Document Title

Summary of the ecotoxicological studies
ACL + DFF SC 600 (500 + 100) G

Data Requirement(s)

Regulation (EC) No 1107/2009 & Regulation (EU) No 284/2013

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Section 10: Ecotoxicological studies

According to the Guidance Document SANCO/10181/2013 for applicants on preparing dossiers for the approval of a chemical active substance

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Version history

Date [yyyy-mm-dd]	Data points containing amendments or additions[1] and brief description	Document identifier and version number
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[1] It is suggested that applicants adopt a similar approach to showing revisions and version history as outlined in SANCO/10180/2013 Chapter 4, 'How to revise an Assessment Report'

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CP 10 ECOTOXICOLOGICAL STUDIES ON THE PLANT PROTECTION PRODUCT

Aclonifen was included in Annex I to Council Directive 91/414/EEC in 2008 (Directive 2008/116/EC, Entry into Force on 01 August 2009).

Diflufenican was included on Annex I of Directive 91/414/EEC on 1 January 2009 under Inclusion Directive 2008/66/EC and implemented under Regulation (EU) No 540/2011. The Annex I Inclusion Directives for diflufenican (2008/66/EC) provide specific provisions under Part B which need to be considered by the applicant in the preparation of their submission and by the MS prior to granting an authorisation. For the implementation of the uniform principles of Annex VI the conclusions of the review report on diflufenican and in particular Appendices I and II thereof, as finalised in the Standing Committee on the Food Chain and Animal Health on 14/03/2008 and on 16/06/2000, respectively, shall be taken into account.

The formulation Aclonifen + Diflufenican SC 600 (500+100 g/L) (or ACL + DFF SC 600 (500 + 100) G), is a suspension concentrate formulation containing 500 g/L of aclonifen and 100 g/L of diflufenican. This formulation is registered throughout Europe under trade names such as Mateno Duo SC 600 (Product code specification #102000029998). This formulation was not a representative product under the previous dossier submitted for Annex I inclusion.

This present dossier in support of approval renewal includes all the data submitted at the time of the Annex I inclusion, in summaries updated and re-evaluated as necessary to take account of current validity criteria and data requirements.

Use pattern considered in this risk assessment

Table 10-1: Intended application pattern ACL + DFF SC 600 (500 + 100) G

Crop	Timing of application (range)	Number of applications	Application interval [days]	Maximum label rate (range) [L/ha]	Maximum application rate, individual treatment (ranges) [g/ha]
Winter wheat, winter triticale	00-13 Pre and post emergence	1	-	0.7	350
Winter wheat, winter rye	00-13 Pre and post emergence	1	-	0.35	175

Definition of the residue for risk assessment

Justification for the residue definition for risk assessment is provided in MCA Sec.7, Point 7.4.1 and MCA Sec. 6, Point 6.7.1.

Table 10-2: Definition of the residue for risk assessment

Compartment	Compound / Code
Soil	Aclonifen
Groundwater	Aclonifen
Surface water	Aclonifen
Plant material	Aclonifen

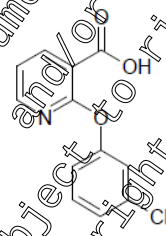
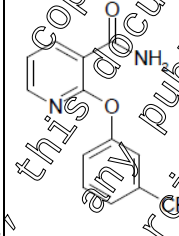
Consideration of metabolites

A list of metabolites found in environmental compartments is provided below. The need for conducting a metabolite-specific risk assessment in the context of the evaluation of ACL + DFF SC 600 (500 + 100) G is indicated in the table.

None of the metabolites of aclonifen is considered as ecotoxicologically relevant. None of the metabolites poses a higher risk to terrestrial and aquatic organisms than the parent compound.

Although risk assessment of the metabolites of diflufenican is indicated, this has not been detailed in this report as diflufenican is still under EU review. Risk assessment of the metabolites of diflufenican in soil and aquatic organisms is covered by this review.

Table 10-3: Metabolites of diflufenican

Metabolite	Chemical structure	Molar mass	Maximum occurrence in compartments	Risk assessment required?
AE B107137 (M&B38181)		283 g/mol	Occurrence >10% in aerobic soil degradation study >10% (entire system) aerobic aquatic degradationstudy	Yes, soil and aquatic organisms
AE 0542291 (M&B43625)		282 g/mol	Occurrence >10% in aerobic soil degradation study	Yes, soil and aquatic organisms

CP 10.1 Effects on birds and other terrestrial vertebrates

CP 10.1.1 Effects on birds

A summary of the avian toxicity endpoints for aclonifen and diflufenican is provided in the following table. Details and a full description of the toxicity studies for aclonifen used in this risk assessment can be found in Document M-CA 8 of this dossier. Details and a full description of toxicity studies for diflufenican are provided in the relevant EU DAR (2006).

Table 10.1-1: Avian endpoints used in risk assessment

Test item	Risk assessment	Type of exposure	Endpoint	Reference
Aclonifen	Acute risk assessment	Acute oral toxicity on bobwhite quail	**LD_{50} >2000 mg a.s./kg bw**	KCA 8.1.1.1/01 M-172009-01-1 [redacted] 1999
	Long-term risk assessment	Subchronic, 6 week dietary (reproduction) on Japanese quail	NOAEC = 1000 ppm **NOAEL = 141 mg a.s./kg bw/day**	KCA 8.1.1.3/01 M-174897-01-1 [redacted], H. 1995
Diflufenican	Acute risk assessment	Acute oral toxicity on bobwhite quail	**LD_{50} >2150 mg/kg bw**	EFSA Scientific Report 122 (2007), 1-84
	Long-term risk assessment	Subchronic, 20 week dietary (reproduction) on bobwhite quail	**NOEL = 91.84 mg/kg**	EFSA Scientific Report 122 (2007), 1-84

Endpoints in **bold** were used in the risk assessment

Toxicity of the formulation

Aclonifen is of low acute oral toxicity to bobwhite quail with LD_{50} values in excess of 2000 mg a.s./kg bw. Similarly, diflufenican is of low toxicity with an LD_{50} value for acute oral toxicity to bobwhite quail of greater than 2150 mg a.s./kg bw.

With regard to animal welfare, acute oral studies with formulations are not routinely conducted on birds, but only with the active ingredients. If substances are non-toxic to birds, the LD_{50} data of the active ingredient can be used to reliably predict the toxicity of the formulation.

Taking into consideration that the LD_{50} values for aclonifen and diflufenican confirm that both active substances are non-toxic to birds, it is safe to assume that the product is also non-toxic to birds. Furthermore, studies conducted with mammals indicate that the formulation is not more toxic than expected based on concentration additivity of its active substances.

Therefore, it is justified to waive the acute test with the formulation in birds.

Summary of the risk assessment for birds

The risk assessment for effects of ACL + DFF SC 600 (500 + 100) G on birds was performed in accordance with the "European Food Safety Authority; Guidance Document on Risk Assessment for Birds & Mammals" (EFSA 2009)[1] (subsequently referred to as the Guidance document (EFSA 2009)). The risk assessment demonstrated acceptable acute and long-term dietary exposure risks following the proposed uses and based on the 'worst-case' screening step.

The risk from consumption of contaminated water was assessed for aclonifen and diflufenican. The acute and long-term risk from drinking water exposure was considered to be acceptable.

[1] European Food Safety Authority; Guidance Document on Risk Assessment for Birds & Mammals on request from EFSA. EFSA Journal .2009; 7(12):1438. [139 pp.] doi:10.2903/j.efsa.2009.1438. Avalable online: www.efsa.europa.eu

Aclonifen and diflufenican have a log P_{ow} of 4.37 and 4.2, respectively, which is higher than the trigger value of 3 and hence an assessment of the risk from secondary poisoning was required. The secondary poisoning risk for earthworm-eating and fish-eating birds from the proposed uses of ACL + DFF SC 600 (500 + 100) G was shown to be acceptable.

An acceptable risk for birds from the combined exposure to both active substances in the product can be concluded.

Risk assessment for birds

The following avian risk assessment has been conducted in line with EFSA's Bird and Mammal Guidance Document (EFSA Journal 2009; 7(12):1438), referred to in the following as EFSA (2009). No short-term risk assessment is required under EFSA (2009) as this is assumed to be covered by the acute and reproductive (long-term) risk assessment and these are conducted in the sections below.

To achieve a concise risk assessment, the risk envelope approach is applied. The assessment for the use pre- and post-emergence application (BBCH 00 – 13) of 1 x 0.35 kg a.s./ha in winter cereals (winter wheat, winter triticale) also covers the risk for birds from use at lower application rates.

The main potential route of exposure for birds to foliar applied agrochemicals is considered to be through the ingestion of residues on contaminated food, e.g. vegetation, insects and earthworms. The intended GAP for ACL + DFF SC 600 (500 + 100) G is presented in Table 10-1 above.

Direct exposure of ACL + DFF SC 600 (500 + 100) G to birds is considered unlikely since at the time of application, and for a period thereafter most birds will leave the immediate vicinity of spray operations in response to the degree of human disturbance. The greatest levels of exposure will arise in the case of birds foraging in the foliage of the crops some hours after application.

DIETARY RISK ASSESSMENT

Screening assessment

The first, or screening, step assesses the risk based on a worst-case approach. The risk is considered acceptable, if the 'Toxicity Exposure Ratio' (TER) value pass the trigger values of ≥10 for acute exposure and ≥5 for chronic exposure. If the TER values do not pass the trigger values in certain areas, a Tier 1 risk assessment based on more relevant and realistic conditions is performed in those particular areas.

Calculation of Daily dietary Dose (DDD)

The daily dietary dose (DDD) for a single application is given by the following equation:

$$DDD_{single\ application} = Application\ Rate\ [kg/ha] x\ Shortcut\ Value\ x\ TWA$$

The Time Weighted Average factor (TWA) is only considered for the long-term exposure. The long-term risk assessment can be based on a TWA = 0.53 (estimates time-weighted exposure over 21 days, assuming a default DT_{50} of 10 days).

Calculation of Toxicity Exposure Ratio (TER)

The assessment of the risks to birds is performed for both acute and long-term exposures using endpoints derived from acute and reproduction studies with birds.

The calculation of acute and long-term toxicity-exposure-ratios (TER) is defined as follows:

Acute risk assessment: $TER_A = LD_{50}/DDD$

Reproductive risk assessment: $TER_{LT} = NOAEL/DDD$

Screening step

According to EFSA (2009), an 'indicator species' is used in a screening step to eliminate all those substances that clearly pose a low risk to birds. This 'indicator species' is not a real species, but, by virtue of its size and feeding habits, is considered to have a higher exposure than (i.e. to be protective of) other species that may occur in a particular crop at a particular time.

For application to the crop relevant for this dossier, winter wheat, the small omnivorous bird should be considered in the screening step using the relevant shortcut values for acute and long-term risk assessments. The shortcut value consists of the food intake rate of the species of concern, its body weight, the concentration of a substance in/on fresh diet and the fraction of diet obtained in the treated area.

The formulation ACL + DFF SC 600 (500 + 100) G is applied as a spray liquid pre- and early post-emergence. Residues in vegetation are therefore negligible. There is however the potential for ACL + DFF SC 600 (500 + 100) G to reach bare soil, and hence the crop group 'Bare soil' is also included at the screening stage. For bare soil, the small granivorous bird should be considered according to EFSA (2009). Green plants (weeds) are only present before they are removed during cultivation and drilling process.

Table 10.1-2: Avian indicator species and shortcut values for the screening assessment

Crop	Indicator species	Shortcut value (SV)	
		Acute assessment	Reproductive assessment
Bare soil	Small granivorous bird	240	11.4
Winter wheat	Small omnivorous bird	158.8	64.8

Table 10.1-3: Avian screening acute assessment for the proposed uses of ACL + DFF SC 600 (500 + 100) G

Intended use		Winter cereals, BBCH 00-13, pre- and post-emergence					
Active substance/product		Aclonifen					
Application rate (g/ha)		350					
Crop	**Indicator Species**	**Toxicity (mg a.s./ kg bw)**	**Appl. rate (kg/ha)**	**SV**	**DDD**	**TER_A**	**Annex VI trigger**
Bare soil	Small granivorous bird	>2000	0.35	24.7	8.65	>231.4	10
Winter wheat	Small omnivorous bird			158.8	55.58	>35.98	
Active substance/product		Diflufenican					
Application rate (g/ha)		70					
Crop	**Indicator Species**	**Toxicity (mg a.s./ kg bw)**	**Appl. rate (kg/ha)**	**SV**	**DDD**	**TER_A**	**Annex VI trigger**

Bare soil	Small granivorous bird	>2150	0.070	24.7	1.73	>1243	10
Winter wheat	Small omnivorous bird			158.8	11.12	>193.4	

SV: Shortcut Value
TER: Toxicity Exposure Ratio
DDD: Daily Dietary Dose

The screening assessment for the acute risks to birds from exposure to ACL + DFF SC 600 (500 + 100) G after use according to the recommended GAP demonstrate that the risks are acceptable, with the TER_A value calculated to be greater than the Annex VI trigger of 10, indicating a low potential acute risk to birds from the exposure of ACL + DFF SC 600 (500 + 100) G. In this occasion, a first-tier assessment was not required.

For the long-term (reproduction) assessment, in accordance with the recommendations of EFSA (2009), the acute oral LD_{50} used in the acute avian assessment was divided by 10 to obtain the $LD_{50}/10$. This was compared to the lowest NOAEL from the avian reproduction studies and the lowest of the $LD_{50}/10$ and NOAEL values was used in the screening assessment.

Aclonifen:

Acute oral LD_{50} from acute avian assessment >2000 mg a.s./kg bw

$LD_{50}/10$ = >200 mg a.s./kg bw

Lowest NOAEL from avian reproduction studies = 141 mg a.s./kg bw/d

The NOAEL was less than the $LD_{50}/10$, therefore the NOAEL from the avian reproduction studies was used in the long-term assessment.

Diflufenican:

Acute oral LD_{50} from acute avian assessment >2150 mg a.s./kg bw

$LD_{50}/10$ = >215 mg a.s./kg bw

Lowest NOAEL from avian reproduction studies = 91.84 mg a.s./kg bw/d

The NOAEL was less than the $LD_{50}/10$, therefore the NOAEL from the avian reproduction studies was used in the long-term assessment.

Table 10.1.4: Avian screening long-term assessment for the proposed uses of ACL + DFF SC 600 (500 + 100) G

Intended use		Winter cereals, BBCH 00-13, pre- and post-emergence						
Active substance/product		Aclonifen						
Application rate (g/ha)		350						
Crop	**Indicator Species**	**Toxicity (mg a.s./kg bw/d)**	**Appl. rate (kg/ha)**	**SV**	**TWA**	**DDD**	**TER_{LT}**	**Annex VI trigger**
Bare soil	Small granivorous bird	>141	0.35	11.4	0.53	2.11	>66.68	5
Winter wheat	Small omnivorous bird			64.8	0.53	12.02	>11.73	
Active substance/product		Diflufenican						

Application rate (g/ha)		70						
Crop	**Indicator Species**	**Toxicity (mg a.s./kg bw/d)**	**Appl. rate (kg/ha)**	**SV**	**TWA**	**DDD**	**TER_{LT}**	**Annex VI trigger**
Bare soil	Small granivorous bird	91.84	0.070	11.4	0.53	0.42	217.15	5
Winter wheat	Small omnivorous bird			64.8	0.53	2.40	38.2	

SV: Shortcut Value
TWA: Time Weighted Average factor
TER: Toxicity Exposure Ratio
DDD: Daily Dietary Dose

The screening assessment for the long-term risks to birds from exposure to ACL + DFF SC 600 (500 + 100) G after use according to the recommended GAP demonstrate that the risks are acceptable, with the TER_{LT} value calculated to be greater than the Annex VI trigger of 5, indicating a low potential long-term risk to birds from the exposure of ACL + DFF SC 600 (500 + 100) G. In this occasion, a first-tier assessment was not required.

Assessment of combined toxicity

An assessment of combined toxicity of the active substances present in ACL + DFF SC 600 (500 + 100) G has been made according to current EFSA Guidance. In post-AR zonal and country specific assessments, the combined toxicity of all relevant products will be performed according to zonal or country guidance.

When a product contains more than one active substance, an additional assessment on combined toxicity risk has to be presented. It is considered that a quantitative toxicity risk assessment according to concentration addition is not needed if one of the following points applies:

- The risk assessment for all active substances in the product passes with a high margin of safety
- One active substance clearly drives the risk assessment

These conditions are assessed following a step-wise approach. A detailed description of this approach is presented in a separate document ([redacted], [redacted], [redacted], 2016, M-571377-02-1). Note that for the calculation only the scenario with the lowest TER values was considered (most critical scenario). This safety covers all other scenarios.

1st step: Margin of safety

Condition: all TER values are >Trigger × n

Where:

n = number active substances in the mixture

2nd step: Risk per fraction

Condition: One a.s. contributes to ≥90% of the predicted combined toxicity of the product.

Assessment: The contribution of each individual a.s. to the combined toxicity (risk per fraction, rpf) is estimated based on the following equation:

$$rpf_{a.s.1} = \frac{1}{TER_{a.s.1}} / (\frac{1}{TER_{a.s.1}} + \frac{1}{TER_{a.s.2}} \ldots + \frac{1}{TER_{a.s.i}})$$

The estimation is based on TER values from the same refinement level to assure comparability.

3rd step: TER_{MIX} calculation

Condition: The combined toxicity is acceptable if $TER_{MIX} \geq 10$ (acute) or ≥ 5 (long-term).

Assessment: The combined toxicity risk (TER_{MIX}) with concentration-addition is estimated based on the following equation:

$$TER_{mix} = 1 / (\frac{1}{TER_{a.s.1}} + \frac{1}{TER_{a.s.2}} \ldots \frac{1}{TER_{a.s.i}})$$

Table 10.1-5: Combined toxicity assessment - birds

Intended use	Winter cereals, BBCH 00 - 13					
Active substances	Aclonifen (ACL) + Diflufenican (DFF)					
Application rate (L/ha)	0.7					
Scenario / Indicator species	**TER values**		**Trigger**	**1st step (all TER ≥trigger × n)**	**2nd step (Rpfmax)**	**3rd step (TER_{MIX})**
	ACL	**DFF**				
Acute / Small granivorous bird / pre-emergence	>231.4	>1234	≥10	Yes	Not needed	Not needed
Acute / Small omnivorous bird / post-emergence	>35.98	>193.4	≥10	Yes	Not needed	Not needed
Long-term / Small granivorous bird / pre-emergence	>66.68	>217.15	≥5	Yes	Not needed	Not needed
Long-term / Small omnivorous bird / post-emergence	>11.73	38.2	≥5	Yes	Not needed	Not needed

An acceptable acute and chronic risk for birds from the combined exposure to all active substances in the product can be concluded due to a high margin of safety (all TER values > trigger × n).

DRINKING WATER RISK ASSESSMENT

Exposure of birds or mammals via drinking water is not explicitly included in the DDD calculations of the dietary risk assessment. Therefore, in line with EFSA (2009) an approach is presented that allows estimating the possible risk arising from uptake of contaminated drinking water for two basic scenarios. Due to the incidental nature of occurrence of drinking water reservoirs on agricultural fields (as compared to the contamination of food items growing or dwelling on those fields), a separate assessment of this exposure route is considered appropriate at least on the first-tier level.

Most birds and mammals can in principle satisfy (at least parts of) their daily water demand via uptake of food. However, this potential depends on the water content of the diet items, which is lowest for

seeds. Therefore, the assessment methodology for the risk to birds and mammals of pesticides in drinking water as provided below uses small granivorous animals as indicator species at Tier 1.

EFSA (2009) identifies two scenarios as relevant for assessing the risk of pesticides via drinking water to birds and mammals:

- *Leaf scenario:* Birds taking water that is collected in leaf whorls after application of a pesticide to a crop and subsequent rainfall or irrigation.
- *Puddle scenario:* Birds and mammals taking water from puddles formed on the soil surface of a field when a (heavy) rainfall event follows the application of a pesticide to a crop or bare soil.

A leaf scenario is clearly the worst-case situation. It is relevant for spray applications only and according to EFSA (2009) should be considered for the following crop types and growth stages:

- Leaf vegetables (forming heads) at principal growth stage 4 until harvest (classification according to BBCH52).
- Other leaf vegetables (e.g. cauliflower) at principal growth stage 4 or later, with a morphology that facilitates collection of rain/irrigation water in reservoirs that are large enough and easily accessible to attract birds and sufficiently stable over some hours.

A leaf scenario is not deemed relevant for small mammals.

As the proposed use for ACL + DFF SC 600 (500 + 100) G does not include any leaf scenario listed above, the only relevant source of exposure is the puddle scenario.

EFSA (2009) indicates that no specific calculations of exposure and TER are necessary when the ratio of the effective application rate (g/ha) to the relevant endpoint (mg a.s./kg bw/d) does not exceed 50 in the case of less sorptive substances (K_{oc} <500 L/kg) or 3000 in the case of more sorptive substances (K_{oc} >500 L/kg).

Rather than the effective application rate, the maximum application rate for aclonifen (350 g a.s./ha) and for diflufenican (70 g a.s./ha) will be used as a worse case consideration. The mean K_{oc} value for aclonifen is 5727 L/kg and as it is >500 L/kg the trigger of 3000 is acceptable. The mean K_{oc} value for diflufenican is 3417 L/kg and as it is >500 L/kg the trigger of 3000 is acceptable

Table 10.1-6: Application rate to endpoint ratios for the proposed uses of ACL + DFF SC 600 (500 + 100) G

Intended use		Winter cereals, BBCH 00-13, pre- and post-emergence		
Active substance/product		Aclonifen		
Application rate (g/ha)		350		
Risk assessment	**Application rate (g a.s./ha)**	**Endpoint (mg a.s./kg bw/d)**	**Ratio**	**Trigger**
Acute	350	>2000	<0.18	3000
Long-term		>141	<2.48	
Active substance/product		Diflufenican		
Application rate (g/ha)		70		
Risk assessment	**Application rate (g a.s./ha)**	**Endpoint (mg a.s./kg bw/d)**	**Ratio**	**Trigger**
Acute	70	>2150	<0.03	3000

Long-term		91.84	0.76	

As the ratios of application rate to endpoint are lower than 3000 for both the acute and long-term assessment no specific calculations of exposure to birds via drinking water are necessary. An acceptable risk can be concluded from contaminated drinking water as a result of the proposed use of ACL + DFF SC 600 (500 + 100) G.

BIOACCUMULATION AND FOOD CHAIN BEHAVIOUR

Plant protection products with high bioaccumulation potential could theoretically bear a risk of secondary poisoning for birds, if contaminated prey such as fish or earthworms are eaten. According to EFSA (2009), for organic chemicals, a log P_{ow} >3 is used to indicate whether there might be a potential for bioaccumulation and should be assessed for the risk of biomagnification in terrestrial food chains. The log P_{ow} of aclonifen was determined to be 4.37 (see Document M-CA2, Section CA 2.7, Physical and chemical properties) and for diflufenican was determined to be 4.2 (EFSA Scientific Report (2007) 122, 1-84, Conclusion on the peer review of diflufenican). Therefore, a risk assessment for a generic earthworm-eating birds and a generic fish-eating bird has been performed to evaluate the risk of secondary poisoning from the use of ACL + DFF SC 600 (500 + 100) G.

a) Food chain from earthworm to earthworm-eating birds:

Dry soil approach

The bioconcentration factor for the earthworm ($BCF_{earthworm}$) was estimated according to the following equation (from the works of Jager, 1998):

$$BCF_{earthworm} = \frac{0.84 + 0.012 K_{ow}}{f_{oc} \times K_{oc}}$$

Where:

K_{oc} = Organic carbon adsorption coefficient

f_{oc} = Organic carbon content of soil (default value of 0.02 used)

Table 10.1-7: Calculation of $BCF_{earthworm}$ for ACL + DFF SC 600 (500 + 100) G

Aclonifen			
K_{ow}	**f_{oc}**	**K_{oc}**	**$BCF_{earthworm}$**
23442[1]	0.02	5727[2]	2.4463
Diflufenican			
K_{ow}	**f_{oc}**	**K_{oc}**	**$BCF_{earthworm}$**
15849[3]	0.02	3417[3]	2.795

1: See Document CA-2, Section CA 2.7
2: See Document CA7, Section CA 7.1.3.1
3: EFSA Scientific Report 122 (2007), 1-84

The calculated BCF value along with the PEC_{soil} from the proposed use in winter wheat was used to estimate the residue level in earthworms ($PEC_{earthworm}$) using the following equation:

$$PEC_{earthworm} = PEC_{soil} \times BCF_{earthworm}$$

The residue ($PEC_{earthworm}$) was converted into a daily dose by multiplying with the default value for birds 1.05 (calculated on the basis of a 100 g bird eating 104.6 g earthworm fresh per day), according to Smit (2005). The TER_{LT} was then calculated from the daily dose and the long-term NOAEL.

Table 10.1-8: Food chain from earthworm to earthworm-eating birds assessment for the proposed use of ACL + DFF SC 600 (500 + 100) G

Aclonifen						
NOAEL (mg/kg bw/d)	**PEC_{soil}**	**$BCF_{earthworm}$**	**$PEC_{earthworm}$**	**Daily dose (mg/kg bw/d)**	**TER_{LT}**	**Trigger**
>141	0.5113	2.463	1.259	1.322	106.7	5
Diflufenican						
NOAEL (mg/kg bw/d)	**PEC_{soil}**	**$BCF_{earthworm}$**	**$PEC_{earthworm}$**	**Daily dose (mg/kg bw/d)**	**TER_{LT}**	**Trigger**
91.84	0.405	2.795	0.998	1.048	87.7	5

The risk from the proposed use of ACL + DFF SC 600 (500 + 100) G in winter wheat was above the TER_{LT} trigger value of 5, indicating the risk to earthworm-eating birds was acceptable.

b) Food chain from fish to fish-eating birds

The BCF (whole-body) for fish, experimentally determined for the active ingredient aclonifen is 1349 L kg^{-1} ([redacted] 2019, M-667576-02-1, KCA 8.2.2.3/03).

The BCF (whole-body) for fish, experimentally determined for the active ingredient diflufenican is 1596 L kg^{-1} (EFSA Scientific Report 122 (2007), 1-84).

The residue in fish was estimated according to the following equation with the TWA from the reproductive assessment being used:

$$PEC_{fish} = PEC_{sw} \times TWA \times BCF$$

The residue (PEC_{fish}) was converted into a daily dose by multiplying with the default value for birds 0.159 (calculated on the basis of a 1000 g bird eating 159 g fresh fish per day) according to Smit (2005). The TER_{LT} was then calculated from the daily dose and the long-term NOAEL.

Table 10.1-9: Food chain from fish to fish-eating birds assessment for the proposed uses of ACL + DFF SC 600 (500 + 100) G

Aclonifen							
NOAEL (mg/kg bw/d)	**PEC_{sw} (mg/L)**	**TWA**	**BCF**	**PEC_{fish}**	**Daily dose (mg/kg bw/d)**	**TER_{LT}**	**Trigger**
>141	0.0167	0.53	1349	11.94	1.90	74.1	5
Diflufenican							
NOAEL (mg/kg bw/d)	**PEC_{sw} (mg/L)**	**TWA**	**BCF**	**PEC_{fish}**	**Daily dose (mg/kg bw/d)**	**TER_{LT}**	**Trigger**

91.84	0.0042[1]	0.53	1596	3.55	0.57	162.6	5

1: Maximum PEC_{sw} from FOCUS Step 1

The TER_{LT} is above the relevant trigger value of 5 demonstrating that there is no unacceptable long-term risk to birds *via* the food chain from fish to fish-eating birds from the proposed uses of ACL + DFF SC 600 (500 + 100) G.

c) Biomagnification in terrestrial food chains

Absorption, distribution, metabolism and excretion (ADME) studies with aclonifen have shown that the substance was rapidly absorbed and excreted with the major route of excretion via the urine (62-65% of the administered dose) with the rest excreted in the faeces (Document M-CA5, Section CA 5.1.1). After absorption from the gastro-intestinal tract the compound was extensively and rapidly metabolised prior to excretion. Due to the high metabolism and excretion rate of aclonifen no indication of accumulation in the tissues was observed. It can therefore be assumed that there is no biomagnification along the food chain and, as such, in accordance with EFSA (2009), no further assessment of the potential for biomagnification in terrestrial food chains is required.

Absorption, distribution, metabolism and excretion (ADME) studies with diflufenican have shown that the substance was adsorbed orally and excreted with a whole body half-life of 50 to 60 hours (2.5 days). The proportion of ingested dose was 58 to 71% (males and females, respectively) with preferential distribution to high adipose (fatty) tissues, suggesting a potential for long term accumulation.

Therefore, the potential for biomagnification in terrestrial food chains should be considered. According to EFSA (2009) the food-to-organism is estimated according to the following equation:

$$BAF_{organism\text{-}food} = \frac{\alpha \times FIR}{k_2}$$

Where:
α = fraction of ingested dose absorbed =0.71 (71%)
k_2 = ln(2)/t1/2 = 0.693 / 2.5d = 0.277
FIR = food ingestion rate relevant to body weight = 0.3*
* 0.3 is appropriate value for carnivorous / insectivorous species (EFSA, 2009 and SANCO/4145/2000)

Using the above, the BAF was estimated to be <1 (0.77), therefore, no further assessment of the potential for biomagnification in terrestrial food chains is required.

CP 10.1.1.1 Acute oral toxicity

No studies were performed on the representative formulation as it was considered that the data generated for the active substances, aclonifen and diflufenican, was sufficient to reliably predict the toxicity of the formulation. Details of the studies performed on aclonifen are referenced in Document M-CA 8 of this dossier and for diflufenican in the relevant EU DAR (2006).

CP 10.1.1.2 Higher tier data on birds

No further data are required as no unacceptable risk to birds is anticipated according to the screening risk assessment.

CP 10.1.2 Effects on terrestrial vertebrates other than birds

A summary of the mammalian toxicity endpoints for aclonifen is provided in the following table. Details and a full description of the toxicity studies used in this risk assessment can be found in Document M-CA 5 of this dossier and for diflufenican in the relevant EU DAR (2006).

Effects on mammals of ACL + DFF SC 600 (500 + 100) G were not evaluated as part of the EU assessment of aclonifen and diflufenican. Details of the studies performed on ACL + DFF SC 600 (500 + 100) G are referenced in Document M-CA 5 of this dossier.

Table 10.1-10: Mammalian endpoints used in risk assessment

Test item	Type of exposure		Endpoint	Reference
ACL + DFF SC 600 (500 + 100) G	Acute risk assessment	Acute oral toxicity on rat	LD_{50} >2000 mg/kg bw (>985.8 mg a.s./kg bw)	KCP 7.1.1/01 M-557590-01-1 [redacted] 2016
Aclonifen	Acute risk assessment	Acute oral toxicity on rat	**LD_{50} >5000 mg a.s./kg bw**	KCA 5.2.1/01 M-174876-01-1 [redacted] 1981
	Long-term risk assessment	2-generation study on rat	**NOAEL = 35 mg a.s./kg bw/d**	KCA 5.6.1/01 M-174748-01-1 [redacted], 1985
Diflufenican	Acute risk assessment	Acute oral toxicity on rat	**LD_{50} >5000 mg a.s./kg bw**	EFSA Scientific Report 122 (2007), 1-84
	Long-term risk assessment	2-generation study on rat	**NOAEL = 35.5 mg/kg bw/d** (parental effects)	EFSA Scientific Report 122 (2007), 1-84

Endpoints in **bold** were used in the risk assessment

Summary of risk assessment for mammals

The risk assessment for effects of ACL + DFF SC 600 (500 + 100) G on mammals was performed in accordance with the "European Food Safety Authority, Guidance Document on Risk Assessment for Birds & Mammals" (EFSA 2009)[2], (subsequently referred to as the Guidance document (EFSA 2009)). The risk assessment demonstrated acceptable acute dietary exposure risks following the proposed uses and based on the 'worst-case' screening step. Unacceptable long-term risks were shown following the 'worst-case' screening step, however following a first tier assessment, acceptable risk was demonstrated.

The risk from consumption of contaminated water was assessed for aclonifen and diflufenican. The acute and long-term risk from drinking water exposure was considered to be acceptable. No risk is discernible for mammals drinking contaminated water and also the secondary poisonin

Aclonifen and diflufenican have a log P_{ow} of 4.37 and 4.2, respectively, which is higher than the trigger value of 3 and hence an assessment of the risk from secondary poisoning was required. The secondary poisoning risk for earthworm-eating and fish-eating mammals from the proposed uses of ACL + DFF SC 600 (500 + 100) G was shown to be acceptable.

[2] European Food Safety Authority; Guidance Document on Risk Assessment for Birds & Mammals on request from EFSA. EFSA Journal .2009; 7(12):1438. [139 pp.] doi:10.2903/j.efsa.2009.1438. Avalable online: www.efsa.europa.eu

An acceptable risk for mammals from the combined exposure to both active substances in the product can be concluded.

Risk assessment for mammals

The following mammalian risk assessment has been conducted in line with EFSA's Bird and Mammal Guidance Document (EFSA Journal 2009; 7(12):1438), referred to in the following as EFSA (2009). No short-term risk assessment is required under EFSA (2009) as this is assumed to be covered by the acute and reproductive (long-term) risk assessment and therefore these are conducted in the sections below.

The main potential route of exposure for mammals to foliar applied agrochemicals is considered to be through the ingestion of residues on contaminated food, e.g. vegetation, insects and earthworms. The intended GAP for ACL + DFF SC 600 (500 + 100) G is presented in Table 10-1 above.

Direct exposure of ACL + DFF SC 600 (500 + 100) G to mammals is considered unlikely since at the time of application, and for a period thereafter, most mammals will leave the immediate vicinity of spray operations in response to the degree of human disturbance. The greatest levels of exposure will arise in the case of mammals foraging in the foliage of the crops some hours after application.

To achieve a concise risk assessment, the risk envelope approach is applied. Here all following assessments have been made for the use of ACL + DFF SC 600 (500 + 100) G in winter wheat using an application rate of 350 g a.s./ha as this will also cover the risks from the use at lower application rates.

Toxicity of the formulation

A comparison of the acute LD_{50} valued derived for the formulation with the LD_{50} value calculated from the toxicity data of the active substances indicates that the formulation is not more toxic than expected based on its active ingredient content. Therefore, the risk assessment will be based on the active substances.

	Aclonifen	Diflufenican
Content within the product [%] [1]	40.7	8.13
LD_{50} [mg a.s./kg bw]	>5000	>5000
LD_{50} – mixed toxicity [mg product/kg bw]	10240	

1: Based on a nominal active ingredient content of 500 g/L ACL and 100 g/L DFF and a formulation density of 1.230 g/mL

Calculation of acute mixed toxicity determined according to Finney (EFSA, 2009)

Selection of relevant endpoint for long-term/reproductive risk assessment

The EFSA Scientific Report for Aclonifen (2008)[3] identified a NOAEL of 8 mg a.s./kg bw/d based on the 2-generation reproductive toxicity study on rats ([redacted], 1985, KCA 5.6.1/01) as the relevant endpoint for the long-term/reproductive risk assessment. Since this time more detailed guidance for the

[3] EFSA Scientific Report (2008) 149, 1-80, Conclusion on the peer review of aclonifen

risk assessment has been developed (EFSA, 2008[4] and EFSA, 2009[5]) and as such a re-assessment of the relevant endpoint has been undertaken ([redacted] 2019, KCA 8.1.2.2/01).

Based on this re-assessment, the relevant endpoint for the long-term/reproductive risk assessment is concluded to be 35 mg a.s./kg bw/d.

DIETARY RISK ASSESSMENT

Screening assessment

The first, or screening, step assesses the risk based on a worst-case approach. The risk is considered acceptable, if the 'Toxicity Exposure Ratio' (TER) value pass the trigger values of ≥10 for acute exposure and ≥5 for chronic exposure. If the TER values do not pass the trigger values in certain areas, a Tier 1 risk assessment based on more relevant and realistic conditions is performed in those particular areas.

Calculation of Daily dietary Dose (DDD)

The daily dietary dose (DDD) for a single application is given by the following equation:

$$DDD_{single\ application} = Application\ Rate\ [kg/ha] x\ Shortcut\ Value\ x\ TWA$$

The Time Weighted Average factor (TWA) is only considered for the long-term exposure. The long-term risk assessment can be based on a TWA = 0.53 (estimates time-weighted exposure over 21 days, assuming a default DT_{50} of 10 days).

Calculation of Toxicity Exposure Ratio (TER)

The assessment of the risks to mammals is performed for both acute and long-term exposures using endpoints derived from acute and reproduction studies with mammals.

The calculation of acute and long-term toxicity-exposure-ratios (TER) is defined as follows:

Acute risk assessment: $TER_A = LD_{50}/DDD$

Reproductive risk assessment: $TER_{LT} = NOAEL/DDD$

Screening step

According to EFSA (2009), an 'indicator species' is used in a screening step to eliminate all those substances that clearly pose a low risk to mammals. This 'indicator species' is not a real species but, by virtue of its size and feeding habits, is considered to have a higher exposure than (i.e. to be protective of) other species that may occur in a particular crop at a particular time.

For application to the crop relevant for this dossier, winter wheat, the small herbivorous mammal should be considered in the screening step using the relevant shortcut values for acute and long-term risk assessments. The shortcut value consists of the food intake rate of the species of concern, its body

[4] EFSA Scientific Opinion of the Panel on Plant protection products and their Residues. The EFSA Journal (2008) 734, 1-181

[5] European Food Safety Authority; Guidance Document on Risk Assessment for Birds & Mammals on request from EFSA. EFSA Journal 2009; 7(12):1438. doi:10.2903/j.efsa.2009.1438. Available online: www.efsa.europa.eu

weight, the concentration of a substance in/on fresh diet and the fraction of diet obtained in the treated area.

The formulation ACL + DFF SC 600 (500 + 100) G is applied as a spray liquid pre-emergent to bare soil immediately after soil cultivation. Residues in vegetation are therefore negligible. Green plants (weeds) are only present before they are removed during cultivation and drilling process. There is therefore the potential for aclonifen to reach bare soil, and hence the crop group 'Bare soil' is also included at the screening stage. For bare soil, the small granivorous mammal should be considered according to EFSA (2009).

Table 10.1-11: Mammalian indicator species and shortcut values for the screening assessment

Crop	Indicator species	Shortcut value (SV)	
		Acute assessment	Reproductive assessment
Bare soil	Small granivorous mammal	14.4	6.6
Winter wheat	Small herbivorous mammal	118.4	48.3

Table 10.1-12: Mammalian screening acute assessment for the proposed uses of ACL + DFF SC 600 (500 + 100) G

Intended use		Winter cereals, BBCH 00-13, pre- and post-emergence					
Active substance/product		Aclonifen					
Application rate (g/ha)		350					
Crop	**Indicator Species**	**Toxicity (mg a.s./ kg bw)**	**Appl. rate (kg/ha)**	**SV**	**DDD**	**TER_A**	**Annex VI trigger**
Bare soil	Small granivorous mammal	5000	0.35	14.4	5.04	992.1	10
Winter wheat	Small herbivorous mammal			118.4	41.44	120.7	
Active substance/product		Diflufenican					
Application rate (g/ha)		70					
Bare soil	Small granivorous mammal	5000	0.070	14.4	1.01	4960	10
Winter wheat	Small herbivorous mammal			118.4	8.29	603.3	

SV: Shortcut Value
TER: Toxicity Exposure Ratio
DDD: Daily Dietary Dose

The screening assessment for the acute risks to mammals from exposure to ACL + DFF SC 600 (500 + 100) G after use according to the recommended GAP demonstrate that the risks are acceptable, with the TER_A value calculated to be greater than the Annex VI trigger of 10, indicating a low potential acute risk to mammals from the exposure of ACL + DFF SC 600 (500 + 100) G. In this occasion, a first-tier assessment was not required.

Table 10.1-13: Mammalian screening long-term assessment for the proposed uses of ACL + DFF SC 600 (500 + 100) G

Intended use		Winter cereals, BBCH 00-13, pre- and post-emergence						
Active substance/product		Aclonifen						
Application rate (g/ha)		350						
Crop	**Indicator Species**	**Toxicity (mg a.s./kg bw/d)**	**Appl. rate (kg/ha)**	**SV**	**TWA**	**DDD**	**TER_{LT}**	**Annex VI trigger**
Bare soil	Small granivorous mammal	35	0.35	6.6	0.53	1.22	28.59	5
Winter wheat	Small herbivorous mammal			48.30	0.53	8.96	**3.91**	
Active substance/product		Diflufenican						
Application rate (g/ha)		70						
Bare soil	Small granivorous mammal	35.5	0.070	6.6	0.53	0.24	144.98	5
Winter wheat	Small herbivorous mammal			48.30	0.53	1.79	19.81	

SV: Shortcut Value
TWA: Time Weighted Average factor
DDD: Daily Dietary Dose
TER: Toxicity Exposure Ratio
TER values in **bold** are indicating unacceptable risks

The screening assessment for the long-term risks to mammals from exposure to ACL + DFF SC 600 (500 + 100) G after use according to the recommended GAP demonstrates that the risks from the potential application to bare soil are acceptable, with the TER_{LT} value for both aclonifen and diflufenican calculated to be greater than the Annex VI trigger of 5, indicating a low potential long-term risk to mammals from the exposure of ACL + DFF SC 600 (500 + 100) G.

Similarly, the screening assessment for the long-term risks to mammals from exposure to ACL + DFF SC 600 (500 + 100) G after use according to the recommended GAP demonstrates that the risks from the potential application to winter wheat are acceptable for diflufenican with the TER_{LT} value for diflufenican calculated to be greater than the Annex VI trigger of 5, indicating a low potential long-term risk to mammals.

However the TER_{LT} for the use of aclonifen in winter wheat showed an unacceptable risk at the screening stage and therefore, a first-tier assessment was required.

In the first-tier assessment, more realistic exposure estimates along with a 'generic focal species' is used. In accordance with EFSA (2009), a 'generic focal species' is not a real species. However it is considered to be representative of all those species potentially at risk, i.e. it is based on ecological knowledge of a range of species that could be at risk. It has a high food intake rate and may consume a mixed diet rather than just one as for the indicator species. The diet is not real but is considered to be representative of the species represented and hence EFSA (2009) employs a quartile approach where only the 2, 3 or 4 largest food types have been extrapolated to either 25% or 50% of the total diet. The 'generic focal species' is also considered to be a representative of the types of birds or mammals that occur across Member States.

Table 10.1-14: Mammalian first tier long-term assessment for the proposed uses of ACL + DFF SC 600 (500 + 100) G

Intended use		Winter cereals, BBCH 00-13, pre- and post-emergence					
Active substance/product		Aclonifen					
Intended use		Winter cereals, BBCH 00-13, pre- and post-emergence					
Scenario	**Generic focal spp.**	**SV**	**TWA**	**DDD (mg a.s./kg bw)**	**Endpoint (mg a.s./kg bw)**	**TER_{LT}**	**Trigger value**
BBCH <10	Small omnivorous mammal "mouse"	5.7	0.53	1.06	35	33.10	5
BBCH 10-19	Small insectivorous mammal "shrew"	4.2		0.78		44.92	
Early shoots	Large herbivorous mammal "lagomorph"	22.3		4.14		8.46	
BBCH 10-29	Small omnivorous mammal "mouse"	7.8		1.45		24.19	

SV: Shortcut Value
TWA: Time Weighted Average factor
DDD: Daily Dietary Dose
TER: Toxicity Exposure Ratio

Following a Tier 1 assessment, the TER_{LT} values from the use in winter wheat were shown to be greater than the Annex VI trigger of 5, indicating a low potential long-term risk to mammals from the exposure of ACL + DFF SC 600 (500 + 100) G.

Assessment of combined toxicity

An assessment of combined toxicity of the active substances present in ACL + DFF SC 600 (500 + 100) G has been made according to current EFSA Guidance. In post-AIR zonal and country specific assessments, the combined toxicity of all relevant products will be performed according to zonal or country guidance.

When a product contains more than one active substance, an additional assessment on combined toxicity risk has to be presented. It is considered that a quantitative toxicity risk assessment according to concentration addition is not needed if one of the following points applies:

- The risk assessment for all active substances in the product passes with a high margin of safety
- One active substance clearly drives the risk assessment

These conditions are assessed following a step-wise approach. A detailed description of this approach is presented in a separate document ([redacted], [redacted], [redacted], 2016, M-571377-02-1). Note that for the calculation only the scenario with the lowest TER values was considered (most critical scenario). This safely covers all other scenarios.

1st step: Margin of safety

Condition: all TER values are >Trigger × n

Where:

n = number active substances in the mixture

2nd step: Risk per fraction

Condition: One a.s. contributes to ≥90% of the predicted combined toxicity of the product.

Assessment: The contribution of each individual a.s. to the combined toxicity (risk per fraction, rpf) is estimated based on the following equation:

$$rpf_{a.s.1} = \frac{1}{TER_{a.s.1}} / (\frac{1}{TER_{a.s.1}} \frac{1}{TER_{a.s.2}} \ldots + \frac{1}{TER_{a.s.i}})$$

The estimation is based on TER values from the same refinement level to assure comparability.

3rd step: TER_{MIX} calculation

Condition: The combined toxicity is acceptable if $TER_{MIX} \geq 10$ (acute) or ≥ 5 (long-term).

Assessment: The combined toxicity risk (TER_{MIX}) with concentration-addition is estimated based on the following equation:

$$TER_{mix} = 1 / (\frac{1}{TER_{a.s.1}} + \frac{1}{TER_{a.s.2}} \ldots + \frac{1}{TER_{a.s.i}})$$

Table 10.1-15: Combined toxicity assessment – mammals

Intended use	Winter cereals, BBCH 00 - 13					
Active substances	Aclonifen (ACL) + Diflufenican (DFF)					
Application rate (L/ha)	0.35					
Scenario / Indicator species	**TER values**		**Trigger**	**1st step (all TER ≥trigger × n)**	**2nd step (Rpfmax)**	**3rd step (TER_{MIX})**
	ACL	**DFF**				
Acute / Small granivorous mammal / pre-emergence	992.1	4960	10	Yes	Not needed	Not needed
Acute / Small herbivorous mammal / post-emergence	120.3	603.3		Yes	Not needed	Not needed
Long-term / Small granivorous mammal / pre-emergence	28.59	144.98	5	Yes	Not needed	Not needed
Long-term / Small herbivorous mammal / post-emergence	**3.91**	19.81		No	0.96 (ACL)	Not needed

An acceptable acute and chronic risk for mammals from the combined exposure to all active substances in the product can be concluded due to a high margin of safety (all TER values > trigger × n) for the acute risk. For the combined long-term toxicity risk, aclonifen is shown to contribute ≥90% of the predicted combined toxicity of the product. Therefore, no further combined toxicity risk assessment is required.

DRINKING WATER RISK ASSESSMENT

Exposure of birds or mammals via drinking water is not explicitly included in the DDD calculations of the dietary risk assessment. Therefore, in line with EFSA (2009) an approach is presented that allows estimating the possible risk arising from uptake of contaminated drinking water. A leaf scenario is deemed not relevant for small mammals and hence only the puddle scenario has been assessed.

EFSA (2009) indicates that no specific calculations of exposure and TER are necessary when the ratio of the effective application rate (g/ha) to the relevant endpoint (mg a.s./kg bw/d) does not exceed 50 in the case of less sorptive substances (K_{oc} <500 L/kg) or 3000 in the case of more sorptive substances (K_{oc} >500 L/kg).

Rather than the effective application rate, the maximum application rate for aclonifen (350 g a.s./ha) and for diflufenican (70 g a.s./ha) will be used as a worse case consideration. The mean K_{oc} value for aclonifen is 5727 L/kg and as it is >500 L/kg the trigger of 3000 is acceptable. The mean K_{oc} value for diflufenican is 3417 L/kg and as it is >500 L/kg the trigger of 3000 is acceptable.

Table 10.1-16: Application rate to endpoint ratios for the proposed uses of ACL + DFF SC 600 (500 + 100) G

Intended use		Winter cereals, BBCH 00-13, pre- and post-emergence		
Active substance/product		Aclonifen		
Application rate (g/ha)		350		
Risk assessment	**Application rate (g a.s./ha)**	**Endpoint (mg a.s./kg bw/d)**	**Ratio**	**Trigger**
Acute	350	>5000	<0.07	3000
Long-term		35	10	
Active substance/product		Diflufenican		
Application rate (g/ha)		70		
Risk assessment	**Application rate (g a.s./ha)**	**Endpoint (mg a.s./kg bw/d)**	**Ratio**	**Trigger**
Acute	70	>5000	<0.014	3000
Long-term		35.5	1.97	

As the ratios of application rate to endpoint are lower than 3000 for both the acute and long-term assessment no specific calculations of exposure to mammals via drinking water are necessary. An acceptable risk can be concluded from contaminated drinking water as a result of the proposed use of ACL + DFF SC 600 (500 + 100) G.

BIOACCUMULATION AND FOOD CHAIN BEHAVIOUR

Plant protection products with high bioaccumulation potential could theoretically bear a risk of secondary poisoning for mammals if contaminated prey such as fish or earthworms are eaten. According to EFSA (2009), for organic chemicals, a log P_{ow} >3 is used to indicate whether there might be a potential for bioaccumulation and should be assessed for the risk of biomagnification in terrestrial food chains. The log P_{ow} of aclonifen was determined to be 4.37 (see Document M-CA2, Section CA 2.7, Physical and chemical properties) and for diflufenican was determined to be 4.2 (EFSA Scientific Report (2007) 122, 1-84, Conclusion on the peer review of diflufenican). Therefore, a risk assessment for a generic earthworm-eating mammals and a generic fish-eating mammal has been performed to evaluate the risk of secondary poisoning from the use of ACL + DFF SC 600 (500 + 100) G.

a) Food chain from earthworm to earthworm-eating mammals:

Dry soil approach

The bioconcentration factor for the earthworm ($BCF_{earthworm}$) was estimated according to the following equation (from the works of Jager, 1998):

$$BCF_{earthworm} = \frac{0.84 + 0.012K_{ow}}{f_{oc}\ x\ K_{oc}}$$

Where:

K_{oc} = Organic carbon adsorption coefficient

f_{oc} = Organic carbon content of soil (default value of 0.02 used)

Table 10.1-17: Calculation of $BCF_{earthworm}$ for ACL + DFF SC 600 (500 + 100) G

Aclonifen			
K_{ow}	**f_{oc}**	**K_{oc}**	**$BCF_{earthworm}$**
23442[1]	0.02	5727[2]	2.463
Diflufenican			
K_{ow}	**f_{oc}**	**K_{oc}**	**$BCF_{earthworm}$**
15849[3]	0.02	3417[3]	2.795

1: See Document CA-2, Section CA 2.7
2: See Document CA7, Section CA 7.1.3.1
3: EFSA Scientific Report 125 (2007), 1-84

The calculated BCF value along with the PEC_{soil} from the proposed use in winter wheat was used to estimate the residue level in earthworms ($PEC_{earthworm}$) using the following equation:

$$PEC_{earthworm} = PEC_{soil} \times BCF_{earthworm}$$

The residue ($PEC_{earthworm}$) was converted into a daily dose by multiplying with the default value for mammals 1.28 (calculated on the basis of a 10 g mammal eating 12.8 g earthworm fresh per day), according to Smit (2005). The TER_{LT} was then calculated from the daily dose and the long-term NOAEL.

Table 10.1-18: Food chain from earthworm to earthworm-eating mammals assessment for the proposed use of ACL + DFF SC 600 (500 + 100) G

Aclonifen						
NOAEL (mg/kg bw/d)	**PEC_{soil}**	**$BCF_{earthworm}$**	**$PEC_{earthworm}$**	**Daily dose (mg/kg bw/d)**	**TER_{LT}**	**Trigger**
35	0.5113	2.463	1.259	1.612	22	5
Diflufenican						
NOAEL (mg/kg bw/d)	**PEC_{soil}**	**$BCF_{earthworm}$**	**$PEC_{earthworm}$**	**Daily dose (mg/kg bw/d)**	**TER_{LT}**	**Trigger**
35	0.405	2.795	1.132	1.449	25	5

The risk from the proposed use of ACL + DFF SC 600 (500 + 100) G in winter wheat was above the TER_{LT} trigger value of 5, indicating the risk to earthworm-eating birds was acceptable.

b) Food chain from fish to fish-eating mammals

The BCF (whole-body) for fish, experimentally determined for the active ingredient aclonifen is 1349 L kg^{-1} ([redacted] 2019, M-667576-02-1, KCA 8.2.2.3/03).

The BCF (whole-body) for fish, experimentally determined for the active ingredient diflufenican is 1596 L kg^{-1} (EFSA Scientific Report 122 (2007), 1-84).

The residue in fish was estimated according to the following equation with the TWA from the reproductive assessment being used:

$$PEC_{fish} = PEC_{sw} \times TWA \times BCF$$

The residue (PEC_{fish}) was converted into a daily dose by multiplying with the default value for mammals 0.142 (calculated on the basis of a 3000 g mammal eating 425 g fresh fish per day) according to Smit (2005). The TER_{LT} was then calculated from the daily dose and the long-term NOAEL.

Table 10.1-19: Food chain from fish to fish-eating mammals assessment for the proposed uses of ACL + DFF SC 600 (500 + 100) G

Aclonifen							
NOAEL (mg/kg bw/d)	**PEC_{sw} (mg/L)**	**TWA**	**BCF**	**PEC_{fish}**	**Daily dose (mg/kg bw/d)**	**TER_{LT}**	**Trigger**
35	0.0167[1]	0.53	1349	11.94	1.70	21	5
Diflufenican							
NOAEL (mg/kg bw/d)	**PEC_{sw} (mg/L)**	**TWA**	**BCF**	**PEC_{fish}**	**Daily dose (mg/kg bw/d)**	**TER_{LT}**	**Trigger**
35.5	0.0042[1]	0.53	1596	3.00	0.426	83	5

1: Maximum PEC_{sw} from FOCUS Step 1

The TER_{LT} is above the relevant trigger value of 5 demonstrating that there is no unacceptable long-term risk to birds *via* the food chain from fish to fish-eating birds from the proposed uses of ACL + DFF SC 600 (500 + 100) G.

c) Biomagnification in terrestrial food chains

Absorption, distribution, metabolism and excretion (ADME) studies with aclonifen have shown that the substance was rapidly absorbed and excreted with the major route of excretion via the urine (62-65% of the administered dose) with the rest excreted in the faeces (Document M-CA5, Section CA 5.1.1). After absorption from the gastro-intestinal tract the compound was extensively and rapidly metabolised prior to excretion. Due to the high metabolism and excretion rate of aclonifen no indication of accumulation in the tissues was observed. It can therefore be assumed that there is no biomagnification along the food chain and, as such, in accordance with EFSA (2009), no further assessment of the potential for biomagnification in terrestrial food chains is required.

Absorption, distribution, metabolism and excretion (ADME) studies with diflufenican have shown that the substance was adsorbed orally and excreted with a whole body half-life of 50 to 60 hours (2.5 days). The proportion of ingested dose was 58 to 71% (males and females, respectively) with preferential distribution to high adipose (fatty) tissues, suggesting a potential for long term accumulation.

Therefore, the potential for biomagnification in terrestrial food chains should be considered. According to EFSA (2009) the food-to-organism is estimated according to the following equation:

$$BAF_{organisms,\ food} = \frac{\alpha \times FIR}{k_2}$$

Where:
α = fraction of ingested dose absorbed =0.71 (71%)
k_2 = ln(2)/t1/2 = 0.693 / 2.5d = 0.277
FIR = food ingestion rate relevant to body weight = 0.3*
* 0.3 is appropriate value for carnivorous / insectivorous species (EFSA 2009 and SANCO/4145/2000)

Using the above, the BAF was estimated to be <1 (0.77), therefore, no further assessment of the potential for biomagnification in terrestrial food chains is required.

CP 10.1.2.1 Acute oral toxicity to mammals

Please refer to the mammalian toxicology section; Document M-CP 7.1.1.

CP 10.1.2.2 Higher tier data on mammals

No further data are required as no unacceptable risk to mammals is anticipated according to the screening and/or first tier risk assessments.

CP 10.1.3 Effects on other terrestrial vertebrate wildlife (reptiles and amphibians)

The available and relevant data covering potential effects of aclonifen and diflufenican on terrestrial vertebrates are presented under Section CP 10.1.1 for birds and Section CP 10.1.2 for mammals. A literature review (Document M-CA9) did not reveal any relevant studies addressing the effects of aclonifen on terrestrial vertebrate wildlife.

Regarding assessment of potential effects on reptiles and amphibians neither guidance documents nor testing guidelines are available at present. Therefore, no additional data on terrestrial vertebrate wildlife is presented here.

CP 10.2 Effects on aquatic organisms

A summary of the aquatic toxicity endpoints for ACL + DFF SC 600 (500 + 100) G, aclonifen and diflufenican are provided in Table 10.2-1, Table 10.2-2 and Table 10.2-3, respectively. Details and a full description of the aquatic toxicity studies for aclonifen used in this risk assessment can be found in Document M-CA 8 of this dossier. Details and a full description of toxicity studies for diflufenican are provided in the EU DAR (2006).

Table 10.2-1: Summary of the effects of ACL + DFF SC 600 (500 + 100) G on aquatic organisms

Test item	Test species	Type of exposure	Endpoint	Reference
Acute toxicity to fish				
ACL + DFF SC 600 (500 + 100) G	Rainbow trout (*Oncorhynchus mykiss)*	Acute, 96-hour, static	LC_{50} = 2.3 mg formulation/L (nom) LC_{50} = 1.39 mg formulation/L (geom)	KCP 10.2.1/01 M-568874-01-1 [redacted], 2016
Acute toxicity to aquatic invertebrates				
ACL + DFF SC 600 (500 + 100) G	*Daphnia magna*	Acute 48-hour, static	EC_{50} = 2.47 mg/L (nom)	KCP 10.2.1/02 M-565104-01-1 [redacted], 2016
Effects on growth of green algae				
ACL + DFF SC 600 (500 + 100) G	*Pseudokirchneriella subcapitata*	Short-term, 72-hour, static	**E_rC_{50} = 0.00364 mg prod./L (nom)** E_yC_{50} = 0.00151 mg prod./L (nom)	KCP 10.2.1/03 M-666322-02-1 [redacted] (2019)
Effects on aquatic macrophytes				
ACL + DFF SC 600 (500) + 100) G	*Lemna gibba*	7-day, semi-static	$E_rC_{50\ frond}$ = 27.6 µg prod./L (nom) **$E_rC_{50\ weight}$ = 24.6 µg prod.//L (nom)** $E_yC_{50\ frond}$ = 8.45 µg prod./L (nom) $E_yC_{50\ weight}$ = 7.08 µg prod./L (nom) NOE_rC = 3.13 µg prod./L (nom) LOE_rC = 6.25 µg prod./L (nom)	KCP 10.2.1/04 M-666321-01-1 [redacted](2019)

Endpoints in **bold** were used in the risk assessment
nom: nominal test concentrations
gmm: geometric mean measured test concentrations

Table 10.2-2: Summary of the effects of aclonifen on aquatic organisms

Test item	Test species	Type of exposure	Endpoint	Reference
Acute toxicity to fish				
Aclonifen	Rainbow trout (*Oncorhynchus mykiss)*	Acute, 96-hour, static	**LC_{50} = 0.67 mg/L (nom)**	KCA 8.2.1/01 M-174317-01-1 [redacted], M.T., 1991
Long-term and chronic toxicity to fish				
Aclonifen	Fathead minnow (*Pimephales promelas*)	Long-term, 35-day ELS	**35-day $NOEC_{survival}$ = 0.0425 mg/L (mm)** 35-Day $NOEC_{growth}$ ≥0.106 mg/L (mm) 35-Day $EC_{10,survival}$ = ND 35-Day $EC_{10,growth}$ = ND	KCA 8.2.2.1/01 M-626723-01-1 [redacted], 2018
Bioconcentration in fish				
Aclonifen	Rainbow trout (*Oncorhynchus mykiss)*	Long-term, bioaccumulation	**BCF_{Kg1} = 1349 L/kg**	KCA 8.2.2.3/03 M-667676-02-1 [redacted] 2019
Acute toxicity to aquatic invertebrates				
Aclonifen	*Daphnia magna*	Acute, 48-hour, static	**EC_{50} = 1.2 mg/L (mm).**	KCA 8.2.4.1/01 M-174321-01-1 [redacted] 1991
Long-term and chronic toxicity to aquatic invertebrates				
Aclonifen	*Daphnia magna*	Long-term, 21-day, semi-static	**21-Day $NOEC_{body\ length}$ = 0.0142 mg/L (twa)** 21-Day $EC_{10,body\ length}$ = 0.0193 mg/L (twa)	KCA 8.2.5.1/02 M-573305-02-1 [redacted] 2017
Development and emergence in *Chironomus riparius*				
Aclonifen	*Chironomus riparius*	Long-term, 21-day, static	**21-Day spiked water $NOEC_{emergence}$ = 0.472 mg/L (im)** 21-Day spiked water $EC_{10,emergence}$ = ND	KCA 8.2.5.3/01 M-174918-01-1 [redacted], 1996
Sediment dwelling organisms				
Aclonifen	*Chironomus riparius*	Long-term, 28-day, static	**28-Day spiked sediment $NOEC_{emergence}$ = 32 mg/kg (nom)** 28-Day spiked sediment $EC_{10,\ emergence}$ = 36 mg/kg (nom)	KCA 8.2.5.4/01 M-227300-01-1 [redacted], I., 2004 & KCA 8.2.5.4/02 M-674905-01-1 [redacted], 2019
Effects on growth of green algae				

Test item	Test species	Type of exposure	Endpoint	Reference
Aclonifen	*Desmodesmus subspicatus*	Short-term, 96-hour, static	$NOEC_{growth\ rate}$ (0 – 96h) = 0.0000811 mg/L (mm) E_rC_{10} (0 – 96h) = 0.0104 mg/L (mm) **E_rC_{50} (0 – 96h) = 0.0203 mg/L (mm)** $NOEC_{yield}$ (0 – 96h) = 0.0000811 mg/L (mm) E_yC_{10} (0 – 96h) = 0.0244 mg/L (mm) E_yC_{50} (0 – 96h) = 0.0107 mg/L (mm)	KCA 8.2.6.1/03 M-574872-02-1 [redacted], 2016
Effects on aquatic macrophytes				
Aclonifen	*Lemna gibba*	14-day, semi-static	$NOEC_{growth\ rate,\ dry\ weight}$ = 0.00200 mg/L (mm) E_rC_{10} (0 – 14d)$_{dry\ weight}$ = 0.000265 mg/L (mm) **E_rC_{50} (0 – 14d)$_{dry\ weight}$ = 0.0136 mg/L (mm)**	KCA 8.2.7/01 M-171423-01-1 [redacted], J.R., 1998 & KCA 8.2.7/02 M-255537-01-1 [redacted] 2005
Primary producers (algae & macrophytes)				
Aclonifen	Species sensitive distribution utilizing 12 species	-	**HC_5 = 0.000595 mg a.s./L**	See justification

Endpoints in **bold** were used in the risk assessment
ND: not determined
nom: nominal test concentrations
mm: mean measured test concentrations
twa: time-weighted average measured test concentrations
im: initial measured test concentrations

Justification of new endpoints - aclonifen

Where endpoints differ from the EFSA Scientific Report for Aclonifen (2008), justifications are provided below:

Long-term and chronic toxicity to fish

The study referenced in the DAR (2006) and EFSA Scientific Report 149 (2008), [redacted] (1997) KCA 8.2.2.1/01, is considered as not valid due to a failure to meet all relevant validity criteria given in the updated OECD 210 (2013) test guideline. A new study, [redacted] (2018), KCA 8.2.2.1/03, is presented which satisfies all current guideline validity criteria.

The NOEC for the new study is 42.5 µg/L and is considered the relevant endpoint for use in risk assessment.

A detailed justification for this updated endpoint is presented in KCA 8.2.2.1/04.

Bioconcentration in fish

The study referenced in the DAR (2006) and EFSA Scientific Report 149 (2008), [redacted] (1995) KCA 8.2.2.3/01, is considered as not valid due to a failure to meet all relevant validity criteria given in

the updated OECD 305-I (2012) test guideline. A new study, [redacted] (2019), KCA 8.2.2.3/03, is presented which satisfies all current guideline validity criteria.

A BCF_{KgL} of 1349 L/kg was determined in the current study and is considered the relevant endpoint for use in risk assessment.

A detailed justification for this updated endpoint is presented in KCA 8.2.2.3/04.

Long-term and chronic toxicity to aquatic invertebrates

The study referenced in the DAR (2006) and EFSA Scientific Report 149 (2008), [redacted] (1991) KCA 8.2.5.1/01, is not valid due to a failure to meet the validity criteria relating to the mean number of living offspring per parent animal of the updated OECD 211 (2012) test guideline. A new study, [redacted] (2017), KCA 8.2.5.1/02, is presented which satisfies all current guideline validity criteria.

The NOEC for the new study is 14.2 µg/L and is considered the relevant endpoint for use in risk assessment.

Effects on growth of green algae

The study referenced in the DAR (2006) and EFSA Scientific Report 149 (2008), [redacted] (1990) KCA 8.2.6.1/01, is not valid due to a failure to meet the validity criteria relating to the coefficient of variation of sectional growth rates in control cultures of the updated OECD 201 (2011) test guideline. A new study, [redacted] (2016), KCA 8.2.6.1/03, is presented which satisfies all current guideline validity criteria.

The E_rC_{50} (0 – 96h) of 0.0203 mg/L determined in this new study is considered the relevant endpoint for use in risk assessment.

Effects on aquatic macrophytes

The E_rC_{50} = 0.012 mg/L and the E_bC_{50} = 0.006 mg a.s./L as listed in the DAR (2006) and EFSA Scientific Report 149 (2008) for *Lemna* were erroneously labelled as growth rate and biomass related endpoints although in fact no calculations for these response variables had been conducted in the frame of the study report.

In order to fulfil the current requirements as set out in Regulation 283/2013 and OECD 221, which ask for the EC_{50} for growth rate of both endpoints, i.e. frond number and dry weight of plants, the endpoints of the original study by [redacted] (KCA 8.2.7/01) were re-calculated by [redacted] (KCA 8.2.7/02). The resulting 14d-E_rC_{50} = 13.6 µg/L for dry weight is considered the relevant endpoint for use in risk assessment.

Primary producers (algae & macrophytes)

Since EFSA Scientific Report 149 (2008) was published, the use of growth endpoints for primary producers in the construction of Species Sensitivity Distributions (SSDs) has gained wide acceptance and is supported by the EFSA Aquatic Guidance Document[6] and EFSA's Outcome of the Pesticides Peer Review Meeting on general recurring issues in ecotoxicology (EFSA, 2015)[7].

[6] EFSA PPR Panel (EFSA Panel on Plant Protection Products and their Residues), 2013. Guidance on tiered risk assessment for plant protection products for aquatic organisms in edge-of-field surface waters. EFSA Journal 2013;11(7):3290, 268 pp. doi:10.2903/j.efsa.2013.3290

[7] EFSA (European Food Safety Authority), 2015. Technical report on the outcome of the pesticides peer review meeting on general recurring issues in ecotoxicology. EFSA supporting publication 2015:EN-924. 62 pp.

An SSD curve has been constructed using the data generated in 12 aquatic primary producer species and resulted in a HC_5 = 5.95 µg a.s./L which is considered the relevant endpoint for use in risk assessment for primary producers.

Table 10.2-3: Summary of the effects of diflufenican and relevant metabolites on aquatic organisms

Test species	Test item	Type of Exposure	Endpoint	Reference
Acute toxicity to fish				
Common carp (*Cyprinus carpio*)	Diflufenican	Acute, 96-hour, static	96-Hour LC_{50} = 0.0985 a.s. mg/L (mm)	EFSA Scientific Report 122 (2007), 1-84
Long-term toxicity to fish (early life stage)				
Fathead minnow (*Pimephales promelas*)	Diflufenican	Long-term, 35-day ELS, flowthrough	35d (ELS) NOEC = 0.015 mg a.s./L (mm)	EFSA Scientific Report 122 (2007), 1-84
Acute toxicity to aquatic invertebrates				
Daphnia magna	Diflufenican	Acute, 48-hour, static	48-hr EC_{50} > 0.24 mg/L (mm)	EFSA Scientific Report 122 (2007), 1-84
Long-term and chronic toxicity to aquatic invertebrates				
Daphnia magna	Diflufenican	Long-term, 21-day, semi-static	21-d NOEC = 0.052 mg/L (mm)	EFSA Scientific Report 122 (2007), 1-84
Development and emergence in *Chironomus riparius*				
Chironomus riparius	Diflufenican	Long-term, 28-day, static	28-d spiked water NOEC = 0.100 mg a.s./L (nom)	EFSA Scientific Report 122 (2007), 1-84
Sediment dwelling organisms				
Chironomus riparius	Diflufenican	Long-term, 28-day, static	28-d spiked sediment NOEC = 2.0 mg/kg sed dw (nom)	EFSA Scientific Report 122 (2007), 1-84
Effects on growth of green algae				
Desmodesmus subspicatus	Diflufenican	Short-term, 72-hour, static	72-hr E_rC_{50} = 0.00045 mg a.s./L (mm)	See justification
Effects on aquatic macrophytes				
Lemna gibba	Diflufenican	14-day, semi-static	E_rC_{50} (0 – 14 d) = 0.039 mg a.s./L (mm)	EFSA Scientific Report 122 (2007), 1-84

nom. nominal test concentrations
mm. mean measured test concentrations

Justification of new endpoints - diflufenican

Where endpoints differ from the EFSA Scientific Report for diflufenican (2007), justifications are provided below:

Effects on growth of green algae

The study referenced in the DAR (2006) and EFSA Scientific Report 149 (2007), 1-84, [redacted] (1997) used the E_bC_{50} value of 0.00025 mg a.s./L (nominal) for the aquatic risk assessment for algae. According to the current guidance document the growth rate is the preferred endpoint. The E_rC_{50} value (0–72h) of 0.00045 mg a.s./L (nominal) is considered the relevant endpoint for use in risk assessment.

Summary of the Risk Assessment for aclonifen on aquatic organisms

The risk assessment for effects of ACL + DFF SC 600 (500 + 100) G on aquatic organisms was performed in accordance with the "Guidance of tiered risk assessment for plant protection products for aquatic organisms in edge-of-field surface waters" (EFSA Journal 2013; 11(7):3290)[8].

Based on the maximum FOCUS Step 3 PECs, PEC:RAC ratios were shown to be less than 1 for fish (acute and prolonged), invertebrates (acute) and sediment dwelling organisms, indicating acceptable risk. However, for invertebrates (chronic), algae and aquatic macrophytes, some PEC:RAC ratios were in excess of 1 and hence for these organisms the risk assessment required refinement.

In view of the substantial amount of data available for primary producers (algae and macrophytes) and the comparability of growth rate endpoints between algae and aquatic macrophytes, it was possible to calculate an HC_5 based on the available E_rC_{50} data for primary producers.

Following the refinement of the endpoint for primary producers, an acceptable risk was still not shown and hence mitigation methods were suggested. As the RAC for invertebrates (chronic) was lower than the refined RAC for primary producers, mitigation required for invertebrates (chronic) covered the risk for the less sensitive species also.

Risk was shown to be acceptable for aquatic organisms for the proposed uses of ACL + DFF SC 600 (500 + 100) G when a 3-metre no-spray buffer with no nozzle reduction was applied or alternatively 50% drift reduction without requirement for a no-spray buffer.

Risk assessment for aquatic organisms - aclonifen

The following risk assessment has been conducted in line with the "Guidance of tiered risk assessment for plant protection products for aquatic organisms in edge-of-field surface waters" (EFSA Journal 2013; 11(7):3290), hereafter referred to as EFSA Aquatic Guidance Document, 2013.

Exposure

Aquatic organisms may be exposed to ACL + DFF SC 600 (500 + 100) G through spray drift, run-off and drainage from the application site into adjacent water bodies. Exposure of aquatic organisms from these routes was estimated by calculating Predicted Environmental Concentrations in surface water (PEC_{sw}) and sediment (PEC_{sed}) for aclonifen. The predicted concentrations of aclonifen were calculated at FOCUS Steps 1, 2 and 3 using FOCUS version 3.2 software.

[8] EFSA PPR Panel (EFSA Panel on Plant Protection Products and their Residues), 2013. Guidance on tiered risk assessment for plant protection products for aquatic organisms in edge-of-field surface waters. EFSA Journal 2013;11(7):3290, 268 pp. doi:10.2903/j.efsa.2013.3290

To achieve a concise risk assessment, the risk envelope approach is applied. Here all following assessments have been made for the use of ACL + DFF SC 600 (500 + 100) G in winter cereals using an application rate of 350 g a.s./ha as this will also cover the risks from the use at lower application rates.

Regulatory Acceptable Concentration (RAC_{sw}) values based on the toxicity endpoints from the most sensitive species were compared to the maximal PEC_{sw} and sediment PEC_{sed} values derived from the FOCUS Step 1, 2 and 3 values for aclonifen. Full details of the calculation of the PEC values are provided in Document M-CP9.

Table 10.2-4: Aquatic organisms: acceptability of risk (PEC/RAC <1) for aclonifen for each organism group for the application of ACL + DFF SC 600 (500 + 100) G in winter cereals

Group		Fish acute	Fish prolonged	Invert. acute	Invert. prolonged	Sed. Dwell. Prolonged	Green Algae	Aquatic macrophyte		Sed. Dwell. Prolonged
Test species		*Oncorhynchus mykiss*	*Pimephales promelas*	*Daphnia magna*	*Daphnia magna*	*Chironomus riparius*	*Desmodesmus subspicatus*	*Lemna gibba*		*Chironomus riparius*
Endpoint		LC_{50}	NOEC	EC_{50}	NOEC	NOEC	EC_{50}	E_rC_{50}		NOEC
(µg/L)		670	42.5	1200	14.2	472	24.6	13.6		32000
AF		100	10	100	10	10	10	10		10
RAC (µg/L)		6.7	4.25	12	1.42	47.2	2.46	1.36		3200
FOCUS Scenario	PEC $_{sw-max}$ (µg/L)								PEC $_{sed-max}$ (µg/kg)	
Step 1										
	16.7	**2.493**	**3.929**	**1.392**	**11.761**	0.354	**8.227**	**12.279**	774	0.242
Step 2										
N-Europe	6.97	**1.040**	**1.640**	0.581	**4.908**	0.148	**3.433**	**5.125**	390	0.122
S-Europe	5.67	0.846	**1.334**	0.473	**3.993**	0.120	**2.793**	**4.169**	315	0.098
Step 3										
D1/ditch	2.22	0.331	0.522	0.185	**1.563**	0.047	**1.094**	**1.632**	-	-
D1/stream	1.95	0.291	0.459	0.163	**1.373**	0.041	0.961	**1.434**	-	-
D2/ditch	2.23	0.333	0.525	0.186	**1.570**	0.047	**1.099**	**1.640**	-	-
D2/stream	1.98	0.296	0.466	0.165	**1.394**	0.042	0.975	**1.456**	-	-
D3/ditch	2.19	0.327	0.515	0.183	**1.542**	0.046	**1.079**	**1.610**	-	-
D4/pond	0.076	0.011	0.018	0.006	0.054	0.002	0.037	0.056	-	-
D4/stream	1.90	0.284	0.447	0.158	**1.338**	0.040	0.936	**1.397**	-	-
D5/pond	0.076	0.011	0.018	0.006	0.054	0.002	0.037	0.056	-	-
D5/stream	2.05	0.306	0.482	0.171	**1.444**	0.043	**1.010**	**1.507**	-	-
D6/ditch	2.22	0.331	0.522	0.185	**1.563**	0.047	**1.094**	**1.632**	-	-
R1/pond	0.105	0.016	0.025	0.009	0.074	0.002	0.052	0.077	-	-
R1/stream	1.44	0.215	0.339	0.120	**1.014**	0.031	0.709	**1.059**	-	-
R3/stream	2.01	0.300	0.473	0.168	**1.415**	0.043	0.990	**1.478**	-	-
R4/stream	1.45	0.216	0.341	0.121	**1.021**	0.031	0.714	**1.066**	-	-

AF: Assessment factor; PEC: Predicted environmental concentration; RAC: Regulatory acceptable concentration
PEC/RAC ratios above the relevant trigger of 1 are shown in **bold** indicating unacceptable risk

Based on the maximum FOCUS Step 3 PECs, the above calculations show PEC:RAC ratios in excess of 1 for invertebrates (chronic), algae and aquatic macrophytes. For these organisms a refined risk assessment is presented below.

Refined risk assessment

Calculation of the species-sensitivity distribution (SSD) with growth rate endpoints for all primary producer species

In view of the substantial amount of data available for primary producers (algae and macrophytes) and the comparability of growth rate endpoints between algae and aquatic macrophytes, it is considered acceptable to calculate an HC_5 based on the available E_rC_{50} data for primary producers.

The SSD was calculated following the recommendations of the EFSA Aquatic Guidance Document, 2013 using the DEFRA webfram tool (https://webfram.com/home.aspx). In particular, unbound values should not be included in an SSD, however in cases where the unbound value relates to a species for which no other data is available, the unbound value can be used (without the < or > sign) if it is outside the range of all other available toxicity values.

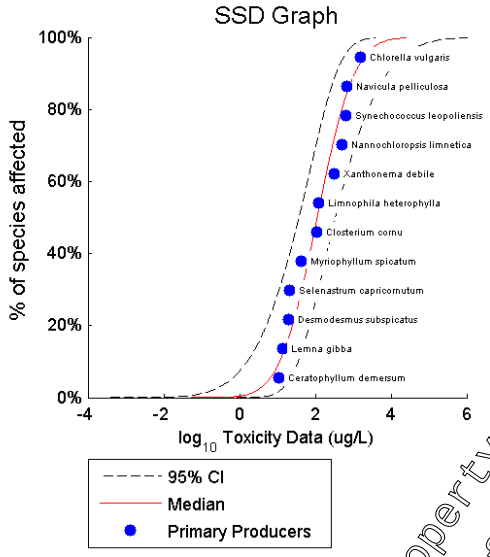
In the following table all primary producer E_rC_{50} endpoints are listed, along with the applicability of these for use in the calculation of the SSD.

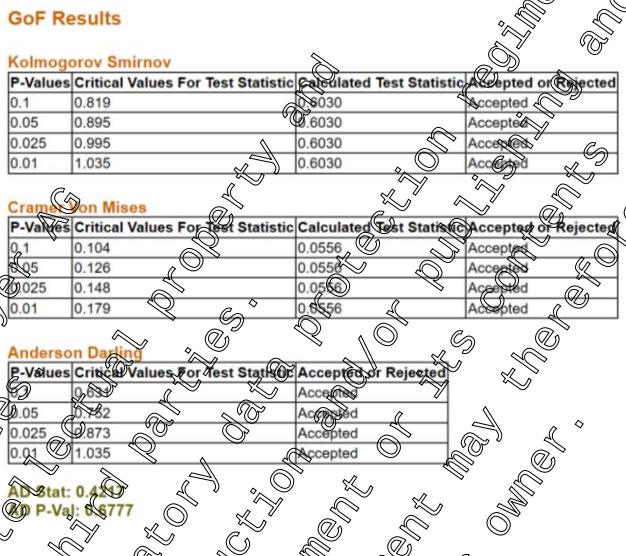
Table 10.2-5: Primary producer endpoints and applicability to SSD calculation

Reference	Species	Endpoint, E_rC_{50} (µg a.s./L)
KCA 8.2.7/03	*Ceratophyllum demersum*	10.8
KCA 8.2.7/01 KCA 8.2.7/02	*Lemna gibba*	13.6
KCA 8.2.6.1/03	*Desmodesmus subspicatus*	20.3
KCA 10.2.1/04	*Selenastrum capricornutum*	21.482
KCA 8.2.7/09 KCA 8.2.7/10	*Myriophyllum spicatum*	42.01
KCA 8.2.7/05	*Cabomba caroliniana*	> 79.5[1]
KCA 8.2.7/07	*Heteranthera zosterifolia*	> 98.5[1]
KCA 8.2.6.2/02	*Closterium cornu*	112
KCA 8.2.7/06	*Limnophila heterophylla*	122
KCA 8.2.7/08	*Egeria densa*	> 221[1]
KCA 8.2.7/04	*Elodea canadensis*	> 306[1]
KCA 8.2.6.2/02	*Xanthonema debile*	319
KCA 8.2.6.2/02	*Nannochloropsis limnetica*	513
KCA 8.2.6.2/03	*Synechococcus leopoliensis*	644
KCA 8.2.6.2/04	*Navicula pelliculosa*	672
KCA 8.2.6.2/07	*Chlorella vulgaris*	> 1583[2]
HC_5		**5.95**

[1] unbound endpoint within the range of available toxicity values, not used in SSD calculation
[2] unbound value outside of the range of available toxicity value, used in SSD calculation

Table 10.2-6: Updated SSD curve based on growth rate endpoints for all species (HC_5 = 5.95 µg/L)





In accordance with the recommendations of EFSA, 2103, the SSD-RAC for primary producers was calculated using the median HC_5 of 5.95 µg a.s./L and applying an Assessment Factor (AF) of 3. The resultant SSD-RAC was calculated to be 1.98 µg a.s./L.

Table 10.2-7: Primary producers: acceptability of risk (PEC/RAC <1) for aclonifen based on refined toxicity data for primary producers (HC_5 = 5.95 µg a.s./L) for the application of ACL + DFF SC 600 (500 + 100) G in winter cereals

Group		**Primary Producers**
Test species		**SSD**
Endpoint (µg/L)		HC_5 5.95
AF		3
RAC (µg/L)		1.98
FOCUS Scenario	**$PEC_{gl\text{-}max}$ (µg/L)**	
Step 1		
	16.7	**8.420**
Step 2		
N-Europe	6.97	**3.514**
S-Europe	5.67	**2.859**
Step 3		
D1/ditch	2.22	**1.119**
D1/stream	1.95	0.983
D2/ditch	2.23	**1.124**
D2/stream	1.98	0.998
D3/ditch	2.19	**1.104**
D4/pond	0.076	0.038
D4/stream	1.90	0.958
D5/pond	0.076	0.038
D5/stream	2.05	**1.034**
D6/ditch	2.22	**1.119**
R1/pond	0.105	0.053
R1/stream	1.45	0.726
R3/stream	2.01	**1.013**

R4/stream	1.45	0.731

AF: Assessment factor; PEC: Predicted environmental concentration; RAC: Regulatory acceptable concentration
PEC/RAC ratios above the relevant trigger of 1 are shown in **bold** indicating unacceptable risk

Following the refinement of the endpoint for primary producers, an acceptable risk was still not shown and hence mitigation methods are suggested. The RAC for invertebrates (prolonged) of 1.42 µg/L (Table 10.2-4) was lower than the refined RAC for primary producers (1.98 µg/L) and hence the mitigation required for invertebrates (prolonged) will cover the risk for the less sensitive species also.

Table 10.2-8: Aquatic organisms: acceptability of risk (PEC/RAC <1) for aclonifen based on toxicity data for invertebrate chronic (NOEC = 14.2 µg a.s./L) for the application of ACL + DFF SC 600 (500 + 100) G in winter cereals considering mitigation methods

Intended use		**Wheat**						**RAC (µg/L)**					
Active substance		**Aclonifen**						**1.42**					
Application rate (g/ha)		**1 x 350 g/ha**						**PEC/RAC ratio**					
Nozzle reduction	**No-spray buffer (m)**	**0**	**5**	**10**	**20**	**10**	**20**	**0**	**5**	**10**	**20**	**10**	**20**
	Vegetated filter strip (m)	**-**	**-**	**-**	**-**	**10**	**20**	**-**	**-**	**-**	**-**	**10**	**20**
None	D1/ditch	2.2200	0.6020	0.3190	0.1660	0.3190	0.1660	**1.56**	0.42	0.22	0.12	0.22	0.12
50%		1.1100	0.3010	0.1590	0.0830	0.1590	0.0830	0.78	0.21	0.11	0.06	0.11	0.06
75%		0.5550	0.1500	0.0800	0.0410	0.0800	0.0410	0.39	0.11	0.06	0.03	0.06	0.03
90%		0.2220	0.0600	0.0320	0.0170	0.0320	0.0170	0.16	0.04	0.02	0.01	0.02	0.01
None	D1/stream	1.9500	0.7100	0.3760	0.1950	0.3760	0.1950	**1.37**	0.50	0.26	0.14	0.26	0.14
50%		0.9720	0.3550	0.1880	0.0980	0.1880	0.0980	0.68	0.25	0.13	0.07	0.13	0.07
75%		0.4850	0.1770	0.0940	0.0490	0.0940	0.0490	0.34	0.12	0.07	0.03	0.07	0.03
90%		0.1940	0.0710	0.0380	0.0200	0.0380	0.0200	0.14	0.05	0.03	0.01	0.03	0.01
None	D2/ditch	2.2300	0.6030	0.3190	0.1660	0.3190	0.1660	**1.57**	0.42	0.22	0.12	0.22	0.12
50%		1.1100	0.3010	0.1600	0.0830	0.1600	0.0830	0.78	0.21	0.11	0.06	0.11	0.06
75%		0.5560	0.1500	0.0800	0.0410	0.0800	0.0410	0.39	0.11	0.06	0.03	0.06	0.03
90%		0.2220	0.0600	0.0320	0.0170	0.0320	0.0170	0.16	0.04	0.02	0.01	0.02	0.01
None	D2/stream	1.9800	0.7230	0.3830	0.1990	0.3830	0.1990	**1.39**	0.51	0.27	0.14	0.27	0.14
50%		0.9900	0.3610	0.1910	0.0990	0.1910	0.0990	0.70	0.25	0.13	0.07	0.13	0.07
75%		0.4940	0.1800	0.0960	0.0500	0.0960	0.0500	0.35	0.13	0.07	0.04	0.07	0.04
90%		0.1980	0.0720	0.0380	0.0200	0.0380	0.0200	0.14	0.05	0.03	0.01	0.03	0.01
None	D3/ditch	2.1900	0.5940	0.3150	0.1630	0.3150	0.1630	**1.54**	0.42	0.22	0.11	0.22	0.11
50%		1.1000	0.2970	0.1570	0.0820	0.1570	0.0820	0.77	0.21	0.11	0.06	0.11	0.06
75%		0.5470	0.1480	0.0790	0.0410	0.0790	0.0410	0.39	0.10	0.06	0.03	0.06	0.03
90%		0.2190	0.0590	0.0310	0.0160	0.0310	0.0160	0.15	0.04	0.02	0.01	0.02	0.01
None	D4/pond	0.0760	0.0650	0.0470	0.0310	0.0470	0.0310	0.05	0.05	0.03	0.02	0.03	0.02
50%		0.0380	0.0330	0.0230	0.0160	0.0230	0.0160	0.03	0.02	0.02	0.01	0.02	0.01
75%		0.0190	0.0160	0.0120	0.0080	0.0120	0.0080	0.01	0.01	0.01	0.01	0.01	0.01
90%		0.0080	0.0070	0.0050	0.0040	0.0050	0.0040	0.01	0.00	0.00	0.00	0.00	0.00
None	D4/stream	1.9800	0.6940	0.3680	0.1910	0.3680	0.1910	**1.34**	0.49	0.26	0.13	0.26	0.13
50%		0.9500	0.3460	0.1840	0.0950	0.1840	0.0950	0.67	0.24	0.13	0.07	0.13	0.07
75%		0.4740	0.1730	0.0920	0.0480	0.0920	0.0480	0.33	0.12	0.06	0.03	0.06	0.03
90%		0.1900	0.0690	0.0370	0.0320	0.0370	0.0320	0.13	0.05	0.03	0.02	0.03	0.02
None	D5/pond	0.0760	0.0650	0.0470	0.0310	0.0470	0.0310	0.05	0.05	0.03	0.02	0.03	0.02

50%		0.0380	0.0330	0.0240	0.0160	0.0240	0.0160	0.03	0.02	0.02	0.01	0.02	0.01
75%		0.0190	0.0160	0.0120	0.0080	0.0120	0.0080	0.01	0.01	0.01	0.01	0.01	0.01
90%		0.0080	0.0070	0.0050	0.0030	0.0050	0.0030	0.01	0.00	0.00	0.00	0.00	0.00
None	D5/stream	2.0500	0.7480	0.3970	0.2060	0.3970	0.2060	**1.44**	0.53	0.28	0.15	0.28	0.15
50%		1.0200	0.3740	0.1980	0.1030	0.1980	0.1030	0.72	0.26	0.14	0.07	0.14	0.07
75%		0.5120	0.1870	0.0990	0.0510	0.0990	0.0510	0.36	0.13	0.07	0.04	0.07	0.04
90%		0.2050	0.0750	0.0400	0.0210	0.0400	0.0210	0.14	0.05	0.03	0.01	0.03	0.01
None	D6/ditch	2.2200	0.6000	0.3180	0.1650	0.3180	0.1650	**1.56**	0.42	0.22	0.12	0.22	0.12
50%		1.1100	0.3000	0.1590	0.0830	0.1590	0.0830	0.78	0.21	0.11	0.06	0.11	0.06
75%		0.5530	0.1500	0.0790	0.0620	0.0790	0.0620	0.39	0.11	0.06	0.04	0.06	0.04
90%		0.2210	0.0620	0.0620	0.0620	0.0620	0.0620	0.16	0.04	0.04	0.04	0.04	0.04
None	R1/pond	0.1050	0.1010	0.0950	0.0900	0.0480	0.0320	0.07	0.07	0.07	0.06	0.03	0.02
50%		0.0920	0.0910	0.0880	0.0850	0.0400	0.0210	0.06	0.06	0.06	0.06	0.03	0.01
75%		0.0860	0.0850	0.0840	0.0830	0.0360	0.0190	0.06	0.06	0.06	0.06	0.03	0.01
90%		0.0830	0.0820	0.0820	0.0810	0.0340	0.0170	0.06	0.06	0.06	0.06	0.02	0.01
None	R1/stream	1.4400	0.5270	0.4770	0.4770	0.2790	0.1450	**1.01**	0.37	0.34	0.34	0.20	0.10
50%		0.7220	0.4770	0.4770	0.4770	0.2140	0.1110	0.51	0.34	0.34	0.34	0.15	0.08
75%		0.4770	0.4770	0.4770	0.4770	0.2140	0.1110	0.34	0.34	0.34	0.34	0.15	0.08
90%		0.4770	0.4770	0.4770	0.4770	0.2140	0.1110	0.34	0.34	0.34	0.34	0.15	0.08
None	R3/stream	2.0100	0.7320	0.5130	0.5130	0.3880	0.2010	**1.42**	0.52	0.36	0.36	0.27	0.14
50%		1.0000	0.5130	0.5130	0.5130	0.2340	0.1230	0.70	0.36	0.36	0.36	0.16	0.09
75%		0.5130	0.5130	0.5130	0.5130	0.2340	0.1230	0.36	0.36	0.36	0.36	0.16	0.09
90%		0.5130	0.5130	0.5130	0.5130	0.2340	0.1230	0.36	0.36	0.36	0.36	0.16	0.09
None	R4/stream	1.4500	0.7010	0.7010	0.7010	0.3160	0.1650	**1.02**	0.49	0.49	0.49	0.22	0.12
50%		0.7260	0.7010	0.7010	0.7010	0.3160	0.1650	0.51	0.49	0.49	0.49	0.22	0.12
75%		0.7010	0.7010	0.7010	0.7010	0.3160	0.1650	0.49	0.49	0.49	0.49	0.22	0.12
90%		0.7010	0.7010	0.7010	0.7010	0.3160	0.1650	0.49	0.49	0.49	0.49	0.22	0.12

PEC: Predicted environmental concentration; RAC: Regulatory acceptable concentration; PEC/RAC ratios above the relevant trigger of 1 are shown in **bold**

Based on the NOEC of 14.2 mg/L for invertebrates (prolonged), a 5-metre no-spray buffer with no nozzle reduction or alternatively 50% drift reduction without requirement for a no-spray buffer would be sufficient to mitigate the risk for the intended use in winter cereals.

Summary of risk assessment for the formulated product

An assessment of the risk of the formulated product to aquatic organisms was undertaken using PEC_{sw} values calculated assuming spray drift only. The initial assessment indicated no unacceptable risks to fish or invertebrates, however PEC/RAC ratios in excess of 1 were calculated for algae and aquatic macrophytes. Following suitable mitigation measures it was shown that the risk to algae and aquatic macrophytes could be reduced to acceptable levels.

As the formulation is a dual active plant protection product, Regulation (EC) No 1107/2009 requires that 'interaction between the a.s., safeners, synergists and co-formulants shall be taken into account' in the evaluation and authorisation. As a first step, the toxicity of the formulation on aquatic organisms is compared to the expected toxicity if additivity is assumed using the MDR approach as defined in EFSA aquatic guidance document (2013).

The toxicity of the formulation was similar to the expected toxicity based on additivity as the MDR for fish, algae, aquatic plants and invertebrates was between 0.2 and 5.

When a product contains more than one active substance, an additional assessment on combined toxicity risk has to be presented. A quantitative toxicity risk assessment according to concentration addition is not needed if the risk assessment for all active substances in the product passes with a high margin of safety or if one active substance clearly drives the risk assessment. These conditions are assessed following a step-wise approach.

An acceptable risk for all aquatic organisms (fish, invertebrates, algae, macrophytes and sediment dwellers) was shown, therefore, no further combined toxicity assessment is required.

Risk assessment for the formulated product

The predominant route of exposure for aquatic organisms to the formulated product will be via spray drift into adjacent water bodies. Exposure of aquatic organisms from this route was estimated by calculating Predicted Environmental Concentrations in surface water (PEC_{sw}) using the proposed application rate and spray drift values published by [redacted] (2001)[9] for a single application to field crops.

To achieve a concise risk assessment, the risk envelope approach is applied. Here all following assessments have been made for the use of ACL + DFF SC 600 (500 + 100) G in winter cereals using an application rate of 0.7 L/ha as this will also cover the risks from the use at lower application rates.

Regulatory Acceptable Concentration (RAC_{sw}) values based on the toxicity endpoints from testing of the formulated product (see Table 10.2-1) were compared to the maximal PEC_{sw} value derived from spray drift for ACL + DFF SC 600 (500 + 100) G.

Table 10.2-9: Aquatic organisms: acceptability of risk (PEC/RAC <1) for each organism group for the application of ACL + DFF SC 600 (500 + 100) G in winter cereals

Group		Fish acute	Invert. acute	Algae	Aquatic macrophyte
Test species		***Oncorhynchus mykiss***	***Daphnia magna***	***P. subspicatus***	***Lemna gibba***
Endpoint		LC_{50}	EC_{50}	E_rC_{50}	E_rC_{50}
(µg/L)		1390	2470	3.64	24.6
AF		100	100	10	10
RAC (µg/L)		13.9	24.7	0.364	2.46
Drift (%)	**PEC_{sw} (µg/L)**				
2.77	7.95	0.57	0.32	**21.84**	**3.23**

AF: Assessment factor; PEC: Predicted environmental concentration; RAC: Regulatory acceptable concentration
PEC/RAC ratios above the relevant trigger of 1 are shown in **bold** indicating unacceptable risk

[9] [redacted] (2001): New basic drift values in the authorization procedure for plant protection products. In: [redacted] (eds), Workshop on Risk Assessment and Risk Mitigation Measures in the Context of the Authorization of Plant Protection Products (WORMM). Mitt Biol Bundesanst Land-Forstwirtsch Berlin-Dahlem, 383, 133-141

Based on the maximal PEC_{sw} value derived from spray drift for ACL + DFF SC 600 (500 + 100) G, the above calculations show PEC:RAC ratios in excess of 1 for algae and aquatic macrophytes. For these organisms a refined risk assessment is presented below.

Refined risk assessment

In order to refine the risk assessment, mitigation measures such as no-spray buffers and nozzle drift reduction are suggested. The RAC for algae of 0.364 µg/L (Table 10.2-9) was significantly lower than the RAC for aquatic macrophytes (2.46 µg/L) and hence the mitigation required for algae will cover the risk for the less sensitive species also.

Table 10.2-10: Aquatic organisms: acceptability of risk (PEC/RAC <1) based on toxicity data for algae (RAC = 0.364 µg/L) for the application of ACL + DFF SC 600 (500 + 100) G in winter cereals considering mitigation methods

Predicted Environmental Concentrations (PEC_{sw}) (µg/L)					
Nozzle reduction	**No-spray buffer (m)**				
	0	**5**	**10**	**15**	**20**
None	7.95	1.64	0.83	0.57	0.43
50%	3.98	0.82	0.42	0.29	0.22
75%	1.99	0.41	0.21	0.14	0.11
90%	0.80	0.16	0.08	0.06	0.04
PEC/RAC ratios					
Nozzle reduction	**No-spray buffer (m)**				
	0	**5**	**10**	**15**	**20**
None	**21.84**	**4.51**	**2.28**	**1.57**	**1.18**
50%	**10.92**	**2.25**	**1.14**	0.78	0.59
75%	**5.46**	**1.13**	0.57	0.39	0.30
90%	**2.18**	0.45	0.23	0.16	0.12

PEC/RAC ratios above the relevant trigger of 1 are shown in **bold** indicating unacceptable risk

Based on the RAC of 0.364 µg/L for algae, the following methods would be sufficient to mitigate the risk for the intended use in winter cereals:

a) 5-metre no-spray buffer with 90% nozzle reduction
b) 10-metre no-spray buffer with 75% nozzle reduction
c) 15-metre no-spray buffer with 50% nozzle reduction

Combinations of active substances in formulations

An assessment of combined toxicity of the active substances present in ACL + DFF SC 600 (500 + 100) G has been made according to current EFSA Guidance. In post-AIR zonal and country specific assessments, the combined toxicity of all relevant products will be performed according to zonal or country guidance.

Regulation (EC) No 1107/2009 requires that 'interaction between the a.s., safeners, synergists and co-formulants shall be taken into account' in the evaluation and authorisation.

As a first step, the toxicity of the formulation on aquatic organisms (fish, aquatic invertebrates, algae and macrophytes) is compared to the expected toxicity if additivity is assumed. This is performed using the MDR (model deviation ratio) approach as defined in the EFSA Aquatic Guidance document.

The observed and calculated mixture toxicities are considered in agreement if the MDR is between 0.2 and 5. If the MDR is >5 synergistic (more than additive) mixture toxicity is indicated. Antagonistic (less than additive) mixture toxicity is indicated if the MDR is <0.2.

Table 10.2-11: Calculation of the acute mixed toxicity of ACL+DFF SC 600 to the most sensitive organisms according to Finney additivity assumption

Content within product (%)	Aclonifen: 41.1%				
	Diflufenican: 8.21%				
Species	**Endpoint (mg/L)**		**Formulation**		**MDR**
	Aclonifen	**Diflufenican**	**Expected**	**Measured**	
Green algae	0.0069	0.00045	0.0041	0.0036	1.14
Aquatic plants	0.0136	0.039	0.0309	0.0246	1.26
Aquatic invertebrates	1.2	0.24	1.4607	2.47	0.59
Fish	0.67	0.0985	0.6961	2.3	0.30

The MDR are 0.30 for fish, 0.59 for aquatic invertebrates and are 1.14 and 1.12 for algae and macrophytes, respectively. According to the Aquatic Guidance document, the toxicity of the formulation is similar to the expected one based on additivity when the MDR is between 0.2 and 5.

Assessment of combined toxicity

When a product contains more than one active substance, an additional assessment on combined toxicity risk has to be presented. It is considered that a quantitative toxicity risk assessment according to concentration addition is not needed if one of the following points applies:

- The risk assessment for all active substances in the product passes with a high margin of safety
- One active substance clearly drives the risk assessment

These conditions are assessed following a step-wise approach. A detailed description of this approach is presented in a separate document ([redacted], 2016, M-571377-02-1). The assessment is based on RQ values (risk quotient RQ = PEC/RAC). Note that RQ values which actually pass the risk assessment are used and if different mitigation measures result in an acceptable risk, the highest RQ value per individual substance is used). This safely covers all other scenarios.

1st step: Margin of safety

Condition: all RQ values are <1/n

Where:

n = number active substances in the mixture

2nd step: Risk per fraction

Condition: One a.s. contributes to ≥90% of the predicted combined toxicity of the product.

Assessment: The contribution of each individual a.s. to the combined toxicity (risk per fraction, rpf) is estimated based on the following equation:

$$rpf_{a.s.1} = RQ_{a..s.1} /(RQ_{a.s.1} + RQ_{a.s.2} ... + RQ_{a..s.i})$$

The estimation is based on RQ values from the same FOCUS Step to assure comparability.

3rd step: RQ_{MIX} calculation

Condition: The combined toxicity is acceptable if $RQ_{MIX} \leq 1$.

Assessment: The combined toxicity risk (RQ_{MIX}) with concentration-addition for aquatic organisms is estimated based on the following equation:

$$RQ_{mix} = \sum_{i=1}^{n} \frac{PEC_i}{RAC_i}$$

RQ values (PEC/RAC) for aclonifen used in the combined toxicity assessment are shown in Table 10.2-4.

RQ values (PEC/RAC) for diflufenican used in the combined toxicity assessment are calculated based on studies conducted on diflufenican. Full details of these studies are provided in the relevant EU DAR and are outlined in the EFSA Scientific Report 149 (2008), 1-80. PECsw-max values FOCUS Step 2 and 3 PEC values used in this combined risk assessment can be found in Document M-CP9 and KCP 10.1 (M-604961-01-1) of this dossier..

Table 10.2-12: RQ ($PEEC_{sw}$:RAC_{sw}) ratios for diflufenican for the application of ACL + DFF SC 600 (500 + 100) G in winter wheat

Group		Fish acute	Fish prolonged	Invert. acute	Invert prolonged	Sediment dweller prolonged	Algae	Aquatic macrophyte
Test species		*Cyprinus carpio*	*Pimephales promelas*	*Daphnia magna*	*Daphnia magna*	*Chironomus riparius*	*D. subspicatus*	*Lemna gibba*
Endpoint		LC_{50}	NOEC	EC_{50}	NOEC	NOEC	E_rC_{50}	E_rC_{50}
(µg/L)		98.3	15	240	50	100	0.45	39
AF		100	10	100	10	10	10	10
RAC (µg/L)		0.985	1.5	2.4	5.2	10	0.045	3.9
FOCUS Scenario	**PEC gl-max (µg/L)**							
Step 2								
N-Europe	2.2201	2.254	1.1480	0.925	0.427	0.222	49.336	0.569
Step 3								
D2/ditch	0.4928	0.500	0.329	0.205	0.095	0.049	10.951	0.126

AF: Assessment factor

The following assessment of combined toxicity is based on FOCUS Step 2/3 PEC_{sw} values considering pre and postemergence.

Table 10.2-13: Combined toxicity assessment – aquatic organisms

Intended use:	Winter cereals, BBCH 00 - 13

Active substances:	Aclonifen (ACL) + Diflufenican (DFF)						
Application rate (g/ha):	0.35						
	Fish acute	**Fish prolonged**	**Invert. acute**	**Invert. prolonged**	**Sediment dweller prolonged**	**Algae**	**Aquatic macrophytes**
RQ (risk quotient) value = PEC/RAC[a]							
ACL	1.040 (0.333)	1.640 (0.525)	0.581 (0.186)	4.908	0.148	3.433	5.125
DFF	2.254 (0.500)	1.148 (0.329)	0.925 (0.205)	0.427	0.222	49.336	0.569
Trigger	1	1	1	1	1	1	1
1/n	0.5	0.5	0.5	0.5	0.5	0.5	0.5
1st step (all RQ <1/n)	No	No	No	No	Yes	No	No
2nd step (Rpfmax)	0.684 (DFF)	0.588 (ACL)	0.614 (DFF)	0.920 (ACL)	Not required	0.935 (DFF)	0.900 (ACL)
3rd step (RQ_{MIX})[b]	0.833	0.854	0.391	Not required	Not required	Not required	Not required

FOCUS Step 2 (NEU) RQ values quoted
RQ values in parenthesis = worst case FOCUS Step 3 values (Table 10.2-4 and Table 10.2-12)
a Differing RQ values used for rpf calculations to fulfil the criterion of identical exposure levels
b 3rd step (RQ_{MIX}) calculated using FOCUS Step 3 values

An acceptable risk for sediment dwellers is shown at 1st step (all RQ values < trigger × n). After 2nd step (one a.s. contributes to ≥90% of the predicted combined toxicity of the product) acceptable risks were shown for chronic invertebrates, green algae and aquatic macrophytes. Following the 3rd step (combined toxicity risk (RQ_{MIX}) with concentration-addition) an acceptable risk was shown for acute and chronic fish and acute invertebrates. Therefore, no further combined toxicity assessment is required.

CP 10.2.1 Acute toxicity to fish, aquatic invertebrates, or effects on aquatic algae and macrophytes

Studies on the toxicity of the formulation ACL + DFF SC 600 (500 + 100) G to fish, aquatic invertebrates and algae have been conducted and presented below.

Data Point:	KCP 10.2.1/01
Report Author:	[redacted]; [redacted]
Report Year:	2016
Report Title:	Acute toxicity of aclonifen + diflufenican SC 600 to rainbow trout (Oncorhynchus mykiss) under static conditions - Final report -
Report No:	EBDCN140
Document No:	M-568874-01-1
Guideline(s) followed in study:	EU Directive 91/414/EEC Regulation (EC) No. 1107/2009 US EPA OCSPP 850.1075
Deviations from current test guideline:	Current guideline: OECD 203, 2019 No Deviation
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The acute toxicity of aclonifen + diflufenican SC 600 to rainbow trout, *Oncorhynchus mykiss*, was determined in a 96-hour static exposure. Test solutions were prepared by direct addition of test substance to dilution water. Ten rainbow trout per test group were exposed to an untreated control and nominal formulation concentrations of 0.25, 0.50, 1.0, 2.0 and 4.0 mg/L. The total test period was 96 hours. Samples for analytical confirmation of actual exposure concentrations were taken at the start and after 96 hours of exposure.

Dissolved oxygen, pH, and temperature were measured in the controls and each test concentration at the beginning of the test and end of the test. Daily observations were made of mortality and treatment related effects.

Measured concentrations of test exposure solutions at the start of the test range from 81 to 93% of nominal. At 96 hours, concentrations ranged from 36 to 49% of nominal. Overall, geometric mean measured concentrations ranged from 55 to 67% of nominal. As a consequence, analytical results were calculated on the basis of geometric mean measured concentrations.

Based on geometric mean concentrations of aclonifen the 96-hour LC_{50} of Aclonifen + diflufenican SC 600 to rainbow trout, *Oncorhynchus mykiss*, was estimated to be 1.39 mg formulation/L (confidence limits 1.11 – 1.75 mg/L). The NOEC, based on mortality, was 0.287 mg formulation/L.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test Item:** aclonifen + diflufenican SC 600
   Batch no.: 2015-010653
   Active Ingredient / Purity: Aclonifen: 41.1% w/w (505 g/L)
   Diflufenican: 8.21% w/w (101.0 g/L)

Appearance:	Yellow suspension
Storage:	Ambient
Expiry date:	12 January 2017

2.	**Test Organism:**	Rainbow trout, *Oncorhynchus mykiss*
	Mean length:	57.0 mm (± 3.3 mm)
	Mean wet weight:	1.55g (± 0.19g)
	Source:	[redacted]
		Fish were acclimated for at least 14 days
		Mortalities in 7 days prior to test start were 0%
	Feeding:	Commercial trout pellets and live brine shrimp daily. Discontinued 48 hours prior to study start
3.	**Test water:**	Dilution water (soft processed water) consists of dechlorinated municipal water blended with reverse osmosis water designed to produce soft (40 to 60 mg/L as $CaCO_3$) water. Dilution water is stored in polypropylene or PVC holding tanks and intensely aerated before use
	Total hardness:	234- 250 mg/L as $CaCO_3$

B. STUDY DESIGN AND METHODS

1. In-life phase: 5 – 9 May 2016

2. Exposure conditions

Test vessels:	38 litre capacity glass aquaria, containing 30 L test solution.
Experimental design:	Five test concentrations (0.25, 0.50, 1.0, 2.0 and 4.0 mg formulation/L) plus one control 10 fish per single replicate per test concentration
Loading:	0.52 g/L
Temperature:	13.5 – 13.7°C
pH:	7.9 – 8.2
Dissolved oxygen:	>6.9 mg O_2/L
Aeration:	Not specified
Photoperiod:	16 h light: 8 h dark

3. Administration of the test item

The highest nominal test exposure concentration (4.0 mg formulation/L) was prepared by addition of test material to dilution water, followed by 1 minute of vigorous stirring. Lower test concentrations were prepared by dilution of the 4.0 mg/L test solution. No precipitation was observed.

Each exposure concentration and the control comprised 1 replicate each containing 10 fish.

4. Measurements and observations

Observations for mortality were made after 4 hours and daily thereafter (24, 48, 72 and 96 hours). Mortality was defined as absence of respiratory movement and absence of response to physical stimulation.

Temperature, pH and dissolved oxygen were measured at the start of the test and daily thereafter. Total hardness was determined in the control and selected concentrations at the start and end of the test.

Samples were taken from the aqueous stock solutions and from each test vessel prior to the addition of fish, after 96 hours. Analysis was performed by LC-MS/MS with a UV detector.

5. Statistics/Data evaluation

The LC50 values were calculated using CETIS statistical software and were determined by the characteristics of the data, i.e. the number of concentrations in which survival was between 0 and 100% and the 95% confidence intervals. The NOEC and LOEC were empirically determined based upon observation data including lethal and sublethal effects.

II. RESULTS AND DISCUSSION

A: ANALYTICAL VERIFICATION

Measured concentrations of test exposure solutions at the start of the test range from 81 to 93% of nominal. At 96 hours concentrations ranged from 36 to 49% of nominal. As a consequence, analytical results were calculated on the basis of geometric mean measured concentrations.

The validated method is summarised in Document M-CP5 (CP 5.1.2/1).

The results of analysis of test solutions is summarised below.

Table: Measured concentrations of Aclonifen + diflufenican SC 600 (aclonifen)

Nominal concn (mg formulation/L)	Nominal concn (mg a.s./L)	0 hours		96 hours		Geometric mean measured concn (mg a.s./L)	% of nominal
		Measured concn (mg/L)	% of nominal	Measured concn (mg/L)	% of nominal		
Control	-	<0.5	-	<0.5	-	-	-
0.25	0.103	0.0876	85	0.037	36	0.0569	55
0.50	0.206	0.167	81	0.083	40	0.118	57
1.0	0.411	0.346	84	0.175	43	0.246	60
2.0	0.822	0.752	92	0.281	34	0.460	56
4.0	1.64	1.52	93	0.799	49	1.10	67

LoQ = Limit of quantification, 0.015 mg aclonifen/L

B: BIOLOGICAL DATA

The highest geometric mean measured concentration showing no mortality and the lowest at which there was 100% mortality was 0.118 and 1.10 mg/L, respectively. The cumulative mortality of rainbow trout after 4, 24, 48, 72 and 96 hours are presented in the following table:

Table: Cumulative mortality for rainbow trout from the exposure to Aclonifen + diflufenican SC 600

Geomtric mean measured concentration (mg a.s./L)	Geomtric mean measured concentration (mg formulation/L)	Cumulative mortality (%)				
		4h	24h	48h	72h	96h
Control	-	0	0	0	0	0
0.0569	0.138	0	0	0	0	0
0.118	0.287	0	0	0	0	0
0.246	0.599	0	0	0	0	1 (10)
0.460	1.12	0	0	1 (10)	1 (10)	2 (20)
1.10	2.68	0	2 (20)	5 (50)	10 (100)	10 (100)

Treatment related effects, other than death, were dark colouration, vertical orientation, laboured respiration and loss of equilibrium. Treatment related effects were sustained and progressive and were seen at exposure concentrations 0.118, 0.246, 0.460 and 1.10 mg/L by the end of the test. All surviving fish in these concentrations showed some symptoms. No symptoms were observed at the lowest treatment (0.0569 mg/L). Consequently, the NOEC based on treatment related effects was determined as the lowest measured concentration (0.0569 mg/L).

All chemical and physical parameters in the definitive test were within expected ranges.

Based on the observed mortality, the LC_{50} values at each observation point were determined to be:

Table: EC_{50} values from the exposure of rainbow trout *Oncorhynchus mykiss* to Aclonifen + diflufenican SC 600

Time (Hours)	Nominal (formulation)		Geometric mean measured (formulation)	
	LC_{50} (mg formulation/L)	95% confidence limits (mg/L)	LC_{50} (mg formulation/L)	95% confidence limits (mg/L)
24	>4.00[a]	-	>2.68[a]	-
48	4.00[b]	-	2.68[b]	-
72	2.64[c]	2.31 – 3.04	1.61[c]	1.39 – 1.85
96	2.30[c]	1.85 – 2.86	1.39[c]	1.11 – 1.75
NOEC (mortality)	0.50		0.287	
NOEC (treatment related effects)	0.25	-	0.138	-

a Linear interpolation
b Trimmed Spearman-Kärber
c Spearman-Kärber

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 203, 2019)	Achieved

Mortality in controls	<10%	0%
Dissolved oxygen concentration at the end of the test	>3 mg/L	>6.9 mg/L
Analytical measurement of test concentrations	Compulsory	Performed

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint	Nominal concentration (mg formulation /L)	Geometric mean measured concentration (mg formulation/L)
LC_{50} (96 hours)	2.30	1.39
95% confidence limits	1.85 – 2.86	1.11 – 1.75
NOEC (mortality)	0.50	0.287

III. CONCLUSION

Based on geometric mean concentrations of aclonifen the 96-hour LC_{50} of Aclonifen + diflufenican SC 600 to rainbow trout, *Oncorhynchus mykiss*, was estimated to be 1.39 mg formulation/L (confidence limits 1.11 – 1.75 mg/L). The NOEC, based on mortality, was 0.287 mg formulation/L.

[redacted] *2016*

Assessment and conclusion by applicant:

All validity criteria were satisfied and therefore this study can be considered to be valid.

Measured results varied by more than 20% from nominal, therefore, results were based on geometric mean measured concentrations.

The 96-hour LC_{50} of Aclonifen + diflufenican SC 600 to rainbow trout, *Oncorhynchus mykiss*, was estimated to be 1.39 mg/L (confidence limits 1.11 – 1.75 mg/L). The NOEC, based on mortality, was 0.287 mg/L.

Assessment and conclusion by RMS:

Data Point:	KCP 10.2.1/02
Report Author:	[redacted]; [redacted]
Report Year:	2016
Report Title:	Acute toxicity of aclonifen + diflufenican SC 600 to Daphnia magna under static conditions - Final report -
Report No:	EBDCN191
Document No:	M-565104-01-1
Guideline(s) followed in study:	OCSPP Guideline 850.1010, OECD Guideline 202. The afore mentioned guidelines were harmonized for various test parameters (i.e. temperature, light, etc.) to achieve optimal environmental conditions for the test organisms. Scientific discretion was implemented where guideline parameters do not fully converge
Deviations from current test guideline:	Current guideline: OECD 202, 2004 No Deviation
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The acute toxicity of aclonifen + diflufenican SC 600 to *Daphnia magna*, was determined in a 48-hour, static exposure. Test solutions were prepared by direct addition of test substance to dilution water. Twenty *Daphnia* per test group were exposed to an untreated control and nominal formulation concentrations of 0.31, 0.63, 1.25, 2.5, 5 and 10 mg/L. The total test period was 48 hours. Samples for analytical confirmation of actual exposure concentrations were taken at the start and after 48 hours of exposure.

Dissolved oxygen, pH, and temperature were measured in the controls and each test concentration at the beginning of the test and daily thereafter. Daily observations were made of immobilisation.

Analytical results indicate that intended exposure concentrations were substantially achieved (85 – 95% of nominal) and were adequately maintained during the test (80 – 95% of nominal initial measured concentration and 89 – 97% of nominal after 48 hours exposure).

The 48-hour EC_{50} of aclonifen + diflufenican SC 600 to *Daphnia magna* was determined to be 2.47 mg/L (confidence limits 2.14 – 2.73 mg formulation/L). The NOEC was 0.50 mg/L. Results were based on nominal concentrations.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test Item:**	aclonifen + diflufenican SC 600
Batch no.:	2015-010653
Active Ingredient / Purity:	Aclonifen: 41.1% w/w (505 g/L) Diflufenican: 8.21% w/w (101.0 g/L)

	Appearance:	Yellow suspension
	Storage:	Ambient
	Expiry date:	12 January 2017
2.	**Test Organism:**	*Daphnia magna*
	Age:	Juvenile *Daphnia* <24 hours old at start of the test
	Source:	[redacted]
	Feeding:	Adult cultures fed daily with green alga, *Pseudokirchneriella subcapitata*, and/or Tetrafin® flake food. No feeding during test.
3.	**Test water:**	Dilution water (hard processed water) consists of spring water blended with reverse osmosis water designed to produce hard (160 to 180 mg/L as $CaCO_3$) water. Spring water collected from a spring box, passed through a multimedia filter, a 5-micron bag filter, granular activated carbon filters, a 1-micron cartridge filter and finally an ultraviolet sterilizer. Dilution water is stored in polypropylene or PVC holding tanks and intensely aerated before use
	Total hardness:	Nominally 160 - 180 mg/L as $CaCO_3$

A. STUDY DESIGN AND METHODS

1. In-life phase: 29 to 31 March 2016

2. Exposure conditions

Test vessels:	Borosilicate glass beakers (250 mL) with 200 mL fill volume
Experimental design:	Five test concentrations (0.25, 0.50, 1.0, 2.0 and 4.0 mg formulation/L) plus one control; 4 replicates each containing 5 *Daphnia*
Loading:	*ca.* 40 mL of media per *Daphnia*
Temperature:	19.7 – 20.4°C
pH:	8.1 – 8.4
Dissolved oxygen:	8.3 – 9.4 mg O_2/L
Total hardness:	164 – 176 mg/L as $CaCO_3$
Aeration:	None
Photoperiod:	16 h light: 8 h dark

3. Administration of the test item

The highest nominal test exposure concentration (4.0 mg formulation/L) was prepared by addition of test material to dilution water. Lower test concentrations were prepared by serial dilution of the 4.0 mg/L test solution. No precipitation was observed.

Each exposure concentration and the control comprised 4 replicates each containing 5 *Daphnia*.

4. Measurements and observations

The number of immobilised daphnids was assessed after 4, 24 and 48 hours from the beginning of the test. The criterion for the effect (immobility) was the inability to swim within 15 seconds after gentle prodding with a glass rod.

Temperature, dissolved oxygen concentrations and pH values were measured in all the test groups and the control vessels at the beginning and end of the test. The total hardness and alkalinity were also measured at the start and end of the test. Measurements were from the excess solutions remaining after filling test vessels at the start of the test and from pooled replicates at the end of the test.

Samples were taken from each test concentration and control for analysis. The samples were collected at 0 hours from fresh test solutions and at the end of the test from pooled replicates of the aged test solutions.

5. Statistics/Data evaluation

The EC50 values were calculated using CETIS statistical software and were determined by the characteristics of the data, i.e. the number of concentrations in which immobilizations were between 0 and 100% and the 95% confidence intervals. The NOEC and LOEC were empirically determined based upon observation data including lethal and sublethal effects.

II. RESULTS AND DISCUSSION

A: ANALYTICAL VERIFICATION

Analytical results indicate that intended exposure concentrations were substantially achieved (85 - 95% of nom–nal) and were adequately maintained during the test (80 – 95% of nominal initial measured concentration and 89 – 97% of nominal after 48 hours exposure).

The validated method is summarised in Document M-CP5 (CP 5.1.2/2).

The results of analysis of test solutions is summarised below:

Table: Measured concentrations of aclonifen in aclonifen + diflufenican SC 600 formulation

Nominal concn (mg formulation/L)	Nominal concn (mg a.s./L)	0 hour		48 hours		Mean measured concn aclonifen (mg a.s./L)	% of nominal
		Measured concn (mg/L)	% of nominal	Measured concn (mg/L)	% of nominal		
Control		<LoQ	-	<LoQ	-	<LoQ	-
0.25	0.103	0.0829	80	0.918	89	0.874	85
5.0	0.206	0.181	88	0.194	94	0.187	91
1.0	0.411	0.384	93	0.400	97	0.392	95
2.0	0.822	0.780	95	0.781	95	0.780	95
4.0	1.64	1.52	92	1.49	91	1.50	92

LoQ = Limit of quantification, 0.015 mg aclonifen/L

B: BIOLOGICAL DATA

The number of immobilized daphnids and the percentage of immobilization at 24 and 48 hours of exposure are presented in the following table:

Table: Numbers of mobile, immobile and floating *Daphnia magna* after exposure to aclonifen + diflufenican SC 600 formulation

Nominal concn (mg/L)	4 hours		24 hours		48 hours	
	Sublethal	Immobile (%)	Sublethal	Immobile (%)	Sublethal	Immobile (%)
Control	0/20	-	0/20	0	0/20	0
0.25	0/20	-	0/20	0	0/20	0
5.0	0/20	-	0/20	0	0/20	0
1.0	0/20	-	1/20	0	20/20	0
2.0	0/20	-	19/20	0	14/14	30
4.0	0/20	-	19/19	5	0/0	100

All chemical and physical parameters in the definitive test were within expected ranges.

Based on the observed immobilisation the EC_{50} values at each observation point were determined to be:

Table: EC_{50} values from the exposure of *Daphnia magna* to aclonifen + diflufenican SC 600 formulation

Time (Hours)	Nominal concentration (mg formulation/L)	
	EC_{50} (mg/L)	95% confidence limits (mg/L)
48	2.47	2.14 – 2.73
NOEC (48 hours)	0.50	-

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 202, 2004)	Achieved
Mortality in controls	<10%	0%
Dissolved oxygen concentration at the end of the test	>3 mg/L	>8.3 mg/L

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint	Nominal concentration (mg formulation/L)
EC_{50} (48 hours)	2.47
95% confidence limits	2.14 – 2.73
NOEC	0.50

III. CONCLUSION

The 48-hour EC_{50} of aclonifen + diflufenican SC 600 to *Daphnia magna* was determined to be 2.47 mg/L (confidence limits 2.14 – 2.73 mg formulation/L), based on nominal test concentrations. The NOEC was 0.50 mg/L.

[redacted] *(2016)*

Assessment and conclusion by applicant:

Study is acceptable

The 48-hour EC_{50} to *Daphnia magna* was determined to be 2.47 mg/L (confidence limits 2.14 – 2.73 mg/L).

Assessment and conclusion by RMS:

Data Point:	KCP 10.2.1/05
Report Author:	[redacted]
Report Year:	2019
Report Title:	1st final report amendment - Aclonifen + diflufenican SC600 (500 + 100 g/L) - Toxicity to Pseudokirchneriella subcapitata in an algal growth inhibition test
Report No:	1449/1210
Document No:	M-666322-02-1
Guideline(s) followed in study:	OECD Guidelines for the Testing of Chemicals, Section 2, No. 201: "Freshwater Alga and Cyanobacteria, Growth Inhibition Test", adopted March 23, 2006, corrected July 28, 2011 Commission Regulation (EC) No 761/2009, Annex, Part C, C.3.: "Freshwater Algae and Cyanobacteria, Growth Inhibition Test", Official Journal of the European Union (EN), dated August 24, 2009 SANCO/3029/99 rev.4 11/07/00: Residues: Guidance for generating and reporting methods of analysis in support of pre-registration data requirements for Annex II (part A; Section 4) and Annex III (part A; Section 5) of directive 91/414
Deviations from current test guideline:	Current guideline: OECD 201, 2011. No Deviation
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The effects of aclonifen + diflufenican SC 600 (500 + 100 g/L) on the unicellular green alga, *Pseudokirchneriella subcapitata* (currently known as *Raphidocelis subcapitata*), was determined in a 72-hour exposure. Algae were exposed to an untreated control, and nominal aclonifen + diflufenican SC 600 concentrations of 0.375, 0.75, 1.5, 3.0 and 6.0 µg test item/L. The total test period was 72 hours.

Samples for analytical confirmation of actual exposure concentrations of aclonifen and diflufenican were taken at the start and after 72 hours of exposure. Temperature and pH were measured in the control and each test concentration at the beginning and end of the test. Algal cell density was determined daily by spectrophotometric measurement.

In the freshly prepared test media at the start of the test the average recoveries of the active ingredient aclonifen ranged between 91 – 115% of nominal. In the aged test media after 24, 48 and 72 hours test duration the average recoveries for the concentrations of 0.375 to 6.0 µg test item/L ranged between 85 – 102% of nominal.

In the freshly prepared test media at the start of the test the average recoveries of the active ingredient diflufenican ranged between 64 – 114% of nominal. The lower initial mean recovery of 64 and 68% at the nominal test concentrations of 6.0 and 1.5 µg test item/L were considered to be due to sampling errors since correct dosing was confirmed by the aclonifen measurements (115 and 91% initial mean). Therefore, the diflufenican recovery <80% at day 0 at 6.0 and 1.5 µg/L are not taken into consideration. In the aged test media after 24, 48 and 72 hours test duration the average recoveries for the concentrations of 0.375 up to 6.0 µg test item/L ranged between 74 – 89% of nominal.

The 72-hour EC_{50} for growth rate (ErC_{50}), based on nominal concentrations, was 3.64 µg formulation/L (95% confidence limits 3.49 – 3.80 µg formulation/L). The 72-hour NOEC for growth rate was 0.375 µg formulation/L and the corresponding LOEC was 0.750 µg formulation/L, based on nominal test concentrations.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test Item:** Aclonifen + diflufenican SC 600
   Batch no.: 2018-007429
   Active Ingredient / Purity: Aclonifen: 39.9% w/w (492 g/L)
   Diflufenican: 8.52% w/w (105.0 g/L)
   Appearance: Yellow liquid
   Storage: At 20 ± 5 °C, in the dark.
   A storage condition from 2 - 30 °C is also acceptable
   Expiry date: 28 August 2020

2. **Test Organism:** *Pseudokirchneriella subcapitata* (currently known as *Raphidocelis subcapitata*)
   Strain: 61.81 SAG
   Source: [redacted]
   Initial density: 0.5 x 10^4 cells/mL

	Culture and test conditions:	Incubated in a water bath. Light intensity was 4870 lux (mean measured)
3.	**Treatment:**	Sterile OECD medium
4.	**Test vessels:**	50 mL Erlenmeyer flasks, containing 50 mL test solution continuously stirred with magnetic stirrers, covered with air permeable class dishes
	Test water:	1 ×Sterile AAP medium
	Shaking:	100 rpm
5.	**Environmental conditions:**	
	Temperature:	22.1 – 22.2°C
	pH	8.1 – 9.2 (control) 7.9 – 9.0 (test item treatments)
	Photoperiod	Continuous illumination, 4450 – 5350 lux (mean 4870 lux)

B. STUDY DESIGN AND METHODS

1. In-life phase: 7 to 10 May 2019

2. Exposure conditions

The test started (0 hours) by addition of 0.5×10^4 cells/mL to each test vessel. Algae were from laboratory cultures. The test was performed with six replicates per control and three replicates per test item concentration. Additionally, one replicate of each test concentration and control was prepared without algae to provide a 'blank' for spectrophotometric measurements.

3. Administration of the test item

A stock solution of 10 mg test item/L was prepared by dissolving 10.1 mg test item into 1010 mL test water with intense stirring for 30 minutes. Volumes of this stock solution were used to prepare the highest test item concentration. Volumes of the highest test concentration were diluted with test water to prepare the lower test concentrations. The test media were prepared just before introduction of the algae (start of the test). Test vessels were randomly allocated in the test water bath

4. Measurements and observations

The cell density in each replicate was determined daily (days 1, 2 and 3) during the test period by spectrophotometric measurement. The algal cell densities were calculated by subtracting the absorption of the blanks, from each of the measured absorption of the test media (with algae). To check for any effect of the test item on the morphology of the algal cells, at least one sample from all test item concentration was taken after 72 hours of exposure. The shape of the treated algal cells compared to the control was examined microscopically.

The temperature was measured daily in a flask filled with water and incubated under the same conditions as the test flasks. The pH was measured in all test treatments and the control on day 0 and 3 (start and end of test).

Duplicate samples from the freshly prepared test media of all test concentrations and the control were taken at the start of the test. For the determination of the stability of the test item under the test conditions

and of the maintenance of the test item concentrations during the test period, duplicate samples from the test media of all test concentrations and the control were collected at the end of the test (after 72 hours) by pooling replicates of each treatment. Additionally, samples were taken after 24 and 48 hours by taking aliquots of each replicate which were pooled per treatment group.

All samples were stored in a freezer (≤ -20 °C), protected from light, until analysis was performed.

The concentrations of the active ingredients aclonifen and diflufenican of the test item (aclonifen + diflufenican SC 600) were analysed in the duplicate test media samples from all test concentrations, in the duplicate control samples and in the additional control samples, from all sampling times (0, 24, 48 and 72 hours). Samples were analysed by LC-MS/MS.

5. Statistics/Data evaluation

Based on the calculated cell densities, the 72-h E_rC_{50} and EC_{50}, the corresponding EC_{20} and EC_{10} values and, where possible, their 95%-confidence limits were calculated by Probit analysis.

For the determination of the 72-h LOEC and NOEC, the calculated growth rates and yields at each test concentration were tested for significant differences compared to the control values by Williams t-test (yield and growth rate), respectively. The software used to perform the statistical analysis was ToxRat Professional, Version 3.2.1, ToxRat Solutions GmbH.

D. RESULTS AND DISCUSSION

A: ANALYTICAL VERIFICATION

In the freshly prepared test media at the start of the test the average recoveries of the active ingredient aclonifen ranged between 91 – 115% of nominal. In the aged test media after 24, 48 and 72 hours test duration the average recoveries for the concentrations of 0.375 to 6.0 µg test item/L ranged between 85 – 102% of nominal.

In the freshly prepared test media at the start of the test the average recoveries of the active ingredient diflufenican ranged between 64 – 114% of nominal. The lower initial mean recovery of 64 and 68% at the nominal test concentrations of 6.0 and 1.5 µg test item/L were considered to be due to sampling errors since correct dosing was confirmed by the aclonifen measurements (115 and 91% initial mean). Therefore, the diflufenican recovery <80% at day 0 at 6.0 and 1.5 µg/L are not taken into consideration. In the aged test media after 24, 48 and 72 hours test duration the average recoveries for the concentrations of 0.375 up to 6.0 µg test item/L ranged between 74 – 89% of nominal.

Initial measured concentrations of diflufenican ranged from 88 to 120% and at the end of the test (72 hours) from 99 to 106% of nominal. The overall mean measured concentrations ranged from 94 to 109% of nominal. As the measured results remained within 100 ± 20% test results were calculated using nominal concentrations.

The validated method is summarised in Document M-CP5 (CP 5.1.2/11).

Table: Summary of analytical results

Nominal concn	Measured concn as % of nominal[a]	
	Aclonifen	Diflufenican

(µg test item/L)	0 h (fresh	24-72h (aged)	0 h (fresh	24-72h (aged)
Control	-	-	-	-
0.375	98	94	84	88
0.75	98	87	114	78
1.5	91	85	68[b]	74
3.0	106	102	90	89
6.0	115	94	64[b]	84

a Mean of all measured samples per treatment group
b Correct dosing is confirmed by aclonifen measurement

B: BIOLOGICAL DATA

Growth inhibition

The 72-hour E_rC_{50} value was calculated to be 3.64 µg test item/L. The 72-h E_rC_{10} value was calculated to be 1.29 µg test item/L. The 72-h NOE$_r$C was determined to be 0.375 µg test item/L and the associated 72-h LOE$_r$C was 0.75 µg test item/L.

Mean area under the curve, yield and growth rates are presented in the following table.

Table: Mean yield, cumulative biomass and growth rate after 72 hours of exposure

Nominal concentration (µg formulation/L)	Mean yield (cells/mL×10^4)	Mean area under growth curve	Mean growth rate	% inhibition (of mean growth rate)
Control	118.95	118.45	1.824	-
0.375	114.23	110.76	1.801	1.2
0.75	97.16	96.66*	1.754	3.8*
1.5	67.60	67.10*	1.635*	10.4*
3.0	11.15	10.65*	1.026	43.7*
6.0	2.387	1.887*	0.520	71.5*

Statistically significant from control (Williams t-tTest, α = 0.05, one-sided)
Negative values in '% inhibition' indicate an increase in growth relative to the control

Microscopic examination of the algal cells after 72 hours did not show any difference between the algae from any test treatment (up to nominal test concentration of 6 µg test item/L) and the control.

All chemical and physical parameters in the definitive test were within expected ranges.

Based on the calculated growth rate, the ErC_{10}, ErC_{20} and ErC_{50} values at 72 hours were determined to be:

Table: EC_{50} values from the exposure of green alga *Pseudokirchneriella subcapitata* (currently known as *Raphidocelis subcapitata)* to aclonifen + diflufenican SC 600

Endpoint (96 hours)	Based on nominal test concn (µg formulation/L)	95% confidence limits (µg formulation/L)
ErC_{10}	1.29	1.17 – 1.40
ErC_{20}	1.84	1.71 – 1.96
ErC_{50}	3.64	3.49 – 3.80
NOEC	0.375	-
LOEC	0.750	-

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 202, 2004)	Achieved
Biomass in control should increase exponentially by factor of ≥16 within 72-h test period.	≥16	238
Mean coefficient of variation for section by section specific growth rates (days 0-1, 1-2 & 2-3) for 72-h test) in controls must be ≤35%	≤35%	17.4%
Coefficient of variation of average specific growth rates during whole test period in replicate controls must be ≤7%	≤7%	1.0%

Study was valid according to the validity criteria set out in the OECD 201 guideline (2011) and is therefore considered an acceptable study.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint	Nominal concentration (µg formulation/L)	95% confidence limit
E_rC_{10}	1.29	1.17 – 1.40
E_rC_{20}	1.84	1.71 – 1.96
E_rC_{50}	3.64	3.49 – 3.80
NOEC	0.375	
LOEC	0.750	

III. CONCLUSION

The 72-hour EC_{50} for growth rate (E_rC_{50}), based on nominal concentrations, was 3.64 µg formulation/L (95% confidence limits 3.49 – 3.80 µg formulation/L). The 72-hour NOEC for growth rate was 0.375 µg formulation/L and the corresponding LOEC was 0.750 µg formulation/L, based on nominal test concentrations.

[redacted] *(2019)*

Assessment and conclusion by applicant:

All validity criteria were satisfied and therefore this study can be considered to be valid.

The 72-hour ErC50 of aclonifen + diflufenican SC 600 (aclonifen 600 g/L) to green alga, *Pseudokirchneriella subcapitata* (currently known as *Raphidocelis subcapitata*), was calculated to be 3.64 µg formulation/L (95% confidence limits 3.49 and 3.80 µg formulation/L), based on nominal concentrations. The NOEC was 0.375 µg formulation/L, based nominal concentrations.

Assessment and conclusion by RMS:

Assessment and conclusion by RMS:

Data Point:	KCP 10.2.1/04
Report Author:	[redacted]; [redacted]
Report Year:	2019
Report Title:	Aclonifen + diflufenican SC 600 (500 + 100 G/L) - Toxicity to the aquatic plant Lemna gibba in a semi-static growth inhibition test
Report No:	144971240
Document No:	M-666321-01-1
Guideline(s) followed in study:	Commission Regulation (EC) No 761/2009, Annex, Part C, C.26 "Lemna sp. Growth Inhibition Test", Official Journal of the European Union (EN), dated August 24, 2009 OECD Guidelines for the Testing of Chemicals, No. 221: "Lemna sp. Growth Inhibition Test", adopted March 23, 2006 SANCO/3029/99 rev.4 11/07/00: Residues: Guidance for generating and reporting methods of analysis in support of pre-registration data requirements for Annex II (part A; Section 4) and Annex III (part A; Section 5) of directive 91/414
Deviations from current test guideline:	Current guideline: OECD 221, 2006 No Deviation
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary:

The effects of aclonifen + diflufenican SC 600 (500 + 100 g/L on the growth and reproduction of the aquatic monocotyledonous plant, *Lemna gibba,* were investigated in an exposure to nominal concentrations of 3.13, 6.25, 12.5, 25 and 50 µg formulation/L in a semi-static test. The inhibition of growth in relation to control cultures was determined over a test period of 7 days

At experimental start each test vessel was inoculated with 12 fronds. There were three replicate test vessels for each test level including the controls. Growth was determined by frond counts on Days 0, 3, 5 and 7 and frond dry weights from day 0 and day 7. The effect was expressed in terms of percent inhibition in frond number and biomass relative to the blank control on day seven of the study.

The quantification of the two active ingredients of the test item aclonifen + diflufenican SC600 (500 + 100 g/L) in the test samples was performed using liquid chromatography with MS/MS detection.

Aclonifen: In the freshly prepared test media at the start of the test the average recoveries of the active ingredient aclonifen ranged between 93 – 113% of nominal. In the aged test media after 3, 5 and 7 days

test duration the average recoveries of the active ingredient aclonifen ranged between 91 – 109% of nominal.

Diflufenican: In the freshly prepared test media at the start of the test the average recoveries of the active ingredient diflufenican ranged between 91 – 108% of nominal. In the aged test media after 3, 5 and 7 days test duration the average recoveries of the active ingredient diflufenican ranged between 89 – 109% of nominal.

The 7-day E_rC_{50} was calculated to be 27.6 and 24.6 µg test item/L for frond number and dry weight, respectively. The 7-day NOE_rC and the LOE_rC were determined to be 3.13 and 6.25 µg test item/L for frond number and dry weight, respectively.

I. MATERIALS AND METHODS

A. MATERIALS

1.	**Test material:**	Aclonifen + diflufenican SC 600 (500 + 100 g/L)
	Batch no.:	2018-0 7429
	Active ingredient/Purity:	Aclonifen: 39.9% w/w (492.0 g/L) Diflufenican: 8.52% w/w (105.0 g/L)
	Appearance	Yellow liquid
	Storage	At 20 ± 5 °C, in the dark (a storage condition from 2 – 30 °C is also acceptable)
	Expiry:	28 August 2020
2.	**Test organism:**	*Lemna gibba*
	Strain:	G3
	Source:	[redacted]
3.	**Treatment:**	Nominal test concentrations of control and 3.13, 6.25, 12.5, 25.0 and 50 µg formulation/L
4.	**Test vessels:**	250 mL glass vessels with 200 mL test solution, covered with watch glasses
	Test water:	20 × AAP medium
5.	**Environmental conditions:**	
	Temperature:	22.9 – 24.2 °C
	pH:	7.4 – 7.5 (freshly prepared control medium at test start) 8.1 – 8.9 (aged control medium at end of test and test medium renewals) 7.5 – 7.6 (freshly prepared test concentrations at test start) 8.1 – 8.9 (aged test concentrations at end of test and test medium renewals)
	Photoperiod:	Continuous illumination, 7897, range 7160 - 8360 lux

B. STUDY DESIGN AND METHODS

1.	**In-life phase:**	03 to 13 May 2019

2. **Test organism assignment and treatment**

Colonies consisting of 3 plants with a total of 12 fronds were transferred from the inoculum culture to each test vessel, with three replicates per treatment. The test vessels were placed in a random order and were repositioned each test medium renewal day to minimize differences in light intensity. A semi-static test procedure was used and the test media were renewed on day 3 and.

The initial dry weight of a sample of fronds similar to those used to inoculate the test vessels was determined.

3. Dose preparation

A stock solution of 20 mg test item/L was prepared by dissolving at approximately 20.0 mg test item into 1000 mL test water on days 0, 3 and 5 followed by intense stirring for 10 minutes. Volumes of this stock solution were diluted with test water to prepare the test media of the highest test concentration. Volumes of this concentration were used to prepare the several lower test concentrations. The test media were prepared just before introduction of the *Lemna* (start of the test) and each test medium renewal.

4. Measurements and observations

Frond counts were made on Days 0, 3, 5 and 7 and the appearance of colonies was observed. Following day 3 and 5 observations, the fronds were transferred to newly prepared solutions. At test termination (day 7) frond densities for each treatment and control replicate vessel were determined. Frond dry weight was determined by drying fronds at 60°C to a constant weight.

The temperature was measured daily in a test vessel filled with water and incubated under the same conditions as the test vessels. Light intensity was measured once during the test. The pH-values were measured in all freshly prepared and aged test media.

At the beginning and end of each renewal period (i.e., day 0, 3, 5 and 7), a sample was removed from each treatment and the control solution to be analysed for aclonifen and diflufenican concentration. On day 3 and 5 samples of old and new test solutions were analysed. Samples were analysed by LC-MS/MS.

5. Statistics

Growth rates were derived from frond number counts on days 0, 3, 5 and 7, as well as dry weights measured at experimental start and study termination. In addition, yield measurements were calculated based on the same parameters.

The E_rC_{50} and the E_yC_{50}, the corresponding EC_{20} and EC_{10} values and where possible their 95% confidence limits were calculated by Probit analysis. For the determination of the 7-day $LOE_{y/r}C$ and $NOE_{y/r}C$ values significant differences at the test concentrations compared to the control values were tested by the Dunnett's t-test (frond number and dry weight). The software used to perform the statistical analysis was ToxRat Professional, Version 3.2.1, ToxRat Solutions GmbH. .

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

The quantification of the two active ingredients of the test item aclonifen + diflufenican SC600 (500 + 100 g/L) in the test samples was performed using liquid chromatography with MS/MS detection.

Aclonifen: In the freshly prepared test media at the start of the test the average recoveries of the active ingredient aclonifen ranged between 93 – 113% of nominal. In the aged test media after 3, 5 and 7 days test duration the average recoveries of the active ingredient aclonifen ranged between 91 – 109% of nominal.

Diflufenican: In the freshly prepared test media at the start of the test the average recoveries of the active ingredient diflufenican ranged between 91 – 108% of nominal. In the aged test media after 3, 5 and 7 days test duration the average recoveries of the active ingredient diflufenican ranged between 89 – 109% of nominal.

The validated method is summarised in Document M-CP5 (CP 5.4.2/12).

Table: Summary of analytical results

Nominal concn (µg test item/L)	Measured concn as % of nominal[a]			
	Aclonifen		Diflufenican	
	0 h (fresh)	3-7d (aged)	0 h (fresh	3-7d (aged)
Control	-	-	-	-
0.375	113	109	108	109
0.75	107	106	100	106
1.5	99	101	92	99
3.0	96	97	91	99
6.0	93	91	94	89

a Mean of all measured samples per treatment group

B. BIOLOGICAL DATA

Frond density

Frond production, biomass (dry weight) and observations of the fronds recorded during the 7-day exposure to aclonifen + diflufenican SC 600 are presented below.

Table: Mean frond numbers and biomass over 7-day exposure to aclonifen + diflufenican SC 600 (500 + 100 g/L)

Nominal concn (µg test item/L)	Frond number	Growth rate (fronds/h)	% inhibition	Frond dry weight	Growth rate (dry weight, 0 – 7 days)	% inhibition
Control	344.7	0.480		41.0	0.492	-
3.13	363.7	0.487	-1.6	40.1	0.490	0.5
6.25	219.3	0.415	13.5*	21.8	0.402	18.3*
12.5	99.3	0.301	37.3*	9.4	0.282	42.7*
25.0	58.3	0.224	52.9*	5.5	0.206	58.2*
50.0	49.3	0.202	57.9*	5.9	0.216	56.1*

Negative % inhibition indicates growth relative to the control
* Statistically significant difference from control (Dunnett's t-test, α = 0.05, one-sided smaller)

Shape of fronds

The appearance of the fronds was recorded daily. The fronds showed deviations from the control replicates after 7 days in the following test treatments; 3.13 µg test item/L had slightly shortened roots, the 6.25 and 12.5 µg test item/L had slightly gibbous growth, necrosis and chlorosis, shortened roots

and separated fronds and the 25.0 and 50 µg test item/L showed gibbous growth, necrosis, separated fronds and chlorosis.

A. VALIDITY CRITERIA

Validity criterion	Required (OECD 221, 2006)	Achieved
Doubling time of frond number in the control <2.5 days (60 h), corresponding to approximately 7-fold increase in 7 days	<2.5d	1.4 days (approx. 34 hours)
Control coefficient of variation for yield and growth	≤35%	5.7% yield (frond number) 1.7% growth rate (frond number) 9.7% yield (frond dry weight) 2.7% growth rate (frond dry weight)

All biological validity criteria for OECD and OCSPP guidelines were met, therefore, this study may be considered acceptable.

B. TOXICITY ENDPOINTS

Table: Summary of endpoints

Parameter	Nominal concn (µg test item/L)			
	Growth rate (frond number)	95% confidence limit	Growth rate (dry weight)	95% confidence limit
EC_{10}	3.84	3.51-4.21	2.89	2.64-3.17
EC_{20}	7.56	7.10-8.04	6.03	5.66-6.42
EC_{50}	27.6	26.4-28.8	24.6	23.6-25.7
NOEC	3.13		3.13	-
LOEC	6.25	-	6.25	-

III. CONCLUSION

The influence of actinifen + diflufenican SC600 (500 + 100 g/L) on the growth of the freshwater plant *Lemna gibba* was assessed in a semi-static concentration-response test. The 7-day E_rC_{50} was calculated to be 27.6 and 24.6 µg test item/L for frond number and dry weight, respectively. The 7-day NOE_rC and the LOE_rC were determined to be 3.13 and 6.25 µg test item/L for frond number and dry weight, respectively. All reported results refer to nominal concentrations of the test item.

[redacted] *(2019)*

Assessment and conclusion by applicant:

All biological validity criteria for OECD and OCSPP guidelines were met, therefore, this study may be considered acceptable

The 7-day E_rC_{50} was calculated to be 27.6 and 24.6 µg test item/L for frond number and dry weight, respectively. The 7-day NOE_rC and the LOE_rC were determined to be 3.13 and 6.25 µg test item/L for frond number and dry weight, respectively. Results calculated based on nominal concentrations of test item.

Assessment and conclusion by RMS:

CP 10.2.2 Additional long-term and chronic toxicity studies on fish, aquatic invertebrates and sediment dwelling organisms

No studies were necessary based on the current data requirements. Please refer to Document MCA, Section 8.2.

CP 10.2.3 Further testing on aquatic organisms

No further studies were necessary based on current data requirements.

CP 10.3 Effects on arthropods

CP 10.3.1 Effects on bees

Studies on the toxicity to bees have bee carried out with aclonifen + diflufenican SC 600, aclonifen and diflufenican. The available bee toxicity data are summarised in the following table.

Table 10.3-1: Summary of toxicity data to bees

Test item	Test species	Time-scale Test type / substrate	Endpoint	Reference
ACL + DFF SC 600 (500 + 100) G	Honey bee *Apis mellifera* L.	48 h Acute oral	**LD_{50} >220.6 µg product/bee**	KCP 10.3.1.1.1/01 KCP 10.3.1.2/01 M-566650-01-1 [redacted] 2016
Aclonifen	Honey bee *Apis mellifera* L.	48 h Acute oral	**LD_{50} >106.8 µg a.s./bee**	KCA 8.3.1.1.1/01 KCA 8.3.1.2/01 M-174936-01-1 [redacted], 1999
Diflufenican	Honey bee *Apis mellifera* L.	48 h Acute oral	**LD_{50} > 112.3 µg a.s./bee**	EFSA Scientific Report 122 (2007), 1-84
ACL + DFF SC 600 (500 + 100) G	Honey bee *Apis mellifera* L.	48 h Acute contact	**LD_{50} >200.0 µg product/bee**	KCP 10.3.1.1.1/01 KCP 10.3.1.2/01 M-566650-01-1 [redacted] 2016
Aclonifen	Honey bee *Apis mellifera* L.	48 h Acute contact	**LD_{50} >100 µg a.s./bee**	KCA 8.3.1.1.1/01 KCA 8.3.1.2/01 M-174936-01-1 [redacted], 1999
Diflufenican	Honey bee *Apis mellifera* L.	48 h Acute contact	**LD_{50} >100 µg a.s./bee**	EFSA Scientific Report 122 (2007), 1-84

Aclonifen	Honey bee *Apis mellifera* L.	22 d repeated exposure Larval toxicity	**NOED = 40.0 µg a.s./bee larva**	KCA 8.3.1.3/02 M-578600-01-1 [redacted], 2017
Aclonifen SC 600 (BANDUR SC 600)	Honey bee *Apis mellifera* L.	10 d chronic oral	**NOED = 36.55 kg a.s./bee/day**	KCP 10.3.1.2/01 M-601664-01-1 [redacted], 2017 & KCP 10.3.1.2/02 M-567602-01-1 [redacted], 2017
Diflufenican SC 500	Honey bee *Apis mellifera* L.	10 d chronic, adult feeding	NOEC ≥ 12.5 mg a.s./kg food LC_{50} >12.5 mg a.s./kg food **NOEDD = 0.46 µg a.s./bee/d** LDD_{50} >0.46 µg a.s./bee/d	KCP 10.3.1.5/02 M-539946-02-1 [redacted], 2015
Higher tier studies (tunnel test, field studies)				
Diflufenican SC 500	Honey bee *Apis mellifera* L.	Semi-field honey bee brood study (according to OECD 75; forced exposure conditions) in *Phacelia*; application during full-bloom and bees actively foraging	No effects on the survival of adult bees and honeybee pupae, foraging activity, behaviour, colony development and colony strength as well as on the bee brood at 120 g a.s./ha	KCP 10.3.1.5/01 M-551531-02-1 [redacted], 2016,
Diflufenican SC 500	Honey bee *Apis mellifera* L.	Honey bee brood feeding (following Oomen et al., (1992))	No adverse effects on mortality and brood development (brood termination rate, brood index, compensation index) at 300 ppm a.s. (0.3 g a.s./L; 0.72 g product/L)	KCP 10.3.1.6/01 M-478913-01-1 [redacted], 2014

Values in **bold** used in risk assessment

Summary of the risk assessment for ACL + DFF SC 600 (500 + 100) G and bees

The risk assessment for effects of ACL + DFF SC 600 (500 + 100) G on bees was performed in accordance with the recommendation of the 'Guidance Document on Terrestrial Ecotoxicology', as provided by the Commission Services (SANCO/10329/2002 rev.2 (final), October 17, 2002).

The risk assessment showed no unacceptable acute or chronic risks arising from the use of ACL + DFF SC 600 (500 + 100) G according to the proposed GAP.

Risk assessment for bees

The evaluation of the risk for bees was performed in accordance with the recommendations of the "Guidance Document on Terrestrial Ecotoxicology", as provided by the Commission Services (SANCO/ 10329/2002 rev.2 (final), October 17, 2002).

Bayer recognizes the need to review the bee pollinator risk assessment based on scientific progress. However, the EFSA Bee Guidance Document issued in 2013 has not been noted and therefore is not a realistically feasible way forward for assessing the chronic risk to honeybees. Therefore, the risk assessment below has been conducted following the EPPO 2010 scheme which provides a comparable level of protection to the EFSA approach and is based on the current scientific state of the art for bee pollinator risk assessment.

Application scenario

According to the GAP table, ACL + DFF SC 600 (500 + 100) G is proposed to be applied to winter cereals at 0.35 or 0.175 kg a.s./ha (1 application), during BBCH 00-13. The following assessments have been made for the use of ACL + DFF SC 600 (500 + 100) G in winter cereals using an application rate of 0.35 kg a.s./ha as this will also cover the risks from the use at lower application rates.

Risk assessment for the formulated product

As a first step, predicted (surrogate) endpoints for the formulated product were calculated based on the available data for the active substances. Where measured formulation data was available, the predicted endpoints were compared to the measured endpoints following the approach as defined in the EFSA Aquatic Guidance document.

If the measured formulation endpoint is lower than the predicted endpoint then the measured formulation endpoint should be used in the risk assessment. If the predicted endpoint is the lower value, then this should be used in the risk assessment. In cases where there is no measured formulation endpoint, the predicted endpoint should be used in the risk assessment.

Table 10.3-2: Calculation of the mixed toxicity of ACL + DFF SC 600 (500 + 100) G for honey bees

Endpoints	Active substance content (%)	Acute contact LD_{50} (ug a.s./bee)	Acute oral LD_{50} (ug a.s./bee)
Aclonifen	41.1	100	106.8
DFF	8.21	100	112.3
Predicted mix	-	202.8	218.4
Measured formulation	-	200	220

The predicted acute oral and contact mixture toxicity endpoints closely reflect the measured acute formulation study data, supporting the assumption that endpoints generated for the active substances are comparable to and reliably predict the relevant endpoints for the formulated product.

Hazard Quotient for bees

Acute contact and oral hazard quotients (Q_H) for the maximum proposed application rate of ACL + DFF SC 600 (500 + 100) G are presented in the following table:

Table 10.3-3: Acute risk assessment for bees arising from the use of ACL + DFF SC 600 (500 + 100) G in winter cereals

Intended use	Winter cereals, 0.7 L prod./ha, BBCH 00 - 13

Active substance	Aclonifen		
Application rate (g a.s./ha)	1 × 350		
Test design	**LD_{50} (µg/bee)**	**Single application rate (g a.s./ha)**	**Q_{HO}, Q_{HC} criterion: Q_H ≤50**
Oral toxicity	>106.8	350	<3.3
Contact toxicity	>100		<3.5
Active substance	Diflufenican		
Application rate (g a.s./ha)	1 × 70		
Test design	**LD_{50} (µg/bee)**	**Single application rate (g a.s./ha)**	**Q_{HO}, Q_{HC} criterion: Q_H ≤50**
Oral toxicity	>112.3	70	<0.6
Contact toxicity	>100		<0.7
Active substance	ACL + DFF SC 600 (500 + 100) G		
Application rate (g a.s./ha)	1 × 861[1]		
Test design	**LD_{50} (µg/bee)**	**Single application rate (g a.s./ha)**	**Q_{HO}, Q_{HC} criterion: Q_H ≤50**
Oral toxicity	>220.6	861	<3.9
Contact toxicity	>200		<4.3

Q_{HO}, Q_{HC}: Hazard quotients for oral and contact toxicity, Q_H values in **bold** breach the relevant trigger

1 Based on an application rate of 0.7 l product/ha and a formulation density of 1.230 g/mL

Hazard quotients for both oral and contact toxicity were below the trigger value of 50, indicating no unacceptable risks to honey bees from the use of ACL + DFF SC 600 (500 + 100) G according to the GAP.

Chronic risk assessment

The chronic oral and development risks to honeybee adults and larvae have been evaluated in accordance with the EPPO guidance (EPPO 2010). These long-term assessments are considered to address potential exposure via nectar and pollen from the treated crop and flowering weeds and encompass potential exposure from systemic activity.

Chronic risk to honeybee adults

In accordance with the revised EPPO scheme (OEPP/EPPO 2010) the chronic risk to adult bees and larvae can be evaluated by comparing the NOED to an estimate of daily residue consumption to give a toxicity exposure ratio (TER). The EPPO assessment trigger value is 1, whereby a TER >1 indicates a low risk based on the use evaluated. Daily residue consumption data are available from the draft EFSA guidance (2013)[10]. The worst-case screening SV for adult bees and larvae, taken from the EFSA guidance (2013), is applied in the assessment. The equation applied in the risk assessment is as follows and the TER calculations are presented in Table 10.3-4:

$$TER = \frac{NOED}{\text{Application rate (kg a.s./ha) x consumption (SV)}}$$

[10] "Guidance on the risk assessment of plant protection products on bees (*Apis mellifera*, *Bombus* spp. and solitary bees)" EFSA Journal 2013; 11(7):3295

Table 10.3-4: Chronic risk assessment for bees arising from the use of ACL + DFF SC 600 (500 + 100) G in winter cereals

Intended use		Winter cereals, 0.7 L prod./ha, BBCH 00 - 13			
Active substance		Aclonifen			
Application rate (kg a.s./ha)		1 × 0.35			
Assessment	**Toxicity[1]**	**Single application rate (kg a.s./ha)**	**Daily consumption (SV)[2]**	**Daily exposure[3]**	**TER criterion: >1**
Adult - chronic	36.55	0.35	7.6	4.56	8.0
Larvae - chronic	40		4.4	2.64	15.2
Active substance		Diflufenican			
Application rate (kg a.s./ha)		1 × 0.07			
Assessment	**Toxicity[1]**	**Single application rate (kg a.s./ha)**	**Daily consumption (SV)[2]**	**Daily exposure[3]**	**TER criterion: >1**
Adult - chronic	0.46	0.07	7.6	0.037	12.4

TER values in **bold** breach the relevant trigger
[1]: NOEDD (µg a.s./bee/day) for adults; NOED (µg a.s./larva) for larvae
[2]: Worst-case short-cut value (90th percentile) for daily exposure (downwards spraying) from the EFSA Guidance (EFSA, 2013)
[3]: Daily exposure expressed as µg a.s./bee/day for adults and µg a.s./larva for larvae

Hazard quotients for both adult and larval chronic toxicity from aclonifen were above the trigger value (>1), indicating no unacceptable risks to honey bees. Similarly, the hazard quotient for adult chronic toxicity from diflufenican was above the trigger value (>1), indicating no unacceptable risk to adult honey bees.

No studies were available to determine the chronic effects of diflufenican on honey bee larvae. However, there are two higher tier studies available which assess the effects of diflufenican SC 500 on honey bee brood in semi-field conditions.

The brood feeding test ([redacted], 2014) showed that no biologically relevant adverse effects on mortality and brood development (brood termination rate, brood index, compensation index) at 0.3 g a.s./L (single test concentration). Additionally, a semi-field brood study (Hecht-Rost, 2016) conducted aaccording to OECD 75; with forced exposure conditions during full-bloom and with bees actively foraging) showed no effects on the survival of adult bees and honeybee pupae, foraging activity, behaviour, colony development and colony strength as well as on the bee brood at 120 g a.s./ha.

Based on the results of these studies, it can be concluded that diflufenican does not adversely affect honey bees and honey bee brood when applied at a rate of up to 120 g a.s./ha during honey bees actively foraging on a bee-attractive, flowering crop. This is greater than the proposed highest application rate of diflufenican according to the GAP of 70 g a.s./ha indicating there is no unacceptable risk to adult or larval honey bees.

Therefore, it is therefore considered that ACL + DFF SC 600 (500 + 100) G does not pose any unacceptable risk due to chronic oral exposure when applied according to the GAP.

CP 10.3.1.1 Acute toxicity to bees

CP 10.3.1.1.1 Acute oral toxicity to bees

Data Point:	KCP 10.3.1.1.1/01
Report Author:	[redacted]
Report Year:	2016
Report Title:	Aclonifen + diflufenican SC 600 (500+100) G: Effects (acute contact and oral) on honey bees (Apis mellifera L.) in the laboratory – Final report
Report No:	112511035
Document No:	M-566650-01-1
Guideline(s) followed in study:	OECD 213 and 214 (1998), US EPA OCSPP 850.3020, 850.supp.
Deviations from current test guideline:	Current guideline: OECD 213/214, 1998 Test item and reference item applied as 1.5 µL droplet to ensure more reliable dispersion of test item. Test facility has experience to confirm this deviation does not affect outcome of studies and hence deviation is acceptable
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

An acute test was conducted to determine the acute oral and contact effect of aclonifen + diflufenican SC 600 on mortality and behaviour of the honey bee, *Apis mellifera*. The test was a limit test conducted at the nominal test concentration of 200 µg formulation/bee over 48 hours and included a control plus four concentrations of the toxic standard (dimethoate). Bees were assessed for mortality and any behavioural effects.

The contact test was 48 hours duration. At the end of the contact toxicity test (48 hours after application), there was 2% mortality at 200.0 µg product/bee. No test item related behavioural abnormalities were observed. There was 0% mortality in the control. Since only 2% mortality occurred in the 200.0 µg product/bee group, the contact LD_{50} can be considered as >200 µg product/bee.

The oral test was 48 hours duration. The maximum nominal test level of aclonifen + diflufenican SC 600 (500+100) G (*i.e.* 200 µg formulation/bee) corresponded to an actual intake of 220.6 µg formulation/bee. This dose level led to no mortality after 48 hours. No test item related behavioural abnormalities were observed. There was 0% mortality in the control. Since no mortality occurred in the 220.6 µg formulaton/bee group, the oral LD_{50} can be considered as >220.6 µg formulation/bee.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test Item:** aclonifen + diflufenican SC 600 (500 + 100)

Batch no.:	2015-010653
Active Ingredient / Purity:	Aclonifen (AE F068300): 44.1% w/w (505.1 g/L) Diflufenican (AE F088657): 8.21% w/w (101.0 g/L)
Appearance:	Yellow suspension
Storage:	25 ± 5°C (storage conditions +2°C to +30°C also acceptable)
Expiry date:	12 January 2017

2.	**Reference item:**	BAS 152 11 I
	Batch no.:	FRE-001226
	Active Ingredient / Purity:	400 g/L dimethoate (420.3 g/L analysed) Dose levels calculated using 420.3 g/L

3.	**Test Organism:**	Young female worker honey bee, *Apis mellifera* L.
	Age:	Not specified
	Source:	Disease-free, queen right colonies bred at test facility
	Feeding:	50% w/v sucrose solution *ad libitum;* was given directly after treatment via syringes inserted into cages *via* an opening in the top of the test units and from which bees accessed the food directly. No replacement of the food was necessary during the experimental period (48 h).

A. STUDY DESIGN AND METHODS

1. In-life phase: 23 to 26 May 2016

2. Exposure conditions

Test vessels:	Stainless steel cages 8 cm x 6 cm x 4 cm (length x height x width) with transparent pane for observation and perforated board on bottom of cage to allow sufficient air supply
Experimental design:	*Contact:* Control (tap water with 0.5% Adhäsit, applied after anaesthetisation with CO_2), test item 200 µg formulation/bee; Dimethoate (toxic standard) 0.10, 0.15, 0.20, 0.30 µg a.s./bee *Oral:* Control (50% w/v aqueous sucrose solution); test item 200 µg formulation/bee; Dimethoate (toxic standard) 0.05, 0.08, 0.15, 0.30 µg a.s./bee
Replicates:	5 replicates per test item dose level, controls and toxic standard, consisting of 10 bees
Temperature:	24.2 – 26.1°C
Relative humidity:	44.5 – 58.0%
Photoperiod:	Darkness (except during observation)

3. Administration of the test item

Contact toxicity test

Test substance was dissolved in tap water with 0.5% Adhäsit. Bees were anaesthetised with CO_2 until completely immobilised immediately before application of test treatments. A single 5 µL droplet was placed on the dorsal thorax of each bee using a calibrated pipette. After application bees were returned to test cages and f fed *ad libitum* with untreated 50% aqueous sucrose solution.

Oral toxicity test

Test substance and reference item were applied in 50% w/v aqueous sucrose solution. The treated food was offered in syringes, which were weighed before and after introduction into the cages (duration of uptake was 1 hour 10 minutes for the test item treatments). After a maximum of 1 hour 10 minutes, the uptake was complete and the syringes containing the treated food were removed, weighed and replaced by ones containing fresh, untreated food. The mean target dose levels (*e.g.* 200 µg product/bee nominal) would have been obtained if exactly 20 mg/bee of the treated food were ingested. In practice, uptake of the treated 50% w/v sucrose solutions differed slightly from the nominal 20 mg/bee and results are given based on the measured consumption (220.6 µg product/bee).

4. Measurements and observations

Observation of the bees was undertaken at the following times:

- 4 hours (first day)
- 24 hours, 48 hours following days

Any cases of mortality and/or poisoning or behavioural abnormalities of the bees (e.g. food refusal, apathy, moving coordination problems) were recorded.

5. Statistics/Data evaluation

Results obtained from the bees treated with the test item and the reference items were compared to those obtained from the control in both the contact and oral tests. The contact and oral LD50 values of the reference item were estimated using the binomial distribution (according to [redacted] 1977). It was not necessary to correct the test item and the reference item mortality, since no control mortality occurred in either the contact or oral toxicity tests.

The NOED was estimated using Fisher's Exact Test (pairwise comparison, one-sided greater, $\alpha = 0.05$), which is a distribution-free test and does not require testing for normality or homogeneity prior to analysis.

The software used to perform the statistical analysis was ToxRat® Professional, Version 3.2.1, ToxRat® Solutions GmbH.

II. RESULTS AND DISCUSSION

A: ANALYTICAL VERIFICATION

No analytical verification of the dosing solutions was performed.

B: BIOLOGICAL DATA

Contact toxicity test

No behavioural abnormalities were observed.

Table: Mean mortality and behavioural abnormalities of the bees in the contact toxicity test

Dose (µg formulation/bee)	Mortality (%)	
	24h	48h
Control	0	0
Test substance		
200	0	2
Toxic standard		
0.10	0	2
0.15	4	10
0.20	32	44
0.30	80	90

Oral toxicity test

No behavioural abnormalities were observed.

Table: Mean mortality and behavioural abnormalities of the bees in the oral toxicity test

Dose (µg formulation/bee)	Intake (µg formulation/bee)	Mortality (%)	
		24h	48h
Control	-	0	0
Test substance			
200	220.6	0	0
Toxic standard			
0.05	0.06	0	0
0.08	0.08	0	0
0.15	0.16	52	60
0.30	0.32	98	100

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 213/214, 1998)	Achieved
Mortality in controls	≤10%	0% (contact test) 0% (oral test)
Oral LD_{50} of the toxic standard (dimethoate)	0.10 – 0.35 µg a.i./bee	0.16 µg a.i./bee after 48h
Contact LD_{50} of the toxic standard (dimethoate)	0.10 – 0.30 µg a.i./bee	0.23 µg a.i./bee after 48h

The OECD 213/214 validity criteria regarding control mortality were met. Therefore, it is considered that this study is valid for risk assessment purposes.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoints (µg formulation/bee)	24h	48h

		LD_{50}	95% confidence interval	LD_{50}	95% confidence interval
Contact	Test substance	>200	-	>200	-
	Toxic standard	0.23	0.20 – 0.30	0.21	0.15 – 0.30
Oral	Test substance	>220.6	-	>145.36	-
	Toxic standard	0.16	0.08 – 0.32	0.14	0.08 – 0.32

III. CONCLUSION

The toxicity of aclonifen + diflufenican SC 600 was tested in both an acute contact and an oral toxicity test on honey bees. The LD_{50} (48 h) was >200 µg formulation/bee in the contact toxicity test. The LD_{50} (48 h) was >220.6 µg formulation/bee in the oral toxicity test.

[redacted] *(2016)*

Assessment and conclusion by applicant:

The OECD 213/214 validity criteria regarding control mortality were met. Therefore, it is considered that this study is valid for risk assessment purposes.

The toxicity of aclonifen + diflufenican SC 600 was tested in both an acute contact and an oral toxicity test on honey bees. The LD_{50} (48 h) was >200 µg formulation/bee in the contact toxicity test. The LD_{50} (48 h) was >220.6 µg formulation/bee in the oral toxicity test.

Assessment and conclusion by RMS:

CP 10.3.1.1.2 Acute contact toxicity to bees

Please refer to section 10.3.1.1.1 for relevant study data.

CP 10.3.1.2 Chronic toxicity to bees

Data Point:	KCP 10.3.1.2/01
Report Author:	[redacted]
Report Year:	2017
Report Title:	Aclonifen SC 600 - Assessment of effects on the adult honey bee, Apis mellifera L., in a 10 day chronic feeding test under laboratory conditions
Report No:	S15-00363
Document No:	M-601664-01-1
Guideline(s) followed in study:	Regulation (EC) No 1107/2009 (2009) Directive 2003-01 (Canada/PMRA) US EPA OCSPP 850.SUPP
Deviations from current test guideline:	Current guideline: OECD 245, 2017 No Deviation
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Data Point:	KCP 10.3.1.2/02
Report Author:	[redacted]
Report Year:	2016
Report Title:	Final report: Aclonifen SC 600 - Assessment of effects on the adult honey bee, apis mellifera L. in a 10 day chronic feeding test under laboratory conditions
Report No:	P62164704
Document No:	M-567602-01-1
Guideline(s) followed in study:	Regulation (EC) No 1107/2009 of the European Parliament and the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC European Commission Guidance Document for Generating and Reporting Methods of Analysis in Support of Pre-Registration data Requirements for Annex II (part A, Section 4) and Annex III (part A, section 5) of directive 91/414, SANCO/3029/99 rev. 4, 11/07/00 Guidance document on residue analytical methods, SANCO/825/00/rev. 8.1, European Commission, Directorate General Health and Consumer Protection 16/11/2010 US EPA Residue Chemistry Test Guideline OCSPP 860.1340: Residue Analytical Method
Deviations from current test guideline:	Current guideline: OECD 245, 2017 No Deviation
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The study was conducted to determine possible effects of aclonifen SC 600 on the honey bee, *Apis mellifera* L. after 10-day chronic feeding test under laboratory conditions. The test included a control, a solvent control, the test item (target 1050, 1366, 1775, 2308 and 3000 mg a.s./kg) and reference item groups (dimethoate, 0.90 mg a.s./kg). Additionally, bees were assessed for any behavioural effects.

Analysis of the feeding solutions determined mean measured concentrations ranged from 93 to 98% of nominal.

The mean consumption of feeding solution per day (corrected for evaporation), the mean uptake of test item per day over the 10-day test period and the accumulated mean uptake of test item are presented in the following table. After 10 days of continuous exposure the accumulated mean uptake of aclonifen SC 600 at the treatment levels of 1050, 1366, 1775, 2308 and 3000 mg a.s./kg feeding solution was 268.54, 365.52, 421.24, 562.54 and 712.08 µg a.s./bee, respectively. The corresponding average daily dose was therefore 26.85, 36.55, 42.12, 56.25 and 71.21 µg a.s./bee/day.

The overall mean daily consumption of feeding solution (i.e. the average consumption/bee over 10 days) in the test item treatment groups was reduced compared to the overall mean daily consumption of feeding solution in the solvent control group (25.6, 26.8, 24.0, 24.4 and 23.7 mg/bee/day at 1050, 1366, 1775, 2308 and 3000 mg a.s./kg feeding solution, respectively, compared to 33.8 mg/bee/day in the solvent control group).

The NOEC for mortality after 10 days of continuous exposure was determined to be 1366 mg a.s./kg feeding solution. The corresponding NOEDD, based on the actual consumption of the respective feeding solutions, was determined to be 36.55 µg a.s./bee/day.

The LC_{50} after 10 days of continuous exposure was determined to be >3000 mg a.s./kg feeding solution. The corresponding LDD_{50}, based on the actual consumption of the respective feeding solutions, was determined to be >71.21 µg a.s./bee/day.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test Item:** Aclonifen SC 600
   Batch no.: LV56005993
   Active Ingredient / Purity: 596.7 g/L (49.5% w/w) analysed
   Appearance: Yellow liquid
   Storage: Room temperature in the dark
   Expiry date: 9 February 2017

2. **Reference item:** Perfekthion / BAS 152 11 I
   Batch no.: FRE-000926
   Active Ingredient / Purity: Dimethoate (400 g/L)

3.	**Test Organism:**	Young adult worker honey bees (*Apis mellifera* L.)
	Age:	1 - 2 days old (newly hatched)
	Source:	[redacted]
	Feeding:	*ad libitum* with 50% (w/v) aqueous sucrose solution

B. STUDY DESIGN AND METHODS

1. In-life phase: 9 – 23 June 2015

2. Exposure conditions

Test vessels: Easy to clean and well-ventilated stainless steel cages were used. The size of the test cages (approximately 8 x 4 x 6 cm) provided adequate space for the bees. Each test unit was labelled with the study number and all necessary additional information to ensure unique identification. The units were equipped with a transparent pane to enable observation. The bottom of the cage was perforated steel to enable sufficient air supply. Cages were lined with filter paper

Experimental design: Control, solvent control (0.1% xanthan); test item 1050, 1366, 1775, 2308 and 3000 mg a.s./kg feeding solution;

Dimethoate 0.9 mg a.s./kg feeding solution

Replicates: Four replicates of 10 bees were used. Therefore, a total number of 40 bees for each control, test item concentration treatment and for the reference treatment were set up

Additionally, 4 test units without bees but with full food syringes for daily dose verification

Temperature: Target: 33 ± 2 °C; Achieved: 31.7 – 34.6°C

Relative humidity: Target: 60 ± 10%; Achieved: 36.7 – 64.2%

Photoperiod: Darkness (except during application and observation)

3. Administration of the test item

Dose preparation

Stock solutions of the test item in 50% (w/v) sucrose solution containing 0.1% xanthan were prepared with deionised water and stored refrigerated (6 ± 2°C) for a maximum of 4 days. Reference treatment stock solution was prepared using deionised water at the start of the test and stored refrigerated (6 ± 2°C). Further dilution of the stock solution to achieve desired concentrations was carried out on the day of use. Definitive solutions were freshly prepared daily from stock solution with 50% (w/v) aqueous sucrose solution for the reference treatments and with 50% (w/v) aqueous sucrose solution plus 0.1% xanthan for the test item treatments.

Oral treatment

Approximately 3-4 mL feeding solution was offered to the test organisms of each test unit in plastic syringes (approx. 5mL). The tip of each feeder was removed to all bees had access to the feeding solution. Syringes were replaced every day throughout the 10-day test period with freshly prepared feeding solutions. Therefore, the bees were continuously exposed to the feeding solution over a ten day period (D0 - D10). The amount of food consumed was determined by weighing the syringes before being introduced into the test units and after they were replaced by new ones in order to enable the calculation of the amount of effectively ingested dose. The dose consumed per bee was calculated by dividing the consumed amount of aqueous sucrose solution by the number of surviving bees. Food consumption was corrected accounting the loss by evaporation.

4. Measurements and observations

Mortality and behavioural abnormalities were assessed daily from D1 to D10. Any immobile bees not reacting at contact with a fine brush were considered dead. At the feeding time, dead bees were systematically removed from the cages.

Behavioural abnormalities in the test item treatment (e.g. moribund, affected, cramps, apathy or regurgitating) were assessed during the course of the study. Bees in the reference group were not assessed for behavioural abnormalities as it was assumed that moribund bees would die by the end of the study.

Analytical samples and retain samples of the feeding solutions in the control and test item treatments were taken daily after preparation of feeding solutions. The weight of each samples was determined and recorded. No samples of reference feeding solutions were taken. Samples were stored frozen (-18oC) with 45 minutes of sampling until required for analysis.

Analytical determination was conducted by [redacted]

5. Statistics/Data evaluation

The percent cumulative mortality was calculated for each treatment group and was corrected for control mortality according to the formula of [redacted] (1947).

Fisher's Exact Test with Bonferroni Correction (one-sided greater, $\alpha = 0.05$) was used to evaluate whether there are significant differences between the mortality data of the solvent control and the test item treatment group and to determine the NOEC and NOEDD based on mortality. Probit analysis using linear maximum likelihood regression was used to calculate the LC10, LC20, LDD10 and LDD20.

Statistical calculations were made by using the statistical program TOXRAT Professional 3.2.1.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

The mean measured concentrations determined in the feeding solutions ranged from 93 to 98% of nominal.

Table: Analytical verification of feeding solutions

Nominal concentration (mg a.s./kg)	Measured concentration (g/L)	% of nominal concentration
Control	<LoQ	-
1050	1028	98
1366	1337	98
1775	1651	93
2308	2216	96
3000	2849	95

LoQ (limit of quantification) = 0.01 mg/kg
LoD (limit of detection) = 0.005 mg/kg

The validated method is summarised in Document M-CP5 (CP 5.1.2/13).

B. BIOLOGICAL DATA

In the test item group, a cumulative mortality of 5, 12.5, 15, 32.5 and 45% was observed at the concentrations of 1050, 1366, 1755, 2308 and 3000 mg a.i./kg feeding solution respectively. Mortality was statistically significantly different when compared to the control at 1775, 2308 and 3000 mg a.s./kg. The mortality in the dimethoate reference treatment (nominally 0.90 mg a.s./kg) was 100% by the end of the 10-day exposure period.

In the test item treatment groups, some affected bees were observed from assessment day 4 to 10 at all tested concentrations. A few apathetic and moribund bees were observed in the three highest concentrations of 1775, 2308 and 3000 mg a.s./kg feeding solution.

Table: Food uptake and mortality at the end of the test

Nominal test concentration (mg/kg)	Cumulative mortality (%)									
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
Control	0	0	0	0	2.5	2.5	2.5	2.5	5	5
Solvent control[2]	0	0	0	0	0	0	0	0	0	0
Reference item (Dimethoate)[1]	0	0	0	0	15.4	43.6	74.4	92.3	97.4	100
1050	0	0	0	0	0	0	0	5	5	5
1366	0	0	0	0	2.5	2.5	2.5	10	12.5	12.5
1775	0	0	0	0	2.5	5	5	7.5	15	15*
2308	0	0	0	7.5	10	10	12.5	17.5	22.5	32.5*
3000	0	0	0	0	0	5	10	27.5	40	45*

1 – mortality corrected for corresponding control mortality ([redacted] 1947)

* Statistically significant difference compared to the control; Fisher's Exact Test ([redacted] corrected, one-sided, α = 0.05

Food consumption and uptake of test item

The mean consumption of feeding solution per day (corrected for evaporation), the mean uptake of test item per day over the 10-day test period and the accumulated mean uptake of test item are presented in the following table. After 10 days of continuous exposure the accumulated mean uptake of aclonifen SC 600 at the treatment levels of 1050, 1366, 1775, 2308 and 3000 mg a.i./kg feeding solution was 268.54, 365.52, 421.24, 562.54 and 712.08 µg a.i./bee, respectively. The corresponding average daily dose was therefore 26.85, 36.55, 42.12, 56.25 and 71.21 µg a.i./bee/day.

The overall mean daily consumption of feeding solution (i.e. the average consumption/bee over 10 days) in the test item treatment groups was reduced compared to the overall mean daily consumption of feeding solution in the solvent control group (25.6, 26.8, 24.0, 24.4 and 23.7 mg/bee/day at 1050, 1366, 1775, 2308 and 3000 mg a.i./kg feeding solution, respectively, compared to 33.8 mg/bee/day in the solvent control group).

Table: Mean food consumption and test item uptake over the 10-day test exposure

Nominal test concentration (mg/kg)	Mean consumption of feeding solution (mg/bee)	Mean uptake of active ingredient (µg a.s./bee/day)	Accumulated mean uptake of active ingredient (µg a.s./bee/day)
Control	30.3	-	-
Solvent control	33.8	-	-
Reference item	16.9	0.02	0.17
1050	25.6	26.85	268.54
1366	26.8	36.55	365.52
1775	24.0	42.12	421.24
2308	24.4	56.25	562.54
3000	23.7	71.21	712.08

Reference item = dimethoate (0.090 mg/kg)

The NOEC for mortality after 10 days of continuous exposure was determined to be 1366 mg a.s./kg feeding solution. The corresponding NOEDD, based on the actual consumption of the respective feeding solutions, was determined to be 36.55 µg a.s./bee/day.

The LC_{50} after 10 days of continuous exposure was determined to be >3000 mg a.s./kg feeding solution. The corresponding LDD_{50} based on the actual consumption of the respective feeding solutions, was determined to be >71.21 µg a.s./bee/day.

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 245, 2017)	Achieved
Average mortality in control treatment	≤15%	10%
Average mortality in reference item treatment	≥50%	100%

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint		LC_x [95% Confidence Limits]
Day 10	NOEC[1]	1366 mg a.s/kg feeding solution
	NOEDD[1,2]	36.55 µg a.i./bee/day
	LC_{10}	1342.01 mg a.i./kg feeding solution [953.94 – 1594.29]
	LC_{20}	1824.40 mg a.i./kg feeding solution [1516.96-2109.65]
	LC_{50}	>3000 mg a.i./kg feeding solution (n.d.)
	LDD_{10}	34.29 µg a.i./bee/day [25.30 – 40.04]
	LDD_{20}	45.21 µg a.i./bee/day [38.30 – 51.52]
	LDD_{50}	> 71.21 µg a.i./bee/day (n.d.)

1 = based on mortality (not significantly different compared to control)
2 = based on actual doses
(n.d) = not determined due to mathematical or inappropriate data

III. CONCLUSION

Continuous *ad libitum* feeding of honey bees in the laboratory over a period of 10 consecutive days with the test item aclonifen SC 600 at the treatment levels of 1050, 1366, 1775, 2308 and 3000 mg a.s./kg feeding solution resulted in dose-dependent effects regarding mortality.

The NOEC for mortality after 10 days of continuous exposure was determined to be 1366 mg a.s./kg feeding solution. The corresponding NOEDD, based on the actual consumption of the respective feeding solutions, was determined to be 36.55 µg a.s./bee/day.

The LC_{50} after 10 days of continuous exposure was determined to be >3000 mg a.s./kg feeding solution. The corresponding LDD_{50}, based on the actual consumption of the respective feeding solutions, was determined to be >71.21 µg a.s./bee/day.

[redacted] *(2017)*

Assessment and conclusion by applicant:

All validity criteria were satisfied and therefore this study can be considered to be valid.

The NOEC for mortality after 10 days of continuous exposure was determined to be 1366 mg a.s./kg feeding solution. The corresponding NOEDD, based on the actual consumption of the respective feeding solutions, was determined to be 36.55 µg a.s./bee/day.

The LC_{50} after 10 days of continuous exposure was determined to be >3000 mg a.s./kg feeding solution. The corresponding LDD_{50}, based on the actual consumption of the respective feeding solutions, was determined to be >71.21 µg a.s./bee/day.

Assessment and conclusion by RMS:

Data Point:	KCP 10.3.1.2/03
Report Author:	[redacted]
Report Year:	2015
Report Title:	Diflufenican SC 500A G - Assessment of effects on the adult honey bee, apis mellifera L., in a 10 days chronic feeding test under laboratory conditions - Final report amendment 1
Report No:	M-539946-02-1
Document No:	M-539946-02-1
Guideline(s) followed in study:	OECD Guideline No. 213 (1998), CEB No. 230 (2012) and OECD Guideline Proposal (2013) US EPA OCSPP Guideline No. 850.SUPP
Deviations from current test guideline:	Current guideline: OECD 213/214, 1998 No Deviation
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

Honey bees were exposed to a 50 % aqueous sucrose solution containing one concentration of Diflufenican SC 500A G by continuous and ad libitum feeding over a period of 10 days. The control group was fed with untreated sucrose solution (C). Mortality and sub-lethal effects were assessed daily during the 10-day exposure period. The chronic effects of Diflufenican SC 500A G. were evaluated by comparing the results of the test item group to those of the solvent control group

The NOEC for mortality after 10 days of continuous exposure was determined to be 12.5 mg a.s. diflufenican/kg feeding solution. The corresponding NOEDD, based on the actual consumption of the respective feeding solutions, was determined to be 0.46 µg a.s./bee/day.

The LC_{50} after 10 days of continuous exposure was determined to be > 12.5 mg a.s. diflufenican/kg feeding solution. The corresponding LDD_{50} (Lethal Dietary Dose), based on the actual consumption of the respective feeding solutions, was determined to be > 0.46 µg a.s./bee/day.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Item:	Diflufenican SC 500A G
Batch no.:	EV5400173
Active Ingredient / Purity:	500 g/L (nominal), 493.8 g/L, according to certificate of analysis
Appearance:	Beige liquid
Storage:	Room temperature in the dark
Expiry date:	14 June 2015

2.	**Reference item:**	Perfekthion (BAS 152 11)
	Batch no.:	FRE-000926
	Active Ingredient / Purity:	400 g/L (nominal), 400.9 g/L according to certificate of analysis
3.	**Test Organism:**	Honey bee, *Apis mellifera* L. from a
	Age:	Young adult worker bees (newly hatched, 1-4 days old)
	Source:	[redacted]
	Feeding:	Day 15 following application 1.5L commercial ready-to-use syrup (Apiinvert) was supplied to each colony. During 4th colony assessment it was noted that insufficient nectar/honey stores were available. Consequently, a small amount of supplemental food was supplied to all colonies to prevent starvation and a decline in colonies. It was considered that very limited natural resources available to colonies at the open field location led to shortage of food. Water was offered in each tunnel via a drinking trough, except during test item application

A. STUDY DESIGN AND METHODS

1. In-life phase: 5 to 20 August 2014

2. Exposure conditions

Test units	Stainless steel cages (8 cm x 4 cm base, x 6 cm height). Front of cages was transparent panel to allow observations. Bottom of cages was perforated board to allow sufficient air supply. Test cages were lined with filter paper
Temperature:	32.2 – 33.8°C
Relative humidity:	54.0 – 62.7%
Light	Constant darkness

3. Administration of the test item

The test item diflufenican SC 500A G was dissolved in tap water in order to obtain a stock solution. The amount of test item needed for the daily preparation of the stock solution was weighed in advance and then stored tightly closed under cool and dark conditions in the refrigerator (6 ± 2 °C) until use. The definitive test item feeding solution was prepared daily by diluting the stock solution with 50% (w/v) aqueous sucrose solution.

The reference item feeding solution, Perfekthion, was dissolved in tap water to obtain a stock solution (S1). This stock solution was diluted with tap water to obtain a solution (S2). Solution S2 was prepared

three times on application days 1, 4 and 7 and was stored under cool and dark conditions (refrigerator). The feeding solution was prepared by diluting solution S2 with 50% (w/v) aqueous sucrose solution.

For the control feeding solution a 50% aqueous sucrose solution was prepared and stored under cool conditions in the dark (refrigerator) for a maximum period of two days.

The feeding solutions were offered *ad libitum* to each cage of 10 bees in plastic syringes (Omnifix®, 5 mL). The tip of each syringe was removed so that the bees had access to the feeding solution. Every morning the syringes of all test cages (i.e. test item and control) were replaced by new syringes, filled with freshly prepared feeding solution. The weight of the syringes was determined before and after feeding on the next day in order to determine the mean food consumption of the bees per replicate.

4. Method

Four days prior to test start, brood combs containing capped cells with an expected hatch on the same day were taken out of a honey bee colony and transferred into the climatic chamber. A pollen and honey comb was placed beside the brood combs as food for the hatched bees. One day prior to test start the 1 - 3 days old bees were picked off the combs, transferred to the test cages and kept under test conditions until test start. Moribund bees were rejected and replaced by healthy bees before starting the test.

During the entire test period the bees were kept in cages.

5. Measurements and observations

Samples of the feeding solutions prepared freshly every day throughout the 10-days continuous feeding period were taken daily for subsequent chemical analysis in order to reveal the actual concentration of the test item. All samples were stored deep frozen (-18°C) immediately after sampling and maintained in a deep frozen condition and adequately separated during storage and shipment for subsequent chemical analysis.

Mortality

Mortality was recorded daily as number of dead individuals per cage. Percent values per treatment group and day were calculated on the basis of the number of introduced test organisms on day 1 and the accumulated number of dead individuals on the different assessment days.

The mortality was corrected for corresponding control mortality according to the formula of Schneider-Orelli (1947).

Food consumption

The daily consumption of feeding solution per bee was calculated by dividing the total daily consumption per replicate by the number of living bees at the beginning of the respective feeding interval.

For each treatment group, the mean consumption of feeding solution/bee/day was calculated by averaging the replicate values.

Data on food consumption were calculated for each treatment group as mean consumption of feeding solution [mg/bee/day], overall mean consumption of feeding solution [mg/bee/day], overall mean uptake of test item [µg a.s./bee/day] and accumulated mean uptake of test item [µg a.s./bee].

5. Statistics/Data evaluation

For the statistical comparison of the food consumption, non-rounded mean values per replicate over the entire test period were taken. Data of food consumption were statistically analysed by using the Student-t-test (left-sided, $\alpha = 0.05$) depending on the results of the pre-test of Shapiro Wilks and F-Test ($\alpha = 0.05$).

Statistical calculations were made by using the statistical program TOXRAT Professional 2.10.

II. RESULTS AND DISCUSSION

A: ANALYTICAL VERIFICATION

The mean measured concentration of diflufenican in the feeding solution was 107% of nominal. No residues of diflufenican (>Lod) were found in any of the control samples. The limit of quantification (LoQ), defined as the lowest validated fortification level of diflufenican was 0.01 mg/kg in the feeding solution. The corresponding limit of detection (LoD) was 0.003 mg/kg.

Treatment	Nominal concentration (mg/kg)	Mean measured concentration (mg/kg)	% of nominal
Control	-	<LoD	-
Diflufenican SC 500A G	12.5	13.4	107

The validated method is summarised in Document M-CP5 (CP 5.1.2/10).

B: BIOLOGICAL DATA

Mortality

The mortality in the control group was 2.5% after 10 days and thus remained within validity criterion of 15% mortality.

Mortality in the reference item treatment group increased during the test period and reached 65% (corrected 64.1%) after 10 days. Thereby exceeding the 50% mortality threshold set as validity criterion and confirming the test is suitable to determine toxic effects in a chronic exposure scenario.

After 10 days of continuous exposure to the concentration level of 12.5 mg a.s. diflufenican/kg feeding solution, no mortality could be observed (corrected -2.6%). The LDD_{50} value of >0.46 µg a.s./bee/day and a NOEDD at 0.46 µg a.s./bee/day was determined.

Table: Summary of mortality

Treatment (mg a.s./kg)	Cumulative mortality (%)									
	Assessment day									
	1	2	3	4	5	6	7	8	9	10
Control	0	0	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Diflufenican SC 500A G (12.5)	0	0	0	0	0	0	0	0	0	0
Perfekthion (0.9)	0	0	2.5	7.5	7.5	12.5	25	37.5	50	65
	Corrected cumulative mortality (%)									

Diflufenican SC 500A G (12.5)	0	0	-2.6	-2.6	-2.6	-2.6	-2.6	-2.6	-2.6	-2.6
Perfekthion (0.9)	0	0	0	5.1	5.1	10.3	23.1	35.9	48.7	64.1

Sub-lethal effects

In the control group no sub-lethal effects were observed. In the test item treatment group at the concentration level of 12.5 mg a.s. diflufenican/kg feeding solution only one single affected bee was observed on assessment day 1.

C: VALIDITY CRITERIA

The study was not conducted according to a standard guideline but was based on OECD guideline 213 (1998), CEB no. 230 (2012) and OECD guideline proposal (2013). As such no validity criteria were specified. However, mean control mortality was ≤15% (actual 2.5%) and mean reference toxicant mortality was ≥50% (actual 65%). Therefore it is considered that this study is valid for risk assessment purposes.

D: TOXICITY ENDPOINTS

Table: Summary of endpoints

Mortality		Food consumption	
LD_{50}	>12.5 mg/kg feeding solution	LC_{50}	>12.5 mg/kg feeding solution
NOEC	12.5 mg/kg feeding solution	NOEC	12.5 mg/kg feeding solution
NOEDD	0.46 µg a.s./bee/day	LDD_{50}	>0.46 µg a.s./bee/day

III. CONCLUSION

After 10 days of continuous feeding, a cumulative control mortality of 2.5% was observed. There was no mortality at the single test exposure concentration of 12.5 mg a.s. diflufenican/kg feeding solution (corrected -2.6%) at the final assessment.

In the reference item treatment group, mortality increased during the test period and reached 65% (corrected 64.1%) after ten days, thereby demonstrating the test was suitable to determine toxic effects in a chronic exposure scenario.

In the test item treatment group (12.5 mg a.s. diflufenican/kg feeding solution) sub-lethal effects were only observed at the first assessment in a single bee.

The overall mean daily consumption of feeding solution in the test item treatment group was not statistically significantly different when compared to the untreated control group (36.7 mg/bee/day at 12.5 mg a.s. diflufenican/kg feeding solution, compared to 39.4 mg/bee/day in the control group). In the toxic reference item group, the overall mean daily consumption of feeding solution was 28.1 mg/bee/day.

At the end of the 10-day exposure period, the mean accumulated uptake of the test item at the concentration level 12.5 mg a.s. diflufenican/kg feeding solution was 4.60 µg a.s./bee (based on the actual consumption of feeding solution by the honey bees). The corresponding daily mean uptake was therefore 0.46 µg a.s./bee/day.

The NOEC for mortality after 10 days of continuous exposure was determined to be 12.5 mg a.s. diflufenican/kg feeding solution. The corresponding NOEDD, based on the actual consumption of the respective feeding solutions, was determined to be 0.46 µg a.s./bee/day.

The LC_{50} after 10 days of continuous exposure was determined to be > 12.5 mg a.s. diflufenican/kg feeding solution. The corresponding LDD_{50} (Lethal Dietary Dose), based on the actual consumption of the respective feeding solutions, was determined to be > 0.46 µg a.s./bee/day.

The concentration tested in this study was limited by the maximum solubility of the test item. Since this concentration caused no mortality, the generated end-points are limited by solubility of the test item.

[redacted] *(2015)*

Assessment and conclusion by applicant:

The study was not conducted according to a standard guideline but was based on OECD guideline 213 (1998), CEB no. 230 (2012) and OECD guideline proposal (2013). As such no validity criteria were specified. However, mean control mortality was ≤15% (actual 2.5%) and mean reference toxicant mortality was ≥50% (actual 65%). Therefore, it is considered that this study is valid for risk assessment purposes.

The NOEC for mortality after 10 days of continuous exposure was determined to be 12.5 mg a.s. diflufenican/kg feeding solution. The corresponding NOEDD, based on the actual consumption of the respective feeding solutions, was determined to be 0.46 µg a.s./bee/day.

The LC_{50} after 10 days of continuous exposure was determined to be > 12.5 mg a.s. diflufenican/kg feeding solution. The corresponding LDD_{50} (Lethal Dietary Dose), based on the actual consumption of the respective feeding solutions, was determined to be > 0.46 µg a.s./bee/day.

Assessment and conclusion by RMS:

CP 10.3.1.3 Effects on honey bee development and other honey bee life stages

No data available on formulated product.

CP 10.3.1.4 Sub-lethal effects

No data available on formulated product.

CP 10.3.1.5 Cage and tunnel tests

Data Point:	KCP 10.3.1.5/01
Report Author:	[redacted]; [redacted]; [redacted]
Report Year:	2019
Report Title:	Amended final report: Semi-field brood study to evaluate potential effects of diflufenican on brood development of honeybees (Apis mellifera L.)
Report No:	1940025
Document No:	M-551531-02-1
Guideline(s) followed in study:	Regulation (EC) No. 1107/2009 Directive 2003-01 (Canada/PMRA) US EPA OCSPP Not Applicable OECD Guidance Document No. 75 (2007)
Deviations from current test guideline:	OECD GD 75 (2007) Minor deviations relating to assessment dates and recording of meteorlogical data. These deviations are not considered to have affected the integrity or outcome of the study
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

To assess the potential effects of Diflufenican SC 500A G on honey bee colonies including brood development, 287 g product/ha (corresponding to 120 g diflufenican/ha), tap water for the control and a reference item were applied to a full-flowering and highly bee-attractive crop (*Phacelia tanacetifolia*) under semi-field (tunnel) conditions during bee-flight.

No biological relevant adverse effects on mortality of worker bees or pupae were observed. Foraging activity, behaviour, nectar- and pollen storage as well as queen survival were not affected. No effects on colony development, colony strength or bee brood were observed.

Based on the results of this study it can be concluded that Diflufenican SC 500A G applied at a nominal rate of 287 g product/ha (120 g diflufenican/ha) during honeybee flight does not adversely affect honeybee colonies.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test Item:** Diflufenican SC 500A G
   Batch no.: EV54001703
   Active Ingredient / Purity: 493.8 g diflufenican/L
   Density: 1.179 g/cm^3
   Appearance: Beige suspension
   Storage: ambient (+2 °C to +30 °C), dark
   Expiry date: 14 June 2015

2. **Reference item:** Insegar 25 WP

Batch no.:	SM02K434
Active Ingredient / Purity:	Fenoxycarb, 25.0% w/w

3. Test Organism: Honey bee, *Apis mellifera* L.

Age: Not specified

Source: [redacted]

Colony details: For the test, honeybee colonies with the following properties were used:

- Colonies with ten combs, three to five brood combs and sufficient food supply
- Colonies were produced at the same time
- Sister queens from 2014 were used to ensure colonies which are as equal as possible
- At the first brood assessment (DAA -2/BFD 0) colonies contained mean numbers of 7,166, 7,518 and 6,630 worker bees, 5,950, 6,000 and 6,100 brood cells, 24,950, 20,533, and 19,850 food cells were used per treatment group (control, test item and reference item).
- Honeybees were free of clear visual symptoms of disease (e.g. Varroatosis, Nosemosis, Amoebiosis, Chalkbrood, Sacbrood, American or European foulbrood) or pests (Varroa destructor).
- Colonies were free of unusual occurrences (e.g. presence of dark "bald" honeybees, "crawlers" or flightless honeybees, unusual brood patterns, or brood age structure)

At least four weeks before the start of the test, no medical treatment was done. The used colonies were as homogeneous as practicable possible consisting of a natural distribution of all worker ages. All hives were equipped with a dead bee trap at the entrance to count the number of dead honeybees that were carried out of the hives. The colonies were set-up in the tunnels in the evening of 21 August 2014, shortly before full flowering of the crop, fourteen days before application. The entrances of the colonies were adjusted towards south or east. The colonies were kept in the tunnels for a period of six days after the application and then relocated to the monitoring site in the evening of 10 September 2014.

A. STUDY DESIGN AND METHODS

1. In-life phase: 17 July – 29 September 2014

2. Exposure conditions

Test tunnels
Semi-circular tunnels (18m x 6m x 2.9m, length x width x height) constructed of tubular steel frame with light transparent gauze fabric (mesh size: 2 mm). Tunnels were placed over flowering plants before experimental start date with a distance of 1.5 - 3m between tunnels

Plants
Phacelia tanacetifolia. Flowering Phacelia is highly attractive to honey bees.
Seeds were sown before study start at a rate of 14 kg/ha. Plots of ca. 75m2 prepared per replicate prior to setting of tunnels

Set up of plots
Before the set-up of the tunnels, two areas of *P. tanacetifolia* with ~2.5 m width and ~16.8 m length were cut out of the crop, separated by a path of 0.6 m, resulting in an effective crop area in the tunnel of ~84 m². Linen sheets with a width of 0.6 m were spread out at the inner walls of the short sides and on the path in the middle of the tunnel to aid the collection and counting of dead honeybees on the ground.

Location of test field
[redacted]

Temperature: Natural conditions, recorded whole experimental time
Relative humidity: Natural conditions, recorded whole experimental time
Precipitation Natural conditions, recorded whole experimental time
Wind Natural conditions, recorded during test item application
Clouding Natural conditions, recorded during test item application

3. Administration of the test item

The application rate of the test item and the reference item used in this study are presented in the following table:

Treatment	Target rate product/ha	Target rate a.s./ha	Spray volume [L/ha]
Control (tap water)	None	None	400.0
Test item (Diflufenican SC 500A G)	287 g[1]	120 g	400.2
Reference item (Insegar 25 WG)	1200 g[2]	300 g	401.2

[1]: Based on analysed content of a.s.
[2]: Based on the nominal content of a.s.

The application took place during honeybee flight at full flowering of the crop on 04 September 2014. For the application a calibrated boom sprayer was used according to good agricultural practice.

The application order was control, test item and reference item. During application the hives were covered by plastic sheets in order to protect them from direct spray residues. The test and the reference item were pre-weighted at the laboratory and added to the respective amount of water shortly before application. Homogeneity of spray solutions was obtained by thorough stirring and mixing immediately before application.

After the application in each tunnel the effective applied spray volume was determined by the calibrated flow meter of the boom sprayer. Deviations regarding the target and actually applied spray solutions ranged between 0.0% and 0.6% for the control, -0.1% and 1.1% for the test item, and -0.6% and 0.0% for the reference item and were therefore within the acceptable spray tolerance of ±10%.

The following criteria were met for the application:

- *P. tanacetifolia* was close to full bloom (BBCH 62 – 64)
- Wind speed outside the tunnels was < 2 m/s
- Crop was dry
- Mean foraging activity per treatment group was ≥ 10 honeybees/m²

4. Measurements and observations

Mortality

Mortality of honeybees was assessed on linen sheets (area approximately 18 m²) which were spread out at the front, middle and back of the tunnels. Additionally the dead honeybees were noted and counted in the dead bee traps which were attached to the entrance of the hives. The assessments were done according to the time table presented below. Honeybees were separated into dead adult worker bees, larvae, pupae and males. At each assessment day the dead honeybees were removed.

As the crop started to fade on DAA 6 (used test item was an herbicide), the colonies were transported to the monitoring site on the same day in the evening. Therefore the first mortality assessment at the monitoring site was conducted on DAA 7.

Mortality evaluation intervals were as follows:

Time of the test	DAA	Evaluation of mortality
12 to 1 days before application[1]	-12 to -1	Once a day approximately at the same time in the morning
On the day of application	0ba	Up to approximately 2.5 hours before application[2]
	0aa	2 h after application in the evening after daily flight activity of the bees
During exposure period in the tunnels[3]	1 to 6	Once a day approximately at the same time in the morning
Up to day 25 after application (only dead bee traps)	7 to 25	Once a day approximately at the same time in the morning

DAA = Days after treatment (DAA 0 = 04.09.2014)
[1]: As a period of 5 days before application was considered to be sufficient, only data from DAA -5 on were evaluated. Hence the mortality from DAA -12 to -6 are not presented;
[2]: Due to technical reason, the assessments were not performed shortly before the application but approximately 1.5 to 2.5 hours before the application.
[3]: On the day of transporting the colonies to the monitoring site (DAA 6), the mortality was assessed also in the evening. This was done in order not to miss the dead bees carried out of the hives between the assessments done in the

morning and the time of transport. For the interpretation and evaluation of the evening bees were added to the mortality assessed on the following day (DAA 7).

Foraging activity

Foraging activity was recorded on visual estimated areas of 1 m² at three different places in each tunnel according to the time schedule presented below. During each assessment the numbers of honeybees foraging on flowering *P. tanacetifolia* were counted for approximately 15 seconds per visual estimated area. At each assessment, the square to be observed was chosen randomly. On DAA -1 no foraging activity assessments were accidentally performed.

As the crop started to fade on DAA 6 (used test item was an herbicide), the colonies were transported to the monitoring site on the same day in the evening. Thus the foraging activity assessments stopped on DAA 6.

Time of the test	DAA	Evaluation of foraging activity
Over five days before application[1]	-12 to -1	Once a day at bee flight
On the day of application	0ba	Shortly before application
	0aa	4 times within the first hour after application 2 h after application 4 h after application 6 h after application
On the day following application	1	Three times during bee flight (morning, late noon, early afternoon)
During exposure period in the tunnels	2 to 6	Once a day at bee flight

DAA = Days after treatment (DAA 0 = 04.09.2014)
[1]: As a period of 3 days before application was considered to be sufficient, only data from DAA -5 on were evaluated (excluded DAA -4 and DAA -1). Hence the foraging activity data from DAA -12 to -6 are not presented;

Behaviour

The behaviour of the honeybees was observed in parallel to the foraging activity assessments as well as during emptying the dead bee traps. At least the following parameters were checked in comparison to the control:

- Aggressiveness
- Intensive flying activity without landing on the crop
- Clustering at the bee hive entrance
- Landing on the covering gauze
- Intoxication symptoms (e.g. paralysed bees, cramping bees)

Condition of the colonies

To assess potential effects of the test item on the condition of the colonies the following parameters were assessed six times during the field phase of the study:

- Strength of the colonies (number of honeybees)
- Presence of a healthy queen (e.g. presence of eggs)
- Comb area containing eggs, larvae and capped cells

- Comb area containing pollen and nectar

The colony assessments were conducted according to the Liebefeld method of [redacted]. (1987) and [redacted] (1999) as well as according to [redacted] (2008). For this purpose the comb was visually divided in areas of 1 dm². This was done for both sides of all combs of each hive. According to [redacted] (1987) and [redacted] (1999) one square of 100 cm² covered densely with honeybees represents approximately 130 worker bees or 400 worker bee cells, respectively. One square of male brood contains approximately 230 cells ([redacted] 2008). The absolute number of honeybees and cells filled with brood or food per colony were calculated by multiplying the number of estimated squares by 130 (for honeybees), by 400 (for worker bee cells containing brood such as eggs and larvae or food such as pollen and honey) or by 230 (male brood cells).

The assessments took place in parallel to the detailed brood assessments as presented below and also on DAA 25 (=BFD 27).

Development of bee brood

The assessments of the development of the honeybee brood were performed according to the OECD 75 guidance document (2007) and [redacted] (2012).

The development of the honeybee brood in individual marked cells was observed using digital image processing software. At the first BFD assessment (BFD 0, = brood area fixing day 0) before the application one or two brood combs out of each colony were chosen and a digital photo (non-GLP) was taken after the combs were marked distinctively in order to prevent any confusion. Afterwards the pictures were evaluated by using the software. For this purpose all pictures of all BFDs were adjusted by means of the software to guarantee that each single cell of each comb side could be recovered. After adjusting the pictures of BFD0, 304 to 345 cells filled with eggs were marked per colony. For every following BFD-assessment the software recovered exactly the cells which were marked on BFD 0. For the assessments at BFD 6 to 23 the content of the single cells was identified and marked with the symbols suggested in the OECD 75 guidance document (2007) by using the software (Table 8). Thereby the development of each individually marked cell throughout the duration of the Field Phase of the study could be determined (pre-imaginal developmental period of worker honeybees is normally 21 days). A successful brood development could be assumed at the last assessment date when cells were empty due to hatching of adult bees or again filled with eggs, young larvae, pollen or nectar after hatching. In contrast, a termination of the brood in the marked cells could be presumed if a cell was empty during BFD 6 to BFD 16 or if the cell contained an earlier brood stage than expected, or if the cell was filled with pollen or nectar.

After the BFD-assessments the determined brood stages of the marked cells were transformed into evaluation values and the brood termination rates, the brood indices and the brood compensation indices were calculated with the software.

The expected brood stage for every assessment is given in the following table:

Assessment date	**Determined brood stage in marked cells**
BFD 0 (= 2 days before application, DAA -2)	Eggs
Assessment date	**Expected brood stage in marked cells**
BFD 6 (= DAA 4)	Young to old larvae
BFD 10 (= DAA 8)	Capped cells

BFD 16 (= DAA 14)	Capped cells shortly before hatch
BFD 23 (= DAA 21)	Empty cells or cells containing eggs or pollen/nectar

Category at assessment	Classification of cell content	Evaluation values
Egg stage	1	1
Young larvae (L1-L2)	2	2
Old larvae (L3-L5)	3	3
Pupal stage (capped cells)	4	4
Empty after successful hatch or again filled with brood (egg/young larva) or with pollen/ nectar at BFD 21	-	5
Cell filled with not expected (earlier) brood stage or filled with pollen/nectar between BFD 6 and BFD 14 due to termination of the development	-	0
Empty	e	0
Dead larvae / pupae	7	0
Honey	n	0
Pollen	p	0

- No classification

5. Statistics/Data evaluation

Brood termination rate

For the calculation of the brood termination rate the observed cells were classified as follows:

1. Successful development: The bee brood in the observed cell reached the expected brood stage at the different BFD-assessments. On BFD 23 it was found empty or contained an egg or was filled with pollen or nectar after hatch of the adult bee.
2. Bee brood termination: The bee brood in the observed cell did not reach the expected brood stage at one of the BFD-assessments between BFD 6 to BFD 23 or the cell was empty or filled with food between BFD 6 to BFD 16.

The termination rate was determined for each colony separately and the mean value per treatment group was calculated.

Brood index

The brood-index is an indicator of the bee brood development and facilitates a comparison between different treatments.

The brood-index was calculated for each BFD-assessment and colony. Therefore the brood development in each cell was checked at each BFD-assessment starting from BFD 0 up to BFD 23. The cells were classified from 1 to 5 in cases where the cells contained the expected brood stage at the respective BFD-assessment. For BFD-assessments where cells did not contain the expected brood stage between BFD 6 to BFD 23 or when eggs or larvae in the cells were empty or replaced by food between BFD 6 to BFD 16 the cell was counted as 0 and also on all following BFD-assessments, irrespective if the cell was filled with brood again.

For the final calculation of the brood-index of each BFD-assessment and replicate, the transformation values of all individual cells were summed up and divided by the total number of observed cells. Additionally the average brood-index per treatment group was calculated.

Brood compensation index

The brood compensation-index is an indicator for the recovery of a colony and was also calculated for each BFD-assessment and replicate. The cells were classified from 1 to 5 as described for the brood-index, solely based on the identified growth stage of the BFD-assessments. In contrast to the brood-index, cells refilled with brood were taken into account for the calculation.

By that the compensation of bee brood losses was included in the calculation of the indices. If the brood in a respective cell was cleared and the cell was again filled with pollen or nectar the cell was counted as 0, as long as it was not refilled with brood. For the final calculation of the brood compensation-index of each BFD-assessment and replicate, the evaluation values of all individual cells were summed up and divided by the total number of observed cells. Additionally the average brood compensation-index per treatment group was calculated.

Statistics

The endpoints for statistics were the evaluation of mortality, overall foraging activity, brood termination rate (% terminated eggs/colony), brood-index and brood compensation-index. The arithmetic mean and the standard deviation per replicate and treatment group were calculated. Due to the fact that the colony assessment data are estimated values no statistics were done for this parameter. However, the arithmetic mean and the standard deviation per replicate and treatment group were calculated.

Data of mortality, foraging activity and the development of the bee brood were tested with Shapiro–Wilk test for testing of normality, Bartlett's test to assess the homogeneity of variances, followed by ANOVA and (if ANOVA showed differences among the treatment groups) by Dunnett's test (α-corrected, normal distributed and variance homogenous) or Kruskal-Wallis analysis and (if Kruskal-Wallis analysis showed differences among the treatment groups) by Wilcoxon–Mann–Whitney test (U-test; α-corrected, not normal distributed and/or not variance homogenous). Significance level of α = 0.05.

As due to unfavourable weather conditions there was no or only very little foraging activity on DAA -4 and as the foraging activity assessments on DAA -1 were accidentally not recorded these respective two days were not considered for any statistical evaluation.

Statistics were conducted with R (version 3.0.3).

II. RESULTS AND DISCUSSION

A: ANALYTICAL VERIFICATION

No analytical verification of the dosing solutions was performed.

B: BIOLOGICAL DATA

Mortality

The mean mortalities of adult worker bees for all treatment groups in the period DAA -5 to DAA 25 are shown in the following table:

Table: Mean adult worker bee mortalities of the different treatment groups

Date [dd.mm.yyyy]	DAA	Control [n]		Test item [n]			Reference item [n]			Statistical (multiple) comparison between all treatment groups
		Mean	SD	Mean	SD	stat.	Mean	SD	stat.	
30.08.2014	-5	32.0	10.5	29.8	17.8	n.s. #	36.5	6.6	n.s. #	n.s. #
31.08.2014	-4	28.0	14.3	23.3	13.3	n.s. #	23.8	10.2	n.s. #	n.s. #
01.09.2014	-3	21.5	5.4	23.8	12.0	n.s. #	19.8	6.2	n.s. #	n.s. #
02.09.2014	-2	34.3	2.1	38.3	14.8	n.s. ◊	35.3	18.8	n.s. ◊	n.s. ◊
03.09.2014	-1	37.0	7.1	27.0	11.4	n.s. #	33.8	3.6	n.s. #	n.s. #
04.09.2014	0ba	44.3	6.8	42.5	19.8	n.s. #	56.8	27.0	n.s. #	n.s. #
Mean DAA -5 to 0ba[1]		**32.8**	**10.5[3]**	**30.8**	**15.3**	n.s. ◊	**34.3**	**17.7**	n.s. ◊	n.s. ◊
04.09.2014	0aa	15.8	5.1	26.0	15.5	n.s. #	25.5	13.3	n.s. #	n.s. #
	0aa	17.8	10.5	15.0	3.3	n.s. #	21.0	13.7	n.s. #	n.s. #
	Σ 0aa	33.5	14.0	41.0	18.5	n.s. ◊	46.5	25.5	n.s. ◊	n.s. ◊
05.09.2014	1	14.0	4.3	23.3	15.8	n.s. #	17.3	14.5	n.s. ◊	n.s. ◊
06.09.2014	2	60.8	19.6	38.0	22.3	n.s. ◊	43.0	20.1	n.s. ◊	n.s. ◊
07.09.2014	3	62.3	35.7	40.8	18.5	n.s. #	38.3	20.3	n.s. #	n.s. #
08.09.2014	4	53.3	28.2	51.3	17.0	n.s. #	43.8	21.7	n.s. #	n.s. #
09.09.2014	5	47.3	11.9	36.0	18.0	n.s. #	34.8	22.2	n.s. #	n.s. #
10.09.2014	6	63.8	18.4	46.5	15.8	n.s. #	62.0	41.1	n.s. #	n.s. #
Mean DAA 0aa to 6[1]		**47.8**	**25.4[3]**	**39.5**	**18.0**	n.s. ◊	**40.8**	**25.4**	n.s. ◊	n.s. ◊
11.09.2014	7	4.5	4.6	1.3	0.5	n.s. ◊	3.3	2.8	n.s.	n.s. ◊
12.09.2014	8	8.5	7.9	3.3	3.0	n.s. #	3.5	3.9	n.s. #	n.s. #
13.09.2014	9	10.0	4.2	2.3	2.2	n.s. #	9.5	5.0	n.s. #	n.s. #
14.09.2014	10	18.8	13.6	6.5	7.0	n.s. ◊	9.8	11.0	n.s. ◊	n.s. ◊
15.09.2014	11	18.8	22.4	6.5	2.4	n.s. ◊	15.3	7.9	n.s. ◊	n.s. ◊
16.09.2014	12	13.8	24.9	7.0	2.4	n.s. ◊	19.0	22.7	n.s. ◊	n.s. ◊
17.09.2014	13	29.3	39.8	4.3	2.4	n.s. ◊	28.5	35.9	n.s. ◊	n.s. ◊
18.09.2014	14	14.0	16.4	9.8	3.9	n.s. ◊	17.0	14.7	n.s. ◊	n.s. ◊
19.09.2014	15	25.8	9.7	21.3	16.1	n.s. #	22.8	13.1	n.s. #	n.s. #
20.09.2014	16	21.3	10.0	15.0	8.0	n.s. #	23.8	17.1	n.s. #	n.s. #
21.09.2014	17	12.0	6.2	12.3	6.3	n.s. #	19.3	10.9	n.s. #	n.s. #
22.09.2014	18	15.3	6.1	8.0	10.1	n.s. #	17.8	13.0	n.s. #	n.s. #
23.09.2014	19	19.0	12.1	11.8	9.6	n.s. ◊	16.0	6.7	n.s. ◊	n.s. ◊
24.09.2014	20	24.8	8.8	12.5	7.3	n.s. #	17.0	9.8	n.s. #	n.s. #
25.09.2014	21	27.0	27.2	12.5	8.4	n.s. #	21.8	11.8	n.s. #	n.s. #
26.09.2014	22	35.8	15.0	26.8	13.8	n.s. #	33.0	29.9	n.s. #	n.s. #
27.09.2014	23	19.5	6.4	20.3	9.0	n.s. ◊	18.5	11.3	n.s. ◊	n.s. ◊
28.09.2014	24	9.5	9.0	16.5	8.9	n.s. ◊	11.3	9.7	n.s. ◊	n.s. ◊
29.09.2014	25	13.3	6.8	17.0	9.8	n.s. ◊	11.8	18.2	n.s. ◊	n.s. ◊
Mean DAA 7 to 25[2]		**18.0**	**16.0**	**11.3**	**9.8**	n.s. ◊	**16.8**	**15.5**	n.s. ◊	n.s. ◊
Mean DAA 0aa to 25		**26.0**	**23.1[3]**	**18.9**	**17.7**	n.s. ◊	**23.2**	**21.4**	n.s. ◊	n.s. ◊

[1]: mortality in dead bee traps and on linen sheets; [2]: mortality in dead bee traps; [3]: standard deviation calculated for the individual values of the respective group; DAA = days after application (ba = before application, aa = after application); SD = standard deviation; * test item / reference item mortality statistically significantly different compared to the control mortality ($p<0.05$); n.s. = test item / reference item mortality statistically not significantly different compared to the control mortality ($p>0.05$); # = ANOVA / Dunnett's test; ◊ = Kruskal-Wallis analysis / Wilcoxon–Mann–Whitney test

Foraging activity

The foraging activity of the honeybees was assessed on DAA -5 to DAA 6. The course of the foraging activity was comparable for all treatment groups over the whole pre-exposure and exposure period.

Table: Mean foraging activities of the different treatment groups

Date [dd.mm.yyyy]	DAA	Control [bees/m²/ 15 sec]		Test item [bees/m²/15 sec]			Reference item [bees/m²/15 sec]			Statistical (multiple) comparison between all treatment groups
		mean	±SD	mean	±SD	stat.	mean	±SD	stat.	
30.08.2014	-5	22.8	2.9	19.6	1.9	n.s. #	20.3	1.8	n.s. #	n.s. #
31.08.2014	-4	0.1	0.2	0.3	0.3	n.s. ◊	0.1	0.2	n.s. ◊	n.s. ◊
01.09.2014	-3	7.5	2.1	9.7	2.7	n.s. ◊	8.4	2.6	n.s. ◊	n.s. ◊
02.09.2014	-2	15.1	5.3	15.3	5.4	n.s. #	14.2	3.1	n.s. #	n.s. #
03.09.2014	-1	NA	NA	NA	NA	--	NA	NA	--	--
04.09.2014	0ba	10.7	0.4	11.7	1.2	n.s. ◊	12.8	1.7	* ◊	* ◊
Mean DAA -5 to 0ba[1]		**14.0**	**6.6**	**14.0**	**4.8**	**n.s. ◊**	**13.9**	**4.9**	**n.s. ◊**	n.s. ◊
04.09.2014	0aa[2]	11.4	4.4	11.7	5.1	n.s. ◊	11.4	3.7	n.s. ◊	n.s. ◊
05.06.2014	1[2]	16.9	5.3	16.0	4.3	n.s. ◊	20.1	7.7	n.s. ◊	n.s. ◊
06.09.2014	2	12.8	4.3	14.0	4.6	n.s. #	13.1	3.1	n.s. #	n.s. #
07.09.2014	3	14.8	2.9	15.8	1.2	n.s. #	12.7	3.1	n.s. #	n.s. #
08.09.2014	4	13.7	4.4	15.5	2.6	n.s. #	16.5	2.7	n.s. #	n.s. #
09.09.2014	5	13.4	3.4	14.6	2.1	n.s. #	13.7	1.4	n.s. #	n.s. #
10.09.2014	6	10.7	2.2	12.7	3.3	n.s. #	14.1	2.1	n.s. #	n.s. #
Mean DAA 0aa to 6		**13.4**	**3.5**	**14.3**	**2.8**	**n.s.**	**14.4**	**3.7**	**n.s.**	n.s.

DAA = days after application (ba = before application, aa = after application); [1]: due to a low foraging activity DAA -4 was not considered in any calculation; [2]: mean foraging activity on DAA 0aa (7 assessments) and DAA 1 (3 assessments); SD = standard deviation (calculated for every single assessment (three locations/tunnel)/day); NA = not assessed; * test item / reference item foraging activity statistically significantly different compared to the control foraging activity ($p \leq 0.05$); n.s. = test item / reference item foraging activity statistically not significantly different compared to the control foraging activity ($p > 0.05$); # = ANOVA / Dunnett's test; ◊ = Kruskal-Wallis analysis / Wilcoxon–Mann–Whitney test; -- = not applicable

Behaviour and conspicuous observations

The behaviour of the honeybees was generally inconspicuous. No abnormal behaviour was observed at the mortality or foraging activity assessments.

Development of colony strength

The mean strength of the colonies at pre-application assessment (BYD 0) was 7,166 ± 1,290 worker bees/colony in the control, 7,518 ± 1,057 worker bees/colony in the test item group and 6,630 ± 3,154 worker bees/colony in the reference item group and thus on a similar level in all treatment groups. For a better comparison of the treatment groups the initial values were set to 100%.

At the following two colony assessments (DAA 4 and DAA 8) the mean estimated numbers of worker bees of the control and test item group were at similar levels. Thereafter, i.e. on DAA 14 the strength of the test item group was at a level slightly below that of the control group, increased thereafter again above the control level (DAA 21) and decreased again at the final assessment on DAA 25 (5,330 ± 1,834 worker bees). At the same time, both the control and the reference item group showed decreasing tendencies and ended up with 5,168 ± 707 worker bees (control) and 4,534 ± 2,035 worker bees (reference item). At this point in time of the year the colonies are naturally reducing the numbers of worker bees so this observation was considered to be normal.

The variation of the strength in the control and the test item group indicate that an adverse effect of the test item on the colony strength can be excluded.

Table: Development of the mean colony strength

Date	BFD (DAA)	Control group			Test item group			Reference item group		
		absolute [n][1]		relative[2]	absolute [n][1]		relative[2]	absolute [n][1]		relative[2]
		mean	± SD		mean	± SD		mean	± SD	
02.09.2014	0 (-2)	7166	1290	100%	7518	1057	100%	6639	3154	100%
08.09.2014	6 (4)	6971	1661	97%	6933	1073	92%	7020	2452	106%
12.09.2014	10 (8)	6906	1174	96%	7193	1355	96%	7410	2814	112%
18.09.2014	16 (14)	6598	1423	92%	5958	1249	79%	6809	2433	103%
25.09.2014	23 (21)	6061	889	85%	6698	1858	89%	5753	2076	87%
29.09.2014	27 (25)	5168	707	72%	5330	1834	71%	4534	2035	68%

DAA = days after application; BFD = brood area fixing day; [1]: absolute mean number of egg cells of the colonies ± standard deviation; [2]: relative mean number of egg cells of the colonies, the number of egg cells was set as 100 % at the first assessment

Development of the bee brood

Brood termination rate

The mean termination-rates at the last brood assessment at BFD 23 were 30.4 % ± 15.7 % for the control group, 27.1 ± 13.1 % for the test item group, and 80.8 ± 21.5 % for the reference item group. The high standard deviations suggest that the termination-rates of the replicates within each treatment group were fluctuating. The termination-rates at BFD 23 of the control group ranged from 14.8 % to 47.4 %, that of the test item group from 17.7 % to 42.0 % and the termination-rates of the reference item group ranged from 49.6 % to 97.8 %. However, the test item data showed no adverse effects on the development of the bee brood

No statistically significant difference was found between the control and the test item group, whereas the reference item group showed a statistically significant increase compared to the control group at BFD 6, 16 and 23 ($p<0.05$, ANOVA, Dunnett's test).

Table: Mean brood termination-rates of the different treatment groups

Test Group	BFD 6	BFD 10	BFD 16	BFD 23
Control	23.71% [14.06%]	27.27% [14.69%]	30.33% [15.53%]	30.41% [15.65%]
Test	22.73% [13.27%]	24.98% [13.44%]	27.05% [13.08%]	27.05% [13.08%]
Reference	66.61%* [31.46%]	67.99% [32.37%]	80.78%* [21.54%]	80.78%* [21.54%]

BFD = brood area fixing day (BFD0 = 02.09.2014); [standard deviation]; * statistically significantly different compared to the control group ($p<0.05$, ANOVA, Dunnett's test)

Brood-index

The brood-indices correlate with the brood termination-rates in a way that the higher the brood termination-rates, the lower will be the brood-indices and vice versa. Consequently, the mean brood-index of the test item group was higher at all BFDs when compared to the control and not statistically

significant different. Thus regarding the brood indices the test item did not cause adverse effects on the development of the bee brood.

Table: Mean brood-indices of the different treatment groups

Test group	BFD 0	BFD 6	BFD 10	BFD 16	BFD 23
Control	1.00	2.50	2.91	2.79	3.48
Test	1.00	2.51	3.01	2.92	3.65
Reference	1.00	1.16	1.28	0.77*	0.96*
Expected brood stages	1	2-3	4	4	5

Brood stages (if no termination of brood occurred): 1 ≙ egg, 2 ≙ young larvae, 3 ≙ old larvae, 4 ≙ pupae, 5 ≙ successful hatch (empty cell, egg, food); BFD = brood area fixing day (BFD0 = 02.09.2014); * statistically significantly different compared to the control group ($p<0.05$, ANOVA, Dunnett's test)

Brood compensation-index

The brood compensation-index is an indicator for the recovery of the colony. Generally, the mean brood compensation-indices of all treatment groups were slightly higher than the corresponding brood-indices indicating that cells with terminated brood were refilled with new eggs. The mean brood compensation-indices of the control were lower compared to the test item colonies, indicating that the test item caused no adverse effects on the bee brood development.

Table: Mean brood compensation-indices of the different treatment groups

Test group	BFD 0	BFD 6	BFD 10	BFD 16	BFD 23
Control	1.00	2.58	3.10	3.22	4.18
Test	1.00	2.62	3.22	3.37	4.28
Reference	1.00	1.42	1.44*	1.22*	1.90
Expected brood stages	1	2-3	4	4	5

Brood stages (if no termination of brood occurred): 1 ≙ egg, 2 ≙ young larvae, 3 ≙ old larvae, 4 ≙ pupae, 5 ≙ successful hatch (empty cell, egg, food); BFD = brood area fixing day (BFD0 = 02.09.2014); * statistically significantly different compared to the control group ($p<0.05$, ANOVA, Dunnett's test)

C: VALIDITY CRITERIA

The study was based on OECD guidance document 75 (2007). Validity criteria were qualitative; control mortality should not be considerable and there should be a high number of impacted bees in the reference test treatments.

Over the course of the study (day 0 – 27) there was a daily mean mortality of 26.0, from this it can be inferred that the validity criterion for control mortality was met.

The reference item treatment showed significant impact compared to the control in relation to brood termination rate, brood compensation rate and brood index, and from this it can be inferred that the validity criterion for considerable impact in the reference test treatment was met.

Therefore, it is considered that this study is valid for risk assessment purposes.

III. CONCLUSION

To assess the potential effects of Diflufenican SC 500A G on the brood development of honeybees (*Apis mellifera* L.), Diflufenican SC 500A G was applied at a nominal rate of 287 g product/ha (120 g diflufenican/ha) on *Phacelia tanacetifolia* during honeybee flight under semi-field conditions in summer 2014.

The application of Diflufenican SC 500A G did not cause any effects on the survival of adult honeybees, honeybee pupae, foraging activity, behaviour, colony development and colony strength as well as on the bee brood.

Thus this study demonstrates that Diflufenican SC 500A G applied at a nominal rate of 287 g product/ha (120 g diflufenican /ha) during honeybee flight did not adversely affect honeybee colonies.

[redacted] *(2016)*

Assessment and conclusion by applicant:

The study was based on OECD guidance document 75 (2007) and all relevant validity criteria were satisfied.

Therefore, it is considered that this study is valid for risk assessment purposes.

No biological relevant adverse effects on mortality of worker bees or pupae were observed. Foraging activity, behaviour, nectar- and pollen storage as well as queen survival were not affected. No effects on colony development, colony strength or bee brood were observed.

Based on the results of this study, it can be concluded that Diflufenican SC 500A G applied at a nominal rate of 287 g product/ha (120 g diflufenican /ha) during honeybee flight does not adversely affect honeybee colonies.

Assessment and conclusion by RMS:

CP 10.3.1.6 Field tests with honeybees

Data Point:	KCP 10.3.1.6/01
Report Author:	[redacted]
Report Year:	2014
Report Title:	Diflufenican SC 500A G: effects on honey bee brood (Apis mellifera L.) - Brood feeding test
Report No:	79071031
Document No:	M-478913-01-1
Guideline(s) followed in study:	[redacted]1992 US EPA OCSPP Guideline No. 850.SUPP
Deviations from current test guideline:	Current guideline: Not applicable No deviations from study plan
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

A bee brood test was conducted, in order to assess the effect of Diflufenican SC 500 to the honey bee brood using 0.72 g/L test item, equivalent to an active substance concentration of 0.3 g a.s./L. An untreated control and a toxic reference were included in the study.

Three bee colonies were used per treatment group. The test item and reference item solutions were mixed with ready-to-use sugar syrup (Apiinvert) and applied to the bee colonies via a feeding trough, which was put directly into the colony on top of the second magazine. Pure sugar syrup (Apiinvert) was used for the controls. Ontogenesis of a defined number of honey bee eggs, young- and old larvae was observed for a period of 21 days following the application for each treatment group and colony. This was assessed one day before the application. For each subsequent brood assessment (BFDn) the same comb(s) was (were) selected from the respective colony in order to investigate the progress of brood development. Ontogenesis of the bee brood was observed for a period of 21 days after application (*i.e.* 22 days following BFD0). Mortality of adult bees and pupae was also assessed

The mean termination rate of eggs, the development success of the young larvae, the mean termination rate of old larvae, the brood termination rate over all stages and adult bee mortality were not statistically significantly different when compared to the control group. No effects of the test item on honey bee pupae and larvae were observed.

The reference item treatment resulted in a statistically significant increase of unsuccessful egg and young larvae development. The mean brood termination rate over all stages was statistically significantly higher than in the control confirming the sensitivity of the test system and the validity of the test conditions.

Overall, it can be concluded that the administration of diflufenican SC 500A G fortified sugar syrup (300 ppm diflufenican) to honey bee colonies does neither adversely affect honey bee colonies nor bee brood development.

I. MATERIALS AND METHODS

A. MATERIALS

1.	**Test Item:**	Diflufenican SC 500A G
	Batch no.:	EV54001569
	Active Ingredient / Purity:	42.2% w/w (497.5 g/L), according to certificate of analysis
	Appearance:	Light beige liquid
	Storage:	Room temperature in the dark
	Expiry date:	28 September 2014
2.	**Reference item:**	Insegar (Fenoxycarb)
	Batch no.:	L160112
	Active Ingredient / Purity:	250 g/kg (nominal), 257 g/kg according to certificate of analysis
3.	**Test Organism:**	Young adult worker honey bee, *Apis mellifera* L.
	Age:	All ages and all stages
	Source:	[redacted]
	Feeding:	Natural food and water. No additional food provided during the study

A. STUDY DESIGN AND METHODS

1.	**In-life phase:**	24 June to 18 July 2013
2.	**Exposure conditions**	
	Setting of the bees	In the evening, 28 days before application, *ca.* 50 m behind the IBACON building on a meadow to enable the bees to get familiar with the new environment and to lower the set-up related mortality to a normal extent. The bee colonies were removed to a 2nd location on day 13 following the application. The colonies remained at this second location until the end of the trial
	Test sites	Uncultivated fields and hedgerows, surrounding area with typical agricultural use, mainly arable crops and meadows
	Location of 2nd field	*ca.* 2 km distance to the first test site
	Location of test field	[redacted] Germany
	Temperature:	Natural conditions, recorded whole experimental time
	Relative humidity:	Natural conditions, recorded whole experimental time
	Precipitation	Natural conditions, recorded whole experimental time
	Wind	Natural conditions, recorded during test item application
	Clouding	Recorded daily during exposure phase

3. Administration of the test item

One single application per colony containing 1 L contaminated (test item and reference item) or untreated commercial ready-to-use sugar syrup (Apiinvert) per colony was used. This was applied during the afternoon in order to prevent robbery. Diflufenican SC 500A G was dissolved in 1 L ready-to-use sugar syrup (Apiinvert) per colony, equivalent to an active substance concentration of 0.30 g diflufenican a.s./L (300 ppm). The reference item (Insegar; 25 % fenoxycarb) was dissolved in 1 L ready-to-use sugar syrup (Apiinvert) per colony, equivalent to a nominal active substance concentration of 0.75 g fenoxycarb a.s./L. For the control 1L untreated ready-to-use sugar syrup (Apiinvert) per colony was supplied.

The ready prepared sugar solutions were offered per colony in a feeding trough (for feeding bees according to routine bee keeping practice). The trough was put into an empty magazine on top of the populated bee magazines. The bees had free access to the feeding trough, with feeding being started during the afternoon (14:40 to 16:10 h). The feeding troughs and empty magazines were removed after complete uptake of the feeding solutions. The bees did not ingest the offered food completely 24 hours after application in most of the colonies, therefore the feeding troughs remained in the hives until food uptake was complete (max. 47 hrs for some colonies in the control and test item treated group). Each feeding trough was weighed before introduction to the bee colonies and after uptake of the contaminated food to determine the exact amount of ingested food by the bee colony.

4. Measurements and observations

Mortality

Dead bees were collected from deadbee traps (wooden boxes, approximately 50 cm x 50 cm x 15 cm, upper side covered by wire mesh with a mesh size of *ca.* 1 cm, according to an IBACON design), placed in front of each colony. Dead bees, removed from the colonies by worker bees, were dropped in the trap as they try to fly through the mesh cover. The collected dead bees were separated during counting into adult worker bees, larvae and pupae. Inspection intervals were once per day from day -3 to day 21 after application of test item.

Behavioural abnormalities

Behavioural abnormalities of the bees at the colony entrance *e.g.* intensive cleaning, restlessness or moving coordination problems were recorded daily at the same time as mortality observations.

Development of bee brood

The honey bee brood was assessed at different expected stages during the development, covering one complete development period of the honey bee (*i.e.* one complete honey bee brood cycle, 21 days). The development of the bee brood in individually marked cells was observed by photographing the combs. At the assessment before the application (=BFD0; Brood Area Fixing Day), one (or several) brood comb(s) with an appropriate amount of eggs, young- and old larvae was (were) selected from of each colony under investigation and a digital photo of this (these) brood comb(s) was (were) taken, respectively. The comb was labelled with two pins as a mark for orientation and retrieval of marked positions on the comb. 150 cells containing eggs, 150 cells with young larvae and 150 cells with old larvae were selected, automatically numbered and marked by using an image analysis program (ImageJ). For each subsequent brood assessment (BFDn), the same comb(s) per individual colony was (were)

selected, and another digital photo was taken and saved. For each photo, the orientation points were marked again, in order to allow for an automated allocation of the previous marked cells. After retrieval of the cells for each assessment date, the cell content was assigned allowing continuous photo-documentation, starting with the first brood fixing date (BFD0) and continuing until the end of the assessment period. After completion of the cell assessments, a gallery of the cells was automatically generated. Therefore, the development of each individually marked cell throughout the duration of the test could be determined.

The different brood stages on the assessment dates were transcribed into indices (*e.g.* 0=empty; 1=egg; 2=young larvae, 3=old larvae; 4=pupa; 5=nectar; 6=pollen; 7=dead; 8=not classified) to calculate the termination rate.

If not enough development stages were found on one side of the selected comb per individual colony, the brood on the second side of this comb or an additional comb was selected, photographed and inspected accordingly. In all treatment groups and replicates, 150 cells were marked.

Bee brood assessment evaluation was conducted as follows:

Assessment date	Expected brood stage		
1 day pre application (BFDO)	Eggs (1)	Young larvae (2)	Old larvae (3)
5 days after BFDO	Young (2) to old (3)	Old larvae (3) or capped cells (4)	Capped cells (4)
9 days BFDO	Capped cells (4)	Capped cells (4)	Capped cells (4)
16 days after BFDO	Capped cells (4) shortly before hatch	All development stages, empty or food	Capped cells or all development stages, empty or food containing cells
22 days after BFDO	Empty cells or cells with eggs or young larvae or food	-	-

BFDO = Brood Area Fixing Day

Brood termination rate

Failure or incomplete development in individual cells was quantitatively assessed. For the calculation of the brood termination rate, the observed cells were split into 2 categories:

• the bee brood in the observed cell reached the expected brood stage at the different assessment days or was found empty or containing an egg or a small larva after hatch of the adult on BFD +22 (egg stage) or BFD +16 (young and old larvae) = successful development.

• the bee brood in the observed cell did not reach the expected brood stage at one of the assessment days, was empty or food/nectar was stored in the cell during BFD +5 to BFD +16 (eggs) or BFD +9 (larvae) = termination of the bee brood development.

If the cell did not contain the expected brood stage during BFD +5 and BFD +22 (eggs) or BFD +9 (larvae), the termination index of the cell was counted as 0. If the cell showed a successful bee brood development, the cell was counted as 1 (=successful development) at the end of the assessments.

The percentage of brood which did not successfully develop to an adult bee was determined by dividing the number of unsuccessfully developed cells by the total number of observed cells of the different brood stages and multiplied by 100.

5. Statistics/Data evaluation

The data were tested for normal distribution using Shapiro-Wilk`s test and homogeneity of variance using Levene`s test.

Mortality: A pairwise comparison (α = 0.05) was conducted for the mortality data (two-sided before application and one-sided greater, after application) using Student t-test for homogeneous variances.

Brood Development: A pairwise comparison (one-sided greater, α = 0.05) was conducted for the comparison of the brood data (egg and larvae termination rates), using Student t-test for homogenous variances.

The software used to perform the statistical analysis was Tox-Rat Professional, Version 2.10.05, ® ToxRat Solutions GmbH).

II. RESULTS AND DISCUSSION

A: ANALYTICAL VERIFICATION

No analytical verification of the dosing solutions was performed.

B: BIOLOGICAL DATA

Mortality

The starting conditions for the experiment were considered as ideal and equivalent for all colonies. During the three days before application the mean number of dead bees found in the traps was low and ranged from 6.6 to 14.2 dead bees per colony per day amongst the different groups. There was no statistically significant difference of the mortality of the adult worker bees between the treatment groups; Student t-test, pairwise, α = 0.05, two-sided.

Following the treatment with diflufenican SC 500A G, no direct (acute) toxicity occurred after ingestion of the test item treated sugar syrup. There was no increased mortality level in the test item treated replicates at any point in time of the test. Until test termination, the number of dead worker bees found in the dead bee traps in the colonies of the test item treated groups was comparable or even lower to the control group. On each of the assessment days, no statistically significant difference in the number of dead bees in the test item group was detectable when compared to the control values (Student t-test, pair-wise comparison to the control, one-sided greater, α = 0.05).

Over the entire post-application period from day 0 to day 21, a mean of 7.8 dead bees/colony/day was found in the dead bee traps of the test item treated colonies (300 ppm). In comparison a mean of 8.5 dead bees per colony/day was found in the control group. A comparison of the overall mean number of dead bees per treatment group for the entire post-application period (day 0 to day 21) did not show a statistically significant difference between the control and the test item treatment (Student t-test, pair-wise comparison to the control, one-sided greater, α = 0.05).

Treatment with the reference item Insegar (0.75 g a.s./L fenoxycarb) resulted during the post-application period (day 0 to day 21) in a slightly increased number of dead bees (18.7 dead bees per day per colony). This increased number of dead bees was statistically significantly higher when compared to the control (Student t-test, pairwise, one-sided greater, $\alpha = 0.05$).

Table: Summary of mortality date for worker bees

Day	Mean mortality (+ SD)		
	Control	Diflufenican SC 500A G	Reference item
-3 to -1 (before application)	8.9 (12.5)	6.6 (5.0)	14.2 (14.9)
0 – 21 (after application)	8.5 (6.3)	7.8 (7.6)	18.7 (26.4)

Mean of 3 colonies per treatment group

Mortality of pupae and larvae

Before application the number of dead pupae before the start of the test three days before application was low with a mean of 0.1 to 2.9 pupae/colony/day. No statistically significant difference amongst the treatment group could be detected (Student t-test, pairwise comparison, $\alpha = 0.05$, two-sided). Therefore, the conditions to start assessments for detection of effects on pupae/larvae mortality can be considered as ideal.

After application, the number of dead larvae found during the trial was low. Therefore, the displayed mortality values reflect the pupae and larvae mortality together. During the entire period from day 0, following the application, until day 21, a mean of 1.1 dead pupae/larvae per day and colony was found in the Diflufenican SC 500A G treatment group (300 ppm). In the control group, during the same time period, a mean of 1.7 dead pupae/larvae per day and colony was found. There was no statistically significant difference in the number of dead pupae/larvae between the colonies of the test item group and the colonies of the control group (Student t-test, pairwise comparison, $\alpha = 0.05$, one-sided greater).

Application of the reference item Insegar (0.75 g fenoxycarb/L) did not result in an increased number of dead pupae/larvae after application. During the same time period, a mean of 0.8 dead pupae/larvae per day and colony was found. This was not statistically significant compared to the value of the control colonies (Student t-test, pairwise comparison, $\alpha = 0.05$, one-sided greater).

Table: Summary of mortality date for pupae and larvae

Day	Mean mortality (+ SD)		
	Control	Diflufenican SC 500A G	Reference item
-3 to -1 (before application)	0.1 (0.2)	0.8 (0.8)	2.9 (3.2)
0 – 21 (after application)	1.7 (2.5)	1.1 (1.3)	0.8 (1.6)

Mean of 3 colonies per treatment group

Behavioural abnormalities

No behavioural impairments were noted at any time in the test or reference item treatment group until the end of the test.

Colony strength

The preliminary brood check indicated healthy colonies with all brood stages present and a sufficient supply with nectar and pollen. In all colonies the presence of a live and healthy queen or fresh laid eggs were observed. All stages of brood (eggs, larvae and capped brood) were found during the pre-

application check in all colonies in all treatment groups. In addition, sufficient nectar- and pollen stores were found in each colony as an indication of normal behaviour.

Development of bee brood

Following the assessment of single cells from the egg stage to the successfully hatched worker bee, the mean termination rate in the test item group was 40.2 % compared to 9.6 % in the control group. This higher mean termination rate in the test item group was not statistically significantly different (Student t-test, pairwise comparison, one-sided greater) when compared to control.

Comparing the development success of the young larvae after treatment with the test item to the corresponding control values, a slightly higher mean termination rate in the test item group was observed. In the test item group, 31.1 % of the marked young larvae did not reach the adult stage, whereas the termination rate in the control group was 24.4 %. When subjecting the data to statistical analysis (Student t-test, pairwise comparison, one-sided greater), the difference was found not to be statistically significant.

No statistically significant effect of the test item on old larvae was found. 22.2 % of the marked old larvae in the test item colonies have not completed their development, compared to 3.3 % in the control group (Student t-test, pairwise comparison, one-sided greater).

Treatment with the reference item Insegar resulted in a statistically significant loss of brood development of the marked eggs, young and old larvae, finally resulting in a termination rate of 99.8 % (eggs and young larvae) and 26.9 % (old larvae), respectively. The termination rates of the eggs and young larvae were statistically significantly different when compared to the corresponding control values (Student t-test, pairwise comparison, one-sided greater).

Table: Bee brood termination rate

Treatment	Eggs				Young larvae				Old larvae			
	BFD 0	BFD0 +22			BFD 0	BFD0 +22			BFD 0	BFD0 +22		
		TC	%	Mean %		TC	%	Mean %		TC	%	Mean %
Control	150	22	14.7	9.6	150	3	2.0	24.4	150	2	1.3	3.3
	150	13	8.7		150	28	18.7		150	8	5.3	
	150	8	5.3		150	79	52.7		150	5	3.3	
8.87DFF SC500A G	150	116	77.3	40.2	150	6	4.0	31.1	150	13	8.7	222
	150	50	33.3		150	59	39.3		150	8	5.3	
	150	15	10.0		150	75	50.0		150	79	52.7	
Reference item	150	150	100	99.8 *	150	150	100	99.8 *	150	7	4.7	26.9
	150	149	99.3		150	150	100		150	62	41.3	
	150	150	100		150	149	99.3		150	52	34.7	

TC: Terminated cells
BFD0: Brood Fixing Day 0
* Statistically significant compared to control (Student t-test, pairwise comparison, one-sided greater, α = 0.05)

C: VALIDITY CRITERIA

The study was not conducted according to a standard guideline but was based on Oomen et al (Method for honey bee brood feeding test with insect growth-regulating insecticides, OEPP/EPPO bulletin 22:613-616 (1992)). As such no validity criteria were specified. Validity set out in the study were

qualitative, stating that control mortality should not be considerable and that there should be a high number of impacted bees in the reference test treatments.

Mean control mortality of the adult bees from day 0 after application to day 21 ranged from 2.7 to 26.7 dead bees per colony. As the overall mean mortality in the control group after application was low (8.5 dead bees/colony/day), this value can be empirically regarded to be within the range of normal mortality levels of colonies under field conditions. In addition, a mean of 1.7 dead pupae per colony per day were found during the 21 days post-application period. This value can be considered to represent a biologically typical number of dead pupae over a period of 21 days.

The reference item treatment showed high number of impacted bee brood, which resulted in 75.5% mean loss of the initial observed cells (99.8% eggs, 99.8% young larvae and 26.9% old larvae stages, respectively). The termination rate of the eggs and young larvae termination rates were statistically significantly higher compared to the control. Thus, the reference item values were sufficiently high to demonstrate the sensitivity of the test system and the validity of the test conditions.

Therefore, it is considered that this study is valid for risk assessment purposes.

D: TOXICITY ENDPOINTS

Table: Summary of endpoints

Treatment		Control	Diflufenican SC 500A G	Reference item
Rate per L sugar solution (product)			0.72 g/L	3.0 g/L
Rate per L sugar solution (a.s.)			0.30 g a.s./L	0.75 g a.s./L
Termination rate of eggs (%)		9.6	40.2	99.8*
Termination rate of young larvae (%)		24.4	31.1	99.8*
Termination rate of old larvae (%)		3.3	22.2	26.9
Mean brood termination rate over all stages (%)		12.4	31.2	75.5*
Mean mortality of worker bees/colony/day	pre-application	8.9	6.6	14.2
	post-application	8.5	7.8	18.7*
Mean mortality of pupae/colony/day	pre-application	0.1	0.8	2.9
	post-application	1.7	1.1	0.8
Mean no. bees before application		16770	13455	13860

* Significant difference from control (students t-test, $\alpha = 0.05$, pair-wise comparison two-sided before application and one-sided greater after application

III. CONCLUSION

The mean termination rate of eggs was higher in the test item treatment group (40.2 %) when compared to the values of the control group (9.6 %), but this difference was not statistically significantly different when compared to the control group.

The development success of the young larvae in the test item treatment group was slightly lower and resulted in a mean termination rate of 31.1 % compared to 24.4 % in the control group. This difference was not statistically significant compared to the control group.

The mean termination rate of old larvae was higher in the test item treatment group (22.2 %) when compared to the values of the control group (3.3 %). The difference was not statistically significantly different to the control value.

The same holds true when considering the brood termination rate over all stages: the mean termination rate over all stages was slightly higher in the test item treatment group (31.2 %) when compared to the values of the control group (12.4 %), but again, the difference was not statistically significantly different.

Adult bee mortality in the test item treatment group was slightly lower (mean of 7.8 dead bees per day) and not statistically significantly different when compared to the control group (8.5 dead bees per day).

No effects of the test item on honey bee pupae and larvae were observed.

In contrast, the reference item treatment (Insegar, a.s. = fenoxycarb) resulted in a statistically significant increase of unsuccessful egg- and young larvae development, moreover, the mean brood termination rate over all stages was statistically significantly higher than in the control, which all confirmed the sensitivity of the test system and the validity of the test conditions.

Overall, it can be concluded according to the results of this study that the administration of Diflufenican SC 500A G fortified sugar syrup (300 ppm diflufenican) to honey bee colonies does neither adversely affect honey bee colonies nor bee brood development.

[redacted] *(2014)*

Assessment and conclusion by applicant:

The study was not conducted according to a standard guideline but was based on [redacted] (1992). As such no validity criteria were specified. Validity set out in the study were qualitative. There was no significant difference in mortality between the control and the test treatment, and from this it can be inferred that the validity criterion for control mortality was met.

The reference item treatment showed significant impact compared to the control in relation to brood termination rate, brood compensation rate and brood index, and from this it can be inferred that the validity criterion for considerable impact in the reference test treatment was met.

Therefore, it is considered that this study is valid for risk assessment purposes.

The mean termination rate of eggs, the development success of the young larvae, the mean termination rate of old larvae, the brood termination rate over all stages and adult bee mortality were not statistically significantly different when compared to the control group.

No effects of the test item on honey bee pupae and larvae were observed.

The reference item treatment resulted in a statistically significant increase of unsuccessful egg and young larvae development. The mean brood termination rate over all stages was statistically significantly higher than in the control confirming the sensitivity of the test system and the validity of the test conditions.

Overall, it can be concluded that the administration of diflufenican SC 500A G fortified sugar syrup (300 ppm diflufenican) to honey bee colonies does neither adversely affect honey bee colonies nor bee brood development.

Assessment and conclusion by RMS:

CP 10.3.2 Effects on non-target arthropods other than bees

A summary of the non-target arthropod toxicity endpoints for ACL + DFF SC 600 (500 + 100) G is provided in the following table.

Table 10.3-5: Non-target arthropod endpoints used in risk assessment

Test item	Test species	Time-scale Test type / substrate	Endpoint	Reference
ACL + DFF SC 600 (500 + 100) G	*Aphidius rhopalosiphi*	48 h Mortality Glass plate (2D)	**LR_{50} >700 mL formulation/ha**	KCP 10.3.2.1/01 M-574009-02-1 [redacted] 2019
		48 h Reproduction Glass plate (2D)	**ER_{50} >700 mL formulation/ha**	
ACL + DFF SC 600 (500 + 100) G	*Typhlodromus pyri*	14-d Mortality Glass plate (2D)	**LR_{50} = 204 mL formulation/ha**	KCP 10.3.2.1/02 M-572996-02-1 [redacted] 2019
		14-d Reproduction Glass plate (2D)	**ER_{50} = 124 mL formulation/ha**	
ACL + DFF SC 600 (500 + 100) G	*Typhlodromus pyri*	14-d Mortality Leaf disc (2D)	LR_{50} >700 mL formulation/ha	KCP 10.3.2.2/01 M-583199-02-1 [redacted] 2019
		14-d Reproduction Leaf disc (2D)	**ER_{50} 409 mL formulation/ha**	
ACL + DFF SC 600 (500 + 100) G	*Aleochara bilineata*	44-d Reproduction Natural soil (2D)	**ER_{50} >700 mL formulation/ha** NOER ≥700 mL formulation/ha	KCP 10.3.2.2/02 M-588143-01-1 [redacted] 2019
ACL + DFF SC 600 (500 + 100) G	*Typhlodromus pyri*	14-d Mortality Maize plant (3D) aged residues	700 mL formulation/ha Mortality of 0.0% at 0 DAT and 2.0% at 14 DAT	KCP 10.3.2.3/01 M-596590-01-1 [redacted], 2017
		14-d Reproduction Maize plant (3D) aged residues	700 mL formulation/ha Reduction of 8.1% reproduction at 0 DAT and 1.6% at 14 DAT	

Values in **bold** used in risk assessment

Summary of the risk assessment for ACL + DFF SC 600 (500 + 100) G on non-target terrestrial arthropods

The evaluation of the risk for non-target arthropods was performed in accordance with the recommendations of the "Guidance Document on Terrestrial Ecotoxicology", as provided by the Commission Services (SANCO/10329/2002 rev.2 (final), October 17, 2002), and in consideration of the recommendations of the guidance document ESCORT 2.

Predicted environmental rates (PER), in-field and off-field, were determined (according to ESCORT 2, [redacted]2000[11]) for the proposed uses of ACL + DFF SC 600 (500 + 100) G.

Based on the hazard quotients calculated based on Tier I (glass plate) LR_{50} values for *Typhlodromus pyri*, ACL + DFF SC 600 (500 + 100) G poses an unacceptable risk to the indicator species following the proposed uses as the calculated in-field HQ values were greater than the trigger value of 2.

A Tier II extended laboratory study was conducted with *Typhlodromus pyri* and also with an additional ground/leaf dwelling arthropod species, *Aleochara bilineata*.

Risk assessment following from these higher tier extended laboratory studies indicated an acceptable risk for *Aleochara bilineata*. Therefore, there is no risk on mortality and reproduction on additional soil-/foliage-dwelling non-target arthropods from the in-field scenario. However greater than 50% effects were observed in the study performed on *T. pyri* at application rates that were lower than the in-field PER thereby indicating unacceptable risk to *T.pyri*.

Consequently an aged residue test (3D) was conducted to determine the extent and duration of residual activity of ACL + DFF SC 600 (500 + 100) G on *T. pyri* following contact exposure to fresh, dry and semi-field aged spray residues on potted maize plants. Fresh dried or aged residues of ACL + DFF SC 600 (500 + 100) G had no significant effect on mortality or the reproductive capacity of *T.pyri* when applied at a rate of 0.7 L product/ha.

An acceptable risk to non-target arthropods can therefore be concluded from the use ACL + DFF SC 600 (500 + 100) G according to the proposed uses in the GAP.

Application scenario

According to the GAP table, ACL + DFF SC 600 (500 + 100) G is proposed to be applied to winter cereals at 0.7 or 0.35 L/ha (1 application), during BBCH 00-13. The following assessments have been made for the use of ACL + DFF SC 600 (500 + 100) G in winter cereals using an application rate of 0.7 L/ha as this will also cover the risks from the use at lower application rates.

Risk assessment for other non-target arthropods

The risk assessment for non-target arthropods has been conducted in line with ESCORT 2 ([redacted]2000).

In-field

Non-target arthropods can be exposed to residues from ACL + DFF SC 600 (500 + 100) G by direct contact either as a result of over-spray or through contact with residues on soil or in food items. The product, ACL + DFF SC 600 (500 + 100) G, is applied at a proposed maximum application of 0.7 L/ha.

11 [redacted](2000) Guidance document on regulatory testing and risk assessment procedures for plant protection products with non-target arthropods. From the ESCORT 2 workshop (European Standard Characteristics Of non-target arthropod Regulatory Testing)

The in-field exposure (predicted environmental residue, PER) is calculated according to ESCORT 2 using the following equation:

$$In - field\ PER = Application\ rate\ x\ MAF$$

The MAF is a generic multiple application factor, which is used to take into account the potential build-up of applied active substances between applications and is based on the application interval, the DT50 value on foliage and the number of applications. As ACL + DFF SC 600 (500 + 100) G is only applied once, the MAF value is 1.0 and hence the in-field PER is 0.7 L/ha (700 mL/ha).

Off-field

Risk assessment of areas immediately surrounding the crop is considered important since these areas represent potential natural reservoirs for immigration, emigration and reproduction of arthropod species and provide increased species diversity in the natural community. Exposure of non-target arthropods living in off-field areas to ACL + DFF SC 600 (500 + 100) G will mainly be due to spray drift from field applications. Off-field areas are assumed to be densely vegetated, and thus, spray drift is unlikely to reach bare ground.

The off-field exposure (predicted environmental residue, PER) is calculated according to ESCORT 2 using the following equation:

$$Off - field\ PER = Application\ rate\ x\ MAF\ x\ \frac{drift\ factor}{vegetation\ distribution\ factor}\ x\ correction\ factor$$

Vegetation distribution factor: The model used to estimate spray drift was developed for drift onto a two-dimensional water surface and, as such, does not account for interception and dilution by three-dimensional vegetation in off-crop areas. Therefore, a vegetation distribution or dilution factor is incorporated into the equation when calculating PERs to be used in conjunction with toxicity endpoints derived from two-dimensional (glass plate or leaf disc) studies. A dilution factor of 10 is recommended by ESCORT 2 and will be associated to endpoints from studies in 2D systems, while in case of 3D systems no vegetation distribution factor is used.

Drift factor: The drift factor value (%) at different distances varies depending on the crop and total number of applications; since a single application is intended, the drift value at 90th percentile of 2.77% in field crops at 1 m distance is used (Appendix VI, ESCORT 2, Candolfi et al. 2000).

Correction factor: As recommended by ESCORT 2, correction factors of 10 and 5 are used respectively for Tier I and Tier II assessments.

Table 10.3-6: Calculation of Tier I off-field PER value for ACL + DFF SC 600 (500 + 100) G

Crop	Max single application rate (mL/ha)	Drift factor %	Vegetation distribution factor	Correction factor	MAF	Off-field PER (mL/ha)
Tier I assessment (based on 2D studies)						
Winter cereals	700	2.77	10	10	1.0	19.39

Calculation of the Tier I in-field and off-field Hazard Quotients (HQ)

The risk to non-target arthropods is assessed using the approach recommended in the published ESCORT 2 document ([redacted] 2001 and SANCO/10329/2002).

The potential risk of ACL + DFF SC 600 (500 + 100) G to non-target arthropods was assessed by calculation of the hazard quotient (HQ) using the equation below. The input values were based on the predicted environmental residue (PER) and the lowest lethal rate (LR_{50}) values for both sensitive species exposed to ACL + DFF SC 600 (500 + 100) G.

$$HQ = \frac{PER}{LR_{50}}$$

The HQ values based on Tier I laboratory studies are evaluated against a trigger value of 2. If values are above the trigger a risk to non-target arthropods is indicated and further higher-tier assessment to address the potential risk is required. The resulting $HQ_{in\text{-}field}$ and $HQ_{off\text{-}field}$ values for non-target arthropods are presented in the following table.

Table 10.3-7: Tier I In-field and Off-field HQs for non-target arthropods exposed to ACL + DFF SC 600 (500 + 100) G

Species	LR_{50} (mL/ha)	In-field PER (mL/ha)	$HQ_{in\text{-}field}$	Off-field PER (mL/ha)	$HQ_{off\text{-}field}$	Trigger
Aphidius rhopalosiphi	>700	700	<1.0	19.39	0.0277	2
Typhlodromus pyri	204		**3.43**		0.0950	

Values in **bold** indicate unacceptable risks

The in-field and off-field HQ values for *Aphidius rhopalosiphi* and the off-field HQ values for *Typhlodromus pyri* were below the Tier I trigger value of 2. However the in-field HQ values for *Typhlodromus pyri* were below the Tier I trigger value and hence a Tier II assessment is necessary and is presented below.

Tier II in-field assessment (extended laboratory study)

As the HQ value for *Typhlodromus* exceeds the trigger value for the in-field habitats, higher-tier testing is required. According to ESCORT 2 one additional species should be tested if the HQs are only exceeded for the in-field risk assessment. In the case of ACL + DFF SC 600 (500 + 100) G (early spray application on bare soil) *Aleochara bilineata* should preferably be used.

Table 10.3-8: Tier II assessment of the in-field risk for non-target organisms exposed to ACL + DFF SC 600 (500 + 100) G

Species	Rate with ≤ 50% effect (mL/ha)	In-field PER (mL/ha)	In-field PER below rate with ≤ 50% effect?
Typhlodromus pyri	409	700	No
Aleochara bilineata	>700		Yes

These Tier II extended laboratory studies indicated an acceptable risk for *Aleochara bilineata*. Therefore, there is no risk on mortality and reproduction on additional soil-/foliage-dwelling non-target arthropods from the in-field scenario. However, greater than 50% effects were observed in the study

performed on *T. pyri* at application rates that were lower than the in-field PER thereby indicating unacceptable risk to *T. pyri*.

Higher-tier risk assessment

To demonstrate that effects on predatory mites due to an exposure on treated plants will not be long-lasting, an aged residue study (M-596590-01-1) was conducted with maize plants at an application rate of 0.7 L/ha. Fresh dried or aged residues of ACL + DFF SC 600 (500 + 100) G did not have a significant effect on either the mortality or the reproductive capacity of *T.pyri* when applied at a rate of 0.7 L product/ha.

It can therefore be concluded that no long-lasting effects on foliage dwelling non-target arthropods with a sensitivity similar to *Typhlodromus pyri* are to be expected from the exposure to ACL + DFF SC 600 (500 + 100) G according to the proposed use pattern.

Concerning the effects on soil dwelling non-target arthropods the study on *Aleochara bilineata* indicated no adverse effects at the maximum intended application rate of 0.7 L/ha (700 mL/ha).

Therefore, it can be concluded that the application of ACL + DFF SC 600 (500 + 100) G with application rates up to 0.7 L/ha (700 mL/ha) will not result in unacceptable adverse effects on non-target arthropods.

CP 10.3.2.1 Standard laboratory testing for non-target arthropods

Data Point:	KCP 10.3.2.1/01
Report Author:	[redacted]
Report Year:	2019
Report Title:	Amendment no. 1 to toxicity to the parasitoid wasp Aphidius rhopalosiphi (Hymenoptera: Braconidae) using a laboratory test diflufenican + aclonifen SC 600 (100+500 g/L)
Report No:	CW16009-A1
Document No:	M-574009-02-1
Guideline(s) followed in study:	EU Directive 91/414/EEC Regulation (EC) No. 1107/2009 US EPA OCSPP Not Applicable
Deviations from current test guideline:	Current guideline: [redacted] (2000) No Deviation
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

A study was conducted to determine the effect of aclonifen + diflufenican SC 600 on mortality of the parasitoid wasp, *Aphidius rhopalosiphi* (Hymenoptera, Braconidae), after 48 hours of exposure on a treated glass surface. Additionally, an assessment for significant sublethal effects (parasitisation activity) was made.

The nominal exposure concentrations were 70, 124, 221, 394 and 700 mL formulation/ha in 200 L deionised water. The toxic reference item (dimethoate) was prepared at the nominal exposure concentration of 0.095 mL product/ha in 200 L deionised water. The control comprised deionised water only. Adult parasitoids were exposed to dried spray residues on glass plates for 48 hours. Mortality was assessed after 2, 24 and 48 hours of exposure. Four replicates each containing 15 parasitoids were used per treatment group. For the reproduction assessment 15 surviving females were removed from the exposure units and their reproductive capacity was assessed by confining them individually over untreated barley plants infested with the host cereal aphids, *Rhopalosiphum padi*. The females were removed after 24 hours and the aphid-infested plants left for further 141 days before the numbers of aphid mummies that had developed were assessed.

After 48 hours 3.3% of the wasps were found dead in the control group. Corrected mortality in the treatment groups were not statistically significantly different compared to the control. In the reference item group, all wasps were dead after 48 hours of exposure. The NOER (no observed effect rate) for mortality was ≥700 mL formulation/ha. The LR_{50} was estimated to be >700 mL formulation/ha.

The mean number of mummies per female in the control group was 39.5. The reduction in reproductive success relative to the control was not no statistical significance compared to the control in any treatment group. The NOER (no observed effect rate) for reproduction was ≥700 mL formulation/ha. The ER_{50} was estimated to be >700 mL formulation/ha.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test Item:** Aclonifen + diflufenican SC 600
   Lot no.: TOX 20193-00
   Active Ingredient / Purity: Aclonifen: 41.1% w/w (505.1 g/L)
   Diflufenican: 8.21% w/w (100.0 g/L)
   Appearance: Yellow suspension
   Storage: 25 ± 2 °C (storage conditions from +2 °C to +30 °C are acceptable)
   Expiry date: 12 January 2017

2. **Reference item:** Dimethoate EC 400 g/L
   Batch no.: BAS 152 11 I
   Active Ingredient / Purity: Dimethoate, 420.3 g/L

3. **Test Organism:** *Aphidius rhopalosiphi*
   Age: Adults, less than 48 hours old
   Source: [redacted]
   Acclimatisation: Approximately 2 days under test conditions
   Feeding: Honey in water (1:3)

A. STUDY DESIGN AND METHODS

1. In-life phase:	15 – 29 February 2016

2. Exposure conditions

Test vessels:	*Exposure units:* 2 treated glass plates (10 cm × 10 cm) with corresponding glass covers and an untreated acrylic frame (internal dimensions 92 × 92 × 14 mm) with 3 × ventilation holes covered with gauze *Post-exposure units:* potted barley plants, infested with the host aphids of all developmental stages (*Rhopalosiphum padi*) was enclosed by a polyacrylic cylinder (185 mm high, 85-120 mm diameter) with a fine gauze on the top. Cylinder was vented to avoid condensation on walls
Experimental design:	7 experimental groups: control (tap water), test item (5 groups, nominally 70, 124, 221, 394 and 700 mL formulation/ha in 200 L deionised water) and toxic standard (dimethoate).
Replicates:	exposure period: 4 exposure units (replicates) per treatment group
Loading:	15 wasps per replicate
Temperature:	Temperature controlled rooms at 19.0 – 21.0 °C
Relative humidity:	60 - 90% Note: short deviations in test conditions (<2h) were not considered to affected study outcome. Conditions were continuously monitored and recorded by data logger
Photoperiod:	16 h light: 8 h dark
Light intensity:	mortality phase: 677 - 1170 lux parasitisation phase: 620 – 857 lux reproduction phase: 14200 - 16590 lux
Ventilation:	Ventilation holes covered with gauze (80 µm mesh size)

3. Administration of the test item

Dose preparation

Suspensions of test and reference item were prepared on the day of application. The test item and reference item test concentrations were prepared in deionised water. The nominal exposure concentrations were 70, 124, 221, 394 and 700 mL formulation/ha in 200 L deionised water. The reference item was prepared at the nominal exposure concentration of 0.095 mL product/ha in 200L deionised water. The control comprised deionised water only. The test item was applied to an inert substrate (glass plate) using a linear cabinet track sprayer, at a rate of 200 L deionised water/ha. After spray coating had dried the glass plates were removed to corresponding frames.

Test organism assignment and exposure

The study encompassed 7 treatment groups (5 x test item, 1 x control, 1 x reference item) with 4 replicates each containing 15 adult parasitoid wasps. The parasitoid wasps were exposed to dried

residues on treated glass plates. Survival of the parasitoid wasps was assessed 2, 24 and 48 hours after application.

For the reproduction assessment 15 females per test group were impartially selected from surviving females and were then confining individually over untreated barley plants infested with the host cereal aphids, *Rhopalosiphum padi*. The females were removed after 24 hours and the aphid-infested plants left for further 11 days before the numbers of aphid mummies that had developed were assessed.

4. Measurements and observations

Observations of mortality were recorded approximately 2, 24 and 48 hours after test initiation. Wasps were defined as live (alive and apparently unaffected), affected (any abnormal behaviour / reduced co-ordination), moribund (unable to walk but still moving legs/antennae), dead (no longer moving). The number of parasitoid wasps affected were recorded.

Number of aphid mummies were counted 11 days after the 24 hour parasitisation period. Due to the high mortality no reproduction testing was performed with the reference item.

5. Statistics/Data evaluation

For the three assessment dates (2 h, 24 h, 48 h) the number of moribund and dead wasps was summed up each replicate and calculated as percentage. A mean value of the replicates was calculated. Corrected mortality was obtained by comparing the values observed in the treated samples with those in the control samples, according to the formula of [redacted] (1947).

Reproductive performance was calculated for each replicate and expressed as mummies (m) per female (fem).

The mortality data were analysed for significance using the Fisher Exact test (one-sided with [redacted] adjustment; $\alpha = 0.05$), which is a distribution-free test method and does not require testing for normality or homogeneity of variance prior analysis.

The reproduction data were tested for normal distribution using the Shapiro-Wilk test and for homogeneity of variance using the Levene test. As the reproduction data in this study were not normally distributed the Wilcoxon test (one-sided with Bonferroni-Holm adjustment; $\alpha = 0.05$) was used.

The computer program SAS (Version 9.4, 2002-2012) was used to perform the statistical analyses.

II. RESULTS AND DISCUSSION

A: ANALYTICAL VERIFICATION

Analytical verification was not required.

B: BIOLOGICAL DATA

Mortality

After 48 hours 3.3% of the wasps were found dead in the control group. In the groups treated with 70, 124, 221 and 394 mL product/ha 1.7%, -1.7%, 3.4% and 1.7% corrected mortality were detected, respectively. In the highest test item rate of 700 mL product/ha a corrected mortality of 5.2% was observed. These were not statistically significantly different compared to the control. In the reference

item group, all wasps were dead after 48 h of exposure. The NOER (no observed effect rate) for mortality was ≥700 mL product/ha. The LR_{50} was estimated to be >700 mL product/ha.

Table: Effects of aclonifen + diflufenican SC 600 on mortality of the parasitoid wasp, *Aphidius rhopalosiphi*, exposed to fresh dried residue in the laboratory

Treatment (mL formulation/ha)	Mortality after 48h (%)		P-value*
	Uncorrected	Corrected	
Control	3.3	-	-
70	5.0	1.7	1.0
124	1.7	-1.7	1.0
221	6.7	3.4	1.0
394	5.0	1.7	1.0
700	8.3	5.2	1.0
Reference item	100	100	-

*: Significant difference compared to control (Fisher's Exact test, one-sided, p-values adjusted according to Bonferroni-Holm)

Reproduction

The mean number of mummies per female in the control group was 39.5. This compared to 30.9 mummies/female in the 70 mL product/ha rate of the test item, 33.4 mummies/female in the 124 mL product/ha rate, 38.7 mummies/female in the 221 mL product/ha rate, 26.5 mummies/female in the 394 mL product/ha rate and 34.8 mummies/female in the 700 mL product/ha rate of diflufenican + aclonifen SC 600 g/L.

The reduction in reproductive success relative to the control at the 70, 124 and 221 mL product/ha rate was 21.6%, 15.4% and 1.9%, respectively. A reduction of 32.8% at the 394 ml product/ha rate and of 11.8% at the at the 700 mL product/ha rate of diflufenican + aclonifen SC 600 g/L was detected. There was no statistical significance compared to the control. The NOER (no observed effect rate) for reproduction was ≥700 mL product/ha. The ER_{50} was estimated to be >700 mL product/ha.

Table: Effects of aclonifen + diflufenican SC 600 on reproduction of the parasitoid wasp, *Aphidius rhopalosiphi*, exposed to fresh dried residue in the laboratory

Nominal Application Rate (mL formulation/ha)	Reproduction		
	Rate (mummies per female)	Reduction relative to control (%)	P-value*
Control	39.5	-	
70	30.9	21.6	0.426
124	33.4	15.4	0.449
221	38.7	1.9	0.442
394	26.5	32.8	0.249
700	34.8	11.8	0.418
Reference item	n.a.	n.a.	

*: Significant difference compared to control group (Wilcoxon, one-sided, p-values adjusted according to [redacted]

[redacted]

C. VALIDITY CRITERIA

Validity criterion	Required	Achieved
Control mortality	≤13%	3.3%
Reference item mortality	≥50%	100%
Mean reproduction per female in control	≥5	39.5
No. wasps in control producing no mummies	≤2	2

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint	Nominal Application Rate (mL formulation/ha)
$LR_{50\ mortality}$	>700
$ER_{50\ reproduction}$	>700
NOEC	≥700

III. CONCLUSION

The LR_{50} was estimated to be >700 mL product/ha. The NOER for mortality was ≥700 mL formulation/ha.

[redacted] *(2019)*

Assessment and conclusion by applicant:

All validity criteria were satisfied and therefore this study can be considered to be valid.

The LR_{50} was estimated to be >700 mL product/ha. The NOER for mortality was ≥700 mL formulation/ha.

The ER_{50} was estimated to be > 700 mL product/ha. The NOER for reproduction was ≥700 mL formulation/ha.

Assessment and conclusion by RMS:

Data Point:	KCP 10.3.2.1/02
Report Author:	[redacted]
Report Year:	2019
Report Title:	Amendment No. 1 to toxicity to the predatory mite Typhlodromus pyri (Acari: Phytoseiidae) using a laboratory test - Diflufenican + aclonifen SC 600 (100+500 g/L)
Report No:	CW16/008-A1
Document No:	M-572996-02-1
Guideline(s) followed in study:	EU Directive 91/414/EEC Regulation (EC) No. 1107/2009 US EPA OCSPP Not Applicable
Deviations from current test guideline:	Current guideline: Blumel et al. (2000) No Deviation
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

A study was conducted to determine the effect of aclonifen + diflufenican SC 600 on mortality and reproduction of the predatory mite, *Typhlodromus pyri* (Acari: Phytoseiidae), after 14 days of exposure on a treated glass surface.

The nominal exposure concentrations were 70, 124, 221, 394 and 700 mL formulation/ha in 200 L deionised water. The reference item was prepared at the nominal exposure concentration of 11.9 mL formulation/ha in 200 L deionised water. The control comprised deionised water only.

On days 4, 7, 10, 12 and 14 the number of dead and living mites was counted. In addition, from day 7 the number of females, males, eggs and juveniles was counted

The mortality / escaping rate in the control group up to day 7 after treatment was 4.0%. At the lowest test item rate of 70 mL formulation/ha, a corrected mortality of 7.3% was observed, which was not statistically significantly different compared to the control. All other test item rates showed statistical

significance. The LR_{50} was calculated to be 204 mL formulation/ha. The NOER (no observed effect rate) for mortality was 70 mL formulation/ha.

Reproduction was assessed for the two lowest rates of diflufenican + aclonifen SC 600 g/L, 70 and 124 mL formulation/ha. At the 70 mL formulation/ha rate, the reproduction was reduced by 45.7% and by 46.3% at the 124 mL formulation/ha rate, both were statistically significantly different compared to the control. The ER_{50} was estimated to be < 70 mL formulation/ha. The NOER (no observed effect rate) for reproduction was > 124 mL formulation/ha.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test Item:** Aclonifen + diflufenican SC 600
   Lot no.: TOX 20193-00
   Active Ingredient / Purity: Aclonifen: 41.1% w/w (505.1 g/L)
   Diflufenican: 8.21% w/w (101.0 g/L)
   Appearance: Yellow suspension
   Storage: 25 ± 2 °C (storage conditions from +2 °C to +30 °C are acceptable)
   Expiry date: 12 January 2017

2. **Reference item:** Dimethoate EC 400 g/L
   Batch no.: BAS 152 11 I
   Active Ingredient / Purity: Dimethoate, 420.3 g/L

3. **Test Organism:** *Typhlodromus pyri*
   Age: Protonymphs
   Source: [redacted]
   Acclimatisation: Approximately 2 days under test conditions
   Feeding: Pollen mix (Birch:Pine, 1:1)

A. STUDY DESIGN AND METHODS

1. **In-life phase:** 1 - 15 April 2016

2. Exposure conditions

Test vessels: Two glass cover slides (24 x 60 mm) put together so that their longitudinal sides touch and leave a narrow gap. Floral foam (approx. 48 x 60 x 40 mm) in a plastic tray with water which was sucked up by the floral foam. On top of the floral foam was tissue paper and cover slides. To avoid mites escaping a barrier of sticky material was placed in the middle of the cover slides. Each exposure unit was transferred to a plastic tray filled with deionised water. The narrow gap between the two cover slides was filled with water by capillary forces to provide mites with

	water. The units were prepared one day before application to ensure floral foam was saturated with water
Experimental design:	7 experimental groups: control (tap water), test item (5 groups, nominally 70, 124, 221, 394 and 700 mL formulation/ha in 200L deionised water) and toxic standard (dimethoate)
Replicates:	5 exposure units (replicates) per treatment group
Loading:	20 mites per replicate
Temperature:	Temperature controlled rooms at 24.0 – 25.0 °C
Relative humidity:	60 – 72% Note: short deviations in test conditions (<2h) were not considered to affected study outcome. Conditions were continuously monitored and recorded by data logger
Photoperiod:	16 h light: 8 h dark
Light intensity:	110 - 415 lux

3. Administration of the test item

Dose preparation

Suspensions of test and reference item were prepared on the day of application. The test item and reference item test concentrations were prepared in deionised water. The nominal exposure concentrations were 70, 124, 221, 394 and 700 mL formulation/ha in 200 L deionised water. The reference item was prepared at the nominal exposure concentration of 11.9 mL formulation/ha in 200L deionised water. The control comprised deionised water only. The test item was applied to an inert substrate (glass plate) using a linear cabinet track sprayer, at a rate of 200 L deionised water/ha. After spray coating had dried (partly) the glass plates were removed back to plastic trays.

Test organism assignment and exposure

The study encompassed 7 treatment groups (5 x test item, 1 x control, 1 x reference item) with 4 replicates each containing 20 protonymph predatory mites. The predatory mites were exposed within 1.5 hours after application of test item. Pollen mix (birch + pine) was supplied as food.

4. Measurements and observations

On days 4, 7, 10, 12 and 14 the number of dead and living mites was counted. Dead mites were removed with a fine brush. The number of escaped mites was calculated, and food was replenished. In addition, from day 7 the number of females, males, eggs and juveniles was counted. Eggs and juveniles were removed. Food was replenished at each observation point.

5. Statistics/Data evaluation

The number of living and dead mites was counted and recorded on the assessment dates. At day 7 of the study, the number of dead and escaped mites was summed up for each replicate and calculated as percentage. A mean value of the five replicates was calculated. Mites that could not be found on the test units or which stuck in the glue barrier were recorded as escapees and added to those which had died. The corrected mortality was obtained by comparing the values observed in the treated samples with those in the control samples, according to the formula of [redacted] (1947).

Reproductive performance was determined by counting the number of females and eggs.

The mortality data were analysed for significance using the Fisher Exact test (one-sided with Bonferroni-Holm adjustment; α = 0 .05), which is a distribution-free test method and does not require testing for normality or homogeneity of variance prior analysis.

The reproduction data were tested for normal distribution using the Shapiro-Wilk test and for homogeneity of variance using the Levene test. As the reproduction data in this study were normally distributed and homogenous one-way ANOVA and the Williams test (one-sided; α = 0.05) were used. The LR_{50} value was calculated using Probit analysis.

The computer program SAS (Version 9.4, 2002-2012) was used to perform the statistical analyses.

II. RESULTS AND DISCUSSION

A: ANALYTICAL VERIFICATION

Analytical verification was not required.

B: BIOLOGICAL DATA

Mortality

The mortality / escaping rate in the control group up to day 7 after treatment was 4.0%. At the lowest test item rate of 70 mL formulation/ha, a corrected mortality of 7.3% was observed, which was not statistically significantly different compared to the control. All other test item rates showed statistical significance with 28.1% corrected mortality at the 124 mL formulation/ha rate and 66.7%, 76.0% and 83.3% at the 221, 394 and 700 mL formulation/ha rates, respectively.

The LR_{50} was calculated to be 204 mL formulation/ha.

The NOER (no observed effect rate) for mortality was 70 mL formulation/ha.

In the reference item group a corrected mortality of 91.7% was observed on day 7 of the study.

Table: Effects of aclonifen + diflufenican SC 600 on mortality of the predatory mite, *Typhlodromus pyri*, exposed to fresh dried residue in the laboratory

Treatment (mL formulation/ha)	Mortality after 7days (%)		P-value*
	Uncorrected	Corrected	
Control	4	-	-
70	11	7.3	0.052
124	31	28.1	<0.001*
221	68	66.7	<0.001*
394	77	76.0	<0.001*
700	84	83.3	<0.001*
Reference item	92	91.7	-

*: Significant difference compared to control (Fisher's Exact test, one-sided, α = 0.05, p-values adjusted according to Bonferroni-Holm)

Reproduction

Reproduction was assessed for the two lowest rates of diflufenican + aclonifen SC 600 g/L, 70 and 124 mL formulation/ha. The mean number of offspring produced per female in the control group was 5.03. This compared to 2.73 eggs/female in the 70 mL formulation/ha rate of the test item and 2.70 eggs/female in the 124 mL formulation/ha rate (all rates refer to diflufenican + aclonifen SC 600 g/L). At the 70 mL formulation/ha rate, the reproduction was reduced by 45.7% and by 46.3% at the 124 mL formulation/ha rate, both were statistically significantly different compared to the control.

The ER_{50} was estimated to be < 70 mL formulation/ha.

The NOER (no observed effect rate) for reproduction was > 124 mL formulation/ha.

Table: Effects of aclonifen + diflufenican SC 600 on parasitisation efficiency of the predatory mite, *Typhlodromus pyri*, exposed to fresh dried residue in the laboratory

Nominal Application Rate (mL formulation/ha)	Reproduction		
	Rate (mummies per female)	Reduction relative to control (%)	P-value*
Control	5.03	-	
70	2.73	45.7	0.006*
124	2.70	46.3	0.007*
221	n.a.	n.a.	-
394	n.a.	n.a.	-
700	n.a.	n.a.	-
Reference item	n.a.	n.a.	-

*: Significant difference compared to control group (one-way ANOVA, Williams one-sided)

C. VALIDITY CRITERIA

Validity criterion	Required	Achieved
Control mortality	≤20%	4.0%
Reference item mortality (corrected)	≥50%	91.7%
Mean no. of eggs/female in control (from day 7)	≥4	5.03

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint	Nominal Application Rate (mL formulation/ha)
$ER_{50\ mortality}$	204
$NOEC_{\ mortality}$	70
$ER_{50\ reproduction}$	>124
$NOEC_{\ reproduction}$	<70

III. CONCLUSION

The LR_{50} was estimated to be 204 mL formulation/ha. The NOEC for mortality was 70 mL formulation/ha.

The ER_{50} was estimated to be >124 mL formulation/ha. The NOEC for reproduction was 70 mL formulation/ha.

[redacted] (2019)

Assessment and conclusion by applicant:

All validity criteria were satisfied and therefore this study can be considered to be valid.

The LR_{50} was estimated to be 204 mL formulation/ha. The NOEC for mortality was 70 mL formulation/ha.

The ER_{50} was estimated to be >124 mL formulation/ha. The NOEC for reproduction was 70 mL formulation/ha.

Assessment and conclusion by RMS:

CP 10.3.2.2 Extended laboratory testing, aged residue studies with non-target arthropods

Data Point:	KCP 10.3.2.2.01
Report Author:	[redacted]
Report Year:	2019
Report Title:	Amendment no. 1 to toxicity to the predatory mite Typhlodromus pyri (Acari: Phytoseiidae) using an extended laboratory test on bean diflufenican + aclonifen SC 600 (100+500 g/L)
Report No:	CW16/059-A1
Document No:	M-583199-02-1
Guideline(s) followed in study:	EU Directive 91/414/EEC; Regulation (EC) No. 1107/2009; US EPA OCSPP not applicable; BLUEMEL ET AL. (2000) modified; CANDOLFI ET AL. (2001)
Deviations from current test guideline:	Current guideline: Blumel et al. (2000) No Deviation
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

A study was conducted to determine the effect of aclonifen + diflufenican SC 600 on mortality and reproduction of the predatory mite, *Typhlodromus pyri* (Acari: Phytoseiidae), after 14 days of exposure on a treated glass surface.

The nominal exposure concentrations were 70, 124, 221, 394 and 700 mL formulation/ha in 200 L deionised water. The reference item was prepared at the nominal exposure concentration of 119 mL formulation/ha in 200L deionised water. The control comprised deionised water only.

On days 4, 7, 10, 12 and 14 the number of dead and living mites was counted. In addition, from day 7 the number of females, males, eggs and juveniles was counted

The mortality / escaping rate in the control group up to day 7 after treatment was 4.0%. At the lowest test item rate of 70 mL formulation/ha, a corrected mortality of 7.3% was observed, which was not statistically significantly different compared to the control. All other test item rates showed statistical significance. The LR_{50} was calculated to be 204 mL formulation/ha. The NOER (no observed effect rate) for mortality was 70 mL formulation/ha.

Reproduction was assessed for the two lowest rates of diflufenican + aclonifen SC 600 g/L, 70 and 124 mL formulation/ha. At the 70 mL formulation/ha rate, the reproduction was reduced by 45.7% and by 46.3% at the 124 mL formulation/ha rate, both were statistically significantly different compared to the control. The ER_{50} was estimated to be < 70 mL formulation/ha. The NOER (no observed effect rate) for reproduction was > 124 mL formulation/ha.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test Item:** Aclonifen + diflufenican SC 600
   Lot no.: TOX 20193-00
   Active Ingredient / Purity: Aclonifen: 41.0% w/w (504.2 g/L)
   Diflufenican: 8.00% w/w (99.69 g/L)
   Appearance: Yellow dispersion
   Storage: 25 ± 2 °C (storage conditions from +2 °C to +30 °C are acceptable)
   Expiry date: 29 November 2018

2. **Reference item:** Dimethoate EC 400 g/L
   Batch no.: BAS 152 11 I
   Active Ingredient / Purity: Dimethoate, 420.3 g/L

3. **Test Organism:** *Typhlodromus pyri*
   Age: Protonymphs, <24 hours old at study start
   Source: [redacted]
   Acclimatisation: Approximately 2 days under test conditions
   Feeding: Pollen mix (Birch:Pine, 1:1)

A. STUDY DESIGN AND METHODS

1.	**In-life phase:**	9 to 23 December 2016

2. Exposure conditions

Test vessels:	A treated *Phaseolus vulgaris* leaf disc was laid on a layer of wet filter paper on top of a water soaked floral foam. A circle of insect glue (approx. 40 mm) was formed on the leaves. Units were placed on a plastic tray such that the filter paper was constantly provided with deionised water
Experimental design:	7 experimental groups: control (tap water), test item (5 groups, nominally 70, 124, 221, 394 and 700 mL formulation/ha in 200L deionised water) and toxic standard (dimethoate)
Replicates:	5 exposure units (replicates) per treatment group
Loading:	20 protonymphs per replicate
Temperature:	Temperature controlled rooms at 24.0 – 25.0 °C
Relative humidity:	60 – 72% Note: short deviations in test conditions (<2h) were not considered to affected study outcome. Conditions were continuously monitored and recorded by data logger
Photoperiod:	16 h light: 8 h dark
Light intensity:	110 – 415 lux

3. Administration of the test item

Dose preparation

Suspensions of test and reference item were prepared on the day of application. The test item and reference item test concentrations were prepared in deionised water. The nominal exposure concentrations were 70, 124, 221, 394 and 700 mL formulation/ha in 200 L deionised water. The reference item was prepared at the nominal exposure concentration of 47.6 mL formulation/ha in 200L deionised water. The control comprised deionised water only.

The test item was applied to the upper side of detached *Phaseolus vulgaris* leaf disc using a linear cabinet track sprayer, at a rate of 200 L deionised water/ha.

Test organism assignment and exposure

The study encompassed 7 treatment groups (5 x test item, 1 x control, 1 x reference item) with 4 replicates each containing 20 protonymph predatory mites. The predatory mites were exposed within 1.5 hours after application of test item. Pollen mix (birch + pine) was supplied as food.

4. Measurements and observations

On days 4, 7, 10, 12 and 14 the number of dead and living mites was counted. Dead mites were removed with a fine brush. The number of escaped mites was calculated, and food was replenished. In addition, from day 7 the number of females, males, eggs and juveniles was counted. Eggs and juveniles were removed. Food was replenished at each observation point.

5. Statistics/Data evaluation

The number of living and dead mites was counted and recorded on the assessment dates. At day 7 of the study, the number of dead and escaped mites was summed up for each replicate and calculated as percentage. A mean value of the five replicates was calculated. Mites that could not be found on the test units or which stuck in the glue barrier were recorded as escapees and added to those which had died. The corrected mortality was obtained by comparing the values observed in the treated samples with those in the control samples, according to the formula of Schneider-Orelli (1947).

Reproductive performance was determined by counting the number of females and eggs.

The mortality data were analysed for significance using the Fisher Exact test (one-sided with Bonferroni-Holm adjustment; α = 0 .05), which is a distribution-free test method and does not require testing for normality or homogeneity of variance prior analysis.

The reproduction data were tested for normal distribution using the Shapiro-Wilk test and for homogeneity of variance using the Levene test (α = 0 .05). As the reproduction data in this study were normally distributed but not homogenous the Welch test (α = 0.05) was used. The ER_{50} value was calculated using Probit analysis.

The computer program SAS (Version 9.4, 2002-2012) was used to perform the statistical analyses.

II. RESULTS AND DISCUSSION

A: ANALYTICAL VERIFICATION

Analytical verification was not required.

B: BIOLOGICAL DATA

Mortality

The mortality / escaping rate in the control group up to day 7 after treatment was 14.0%. In all test item rates no statistically significantly different mortality compared to the control was found (Fisher`s Exact test (one-sided, α = 0.05). The corrected mortality was below 11%.

The NOER (no observed effect rate) for mortality was ≥700 mL product/ha.

The LR_{50} (lethal rate causing 50% mortality) was estimated to be >700 mL product/ha.

In the reference item group 92% of the mites were dead on day 7 of the study.

Table: Effects of aclonifen + diflufenican SC 600 on mortality of the predatory mite, *Typhlodromus pyri*, exposed on detached bean leaves (*Phaseolus vulgaris*)

Treatment (mL formulation/ha)	Mortality after 7days (%)		P-value*
	Uncorrected	Corrected	
Control	14	-	-
70	21	8.1	0.528
124	14	0	1.000
221	17	3.5	1.000
394	8	-7.0	1.000
700	23	10.5	0.361
Reference item	92	90.7	-

*: Significant difference compared to control (Fisher's Exact test, one-sided, α = 0.05, p-values adjusted according to [redacted])

Reproduction

The mean number of offspring produced per female in the control group was 5.0. This compared to 4.4 eggs/female in the 70 mL product/ha rate of the test item, 4.2 eggs/female in the 124 mL product/ha rate, 3.1 eggs/female in the 221 mL product/ha rate, 2.6 eggs/female in the 394 and 2.2 eggs/female in the 700 mL product/ha rate (all rates refer to mL product/ha rate).

The reproduction was reduced by 12.1% at the 70 mL product/ha rate and by 15.8% at the 124 mL product/ha rate. At the higher test item rates of 221, 394 and 700 mL product/ha, the reduction was 37.5%, 48.5% and 57.3%, respectively. All tested test item rates showed statistical significance in reduction compared to the control, except the lowest test item rate of 70 mL product/ha (Welch test; one-sided, α = 0.05).

The NOER (no observed effect rate) for reproduction was 70 mL product/ha.

The ER_{50} (effect rate causing 50% effects on reproduction) was calculated to be 409 mL product/ha.

Table: Effects of aclonifen + diflufenican SC 600 on reproduction of the predatory mite, *Typhlodromus pyri*, exposed on detached bean leaves (*Phaseolus vulgaris*)

Nominal Application Rate (mL formulation/ha)	Reproduction		P-value*
	Rate (mummies per female)	Reduction relative to control (%)	
Control	5.0	-	-
70	4.4	12.1	0.080
124	4.2	15.8	0.020*
221	3.1	37.5	0.012*
394	2.6	48.5	<0.001*
700	2.2	57.3	<0.001*
Reference item	n.a.	n.a.	-

*: No significant difference compared to control group (Welch test, one-sided)

C. VALIDITY CRITERIA

Validity criterion	Required	Achieved
Control mortality	≤20%	14.0%
Reference item mortality (corrected)	≥50%	90.7%
Mean no. of eggs/female in control (from day 7)	≥4	5.0

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint	Nominal Application Rate

	(mL formulation/ha)
LR_{50} mortality	>700
NOEC mortality	≥700
ER_{50} reproduction	409
NOEC reproduction	70

The LR_{50} was estimated to be >700 mL product/ha. The NOER for mortality was ≥ 700 mL product/ha.

The ER_{50} was calculated to be 409 mL product/ha. The NOER for reproduction was 70 mL product/ha.

The figures obtained fulfil the validity criteria of the laboratory method for exposure on glass plates ([redacted] 2000).

[redacted] (2019)

Assessment and conclusion by applicant:

All validity criteria were satisfied and therefore this study can be considered to be valid.

The LR_{50} was estimated to be >700 mL formulation/ha. The NOEC for mortality was ≥700 mL formulation/ha.

The ER_{50} was estimated to be 409 mL formulation/ha. The NOEC for reproduction was ≥700 mL formulation/ha.

Assessment and conclusion by RMS:

Data Point:	KCP 10.3.2.2.02
Report Author:	[redacted]
Report Year:	2017
Report Title:	Chronic toxicity of diflufenican + aclonifen SC 600 (100+500 g/L) to the rove beetle Aleochara bilineata (Coleoptera:staphylinidae)under extended laboratory conditions – Final report
Report No:	CW16/062
Document No:	M-588143-01-1
Guideline(s) followed in study:	[redacted] (2000); [redacted] (2001)
Deviations from current test guideline:	Current guideline: [redacted] (2000) During the parasitation phase, temperature increased slightly to 22.4 °C for 2 h and relative humidity decreased to 56% for 6.5 h. This had no negative impact on the outcome of the study as all validity criteria were met
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

A study was conducted to determine the effect of aclonifen + diflufenican SC 600 on reproduction of the rove beetle, *Aleochara bilineata* (Coleoptera staphylinidae), after 44 days of exposure.

The nominal exposure concentrations were 70, 124, 221, 394 and 700 mL formulation/ha in 400 L deionised water. The reference item was prepared at the nominal exposure concentration of 3569.5 mL formulation/ha in 40 0L deionised water. The control comprised deionised water only.

Adults of *Aleochara bilineata* (1-7 days old at study start) were exposed in 4 replicates of 20 beetles (per test group) to the spray residues of the test item, reference item and control, respectively. On day 7, 14, and 21 approximately 500 pupae of *Delia antiqua* were buried into the soil of each replicate to be parasitized. During the assessments the beetles were fed with deep frozen larvae of *Tenebrio molitor*.

The number of hatched beetles of the F1 generation was recorded over a period of 44 days. From these data the endpoint reproductive capacity was calculated.

The ER_{50} was estimated to be >700 mL formulation/ha. The NOER (no observed effect rate) for reproduction was ≥700 mL formulation/ha.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test Item:** Aclonifen + diflufenican SC 600
   Lot no.: TOX 20193-01
   Active Ingredient / Purity: Aclonifen: 41.0% w/w (504.2 g/L)
   Diflufenican: 8.10% w/w (99.69 g/L)
   Appearance: Yellow dispersion
   Storage: 25 ± 2 °C (storage conditions from +2 °C to +30 °C are acceptable)
   Expiry date: 29 November 2018

2. **Reference item:** Dimethoate EC 400 g/L
   Batch no.: BAS 152 11 I
   Active Ingredient / Purity: Dimethoate, 420.3 g/L

3. **Test Organism:** *Aleochara bilineata*
   Age: Adult (1-7 days oldat study start)
   Source: [redacted]
   Acclimatisation: Onion fly pupae (parasitized with *Aleochara bilineata*) obtained from the breeder were put in hatching cages.

	Freshly emerged beetles were collected daily and put into plastic boxes (approx. 24 x 24 x 10.5 cm) closed with gauze, mesh size 80 µm) containing wet tissue paper. Until the start of the study the test organisms were maintained under test conditions (18 - 22 °C and 60 - 90% relative humidity). Food (larvae of *Tenebrio molitor*) was provided daily
Feeding:	Larvae of *Tenebrio molitor* L. (yellow mealworm), were obtained from [redacted] and were deep frozen directly after delivery

A. STUDY DESIGN AND METHODS

1. In-life phase:	18 January to 9 April 2017
2. Exposure conditions	
Test vessels:	Bucket (16.5 cm, 13 cm high) with a lid with a gauze covered opening (10 - 12 cm, mesh size 80 µm) and filled with LUFA 2.1 soil up to a height of about 4.0 - 5.5 cm. Soil was silty sand (according to German DIN) from [redacted]
Experimental design:	7 experimental groups: control (tap water), test item (5 groups, nominally 70, 124, 221, 394 and 700 mL formulation/ha in 400 L deionised water) and toxic standard (dimethoate)
Replicates:	4 exposure units (replicates) per treatment group
Loading:	10 male + 10 female adult beetles per replicate
Temperature:	Temperature controlled rooms at 19.0 – 22.0 °C
Relative humidity:	60 – 89% Note: short deviations in test conditions (<2h) were not considered to affected study outcome. Conditions were continuously monitored and recorded by data logger. During parasitisation phase the temperature increased slightly to 22.4 °C for 2 h and the relative humidity decreased to 56% for 6.5 h.
Photoperiod:	16 h light: 8 h dark
Light intensity:	161 - 561 lux

3. Administration of the test item

Dose preparation

Suspensions of test and reference item were prepared on the day of application. The test item and reference item test concentrations were prepared in deionised water. The nominal exposure concentrations were 70, 124, 221, 394 and 700 mL formulation/ha in 400 L deionised water/ha. The reference item was prepared at the nominal exposure concentration of 3569.5 mL formulation/ha in 400 L deionised water/ha. The control comprised deionised water only.

The test item was applied to entire exposure unit using a linear cabinet track sprayer, at a rate of 400 L deionised water/ha. After spray coating had dried (partly) the glass plates were removed back to plastic trays.

Test organism assignment and exposure

The study encompassed 7 treatment groups (5 x test item, 1 x control, 1 x reference item) with 4 replicates each containing 10 male + 10 female rove beetles. Sex of the beetles was determined prior to study start by observation of mating behaviour on study day 0. For sexing only pairs which mated for at least 10 seconds were distinguished as male and female and transferred to glass jars.

Directly after treatment ten pairs of male and female adult beetles were added impartially to each exposure unit by placing them on the treated substrate. The units were closed with gauze lids and transferred to a controlled environmental room.

Approximately one hour after application the beetles were fed and then in 2 to 3 day intervals up to day 28 after application. The food (larvae of *Tenebrio molitor*) was placed on the surface of the soil.

At day 7, 14 and 21 after application approximately 500 onion fly pupae (*Delia antiqua*) were added and carefully mixed with the substrate of each exposure unit so that the pupae were distributed homogeneously within the unites and completely covered with substrate.

4. Measurements and observations

The number of pupae were determined by weight. At day 28 beetles were removed from exposure units and discarded. Soil, containing parasitized onion fly pupae were dried for 7 days by removing lids from exposure units.

At day 35, pupae were removed from substrate by sieving and by flushing with water. After drying pupae were placed in hatching cages (each replicate separately) and incubated. The number of hatched beetles was recorded on a daily basis. Test was terminated when hatching rate in the control group was <2 beetles per replicate per day.

5. Statistics/Data evaluation

The mean number of offspring per female was calculated for each treatment with standard deviation. Observations in the treatment groups were expressed relative to the water control group.

The reproduction data were tested for normal distribution using the Shapiro-Wilk test and for homogeneity of variance using the Levene test (α = 0 .05). The Wilcoxon test (one-sided with Bonferroni-Holm adjustment) was used as uncorrected p-values were very close together.

The computer program SAS (Version 9.4) was used to perform the statistical analyses.

II. RESULTS AND DISCUSSION

A: ANALYTICAL VERIFICATION

Analytical verification was not required.

B: BIOLOGICAL DATA

The mean number of hatched beetles per replicate and the parasitisation rate are summarised below:

Table: Mean number hatched rove beetles *Aleochara bilineata*, and parasitisation rate per replicate

Nominal Application Rate (mL formulation/ha)	Mean no. hatched beetles of F1 generations per replicate	Hatched beetles per introduced female (SD)	No. hatched beetles per host pupae (SD)	Parasitisation rate (%)	Reduction in reproductive capacity R_r (%)
Control	787	78.7 (2.80)	0.525 (0.019)	52.48	-
70	853	85.3 (1.87)	0.569 (0.012)	56.88	-8.4
124	893	89.3 (5.35)	0.596 (0.036)	59.55	-13.5
221	870	87.0 (5.41)	0.580 (0.036)	57.97	-10.4
394	911	91.1 (3.13)	0.607 (0.021)	60.72	-15.7
700	898	89.8 (3.39)	0.599 (0.023)	59.85	-14.0
Reference item	5	0.5 (0.20)	0.003 (0.0001)	0.33	99.4

SD: Standard deviation

Negative value indicates increase relative to control

In all test item groups, no statistically significant reduction of reproductive capacity compared to the control group was found (Wilcoxon test, one-sided).

The mean number of hatched beetles per replicate in the control group was 787 and the parasitation rate (relation of number of hatched beetles to the number of introduced pupae) was 52.48%.

In the highest test item rate of 700 mL product/ha the mean number of hatched beetles per replicate was 898 and the parasitation rate was 59.85%. In the reference item group a reduction of reproductive capacity of 99.4% was detected.

The NOER (no observed effect rate) was ≥700 mL product/ha.

The ER_{50} (effect rate causing 50% effects) was estimated to be > 700 mL product/ha.

Table: Effects of aclonifen + diflufenican SC 600 on reproduction of the rove beetle, *Aleochara bilineata*

Nominal Application Rate (mL formulation/ha)	Reproduction		P-value*
	Hatched beetles per introduced female (mean)	Reduction relative to control (%)	
Control	87.7	-	
70	85.3	-8.4	0.076
124	89.3	-13.6	0.076
221	87.0	-10.4	0.076
394	91.1	-15.7	0.076
700	89.8	-14.0	0.076
Reference item	0.5	99.7	

*: Significant difference compared to control group (Wilcoxon, one-sided) p-values adjusted according to Bonferroni-Hom

Negative value indicates increase relative to control

C. VALIDITY CRITERIA

Validity criterion	Required	Achieved
Average no. hatched beetles in F1 generation per replicate in control	>400	787
Reduction in reproductive capacity of reference item relative to control	≥50%	99.4%

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint	Nominal Application Rate (mL formulation/ha)
$ER_{50\ reproduction}$	>700
$NOER_{reproduction}$	≥700

III. CONCLUSION

The ER_{50} was calculated to be >700 mL product/ha. The NOER for reproduction was ≥700 mL product/ha.

The figures obtained fulfil the validity criteria of the extended laboratory method (Grimm *et al*, 2000).

[redacted] *(2017)*

Assessment and conclusion by applicant:

All validity criteria were satisfied and therefore this study can be considered to be valid.

The ER_{50} was calculated to be >700 mL product/ha. The NOER for reproduction was ≥700 mL product/ha.

Assessment and conclusion by RMS:

CP 10.3.2.3 Semi-field studies with non-target arthropods

Data Point:	KCP 10.3.2.3/01
Report Author:	[redacted]
Report Year:	2017
Report Title:	Effects of diflufenican + aclonifen SC 600 (100+500 g/L) on the predatory mite Typhlodromus pyri SCHEUTEN in an extended laboratory test (under semi-field conditions aged residues on maize plants)
Report No:	17 48 NTR 0004
Document No:	M-596590-01-1
Guideline(s) followed in study:	EU Directive 91/414/EEC; Regulation (EC) No 1107/2009; US EPA OCSPP Not Applicable
Deviations from current test guideline:	Current guideline: IOBC guideline (Blümel et al. (2000) modified No Deviation
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The test item diflufenican + aclonifen SC 600 (100+500 g/L) was tested under extended laboratory conditions after contact exposure of protonymphs of the predatory mite *Typhlodromus pyri* to fresh dried or under semi-field conditions aged spray residues at different exposure times (0 and 14 days after treatment). The test item was applied with a rate of 0.7 L product/ha in 400 L deionised water/ha on potted maize plants (*Zea maize*). The control group was treated with deionised water, in the same way as the test item. Dimethoate EC 400 (30 mL product/ha, nominally equivalent to 12 g a.s./ha, in 400 L deionised water/ha) was used as a toxic reference item, applied under semi-field conditions on potted maize plants for exposure started on day 0 and under laboratory conditions on excised untreated maize leaves at a rate of 15 mL product/ha (nominally equivalent to 6 g a.s./ha, in 200 L deionised water/ha) for exposure started on day 14. Aging of the spray residues on potted maize plants took place under semi-field conditions rain protected under a UV-permeable roof from the application until the start of the 2nd bioassay on day 14.

Protonymphs of the predatory mite *Typhlodromus pyri* Scheuten were exposed in 5 replicates per treatment group and 20 mites per replicate to the residues of the test item, reference item and control treatments, respectively.

The number of surviving, dead and escaped predatory mites was recorded over a period of 7 days. Additionally, effects on reproduction were investigated (number of eggs per surviving female, assessed 9, 11 and 14 days after application) for bioassays started on day 0 and 14.

The ER_{50} was estimated to be >700 mL formulation/ha. The NOER (no observed effect rate) for reproduction was ≥700 mL formulation/ha.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test Item:** Aclonifen + diflufenican SC 600
   Lot no.: TOX 20193-01
   Active Ingredient / Purity: Aclonifen: 41.0% w/w (504.2 g/L)
   Diflufenican: 8.10% w/w (99.69 g/L)
   Appearance: Yellow dispersion
   Storage: 25 ± 2 °C (storage conditions from +2 °C to +30 °C are acceptable)
   Expiry date: 29 November 2018

2. **Reference item:** Dimethoate EC 400 g/L
   Batch no.: BAS 152 11 I
   Active Ingredient / Purity: Dimethoate, 405.2 g/L

3. **Test Organism:** *Typhlodromus pyri* SCHEUTEN, (Acari: Phytoseiidae
   Age: <24 hour old protonymphs
   Source: [redacted]
   Acclimatisation: Eggs of the predatory mite were placed in hatching cages on moistened filter paper placed on the top of a water-saturated sponge placed in a cage filled with tap water up to a height of approx. 15 mm
   Feeding: Pollen: pine (*Pinus nigra*) and birch (*Betula pendula*), 1:1

A. STUDY DESIGN AND METHODS

1. **In-life phase:** 30 May to 27 June 2017

2. Exposure conditions

Test vessels: Bucket (16.5 cm, 13 cm high) with a lid with a gauze covered opening (10 - 12 cm, mesh size 80 µm) and filled with LUFA 2.1 soil up to a height of about 4.0 - 5.5 cm.
Soil was silty sand (according to German DIN) from Landwirtschaftliche Untersuchungs- und Forschungsanstalt Speyer, Germany

Experimental design:	Control - deionised water 400 L/ha (1 application (day 0) under semi-field conditions) Reference item – dimethoate EC400 (30 mL/ha in 400 L/ha (1 application (day 0) under semi-field conditions) Reference item – dimethoate EC400 (15 mL/ha in 200 L/ha (1 application (day 14) under laboratory conditions) Test item - aclonifen + diflufenican SC 600 (0.7 L/ha in 400 L/ha (1 application (day 0) under semi-field conditions.
Replicates:	5 exposure units (replicates) per treatment group
Loading:	20 mites per replicate, 100 mites per treatment group
Temperature:	23 – 26 °C
Relative humidity:	67 – 72%
Photoperiod:	16 h light: 8 h dark
Light intensity:	1940 - 2020 lux

3. Administration of the test item

Dose preparation

Suspensions of test and reference item were prepared on the day of application. The test item and reference item test concentrations were prepared in deionised water. The nominal exposure concentration was 0.7 L formulation/ha in 400 L deionised water/ha. The reference item was prepared at the nominal exposure concentration of 30 mL formulation/ha in 400 L deionised water/ha (day 0) and 15 mL formulation/ha in 200 L deionised water/ha (day 14). The control comprised deionised water only.

Semi-field (outdoor) conditions for application (control, test item and reference item treatment) on day 0

The application of the spray solutions was carried out under semi-field (outdoor) conditions. The test solution was sprayed onto potted maize plants using spray equipment for small plot applications (plot-sprayer). Prior to spraying the potted maize plants of each treatment were set up in a 25 m2 application plot. The plants in the containers were placed in a row on a stripe in the middle of the plot with a minimum space of 50 cm between containers to prevent an overlay between plants during application.

Before the application, the end of the leaves of the maize plants were marked to ensure that only treated maize leaves were cut before start of each bioassay. Potential development of new leaves after the application was monitored in order that new leaves were not used as test substrate.

Laboratory conditions for the application of the reference item on day 14

At bioassay day 14, the reference item was freshly applied on untreated detached maize leaves under laboratory conditions. Four glass plates of known surface area were sprayed with deionised water.

Test organism assignment and exposure

The study encompassed 7 treatment groups (5 x test item, 1 x control, 1 x reference item) with 4 replicates each containing 10 male + 10 female rove beetles. Sex of the beetles was determined prior

to study start by observation of mating behaviour on study day 0. For sexing only pairs which mated for at least 10 seconds were distinguished as male and female and transferred to glass jars.

Directly after treatment ten pairs of male and female adult beetles were added impartially to each exposure unit by placing them on the treated substrate. The units were closed with gauze lids and transferred to a controlled environmental room.

Approximately one hour after application the beetles were fed and then in 2 to 3 day intervals up to day 28 after application. The food (larvae of *Tenebrio molitor*) was placed on the surface of the soil.

At day 7, 14 and 21 after application approximately 500 onion fly pupae (*Delia antiqua*) were added and carefully mixed with the substrate of each exposure unit so that the pupae were distributed homogeneously within the unites and completely covered with substrate.

4. Measurements and observations

The number of pupae were determined by weight. At day 28 beetles were removed from exposure units and discarded. Soil, containing parasitized onion fly pupae were dried for 7 days by removing lids from exposure units.

At day 35, pupae were removed from substrate by sieving and by flushing with water. After drying pupae were placed in hatching cages (each replicate separately) and incubated. The number of hatched beetles was recorded on a daily basis. Test was terminated when hatching rate in the control group was <2 beetles per replicate per day.

5. Statistics/Data evaluation

The mean number of offspring per female was calculated for each treatment with standard deviation. Observations in the treatment groups were expressed relative to the water control group.

The reproduction data were tested for normal distribution using the Shapiro-Wilk test and for homogeneity of variance using the Levene test ($\alpha = 0.05$). The Wilcoxon test (one-sided with Bonferroni-Holm adjustment) was used as uncorrected p-values were very close together.

The computer program SAS (Version 9.4) was used to perform the statistical analyses.

II. RESULTS AND DISCUSSION

A: ANALYTICAL VERIFICATION

Analytical verification was not required.

B: BIOLOGICAL DATA

The mean number of hatched beetles per replicate and the parasitisation rate are summarised below:

Table: Mean number hatched rove beetles *Aleochara bilineata*, and parasitisation rate per replicate

Nominal Application Rate (mL formulation/ha)	Mean no. hatched beetles of F1 generations per replicate	Hatched beetles per introduced female (SD)	No. hatched beetles per host pupae (SD)	Parasitisation rate (%)	Reduction in reproductive capacity R (%)
Control	787	78.7 (2.80)	0.525 (0.019)	52.48	
70	853	85.3 (1.87)	0.569 (0.012)	56.88	-8.4
124	893	89.3 (5.35)	0.596 (0.036)	59.55	-13.5
221	870	87.0 (5.41)	0.580 (0.036)	57.97	-10.4
394	911	91.1 (3.13)	0.607 (0.021)	60.72	-15.7
700	898	89.8 (3.39)	0.599 (0.023)	59.85	-14.0
Reference item	5	0.5 (0.20)	0.003 (0.0001)	0.33	99.4

SD: Standard deviation

Negative value indicates increase relative to control

In all test item groups, no statistically significant reduction of reproductive capacity compared to the control group was found (Wilcoxon test, one-sided).

The mean number of hatched beetles per replicate in the control group was 787 and the parasitisation rate (relation of number of hatched beetles to the number of introduced pupae) was 52.48%.

In the highest test item rate of 700 mL product/ha the mean number of hatched beetles per replicate was 898 and the parasitisation rate was 59.85%. In the reference item group a reduction of reproductive capacity of 99.4% was detected.

The NOER (no observed effect rate) was >700 mL product/ha.

The ER_{50} (effect rate causing 50% effects) was estimated to be >700 mL product/ha.

Table: Effects of aclonifen + diflufenican SC 600 on reproduction of the rove beetle, *Aleochara bilineata*

Nominal Application Rate (mL formulation/ha)	Reproduction		P-value*
	Hatched beetles per introduced female (mean)	Reduction relative to control (%)	
Control	87.7	-	
70	85.3	-8.4	0.076
124	89.3	-13.6	0.076
221	87.0	-10.4	0.076
394	91.1	-15.7	0.076
700	89.8	-14.0	0.076
Reference item	0.5	99.7	

*: Significant difference compared to control group (Wilcoxon, one-sided) p-values adjusted according to [redacted]

Negative value indicates increase relative to control

C. VALIDITY CRITERIA

Validity criterion	Required	Achieved
Average no. hatched beetles in F1 generation per replicate in control	≥400	787
Reduction in reproductive capacity of reference item relative to control	≥50%	99.4%

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint	Nominal Application Rate (mL formulation/ha)
$ER_{50\ reproduction}$	>700
$NOER_{\ reproduction}$	≥700

III. CONCLUSION

The ER_{50} was calculated to be >700 mL product/ha. The NOER for reproduction was ≥700 mL product/ha.

The figures obtained fulfil the validity criteria of the extended laboratory method ([redacted] 2000).

[redacted] *(2017)*

Assessment and conclusion by applicant:

All validity criteria were satisfied and therefore this study can be considered to be valid.

The ER_{50} for rove beetle *Aleochara bilineata* was calculated to be >700 mL product/ha. The NOER for reproduction was ≥700 mL product/ha.

Assessment and conclusion by RMS:

CP 10.3.2.4 Field studies with non-target arthropods

No data available on formulated product.

CP 10.3.2.5 Other routes of exposure for non-target arthropods

No data available on formulated product.

CP 10.4 Effects on non-target soil meso- and macrofauna

Studies on the toxicity to earthworms and other non-target soil organisms (meso- and macro-fauna) have been carried out with aclonifen and diflufenican and with ACL + DFF SC 600 (500 + 100) G. Full details of these studies are outlined in document KCA 8 and the relevant EU DAR. New studies are summarised below.

CP 10.4.1 Earthworms

A summary of the relevant endpoints for the effects of ACL + DFF SC 600 (500 + 100) G on earthworms is provided in the following table.

Table 10.4-1: Earthworm endpoints used in risk assessment

Test item	Test species	Time-scale Test type / Application method	Endpoint	Reference
ACL + DFF SC 600 (500 + 100) G	*Eisenia fetida*	56-d chronic Reproduction incorporation into soil, 10% peat content	**NOEC = 381.7 mg test item/kg** EC_{10} = 447.9 mg test item/kg	KCP 10.4.1.1/01 M-581081-01-1 [redacted], 2017
Aclonifen (tested as Aclonifen SC 600 G)	*Eisenia fetida*	56-d chronic Reproduction, incorporation into soil	**$NOEC_{corr}$ = 15.6 mg a.s./kg**[1]	KCP 10.4.1.1/02 M-580432-02-1 [redacted], 2019
Diflufenican	*Eisenia fetida*	14 d, acute Mixed into substrate, 10% peat content	LC_{50} > 1000 mg/kg dw $LC_{50,corr}$ > 500 mg/kg dw[1,2]	EFSA Scientific Report 122 (2007), 1-84
Diflufenican	*Eisenia fetida*	56 d, chronic Mixed into substrate, 10% peat content	NOEC = 1000 mg/kg dw **$NOEC_{corr}$ = 500 mg/kg dw**[1]	EFSA Scientific Report 122 (2007), 1-84
AE B107137	*Eisenia fetida*	14 d, acute Mixed into substrate, 10% peat content	LC_{50} >1000 mg/kg dw $LC_{50,corr}$ >500 mg/kg dw[1,2]	EFSA Scientific Report 122 (2007), 1-84

AE 0542291	*Eisenia fetida*	14 d, acute Mixed into substrate, 10% peat content	$LC_{50,\ corr}$ >500 mg/kg dw[1,2]	EFSA Scientific Report 122 (2007), 1-84

Values in **bold** used in risk assessment
[1]: Corrected value derived by dividing the endpoint by a factor of 2 in accordance with SANCO/10329/2002
[2]: This study design and endpoint is no longer required for the registration of active ingredients in the EU

Summary of the risk assessment for ACL + DFF SC 600 (500 + 100) G and earthworms

The chronic toxicity endpoint for earthworms exposed to ACL + DFF SC 600 (500 + 100) G was used to calculate the toxicity exposure ratio (TER) value in accordance with the Terrestrial Guidance Document (SANCO/10329/2002)[12] and EFSA Journal 2017; 15(2):4690[13]. The TER_{LC} value for aclonifen was above the trigger value of 5 in accordance with the proposed uses and therefore, the risk was considered to be acceptable.

Application scenario

According to the GAP table, ACL + DFF SC 600 (500 + 100) G is proposed to be applied to winter cereals at 0.7 or 0.35 L/ha (1 application) during BBCH 00-13. The following assessments have been made for the use of ACL + DFF SC 600 (500 + 100) G in winter cereals using an application rate of 0.7 L/ha as this will also cover the risks from the use at lower application rates.

Risk assessment for earthworms

The risk assessment for earthworms has been conducted in line with the Terrestrial Guidance Document (SANCO/10329/2002) and EFSA Journal 2017; 15(2):4690.

Details on the predicted environmental concentrations (standard field calculations) in soil (PEC_{soil}) for aclonifen and diflufenican are presented in Document M-CP9 ~~and for diflufenican these are presented in EFSA Scientific Report 122 (2007), 1-84~~. The PEC was calculated using a standard approach with 5 cm mixing depth, soil density of 1.5 kg/L. Crop interception was not considered. No degradation data is available for the product, therefore, TWA, plateau and accumulated concentrations were not calculated, and tillage depth is not relevant.

The relevant earthworm reproduction study performed on ACL + DFF SC 600 (500 + 100) G ([redacted], 2017, K-CP 10.4.1.1/01) and on aclonifen (ACL + DFF SC 600 (500 + 100) G ([redacted], 2017, K-CP 10.4.1.1/02) determined both EC_{10} and NOEC values. In accordance with EFSA's Outcome of the Pesticides Peer Review Meeting on general recurring issues in ecotoxicology (EFSA, 2019)[14], as the NOEC was lower than the EC_{10}, the NOEC was used in the risk assessment.

[12] European Commission (EC), 2002. Guidance document on terrestrial ecotoxicology under Council Directive 91/414/EEC (SANCO/10329/2002) revision 2, final. 1–39.
[13] EFSA PPR Panel (EFSA Panel on Plant Protection Products and their Residues), [redacted] 2017. Scientific Opinion addressing the state of the science on risk assessment of plant protection products for in-soil organisms. EFSA Journal 2017;15(2):4690, 225 pp. doi:10.2903/j. efsa.2017.4690
[14] EFSA (European Food Safety Authority), 2019. Technical report on the outcome of the Pesticides Peer Review Meeting on general recurring issues in ecotoxicology. EFSA supporting publication 2019:EN-1673. 117 pp. doi:10.2903/sp.efsa.2019.EN-1673

The log P_{ow} for aclonifen and diflufenican are >2 (4.37 and 4.05, respectively) and the organic carbon content of the artificial soil used in the earthworm reproduction studies was high (10% peat content) and hence, in line with the EU Guidance Document on Terrestrial Ecotoxicology (SANCO/10329/2002) the NOEC has to be divided by 2 for use in the risk assessment. Endpoints were further corrected to express $NOEC_{corr.}$ in terms of product density and active ingredient content. This gives a $NOEC_{corr}$ of 190.9 mg test item/kg for ACL + DFF SC 600 (500 + 100) G and a $NOEC_{corr}$ of 15.6 and 500 mg a.s./kg for aclonifen and diflufenican, respectively.

Table 10.4-2: Earthworm toxicity exposure ratios for the proposed uses of ACL + DFF SC 600 (500 + 100) G

Intended use		Winter cereals, 0.7 L prod./ha, BBCH 00 - 13		
Test item	**Maximum PEC_{soil} (mg prod./kg soil dw)**	**Corrected endpoint * (mg prod./kg soil dw)**	**TER_{LT}**	**Trigger value**
ACL + DFF SC 600 (500 + 100) G	1.148[1]	94[2]	82	5
Test item	**Maximum PEC_{soil} (mg a.s./kg soil dw)**	**Corrected endpoint * (mg a.s./kg soil dw)**	**TER_{LT}**	
Aclonifen	0.5113	15.6	31	
Diflufenican	0.405	500	1235	

* In line with the EU Guidance Document on Terrestrial Ecotoxicology (SANCO/10329/2002) the NOEC may be divided by 2 for use in the risk assessment where log P_{ow} is >2 and the organic carbon content of the artificial soil used is high (10% peat content)

1: Based on application rate of 1 x 0.7 L product/ha, 0% crop interception and a product density of 1.230 g/mL = 861 g/ha

2: Corrected endpoint expressed in terms of the active ingredient content, accounting for product density and active ingredient content of product

The long-term TER values are above the risk assessment trigger value of 5. It is concluded that the risks to earthworms are acceptable when ACL + DFF SC 600 (500 + 100) G is used according to the recommended GAP.

CP 10.4.1.1 Earthworms sub-lethal effects

Data Point:	KCP 10.4.1.1/01
Report Author:	[redacted]
Report Year:	2017
Report Title:	Aclonifen + diflufenican SC 600 (500+100) G: Effects on survival, growth and reproduction of the earthworm Eisenia andrei tested in artificial soil
Report No:	16 10 48 265 S
Document No:	M-581081-01-1
Guideline(s) followed in study:	EU Directive 91/414/EEC; Regulation (EC) No 1107/2009 (2009); US EPA OCSPP Not Applicable
Deviations from current test guideline:	Current guideline: OECD 222 (2016) No Deviation
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary:

The effects of aclonifen + diflufenican SC 600 (500+100) G on the mortality, body weight, feeding activity and reproduction of adult *Eisenia fetida* were investigated in a laboratory study lasting eight weeks. Nominal exposure concentrations were 12, 21.4, 38.0, 67.7, 120.5, 214.4, 381.7, 679.4 mg test item/kg soil dry weight (with a spacing factor of 1.78). Control substrate was untreated.

The reference item, carbendazim SC 500, was tested in a separate study at 5 and 10 mg product/kg soil dry weight. .

The mortality of adult worms was 0 – 5.0% in the treated groups and 2.5% in the control group. No statistically significant mortality compared to the control was observed at any test item concentration (Multiple Sequentially-rejective Fisher Test After Bonferroni-Holm, α = 0.05, one-sided greater). No pathological symptoms and no effects on behaviour (including feeding activity) of the worms were observed during the test.

The weight change of adult worms ranged between 28.2 and 31.6% in the treated groups and 30.3% in the control group. The test item caused no statistically significant change in biomass (change in fresh weight after 4 weeks relative to initial fresh weight) compared to the control groups at any concentration tested (Williams-t-test, α = 0.05, two-sided).

Statistically significant effects (Williams-t-test, α = 0.05, one-sided smaller) on the number of juveniles compared to the control group were recorded at a concentration of 679.4 mg test item/kg d.w.

Based on the statistical evaluation of these results, the No-Observed-Effect-Concentration (NOEC) of aclonifen + diflufenican SC 600 (500+100) G for reproduction was determined to be 381.7 mg test item/kg d.w., and the Lowest-Observed-Effect-Concentration (LOEC) for reproduction was determined to be 679.4 mg test item/kg d.w.

The EC_{10} and EC_{20} values for reproduction were calculated to be 447.9 and 500.8 mg test item/kg soil d.w., respectively (based on Logit analysis).

I. MATERIALS AND METHODS

A. MATERIALS

1.	**Test Item:**	Aclonifen + diflufenican SC 600 (500+100) G
	Batch no.:	2015-010653
	Active Ingredient / Purity:	Aclonifen 500 g/L (nominal); 41.1% w/w, 505.1 g/L (analysed) Diflufenican 100 g/L (nominal); 8.21% w/w, 101.0 g/L (analysed)
	Appearance:	Yellow suspension
	Expiry date:	12 January 2017
	Storage:	In original container, between +2 and +30 °C in the dark
2.	**Reference item:**	Maypon Flow (carbendazim SC 500)
	Batch no:	Not known
	Active Ingredient / Purity:	500 g/L nominal
3.	**Test Species:**	*Eisenia andrei* ([redacted] 1972)
	Source:	[redacted]
	Age:	Approximately 3 months, with clitellum 250 – 433 mg/worm (fresh weight)
	Acclimatisation:	24 hours, in artificial soil, under test conditions (with food)

B. STUDY DESIGN AND METHODS

1.	**In-life phase:**	15 November 2016 to 10 January 2017
2.	**Exposure conditions**	
	Test vessels:	Plastic boxes (16.5 x 12 x 6 cm, internal dimensions) with lids pervious lids to light and air. Each container was filled with 675 g of the prepared soil (approximately 500 g (dry weight) artificial soil
	Test soil:	Artificial soil according to OECD 207 with 10% peat content
	Treatment:	12, 21.4, 38.0, 67.7, 120.5, 214.4, 381.7, 679.4 mg test item/kg soil dry weight Control substrate was untreated Reference item, (separate study) 5 and 10 mg carbendazim SC 500/kg soil dry weight
	Number of replicates:	4 per treatment group, 8 for control group
	Number of individuals:	10 per replicate, i.e. 40 individuals per treatment group, 80 for control group
	Test duration:	8 weeks
	Temperature:	Exposure room: 18 – 20.9 °C
	Light regime:	16 h light: 8 h dark
	Light intensity:	580 lux

pH:	6.05 – 6.17 (test start)
	5.81 – 5.89 (test end)
Water content:	27.2 – 30.1% (52.9 – 58.5% of the water holding capacity)

3. Administration of the test item

Dose preparation and dosing

Test solutions were made by dispersing weighed amounts of the test item in deionised water, immediately prior to application. The test item was dispersed in sufficient deionised water such that the addition of the test solutions to the test substrate resulted in a final water content of 40-60% of WHC. The treated substrate was thoroughly mixed separately for each replicate using a laboratory mixer immediately after application.

Test organism exposure

Acclimatised test worms were washed with deionised water, gently dried and individually weighed, then impartially assigned to the test vessels (start of exposure). Test vessels were randomly distributed in a controlled-environment test room. The physico-chemical parameters of the artificial soil (water content, pH; analysed from pooled samples of each treatment group separately) were determined.

After 24 hours and then weekly for the initial 4 weeks of exposure, the test organisms were fed with initially 5 g manure per test vessel on the soil surface which was sprinkled with 5 mL deionised water (weekly amount of 5 g manure according to feeding activity).

4. Measurements and observations

At test start: individual fresh weight (mg/worm) behaviour of earthworms determination of physico-chemical parameters (water content, pH) of the artificial soil were determined. Weekly observation of behavioural and pathological symptoms (including the feeding activity).

Four weeks after the start of exposure (end of adult earthworm exposure) surviving adult earthworms were removed, counted, washed and weighed.

Eight weeks after the start of exposure (end of juvenile earthworm exposure and test end) juvenile earthworms were separated and counted. Observation of behavioural and pathological symptoms (including morphological alterations) determination of physico-chemical parameters (water content, pH) of the artificial soil were taken.

5. Statistics/Data evaluation

The endpoints were mortality, change of biomass (difference in fresh weight of surviving worms between test start and four weeks after treatment) and reproduction (the number of juveniles present). The arithmetic mean and the standard deviation per treatment and per control for reproduction, mortality and biomass were calculated. The statistical analysis was performed with the software ToxRat Professional 3.2.1 (Ratte 2015).

The EC_{10} and EC_{20} values (number of juveniles) were calculated by Logit analysis using linear max. likelihood regression. Confidence limits (95%) of the ECx values were computed by normal approximation. The Multiple Sequentially-rejective Fisher Test After Bonferroni-Holm and the Williams-t-test were used to compare the control with the independent test item groups. For statistical

evaluation of the biomass change, the changed mean fresh weight of surviving worms per replicate was used.

II. RESULTS AND DISCUSSION

Analytical verification

No analytical verification of the dose solutions was performed.

Biological data

The mortality of adult worms was 0 – 5.0% in the treated groups and 2.5% in the control group. No statistically significant mortality compared to the control was observed at any test item concentration (Multiple Sequentially-rejective Fisher Test After [redacted], α = 0.05, one-sided greater). No pathological symptoms and no effects on behaviour (including feeding activity) of the worms were observed during the test.

The weight change of adult worms ranged between 28.2 and 31.6% in the treated groups and 30.3% in the control group. The test item caused no statistically significant change in biomass (change in fresh weight after 4 weeks relative to initial fresh weight) compared to the control groups at any concentration tested (Williams-t-test, α = 0.05, two-sided).

Statistically significant effects (Williams-t-test, α = 0.05, one-sided smaller) on the number of juveniles compared to the control group were recorded at a concentration of 679.4 mg test item/kg d.w.

Table: Effect of aclonifen + diflufenican SC 600 (500+100) G on earthworms (*Eisenia fetida*) in a 56-day reproduction study

Treatment (mg test item/kg)	Adults mortality (mean after 4 weeks)[1] (%)	Mean body weight after 28 days (mg/worm)	Body weight change[2] (% of initial)	Reproduction (no. of juveniles)	Reproduction (% of control)
Control	2.5	390.7	30.3	135.1	-
12	2.5	387.0	28.4	134.0	99.2
21.4	2.5	383.5	28.5	129.0	95.5
38.0	5.0	392.3	30.1	132.3	97.9
67.7	0	386.0	28.3	141.0	104.3
120.5	2.5	395.6	31.6	150.5	111.4
214.4	5.0	395.2	30.8	134.8	99.7
381.7	2.5	389.6	29.7	141.0	104.3
679.4	0	391.4	28.2	41.0[3]	30.3[3]

1: Not statistically significantly different compared to the control for mortality (Multiple Sequentially-rejective Fisher Test after [redacted], α = 0.05, one-sided greater)

2: Not statistically significantly different compared to the control for biomass (Williams t-test, α = 0.05, two-sided)

3: Statistically significantly different compared to the control for reproduction (Williams t-test for reproduction, α = 0.05, one-sided, smaller)

Based on the statistical evaluation of these results, the No-Observed-Effect-Concentration (NOEC) for reproduction was determined to be 381.7 mg test item/kg d.w., and the Lowest-Observed-Effect-Concentration (LOEC) for reproduction was determined to be 679.4 mg test item/kg d.w. The EC_{10} and EC_{20} values for reproduction were calculated to be 447.9 and 500.8 mg test item/kg soil d.w., respectively (based on Logit analysis).

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 222, 2016)	Achieved
Adult mortality (after 4 weeks)	≤10%	2.5%
Reproduction (worms per container)	≥30	268
Reproduction (coefficient of variation)	≤30%	17.4%

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint	Nominal Application Rate (mg test item/kg soil d.w)
$NOEC_{mortality}$	≥679.4
$NOEC_{biomass}$	≥679.4
$NOEC_{reproduction}$	381.7
$EC_{10\ reproduction}$	447.9
$EC_{20\ reproduction}$	500.8

III. CONCLUSION

Based on the statistical evaluation of these results, the No-Observed-Effect-Concentration (NOEC) of aclonifen + diflufenican SC 600 (500+100) G for reproduction was determined to be 381.7 mg test item/kg d.w., and the Lowest-Observed-Effect-Concentration (LOEC) for reproduction was determined to be 679.4 mg test item/kg d.w.

The EC_{10} and EC_{20} values for reproduction were calculated to be 447.9 and 500.8 mg test item/kg soil d.w., respectively (based on Logit analysis).

[redacted] *(2017)*

Assessment and conclusion by applicant:

Validity criteria according to OECD 222 (2016) were satisfied and therefore this study can be considered to be valid .

Based on the statistical evaluation of these results, the No-Observed-Effect-Concentration (NOEC) of aclonifen + diflufenican SC 600 (500+100) G for reproduction was determined to be 381.7 mg test item/kg d.w., and the Lowest-Observed-Effect-Concentration (LOEC) for reproduction was determined to be 679.4 mg test item/kg d.w.

EFSA's Outcome of the Pesticides Peer Review Meeting on general recurring issues in ecotoxicology (EFSA, 2019)[15] recommends that the lowest of the EC_{10} and NOEC values be used for risk assessment purposes. In this study, as the NOEC was lower than the EC_{10}, the NOEC of 381.7 mg test item/kg soil should be used for risk assessment.

Assessment and conclusion by RMS:

Data Point:	KCP 10.4.1.1/02
Report Author:	[redacted]
Report Year:	2019
Report Title:	Amendment no. 1: Aclonifen SC 600 G: Effects on survival, growth and reproduction of the earthworm Eisenia andrei tested in artificial soil
Report No:	16 10 48 169 S
Document No:	M-580432-02-1
Guideline(s) followed in study:	OECD 222 (2004), ISO 11268-2 (1998)
Deviations from current test guideline:	Current guideline: OECD 222, 2016 No Deviation
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary:

The effects of aclonifen SC 600 G on the mortality, body weight and reproduction of adult *Eisenia fetida* were investigated in a laboratory study lasting eight weeks.

Adult earthworms (*Eisenia andrei*, about 3 months old) were exposed to 20, 35, 63, 112, 200, 355, 631 and 1122 mg test item/kg dry weight mixed into artificial soil. Mortality and biomass change were determined after 4 weeks and reproduction was determined after 8 weeks.

Aclonifen SC 600 G showed statistically significant adverse effects on survival of the earthworm *Eisenia andrei* in artificial soil at 1122 mg test item/kg soil dry weight, i.e. the highest concentration tested.

Statistically significantly adverse effects on biomass were determined at 355, 631 and 1122 mg test item/kg soil dry weight. The test item showed statistically significant adverse effects on reproduction at

[15] EFSA (European Food Safety Authority), 2019. Technical report on the outcome of the Pesticides Peer Review Meeting on general recurring issues in ecotoxicology. EFSA supporting publication 2019:EN-1673. 117 pp. doi:10.2903/sp.efsa.2019.EN-1673

112, 200, 355, 631 and 1122 mg test item/kg d.w. Therefore, the No-Observed-Effect-Concentration (NOEC) for reproduction was determined to be 63 mg test item/kg d.w., and the Lowest-Observed-Effect-Concentration (LOEC) for reproduction was determined to be 112 mg test item/kg d.w.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test Item:** Aclonifen SC 600 G
   Batch no.: EV56005993
   Active Ingredient / Purity: Aclonifen 600 g/L (nominal); 49.5% w/w, 596.7 g/L (analysed)
   Appearance: Yellow suspension
   Expiry date: 09 February 2017
   Storage: 25 ± 5 °C

2. **Reference item:** Maypon Flow
   Batch no: Not reported
   Active Ingredient / Purity: Carbendazim, SC 500

3. **Test Species:** earthworm *Eisenia andrei* ([redacted] 1972)
   Source: [redacted]
   Age: Adult worms (approximately 3 months old with clitellum)
   Acclimatisation: Approximately 24 hours in the artificial substrate (with food)
   Feeding: Air-dried and finely ground horse manure

B. STUDY DESIGN AND METHODS

1. In-life phase: 27 July – 21 September 2016

2. Exposure conditions

Test vessels: Plastic vessel of Bellaplast (inside dimensions: about 16.5 cm x 12 cm x 6 cm) with a lid pervious to air and light

Test soil: Artificial soil

10% sphagnum peat; origin: [redacted] classified according to DIN 11540 (as close to pH 5.5-6.0 as possible, no visible plant remains, finely ground, dried to measured moisture content)

20% kaolin clay (kaolinite content > 30%); type: Kaolin W, origin: [redacted]

	0.5% calcium carbonate; origin: [redacted]
	69.5% industrial quartz sand; type: Millisil W3, origin: [redacted] (fine sand is dominant with more than 50% of the particles between 50 and 200 µm)
	deionised water
Treatment:	Control
	20, 35, 63, 112, 200, 355, 631, 1122 mg test item/kg soil dry weight
	Reference item 5 and 10 mg product/kg soil dry weight (tested in a separate study)
Number of replicates:	8 for the control and 4 per treatment group
Number of individuals:	10 per replicate, i.e. 80 individuals for the control and 40 individuals per treatment group
Test duration:	8 weeks
Temperature:	18.7 – 20.8 °C
Light regime:	16 h light: 8 h dark
Light intensity:	550 lux
pH:	5.62 – 6.03
Water content:	34.2 – 35.0% (54.5 – 56.7% of the water holding capacity)

3. Administration of the test item

Dose preparation and dosing

Test solutions were made by dispersing weighed amounts of the test item in deionised water, immediately prior to application. The test item was dispersed in sufficient deionised water such that the addition of the test solutions to the test substrate resulted in a final water content of 40-60% of WHC. The treated substrate was thoroughly mixed using a laboratory mixer immediately after application.

Test organism exposure

One day before test start, the dry artificial soil was pre-moistened by adding deionised water to obtain approximately half of the final water content. Earthworms were acclimatised in a separate batch of the artificial soil (mixed with horse manure) for approximately 24 hours before test start.

On the day of the test start, the test item was introduced by dispersing the quantity of test item required to obtain the desired test concentration in the volume of water required to hydrate the soil to 40-60% of its WHC. The control substrate contained the corresponding amount of deionised water only. Each test vessel was then filled with the treated soil. After a randomising procedure according to the worm fresh weight, selected groups of 10 worms were then randomly assigned to each treatment group. The individually weighed worms (10 worms/vessel) were placed on the surface of the soil. After approximately thirty minutes, the test vessels were closed with perforated transparent lids, which allowed gas exchange between substrate and atmosphere and access of light, but prevented worms from escaping. The test vessels were then set up at random in a controlled-environment test room.

4. Measurements and observations

After four weeks, the adult worms were removed from the test vessels. The number of surviving worms (adult mortality) and their biomass change were determined, behaviour (including feeding activity) and pathological symptoms were recorded. The adult worms were discarded after counting and weighing. Subsequently, the soil of each vessel was mixed carefully with 5 g manure. This was the last feeding occasion of the experiment. The test was then continued for another four weeks. The final assessment included counting of juveniles per test vessel, determination of the water content and pH measurements of the artificial soil. Juveniles were counted by manual inspection of the substrate.

5. Statistics/Data evaluation

The endpoints were mortality, change of biomass (difference in fresh weight of surviving worms between test start and four weeks after treatment) and reproduction (the number of juveniles present). The arithmetic mean and the standard deviation per treatment and per control for reproduction, mortality and biomass were calculated. The statistical analysis was performed with the software ToxRat Professional 3.2.1 (Ratte 2015). The EC_{10} and EC_{20} values (number of juveniles) were calculated by Weibull analysis using linear max. likelihood regression. Confidence limits (95%) of the ECx values were computed by normal approximation. The Multiple Sequentially-rejective Fisher Test After [redacted], Welch-t-test after [redacted] and the Williams-t-test were used to compare the control with the independent test item groups. For statistical evaluation of the biomass change, the changed mean fresh weight of surviving worms per replicate was used.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

No analytical verification of the dose solutions was performed.

B. BIOLOGICAL DATA

The mortality of adult worms was 0 – 60% in the treated groups and 0% in the control group. Statistically significant mortality compared to the control was observed at 1122 mg test item/kg d.w. (Multiple Sequentially-rejective Fisher Test After [redacted], α = 0.05, one-sided greater). No pathological symptoms and no further effects on behaviour of the worms were observed. The feeding activity of adult worms was reduced at 355, 631 and 1122 mg test item/kg soil d.w.

The weight change of adult worms ranged between -31.8 and 20.5% in the treated groups and 19.8% in the control group. The test item caused statistically significant change in biomass (change in fresh weight after 4 weeks relative to initial fresh weight) compared to the control groups were recorded at concentrations of 355, 631 and 1122 mg test item/kg d.w. (Welch-t-test after [redacted], α = 0.05, two-sided).

Statistically significant effects (Williams-t-test, α = 0.05, one-sided smaller) on number of juveniles compared to the control group were recorded at concentrations of 112, 200, 355, 631 and 1122 mg test item/kg d.w.

Table: Effect of aclonifen SC 600 G on earthworms (*Eisenia fetida*) in a 56-day reproduction study

Treatment (mg/kg d.w)	Mortality after 4 weeks (%)	Mean biomass change after 4 weeks (mg)	Reproduction (no. of juveniles / replicate after 8 weeks)
Control	0.0	88.7	141.5
20	0.0	92.8	129.8

35	0.0	87.0	140.5
63	0.0	85.9	121.8
112	0.0	89.2	108.0[3]
200	0.0	78.8	116.5[3]
355	0.0	17.8[2]	109.5[3]
631	0.0	-34.4[2]	62.5[3]
1122	60.0[1]	-144.0[2]	0.0[3]

[1]: Significantly different compared to the control (Multiple Sequentially-rejective Fisher Test After Bonferroni-Holm, α = 0.05, one-sided greater)
[2]: Significantly different compared to the control (Welch-t-test after Bonferroni-Holm, α = 0.05, two-sided)
[3]: Significantly different compared to the control (Williams-t-test, α = 0.05, one-sided smaller)

Based on the statistical evaluation of these results, the No-Observed-Effect-Concentration (NOEC) for reproduction was determined to be 63 mg test item/kg soil d.w. and the Lowest-Observed-Effect-Concentration (LOEC) for reproduction was determined to be 112 mg test item/kg soil d.w. The EC_{10} and EC_{20} values for reproduction were calculated to be 144 and 240 mg test item/kg soil d.w., respectively.

In the reference test, the number of juveniles was reduced by 39 and 96% by the toxic standard Maypon Flow (Carbendazim, SC 500) at concentrations of 5 and 10 mg product/kg d.w. in comparison to the control. Therefore, the observed effects assure a high sensitivity of the test system.

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 222, 2016)	Achieved
Mortality	≤ 10%	0%
Reproduction (worms per container)	≥ 30	141.5
Reproduction (coefficient of variation)	≤ 10%	14.7%

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint	Nominal Concentration (mg/kg d.w.)
$NOEC_{mortality}$	631
$NOEC_{biomass\ change}$	200
$NOEC_{reproduction}$	63
$LOEC_{reproduction}$	112
EC_{10} [95% confidence limits]	144 [59 – 352]
EC_{20} [95% confidence limits]	240 [130 – 442]

III. CONCLUSION

Aclonifen SC 600 G showed statistically significant adverse effects on survival of the earthworm *Eisenia andrei* in artificial soil at 1122 mg test item/kg soil dry weight, i.e. the highest concentration tested.

Statistically significantly adverse effects on biomass were determined at 355, 631 and 1122 mg test item/kg soil dry weight. The test item showed statistically significant adverse effects on reproduction at 112, 200, 355, 631 and 1122 mg test item/kg d.w. Therefore, the No-Observed-Effect-Concentration (NOEC) for reproduction was determined to be 63 mg test item/kg d.w. and the Lowest-Observed-Effect-Concentration (LOEC) for reproduction was determined to be 112 mg test item/kg d.w.

[redacted] (2017)

Assessment and conclusion by applicant:
All validity criteria were satisfied and therefore this study can be considered to be valid.

Based on the most relevant biological endpoint of reproduction, the NOEC was determined to be 63 mg test item/kg soil and the EC_{10} was determined to be 144 mg test item/kg soil.

EFSA's Outcome of the Pesticides Peer Review Meeting on general recurring issues in ecotoxicology (EFSA, 2019)[16] recommends that the lowest of the EC_{10} and NOEC values be used for risk assessment purposes. In this study, as the NOEC was lower than the EC_{10}, the NOEC of 63 mg test item/kg soil should be used for risk assessment.

For use in the risk assessment, as the log P_{ow} for aclonifen is greater than 2 and the organic carbon content of the artificial soil was high (10% peat content), in line with the EU Guidance Document on Terrestrial Ecotoxicology (SANCO/10329/2002) the endpoints have to be divided by 2.

Results have also been calculated in terms of the active ingredient content, assuming an active ingredient content of 49.5% w/w.

Table: Summary of endpoints

Endpoint	Concentration (mg/kg d.w.)	Concentration corrected for log P_{ow} (mg/kg d.w.)	Concentration corrected for log P_{ow} and active ingredient content (mg a.s./kg d.w.)
$NOEC_{mortality}$	631	316	156
$NOEC_{biomass\ change}$	200	100	49.5
$NOEC_{reproduction}$	63	31.5	15.6
$LOEC_{reproduction}$	112	56	27.7
EC_{10} [95% confidence limits]	144 [59 – 352]	72 [29.5 - 176]	35.6 [14.6 – 87.1]
EC_{20} [95% confidence limits]	240 [130 – 442]	120 [65 – 221]	59.4 [32.2 – 109]

[16] EFSA (European Food Safety Authority), 2019. Technical report on the outcome of the Pesticides Peer Review Meeting on general recurring issues in ecotoxicology. EFSA supporting publication 2019:EN-1673. 117 pp. doi:10.2903/sp.efsa.2019.EN-1673

Assessment and conclusion by RMS:

CP 10.4.1.2 Earthworms field studies

No data available on formulated product.

CP 10.4.2 Effects on non-target soil meso- and macrofauna (other than earthworms)

A summary of the relevant endpoints for the effects of ACL + DFF SC 600 (500 + 100) G on non-target soil meso- and macrofauna (other than earthworms) is provided in the following table. Full details of these studies are provided in the respective EU DAR and related documents. Studies with ACL + DFF SC 600 (500 + 100) G are outlined below.

The log P_{ow} values for aclonifen and diflufenican are greater than 2 and therefore an additional factor of 2 which covers the possible sorption of high log P_{ow} substances to soil was applied to the study endpoints.

Table 10.4-3: Non-target soil meso- and macrofauna (other than earthworms) endpoints used in risk assessment

Test item	Test species	Time-scale Test type	Endpoint	Reference
ACL + DFF SC 600 (500 + 100) G	*Folsomia candida*	28 d, chronic Mixed into substrate, 5% peat content	NOEC = 178 mg prod./kg dw EC_{10} = 155 mg prod./kg dw $NOEC_{corr}$ = 45.9 mg a.s./kg dw[1] **$EC_{10\ corr}$ = 38.2 mg a.s./kg dw[1]**	KCP 10.4.2.1/02 M-581084-01-1 [redacted], 2017
ACL + DFF SC 600 (500 + 100) G	*Hypoaspis aculeifer*	14 d, chronic Mixed into substrate, 5% peat content	NOEC = 562 mg prod./kg dw EC_{10} = 456 mg prod./kg dw $NOEC_{corr}$ = 139 mg a.s./kg dw[1] **$EC_{10\ corr}$ = 112 mg a.s./kg dw[1]**	KCP 10.4.2.1/01 M-580225-01-1 [redacted], 2017
Aclonifen (tested as Aclonifen SC 600 G)	*Folsomia candida*	28 d, chronic Mixed into substrate, 5% peat content	NOEC = 316 mg prod./kg dw EC_{10} = 311 mg prod./kg dw $NOEC_{corr}$ = 78 mg a.s./kg dw[2] **$EC_{10\ corr}$ = 77 mg a.s./kg dw[2]**	KCP 10.4.2.1/03 M-404393-01-1 [redacted], 2011 & KCP 10.4.2.1/04 M-675907-01-1 [redacted], 2019
Aclonifen (tested as Aclonifen SC 600 G)	*Hypoaspis aculeifer*	14-d, chronic Mixed into substrate, 5% peat content	NOEC = 562 mg prod./kg dw EC_{10} = N.D. **$NOEC_{corr}$ = 139 mg a.s./kg dw[2]** $EC_{10\ corr}$ = N.D.	KCP 10.4.2.1/05 M-404537-01-1 [redacted] 2011
Diflufenican (tested as DFF SC 500)	*Folsomia candida*	28 d, chronic Mixed into substrate, 5% peat content	NOEC ≥1000 mg prod./kg dw NOEC ≥438 mg a.s./kg dw **$NOEC_{corr}$ ≥219 mg a.s./kg dw[3]**	EFSA Scientific Report 122 (2007), 1-84

Diflufenican (tested as DFF SC 500)	*Hypoaspis aculeifer*	14 d, chronic Mixed into substrate, 5% peat content	NOEC ≥1000 mg prod./kg dw EC_{10} = N.D. **$NOEC_{corr}$ ≥213 mg a.s./kg dw[4]** $EC_{10\ corr}$ = N.D.	KCP 10.4.2.1/06 M-533188-01-1 [redacted], 2015

Values in **bold** used in risk assessment[1]

N.D.: Not determined

1: Corrected value derived by dividing the endpoint by a factor of 2 in accordance with SANCO/10329/2002 and correcting for a total active substance content of 49.31% w/w
2: Corrected value derived by dividing the endpoint by a factor of 2 in accordance with SANCO/10329/2002 and correcting for an active substance content of 49.4% w/w
3: Corrected value derived by dividing the endpoint by a factor of 2 in accordance with SANCO/10329/2002
4: Corrected value derived by dividing the endpoint by a factor of 2 in accordance with SANCO/10329/2002 and correcting for an active substance content of 42.6% w/w

Summary of the risk assessment for ACL + DFF SC 600 (500 + 100) G and non-target soil meso- and macrofauna (other than earthworms)

The chronic toxicity endpoints for *Hypoaspis aculeifer* and *Folsomia candida* exposed to ACL + DFF SC 600 (500 + 100) G was used to calculate the toxicity exposure ratio (TER) values in accordance with the Terrestrial Guidance Document (SANCO/10329/2002)[17] and EFSA Journal 2017; 15(2):4690[18].

The TER_{LT} values for aclonifen were above the trigger value of 5 in accordance with the proposed uses and therefore, the risk was considered to be acceptable.

Aclonifen and diflufenican did not have a significant adverse effect on organic matter decomposition at application rates higher than that for the proposed use of ACL + DFF SC 600 (500 + 100) G. It is concluded that the risks to soil organisms involved in the breakdown of organic matter are acceptable when ACL + DFF SC 600 (500 + 100) G is used according to the recommended GAP.

Application scenario

According to the GAP table, ACL + DFF SC 600 (500 + 100) G is proposed to be applied to winter cereals at 0.7 or 0.35 L/ha (1 application) during BBCH 00-13. The following assessments have been made for the use of ACL + DFF SC 600 (500 + 100) G in winter cereals using an application rate of 0.7 L/ha as this will also cover the risks from the use at lower application rates.

Risk assessment for other non-target soil meso- and macrofauna (other than earthworms)

The risk assessment for non-target soil meso- and macrofauna (other than earthworms) has been conducted in line with the Terrestrial Guidance Document (SANCO/10329/2002) and EFSA Journal 2017;15(2):4690.

[17] European Commission (EC), 2002. Guidance document on terrestrial ecotoxicology under Council Directive 91/414/EEC (SANCO/10329/2002) revision 2, final. 1–39.

[18] EFSA PPR Panel (EFSA Panel on Plant Protection Products and their Residues), [redacted] 2017. Scientific Opinion addressing the state of the science on risk assessment of plant protection products for in-soil organisms. EFSA Journal 2017;15(2):4690, 225 pp. doi:10.2903/j. efsa.2017.4690

Details on the predicted environmental concentrations (standard field calculations) in soil (PEC_{soil}) for aclonifen and diflufenican are presented in Document M-CP9 ~~and for diflufenican these are presented in EFSA Scientific Report 122 (2007), 1-84~~. The PEC was calculated using a standard approach with 5 cm mixing depth, soil density of 1.5 kg/L. Crop interception was not considered. No degradation data is available for the product, therefore, TWA, plateau and accumulated concentrations were not calculated, and tillage depth is not relevant.

Table 10.4-4: Non-target soil meso- and macrofauna (other than earthworms) toxicity exposure ratios for the proposed uses of ACL + DFF SC 600 (500 + 100) G

Intended use		Winter cereals, 0.7 L prod./ha, BBCH 00 - 13		
Test item		ACL + DFF SC 600 (500 + 100) G		
Test species	**Maximum PEC_{soil} (mg/kg soil dw)**	**Corrected endpoint (mg a.s./kg soil dw)**	**TER_{LT}**	**Trigger value**
Folsomia candida	1.148[1]	382	333	5
Hypoaspis aculeifer		112	97.6	
Test item		Aclonifen (tested as Aclonifen SC 600)		
Application rate (g a.s./kg)		1 × 350		
Folsomia candida	0.5113	112	219	5
Hypoaspis aculeifer		139	272	
Test item		Diflufenican (tested as Diflufenican SC 500)		
Application rate (g a.s./kg)		1 × 70		
Folsomia candida	0.405	219	541	5
Hypoaspis aculeifer		213	526	

1: Based on application rate of 1 x 0.7 L product/ha, 0% crop interception and a product density of 1.230 g/mL = 861 g/ha

The long-term TER values were above the risk assessment trigger value of 5. It is concluded that the risks to non-target soil meso- and macrofauna (other than earthworms) are acceptable when ACL + DFF SC 600 (500 + 100) G is used according to the recommended GAP.

Overall conclusions

All TER values for earthworms and other soil macro-organisms are above the trigger of concern. Therefore, no unacceptable risk to non-target soil organisms is expected using the product according to the proposed GAP.

CP 10.4.2.1 Species level testing

Data Point:	KCP 10.4.2.1/01
Report Author:	[redacted]
Report Year:	2017
Report Title:	Aclonifen + diflufenican SC 600 (500+100) G: Effects on mortality and reproduction of the predatory mite Hypoaspis aculeifer tested in artificial soil
Report No:	16 10 48 264 S
Document No:	M-580225-01-1
Guideline(s) followed in study:	EU Directive 91/414/EEC Regulation (EC) No 1107/2009 (2009) US EPA OCSPP Not Applicable
Deviations from current test guideline:	Current guideline: OECD 226, 2016 No Deviation
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

A study was conducted to determine the effect of aclonifen + diflufenican SC 600 (500+100) G on mortality and reproduction of the predaceous mite *Hypoaspis aculeifer*.

Ten adult, fertilized, female *Hypoaspis aculeifer* per replicate (8 control replicates and 4 replicates for each test item concentration) were exposed to control and treatments. Concentrations of 18, 32, 56, 100, 178, 316, 562 and 1000 mg test item /kg dry weight artificial soil were tested.

After a period of 14 days, the surviving adults and the living juveniles were counted.

The test item aclonifen + diflufenican SC 600 (500+100) G showed no statistically significantly adverse effects on adult mortality of the predatory mite *Hypoaspis aculeifer* at all tested concentrations. The test item showed no statistically significantly adverse effects on reproduction of *Hypoaspis aculeifer* up to and including 316 mg test item/kg soil dry weight. At a test concentration of 562 and 1000 mg test item/kg soil dry weight statistically significant effects on reproduction were observed.

Therefore, the No-Observed-Effect-Concentration (NOEC) and Lowest-Observed-Effect-Concentration (LOEC) for mortality were determined to be ≥1000 and >1000 mg test item/kg soil d.w., respectively. The NOEC and LOEC for reproduction were determined to be 316 and 562 mg test item/kg soil d.w., respectively.

The EC_{10} and EC_{20} values for reproduction were calculated to be 456 and 839 mg test item/kg soil dry weight, respectively.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test Item:** aclonifen + diflufenican SC 600 (500+100) G
   Batch no.: 2015-010653

Active Ingredient / Purity:	Aclonifen, 505.1 g/L (41.1%w/w) Diflufenican, 101.0 g/L (8.21% w/w)
Expiry date:	12 January 2016
Appearance:	Yellow suspension
Storage:	25 ± 5 °C

2. Test Organism: *Hypoaspis aculeifer* (Acari: Laelapidae)

Age: Adult, from a synchronised culture with age difference of 2 days

Source: [redacted]

Feeding: *Tyrophagus putrescentiae* (cheese mites)

B. STUDY DESIGN AND METHODS

1. In-life phase: 10 August to 1 September 2016

2. Exposure conditions

Test vessels: 100 mL SCHOTT-bottle with screw cap (inside dimensions: 4 cm in diameter, 11 cm high; supplier: VWR International)

Test soil: Artificial soil was prepared according to the guideline with the following constituents (percentage distribution on dry weight basis):

- 74.8% fine quartz sand (Millisil W3, fine sand with >50% of the particles between 50 and 200 µm)
- 5% Sphagnum peat, air-dried and finely ground
- 20% Kaolin clay (content of Kaolinite: $Al_2Si_2O_5(OH)_4$) >30%)
- approximately 0.2% Calcium carbonate ($CaCO_3$) (for the adjustment to pH to 6.0 ± 0.5)

Experimental design: Control and five test item groups (18, 32, 56, 100, 178, 316, 562 and 1000 mg test item/kg dry soil)

Replicates: Control – 8 replicates (+2 replicates for determination of water content & pH, without predatory mites);
Test item treatments – 4 replicates (+2 replicates for determination of water content & pH, without predatory mites)

Loading: 10 adult, fertilized female *Hypoaspis aculeifer* per replicate
Total 80 mites per control group
Total 40 mites per treatment group

Feeding: Before and during the test, the predatory mites were fed every 2 – 3 days with *Tyrophagus putrescentiae* (SCHRANK) reared in the [redacted]

Temperature: 20 ± 2 °C

Photoperiod: 16-hour light : 8-hour darkness
Light intensity: 507 lux

3. Administration of the test item

Dose preparation

All test item solutions were prepared freshly on the day of the application.

An exact weighed amount of the test item was mixed with deionised water to make a stock solution, without addition of solubility mediators, immediately before application. This stock solution was stepwise diluted with deionised water to prepare 7 further test solutions (serial dilution, spacing factor 4√10). Afterwards the test solutions were thoroughly mixed with the artificial soil by means of a hand stirrer. The preparation of the test substrate was performed in the following order: first the untreated control and thereafter the test item treated groups with increasing concentrations. Afterwards the treated artificial soil of each application rate and the control was portioned out. Each test vessel of the 8 control replicates and the 4 treatment replicates of each concentration plus 2 replicates for measurement purpose was filled up with 20 g dry weight artificial soil avoiding compression of the artificial soil. The remaining artificial soil was disposed.

Test organism assignment and exposure

The test was started with adult females of the soil mite species *Hypoaspis aculeifer* (CANESTRINI) which were taken from a synchronised culture with an age difference of 2 days. At day 35 after transfer of the parental females to the rearing vessels for egg-laying, the mites of the synchronised culture were suitable for the test.

At test start (within 2 h after treatment of the soil), adult females of the synchronised culture were transferred to the prepared test vessels which contained untreated (control) or test item treated artificial soil (20 g soil dry weight) with a water content of 40-60% of the maximum water holding capacity (WHC). Per test vessel 10 adult females were introduced by means of a moistened brush (= start of exposure). Afterwards the food mite *Tyrophagus putrescentiae* was added (approximately 20 mg per vessel), the test vessels were tightly closed and randomly set up in a controlled environment test room. The test was carried out under a controlled light-dark cycle. The water content of the soil substrate in the test vessels was determined at test start (after application) and at day 14 after application and was maintained throughout the test by reweighing the additional test vessels. Compensation of water loss was not necessary. The vessels were briefly opened every 2 - 3 days for aeration and feeding.

4. Measurements and observations

During the test the test vessels were briefly opening for aeration every 2 - 3 days and checking of the water content by reweighing the additional vessels. Daily checking of the food mites and replenishing if necessary.

14 days after start of exposure the physico-chemical parameters (water content, pH) for the each treatment group were determined and surviving mites and juveniles of *Hypoaspis aculeifer* were extracted from each test replicate using a temperature gradient. The extraction was conducted for 48 hours and during this time adult and juvenile mites moved down through the soil substrate away from the heat source, until they fell from the substrate into the funnel / fixing liquid. Following extraction,

all juveniles and adults present in the fixing liquid were counted. Any adult mites not found after extraction were recorded as dead. From these data the mortality of the adult females and the reproductive output were calculated.

5. Statistics/Data evaluation

The statistical analysis was performed with the software ToxRat 3.2.1 ([redacted] 2015). Multiple Sequentially-rejective Fisher Test with [redacted] correction and Williams-t-test were used to compare the control with the independent test item groups. Logit analysis was used for the ECx calculation.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

Analytical verification was not required.

B. BIOLOGICAL DATA

In the control group a parental mortality of 0.0% was observed. The mortality in the test item treatment groups ranged between 0.0 and 10.0%. Fourteen days after introduction of the parental mites into the test vessels, the mean number of juveniles was 287.5 in the control and 294.5, 297.0, 296.5, 276.3, 295.5, 267.3, 248.5 and 219.5 at concentrations 18, 32, 56, 100, 178, 316, 562 and 1000 mg test item/kg soil d.w., respectively.

Table: Effects of aclonifen + diflufenican SC 600 (500+100) G on mortality and reproduction of *Hypoaspis aculeifer*

Nominal Concentration (mg/kg)	Mortality (%)	Reproduction (juveniles/vessel)	Reproduction (% of control)
Control	0	287.5	-
18	5	294.5	102
32	0	297.0	103
56	5	296.5	103
100	10	276.3	96
178	0	295.5	103
316	2.5	267.3	93
562	5	248.5*	86
1000	5	219.5*	76

*: Statistical significance (Williams t-test for reproduction, one-sided smaller, $\alpha = 0.05$)

In a separate study the EC_{50} (reproduction) of the reference item dimethoate (EC 400 g/L, nominal) was calculated to be 3.1 mg a.s./kg soil d.w. The results of the reference test demonstrate the sensitivity of the test.

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 226, 2016)	Achieved

Control mortality	≤20%	0%
Mean number of juveniles per control replicate	≥50	287.5
Coefficient of variation for juveniles/control replicate	≤30%	6.4%

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint	Nominal concentration (mg test item/kg)
$LC_{50\ mortality}$	>1000
$NOEC_{mortality}$	≥1000
$LOEC_{mortality}$	>1000
$EC_{50\ reproduction}$	>1000
$EC_{20\ reproduction}$	839
$EC_{10\ reproduction}$	456
$NOEC_{reproduction}$	562
$LOEC_{reproduction}$	1000

III. CONCLUSION

There were no significant differences in reproduction between control and all concentrations up to 562 mg test item/kg dry weight artificial soil.

Therefore the No-Observed-Effect-Concentration (NOEC) for reproduction is 562 mg test item/kg dry weight artificial soil. The Lowest-Observed-Effect- Concentration (LOEC) for reproduction is 1000 mg test item/kg dry weight artificial soil.

[redacted] *(2017)*

Assessment and conclusion by applicant:

All validity criteria were satisfied and therefore this study can be considered to be valid.

The No-Observed-Effect-Concentration (NOEC) and EC_{10} for reproduction are 562 and 456 mg product/kg dry weight artificial soil respectively.

For use in the risk assessment, as the log P_{ow} values for aclonifen and diflufenican are greater than 2, in line with the EU Guidance Document on Terrestrial Ecotoxicology (SANCO/10329/2002), endpoints have to be divided by 2. The $NOEC_{corr}$ and $EC_{10\ corr}$ were therefore 281 and 228 mg product /kg dry weight artificial soil respectively.

In terms of active substance content, based on an aclonifen content of 41.1% w/w and a diflufenican content of 8.21% w/w, the $NOEC_{corr}$ and $EC_{10\ corr}$ were estimated to be 139 and 112 mg a.s./kg respectively.

EFSA's Outcome of the Pesticides Peer Review Meeting on general recurring issues in ecotoxicology (EFSA, 2019)[19] recommends that the lowest of the EC_{10} and NOEC values be used for risk assessment purposes. In this study, as the EC_{10} was lower than the NOEC, the EC_{10} should be used for risk assessment.

Assessment and conclusion by RMS:

Data Point:	KCP 10.4.2.1/02
Report Author:	[redacted]
Report Year:	2017
Report Title:	Aclonifen + diflufenican SC 600 (500+100) G: Effects on mortality and reproduction of the collembolan species Folsomia candida tested in artificial soil
Report No:	16 10 48 2636
Document No:	M-581084-01-1
Guideline(s) followed in study:	EU Directive 91/414/EEC; Regulation (EC) No 1107/2009 (2009); US EPA OCSPP Not Applicable
Deviations from current test guideline:	Current guideline: OECD 226, 2016 No Deviation
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

A study was conducted to determine the effect of aclonifen + diflufenican SC 600 (500+100) G on mortality and reproduction of the collembolan *Folsomia candida*.

Ten juvenile (9-12 day old) collembola *Folsomia candida* per replicate (8 control replicates and 4 replicates for each test item concentration) were exposed to control and treatments. Concentrations of 18, 32, 56, 100, 178, 316, 562 and 1000 mg test item /kg dry weight artificial soil were tested.

After a period of 4 weeks, the surviving adults and the living juveniles were counted.

The test item aclonifen + diflufenican SC 600 (500+100) G showed statistically significant adverse effects on adult mortality of the collembolan *Folsomia candida* in artificial soil at a concentration of 1000 mg test item/kg d.w.

[19] EFSA (European Food Safety Authority), 2019. Technical report on the outcome of the Pesticides Peer Review Meeting on general recurring issues in ecotoxicology. EFSA supporting publication 2019:EN-1673. 117 pp. doi:10.2903/sp.efsa.2019.EN-1673

The No-Observed-Effect-Concentration (NOEC) for mortality was determined to be 562 mg test item/kg soil d.w. and the Lowest-Observed-Effect-Concentration (LOEC) for mortality was determined to be 1000 mg test item/kg soil d.w.

The test item caused a significant reduction of reproduction of the collembolan *Folsomia candida* in artificial soil at concentrations of 316, 562 and 1000 mg test item/kg d.w. Therefore, the No-Observed-Effect-Concentration (NOEC) for reproduction was determined to be 178 mg test item/kg soil d.w. and the Lowest-Observed-Effect-Concentration (LOEC) for reproduction was determined to be 316 mg test item/kg soil d.w.

The EC_{10} and EC_{20} values for reproduction were calculated to be 155 and 234 mg test item/kg soil d.w., respectively.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test Item:** aclonifen + diflufenican SC 600 (500+100) G
   Batch no.: 2015010653
   Active Ingredient / Purity: Aclonifen, 505.1 g/L (41.1% w/w)
   Diflufenican, 101.0 g/L (8.21% w/w)
   Expiry date: 12 January 2016
   Appearance: Yellow suspension
   Storage: 25 ± 5°C

2. **Test Organism:** *Folsomia candida* (Willem)
   Age: Juvenile, from a synchronised culture aged 9-12 days old
   Source: [redacted]
   Feeding: Granulated dry yeast

B. STUDY DESIGN AND METHODS

1. **In-life phase:** 17 November to 15 December 2016

2. Exposure conditions

Test vessels: Glass container (approx. 150 mL) covered with a lid, surface area of soil: 18.9 cm^2

Test soil: Artificial soil was prepared according to the guideline with the following constituents (percentage distribution on dry weight basis):

- 74.7% fine quartz sand (Millisil W3, fine sand with >50% of the particles between 50 and 200 µm)
- 5% Sphagnum peat, air dried and finely ground
- 20% Kaolin clay (content of Kaolinite: $Al_2Si_2O_5(OH)_4$) >30%)

- approximately 0.3% Calcium carbonate ($CaCO_3$) (for the adjustment to pH to 5.5-6.0)

Experimental design:	Control and five test item groups (18, 32, 56, 100, 178, 316, 562 and 1000 mg test item/kg dry soil)
Replicates:	Control – 8 replicates (+2 replicates for determination of water content & pH, without predatory mites); Test item treatments – 4 replicates (+2 replicates for determination of water content & pH, without predatory mites)
Loading:	10 juvenile (9-12 day old) collembola, *Folsomia candida* per replicate Total 80 mites per control group Total 40 mites per treatment group
Feeding	Acclimation: granulated dry yeast, supplied twice a week During study: approximately 2 mg granulated dry yeast at the start of the test and after 14 days
Temperature:	20 ± 2 °C
Photoperiod:	16-hour light : 8-hour darkness
Light intensity:	Diffuse artificial light

3. Administration of the test item

Dose preparation

Two days before the start of the test, the dry artificial soil was pre-moistened by adding deionised water to obtain approximately half of the final water content. On the day of the test start, the test item was introduced by dispersing the quantity of test item required to obtain the desired test concentration in the volume of water required to hydrate the soil to 40 – 60% of its WHC. The control substrate contained the corresponding amount of deionised water only. After thorough mixing, 30 g (wet weight) of the test substrate was placed into each vessel, avoiding compression.

Test organism assignment and exposure

The test was started using juvenile collembolans, *Folsomia candida*, well-fed and 9 - 12 days old. Test organisms of a uniform age were obtained by transferring egg clusters from the breeding containers to fresh containers of fresh substrate 12 days before starting the experiment. After 72 hours these egg clusters were removed from the containers and the juveniles that had hatched during the preceding 72 hours were fed with granulated dry yeast. After a further 9 days the test organisms were collected and used for the test. Ten test organisms were introduced to each vessel, using an aspirator. After addition of the test organisms, the test vessels were positioned randomly in a controlled-environment test room, and these positions were re-randomized weekly. The test containers were tightly covered with a lid and briefly opened twice a week for aeration. The test organisms were fed twice during the experiment (at the start of the test and after 14 days) with approximately 2 mg of granulated dry yeast per test vessel.

4. Measurements and observations

The pH and water content of the test substrate were determined at the start and at the end of the test. The water content was checked weekly by reweighing the additional test vessels. Water loss was compensated if exceeding 2% of the initial water content.

Twice a week the test vessels were briefly opened for aeration. 2 weeks after start of exposure all treatment were fed with approximately 2 mg of granulated dry yeast per test vessel

Four weeks after introducing the test organisms the parental and juvenile collembolans in the test and control vessels were counted. The test substrate of each replicate was poured into an individual container and the test organisms were floated off the substrate by the addition of water. To improve the contrast between the white collembolans and surrounding water surface, the water was stained dark with ink. After gentle stirring the numbers of parental and juvenile collembolans floating on the surface were determined. Missing parental collembolans were assumed to have died during the test period. Surviving adults and juveniles were counted using a digital image processing system. The extraction efficiency of the extraction method was determined to be 97% in a separate extraction run using vessels containing a known number of juveniles kept in untreated test substrate.

5. Statistics/Data evaluation

Mortality (number of dead adults) as % for each treatment group was calculated. Missing collembolans were counted as dead. The reproductive output for each test item treatment group was calculated as % compared to control.

The statistical analysis was performed with the software ToxRat Professional 3.2.1 [redacted] 2015). Multiple Sequentially-rejective Fisher Test after [redacted] and Williams-t-test were used to compare the control with the independent test item group. The EC_x values were calculated by Probit analysis using linear maximum likelihood regression. Confidence limits (95%) of the EC_x values were computed by normal approximation.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

Analytical verification was not required.

B. BIOLOGICAL DATA

Mortality rates of 0 - 30.0% were recorded in the test item treatment groups. 3.8% parental mortality was observed in the control. Statistically significant effects on mortality compared to the control were observed at a concentration of 1000 mg test item/kg soil dry weight (Multiple Sequentially-rejective Fisher Test after [redacted] $\alpha = 0.05$, one-sided greater). No effects on behaviour of the collembolans were observed during the test.

The No-Observed-Effect-Concentration (NOEC) for mortality was determined to be 562 mg test item/kg soil d.w.

The mean number of juvenile collembolans counted four weeks after introduction of the parental collembolans into the test vessels was 714 in the control and 694, 710, 744, 706, 662, 471, 296 and 212 at concentrations of 18, 32, 56, 100, 178, 316, 562 and 1000 mg test item/kg soil d.w., respectively.

Statistically significant effects (Williams-t-test, α = 0.05, one-sided smaller) on the number of juveniles compared to the control group were recorded at concentrations of 316, 562 and 1000 mg test item/kg soil d.w.

The No-Observed-Effect-Concentration (NOEC) for reproduction was determined to be 178 mg test item/kg soil d.w.

The EC_{10} and EC_{20} values for reproduction were calculated to be 155 and 234 mg test item/kg soil d.w. respectively (based on Probit analysis.

Table: Effects of aclonifen + diflufenican SC 600 (500+100) G on mortality and reproduction of *Folsomia candida*

Nominal Concentration (mg/kg)	Mortality (%)	Reproduction (juveniles/vessel)	Reproduction (% of control)
Control	3.8	714	-
18	2.5	694	97
32	5.0	710	99
56	5.0	744	104
100	2.5	706	99
178	0	662	93
316	5.0	471	66**
562	10.0	296	41**
1000	30.0*	212	30**

*: Statistical significance (Multiple Sequentially-rejective Fisher Test After Bonferroni-Holm, one-sided-greater, α = 0.05)

**: Statistical significance (Williams-t-test, one-sided-smaller, α = 0.05)

In a separate study, the EC_{50} (reproduction) of the reference item boric acid was calculated to be 104 mg/kg soil dry weight. The results of the reference test demonstrate the sensitivity of the test system.

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 226, 2016)	Achieved
Mean adult control mortality	≤20%	3.8%
Mean number of juveniles per control replicate	≥100	714
Coefficient of variation for mean no. juveniles/control replicate	≤30%	11.3%

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint	Nominal concentration (mg test item/kg)

$LC_{50\ mortality}$	>1000
$NOEC_{mortality}$	562
$LOEC_{mortality\ n}$	1000
$EC_{50\ reproduction}$	n.c.
$EC_{20\ reproduction}$	234 (183 – 29)
$EC_{10\ reproduction}$	155 (110 – 217)
$NOEC_{reproduction}$	178
$LOEC_{reproduction}$	316

n.c.: Not calculable. <50% reduction in reproduction at all test concentrations

III. CONCLUSION

The test item aclonifen + diflufenican SC 600 (500+100) G showed statistically significant adverse effects on adult mortality of the collembolan *Folsomia candida* in artificial soil at a concentration of 1000 mg test item/kg d.w.

The No-Observed-Effect-Concentration (NOEC) for mortality was determined to be 562 mg test item/kg soil d.w. and the Lowest-Observed-Effect-Concentration (LOEC) for mortality was determined to be 1000 mg test item/kg soil d.w.

The test item caused a significant reduction of reproduction of the collembolan *Folsomia candida* in artificial soil at concentrations of 316, 562 and 1000 mg test item/kg d.w. Therefore, the No-Observed-Effect-Concentration (NOEC) for reproduction was determined to be 178 mg test item/kg soil d.w. and the Lowest-Observed-Effect-Concentration (LOEC) for reproduction was determined to be 316 mg test item/kg soil d.w.

The EC_{10} and EC_{20} values for reproduction were calculated to be 155 and 234 mg test item/kg soil d.w., respectively.

[redacted] *(2017)*

Assessment and conclusion by applicant:

All validity criteria were satisfied and therefore this study can be considered to be valid.

The No-Observed-Effect-Concentration (NOEC) and EC_{10} for reproduction are 178 and 155 mg product/kg dry weight artificial soil respectively.

For use in the risk assessment, as the log P_{ow} values for aclonifen and diflufenican are greater than 2, in line with the EU Guidance Document on Terrestrial Ecotoxicology (SANCO/10329/2002), endpoints have to be divided by 2. The $NOEC_{corr}$ and $EC_{10\ corr}$ were therefore 89 and 77.5 mg product /kg dry weight artificial soil respectively.

In terms of active substance content, based on an aclonifen content of 41.1% w/w and a diflufenican content of 8.21% w/w, the $NOEC_{corr}$ and $EC_{10\ corr}$ were estimated to be 43.9 and 38.2 mg a.s./kg respectively.

EFSA's Outcome of the Pesticides Peer Review Meeting on general recurring issues in ecotoxicology (EFSA, 2019)[20] recommends that the lowest of the EC_{10} and NOEC values be used for risk assessment purposes. In this study, as the EC_{10} was lower than the NOEC, the EC_{10} should be used for risk assessment.

Assessment and conclusion by RMS:

Data Point:	KCP 10.4.2.1/03
Report Author:	[redacted]
Report Year:	2011
Report Title:	Aclonifen SC 600 G: Influence on the reproduction of the collembolan species Folsomia candida tested in artificial soil
Report No:	FRM-COLL-112/10
Document No:	M-404393-01-1
Guideline(s) followed in study:	OECD 232 adopted, September 07, 2009. OECD Guidelines for Testing Chemicals - Collembolan Reproduction Test in Soil
Deviations from current test guideline:	Current Guideline: OECD 232, 2016 Due to technical reasons the soil was premoistened at test start instead of 2 to 7 days before start of the test. No influence on the study.
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

A study was conducted to determine the effect of aclonifen SC 600 G on the mortality and reproduction of the collembolan species *Folsomia candida*. 10 collembolans (11-12 days old) per replicate (8 replicates for the control group and 4 replicates for each treatment group) were exposed to control (water treated), 100, 178, 316, 562 and 1000 mg test item/kg artificial soil dry weight at 20 ± 2°C, 400 – 800 lux, 16h light : 8h dark. During the study, they were fed with granulated dry yeast.

Mortality and reproduction were determined after 28 days. In the control group 8.8% of the adult *Folsomia candida* died which is below the allowed maximum of ≤20% mortality.

Concerning the number of juveniles, statistical analysis (William's-t test, one-sided smaller, $\alpha = 0.05$) revealed no significant difference between control and the treatment groups with 100 to 316 mg test

[20] EFSA (European Food Safety Authority), 2019. Technical report on the outcome of the Pesticides Peer Review Meeting on general recurring issues in ecotoxicology. EFSA supporting publication 2019:EN-1673. 117 pp. doi:10.2903/sp.efsa.2019.EN-1673

item/kg artificial soil dry weight. Therefore, the No Observed Effect Concentration (NOEC) for reproduction is 316 mg test item/kg artificial soil dry weight. The Lowest Observed Effect Concentration (LOEC) for reproduction is 562 mg test item/kg artificial soil dry weight.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test Item:** Aclonifen SC 600 G

   Batch no.: EV54001166

   Active Ingredient / Purity: Aclonifen, 595.0 g/L (49.4%w/w)

   Expiry date: 16 February 2013

   Appearance: Yellow suspension

   Storage: 25 ± 5 °C

2. **Test Organism:** *Folsomia candida* (Collembolan, Isotomidae)

   Age: Collembolans from a synchronised culture at an age of 11-12 days

   Source: [redacted]

   Feeding: Bakers dry yeast

B. STUDY DESIGN AND METHODS

1. In-life phase: 22 February – 23 March 2011

2. Exposure conditions

Test vessels: Glass vessels (volume 140 mL, diameter 5 cm at the bottom, height 7 cm). The test vessels were covered with glass lids to prevent the collembolans from escaping but allowing aeration during the test period

Test soil: Artificial soil was prepared according to the guideline with the following constituents (percentage distribution on dry weight basis):

74.8% fine quartz sand (sort F 36, particle size 0.2 – 0.05 mm = 91.35%)

5% Sphagnum peat, air dried and finely ground

20% Kaolin clay (content of Kaolinite: $Al_2Si_2O_5(OH)_4$) = 30.2%)

approximately 0.2% Calcium carbonate ($CaCO_3$) (for the adjustment to pH to 6.0 ± 0.5)

Experimental design: Control and five test item groups (100, 178, 316, 562 and 1000 mg a.s./kg dry soil)

Replicates:	Control – 8 replicates; Test item treatments – 4 replicates
Loading:	10 collembolans per replicate
Temperature:	20 ± 2 °C
Photoperiod:	16-hour light: 8-hour darkness
Light intensity:	400 – 800 lux

3. Administration of the test item

Dose preparation

All test item solutions were prepared freshly on the day of the application.

- Stock solution (= solution 1) 2.4991 g test item filled up to a volume of 250 mL with deionised water (1000 mg test item/kg dry weight artificial soil).
- Solution 2: 141 mL solution 1 was filled up to 250 mL with deionised water (562 mg test item/kg dry weight artificial soil)
- Solution 3: 141 mL solution 2 was filled up to 250 mL with deionised water (316 mg test item/kg dry weight artificial soil)
- Solution 4: 141 mL solution 3 was filled up to 250 mL with deionised water (178 mg test item/kg dry weight artificial soil)
- Solution 5: 140 mL solution 4 was filled up to 250 mL with deionised water (100 mg test item/kg dry weight artificial soil)

A uniform volume of 50 mL was used for all application solutions (starting with the lowest application rate and ending with the highest application rate). The test item was thoroughly mixed into 500 g artificial dry weight artificial soil of each application rate using a laboratory mixer (Krefft). The control group was treated first in the same way as described above but with 50 mL deionised water only. Afterwards the treated artificial soil of each application rate and the control was portioned out. Each test vessel of the 8 control replicates and the 4 treatment replicates of each concentration plus the one for measurement purpose was filled up with 30 g dry weight artificial soil avoiding compression of the artificial soil. The remaining artificial soil was disposed.

Test organism assignment and exposure

Directly after application the 11-12 days old collembolans from the synchronised cohort were collected with an exhauster into small glass tubes. They were counted to ensure that 10 non-damaged individuals were introduced. Then the 10 collembolans were placed on the soil surface of the treated soil and the untreated control respectively. The sequence of inserting the test organisms into the test vessels followed a computer-generated random design.

4. Measurements and observations

Directly after the addition of the collembolans, they were fed with granulated dry yeast. Feeding was also done 14 days after test start. Approximately 2 mg (one spatula tip) per test vessel was added per feeding date.

At test start each test vessel was weighed for the determination of water loss. After 14 days the loss of water was determined by reweighing the test vessels. The vessels were re-wetted with the approximately

2-fold amount of the missing water. The test vessels were set up randomised in a climatic test room. After 7, 14 and 21 days the test vessels were re-randomised.

After 28 days, the soil of each replicate was transferred into a plastic vessel (volume: 200 mL; surface: 75 cm^2). Each portion was stirred up with 80 mL of deionised water and the collembolans drifted to the surface. The water was coloured with 10 mL black ink in order to increase the contrast between the water and the white collembolans. From each vessel a digital image was taken. Each digital image was checked by visual inspection. In a first step the adult collembolans were visually counted and marked on the digital image. In a second step the automatically counted juveniles were checked for mistakes and the counting was manually corrected if necessary. These procedures were carried out with the LemnaTec Scanalyzer, Software: [redacted]

5. Statistics/Data evaluation

Endpoints of the test were mortality of the adult collembolans in comparison to the initially placed test organisms expressed in % and the number of offspring hatched from the eggs and surviving until the end of the test period per test vessel (reproduction). Missing adults (compared to the number of initially placed test organisms) were considered to be dead, since dead collembolans cannot be extracted.

Data of reproduction were tested for normal distribution and homogeneity of variance using Kolmogorov - Smirnov -Test and Cochran's -Test ($\alpha = 0.05$) respectively. Data of reproduction were normally distributed and homogeneity of variances was given. Therefore William's-t test (one-sided-smaller, $\alpha = 0.05$) was used to determine NOEC and LOEC values.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

Analytical verification was not required.

B. BIOLOGICAL DATA

In the control group 8.8% of the adult collembolans died which is below the allowed maximum of ≤20% mortality.

Concerning the number of juveniles, a statistically significant effect (William's-t test, one-sided-smaller, $\alpha = 0.05$) was found in the treatment groups with 562 and 1000 mg test item/kg artificial soil dry weight. The No Observed Effect Concentration ($NOEC_{reproduction}$) is 316 mg test item/kg artificial soil dry weight. The Lowest Observed Effect Concentration ($LOEC_{reproduction}$) is 562 mg test item/kg artificial soil dry weight.

Table: Effects of aclonifen SC 600 G on mortality and reproduction of *Folsomia candida*

Nominal concentration (mg/kg)	Mortality (%)	Reproduction (juveniles/vessel)	Reproduction (% of control)
Control	8.8	1053.3	-
200	7.5	1154.3	109.6
178	10.0	1086.8	103.2
316	7.5	1056.5	100.3

Nominal concentration (mg/kg)	Mortality (%)	Reproduction (juveniles/vessel)	Reproduction (% of control)
562	15.0	657.5	62.4[1]
1000	25.0	455.0	43.2

1 Statistical significance (Williams t-test, one-sided smaller, α = 0.05)

In a separate, non-GLP reference item test, boric acid showed an EC_{50} for reproduction of 91 mg/kg which was within the recommended range of the guideline of about 100 mg/kg dry weight artificial soil. This shows that the test organisms are sufficiently sensitive.

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 232, 2016)	Achieved
Control mortality	≤ 20%	8.8%

The validity criterion was satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint	Nominal concentration (mg test item/kg)
$LC_{50\ mortality}$	1000
$NOEC_{reproduction}$	316
$LOEC_{reproduction}$	562

III. CONCLUSION

There were no significant differences in reproduction between control and the treatment groups with 100 to 316 mg test item/kg artificial soil dry weight.

Therefore, the No Observed-Effect-Concentration (NOEC) for reproduction is 316 mg test item/kg dry weight artificial soil. The Lowest Observed-Effect- Concentration (LOEC) for reproduction is 562 mg test item/kg dry weight artificial soil.

[redacted] *(2011)*

Data Point:	KCP 10.4.2.1/06
Report Author:	[redacted]
Report Year:	2019
Report Title:	Aclonifen SC 600 G: Influence on the reproduction of the collembolan species Folsomia candida tested in artificial soil - Statistical re-analysis of [redacted], 2011 (M-404393-01-1) study
Report No:	VC/19/027/003
Document No:	M-675907-01-1
Guideline(s) followed in study:	Not applicable. Report is a re-evaluation of previously generated study data
Deviations from current test guideline:	Not applicable
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The report for the collembolan reproduction test of Aclonifen SC 600 G to Folsomia candida (M-404393-01-1, [redacted], 2011) only provided LOEC and NOEC values for the test item. Data from the study has been re-analysed in order to provide L/EC_{10}, L/EC_{20} and L/EC_{50} values.

Statistical analyses of the available data resulted in the calculation of the following L/EC_x values:

Parameter	Reproduction			Survival		
	EC_{10}	EC_{20}	EC_{50}	LC_{10}	LC_{20}	LC_{50}
Value (mg/kg)	311.268	429.338	794.310	736.11	1058.61	n.d
Lower 95%-cl	228.355	315.847	529.884	482.58	788.82	n.d
Upper 95%-cl	424.286	584.331	1168.703	1062.64	1238.95	n.d

n.d.: not determined due to mathematical reasons (inappropriate data) or value is beyond the tested concentrations

All computations were carried out in ToxRat Professional version 3.3.0 (ToxRat Solutions GmbH, 2018).

[redacted] *(2019)*

Assessment and conclusion by applicant

All validity criteria were satisfied and therefore this study can be considered to be valid.

The No-Observed-Effect-Concentration (NOEC) for reproduction is 316 mg product/kg dry weight artificial soil.

L/EC_{10}, L/EC_{20} and L/EC_{50} values were determined following re-analysis of the original study data and are summarised below.

Parameter	Reproduction			Survival		
	EC_{10}	EC_{20}	EC_{50}	LC_{10}	LC_{20}	LC_{50}
Value (mg prod./kg)	311.268	429.338	794.310	736.11	1058.61	n.d

Lower 95%-cl	228.355	315.847	529.884	482.58	788.82	n.d
Upper 95%-cl	424.286	584.331	1168.703	1062.64	238.95	n.d

n.d.: not determined due to mathematical reasons (inappropriate data) or value is beyond the tested concentrations

EFSA's Outcome of the Pesticides Peer Review Meeting on general recurring issues in ecotoxicology (EFSA, 2019)[21] recommends that the lowest of the EC_{10} and NOEC values be used for risk assessment purposes. In this study, as the EC_{10} was lower than the NOEC, the EC_{10} should be used for risk assessment.

For use in the risk assessment, as the log P_{ow} for aclonifen is greater than 2, in line with the EU Guidance Document on Terrestrial Ecotoxicology (SANCO/10329/2002), endpoints have to be divided by 2. The $EC_{10\ corr}$ was therefore 281 mg product /kg dry weight artificial soil.

In terms of the active ingredient content, based on an active ingredient content of 49.4%, the $EC_{10\ corr}$ was estimated to be 77 mg aclonifen/kg.

Assuming a soil incorporation depth of 5 cm and a bulk soil density of 1.5, the $EC_{10\ corr}$ was estimated to be 58 kg a.s./ha.

Assessment and conclusion by RMS:

Data Point:	KCP 10.4.2.1/04
Report Author:	[redacted]
Report Year:	2011
Report Title:	Aclonifen SC 600 G: Influence on mortality and reproduction on the soil mite species Hypoaspis aculeifer tested in artificial soil
Report No:	KBA-HR-45/11
Document No:	M-404537-01-1
Guideline(s) followed in study:	OECD 226 from October 03, 2008: OECD guideline for the Testing of Chemicals - Predatory mite (Hypoaspis (Geolaelaps) aculeifer) reproduction test in soil
Deviations from current test guideline:	Current guideline: OECD 226, 2008 Transfer of the test animals was finished within three hours after the application of the test item rather than within two hours due to technical reasons. This has no impact on this study.
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

[21] EFSA (European Food Safety Authority), 2019. Technical report on the outcome of the Pesticides Peer Review Meeting on general recurring issues in ecotoxicology. EFSA supporting publication 2019:EN-1673. 117 pp. doi:10.2903/sp.efsa.2019.EN-1673

Executive Summary

A study was conducted to determine the effect of aclonifen SC 600 G on mortality and reproduction of the predaceous mite *Hypoapsis aculeifer*.

Ten adult, fertilized, female *Hypoaspis aculeifer* per replicate (8 control replicates and 4 replicates for each test item concentration) were exposed to control and treatments. Concentrations of 100, 178, 316, 562 and 1000 mg test item/kg dry weight artificial soil were tested.

After a period of 14 days, the surviving adults and the living juveniles were counted.

In the control group 3.8% of the adult *Hypoaspis aculeifer* died which is below the allowed maximum of ≤20% mortality. The LC_{50} could not be calculated and is considered to be > 1000 mg test item/kg dry weight artificial soil.

Concerning the number of juveniles, statistical analysis (Williams t-test, one-sided smaller, $\alpha = 0.05$) revealed no significant difference between control and all concentrations up to 562 mg test item/kg dry weight artificial soil. Therefore, the No-Observed-Effect-Concentration (NOEC) for reproduction is 562 mg test item/kg dry weight artificial soil. The Lowest-Observed-Effect-Concentration (LOEC) for reproduction is 1000 mg test item/kg dry weight artificial soil.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Item: Aclonifen SC 600 G

Batch no.: EVS4001166

Active Ingredient / Purity: Aclonifen, 595.0 g/L (49.4% w/w)

Expiry date: 16 February 2013

Appearance: Yellow suspension

Storage: 25 ± 5°C

2. Test Organism: Hypoaspis aculeifer (Acari: Laelapidae)

Age: Adult, fertilized females were used as test organisms in the study 29 days after start of egg laying

Source: [redacted]

Feeding: Tyrophagus putrescentiae (cheese mites)

B. STUDY DESIGN AND METHODS

1. In-life phase: 22 February – 21 March 2011

2. Exposure conditions

Test vessels:	Glass vessels (Weck Mini-Sturzglas, volume 140 mL, diameter 5 cm at the bottom, height 7 cm). The test vessels were covered with glass lids to prevent *Hypoaspis aculeifer* from escaping but allowing aeration during the test period.
Test soil:	Artificial soil was prepared according to the guideline with the following constituents (percentage distribution on dry weight basis): 74.8% fine quartz sand (sort F 36, particle size 0.2 – 0.05 mm = 91.35%) 5% Sphagnum peat, air dried and finely ground 20% Kaolin clay (content of Kaolinite: $Al_2Si_2O_5(OH)_4$) = 30.2%) approximately 0.2% Calcium carbonate ($CaCO_3$) for the adjustment to pH to 6.0 ± 0.5)
Experimental design:	Control and five test item groups (100, 178, 316, 562 and 1000 mg a.s./kg dry soil)
Replicates:	Control – 8 replicates; Test item treatments – 4 replicates
Loading:	10 adult, fertilized female *Hypoaspis aculeifer* per replicate
Temperature:	20 ± 2 °C
Photoperiod:	16-hour light / 8-hour darkness
Light intensity:	400 – 800 lux

3. Administration of the test item

Dose preparation

All test item solutions were prepared freshly on the day of the application.

- Stock solution (= solution 1) 2.4991 g test item filled up to a volume of 250 mL with deionised water (1000 mg test item/kg dry weight artificial soil).
- Solution 2: 141 mL solution 1 was filled up to 250 mL with deionised water (562 mg test item/kg dry weight artificial soil)
- Solution 3: 141 mL solution 2 was filled up to 250 mL with deionised water (316 mg test item/kg dry weight artificial soil)
- Solution 4: 141 mL solution 3 was filled up to 250 mL with deionised water (178 mg test item/kg dry weight artificial soil)
- Solution 5: 140 mL solution 4 was filled up to 250 mL with deionised water (100 mg test item/kg dry weight artificial soil)

A uniform volume of 50 mL was used for all application solutions (starting with the lowest application rate and ending with the highest application rate). The test item was thoroughly mixed into 500 g artificial dry weight artificial soil of each application rate using a laboratory mixer (Krefft). The control group was treated first in the same way as described above but with 50 mL deionised water only. Afterwards the treated artificial soil of each application rate and the control was portioned out. Each test vessel of the 8 control replicates and the 4 treatment replicates of each concentration plus the one for

measurement purpose was filled up with 20 g dry weight artificial soil avoiding compression of the artificial soil. The remaining artificial soil was disposed.

Test organism assignment and exposure

Directly after application of the test item, the adult, fertilized, female (29 days after start of egg laying for three days) were exposed to the control and treatment vessels. This was achieved by putting 10 females individually onto the surface of the artificial soil using a fine brush.

4. Measurements and observations

Directly after the addition of the *Hypoaspis aculeifer*, they were fed with cheese mites (*Tyrophagus putrescentiae*). During the test the soil mites were fed 3, 7 and 10 days after test start with the cheese mites.

Each test vessel was weighed for the determination of water loss. Seven days after test start water loss was determined and replenished.

After a period of 14 days, the surviving adults and the living juveniles per test vessel were extracted, applying a temperature gradient. All *Hypoaspis aculeifer* (adult, females and juveniles) were counted under a binocular.

5. Statistics/Data evaluation

The calculation of mean, standard deviation and % mortality of the control and treatment groups with Excel sheets (Microsoft Excel 2003) were documented in the raw data.

For the reproduction, for normal distribution and homogeneity of variance using Kolmogorroff-Smirnov Test and Cochran-Test ($\alpha = 0.05$), respectively were used. Data of reproduction were normally distributed and homogeneity of variances was given. Therefore, Williams t-test (one-sided smaller, $\alpha = 0.05$) was used to determine NOEC and LOEC values. Probit analysis was used to determine the EC_{50} value.

The software used to perform the statistical analysis was ToxRat Pro 2.10 (released February 19, 2009); (Ratte, 2001-2009).

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

Analytical verification was not required.

B. BIOLOGICAL DATA

In the control group 3.8% of the adult *Hypoaspis aculeifer* died which is below the allowed maximum of ≤20% mortality. The LC_{50} could not be calculated and is considered to be >1000 mg test item/kg dry weight artificial soil.

Concerning the number of juveniles, statistical analysis (Williams t-test, one-sided smaller, $\alpha = 0.05$) revealed no significant differences between control and the all concentrations tested. Therefore, the No Observed-Effect-Concentration (NOEC) for reproduction is 562 mg test item/kg dry weight artificial soil. The Lowest-Observed-Effect- Concentration (LOEC) for reproduction is 1000 mg test item/kg dry weight artificial soil.

Table: Effects of aclonifen SC 600 G on mortality and reproduction of *Hypoaspis aculeifer*

Nominal concentration (mg/kg)	Mortality (%)	Reproduction (juveniles/vessel)	Reproduction (% of control)
Control	3.8	333.0	-
100	7.5	331.5	99.5
178	0.0	335.0	100.6
316	5.0	335.8	100.8
562	2.5	343.5	103.2
1000	2.5	301.8[1]	90.6

[1]: statistical significance (Williams t-test, one-sided smaller, $\alpha = 0.05$)

In a separate non-GLP reference item test, Dimethoate showed a LC_{50} of 4.051 mg a.s./kg and a $NOEC_{reproduction}$ of 3.156 mg a.s./kg. The EC_{50} for reproduction was 6.445 mg a.s./kg which was within the recommended range of the guideline of 3.0 – 7.0 mg a.s./kg dry weight artificial soil. This shows that the test organisms are sufficiently sensitive

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 226, 2016)	Achieved
Control mortality	≤20%	3.8%
Mean number of juveniles per control replicate	≥50	333
Coefficient of variation for juveniles/control replicate	≤30%	4.4%

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint	Nominal Concentration (mg test item/kg)
$LC_{50\ mortality}$	1000
$NOEC_{reproduction}$	562
$LOEC_{reproduction}$	1000

III. CONCLUSION

There were no significant differences in reproduction between control and all concentrations up to 562 mg test item/kg dry weight artificial soil.

Therefore, the No-Observed-Effect-Concentration (NOEC) for reproduction is 562 mg test item/kg dry weight artificial soil. The Lowest-Observed-Effect- Concentration (LOEC) for reproduction is 1000 mg test item/kg dry weight artificial soil.

[redacted] *(2011)*

Assessment and conclusion by applicant:

All validity criteria were satisfied and therefore this study can be considered to be valid.

The No-Observed-Effect-Concentration (NOEC) for reproduction is 562 mg aclonifen SC 600 G /kg dry weight artificial soil.

Effects on reproduction at the highest test concentration of 1000 mg aclonifen SC 600 G /kg dry weight artificial soil were less than 10%. It was not therefore possible to determine EC_{10}, EC_{20} or EC_{50} values.

In terms of the active ingredient content, assuming a product density of 1.2 and an active ingredient content of 60%, the NOEC was estimated to be 281 mg aclonifen/kg.

Assuming a soil incorporation depth of 5 cm and a bulk soil density of 1.5, the NOEC was estimated to be 210 kg a.s./ha.

Assessment and conclusion by RMS:

Data Point:	KCP 10.4.2.1/05
Report Author:	[redacted]
Report Year:	2015
Report Title:	Diflufenican SC 500 G: Effects on reproduction of the predatory mite Hypoaspis aculeifer in artificial soil with 5 percent peat
Report No:	105761089
Document No:	M-533188-01-1
Guideline(s) followed in study:	EU Directive 91/414/EEC; Regulation (EC) No 1107/2009 (2009); US EPA OCSPP not applicable; OECD 226: Guidelines for the testing of chemicals - Predatory Mite (Hypoaspis (Geolaelaps) aculeifer) reproduction test in soil, adopted October 03, 2008
Deviations from current test guideline:	Current guideline: OECD 226, 2016 No Deviation
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

A study was conducted to determine the effect of Diflufenican SC 500 G on mortality and reproduction of the predaceous mite *Hypoapsis aculeifer*.

Ten adult, fertilized, female *Hypoaspis aculeifer* per replicate (8 control replicates and 4 replicates for each test item concentration) were exposed to control and treatments. Concentrations of 100, 178, 316, 562 and 1000 mg test item/kg dry soil were tested.

After a period of 14 days, the surviving adults and the living juveniles were counted.

Diflufenican SC 500 G caused no statistically significant effects on mortality or reproduction of *Hypoaspis aculeifer* up to and including the concentration of 1000 mg test item/kg soil.

Therefore, the overall No Observed Effect Concentration (NOEC) was determined to be ≥1000 mg test item/kg soil. The overall Lowest Observed Effect Concentration (LOEC) was estimated to be >1000 mg test item/kg soil.

I. MATERIALS AND METHODS

A. MATERIALS

1.	**Test Item:**	Diflufenican SC 500 G
	Batch no.:	2015-005338-01
	Active Ingredient / Purity:	Diflufenican: 500 g/L (nominal), 502.6 g/L (42.6% w/w) (analysed)
	Expiry date:	22 June 2017
	Appearance:	light beige liquid
	Storage:	+2° C to +30° C, in the dark
2.	**Test Organism:**	*Hypoaspis aculeifer* (Canestrini 1883)
	Age:	Adult females (from a synchronized cohort)
	Source:	[redacted]
	Feeding:	*Tyrophagus putrescentiae* (cheese mites)

B. STUDY DESIGN AND METHODS

1. **In-life phase:** 20 July – 05 August 2015

2. Exposure conditions

Test vessels: Glass containers (volume: 100 mL; diameter: 5 cm), tight screw top closure to avoid water evaporation, filled with approximately 20 g ± 1.0 g artificial soil dry weight.

Test soil: According to OECD 226:

- 5% Sphagnum-peat, air-dried and finely ground (with no visible plant remains) ([redacted])
- 20% Kaolin clay (Kaolinite content > 30%; [redacted])
- 74.8% fine quartz-sand (F34) (depending on the amount of $CaCO_3$ needed, more than 50% by mass of particle size 0.05 mm to 0.2 mm; [redacted])
- 0.2% Calcium carbonate ($CaCO_3$) extra pure ([redacted]) to adjust pH to 6.0 ± 0.5.

The artificial soil was moistened to approximately half of the final water content 3 days before the application. The additional

	water required to achieve the final water content was added when applying the test item.
Maximum Water Holding Capacity (WHC):	42% of the dry weight
Experimental design:	Control and five test item groups (100, 178, 316, 562 and 1000 mg test item/kg dry soil)
Replicates:	4 per treatment group and 8 for the control. 1 additional container per treatment to check the pH and water content of the test substrate after 14 days
Loading:	10 adult female mites per unit
Feeding	Cheese mites (*Tyrophagus putrescentiae*, cultured by ibacon) were placed on the soil surface: 2 spatulas on day 0, 2 and 4, 1 spatula on day 7 and 9, ½ spatula on day 11.
Temperature:	18 °C to 22 °C
Photoperiod:	16-hour light : 8-hour darkness
Light intensity:	400 to 800 lux

3. Administration of the test item

Dose preparation

A stock solution was prepared by weighing 750.0 mg of Diflufenican SC 500 G using an analytical balance. The test item was transferred into a glass beaker and deionised water was added to obtain a final net weight of 100.9 g. The resulting suspension contained a concentration of 7.4331 mg test item/g. A dilution series was prepared and 26.9 g of the stock solution or of the corresponding dilutions were added to artificial soil equivalent to 200 g dry weight to prepare the target concentrations in the soil.

The control was not treated and was moistened with deionised water. The soil for each treatment group was mixed with a laboratory mixer to ensure a homogeneous distribution. Each group was treated in one batch and then split into the replicates.

Test organism assignment and exposure

The test organisms were collected with a fine brush, put into a small glass tube, counted to ensure that 10 adult females were introduced and placed onto the surface of the treated artificial soil

4. Measurements and observations

All vessels including the additional containers were ventilated on days 2, 4, 7, 9 and 11 by opening the lids for a short period.

Water content was checked on day 7 after application by reweighing the additional test containers. Loss of water was not compensated as it did not deviate by more than 2% from the initial water content.

After 14 days exposure the soil was filled into Millipore pots with attached plastic containers for collecting the escaping mites. These extraction units were placed in a Kempson extractor. The soil including the mites was exposed to a temperature of approximately 25 °C and 30 °C for approximately 2 days. Escaping mites were collected in a fixing liquid, cooled at a temperature of approximately 16 °C. The fixing liquid contained glycol and a detergent.

Adult animals were counted once visually, juvenile animals were counted twice under binocular microscopes. None of the replicate counts deviated more than 10% from their mean value.

The number of surviving adult female predatory mites 14 days after test initiation was recorded (counted after extraction). Missing adult predatory mites were recorded as dead as it was assumed they would have died and degraded during the test period.

The living predatory mites were observed for differences in morphology or any abnormalities at experimental end.

The number of juvenile mites at day 14 after application was counted after extraction.

5. Statistics/Data evaluation

Mortality data were statistically analysed using Fisher's Exact Binomial Test (multiple comparison, with Bonferroni Correction, α = 0.05, one-sided greater).

Reproduction data were tested for normal distribution and homogeneity of variance using Shapiro-Wilk's test and Levene's test (α = 0.05). As data were normally distributed and homogeneous, the further statistical evaluation was performed using Williams t-test (multiple comparison, α = 0.05, one-sided smaller).

The determination of the NOEC and LOEC values was based on the results of the statistical evaluation. The EC_{10} and EC_{20} values could not be determined due to mathematical reasons.

The software used to perform the statistical analysis was ToxRat Professional, Version 2.10.05, ToxRat® Solutions GmbH.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

Analytical verification was not required.

B. BIOLOGICAL DATA

Mortality of *Hypoaspis aculeifer* in the test item treated groups ranged from 3% to 18%. The values were not statistically significantly different compared to the control, where 5% of the adult mites were dead (Fisher's Exact Test, α = 0.05, one-sided greater). Therefore the No Observed Effect Concentration (NOEC) for mortality was determined to be ≥1000 mg test item/kg soil. The LOEC for mortality was estimated to be >1000 mg test item/kg soil.

No differences in morphology of the mites between the test item treated groups and the control were observed.

There were no statistically significant effects on reproduction of *Hypoaspis aculeifer* up to and including the highest test concentration of 1000 mg test item/kg soil (Williams t-test, α = 0.05, one-sided smaller). Therefore, the No Observed Effect Concentration (NOEC) was determined to be ≥1000 mg test item/kg soil and the LOEC was estimated to be >1000 mg test item/kg soil. The EC_{10} and EC_{20} values could not be determined due to mathematical reasons.

Table: Effects of Diflufenican SC 500 G on mortality and reproduction of *Hypoaspis aculeifer*

Nominal Concentration (mg/kg)	Mortality (%)	Reproduction (juveniles/replicate)	Reproduction (% of control)
Control	5	209	-
100	13	270	129
178	18	208	99.7
316	5	174	83.4
562	8	215	103
1000	3	172	82.1

In a separate study the EC_{50} (reproduction) of the reference item dimethoate (EC 400 g/L, nominal) was calculated to be 3.9 mg a.s./kg soil d.w. The results of the reference test demonstrate the sensitivity of the test.

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 226, 2016)	Achieved
Control mortality	≤20%	5%
Mean number of juveniles per control replicate	≥50	149 – 260
Coefficient of variation for juveniles/control replicate	≤30%	15.8%

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint	Nominal concentration (mg test item/kg)
$NOEC_{mortality}$	≥1000
$LOEC_{mortality}$	>1000
$NOEC_{reproduction}$	≥1000
$LOEC_{reproduction}$	>1000

III. CONCLUSION

Diflufenican SC 500 G caused no statistically significant effects on mortality or reproduction of *Hypoaspis aculeifer* up to and including the concentration of 1000 mg test item/kg soil.

Therefore, the overall No Observed Effect Concentration (NOEC) was determined to be ≥1000 mg test item/kg soil. The overall Lowest Observed Effect Concentration (LOEC) was estimated to be >1000 mg test item/kg soil.

[redacted] *(2015)*

Assessment and conclusion by applicant:

All validity criteria were satisfied and therefore this study can be considered to be valid.

The No-Observed-Effect-Concentration (NOEC) for reproduction was ≥1000 mg product/kg dry weight artificial soil. EC_{10} and EC_{20} values could not be determined due to mathematical reasons.

For use in the risk assessment, as the log P_{ow} for diflufenican is greater than 2, in line with the EU Guidance Document on Terrestrial Ecotoxicology (SANCO/10329/2002), endpoints have to be divided by 2. The $NOEC_{corr}$ was therefore ≥500 mg product /kg dry weight artificial soil.

In terms of active substance content, based on a diflufenican content of 42.6% w/w, the $NOEC_{corr}$ was estimated to be ≥213 mg a.s./kg.

Assessment and conclusion by RMS:

CP 10.4.2.2 Higher tier testing

No data available on formulated product.

CP 10.5 Effects on soil nitrogen transformation

A summary of the endpoints related to the effects on soil nitrogen transformation is provided in the following table. Details and a full description of the studies performed on the active substance, aclonifen, used in this risk assessment can be found in Document M-CA 8 of this dossier.

Table 10.5-1: Summary of data on the effects of aclonifen and ACL + DFF SC 600 (500 + 100) G to soil nitrogen transformation

Test item	Time scale	Endpoint	Reference
ACL + DFF SC 600 (500 + 100) G	28 days	No adverse effect after 28 days at a maximum tested concentration of 5.74 kg a.s./ha (7.65 mg a.s./kg)	KCP 10.5.1/01 M-578471-01-1 [redacted] 2017
Aclonifen	28 days	No adverse effect after 28 days at a maximum tested concentration of 15 kg a.s./ha (20 mg a.s./kg)	KCA 8.5/01 M-218214-01-2 [redacted], 1984
AE B107137	28 days	No adverse effect after 28 days at a tested concentration of 0.375 kg parent/ha (0.359 mg/kg dw)	EFSA Scientific Report 122 (2007), 1-84
AE 0542291	28 days	No adverse effect after 28 days at a tested concentration of 0.375 kg parent/ha (0.358 mg/kg dw)	EFSA Scientific Report 122 (2007), 1-84

Application scenario

According to the GAP table, ACL + DFF SC 600 (500 + 100) G is proposed to be applied to winter cereals at 0.7 or 0.35 L/ha (1 application), during BBCH 00-13. The following assessments have been made for the use of ACL + DFF SC 600 (500 + 100) G in winter cereals using an application rate of 0.7 L/ha as this will also cover the risks from the use at lower application rates.

Risk assessment for Soil Nitrogen Transformation

The risk to soil microbial processes has been assessed in accordance with the Terrestrial Guidance Document (SANCO/10329/2002).

To assess the risk to soil microbial processes, the PEC_{soil} is compared to the No Effect Concentration determined from a suitable laboratory study. If the PEC_{soil} is lower than the No Effect Concentration then the risks to soil micro-organisms is considered acceptable.

Table 10.5-2: Assessment of effects on soil microbial processes

Test item	Concentration where effects <25% are seen. (mg a.s./kg)	PEC_{soil} (mg/kg)	PEC_{soil} < Endpoint
ACL + DFF SC 600 (500 + 100) G	7.65	1.148	Yes
Aclonifen	20	0.5113	Yes
AE B107137	0.359	0.013	Yes
AE 0542291	0.358	0.020	Yes

The PEC_{soil} was lower than the no effect concentration indicating the risks to soil organisms from the proposed uses of ACL + DFF SC 600 (500 + 100) G are acceptable.

Studies on the effect of the formulation ACL + DFF SC 600 (500 + 100) G on soil nitrogen transformation have been conducted and presented below.

Data Point:	KCP 10.5/01
Report Author:	[redacted]
Report Year:	2017
Report Title:	Aclonifen + diflufenican SC 600 (500+100) G: Effects on the activity of soil microflora (nitrogen transformation test)
Report No:	16 10 48 086 N
Document No:	M-578471-01-1
Guideline(s) followed in study:	EU Directive 91/414/EEC Regulation (EC) No. 1107/2009 (2009) US EPA OCSPP Not Applicable
Deviations from current test guideline:	Current guideline: OECD 213,214, 1998 Test item and reference item applied as 1 × 5 µL droplet to ensure more reliable dispersion of test item. Test facility has experience to confirm this deviation does not affect outcome of studies and hence deviation is acceptable
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The effect of aclonifen + diflufenican SC 600 (500+100) G on nitrogen turnover was investigated by determining ammonium, nitrate and nitrite-nitrogen concentrations in soil amended with Lucerne meal. The test item was applied at 2 rates to 2 soils to give 1.15 mg formulation/kg soil (0.7 L test item/ha) and 5.74 mg formulation/kg soil (3.5 L test item/ha). In a separate study, a reference substance, dinoterb, was used.

A loamy sand soil (DIN 4220) was exposed for 28 days to 1.15 mg test item/kg soil dry weight and 5.74 mg test item/kg soil dry weight. Application rates were equivalent to 0.7 L test item/ha and3.5 L kg test item/ha. The nitrogen transformation was determined in soil enriched with Lucerne meal (concentration in soil 0.5%). NH_4-nitrogen, NO_3- and NO_2-nitrogen were determined by an autoanalyzer at 0, 7, 14 and 28 days after treatment).

Aclonifen + diflufenican SC 600 (500+100) G caused no adverse effects (difference to control <25%, OECD 216) on the soil nitrogen transformation (expressed as NO_3-N-production) at the end of the 28 day incubation period. The study was performed in a field soil at concentrations up to 5.74 mg test item/kg soil dry weight, which was equivalent to application rates up to 3.5 L test item/ha (highest tested concentration).

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test Item:** Aclonifen + diflufenican SC 600 (500+100) G
   Batch no.: 2015-010653
   Active Ingredient / Purity: Aclonifen: 505.1 g/L (41.1% w/w)

		Diflufenican: 101.0 g/L (8.21% w/w)
	Appearance:	Yellow suspension
	Expiry date:	12 January 2016
	Storage:	25 ± 5 °C, +2 °C to +30 °C are also acceptable
2.	**Reference item:**	Dinoterb (tested in a separate study to verify sensitivity of the test system)
3.	**Test Soil:**	Loamy sand
	Source:	[redacted]
	Pre-treatment:	The soils used in the study were obtained from [redacted]. An agriculturally utilised soil was selected. The supply, including the data of relevant soil analyses, was conducted by [redacted]. The soil was removed to a depth of 20 cm as mixed sample. The soil was dried at room temperature, passed through a 2 mm mesh sieve and then stored at a temperature of approx. 4 °C in containers under aerobic conditions in the dark. Before application, the soil was adapted to test conditions.

A. STUDY DESIGN AND METHODS

1.	**In-life phase:**	7 September to 5 October 2016
2. Exposure conditions		
	Experimental design:	Two test concentrations (1.15 mg test item/kg dry soil and 5.74 mg test item/kg dry soil, equivalent to 0.7 L formulation/ha and 3.5 L formulation/ha respectively, plus one control; three replicates of each
	Temperature:	19.7–20.9 °C
	Moisture content:	36.55 ± 5% of water holding capacity (WHC)

3. Administration of the test item

The test item was mixed with deionised water and the test solution was subsequently mixed with the soil by means of a hand-stirrer. Water was added to the soil to achieve a water content of approximately 45% of WHC.

The incubation of the soil samples was performed as a series of individual and equally sized subsamples of each treatment group. Soil (200 g dry weight) per test vessel was weighed and mixed with 0.5% (1.0 g/200 g soil d.w.) Lucerne meal by hand-stirrer (C/N ratio of the Lucerne meal was 13.2/1). One additional soil sample (without Lucerne meal) was used for determination of the initial NO_3-N-content. The initial NO_3-N-content was 2.38 mg /100 g soil d.w.

4. Measurements and observations

The incubation of the prepared soil was carried out in wide mouth glass flasks (500 mL). The screw caps of the flasks used permitted an air exchange. The water content of the soil in each test vessel was

determined at test start (after application) and adjusted once a week to the required range of 40 - 50% of WHC. The pH-values of the soil used in the test were measured at test start (after application) and at the final sampling on day 28.

A sample of each replicate of each treatment was taken at intervals of 3 hours, 7, 14 and 28 days and the nitrogen transformation of the soil was determined. For calculation of the test concentrations (mg/kg soil d.w.) a soil depth of 5 cm and a soil bulk density of 1.5 g dry weight/cm^3 were assumed for conversion of soil volume to soil dry weight. The mean nitrogen-content, standard deviation and coefficient of variation were calculated for each treatment group and sampling date. The nitrogen transformation rate per time interval and the nitrogen transformation rate/time interval/day were calculated for each treatment group.

5. Statistics/Data evaluation

A statistical evaluation of the test results was performed by means of a 2-sided Student-t-test (for homogeneous variances at 5% significance level).

II. RESULTS AND DISCUSSION

No adverse effects of aclonifen + diflufenican SC 600 (500+100) G on nitrogen transformation in soil could be observed at both test concentrations (1.15 mg test item/kg dry soil and 5.74 mg test item/kg dry soil) during the 28-day experiment. Differences from the control of -0.3% (test concentration 1.15 mg test item/kg dry soil) and +4.3% (test concentration 5.74 mg test item/kg dry soil) were measured at the end of the 28-day incubation period (time interval 14-28).

Table: The effect of aclonifen + diflufenican SC 600 (500+100) G on ammonium-nitrogen and nitrate-nitrogen concentrations (mg/kg soil) in a loamy sand soil

Time interval (days)	Control	1.15 mg test item/kg soil dry weight equivalent to 0.7 L test item/ha		5.74 mg test item/kg soil dry weight equivalent to 3.5 L test item/ha	
	Nitrate-N	Nitrate-N	% of control	Nitrate-N	% of control
0-7	4.12 ± 0.01	4.13 ± 0.26	+0.2	4.33 ± 0.45	+5.1
7-14	1.50 ± 0.28	1.81 ± 0.16	+20.6	1.30 ± 0.17	-13.7
14-28	0.93 ± 0.16	0.93 ± 0.28	-0.3	0.97 ± 0.28	+4.3

Rate: Nitrate-N in mg/kg soil dry weight/time interval/day, mean of 3 replicates and standard deviation
No statistically significant difference to the control (Student-t-test for homogeneous variances, 2-sided, $p \leq 0.05$)

In a separate study the reference item (Dinoterb) caused an inhibition of nitrogen transformation of -37.0% and a stimulation of nitrogen transformation of +37.6% at 6.80 mg and 27.00 mg Dinoterb per kg soil dry weight, respectively, determined 28 days after application (time interval 14-28 days).

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 216, 2010)	Achieved
Variation between controls	≤15%	3.2%

The validity criterion was satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint	Effect
Nitrogen transformation	No adverse effect (<25%) after 28 days at a maximum tested concentration of 5.75 kg formulation/ha (equivalent to field application rate of 3.5 L formulation/ha)

III. CONCLUSION

Aclonifen + diflufenican SC 600 (500+100) G caused no adverse effects (difference to control <25%, OECD 216) on the soil nitrogen transformation (expressed as NO_3-N-production) at the end of the 28 day incubation period. The study was performed in a field soil at concentrations up to 5.74 mg test item/kg soil dry weight, which are equivalent to application rates up to 3.5 L test item/ha.

[redacted] *(2017)*

Assessment and conclusion by applicant:

All validity criteria were satisfied and therefore this study can be considered to be valid.

Aclonifen + diflufenican SC 600 (500+100) G caused no adverse effects (difference to control <25%, OECD 216) on the soil nitrogen transformation (expressed as NO3-N-production) at the end of the 28-day incubation period. The study was performed in a field soil at concentrations up to 5.74 mg test item/kg soil dry weight, which are equivalent to application rates up to 3.5 L test item/ha (the highest tested concentration)

Assessment and conclusion by RMS:

CP 10.6 Effects on terrestrial non-target higher plants

The effects of ACL + DFF SC 600 (300 + 100) G on non-target plants has been studied under greenhouse conditions. A summary of the endpoints for all tested plant species is provided in the following table.

Table 10.6-1: Summary of data on the effects of ACL + DFF SC 600 (500 + 100) G on non-target plants

Test design	Test species	ER_{50} (mL product/ha)	Reference
21 d seedling emergence	*Beta vulgaris* d	**ER_{50} dry weight = 54.9 mL/ha**	KCP 10.6.2/01 M-574745-01-1 [redacted], 2016
	Brassica napus d	ER_{50} all parameter >57.53 mL/ha	
	Cucumis sativus d	ER_{50} all parameter >132.33 mL/ha	
	Glycine max d d	ER_{50} all parameter >132.33 mL/ha	

	Helianthus annuus	ER_{50} all parameter >57.53 mL/ha	
	Lycopersicon esculentum d	ER_{50} all parameter >57.53 mL/ha	
	Allium cepa m	ER_{50} all parameter >57.53 mL/ha	
	Avena sativa m	ER_{50} dry weight = 127.8 mL/ha	
	Triticum aestivum m	ER_{50} all parameter >132.53 mL/ha	
	Zea mays m	ER_{50} all parameter >132.53 mL/ha	
21 d vegetative vigour	*Beta vulgaris* d	**ER_{50} shoot dry weight = 55.21 mL/ha**	KCP 10.6.2/02 M-574750-01-1 [redacted], 2016
	Brassica napus d	ER_{50} shoot dry weight >57.53 mL/ha	
	Cucumis sativus d	ER_{50} shoot dry weight >132.53 mL/ha	
	Glycine max d d	ER_{50} shoot dry weight >132.53 mL/ha	
	Helianthus annuus	ER_{50} shoot dry weight >57.53 mL/ha	
	Lycopersicon esculentum d	ER_{50} shoot dry weight >57.53 mL/ha	
	Allium cepa m	ER_{50} shoot dry weight >57.53 mL/ha	
	Avena sativa m	ER_{50} shoot dry weight >132.53 mL/ha	
	Triticum aestivum m	ER_{50} shoot dry weight >132.53 mL/ha	
	Zea mays m	ER_{50} shoot dry weight >132.53 mL/ha	

Endpoints in **bold** were used in the risk assessment
d: Dicotyledon species
m: Monotcoyledon species

Summary of the Risk assessment for Terrestrial Non-Target Higher Plants

The risk assessment for effects of ACL + DFF SC 600 (500 + 100) G on non-target terrestrial plants was performed in accordance with the EU Guidance Document on Terrestrial Ecotoxicology (SANCO/10329/2002).

Application scenario

According to the GAP table, ACL + DFF SC 600 (500 + 100) G is proposed to be applied to winter cereals at 0.7 or 0.35 L/ha (1 application), during BBCH 00-13. The following assessments have been made for the use of ACL + DFF SC 600 (500 + 100) G in winter cereals using an application rate of 0.7 L/ha as this will also cover the risks from the use at lower application rates.

Risk assessment for Terrestrial Non-Target Higher Plants

The potential risk to non-target terrestrial plants from the proposed uses of ACL + DFF SC 600 (500 + 100) G has been evaluated using the recommendations presented in the EU Guidance Document on Terrestrial Ecotoxicology (SANCO/10329/2002 rev.2 final, 2002). It is restricted to off-field situations, as non-target plants are non-crop plants located outside the treated area.

At an application rate of 0.35 L product/ha, acceptable risk to non-target plants following application of aclonifen + diflufenican SC 600 to winter cereals according to the proposed GAP were shown.

At the higher application rate of 0.7 L product/ha, TER values for both seedling emergence and vegetative vigour were below the trigger value of 5 and hence risk mitigation measures are required.

The deterministic risk assessment based on the lowest ER_{50} observed for *Beta vulgaris* in a seedling emergence and a vegetative vigour study, resulted in an acceptable risk for an application rate of 0.7 L product/ha provided that appropriate risk mitigation measures are applied. These would be a 5 m in-crop buffer or alternatively 50% drift reducing nozzles without buffer could be applied.

Deterministic risk assessment

According to the Terrestrial Guidance Document the risk to non-target plants is evaluated by comparing the lowest ER_{50} from the laboratory studies with the calculated Predicted Environmental Rates ($PER_{off-field}$). A trigger of 5 can be accepted if at least 6 plant species have been tested.

For ACL + DFF SC 600 (500 + 100) G, a broad database is available for non-target terrestrial plants.

Vegatative vigour tests and/or seedling emergence tests with a variety of dicotyledenous and monocotlyedenous non-target plants species have been completed: *Allium cepa* (onion), *Avena sativa* (oat), *Beta vulgaris* (sugar beet), *Brassica napus* (oilseed rape), *Cucumis sativus* (cucumber), *Glycine max* (soybean), *Helianthus annus* (sunflower), *Lycopersicon esculentum* (tomato), *Triticum aestivum* (wheat), and *Zea mays* (corn). These tests were conducted under greenhouse conditions.

Off-field predicted environmental rates (PER) were calculated according to SANCO 10329/2002 considering a distance of 1 m from the field edge, and TER values compared to a trigger value of 5.

Table 10.6-2: Assessment of the risk for non-target plants due to the use of ACL + DFF SC 600 (500 + 100) G in winter cereals – Deterministic risk assessment

Intended use		Winter cereals, BBCH 00 - 13			
Test item		ACL + DFF SC 600 (500 + 100) G			
MAF		1.0			
Distance fron field edge		1 m			
Test species	**ER_{50} (mL/ha)**	**Drift rate (%)**	**$PER_{off-field}$ (mL/ha)**	**TER**	**Trigger value**
Application rate = 0.35 L product/ha					
Beta vulgaris Seedling emergence	54.90	2.77	9.70	5.66	5
Beta vulgaris Vegetative vigour	55.21			5.69	
Application rate = 0.7 L product/ha					
Beta vulgaris Seedling emergence	54.90	2.77	19.39	**2.83**	5
Beta vulgaris Vegetative vigour	55.21			**2.85**	

TER values shown in **bold** fall below the relevant trigger

At an application rate of 0.35 L product/ha, acceptable risk to non-target plants was shown as TER values for both seedling emergence and vegetative vigour were above the trigger value of 5. However, at the high application rate of 0.7 L product/ha, TER values for both seedling emergence and vegetative vigour are below the trigger value of 5 and hence risk mitigation measures are required.

Risk mitigation measures

The deterministic risk assessment did not pass the trigger for the application rate of 0.7 L product/ha, indicating a need for further assessment under consideration of risk mitigation measures in order to reduce the off-field exposure. These mitigation measures correspond to unsprayed in-field buffer strips of a given width and/or the usage of drift reducing nozzles. The results of the risk assessment using typical mitigation measures (no-spray buffer zones of 5 or 10 m, drift-reducing nozzles with reduction by 50%, 75%, or 90%) are summarised in the following tables.

Table 10.6-3: Assessment of the risk for non-target plants due to the use of ACL + DFF SC 600 (500 + 100) G in winter cereals (1 x 0.7 L/ha) – Deterministic risk assessment considering risk mitigation

Buffer strip (m)	Drift rate (%)	$PER_{off\text{-}field}$ (mL/ha)	$PER_{off\text{-}field}$ 50% drift red. (mL/ha)	$PER_{off\text{-}field}$ 75% drift red. (mL/ha)	$PER_{off\text{-}field}$ 90% drift red. (mL/ha)
1	2.77	19.39	9.70	4.85	1.94
5	0.57	3.99	2.00	1.00	0.40
10	0.29	2.03	1.02	0.51	0.20
Seedling emergence ER_{50} = 54.9 mL/ha		**TER Trigger: TER ≥ 5**			
1		**2.83**	5.66	11.33	28.31
5		13.76	27.52	55.04	137.59
10		27.04	54.09	108.18	270.44
Vegetative vigour ER_{50} = 55.21 mL/ha		**TER Trigger: TER ≥ 5**			
1		**2.85**	5.69	11.39	28.47
5		13.84	27.67	55.35	138.37
10		27.20	54.39	108.79	271.97

TER values shown in **bold** fall below the relevant trigger.

The deterministic risk assessment based on the lowest ER_{50} observed for *Beta vulgaris* in a seedling emergence and a vegetative vigour study, resulted in an acceptable risk for an application rate of 0.7 L product/ha provided that appropriate risk mitigation measures are applied. These would be a 5 m in-crop buffer or alternatively 50% drift reducing nozzles without buffer could be applied.

CP 10.6.1 Summary of screening data

Not required as the formulated product has been evaluated for phytotoxicity (see point CP 10.6.2 below).

CP 10.6.2 Testing on non-target plants

Studies on the effect of the formulation ACL + DFF SC 600 (500 + 100) G on non-target plants have been conducted and presented below.

Executive Summary

A study was conducted to determine the effect of diflufenican + aclonifen SC 600 on seedling emergence in seven terrestrial non-target plant species representing eight non-target terrestrial plant families.

The test was run over 21 days using six application rates applied as a soil spray per test species. The nominal application rates were 2.06, 4.73, 10.88, 25.01, 57.53 and 132.33 mL product/ha plus a control. Percentage of emergence and visual phytotoxicity ratings (e.g. chlorosis, necrosis, abnormal growth) were recorded on days 7, 14 and 21. Mortality and growth (fresh weight) was determined on day 21.

Six dicotyledonous and four monocotyledonous species were cultivated in soil. Diflufenican + aclonifen SC 600 (100 + 500 g/L) was applied to the soil surface by spray application after seeding at 2.06, 4,73, 10.88, 25.01 and 57.53 mL product/ha for the plant species *Beta vulgaris, Brassica napus, Helianthus annuus, Lycopersicon esculentum* and *Allium cepa* and at 4,73, 10.88, 25.01, 57.53 132.33 mL product/ha for the plant species *Cucumis sativus, Glycine max, Avena sativa, Triticum aestivum* and *Zea mays*. Results were compared to the deionised water treated control. In each treatment group a total number of 20 seeds were sown.

An application of diflufenican + aclonifen SC 600 (100 + 500 g/L) resulted in no statistically significantly effects on the parameter seedling emergence and post-emergence mortality for any of the plant species tested.

Statistically significantly effects on the parameter shoot dry weight could be observed for the plant species *Beta vulgaris, Cucumis sativus, Avena sativa* and *Triticum aestivum.*

The NOER for *Beta vulgaris* and *Cucumis sativus* was 25.01 mL product/ha. The respective LOER was 57.53 mL product/ha.

The NOER for *Avena sativa* and *Triticum aestivum* was 57.53 mL product/ha. The respective LOER was 132.33 mL product/ha.

The ER_{50} for *Beta vulgaris* with corresponding 95% confidence limits was 54.9 (44.8 – 77.1) mL product/ha and for *Avena sativa* with corresponding 95% confidence limits was 127.8 (102.0 - 685.52) mL product/ha. For all remaining plant species no ER_{50} could be calculated due to a lack of ≥50% inhibition.

I. MATERIALS AND METHODS

A. MATERIALS

1.	**Test Item:**	Diflufenican + Aclonifen SC 600 (100 + 500 g/L)
	Batch no.:	2015-010653
	Active Ingredient / Purity:	Aclonifen: 505.1g/L (41.1% w/w) Diflufenican: 101.0 g/L (8.21% w/w)
	Expiry date:	12 January 2017
	Appearance:	Yellow liquid
	Storage:	Ambient (+5 to +30 °C) in the dark

2. Test species: 6 dicotyledoneae and 4 monocotyledoneae species were chosen representing 8 plant families. Untreated seeds from commercial suppliers were used, care was taken that within species only seeds of the same size were used

	Family	Species	Common name
Dicotyledonae	Brassicaceae	*Brassica napus*	Oilseed Rape
Dicotyledonae	Curcubitaceae	*Cucumis sativus*	Cucumber
Dicotyledonae	Amaranthaceae	*Beta vulgaris*	Sugar beet
Dicotyledonae	Fabaceae	*Glycine max*	Soybean
Dicotyledonae	Solanacae	*Lycopersicon esculentum*	Tomato
Dicotyledonae	Asteraceae	*Helianthus annus*	Sunflower
Monocotyledonae	Amaryllidaceae	*Allium cepa*	Onion
Monocotyledonae	Poaceae	*Avena sativa*	Oat
Monocotyledonae	Poaceae	*Zea mays*	Maize
Monocotyledonae	Poaceae	*Triticum aestivum*	Wheat

B. STUDY DESIGN AND METHODS

1. In-life phase: 27 April to 13 September 2016

2. Exposure conditions

Test vessels: Pots of diameter 15 cm were filled with approx. 1.5 kg test soil

Soil: Specially mixed soil substrate (silty sand) was provided by EBRD GmbH & Co. KG and was used for cultivation of the plant species. A sample of this substrate was characterised (non-GLP). It was composed of:

- 84.9% sand
- 10.8% silt
- 4.3% clay

with a pH of 7.4

- total organic carbon content of < 0.3%
- electronic conductivity of 62.5 µS/cm

Experimental design: Control, test item (6 applications: nominally 2.06, 4.73, 10.88, 25.01, 57.53 and 132.33 mL product/ha)

Replicates: 6 pots each containing 5 seeds per treatment group

Temperature: Air temperature: 20.6 - 45.7 °C (Target: 12 °C – 32 °C)

Relative humidity: 27.3 - 87.8% (target 45 – 95%)

Photoperiod: 16h light:8h dark

Light intensity: Light intensity measured once a week (min/max): 14882 – 19473 lux (target: at least 10000 lux)

Irrigation: Bottom watering was done to the plant saucer of each pot, providing the plant root with a sufficient water supply. The water supply was controlled and water was replenished regularly. Plants were fertilised with a 2‰ nutrient solution of

"Wuxal Flüssigdünger" diluted in tap water (day 12 after application). A volume of approximately 100 mL of this solution was added to every plant saucer of every pot

3. Administration of the test item

The test item was directly weighed in for the highest test item solution of 132.33 mL product/ha two days before application and dissolved in deionised water at the day of application. For the lower treatment rates (2.06 to 57.53 mL product/ha), aliquots were taken from the highest test item rate and dissolved in deionised water, respectively. Deionised water was used for the control treatment.

The application was conducted with a laboratory track-sprayer ([redacted]). The track-sprayer was calibrated with deionised water before the application by adjusting the spray pressure (2.5 bar), application speed (2 km/h), type of nozzle (8001 EVS, TeeJet) and distance to the target (45.0 cm) to provide an output of 200 L ± 10% per ha. The applied amount was determined by weighing two glass plates (each of 30 x 45 cm) as a reference before and immediately after treatment.

Species	Plants/rep	Rep/TG	no. of TG*
Dicotyledonous Species			
Brassica napus	2	10	6
Cucumis sativus	2	10	6
Beta vulgaris	2	10	6
Gossypium hirsutum	2	10	6
Daucus carota	2	10	6
Hordeum vulgare	2	10	6
Triticum aestivum	2	10	6
Monocotyledonous Species			
Allium cepa	4	5	6
Avena sativa	4	5	6
Triticum aestivum	4	5	6
Zea mays	2	10	6

TG – treatment group
Rep - replicate
* 5 test item treatment groups + 1 control group

In total 20 seeds per species and treatment group were sown. Plants were grown in a greenhouse located in Neulingen-Göbrichen, Germany near the test facility.

The pots were set up sorted per treatment group and within each treatment group after application. All pots were repositioned at the first and second assessment day to minimise variability in growth conditions. Heating, cooling, shading and ventilation was controlled to obtain the recommended air temperature and relative humidity. An independent set of high pressure sodium lamps above each cultivation table insured an appropriate exposure to light.

4. Measurements and observations

Air temperature and relative air humidity were measured continuously with a calibrated data logger in the shade and at plant height. Light intensity was measured once a week with a mobile luxmeter at plant level.

Duplicate samples from the freshly prepared and continuously stirred stock solution were taken before application for verification of test item concentrations. Samples were stored, sealed tightly, in glass flasks and deep frozen (≤ -18 °C) immediately after sampling and until analysis at the test facility.

The number of emerged seeds per replicate (= pot) were assessed after 7, 14 and 21 days (after ≥50% of the seedlings in the control group had emerged).

The cumulative number of dead plants per replicate (= pot) were recorded for each assessment day. Dead plants were removed in order to avoid formation of mould.

Symptoms of phytotoxicity were assessed visually after 7, 14 and 21 days using a system based on EPPO guideline 1/135(4) (2014). A gradual rating was assigned to characterise changes in plant morphology including necrosis, chlorosis or any other characteristic that was clearly a response of the plants to the treatment. The ratings range from phytotoxicity grade 1 to 5, with 1 indicating normal plant appearance and 5 indicating plants being totally affected by the observed symptoms.

Growth stage of the plants (= BBCH stage) was assessed and recorded per replicate (= pot) of all treatment groups at the last assessment day.

At the end of the observation period (21 days after ≥50% seedling emergence in the control), the surviving plants were clipped at soil level for determination of shoot dry weight. The weight of the above-ground shoot portion of all surviving plants per replicate was measured after drying at 60 °C until constant weight was reached.

5. Statistics/Data evaluation

The data of seedling emergence and plant survival were tested with the Fisher`s Exact Binomial Test with Bonferroni Correction.

The data of shoot dry weight was tested for normality and homoscedasticity using Shapiro-Wilk`s Test and Levene-Test followed by a William`s t-test in case that both requirements were fulfilled and the trend analysis by contrast was significant.

If the trend analysis by contrast was not significant the Dunnett`s t-test was conducted. If the data were not normal distributed but homogenous and the trend analysis by contrast was significant, the Jonckheere-Terpstra test was conducted.

The significance level was set to $\alpha = 0.05$ for all tests.

The outlier-test after Dixon and Hartley was used for the parameter shoot dry weight of the plant species *Avena sativa* and *Helianthus annuus*.

The effect rates with their 95% confidence limits were calculated by Probit analysis using linear max. likelihood regression, where possible.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

The analysed concentration of diflufenican in the highest test item solution corresponded to 86% of the target concentration.

The validated method is summarised in Document M-CP5 (CP 5.1.2/13).

B. BIOLOGICAL DATA

Seedling Emergence: No statistically significant effects on the parameter seedling emergence could be observed for any of the plant species tested after 21 days.

Post-Emergence Mortality: No statistically significant effects on post-emergence mortality could be determined for any of the plant species tested. Mortality was observed for the plant species *Allium cepa* and *Avena sativa*. The most sensitive species was *Allium cepa* with 16.7% mortality at 57.33 mL product/ha after 21 days.

Phytotoxicity: Symptoms of phytotoxcicity were observed for all plant species except *Helianthus annuus* and *Lycopersicon esculentum* after 21 days. The observed symptoms were stunted growth, chlorosis and necrosis. Slight symptoms (median 2) were observed for *Brassica napus* at 25.01 and 57.53 mL product/ha, for *Beta vulgaris* and *Allium cepa* at 57.53 mL product/ha and for *Glycine max, Triticum aestivum* and *Zea mays* at 132.33 mL product/ha. Moderate symptoms (median 3) were observed for *Cucumis sativus* and *Avena sativa* at 132.33 mL product/ha.

Growth Stage: No differences in the BBCH growth stages compared to the control were observed for any of the plant species tested after 21 days.

Shoot Dry Weight: An application of diflufenican + aclonifen SC 600 (100 + 500 g/L) resulted in statistically significant effects on shoot dry weight for the plant species *Beta vulgaris, Avena sativa, Triticum aestivum* (Williams t-test, one-sided smaller, $p \leq 0.05$) and *Cucumis sativus* (Jonckheere-Terpstra test, one-sided smaller, $p \leq 0.05$). A statistically significant effect occurred for *Lycopersicon esculentum* (Dunnett`s t-test, one-sided smaller, $p \leq 0.05$) at 2.06 mL product/ha. This statistically significant effect was considered as not treatment related since the replicate values were still within the range covered by the control.

The highest inhibition of shoot dry weight compared to the control was observed for *Beta vulgaris* with 53.1% at 57.53 mL product/ha followed by *Avena sativa* with 52.3% and *Cucumis sativus* with 45.6% at 132.33 mL product/ha, respectively.

An application of diflufenican + aclonifen SC 600 (100 + 500 g/L) resulted in no statistically significantly effects on the parameter seedling emergence and post-emergence mortality for any of the plant species tested.

Statistically significantly effects on the parameter shoot dry weight could be observed for the plant species *Beta vulgaris, Cucumis sativus, Avena sativa* and *Triticum aestivum.*

The NOER for *Beta vulgaris* and *Cucumis sativus* was 25.01 mL product/ha. The respective LOER was 57.53 mL product/ha.

The NOER for *Avena sativa* and *Triticum aestivum* was 57.53 mL product/ha. The respective LOER was 132.33 mL product/ha.

The ER_{50} for *Beta vulgaris* with corresponding 95% confidence limits was 54.9 (44.8 – 77.1) mL product/ha and for *Avena sativa* with corresponding 95% confidence limits was 127.8 (102.0 - 685.52)

mL product/ha. For all remaining plant species no ER_{50} could be calculated due to a lack of inhibition ≥50%.

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 208, 2006)	Achieved
Control seedling emergence	≥70%	85 – 100%
Control plant survival	≥90%	100%

The control seedlings of each species did not exhibit visible phytotoxic effects (e.g. chlorosis, necrosis, wilting, leaf and stem deformations) and control plants exhibited only normal variation in growth and morphology for that particular species.

The environmental conditions for each particular species were identical and growing media contained the same amount of soil matrix, support media, or substrate from the same source.

Therefore, all validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Species	Seedling emergence		Post-emergence mortality		Shoot dry weight	
	NOER	ER_{50} (95% CI)	NOER	ER_{50} (95% CI)	NOER	ER_{50} (95% CI)
Dicotyledonous species						
Beta vulgaris	≥57.53[a]	>57.53[b]	≥57.53[a]	>57.53[b]	25.01	54.9 (44.8 – 77.1)
Brassica napus	≥57.53[a]	>57.53[b]	≥57.53[a]	>57.53[b]	≥57.53[a]	>57.53[b]
Cucumis sativus	≥132.33[a]	>132.53[b]	≥132.33[a]	>132.53[b]	25.01	>132.53[b]
Glycine max	≥132.33[a]	>132.53[b]	≥132.33[a]	>132.53[b]	≥132.33[a]	>132.53[b]
Helianthus annuus	≥57.53[a]	>57.53[b]	≥57.53[a]	>57.53[b]	≥57.53[a]	>57.53[b]
Lycopersicon esculentum	≥57.53[a]	>57.53[b]	≥57.53[a]	>57.53[b]	≥57.53[a]	>57.53[b]
Monocotyledonous species						
Allium cepa	≥57.53[a]	>57.53[b]	≥57.53[a]	>57.53[b]	≥57.53[a]	>57.53[b]
Avena sativa	≥132.33[a]	>132.53[b]	≥132.33[a]	>132.53[b]	57.53	127.8 (10.2 – 686)
Triticum aestivum	≥132.33[a]	>132.53[b]	≥132.33[a]	>132.53[b]	57.53	>132.53[b]
Zea mays	≥132.33[a]	>132.53[b]	≥132.33[a]	>132.53[b]	≥132.33[a]	>132.53[b]

a: NOER could not be determined due to a lack of statistically significant differences but can be regarded as above the highest rate tested, 57.53 or 132.33 mL product/ha

b: ER_{50} could not be calculated due to a lack of inhibition ≥50% but can be regarded as above the highest rate tested, 57.53 or 132.33 mL product/ha

III. CONCLUSION

An application of diflufenican + aclonifen SC 600 (100 + 500 g/L) resulted in no statistically significantly effects on the parameter seedling emergence and post-emergence mortality for any of the plant species tested.

Statistically significantly effects on the parameter shoot dry weight could be observed for the plant species *Beta vulgaris, Cucumis sativus, Avena sativa* and *Triticum aestivum.*

The NOER for *Beta vulgaris* and *Cucumis sativus* was 25.01 mL product/ha. The respective LOER was 57.53 mL product/ha.

The NOER for *Avena sativa* and *Triticum aestivum* was 57.53 mL product/ha. The respective LOER was 132.33 mL product/ha.

The ER_{50} for *Beta vulgaris* with corresponding 95% confidence limits was 54.9 (44.8 – 77.1) mL product/ha and for *Avena sativa* with corresponding 95% confidence limits was 127.8 (102.0 - 685.52) mL product/ha. For all remaining plant species no ER_{50} could be calculated due to a lack of ≥50% inhibition.

[redacted] *(2016)*

Assessment and conclusion by applicant:

All validity criteria were satisfied and therefore this study can be considered to be valid.

It was not possible to calculate ER_{50} values for most tested plant species due to a lack of ≥50% inhibition. Only 2 of the 10 tested plant species exhibited >50% inhibition, for these the ER_{50} for *Beta vulgaris* with corresponding 95% confidence limits was 54.9 (44.8 – 77.1) mL product/ha and for *Avena sativa* with corresponding 95% confidence limits was 127.8 (102.0 - 685.52) mL product/ha.

Assessment and conclusion by RMS:

Data Point:	KCP 10.6.2/02
Report Author:	[redacted]
Report Year:	2016
Report Title:	Diflufenican + aclonifen SC 600 (100 + 500 g/L): Effects on the vegetative vigour of ten non-target terrestrial plant species (tier 2)
Report No:	S16-00144
Document No:	M-574750-01-1
Guideline(s) followed in study:	EU Directive 91/414/EEC Regulation (EC) No. 1107/2009 US EPA OCSPP 850.4150 (2012) OECD 227 (2006)
Deviations from current test guideline:	Current guideline: OECD 227 (2006) Temperature and relative humidity went outside the recommended test conditions. However, these deviations had no influence on the outcome of the study, since all plants were kept in one greenhouse and all had the same growth conditions and no control mortality was observed.
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

A study was conducted to determine the effect of diflufenican + aclonifen SC 600 on vegetative vigour in ten terrestrial non-target plant species representing eight non-target terrestrial plant families under greenhouse conditions.

In this rate response test ten plant species in the 2-4 leaf stage (BBCH growth stages 12-14) were treated with diflufenican + aclonifen SC 600 (100 + 500 g/L) at 2.06, 4.73, 10.88, 25.01, 57.53 and 132.33 mL product/ha in different combinations. Deionised water was used for the control treatment. Each treatment group consisted of a total of 20 plants which were applied by spray application.

The plants were evaluated for effects of the test item 7, 14 and 21 days after application. BBCH growth stage and shoot dry weight were assessed on day 21.

Six dicotyledonous and four monocotyledonous species were cultivated in soil. Diflufenican + aclonifen SC 600 (100 + 500 g/L) was applied to the soil surface by spray application after seeding at 2.06, 4,73, 10.88, 25.01 and 57.53 mL product/ha for the plant species *Beta vulgaris, Brassica napus, Helianthus annuus, Lycopersicon esculentum* and *Allium cepa* and at 4,73, 10.88, 25.01, 57.53 132.33 mL product/ha for the plant species *Cucumis sativus, Glycine max, Avena sativa, Triticum aestivum* and *Zea mays*. Results were compared to the deionised water treated control. In each treatment group a total number of 20 seeds were sown.

An application of diflufenican + aclonifen SC 600 (100 + 500 g/L) resulted in no mortality for any of the plant species tested except *Allium cepa* with 10.0%.

Statistically significantly effects on the parameter shoot dry weight could be observed for the plant species *Beta vulgaris*. The LOER for *Beta vulgaris* was 10.88 mL product/ha. The respective NOER was 4.73 mL product/ha.

The ER_{50} for *Beta vulgaris* with corresponding confidence limits was 55.21 (35.03 – 94.00) mL product/ha. For all remaining species no ER_{50} could be calculated due to a lack of inhibition equal or above 50%.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Item: Diflufenican + Aclonifen SC 600 (100 + 500 g/L)

Batch no.: 2015-010653

Active Ingredient / Purity: Aclonifen: 505.1g/L (41.1% w/w)
Diflufenican: 101.0 g/L (8.21% w/w)

Expiry date: 12 January 2017

Appearance: Yellow liquid

Storage: Ambient (+5 to +30 °C) in the dark

2. Test species: 6 dicotyledoneae and 4 monocotyledoneae species were chosen representing 8 plant families. Untreated seeds from commercial suppliers were used, care was taken that within species only seeds of the same size were used

	Family	Species	Common name
Dicotyledonae	Brassicaceae	*Brassica napus*	Oilseed Rape
Dicotyledonae	Curcubitaceae	*Cucumis sativus*	Cucumber
Dicotyledonae	Amaranthaceae	*Beta vulgaris*	Sugar beet
Dicotyledonae	Fabaceae	*Glycine max*	Soybean
Dicotyledonae	Solanacae	*Lycopersicon esculentum*	Tomato
Dicotyledonae	Asteraceae	*Hellianthus annus*	Sunflower
Monocotyledonae	Amaryllidaceae	*Allium cepa*	Onion
Monocotyledonae	Poaceae	*Avena sativa*	Oat
Monocotyledonae	Poaceae	*Zea mays*	Maize
Monocotyledonae	Poaceae	*Triticum aestivum*	Wheat

B. STUDY DESIGN AND METHODS

1. In-life phase: 13 June – 8 July 2016

2. Exposure conditions

Test vessels: Pots of diameter 15 cm were filled with approx. 1.5 kg test soil.

Soil: Specially mixed soil substrate (silty sand) was provided by [redacted] and was used for cultivation of the plant species. A sample of this substrate was characterised (non-GLP). It was composed of:

84.9% sand
10.8% silt
4.3% clay
with a pH of 7.4
total organic carbon content of < 0.3%
electronic conductivity of 62.5 µS/cm

Experimental design: Control, test item (6 applications: nominally 2.06, 4.73, 10.88, 25.01, 57.53 and 132.33 mL product/ha)

Replicates: 6 pots each containing 5 seeds per treatment group

Temperature: Air temperature: 20.7 – 41.3 °C (Target: 12 °C – 32 °C)

Relative humidity: 33.3 – 94.6% (target 45 – 95%)

Photoperiod: 16h light: 8h dark

Light intensity: Light intensity measured once a week (min/max): 12735 - 18289 lux (target: at least 10000 lux)

Irrigation: Bottom watering was done to the plant saucer of each pot, providing the plant root with a sufficient water supply. The water supply was controlled and water was replenished regularly. Plants were fertilised with a 2‰ nutrient solution of "Wuxal Flüssigdünger" diluted in tap water (day 12 after application). A volume of approximately 100 mL of this solution was added to every plant saucer of every pot

3. Administration of the test item

The test item was directly weighed in for the highest test item solution of 132.33 mL product/ha two days before application and dissolved in deionised water at the day of application. For the lower treatment rates (2.06 to 57.53 mL product/ha), aliquots were taken from the highest test item rate and dissolved in deionised water, respectively. Deionised water was used for the control treatment.

The application was conducted with a laboratory track-sprayer ([redacted]). The track-sprayer was calibrated with deionised water before the application by adjusting the spray pressure (2.5 bar), application speed (2 km/h), type of nozzle (80015 EVS, TeeJet)

and distance to the target (45.0 cm) to provide an output of 200 L ± 10% per ha. The applied amount was determined by weighing two glass plates (each of 30 x 45 cm) as a reference before and immediately after treatment.

Species	Plants/rep	Rep/TG	no. of TG*
Dicotyledonous Species			
Brassica napus	2	10	6
Cucumis sativus	2	10	6
Beta vulgaris	2	10	6
Gossypium hirsutum	2	10	6
Daucus carota	2	10	6
Hordeum vulgare	2	10	6
Triticum aestivum	2	10	6
Monocotyledonous Species			
Allium cepa	4	5	6
Avena sativa	4	5	6
Triticum aestivum	4	5	6
Zea mays	2	10	6

TG – treatment group

Rep - replicate

* 5 test item treatment groups + 1 control group

In total 20 seeds per species and treatment group were sown. Plants were grown in a greenhouse located in [redacted] near the test facility.

The pots were set up sorted per treatment group and within each treatment group after application. All pots were repositioned at the first and second assessment day to minimise variability in growth conditions. Heating, cooling, shading and ventilation was controlled to obtain the recommended air temperature and relative humidity. An independent set of high pressure sodium lamps above each cultivation table insured an appropriate exposure to light .

4. Measurements and observations

Air temperature and relative air humidity were measured continuously with a calibrated data logger in the shade and at plant height. Light intensity was measured once a week with a mobile luxmeter at plant level.

Duplicate samples from the freshly prepared and continuously stirred stock solution were taken before application for verification of test item concentrations. Samples were stored, sealed tightly, in glass flasks and deep frozen (≤ -18 °C) immediately after sampling and until analysis at the test facility.

The number of emerged seeds per replicate (= pot) were assessed after 7, 14 and 21 days (after ≥50% of the seedlings in the control group had emerged).

The cumulative number of dead plants per replicate (= pot) were recorded for each assessment day. Dead plants were removed in order to avoid formation of mould.

Symptoms of phytotoxicity were assessed visually after 7, 14 and 21 days using a system based on EPPO guideline 1/135(4) (2014). A gradual rating was assigned to characterise changes in plant morphology including necrosis, chlorosis or any other characteristic that was clearly a response of the plants to the treatment. The ratings range from phytotoxicity grade 1 to 5; with 1 indicating normal plant appearance and 5 indicating plants being totally affected by the observed symptoms.

Growth stage of the plants (= BBCH stage) was assessed and recorded per replicate (= pot) of all treatment groups at the last assessment day.

At the end of the observation period (21 days after ≥50% seedling emergence in the control) the surviving plants were clipped at soil level for determination of shoot dry weight. The weight of the above-ground shoot portion of all surviving plants per replicate was measured after drying at 60 °C until constant weight was reached.

5. Statistics/Data evaluation

The data of mortality were tested with the Fisher`s Exact Binomial Test with Bonferroni Correction.

The data of shoot dry weight were tested for normality and homoscedasticity using Shapiro-Wilk`s Test and Levene's-Test followed by a Welch t-test with Bonferroni-Holm adjustment in case the data were non-homogenous. The William`s test was conducted in case that both requirements were fulfilled. The significance level was set to $\alpha = 0.05$ for all tests.

The effect rates with their 95% confidence limits were calculated by Probit analysis using linear max. likelihood regression, where possible.

Statistical analysis was performed using the program ToxRatPro Version 3.2.1.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

The analysed concentration of diflufenican in the highest test item solution corresponded to 92% of the target concentration.

The validated method is summarised in Document M-CP5 (CP 5.1.2/14).

B. BIOLOGICAL DATA

Mortality: No mortality occurred for any species tested except *Allium cepa* with 10.0% at 4.73 mL product/ha.

Phytotoxicity: The phytotoxic symptoms were leaf deformation, chlorosis and necrosis. Phytotoxic effects up to a grade of 3 (moderate symptoms) were observed for all dicotyledonous species. All monocotyledonous species were less sensitive and showed either nor phytotoxicity or only slight symptoms in case of *Zea mays*.

Growth Stage: No difference in the BBCH growth stages compared to the control were observed for any of the plant species tested on the last assessment day (day 21).

Shoot Dry Weight: An application of diflufenican + aclonifen SC 600 (100 + 500 g/L) resulted in statistically significant effects on shoot dry weight for the plant species *Beta vulgaris* (Williams test, one-sided smaller, $p \leq 0.05$) with 45.2% at the highest test item rate of 57.53 mL product/ha.

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 227, 2006)	Achieved
Control seedling emergence	≥70%	92 – 100%
Control plant survival	≥90%	100%

The control seedlings of each species did not exhibit visible phytotoxic effects (e.g. chlorosis, necrosis, wilting, leaf and stem deformations) and control plants exhibited only normal variation in growth and morphology for that particular species.

The environmental conditions for each particular species were identical and growing media contained the same amount of soil matrix, support media, or substrate from the same source.

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Species	Shoot dry weight	
	NOER	ER_{50} (95% CI)
Beta vulgaris	4.73	55.21 (35.0 – 94.0)
Brassica napus	≥57.53	>57.53
Cucumis sativus	≥132.33	>132.53
Glycine max	≥132.33	>132.53
Helianthus annuus	≥57.53	>57.53
Lycopersicon esculentum	≥57.53	>57.53
Allium cepa	≥57.53	>57.53
Avena sativa	≥132.33	>132.53
Triticum gestivum	≥132.33	>132.53
Zea mays	≥132.33	>132.53 [b]

III. CONCLUSION

An application of diflufenican + aclonifen SC 600 (100 + 500 g/L) resulted in no mortality for any of the plant species tested except *Allium cepa* with 10.0%.

Statistically significantly effects on the parameter shoot dry weight could be observed for the plant species *Beta vulgaris*. The LOER for *Beta vulgaris* was 10.88 mL product/ha. The respective NOER was 4.73 mL product/ha. The ER_{50} for *Beta vulgaris* with corresponding confidence limits was 55.21 (35.03 – 94.00) mL product/ha. For all remaining species no ER_{50} could be calculated due to a lack of inhibition equal or above 50%.

[redacted] (2016)

Assessment and conclusion by applicant:

All validity criteria were satisfied and therefore this study can be considered to be valid.

Application of diflufenican + aclonifen SC 600 (100 + 500 g/L) resulted in no mortality for any of the plant species tested, except *Allium cepa* (10.0% mortality).

The ER_{50} for *Beta vulgaris* with corresponding confidence limits was 55.21 (35.03 – 94.00) mL product/ha for shoot dry weight.

For all remaining species no ER_{50} could be calculated due to a lack of inhibition equal or above 50%.

Assessment and conclusion by RMS:

CP 10.6.3 Extended laboratory studies on non-target plants

No further testing on, or assessment of risk to, other non-target plants is considered necessary.

CP 10.6.4 Semi-field and field tests on non-target plants

No further testing on, or assessment of risk to, other non-target plants is considered necessary.

CP 10.7 Effects on other terrestrial organisms (flora and fauna)

No further testing on, or assessment of risk to, other non-target plants is considered necessary.

CP 10.8 Effects on biological methods for sewage treatment

A summary of the endpoints related to the effects on biological methods for sewage treatment is provided in the following table. Details and a full description of the studies performed on the active substance, aclonifen, used in this risk assessment can be found in Document M-CA 8 of this dossier.

Table 10.8-1: Summary of data on the effects of aclonifen and ACL + DFF SC 600 (500 + 100) G on biological methods for sewage treatment

Test item	Test species	Time scale	Endpoint	Reference
Aclonifen	Activated sewage sludge	3 hours	EC_{50} >100 mg a.s./L	KCA 8.8/03 M-664091-01-1 [redacted]

Test item	Test species	Time scale	Endpoint	Reference
	micro-organisms			[redacted], 2019
Diflufenican	Activated sewage sludge micro-organisms	3 hours	EC_{50} >1000 mg a.s./L	EFSA Scientific Report 122, (2007) 1-84

Risk assessment for biological methods for sewage treatment

The risk to biological methods for sewage treatment has been assessed for aclonifen and diflufenican.

The most recent study for aclonifen ([redacted], 2019, KCA 8.8.03) was conducted according to the latest update to an internationally accepted test design and hence it is considered to be the most appropriate study for the risk assessment. No inhibition of respiration of activated sewage sludge was observed up to 100 mg aclonifen/L and the EC_{50} may be considered to be >100 mg aclonifen/L. This value is considered appropriate for risk assessment.

Based on the maximum predicted surface water PEC (16.7 µg aclonifen/L, FOCUS Step 1) given in Document M-CP 9, Section CP 9.2.5, the effects reported in the Spoo-Klöppel study indicate that adverse effects on biological sewage treatment plants are not to be expected.

In the study for diflufenican (EFSA Scientific Report 122, 1-84) no inhibition of respiration of activated sewage sludge was observed up to the highest tested concentration of 1000 mg diflufenican/L (and the EC_{50} may be considered to be >1000 mg diflufenican/L). This value is considered appropriate for risk assessment.

Based on the maximum predicted surface water PEC (4.48 µg diflufenican/L, FOCUS Step 1) given in Document M-CP9, the effects reported indicate that adverse effects on biological sewage treatment plants are not to be expected.

Further studies on the effect of the formulation ACL + DFF SC 600 (500 + 100) G on biological methods for sewage treatment are not considered necessary.

CP 10.9 Monitoring data

No data available.