ -N (µg/g soil)		
		S1	S2	S3	S1	S2	S3	S1	S2	S3
Control	Control	643 _a	591 _a	545 _a	17 _a	16 _a	16 _a	11 _a	10 _a	11 _a
NH ₄ -N		666 _a	611 _a	562 _a	32 _b	33 _b	31 _b	11 _a	9 _a	9 _a
NO ₃ —N		658 _a	584 _a	549 _a	19 _a	18 _a	20 _a	34 _b	30 _b	30 _b
Control	2% lupin	1911 _b	1429 _b	1005 _b	91 _c	75 _c	60 _c	35 _c	28 _c	21 _c
NH ₄ -N		2537 _c	2018 _c	1599 _c	105 _d	88 _d	75 _d	32 _c	24 _c	18 _c
NO ₃ —N		3031 _d	2629 _d	2175 _d	107 _d	89 _d	79 _d	66 _d	55 _d	47 _d
		Day 40								
		NH ₄ + -N (µg/g soil)			NO ₃ —N (µg/g soil)					
		S1	S2	S3	S1	S2	S3			
Control	Control	4 _a	5 _a	5 _a	9 _a	9 _a	8 _a			
NH ₄ -N		26 _b	20 _b	22 _b	10 _a	11 _a	12 _a			
NO ₃ —N		16 _a	13 _a	15 _c	52 _b	51 _b	47 _b			
Control	2% lupin	74 _c	60 _c	49 _d	47 _b	35 _c	26 _c			
NH ₄ -N		137 _d	87 _d	78 _e	43 _b	38 _c	29 _c			
NO ₃ —N		132 _d	84 _d	75 _e	75 _c	63 _d	57 _d			

Values are means of four replicates.

S1, S2 and S3 correspond to $\text{EC}_{1:5}$ 0.21, 0.51 and 0.85 dS/m, respectively.

Values in the same column followed by the same letter are not significantly different at $p < 0.05$ (ANOVA)

Table 9.5-4: Microbial biomass C, N and P and available P in the sandy loam soil with or without lupin residues of 50 µg N/g soil added.

Nitrogen	Residue	Day 15			Day 30			Day 45		
		Microbial biomass C (µg/g soil)								
		S1	S2	S3	S1	S2	S3	S1	S2	S3
Control	Control	295 _a	252 _a	215 _a	250 _a	215 _a	171 _a	184 _a	150 _a	121 _a
NH ₄ -N		311 _a	268 _a	204 _a	261 _a	227 _a	182 _a	195 _a	171 _a	125 _a
NO ₃ —N		383 _b	316 _b	271 _b	315 _b	261 _b	170 _a	261 _b	172 _a	111 _a
Control	2% lupin	651 _c	624 _c	592 _c	361 _c	311 _c	256 _b	144 _c	129 _a	117 _a
NH ₄ -N		764 _d	712 _d	659 _d	599 _d	534 _d	475 _c	445 _d	401 _b	351 _b
NO ₃ —N		850 _e	809 _e	770 _e	663 _e	611 _e	570 _d	462 _d	405 _b	362 _b
		Microbial biomass N (µg/g soil)								
		S1	S2	S3	S1	S2	S3	S1	S2	S3
Control	Control	27 _a	22 _a	18 _a	17 _a	12 _a	10 _a	11 _a	9 _a	4 _a
NH ₄ -N		30 _a	23 _a	20 _a	22 _a	17 _a	13 _a	14 _a	9 _a	7 _a
NO ₃ —N		41 _b	35 _b	30 _b	29 _b	24 _b	19 _b	16 _a	13 _a	8 _a
Control	2% lupin	98 _c	87 _c	74 _c	85 _c	74 _c	65 _c	75 _b	68 _b	59 _b
NH ₄ -N		115 _d	93 _c	78 _c	98 _d	79 _c	65 _c	86 _c	71 _b	62 _b
NO ₃ —N		128 _e	110 _d	95 _d	110 _e	91 _d	80 _d	91 _c	79 _c	70 _c
		Microbial biomass P (µg/g soil)								
		S1	S2	S3	S1	S2	S3	S1	S2	S3
Control	Control	3.0 _a	2.7 _a	2.5 _a	2.6 _a	2.2 _a	1.5 _a	2.0 _a	1.5 _a	0.9 _a
NH ₄ -N		3.1 _a	2.8 _a	2.4 _a	2.6 _a	2.3 _a	1.7 _a	2.2 _a	1.8 _b	1.0 _a
NO ₃ —N		4.5 _b	4.0 _b	3.5 _b	4.1 _b	3.7 _b	3.2 _b	3.6 _b	3.2 _c	2.7 _b
Control	2% lupin	15.1 _c	13.4 _c	11.0 _c	11.5 _c	19.1 _c	18.2 _c	8.3 _c	7.1 _d	6.5 _c
NH ₄ -N		19.0 _d	16.2 _c	14.9 _c	15.1 _c	13.0 _c	11.5 _c	12.5 _c	10.0 _d	8.6 _c
NO ₃ —N		23.3 _e	20.1 _d	17.8 _d	19.2 _d	16.1 _d	13.5 _d	16.6 _d	14.5 _e	11.1 _d

Nitrogen	Residue	Day 15			Day 30			Day 45		
		Microbial biomass C (µg/g soil)								
		S1	S2	S3	S1	S2	S3	S1	S2	S3
		Available P (µg/g soil)								
		S1	S2	S3	S1	S2	S3	S1	S2	S3
Control	Control	1.3 _a	1.0 _a	0.7 _a	1.2 _a	1.0 _a	0.8 _a	1.2 _a	1.0 _a	0.6 _a
NH ₄ -N		1.3 _a	1.1 _a	0.8 _a	1.1 _a	0.9 _a	0.7 _a	1.0 _a	0.8 _a	0.6 _a
NO ₃ —N		1.7 _a	1.4 _a	1.0 _a	1.3 _a	1.0 _a	0.8 _a	1.0 _a	0.8 _a	0.5 _a
Control	2% lupin	2.5 _b	2.1 _b	1.5 _b	4.3 _b	3.5 _b	2.7 _b	6.3 _b	5.5 _b	5.0 _b
NH ₄ -N		2.4 _b	2.2 _b	1.4 _b	4.5 _b	3.6 _b	2.9 _b	6.6 _b	5.7 _b	5.1 _b
NO ₃ —N		5.7 _c	4.0 _c	2.1 _c	6.5 _c	5.2 _c	4.5 _c	8.3 _c	7.5 _c	5.7 _b

Values are means of four replicates.

S1, S2 and S3 correspond to EC_{1:5} 0.21, 0.51 and 0.85 dS/m, respectively.

Values in the same column followed by the same letter are not significantly different at $p < 0.05$ (ANOVA)

III. CONCLUSION :

A laboratory experiment was conducted to investigate the effects of NH₄-N or NO₃-N on microbial activity and biomass and N and P availability in a saline sandy loam. Three levels of salinity were imposed in the sandy loam using solutions of Na⁺ and Ca²⁺. Soil was amended with or without 2% (w/w) lupin residues (C/N ratio 15.4). With no residue added, the concentration of available N and P remained unchanged over 45 days. Soil respiration and microbial biomass C, N, and P decreased with increasing salinity, but significantly increased with addition of lupin residues. The greater C availability in the lupin residue amended saline sandy loam stimulated microbial activity and biomass with greater N demand, thus promoted immobilisation of NO₃. Hence, N and P availability increased in the saline sandy loam.

HSE COMMENTS :

This study is from the scientific literature and does not conform to standardised test guidelines, nor is it GLP compliant. The study's methodology, reporting and analysis have been considered with regard to how well they meet common standards of scientific practice, based on 'expert judgement'.

The study has been well conducted in accordance with scientific principles and the same lupin species (*Lupinus albus*) has been used as the active substance is derived from. However details on the test substance such as batch number and purity are not available, which raises some uncertainty as to the relevance of the study for the assessment of this active substance. Addition of lupin residue led to significant increases in soil respiration and microbial biomass. The risk assessment for soil micro-organisms is typically based on results of the nitrogen transformation test, thus assessing functionality as oppose to microbial biomass. This study indicates that residues of *L. albus* do not negatively impact soil micro-organisms, when incorporated at a rate of 2% to soil. HSE considers that the results may be used as supporting evidence in the risk assessment for soil micro-organisms.

Reliability : Reliable with reservations.

(Studies are graded as one of the following categories: 1) Reliable, 2) Reliable with reservations, 3) Unreliable. Categorisation is based on the evaluator's subjective assessment of the quality of the methods, reporting and analysis as presented in the study.)

Reference:	K-CA 8.5/03
Report Title:	Soil Fungal and Bacterial Populations in White Lupin (<i>Lupinus albus</i>) – Maize (<i>Zea mays</i> L) Cropping System Amended With Minjingu Phosphate Rock.
Author(s) & Year:	Lelei, J. J. and Onwonga, R. N. (2014)
Document No	Journal of Agriculture and Ecology Research International 1(1): 1-17, 2014; Article no. JAERI.2014.001
Substance used:	White lupin (<i>Lupinus albus</i>)
Method of analysis:	No analysis conducted
Guideline(s):	No, study from published literature
Deviations:	N/A, study from published literature
GLP or GEP:	No, study from published literature

Acceptability:	Supplementary
Study relied upon:	Yes, however data protection not applicable to published literature studies

I. MATERIALS AND METHODS

A. MATERIALS

Test Material White lupin (*Lupinus albus*)

Lot/Batch #: Not reported

Purity: Not reported

Description: Not reported

Stability of test compound: Not reported

Reanalysis/Expiry date: Not reported

Density: Not reported

Treatments

Test rates: Control i.e. fallow – maize rotation with triple super phosphate fertilizer applied, fallow – maize rotation with MPR (Minjingu Phosphate Rock) applied, white lupin – maize rotation with MPR applied and maize/white lupin intercrop with MPR applied

Reference item: None

Solvent/vehicle: None

Analysis of test concentrations: N/A

Test organisms

Species: Soil fungi and bacteria

Age: N/A

Source: N/A

Feeding: N/A

Test design

Replication: 4 replicates

Plot sizes:	3.75 m × 4.8 m
Duration of test:	Four seasons: long (LRS) and short (SRS) rain seasons of 2010 and 2011

Environmental test conditions

Location:	Farmer's field in Njoro sub- County, Kenya (longitude 35°23' and 35°35' East and Latitude 0°13' and 1°10' south; 2200 m asl)
Total rainfall:	918 mm (2010) and 982 mm (2011)
Air temperature:	Min: 17.6°C Max: 19.1°C
Soil classification:	Mollic Phaeozems
Soil characteristics:	Neutral in pH (pH water 6.4), Moderate in organic C (15 g kg ⁻¹) High in total N (3.5 g kg ⁻¹) Low in Olsen extractable P (14.2 mg kg ⁻¹) Exchangeable bases (cmol kg ⁻¹); Ca (6.5), Mg (0.72) and K (1.42) Clay loam in texture (%); sand (36), silt (29.6), and clay (34)

B. STUDY DESIGN AND METHODS

The experiment was laid out in a randomized complete block design with four replications. The plot sizes measured 3.75 m × 4.8 m. Space for foot paths (0.5 m) between the plots and blocks (1 m) was provided. The treatments were; (i) control i.e. fallow (F) – maize (M) rotation with triple super phosphate fertilizer (TSP) applied (MTSP – F), (ii) fallow – maize rotation with MPR applied (MMPR – F), (iii) white lupin (L) – maize rotation with MPR applied (MMPR – L) and (iv) maize / white lupin intercrop with MPR applied (M/LMPR – F).

Land was prepared manually using hand hoes. MPR was incorporated to a depth of 0–0.15 m along the planting furrows two weeks before planting. TSP was applied at planting by banding. Both P sources were applied at the rate of 60 kg P ha⁻¹. MPR was applied only once during the entire experimental period while TSP was applied twice i.e., at planting of maize in the LRS of 2010 and 2011. Nitrogen was applied at the rate of 75 kg N ha⁻¹ as calcium ammonium nitrate (CAN) fertilizer to all plots, split into two applications; 45 and 30 kg N ha⁻¹ at planting and at topdressing (a month after planting), respectively.

Long rain season: Maize (*Zea mays* L., Hybrid, 513) was sown during the LRS of 2010 and 2011 in all treatments, at 75 cm × 30 cm spacing. Two maize seeds were sown into each planting hole and thinned to one plant 30 days after sowing (DAS). In M/LMPR – F treatment, two white lupin (*Lupinus albus*) seeds were sown between the rows of maize i.e., one row of white lupin between two rows of maize in the LRS of 2010 and 2011. Thinning to one plant (maize and white lupin) was done a month after sowing.

Short rain season: White lupin was sown at the rate of two seeds per hole at spacing of 75 × 30 cm in the treatment MMPR- L during the SRS of both 2010 and 2011. Thinning to one plant per hole was done a month after sowing. The MTSP- F, M /LMPR – F and (MMPR –F) were left under a weedy fallow in the SRS of both years.

White lupin residues and weeds were chopped into 5–20 cm small pieces spread across the plots and incorporated in soil to a depth of 15 cm during land preparation for maize planting, using hand hoes. Maize residues were removed from field after harvest of grains.

Composite soil samples for determination of initial physical and chemical properties were collected from three profile pits (0–60 cm depth) before application of treatments, put in polythene bags and sealed. Thereafter, soil samples were collected from the top soil (0–20 cm) at seedling, 50% flowering and maturity stages of maize (LRS) and white lupin (SRS) for enumeration of soil fungi and bacteria. Soil samples from the weedy fallow in the SRS were collected at the same time period as lupin samples. The samples were obtained randomly from four locations in each plot between the plants within a row and bulked to get one composite sample. The samples were put in sterile polythene bags and sealed with rubber bands. In the laboratory, roots were removed from soil by sieving. Samples for analysis of initial physical and chemical properties were air dried in the laboratory while those for microbial analysis were stored at 4°C and analysed within 3 days.

Fungi and bacteria were enumerated by serial dilution plate method using potato dextrose agar (PDA) for fungi and nutrient agar (NA) media for bacteria. The inoculated petri plates were incubated in a sterile culture room at 25 ± 1°C. Colony forming units (CFU) were estimated by counting the number of colonies under a digital counter, after seven days for fungi and two days for bacteria. Number of bacteria and fungi in 1 g of soil was calculated.

Analysis of variance (ANOVA) was used to detect statistical variation in fungal and bacterial population in the different treatments. The SPSS software appropriate for a randomized complete block design was used. Tukey's Honestly Significant Difference ($P = 0.05$) was used for mean separation.

II. RESULTS AND DISCUSSION

The population of bacteria increased from maize seedling to 50% flowering and declined at maturity in treatments; M_{TSP} - F (control), M_{MPR} –F and M_{MPR} - L, in LRS and in M/L_{MPR} – F, M_{TSP} - F and M_{MPR} –F in SRS of both 2010 and 2011.

In both years, bacterial numbers declined at 50% flowering in M/L_{MPR} – F in LRS and M_{MPR} – L in SRS followed by a slight increase towards maturity. A paired sample t-test showed that the number of bacteria was significantly ($P = 0.05$) higher in the LRS of 2011 than SRS of 2011 at seedling ($t = 0.043$) and maturity ($t = 0.03$). There were no significant differences in bacterial numbers in the LRS and SRS of 2010.

Table 9.5-5: Population of bacteria (CFU $\times 10^{-6}$ g $^{-1}$ dry soil) at different sampling times and treatments

Treatment	Long rain season						Short rain season					
	Seedling		50% flowering		Maturity		Seedling		50% flowering		Maturity	
	2010	2011	2010	2011	2010	2011	2010	2011	2010	2011	2010	2011
M_{TSP} - F	3.9 ^a	4.1 ^a	5.5 ^a	5.3 ^a	1.5 ^b	1.7 ^b	3.1 ^a	3.3 ^a	3.4 ^a	3.5 ^a	3.1 ^a	0.7 ^b
M_{MPR} –F	3.1 ^b	2.2 ^c	4.9 ^b	2.6 ^b	1.8 ^b	1.1 ^c	2.5 ^b	1.9 ^c	2.8 ^b	2.1 ^c	1.9 ^c	0.4 ^b
M_{MPR} - L	3.3 ^b	3.1 ^b	4.7 ^b	3.2 ^b	1.7 ^b	2.2 ^a	2.9 ^b	2.4 ^b	1.1 ^c	1.1 ^d	1.2 ^c	1.3 ^a
M/L_{MPR} – F	2.6 ^c	2.7 ^b	1.9 ^c	1.4 ^c	2.6 ^a	2.4 ^a	3.3 ^a	2.5 ^b	3.5 ^a	2.7 ^b	2.9 ^b	1.2 ^a

^{a-d}Means in a column followed by the same letter are not significantly different at $P = 0.05$, using the Tukey mean separation procedure.

Fungal numbers increased from seedling to 50% flowering and declined at maturity in all treatments in the LRS and M_{MPR} - L in the SRS of both years.

During the SRS of 2010 and 2011, the fungal numbers gradually declined with sampling periods in treatments M_{TSP} - F, M_{TSP} - F and M/L_{MPR} – F which were under weedy fallow. The t-test values for comparison of fungal numbers between the LRS of 2010 and SRS of 2010 were 0.05, 0.038 and 0.192 with the corresponding values for LRS of 2011 and SRS of 2011 being 0.019, 0.49 and 0.017. T-tests failed to reveal statistically significant differences in fungal numbers between SRS of 2010 and LRS of 2010 at crop maturity only. Fungal numbers were generally higher in the second year.

Table 9.5-6: Population of fungi (CFU $\times 10^{-6}$ g⁻¹ dry soil) at different sampling times and treatments

Treatment	Long rain season						Short rain season					
	Seedling		50% flowering		Maturity		Seedling		50% flowering		Maturity	
	2010	2011	2010	2011	2010	2011	2010	2011	2010	2011	2010	2011
M _{TSP} - F	4.9 ^a	4.4 ^d	6.9 ^a	5.6 ^c	2.4 ^a	1.5 ^b	1.3 ^b	1.9 ^c	0.5 ^b	1.7 ^d	0.1 ^b	0.9 ^c
M _{MPR} –F	4.1 ^c	5.2 ^c	5.3 ^b	4.6 ^d	1.8 ^b	1.6 ^b	1.3 ^b	2.5 ^c	0.4 ^b	2.3 ^c	0.2 ^b	0.5 ^d
M _{MPR} - L	4.2 ^c	6.1 ^b	5.1 ^b	6.9 ^b	1.4 ^b	2.5 ^a	2.4 ^a	5.1 ^a	4.2 ^a	5.4 ^a	2.1 ^a	2.1 ^a
M/L _{MPR} – F	4.4 ^b	7.2 ^a	7.1 ^a	10.1 ^a	1.5 ^b	2.4 ^a	1.4 ^b	3.7 ^b	0.5 ^b	3.6 ^b	0.4 ^b	1.5 ^b

^{a-d}Means in a column followed by the same letter are not significantly different at P = 0.05, using the Tukey mean separation procedure.

The correlation between number of fungi and bacteria was -0.95 at 50% flowering of lupin in the SRS of 2010 and 0.97 at the termination of the experiment in the SRS of 2011. Negative correlation at 50% flowering indicates that as the population of fungi increased bacterial numbers were declining. Positive correlation between fungi and bacteria at the termination of the experiment shows that both organisms increased and none was suppressed.

III. CONCLUSION

This paper was conducted to determine fungal and bacterial populations under white lupin (*Lupinus albus*) – maize (*Zea mays* L) cropping system amended with Minjingu Phosphate Rock (MPR).

White lupin-maize cropping system with application of MPR increased soil bacterial and fungal population, an indication of improved soil health and hence cropping system sustainability.

HSE COMMENTS:

This study is from the scientific literature and does not conform to standardised test guidelines, nor is it GLP compliant. The study's methodology, reporting and analysis have been considered with regard to how well they meet common standards of scientific practice, based on 'expert judgement'.

The study has been well conducted in accordance with scientific principles and the same lupin species (*Lupinus albus*) has been used as the active substance is derived from. However there is no information relating to the batch or purity of the test item which limits the reliability of the study. The study indicates that soil bacterial and

fungal populations were increased in lupin-maize cropping systems, however no measurements were taken to investigate the functionality of these populations, such as measuring nitrogen or carbon transformation. As such the study is of limited use in the risk assessment for soil micro-organisms but may still be considered as supporting information.

Reliability: Reliable with reservations.

(Studies are graded as one of the following categories: 1) Reliable, 2) Reliable with reservations, 3) Unreliable. Categorisation is based on the evaluator's subjective assessment of the quality of the methods, reporting and analysis as presented in the study.)

Reference:	K-CP 10.6.2/01
Report Title:	Effects of prior crop residue management on microbial properties and crop residue decomposition
Author(s) & Year:	Cookson, W.R., Beare, M.H., and Wilson, P.E. (1998)
Document No	Applied Soil Ecology 7 (1998) 179 – 188
Substance used:	White lupin residues (<i>Lupinus albus</i> L.)
Method of analysis:	No analysis conducted
Guideline(s):	No, study from published literature
Deviations:	N/A, study from published literature
GLP or GEP:	No, study from published literature
Acceptability:	Supplementary
Study relied upon:	Yes, however data protection not applicable to published literature studies

I. MATERIALS AND METHODS

A. MATERIALS

Test Material	Wheat (<i>Triticum aestivum</i>), barley (<i>Hordeum vulgare</i>), and lupin (<i>Lupinus albus</i>) residues
Test site	
Location:	AgResearch Lincoln Research Farm (43°38'S, 172°30'E)
Soil:	Wakanui silt loam (USDA)
Use:	Immediately prior to the test, part of a straw management trial
Test Design	
Test vessel:	25 x 12 cm fibreglass-nylon litterbags with 1.8 mm mesh
Litterbag contents:	5 to 10 g pre-weighed, oven-dried stems and leaves
Replicates:	7 replicates of wheat and barley, 2 of lupin
Duration:	90 days (for tests concerning lupin)

B. STUDY DESIGN AND METHODS

A litterbag decomposition technique was used to describe the composition and activity of decomposer communities and their capacity to degrade crop residues of differing quality under the different residue management practices of burning, removing, or incorporating residues in wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), and lupin (*Lupinus albus*).

Soil at the test site was a Wakanui silt loam (USDA) with a pH of 5.3. Soil contained 3.1% total C, 0.33% total N, and 20.0 µg/g Olsen P. For four years prior to the initiation of this study, the site was maintained as a perennial rye grass / white clover pasture. Immediately prior to the test, the site was being used as part of a straw management trial. To the test site were added the fungicides Tilt and Sportak, the herbicides Trimec and Versatill, and the product Raingard. All test plots were fertilised at post-emergence with 100 kg N/ha using Cropmaster 20 or ammonium sulphate.

One of three crop residue management treatments were evaluated: Burned: post-harvest residues were burned followed by ploughing; Removed: post-harvest residues cut to a height of ca. 6 cm, baled and removed; Incorporated: post-harvest residues of ca. 6 cm were mulched and incorporated by ploughing.

Standing dead residues of wheat and barley, and volunteer seedlings of lupin were collected from research plots adjacent to the trial site, and 5 to 10 g pre-weighed, oven-dried (60°C) stems and leaves were placed into 25 x 15 cm fibreglass-nylon litterbags with 1.8 mm mesh. Seven wheat, seven barley, and two lupin litterbags were buried vertically in each plot at a depth of 30 cm. Ten residue bags were transported to the field, then returned to the laboratory and reweighed to correct for small amounts of residue mass loss due to transport and handling.

Wheat, barley, and lupin residues were harvested 28 and 90 days after burial. Residues remaining on each sample date were extracted from the litterbag, shaken gently over a 1-mm sieve to remove the majority of the soil and total wet weights recorded. Subsamples of 2.0 g were removed and oven-dried at 60°C for 48 hours to determine gravimetric water contents.

Potential microbial activity of the residues was determined using a substrate-induced respiration (SIR) method adapted for use on plant residues. This involved adding 3 mL glucose solution to a 2 to 4 g sub-sample of field-moist residues in a conical flask. This was incubated at 23°C for four hours, and the respired CO₂ was trapped and corrected for controls.

Total and viable fungal and bacterial populations were quantified using a fluorescent staining technique. Sub-samples (2.0 g) of the litterbags were homogenised in a blender and diluted using 100 mL sterilised water. A 10-μL aliquot was spread onto a glass slide, air dried, and heat fixed. Differential fluorescent stain and fluorescent brighter were added and the slides were incubated at room temperature for 60 minutes. Direct counts of red and blue fluorescing bacterial cells and fungal hyphae were made using an epifluorescent microscope.

Remaining residues from each litterbag were oven-dried at 60°C for 48 hours and finely ground to 0.5 mm. Sub-samples of 0.5 g were ashed in a muffle furnace to correct all dry weight estimates to an ash-free dry weight basis (AFDW). Daily decay rate constants (k) for the residues were calculated using the single negative exponential decay function. The total N content of the residues was determined by Kjeldahl digestion. The lignin content of the initial residues was determined by proximate analyses.

Calculated ratios and percentages were arcsine-square root transformed prior to statistical analyses. Treatment and interaction effects were determined using the analysis of variance procedure in GENSTAT. Significant differences among

transformed means were assessed by LSD test. linear regression analyses were performed on untransformed data.

II. RESULTS AND DISCUSSION

Mass loss from lupin residues averaged across the three residue management practices was 75.1% during the first 90 days of decomposition. There was no statistically significant difference in the mass loss between the three practices. Overall, the percentage of initial mass remaining after 90 days of decay increased with increases in the initial lignin:N ratio of the residues (wheat (38.3%) > barley (42.3%) > lupin (75.1%)).

Table 9.5-7: Effects of straw management practices on the mass loss and decay rates of residues after 90 days of decomposition

Crop practice	Wheat		Barley		Lupin	
	% AFDW ¹ remaining	Decay rate (k/d x10 ³)	% AFDW ¹ remaining	Decay rate (k/d x10 ³)	% AFDW ¹ remaining	Decay rate (k/d x10 ³)
Burned	63.6	5.02	59.4	6.28	23.6	ND
Removed	67.1	4.76	55.2	6.29	24.3	ND
Incorporated	54.4*	7.18*	58.5	6.42	26.9	ND

¹ Ash-free dry weight * Within columns, statistically significantly different (p < 0.05)

ND: Not determined

After 90 days of decay, the average percent N remaining in lupin residues (20.3%) was significantly lower than wheat (73.3%) and barley (71.8%). Residue management practice did not have any significant effects on the N content of lupin residues in the study.

Table 9.5-8: Effects of straw management practices on the percentage of initial N remaining in residues after 14 and 90 days of decomposition

Crop practice	Wheat		Barley		Lupin	
	Day 14	Day 90	Day 14	Day 90	Day 14	Day 90
Burned	111	80	110	76	ND	16
Removed	115	77	122	67	ND	18
Incorporated	130*	63*	116	71	ND	27

* Within columns, statistically significantly different ($p < 0.05$) ND: Not determined

The average rate of SIR from lupin residues was 2.2 to 2.4 and 1.1 to 1.3 times that of wheat and barley residues after 28 and 90 days of decay, respectively. When data from all samples dates were considered, no significant effects of prior residue management on the SIR rates of lupin were found. However when analysed separately, the SIR results from day 90 indicated significant effects of residue type ($p < 0.005$), management practice ($p < 0.05$), and their interaction ($p < 0.01$).

Table 9.5-9: Effects of straw management practices on substrate-induced respiration (SIR) rates ($\mu\text{g CO}_2/\text{g AFDW/h}$) of residues after 28 and 90 days of decomposition

Crop practice	Wheat		Barley		Lupin	
	Day 28	Day 90	Day 28	Day 90	Day 28	Day 90
Burned	765	569	758	632	1923	651
Removed	691	537	861	550	2063	631
Incorporated	938*	768*	901	575	1849	902*

* Within columns, statistically significantly different ($p < 0.05$)

After 28 days of decay, viable bacteria averages 14, 26, and 38 x 10⁹ cells/g AFDW on wheat, barley, and lupin residues, respectively. Similar differences were recorded for total fungal hyphae, averaging 1475, 2686, and 6405 m/g AFDW on wheat, barley, and lupin residues, respectively.

Table 9.5-10: Effects of straw management practices on counts of viable bacteria (x 10⁹ cells/g AFDW) and total fungi (mg/AFDW) of residues after 28 days of decomposition

Crop practice	Wheat		Barley		Lupin	
	Viable bacteria	Total fungi	Viable bacteria	Total fungi	Viable bacteria	Total fungi
Burned	13.9	1220	27.4	2829	25.3	6667
Removed	9.3	1110	21.9	1669	36.7	6205
Incorporated	19.0	2095	28.0	3559	51.9	6343

III. CONCLUSION:

The lack of management effects on microbial activity and residue decomposition of lupin residues is most probably related to differences in its initial chemical composition. Lupin had the highest initial N concentration and the lowest initial lignin concentration of the three residues investigated in this study. The results of this study suggest that the initial lignin:N ratio is an important delimiter of residue decay for the residue types investigated

Decomposition of the three residue types in this study appeared to depend on their initial chemical composition. It is thought that previous exposure to wheat straw is likely to have conditioned the microbial community to more readily degrade some of the chemical constituents. In the burned and removed treatments, where prior conditioning to wheat residues was minimal, on average 65, 57, and 24% of the initial wheat, barley, and lupin residues, respectively, remained after 90 days of decay.

HSE COMMENTS

This study is from the scientific literature and does not conform to standardised test guidelines, nor is it GLP compliant. The study's methodology, reporting and analysis have been considered with regard to how well they meet common standards of scientific practice, based on 'expert judgement'.

The study has been well conducted in accordance with scientific principles and the species of lupin tested is the same as that used to derive the active substance under assessment. However, it is noted that it was lupin seedlings that were incorporated into soil. It is not clear whether any seeds were included in the residues. This raises some uncertainty considering the active substance is derived from seeds of *L. albus*. Nonetheless, this study provides support that incorporation of lupin seedling residues into soil does not negatively affect soil microbial population composition, nor does it adversely affect microbial activity with regard to decomposition processes. The highest level of decomposition in the litterbags (greatest mass loss compared to wheat and barley residue treatments) and the highest level of substrate-induced respiration was observed in the lupin residue treatment group. Additionally the populations of bacteria and fungi were higher in the lupin treated group. The results may be considered as supporting evidence in the risk assessment.

Reliability: Reliable with reservations

(Studies are graded as one of the following categories: 1) Reliable, 2) Reliable with reservations, 3) Unreliable. Categorisation is based on the evaluator's subjective assessment of the quality of the methods, reporting and analysis as presented in the study.)

B.9.6. Effects on terrestrial non-target higher plants

B.9.6.1. Summary of screening data

None submitted or required.

B.9.6.2. Testing on non-target plants

Reference:	K-CA 8.6.1/01
Report Title:	PROBLAD: Vegetative Vigour Limit Test for Non Target Plants on Six Plant Species.
Author(s) & Year:	Peterek (2011)
Document No	CEV SA, Unpublished report No. : S10-02560
Substance used:	PROBLAD PLUS (Batch: 201009, 20% BLAD, 1.30 g/cm ³)
Method of analysis:	No analysis conducted
Guideline(s):	OECD 227 (2006)
Deviations:	Yes, see HSE comments
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

I. MATERIALS AND METHODS**A. MATERIALS**

Test Material	PROBLAD PLUS
Lot/Batch #:	Batch no. 201009
Actual content of active ingredients:	BLAD content 20.0% w/w (according to certificate of analysis)
Treatments	
Test rates:	Limit test at 2000 mL/ha
Control:	Demineralised water
Application method:	Laboratory track sprayer, calibrated to produce a spray output of 300 L/ha ± 10%

Test organisms

Species:	Oilseed rape; <i>Brassica napus</i> (dicotyledonous)
	Cucumber; <i>Cucumis sativus</i> (dicotyledonous)
	Lettuce; <i>Lactuca sativa</i> (dicotyledonous)
	Tomato; <i>Solanum lycopersicum</i> (dicotyledonous)
	Onion ; <i>Allium cepa</i> (monocotyledonous)
	Maize ; <i>Zea mays</i> (monocotyledonous)

Test design

Vessels:	Plastic pots approx.. 15 cm diameter.
Test soil:	Artificial soil substrate (heat treated natural soil mixed with sand). 69.12% sand, 17.13% clay and 13.75% silt. Total organic carbon content of 2.1%.
Replication:	15 replicates/treatment, 1 pot per replicate (for <i>Allium cepa</i> , 6 replicates/treatment, 1 pot per replicate).
Sampling interval:	7, 14 and 21 days after application.
Duration of test:	21 days

Environmental test conditions

Temperature:	11.0–32.5°C
Humidity	33.5–82.0%
pH of soil:	8.1
Photoperiod:	16 h light, 8 h dark. 11500–30000 lux.

B. STUDY DESIGN AND METHODS

The plant species tested included two monocotyledons: maize (*Zea mays*) and onion (*Allium cepa*); and four dicotyledons: oilseed rape (*Brassica napus*), cucumber (*Cucumis sativus*), lettuce (*Lactuca sativa*) and tomato (*Solanum lycopersicum*). The seeds had not been pre-treated with fungicides or insecticides and no plant protection products have been used.

All the species were grown in plastic pots with approximately 15 cm in diameter. The number of emerged seeds were reduced to two (or five for onion) seedlings per pot after the emergence had been recorded. Each treatment group consisted of total 30 plants. The soil used as support medium in the test was an artificial soil substrate (heat treated natural soil mixed with sand), consisting of 69.12% sand, 17.13% clay and 13.75% silt with a pH of 8.1 and a total organic matter content of 2.1%. The control and test item pots were irrigated with water from a source located at the

bottom of each pot. A cotton strip connected soil and water source. The solution consumed was controlled regularly and missing water supplemented. No plant protection measures were performed over the whole testing period

The trial comprised one application rate and a demineralised water control. The spray cabin was calibrated before the applications by adjusting the pressure, application speed and nozzle type. At least three calibration runs were conducted until each resulted in an output of 300 L/ha \pm 10%. The test item was dispersed in demineralised water as a carrier and the solution was visually checked to ensure the whole test item had dispersed. The control pots were first sprayed with demineralised water only, followed by the pots sprayed with test item solution.

The test was performed in a greenhouse under the following conditions: temperature of 11.0 to 32.5°C, humidity of 33.5 to 82% and 16 hours of light per day. Pots were moved at random at day 7 and 14 after application to compensate any possible lack of uniformity in environmental conditions.

The duration of the test was 21 days. The plants were assessed at 7, 14, and 21 days after application for mortality and visual phytotoxicity expressed as a percentage of healthy untreated control plants. At the end of the observation period, surviving plants were clipped at soil level. The number of living plants/replicate and the plant height of each single plant/ replicate were determined. The weight of the above-ground (shoot) portion of all surviving plants of each replicate was measured. The dry weight was determined after drying at 80°C until constant weight is reached as a pooled sample.

For the statistical analyses F-test, Mann-Whitney-U-Test or Satterthwaite t-Test were used.

II. RESULTS AND DISCUSSION

The results of the study are summarised in the following table.

Table 9.6.2-1: Summary of effects of PROBLAD PLUS on phytotoxicity, plant height and dry biomass

Test species	Mean Phytotoxicity (%)¹	Inhibition of Plant Height (%)	Inhibition of dry biomass (%)
Oilseed rape (Brassica napus)	0.0	5.1	4.6
Cucumber (Cucumis sativus)	10.0	16.3*	18.8*
Lettuce (Lactuca sativa)	2.7	9.0	8.4
Tomato (Solanum lycopersicum)	1.3	-5.8	10.0
Onion (Allium cepa)	0.0	12.7	8.9
Maize (Zea mays)	2.0	0.5	-7.6

*statistically significant different compared to the control

¹ Calculated with the highest % value per replicate

Validity criteria were fulfilled for the six species tested. Phytotoxicity was observed only in cucumber (*Cucumis sativus*), although % values were relatively low. Lettuce (*Lactuca sativa*), tomato (*Solanum lycopersicum*) and maize (*Zea mays*) showed phytotoxic symptoms, at low % values, only on day 14 and 21. Oilseed rape (*Brassica napus*) and onion (*Allium cepa*) showed no phytotoxic symptoms at any day. The dicotyledonous species *Cucumis sativus* showed a significant effect on the plant height after application of the test item, although the inhibition was relatively low. Also, only *Cucumis sativus* showed significant effect on biomass, with a relatively low inhibition of 18.8%.

On some days the temperature and humidity were outside the recommended ranges (target temperature 12°C to 32°C and humidity 45% to 95%; actual temperature 11.0°C to 32.5°C and humidity 33.5% to 82.0%). This deviation from the Study Plan was due to a technical reason and it is considered to have no impact on the study as all validity criteria were met for all species.

Statistical analysis was carried out using F-Test, Mann-Whitney-U-Test (Exact) or the t-Test Satterthwaite rather than the tests stated in the Study Plan. This deviation from the Study Plan was due to a handling reason and it is considered to have no impact on the study.

Validity criteria:

The validity criteria in accordance with OECD 227 (2006) were met:

Validity criterion	Required	Obtained
Seedling emergence	Seedling emergence in all conditions should be at least 70%	> 70%
Phytotoxic effects in the controls	Control plants should not exhibit visible phytotoxic effects. Plants only exhibit normal variation in growth and morphology.	0% phytotoxicity in controls
Mean control plant survival	Mean plant survival in the control should be at least 90% for the duration of the study.	> 90%
Environmental conditions	Environmental conditions for a particular species are identical, and the growing media should contain the same amount of soil/substrate, from the same source.	Identical conditions for all species and treatments.

III. CONCLUSION

Cucumis sativus was the most sensitive species tested for all three variables (phytotoxicity, biomass and plant height). This species showed 10% phytotoxicity at assessment day 21, 16.3% reduction in plant height and 18.8% reduction in biomass when exposed to PROBLAD PLUS at 2000 mL product/ha.

Therefore, the ER₅₀ (biomass) for all six species is 2000 mL product/ha.

HSE COMMENTS

This study has been conducted in accordance with GLP and follows OECD 227 (2006 guidelines).

The study report states that the validity criteria for seedling emergence and control plant survival were met, as the seedling emergence was > 70% and the survival was > 90%. However the actual seedling emergence and survival results were not documented to verify this. The assumption is made that the validity criteria were met, although clarification may be required.

Analytical verification of the test item solution has not been conducted, despite being recommended in OECD 227. However as only a single test rate was used, and this was prepared by directly dispersing the test item in water, rather than dilution of a stock solution, it is assumed that the nominal concentration was achieved.

The guideline recommends the use of a reference item to verify the sensitivity of the test system. This does not appear to have been conducted in this study and no justification for this omission has been provided. However a reference test is not an explicit requirement so this is not detrimental to the study outcome.

There were deviations in the environmental conditions from the guideline recommended ranges. Firstly, the humidity fell below the lower guideline limit of 45% to a minimum of 33.5% on day 6 and 7. The humidity fell below 45% on a total of 5 days during the study but the length of these deviations is not known. Secondly, the temperature dropped slightly below the guideline minimum of 12°C to a minimum of 11°C and slightly exceeded the maximum temperature of 32°C to a maximum of 32.5°C. Temperature dropped below the lower limit on one day only, and exceeded the upper limit on two days. The actual lengths of the deviations are not known. Since the validity criteria were met and there were no abnormal effects on growth or survival in the control group, these deviations are not thought to have affected the study outcome and can be considered minor.

The most sensitive species was *Cucumis sativus*, which showed 10% effect on phytotoxicity, 16.3% inhibition in plant height and 18.8% inhibition in biomass. The inhibition in plant height and biomass were statistically significantly different to the control. Less than 50% effects were observed across all measured parameters.

The endpoint suitable for use in risk assessment is therefore:

- < 50% effects on phytotoxicity at concentrations up to 2000 mL/ha

Reference:	K-CA 8.6.2/01
Report Title:	PROBLAD PLUS: Effects on the Vegetative Vigour and on the Seedling Emergence and Growth of Six Non-Target Terrestrial Plant Species under Greenhouse Conditions.
Author(s) & Year:	Huerta, F. (2020)
Document No	CEV SA. Report number S20-05408
Substance used:	PROBLAD PLUS. (Batch: CF01907-001, 21.2% BLAD, 1.20 g/cm ³)

Method of analysis:	Enzyme-Linked Immunosorbent Assay (ELISA). Method ref. S18-08251
Guideline(s):	OECD 227 (2006) and OECD 208 (2006)
Deviations:	Yes, see HSE comments
GLP or GEP:	Yes
Acceptability:	Yes
Study relied upon:	Yes

I. MATERIALS AND METHODS

A. MATERIALS

Test Material PROBLAD PLUS

Lot/Batch # : CF01907-001

Purity : 100% active substance; nominally 20% lead component BLAD protein (actual = 21.2%, according to certificate of analysis)

Treatments

Test rates : 3.2 L/ha in 200 L/ha volume (limit test)

Solvent/vehicle : Distilled water

Negative control : Distilled water

Test organisms

Species : Dicotyledonous species : *Brassica napus* (oilseed rape), *Cucumis sativus* (cucumber), *Lactuca sativa* (lettuce) and *Lycopersicon esculentum* (tomato).

Monocotyledonous species : *Zea mays* (maize) and *Allium cepa* (onion).

Seed at day 0 (seedling emergence test); seedlings at BBCH growth stage 12-14 (vegetative vigour test)

Seeds were untreated.

Source : Commercial suppliers

Test design

Test vessel :	Plant pots (Ø15.1 cm, capacity 1.5 L) were filled with the test soil to 2 cm below the top
Test medium :	Artificial Sandy Loam soil; 4.00% Silt, 14.72% Clay, 81.28% Sand; ToC 1.26%; pH 7.26
Replication :	20 replicates, except <i>Allium cepa</i> (onion) 10 replicates
No. Plants/vessel :	Two plants per test vessel, except <i>Allium cepa</i> (onion) four plants per vessel
Duration of test :	Seedling emergence test: 21 days from 50% species control plant emergence (except tomato: 20 days and onion: 22 days) Vegetative vigour test : 21 days

Environmental test conditions

Temperature :	19.7–34.3°C (continuous measurement using data logger)
Relative humidity :	51–97% (continuous measurement using data logger)
Photoperiod :	16 hours light:8 hours dark
Light intensity :	374.2–421.1 $\mu\text{E}/\text{m}^2/\text{s}$ (measured at 7, 10 and 21 days after application, at plant level)

B. STUDY DESIGN AND METHODS

Experimental dates: 27 July 2020 to 27 November 2020.

Application

Test item solutions were prepared as close as possible to the time of application. Distilled water was used as the solvent for the test item. 19.200 g of test item was used to prepare the test solution.

Application was conducted with a laboratory track-sprayer (Schachtner, Ludwigsberg, Germany) and Teejet 8002 EVS nozzle. The track-sprayer was calibrated with water before application. The spray pressure and distance were adjusted to provide the target output of 200 L/ha ($\pm 10\%$). Three calibration runs were conducted by

weighing five metal plates before and after treatment. In the calibration runs the minimum spray volume was 200 L/ha and the max was 204 L/ha. The actual delivery rates in the experimental phase were 206 L/ha (3.00% deviation from target) for the control and 188.00 L/ha (-6% deviation from target) for the test item.

Vigour Test

Four dicotyledonous and two monocotyledonous species were cultivated in soil. PROBLAD PLUS was applied at a limit application rate (3.2 L/ha) in a 200 L/ha water volume. Seeds were sown in excess for each replicate plot and the number of emerged seedlings was reduced to the required number of plants before application. In each treatment group a total of 40 plants at BBCH growth stage 12–14 were applied. The test duration was 21 days following application. During this period, plants were assessed for mortality and phytotoxicity symptoms on days 7, 14 and 21. The effects on plant shoot height and shoot dry weight were determined on day 21. Results were compared to the distilled water treated control.

Seedling Emergence Test

Seeds of six dicotyledonous and four monocotyledonous species were sown into soil. PROBLAD PLUS was applied at a limit application rate (3.2 L/ha) in a 200 L/ha application volume. In each treatment group a total of 40 seeds were sown. Plants were assessed for seedling emergence, plant survival, growth stage, and phytotoxicity symptoms on days 7, 14 and 21 after 50% of the seeds in the control had emerged in each species. The effects on plant shoot height and shoot dry weight were determined on nominally day 21. The species *Lycopersicon esculentum* (tomato) and *Allium cepa* (onion) were assessed for seedling emergence, plant survival, growth stage, and phytotoxicity symptoms on days 6, 13 and 20 and 8, 15 and 22 after 50% of the seeds in the control had emerged, respectively. The effects on plant shoot height and shoot dry weight were determined on day 20 for *Lycopersicon esculentum* (tomato) and on day 22 for *Allium cepa* (onion). (See Amendments and Deviations Chapter). Results were compared to the distilled water treated control.

Assessment of shoot height and dry weight

Shoot height was evaluated per plant on the last assessment day. The length of above-ground vegetation was measured for each surviving plant from the soil surface to the apical tip or highest part on the last assessment day. The inhibition in shoot height compared to the control group was calculated in percent for each test item group.

For assessment of shoot dry weight, surviving plants were clipped at soil level on the last assessment day and dried at 60°C until constant weight was reached. The shoot

dry weight was determined per replicate. The inhibition in shoot dry weight compared to the control group was calculated in percent for each test item group.

Assessment of phytotoxicity

Symptoms of phytotoxicity were assessed visually for all living plants using a system based on EPPO guideline 1/135(4) (2014). On each assessment day plants were assigned a rating to describe how healthy they looked per replicate, taking into account necrosis, chlorosis and other characteristics that could be treatment related. Ratings ranged from 0 to 100%:

0% = indicating no injury or effect on plants;

up to 20% = indicating plants with slight symptom(s);

up to 40% = indicating plants with moderate symptom(s);

up to 60% = indicating plants with severe symptom(s);

up to 80% = indicating symptom(s) on total plant;

up to 90% = indicating moribund plants;

100% = indicating dead plants

Analytical methods

Samples of the spray solution used to treat at nominally 3.2 L/ha were sampled and analysed using the validated analytical method (enzyme linked immunosorbent assay; ELISA).

Statistical analysis

Statistical analysis of data was performed using the ToxRat Solutions program (ToxRat® Professional Version 3.2.1). Mean seedling emergence, mean mortality, mean final heights and mean final shoot dry weight were compared using a suitable statistical test in order to obtain the NOER and LOER values.

Comparison between each rate of the test item assayed and the control, was performed for all the plant species. For quantal data Fisher's Exact Binomial Test ($\alpha = 0.05$) was performed. For metric data STUDENT-t Test ($\alpha = 0.05$) for Homogeneous Variances was performed.

II. RESULTS AND DISCUSSION

Validity criteria:

Validity criteria according to the OECD 208 and 227 guidelines (2006) were met.

Validity criterion	Required	Obtained
OECD 208 (2006): Seedling emergence and seedling growth test		
Control seedling emergence	Seedling emergence in the controls should be at least 70%	87.5% to 100.0%
Phytotoxic effects in the controls	Control plants should not exhibit visible phytotoxic effects. Plants only exhibit normal variation in growth and morphology.	The control seedlings did not exhibit visible phytotoxic effects, only normal variation in growth and morphology for that particular species.
Survival of emerged control seedlings	Mean plant survival in the control should be at least 90% for the duration of the study.	100% control plant survival
Environmental conditions	Environmental conditions for a particular species are identical, and the growing media should contain the same amount of soil/substrate, from the same source.	The environmental conditions for each particular species were identical and growing media contained the same amount of soil matrix (all pots were filled manually until the same level, to 2 cm below the top of the container), support media, or substrate from the same source.
OECD 227 (2006): Vegetative vigour test		
Seedling emergence	Seedling emergence in all conditions should be at least 70%	77.1% to 95.5%
Phytotoxic effects in the controls	Control plants should not exhibit visible phytotoxic effects. Plants only exhibit normal variation in growth and morphology.	The control seedlings did not exhibit visible phytotoxic effects, only normal variation in growth and morphology for that particular species.

Validity criterion	Required	Obtained
Mean control plant survival	Mean plant survival in the control should be at least 90% for the duration of the study.	100% control plant survival
Environmental conditions	Environmental conditions for a particular species are identical, and the growing media should contain the same amount of soil/substrate, from the same source.	The environmental conditions for each particular species were identical and growing media contained the same amount of soil matrix (all pots were filled manually until the same level, to 2 cm below the top of the container), support media, or substrate from the same source.

Analytical results:

Analysis of the stock solutions via ELISA detection method resulted in 102% of nominal concentration required to apply at 3.2 L/ha. This, along with demonstrated calibration of the spray equipment to deliver at 200 L/ha, confirm that treatment rates were sufficiently close to nominal. All endpoints are therefore described in terms of nominal treatment rate.

Seedling emergence:**Table 9.6.2-2: Mean emergence data**

Treatment	Mean Emergence %					
(L/ha)	Brassica napus	Cucumis sativus	Lactuca sativa	Lycopersicon esculentum	Zea mays	Allium cepa
Control (0)	95.00	100.00	95.00	100.00	92.50	87.50
3.200	95.00	95.00	100.00	97.50	95.00	85.00

Post-emergence mortality :**Table 9.6.2-3: Mean mortality data, vegetative vigour test**

Treatment	Mean Mortality %					
(L/ha)	Brassica napus	Cucumis sativus	Lactuca sativa	Lycopersicon esculentum	Zea mays	Allium cepa
Control (0)	0.00	0.00	0.00	0.00	0.00	0.00
3.200	0.00	0.00	0.00	0.00	0.00	0.00

Table 9.6.2-4: Mean mortality data, seedling emergence test

Treatment	Mean Mortality %					
(L/ha)	Brassica napus	Cucumis sativus	Lactuca sativa	Lycopersicon esculentum	Zea mays	Allium cepa
Control (0)	0.00	0.00	0.00	0.00	0.00	0.00
3.200	0.00	0.00	0.00	0.00	0.00	0.00

Phytotoxicity :

In both the seedling emergence and vegetative vigour tests no visual phytotoxic symptoms were observed in either control or treatment group plants.

Growth stage:

In observations on days 7, 14 and test end day there were no notable differences in the growth stage of control and treatment plants.

Shoot height:**Table 9.6.2-5: Mean shoot height (cm), vegetative vigour test**

Treatment (L/ha)	Mean value [cm]	± SD [cm]	Inhibition (% compared to control)
Brassica napus (oilseed rape)			
Control (0)	55.68	4.572	--
3.200	58.58	6.269	-5.21
Cucumis sativus (cucumber)			
Control (0)	186.30	15.800	--
3.200	194.10	12.997	-4.19
Lactuca sativa (lettuce)			
Control (0)	27.61	1.615	--
3.200	25.95*	2.605	6.02
Lycopersicon esculentum (tomato)			
Control (0)	79.83	5.511	--
3.200	81.60	7.303	-2.22
Zea mays (Maize)			
Control (0)	193.35	6.310	--
3.200	192.39	9.607	0.50
Allium cepa (onion)			
Control (0)	46.98	2.994	--
3.200	47.55	3.251	-1.22

* Significantly different from the control at the 0.05 probability level

Table 9.6.2-6: Mean shoot height (cm), seedling emergence test

Treatment (L/ha)	Mean value [cm]	± SD [cm]	Inhibition (% compared to control)
Brassica napus (oilseed rape)			
Control (0)	37.91	3.759	--
3.200	36.70	3.307	3.20
Cucumis sativus (cucumber)			
Control (0)	62.70	7.459	--
3.200	61.18	12.846	2.43
Lactuca sativa (lettuce)			
Control (0)	15.33	1.885	--
3.200	16.28	1.256	-6.20
Lycopersicon esculentum (tomato)			
Control (0)	35.79	4.553	--
3.200	37.74	5.134	-5.45
Zea mays (Maize)			
Control (0)	121.37	11.415	--
3.200	129.15	11.412	-6.41
Allium cepa (onion)			
Control (0)	28.02	3.105	--
3.200	29.83	2.319	-6.48

Shoot dry weight :**Table 9.6.2-7: Mean shoot dry weight (g), vegetative vigour test**

Treatment (L/ha)	Mean value [g]	± SD [g]	Inhibition (% compared to control)
Brassica napus (oilseed rape)			
Control (0)	13.58	3.014	--
3.200	12.63	2.724	6.97
Cucumis sativus (cucumber)			
Control (0)	18.79	2.724	--
3.200	19.22	3.014	-2.33
Lactuca sativa (lettuce)			
Control (0)	4.22	1.389	--
3.200	4.15	1.111	1.72
Lycopersicon esculentum (tomato)			
Control (0)	13.13	2.347	--
3.200	12.26	2.663	6.60
Zea mays (Maize)			
Control (0)	14.34	1.399	--
3.200	14.95	1.730	-4.20
Allium cepa (onion)			
Control (0)	1.13	0.278	--
3.200	1.44	0.349	-27.23

Table 9.6.2-8: Mean shoot dry weight (g), seedling emergence test

Treatment (L/ha)	Mean value [g]	± SD [g]	Inhibition (% compared to control)
Brassica napus (oilseed rape)			
Control (0)	4.32	0.699	--
3.200	4.75	0.931	-9.94
Cucumis sativus (cucumber)			
Control (0)	4.59	0.927	--
3.200	5.32	1.319	-15.81
Lactuca sativa (lettuce)			
Control (0)	0.84	0.311	--
3.200	0.97	0.201	-16.19
Lycopersicon esculentum (tomato)			
Control (0)	2.23	0.391	--
3.200	2.62	0.422	-17.81
Zea mays (Maize)			
Control (0)	7.58	1.295	--
3.200	7.88	1.589	-3.96
Allium cepa (onion)			
Control (0)	0.19	0.041	--
3.200	0.21	0.043	-14.30

II. CONCLUSIONS

Vigour Test :

It can be concluded that PROBLAD PLUS at the limit application rate had no significant effects on mortality, or on shoot dry weight, and it has a slight, but statistically significant, effect on shoot height (lettuce) but effect was lower than 25% (a 6% decrease versus mean control height). There were no notable phytotoxic symptoms or plant growth stage.

The ER₂₅ and ER₅₀ values for mortality, phytotoxicity, shoot height and shoot dry weight were nominally > 3.2 L test item/ha.

Seedling Emergence Test:

It can be concluded that PROBLAD PLUS at the limit application rate had no significant effects on emergence, on mortality, growth stage, on shoot height and on shoot dry weight. There were no notable phytotoxic symptoms or plant growth stage.

The ER₂₅ and ER₅₀ values for emergence, mortality, phytotoxicity, shoot height and shoot dry weight were nominally > 3.2 L test item/ha.

Table 9.6.2-9: Endpoints from the seedling emergence and vegetative vigour tests considering all parameters assessed (based on nominal rates)

Family	Species	Common Name	PROBLAD PLUS [L/ha]			
			NOER	LOER	ER ₂₅	ER ₅₀
Dicotyledonous species						
Brassicaceae	Brassica napus	Oilseed rape	≥ 3.200	> 3.200	> 3.200	> 3.200
Cucurbitaceae	Cucumis sativus	Cucumber	≥ 3.200	> 3.200	> 3.200	> 3.200
Asteraceae	Lactuca sativa	Lettuce	--	3.200	> 3.200	> 3.200
Solanaceae	Lycopersicon esculentum	Tomato	≥ 3.200	> 3.200	> 3.200	> 3.200
Monocotyledonous species						
Poaceae	Zea mays	Maize	≥ 3.200	> 3.200	> 3.200	> 3.200
Amaryllidaceae	Allium cepa	Onion	≥ 3.200	> 3.200	> 3.200	> 3.200

Huerta, F. (2020).

HSE COMMENTS :

This study has been conducted in accordance with GLP and follows OECD 208 and OECD 227 guidelines with no major deviations. The validity criteria have been met.

There are a couple of minor guideline deviations. Firstly, in both OECD 208 and OECD 227, the recommended light intensity is $350 \pm 50 \mu\text{E}/\text{m}^2/\text{s}$. In this study the light intensity ranged from $374.2 - 421.1 \mu\text{E}/\text{m}^2/\text{s}$ thus slightly exceeding the upper limit of the guideline recommendation. Since the validity criteria were met and there were no unexpected effects on the control plants, this deviation is considered to be minor and to not have affected the study outcome.

Additionally, the OECD 227 guideline states that a reference test may be conducted at regular intervals to verify the performance of the test. No reference test has been conducted, nor are any historical control biomass or growth measurement results reported here to aid in evaluating the performance of the test system. However, a reference test is not an explicit guideline requirement and the study validity criteria were met, so this omission is not cause to invalidate the study.

The study was conducted as a limit test with one test item concentration of 3.200 L/ha, which is the maximum recommended application rate of PROBLAD PLUS (equivalent to 4.016 kg/ha). Statistical evaluation of the ER_{50} value was therefore not possible but since $< 50\%$ effects occurred at the limit dose, the ER_{50} was estimated to be $> 3.200 \text{ L/ha}$. No observations of phytotoxic effects were made for any of the tested species at the limit dose.

Analytical verification of the spray solution has confirmed that the target rate was achieved, since the recovery of the test item was 102% of the nominal value. The validity of the analytical method has been considered in Volume 3 CA B5. The following conclusion was reached: "The analytical method for the determination of PROBLAD PLUS in W4 can be considered fully validated in accordance with SANTE/2020/12830 rev. 1".

The endpoint suitable for use in risk assessment is:

- ER_{50} (emergence, mortality, phytotoxicity, shoot height and shoot dry weight; all tested species): $> 3.200 \text{ L PROBLAD PLUS/ha}$.

B.9.7. Effects on other terrestrial organisms (flora and fauna)

A literature study has been provided investigating the bactericide-like effects of *Lupinus* alkaloids.

Reference:	K-CA 8.7/01
Report Title:	Bactericide-like effect of <i>Lupinus</i> alkaloids
Author(s) & Year:	de la Vega, R. et al. (1996)
Document No	Industrial Crops and Products 5 (1996) 141-148
Substance used:	<i>Lupinus</i> alkaloids: Lupanine, lupinine, sparteine and gramine
Method of analysis:	No analysis conducted
Guideline(s):	No, study from published literature
Deviations:	N/A, study from published literature
GLP or GEP:	No, study from published literature
Acceptability:	Supplementary
Study relied upon:	Yes, however data protection not applicable to published literature studies

I. MATERIALS AND METHODS

A. MATERIALS

Test Material	Lupanine, lupinine, sparteine and gramine
Lot/Batch #:	Not reported
Purity:	Not reported
Description:	Not reported

Stability of test compound:	Not reported
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Reanalysis/Expiry date:	Not reported
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Density:	Not reported
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Treatments

Test rates:	Not reported
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Reference item:	Not reported
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Solvent/vehicle:	Not reported
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Analysis of test concentrations:	Not reported
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Test organisms

Species:	<i>Pseudomonas syringae</i> P.V. <i>phaseolicola</i> ; <i>Pseudomonas syringae</i> P.V. <i>tomato</i> ; <i>Pseudomonas putida</i> ; <i>Erwinia carotovora</i> var. <i>carotovora</i>
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Source:	n/a
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Test design

Test vessel:	10-mL Petri dishes containing 100 mL test media
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Replication:	10 replicates
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Duration of test:	8 days
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Environmental test conditions

Temperature:	Not reported
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Relative humidity	Not reported
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Photoperiod:	Not reported
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B. STUDY DESIGN AND METHODS

The bactericidal (total inhibition) and bacteriostatic (softened growth in relation with control group) properties of crude alkaloid extracts from *L. albus*, containing mainly lupanine, and crude alkaloid extracts from *L. luteus*, containing mainly lupanine, were tested along with pure alkaloids, sparteine and gramine from a commercial source.

Gramine and sparteine were added separately to 100 mL of melted Ring B medium in order to obtain concentrations of 5, 10, 15, 20, 25, 30, 35 and 40 mM of these alkaloids. In the same way the *L. albus* and *L. luteus* extracts were added to the

medium to obtain the same concentrations (5-40 mM) of lupanine and lupinine, respectively. For this the percentage of lupanine in the *L. albus* alkaloid extract and the percentage of lupinine in the *L. luteus* alkaloid extract were considered, both obtained with the above quantification methodology. Both alkaloid extracts were prepared following the crude alkaloid procedure.

Each 100 mL of medium containing one different concentration was distributed in 10 Petri dishes (10 mL/dish), which were left overnight under UV light.

Therefore, the experiments were carried out with 10 replicates. The Petri dishes were divided into four equal parts. On each part, one of the four bacteria species was inoculated. For sowing the bacteria, 0.01 mL of the culture with 3×10^8 cells/mL (following the McFarlan turbidity scale) was dropped on the Petri dish. The Petri dishes were incubated at 27°C for 8 days and the growth of the colonies was measured in mm at 4 and 8 days.

Statistical analysis was performed with program BMDP 1R of the series BMDP Statistical Software and MLP (maximum likelihood program release 3.08) in a CYBER 380 computer.

II. RESULTS AND DISCUSSION

The results show that, of the quinolizidine lupine alkaloids, lupanine was the most abundant in *L. albus*, *L. mutabilis* and *L. angustifolius*. In *L. albus* multiflorine, albine and sparteine were also present. Sparteine was the second most common in *L. mutabilis* and also 4-OH-lupanine appears in this species. A considerable amount of angustifoline was found in *L. angustifolius*. In all three species isolupanine and 13-OH-lupanine were detected.

In the species *L. luteus* and *L. hispanicus* the dominant alkaloid was lupinine. Also, sparteine was present in the ecotypes of *L. luteus* studied. The alkaloid epilupinine appears only in *L. hispanicus* ssp *bicolor*.

In both subspecies of *L. hispanicus* (ssp *hispanicus* and ssp *bicolor*) the indolic alkaloid, gramine was present. This compound was also found in *L. luteus* but not in the other species of lupins studied. Triptophol was present in the two species analysed.

From all the lupin species studied *L. albus* and *L. luteus* were selected for the experiments with bacteria, because of their high content in lupanine and lupinine respectively.

The response of each type of bacteria to the presence of an alkaloid in the growth medium was significantly different ($P < 0.05$). The observation of the results obtained show four different situations:

- (a) The colony cultivated in a medium with an alkaloid presents the same growth than the control (e.g., *P. putida* incubated with 5 mM of lupanine).
- (b) The colony cultivated in a medium with an alkaloid grows more than the control (e.g., *P. putida* incubated with 10, 15 or 20 mM of sparteine).
- (c) The colony cultivated in a medium with an alkaloid grows less than the control (bacteriostatic effect) (e.g., *P. putida* incubated with 5 mM of lupinine).
- (d) The colony cultivated in a medium with an alkaloid is totally inhibited (e.g., *P. putida* incubated with 10 mM of lupinine).

Lupinine was observed to be the most effective alkaloid to achieve the inhibition of the growth in the four bacteria assayed. At a concentration of 5 mM the growth of the bacteria is completely stopped except for *P. putida*, which required a concentration of 10 mM is needed to stop completely the growth of the colonies.

Considering the possibility of using any of the four alkaloids as a natural bactericide, it can be concluded that lupinine is the alkaloid with more possibilities to control the four types of bacteria studied. Gramine could also be considered effective in the control of *P. phaseolicola* and *P. tomato*. In both cases the growth is completely inhibited in concentrations lower than 10 mM.

III. CONCLUSION

This paper was conducted to assess the effect of exposure to a number of lupine alkaloids against a number of pathogenic bacteria.

Crude alkaloid extracts from *Lupinus albus* and *Lupinus luteus* containing mainly lupanine and lupinine, respectively, were assayed at different concentrations, along with the individual bactericidal effects of sparteine and gramine.

The results indicated that lupinine showed the highest bactericidal effect on the four bacteria species studied, with concentrations between 5 and 10 mM needed to completely stop the bacteria growth. Gramine was also observed to be effective in the control of *P. phaseolicola* and *P. tomato*.

HSE COMMENTS

This study does not conform to standardised test guidelines and is not GLP compliant. The study has been considered with regard to how well it meets common standards of scientific practice, based on 'expert judgement'.

The methodology, study design and statistical analysis are well described and are in accordance with general scientific principles. Limited information is provided on the

test items and no batch number or purity is provided for any tested alkaloids. Bactericidal effects were greatest in the lupinine treatment. *Lupinus albus*, from which the active substance is derived is known to be low in alkaloids, and lupinine was not the dominant alkaloid in *Lupinus albus*. This indicates that residues of *Lupinus albus* extracts may have lower bactericidal effects than residues of other species. However, the results of the study are of limited relevance in the risk assessment of aqueous extract from the germinated seeds of sweet *Lupinus albus*, since only single compounds have been tested, rather than the whole lupin seed extract.

Reliability : Reliable with reservations

(Studies are graded as one of the following categories : 1) Reliable, 2) Reliable with reservations, 3) Unreliable. Categorisation is based on the evaluator's subjective assessment of the quality of the methods, reporting and analysis as presented in the study.)

B.9.8. Effects on biological methods for sewage treatment

No data submitted or required. PROBLAD PLUS has been demonstrated to be readily biodegradable in accordance with OECD 301 and so no effect on aquatic micro-organisms is foreseen.

B.9.9. Monitoring data

No data submitted or required.

B.9.10. Biological activity of metabolites potentially occurring in groundwater

No data submitted or required. No potentially relevant metabolites have been identified.

B.9.11. References relied on

Scientific peer-reviewed open literature report

Literature reviews have been carried out for the active substance aqueous extract from the germinated seeds of sweet *Lupinus albus*. The reviews have been conducted in accordance with Article 8 (5) of assimilated 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 reviews 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 aqueous extract from the germinated seeds of sweet *Lupinus albus*. The dates of the conducted searches are shown in Table 9.11-1.

Table 9.11-1: Dates of the conducted literature searches

Date of initial search for ‘BLAD’ and ‘PROBLAD’. Report CEV/02/01-LRR1	5 May 2016
Updated search following EU RMS evaluation of literature search. Report: CEV/02/01-LRR2	4 June 2018
Literature search for quinolizidine alkaloids. Report: CEV/02/01-LRR3	11 November 2019
Top-up search	22 July 2024

An initial literature review, CEV/02/01-LRR1, was conducted before submission to the EU in 2016. During the evaluation, the active substance was renamed in accordance with the Botanical Guidance (SANCO/11470/2012– rev. 8) and so additional search terms including the botanical name were required in the literature search. Additionally, whilst it was noted that the literature review was acceptable and in accordance with EFSA journal 2011; 9(2):2092, it was found to be incomplete with regards to the ecotoxicology section. A second literature review, CEV/02/01-LRR2, conducted in 2018 aimed to address these deficiencies by including ecotoxicology-specific search terms and the botanical name. A third literature review, CEV/02/01-LRR3, was conducted to identify published literature on quinolizidine alkaloids. A top-up search was requested by HSE to address the gap between the initial literature search in 2018/2019 and 6 months prior to the submission of the dossier in July 2022.

The process of selecting relevant scientific peer reviewed open literature is summarised as follows:

- A search was conducted across scientific source databases (detailed in Table 9.11-2) using the search terms listed below.

- An initial rapid assessment of relevance was made based on title and abstracts.
- If the publication fulfilled the initial assessment of relevance, the full text of the publication was analysed.
- The reliability of relevant studies was considered in accordance with Klimisch et al. (1997).
- Reliable and relevant studies are included in the dossier.

Databases searched

All of the databases searched and the justification, provided by the applicant, for choosing each one has been summarised in the table below. Eleven databases were searched in the CEV/02/01-LRR1 and CEV/02/01-LRR2 and in the top-up search. These searches considered studies published since 2005. Only the eight STN databases were searched in CEV/02/01-LRR3, which considered studies published since 2006. This covers the minimum requirement of ten years prior to the date of the search.

Table 9.11-2: Databases searched and justification for choice

Provider	Database	Justification
STN International	ANABSTR – Analytical abstracts	The Analytical Abstracts database covers worldwide literature on analytical chemistry. Amongst others subject coverage includes Agriculture, Environment, and Food. Sources include journals, books, conference proceedings, reports, and standards. Bibliographic information, indexing terms, abstracts, chemical names, and CAS Registry Numbers are all searchable.
	BIOSIS	BIOSIS Previews® is the largest and most comprehensive life science database in the world. Amongst others, subject coverage includes agriculture, biochemistry, biophysics, botany, environmental biology, physiology, toxicology. Sources include periodicals, journals, conference proceedings, reviews, reports, patents, and short

Provider	Database	Justification
		communications. Nearly 6,000 life source journals, 1,500 international meetings as well as review articles, books, and monographs are reviewed for inclusion. Bibliographic information, indexing terms, abstracts, and CAS Registry Numbers are all searchable.
	CAPLUS Chemical Abstracts Plus	The Chemical Abstracts (CA) database covers all areas of biochemistry, chemistry and chemical engineering, and related sciences. Sources include over 8,000 journals, patents from 38 national patent offices and two international patent organizations, technical reports, books, conference proceedings, and dissertations. Electronic only journals and Web preprints are also covered. Bibliographic terms, indexing terms, roles, CAS Registry Numbers, International Patent Classification, and abstracts are searchable.
	EMBASE Excerpta Medica	The Excerpta Medica 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 for EMBASE include more than 4,000 journals from approximately 70 countries, monographs, conference proceedings, dissertations, and reports.
	MEDLINE	The MEDLINE database is produced by the U.S. National Library of Medicine and contains more than 22.6 million records. The database contains information on all areas of medicine. Sources include journals and chapters in books or symposia.
	RTECS	Registry of Toxic Effects of Chemical Substances contains factual toxicity data for commercially important substances from research and government reports. Coverage includes irritation

Provider	Database	Justification
		data, federal standards and regulations, mutagenicity, tumorigenic effects, acute toxicity and multiple dose toxicity data, carcinogenicity reviews. Sources include journal articles, government reports, and unpublished EPA test submissions (TSCATS).
	SCISEARCH Science Citation Index	Science Citation Index, one of the largest multidisciplinary scientific databases, is an international index to the literature covering virtually every subject area within the broad fields of science, technology, and biomedicine. Records include references from over 5,600 scientific, technical, and medical journals are contained in the database.
	TOXCENTER	Toxicology Center covers the pharmacological, biochemical, physiological, and toxicological effects of drugs and other chemicals. TOXCENTER is composed of the following subfiles: BIOSIS (1969 to date), Caplus (1907 to date), IPA (1970 to date), and MEDLINE (1953 to date). 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. Records contain bibliographic data, abstracts, indexing terms, chemical names, and CAS Registry Numbers.
US National Library of Medicine National Institutes of Health	PUBMED	PubMed is a database comprising more than 21 million citations for biomedical literature from MEDLINE, life science journals, and online books. Citations may include links to full-text content from PubMed Central and publisher web sites.

Provider	Database	Justification
Elsevier BV	ScienceDirect	ScienceDirect is a data base of scientific literature published by Elsevier B.V. This database contains more than 10 million journal articles and book chapters from more than 2500 journals and 11,000 books.
John Wiley and Sons Inc	Wiley Online Library	Wiley Online Library features 1500 journals, over 13,000 Online Books, and hundreds of multi-volume reference works, databases and other resources

Databases used in the top-up search are summarised in the table below.

Table 9.11-3: Databases used in top-up search and justification for choice

Database	Justification
AGRICOLA Agriculture Online Access	Agricola is a multidisciplinary database with worldwide coverage of agriculture and related fields, including Agriculture, Animal Science, Chemistry, Entomology, Food Science, Forestry, Genetics, Life Sciences, Natural Resources, Nutrition, Pesticides, Plant Diseases, Soil Science etc., with over 8.2 million records. Sources include bibliographies, serial articles, book chapters, monographs, computer files, serials, maps, audiovisuals, reports, catalogs and chemical libraries from suppliers worldwide. Records contain bibliographic information, geographic terms, controlled terms, and supplementary terms that include GenBank Numbers from 1979 to date.
BIOSIS BIOSIS Previews	BIOSIS Previews® is a multidisciplinary database with worldwide coverage of research on all biological and biomedical topics, including Agriculture, Anatomy, Behaviour, Biochemistry, Botany, Cell Biology, Environmental Biology, Experimental Clinical Medicine, Genetics, Immunology, Microbiology, Pathology, Pharmacology, Physiology, Toxicology etc., with over 27.8 million records. Sources include more than 5,000 journals, U.S. patents, reports, meeting abstracts and papers, reviews and books. Records contain bibliographic data, indexing information, and abstracts from 1926 to date.

CABA CAB Abstracts	CAB Abstracts is a multidisciplinary database with coverage that includes Agriculture, Agricultural chemicals (fertilizers, pesticides, and veterinary pharmaceuticals), Animal sciences and production, Crop protection, Crop sciences and production, Environment, Food science and technology, Forestry, Genetics, Human medicine (fungal, parasitic, diseases and diseases caused or, transmitted by insects), Human nutrition, Soils and fertilizers, etc., with over 11.1 million records. Sources include over 9,000 serial journals, review journals, symposia, annual reports conference proceedings, general reports newsletters, books discussion papers, handbooks theses and bulletins. Records contain bibliographic information, abstracts, and indexing information, including CAS Registry Numbers® from 1973 to date.
EMBASE	Embase™ is a multidisciplinary database with coverage that includes Biological science relevant to human medicine, Biochemistry, Clinical and experimental medicine, Drugs, Environmental science, Pharmacology and drug therapy, Public health etc., with over 34.4 million records. Sources include over 8,500 journals, including MEDLINE® titles, books, conference proceedings and reports. Records contain bibliographic information, controlled terms, drug trade names and their manufacturers, medical device trade names and manufacturers, abstracts and CAS Registry Numbers® from 1947 to date.
FSTA Food Science and Technology Abstracts®	Food Science and Technology Abstracts® is a database that covers all areas of food sciences including Biotechnology, Hygiene and toxicology, Physiology, Plant pathology, Soils, etc., with over 1.79 million records. Sources for FSTA include about 4,500 journals, books, conference proceedings, reports, patents, pamphlets, legislation and dissertations. Bibliographic information, indexing terms and abstracts from 1969 to date.
HCAPLUS Chemical Abstracts Plus	Chemical Abstracts Plus is a database that covers areas of Analytical chemistry, Applied chemistry, Biochemistry, Chemical engineering, Macromolecular chemistry and Organic chemistry with over 62.2 million records. Sources include thousands of journals, patents, conference proceedings, electronic-only journals, books, dissertations, reviews, technical disclosures, web pre-prints, meeting abstracts. Records contain bibliographic information, indexing, available abstracts and claims from worldwide patent authorities from 1907 to date, with over 180,000 pre-1907 records.

MEDLINE MEDlars onLINE	MEDLINE is a database that covers all areas in the broad field of biomedicine. Sources include about 5,300 journals, citations from Index Medicus, index to Dental Literature, HealthSTAR database, International Nursing Index, OLDMEDLINE, data from NLM's Cumulated Index Medicus and from Current List to Medical Literature. Bibliographic information, abstracts, and CAS Registry Numbers from 1946 to date.
NTIS National Technical Information Service	Geological Reference File is a multidisciplinary database covering subjects as Agriculture and food, Atmospheric sciences, Chemistry, Environmental pollution and control, Medicine and biology, Natural resources and earth sciences etc., with over 3 million records. Sources include reports and publications on research, development, and engineering projects, sponsored by U.S. and non-U.S. governments. Records contain bibliographic information, abstracts and indexing terms from 1964 to date.
PQSCITECH ProQuest Science & Technology	ProQuest Science & Technology is a multidisciplinary database covering subjects as AIDS & cancer research, Agrology, Animal behaviour, Bacteriology, Biological membranes, Biotechnology (agricultural, medical, environmental, marine & pharmaceutical), Ecology, Entomology, Genetics (plant, animal, & human), Health & safety science, Human genome research, Immunology, Microbiology, Molecular biology, Mycology, Neurosciences, Protozoology, Risk assessment, Toxicology, Virology, Zoology etc., with over 34.3 million records. Sources include journals, patents (until 2016), conferences and books. Records contain bibliographic information, abstracts and indexing terms from 1962 to date.
SCISEARCH Science Citation Index	Science Citation Index is a multidisciplinary database covering subjects as Agriculture, Pharmacology, Anatomy, Environmental sciences, Genetics, Plant sciences, Immunology, Biology, Reproductive systems, Biotechnology, Chemistry, Medicine, Neuroscience, Ecology, Oncology, Zoology etc., with over 47.7 million records. Sources include approximately 8,600 scientific, technical, and medical journals, patents (until 2016), conferences and books. Records contain bibliographic information, abstracts, author keywords, and KeyWords Plus® from 1974 to date.

TOXCENTER Toxicology Center Database	Toxicology Center Database is a multidisciplinary database covering subjects as Air Pollution, Animal Venom, Antidotes, Carcinogenesis via Chemicals, Chemically Induced Diseases, Drug Evaluations, Environmental Pollution, Food Contamination, Mutagenesis, Occupational Hazards, Pesticides and Herbicides, Toxicological Analysis etc., with over 16.2 million records. 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. Records contain bibliographic information, abstracts, and indexing information, including CAS Registry Numbers® from 1907 to date.
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Search parameters

Patent literature was not considered to be relevant to the results of this search, so the results were filtered to remove these from by limiting the output to non-patent document types.

CEV/02/01-LRR1, 2016:

For the search conducted in 2016, the following search terms for substances were used:

For the ScienceDirect, Pubmed and Wiley Online Library platforms the terms 'BLAD', 'PROBLAD', 'PROBLAD PLUS' and the CAS number '1219521-95-5', lupinene, β -coaglutin and vicilin were used. A search was performed on lupinene, β -coaglutin and vicilin since they are closely related proteins to BLAD (the lead component). In order to refine the large number of articles identified, the additional search terms below were included:

Tox OR hazard OR adverse OR health OR NOAEL OR NOEL OR LOAEL OR LOEL OR BMD OR "vivo" OR "vitro" or 'storage stability' OR storage OR stability OR metabolic OR metabolism OR degradation OR breakdown OR 'residues' OR residue OR 'processing' OR hydrolysis OR rotation OR plant OR crop OR feed OR animal OR livestock OR hen OR cattle OR ruminant OR goat OR cow OR pig OR 'risk assessment' OR consume OR exposure or 'soil' OR 'water' OR 'air' OR environment OR fate OR endocrine disrupt OR bioaccumulation OR biomagnification OR bioconcentration OR poison OR effect.

A large number of articles present from the search term 'BLAD' were actually from the word 'bladder' and had no relation to the active substance 'BLAD'. An additional field was therefore applied to the search.

NOT bladder NOT Urinary NOT incontinence NOT Pelvic floor NOT Urethral NOT urology NOT urological NOT urinary tract NOT renal.

For the STN databases, the terms 'BLAD', 'PROBLAD', 'PROBLAD PLUS' and the CAS number '1219521-95-5' were used. A further search on 'lupinene', 'β-coaglutin' and 'vicilin' was performed. A large number of articles were produced in the Wiley online search, so further refinement was conducted using the following search terms:

Tox OR hazard OR adverse OR health OR NOAEL OR NOEL OR LOAEL OR LOEL OR BMD OR vivo OR vitro or storage stability OR storage OR stability OR metabolic OR metabolism OR degradation OR breakdown OR residues OR residue OR processing OR hydrolysis OR rotation OR plant OR crop OR feed OR animal OR livestock OR hen OR cattle OR ruminant OR goat OR cow OR pig OR risk assessment OR consume OR exposure or soil OR water OR air OR environment OR fate OR endocrine disrupt OR bioaccumulation OR biomagnification OR bioconcentration OR poison OR effect.

CEV/02/01-LRR2, 2018:

Following the change of active substance name during the EU assessment, the revised literature search was conducted with the following substance search terms for all databases: 'Lupinus albus', 'Lupinus albus (sweet lupin) seeds', 'Sweet lupine', 'Sweet lupin', 'Lupines', and 'Lupinus albus seeds'.

In addition, a further search using the terms from the initial search was conducted, but the following ecotoxicology search terms were included for refinement of the results of the 'BLAD', 'β-coaglutin' and 'vicilin' searches:

'AND birds OR mammals OR reptiles OR amphibians OR fish OR fishes OR daphnia OR algae OR bees OR arthropods OR soil organisms OR terrestrial plants OR effects on vertebrates birds, mammals OR feed OR diet OR seeds OR soil microorganisms OR vertebrates OR broiler chickens OR bacteria OR nitrogen transformation OR residues'.

CEV/02/01-LRR3, 2019:

For each substance identified, the search terms were chemical name, other names, IUPAC name and CAS number, if available. CAS numbers were also used to search registry fields. If CAS numbers were not already known, a chemical structure search in the STN Registry file was used to determine registry numbers for this search.

Search terms for the quinolizidine alkaloids:

Table 9.11-3 4: **Search terms used in CEV/02/01-LRR3, 2019**

Code number / name	Chemical name, other names, IUPAC name and CAS number
Lupanine	(1S,2R,9S,10S)-7,15-diazatetracyclo[7.7.1.02,7.010,15]heptadecan-6-one 550-90-3
13 α -OH-lupanine	4356-43-8
Lupinine	486-70-4
Sparteine	90-39-1

Top-up search:

A single concept search was carried out for the search terms used in the June 2018 literature search and November 2019 literature search. No endpoint specific search terms were used. Patent literature was not considered to be relevant to the results of the search. The search was therefore filtered to non-patent document types.

Relevance and reliability

The relevance of literature studies has been 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 and not another. Specific criteria for the assessment of relevance from an ecotoxicology perspective were defined as follows:

1. Well defined test material
2. Relevant test species used
3. Relevance to standard test guidelines

The initial check of relevance was conducted by reference to the study titles and, if necessary, the abstracts. Studies deemed to meet the relevance criteria, or be of unknown relevance following review of the abstract, were then obtained as full papers for assessment of relevance in detail, and their reliability. The reliability of the

study has been defined as the inherent quality of a study, thus the criteria will always be the same in whatever framework reliability is evaluated, the reporting quality of the methodology, experimental procedure, and results (i.e. free from bias, findings reflect true facts), and the reproducibility of the study. The reliability of studies deemed to be relevant was conducted according to Klimisch et al. 1997.

For the top-up search, the following relevance criteria were employed:

1. Well defined test material (including purity/content).
2. Relevant test species (Mammalian related studies are evaluated by toxicology. For the risk assessment of birds, fish, aquatic invertebrates or macrophytes, sediment dwelling organisms, algal growth, arthropods, soil meso- and macrofauna and non-target plants – all species are evaluated).
3. Well defined application method and rates, and exposure route is clearly defined and environmentally relevant.
4. Effects are related to single test item, and a quantitative relationship exists between the reported endpoint and risk assessment endpoints.
5. Study conditions should not differ significantly from recommended protocols.
6. Relevant endpoints for ecotoxicological risk assessment.
7. The article contains adequate information about the persistency or bioconcentration of the substance of concern.
8. The article contains adequate information about the toxicity of the substance of concern to non-target organisms living in the environment.
9. The publication represents field observations on individual mortalities of terrestrial vertebrates related to the use of the substance of concern.
10. The article contains adequate information on the effect of the substance of concern on biological activity used for sewage clearance.
11. The article reports on residues of the substance of concern in plant moieties adequate for the assessment of the exposure of bees.
12. The article provides information that elucidates the mechanism of effects or explores on the environmental behaviour of the substance of concern.
13. The article's relevance for the hazard assessment or risk characterization clear after full-text analysis.

Results

The results of the search method employed in the three searches are presented in the tables below.

Table 9.11-4-5: Results of the study selection process for BLAD and PROBLAD PLUS CEV/02/01-LRR1

Database/Platform:	STN	Pubmed	Science Direct	Wiley online library
Date of the search:	05/05/2016	05/05/2016	05/05/2016	05/05/2016
Date span of the search:	2006-Present	2006-Present	2006-Present	2006-Present
Date of the latest database update includes in the search:	05/05/2016	05/05/2016	05/05/2016	05/05/2016
Total number of summary records retrieved:	56	56	898	2635
Total number of summary records retrieved after removing duplicates from all searches	2556			
Number of articles excluded from search results after rapid assessment for relevance	2554			
Total number of full text documents assessed in detail	2			
Number of studies excluded after detailed assessment	0			
Number of studies to be included in the dossier	2			

Table 9.11-5 6: Results of the study selection process for further search
CEV/02/01-LRR1

Database/Platform:	STN	Pubmed	Science Direct	Wiley online library
Date of the search:	05/05/2016	05/05/2016	05/05/2016	05/05/2016
Date span of the search:	2006-Present	2006-Present	2006-Present	2006-Present
Date of the latest database update includes in the search:	05/05/2016	05/05/2016	05/05/2016	05/05/2016
Total number of summary records retrieved:	228	154	775	591
Total number of summary records retrieved after removing duplicates from all searches	1303			
Number of articles excluded from search results after rapid assessment for relevance	1278			
Total number of full text documents assessed in detail	25			
Number of studies excluded after detailed assessment	22			
Number of studies to be included in the dossier	2 (both of which appeared in previous BLAD/PROBLAD PLUS search)			

Two studies were identified as relevant to the dossier but were not relevant to the ecotoxicology section.

Table 9.11-6 7: Results of the study selection process for CEV/02/01-LRR2

Database/Platform:	STN	Pubmed	Science Direct	Wiley online library
Date of the search:	04/06/2018	04/06/2018	04/06/2018	04/06/2018
Date span of the search:	01/01/2006 – 31/12/2018	01/01/2006 – 31/12/2018	01/01/2006 – 31/12/2018	01/01/2006 – 31/12/2018
Date of the latest database update includes in the search:	04/06/2018	04/06/2018	04/06/2018	04/06/2018
Total number of summary records retrieved:	798	1340	5226	1623
Total number of summary records retrieved after removing duplicates from all searches	4185			
Number of articles excluded from search results after rapid assessment for relevance	4171			
Total number of full text documents assessed in detail	14			
Number of studies excluded after detailed assessment	2			
Number of studies to be included in the dossier	12			

All of the twelve studies identified were relevant to the ecotoxicology section. These are summarised in the table below.

Table 9.11-7-8: Results to be included in the dossier CEV/02/01-LRR2

Author	Year	Title	Reference	Full text reviewed	Meet Relevance/Reliability Criteria		Comments
				Y or N	Y, N or N/A	Result of reliability/relevance assessment/ justification if not reliable	
Viveros, A.; Centeno, C.; Arijia, I.; Brenes, A.	2007	Cholesterol-lowering effects of dietary lupin (<i>Lupinus albus</i> var multolupa) in chicken diets	Poultry Science 86: 2631-2638	Y	Y	Klimisch score 3	In the study, the effects of different concentrations of lupin seeds (0, 200 and 400g/kg) in chicken diets on performance and biochemical factors was assessed. In the study no adverse signs of toxicity were reported for birds receiving lupin seed in their diet other than growth reduction which was attributed to low palatability of seeds. The conclusion indicated that lupin seed intake may be effective in lowering the cholesterol absorption and may have a potential application as a cholesterol-reducing agent.

							This Paper is discussed in MCA Section 8.
Not stated	2009	Criteria for safe use of plant ingredients in diets for aquaculture fish*	Opinion of the Panel on Animal feed of the Norwegian Scientific Committee for Food Safety*	Y	Y	Klimisch score 3	The Norwegian food safety committee concluded sweet lupin meal may be included in diets for rainbow trout up to 30-40% of the total diet without significantly influencing growth and nutrient utilisation. This Paper is discussed in MCA Section 8.
Yones, A. M. A. M.	2010	Effect of lupin kernel meal as plant protein source in diets of red hybrid tilapia (<i>Oreochromis niloticus</i> x <i>O. mossambicus</i>) on growth performance and nutrient utilisation*	African Journal of Biological Science 6:(1) 1-16*	Y	Y	Klimisch score 3	Lupin meal was added to tilapia diets and was shown to be utilised efficient with diets containing up to 50% lupin meal. This Paper is discussed in MCA Section 8.

Molina-Poveda, C.; Lucas, M.; Jover, M.	2013	Evaluation of the potential of Andean lupin meal (<i>Lupinus mutabilis</i> Sweet) as an alternative to fish meal in juvenile <i>Litopenaeus vannamei</i> diets	Aquaculture 410-411: 148-156	Y	Y	Klimisch score 3	The study showed no significant effect on growth when diet of the shrimp included up to 50% lupin meal. At 75 and 100% lupin meal content growth rate resulted in a lower growth rate. Mortality in the study was low (< 20%) and was not shown to be affected by the treatments. This Paper is discussed in MCA Section 8.
Borquez, A.; Serrano, E.; Dantagnan, P.; Carrascosa, J.; Hernandez, A.	2010	Feeding high inclusion of whole grain white lupin (<i>Lupinus albus</i>) to rainbow trout (<i>Oncorhynchus mykiss</i>); effects on growth nutrient digestibility, live and intestine	Aquaculture Research 42:(8) 1067-1078	Y	Y	Klimisch score 3	Study used to highlight that lupin is a suitable fish food and so poses low toxicity to fish. The results indicated that lupin could be included up to 50% of the fish diet without negative effects on growth. This Paper is discussed in MCA Section 8.

		histology and muscle fatty acid composition					
Kaczmarek, S. A.; Hejdysz, M.; Kubis, M.; Rutkowski, A.	2016	Influence of graded inclusion of white lupin (<i>Lupinus albus</i>) meal on performance, nutrient digestibility and intestinal morphology of broiler chickens	British Poultry Science 57:(3) 364-374	Y	Y	Klimisch score 3	The study was conducted with 480 broiler chick. Six treatment diet groups consisting of 100, 150, 200, 250 and 300g lupin meal/kg of white lupin meal. The birds were fed the experimental diets over 35 days after which the condition of the birds were assessed. Birds fed a diet consisting of 200 and 300g lupin meal/kg diet had a lower body weight gain than the control birds and this also correlated with a lower feed intake rate compared to the control. This Paper is discussed in MCA Section 8.
Elgharbaly, A.; Marschner, P.	2011	Microbial activity and biomass and N and P availability in a saline sandy loam	European Journal of Soil Biology 47:(5) 310-315	Y	Y	Klimisch score 3	The study evaluated the effects on the addition of lupin residues on soil microbial action and microbial biomass. This Paper is discussed in MCA Section 8.

		amended with inorganic N and lupin residues					
Zhang, Y.; Øverland, M.; Xie, S.; Dong, Z.; Lv, Z.; Xu, J.; Storebakken, T.	2012	Mixtures of lupin and pea protein concentrates can efficiently replace high-quality fish meal in extruded diets for juvenile black sea bream (<i>Acanthopagrus schlegelii</i>)	Aquaculture 354-355: 68-74	Y	Y	Klimisch score 3	The maximum lupin concentration consisted of 50% lupin protein in the fish diet. After 60 days, the average weight gain of the fish was not shown to have been affected negatively by the inclusion of the lupin diet. This Paper is discussed in MCA Section 8.
Ephrem, N.; Tegegne, F.; Mekuria, Y.	2015	Nutrient intake, digestibility and growth performance of Washera lambs	Small Ruminant Research 130: 101-107	Y	Y	Klimisch score 3	The study evaluated the effects of supplementing a hay based diet with sweet lupin on the growth of lambs. In the treatments sweet lupin was added up to 245g and was shown to significantly improve feed conversion efficiency.

Yeheyis , L.		supplemented with graded levels of sweet blue lupin (<i>Lupinus angustifolius</i> L.) seed					This Paper is discussed in MCA Section 8.
Kim, J. C.; Heo, J. M.; Mullan, B. P.; Pluske, J. R.	2012	Performance and intestinal responses to dehulling and inclusion level of Australian sweet lupins (<i>Lupinus angustifolius</i> L.) in diets for weaner pigs	Animal Feed Science and Technology 172:(3-4) 201-209	Y	Y	Klimisch score 3	The study concluded that inclusion of sweet lupins up to 240g/kg diet without negative effect on pig growth or digestion. This Paper is discussed in MCA Section 8.
Ravindr an, G.; Ravindr an, V.; Bryden, W. L.	2006	Total and ileal digestible tryptophan contents of feedstuffs for	Journal of the Science of Food and Agriculture 86: 1132-1137	Y	Y	Klimisch score 3	The study looked at the effect of adding lupin to the diet of broiler chickens in terms of their condition and digestibility of the diet. This Paper is discussed in MCA Section 8.

		broiler chickens					
Volek, Z.; Maroun ek, M.	2009	Whole white lupin (<i>Lupinus albus</i> cv. Amiga) seeds as a source of protein for growing-fattening rabbits	Animal Feed Science and Technology 152:(3-4) 322-329	Y	Y	Klimisch score 3	Study provides supporting evidence of the low toxicity of lupin to mammals. Rabbits were fed a diet containing 150g sweet lupin and no effect on growth or condition were noted. This Paper is discussed in MCA Section 8.

* References not found in search but were referenced within papers reviewed within the search. These articles were thought to be relevant and therefore included in this literature review

Table 9.11-8-9: Results of the study selection process for the CEV/02/01-LRR3 search

Total number of summary records retrieved from search	1418
Total number of summary records retrieved after removing duplicates from all database searches	1169
Number of summary records excluded after rapid assessment for relevance (by title/abstract)	1163
Number of publications excluded after detailed assessment of full-texts (i.e. not relevant or not sufficiently reliable)	3
Number of publications not excluded after detailed assessment and included in the dossier	3

The studies that were excluded are summarised in the table below, along with the reason for not including them in the dossier.

Of the three studies that were included in the dossier, two were relevant to the ecotoxicology assessment and have been included in the Volume 3CA B9. These are summarised in the table below:

Table 9.11-9 10: Results to be included in the dossier CEV/02/01-LRR3

Data requirement	Author	Year	Title	Source
CA 8.2.3/01	Serrano, E.; Storebakken, T.; Borquez, A.; Penn, M.; Shearer, K. D.; Dantagnan, P.; Mydland, L. T.	2011	Histology and growth performance in rainbow trout (<i>Oncorhynchus mykiss</i>) in response to increasing dietary concentration of sparteine, a common alkaloid in lupins	Aquaculture Nutrition (2011), 18(3), 313-320

CA 8.2.3/02	Serrano, E.; Storebakken, T.; Penn, M.; Landsverk, T.; Hansen, J.O.; Mydland, L. T. Editor(s): Palta, J. A.; Berger, J. D.	2008	Responses in rainbow trout (<i>Oncorhynchus mykiss</i>) to increasing dietary dose of lupinine alkaloid.	Lupins for health and wealth. Proceedings of the 12th International; Lupin Conference, Fremantle, Western Australia, 14-18 September 2008 (2008), pp. 94-99, 23 refs.
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Table 9.11-11: Summary of the top-up search results following rapid assessment

	Number	Justification
Total number of summary records retrieved from search(after automatic STN duplicates removal) ¹	839	-
Total number of summary records retrieved after manually removing duplicates from all database searches ²	594	-
Number of summary records excluded after rapid assessment for relevance (by title/abstract)	594	-
Number of publications excluded after detailed assessment of full-texts (i.e. not relevant or not sufficiently reliable)	0	N/A
Number of publications not excluded after detailed assessment and included in the dossier	0	N/A
Number of additional publications included in the dossier as supporting information	0	N/A

¹ Duplicate removal was performed within STN, removing duplicates that fully match between the databases searched.

² Additional duplicates between databases and searches were manually removed prior to rapid assessment.

No publications were deemed relevant based on their titles or abstracts and thus no detailed assessment was conducted or required.

Conclusions

A variety of databases have been searched and the search criteria applied are considered to be suitable. Reliable and relevant literature has been presented in the dossier for evaluation. HSE agrees with the exclusion of additional literature based on guidance in EFSA journal 2011. However the literature search does not span the 10 year period prior to submission. The latest literature search was conducted in 2019 and the dossier was submitted to HSE in 2021. Further information may be requested from the applicant. 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.

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
K-CA 8.1.1.1/01	Arslan, C. & Seker, E.	2002	The effects of processed white lupin seeds (<i>Lupinus albus</i> L.) on growth performance of Japanese quail. Revue Méd.Vét. 153, 10, 643-646.	Y	N	N/A	N/A	N/A
K-CA 8.1.1.1/02	Rubio, L et al.	2003	Effects of feeding growing broiler chickens with practical diets containing sweet lupin (<i>Lupinus angustifolius</i>) seed meal. British Poultry Science. Volume 44, Issue 3	Y	N	N/A	N/A	N/A

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
K-CA 8.1.1.1/03	Viveros, A. et al.	2007	Cholesterol-lowering effects of dietary lupin (<i>Lupinus albus</i> var Multolupa) in chicken diets . Poultry Science 86:2631-2638 doi: 10.3382/ps.2007-00128	Y	N	N/A	N/A	N/A
K-CA 8.1.1.1/04	Kaczmarek, S.A. et al.	2016	Influence of graded inclusion of white lupin (<i>Lupinus albus</i>) meal on performance, nutrients digestibility and intestinal morphology of broiler chickens. British Poultry Science, DOI: 10.1080/00071668.2016.1171295	Y	N	N/A	N/A	N/A

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
K-CA 8.1.1.1/05	Ravindran, G. et al.	2006	Total and ileal digestible tryptophan contents of feedstuffs for broiler chickens. J Sci Food Agric 86:1132-1137 (2006)	Y	N	N/A	N/A	N/A
K-CA 8.1.1.1/06	Olkowski, B. I. et al.	2005	Feeding High Levels of Lupine Seeds to Broiler Chickens: Plasma Micronutrient Status in the Context of Digesta Viscosity and Morphometric and Ultrastructural Changes in the Gastrointestinal Tract. Poultry Science 84:1707–1715	Y	N	N/A	N/A	N/A

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
K-CA 8.1.2.2/01	Ephrem, N. et al.	2015	Nutrient intake, digestibility and growth performance of Washera lambs supplemented with graded levels of sweet blue lupin (<i>Lupinus angustifolus</i> L.) seed. Small Ruminant Research 130 (2015) 101-107.	Y	N	N/A	N/A	N/A
K-CA 8.1.2.2/02	Kim, J. C.	2012	Performance and intestinal responses to dehulling and inclusion level of Australian sweet lupins (<i>Lupinus angustifolius</i> L.) in diets for weaner pigs. Animal feed science and technology. 201-209 172.	Y	N	N/A	N/A	N/A

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
K-CA 8.1.2.2/03	Volek, Z. & Marounek, M.	2009	Whole white lupin (<i>Lupinus albus</i> cv. Amiga) seeds as a source of protein for growing-fattening rabbits. Animal Feed Science and Technology 152 (2009) 322–329	Y	N	N/A	N/A	N/A
K-CA 8.2.1/01	[REDACTED]	2011	Assessment of toxic effects of PROBLAD on Rainbow Trout (<i>Oncorhynchus mykiss</i>) (Teleostei, Salmonidae). Company report no. S10-02621 [REDACTED] GLP, Unpublished	Y	Y	Data for first approval Not used in risk assessment, supporting information only	CEV	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
K-CA 8.2.2/01	Zhang, Y. et al.	2012a	Optimal inclusion of lupin and pea protein concentrates in extruded diets for rainbow trout (<i>Oncorhynchus mykiss</i>). Aquaculture 344-349 (2012) 100–113	Y	N	N/A	N/A	N/A
K-CA 8.2.2/02	Hemre et al.	2009	Criteria for safe use of plant ingredients in diets for aquacultured fish (Opinion of the Panel on Animal Feed of the Norwegian Scientific Committee for Food Safety) Note that the present summary includes details on lupins. The paper also analyses the effect of the use of soybean, pea, canola, sunflower, cotton seed, wheat gluten, potato protein. 1365-2109 in Aquaculture Research, 2010, 1-12	N	N	N/A	N/A	N/A

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
K-CA 8.2.2/03	Yones, A. M.	2010	Effect of lupin kernel meal as plant protein source in diets of Red hybrid tilapia (<i>Oreochromis niloticus</i> x <i>O. mossambicus</i>) on growth performance and nutrients utilisation. African J. Biol. Sci., 6 (1) : 1-16 (2010) ISSN 1687-4870	Y	N	N/A	N/A	N/A
K-CA 8.2.2/04	Molina-Poveda, C. et al.	2013	Evaluation of the potential of Andean lupin meal (<i>Lupinus mutabilis</i> Sweet) as an alternative to fish meal in juvenile <i>Litopenaeus vannamei</i> diets. Aquaculture 410-411 (2013) 148-156	Y	N	N/A	N/A	N/A

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
K-CA 8.2.2/05	Borquez et al.,. 2010.	2010	Feeding high inclusion of whole grain white lupin (<i>Lupinus albus</i>) to rainbow trout (<i>Oncorhynchus mykiss</i>) : effects on growth, nutrient digestibility, liver and intestine histology and muscle fatty acid composition. 1365-2109 in Aquaculture Research, 2010, 1-12.	Y	N	N/A	N/A	N/A
K-CA 8.2.2/06	Zhang, Y. et al.	2012b	Mixtures of lupin and pea protein concentrates can efficiently replace high-quality fishmeal in extruded diets for juvenile black sea bream (<i>Acanthopagrus schlegeli</i>) Aquaculture 354-355 (2012) 68-74	Y	N	N/A	N/A	N/A

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
K-CA 8.2.2/07	Farhangi, M. & Carter, C. G.	2001	Growth, physiological and immunological responses of rainbow trout (<i>Oncorhynchus mykiss</i>) to different dietary inclusion levels of dehulled lupin (<i>Lupinus angustifolius</i>) Aquaculture Research, 2001, 32 (Suppl. 1), 329-340	Y	N	N/A	N/A	N/A
K-CA 8.2.2/08	Glencross, B. D.	2001	Feeding lupins to fish : A review of the nutritional and biological value of lupins in aquaculture feeds. P. 126. Department of Fisheries—Research Division, Government of Western Australia	N	N	N/A	N/A	N/A

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
K-CA 8.2.3/01	Serrano, E. et al.	2011	Histology and growth performance in rainbow trout (<i>Oncorhynchus mykiss</i>) in response to increasing dietary concentration of sparteine, a common alkaloid in lupins Aquaculture Nutrition, doi: 10.1111/j.1365-2095.2011.00899.x	Y	N	N/A	N/A	N/A
K-CA 8.2.3/02	Serrano, E. et al.	2008	Responses in rainbow trout (<i>Oncorhynchus mykiss</i>) to increasing dietary dose of lupinine alkaloid J.A. Palta and J.B. Berger (eds). 2008. 'Lupins for Health and Wealth' Proceedings of the 12 th International Lupin Conference ISBN 0-86476-153-8	Y	N	N/A	N/A	N/A

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
K-CA 8.2.4.1/01	Weber, K.	2011	Assessment of Toxic Effects of PROBLAD on <i>Daphnia magna</i> using the 48 h Acute Immobilisation Test Company report no. S10-02622 Eurofins Agrosciences Services GmbH GLP, Unpublished	N	Y	Data for first approval Not used in risk assessment	CEV	N
K-CA 8.2.4.1/02	Gerke, A.K. and Schneider, S.Z.	2019	PROBLAD PLUS: A 48-hour static-renewal acute toxicity test with the cladoceran (<i>Daphnia magna</i>). Company report no. 896A-101 Eurofins EAG Agroscience, LLC, USA GLP, Unpublished	N	Y	Data for first approval	CEV	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
K-CA 8.2.5.1/01	Gerke, A.K. and Schneider, S.Z.	2019	PROBLAD PLUS: A semi-static life-cycle toxicity test with the cladoceran (<i>Daphnia magna</i>). Company report no. 896A-102 Eurofins EAG Agrosience, LLC, USA GLP, Unpublished	N	Y	Data for first approval	CEV	N
K-CA 8.2.6.1/01	Falk, S.	2011	PROBLAD: Testing of Effects to the single cell green alga <i>Desmodesmus subspicatus</i> in a 72 h static test Company report no. S10-02623 Eurofins Agrosience Services GmBH GLP, Unpublished	N	Y	Data for first approval Not used in risk assessment	CEV	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
K-CA 8.2.6.1/02	Arnie, J.R.	2019	PROBLAD PLUS: A 72-hour toxicity test with the freshwater alga (<i>Raphidocelis subcapitata</i>). Company report no. 896P-101 Eurofins EAG Agrosience, LLC, USA GLP, Unpublished	N	Y	Data for first approval	CEV	N
K-CA 8.3.1.1.1/01	Kling, A.	2010	PROBLAD: Acute oral and contact toxicity to the Honey bee, <i>Apis mellifera</i> L., in the laboratory. Company report no. S10-02558 Eurofins Agrosience Services GmBH GLP, Unpublished	N	Y	Data for first approval	CEV	N

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K-CA 8.3.1.1.1/02	Aguilar-Alberola J.A.	2019	PROBLAD PLUS: Acute oral toxicity to the Honey bee, <i>Apis mellifera</i> L., under laboratory conditions Company report no. S19-21875 Trialcamp S.L.U., Spain GLP, Unpublished	N	Y	Data for first approval	CEV	N
K-CA 8.3.1.1.2/02	Aguilar-Alberola J.A.	2020	PROBLAD PLUS: Acute oral and contact Toxicity to the Bumblebee <i>Bombus terrestris</i> L., under Laboratory Conditions Company report no. S20-00599 Trialcamp S.L.U., 46290 Alcàsser (Valencia), Spain GLP, Unpublished	N	Y	Data for first approval Not used in risk assessment	CEV	N

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K-CA 8.3.1.2/01	Harkin, S.	2015a	PROBLAD PLUS: Chronic toxicity test for adult honeybees (<i>Apis mellifera</i> L.) Company report no. B2CF1000 FERA GLP, Unpublished	N	Y	Data for first approval Not used in risk assessment	CEV	N
K-CA 8.3.1.2/02	Aguilar-Alberola J.A.	2019	PROBLAD PLUS: Chronic oral toxicity test (10-day feeding) to the honey bee, <i>Apis mellifera</i> L. under laboratory conditions Company report no. S19-21016 Trialcamp S.L.U., Spain GLP, Unpublished	N	Y	Data for first approval Not used in risk assessment	CEV	N

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K-CA 8.3.1.3/01	Harkin, S.	2015b	PROBLAD PLUS: In vitro chronic toxicity to larval stage honeybee (<i>Apis mellifera</i> L.) Company report no. B2CF2000 FERA GLP, Unpublished	N	Y	Data for first approval Not used in risk assessment	CEV	N
K-CA 8.3.1.3/02	Aguilar-Alberola J.A.	2019	PROBLAD PLUS: Honey bee (<i>Apis mellifera</i> L.) larval toxicity test following repeated exposure under laboratory conditions Company report no. S19-21015 Trialcamp S.L.U., Spain GLP, Unpublished	N	Y	Data for first approval Not used in risk assessment	CEV	N

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K-CA 8.3.2.1/01	Klug, T.	2010a	PROBLAD-Toxicity to the Aphid Parasitoid, <i>Aphidius rhopalosiphi</i> De Stefani-Perez (Hymenoptera, Brachonidae,) in the Laboratory Company report no. S10-02555 Eurofins Agrosience Services GmbH GLP, Unpublished	N	Y	Data for first approval	CEV	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
K-CA 8.3.2.1/02	Stevens, J.	2014	PROBLAD PLUS – A rate-response extended laboratory bioassay of the effects of fresh residues on the parasitic wasp <i>Aphidius rhopalosiphi</i> (Hymenoptera, Braconidae) Company report no: CEV-14-1 Mambo-Tox Ltd. GLP, Unpublished	N	Y	Data for first approval Not used in risk assessment	CEV	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
K-CA 8.3.2.2/01	Klug, T.	2010b	PROBLAD: Toxicity to the Predatory Mite, Typhlodromus pyri Scheuten (Acari, Phytoseiidae) in the Laboratory (Rate Response Test) Company report no. S10-02556 Eurofins Agroscience Services GmbH GLP, Unpublished	N	Y	Data for first approval	CEV	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
K-CA 8.3.2.2/02	Fallowfield, L.	2014	PROBLAD PLUS – A rate-response extended laboratory bioassay of the effects of fresh residues on the predatory mite <i>Typhlodromus pyri</i> (Acari: Phytoseiidae) Company report no. CEV-14-2 Mambo-Tox Ltd. GLP, Unpublished	N	Y	Data for first approval Not used in risk assessment	CEV	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
K-CP 8.3.2.2/01	Vaughan, R.	2017	<p>PROBLAD PLUS – A rate-response extended laboratory test to determine effects on the green lacewing <i>Chrysoperla carnea</i> (Neuroptera, Chrysopidae)</p> <p>Mambo-tox Ltd. Southampton, UK</p> <p>Company report no. CEV-17-1</p> <p>GLP, Unpublished</p>	N	Y	<p>Data for first approval</p> <p>Not used in risk assessment</p>	CEV	N

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K-CA 8.4.1/01	Ganssmann, M.	2010a	PROBLAD-Acute Toxicity on Earthworms, <i>Eisenia fetida</i> using an Artificial Soil Test Company report no. S10-02557 Eurofins Agrosience Services GmbH GLP, Unpublished	N	Y	Data for first approval Not evaluated or used in risk assessment	CEV	N
K-CA 8.4.1/02	Friedrich, S.	2017	Effect of PROBLAD PLUS on the earthworm <i>Eisenia andrei</i> in artificial soil. Company report no. 17 48 TEC 0013 GLP, Unpublished	N	Y	Data for first approval	CEV	N

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K-CA 8.4.1/03	Anton, B.	2020	PROBLAD PLUS: Sublethal Toxicity to the Earthworm <i>Eisenia andrei</i> (Oligochaeta, Lumbricidae) in Artificial Soil with 10 % Peat. Company report no. S20-00600 Trialcamp S.L.U., 46290 Alcàsser (Valencia), Spain GLP, Unpublished	N	Y	Data for first approval	CEV	N
K-CP 10.4.2/02	van Vliet, P.C.J et al.	2000	Soil biota and crop residue decomposition during summer and autumn in south-western Australia Applied Soil Ecology 14 (2000) 111-124	N	N	N/A	N/A	N/A

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K-CA 8.5/01	Ganssmann, M.	2010b	PROBLAD- Assessment of the Side Effects on the Activity of the Soil Microflora Company report no. S10-02559 Eurofins Agrosience Services GmbH GLP, Unpublished	N	Y	Data for first approval	CEV	N
K-CA 8.5/02	Elgharably, A. and Marschner, P	2011	Microbial activity and biomass and N and P availability in a saline sandy loam amended with inorganic N and lupin residues. European Journal of Soil Biology 47 310 – 315.	N	N	N/A	N/A	N/A

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K-CA 8.5/03	Lelei, J. J. and Onwonga, R. N.	2014	Soil Fungal and Bacterial Populations in White Lupin (<i>Lupinus albus</i>) – Maize (<i>Zea mays</i> L) Cropping System Amended With Minjingu Phosphate Rock. Journal of Agriculture and Ecology Research International 1(1): 1-17, 2014; Article no. JAERI.2014.001	N	N	N/A	N/A	N/A
K-CP 10.6.2/01	Cookson, W.R. et al.	1998	Effects of prior crop residue management on microbial properties and crop residue decomposition Applied Soil Ecology 7 (1998) 179 – 188	N	N	N/A	N/A	N/A

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K-CA 8.6.1/01	Peterek, S.	2011	PROBLAD: Vegetative Vigour Limit Test for Non Target Plants on Six Plant Species Company report no. S10-02560 Eurofins Agroscience Services GmbH GLP, Unpublished	N	Y	Data for first approval	CEV	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
K-CA 8.6.2/01	Huerta, F.	2020	<p>PROBLAD PLUS: Effects on the Vegetative Vigour and on the Seedling Emergence and Growth of Six Non-Target Terrestrial Plant Species under Greenhouse Conditions</p> <p>Company report no. S20-05408</p> <p>Trialcamp S.L.U., 46290 Alcàsser (Valencia), Spain</p> <p>GLP, Unpublished</p>	N	Y	Data for first approval	CEV	N