Annex I to the CLH report

Proposal for Harmonised Classification and Labelling

Based on Regulation (EC) No 1272/2008 (CLP Regulation), Annex VI, Part 2

International Chemical Identification:

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

EC Number: **Not allocated**

CAS Number: **Not allocated**

Index Number: **Not allocated**

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1 PHYSICAL HAZARDS

1.1 Explosives

Explosive properties (CA 2.11)	EEC A.14	PROBLAD PLUS Lot: 16102012 20% BLAD	PROBLAD PLUS is not explosive	**Acceptable**	GLP	Cage, 2013 (MIB0036)

1.2 Flammable gases (including chemically unstable gases)

No studies available.

1.3 Oxidising gases

No studies available.

1.4 Gases under pressure

No studies available.

1.5 Flammable liquid

Flash point (CA 2.10)	OPPTS 830.7000 (Equivalent to EEC A.9)	PROBLAD PLUS Lot#: 040511; Batch 1 20% BLAD	>100°C. The test item is not flammable	**Acceptable**	GLP	Wo, 2012b (32388)

1.6 Flammable solids

No studies available.

1.7 Self-reactive substances

No studies available.

1.8 Pyrophoric liquids

No studies available.

1.9 Pyrophoric solid

No studies available.

1.10 Self-heating substances

No studies available.

1.11 Substances which in contact with water emit flammable gases

No studies available.

1.12 Oxidising liquids

1.12.1 Study 1

Oxidizing properties (CA 2.13)	US federal register Vol 44 (1979) and OPPTS 830.6314	PROBLAD PLUS Lot#: 040511; Batch 1 20% BLAD	No oxidising or reducing potential reported except when tested with potassium permanganate where a reaction was observed within two minutes. PROBLAD PLUS is an aqueous solution containing the lead component BLAD. BLAD is a 20 kDa polypeptide of β-conglutin, or characterized as a fragment of the amino acid sequence of β-conglutin. As such it is not expected to be oxidizing. No other components of PROBLAD PLUS contain groups that would imply oxidising properties such as nitrates, metal oxides, hypofluorites, difluoroaminopolynitroaryls, perchlorates, bromates and iodites. Therefore PROBLAD PLUS will not be oxidising.	**Acceptable**	GLP	Wo, 2012b (32388)

1.12.2 Study 2

Oxidizing properties (CA 2.13)	EEC A.21	PROBLAD PLUS Lot: 16102012 20% BLAD	PROBLAD PLUS is not oxidising	**Acceptable**	GLP	Cage, 2013 (MIB0036)

1.13 Oxidising solids

No studies available.

1.14 Organic peroxides

No studies available.

1.15 Corrosive to metals

Corrosivity to metals (CA 2.14)	ASTM G31-12a	PROBLAD PLUS Lot: C191907-001 20% BLAD	Degree of corrosion observed after 72h exposure of the metal to PROBLAD PLUS at 55 °C: *Steel SAE 1020*: $1.04x10^{-1}$ mm/year *Aluminium*: $2.64x10^{-1}$ mm/year *Copper*: $1.03x10^{-3}$ mm/year PROBLAD PLUS is not corrosive to metals, with a corrosion rate below the classification threshold of 6.25 mm/year (at 55 °C) for all of the tested metals.	**Acceptable** The test duration (72 h) was substantially shorter than the prescribed minimum of 168 h. Weight loss was used as corrosion criterion, assuming uniform corrosion. As the observed weight loss was still about a factor of 40 below the minimum uniform loss allowed after one week, it is not reasonably expected that, if the test duration would have been extended by a factor of 2.3 as required, the threshold would have been exceeded.	GLP	Angela, 2021 (Report no. 07223/20)

2 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

Sweet Lupin (seeds), *Lupinus albus* L., germ., ext. [PROBLAD PLUS] is a plant extract with fungicidal properties that can be used on food and non-food crops. The product is extracted from the germinated seeds of sweet lupines (*Lupinus albus*) where after filtration and concentration it is formulated into PROBLAD PLUS. The lead component BLAD, which forms 20% w/w of the PROBLAD PLUS formulation, is not isolated during the preparation of the product. The testing summarised in the dossier has therefore been conducted with PROBLAD PLUS.

As PROBLAD PLUS contains the naturally occurring polypeptide component, BLAD (the lead component), the protein will be broken down in the digestive tract, enter the amino acid pool and be consumed into normal catabolic processes. As susceptible to proteolytic degradation as it is, radiolabelling of the test article is not possible. Therefore, studies on absorption, distribution, metabolism and excretion in mammals have not been undertaken.

3 HEALTH HAZARDS

Acute toxicity

3.1 Acute toxicity - oral route

3.1.1 Animal data

3.1.1.1 Study 1

Study reference: CA 5.2.1/01, B.6.2.1, study 1

reference	:	Up-and-down procedure	exposure	:	gavage
Report number	:	31002, 2012a	doses	:	5000 mg/kg bw
test substance		PROBLAD PLUS, Material nr 201009, batch 7, purity: 20% BLAD	GLP statement	:	yes
species	:	Rat, Sprague-Dawley, females	guideline	:	in accordance with OECD 425
group size	:	1 animal, followed by 2 animals	acceptability	:	acceptable

Study design

A. Materials:

1. Test Material: PROBLAD PLUS

Description: Brown in a liquid formulation

Lot/Batch No.: Material no.: 201009, batch: 7

Purity: 20% BLAD (the lead component) (80% other ingredients)

CAS No.: n/a

Stability of test compound: No stability data given, assumed to be stable for the duration of the study (refer to CP Section 2)

2. Vehicle and/or positive control: None, test article administered as received / none

3. Test animals:

Species: Rat

Strain: Sprague Dawley

Age: ♀: 11 weeks

Weight at dosing: ♀: 213 – 247 g

Source: Ace Animals, Inc. Boyertown, PA

Acclimation period: 22 to 23 days

B. Study Design and Methods:

1. In life dates: 21 October 2010 to 11 November 2010

2. Animal assignment and treatment: An initial limit dose of 5000 mg/kg bw was administered to one healthy female rat by oral gavage using a dosing volume of 4 mL/kg to correct for specific gravity. Due to the absence of mortality in this animal, two additional females received the same dose level, simultaneously. Since these animals survived, no additional animals were tested. Females were selected for the test because they are frequently more sensitive in terms of toxicity than males. All animals were observed for mortality, signs of gross toxicity and behavioural changes at least once daily for 14 days after dosing. Body weights were recorded prior to administration and again on days 7 and 14 (termination) following dosing. Necropsies were performed on all animals at terminal sacrifice.

3. Statistics: n/a

Results

Mortality:

All animals survived to the scheduled necropsy.

Table B.6.2.1-1: Doses, mortality/ animals treated

Dose (mg/kg bw)	♂	♀	Combined
5000	-	0/3	0/3

Clinical observations:

No gross signs of clinical toxicity, adverse pharmacologic effects or abnormal behaviour were observed.

Body weight:

Over the entire period (0 to 14 d) all animals gained weight (between 23 to 25%). However, during the 7 to 14 day interval 1/3 animals lost weight; this animal however gained weight at a greater rate during the interval 0 to 7 days, compared to the other animals.

Pathology:

No gross abnormalities were observed.

Conclusion

Under the conditions of this study the acute oral LD_{50} is >5000 mg/kg bw in female rats. According to Annex I of Regulation (EC) No. 1272/2008, the test article has no obligatory labelling requirement for acute oral toxicity and classification is not required.

An explanation as to why a dose of 5000 mg/kg bw was used instead of 2000 mg/kg bw is not given in the study report. However, the applicant indicated that the study was conducted in accordance with the US OPPTS 870.1100 test guideline, which has an upper limit dose of 2000-5000 mg/kg bw.

3.2 Acute toxicity - dermal route

3.2.1 Animal data

3.2.1.1 Study 1

Study reference: CA 5.2.2/01, B.6.2.2, study 1

reference	:	Acute dermal toxicity	exposure	:	Dermal application
Report number	:	31003, 2012b	doses	:	2000 mg/kg bw
test substance		PROBLAD PLUS, Material nr 201009, batch 7, purity: 20% BLAD	GLP statement	:	Yes
species	:	Rat, Sprague-Dawley	guideline	:	in accordance with OECD 402
group size	:	5/sex	acceptability	:	acceptable

Study design

A. Materials:

1. Test Material: PROBLAD PLUS

Description: Brown in a liquid formulation

Lot/Batch No.: Material no.: 201009, batch: 7

Purity: 20% BLAD (the lead component) (80% other ingredients)

CAS No.: n/a

Stability of test compound: No stability data given, assumed to be stable for the duration of the study (refer to CP Section 2)

2. Vehicle and/or positive control: None, test article administered as received / none

3. Test animals:

Species: Rat

Strain: Sprague Dawley

Age: 10 – 11 weeks

Weight at dosing: ♂: 310 – 421 g, ♀: 202 – 255 g

Source: Ace Animals, Inc. Boyertown, PA

Acclimation period: 24 days

B. Study Design and Methods:

1. In life dates: 21 October 2010 to 6 December 2010

2. Animal assignment and treatment: On the day prior to application, a group of rats were prepared by clipping the dorsal area and the trunk. After clipping and prior to application, the animals

were examined for health, weighing (initial) and the skin checked for any abnormalities.
A dose of 2000 mg/kg bw was applied evenly at a volume of ~1.6 mg/kg bw over a dose area of ~10% of the body surface area and covered with a gauze pad. The gauze pad and entire trunk of each animal were then wrapped with Durapore tape. After 24 hours of exposure the test material was removed and the test site was gently cleaned. All animals were observed for mortality, signs of gross toxicity and behavioural changes at least once daily for 14 days after dosing. Body weights were recorded prior to administration and again on days 7 and 14 (termination) following dosing. Necropsies were performed on all animals at terminal sacrifice.

3. Statistics: n/a

Results

Mortality:

All animals survived to the scheduled necropsy.

Table B.6.2.2-1: Doses, mortality/ animals treated

Dose (mg/kg bw)	♂	♀	Combined
2000	0/5	0/5	0/10

Clinical observations:

Clinical signs of toxicity were limited to red nasal discharge on day 2 in 3/5 males and 1/5 females. No signs of dermal irritation were observed.

Body weight:

Over the entire period (0 to 14d) all animals gained weight (between 11 to 17% and 9 to 13% for males and females, respectively). However, during the 0 to 7 day interval 1/5 females lost weight (-2%); this animal however gained weight at a greater rate during the interval 7 to 14 days, compared to the other animals.

Pathology:

No gross abnormalities were observed.

Conclusion

Under the conditions of this study the acute dermal LD_{50} is >2000 mg/kg bw in male and female rats. According to Annex I of Regulation (EC) No. 1272/2008, the test article has no obligatory labelling requirement for acute oral toxicity and classification is not required.

3.3 Acute toxicity - inhalation route

3.3.1 Animal data

3.3.1.1 Study 1

Study reference: CA 5.2.3/01, B.6.2.3, study 1

reference	:	Acute inhalation study	exposure	:	4 h inhalation, nose-only
Report number	:	30998, 2012c	doses	:	5.34 mg/L
test substance		PROBLAD PLUS, Material nr 201009, batch 7 and 1, purity: 20% BLAD	GLP statement	:	yes
species	:	Rat, Sprague-Dawley	guideline	:	in accordance with OECD 403
group size	:	5/sex	acceptability	:	acceptable

Study design

A. Materials:

1. Test Material: PROBLAD PLUS

Description: Brown in a liquid formulation

Lot/Batch No.: Material no.: 201009, batch: 7 (pre-test trials)

Material no.: 201009, batch: 1 (main study)

Purity: 20% BLAD (the lead component) (80% other ingredients)

CAS No.: n/a

Stability of test compound: No stability data given, assumed to be stable for the duration of the study (refer to CP Section 2)

2. Vehicle and/or positive control: Distilled water, test article administered as received / none.

3. Test animals:

Species: Rat

Strain: Sprague Dawley

Age: 9 to 10 weeks

Weight at dosing: ♂: 277 to 304 g, ♀: 180 to 205 g

Source: SAGE™ Labs, Boyertown, PA

Acclimation period: 14 days

B Study Design and Methods:

1. In life dates: 10 January 2011 to 08 February 2011

2. Animal assignment and treatment: After establishing the desired generation procedures during pre-test trials, 10 rats (5/sex) were exposed to the test atmosphere for 4 hours at a concentration of 5.34 mg/L. Chamber concentration and particle size distributions of the test article were determined periodically during the exposure period. Animals were observed for mortality, signs of gross toxicity and behavioural changes once daily for 14 days following exposure. Body weights were recorded prior to exposure and again on days 1, 3, 7 and 14. Necropsies were performed on all animals at terminal sacrifice.

3. Generation of the test A nose-only inhalation chamber with an internal volume of ~28 L was used for

atmosphere / chamber description:	exposure. Animals were individually housed in holding tubes, with both water and food withheld during the exposure period. The test material was diluted in distilled water to a 50% (w/w) mixture. The test atmosphere was generated using a ¼ inch atomiser. Compressed air was supplied at 30 psi. The test material was metered to the atomisation nozzle using a peristaltic pump. Gravimetric samples were withdrawn from the animals breathing zone at 6 intervals during the exposure period. Particle size distribution was determined by sampling twice during the exposure period. The filter paper collection stages were weighed before and after sampling to determine the mass collected upon each stage.
4. Statistics:	n/a

Results

Analytical measurements:

The gravimetric chamber concentration was 5.34 mg/L and the nominal chamber concentration was 202.7 mg/L (50% w/w mixture of test substance in distilled water). Based on graphic analysis of the particle size distribution as measured with an Andersen Cascade Impactor, the mass median aerodynamic diameter was estimated to be 2.7 µm.

Mortality:

All animals survived to the scheduled necropsy.

Table B.6.2.3-1: Doses, mortality/ animals treated

Target dose (mg/L) (Actual dose (mg/L)	♂	♀	Combined
5.00 (5.34)	0/5	0/5	0/10

Clinical observations:

Clinical signs were limited to one animal of each sex displaying signs of hypoactivity and 7 animals (3M / 4F) exhibiting abnormal respiration. All affected animals recovered by day 8 and appeared normal for the remainder of the observation period.

Body weight:

Whilst all animals lost weight by day 1, all animals showed a continued weight gain during day 314.

Pathology:

No gross abnormalities were observed.

Conclusion

Under the conditions of this study the acute inhalation LD_{50} is >5.34 mg/L in male and female rats. According to Annex I of Regulation (EC) No. 1272/2008, the test article has no obligatory labelling requirement for acute inhalation toxicity and classification is not required.

3.4 Skin corrosion/irritation

3.4.1 Animal data

3.4.1.1 Study 1

Study reference: CA 5.2.4/01, B.6.2.4, study 1

reference	:	In vivo skin irritation study	exposure	:	Dermal, semi-occluded
Report number	:	31000, 2012d	doses	:	0.5 mL
test substance	:	PROBLAD PLUS, Material nr 201009, batch 7, purity: 20% BLAD	GLP statement	:	yes
species	:	Rabbit, New-Zealand white	guideline	:	in accordance with OECD 404
group size	:	3 males	acceptability	:	acceptable

Study design

A. Materials:

1. Test Material: PROBLAD PLUS

Description: Brown in a liquid formulation

Lot/Batch No.: Material no.: 201009, batch: 7

Purity: 20% BLAD (the lead component) (80% other ingredients)

CAS No.: n/a

Stability of test compound: No stability data given, assumed to be stable for the duration of the study (refer to CP Section 2)

2. Vehicle and/or positive control: None, test material administered as received / none.

3. Test animals:

Species: Rabbit

Strain: New Zealand white

Age: Young adult

Weight at dosing: Not stated

Source: Robinson Services, Inc., Clemmons, NC

Acclimation period: 7 days

B. Study Design and Methods:

1. In life dates: 30 November 2010 to 3 December 2010

2. Animal assignment and treatment: On the day prior to test material application the fur was clipped from the dorsal area and the trunk. The test material (0.5 mL) was applied as received to a 6 cm^2 dose site of 3 animals. The test site was semi-occluded. After 4 hours of exposure to the test material the test site was gently washed. The test sites were examined 30 to 60 minutes, 24, 48 and 72 h after patch removal. The classification of irritancy was obtained by adding the average erythema and

oedema scores for each scoring interval and diving by the number of evaluation intervals (n=4). Dermal irritation was evaluated by the method of Draize. The animals were observed for signs of gross toxicity and behavioural changes at least once daily during the test period.

Results

Findings are summarised below in Table B.6.2.4-1.

All animals appeared active and healthy during the study. Apart from the dermal irritation noted there were no other signs of gross toxicity, adverse pharmacological effects or abnormal behaviour.

Within 30 to 60 minutes after patch removal, very slight to well defined erythema was noted for all three treated dose sites and very slight oedema was evident at two sites. Signs of erythema had completely resolved within 48 h, with oedema resolved within 24 h.

Table B.6.2.4-1: Individual and mean skin irritation scores according to the Draize scheme

Animal no	Erythema				Oedema				Severity of irritation[1]
	1♂	2♂	3♂	Mean	1♂	2♂	3♂	Mean	
after 0.5 – 1h	1	2	2	**1.7**	0	1	1	**0.7**	2.4
after 24 hr	0	1	1	**0.7**	0	0	0	**0**	0.7
after 48 hr	0	0	0	**0**	0	0	0	**0**	0.0
after 72 hr	0	0	0	**0**	0	0	0	**0**	0.0
mean score	**Erythema**				**Oedema**				-
24 – 72h	0	0.3	0.3	**-**	0	0	0	**-**	-

[1] Primary Dermal Irritation Index reported in study (sum of erythema + oedema scores)

Conclusion

Under the conditions of this study, PROBLAD PLUS is slightly irritating to the skin, based on the primary irritation indices. According to Annex I of Regulation (EC) No. 1272/2008, the test article has no obligatory labelling requirement for skin irritation and classification is not required.

3.5 Serious eye damage/eye irritation

3.5.1 Animal data

3.5.1.1 Study 1

Study reference: CA 5.2.5/01, B.6.2.5, study 1

reference	:	In vivo eye irritation study	exposure	:	Single installation in conjunctival sac
Report number	:	30999, 2012e	doses	:	0.1 mL
test substance	:	PROBLAD PLUS, Material nr 201009, batch 7, purity: 20% BLAD	GLP statement	:	yes
species	:	Rabbit, New Zealand white	guideline	:	in accordance with OECD 405
group size	:	3 females	acceptability	:	acceptable

Study design

A. Materials:

1. Test Material:	PROBLAD PLUS
Description:	Brown in a liquid formulation
Lot/Batch No.:	Material no.: 201009, batch: 7
Purity:	20% BLAD (the lead component) (80% other ingredients)
CAS No.:	n/a
Stability of test compound:	No stability data given, assumed to be stable for the duration of the study
2. Vehicle and/or positive control:	None, test article administered as received / none.
3. Test animals:	
Species:	Rabbit
Strain:	New Zealand white
Age:	Young adult
Weight at dosing:	Not stated
Source:	Robinson Services, Inc., Clemmons, NC
Acclimation period:	5 days

B. Study Design and Methods:

1. In life dates:	6 December 2010 to 13 December 2010
2. Animal assignment and treatment:	Prior to test initiation both eyes of a group of animals were examined using a light source and fluorescein dye. Three healthy animals without pre-existing ocular irritation were selected for the test. Prior to instillation 2-3 drops of ocular anaesthetic were placed into both the treated and control eye of each animal. 0.1 mL of the test material, as supplied was instilled into the conjunctival sac of the right eye of each rabbit. The upper and lower lids were held together for ~1 second before releasing. The other eye of each rabbit remained untreated and served as a control. Ocular irritation was evaluation in accordance with Draize *et al* at 1, 24, 48 and 72 h and at 4 and 7 d post-instillation. The animals were observed for signs of gross toxicity and behavioural changes at least once daily during the test period.

Results

Findings are summarised below in Table B.6.2.5-1.

All animals appeared active and healthy during the study. Apart from the eye irritation noted there were no other signs of gross toxicity, adverse pharmacologic effects or abnormal behaviour.

There was no iritis observed in any treated eye during the study. Within 1 h after the test article instillation all three treated eyes exhibited corneal opacity and positive conjunctivitis. The overall incidence and severity of irritation decreased with time. All animals were free of ocular irritation by day 4.

Using the OECD 405 grading scheme for ocular irritation the mean scores for cornea, iris, conjunctivae and chemosis were 0.3, 0, 1.4, 0.6 and 1.1, respectively. This scoring system takes into account eye effects during the scoring period of 24 – 72 h post dosing.

Table B.6.2.5-1: Eye irritation scores according to the Draize scheme – unwashed eye

Time / Rabbit No.	Cornea (opacity/area)			Iris (value/area)			Conjunctiva-					
							redness			chemosis / discharge		
	1	2	3	1	2	3	1	2	3	1	2	3
1 hr	1/2	1/2	1/1	0/0	0/0	0/0	2	2	2	3/2	3/3	2/2
24 hrs	1/1	1/1	0/4	0/0	0/0	0/0	2	2	2	1/2	1/2	1/2
48 hrs	1/1	0/4	0/4	0/0	0/0	0/0	1	2	1	1/1	1/1	0/0
72 hrs	0/4	0/4	0/4	0/0	0/0	0/0	1	1	1	0/1	0/1	0/0
4 d	0/4	0/4	0/4	0/0	0/0	0/0	0	0	0	0/0	0/0	0/0
7 d	0/4	0/4	0/4	0/0	0/0	0/0	0	0	0	0/0	0/0	0/0
means scores 24-72 hrs[1]	0.7	0.3	0	0	0	0	1.3	1.7	1.3	0.7	0.7	0.3

[1]. Area not taken into account as it was assumed that readings were taken from the densest area

Conclusion

Under the conditions of this study, PROBLAD PLUS is mildly irritating to the eye, based on the MMTS. According to Annex I of Regulation (EC) No. 1272/2008, the test article has no obligatory labelling requirement for eye irritation and classification is not required.

The test should have been terminated earlier, since all animals were free of ocular irritation on Day 4. However, due to a technician error, the clinical observations continued through day 7. This deviation has no impact on the validity or overall conclusion of this study.

3.6 Respiratory sensitisation

No data available.

3.7 Skin sensitisation

3.7.1 Animal data

3.7.1.1 Study 1

Study reference: CA 5.2.6/01, B.6.2.6, study 1

reference	:	Skin sensitisation study	exposure	:	Dermal, occlusive
Report number	:	31004, 2012f	doses	:	0.4 ml; 25, 50, 75 and 100%
test substance	:	PROBLAD PLUS, Material nr 201009, batch 7, purity: 20% BLAD	GLP statement	:	yes
species	:	Guinea pig, Hartley albino	guideline	:	in accordance with OECD 406
group size	:	4 males (pre-test), 20 males (main) and 10 males (controls)	acceptability	:	acceptable

Study design

A. Materials:

1. Test Material:	PROBLAD PLUS
Description:	Brown in a liquid formulation
Lot/Batch No.:	Material no.: 201009, batch: 7
Purity:	20% BLAD (the lead component) (80% other ingredients)
CAS No.:	n/a
Stability of test compound:	No stability data given, assumed to be stable for the duration of the study (refer to CP Section 2)
2. Vehicle and/or positive control:	Distilled water
3. Test animals:	
Species:	Guinea pig
Strain:	Hartley albino
Age:	Young adult
Weight at dosing:	♂: 314 – 387 g
Source:	Elm Hill Breeding Labs, Chelmsford, MA
Acclimation period:	5 - 26 days

B. Study Design and Methods:

1. In life dates:	16 October 2010 to 02 December 2010
2. Preliminary irritation test:	A group of animals (4 ♂) was used to determine the highest non-irritating concentration (HNIC) of the test article prior to the challenge dose. The fur was removed by clipping the dorsal area and flanks of each guinea pig. This area was divided into four test sites (2 sites on each of the midline) on each animal. The test article was applied neat (100%) and also diluted with distilled water to yield w/w concentrations of 75%, 50% and 25%. Each concertation was applied (0.4 mL) to a test site using an occlusive 25 mm Hill Top Chamber. The sites were wrapped with non-allergenic adhesive tape. After 6 h of exposure, the chambers were removed and any residual test article was removed. Approximately 24 h after application, each site was evaluated for erythema according to the following scoring system: 0 No reaction 0.5 Very faint erythema, usually non-confluent* 1 Faint erythema, usually confluent 2 Moderate erythema 3 Severe erythema with or without oedema * very faint erythema is not considered a positive reaction
3. Buehler assay:	On the day before initiation, the dorsal fur of a group of 30 ♂ animals was clipped. After clipping, animals were weighed and the skin was checked for

anomalies.

Once each week for 3 weeks, 0.4 mL of the undiluted test article was applied to the left side of each test animal (20 ♂ in total) using an occlusive 25 mm Hill Top chamber. The chambers were secured in place and wrapped with non-allergenic adhesive tape. After 6 h the chambers were removed and the test sites were gently cleansed. Approximately 24 and 48 h after each induction application readings of were made of local reactions according to the scoring system already detailed above.

Twenty seven days after the first induction dose, 0.4 mL of the test article (undiluted) was applied to a naïve site on the right side of each animal as a challenge dose, using the procedures already described. These sites were evaluated for a sensitisation response approximately 24 and 48 h after the challenge application.

In addition to the test animals, 10 guinea pigs were treated with undiluted test article at challenge only. These animals constituted to the 'naïve control' group.

The sensitivity and specificity of the test system was confirmed from validation data using α-hexylcinnamaldehyde (HCA) as a positive control.

Individual body weights were recorded prior to initiation and again on the day after challenge.

4. Evaluation: For a test article to be considered as contact sensitiser the following criteria were used:

- At the 24 h and/or 48 h scoring interval, ≥15% of the test animals exhibit a positive response (scores ≥0.5) in the absence of similar results in the vehicle control
- The positive reaction at the 24 h interval must persist to 48 h in at least 1 test animal

5. Statistics: Not warranted

Results

Preliminary irritation test:

At doses of 25 and 50% (w/w) no skin reactions were observed. At doses of 75 and 100% (w/w) 2/4 animals exhibited very faint erythema (score of 0.5). From these results the HNIC was 100%, this was used for both the induction and challenge phase.

Buehler assay:

1. Induction phase: Very faint to faint erythema (0.5-1) was noted for all test sites during the induction phase.

2. Challenge phase: Very faint erythema (0.5) was noted for 12 of the 20 test sites 24 h after challenge. Irritation cleared from all affected test sites by 48 h.

In the naïve control animals very faint erythema (0.5) was noted for 3 of 10 naïve control sites 24 h after challenge. Irritation cleared from all affected test

sites by 48 h.

Historical positive control animals (75% (w/w) HCA in mineral oil): 8 out of 10 animals exhibited signs of a sensitisation response (faint to moderate erythema 1-2) 24 h after challenge. Similar indication persisted at 7 of these sites through 48 h; very faint erythema (0.5) was noted for most other sites following challenge, thus demonstrating the validity of the test system.

Historical naïve control animals (75% (w/w) HCA in mineral oil): very faint erythema (0.5) was noted at 3 of 5 naïve control sites 24 h after challenge. Similar irritation persisted at one site through 48 h.

Deficiencies:

Although the Buehler test undertaken only employed 3 application methodology, the 21 day dermal toxicity application showed no evidence suggestive of skin sensitisation. Signs of dermal irritation were limited to very slight scabbing to well-defined erythema and scabbing. Animals did not show any systemic effects which could be attribute to a sensitisation reaction following test article application to skin.

Conclusion

Under the conditions of this study, PROBLAD PLUS does not show evidence of skin sensitisation. According to Annex I of Regulation (EC) No. 1272/2008, the test article has no obligatory labelling requirement for skin sensitisation and classification is not required.

Study 2 – Position paper

Report

R. Boavida Ferreria (2011) Potential allergenicity of lupine seeds (*Lupinus sp*.) with special emphasis on BLAD, an intermediate in the breakdown process of the major storage protein during germination of lupine seeds. CEV SA, Unpublished report No.: CEV110820

Guideline n/a, review report

GLP No

Executive summary

BLAD (the lead component) exhibits an effective broad-spectrum fungicidal activity. This trait is exploited by the fast growing *Lupinus* plantlets, which use BLAD during the most sensitive phase of development to improve their chances of survival in an often harsh environment. When extracted from the cotyledons, BLAD is a very promising fungicide in modern day agriculture due to its unique properties: (*i*) it is a naturally-occurring food and feed item exhibiting a non-toxic mode of action; (*ii*) it exhibits a very effective fungicidal activity towards many fungal species tested; (*iii*) it exhibits both preventive and curative antifungal activities; (iv) its unique mode of action reduces the risk of fungal resistance; (*v*) it exhibits a very high resistance to the sun UV radiation; (*vi*) it is very useful in Integrated Pest Management Programs (IPM); (vii) it does not confer any particular colour, odour or flavour to the treated plants, Overall, there is reasonable certainty that no harm will result to human health or to the environment from the use of BLAD.

The observations that (*i*) BLAD comprises an internal segment of β-conglutin, (*ii*) β-conglutin exhibits a relatively strong homology to the other members of the vicilin family, including well known allergens contained in peanuts and soybeans, and (*iii*) a considerable number of studies concerning the allergenicity of lupine derived products demand an assessment of BLAD's potential allergenicity. It should be noted that there is an even greater number of allergenicity studies for other commonly eaten legumes, such as peanuts, soybeans, peas, beans, *etc*., as well as for many other foodstuffs. In fact, any food containing proteins could be considered potentially capable of eliciting an allergic reaction in humans.

Although the structural features which characterize a food allergen remain largely to be elucidated, a number of physicochemical properties are common to the presently known allergens, *e.g*. allergens typically survive digestion, meaning that they are quite resistant to denaturation and to proteolytic degradation by (gut) proteases.

In this assessment, comparing BLAD as a worst case to a well-established allergen, peanut *Ara h 1*, the following criteria set by Codex Alimentarius (2003) and FAO/WHO (2001) should be met:

(1a): amino acid residue homology ≥35%, or

(1b): identity in one or more sets of ≥6 contiguous amino acid residues, or

(1c): cross-reactivity to known allergens;

(2): high resistance to proteolytic attack; and

(3): ingestion of sufficient amounts.

Discussion of these criteria in relation to BLAD are given below:

(1a): BLAD exhibits a high sequence homology (58%) when compared to a section of *Ara h 1* and of other legume seed storage proteins, especially from the vicilin family. This homology comes as no surprise, since BLAD contains within itself the cupin-2 domain, identified as an allergenic domain in several vicilins and legumins

(1b): Given the 58% sequence homology between BLAD and the corresponding section in *Ara h 1*, the presence of a single ≥6 amino acid residue identity sequence (when compared to other vicilins) suggests a more likely presence of IgE recognition epitopes on the vicilins rather than on BLAD.

(1c): The available literature suggests that the peanut-lupine cross-reactivity allergenic potential is high, but unrelated to lupine β-conglutin (BLAD precursor) (Moneret-Vautrin et al., 1999).

The overall weight of evidence provided under Criteria 1a, 1b and 1c suggests that BLAD exhibits a much smaller potential of allergenicity than legume vicilins.

(2): BLAD exhibits a very high sensitivity to proteases, with no proteolytic fragments leftover, which could act as proteinase inhibitors or anti-nutritional factors.

The evidence provided under Criterion 2 makes it unlikely that BLAD may constitute a serious allergen when compared to other legume globulins, especially *Ara h 1*. BLAD's great susceptibility to proteolytic attack is in agreement with published data indicating that the allergenic potential from lupine seeds seems to be due to γ-conglutin, and less from α- and β-conglutins due to their susceptibility to proteolysis.

It is important to mention that with the exception of BLAD, most α-, β- and γ-conglutin subunits are readily degraded during lupine germination and seedling growth, so that most of them have gone by the time BLAD is collected from 8-days-old (DAG) cotyledons. In addition, any residual γ-conglutin most probably undergoes precipitation under the conditions utilized to extract BLAD from the 8 DAG cotyledons.

(3): Tests performed at CEV laboratories have indicated that on average, fruits like table grapes (typically around 6 g/berry), strawberries (typically around 15 g/berry) and tomatoes/apples (typically around 150 g) will retain 35 µL, 300 µL and 580 µL of the spray solution, respectively, at time of treatment with BLAD. After crop treatment, the calculated amount (concentration) of BLAD for each individual fruit surface will be for a grape berry 8.75 µg (1.46 ppm), for a strawberry 75.00 µg (5.00 ppm) or for a tomato/apple 145.00 µg (less than 1 ppm), respectively. These ppm concentrations are largely overestimated (factor of 60), based on the huge molecular size of BLAD compared to other conventional fungicides. Immunoblotting, a method capable of detecting BLAD in the ng range, has shown that no BLAD residue is present immediately after crop treatment.

Taking Criterion 3 exclusively into account, the extent of BLAD remaining on the plant surface after application at its recommended concentration, and assuming an identical allergenic potential between soybean conglycinins and glycinin on one hand, and BLAD on the other, results into the following comparative scenarios: one edible portion of Tofu (100g), containing *ca*. 8.2g of soybean vicilins plus

legumin, is equivalent to drinking 32.8 mL of BLAD concentrated commercial formulation (PROBLAD PLUS) or 32.8 L of the final spray solution, or equivalent to consuming 56,552 tomatoes or apples, 109,333 strawberries, or 937,143 table grapes immediately after treatment. Similar results were obtained for one serving of boiled peas (100g).

In conclusion the evidence provided above demonstrates that there are no allergenicity-related concerns from the use of BLAD as a biological fungicide in agriculture and/or non-agriculture settings. It is expected that, when used according to the proposed label directions, BLAD's potential harmful effects to humans are negligible, and no adverse effects such as allergenic reactions are expected.

RMS:

In the position paper the following is stated:

The observations that

(i) BLAD comprises an internal segment of β-conglutin,

(ii) β-conglutin exhibits a relatively strong homology to the other members of the 7S or vicilin-like family of seed storage proteins, including well known allergens in peanut and soybean, and

(iii) the considerable number of studies and reports released in recent years concerning the allergenicity of lupine derive products,

call for the following assessment of BLAD's potential allergenicity when used as a fungicide intended for applications to agricultural crops, ornamentals, turf and residential sites.

In addition, in the EU sources of potential allergens for which legislation specifically demands the list in the ingredient list (Commission Directive 2007/68/EC, Annex II) includes *lupine and products thereof.*

In literature, reports of allergic reactions to lupin following ingestion have been growing with the increasing use of lupin proteins in dietary products, either as primary lupin allergy or as a result of cross-reactivity to other legume proteins (especially from peanut). Furthermore, occupational IgE-mediated allergy also has been reported in exposed workers after inhalation or contact with lupin flour. The allergic responses to lupine proteins range from urticaria and angioedema, urticaria contact, oral allergy syndrome and rhinoconjunctivitis to anaphylaxis.

In a (draft) scientific opinion of EFSA on the evaluation of allergenic foods and food ingredients for labelling purposes, it is mentioned that clinical reactions to lupin were triggered in peanut allergic individuals at lupin doses varying from 50 mg to 1.6 gram. Considering the (overestimated) calculated amounts of BLAD on fruit surfaces (grape, strawberry, tomato/apple) and the amounts of these fruits eaten (extracted from PRIMO v.2.0), the values of BLAD ingested based on the amounts calculated to be on the

fruits is between 0.16 and 4.7 mg. These values are below the trigger dose for clinical reactions of 0.05-1.6 gram lupin mentioned in the EFSA scientific opinion.

BLAD (the lead component of PROBLAD PLUS) was assessed according to the criteria set by Codex Alimentarius (2003) and FAO/WHO (2001). According to the data provided for these criteria there are no allergenicity-related concerns from the use of PROBLAD PLUS as a biological fungicide in agriculture.

Following the commenting round, a **data requirement (2.3)** was set to submit the new clinical study, which is evaluated below. In addition, the applicant provided a supplemental report to this study (evaluated as study 4 below).

Study 3 – Clinical study

Report

Anonymous, (2013) Evaluation of the allergenic and cros-allergenic potential of the BLAD polypeptide. CEV SA, Unpublished report No.: CEV

Guideline n/a

GLP Yes

Introduction

International guidelines for the evaluation of the potential allergenicity were developed by the Codex Alimentarius Commission of the FAO and WHO (Codex, 2009). In view of the fungicide PROBLAD PLUS containing the active ingredient BLAD, a 20 kDA polypeptide of β-conglutin, similarity to other allergenic vicilins proteins was investigated in a study which determined the ability of specific and non-specific IgEs, collected from serum of allergic individuals to bind to BLAD, evaluating its likely allergenicity and cros-allergenicity.

Material and methods

Patients: 50 adult patients with symptoms of lupine and/or peanut ingestion who visited the outpatient of Immuno-allergology department from Coimbra University Hospital were invited to participate. Pregnancy, unstable asthma and oral medication with corticosteroids, β-blocking agents or antihistamines were exclusion criteria. All selected individuals were submitted to clinical evaluation, to skin prick tests and specific IgE determination. All the individuals that presented with a wheal and flare reaction >3mm and specific EgE to lupine and/or peanut ≥0.7 kU/L were included in the study. Twenty six patients that satisfied these criteria agreed to participate and gave written informed consent. Detailed clinical information about allergies to lupine, peanut and atopic state were obtained by standardized questionnaire.

Skin prick test (SPT) and specific IgE measurements: All 26 patients were evaluated by SPT with commercial extracts of nuts and leguminosa and prick/prick to lupine. Histamine dichloride (10 mg/mL) and

the glycerol diluent of the STP extracts served as positive and negative controls. SPT responses were expressed as the largest diameter of the wheal reaction in millimetres. Specific IgE levels to peanut and lupine were determined in all patients by the ImmunoCAP assay (Phadia, Uppsala Sweden).

Protein extraction and quantificatoin: PROBLAD PLUS (0.1 g) was diluted in 10 mL pure water. One gram of lupine flour (Lupinus albus) and peanut (Arachis hypogaea) were grounded with liquid nitrogen and incubated with 10% trichloroacetic acid, 60 mM DTT in cold acetone for 1 hour. After centrifugation, pellets were incubated twice with 60 mM DTT in actrone and centrifuged. The pellets were dried and stored. For β-conglutin extraction, the pellet was resuspended and incubated under agitation, filtered and centrifuged. The precipitate was resuspended in globulin solubilising buffer and EGTA, agitated and centrifuged. The solubilised globulins were precipitated and individual globulins were fractionated and purified by AKTA anion exchange chromatography. The protein present in the samples was quantified through modification of the Lowry method.

IgE immunoblot reactivity assay of plasma from lupine and/or peanut allergic patients: Pellets previously obtained were resuspended, precipitated and centrifuged; proteins were separated by SDS-PAGE based on the method of Laemmli. Separated proteins were transferred to Western blot nitrocellulose membrane and immobilized at the membrane by boiling in MilliQ water. For immunoblotting, the nitrocellulose membranes were blocked; incubated with each serum individual lupine and/or peanut allergic patients and washed; incubated with second stage antibody. Immunoreactive polypeptides were detected with the LUMI-PHOS CWB CHEMI SUBSTRAT enhanced chemiluminescent.

Results

Patient characteristics are shown in the following two tables.

Table 1: Clinical data of each selected patient (Pts-patients; sIgE-specific IgE; Lup-lupine; Pean - peanut; SPT –Skin Prick Test).

Pts	Sex	Age	sIgE Lup (kU/L)	sIgE Pean (kU/L)	SPT Lup (mm)	SPT Pean (mm)	Total serum IgE (UI/ml)	Main clinical reactions
0	F	62	12.3		8		355	Anaphylaxis
1	F	34	4.4		7	6	196	Urticaria/ Angioedema
2	F	42	2.0		4		73	Urticaria/ Angioedema
3	F	53	5.9		6		69	Urticaria/Angioedema, anaphylaxis
4	M	18	5.3		4		653	Rush
5	F	44	0.7		6		474	Urticaria/Angioedema, anaphylaxis
6	F	63		0.7	6	3	19	Angioedema, anaphylaxis
7	F	72	1.4	5.0	6	5	191	Urticaria/Angioedema, anaphylaxis
8	F	26		9.0	3		122	Rush
9	F	19	4.3	8.0	6	6	245	Urticaria
10	M	28		12	5	3	157	Urticaria/Angioedema, anaphylaxis
11	F	44		3	4	3	343	Urticaria/Angioedema, oral Pruritus
12	F	25		4.3	5	5	1140	Rush, Urticaria
13	F	45		1.3	6	6	227	Angioedema, tongue and lips pruritus,
14	M	18	1.4	1.7	7	3	362	Urticaria
15	M	46		4.0	5	5	44	Rush, Urticaria
16	M	18		6.0	6	5	623	Urticaria
17	M	18		1.0	4	5	54	Angioedema, tongue and lips pruritus,
18	M	55		3.0		3	1830	Urticaria
19	M	19		12.0	3	4	213	Urticaria/Angioedema
20	F	55		23.4	3.0	3	198	Urticaria
21	F	58		0.9	4	5	78	Urticaria
22	M	22		16.2	3		545	Urticaria
23	F	29		2.0	3	3	173	Urticaria
24	F	50		0.7	4	3	191	Tongue and lips pruritus, Rush
25	M	18		1.2		8	207	Urticaria/Angioedema

Table 2: Sensitization to nuts and leguminosa evaluated by skin prick tests or serum specific IgE.

Pts	soya	almond	bean	walnut	chestnut	pea	hazelnut	pine nut	pistachio	sunflower seed
0	Yes		yes			yes				
1	Yes	yes	yes		Yes	yes	yes			
2					Yes					
3	Yes									
4	Yes		yes			yes				
5										
6	Yes	yes		Yes	Yes		yes	yes		
7	Yes		yes						yes	
8			yes	Yes	Yes		yes			yes
9	Yes	yes	yes		Yes	yes				
10	Yes		yes	Yes	Yes		yes			
11	Yes		yes							yes
12	Yes		yes		Yes	yes				yes
13		Yes	yes						yes	
14			yes		Yes					
15			yes		Yes	yes				yes
16	Yes	yes								yes
17		yes		Yes						
18	Yes									yes
19	Yes		yes	Yes	Yes	yes			yes	
20	Yes		yes	Yes	Yes		yes	yes		yes
21			yes	Yes	Yes		yes	yes		
22	Yes	yes	yes		Yes			yes		
23		yes		Yes					yes	yes
24										yes
25			yes		Yes	yes				yes

Immunoblot analysis was performed to assess if BLAD is specifically recognized by IgE antibodies from patients who are sensitive to lupine and/or peanut. The immunoblot analysis showed a well-defined reaction against total lupine protein, purified β-conglutin and total peanut protein, with some of the sera tested depending on individualized sensitivity. These results are in accordance with the UniCAP value of each tested serum, where the higher value gave a strong intensity of IgE biding.

Conclusion

In this study a signal was produced in the immunoblots corresponding to the individual allergic total protein, whereas no signal was obtained for BLAD with all the 26 tested sera. In conclusion, the selected serum from sensitized patients to either lupine, peanut (or in some situations both sensitizations) and other nuts and legumina did not react with BLAD. Therefore, BLAD is considered not a potential allergen.

Study 4 – Supplement to clinical study

Report

Anonymous, (2013) Evaluation of the allergenic and cros-allergenic potential of the BLAD polypeptide (Supplement to study report no. CHUC-021113; MRID 49276602). CEV SA, Unpublished report No.: CEV

Guideline n/a

GLP Yes

New information in the supplemental report

In the final report of the previous study (shown under study 3 in this section), some of the immunoblotting results only show a faint signal for the positive control. In an attempt to increase the signal, one approach is to use a more concentrated solution of the tested serum.

The methods used were exactly as described in the previous study; the only modification was the dilution of patient serum #2, 3, 166, 17 and 23.

After the conclusion of the study report, 5 adult patients with symptoms to lupin and/or peanut ingestion previously selected and invited to participate in the original study but unable to attend during the assay, were now available and included in this additional testing. The new individuals were submitted for clinical evaluation, skin prick tests and the determination of allergic reaction to nuts and leguminosa as well as specific IgE determination of the food that provoke an allergic reaction.

Results and conclusion

The characteristics of the new patients are shown in the following table.

Table 1: Clinical data of each selected patient (Pts-patients; Lup- lupin; Pean - peanut; SPT –Skin Prick Test).

Pts	Sex	Age	sIgE Lup (kU/L)	sIgE Pean (kU/L)	SPT Lup (mm)	SPT Pean (mm)	Main clinic reactions
26	F	37	4.5	10.6	4	8	Tongue and lips pruritus, anaphylaxis
27	M	21		9.0		5	Urticaria/Angioedema
28	M	43	3.0	11.2	4	8	Urticaria/Angioedema Anaphylaxis
29	F	32	3.0	7.9	3	5	Angioedema, tongue and lips pruritus
30	M	18		22.4		8	Anaphylaxis

Immunoblot analysis was performed on sera from 5 patients from the original study (#2, 3, 16, 17, 23) using twice the concentration of the patients serum and for the 5 new patient sera.

A positive control signal was produced in the immunoblots corresponding to the individual allergic total protein, whereas no signal was obtained for BLAD with all the tested sera.

The selected sera from sensitized patients to either lupin, peanut (or in some situations both sensitizations) did not react with BLAD. Therefore, BLAD is not considered a potential allergen based on the guidance provided in Codex (Codex Alimentarius Commission 2003; Joint FAo/WHO Food Standard programme, appendix III, Guideline for the conduct of food safety assessment of foods derived from recombinant-DNA plants; Appendix IV Annex on the assessment of possible allergenicity) and FAO/WHO (Evaluation of allergenicity of genetically modified foods, 2001).

Following the commenting round, a **data requirement (2.4)** was set to submit additional argumentation on the lack of penetration ability of the epidermis and dermis by proteins whose size or structure is similar to the BLAD protein (including literature references).

In response to this data requirement, the applicant provided the following report:

Study 5 – Dermal penetration of BLAD

Report: Expert opinion on the dermal penetration of BLAD. Gledhill, A., 2019. Report no. 0387776-Tox3.

Guideline n/a

GLP No

The protein BLAD is characterized as a 210 kDa polypeptide which has been shown to be composed of 1736 amino acid residues and which exists in solution in the aqueous formulation.

The skin provides a robust barrier to the exterior environment, protecting against exogenous toxic compounds and pathogenic microorganisms. In order to penetrate the non-viable stratum corneum and reach the epidermis and dermis, chemicals have to either travel between the corneocytes by moving through the lipid matrix (intercellular route) or pass through the corneocytes themselves (intracellular route). Thus dermal penetration depends to a large extent on the physical properties of the chemical. According to EFSA 2017 Guidance on Dermal Absorption, properties that affect absorption include:

- octanol/water partition coefficient
- molecular size
- ionization

For a compound to use the intercellular route it has to have sufficient lipophilicity to dissolve in the lipid matrix and to use the intracellular route it has to be water soluble. The degree of ionization will clearly affect lipophilicity and water solubility. Molecular size affects the ability to pass between or into the tightly packed corneocytes. Since molecular size is not routinely measured, molecular weight is usually used as a surrogate and a molecular weight of >500 Da has been used as a trigger for the use of a lower default dermal absorption value (EFSA Journal 2011;9(7):2294).

The study of dermal penetration has mostly been conducted on drugs and agrochemicals which are generally considered to be drug-like molecules *i.e*. having a molecular weight of <500 Da, moderate to high lipophilicity and water solubility. It is widely accepted that the skin permeation barrier is highly effective for proteins, peptides and other macromolecules (Paudel etal., 2010), mainly due to molecular size.

With the success of transdermal delivery patches for drugs such as nicotine and anti-inflammatories more attention has been given to the potential for transdermal delivery of proteins and peptide therapeutic agents such as insulin (molecular weight 5808 Da) or human growth hormone molecule (molecular weight 22124

Da) which must otherwise be administered by the parenteral (subcutaneous/intravenous) route. The advantages of administration *via* transdermal patch over parenteral administrations are obvious but until recently has not been possible, precisely because of the effectiveness of the skin barrier against proteins and other such molecules. Improvements in technology means that transdermal delivery of pharmaceutical polypeptides such as insulin is becoming feasible but only when co-applied with physical or chemical means to disrupt or bypass the stratum corneum (Chaulagain et al., 2018). This includes the use of electric currents, ultrasound waves, encapsulation in transferomes or microneedles.

Conclusions

It is difficult to provide definitive evidence for the lack of dermal absorption of proteins such as BLAD since the accepted scientific view on the skin barrier indicates dermal absorption studies on large protein molecules are pointless. However, by reference to proteins of pharmaceutical interest, it can be demonstrated that polypeptides such an insulin are not absorbable by the dermal route without significant measures to disrupt or bypass the stratum corneum. Insulin is a *ca.* 6 kDa polypeptide consisting of 51 amino acid residues, so is a smaller molecule than BLAD and is thus considered to provide a worse case assessment with respect to dermal absorption. Therefore, by analogy with a polypeptide such as insulin, it is reasonable to conclude that under normal use conditions BLAD will not reach the epidermis or dermis layers within the skin.

References in this report:

EFSA (European Food Safety Authority), Buist, H., Craig, P., Dewhurst, I., Hougaard Bennekou, S., Kneuer, C., Machera, K., Pieper, C., Court Marques, D., Guillot, G., Ruffo, F. & Chiusolo, A. 2017. Guidance on dermal absorption. *EFSA Journal 2017*;**15(6)**:4873, 60 pp. https://doi.org/10.2903/j.efsa. 2017.4873

EFSA Panel on Plant Protection Products and their Residues (PPR); EFSA Scientific Opinion on the science behind the revision of the guidance document on dermal absorption. *EFSA Journal 2011*;**9(7)**:2294, 73pp. https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2011.2294www.efsa.europa.eu/efsajournal

Paudel, K.S., Mieswski, M., Swadley, C.L., Brogden, N.K, Ghosh, P, Stinchcomb, A.L., Challenges and opportunities in dermal/transdermal delivery. Ther. Deliv. 2010 1 (1) 109-131.

Chaulagain, B., Jain, A., Tiwari, A., Verma A., Jain, S.K., Passive delivery of protein drugs through transdermal route Artificial Cells, Nanomedicine, and Biotechnology (2018) 46:sup1, 472-487, DOI: 10.1080/21691401.2018.1430695.

3.8 Germ cell mutagenicity

3.8.1 In vitro data

3.8.1.1 Study 1

Study reference: CA 5.4.1.1/01, B.6.4.1, study 1

PROBLAD PLUS: Bacterial reverse mutation assay using a treat and plate modification. CEV SA, Unpublished report No.: 8325399 (2016)

Guidelines OECD 471 (1997)

GLP Yes (certified laboratory)

Study design

A. Materials:

1. Test Material: PROBLAD PLUS

Description: Brown viscous liquid

Lot/Batch No.: D3133.0615

Purity: 20.3% BLAD (the lead component) (80% other ingredients)

CAS No.: n/a

Stability of test compound: Confirmed stable for the duration of the study

Solvent used: None, test material administered as received

2. Control materials:

Negative: Purified water

Solvent/final concentration: None (test article used as supplied) / 10%

Positive: -S9	*Strain*	*Mutagen*	*Conc. (µg/mL)*
	TA98	2-nitrofluorene	25
	TA100	4-Nitroquinoline 1-oxide	1
	TA1535	N-methyl-N'-nitro-N- nitrosoguanidine	2.5
	TA1537	Acridine mutagen ICR-191	1
	WP2 *uvrA* (pKM101)	N-methyl-N'-nitro-N- nitrosoguanidine	7.5
Positive: +S9	*Strain*	*Mutagen*	*Conc. (µg/mL)*
	TA98, TA100, TA1537	2-aminoanthracene[1]	2.5
	WP2 *uvrA* (pKM101)		1.5, 5, 10, 20

3. Activation: Aroclor 1254 induced rat liver (protein content: 40 mg/mL) was obtained from MOLTOX®. The composition of the 10% S9 reaction mix was: 100 µL S9, 100 µm Na PBS, 5 µM glucose-6-phosphate, 4 µM NADP (disodium), 8 µM $MgCl_2$, 33 µM KCl

[1] Of note, some researchers have suggested that 2-aminoanthracene should not be used as the only positive control to evaluate S9-mix activity as it has been shown that the chemical may be activated by enzymes other than the microsomal cytochrome P_{450} family. The study report contains the S9-production certificate (MOLTOX®) which confirms that the batch of S9 used in this study was sensitive to other known mutagen that requires metabolic activation, benzo[a]pyrene.

4. Test Organisms: *S. typhimurium* strains: TA98, TA100, TA1535, TA1537

E. coli: WP2*uvr*A.

All test organisms were properly maintained and were checked for appropriate genetic markers (*S. typhimurium*: histine and biotin requirement, rfa mutation, *uvr*B sensitivity, ampicillin-resistance (pKM101). *E.coli*: tryptophan requirement, *uvr*B sensitivity) regularly.

5. Test Concentrations:

a) Preliminary cytotoxicity assay: Not conducted

b) Mutation assays: Treat and plate test 1: 0, 16, 50, 160, 500, 1600, 5000 µg/plate

Treat and plate test 2: 0, 51.2, 128, 320, 800, 2000, 5000 µg/plate

B. Study Design and Methods:

1. In-life dates: 23 July 2015 to 14 August 2015

2. Preliminary cytotoxicity assay: Not conducted

3. Treat and plate mutation assay: The treat and plate methodology was used in preference to the standard plate-incorporation test as the test article is a protein which may cause artefacts due to growth stimulation.

Tubes of bacterial culture were centrifuged at 8 – 10000 *g* to remove culture media. Bacterial pellets were resuspended in 100 mM PBS to give ~ 2 x 10^9 cells/mL. The bacterial suspension was dispensed into aliquots of 1.3 mL ready for treatment. 0.2 mL of a dilution of test chemical, vehicle of 0.05 mL of positive control solution + 0.15 mL of phosphate buffer were added followed by 0.5 mL of 10% S9-mix of buffer solution and the mixture was incubated with shaking for 1 hour at 37 °C.

After incubation, bacteria was collected by centrifugation to remove treatment mixture. Bacteria was then resuspended in 100 mM PBS and centrifuged again. Pellets were then resuspended in PBS to give ~10^8 to 10^9 cells/mL. 0.1 mL of each bacterial suspension was then added in triplicate to 2 mL of molten supplemented agar and poured onto Vogel-Bonner E agar plates. Plates were incubated, protected from light for 2 – 3 days.

4. Toxicity assessment: The background lawns of the plates were examined for signs of toxicity. Other toxicity indicators that may have been noted included a marked reduction in revertants compared to the concurrent vehicle controls and/or a reduction in mutagenic response.

4. Statistics: Statistics not warranted

5. Evaluation criteria: The test article was considered mutagenic in this assay if :

- A concentration related increase in revertant numbers which is ≥2-fold (in strains TA98, TA100 or WP2 *uvrA* pKM101) or ≥3-fold (in strains TA1535 or TA1537) above the concurrent vehicle control values.

- The positive trend/effects described above were reproducible.

Results

Analytical determinations:

Analytical determination was deemed unnecessary as the test article was provided pre-formulated. Test article was administered as received using the specific gravity (1.21 g/mL) to adjust test article concentration.

Preliminary cytotoxicity assay:

No preliminary cytotoxicity test was undertaken.

Mutation assay:

Experiment 1: following treatments, evidence of toxicity was observed on the mutation plates treated at 1600 µg/mL and above in strain TA1537 in the absence and presence of S9, and at 5000 µg/mL in strains TA98 and TA100 in the absence of S-9 and in strain TA1535 in the absence and presence of S-9.

Experiment 2: the maximum test concentration of 5000 µg/mL was retained for all strains. Narrowed concentration intervals were employed covering the range 51.2 - 5000 µg/mL, in order to examine more closely those concentrations of PROBLAD PLUS approaching the maximum test concentration and considered therefore most likely to provide evidence of any mutagenic activity. Following these treatments, evidence of toxicity was observed at 2000 and/or 5000 µg/mL in strain TA1537 in the absence and presence of S-9 and in strains TA98, TA100 and TA1535 in the absence of S-9 only.

The test article was completely soluble in the aqueous assay system at all concentrations treated, in each of the experiments performed.

The positive controls induced an acceptable increase in revertant colony numbers, thereby demonstrating the sensitivity and specificity of the test system.

Following PROBLAD PLUS treatments of all the test strains in the absence and presence of S-9, no increases in revertant numbers were observed that were ≥2-fold (in strains TA98, TA100 and WP2*uvrA* pKM101) or ≥3-fold (in strains TA1535 and TA1537) above the concurrent vehicle control. This study was therefore considered to have provided no evidence of any PROBLAD PLUS mutagenic activity in this assay system (refer to Tables B.6.4.1-1 and B.6.4.1-2).

Table B.6.4.1-1: Bacterial (reverse) gene mutation treat and plate data – Experiment 1

Conc	TA98		TA100		TA1535		TA1537		WP2*uvr*A	
(µg/plate)	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0	24.6	21.6	98.6	78.8	15.2	16.6	5.8	4.4	131.4	161.6
16	21.7	26.3	91.0	99.3	12.3	15.3	4.0	5.3	131.0	150.0
50	11.7	28.0	91.0	90.3	14.0	13.3	5.0	3.3	---[1]	---[1]
160	15.0	22.3	94.3	81.0	13.3	12.7	3.3	4.7	132.7	145.3
500	23.0	21.3	98.3	98.0	15.0	15.0	7.3	3.3	197.7	150.3
1600	15.7	23.0	79.7	81.3	8.7	15.7	3.0	1.7	128.3	148.3
5000	12.0[V]	23.0	65.7	103.0	8.7[S]	7.3[S]	[T]	2.0[V]	117.3	152.0
+ve	1066	782.3	931.7	537	1066	133.0	807.3	24.0	514.3	357.7

1 No bacteria present. Likely lost following washing procedure. Strain however acceptable as 5 scorable doses are present
S Slight thinning of bacterial lawn
T Toxic, no revertant colonies

+ve controls:
-S9 (absence of metabolic activation):
TA98: 2-nitrofluorene
TA100: 4-nitroquinoline 1-oxide

Conc (µg/plate)	TA98		TA100		TA1535		TA1537		WP2*uvr*A	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9

V Very thin background bacterial lawn

TA1535: N-methyl-N'-nitro-N-nitrosoguanidine
TA1537: ICR-191 mutagen
WP2*uvr*A: N-methyl-N'-nitro-N-nitrosoguanidine
+S9 (presence of metabolic activation):
All strains: 2-aminoanthracene

Table B.6.4.1-2: Bacterial (reverse) gene mutation treat and plate data – Experiment 2

Conc (µg/plate)	TA98		TA100		TA1535		TA1537		WP2*uvr*A	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0	21.2	22.8	100.2	104.4	16.4	21.4	8.0	7.2	150.4	165.8
51.2	25.7	25.7	95.7	102.3	18.0	25.7	11.0	6.0	166.3	162.3
128	21.7	32.0	108.3	110.3	16.3	20.3	9.3	9.3	164.3	158.3
320	24.7	29.0	105.0	110.0	15.3	25.3	7.7	6.7	161.0	155.0
800	21.0	29.7	111.3	141.0	15.3	22.3	8.0	5.0	181.3	163.3
2000	14.0[S]	25.7	96.0	111.7	22.0	14.7	4.0[S]	5.7	159.7	160.0
5000	14.0[S]	24.7	79.0[S]	106.0	8.7[S]	29.3	4.3[S]	2.0[S]	153.3	156.7
+ve	1280	443	734	427	1113	119	1012	28	877	389

1 No bacteria present. Likely lost following washing procedure. Strain however acceptable as 5 scorable doses are present
S Slight thinning of bacterial lawn
T Toxic, no revertant colonies
V Very thin background bacterial lawn

+ve controls:
-S9 (absence of metabolic activation):
TA98: 2-nitrofluorene
TA100: 4-nitroquinoline 1-oxide
TA1535: N-methyl-N'-nitro-N-nitrosoguanidine
TA1537: ICR-191 mutagen
WP2*uvr*A: N-methyl-N'-nitro-N-nitrosoguanidine
+S9 (presence of metabolic activation):
All strains: 2-aminoanthracene

Table B.6.4.1-3: Bacterial (reverse) gene mutation treat and plate data – Laboratory historical control data

	TA98		TA100		TA1535		TA1537		WP2*uvr*A	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Historical vehicle control strain values										
No. of studies	19	19	19	19	19	19	19	19	17	17
Mean	22	22	94	102	13	13	10	7	176	190
Median	21	21	92	102	13	13	9	6	177	187
95% r.r	5-39	11-53	52-159	62-155	4-25	2.0-33	2-28	1-21	108-252	93-297
Historical positive control strain values										
No. of studies	19	19	19	19	19	19	19	19	17	17
Mean	1190	397	667	322	1280	54	1388	39	512	520
Median	1082	408	639	318	1320	50	1422	37	517	239
95% r.r	610-2499	47-609	119-1500	134-493	588-2128	29-130	99-2550	11-131	225-904	117-404

Ranges calculated in February 2011 by Covance Statistics, using data selected without bias from studies started during the periods given below:

S. typhimurium strains TA98, TA100, TA1535, TA1537 January 2006 to March 2009
E.coli strain WP2*uvr*A pKM101 January 2006 to August 2008

+ve controls:
-S9 (absence of metabolic activation):
TA98: 2-nitrofluorene
TA100: 4-nitroquinoline 1-oxide
TA1535: N-methyl-N'-nitro-N-nitrosoguanidine
TA1537: ICR-191 mutagen
WP2*uvr*A: N-methyl-N'-nitro-N-nitrosoguanidine
+S9 (presence of metabolic activation):
All strains: 2-aminoanthracene
95% r.r.: 95% reference range - calculated from percentiles of the observed distributions

Deficiencies:

None.

Conclusion

It was concluded that PROBLAD PLUS did not induce mutation in four histidine-requiring strains (TA98, TA100, TA1535 and TA1537) of *Salmonella typhimurium*, and one tryptophan-requiring strain (WP2*uvrA* pKM101) of *Escherichia coli* when tested under the conditions of this study. These conditions included treatments at concentrations up to 5000 µg/mL (the maximum recommended concentration according to current regulatory guidelines, and in several cases a toxic treatment concentration), in the absence and presence of a rat liver metabolic activation system (S9) using a modified Treat and Plate methodology.

3.8.1.2 Study 2

Study reference: CA 5.4.1.2/01, B.6.4.1, study 2

PROBLAD PLUS: *In vitro* L5178Y gene mutation assay at the *tk* locus. CEV SA, Unpublished report No.: 8325403 (2015a)

Guidelines	OECD 476 (1997) (also compliant with OECD 490 (28 July 2015)
GLP	Yes (certified laboratory)

Study design

A. Materials:

1.	**Test Material:**	PROBLAD PLUS
	Description:	Brown viscous liquid
	Lot/Batch No.:	D3133.0615
	Purity:	20.3% BLAD (the lead component), (80% other ingredients)
	CAS No.:	n/a
	Stability of test compound:	Confirmed stable for the duration of the study
	Solvent used:	None, test material administered as received using the specific gravity (1.21 g/mL) to adjust test article concentration
2.	**Control materials:**	
	Negative:	Purified water
	Solvent/final concentration:	None (test article used as supplied) / 10%
	Positive: -S9	Methyl methane sulphonate (MMS) – 15 and 20 µg/mL
	Positive: +S9	Benzo[a]pyrene (B[a]P) – 2 and 3 µg/mL
3.	**Activation:**	Aroclor 1254 induced rat liver (protein content: 40 mg/mL) was obtained from MOLTOX®. The composition of the 2% S9 reaction mix was: S9, 180 mg/mL glucose-6-phosphate, 25 mg/mL NADP, 150 mM KCl,
4.	**Test cells:**	L5178Y $tk^{+/-}$ mouse lymphoma cells originated from Dr Donald Clive,

Burroughs Wellcome Co. The selection agent was trifluorothymidine (TFT)

5. Cell culture media: RPMI 1640 medium (supplied with HEPES and L-glutamine) supplemented with heat inactivated horse serum (5% (v/v) for treatment [termed RPMI A] 10% (v/v) for growing post treatment [RPMI 10] and 20% (v/v) growing in microtiter plates [RPMI 20]), 100 units/mL / 100 µg/mL penicillin/streptomycin, 2.5 µg/mL Amophotericin B, 0.5 mg/mL Pluronic (not included in RPMI 20], 0.2 mg/mL sodium pyruvate

6. Test concentrations:

a) Preliminary cytotoxicity assay: 3 h +/-S9: 0, 156.3, 312.5, 625, 1250, 2500, 5000 µg/mL. Single culture used.

b) Mutation assays: 3 h –S9: 0, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000 µg/mL
3 h +S9: 0, 150, 300, 600, 900, 1200, 1250, 1300, 1500, 1800, 2100, 2500 µg/mL. Duplicate cultures used

B. Study Design and Methods:

1. In-life dates: 21 July 2015 to 9 September 2015

2. Preliminary cytotoxicity assay: Cells were exposed to test article formulations, solvent controls for either 3 h in the absence of S9 or 3 h in presence the of S9. Single cultures were used. At the end of treatment cultures were washed, cell culture counted, adjusted (where necessary to 2 x 10^5 cells/mL) transferred to cell culture flasks and incubated for 2 days, with cell adjustment made every 24 hours. At this time cells were sub-cultured to assess cytotoxicity and to initiate the phenotypic expression.
Cloning efficiency was determined by plating ~1.6 cells/well into two 96 well plates. Plates were incubated for 10-14 days. After this period the number of wells without growth of cells was counted.
Osmolality and pH were measured on post-treatment media. As no marked changes are observed, further measurements were not deemed necessary for the mutation experiments.
Solubility of the test article in culture medium was assessed, by eye, at the beginning and end of treatment.

3. Mutation assay: The mutation assay was conducted as detailed above for the preliminary cytotoxicity assay, with the following exceptions:
Duplicate cultures were used along with positive controls included.
Mutation frequency was determined by plating ~2000 cells/well in cell culture medium containing 4 µg TFT/mL. Plates were incubated for 10-14 days. After this period the number of well without growth was counted to provide CE in TFT. Wells with growth in indicated evidence of TFT-resistance mutants.
Colony sizing was performed on negative and positive controls.

4. Toxicity assessment: Toxicity was assessed by RTG – relative total growth. RTG is the measure of cytotoxicity relative to the control that takes into account all cell growth and cell loss during the treatment period and the 2 day expression period (relative

suspension growth) and the cells' ability to clone 2 days after treatment.

5. Acceptance criteria: The assay was considered acceptable if all the following criteria (as defined by Moore *et al*[2]) were met:

- The mean mutant frequencies in the vehicle control cultures fell within the normal range (50 to 170 mutants/10^6 viable cells)
- At least one positive control showed either an absolute increase in mean total MF of at least 300 x 10^{-6} (at least 40% of this should be in the small colony MF), or an increase in small colony mutant frequency of at least 150 x 10^{-6} above the concurrent vehicle control
- The RTG for the positive controls was greater than 10%
- The mean cloning efficiency of the vehicle controls from the Mutation Experiments were between the range 65% to 120%
- The mean SG of the vehicle controls from the Mutation experiments were between the range of 8 to 32 following 3 hour treatments

6. Evaluation criteria: The test article was considered mutagenic in this assay (as defined by Moore *et al*[2]*)* if :

- The MF of any test concentration exceeded the sum of the vehicle control mutant frequency plus GEF
- The linear trend test was statistically significant
- Any observed response is reproducible under the same treatment conditions.

The test article was considered positive in this assay if both of the above criteria were met.

The test article was considered negative in this assay if neither of the above criteria were met.

7. Statistics: Liner trend analysis was only required if the mutant frequency of any test article concentration exceeded the sum of the vehicle control mutant frequency + GEF

Results

Analytical determinations: Analytical determination was deemed unnecessary as the test article was provided pre-formulated. Test article was administered as received using the specific gravity (1.21 g/mL) to adjust test article concentration.

[2] Moore *et al*. (2002). Mouse lymphoma thymidine kinase gene mutation assay: Follow-up International workshop on genotoxicity test procedures, New Orleans, Louisiana, April 2000. *Environmental and Molecular Mutagenesis*, **40**, 292-299

Moore *et al*. (2003). Mouse lymphoma thymidine kinase gene mutation assay: International workshop on genotoxicity test workgroup report – Plymouth, UK 2002. *Mutation Research*, **540**, 127-140.

Moore *et al*. (2006). Mouse lymphoma thymidine kinase gene mutation assay: Follow-up meeting of the international workshop on Genotoxicity testing – Aberdeen, Scotland, 2003 – Assay acceptance criteria, positive controls, and data evaluation. *Environmental and Molecular Mutagenesis*. **47**, 1-5.

Moore *et al*. (2007). Mouse lymphoma thymidine kinase gene mutation assay: Meeting of the International workshop on genotoxicity testing, San Francisco, 2005, recommendations for 24-h treatment. *Mutation. Research*, **627**, 36-40.

Preliminary cytotoxicity assay: The maximum concentration tested in the preliminary test was 5000 µg/mL, a dose level which displayed post-treatment precipitate in the culture medium in both 3 hour treatments, with precipitate also observed at 2500 µg/mL in the presence of S9.

In the absence of S9, toxicity was observed such that the highest concentration analysed (5000 µg/mL) gave 0% RTG. The highest concentration to give a measurable level of toxicity was 2500 µg/mL, which gave 89% RTG. In the presence of S9, toxicity was observed such that the highest concentration analysed (2500 µg/mL) gave 0% RTG. The highest concentration to give a measurable level of toxicity was 1250 µg/mL, which gave 15% RTG.

Mutation assay:

Experiment 1:

-S9:

The maximum concentration tested was 5000 µg/mL, however post-treatment precipitate was observed at concentrations 2000 µg/mL and above. As precipitate was observed at more than one concentration in the lowest precipitating concentrations was retained and the higher precipitating concentrations discarded. No significant toxicity was observed such that the highest concentration analysed (2000 µg/mL) gave 78% relative total growth (RTG), in the presence of precipitate.

In the absence of S9, no increases in mutant frequency (MF) which exceeded the sum of the Global Evaluation Factor (GEF) of 126 + the vehicle control MF, were observed in any treated cultures and no statistically significant linear trend was noted.

+S9:

The maximum concentration tested was 2500 µg/mL, however post-treatment precipitate was observed at concentrations 1500 µg/mL and above. As precipitate was observed at more than one concentration in the lowest precipitating concentrations was retained and the higher precipitating concentrations discarded. The highest concentration selected (1200 µg/mL) was later discarded due to excessive toxicity (RTG <10%). Therefore the highest concentration analysed which gave an acceptable RTG value was 900 µg/mL (14% RTG).

In the presence of S9, there was an increase in mutant frequency which exceeded the sum of the GEF + vehicle control MF at the highest concentration analysed (900 µg/mL) which gave rise to a highly significant linear trend ($p \leq 0.001$). At 900 µg/mL, increases in both small and large colony mutant frequencies were observed, with increases predominantly in small colonies (28% in control *vs.* 78% in at 900 µg/mL) (refer to Table B.6.4.1-4).

Table B.6.4.1-4: Mouse lymphoma toxicity and mutant frequency data from experiment 1

Conc. (µg/mL)	-S9		Conc. (µg/mL)	+S9		
	%RTG	MF		%RTG	MF	Proportion of small colony mutants
0	100	53.57	0	100	57.67	0.28
500	105	51.74	150	109	59.47	-
1000	103	50.34	300	119	61.14	--
1500	113	57.34	600	64	155.66	-
2000PP	78	62.39	900	14	449.12***	0.78
MMS 15	68	262.04	B[a]P 2	89	338.13	0.54
MMS 20	44	432.59	B[a]P 3	53	738.42	0.62

+ve controls: MMS - methyl methanesulphonate; B[a]P - benzo[a]pyrene
PP: precipitate observed at the end of treatment
***Sum of the vehicle control mutant frequency (MF) + GEF (126) being exceeded, with accompanying test for linear trend (one-sided) significant at $p \leq 0.001$

Experiment 2:

-S9:

The maximum concentration tested was 2500 µg/mL. No-treatment precipitate was observed. RTG was reduced to 16%.In the absence of S9, no increases in MF which exceeded sum of the GEF + vehicle control MF were observed in any treated cultures and no statistically significant linear trend was noted.

+S9:

The maximum concentration tested was 1200 µg/mL. No-treatment precipitate was observed. RTG was reduced to 19%. In the presence of S-9, there was an increase in mutant frequency which exceeded sum of the GEF + vehicle control MF at concentrations of ≥1000 µg/mL (giving ≤30% RTG) which gave rise to a highly significant linear trend ($p \leq 0.001$). At the concentrations which exceeded the GEF, increases in both small and large colony mutant frequencies were observed; the increases were predominantly in small colonies (34% in the controls *vs.* 61%, 55% and 62% at concentrations of 1000, 1100 and 1200 µg/mL) (refer to Table B.6.4.1-5).

The positive controls induced an acceptable increase in mutation frequency and an acceptable increase in the number of small colony mutants in both experiments, thereby demonstrating the sensitivity and specificity of the test system.

Table B.6.4.1-5: Mouse lymphoma toxicity and mutant frequency data from experiment 2

Conc. (µg/mL)	-S9		Conc. (µg/mL)	+S9		
	%RTG	MF		%RTG	MF	Proportion of small colony mutants
0	100	59.13	0	100	46.88	0.34
600	76	77.47	300	74	79.30	-
900	108	74.01	400	57	104.33	--
1200	106	73.66	500	49	98.40	-
1500	104	95.85	600	39	127.74	-
1750	89	80.88	700	48	126.07	-
2000	58	111.31	800	42	141.91	-
2250	66	72.07	850	38	129.20	-
2500	16	118.07	900	33	168.13	-
MMS 15	59	439.63	950	28	157.88	-
MMS 20	45	729.04	1000	30	203.14***	0.61
			1100	24	218.12***	0.55
			1200	19	230***	0.62
			B[a]P 2	65	342.06	0.52
			B[a]P 3	58	443.95	0.54

+ve controls: MMS - methyl methanesulphonate; B[a]P - benzo[a]pyrene
PP: precipitate observed at the end of treatment
***Sum of the vehicle control mutant frequency (MF) + GEF (126) being exceeded, with accompanying test for linear trend (one-sided) significant at $p \leq 0.001$

Deficiencies:

None. Although the study was conducted prior to the publication of the new test guideline for the mouse lymphoma *tk* version of the assay (OECD 490 (28 July 2015)), the study is deemed compliant with the new guideline when assessed retrospectively against the new test guideline requirements.

Discussion

The increase in small colony formation observed on two separate occasions in the presence of S9 was deemed to be indicative of a clastogenic (chromosome breakage) mechanism of action[3].

Under the conditions of this study the data constituted a clear positive result in the presence of S9 and indicated the possibility that the increase in MF observed is potentially being driven by a clastogenic type of mechanism.

Conclusion

It is concluded that under the conditions of this assay PROBLAD PLUS induced mutation at the *tk* locus of L5178Y mouse lymphoma cells when tested up to toxic concentrations in the presence of S9 for a 3 hour treatment. Under the same test system, PROBLAD PLUS did not induce mutation at the *tk* locus of mouse lymphoma L5178Y cells in the absence of S9 for a 3 hour treatment when tested up to either a precipitating or a toxic concentration under the experimental conditions employed.

3.8.1.3 Study 3

Study reference: CA 5.4.1.3/01, B.6.4.1, study 3

PROBLAD PLUS: *In vitro* human lymphocyte micronucleus assay. CEV SA, Unpublished report No.: 8325400 (2015)

Guidelines OECD 487 (2014)

GLP Yes (certified laboratory)

Study design

A. Materials:

1. **Test Material:** PROBLAD PLUS
   Description: Brown viscous liquid
   Lot/Batch No.: D3133.0615
   Purity: 20.3% BLAD (the lead component), (80% other ingredients)
   CAS No.: n/a
   Stability of test compound: Confirmed stable for the duration of the study
   Solvent used: None, test material administered as received using the specific gravity (1.21 g/mL) to adjust test article concentration
2. **Control materials:**

[3] Moore, M.M., Clive, D., Hozier, J.C., Howard, B.E., Batson, A.G., Turner, N.T. & Sawyer, J. (1985). Analysis of TFT^r mutants of L5178Y/TK+/- mouse lymphoma cells. *Mutation Research* **151**, pp 161 -174 Applegate, M.L., Moore, M.M., Broder, C.B, Burrell, A., Juhn, G., Kasweck, K.L., Lin, P-F., Wadhams, A. & Hozier, J.C. (1990). Molecular dissection of mutations at the heterozygous thymidine kinase locus in mouse lymphoma cells *PNAS* **87**, pp 51 - 55

Negative:	Purified water
Solvent/final concentration:	None (test article used as supplied) / 10%
Positive: -S9	Mitomycin C (MMC) in purified water at 0.2 and 0.3 µg/mL (3+21 h), 0.1 and 0.2 µg/mL (24+24 h) Vinblastine (VIN) in purified water at 0.04 and 0.06 µg/mL (24+24 h)
Positive: +S9	Cyclophosphamide (CPA) in dimethyl sulphoxide (DMSO) at 2.0 and 3.0 µg/mL

3. Activation: Aroclor 1254 induced rat liver (protein content: 40 mg/mL) was obtained from MOLTOX®. The composition of the 2% S9 reaction mix was: S9, 180 mg/mL glucose-6-phosphate, 25 mg/mL NADP, 150 mM KCl,

4. Test cells: Human peripheral blood was obtained by venipuncture from healthy donors. Cell cycle time was typically 13±2 h

5. Cell culture media: 0.5 mL of whole blood was added to tubes containing 8.1 mL of HEPES-buffered RPMI medium containing 10% (v/v) heat-inactivated foetal calf serum and 0.52% penicillin/streptomycin. Phytohemagglutinin was added at a concentration of 2% to stimulate the lymphocytes to divide.

6. Test concentrations:

a) Preliminary cytotoxicity assay:	3+21 h +/-S9, 24+24 h –S9: 0, 7.256, 12.09, 20.16, 33.59, 55.99, 93.31, 155.5, 259.2, 432, 720, 1200, 2000 µg/mL. Duplicate cultures were used for vehicles and single culture used for test article treatments
b) Mutation assays:	3+21 hour -/+ S-9: 100, 200, 400, 800, 1200, 1600, 2000 µg/mL 24+24 hour –S-9: 100, 200, 400, 800, 1200, 1400, 1600, 1800, 1900, 2000 µg/mL. Quadruplicate cultures were used for vehicles and duplicate cultures were used for test article treatments

B. Study Design and Methods:

1. In-life dates: 22 July 2015 to 28 August 2015

2. Preliminary cytotoxicity assay: Following establishment of cultures (48 hours), S-9 mix or KCl (0.5 mL/culture) was added as appropriate. Cells were exposed to the test article for 3 hours in the absence and presence of S9 (from rats induced with Aroclor 1254). These cultures were sampled at 24 hours after the beginning of treatment (*i.e.* at 72 hours after culture initiation). In addition, a continuous 24-hour treatment (equivalent to approximately 1.5 to 2 times the average generation time of cultured lymphocytes from the panel of donors used in this laboratory) in the absence of S9 was included. These cultures were sampled at 48 hours after the beginning of treatment (*i.e.* at 96 hours after culture initiation).
At the end of treatment, the test article was removed by centrifugation. The cell pellet was washed and resuspended in fresh pre-warmed medium.
Cytochalsin-B (Cyto-B) was added post-wash to cultures to inhibit cytokinesis, resulting in binucleate cells (without effecting karyokinesis). Duplicate cultures

were used for the vehicle control and single cultures were used/test article concentration.

Cytotoxicity was determined by examination of the proportion of mono, bi and multinucleate cells to a minimum of 200 cells/concentration. From this, the replication index (RI) was calculated for each concentration (see Cytotoxicity section below).

3. Micronucleus assay:

Cell treatment: Cells were exposed to the test article formulation, vehicle or positive control as described above, with the exception of quadruplicate cultures used for the vehicle control and duplicate controls used for the positive controls and each test article concentration. Concentrations selected for micronucleus analysis were assessed for cytotoxicity uncoded and for the proportions of mono-, bi- and multinucleate cells to a minimum of 500 cells/culture.

Spindle inhibitor: Cyto-B was added post-wash to cultures to inhibit cytokinesis.

Slide preparation: Slides were prepared by spreading the fixed cultures on clean slides. The slides were immersed in 125 µg/mL acridine orange in phosphate buffered saline (PBS) at pH 6.8 for approximately 10 seconds, washed with PBS (with agitation) for a few seconds before transfer and immersion in a second container of PBS for approximately 10 minutes. Slides were air-dried and stored protected from light at room temperature prior to analysis.

Cytotoxicity: The RI, which indicates the relative number of nuclei compared to controls, was determined using the formula below:

$$RI = \frac{no.\ of\ binucleate\ cells + 2(no.\ of\ multinucleate\ cells)}{total\ no.\ of\ cells\ in\ treated\ cultures}$$

Relative RI (expressed in terms of percentage) for each treated culture was calculated as follows:

$$Relative\ RI\ (\%) = \frac{RI\ of\ treated\ cultures}{RI\ of\ vehicle\ control} \times 100$$

Cytotoxicity (%) was expressed as (100 – Relative RI).

A minimum of 500 cells/culture were examined for cytotoxicity. The intention was to achieve 50-60% cytotoxicity, if this was the limiting factor.

Micronucleus assessment: Slides from the highest selected concentration and two lower concentrations were taken for microscopic analysis.

Immediately prior to analysis, 1-2 drops of PBS were added to the slides before mounting with glass coverslips. One thousand binucleate cells from each culture (2000/concentration) were analysed for micronuclei. The number of cells containing micronuclei and the number of micronuclei per cell on each slide were noted. Observations were recorded on raw data sheets. The microscope

stage co-ordinates of the first six micronucleated cells were recorded.

4. Statistics: Binomial Dispersion Test χ^2 using a statistical significance level of $p \leq 0.05$

5. Evaluation criteria: For valid data, the test article was considered to induce clastogenic and/or aneugenic events if:

1. A statistically significant increase in the frequency of MNBN cells at one or more concentrations was observed.
2. An incidence of MNBN cells at such a concentration that exceeded the normal range in both replicates was observed.
3. A concentration-related increase in the proportion of MNBN cells was observed.

The test article was considered positive in this assay if all of the above criteria were met.

The test article was considered negative in this assay if none of the above criteria were met.

Results

Analytical determinations:

Analytical determination was deemed unnecessary as the test article was provided pre-formulated. Test article was administered as received using the specific gravity (1.21 g/mL) to adjust test article concentration.

Preliminary cytotoxicity assay:

The maximum concentration tested in the preliminary test was 2000 µg/mL, a dose level which displayed no post-treatment precipitate in the culture medium.

No marked toxicity (as measured by replication index) was observed in either of the 3 hour treatments undertaken, with cytotoxicity of 1% and 18% in the absence and presence of S9, respectively. For the 24 hour treatment, 55% cytotoxicity was observed at the highest dose tested.

Micronucleus assay:

Treatment of cells with PROBLAD PLUS for 3+21 hours in the absence and presence of S9 resulted in frequencies of MNBN cells that were generally similar to and not significantly higher than the concurrent vehicle controls at all concentrations analysed. The MNBN cell frequencies of all treated cultures were within the 95th percentile of the current historical control (normal) range under both treatment conditions with the exception of a single culture at 2000 µg/mL for the 3+21 hour -S9 treatment in which the MNBN cell frequency (1.5%) marginally exceeded the normal range of 0.2 to 1.4% but fell within the observed range of 0.2 to 1.5%. There were no indications of a concentration-related response, as indicated by non-significant linear trend tests under both treatment conditions.

Treatment of cells for 24+24 hours in the absence of S9 resulted in frequencies of MNBN cells that were significantly higher ($p \leq 0.05$) than the concurrent vehicle controls at the highest concentration analysed (1600 µg/mL, giving 58% reduction in RI). However, the MNBN cell frequencies of all treated cultures fell within the normal range and the Cochran-Armitage linear trend test was not significant, therefore the statistically significant increase seen at 1600 µg/mL was considered of little or no biological relevance (refer to Table B.6.4.1-6).

Table B.6.4.1-6: Human lymphocyte micronucleus data

3 h (+21 h recovery) –S9			3 h (+21 h recovery) +S9			24 h (+24 h recovery) -S9		
Conc (µg/mL)	Cyto (%)[a]	MNBN freq.[b]	Conc (µg/mL)	Cyto (%)[a]	MNBN freq.[b]	Conc (µg/mL)	Cyto (%)[a]	MNBN freq.[b]
0	-	0.55	0	-	0.35	0	-	0.50
800	8	0.60 NS	800	1	0.45 NS	200	0	0.70 NS
1600	22	0.65 NS	1600	9	0.35 NS	400	18	0.65 NS
2000	19	0.95 NS	2000	11	0.35 NS	1200	36	0.75 NS
MMC 0.2	22	3.55***	CPA 2.0	57	1.60 ***	1600	58	0.95 **
						VIN 0.04	59	3.60 ***

Historical vehicle control ranges (Calculated in February 2014 by CLEH Statistics, for studies started between May 2012 and October 2013)		
Frequency of MNBN cells/cells scored (%):		
Mean: 0.60 Median: 0.64 SD: 0.318 95% reference range: 0.20-1.40 [From 21 studies (61 cultures)]	Mean: 0.60 Median: 0.62 SD: 0.281 95% reference range: 0.20-1.20 [From 22 studies (64 cultures)]	Mean: 0.40 Median: 0.44 SD: 0.230 95% reference range: 0.10-1.10 [From 11 studies (54 cultures)]

** $p \leq 0.05$; *** $p \leq 0.001$; NS Not significant
a cytotoxicity based on replication index
b mean micronucleated binucleate frequency (%)
+ve controls: MMC: Mitomycin C; CPA: Cyclophosphamide; VIN: vinblastine

Deficiencies: None

Conclusion

It is concluded that PROBLAD PLUS did not induce micronuclei in cultured human peripheral blood lymphocytes following treatment for 3 [+21 hour recovery] and 24 hours [+24 hour recovery] in the absence and for 3 hours [+21 hour recovery] in the presence of a rat liver metabolic activation system (S9; 2% v/v) up to a maximum concentration of 2000 µg/mL (the maximum recommended concentration for the *in vitro* micronucleus assay) in the short term treatments or a concentration (1600 µg/mL) limited by toxicity in the longer term treatment.

3.8.2 Animal data

3.8.2.1 Study 1

Study reference: CA 5.4.2.1/01, B.6.4.2, Study 1

PROBLAD PLUS: Rat alkaline comet assay. CEV SA, Unpublished report No.: 8325402 (2015b)

Guidelines OECD 489 (2014)

GLP Yes (certified laboratory)

Study design

A. Materials:

1. Test Material: PROBLAD PLUS

Description: Brown viscous liquid

Lot/Batch No.:	D3133.0615
Purity:	20.3% BLAD (the lead component), (80% other ingredients)
CAS No.:	n/a
Stability of test compound:	Confirmed stable for the duration of the study
Solvent used:	None, test material administered as received using the specific gravity (1.21 g/mL) and dose volume to adjust test article dose level administered

2. Control materials:

Negative:	Purified water
Solvent:	None (test article used as supplied)
Positive:	Ethyl methane sulphonate (EMS) – 200 mg/kg bw

3. Test animals -

Species:	Rat
Strain:	Han Wistar
Age:	49-56 day
Weight at dosing:	Range-finder: ♂: 196-221g; ♀: 142-158g Main experiment: ♂: 192-241g
Source:	Charles River Laboratories, Margate, UK
Acclimation period:	5 days

4. Test article concentrations:

Range-finding test:	Animals received two doses at 2000 mg/kg, separated by 21 h (3 rats/sex) at a dose volume of 1.65 mL/kg bw
Comet assay:	Animals received two doses 0, 500, 1000 or 2000 mg/kg bw separated by 21 h (5 ♂ rats/group) at dose volumes of 1.65, 0.41, 0.83 or 1.65 mL/kg bw, respectively

B. Study Design and Methods:

1. In-life dates:	20 July 2015 to 2 September 2015
2. Treatment and sampling times:	For the comet assay, checks were made to ensure the weight variation for animals prior to dosing was minimal and did not exceed 20% of the mean weight. As no difference in toxicological response was observed between genders in the range-finder experiment, only males were treated in the main study as in general genotoxicity responses between males and females are similar. Animals were dosed orally *via* gavage at doses of 0, 500, 1000, 2000 mg/kg bw. The test article was dosed as received, with the specific gravity used to calculate the dose level, and the dose volume varied to achieved the required dose. Doses were administered at 0 and 21 h, with animals killed at 24 hour, stomach tissue harvested and blood collected *via* the abdominal aorta for clinical chemistry analysis. A positive control was dosed with EMS (200 mg/kg bw) on day 2, with

	sacrifice 3 h post dosing.
3. Clinical chemistry analysis:	Sampled from all vehicle and test article treated animals at necropsy from the abdominal aorta: liver function (alanine aminotransferase, alkaline phosphatase, aspartate aminotransferase, total bilirubin, total cholesterol, albumin, globulin, albumin:globulin, total bilirubin) kidney function (sodium, potassium, calcium, chloride, inorganic phosphorus, creatinine, urea)
2. Sacrifice and pathology:	All control and test article treated animals underwent macroscopic examination for unusual colouration or abnormalities to organs/tissues. Stomach tissue was removed from all animals and histopathology undertaken on stomach samples from all vehicle and test article treated animals. Embedded stomach was sectioned at a nominal 5 µm, stained with haematoxylin and eosin and examined by the Study Pathologist.
3. Tissue and cells sampled:	Stomach was sampled, and single cell suspensions were prepared by mechanical dissociation within 1 h of animal necropsy. Cell suspensions were held on ice until slide preparation.
6. Details of slide preparation:	The cell suspension was mixed with low melting agar and 100 µL/slide of this mixture was plated on to 3 slides/animal and cover slipped. Slides were placed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH adjusted to 10, 1% Triton X-100, 10% DMSO) and left overnight. Following lysis, slides were placed in an electrophoresis tank containing buffer (300 mM NaOH, 1 mM EDTA, pH >13) to allow DNA unwinding (20 minutes) followed by electrophoresis at 0.7 V/cm for 20 minutes. As not all slides could be processed at the same time a block design was employed for the unwinding and electrophoretic steps in order to avoid excessive variation across the groups for each electrophoretic run; i.e. for all animals the same number of triplicate slides were processed at a time. Immediately after electrophoresis slides were washed in neutralisation buffer (0.4 M Tris, pH 7.0 (3 *x* 5 minute washes)). Once neutralised all slides were air dried and stored at room temperature. Immediately prior to scoring 100 µL of ethidium bromide (2 µg/mL) was applied to the slide.
7. Details of scoring:	Slides were coded and scored blind using the Perceptive Instruments Comet IV software linked to fluorescent microscope. The number of 'hedgehogs' observed during comet scoring was recorded. 150 cells/animal/tissue were scored for comet analysis, evenly split over three slides. The comet parameters reported were the %tail intensity (i.e. %DNA in the tail) and the Olive tail moment. The median value/slide was calculated and the mean of the slide medians was calculated to give the mean animal value. The mean of the animal means and standard error of the mean was calculated for each group
8. Cytotoxicity	Histopathology assessment, clinical chemistry findings, %hedgehogs on comet

assessment: slides (high levels of "hedgehogs" indicates the nuclear complex has been significantly fragmented and is considered evidence of excessive DNA damage. Such damage may be due to the cytotoxic nature of the treatment or due to excessive mechanical disruption during cell isolation and has the potential to interfere with Comet analysis).

9. Acceptance criteria: The assay was considered valid if all the following criteria were met:

- The vehicle control data were comparable to laboratory historical control data for each tissue
- The positive control induced responses that were compatible with the laboratory historical control data and produced a statistically significant increase compared to the concurrent vehicle control
- Adequate numbers of cells and doses were analysed
- The high dose was considered to be the MTD, the maximum recommended dose or the maximum practicable dose.

10. Evaluation criteria: The test article was considered to induce DNA damage if:

- A least one of the test doses exhibited a statistically significant increase in tail intensity, compared with the concurrent vehicle control
- The increase was dose related

The test article was considered negative in this assay if neither of the above criteria were met and target tissue was confirmed.

11. Statistics: Tail intensity data was used for statistical analysis.

The positive control group was compared to the vehicle control group using a two-sample t test. The test was interpreted with one-sided risk for increased response with increasing dose.

The vehicle control group the treated groups were analysed separately using one-way analysis of variance (ANOVA). An overall dose response test was performed along with Dunnett's test for pairwise comparisons of each treated group with the vehicle control. For all tissues the test was interpreted with a one-sided risk.

Levene's test for equality of variances between the groups were performed and where evidence of heterogeneity ($p \leq 0.01$) was present, the data were rank transformed prior to analysis.

C. Methods:

1. Clinical observations: Range-finder: each animal was observed prior to, immediate and at 0.5, 1, 2 and 4-6 h post dosing on days 1 and 2.

Main experiment: each animal was observed prior to, immediate and at 1, 2 and 4 h post dosing on day 1. On day 2 each animal was observed prior to and immediately post dose and prior to necropsy.

2. Body weights: Range-finder: day 1 (prior to dosing) and Day 2 (prior to termination).

Main experiment: pre-dose phase (study set-up), Day 1 (prior to dosing) and day

2 (prior to necropsy)

Results

Analytical determinations:

Analytical determination was deemed unnecessary as the test article was provided pre-formulated. Test article was administered as received with the specific gravity used to calculate the dose level, and the dose volume varied to achieve the required dose.

Range finding test:

There were no clinical signs of toxicity observed following dosing at 2000 mg/kg bw/day in either males or females, with all animals gaining weight during the dosing/observation period. As there were no sex related differences in toxicity, the comet assay was conducted in male animals only.

Comet assay:

Clinical observations: No test article related clinical signs of toxicity were observed.

Body weight: No test article related effects on body weight were observed.

Toxicity: No test article related toxicity, as evident by hedgehog occurrence on comet slides were observed.

Clinical chemistry: No test article related clinical chemistry effects were observed.

Necropsy and pathology: Both macroscopic and microscopic observations were deemed to be unremarkable.

Target tissue exposure: There is no bioanalytical method available to detect organ exposure, as PROBLAD PLUS contains the naturally occurring polypeptide component, BLAD, the protein will be broken down, enter the amino acid pool and be consumed into normal catabolic processes. Consequently, radiolabelling of the test article is neither possible nor cost effective. To overcome this, the *in vivo* comet assay investigating DNA damage at the site of contact (the stomach) was deemed to be a valid and appropriate way forward to address any concerns over adequate target organ exposure.

Comet analysis: The positive controls induced an acceptable increase in %tail intensity in stomach, thereby demonstrating the sensitivity and specificity of the test system.

Table B.6.4.2-1: Group mean stomach data

Dose level (mg/kg bw/d)	No. of animals	No .of cells scored	Mean tail intensity[a] (% ±SD)	SEM	Mean tail moment[a] (% ±SD)	SEM	Mean % hedgehogs
0	6	900	0.95 ±1.20	0.49	0.12 ±0.14	0.06	11.85
500	6	900	1.03 ±0.96	0.39	0.13 ±0.13	0.05	9.84
1000	6	750[b]	0.31 ±0.12	0.05	0.04 ±0.01	0.01	8.79
2000	6	900	0.47 ±0.33	0.13	0.05 ±0.04	0.02	13.57
EMS, 200	3	450	9.91 ±0.98	0.56	1.21 ±0.08	0.05	24.34

Historical control data ranges for rat stomach comet Data generated from 13 studies dosed between January 2010 to July 2014					
Vehicle	116	Mean:	2.75 ±1.63	0.29 ±0.20	11.98 ±3.94
		Median:	2.44	0.25	12.00
95% reference range:			0.66-6.01	0.09-0.66	4.43-17.50
Positive	103	Mean:	26.61 ±7.22	3.78 ±1.38	17.14 ±5.19
		Median:	25.66	3.52	17.50
95% reference range:			16.92-39.73	2.06-6.49	9.00-25.00

+ve control: EMS – ethyl methylsulphonate

SEM =- standard error mean

a median values of each slide calculated. The mean of the slide medians were calculated to give the individual mean animal value. The individual mean animal values were averaged to provide group mean

b no cells present on the slides examined, however data available from 5 animals within the group

Deficiencies: None

Conclusion

It is concluded that PROBLAD PLUS did not induce DNA damage in the stomach of male Han Wistar rats following oral gavage dosing at 0 and 21 hour, with harvesting of stomach tissue 3 hours later. The maximum dose administered was 2000 mg/kg bw/day, the maximum recommended dose in accordance with current regulatory guidelines for short-term *in vivo* toxicity testing.

3.9 Carcinogenicity

No long term toxicity and carcinogenicity studies have been conducted and none are considered necessary. PROBLAD PLUS contains 20% BLAD (the lead component). BLAD, is a naturally occurring polypeptide formed during day four to twelve of the germination process of sweet lupines (*Lupinus albus*). BLAD is used in human and animal nutrition, as a food and feed item, it has a non-toxic mode of action which is specific to fungi only (BLAD binds to chitin and chitosan which weakens the cell wall structure and so has been found to be a very effective fungicide against powdery mildew and other diseases) and it is rapidly biodegradable. It is known to be susceptible to proteolytic degradation and the protein will be broken down, enter the amino acid pool and be consumed into normal catabolic processes. A complete genotoxicity test battery confirmed a lack of genotoxic potential. Furthermore, the 90-day study was completely negative and does not give any indication of non-genotoxic carcinogenicity. There is no evidence in the public domain to suggest that proteins similar to BLAD, which contains a segment of β-conglutin (which shares strong homology to other members of the vicilin family (globulin storage protein associated with leguminous seeds such as peas and lentils)) are associated with an increased incidence of cancer. Based on this it can be concluded that the lead component is unlikely to be considered a carcinogen.

3.10 Reproductive toxicity

No reproductive studies (including developmental studies) have been conducted and none are considered necessary. PROBLAD PLUS contains 20% BLAD (the lead component). BLAD, is a naturally occurring polypeptide formed during day four to twelve of the germination process of sweet lupines (*Lupinus albus*). BLAD is used in human and animal nutrition, as a food and feed item, it has a non-toxic mode of action which is specific to fungi only (BLAD binds to chitin and chitosan which weakens the cell wall structure and so has been found to be a very effective fungicide against powdery mildew and other diseases) and it is rapidly biodegradable. It is known to be susceptible to proteolytic degradation and the protein will be broken down, enter the amino acid pool and be consumed into normal catabolic processes. There is no evidence in the public domain to suggest that proteins similar to BLAD, which contains a segment of β-conglutin (which shares strong homology to other members of the vicilin family (globulin storage protein associated with leguminous seeds such as peas and lentils)) are toxic either in a reproductive or developmental capacity. Based on this it can be concluded that the lead component is unlikely to be considered a reproductive or developmental toxin.

3.11 Specific target organ toxicity – single exposure

3.11.1 Animal data

Please refer to sections 3.1 – 3.3

3.12 Specific target organ toxicity – repeated exposure

3.12.1 Animal data

3.12.1.1 Study 1: 90-day oral toxicity in the rat

Study reference: CA 5.3.2/01, B.6.3.2 Study 1

reference	:	90-day oral rat	exposure	:	gavage
Report number	:	8325453, 2016	doses	:	0, 250, 500, 1000 mg/kg bw/day
test substance	:	PROBLAD PLUS, Lot/Batch nr: D3133.0615, purity: 20% BLAD	GLP statement	:	yes
species	:	Rat, Crl:WI(Han)	guideline	:	in accordance with OECD 408
group size	:	10/sex/dose	acceptability	:	acceptable

Study design

A. Materials:

1. Test Material: PROBLAD PLUS

Description: Brown viscous liquid

Lot/Batch No.: D3133.0615

Purity: 20,3% BLAD (the lead component) (80% other ingredients)

CAS No.: n/a

Stability of test compound: Confirmed stable for the duration of the study

2. Vehicle and/or positive control: None, test material administered as received / none.

3. Test animals:

Species: Rat

Strain: Crl:WI(Han)

Age: 7 – 8 weeks

Weight at dosing: ♂: 203 – 253 g; ♀: 143 – 188 g

Source: Charles River Laboratories, Margate, UK

Acclimation period: 7 days

B. Study Design:

1. In life dates: 16 June 2015 to 20 November 2015

2. Animal assignment and treatment:

The animals were assigned to treatment groups on arrival using a total randomisation procedure. Cages were placed in treatment group order across the batteries.

Following the first full weighing (Pre-dose 1/Day -7), group mean body weights and standard deviations were calculated and inspected to ensure there were no unacceptable differences between groups.

Animals were dosed orally *via* gavage once daily for 90 consecutive days, with the dose level varying by dose volume administered (refer to Table B.6.3.2-1).

A control group were orally dosed, with water.

Table B.6.3.2-1: Study design and dose received

Dose level (mg/kg bw/d)	Specific gravity (g/mL)	Dose volume (mL/kg)	Animals assigned	
			♂	♀
0	1.0	0.8	10	10
250	1.21	0.2	10	10
500	1.21	0.4	10	10
1000	1.21	0.8	10	10

3. Statistics:

ANOVA: body weight gains, necropsy (terminal) body weight, haematology, coagulation, clinical chemistry and organ weight variables were analysed separately for each sex.

Levene's test for equality of variances among the groups was performed. Where this showed no evidence of heterogeneity ($p \leq 0.05$) and the group effect from the ANOVA was significant ($p \leq 0.05$), pairwise comparisons with control were made using Dunnett's test. Where Levene's test was significant ($p \leq 0.05$), a rank-transformation was applied to the data prior to analysis. If the group effect of the ANOVA was significant, *t*-tests were used for pairwise comparisons.

C. Methods:

1. Clinical observations: Each animal was given a detailed physical examination at weekly intervals.

2. Post dosing observations: Week 1: once daily at the 1 hour time point post dosing

Week 2 to week 13: once weekly at the 1 hour time point post dosing

3. Functional observation battery:

Observational measurements before removal from the home cage: posture, activity, gait, tremor, convulsion, excessive vocalisation, arousal upon opening cage. These observations were undertaken on all animals before treatment and once a week thereafter, approximately 2 h post dosing.

Observational measurements after removal from the home cage: Ease of removal, ease of handling, excessive vocalisation, tremor, convulsion, palpebral closure, exophthalmus, lacrimation, lacrimation type, salivation, respiration, piloerection, appearance of fur, other These observations were undertaken on all animals before treatment and once a week thereafter, approximately 2 h post dosing.

	Open field measurements: The animal was placed into an arena for 2 minutes and the following measured: Latency to first step, posture, arousal, circling, gait type, gait type severity, stereotypy, tremor, convulsion, other. Number of rears, faecal boli and urine pools. Faecal consistency and the presence of polyuria. These observations were undertaken on all animals before treatment and once a week thereafter, approximately 2 h post dosing. Sensory reactivity to stimuli and grip strength: Approach response, touch response, tail pinch, air righting ability, pupillary response, corneal tactile reflex test, auditory startle response, hindlimb foot splay, forelimb and hindlimb grip strength. These observations were undertaken on all animals during week 13, approximately 2 h post dosing. Motor activity: Assessed in an automated photocell activity recorder for 30 minutes. Activity counts recorded at 2 minute intervals. These observations were undertaken on all animals during week 13, approximately 2 h post dosing.
4. Body weight:	Individual body weights were recorded at randomisation, then weekly thereafter from day 1 (before dosing) and at termination (on animals that were necropsied).
5. Food consumption:	The amount of food consumed by each animal was determined weekly from Week 1. Consumption was calculated as g/animal/day.
6. Ophthalmoscopy examination:	Conducted pre-treatment on all animals and then at week 12/13 on vehicle and high dose group animals
7. Haematology & clinical chemistry:	Conducted on all animals at termination. Haematology: haemoglobin, red blood cell count, packed cell volume (haemocrit), mean cell volume, mean cell haemoglobin, mean cell haemoglobin concentration, reticulocyte count, red cell distribution width, haemoglobin width, total and differential white cell count, platelet count, platelet volume (termed platelet crit), mean platelet volume, platelet distribution width, fibrinogen, prothrombin time, activated partial thromboplastin time, blood smear Clinical chemistry: aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, chloride, total cholesterol, total bilirubin, total protein, albumin, globulin, albumin:globulin ratio, total cholesterol, total bilirubin, sodium, potassium, chloride, calcium, inorganic phosphate, creatinine, urea nitrogen, glucose
8. Urinalysis:	Not conducted
9. Sacrifice and pathology:	Scheduled necropsy were performed on day 91, following an overnight fast. The following tissues were examined microscopically from all control and high dose animals: Adrenals, aorta, bone (femur [+ marrow], sternum [+ marrow]), brain (6 levels), eyes (+ optic nerve), GALT/Peyers patch, gross lesions, gastrointestinal tract (duodenum, jejunum, ileum, caecum, colon, rectum), heart, kidneys, larynx, liver, lymph node (mesenteric, mandibular), lung, mammary gland, muscle

	(quadriceps), nasal cavity, nerve (sciatic), oesophagus, ovary, pancreas, pituitary, prostate, salivary gland (mandibular, sublingual, parotid), sciatic nerve, seminal vesicles, skin, spinal cord (cervical, thoracic, lumber), spleen, stomach, testis (+ epididymis), thymus, thyroid (+ parathyroids), tongue, trachea, urinary bladder, uterus, vagina For low and mid dose group animals only the liver, spleen, kidney and gross lesions, and for females only the brain and spinal cord were examined microscopically. Histopathological examination of the nervous system (brain, sciatic and spinal nerve) was included to assess neurotoxic potential (in addition to the FOB). Potential immunotoxic effects were examined via histopathological assessment of the thymus and spleen along with total and differential white cell counts. Hormone sensitive organs were also histopathologically examined.
10. Organ weights:	The following organs were weighed from all animals: Adrenals, brain, epididymides, heart, kidney, liver, ovary, spleen, testis, thymus, uterus

Results

Observations:

Mortality and clinical signs: All animals survived until the scheduled necropsy. No test article related effects were observed. Clinical signs included: head burrowing (single incidence in one male), salivation (three males exhibiting a single incidence) and raised hair (single female) were noted for a small number of animals only at 1000 mg/kg bw/day. These clinical observations were sporadic and not replicated in the opposite sex. The distribution of these and other clinical observations (*e.g*. thinning/stained fur, noisy respiration) did not suggest a test article-related effect.
Ophthalmoscopic examination: No test article related effects were observed.

Body weight and body weight gain: Overall group mean body weight gain for Weeks 1-13 did not show any statistically significant differences from control and there were no meaningful intergroup differences in weekly mean weight changes during the study.

Food consumption: Food consumption was similar to controls for all treated groups.

Blood analysis:

Haematological findings: No remarkable differences in haematological parameters were observed when test article-treated animals were compared to the controls.
Clinical chemistry findings: No remarkable differences in clinical chemistry parameters were observed when test article-treated animals were compared to the controls.

Functional observation battery:

There was no effect of treatment on weekly measurements (before/upon removal from home cage, in open field) or on end of study motor activity and sensory reactivity. Occasional statistically significant differences from control at 1000

mg/kg/day were isolated (Week 2 and 8 rears, Week 12/13 motor activity TA13 only) and therefore deemed not to be treatment-related. There was nothing in the data to suggest that animal #140 (high dose female) exhibited any adverse neurological effects compared to other animals within the dose group (refer to histopathology section below).

Pathology

Organ weight: There were no test article-related alterations in mean final body weights or mean organ weights.

Table B.6.3.2-2: Absolute organ weights (in grams)

	Males				Females			
	0	**250**	**500**	**1000**	**0**	**250**	**500**	**1000**
Terminal body weight (g)	412.0	393.0	396.2	422.7	244.0	233.9	240.7	250.6
Spleen	0.742	0.687	0.691	0.715	0.542	0.550	0.510	0.587
Liver	9.283	8.875	9.066	9.755	6.164	6.207	6.118	6.436
Adrenal	0.065	0.065	0.061	0.056	0.067	0.067	0.071	0.062
Kidney	2.190	2.079	2.162	2.157	1.459	1.531	1.444	1.504
Testis	3.594	3.441	3.463	3.582	-	-	-	-
Epididymis	1.427	1.431	1.422	1.384	-	-	-	-
Thymus	0.450	0.420	0.368	0.392	0.352	0.294	0.301	0.320
Brain	2.116	2.063	2.027*	2.069	1.963	1.909	1.912	1.933
Ovary	-	-	-	-	0.160	0.154	0.163	0.153
Uterus	-	-	-	-	0.783	0.644	0.656	0.749

Gross pathology: There were no test article-related gross pathology alterations observed at necropsy.

Histopathology: A single female (#140) at 1000 mg/kg bw/d exhibited spinal and brain lesions. This was minimal to slight bilateral symmetrical neuropil vacuolation in the grey matter. In the brain, the following areas were affected: dorsal mid-brain (superior colliculi) and medulla oblongata (several ventral and dorsal nuclei *e.g*. dorsally the vestibular nuclei and ventrally the facial nuclei). In the spinal cord, both dorsal and ventral horns showed increased vacuolation when compared to other females in the study, including those in the control group. No brain or spinal cord changes were observed in males of either control or high dose groups.

Vacuolation of the CNS usually requires transmission electron microscopic examination, ideally on perfused fixed material, to determine the cellular/subcellular location of the vacuoles so an exact localisation of the vacuolation could not be done for the current study.

Artefactual vacuolation is common in sections of the brain and spinal cord due to suboptimal fixation[4]. In contrast, vacuolation associated with genuine pathology is usually accompanied by inflammatory cells[5].

There was no evidence of infiltration by inflammatory cells or inflammatory changes associated with the vacuolation observed in the current study. There were no associated behavioural or clinical signs observed during the in-life phase that could be correlated with the histopathological changes identified in the single affected female. It is important to point out that perfusion fixation represents the gold standard for special neuropathological assessment, rather than immersion fixation of the brain and associated structures. The latter was performed in this study, and whilst it is

[4] Sahota, P.S., Toxicological pathology, Nonclinical safety assessment, pp 907, 911-912, 919-920, CRC Press, Boca Raton (2013)

[5] McInnes E.F., Background Lesions in Laboratory Animals, pp 17-26, Saunders Elsevier, Edinburgh (2012).

generally acceptable as a routine screening method, its limitations are understood and the artefacts produced are recognised. It is important not to over interpret the presence of such an artefact, particularly as it often varies considerably between animals and treatment groups[6].

Although an artefactual nature is likely for the effect seen in the single female at 1000 mg/kg bw/d, a genuine pathological effect cannot be excluded with certainty due to the following:

- A lack of association with other artefactual changes (*e.g.* dark neurons);
- A bilateral symmetrical and unusual distribution, and the absence of the effect in similarly processed brain from other animals in the study;
- This effect has not been seen in control animals from previous Wistar rat studies run at the Test Facility.
- All other microscopic changes reported in the tables were considered incidental or consistent with expected spontaneous changes in this strain and age of rat.

No pathological lesions of the brain and spinal cord were observed in either males of the control or high dose group

Deficiencies:

According to the new data requirements, in the 90-day study potential neurotoxic and immunotoxic effects, genotoxicity and effects potentially related to changes in the hormonal system should be addressed. These were all assessed, except for the genotoxicity. The notifier gave the following explanation:

Whilst there is a requirement to consider genotoxicity by the way of micronuclei formation, as no analytical method is available to detect organ (bone marrow) exposure, this endpoint was assessed by the rat comet assay examining DNA in the stomach (first site of contact).

Conclusion

In this study no effects on either body weight, food consumption, haematology, clinical chemistry, FOB, organ weights or gross pathology were found. During histopathology, the only remarkable finding was one female in the high dose group having slight vacuolation in the brain (multifocal/bilateral, grey matter, mid brain (superior colliculi) and medulla oblongata (dorsal and ventral nuclei)) and vacuolation of the spinal cord (cervical, minimal; lumbar, slight). This finding was not observed in any of the other females in this or in the other dose groups, neither in the males (only control and high dose examined).

Considering that no other neurological findings were observed, including in the FOB, RMS considers this an incidental finding. RMS proposes the highest dose tested (1000 mg/kg bw/day) as the NOAEL, since no test-article related adverse effects were observed in this study.

Following the **expert meeting** (PREV 25, March 2020), the majority of experts considered the NOAEL should be set at 500 mg/kg bw/day based on vacuolation in brain and spinal cord seen in 1 female at the high dose. It was noted that this was a precautionary approach.

[6] Greaves, P. Histopathology of preclinical toxicity studies. Interpretation and relevance in drug safety evaluation. 4th Edition. Chapter 14 Nervous system and special sense organs. pp 801-802. Elsevier, Canada (2012)

3.12.1.2 Study 2: 22-day dermal toxicity in the rat

Study reference: CA 5.3.3/01, B.6.3.3 Study 1

reference	:	22-day dermal toxicity	exposure	:	Dermal, non-occlusive
Report number	:	8297704, 2015	doses	:	0, 100, 300, 1000 mg/kg bw/day
test substance	:	PROBLAD PLUS, Lot/Batch nr: D31-012014, purity: 20.3% BLAD	GLP statement	:	yes
species	:	Rat, Crl:WI(Han)	guideline	:	in accordance with OECD 410
group size	:	5/sex/dose	acceptability	:	acceptable

Study design

A. Materials:

1. Test Material: PROBLAD PLUS

Description: Brown viscous liquid

Lot/Batch No.: D31-012014

Purity: 20.3% BLAD (the lead component) (80% other ingredients)

CAS No.: n/a

Stability of test compound: Confirmed stable for the duration of the study

2. Vehicle and/or positive control: None, test material administered as received / none.

3. Test animals:

Species: Rat

Strain: Crl:WI (Han)

Age: 10 - 12 weeks

Weight at dosing: ♂: 292 – 353 g; ♀: 186 – 226 g

Source: Charles River, Margate, UK

Acclimation period: 14 to 22 days

B. Study Design:

1. In life dates: 04 March 2014 to 03 April 2014

2. Animal assignment and treatment: The animals were assigned to treatment groups on arrival using a total randomisation procedure. Cages were placed in treatment group order across the batteries.

Following the first full weighing (Pre-dose 1/Day -7), group mean body weights and standard deviations were calculated and inspected to ensure there were no unacceptable differences between groups.

Animals were dosed once daily for 22 days (excluding the day of necropsy), with the dose level varying by dose volume administered (refer to Table B.6.3.3-1). The test article was applied to a clipped area of skin (dorsum) for 6 h/d, 7 d/wk.

An area of at least 10% of the total body surface was clipped on the dorsum of the rat. The total surface area for each animal was calculated according to the largest animal in each group using the following formula:

Surface area (cm^2) = K *x* body weight $(g)^{2/3}$ (where K = 9)

(*e.g.* for a 250g rat with a total body surface area of 357 cm^2 the dermal test site would be 35.7 cm^2, an area of approximately 5 cm *x* 7.2 cm)

The test article was spread as uniformly as possible over as much as possible of the dermal test site. A dense gauze patch was placed over the treated skin and retained in place by an elasticated, open-weave, adhesive compression bandage. This was wrapped securely around the torso of the animal. The dressing was considered to be semi-occlusive. The dressing was removed approximately 6 hours after application. The dermal test site of each rat was swabbed with water-moistened cotton wool before the animal was returned to the holding cage.

Table B.6.3.3-1:Study design and dose received

Dose level (mg/kg bw/d)	Specific gravity (g/mL)	Dose volume (mL/kg)	Animals assigned	
			♂	♀
0	1.0	1	5	5
100	1.2646	0.08	5	5
300	1.2646	0.24	5	5
1000	1.2646	0.79	5	5

3. Statistics: ANOVA: body weight gains, necropsy (terminal) body weight, haematology, coagulation, clinical chemistry and organ weight variables were analysed separately for each sex.

Levene's test) for equality of variances among the groups was performed. Where this showed no evidence of heterogeneity ($p \leq 0.05$) and the group effect from the ANOVA was significant ($p \leq 0.05$), pairwise comparisons with control were made using Dunnett's test. Where Levene's test was significant ($p \leq 0.05$), a rank-transformation was applied to the data prior to analysis. If the group effect of the ANOVA was significant, *t*-tests were used for pairwise comparisons.

C. Methods:

1. Clinical observations: All animals were observed at the beginning and end of the working day for signs of ill health or overt toxicity. Each animal was given a detailed physical examination at weekly intervals.

2. Post dose observations: The animals were observed 1 hour after the removal of the dressings and were observed the day after to observe recovery from the previous day. Inflammatory skin reactions were graded using the scale below:

Erythema	**Grade**
No erythema	0
Very slight erythema	1
Well-defined erythema	2
Moderate erythema	3
Severe erythema (beet redness)	4
Oedema	**Grade**
No oedema	0

Very slight oedema	1
Slight oedema (edges of area well-defined by definite raising)	2
Moderate oedema (edges raised approximately 1mm)	3
Severe oedema (raised more than 1 mm and extending beyond dermal test site)	4

3. **Body weights:** Individual body weights were recorded on Day -7, once weekly from Day 1 (before dose) and on the day of (prior to) necropsy.
4. **Food consumption:** The amount of food consumed by each animal was determined twice weekly from Week 1. Consumption was calculated as g/animal/day.
5. **Ophthalmoscopy examination:** Not undertaken.
6. **Haematology & clinical chemistry:** Conducted on all animals at termination.
   Haematology: haemoglobin, red blood cell count, packed cell volume (haemocrit), mean cell volume, mean cell haemoglobin, mean cell haemoglobin concentration, reticulocyte count, red cell distribution width, haemoglobin width, total and differential white cell count, platelet count, mean platelet volume, platelet distribution width, fibrinogen, prothrombin time, activated partial thromboplastin time.
   Clinical chemistry: liver function (aspartate aminotransferase, alanine aminotransferase, γ glutamyl transferase, alkaline phosphatase, total cholesterol, total bilirubin, total protein, albumin, globulin, albumin:globulin ratio), kidney function (sodium, potassium, chloride, calcium, inorganic phosphate, creatinine, urea nitrogen, glucose).
   As some of the females were below 250 g, the required total blood volume could not be collected in-life. Samples for clinical chemistry were therefore withdrawn from the abdominal aorta of all animals at necropsy.
7. **Urinalysis:** Not conducted.
8. **Sacrifice and pathology:** Scheduled necropsy were performed on day 23, following an overnight fast. The following tissues were examined microscopically from all control and high dose animals:
   Adrenals, dosing site (treated skin), gross lesions, kidney, liver, skin (untreated)
   Mammary gland, pituitary gland, testes and epididymis were preserved but not examined.
9. **Organ weights:** The following organs were weighed from all animals:
   Adrenals, kidney, liver, testis (+ epididymis)

Results

Clinical signs and post-dosing observations: No test article related clinical signs of toxicity were observed, with all animals surviving until the scheduled necropsy. No test article related post dosing observations were observed. Signs of

dermal irritation, including very slight to well-defined erythema and scabbing were noted in all test article treated groups, but the severity did not increase with increasing dose.

Table B.6.3.3-2 Dose site observations

	Males				Females			
	0	100	300	1000	0	100	300	1000
Very slight erythema	1	4	1	4	1	3	5	3
Oedema	0	0	0	0	0	0	0	0
Patchy hair regrowth	0	1	1	2	0	2	4	2
Scabbing	1	2	3	4	0	1	4	4
Scarring	0	1	0	0	0	0	0	0

Body weight and body weight gain: No remarkable differences in body weight and body weight gain were observed. Treated males appeared to show a greater degree of body weight loss compared with females and was more noticeable in the high dose group, but this did not achieve statistical significance nor did it exceed 10% relative to controls. None of the group differences in body weight change achieved statistical significance when compared with controls.

Table B.6.3.3-3: Body weight and body weight changes

Parameter	♂ (mg/kg bw/d)				♀ (mg/kg bw/d)			
	0	100	300	1000	0	100	300	1000
Body weight (g)								
Day 1 [% diff]	334.4	312.0 [-7%]	307.6 [-8%]	314.5 [-6%]	200.4	193.9 [-3%]	202.0 [+1%]	207.5 [+4%]
Day 8 [%diff]	334.1	312.9 [-6%]	310.7 [-7%]	313.8 [-6%]	201.6	195.6 [-3%]	202.7 [+0.5%]	204.1 [+1%]
Day 15 [%diff]	341.9	321.7 [-6%]	319.2 [-7%]	317.5 [-8%]	207.6	198.8 [-4%]	209.6 [+1%]	211.0 [+2%]
Day 22 [%diff]	336.8	311.0 [-8%]	307.3 [-9%]	309.0 [-8%]	203.2	193.0 [-5%]	202.7 [0%]	202.7 [0%]
Body weight change								
Days 1-8	-0.4	0.8	3.1	-0.7	1.2	1.7	0.7	-3.4
Days 8-15	7.8	8.8	8.5	3.7	5.9	3.2	6.9	6.9
Days 8-22	2.7	-1.8	-3.4	-4.8	1.6	-2.6	0.0	-1.5
Days 1-22	2.4	-1.0	-0.3	-5.6	2.9	-0.9	0.6	-4.8

[% diff] - % difference *vs*. control

Food consumption: There were no test article related effects on food consumption.

Blood analysis:

Haematological findings: No remarkable differences in haematological parameters were observed when test article-treated animals were compared to the controls.

Clinical chemistry findings: No remarkable differences in clinical chemistry parameters were observed when test article-treated animals were compared to the controls. Where statistically significant changes in clinical chemistry parameters were observed (refer to Table B.6.3.3-3) these were not considered to be treatment related nor toxicologically significant as the changes observed were not consistent across dose groups.

Table B.6.3.3-4: Clinical chemistry – selected parameters (±SD)

Sex - parameter	Dose level (mg/kg bw/d)			
	0	100	300	1000
Clinical chemistry				
♂: Na (mmol/L)	139 ±1.2	141 ±1.0*	141 ±1.0*	141 ±0.9
♂: Ca (mmol/L)	2.58 ±0.029	2.64 ±0.030	2.68 ±0.072*	2.57 ±0.044
♂: Glu (mmol/L)	8.8 ±0.96	9.0 ±0.87	9.2 ±0.85	9.1 ±0.30
♂: Chol (mmol/L)	1.5 ±0.24	1.6 ±0.23	1.7 ±0.36	1.9 ±0.23
♀: Alkaline phosphatase (IU/L)	32 ±5.0	44 ±9.8	49 ±5.4*	35 ±13.1
Haematology				
♂: Neutrophils (10^9/L)	1.28 ±0.320	1.41 ±+0.128	1.42 ±0.176	1.77 ±0.579
♂: Lymphocytes (10^9/L)	5.67 ±1.462	6.32 ±1.559	5.10 ±1.449	5.18 ±1.328
♂: Eosinophils (10^9/L)	0.14 ±0.036	0.08 ±0.014	0.12 ±0.061	0.13 ±0.074

* $p \leq 0.05$

Organ weights: Group mean adrenal weights, adjusted for terminal body weight, were increased by 16%, 18% and 38% for males given 100, 300 or 1000 mg/kg bw/d when compared with concurrent controls. For high dose animals the adrenal weights were statistically significantly increased ($p \leq 0.05$). The adjusted group means reported are estimated after controlling for the effect of the covariate (terminal body weight) on the dependent variable (organ weight). A summary of these weight changes are discussed below (refer to Table B.6.3.3-4). For treated females adrenal weights were comparable to the control group. Histopathological examination was undertaken to better understand the toxicological relevance of the adrenal weight changes in males.

Table B.6.3.3-5: Male Adrenal weights

Parameter	Dose (mg/kg bw/d)			
	0	100	300	1000
Ter. body weight (g)	328.5	305.5	301.8	300.4
Adrenal unadjusted abs. organ weight (g)	0.063	0.063	0.063	0.073
Historical control data[a]:	Mean: 0.058 (n= 195) Range: 0.035 – 0.080 (Sept 2007 – Mar 2011)			
Adrenal wt : ter. body wt (%)	0.0191	0.0208	0.0208	0.0244
Historical control data[a]:	Mean: 0.171 (n= 195) Range: 0.093 – 0.0248 (Sept 2007 – Mar 2011)			
Adjusted for ter. body wt (g) [%increase][b]	0.056	0.065 [+16%]	0.066 [+18%]	0.077* [+38%]

* $p \leq 0.05$
abs absolute
ter terminal

a Laboratory historical control range derived from Han Wistar rats aged 14-26 weeks of age at necropsy. Range: min – max (95% reference range). Refer to Attachments, Historical control data

b As group means are estimated after controlling the effect of terminal body weight, no historical control data are available

Table B.6.3.3-6 Organ weights (in grams)

	Males				Females			
	0	100	300	1000	0	100	300	1000
Terminal body weight (g)	328.5	305.5	301.8	300.4	195.2	189.3	198.7	196.2
Kidney								
- Absolute	1.968	1.850	1.852	1.936	1.278	1.242	1.388	1.308
- Relative to bw	0.5995	0.6056	0.6140	0.6449	0.6539	0.6554	0.6979	0.6667

Liver								
- Absolute	7.411	6.986	6.883	6.727	4.555	4.418	4.923	4.567
- Relative to bw	2.2560	2.2850	2.2757	2.2400	2.3313	2.3327	2.4756	2.3250
Testis/epididymis								
- Absolute	4.802	4.719	4.565	4.554	-	-	-	-
- Relative to bw	1.4605	1.5453	1.5152	1.5133	-	-	-	-

Gross pathology: There were no macroscopic findings suggestive of either local or systemic effects of PROBLAD PLUS.

Histopathology: Findings related to administration of PROBLAD PLUS were noted in the treated skin of males and females given 1000 mg/kg bw/d and in the kidney of males (refer to Table B.6.3.3-5).

In the treated skin, minimal hyperkeratosis was recorded in males and females given 1000 mg/kg bw/d, characterised by a minor increase in thickness of epidermis with increased keratohylaine granules.

In the kidney, hyaline droplets were increased in males given 1000 mg/kg bw/d, characterised by eosinophilic cytoplasmic inclusions in the proximal tubular epithelial cells. The kidney of females given 1000 mg/kg bw/d was similar to controls. Hyaline droplets are a common and variable background finding in male rats. As there were only five animals/sex/group, it is possible that the minor increase recorded at the high dose could be a chance event. However, a relationship to administration of PROBLAD PLUS cannot be excluded.

Examination of the adrenal glands in high dose males revealed no histopathological lesions, with adrenal glands from high dose treated males being comparable to that of the controls.

Table B.6.3.3-5: Selected histopathology findings

Parameter	♂ (mg/kg bw/d)		♀ (mg/kg bw/d)	
	0	1000	0	1000
Hyperkeratosis	5; 5,0,0,0	5; 0,5,0,0	5; 5,0,0,0	5; 1,4,0,0
Kidney, hyaline droplets	5; 0,2,2,1	5; 0,1,1,3	5: 0,0,0,0	5: 0,0,0,0

no. examined;
no finding, minimal, slight, moderate

Discussion

Hyaline droplets appear as eosinophilic cytoplasmic inclusions in the proximal tubular epithelial cells and are a common background finding in the kidney of male rats. They generally represent accumulations of $\alpha_{2\mu}$globulin, a naturally occurring male rat protein. Chemicals which bind to $\alpha_{2\mu}$globulin and form a complex which is more resistant to catabolism will result in accumulations of hyaline droplets. This is a common response of the male rat to xenobiotics and is of little relevance to risk assessment in humans. The minor increase in hyaline droplets in high dose males in this study may have been associated with renal metabolism or excretion of the test article.

Although an increase in adjusted adrenal weights (up +38%) were observed in males, which achieved statistical significance ($p \leq 0.05$) at the high dose, no accompanying adverse histopathology was present.

The primary cause of adrenocortical hypertrophy is increased adrenocorticotrophic hormone (ACTH) stimulation. Such a condition can arise as a result of the stress response, but it may also occur due to deficient adrenocortical insufficiency and represents a serious adverse toxic effect on the function of the adrenal cortex. The latter condition is defined as

adrenocortical insufficiency and there is insufficient information to conclude that the adrenal effects observed in the study were a result of test article related response. To conclude that the adrenocortical hypertrophy observed in this study was due to stress the following arguments are presented (taken from Harvey & Sutcliffe (2010)[7]:

Background:

- The role of the adrenal gland is to secrete glucocorticoid following stimulation *via* ACTH released from the anterior pituitary gland. The control of the entire system is *via* the hypothalamus and there is distinct circadian rhythm monitored *via* the suprachiasmatic nucleus. The hypothalamus receives neural and humoral information and monitors internal and external environment and responds to adverse factors (physical, biochemical or emotional) by triggering the pathway for glucocorticoid production. The hypothalamus produces two neurohormones, *i*) coricotrophin-releasing hormone (CRH) and *ii*) arginine vasopressin (AVP) which stimulate the secretion of ACTH into the bloodstream which in turn promotes synthesis and secretion of glucocorticoid. The sensitivity of the HPA axis to incoming stimuli is modulated by a servo (negative feedback) system through which the release of CRH/AVP and ACTH is negatively regulated by glucocorticoids themselves. The stress response is superimposed upon the circadian tone; the magnitude and duration of the stress response is variable according to the nature and intensity of the stimulus.
- Ideally to confirm the presence of a stress response it would have been ideal to have had the thymus weighed and examined histologically. Atrophy of the thymus would have been an indicator that the adrenal effects observed were not due to adrenocortical insufficiency, with excretion of glucocorticoids with action on the thymus. Unfortunately this was not the case. However, there are other observations that support a stress response, which are attributable to effects on thymus resulting from adrenocortical hypertrophy due to stress.
- An important metabolic action of glucocorticoids is their action in regulating blood glucose, they act as insulin antagonists and cortisol acts to increase blood glucose levels by promoting gluconeogenesis, increasing hepatic glucose output and inhibiting glucose uptake in fat and muscle. They promote the breakdown of carbohydrates and protein (the latter is responsible for adverse effects on growth) and they also have effects on lipid metabolism. They promote water and sodium resorption in the kidney and potassium excretion. As discussed, whilst thymus tissue was not sampled, there was evidence of a stress leukogram (*i.e.* increase of neutrophil count and a decrease of lymphocytes and eosinophils) as a result of systemic stress of the living body all indicate glucocorticoid excess, with full functionality of the adrenal cortex resulting from stress.
    - In this study signs of external stress were evident from the dermal irritation in all treated animals which persisted up until necropsy. When the above is considered in respect of the biochemical and haematological parameters examined, small (non-significant) changes were reported that were deemed consistent with the discussion above. Blood glucose levels in high dose males were increased *vs* the control group (9.1 *vs*. 8.8 mmol/L). Body weight and body weight gain were reduced for all treated males from day 1 onwards. Cholesterol and serum sodium levels were increased. Evidence of the stress leukogram was present, with lymphopenia, eosinopenia and neutrophila.

The minor increase in adrenal weight in the males is consistent with an adaptive response due to minor stress (for example the discomfort/irritation to the skin as the test article caused erythema and scabbing), without compromise in adrenocortical function and not attributable to a direct test article related toxic effect.

[7] Harvey, P. & Sutcliffe, C. (2010). Adrenocortical hypertrophy: establishing cause and toxicological significance. *J. Appl. Toxicol.* **30**, pp 617-626

Conclusion

The dermal exposure to PROBLAD PLUS for 22 consecutive days did not influence body weight (gain), food consumption, haematology or clinical chemistry. Kidney, liver and testis/epididymis weight were not affected; the increase in adrenal weight in top dose males was not significant and not accompanied by histopathological findings. The increase in hyaline droplets in kidneys of the high dose males is not considered relevant for humans as they generally represent accumulation of α2u-globulin (a naturally occurring male rat protein).

The highest dose of 1000 mg/kg bw/day is considered to be the NOAEL for both male and female rats.

Following the **expert meeting** (PREV 25, March 2020), the majority of experts considered the NOAEL should be set at 300 mg/kg bw/day based on increased adrenal weight when adjusted for terminal body weight.

3.13 Aspiration hazard

The UVCB substance does not contain compounds classified as a cat 1 aspiration hazard and the viscosity exceeds the trigger value of 20.5 mm^2/s at 230 mm^2/s (40 °C). In addition, the dossier does not contain any other (human) evidence PROBLAD PLUS may be an aspiration hazard. Therefore, it does not need to be considered for classification in this hazard category.

4 ENVIRONMENTAL HAZARDS

Sweet Lupin (seeds), *Lupinus albus* L., germ., ext. [PROBLAD PLUS] is a plant extract with fungicidal properties that can be used on food and non-food crops. The product is extracted from the germinated seeds of sweet lupines (*Lupinus albus*) where after filtration and concentration it is formulated into PROBLAD PLUS. The major component BLAD, which forms 20% w/w of the PROBLAD PLUS formulation, is not isolated during the preparation of the product. The majority of the testing summarised in the dossier has therefore been conducted with PROBLAD PLUS.

4.1 Degradation

4.1.1 Ready biodegradability (screening studies)

Study reference:

Author: A. Brunswik-Titze

Year: 2015

Title: Biodegradability, CO2-evolution test according to OECD 301 B (July 1992)

Source: CEV S.A, Unpublished report No.: 1035

Detailed study summary and results:

The degradation of BLAD reached 81.4% within a 14 day window and can therefore be considered as readily biodegradable under the conditions of this closed bottle test. Mean biodegradation of the reference substance, sodium benzoate, was 85.4% on Day 14 and 84.6% at the end of the test. The difference of extremes of replicate values of the removal of the test item at the end of the 14 day window was < 20% (9.09%). These data show that the inoculum was viable and exerting normal degradative activity. The degradation of the toxicity control was >25% (71.6%) after 14 days. Therefore, toxic effects of BLAD can be excluded. The test can therefore be considered valid.

Test type:

Guidelines: OECD Guideline for Testing of Chemicals No. 301 B (July 1992) CO2-evolution test

GLP: Yes (certified laboratory)

Test substance:

Description: BLAD, Isolated protein from an extract from plants of the genus Lupinus.

	White powder
Lot/Batch No.:	No. 06.2015
Purity:	>99 % (w/w) BLAD protein
Carbon content:	43.9% (0.439 mg/mg)
ThCO2:	1.61 mg/mg (based on carbon content)
CAS No.:	1219521-95-5
Stability:	2 years at room temperature

Reference Material:

Description:	Sodium benzoate, white powder
Lot/Batch No.:	BCBH0034V
Purity:	Not stated
Carbon content:	58.3% (0.583mg/mg)
ThCO2:	2.137 mg/mg (based on carbon content)
CAS No.:	532-32-1
Stability:	Not stated

Materials and Methods

Test Design

The test was conducted with activated sludge obtained from the municipal wastewater treatment plant at Breisgauer Bucht. The sampling date of the activated sludge was on 07 July 2015 and the dry solid content of the activated sludge was determined as 4.4 g/L by weight measurements after drying at 105°C (mean of triplicate measurements). The activated sludge was washed twice with tap water and one time with mineral medium by settling the sludge, decanting the supernatant and re-suspending the sludge. The mineral medium was prepared from four stock solutions using demineralised water, as described in Table 4.1.1-01.

Table 4.1.1-01 Composition of mineral medium

Prepared from	Compound	Final concentration
Stock solution A	KH_2PO_4 K_2HPO_4 Na_2HPO_4 2 H_2O NH_4Cl	8.5 g/L 21.75 g/L 33.40 g/L 0.5 g/L
Stock solution B	$CaCl_2 \cdot 2\ H_2O$	36.4 g/L
Stock solution C	$MgSO_4 \cdot 7\ H_2O$	22.5 g/L
Stock solution D	$FeCl_3 \cdot 6\ H_2O$	0.25 g/L

The medium was prepared mixing 10 mL of solution A with 900 mL of demineralised water before adding 1 mL of solutions B, C and D. The final solution was made up to 1 L.

Each reaction vessel was filled with 10.2 mL of the prepared activated sludge and made up to 1500 mL volume with the mineral medium. The final solution contained 30 mg/L of dry solids. The reaction vessels were sealed, aerated with CO_2 free air and kept mixed with magnetic stirrers throughout the study. The vessels were left overnight before the addition of the test and reference materials.

Test systems were divided into four study groups. To the inoculum blanks (three replicates) nothing was added. To the tests (three replicates) 68.3 mg of the BLAD protein was added directly to the reaction vessels. To the reference (three replicates) 5.15 mL of 10 g/L stock solution of sodium benzoate was added. For the toxicity control both BLAD and the reference material sodium benzoate was added at the same rate as for the test and the reference.

Table 4.1.1-02 Summary of the amount of test materials added

	Test substance: BLAD	**Reference: Sodium benzoate**
Carbon content (%)	43.9	58.3
$ThCO_2$ (mg/mg)	1.61	2.137
Amount added (mg)	68.3 mg	5.15 mL of 10 g/L = 51.5 mg
Concentration (mg/L)	45.53	34.33
Total carbon concentration (mg/L)	20	20

Each reaction vessel was equipped with 2 gas wash bottles containing 0.2 M NaOH in series to collect the evolved CO_2. Sampling of the traps was made by syringe through butyl rubber septum to prevent loss of CO_2. Inorganic carbon content of the inoculum in the reactors was made at the start and at the end of the incubation period (28 days) using a total carbon analyser. The inorganic carbon in the NaOH traps was measured at 0, 4, 7, 11, 14, 21 and 28 days using a total carbon analyser. The mean CO_2 production from the blank incubate was subtracted from the test and reference reaction vessels and the toxic control for each time point. The amount of CO_2 was then expressed as a % of the maximum theoretical CO_2 production for the test or reference systems. For the 28 day time point the results were also calculated including the inorganic carbon remaining in the reaction vessels.

Results

The validity of the test was confirmed by achieving the following criteria:

- Inorganic carbon in the blank inoculum was less than 5% of the total organic carbon added to the test systems.
- CO_2 in the blank inoculum did not exceed 40 mg/L at the end of the incubation.
- Difference between the replicates at the end of the 10 day window was <20%.
- Degradation of the reference exceeded 60%$ThCO_2$ by day 14.

- More than 25% degradation occurred in the toxicity test and no inhibition observed.

The degradation results are summarised in the Table 4.1.1-03.

Table 4.1.1-03 Summary of biodegradation results as % of maximum theoretical CO_2 production from degradation.

Sample	Rep.	Days after treatment							
		0	**4**	**7**	**11**	**14**	**21**	**28**	**28***
Test flasks (BLAD)	1	0	47.9	61.3	73.9	70.6	87.4	93.7	96.3
	2	0	48.8	66.1	75.3	71.4	86.0	97.7	94.1
	3	0	50.8	66.8	69.9	75.6	83.3	88.0	83.1
Reference flask (Sodium Benzoate)	1	0	59.2	77.6	81.0	80.2	86.7	76.2	75.3
	2	0	62.2	78.1	86.3	87.0	86.7	98.4	99.2
	3	0	64.2	69.6	82.0	78.7	96.0	90.7	88.7
Toxicity control BLAD + Sodium benzoate	-	0	51.3	69.8	78.2	77.3	87.8	89.9	88.6

* Final measurement including inorganic carbon measurements from the reaction vessel.

The degradation of BLAD exceeded 60% after 7 days and therefore meets the criteria for ready biodegradation study (greater than 60% in a 10 day window).

3. Activated sludge inoculum

The effluent of the municipal activated sludge plant of Pforzheim/Germany was taken as inoculums. The effluent was kept under aerobic conditions in the period between sampling and application. The inoculums was filtered through a coarse filter, the first 200 mL being discarded, and was aerated by shaking one week in an Erlenmeyer flask. The initial number of micro-organisms was determined by enumerating the bacterial colonies in 1 mL inoculated test medium after incubation with plant count agar in Petri dishes for 4 days.

4. Mineral medium

The mineral medium was prepared from four stock solutions (1000 fold concentrated) using ultra-pure grade water.

Table 4.1.1-01 Composition of stock solutions used in the preparation of the mineral medium

Prepared from	Compound	Final concentration
Stock solution 1	KH_2PO_4 K_2HPO_4 Na_2HPO_4 NH_4Cl	8.5 mg/L 21.75 mg/L 26.65 mg/L 0.5 mg/L
Stock solution 2	$CaCl_2 \cdot 2\ H_2O$	36.4 mg/L
Stock solution 3	$MgSO_4 \cdot 7\ H_2O$	22.5 mg/L
Stock solution 4	$FeCl_3 \cdot 6\ H_2O$	0.25 mg/L

5. Test system

Four study groups:

Inoculum blank,	0 mg/L PROBLAD PLUS,	0 mg/L Na-Benzoate
Test item,	2 mg/L PROBLAD PLUS,	0 mg/L Na-Benzoate
Reference item	0 mg/L PROBLAD PLUS,	2 mg/L Na-Benzoate
Toxicity control	2 mg/L PROBLAD PLUS,	2 mg/L Na-Benzoate

Study Design:

The test was performed in BOD flasks with ground-in-glass stoppers in single, 2 and 3-fold test assays for each of the 6 measurement dates (0, 4, 7, 11, 14, 21 and 28 days).

A sufficient volume of ultra-pure grade water was prepared in 5-L volumetric flasks. The flasks were filled at first to about three quarters of their volume with water. The water was strongly aerated for *ca* 10 minutes to achieve oxygen saturation and allowed to stand for about 24 h without aeration at test temperature. The O_2 content was measured at the start of the test.

Initially each study group was prepared in 5-L volumetric flasks. The individual salt stock solutions were added (5 mL of each to < 5000 mL), and either the respective test and reference items were added in the final concentrations of 2 mg/L (10 mg/5 L). Subsequently each flask was inoculated with 5 mL of inoculums and the volume made up to 5L with oxygen saturated water.

The contents of the flasks were distributed into the test vessels and the oxygen contents measured. The bottles were incubated in the dark between 19.1 and 22.0ºC. 3 or 2 bottles of each treatment group were removed at each time point for analysis of O_2 content.

Oxygen concentrations were measured with a WTW Microprocessor Oximeter OXI340 and a calibrated electrode.

Evaluation of degradability

For each date of measurement, the exerted BOD was calculated by subtracting the oxygen concentration (mg O_2/L) of the mean initial inoculums blank from that of the other study groups. Then, for each test assay the measured values of the controls were subtracted. This corrected depletion was divided by the concentration (mg/L) of the test item, to obtain BOD as mg oxygen per mg test item. The percentage biodegradation was calculated by dividing the specific BOD by the specific oxygen demand, calculated from the molecular formula in accordance with the OECD guideline.

The COD of PROBLAD PLUS was determined to be 0.676 mg O_2/mg.

The ThOD of the reference item, sodium benzoate, was calculated to be 1.67 mg O_2/mg, and the ThOD of the mixture of sodium benzoate and test item was 1.173 mg O_2/mg.

Results and discussion:

On average the initial oxygen contents of the aerated test media ranged from 8.84-8.90 mg/L. The results of the test are presented in the following table.

Table 4.1.1-02 Percent degradation of PROBLAD PLUS, Na-benzoate and toxicity control

Time [d]	% degradation		
	PROBLAD PLUS	Na-benzoate	Toxicity Control
4	65.9	54.5	45.6
7	82.4	77.8	68.2
11	79.2	81.7	69.9
14	81.4	85.4	71.6
21	93.7	85.8	75.9
28	91.7	84.6	77.6

The results of the test are considered valid due to the following reasons:

- The percentage of the reference item reached 85.4% by day 14
- The residual O_2 content did not fall below 0.5 mg/L
- There was <1.5 mg/L O_2 consumption after 28 days in the inoculums blank
- The difference of extremes of replicate values at the end of the 14 day window was <20% (9.09%)
- The degradation of the toxicity control was >25% (71.6%) after 14 days. Therefore, toxic effects of PROBLAD PLUS can be excluded

Therefore, the test can be considered valid.

Conclusions

The ready biodegradability of BLAD was determined with the closed bottle test. BLAD was tested at a nominal concentration of 45.53 mg/L. At the end of the 28 day period the following biodegradation was determined: BLAD 83.1-96.3%; Sodium-benzoate 75.3-99.2%; Toxicity control 88.6%. Biodegradation of BLAD was greater than 60% in a 10 day window and is therefore considered as readily biodegradable.

RMS comments

This study shows that the major constituent BLAD of Sweet Lupin (seeds), Lupinus albus L., germ.,ext. [PROBLAD PLUS] is readily biodegraded in this standard test. The test is conducted according to the OECD 301 B test guideline, with a positive control test and a toxicity test included and a blank control was also used. The criteria for the OECD 301 B test are met in this study therefore the RMS finds the study acceptable and reliable.

Study reference:

Author: A. Carreira

Year: 2014

Title: Assessment of the ready biodegradability of BLAD with the closed bottle test and SDS-PAGE. CEV, S.A,

Source: Unpublished report No.:CEV-ABB-0914

Detailed study summary and results:

This assessment of ready biodegradability of the BLAD protein was conducted in accordance with the OECD 301 D closed bottle test, however degradation was followed using a specific analytical technique for detection of the BLAD protein and is therefore a true measure of degradation rather than mineralisation.

The investigation was conducted with two different inoculants from two separate waste treatment plants. The study also looked at two different concentration of BLAD protein (100 and 200 mg/L) and also two concentration of inoculum (0.5 mL/L and 5 mL/L). Suitable controls were also used to show the stability of the BLAD protein in mineral medium over the duration of the study without the presence of the inoculum and to show that the inoculum did not interfere with the analytical technique.

The samples were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The equivalent of 25 µg of BLAD from each of the test systems was sampled and the protein precipitated with cold acetone. The pellet was dissolved in buffer and then boiled before being applied to the gels for the SDS-PAGE analysis. After electrophoresis the gels were stained in order to locate the polypeptides in the gels. Quantification was achieved with a densitometer and imaging software in order to integrate the areas of the gels corresponding to the BLAD protein. The results for the 7 and 14 day samples were expressed relative to the equivalent day 0 sample.

The results show that degradation was rapid with very little or no BLAD remaining after 14 days. With the lower concentration of BLAD (100 mg/L) greater than 90% was degraded in 7 days. Degradation may have been a little slower with higher concentration of BLAD (200 mg/ml) but complete degradation was achieved after 14 days (>98%) in all test except the test with lower concentration of inoculum 1, where 3% was remaining after 14 days.

With an average of less than 90% of the BLAD protein remaining after 7 days it is clear that BLAD would be rapidly degraded in the environment and suggests a likely DT_{50} <2 days.

Test type:

Guidelines:	Study was designed in accordance with the OECD 301-D (1992) however degradation was not determined by oxygen consumption. A specific method was used to measure the degradation of the protein. Also, higher concentration of the test substance was used to ensure reliable detection in the analytical system.
GLP:	No

Test substance:

Description:	BLAD, Isolated protein from an extract from plants of the genus Lupinus. White powder
Lot/Batch No.:	No. 06.2015
Purity:	>99 % (w/w) BLAD protein
Carbon content:	43.9% (0.439 mg/mg)
ThCO2:	1.61 mg/mg (based on carbon content)
CAS No.:	1219521-95-5
Stability:	2 years at room temperature

Materials and Methods

Materials

Mineral medium solution

The following Stock solution were prepared

1.	KH_2PO_4	8.50 g/L	
	$KHPO_4$	21.75 g/L	
	$Na_2HPO_4.2H_2O$	33.40 g/L	
	NH_4Cl	0.50 g/L	adjusted to pH 7.4
2.	$CaCl_2.2H_2O$	36.40 g/L	

3. $CaCl_2.2H_2O$ 36.40 g/L

4. $FeCl_3.6H_2O$ 36.40 g/L

One mL of each of the above stock solutions were mixed with 800 mL of ultrapure water and made up to 1 L. The solution was then sterilised by filtration (0.22 µm pore size) and strongly aerated for 20 minutes and then allowed to stand for 20 hours at the test temperature.

Inoculum

Two inocula were used in the study from two distinctly different treatment plants. Inoculum 1 was considered to be more active than inoculum 2. The inoculums were filtered through a course filter, and were pre conditioned by aeration for 5 days at the test temperature without other treatments or additions.

Study Design

The test vessels were prospered under aseptic conditions. Two sets of 50 mL bottles were prepared containing different concentrations of BLAD (100 mg/L and 200 mg/L) by adding the corresponding volume of the 2 mg/mL stock BLAD solution (2.5 ml or 5.0 mL). The bottles were then inoculated with either 0.025 mL or 0.25 mL of the prepared inoculum to create 0.5 mL/L and 5 mL/L solutions in the test vessels. Bottles were completely filled with the sterile mineral medium. In addition to the test vessels, controls vessels were set up containing only BLAD at the two concentrations and also containing only the inoculum at the two concentrations. A summary of the composition of each of the test vessels is shown in Table B.8.2.2-04. For each variant, 5 replicates were prepared for sampling at 0, 7, 14, 18 and 21 days. The bottles were incubated in the dark at 22°C±2°C.

Table 4.1.1-04 The composition of the tests systems is shown in the following table.

Sample No	BLAD (mg/L)	Inoculum	Inoculum concentration (ml/L)
1	100	1	0.5
2	100	1	5.0
3	100	2	0.5
4	100	2	5.0
5	200	1	0.5
6	200	1	5.0
7	200	2	0.5
8	200	2	5.0
9	100	-	-
10	200	-	-

11	-	1	0.5
12	-	1	5.0
13	-	2	0.5
14	-	2	5.0

SDS-PAGE analysis

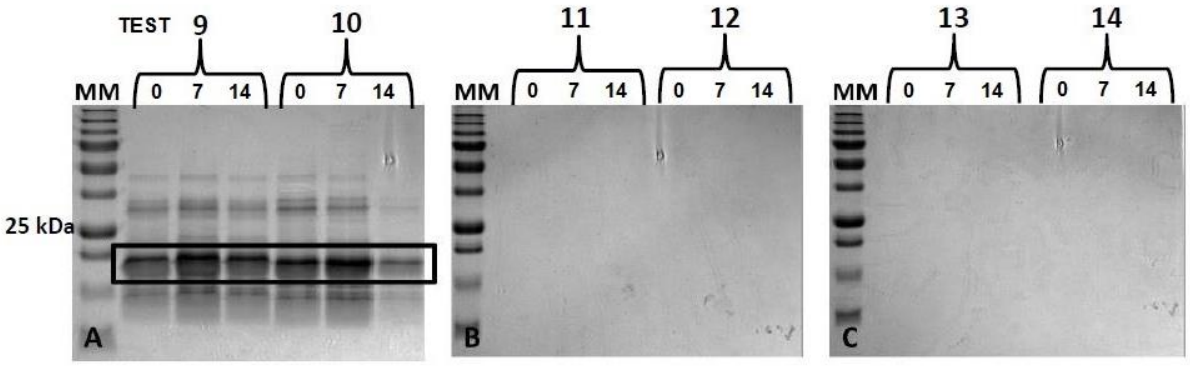
For Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), either 125 µL or 250 µL of each sample to correspond to 25 µg of added BLAD protein, were collected at 3 sampling point. Samples 11, 12, 13 and 14 were used as negative controls of the study. All the samples were precipitated with cold acetone at -20 ºC for 30 minutes. After centrifugation at 11.000 g, the pellets obtained were dissolved in a 3 sample buffer containing 2% (w/v) SDS and 2- β-mercaptoetanol (0.1M), and then boiled for 3 minutes. Proteins were then separated by means of SDS-PAGE in precast 4% to 20% Tris-HCl Ready Gels (Bio-Rad Laboratories, Inc.) based on the method of Laemmli by using the manufacturer´s suggested protocol. Each well was loaded with 25 µg of protein. Precision Plus molecular weight markers (Bio-Rad Laboratories, Inc.) were used as control molecular markers.

The gels were stained with Coomassie Brilliant Blue R250 (CBB R-250). Polypeptides were fixed in TCA 10% (w/v) for 15 minutes. After that, the mini gels were stained for a period longer than 3 hours with a solution containing 0.25% (w/v) CBB R-250, 25% (v/v) 2-propanol and 10% (v/v) glacial acetic acid. The destaining solution composed of 25% (v/v) 2-propanol and 10% (v/v) glacial acetic acid was kept until the polypeptides could be visualised.

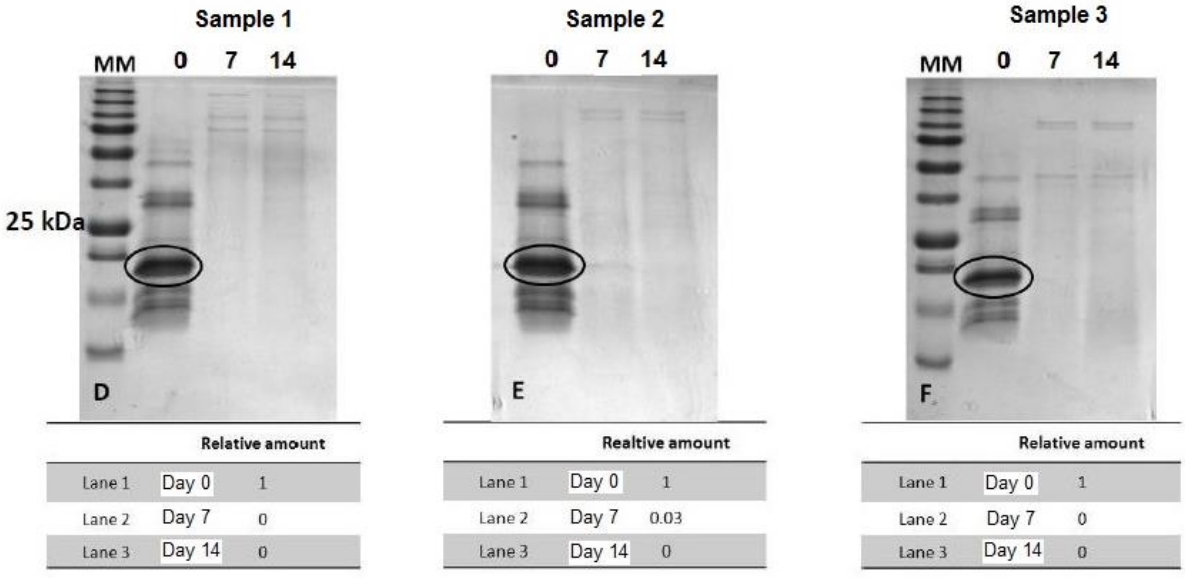
After Coomasie staining procedure, all polyacrylamide gels were submitted to densitometric analysis followed by BLAD's relative quantitation using ImageLab software version 4.1 (Bio-Rad). The software integrates the raw data in a three dimension format, namely, the width and length of the band as x and y respectively, and the colour intensity of the Coomasie protein staining. Therefore the density of each band is measured as the total area under the three dimensional peak. For relative quantitation, BLAD "0 days" sample was used as the reference band and the density peak area value was set with a nominal value of 100%. Therefore all ensuing samples in the same polyacrylamide gel (days 7 and 14) will generate a relative value in comparison with the reference sample.

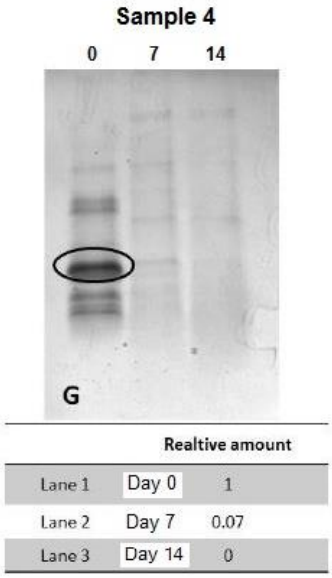
Results and Discussion

The graphic representation of the SDS PAGE gels can be seen below. The controls samples 9 and 10 show that the BLAD protein is stable in the sterile mineral medium without the presence of the inoculum over the 14 days. The control 11 to 14 show that neither of the inoculums contain anything that interferes with the SDS-PAGE analysis.

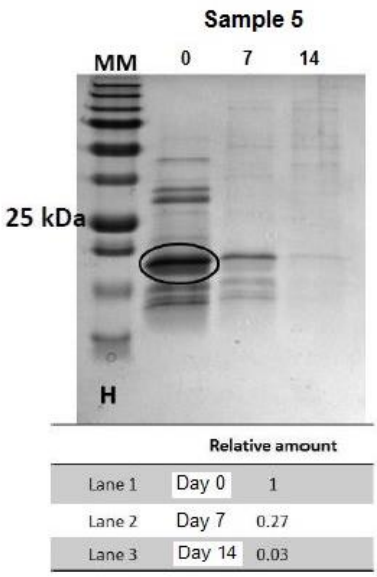


The test samples 1 to 4 show that >90% of the BLAD protein has been degraded after 7 days and has been completely degraded after 14 days when tested at 100 mg/L. With test samples 5 to 8 degradation may have been a bit slower at the higher BLAD protein concentration, especially with inoculum 1 at the lower inoculum concentration (test sample 5) where 27% remained at 7 days and 3% remained at 14 days. However, inoculum 1 at the higher concentration and inoculum 2 at both concentrations were able to completely degrade the BLAD protein after 14 days.

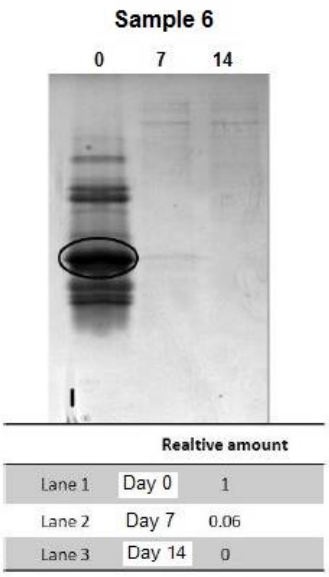




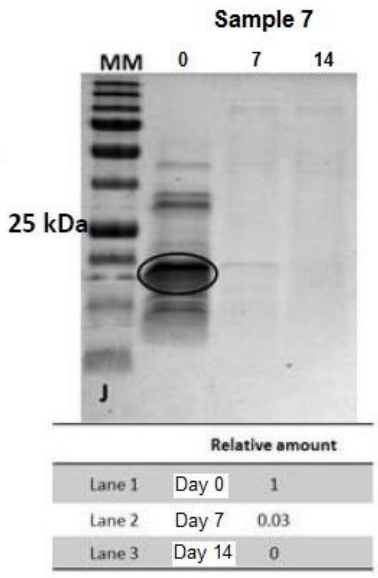
Realtive amount		
Lane 1	Day 0	1
Lane 2	Day 7	0.07
Lane 3	Day 14	0



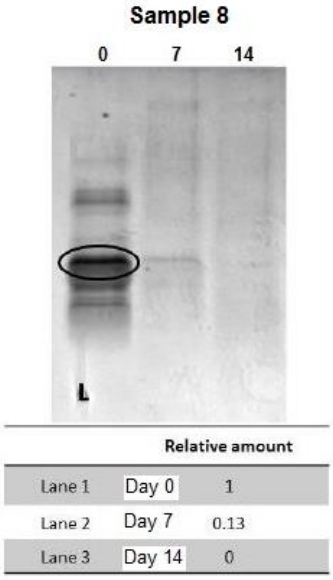
Relative amount		
Lane 1	Day 0	1
Lane 2	Day 7	0.27
Lane 3	Day 14	0.03



Realtive amount		
Lane 1	Day 0	1
Lane 2	Day 7	0.06
Lane 3	Day 14	0



Relative amount		
Lane 1	Day 0	1
Lane 2	Day 7	0.03
Lane 3	Day 14	0



Relative amount		
Lane 1	Day 0	1
Lane 2	Day 7	0.13
Lane 3	Day 14	0

The protein detection technique used in the study has a detection limit of 0.05 µg of protein, therefore considering that each lane represents the equivalent of 25 µg of protein from the test systems, a non-detection represents 0.2% of the initial concentration or >99% degradation. The results are shown in the Table B.8.2.2-05 as % BLAD remaining. With an average of less than 90% of the BLAD protein remaining after 7 days it is clear that degradation is rapid, and suggests a DT_{50} of around 2 days assuming single first order kinetics.

Table 4.1.1-05 Amount of BLAD remaining in the samples after the incubation for 7 an 14 days (by comparison with day 0)

Sample No	BLAD (mg/L)	Inoculum	Inoculum concentration (ml/L)	BLAD remaining after 7 days (%)	BLAD remaining after 14 days (%)
1	100	1	0.5	0	0
2	100	1	5.0	3	0
3	100	2	0.5	0	0
4	100	2	5.0	7	0
5	200	1	0.5	27	3
6	200	1	5.0	6	0
7	200	2	0.5	3	0
8	200	2	5.0	13	0

Conclusions

The study shows that BLAD is rapidly degraded under the conditions of the ready biodegradability test.

RMS comments: This is an internal study from the notifier and not performed under GLP. Therefore, this study is not acceptable as such. Moreover, the report lacks essential information like the source of BLAD, the isolation method of BLAD and the source of the sludge and the concentration of the sludge. The conclusion of the study that BLAD is readily degraded in water with sludge was also observed in study CA 7.2.2.1/01. This study shows that measuring BLAD via SDS-PAGE eletrophoresis is a way to quantify the degradation of BLAD. No samples were taken in between day 0 and 7. This would have given more additional information on the rate of degradation. The RMS proposes to use this study as additional information that BLAD is readily degraded and no major decetable metabolites are formed after 7 days.

Study reference:

Author: Eurofins Agroscience Services GmbH

Year: 2010

Title: PROBLAD: Assessment of ready biodegradability with the closed bottle test. Source: CEV SA, Unpublished report No.: S10-02624

Source: CEV S.A, Unpublished report No.: 1035

Detailed study summary and results:

The degradation of PROBLAD PLUS reached 81.4% within a 14 day window and can therefore be considered as readily biodegradable under the conditions of this closed bottle test. Mean biodegradation of the reference substance, sodium benzoate, was 85.4% on Day 14 and 84.6% at the end of the test. The difference of extremes of replicate values of the removal of the test item at the end of the 14 day window was < 20% (9.09%). These data show that the inoculum was viable and exerting normal degradative activity. The degradation of the toxicity control was >25% (71.6%) after 14 days. Therefore, toxic effects of PROBLAD PLUS can be excluded. Therefore, the test can be considered valid.

Test type:

Guidelines: OECD Guideline for Testing of Chemicals No. 301 D EC method C.4-E (92/69/EEC), biological degradability, determination of the ready degradability, part VI, closed bottle test

GLP: Yes (certified laboratory)

Test substance:

1. Test Material	PROBLAD
Description:	Extract from plants of the genus Lupinus. Brown liquid.
Lot/Batch No.:	No.201009
Purity:	20.0 % (w/w) BLAD protein
CAS No.:	NA
Stability:	Not stated
Note:	PROBLAD containing 20% BLAD is the same as PROBLAD PLUS

Reference Material:

2. Reference substance	Benzoic acid sodium salt
Description	White powder
Lot/Batch No.:	6U006469
Purity:	99.0 % (w/w)
CAS No.:	532-32-1
Stability:	Not stated

The reference item was used at a concentration of 2 mg/L to check the activity of the inoculums.

Materials and Methods

3. Activated sludge inoculum

The effluent of the municipal activated sludge plant of Pforzheim/Germany was taken as inoculums. The effluent was kept under aerobic conditions in the period between sampling and application. The inoculums was filtered through a coarse filter, the first 200 mL being discarded, and was aerated by shaking one week in an Erlenmeyer flask. The initial number of micro-organisms was determined by enumerating the bacterial colonies in 1 mL inoculated test medium after incubation with plant count agar in Petri dishes for 4 days.

4. Mineral medium

The mineral medium was prepared from four stock solutions (1000 fold concentrated) using ultra-pure grade water.

Table 4.1.1-06 Composition of stock solutions used in the preparation of the mineral medium

Prepared from	Compound	Final concentration
Stock solution 1	KH_2PO_4 K_2HPO_4 Na_2HPO_4 NH_4Cl	8.5 mg/L 21.75 mg/L 26.65 mg/L 0.5 mg/L
Stock solution 2	$CaCl_2 \cdot 2\ H_2O$	36.4 mg/L
Stock solution 3	$MgSO_4 \cdot 7\ H_2O$	22.5 mg/L
Stock solution 4	$FeCl_3 \cdot 6\ H_2O$	0.25 mg/L

5. Test system

Four study groups:

Inoculum blank,	0 mg/L PROBLAD PLUS,	0 mg/L Na-Benzoate
Test item,	2 mg/L PROBLAD PLUS,	0 mg/L Na-Benzoate
Reference item	0 mg/L PROBLAD PLUS,	2 mg/L Na-Benzoate
Toxicity control	2 mg/L PROBLAD PLUS,	2 mg/L Na-Benzoate

Study Design:

The test was performed in BOD flasks with ground-in-glass stoppers in single, 2 and 3-fold test assays for each of the 6 measurement dates (0, 4, 7, 11, 14, 21 and 28 days).

A sufficient volume of ultra-pure grade water was prepared in 5-L volumetric flasks. The flasks were filled at first to about three quarters of their volume with water. The water was strongly aerated for *ca* 10 minutes

to achieve oxygen saturation and allowed to stand for about 24 h without aeration at test temperature. The O_2 content was measured at the start of the test.

Initially each study group was prepared in 5-L volumetric flasks. The individual salt stock solutions were added (5 mL of each to < 5000 mL), and either the respective test and reference items were added in the final concentrations of 2 mg/L (10 mg/5 L). Subsequently each flask was inoculated with 5 mL of inoculums and the volume made up to 5L with oxygen saturated water.

The contents of the flasks were distributed into the test vessels and the oxygen contents measured. The bottles were incubated in the dark between 19.1 and 22.0ºC. 3 or 2 bottles of each treatment group were removed at each time point for analysis of O_2 content.

Oxygen concentrations were measured with a WTW Microprocessor Oximeter OXI340 and a calibrated electrode.

Evaluation of degradability

For each date of measurement, the exerted BOD was calculated by subtracting the oxygen concentration (mg O_2/L) of the mean initial inoculums blank from that of the other study groups. Then, for each test assay the measured values of the controls were subtracted. This corrected depletion was divided by the concentration (mg/L) of the test item, to obtain BOD as mg oxygen per mg test item. The percentage biodegradation was calculated by dividing the specific BOD by the specific oxygen demand, calculated from the molecular formula in accordance with the OECD guideline.

The COD of PROBLAD PLUS was determined to be 0.676 mg O_2/mg.

The ThOD of the reference item, sodium benzoate, was calculated to be 1.67 mg O_2/mg, and the ThOD of the mixture of sodium benzoate and test item was 1.173 mg O_2/mg.

Results and discussion:

On average the initial oxygen contents of the aerated test media ranged from 8.84-8.90 mg/L. The results of the test are presented in the following table.

Table 4.1.1-07 Percent degradation of PROBLAD PLUS, Na-benzoate and toxicity control

Time [d]	% degradation		
	PROBLAD PLUS	Na-benzoate	Toxicity Control
4	65.9	54.5	45.6
7	82.4	77.8	68.2
11	79.2	81.7	69.9
14	81.4	85.4	71.6
21	93.7	85.8	75.9
28	91.7	84.6	77.6

The results of the test are considered valid due to the following reasons:

- The percentage of the reference item reached 85.4% by day 14
- The residual O_2 content did not fall below 0.5 mg/L
- There was <1.5 mg/L O_2 consumption after 28 days in the inoculums blank
- The difference of extremes of replicate values at the end of the 14 day window was <20% (9.09%)
- The degradation of the toxicity control was >25% (71.6%) after 14 days. Therefore, toxic effects of PROBLAD PLUS can be excluded

Therefore, the test can be considered valid.

Conclusions

The ready biodegradability of PROBLAD PLUS was determined with the closed bottle test. PROBLAD PLUS was tested at a nominal concentration of 2 mg/L. At the end of the 28 day period the following biodegradation was determined: PROBLAD PLUS 91.7%; Na-benzoate 84.6%; Toxicity control 77.6%. The test item is considered as readily biodegradable.

RMS comments: The study shows that PROBLAD PLUS containing 20% of BLAD is also readily biodegraded. The study was conducted according to the OECD 301 D guidelines. The results show that PROBLAD is readily degraded in this study and complies with the requirements in the OECD guidance. Therefore the RMS agrees with the conclusion that PROBLAD PLUS, a botanical extract of Sweet Lupin (seeds), *Lupinus albus* L., germ., ext., is readily biodegradable and the study is acceptable.

4.1.2 BOD_5/COD

No studies were submitted.

4.1.3 Aquatic simulation tests

No studies were submitted.

4.1.4 Other degradability studies

No studies were submitted.

4.2 Bioaccumulation

4.2.1 Bioaccumulation test on fish

Bioaccumulation in fishes and other organisms is not expected. According to the EFSA Guidance Document (2009), the bioaccumulation potential should be evaluated for substances with a log K_{ow} in excess of 3.

PROBLAD PLUS is readily biodegradable in water and the lead component BLAD has a calculated DT_{50} of 300 days in soil and so the exposure and potential for bioaccumulation will be limited due this rapid break down. PROBLAD PLUS is a complex mixture and consists of numerous components, none of which are isolated during the preparation of the product. It is therefore not relevant to derive a Log Kow for PROBLAD PLUS as a whole. In addition as the main bulk of the identified components occur in soil and water in large concentrations, it would not be relevant to distinguish exposure from PROBLAD PLUS to natural exposure and as such it would not be relevant to estimate potential for bioaccumulation. Furthermore, estimates for many of these components would be very difficult as they are broad classes of compounds and defining their structure would be problematic. As such any further assessment of the potential for bioaccumulation and secondary poisoning from the majority of the PROBLAD PLUS components is not relevant.

The lead component, BLAD is a naturally occurring seed storage protein in germinated sweet lupines. It is a 210 kDa gylco-oligomer which is mainly composed by a 20 kDa protein of β-conglutin, or characterised as a fragment of the amino acid sequence of β-conglutin, therefore, there is no specific molecular or structural formula. As such a Log K_{ow} cannot be estimated using QSAR modelling.

It is possible to estimate partition co-efficient Koc or Kow as shown in environmental fate assessment in Volume 4 (the confidential annex to the draft assessment report drafted for the purpose of authorization of the substance under Reg (EC) 1107/2009/EC), a method of estimating Koc for BLAD is presented based on the molecular weight, number of benzene rings, nitrogen, oxygen and sulphur content. For full details of this calculation please refer to Volume 4. The estimated log K_{OC} for BLAD based on this calculation method ranges from 3.11- 347. This provides an incredibly high K_{OC} of $1x10^{347}$ which indicates a highly immobile substance. As this is unlikely to represent a true K_{OC}, in the environmental fate modelling a default K_{OC} of 10000 L/kg was applied to provide a realistic maximum K_{OC} for BLAD as an immobile substance. An estimated high K_{OC} such as this would also not be considered relevant for a substance which may potentially bioaccumulate due to its strong adsorption to soil/sediment. Therefore it is more representative to base the

calculation on the default K_{OC} of 10000 L/kg to also be consistent with environmental fate modelling. From this the Log K_{OW} for BLAD is estimated to be 4.0 which would trigger a potential for bioaccumulation.

With regard to the other remaining components which are listed in the confidential information in Volume 4 to provide an indication of the potential for bioaccumulation for the active substance, log K_{OW} values have not been determined experimentally but estimated using the QSAR KOWWIN v 1.68. The estimated log Kow values for the two components are -6.5 and 1.71 which are below the trigger of 3 and so the potential risk from secondary poisoning for these components is considered to be low.

The component with an estimated Log Kow of 4.5 is present at a marginal content in PROBLAD PLUS at < 1% and so an assessment of secondary poisoning based on the lead component BLAD (20 %) will be protective of the potential risk from each of the components.

Furthermore, according to Vol.3 CA B6, "*As PROBLAD PLUS contains the naturally occurring polypeptide component, BLAD (the lead component), the protein will be broken down in the digestive tract, enter the amino acid pool and be consumed into normal catabolic processes. It is known to be susceptible to proteolytic degradation. Consequently, radiolabelling of the test article is neither possible nor cost effective.*

4.3 Acute toxicity

4.3.1 Short-term toxicity to fish

CA 8.2.1/01

NEW STUDY

Report

Anonymous, (2011) Assessment of Toxic Effects of PROBLAD on Rainbow Trout (*Oncorhynchus mykiss*) (Teleostei, Salmonidae) CEV SA, Unpublished report No.: S10-02621

Guidelines

OECD 203 (1992)

GLP

Yes (certified laboratory)

Executive Summary

The 96-hour acute toxicity of PROBLAD PLUS to *Oncorhynchus mykiss* was determined in a semi-static system, exposed to nominal test concentrations between 0.1 and 100 mg PROBLAD PLUS/L. Based on nominal concentrations the 96 hour LC_{50} was >100 mg/L and the NOEC was >100 mg PROBLAD PLUS/L. No lethal or sublethal effects were observed in any test item concentration.

Materials and Methods

Test Material

PROBLAD PLUS, active substance content 20.0% w/w, Batch no. 201009

Test Design

Fish were exposed over a period of 96 hours to PROBLAD PLUS at nominal concentrations corresponding to 0.01, 0.1, 1, 10 and 100 mg PROBLAD PLUS/L. One test vessel per treatment level and for the control was prepared. The test was initiated when 7 rainbow trout were impartially selected and distributed to each test vessel. All test vessels were examined at 0, 3, 6, 24, 48, 72 and 96 hours of exposure and mortality was recorded. Effects for this study were based on mortality. No analysis of the test media was conducted and results are based on nominal concentrations.

The fish used in this study were obtained from Fischzucht Wagenhausen Germany. The body length of the fish was 4- 6 cm. Fish were held in the laboratory under conditions comparable to those of the test for >12 days. Feeding stopped during the 24-hour period prior to test initiation and during exposure period. The water temperature was 15– 17 °C. Dissolved oxygen was > 60% throughout the study and the pH of the test media was 6.0 to 8.5.

Since no mortality was observed up to the highest test item concentration of 100 mg PROBLAD PLUS/L, the evaluation of the data did not require statistical analysis.

Results and Discussion

Analysis of the test media was not conducted and effects were based on nominal concentrations. No mortality was observed in any of the test groups, and so the LC_{50} (96 h) was determined to be >100 mg/L

Conclusion

The 96-hour LC_{50} of PROBLAD PLUS to *Oncorhynchus mykiss* in a semi- static system, based on nominal concentrations was >100 mg PROBLAD PLUS/L and the NOEC was >100 mg PROBLAD PLUS/L. No lethal or sub-lethal effects were observed at any test item concentration.

Comment RMS:

Based on the study results and the test conditions, the validity criteria of OECD 203 were fulfilled. No effects on fish were observed during the duration of this test. The report does not contain any method of analysis which is required in order to confirm the exposure of the organisms to the test substance. However, taking into consideration the wide availability of reports on effects of sweet lupin on fishes and that vertebrate experiments must be avoided, the RMS agrees with the approach of the notifier regarding corrected endpoints taking into account possible degradation over the course of the aquatic organisms tests. The corrected endpoint is NOEC >50 mg/L.

4.3.2 Short-term toxicity to aquatic invertebrates

CA 8.2.4.1/01

NEW STUDY

Report

K. Weber (2011) Assessment of Toxic Effects of PROBLAD on *Daphnia magna* using the 48 h Acute Immobilisation Test. CEV SA, Unpublished report No.: S10-02622

Guidelines

OECD 202 (2004)

GLP

Yes (certified laboratory)

Executive Summary

The 48-hour acute toxicity of PROBLAD PLUS (content of BLAD: 20.0%) to *Daphnia magna* was determined in a semi static system, with groups of 20 daphnids per treatment, spanning nominal concentrations between 7.81 and 250 mg/L The 24- and 48-hour immobilisation EC_{50} was >250 and 159.32 mg/L respectively and the 48-hour NOEC was 31.3 mg/L.

Materials and methods

Test Material

PROBLAD PLUS, BLAD content 20.0% w/w, Batch no. 201009

Test Design

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. 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.

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 20.7°C. Dissolved oxygen was above 60% saturation and the total hardness was between 140 – 268 mg/L (as CaCO3). The pH of the test media was 8.16.

Results and Discussion

Details are provided in Table 8.2.4.1. Following 24 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.

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 24 h and 48 h 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.

Table 8.2.4.1/01-1

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

Nominal Concentration (mg PROBLAD PLUS/L)	Number 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
7.81	0	0	0	0	0	0	0	0	0	0
15.6	0	0	0	0	0	0	0	0	0	0
31.3	0	0	0	0	0	0	0	0	1	1
62.5	0	0	0	0	0	1	0	1	1	3
125	1	1	0	0	2	1	3	0	0	4
250	1	1	0	1	3	5	5	0	5	15

Conclusion

Based on nominal concentrations, the 48-hour EC_{50} 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).

Comment RMS: The product seems to have an influence on the oxygen concentration at 250 mg/L. Nevertheless, the testing should have been performed up to a concentration of 100 mg/L as indicated in the EU No. 283/2013. Furtheremore, the RMS agrees that the drop in dissolved oxygen is not significant and it might not have affected the results of the study. However, an analysis of the test concentrations in water is not provided and analytical measurements are required according to OECD 202.Taking into account the limitations to perform analytical methods of the test substance during the experiment on aquatic organisms, the RMS agrees with the approach of the notifier regarding corrected endpoints considering possible degradation over the course of the tests.The corrected endpoint is EC_{50} 79.66 mg/L.

4.3.3 Algal growth inhibition tests

CA 8.2.6.1/01

NEW STUDY

Report

S. Falk (2011) PROBLAD: Testing of Effects of the Single Cell Green Alga *Desmodesmus subspicatus* in a 72 h Static Test. CEV SA, Unpublished report No.: S10-02623

Guidelines

OECD 201 (2006)

GLP

Yes (certified laboratory)

Executive Summary

The 72-hour effects of PROBLAD PLUS on biomass and growth rate of *Desmodesmus subspicatus* were determined in the laboratory. Based on nominal concentrations the 72 h E_yC_{50} for yield was 28.7 mg /L and NOEC 11.1 mg/L. Due to statistical reasons it was not possible to calculate the E_rC_{50} for growth rate.

Materials and methods

Test Material

PROBLAD PLUS, BLAD content 20.0% w/w, Batch no. 201009

Test Design

Algae were exposed over a period of 72 hours to PROBLAD PLUS at nominal concentrations corresponding to 0 (dilution water control), 0.0137, 0.412, 1.23, 3.70, 11.1, 33.3 and 100 mg/L. The culture medium used was a synthetic algal assay growth medium prepared by adding appropriate amounts of nutrient stock solutions to sterile, deionised water. The algal medium used to prepare the exposure solutions was the same as the culture medium. The medium were prepared using deionised water and were equilibrated to test temperature. There were three replicate vessels per treatment and six replicate vessels for the control. The flasks were inoculated with volumes providing the required cell density of 0.5×10^4 cells/mL. Cell numbers were counted using a Neubauer chamber after preparation of a dilution series. Observations of the health of the algal cells were also made at each 24-hour interval.

Analysis of the test substance was not performed, and results were based on nominal concentrations. Measurements of pH were performed at t =0 and t = 3 days and the temperature was measured at day 0, 1, 2 and 3. The pH of the test was 6.71 to 8.91. The temperature was 22.5-24.0 °C throughout the entire study.

The statistical analysis for day 3 was performed for cell number, yield and growth rate using SAS (2002-2008). The data set 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 using Shapiro-Wilks' Test and for homogeneity of variance using the Levene-Test. The E_yC_{50} (for yield) values were determined using Moving average analysis. Due to statistical reasons, values of the concentrations 0.137, 0.412, 1.23 and 3.7 were not taken into account. The EC_{50} of growth rate was not calculable due to statistical reasons.

Results and Discussion

Analytical results

Analysis was not conducted and results were calculated based on the nominal concentrations of 0.137, 0.412, 1.23, 3.70 and 11.1, 33.3 and 100 mg/L.

Biological Results

Details are provided in Table 8.2.6.1/01-1 below.

Table 8.2.6.1/01-1

Cell density of *Desmodesmus subspicatus* after 24, 48 and 72 hours of exposure to PROBLAD PLUS

Nominal concentration (mg/L)	Average cell numbers/mL[a]					
	0 hours	24 hours	48 hours	72 hours	72 hours Inhib. of growth rate (%)	72 hours Inhib. of yield(%)
Control	0.5	1.70	9.98	42.49	0.0	0.0
0.137	0.5	1.43	10.08	45.78	-1.8	-7.8
0.412	0.5	1.48	10.59	49.25	-3.6	-16.1
1.23	0.5	1.29	10.78	47.08	-2.5	-10.9
3.70	0.5	1.55	12.43	55.42	-6.2	-30.8
11.1	0.5	1.42	11.40	55.99	-6.4	-32.8
33.3	0.5	1.54	3.30	4.47	51.4	-90.5
100	0.5	2.08	5.21	7.84	40.5	82.5

[a]Algae counts are divided by 10000. At the start, the cell density was adjusted to 0.5×10^4 cells/mL

Conclusion

The NOEC and LOEC values for growth rate and yield were 11.1 and 33.3 mg /L respectively. The 72 hour E_yC_{50} (yield) was determined to be 28.7 mg /L. The 72 hour EC_{50} value for growth rate could not be calculated due to statistical reasons.

Comment RMS: The validity criteria as per OECD 201 were fulfilled. However, the analysis of the test concentration was not performed. The analysis of the test concentration during the test is a requirement of OECD 201. The RMS agrees with the approach of the applicant on using corrected values of endpoints due

to the limitations to perform analysis of the test substance. The corrected value for this endpoint is 14.35 mg /L.

4.4 Chronic toxicity

Chronic studies to assess the reproductive effects of Sweet Lupin (seeds), *Lupinus albus* L., germ.,ext. [PROBLAD PLUS] to aquatic organisms have not been conducted and are not considered necessary. PROBLAD PLUS contains 20% BLAD (the lead component). BLAD, is a naturally occurring protein formed during day four to twelve of the germination process of sweet lupins (Lupinus albus). Sweet lupins are widely used in aquiculture as a protein rich food source for the fish. The lead component BLAD has a non-toxic mode of action which is specific to fungi only (BLAD binds to chitin and chitosan which weakens the cell wall structure and so has been found to be a very effective fungicide against powdery mildew and other diseases). It is known to be susceptible to proteolytic degradation and the protein will be broken down, enter the amino acid pool and be consumed into normal catabolic processes. There is no evidence in the public domain to suggest that proteins similar to BLAD, which contains a segment of β conglutin (globulin storage protein associated with leguminous seeds such as peas and lentils) are toxic either in a reproductive or developmental capacity. Based on this it can be concluded that the lead component is unlikely to be considered a reproductive or developmental toxin. The other main components of PROBLAD PLUS have been identified and are presented in the confidential information in volume 4. The main proportion of the identified components (e.g. proteins, carbohydrates) occur in soil and aquatic systems in large concentrations. With regard to exposure PROBLAD PLUS has been shown to be readily biodegradable and as such prolonged exposure, relevant for chronic effects will not be relevant.

In addition there is also a wide variety of studies conducted to assess the suitability of lupin meal as a protein source for fish meal in aquaculture. As with the use of lupin as a bird feed, the main focus of the studies is to assess the digestibility and conversion of food uptake into growth by the different fish species. Digestibility of lupins compared to other protein sources can typically be lower due to the presence of indigestible fibre and non-starch polysaccharides. The effect of the lupin diet on growth is therefore attributed to the nutritional content and digestion rather than any sub-chronic effects. A few key studies assessing the effect of lupin diets in different fish species have been summarised in the following table.

Table CA 8.2.2-1
Summary of key literature studies assessing use of lupin meal for fish feed

Study	Species	Overview of method	Estimated exposure	Acute, chronic effects observed	Conclusion
Farhangi & Carter	Rainbow trout	Juvenile trout were weighed and stocked	At the end of the study mean weight	No mortality was observed	No significant effects on

Study	Species	Overview of method	Estimated exposure	Acute, chronic effects observed	Conclusion
(2001)[8]		into 300 L glass tanks in duplicate groups of 38 individuals. Fish were fed twice a day with diets containing 0, 10, 20, 30, 40 and 50 % of dehulled lupins at feeding rate of 2 % body weight for 8 weeks. Fish weight was recorded every 2 weeks and diet adjusted accordingly. Mortality of fish was recorded daily.	for fish was approximately 130 g. Diet was added at a rate of 2 % body weight and so maximum diet added would have been 2.6 g per fish. The highest lupin diet consistent of 50 % lupin and so 1.3 g of lupin was added per fish twice a day by the end of the study. With 38 fish in each tank this would have equated to approximately 50 g of lupin added per 300 L of water (approximate concentration of 167 mg lupin/L).	in any treatment groups. A significant reduction in growth rate was only shown at the highest lupin diet of 50 %, compared to the control.	condition or survival of rainbow trout when lupin was included up to 40 % of diet.
Borquez *et al.* 2010[9]	Rainbow trout	Juvenile trout were randomly assigned to twelve 500 L circular fiberglass tanks (49 fish per tank). Each treatment had three replicate tanks. For experimental diets were used; including a control and three diets containing 30, 40 and 50 % whole white lupin grain. The fish were fed twice a day (the amount was based on visual of feeding behaviour) for 11 weeks.	The fish were fed with each diet twice a day but the amount added was based on the feeding behaviour of fish and so concentration of lupin in the test system cannot be estimated.	Mortality in the study was 2 % and this occurred in the control group. While the diet containing 50 % lupin resulted in the lowest mean weight gain, there was no significant correlation shown between the increase in	The results indicated that lupin could be included up to 50 % of the fish diet without negative effects on growth.

8 Farhangi and Carter, 2001. Growth, physiological and immunological responses of rainbow trout (*Oncorhynchus mykiss*) to different dietary inclusion levels of dehuled lupin (*Lupinus angustifolius*) Aquaculture research, 32 (suppl. 1) 329-340

9 Borquez *et al.* 2010. Feeding high inclusion of whole grain white lupin (*Lupinus albus*) to rainbow trout (*Oncorhynchus mykiss*); effects on growth nutrient digestibility, live and intestine histology and muscle fatty acid composition. Aquaculture research. 1-12

Study	Species	Overview of method	Estimated exposure	Acute, chronic effects observed	Conclusion
				lupin content, feed intake.	
Yones, 2010[10]	Red hybrid tilapia	Juvenile fingerlings were maintained in 1000 L glass tanks with 50 individuals per tank, with three tanks per treatment. Initial mean body weight was approximately 3 g per fish. The fish were fed twice daily at 3 % of their body weight with diets containing either 0, 25, 50 or 60 % lupin seed meal.	Based on a feeding rate of 3 % of body weight, approximately 0.09 g of feed was added per fish twice a day. At each feeding event for 50 individuals in 1000 L water, an estimated 4.5 g of food provided. The maximum lupin diet was 60 % total feed, therefore 2.7 g of lupin was added to each tank twice a day. This is equivalent to 2.7 mg lupin meal/L.	Mortality in the study was not reported. Fingerlings fed 60 % lupin diet showed a lower growth performance than the other groups and showed a lower feed efficacy. Blood characteristics of the fish were comparable between each treatment.	Lupin meal was shown to be utilised efficient with diets containing up to 50 % lupin meal.
Zhang *et al.* 2012a[11].	Black sea bream	Juvenile sea bream were used in a feeding experiment to investigate effects of including lupin protein concentrate in their diet. Two dietary inclusion levels were used, 300 g or 500 g protein/kg diet (30 or 50 % lupin protein). Fifty fish were added to each 500 L tank, with two tanks used per treatment and were fed three times a day for 60 days.	The maximum lupin concentration consisted of 50 % lupin protein in the fish diet. Fish were fed by hand 3 times a day based on feeding behaviour rather than a set amount of feed. Based on this the dose provided to fish is not determined.	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.	The inclusion of lupin protein, up to 50 % lupin content in the diet did not affect fish growth
Zhang *et al*. 2012b[12]	Rainbow trout	Juvenile rainbow trout were used in a feeding experiment to	The maximum lupin concentration consisted of 50 %	After 62 days, the average weight gain	No negative effects of including lupin

[10] Yones, 2010. Effect of lupin kernel meal as plant protein source in diets of red hybrid tilapia (*Oreochromis niloticus* x *O. mossambicus*) on growth performance and nutrient utilisation. African Journal of Biological Science. 6 (1): 1-16. (2010)

[11] Zhang *et al.* 2012. Mixtures of lupin and pea protein concentrates can efficiently replace high-quality fish meal in extruded diets for juvenile black sea bream (*Acanthopagrus schlegeli*). Aquaculture 354-355, 68-74.

Study	Species	Overview of method	Estimated exposure	Acute, chronic effects observed	Conclusion
		investigate effects of including lupin protein concentrate in their diet. Two dietary inclusion levels were used, 300 g or 500 g protein/kg diet (30 or 50 % lupin protein). Forty five fish were added to each 200 L tank, with two tanks used per treatment and were fed three times a day for 62 days.	lupin protein in the fish diet. Fish were fed by hand 3 times a day based on feeding behaviour rather than a set amount of feed.	of the fish was not shown to have been affected negatively by the inclusion of the lupin diet.	in the diet of rainbow trout were shown. The study concluded that any ratio of lupin and pea protein up to a level of 300 g/kg had no effect on fish weight gain.
Molina-Poveda *et al.* 2013.[13]	Shrimp (*Litopenaeus vannamei*)	Two growth trials were conducted with the shrimp in which diets of the shrimp contained 25, 50, 75 and 100 % lupin kernel meal. Eight shrimp were randomly assigned to each 50 L test vessel containing sea water. Six replicates were used be treatment.	The shrimp were fed ad libitum four times a day with the relevant diets. The concentration of lupin in the test vessel can therefore not be estimated.	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.	Lupin can be considered a suitable protein source for shrimp and provide up to 50 % of the dietary protein without negative effect on growth.

[12] Zhang *et al.* 2012. Optimal inclusion of lupin and pea protein concentrates in extruded diets for rainbow trout (*Oncorhynchus mykiss*) Aquaculture 344-349, 100-113

[13] Molina-Poveda, 2013. Evaluation of the potential of Andean lupin meal (*Lupinus mutabilis* Sweet) as an alternative to fish meal in juvenile *Litopenaeus vannamei* diets. Aquaculture. 410-411. 148-156.

Several governmental bodies have also investigated the suitability of lupin based diets in agriculture. The Norwegian committee for food safety conducted a review (2009[14]) of the use of plant ingredients, including lupin, for aquaculture fish and concluded that '*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. 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'.*

A review of the use of lupins as fish feed has been conducted by Glencross (2002)[15] for the Department of Fisheries, Western Australia. The review included an assessment of the suitability of lupins based on their nutritional and biological value for a variety of aquaculture fish species including Rainbow trout, Salmon, Seabream, Pink snapper, Perch, Carp, Tilapia, Turbot. In addition, the use of lupin for feed to shrimp and crayfish was also assessed. The report concluded '*In all aquaculture species for which a nutritional assessment has been made on the value of lupins, they have been shown to be a well-accepted and nutritionally useful ingredient'.*

The LUKAA project (Lupins in UK agriculture and aquaculture) has also extensively investigated the potential for lupins to provide a cost-effective source of vegetable protein which can be utilised as feed for livestock, fish and birds. In their studies with fish they concluded that for the three species assessed, mirror carp, black Nile tilapia and rainbow trout inclusion of up to 25 % white lupin in feed meal had no significant effects on growth performance. Results on studies regarding Sweet lupin seeds as diet for fishes, indicate there are no negative effects on fishes when feeding on this diet.

4.4.1 Potential for endocrine disruption

PROBLAD PLUS has been fully characterised in a five batch analysis presented in Volume 4. The main components are identified to be water, proteins, carbohydrates and lipids. Each of these components are naturally occurring and so it is not possible to distinguish exposure resulting from the breakdown of PROBLAD PLUS with natural background concentrations. These components will contribute a proportion of the diet to fish and as previously noted the precursor to the lead component BLAD is sweet lupin which is used extensively as a food source in agriculture and aquaculture. A review of the toxicity of the components is noted in Volume 4. As the main components will naturally be present in the diet of fish and as such the

14 Opinion of the Panel on Animal feed of the Norwegian Scientific Committee for Food Safety. 2009. Criteria for safe use of plant ingredients in diets for aquaculture fish.

15 Glencross, 2002. Feeding lupins to fish: A review of the nutritional and biological value of lupins in aquaculture feeds. Department of Fisheries, Government of Western Australia.

potential effects on endocrine disruption are not considered relevant. No further indication of the potential for endocrine disrupting properties were identified in the literature search.

4.4.2 Chronic toxicity to aquatic invertebrates

Chronic studies to assess the reproductive effects of Sweet Lupin (seeds), *Lupinus albus* L., germ.,ext. [PROBLAD PLUS] to aquatic organisms have not been conducted and are not considered necessary. PROBLAD PLUS contains 20% BLAD (the lead component). BLAD, is a naturally occurring protein formed during day four to twelve of the germination process of sweet lupins (Lupinus albus). Sweet lupins are widely used in aquiculture as a protein rich food source for the fish. The lead component BLAD has a non-toxic mode of action which is specific to fungi only (BLAD binds to chitin and chitosan which weakens the cell wall structure and so has been found to be a very effective fungicide against powdery mildew and other diseases). It is known to be susceptible to proteolytic degradation and the protein will be broken down, enter the amino acid pool and be consumed into normal catabolic processes. There is no evidence in the public domain to suggest that proteins similar to BLAD, which contains a segment of β conglutin (globulin storage protein associated with leguminous seeds such as peas and lentils) are toxic to aquatic organisms either in a reproductive or developmental capacity. Based on this it can be concluded that the lead component is unlikely to be considered a reproductive or developmental toxin. The other main components of PROBLAD PLUS have been identified and are presented in the confidential information in volume 4. The main proportion of the identified components (e.g. proteins, carbohydrates) occur in soil and aquatic systems in large concentrations. With regard to exposure PROBLAD PLUS has been shown to be readily biodegradable and as such prolonged exposure, relevant for chronic effects will not be relevant.

4.4.3 Chronic toxicity to algae or aquatic plants

Please see 4.4.2

4.5 Acute and/or chronic toxicity to other aquatic organisms

Please see 4.4.2