REGISTRATION REPORT

Part B

Section 9

Ecotoxicology

Detailed summary of the risk assessment

Product code: AG-F8-250 CS

Chemical active substance(s):

Flurochloridone, 250 g/L

Central Zone

Zonal Rapporteur Member State: Poland

CORE ASSESSMENT

(authorisation)

Sponsor: ADAMA Agan Ltd

Applicant: Country organisation/representative of ADAMA Agan Ltd. as reported in Part A

Submission date: January 2020

MS Finalisation date: October 2020 (initial Core Assessment)

March 2021 (final Core Assessment)

August 2021 (evaluation of additional data)

Version history

When	What
January 2020	dRR submitted by the Applicant
October 2020	Initial assessment by the zRMS The report in the dRR format has been prepared by the Applicant, therefore all comments, additional evaluations and conclusions of the zRMS are presented in grey commenting boxes. Minor changes are introduced directly in the text and highlighted in grey. Not agreed or not relevant information are ~~struck through and shaded for transparency~~.
March 2021	Final report (Core Assessment updated following the commenting period) Additional information/assessments included by the zRMS in the report in response to comments recieved from the cMS and the Applicant are highlighted in yellow.
July 2021	Update of the aquatic risk assessment by the Applicant (highlighted in blue)
August 2021	Evaluation of additional information by the zRMS. Additional evlauations, comments and corrections of the Applicants' text made by the zRMS are highlighted in green.

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9 Ecotoxicology (KCP 10)

zRMS comments:

The report in the dRR format has been prepared by the Applicant, therefore all comments, additional evaluations and conclusions of the zRMS are presented in grey commenting boxes. Minor changes are introduced directly in the text and highlighted in grey. Not agreed or not relevant information is struck through and shaded for transparency.

It is noted that part of the risk assessment is based on toxicity endpoints derived at the EU level for representative formulation Racer 25 CS, which composition slightly differs from AG-F8-250 CS, even though the trade name of both formulations is the same. Comparison of the composition of formulation considered during EU review with composition of the current formulation is presented in Part C. The comparison was evaluated by the zRMS phys-chem expert and it was concluded that difference concerns co-formulants that are not classified or have the same classification as replaced components. Furthermore, the difference between particular co-formulants is less than 0.1% and the global difference between compositions of the formulations is less than 10%.
Therefore due to only minor differences between the composition of the representative formulation and current composition of AG-F8-250 CS (Racer 250 CS) endpoints derived at the EU level may be used in support of evaluation of the current formulation.

9.1 Critical GAP and overall conclusions

Table 9.1-1: Table of critical GAPs

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Use-No. *	Member state(s)	Crop and/or situation (crop destination / purpose of crop)	F, Fn, Fpn G, Gn, Gpn or I **	Pests or Group of pests controlled (additionally: developmental stages of the pest or pest group)	**Application**				**Application rate**			PHI (days)	Remarks: e.g. g safener/ synergist per ha	**Conclusion**						
					Method / Kind	Timing / Growth stage of crop & season	Max. number a) per use b) per crop/ season	Min. interval between applications (days)	kg or L product / ha a) max. rate per appl. b) max. total rate per crop/season	g or kg as/ha a) max. rate per appl. b) max. total rate per crop/season	Water L/ha min/max			Birds	Mammals	Aquatic organisms	Bees	Non-target arthropods	Soil organisms	Non-target plants
1	PL	Potato (SOLTU)	F	broadleaved and grass weeds, pre-emergence	Spray application	BBCH 00-09	1 ; 1	n.a	a) 2 L/ha b) 2 L/ha	a) 500 g/ha b) 500 g/ha	200-300	n.a.		A	A	N (algae in D4 and D6 scenario)	A	A	A	R 5 m buffer or 50% DRN
2	PL	Potato (SOLTU)	F	broadleaved and grass weeds, pre-emergence	Spray application	BBCH 00-09	1 ; 1	n.a	a) 1.5 L/ha b) 1.5 L/ha	a) 375 g/ha b) 375 g/ha	200-300	n.a.		A	A	R 10 m VFS [1)]	A	A	A	A

* Use number(s) in accordance with the list of all intended GAPs in Part B, Section 0 should be given in column 1

** F: professional field use, Fn: non-professional field use, Fpn: professional and non-professional field use, G: professional greenhouse use, Gn: non-professional greenhouse use, Gpn: professional and non-professional greenhouse use, I: indoor application

[1)] Conclusion taken based on the risk assessment performed for only scenarios representative for Poland (D3, D4 and R1). In other scenarios the risk may be still unacceptable

Explanation for column 15 – 21 “Conclusion”

A	Acceptable, Safe use
R	Further refinement and/or risk mitigation measures required
C	To be confirmed by cMS
N	No safe use

Remarks table:

(1) Numeration necessary to allow references
(2) Use official codes/nomenclatures of EU
(3) For crops, the EU and Codex classifications (both) should be used; where relevant, the use situation should be described (*e.g.* fumigation of a structure)
(4) F: professional field use, Fn: non-professional field use, Fpn: professional and non-professional field use, G: professional greenhouse use, Gn: non-professional greenhouse use, Gpn: professional and non-professional greenhouse use, I: indoor application
(5) Scientific names and EPPO-Codes of target pests/diseases/ weeds or when relevant the common names of the pest groups (e.g. biting and sucking insects, soil born insects, foliar fungi, weeds) and the developmental stages of the pests and pest groups at the moment of application must be named
(6) Method, e.g. high volume spraying, low volume spraying, spreading, dusting, drench
Kind, e.g. overall, broadcast, aerial spraying, row, individual plant, between the plants - type of equipment used must be indicated
(7) Growth stage at first and last treatment (BBCH Monograph, Growth Stages of Plants, 1997, Blackwell, ISBN 3-8263-3152-4), including where relevant, information on season at time of application
(8) The maximum number of application possible under practical conditions of use must be provided
(9) Minimum interval (in days) between applications of the same product.
(10) For specific uses other specifications might be possible, e.g.: g/m³ in case of fumigation of empty rooms. See also EPPO-Guideline PP 1/239 Dose expression for plant protection products
(11) The dimension (g, kg) must be clearly specified. (Maximum) dose of a.s. per treatment (usually g, kg or L product / ha).
(12) If water volume range depends on application equipments (e.g. ULVA or LVA) it should be mentioned under “application: method/kind”.
(13) PHI - minimum pre-harvest interval
(14) Remarks may include: Extent of use/economic importance/restrictions

9.1.1 Overall conclusions

zRMS comments:

Conclusions presented in points 9.1.1.1 to 9.1.1.7 below were checked by the zRMS and amended where necessary.

9.1.1.1 Effects on birds (KCP 10.1.1), Effects on terrestrial vertebrates other than birds (KCP 10.1.2), Effects on other terrestrial vertebrate wildlife (reptiles and amphibians) (KCP 10.1.3)

The risk assessment for birds and mammals was carried out according to the Guidance Document on Risk Assessment for Birds and Mammals on request from EFSA (EFSA Journal 2009; 7(12): 1438).

Birds

Acute and long-term reproductive Screening Step assessments with resulting TERs above the relevant trigger values indicate a low and acceptable dietary risk for birds exposed to the active substance flurochloridone.

A secondary poisoning assessment indicates a low risk for earthworm- and fish-eating birds. The exposure of birds to drinking water from pools in leaf whorls is not relevant for the proposed uses of AG-F8-250 CS. Detailed risk assessments for birds exposed via drinking water from puddles formed on the field are not triggered.

Thus, treatment with AG-F8-250 CS in accordance with the proposed use patterns in potato poses low risk to birds.

Terrestrial vertebrates other than birds

The acute Screening Step assessment results in TERs above the relevant trigger values, indicating a low and acceptable dietary risk for mammals exposed to the active substance substance flurochloridone.

A secondary poisoning assessment indicates a low risk for earthworm- and fish-eating mammals. Detailed risk assessments for mammals exposed via drinking water from puddles formed on the field are not triggered.

Thus, treatment with AG-F8-250 CS in accordance with the proposed use patterns in potato poses a low risk to terrestrial vertebrates other than birds.

9.1.1.2 Effects on aquatic organisms (KCP 10.2)

The risk assessment for aquatic organisms was carried out according to the Guidance on tiered risk assessment for plant protection products for aquatic organisms in edge-of-field surface waters (EFSA Journal 2013;11(7):3290).

Based on the performed evaluation acceptable acute and chronic risk following application of AG-F8-250 CS at 500 g a.s./ha could be concluded for fish, *Daphnia magna*, sediment-dwellers and aquatic macrophytes with no need for risk mitigation measures, with exception of aquatic macrophytes in R3 scenario, where 10 m vegetated filter strip was required in order to demonstrate acceptable risk.
Available data were sufficient to demonstrate acceptable risk to algae in all R scenarios provided that in R1 scenario 10 m vegetated filter strip is respected, while in R2 and R3 scenarios 20 m vegetated filter strip is applied. In scenario D3 10 meters unsprayed buffer zone to surface water bodies is required.
However, available data were not sufficient to demonstrate acceptable risk to algae in D4 and D6 scenarios from application of higher rate of 500 g a.s./ha, which remains thus unresolved.

Additional modelling performed with consideration of wider buffer zones would not address the risk in D scenarios, where the exposure is driven by drainage and currently there are no efficient mitigation measures enabling reduction of the exposure. The only option would be reduction of the application rate, which potentially may not be possible from the efficacy point of view.

Performed evaluation demonstrated acceptable risk to aquatic organisms from application of AG-F8-250 CS to potatoes at application rate of 375 g a.s./ha, provided that 10 m vegetated filter strip from surface water bodies is respected.

~~Potentially, the risk in R scenarios could be further refined with STEP 4 PEC_{sw} values calculated using VFSmod, which is currently acceptable in Poland (but may be not accepted in other MS). However, this would not address the risk in D scenarios, where the exposure is driven by drainage and currently there are no efficient mitigation measures enabling reduction of the exposure. The only option would be reduction of the application rate, which potentially may not be possible from the efficacy point of view.~~

~~Acceptable risk is indicated for aquatic organisms if a 20 m vegetated buffer distance is accounted for. Drainage entry into surface water bodies has to be excluded.~~

9.1.1.3 Effects on bees (KCP 10.3.1)

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).

Risk assessments with Hazard Quotients for both, acute oral and contact toxicity are below the trigger indicating a low and acceptable risk for bees from exposure to AG-F8-250 CS in accordance with the worst-case use pattern.

9.1.1.4 Effects on arthropods other than bees (KCP 10.3.2)

The risk assessment was conducted according to the ESCORT 2 Guidance Document (2000) and the Guidance Document on Terrestrial Ecotoxicology (2002).

An acceptable in-field risk is indicated based on data for AG-F8-250 CS for the standard test species *Aphidius rhopalosiphi* and *Typhlodromus pyri*.

The off-field risk is indicated to be acceptable based on the available data without the necessity to account for risk mitigation measures.

9.1.1.5 Effects on non-target soil meso- and macrofauna (KCP 10.4), Effects on soil microbial activity (KCP 10.5)

The risk assessment was conducted according to the Guidance Document on Terrestrial Ecotoxicology (2002).

Meso- and macrofauna

With TERs for chronic risk exceeding the relevant trigger values, the intended uses of AG-F8-250 CS do not pose an unacceptable risk to earthworms as well as soil meso- and macrofauna other than earthworms.

Soil microflora

An acceptable risk for soil microbial functions is indicated for the intended worst-case uses of AG-F8-250 CS by Predicted Environmental Concentrations lower than the No Observed Adverse Effect Concentrations (i.e. concentrations causing less than 25% effect on nitrogen transformation or carbon respiration after ≤

100 days).

9.1.1.6 Effects on non-target terrestrial plants (KCP 10.6)

The risk assessment was conducted according to the Guidance Document on Terrestrial Ecotoxicology (2002).

From the worst case application rate of 500 g a.s./ha an acceptable off-field risk is indicated for terrestrial non-target plants exposed towards AG-F8-250 CS in accordance with the intended worst-case use patterns in pre-emergence crops based on the data for vegetative vigour as well as seedling emergence and growth with the necessity to account for risk mitigation requirements as 5 m buffer distance or 50% drift reducing nozzles.
Acceptable risk with no need for risk mitigation measures could be concluded for lower intended application rate of 375 g a.s./ha.

9.1.1.7 Effects on non-target terrestrial plants (KCP 10.6)

No further relevant data available and considered necessary.

9.1.2 Grouping of intended uses for risk assessment

The following table documents the grouping of the intended uses to support application of the risk envelope approach (according to SANCO/11244/2011).

In the first instance, assessments are presented for the risk envelope, i.e. for the maximum single use rate of 2 L product/ha corresponding to 500 g a.s.//ha. For aquatic organisms as well as terrestrial non-target plants, risk assessments are also presented for the reduced rate of 1.5 L product/ha corresponding to 375 g a.s./ha in order to define required risk mitigations as necessary for the respective use rates.

Table 9.1-2: Critical use pattern of AG-F8-250 CS grouped according to organism groups

Grouping according to organism groups			
Group	**Intended uses**	**Relevant use parameters for grouping**	**Relevant parameter or value for sorting**
Terrestrial vertebrates (Birds and Mammals; **9.2** and **9.3**)	According to GAP; refer to Document B0, max. 1x 2.0 L product/ha in pre-emergence crops	Scenarios according to EFSA Birds and Mammals Guidance (2009): **Crop growth stage** ´bare soil´	**BBCH 00-09**: bare soil (pre-emergence), secondary poisoning (earthworm-eating, porewater, fish-eating)
Aquatic organisms (**9.5**)	According to GAP; refer to Document B0, max. 1x 2.0 L product/ha in pre-emergence crops 1x 1.5 L product/ha in pre-emergence crops	Crops according to FOCUS surface water guidance (2015)[1] Use rate	**BBCH 00-09**: default window covering pre-emergence crop Use rate

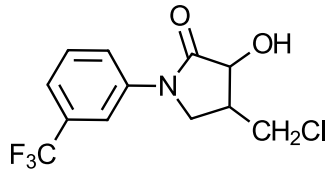
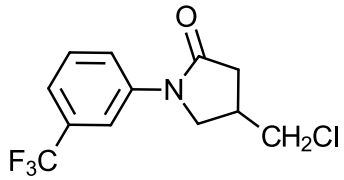
[1] FOCUS (2015): Generic guidance for FOCUS surface water Scenarios. Version 1.4.

Grouping according to organism groups			
Group	**Intended uses**	**Relevant use parameters for grouping**	**Relevant parameter or value for sorting**
Bees (**9.6**)	According to GAP; refer to Document B0, max. 1x 2.0 L product/ha in pre-emergence crops	No distinction required	No distinction required
Terrestrial non-target arthropods other than bees (**9.7**)	According to GAP; refer to Document B0, max. 1x 2.0 L product/ha in pre-emergence crops	No distinction required	No distinction required
Soil meso- and macrofauna / soil microorganisms (**9.8** and **9.9**)	According to GAP; refer to Document B0, max. 1x 2.0 L product/ha in pre-emergence crops	**Crop growth stage**	**BBCH 00-09**: bare soil (pre-emergence) with no crop interception
Non-target terrestrial plants (**9.10**)	According to GAP; refer to Document B0, max. 1x 2.0 L product/ha in pre-emergence crops 1x 1.5 L product/ha in pre-emergence crops	Use rate ~~No distinction required~~	Use rate ~~No distinction required~~

9.1.3 Consideration of metabolites

A list of relevant metabolites found in environmental compartments is provided below. The need for conducting a metabolite-specific risk assessment in the context of the evaluation of AG-F8-250 CS is indicated in the table (refer to dRR Part B8 8.2).

Table 9.1-3 Metabolites of flurochloridone

Metabolite	Chemical structure	Molar mass	Maximum occurrence in compartments	Risk assessment required for exposure via
R406639		293.7 g/mol	Soil: 8.1 % W/S system: 13.9 %	Surface water
R42819		277.7 g/mol	Soil: 10.1 % W/S system: 63.9 %	Surface water

zRMS comments:

Information regarding metabolites of flurochloridone is in line with EU agreed endpoints reported in EFSA Journal 2010;8(12):1869.

9.2 Effects on birds (KCP 10.1.1)

9.2.1 Toxicity data

Avian toxicity studies have been carried out with flurochloridone. Full details of these studies are provided in the respective EU DAR and related documents as well as in Appendix 2 of this document (new studies).

Effects on birds of the formulated product AG-F8-250 CS were not evaluated as part of the EU assessment of flurochloridone.

However, the provision of further data on the formulation is not considered essential, because an increased toxicity of the product is not expected as indicated by acute oral testing in mammals giving a limit dose endpoint (LD_{50} > 2150 mg AG-F8-250 CS/kg bw).

The selection of studies and endpoints for the risk assessment is in line with the results of the EU review process.

Table 9.2-1: Endpoints and effect values relevant for the risk assessment for birds

Species	Substance	Exposure System	Results [a)]	Reference
Bobwhite quail (*Colinus virginianus*)	flurochloridone	Oral 1 d Acute	**LD_{50} > 2150 mg a.s./kg b.w.**	EFSA Conclusion 2010 (Fletcher 1982a)
Bobwhite quail (*Colinus virginianus*)	flurochloridone	Dietary 8 d Short-term	LD_{50} > 590 mg a.s./kg b.w./d	EFSA Conclusion 2010 (Fletcher 1982b)
Mallard duck (*Anas plathyrhynchos*)	flurochloridone	Dietary Reproductive toxicity	**NOEL = 149 mg a.s./kg b.w./d**	EFSA Conclusion 2010 (Frey et al. 2002)

[a)] Relevant toxicity endpoints were selected in accordance with most recent guidance (EFSA 2009) [2]
bold: endpoints relevant for risk assessments

Most recent guidance (EFSA 2009) allows the calculation of geometric means of existing acute toxicity data for the same as well as different species tested for acute avian risk assessments. However, only one acute oral toxicity test is available. The acute risk assessments are therefore based on the EU agreed endpoint (i.e. LD_{50} > 2150 mg a.s./kg b.w.).

The formulated product was not tested with birds as the study with AG-F8-250 CS on rats does not indicate an increased toxicity.

Endpoint from short-term dietary testing is a limit dose endpoint which does not indicate an increased toxicity. Accordingly, the dietary toxicity endpoint for birds is not considered for risk assessments.

The relevant reproductive endpoint from the chronic study has to be compared to the surrogate endpoint for parental toxicity based on the acute oral toxicity ($LD_{50/10}$) and the lower endpoint has to be used for risk assessments in the first instance. As the surrogate endpoint for parental toxicity (i.e. > 215 mg a.s./kg b.w./d) is greater than the EU agreed endpoint which was based on egg production and eggshell thickness at the highest feed concentration of 3000 mg a.s./kg diet, the NOEL of 149 mg a.s./kg b.w./d (corresponding to the feed concentration of 1000 mg a.s./kg diet) is taken into account for reproductive risk assessments.

[2] 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.europe.eu

zRMS comments:

Avian toxicity data provided in Table 9.2-1 are in line with EU agreed endpoints reported in EFSA Journal 2010;8(12):1869.

As LD_{50}/10 of 215 mg/kg bw is higher than NOEL value of 149 mg/kg bw/d, the latter endpoint is relevant for the long-term risk assessment.

As AG-F8-250 CS is a solo formulation of flurochloridone and available data do not indicate that formulation is more toxic than the active compound, the risk assessment based on the active substance toxicity data is considered adequate and sufficient.

Metabolites of flurochloridone

Metabolites of flurochloridone are also occurring in the metabolism of vertebrates, are rapidly excreted and have shown to be of lower toxicity in other organisms, metabolites of the active substance are of no toxicological concern for birds and mammals.

The residues formed in plants (i.e. 5-hydroxy-4-chloromethyl and 3-hydroxy-4-chloromethyl) have also been found in rat metabolism. The metabolite 4-dechloro-hydroxymethyl was found in the application solution in the plant metabolism study in sunflowers (Miaullis et al. 1986) and was considered to be a by-product of the synthesis rather than a real metabolite. Accordingly, the metabolites found in plants are assumed to have been tested in the toxicity studies. Likewise, the metabolite R42819 is a proposed intermediate in the mammalian metabolic pathway.

In addition, more than 97% of the applied dose of the parent was excreted within 72 hours in rats and up to 91% within 96 hours in rabbits.

The environmental metabolites R42819 and R406639 relevant in surface water and sediment have been found to be of distinctly lower toxicity than the parent.

zRMS comments:

The dietary risk assessment from flurochloridone plant metabolites following application to bare soil was deemed not necessary during the EU review of flurochloridone.

In the DAR (February 2006) it is noted that uptake of the active compound by plants was low and therefore birds will be exposed rather to flurochloridone than to its metabolites.

The conclusion derived at the EU level is applicable also for this submission.

9.2.1.1 Justification for new endpoints

Risk assessments are conservatively based on the relevant worst-case EU agreed toxicity endpoints for birds.

9.2.2 Risk assessment for spray applications

The risk assessment is based on the methods presented in the Guidance Document on Risk Assessment for Birds and Mammals on request from EFSA (EFSA Journal 2009; 7(12): 1438; hereafter referred to as EFSA/2009/1438).

9.2.2.1 First-tier assessment (screening/generic focal species)

The results of the acute and reproductive screening step risk assessments are summarised in the following table.

Table 9.2-2: Screening Step assessment of the acute and long-term/reproductive risk for birds due to the use of AG-F8-250 CS in potato at 2.0 L product/ha

Intended use	**Pre-emergence, potato**				
Active substance/product	flurochloridone				
Application rate (g/ha)	1 x 500				
Acute toxicity (mg/kg bw)	> 2150				
TER criterion	10				
Crop scenario Growth stage	**Indicator/generic focal species**	**SV_{90}**	**MAF_{90}**	**DDD_{90} (mg/kg bw/d)**	**TER_a**
BBCH < 10	Small granivorous bird	24.7	1	12.35	> 174.1
Reprod. toxicity (mg/kg bw/d)	149				
TER criterion	5				
Crop scenario Growth stage	**Indicator/generic focal species**	**SV_m**	**MAF_m × TWA**	**DDD_m (mg/kg bw/d)**	**TER_{lt}**
BBCH < 10	Small granivorous bird	11.4	0.53	3.02	49.3

SV: shortcut value; MAF: multiple application factor; TWA: time-weighted average factor; DDD: daily dietary dose; TER: toxicity to exposure ratio. TER values shown in bold fall below the relevant trigger.

zRMS comments:

The dietary risk assessment for flurochloridone presented in Table 9.2-2 above is agreed by the zRMS. Based on the performed screening calculations acceptable acute and long-term dietary risk to birds may be concluded.

It is noted by the zRMS that the available data indicate that flurochloridone is systemic and therefore exposure of birds feeding on seedlings emerging after application of the product cannot be fully ruled out. Unfortunately, such exposure scenario is not covered by EFSA (2009) and for this reason it is not possible to address the risk from seedlings. Nevertheless, in opinion of the zRMS, the exposure via seedlings would be lower than this resulting from directly over-sprayed seeds considered at the screening step and for this reason no unacceptable risk via seedlings is anticipated.

9.2.2.2 Higher-tier risk assessment

As shown in the table above TER_{lt} values are below the relevant trigger value and therefore Higher Tier risk assessment is not required.

9.2.2.3 Drinking water exposure

When necessary, the assessment of the risk for birds due to uptake of contaminated drinking water is conducted for a small granivorous bird with a body weight of 15.3 g (*Carduelis cannabina*) and a drinking water uptake rate of 0.46 L/kg bw/d (*cf.* Appendix K of EFSA/2009/1438).

Leaf scenario

Since AG-F8-250 CS is not intended to be applied on leafy vegetables forming heads or crop plants with comparable water collecting structures at principal growth stage 4 or later, the leaf scenario does not have to be considered for the intended pre-emergence uses.

Puddle scenario

Due to the characteristics of the exposure scenario in connection with the standard assumptions for water uptake by animals, no specific calculations of exposure and TER are necessary when the ratio of effective application rate (in g/ha) to relevant endpoint (in mg/kg bw/d) does not exceed 50 in the case of less sorptive substances (Koc < 500 L/kg) or 3000 in the case of more sorptive substances (Koc ≥ 500 L/kg).

With a K(f)oc of 665 L/kg, flurochloridone belongs to the group of more sorptive substances.

Effective application rate (g/ha)	500 g a.s./ha		
Acute toxicity (mg/kg bw)	> 2150	quotient	< 0.23
Reprod. toxicity (mg/kg bw/d)	149	quotient	3.36

Accordingly, no further calculations are necessary and an acceptable risk from drinking water is indicated.

zRMS comments:

The drinking water risk assessment presented in table above is agreed by the zRMS. Acceptable risk resulting from exposure to the active compound via drinking water may be concluded.

However, performed evaluation does not cover the risk resulting from exposure to pertinent soil metabolites. Respective calculations were thus performed by the zRMS below. In absence of the toxicity data for metabolites, 10 times toxicity of the parent was assumed as unrealistic worst case.

Metabolite	Pseudo application rate [g pm/ha]	Endpoint [mg pm/kg bw] 3)	Quotient	Trigger
R406639	38.1 1)	>215	<0.18	3000
		14.9	2.6	
R42819	44.9 2)	>215	<0.21	50
		14.9	3.0	

1) Calculated with consideration of parent application rate (500 g/ha), molar ratio (0.941) and max occurrence in soil (8.1%)
2) Calculated with consideration of parent application rate (500 g/ha), molar ratio (0.890) and max occurrence in soil (10.1%)
3) 10 times toxicity of the parent as a worst case

Based on calculation presented in table above, acceptable risk to birds exposed to pertinent soil metabolites of fluchloridone via drinking water may be concluded.

9.2.2.4 Effects of secondary poisoning

The log P_{ow} of flurochloridone amounts to 3.36 and thus exceeds the trigger value of 3. A risk assessment for effects due to secondary poisoning is required.

Risk assessment for earthworm-eating birds via secondary poisoning

According to EFSA/2009/1438, the risk for vermivorous birds is assessed for a bird of 100 g body weight with a daily food consumption of 104.6 g.

Bioaccumulation in earthworms is estimated based on predicted concentrations in soil. However, porewater mediated uptake of pesticides seems mainly to be responsible for the effects caused in soft bodied soil organisms. Therefore, the porewater approach in accordance with EFSA (2009) is the more relevant metric for effect and exposure assessments. In this dossier, both assessments are presented.

Risk assessments are presented for the risk envelope as defined in Table 9.1 2 for the pre-emergence (i.e. single application at 2.0 L product/ha on bare soil).

Table 9.2-3: Assessment of the risk for earthworm-eating birds due to exposure to flurochloridone via bioaccumulation in earthworms (secondary poisoning) for the intended use on bare soil (pre-emergence, potato) – dry soil approach

Parameter	flurochloridone	comments
PEC_{soil} (twa = 21 d) [mg/kg soil]	0.6074 ~~0.58~~	0.6074 derived by zRMS
log P_{ow} / P_{ow}	3.36	
Koc	665	
K_{OW}	2291	
foc	0.02	Default
BCF_{worm}	2.13	$BCF_{worm/soil} = (PEC_{worm,ww}/PEC_{soil,dw})$ $= (0.84 + 0.012 \times K_{ow}) / foc \times Koc$
PEC_{worm} [mg a.s./kg bw/d]	1.29 ~~1.24~~	$PEC_{worm} = PEC_{soil} \times BCF_{worm/soil}$
Daily dietary dose [mg/kg bw/d]	1.35 ~~1.3~~	$DDD = PEC_{worm} \times 1.05$
NOEL [mg/kg bw/d]	149.0	
TER_{lt}	110.4 ~~114.6~~	

TER values shown in bold fall below the relevant trigger.

Table 9.2-4: Assessment of the risk for earthworm-eating birds due to exposure to flurochloridone via bioaccumulation in earthworms (secondary poisoning) for the intended use on bare soil (pre-emergence, potato) – porewater approach

Parameter	flurochloridone	comments
$PEC_{porewater}$ (twa = 21 d) [mg/ a.s./L]	0.0571 ~~0.043~~	0.0571 derived by zRMS
PEC_{soil} (twa = 21 d) [mg/kg soil]	0.6074 ~~0.58~~	0.6074 derived by zRMS
log P_{ow} / P_{ow}	3.36	
K_{OW}	2291	
$RHO_{earthworm}$ [kg ww/L]	1	
BCF_{worm}	28.3	$BCF_{worm/water} = (PEC_{worm,ww}/PEC_{porewater}) = (0.84 + 0.012 \times Kow/ RHO_{earthworm})$
F_{solid} [m^3/m^3]	0.6	
RHO_{soil} [kg ww/m^3]	1700	
RHO_{solid} [kg dw/m^3]	2500	
F_{gut} [kg dw/kg ww]	0.1	
$CONV_{soil}$ [kg ww/kg dw]	1.13	
PEC_{worm} [mg a.s./kg bw/d]	1.51 ~~1.14~~	$C_{earthworm} = (BCF_{worm} * PEC_{porewater} + PEC_{soil} * F_{gut} * CONV_{soil})/(1 + F_{gut} * CONV_{soil})$
Daily dietary dose [mg/kg bw/d]	1.6 ~~1.2~~	$DDD = PEC_{worm} \times 1.05$
NOEL [mg/kg bw/d]	149.0	
TER_{lt}	93.1 ~~124.2~~	

TER values shown in bold fall below the relevant trigger.

Risk assessment for fish-eating birds via secondary poisoning

According to EFSA/2009/1438, the risk for piscivorous birds is assessed for a bird of 1000 g body weight with a daily food consumption of 159 g.

Secondary poisoning assessments are conservatively based on the worst-case 21-day FOCUS Step 1 PEC_{SW} of 92.94 µg a.s./L for the intended pre-emergence use in potatoes at a maximum single use rate of 500 g a.s./ha.

Table 9.2-5: Assessment of the risk for fish-eating birds due to exposure to flurochloridone via bioaccumulation in fish (secondary poisoning) for the intended use on bare soil (pre-emergence potato)

Parameter	flurochloridone	comments
PEC_{sw} (twa = 21 d) [mg/L]	0.09294	Worst-case FOCUS Step 1 PEC_{SW}
BCF_{fish}	292.00	
BMF	-	biomagnification factor (relevant for BCF ≥ 2000)
PEC_{fish}	27.14	$PEC_{fish} = PEC_{sw} \times BCF_{fish}$
Daily dietary dose [mg/kg bw/d]	4.32	$DDD = PEC_{fish} \times 0.159$
NOEL [mg/kg bw/d]	149.0	
TER_{lt}	34.5	

TER values shown in bold fall below the relevant trigger.

zRMS comments:

The evaluation of the risk of secondary poisoning to fish-eating birds presented above is agreed by the zRMS.
The evaluation of the risk of secondary poisoning to earthworm-eating birds has been amended by the zRMS with consideration of higher PEC_{soil} values calculated in the course of evaluation performed in area of Section 8.

Overall, acceptable risk of secondary poisoning could be concluded.

No information regarding log Pow of flurochloridone metabolites is available in EFSA Journal 2010;8(12):1869. However, bioaccumulation of these compounds in fish- and earthworm-eating birds has been not considered during the EU review of flurochloridone and in consequence is also not considered in this document.

9.2.2.5 Biomagnification in terrestrial food chains

The toxicokinetic evaluations of flurochloridone indicate a low potential for bioaccumulation (see Peer Review Report). More than 97% of the active substance was excreted within 72 hours in rats and 87 to 91% within 96 hours in rabbits after administration in the ADME studies. The studies show an extensive metabolisation of flurochloridone. Likewise, in fish, the CT_{90} is very short (< 3 days).
Therefore, no further assessment of biomagnification in terrestrial food chains is required.

zRMS comments:

Based on evaluation of the risk of secondary poisoning as well as information regarding metabolism in mammals, biomagnification of flurochloridone in the terrestrial food chain is not expected.

9.2.3 Risk assessment for baits, pellets, granules, prills or treated seed

Not relevant.

9.2.4 Overall conclusions

Acute and long-term reproductive Screening Step assessments with resulting TERs above the relevant trigger values indicate a low and acceptable dietary risk for birds exposed to the active substance flurochloridone.
A secondary poisoning assessment indicates a low risk for earthworm- and fish-eating birds. The exposure of birds to drinking water from pools in leaf whorls is not relevant for the proposed uses of AG-F8-250 CS. Detailed risk assessments for birds exposed via drinking water from puddles formed on the field are not triggered.
Thus, treatment with AG-F8-250 CS in accordance with the proposed use patterns in potato poses low risk to birds.

9.3 Effects on terrestrial vertebrates other than birds (KCP 10.1.2)

9.3.1 Toxicity data

Mammalian toxicity studies have been carried out with flurochloridone. Full details of these studies are provided in the respective EU DAR and related documents as well as in Section 6 (Mammalian Toxicology) of this report.

Effects on mammals of formulation were not evaluated as part of the EU assessment of flurochloridone. New data submitted with this application are listed in Appendix 1 and summarised in Section 6 (Mammalian Toxicology) of this report.

The selection of studies and endpoints for the risk assessment partly deviates from the results of the EU review process.

Table 9.3-1: Endpoints and effect values relevant for the risk assessment for mammals

Species	Substance	Exposure System	Results [a)]	Reference
Rat	Racer ME (AG-F8-250CS)	Oral 14 d Acute	LD_{50} > 5000 mg a.s./kg b.w.	EFSA Scientific Report 2010 (Whittacker, 1986a; (refer to the toxicology section; B6)
Mouse	flurochloridone	Oral 14 d Acute	**LD_{50} = 5000 mg a.s./kg b.w.**	EFSA Scientific Report 2010 (Whittaker, C. J. 1986a)
Rat	flurochloridone	Dietary Reproductive toxicity Long-term, 3 years generation study	NOAEL = 28.6 mg/kg b.w./d [b)]	EFSA Scientific Report 2010 (Downs and Minor 1983)
Rat	flurochloridone	Oral Developmental toxicity	**NOAEL = 20 mg a.s./kg b.w./d**	EFSA Scientific Report 2010 (Nemec M.D. 1984a)
Rabbit	flurochloridone	Oral Developmental toxicity	**NOAEL = 20 mg a.s./kg b.w./d**	EFSA Scientific Report 2010 (Nemec M.D. 1984b)

[a)] Relevant toxicity endpoints were selected in accordance with most recent guidance (EFSA 2009) [3]
[b)] The endpoint in the endpoint table in the Peer Review Report is 27.7 mg a.s./kg b.w./d; however calculations were conducted based on the endpoint of 28.6 mg a.s./kg b.w./d as stated to be the agreed endpoint in the text of the EFSA Journal
bold: endpoints relevant for risk assessments

zRMS comments:

Mammalian toxicity data provided in Table 9.3-1 are in line with EU agreed endpoints reported in EFSA Journal 2010;8(12):1869.

As AG-F8-250 CS is a solo formulation of flurochloridone and available data do not indicate that formulation is more toxic than the active compound, the risk assessment based on the active substance toxicity data is considered adequate and sufficient.

Metabolites of flurochloridone

Metabolites of flurochloridone are also occurring in the metabolism of vertebrates, are rapidly excreted and have shown to be of lower toxicity in other organisms, metabolites of the active substance are of no toxicological concern for birds and mammals.

[3] 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.europe.eu

The residues formed in plants (i.e. 5-hydroxy-4-chloromethyl and 3-hydroxy-4-chloromethyl) have also been found in rat metabolism. The metabolite 4-dechloro-hydroxymethyl was found in the application solution in the plant metabolism study in sunflowers (Miaullis et al. 1986) and was considered to be a by-product of the synthesis rather than a real metabolite. Accordingly, the metabolites found in plants are assumed to have been tested in the toxicity studies. Likewise, the metabolite R42819 is a proposed intermediate in the mammalian metabolic pathway.

In addition, more than 97% of the applied dose of the parent was excreted within 72 hours in rats and up to 91% within 96 hours in rabbits.

The environmental metabolites R42819 and R406639 relevant in surface water and sediment have been found to be of distinctly lower toxicity than the parent.

zRMS comments:

The dietary risk assessment from flurochloridone plant metabolites following application to bare soil was deemed not necessary during the EU review of flurochloridone.

In the DAR (February 2006) it is noted that uptake of the active compound by plants was low and therefore birds will be exposed rather to flurochloridone than to its metabolites.

The conclusion derived at the EU level is applicable also for this submission.

9.3.1.1 Justification for new endpoints

Most recent guidance (EFSA 2009) allows the calculation of geometric means of existing acute toxicity data for the same as well as different species tested for acute mammalian risk assessments. However, only the study providing the EU agreed endpoint of 5000 mg a.s./kg b.w. (LD_{50}) was considered to be valid. Accordingly, this endpoint is taken into account for acute risk assessments.

The study conducted with the formulated product AG-F8-250 CS with an LD_{50} of > 5000 mg product/kg bw does not indicate an increased toxicity (Whittaker 1986a; 818607). Therefore, risk assessments are presented based on the data for the active substance.

The EU agreed reproductive toxicity endpoint (i.e. the NOAEL of 28.6 mg a.s./kg b.w./d) in accordance with EFSA (2009) from multi-generation testing has to be compared to the available data from developmental toxicity studies and the more sensitive endpoint be selected for risk assessments. The lowest endpoint from the teratogenicity studies is a developmental NOAEL of 20 mg a.s./kg b.w./d in rats and rabbits. This endpoint is considered to be relevant for lower tier assessments.

zRMS comments:

In general, the risk assessment should be based on endpoints derived in the course of the EU review of the given substance. As in EFSA Journal 2010;8(12):1869 it is indicated that NOAEL of 28.6 mg a.s./kg bw/d is relevant for the long-term mammalian risk assessment, this values should be taken into account also at the zonal level.

Nevertheless, NOAEL of 20 mg a.s./kg bw/d selected by the Applicant is lower comparing to the EU agreed endpoint and for this reason its consideration for TER calculations is agreed as representing worst case.

9.3.2 Risk assessment for spray applications

The risk assessment is based on the methods presented in the Guidance Document on Risk Assessment for Mammals and Mammals on request from EFSA (EFSA Journal 2009; 7(12): 1438; hereafter referred to as EFSA/2009/1438).

9.3.2.1 First-tier assessment (screening/generic focal species)

The results of the acute and reproductive first-tier risk assessments are summarised in the following tables.

Table 9.3-2: Screening Step assessment of the acute and long-term/reproductive risk for mammals due to the use of AG-F8-250 CS in potato at 2.0 L product/ ha

Intended use	Pre-emergence potato				
Active substance/product	flurochloridone				
Application rate [g/ha]	1x 500				
Acute toxicity [mg/kg bw]	5000				
TER criterion	10				
Crop scenario Growth stage	**Indicator species for screening**	**SV_{90}**	**MAF_{90}**	**DDD_{90} (mg/kg bw/d)**	**TER_a**
BBCH < 10	Small granivorous mammal	14.4	1	7.20	694.4
Reprod. toxicity [mg/kg bw/d]	20.0				
TER criterion	5				
Crop scenario Growth stage	**Indicator species for screening**	**SV_m**	**MAF_m × TWA**	**DDD_m (mg/kg bw/d)**	**TER_{lt}**
BBCH < 10	Small omnivorous mouse	6.6	0.53	1.75	11.4

SV: shortcut value; MAF: multiple application factor; TWA: time-weighted average factor; DDD: daily dietary dose; TER: toxicity to exposure ratio. TER values shown in bold fall below the relevant trigger.

Risk assessments indicate an acceptable acute risk for mammals for exposure towards the active substance flurochloridone.

zRMS comments:

The dietary risk assessment for flurochloridone presented in Table 9.3-2 above is agreed by the zRMS. Based on the performed screening calculations acceptable acute and long-term dietary risk to mammals may be concluded.

It is noted by the zRMS that the available data indicate that flurochloridone is systemic and therefore exposure of mammals feeding on seedlings emerging after application of the product cannot be fully ruled out. Unfortunately, such exposure scenario is not covered by EFSA (2009) and for this reason it is not possible to address the risk from seedlings. Nevertheless, in opinion of the zRMS, the exposure via seedlings would be lower than this resulting from directly over-sprayed seeds considered at the screening step and for this reason no unacceptable risk via seedlings is anticipated.

9.3.2.2 Higher-tier risk assessment

An acceptable risk was already shown using Tier 1 risk assessment and therefore no higher tier risk assessment is required.

9.3.2.3 Drinking water exposure

When necessary, the assessment of the risk for mammals due to uptake of contaminated drinking water is conducted for a small omnivorous mammal with a body weight of 21.7 g (*Apodemus sylvaticus*) and a drinking water uptake rate of 0.24 L/kg bw/d (*cf.* Appendix K of EFSA/2009/1438).

Puddle scenario

Due to the characteristics of the exposure scenario in connection with the standard assumptions for water uptake by animals, no specific calculations of exposure and TER are necessary when the ratio of effective application rate (in g/ha) to relevant endpoint (in mg/kg bw/d) does not exceed 50 in the case of less sorptive substances (Koc < 500 L/kg) or 3000 in the case of more sorptive substances (Koc ≥ 500 L/kg).

With a K(f)oc of 665 L/kg, flurochloridone belongs to the group of more sorptive substances.

Effective application rate (g/ha)	=	500		
Acute toxicity (mg/kg bw)	=	5000	quotient =	0.10
Reprod. toxicity (mg/kg bw/d)	=	20	quotient =	25.0

With a K_{OC} of 665 L/kg and acute and reproductive endpoints of 5000 mg a.s./kg bw and 20 mg a.s./kg bw/d, respectively, and at an intended maximum effective application rate of 500 g a.s./ha (acute and reproductive), no detailed drinking water assessment is triggered for mammals.

zRMS comments:

The drinking water risk assessment presented in table above is agreed by the zRMS. Acceptable risk resulting from exposure to the active compound via drinking water may be concluded.

However, performed evaluation does not cover the risk resulting from exposure to pertinent soil metabolites. Respective calculations were thus performed by the zRMS below. In absence of the toxicity data for metabolites, 10 times toxicity of the parent was assumed as unrealistic worst case.

Metabolite	Pseudo application rate [g pm/ha]	Endpoint [mg pm/kg bw] 3)	Quotient	Trigger
R406639	38.1 1)	500	0.08	3000
		2.0	19.1	
R42819	44.9 2)	500	0.09	50
		2.0	22.5	

1) Calculated with consideration of parent application rate (500 g/ha), molar ratio (0.941) and max occurrence in soil (8.1%)
2) Calculated with consideration of parent application rate (500 g/ha), molar ratio (0.890) and max occurrence in soil (10.1%)
3) 10 times toxicity of the parent as a worst case

Based on calculation presented in table above, acceptable risk to mammals exposed to pertinent soil metabolites of fluchloridone via drinking water may be concluded.

9.3.2.4 Effects of secondary poisoning

The log P_{ow} of flurochloridone amounts to 3.6 and thus exceeds the trigger value of 3. A risk assessment for effects due to secondary poisoning is required.

Risk assessment for earthworm-eating mammals via secondary poisoning

According to EFSA/2009/1438, the risk for vermivorous mammals is assessed for a small mammal of 10 g body weight with a daily food consumption of 12.8 g.

Bioaccumulation in earthworms is estimated based on predicted concentrations in soil. However, porewater mediated uptake of pesticides seems mainly to be responsible for the effects caused in soft bodied soil organisms. Therefore, the porewater approach in accordance with EFSA (2009) is the more relevant metric

for effect and exposure assessments. In this dossier, both assessments are presented.

Risk assessments are presented for the risk envelope as defined in Table 9.1 2 for the pre-emergence (i.e. single application at 2.0 L product/ha on bare soil).

Table 9.3-3: Assessment of the risk for earthworm-eating mammals due to exposure to flurochloridone via bioaccumulation in earthworms (secondary poisoning) for the intended use in potato (pre-emergence) – dry soil approach

Parameter	flurochloridone	comments
PEC_{soil} (twa = 21 d) [mg/kg soil]	0.6074 ~~0.58~~	0.6074 derived by zRMS
log P_{ow} / P_{ow}	3.36	
Koc	665	Mean
K_{OW}	2291	
foc	0.02	Default
BCF_{worm}	2.13	BCFworm/soil = (PECworm,ww/PECsoil,dw) = (0.84 + 0.012 × Kow) / foc × Koc
PEC_{worm} [mg a.s./kg]	1.29 ~~1.24~~	$PEC_{worm} = PEC_{soil} \times BCF_{worm/soil}$
Daily dietary dose [mg/kg bw/d]	1.65 ~~1.59~~	DDD = PEC_{worm} × 1.28
NOEL [mg/kg bw/d]	20	
TER_{lt}	12.1 ~~12.6~~	

TER values shown in bold fall below the relevant trigger.

Table 9.3-4: Assessment of the risk for earthworm-eating mammals due to exposure to flurochloridone via bioaccumulation in earthworms (secondary poisoning) for the intended use in potato (pre-emergence) – porewater approach

Parameter	flurochloridone	comments
$PEC_{porewater}$ (twa = 21 d) [mg/kg soil]	0.0571 ~~0.043~~	0.0571 derived by zRMS
PEC_{soil} (twa = 21 d) [mg/kg soil]	0.6074 ~~0.58~~	0.6074 derived by zRMS
log P_{ow} / P_{ow}	3.36	
Koc	665	Mean
K_{OW}	2291	
$RHO_{earthworm}$ [kg ww/L]	1	
BCF_{worm}	28.3	$BCF_{worm/water} = (PEC_{worm,ww}/PEC_{porewater}) = (0.84 + 0.012 \times Kow/ RHO_{earthworm})$
F_{solid} [m^3/m^3]	0.6	
RHO_{soil} [kg ww/m^3]	1700	
RHO_{solid} [kg dw/m^3]	2500	
F_{gut} [kg dw/kg ww]	0.1	
$CONV_{soil}$ [kg ww/kg dw]	1.13	
PEC_{worm} [mg a.s./kg]	1.51 ~~1.14~~	$C_{earthworm} = (BCF_{worm} * PEC_{porewater} + PEC_{soil} * F_{gut} * CONV_{soil})/(1 + F_{gut} * CONV_{soil})$
Daily dietary dose [mg/kg bw/d]	1.93 ~~1.46~~	DDD = PEC_{worm} × 1.28
NOEL [mg/kg bw/d]	20	
TER_{lt}	10.4 ~~13.7~~	

TER values shown in bold fall below the relevant trigger.

Assessment for fish-eating mammals via secondary poisoning

According to EFSA/2009/1438, the risk for piscivorous mammals is assessed for a mammal of 3000 g body weight with a daily food consumption of 425 g.

Secondary poisoning assessments are conservatively based on the worst-case 21-day FOCUS Step 1 PEC_{SW} of 92.94 µg a.s./L for the intended pre-emergence use in potatoes at a maximum single use rate of 500 g a.s./ha.

Table 9.3-5: Assessment of the risk for fish-eating mammals due to exposure to flurochloridone via bioaccumulation in fish (secondary poisoning) for the intended use in potato (pre-emergence)

Parameter	flurochloridone	comments
PEC_{sw} (twa = 21 d) [mg/L]	0.09294	
BCF_{fish}	292	
BMF	-	biomagnification factor (relevant for BCF ≥ 2000)
PEC_{fish} [mg a.s./kg]	27.14	$PEC_{fish} = PEC_{water} \times BCF_{fish}$
Daily dietary dose [mg/kg bw/d]	3.85	$DDD = PEC_{fish} \times 0.142$
NOEL [mg/kg bw/d]	20.0	
TER_{lt}	5.19	

TER values shown in bold fall below the relevant trigger.

zRMS comments:

The evaluation of the risk of secondary poisoning to fish-eating mammals presented above is agreed by the zRMS. The evaluation of the risk of secondary poisoning to earthworm-eating mammals has been amended by the zRMS with consideration of higher PEC_{soil} values calculated in the course of evaluation performed in area of Section 8.

Overall, acceptable risk of secondary poisoning could be concluded.

No information regarding log Pow of flurochloridone metabolites is available in EFSA Journal 2010;8(12):1869. However, bioaccumulation of these compounds in fish- and earthworm-eating birds has been not considered during the EU review of flurochloridone and in consequence is also not considered in this document.

9.3.2.5 Biomagnification in terrestrial food chains

The toxicokinetic evaluations of flurochloridone indicate a low potential for bioaccumulation (see Peer Review Report). More than 97% of the active substance was excreted within 72 hours in rats and 87 to 91% within 96 hours in rabbits after administration in the ADME studies. The studies show an extensive metabolisation of flurochloridone. Likewise, in fish, the CT_{90} is very short (< 3 days).

Therefore, no further assessment of biomagnification in terrestrial food chains is required.

zRMS comments:

Based on evaluation of the risk of secondary poisoning as well as information regarding metabolism in mammals, biomagnification of flurochloridone in the terrestrial food chain is not expected.

9.3.3 Risk assessment for baits, pellets, granules, prills or treated seed

Not relevant.

9.3.4 Overall conclusions

The acute Screening Step assessment results in TERs above the relevant trigger values, indicating a low and acceptable dietary risk for mammals exposed to the active flurochloridone.

A secondary poisoning assessment indicates a low risk for earthworm- and fish-eating mammals. Detailed risk assessments for mammals exposed via drinking water from puddles formed on the field are not triggered.

Thus, treatment with AG-F8-250 CS in accordance with the proposed use patterns in potato poses a low risk to terrestrial vertebrates other than birds.

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

No additional relevant data available. Refer to the EU reviews of the active substance.

zRMS comments:

As currently there are no agreed rules or criteria for evaluation of the risk to other terrestrial vertebrates like reptiles and amphibians, this issue should be addressed once respective guidance is available and EU agreed endpoints concluded.

9.5 Effects on aquatic organisms (KCP 10.2)

9.5.1 Toxicity data

Studies on the toxicity to aquatic organisms have been carried out with flurochloridone and its relevant metabolites. Full details of these studies are provided in the respective EU DAR and related documents, as well as in Appendix 2 of this document (new studies).

Effects on aquatic organisms of AG-F8-250 CS were evaluated as part of the EU assessment of flurochloridone. New data submitted with this application are listed in Appendix 1 and summarised in Appendix 2.

The selection of studies and endpoints for the risk assessment is in line with the results of the EU review process. However, additional data are provided (refer to the justification provided below under Point 9.5.1.1).

Table 9.5-1: Endpoints and effect values relevant for the risk assessment for aquatic organisms – flurochloridone and relevant metabolites

Species	Substance	Exposure System	Results	Reference
Fish acute				
Oncorhynchus mykiss	flurochloridone	96 h, dynamic	96 h **LC_{50} = 3.0 mg a.s./L**	EFSA Scientific Report 2010 (Douglas et al. 1987)
Lepomis macrochirus	flurochloridone	96 h, s	96 h LC_{50} = 10.2 mg a.s./L	EFSA Scientific Report 2010 (Cohle and Mc Allister 1983)
Fish chronic				
Oncorhynchus mykiss	flurochloridone	28 d, f	**NOEC = 0.36 mg a.s./L** mm	EFSA Scientific Report 2010 (Smith 1990)
Invertebrates acute				
Daphnia magna	flurochloridone	48 h, s	EC_{50} =5.1 mg a.s./L mm	EFSA Scientific Report 2010 (Spare 1983)
Daphnia magna	flurochloridone	48 h, s	**EC_{50} = 3.5 mg a.s./L** mm	EFSA Scientific Report 2010 (Bätscher 2007b)
Invertebrates chronic				
Daphnia magna	flurochloridone	21 d, ss	**NOEC =0.83 mg a.s./L** mm	EFSA Scientific Report 2010 (Stewart et al. 1990)
Sediment dweller chronic				
Chironomus riparius	flurochloridone (trans-isomer)	25 d, s	**NOEC = 0.25 mg a.s./L** nom	EFSA Scientific Report 2010 (Gentle 1997)
Algae				
S. subspicatus	flurochloridone	72 h, s	**E_rC_{50} = 0.0047 mg a.s./L** mm [a)] E_bC_{50} = 0.0021 mg a.s./L mm	EFSA Scientific Report 2010 (Bätscher 2004a)
A. flos-aquae	flurochloridone	72 h, s	**E_rC_{50} = 13.4 mg a.s./L** mm [b)] E_bC_{50} = 8.84 mg a.s./L mm [b)]	DAR, February 2006 ~~EFSA Scientific Report 2010~~ (Wallace and Swarbrick 2001)
S. subspicatus	flurochloridone	24 h, s / 72 h recovery ~~72 h, s~~	E_rC_{50} = 0.32 mg a.s./L mm E_bC_{50} = 0.0206 mg a.s./L mm	EFSA Scientific Report 2010 (Memmert 2006)
S. subspicatus	flurochloridone (cis-isomer)	72 h, s	E_rC_{50} = 0.0069 mg a.s./L mm E_bC_{50} = 0.0045 mg a.s./L mm	EFSA Scientific Report 2010 (Bätscher 2008a)
S. subspicatus	flurochloridone (trans-isomer)	72 h, s	E_rC_{50} = 0.00088 mg a.s./L mm E_bC_{50} =0.00054 mg a.s./L mm	EFSA Scientific Report 2010 (Bätscher 2008b)
S. subspicatus	Metabolite R42819	72 h, s	**E_rC_{50} = 2.3 mg/L** E_bC_{50} = 0.99 mg/L	EFSA Scientific Report 2010 (Bätscher 2004c)

Species	Substance	Exposure System	Results	Reference
S. subspicatus	Metabolite R406639	72 h, s	**E_rC_{50} = 3.3 mg/L** E_bC_{50} = 1.9 mg/L	EFSA Scientific Report 2010 (Bätscher 2004b)
Chlamydomonas reinhardtii	flurochloridone	72 h, s add. recovery period	72 h E_yC_{50} = 17 µg a.s./L 72 h **E_rC_{50} > 25 µg a.s./L** NOEC = 7.7 µg a.s./L (mm) ~~3-day recovery period:~~ ~~$NOAEC_{recovery}$ = 25 µg a.s./L~~	Ref. KCP 10.2.3 Lietdke 2013a; 90015442 Additional information
Chlorella vulgaris	flurochloridone	72 h, s	72 h E_yC_{50} = 3.9 µg a.s./L 72 h **E_rC_{50} = 14.3 µg a.s./L** NOEC = 1.9 µg a.s./L (mm.)	Ref. KCP 10.2.3 Lietdke 2013b; 90015443
Navicula pelliculosa	flurochloridone	72 h, s add. recovery period	72 h E_yC_{50} = 3.4 µg a.s./L 72 h **E_rC_{50} = 12 µg a.s./L** NOEC = 1.5 µg a.s./L (mm) ~~9-day recovery perod:~~ ~~NOAECrecovery = 187 µg a.s./L~~	Ref. KCP 10.2.3 Lietdke 2013c; 90015444
Pseudokircheriella subcapitata	flurochloridone	72 h, s add. recovery period	72 h E_yC_{50} = 1.3 µg a.s./L 72 h **E_rC_{50} = 2.42 µg a.s./L** NOE_rC = 1.00 µg a.s./L (nom) NOE_yC = 0.32 µg a.s./L (nom) ~~4 to 7-day recovery period:~~ ~~**$NOAEC_{recovery}$ = 10.0 µg a.s./L**~~	Ref. KCP 10.2.3 Scheerbaum 2013a; 90015448
Nitzschia communis	flurochloridone	72 h, s add. recovery period	72 h E_yC_{50} = 2.5 µg a.s./L 72 h **E_rC_{50} = 4.45 µg a.s./L** NOEC = 1.00 µg a.s./L (nom) ~~4 to 8-day recovery period:~~ ~~$NOAEC_{recovery}$ = 100 µg a.s./L~~	Ref. KCP 10.2.3 Scheerbaum 2013b; 90015449
Synechococcus leopoliensis	flurochloridone	72 h, s add. recovery period	72 h E_yC_{50} = 2.22 µg a.s./L 72 h **E_rC_{50} = 4.07 µg a.s./L** NOEC = 0.946 µg a.s./L (mm) ~~4 to 7-day recovery period:~~ ~~$NOAEC_{recovery}$ = 14.4 µg a.s./L~~	Ref. KCP 10.2.3 Scheerbaum 2013c; 90015450
Chromulina sp.	flurochloridone	72 h, s add. recovery period	72 h E_yC_{50} = 18.5 µg a.s./L 72 h **E_rC_{50} = 23.3 µg a.s./L** NOEC = 12.5 µg a.s./L (nom) ~~4 to 5-day recovery period:~~ ~~$NOAEC_{recovery}$ = 100 µg a.s./L~~	Ref. KCP 10.2.3 Scheerbaum 2013d; 90016462
Ankistrodesmus falcatus	flurochloridone	72 h, s add. recovery period	72 h E_yC_{50} = 0.516 µg a.s./L 72 h **E_rC_{50} = 0.918 µg a.s./L** NOEC = 0.32 ~~12.5~~ µg a.s./L (nom) ~~4 to 8-day recovery period:~~ ~~**$NOAEC_{recovery}$ = 10 µg a.s./L**~~	Ref. KCP 10.2.3 Scheerbaum 2013e; 90016463
Desmodesmus subspicatus	flurochloridone	72 h, s add. recovery period	72 h E_yC_{50} = 1.14 µg a.s./L 72 h E_rC_{50} = 4.96 µg a.s./L NOEC = 0.393 ~~12.5~~ µg a.s./L (in. meas.) ~~4 to 7-day recovery period:~~ ~~$NOAEC_{recovery}$ = 30.9 µg a.s./L~~	Ref. KCP 10.2.3 Wenzel 2015a; 90016481

Species	Substance	Exposure System	Results	Reference
~~*Desmodesmus subspicatus*~~	~~flurochloridone (trans isomer)~~	~~Pulsed exposure study: 4 pulses~~	~~**NOAEPs** (nominal conc.):~~ ~~**1. 10.5 or 35 µg a.s./L for 24 h**~~ ~~**72 h recovery period**~~ ~~**2. 6.3 or 21 µg a.s./L for 36 h**~~ ~~**36 h recovery period**~~ ~~**3. 4.5 or 15 µg a.s./L for 30 h**~~ ~~**6 h recovery period**~~ ~~**4. 3.0 or 10 µg a.s./L for 36 h**~~ ~~**72 h recovery period**~~	~~Ref. KCP 10.2.3~~ ~~Liedtke 2013d; 90015421~~
~~*Desmodesmus subspicatus*~~	~~flurochloridone (trans isomer)~~	~~Pulsed exposure study: 1 cumulative pulse~~	~~**NOAEPs** (nominal conc.):~~ ~~**1. 1.65 or 5.5 µg a.s./L for 48 h**~~ ~~**2. 1.11 or 3.7 µg a.s./L for 24 h**~~ ~~**3. 0.45 or 1.5 µg a.s./L for 24 h**~~ ~~**4. 0.63 or 2.1 µg a.s./L for 24 h**~~ ~~**5. 1.11 or 3.7 µg a.s./L for 24 h**~~ ~~**6. 0.63 or 2.1 µg a.s./L for 24 h**~~ ~~**72 h recovery period**~~	~~Ref. KCP 10.2.3~~ ~~Liedtke 2013e; 90015432~~
Aquatic plants				
Lemna gibba	flurochloridone	14 d, ss	**EC_{50}** (dry weight) = **0.048 mg a.s./L** $NOE_{b/r}C$ (dry weight) = 0.015 mg a.s./L	EFSA Scientific Report 2010 (Woodyer et al. 2001)
Lemna gibba	flurochloridone (cis-isomer)	7 d, ss	E_rC_{50} (dry weight) = 0.047 mg a.s./L $_{mm}$ E_bC_{50} (dry weight) = 0.013 mg a.s./L $_{mm}$ NOEC (dry weight) < 0.007 mg a.s./L E_rC_{50} (frond number) = 0.104 mg a.s./L $_{mm}$ E_bC_{50} (frond number) = 0.022 mg a.s./L $_{mm}$ NOEC (frond number) < 0.0075 mg a.s./L	EFSA Scientific Report 2010 (Bätscher 2008c)
Lemna gibba	flurochloridone (trans-isomer)	7 d, ss	E_rC_{50} (dry weight) = 0.010 mg a.s./L $_{mm}$ E_bC_{50} (dry weight) < 0.004 mg a.s./L $_{mm}$ NOEC (dry weight) n.d. E_rC_{50} (frond number) = 0.0042 mg a.s./L $_{mm}$ E_bC_{50} (frond number) = 0.020 mg a.s./L $_{mm}$ NOEC (frond number) n.d.	EFSA Scientific Report 2010 (Bätscher 2008d)
Lemna gibba	Metabolite R42819	14 s	**E_rC_{50}** (dry weight) **= 8.2 mg/L** E_bC_{50} (dry weight) =3.2 mg/L	EFSA Scientific Report 2010 (Bätscher 2003)
~~*Lemna minor*~~	~~flurochloridone (trans-isomer)~~	~~Pulsed exposure study: 4 pulses~~	~~NOAEP (nominal conc.):~~ ~~1. 35 µg a.s./L for 24 h~~ ~~72 h recovery period~~ ~~2. 21 µg a.s./L for 36 h~~ ~~36 h recovery period~~ ~~3. 15 µg a.s./L for 30 h~~ ~~6 h recovery period~~ ~~4. 10 µg a.s./L for 36 h~~ ~~7 d recovery period~~	~~Ref. KCP 10.2.3~~ ~~Wenzel 2015b; 90016482~~

Species	Substance	Exposure System	Results	Reference
~~*Myriophyllum spicatum*~~	~~flurochloridone (trans-isomer)~~	~~Pulsed exposure study: 4 pulses~~	~~NOAEP (nominal conc.):~~ ~~1. 35 µg a.s./L for 24 h~~ ~~72 h recovery period~~ ~~2. 21 µg a.s./L for 36 h~~ ~~36 h recovery period~~ ~~3. 15 µg a.s./L for 30 h~~ ~~6 h recovery period~~ ~~4. 10 µg a.s./L for 36 h~~ ~~8 d recovery period~~	~~Ref. KCP 10.2.3~~ ~~Wenzel 2015c; 90016483~~

s: static; ss: semi-static; f: flow-through; nom: based on nominal concentrations; mm: based on mean measured concentrations; im: based on initial measured concentrations; NOAEP: No Observed Adverse Effect (concentration) Pattern
[a)] E_rC_{50} is the preferred endpoint following EFSA (2013)[4]
[b)] as presented in the EU DAR; not provided in the list of endpoints
bold: endpoints relevant for risk assessments

zRMS comments:

Data on toxicity of the active substance and its metabolites to fish, aquatic invertebrates and sediment dwellers provided in Table 9.5-1 are in line with EU agreed endpoints reported in EFSA Journal 2010;8(12):1869.

With regard to toxicity data for algae and aquatic macrophytes – endpoints for standard species derived from standard toxicity studies are in line with data reported in EFSA Journal 2010;8(12):1869 or in the DRAR (February 2006). Respective information has been added to Table 9.5-1 in order to distinguish endpoints reported in the LoEP and in the DAR.

It was also noted that the study by Memmert, 2006 (reported in the LoEP) was performed with 24 hours exposure followed by the recovery period, so respective information has been also added by the zRMS to clarify, that the study was not performed under standard exposure conditions.

In support of evaluation of AG-F8-250 CS studies on toxicity of flurochloridone to additional algal species were provided by the Applicant. Although in general the new active substance studies should not be evaluated at the zonal level, this is allowed in case they are crucial in order to address the risk. As no acceptable risk could be concluded to aquatic species based on Tier 1 endpoints available from the EU review, submission of additional data was justified in line with recommendation of SANCO/10326/2004, rev. 8 (2012). The new studies were evaluated by the zRMS and considered acceptable. However, as recovery should not be taken into account in the risk assessment, part of the studies regarding recovery was not evaluated by the zRMS and information based on this parameter has been struck through in table above. For the study summaries, please refer to Appendix 2. Implementation of the new data into the risk assessment is discussed in point 9.5.1.1 below.

In order to further refine the risk for algae, two pulsed-exposure studies were provided. The studies were evaluated by the zRMS, however they were considered not acceptable for the risk assessment purposes as due to the selected exposure regime, calculation of ECx values was not possible. Potentially, the NOEC values expressed in term of the peak concentration could be used instead of EC_{10} recommended by EFSA Supporting publication 2019:EN-1673, but from study by Liedtke (2013d) NOEC value could be calculated only for two last pulses, while no NOEC could be calculated for pulses 1 and 2 due to effects >50%. In the study by Liedtke (2013e) NOEC values were not calculated at all. Summaries of both studies together with their detailed evaluation by the zRMS may be found in Appendix 2.

The Applicant provided also additional pulsed-exposure studies on effects of flurochloridone trans-isomer on *Lemna gibba* and *Myriophyllum spicatum*. However, neither of the studies was considered by the Applicant in the presented risk assessment, as acceptable risk could be concluded based on Tier 1 endpoint and Step 3/4 exposure data. Taking this into account, the new active substance studies were not necessary to address the risk and were thus not evaluated, in line with of indications of SANCO/10326/2004, rev. 8 (2012). Results of these studies are struck through in Table 9.5-1.

[4] European Food Safety Authority (2013): Scientific Opinion - Guidance Document on tiered risk assessment for plant protection products for aquatic organisms in edge-of-field surface waters. European Food Safety Authority (EFSA), Parma, Italy; EFSA Journal 11(7): 3290

Table 9.5-2: Endpoints and effect values relevant for the risk assessment for aquatic organisms – AG-F8-250 CS

Species	Substance	Exposure System	Results	Reference
S. gairdneri	AG-F8-250 CS (CS formulation containing 250 g a.s./L)	96 h, s [a)]	LC_{50} = 4.13 mg a.s./L	DAR, 2006 (Smith 1989)
Daphnia magna	AG-F8-250 CS (CS formulation containing 250 g a.s./L)	48 h	EC_{50} = 37 mg a.s./L	EFSA Scientific Report 2010 (Bätscher 2007c)
S. capricornutum	AG-F8-250 CS CS formulation containing 250 g a.s./L	96 h, s	E_rC_{50} = 0.044 mg a.s. /L [b)] E_bC_{50} = 0.02 mg a.s./L [b)]	EFSA Scientific Report 2010 (Smyth et al. 1990)

s: static; ss: semi-static; f: flow-through; nom: based on nominal concentrations; mm: based on mean measured concentrations

[a)] as presented in the EU DAR; not provided in the list of endpoints

[b)] it is noted that the values erroneously presented in the List of Endpoints deviate from the correct data as presented in the DAR as well as in the final addendum. The correct E_bC_{50} and E_rC_{50} values as recalculated by the rapporteur based on mean measured concentrations are 0.109 and 0.197 mg a.s./L.

No product data are available for aquatic macrophytes. However, as the data for the other organism groups indicate, the formulated product is of equivalent or even lower toxicity than the active substance. Therefore, product data on the CS formulation were not considered to be necessary and assessments are based on the EU agreed data from testing with the technical active.

zRMS comments:

Data on toxicity of the formulated product (Flurochloridone 25 CS) to fish, aquatic invertebrates and algae provided in Table 9.5-2 are in line with EU agreed endpoints reported in EFSA Journal 2010;8(12):1869.

Based on information available in the DAR (February 2006), it is confirmed that the endpoints for algae should be 0.109 and 0.197 mg a.s./L for E_bC_{50} and E_rC_{50}, respectively

The studies were performed with the representative formulation Racer 25 CS (Flurochloridone 25 CS). As already mentioned in the introductory part of this document, there are only minor differences between the composition of the representative formulation and current composition of AG-F8-250 CS (Racer 250 CS) and for this reason endpoints derived at the EU level may be used in support of evaluation of the current formulation.

9.5.1.1 Justification for new endpoints

In addition to the data evaluated during EU review of the active substance flurochloridone the applicant has conducted further Annex II studies with the active substance flurochloridone (technical grade or isomers) on *Daphnia magna* (confirmatory data) and algal species (aiding higher tier risk assessments).

Risk assessments for the active substance are conducted taking into consideration the respective EU endpoints. Risk assessments are based on the lowest available endpoints as expressed in active substance concentration.

No assessments based on data for the formulated product are considered necessary. Assessments are covered based on the lower endpoints from testing with the technical active.

Initial (lower tier) assessments are based on the lowest EU agreed endpoints. For higher tier assessments, the new data on the active substance provided by the applicant are taken into account.

A new chronic daphnid study was conducted as confirmatory data. However, as the endpoint of this new study is very similar to the EU endpoint and does not modify the conclusion of the evaluation, the risk assessments are based on the EU agreed endpoint.

Risk assessments for the sediment-dwelling midge *Chironomus riparius* are presented despite the fact, that

the study was only accepted as additional information. The study was conducted with a batch containing 99% (w/w) of the trans-isomer. However, the endpoint is in the same order of magnitude as the endpoints for fish and daphnids, which are much less sensitive in comparison to algae and aquatic macrophytes. Therefore, it was argued that the data for fish and daphnids will also cover the potential risk to sediment dwellers.

In addition, at least for algae and aquatic macrophytes, the trans-isomer indicates a higher toxicity. Therefore, the data for *Chironomus* based on this isomer can be considered to likely be also protective for the technical grade substance.

The data on the different isomers are not taken into account for lower tier assessments. Technical flurochloridone is considered to be most important, as it contains a mixture of the isomers as in the initial product with a higher proportion of the trans-isomer for which a higher toxicity was indicated in the studies on algae and *Lemna*. Furthermore, the endpoints for the two isomers are within an order of magnitude (i.e. the results deviate by less than a factor of 10). Finally, higher tier data on algae have been conservatively conducted with the trans-isomer which allows it to address the isomer issue at the higher tier level.

Risk assessments for primary producers (i.e. algae and aquatic macrophytes) are in line with recent guidance preferably based on growth-rate based endpoints (E_rC_{50}).

Species Sensitivity Distribution

A series of single species tests on different algae (n = 8) were performed with technical flurochloridone allowing for a Species Sensitivity Distribution.

A Species Sensitivity Distribution (SSD) assessment based on the ER_{50} estimates was performed by calculating normal distribution of the data sets and plotting 'Fraction affected' against ´log10 Toxicity data´ using ETX 2.0 software[5]. In line with most recent guidance (EFSA 2013)[6] the E_rC_{50} estimates were used as the preferred observational endpoint.

Data from eight algae were considered (see table below).

Multiple single species toxicity data for algae

Test item	Test species (n = 10)	E_rC_{50} [µg a.s./L]	Reference
flurochloridone	*S. subspicatus*	4.7 [a)]	Bätscher 2004a
	A. flos-aquae	13400 [b)]	Wallace and Swarbrick 2001
	C. rheinhardtii	> 25 [c)]	Lietdke 2013a
	C. vulgaris	14.3	Lietdke 2013b
	N. pelliculosa	12	Lietdke 2013c
	P. subcapitata	2.42	Scheerbaum 2013a
	N. communis	4.45	Scheerbaum 2013b
	S. leopoliensis	4.07	Scheerbaum 2013c
	Chromulina sp.	23.3	Scheerbaum 2013d
	A. falcatus	0.918	Scheerbaum 2013e

[a)] corresponding to the EU agreed and worst-case E_rC_{50} for this species
[b)] due to the exceptional insensitivity of *Anabaena*, the endpoint for this species is not taken into account for the SSD
[c)] endpoint not considered as it corresponds to a limit concentration and as the study does not fulfil all validity criteria

The EC_{50} for *Anabaena* was not taken into account. This species proved to be exceptionally insensitive and is therefore considered as an outlier and not included in the SSD. The E_rC_{50} for *Chlamydomonas* was likewise not considered as this is a limit concentration endpoint. Furthermore, the zonal rapporteur did not accept the endpoint from this study as the validity criterion for section-by-section growth rates was slightly

[5] ETX 2.0 – A Program to Calculate Hazardous Concentrations and Fraction Affected Based on Normally Distributed Toxicity Data, P.L.A. van Vlaardingen, T.P. Traas, A.M. Wintersen & T. Aldenberg, RIVM Report 601501028/2004
[6] European Commission. Health & Consumer Protection Directorat – General (2002). Draft Working Document. Guidance Document on Terrestrial Ecotoxicology Under Council Directive 91/414/EEC. SANCO/10329/2002 rev

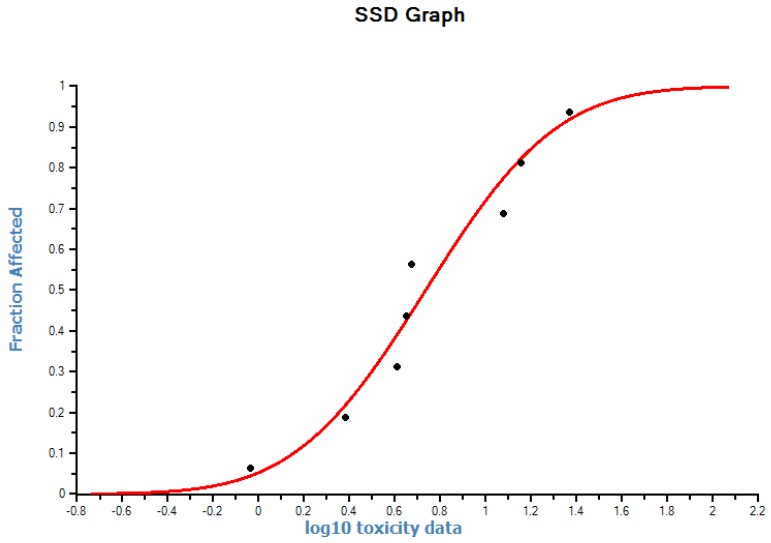
breached in the study. However, the applicant is of the opinion that the validity criteria are applicable for standard test species and that the results of the available test with a slight deviation from the upper limit for the coefficient of variation of section-by-section growth rates (39.7% vs. a maximum of 35%) should be acceptable for a non-standard test species. Anyhow, the study on *Chlamydomonas* is considered as valuable qualitative information supporting the risk assessment by further reducing the uncertainties in regard to inter-species variability of sensitivities. Furthermore, the study including a recovery period corroborates a weight-of-evidence based on the recovery potential of algae (see also below).

SSD over ER_{50} from the relevant growth inhibition data for algae

Parameter:	**E_rR_{50} (n = 8) [µg a.s./L]**
Goodness of fit of toxicity data (normal distribution)	
Anderson-Darling test for normality	Accepted [a)]
Kolmogorov-Smirnov test for normality	Accepted [a)]
Cramer von Mises test for normality	Accepted [a)]
Median HC_5	**0.90**
95% confidence limits	0.19 – 2.00
Mean log toxicity	0.73
Standard deviation	0.45

[a)] acceptable normal distribution at 1% significance level

Thus, the data fulfil the criterion for normal distribution even at the lowest significance level and in accordance with all tests for normality.



Graph 1: SSD over E_rC_{50} from the relevant growth inhibition data for technical flurochloridone

It is noted that the median HC_5 estimate of 0.90 µg a.s./L is comparable to (or even lower than) the most sensitive species of the tested algae (i.e. *A. falcatus* with a lowest E_rR_{50} of 0.918 µg a.s./L) supporting the conclusion that the HC_5 is sufficiently protective for the community of algae.

Thus, in addition to the deterministic approach based on the overall lowest ER_{50}, in a higher tier approach, the exposure estimates are related to the endpoint (median HC_5) from the SSD.

In agreement with the Scientific Opinion of EFSA on the aquatic guidance (2013)[7], an assessment factor of 3 is used in connection with the median HC_5 estimate. Based on this assumption, the resulting Regulatory Acceptable Concentration (**RAC**) would be **0.30 µg a.s./L**. This RAC is lower than the lower tier toxicity estimate for *Scenedesmus* (*Desmodesmus*) *subspicatus* of 4.7 µg a.s./L using a TER risk assessment trigger of 10, identifying this species as a sensitive representative for algae and providing evidence that assessments based on the EU agreed endpoint in combination with the standard assessment factor of 10 is overly conservative.

~~As a further line of evidence supportive of the conclusions drawn in regard to relevant toxicity estimates from the Species Sensitivity Distributions is the calculation of geometric means from the available SSD. The geometric mean is proposed by EFSA (2013) in connection with the standard assessment factor (i.e. 10 in case of algae) for data on less than 8 tested species.~~

~~Building the geometric mean over the E_rC_{50} (n = 8) results in a toxicity endpoint of 5.43 µg a.s./L. This endpoint would translate in a Regulatory Acceptable Concentrations (RAC) of 0.54 µg a.s./L. Accordingly, the assessments based on the median hazardous concentrations for 5% of the species are more conservative than the geometric mean approach. If additionally including the data for *Anabaena* and *Chlamydomonas* (n = 10), the geometric mean E_rC_{50} of > 13.8 µg a.s./L would even calculate to an **RAC of > 1.38 µg a.s./L**. This is supportive of the assumption that the SDD-RAC is overly conservative. It is noted that the geometric mean (unlike the mean) accounts for the distribution of data (i.e. the comparatively high endpoint for *Anabaena* gets less weight) and that unbound endpoints can be considered in context with the geometric mean approach.~~

~~Therefore, as additional line of evidence, alternative assessments are presented based on the geometric mean RAC of > 1.38 µg a.s./L.~~

It is noted that the Applicant understands the Guidance regarding the different approaches based on multiple-single species testing as recommendations which is suggested by the wording in the EFSA document. Thus, the Guidance states that ´if more data […] are available, the Geomean approach could still be applied, but it is recommended to preferably apply the SSD approach´. It is understood that the SSD approach is preferred and uncertainties in connection with probabilistic risk assessment approaches decrease in dependency of the number of species and variety of taxa tested.

It is likewise understood that a standard SSD approach does not support the inclusion of limit concentrations and furthermore, information on distinctly less-sensitive taxa within the overall distribution is not accounted for if these are not within the normal distribution.

This is why the applicant proposes to consider the geometric mean approach as an additional line of evidence. The applicant is of the opinion that the generation of sufficient data principally allowing for an SSD should not result in a bias towards more risk as compared to the geometric mean approach merely through the omittance of additional data not fitting within the distribution of a subset of data, which is a view also supported by the EFSA Guidance document (see below). Thus, it is acknowledged that an increase in number of species tested results in a decrease in uncertainties regarding inter-species variability in sensitivity towards the exposure to the contaminant. As per the proposed scheme, at least 8 endpoints are required in order to justify the application of the SSD approach in context with primary producers, whereas at a lower number the geometric mean approach is in principal applicable. However, the number of test data available as a discriminator by ignoring the nature of data is rather arbitrary even if it might be informed on typical data distributions and variabilities in sensitivities.

This is evidenced also by the reduced requirement of only five data points as a minimum for aquatic vertebrates which is explained by the lower assumed variability within this organism group and due to animal welfare reasons. In any case, this implies that the data distribution should also be considered for decisions on the numbers of endpoints required to either apply the geometric mean or SSD approach or

[7] European Food Safety Authority (2013): Scientific Opinion - Guidance Document on tiered risk assessment for plant protection products for aquatic organisms in edge-of-field surface waters. European Food Safety Authority (EFSA), Parma, Italy; EFSA Journal 11(7): 3290

more generally, that the threshold level for the decision on the appropriate approach (Geomean vs. SSD) is equivocal.

Strictly following the proposed criteria and accepting an inferior data basis for the evaluations using geometric means must be interpreted to be related to a higher level of uncertainty and is likely to give a bias towards more favourable risk evaluation outcomes (as shown below for the actual data). Consequently, the geometric mean approach and the entire data set available (e.g. also including less-sensitive species not fitting within a normal data distribution as well as unbound endpoints) should not be disregarded in the overall risk characterisation as an additional line of evidence.

Evaluating data using both alternative approaches and comparing the resulting RACs is a valuable and more comprehensive measure to inform on the protectiveness of the data available and the uncertainties connected with the risk assessments and setting this into context with lower tier data provides further information. To this effect, the proposal by EFSA to compare RACs following different assessment tiers and comparing Tier 1-RAC, Geomean- or SSD-RAC as well as Tier 3-RAC in the Applicant´s opinion must be interpreted to be valid irrespective of the organism group of concern. The Assessment Factors to be considered are related to the level of (un)certainty. It is acknowledged that a range of AFs is proposed and that among other discriminators, the relative outcome of refinement approaches should be considered to decide on an appropriate AF only in case of aquatic invertebrates and that no such clear suggestion was made in context with other organism groups where assessment factors are less variable. However, it is not comprehensible that RACs derived from increasing tiers should not be lower only in case of invertebrates.

In this context, and likewise deviating from the proposed assessment scheme by EFSA, there is precedents of a more flexible interpretation of the aquatic guidance document (EFSA 2013). For example, for model ecosystem endpoints, the EFSA guidance proposes assessment factors of 2-3 for the Ecological Threshold Option (ETO). It is likewise clearly stated that if more than one study is available, that the most appropriate ´cosm´ should be used for RAC derivation, whereas further reduction of the AF is not encouraged by the Guidance document. Regardless of this and due to the consistency of results between different mesocosms, an AF of 1 was agreed on for the active substance pendimethalin (EFSA 2016[i]). Notably, in this case the Tier 1 RAC for the most sensitive organism group is more favourable than the higher tier endpoint. This example illustrates how a more extensive data basis effectively has reduced uncertainties and that it is justified to make reasoned decisions that might deviate from the standard procedures accounting for all information available.

In line with this argumentation, the proposed ´weight-of-evidence´ is an emphasis on the reduced uncertainty based on the extensive data basis considering all available data as well as the different assessment approaches which in combination are considered to indicate acceptable risk.

It is obvious that the omission of individual data from the data set would lead to different results in the geometric mean as well as SSD assessments and that with increasing numbers of tested species the uncertainty declines that the RAC would not be overprotective.

As presented in the following Table, if only 7 of the species would have been tested, the geometric mean endpoints would have been in a range of > 4.41 (selecting only the 7 most sensitive species within the distribution and by applying the standard assessment factor of 10) to > 24.11 µg a.s./L (based on the 7 least sensitive species) resulting in geometric mean RAC estimates within the range of > 0.44 to > 2.41 µg a.s./L, respectively. That means that all possible outcomes of a less extensive testing strategy within the factually tested species would have resulted in an RAC that is distinctly more favourable than the SSD-RAC of 0.30 µg a.s./L and towards the higher end of the range even well above the predicted concentrations, indicating acceptable risk. Even the lowest value in the range is still higher than the SSD-RAC of 0.30 µg a.s./L.

Multiple single species toxicity data for algae and Tier 2B RAC

Test item	Test species (n = 10)	E_rC_{50} [µg a.s./L]	Reference	Geometric mean E_rC_{50}/RAC [µg a.s./L] (AF = 10)				SSD median HC_5/RAC (AF = 3)
Fluro-chloridone	*A. falcatus*	0.918	Scheerbaum 2013e	> 4.41 (n = 7) **RAC > 0.44**	> 5.43 (n = 8) **RAC > 0.54**	Lowest endpoints not considered	> 13.8 (n = 10) **RAC > 1.38**	0.90 (n = 8) **RAC = 0.30**
	P. subcapitata	2.42	Scheerbaum 2013a			> 24.11 (n = 7) **RAC > 2.41**		
	S. leopoliensis	4.07	Scheerbaum 2013c					
	N. communis	4.45	Scheerbaum 2013b					
	S. subspicatus	4.7 [a]	Bätscher 2004a					
	N. pelliculosa	12	Lietdke 2013c					
	C. vulgaris	14.3	Lietdke 2013b					
	Chromulina sp.	23.3	Scheerbaum 2013d	Highest endpoints not considered	Not considered			Not considered
	C. rheinhardtii	> 25 [b]	Lietdke 2013a					
	A. flos-aquae	13400 [c]	Wallace and Swarbrick 2001					

[a] corresponding to the EU agreed and worst-case E_rC_{50} for this species
[b] endpoint not considered as it corresponds to a limit concentration and as the study does not fulfil all validity criteria
[c] due to the exceptional insensitivity of *Anabaena*, the endpoint for this species is not taken into account for the SSD

Accepting that ´the size of the AF should ideally not result in an SSD-$RAC_{sw;\ ac}$ higher than the Tier 3 RAC derived from effect class 1 and 2 of micro-/ mesocosm studies, nor should it result in an SSD-$RAC_{sw;\ ac}$ lower than the Tier 1 $RAC_{sw;\ ac}$ on the basis of standard test species and/or Geomean-$RAC_{sw;\ ac}$ […] on the basis of the same toxicity data that were used to construct the SSD´, this would suggest that the SSD-RAC should be greater than 0.54 µg a.s./L. The median HC_5 from the SSD was calculated to be 0.90 µg a.s./L. An adapted AF of 2 would still generate an RAC lower than the geometric mean RAC based on the same data set (i.e. 0.45 vs. 0.54 µg a.s./L). If, accordingly, an AF of 1 is applied, the resulting SSD-RAC equals the median HC_5 of 0.90 µg a.s./L. It is also noted that the standard (Tier 1) test species (i.e. the green algae *Scenedesmus subspicatus*) with an E_rC_{50} of 4.7 µg a.s./L translates into an RAC of 0.47 µg a.s./L based on the standard assessment factor of 10 which in turn covers species sensitivity differences within the SSD distribution. The fact that this Tier 1 RAC is still greater than the SSD-RAC likewise supports the conclusion that the SSD based on an assessment factor of 2 or 3 would still overestimate the risk.

The Applicant is of the opinion, that the presented geometric mean RAC of > 1.38 µg a.s./L, should be taken into consideration for an overall weight-of-evidence-based risk characterisation, additionally accounting for the ´recovery potential´ as well as Area Under the Curve (see below). Based on this RAC, an acceptable risk is indicated for all FOCUS R scenarios at a vegetated buffer distance of 20 m and based on standard FOCUS modelling (with the exception of R3 stream if based on standard modelling as provided for the zonal evaluation) and for all relevant scenarios based on VFSmod modelling if a 10 m vegetated buffer distance is accounted for in case of the scenarios relevant in a national context (see risk assessments below).

However, even the SSD-RAC based on an adjusted AF of 1 (0.9 µg a.s./L) fulfilling the prerequisite to be greater than the Geomean-RAC based on the same data set results in acceptable risk indication for the relevant national context (reference is made to the risk assessments based on the ´adapted SSD-RAC). It is emphasized that the SSD-RAC is still considered to be reasonably conservative as it was generated omitting less-sensitive taxa. Besides, the E_rC_{50} for the most sensitive species (i.e. *Ankistrodesmus falcatus*) of 0.918 µg a.s./L is still greater than the adjusted SSD-RACs giving further confidence in the protectiveness of the revised risk assessment approach.

zRMS comments:

First of all it is noted that the geomean approach is not an alternative for HC_5 approach, but is relevant in case data for less than 8 species are available (in case of primary producers). In case data for ≥8 species are available, the HC_5 approach is considered to be more relevant. As for flurochloridone data for 10 species are available with 8 endpoints appropriate for construction of SSD, the geomean approach is not relevant and it thus struck through in the text above.

The SSD approach taken by the Applicant is considered acceptable.

The zRMS agrees with exclusion of the endpoint for *A. flos-aquae*, as in line with indication of the guidance, inclusion of obviously insensitive species may lead to underestimation of the risk.

Exclusion of endpoint for *C. reinhardtii* leads to more conservative approach. Nevertheless, the zRMS is of the opinion that slightly exceeded CV for daily section-by-section specific growth rates (39.7% vs. 35% required by OECD 201) should not be the reason for invalidation of the study , especially two remaining validity criteria were met and it should be kept in mind that they were validated for the standard test species and not additional species not even mentioned in the test guideline. Nevertheless, although the study was not invalidated by the zRMS due to single validity criterion being breached, the study was considered to be not fully reliable due to analytical measurements being performed only for two highest test concentrations. For this reason the study was considered to be relevant as additional information only. For details of the zRMS evaluation of the study, please refer to the comments to the study presented in Appendix 2, KCP 10.2.3/01.

The calculations presented above were checked by the zRMS using ETX 2 tool and obtained results are confirmed with median HC_5 of 0.9 µg a.s./L, leading to RAC of 0.3 µg a.s./L (AF of 3).

Comments on additional information provided by the Applicant (31.08.2021):

After analysis of additional arguments provided by the Applicant the zRMS agrees that consideration of the SSD approach in case of flurochloridone may be not fully appropriate. Actually, it is in opposition to the tiered approach which should be most conservative at Tier 1 and then the conservatism should reasonable decrease at higher tiers due to more extensive testing reducing the uncertainty over the results of the Tier 1 studies. It is quite outstanding situation that higher tier RAC derived with consideration of additional species is actually lower than RAC derived at Tier 1. In case of flurochloridone this could be due to testing of species much more sensitive than standard species, nevertheless, the risk assessment based on SSD-RAC should not result with higher PEC/RAC values comparing to the risk assessment based on Tier 1 data.
Furthermore, as noted by the Applicant above, the following is stated in EFSA (2013):

> *The size of the AF should ideally not result in an SSD-RACsw;ac higher than the tier 3 RAC derived from effect class 1 and 2 of micro-/mesocosms studies nor lower than the tier 1 RACsw;ac on the basis of standard test species and/or the Geomean-RACsw;ac and/or method 3 to 5 (EFSA, 2006a) on the basis of the same toxicity data that were used to construct the SSD.*

Although provided above indications are relevant for invertebrates, the zRMS is of the opinion that they should be equally applicable for all RAC values derived on the basis of the SSD approach. In case of flurochloridone SSD-RAC of 0.30 µg a.s.L is lower than both, Tier 1 RAC and Geomean-RAC, which confirms that its applicability in case of the risk assessment for flurochloridone is questionable.

Taking this into account the zRMS agrees that the risk assessment for algae should be performed with consideration of the Geomean-RAC. It is, however, noted that the Geomean-RAC of 1.38 µg a.s.L based on endpoints available for all algae species tested is higher than the lowest E_rC_{50} of 0.918 µg/L derived for *A. falcatus*, while according to EFSA (2013):

> *If the most sensitive species is more than a factor of 10 (for plants and chronic tests) or 100 (for acute invertebrate and fish test) below the geometric mean of all the tested species, a weight of evidence approach should be applied.*
> *[...]*
> *If the lowest toxicity value is higher than the Geom-RAC value, it is acceptable to use the Geomean approach, otherwise a weight of evidence approach should be applied.*

In opinion of the zRMS before WoE approach is applied, the toxicity data should be analysed in order to exclude insensitive species to obtain unbiased geometric mean value. From information provided in table above it is evident that *Anabaena flos-aquae* was not sensitive to flurochloridone with and endpoint more than 14000 higher than the most sensitive species and more than 500 times higher than the second least sensitive species. Taking this into account the zRMS is of the opinion that endpoint for *A. flos-aquae* should be excluded from calculation of the geometric mean E_rC_{50}. Without this species the geometric mean E_rC_{50} of 6.43 µg a.s./L may be calculated resulting with Geomean-RAC of 0.643 µg a.s./L. This value is considered sufficiently protective since it is lower than endpoint for the most sensitive species.

As the zRMS is not in favour of consideration of different assessment factors than these indicated in the guidance document, the proposal of the Applicant to consider SSD approach with modified AF will not be considered further.

Algal recovery potential

In addition to the 72-hour exposure period, in eight algal tests, recovery periods have been included. In all of these tests, algal recovery was observed indicating that the growth inhibiting effect is reversible; i.e. the active substance has an algistatic but no algicidal effect. The highest rate at which recovery was observed is regarded as ecologically relevant No Observed Adverse Effect Concentration (NOAECrecovery).

The results of these tests for 8 algal species are summarized in the next table.

Recovery potential of algae from multiple single species tests

Test species	Highest concentration tested [µg a.s./L]	ErC50 [µg a.s./L]	EyC50 [µg a.s./L]	NOEC [µg a.s./L]	NOAECrecovery [µg a.s./L]	Test concentration [µg a.s./L]	Recovery period [d]
D. subspicatus	30.9	4.96	1.14	0.393	**30.9**	30.9	7
						8.42	7
						3.28	5
C. reinhardtii	25	> 25	17	7.7	**25**	25	3
N. pelliculosa	187	12	3.4	1.5	**187**	187	9
						51	9
						15	9
						4.7	6
P. subcapitata	10.0	2.42	1.30	0.320 (yield) 1.00 (growth rate)	**10.0**	10.0	7
						3.20	4
N. communis	100	4.45	2.50	1.00	**14.4**	100	8
						31.6	5
						3.16 – 10.0	4
S. leopoliensis	14.4	4.07	2.22	0.946	**14.4**	14.4	7
						7.81	6
						3.13	4
C. nebulosa	100	23.3	18.5	12.5	**100**	100	5
						50.0	4
						25.0	4
A. falcatus	10	0.918	0.516	0.320	**10**	10	8
						3.2	8
						0.320 – 1.00	4

In conclusion, in all single species tests, recovery of algal growth was observed within 3 to 9 days for the highest concentration tested, which in all cases is significantly greater than the worst case maximum PECSW based on FOCUS Step 3 modelling.

In order to make use of the extensive recovery data set available, refined risk assessments are presented based on the overall lowest NOAECrecovery for *Ankistrodesmus falcatus* and *Pseudokirchneriella subcapitata* as an additional line of evidence. As the **NOAEC of 10 µg a.s./L** was the highest concentration tested for the most sensitive species and as for other species recovery was observed at even much higher concentrations, it is considered justified to directly translate the NOAEC into an RAC (i.e. an assessment factor of 1 is assumed).

The assessments based on recovery potential as presented below are intended as additional line of evidence making use of an extensive data set showing that algae are able to recover from short-term effects on growth.

The recovery of test item in the multiple single species tests was within a range of 80 to 120% in which case results are based on nominal values or results were based on mean measured concentrations. As the

effects on algal growth are reversible within 3 to 9 days following exposure (i.e. there is no algicidal effect) and, it can be assumed that the growth inhibition is dependent on the function of exposure over time.

In order to show the time-dependence of effects in the algae studies, the table below presents the increase of biomass or cell density inhibitions over time for the concentration level above the EC50 for the different algae species.

Increase of inhibiting effect over time in single species algae studies for test concentration above EyC50

Reference	Test species	EyC50 [µg a.s./L]	Test concentration [µg a.s./L]	Initial Biomass/ Density a)	Biomass/Density a) of controls (mean)			Biomass/Density a) at test concentration (mean)			Inhibition compared to controls [%] b)		
				0h	24h	48h	72h	24h	48h	72h	0-24h	0-48h	0-72h
Lietdke 2013a	*C. reinhardtii*	17	25 (m)	1.37	3.6	29.7	207.6	3.5	16.1	52.0	4.5	48.0	75.4
Lietdke 2013b	*C. vulgaris*	3.9	4.6 (m)	0.14	2.1	13.1	49.2	1.8	7.3	18.5	15.3	44.8	62.6
Lietdke 2013c	*N. pelliculosa*	3.4	4.7 (m)	1.0	2.6	7.9	40.6	2.5	4.9	8.1	6.3	43.5	82.1
Scheerbaum 2013a	*P. subcapitata*	1.3	3.2 (n)	5089	27747	252080	1919535	14527	23316	26246	58.3	92.6	98.9
Scheerbaum 2013b	*N. communis*	2.5	3.16 (n)	9569	31291	131281	343248	24566	43174	116477	31.0	72.4	68.0
Scheerbaum 2013c	*S. leopoliensis*	2.22	2.45 (m)	77588	174172	433754	1698782	169944	402137	748042	4.4	8.9	58.6
Scheerbaum 2013d	*C. nebulosa*	18.5	25.0 (n)	9656	21863	48449	194469	27017	32302	33618	-42.2	41.6	87.0
Scheerbaum 2013e	*A. falcatus*	0.516	0.320 (n)	10109	29554	110229	359454	25489	78424	289806	20.9	31.8	19.9

n: nominal concentration; m: measured (mean or geomean) concentration; negative value: increased growth compared to controls
a) biomass as determnined over fluorescence measurement in the studies by Liedtke (as relative fluorescence units (x 103 or x 104) and cell densities based on microscopic counting in the studies by Scheerbaum
b) = 100 – (100/(biomass/density of control – initial biomass/density) * (biomass/density of test concentration – initial biomass/density))

With the exception of the non-standard species *Ankistrodesmus falcatus*, for all other algal species a clear increase of the effect magnitude is observed with exposure time. A higher time-dependent inhibition is also visible for the exposure duration up to 48 hours in case of *Ankistrodesmus*. Therefore, the growth inhibition is considered to be dependent on exposure duration.

Comparison of recovery potential of different species

Due to different test concentrations applied in the multiple single species algal tests with recovery phase, a direct comparison of the recovery potential of different species is difficult. *Desmodesmus subspicatus* is one of the most sensitive species tested (as indicated by an NOEC and E_rC_{50} comparable or deviating by a factor of about 5 only, respectively, from the species providing the overall lowest E_rC_{50}; i.e. *Ankistrodesmus falcatus*). The factor is even lower if comparing $E_{b/y}C_{50}$ (2.1/0.516 µg/L = 4 or 2.1/1.14 µg/L = 2 based on comparison with the EU agreed endpoint or the endpoint from the new study by Wenzel (2015a), respectively).

Algal recovery up to the highest concentrations tested was observed in periods between 3 to 9 days in all species.

The period required for recovery at 3.28 µg a.s./L for *Desmodesmus* was 5 days, whereas at 3.2 µg a.s./L *Ankistrodesmus* required 8 days to recover, suggesting a slower recovery than for *Desmodesmus*. However, at a concentration level of 10 µg a.s./L, the effects were reversible likewise following a recovery period of 8 days for *Ankistrodesmus*, whereas at the slightly lower level of 8.42 µg a.s./L, the recovery period of 7 days for *Desmodesmus* was comparable.

Overall, the differences in recovery potential of the various species tested with reversibility of effects in a matter of days even at exaggerated concentration levels are negligible. *Desmodesmus* as one of the most sensitive species is also conservative in regard to the potential for recovery. Besides, it is noted that weight-

~~of evidence argumentations and higher tier risk assessments based recovery endpoints and based on pulsed exposure studies still include an uncertainty factor accounting for inter-species differences.~~

~~The potential of algae and aquatic plants for recovery as well as the time dependence of effect levels is further corroborated by available pulsed exposure studies (see below). The considerations on recovery of different species above corroborate the use of *Desmodesmus* as reasonable and conservative representative of algal taxa for higher tier studies with revised exposure set-up.~~

The data and assessment approach as presented above (grey text) is considered as supportive for the risk assessment. The comprehensive data package as provided by the applicant provides useful information aiding the overall weight-of-evidence risk characterisation.

The applicant acknowledges that the aquatic guidance (EFSA, 2013) allows for the ERO approach, i.e. allowing for intermittent effects on populations of aquatic non-target organisms only in context with model ecosystem data including indirect effects within the aquatic community.

However, it is noted that population level effects, especially in case of short-lived organism groups (as algae), might be considered in context with the magnitude of effects, effect durations as well as with account of relevant exposure also with regard to ecological relevance.

In connection with mesocosm data, acceptable recovery times (refer to effect Class 3A) are defined to take place within a period of 8 weeks. Compared to such extended inhibitions, short-term effects within the range of hours or days and at concentration levels clearly exceeding predicted exposure levels in the field might not be considered ecologically relevant. Besides, comparable, or even more pronounced changes in cell densities of individual taxa (also in relation to other taxa) can likewise be expected in consequence of short-term deviations in other parameters (as e.g. temperature, nutrient availability, etc.). Especially regarding short-term exposure scenarios (as usually represented by typical FOCUS R stream events), NOAEC data on individual taxa might therefore be considered as appropriate effect endpoints provided that effects are only of short duration. In this sense, intermediate growth inhibition with subsequent ´recovery´ might rather be interpreted in context with natural fluctuations of resilient systems than as relevant effects for the population and community level of these organism groups.

Standard lower-tier algal studies consider the growth inhibition observed over the test duration. The endpoint relevant for risk assessments and defining the Regulatory Acceptable Concentration (RAC) is the median effective concentration resulting in 50% growth rate inhibition for the pre-defined study duration of 72 hours. For Tier 1 risk assessments, the E_rC_{50} is related to maximum peak concentrations from FOCUS modelling accounting for an Assessment Factor (AF) of 10. This assessment scheme does not account for the exposure dynamics (i.e. number, magnitude and duration) apart from the single maximum of the predicted exposure scenarios. Whereas the EFSA guidance principally advocates the Ecotoxicologically Relevant Concentration (ERC) as ´conceptual basis for the interface between exposure and effect assessment´, at Tier 1, exposure duration and predicted exposure patterns are not taken into account. This, and the fact that 50% effect levels are acceptable imply that this is sufficiently addressed by the applied AF. It can, however, be assumed that for exposure scenarios with extended exposure duration (i.e. beyond the standard exposure window of 72 hours), more pronounced effects (or lower EC_X) values are to be expected. The ´recovery´ required to re-establish algal cell densities of the level of the original cell population is not considered, nor are potential latent effects.

In light of the argumentation provided above, the provided data on ´recovery´ might be considered to represent the natural resilience or ecologically acceptable fluctuations in algal cell densities and should be taken into consideration for the overall weight-of-evidence. The NOAEC dependent on species (and tested limit concentrations) was found to be between 10 and 187 µg a.s./L with ´recovery´ observed within the comparatively short time-period of only 4 to 9 days.

The risk assessments based on NOAEC are presented in the risk assessments provided below. An AF of 1 is taken into account in the first instance resulting in an $RAC_{recovery}$ of 10 µg a.,s./L which is considered appropriate as:

- The endpoint represents a No Observed Adverse Effect concentration; i.e. is not based on an effect level of 50%.
- The endpoint represents the overall worst-case from single species testing of 8 species.
- The endpoint represents a limit concentration endpoint; i.e. the highest concentration tested with this species

However, in an alternative and more conservative approach, the risk assessments are also presented for an AF of 10 resulting in a worst-case $RAC_{recovery}$ of 1.0 µg a.s./L and additionally consider risk mitigation buffer distances. In addition to the assessments based on standard FOCUS modelling, alternative assessments are amended applying VFSmod as this is considered acceptable by the RMS for national registration.

In further support of this assessment and the conclusion of an acceptable risk, it is noted that at the RAC of 1.0 µg a.s./L in the 72-hour test with *A. falcatus*, growth inhibition only slightly exceeded the 50% effect level (actual: 55.5% growth rate inhibition); i.e. is close to the effect level usually aligned with the AF of 10. Recovery was observed up to the concentration of 1.0 µg a.s./L within ≤ 4 days (distinct sampling day) only. In line with the argumentation provided above, the respective growth inhibition and re-establishment of growth rates are considered to be within ecologically acceptable cell density fluctuations rather than biologically relevant cell density depression with subsequent ´recovery´ in the strict sense of that term.

As an additional line of evidence in the overall risk characterisation, this assessment approach also gives more confidence in the conclusion of an acceptable risk based on other lines of evidence as presented. Even if this approach is not intended in the EFSA Guidance, the fact that within very short time frames, a number of algae species are shown to be resilient towards intermediate cell density reductions with very rapid reestablishment of population levels is an argument that should be accounted for in context with other lines of evidence, particularly as the observed deviations from control densities are debatably not of ecological relevance.

The Applicant considers this line of argumentation as valid (including the quantitative representation in form of risk assessments) as supportive information, especially in context with the critical FOCUS D4 scenario (as relevant for national registration in the concerned Member State) which even under consideration of the lower tier RAC of 0.47 µg a.s./L only marginally fails the assessment trigger of 1 for acceptability of risk (see risk assessments below).

zRMS comments:

In line with current approach in aquatic risk assessment (at all, EU, zonal and national level), recovery is not an option for refinement of the risk, as in the field it would be influenced by the relationship with other species which cannot be accounted for in studies performed with single species, where conditions for recovery are more favourable. In addition to that, under field conditions the organisms are exposed to multiple stressors, including other pesticides, which is difficult to be accounted for even in microcosm/mesocosm studies and not possible to be considered in single species laboratory studies. Taking this into account, the recovery potential is not considered in refinement of the risk and the text above is struck through.

Comments on additional information provided by the Applicant (31.08.2021):

Although the Applicant provided sound justification regarding the recovery potential of algae exposed to flurochloridone, the zRMS maintains its position that recovery is not an option for refinement of the risk for reasons already indicated in the comment above.

It should be noted that zRMS approach is fully in line with requirements in area of aquatic risk assessment relevant for Poland (see list of national agreements of October 2019 available on the website of the Polish Ministry of Agriculture and Rural Development) as well as with conclusions of the Pesticides Peer Review Meeting on general recurring issues in ecotoxicology (point 4.5 of EFSA Supporting publication 2019:EN-1673).

Risk assessment based on recovery will not be considered.

Pulsed exposure studies with algae and aquatic macrophytes

Finally, pulsed exposure studies have been conducted with the trans-isomer of flurochloridone. This isomer was found to be more toxic than the cis-isomer in comparative studies. As the product contains about 3:1 mixture of the trans- and cis-isomer, respectively, testing with the trans-isomer is considered to be a conservative approach.

Algae (i.e. *Desmodesmus subspicatus*) were exposed to two generic exposure patterns argued to be protective of the predicted realistic course of FOCUS exposure concentrations including an assessment factor. In both studies, algae were observed for the potential for recovery following the pulsed exposure period.

Furthermore, the aquatic macrophytes *Lemna minor* and *Myriophyllum spicatum* were tested in one study each with exposure to a series of pulses simulating a generic exposure pattern.

The algae and by Liedtke (2013d and 2013e; Reference codes: 90015421 and 90015432) and macrophyte studies by Wenzel (2015b and 2015c; Reference codes: 90016482 and 90016483) are summarized in more detail in the Appendix below).

The measured course of concentrations over time in the effect studies multiplied by the assessment factor (AF = 10) generates an Environmental Trigger Curve (ETC). This ETC is subsequently can be compared with the predicted FOCUS exposure patterns. An acceptable risk is indicated in case the predicted environmental concentrations over time are below the ETC as tested in the studies.

Two concentration curves have been tested for algae and one for aquatic macrophytes (*Lemna* and *Myriophyllum*). No further refinement is required for aquatic macrophytes where an acceptable risk is presented based on standard toxicity data (see below). The studies summarized in the Appendix, however, are provided as additional information.

The two pulsed exposure studies on algae including recovery phases have been conducted on unicellular green algae (*Scenedesmus subspicatus*), that proved to be most sensitive among a series of tested algae in multiple single species tests and are therefore considered to be protective for algal communities. As complete recovery of growth rates was observed in these studies, the exposure profiles established by measured concentrations were defined as No Observed Adverse Exposure Patterns (NOAEPs).

A detailed comparison of the generic patterns in the effect studies on algae with the relevant FOCUS exposure scenarios are provided by Ranke and Eck (2018; Reference number: 000100958; KCP 10.2.3/14) by means of superposition of exposure in the studies accounting for an additional assessment factor with predicted exposure. Finally, for risk assessments Toxicity/Exposure Ratios (TERs) are calculated based on Areas Under the Curve (AUC) of exposure for relevant FOCUS scenarios.

The expert statement presenting a detailed comparison of the ETC with the predicted FOCUS exposure pattern for the intended use and all relevant FOCUS scenarios and TER calculations is provided along with this submission and is briefly summarized in the Appendix (see under Point KCP 10.2.3 below). Refer also to higher tier risk assessments for algae presented below under Point 9.5.2.

It is acknowledged that the pulsed exposure studies by Liedtke (2013d/e) do not formally fulfil validity criteria and do not provide standard EC_x values to perform risk assessments based on the 50% effect level.

However, it is noted that the studies with pulsed exposure provided are higher tier data with a design significantly deviating from standard Tier 1 studies for which the validity criteria for acceptable testing are defined. Therefore, the Applicant insists, that the criteria are not applicable to the higher tier revised exposure testing.

As in accordance with EFSA guidance, for Tier 2C refinements (i.e. higher tier effect data from revised exposure testing), the principal idea is to generate alternative EC_X (i.e. E_rC_{50}) values to be used for revised risk assessments. This, of course, would require concentration-response testing.

The EFSA Guidance (2013) states that ´higher tier effect assessments do not necessarily need to be performed by simulating constant exposure normally used in standardised lower tier tests but may address the exposure regimes predicted for edge-of-field surface waters´. ´For the derivation of a chronic RAC by means of refined chronic toxicity tests it is proposed to apply an AF of 10 […] to the EC_{50} for plants […; note by the Applicant: this includes algae] under the conditions that the (repeated pulsed) exposure regime […] is realistic to worst case when compared with the relevant predicted (modelled) field exposure profile. […]. The duration of the test is long enough to allow the observation of delayed effects, the refined chronic RAC is compared with the $PEC_{sw, max.}$´.

In deviation to this approach, the studies by Liedtke (2013d/e) were performed at individual exposure patterns which were intended to be protective for a series of predicted FOCUS patterns allowing for an additional Assessment Factor (AF). Accordingly, the tests represent a simulation of worst-case field exposure patterns. Environmental Trigger Curves (ETCs) were generated by accounting for an AF by division of the experimental exposure profile by an appropriate, or even overly conservative factor. As the course of exposure in the ecotoxicological experiments over the respective study duration reflects an NO(A)EC-pattern (or NOAEP) and as additionally AF was considered, this approach is distinctly more conservative as compared to risk assessments based on revised E_rC_{50}. It also directly links the exposure in the field with the exposure in ecotoxicological testing ideally matching the ERC concept.

It also is acknowledged that the pulsed-exposure experiments were not performed with the most sensitive species as informed by multiple-single species testing where *Ankistrodesmus falcatus* turned out to be the most sensitive test species.

With reference to the argumentation provided above (`Algal recovery potential´), the Applicant is of the opinion that the comparable ´recovery´ is still a valid justification for the use of *Desmodesmus* as test species for refined exposure testing. However, and independent of the resilience of the test species, both species differ only by a factor of about 5.1 with regard to median effective concentrations (E_rC_{50} of 4.7 vs. 0.918 µg a.s./L for *D. subspicatus* and *A. falcatus*, respectively) and are therefore in the same order of magnitude regarding sensitivity. *Desmodesmus* actually was among the most sensitive genera tested.

Therefore, and with reference to the conservative Assessment Factor applied which allows for inter-species extrapolation, *Desmodesmus* is considered to be an appropriate test species for revised exposure testing with sufficient protectiveness for the other test species.

Weight-of-evidence risk characterisation

The Applicant is of the opinion that while individual arguments provided in the assessment are connected with some level of uncertainty, that the overall weight-of-evidence indicates safe use of the product when applied in accordance with the intended GAP and the zonal evaluation should account for the combination of lines of evidence provided in connection with the risk mitigation measures proposed.

The overall weight-of evidence is based on:

- The ´recovery´ potential of algae
- Probabilistic risk assessments accounting for a weight-of-evidence based on Tier 1-/SSD- and geometric mean endpoints (Tier 2A/B)
- Tier 2C

zRMS comments:

Consideration of only trans-isomer in higher tier studies is considered acceptable, as this isomer has been demonstrated to be of much higher toxicity comparing to the cis-isomer of flurochloridone.

The position paper by Ranke & Eck (2018) has been evaluated by the zRMS and the general concept of comparison of exposure profiles in FOCUS scenarios and exposure regime in the pulsed exposure studies was agreed. It was, however, noted that in the FOCUS surface water modelling report (Ranke, 2008) the exposure profiles for each relevant scenario were not presented. The general graph presenting summary of exposure profiles in all D and R scenarios on single plots was considered not sufficient to aid validation, as peaks from one scenario covered peaks from another scenario making them not visible and based on the presented graph it could not be confirmed that correct exposure profiles were considered in the position paper.
Nevertheless, in case the FOCUS exposure profiles were correct, their comparison with ETC (ecotoxicological trigger curves, obtained by division of the tested peaks by AF of 10) shows that the exposure regime in the study by Liedke (2013d) covered the exposure pattern predicted for R scenarios. However, the comparison of ETC from study by Liedtke (2013e) clearly demonstrated that the exposure pattern predicted for D scenarios was not covered by the exposure regime.

The pulsed exposure studies by Liedtke (2013d and 2013e) were evaluated by the zRMS and considered not relevant for the risk assessment purposes. Apart of other deficiencies listed in the comments to study summaries in Appendix 2 under KCP 10.2.3/10 and KCP 10.2.3/11, respectively, it was noted that the considered exposure regime was not suitable for calculation of ECx values, required for this type of studies by EFSA aquatic guidance (2013), which clearly indicates the RAC from modified exposure studies (including pulsed exposure studies) must be derived with consideration of E_rC_{50} expressed in term of the peak exposure, while according to EFSA Supporting publication 2019:EN-1673, EC_{10} is more relevant for primary producers in order to exclude effects of recovery and follow ETO option (ERO is not acceptable). In absence of the EC_{10} values, the NOEC could be potentially considered, however in Liedtke (2013d) NOEC could be determined only for pulses 3 and 4, while for pulses 1 and 2 it could not be determined due to significant effects (>50%) seen on algae growth at considered concentrations. In study by Liedtke (2013e) the NOEC values were not calculated at all and growth rates were presented for first pulse and recovery phase at the end of exposure only with no growth rates calculated for exposure phases 2-6.
In general it seems that the overall concept of both studies was to demonstrate recovery of algae at the end of the study following the initial high pulses during first exposure phases followed by lower pulses during later exposure phases. Then, in the risk assessment presented in Ranke & Eck (2018), the maximum time weighted average concentrations (TWAC) and areas under the curve (AUC) for specific time windows in the FOCUS profiles for respective scenarios were calculated and AUC value for the corresponding moving windows over the simulated exposure profiles were compared with the AUC in the corresponding ecotoxicological experiment. This approach included recovery, which is not appropriate, as ERO is not an option at Tier 2, as already described by the zRMS in the commenting box above. Furthermore, as already mentioned above, EFSA (2013) clearly indicates that the endpoints from modified exposure experiments (including pulsed exposure studies) must be expressed in terms of the measured/nominal peak concentrations in the test systems and not as the AUC.

It is also noted that the pulsed exposure studies were performed only with *Desmodesmus subspicatus*, although based on the overall dataset, this species was not most sensitive to flurochloridone, as toxic effects were more pronounced in studies performed with *A. falcatus* (with E_rC_{50} of 0.918 µg a.s.L) or *P. subcapitata* (with E_rC_{50} of 2.42 µg a.s./L). Although the pulsed exposure study with non-standard species (*A. falcatus*) could be potentially difficult to perform due to prolonged duration, the standard species *P. subcapitata* seems to be more relevant to be tested in such an experiment.
The selection of the species for the modified exposure studies was justified by the Applicant using information on recovery, which was similar for *A. falcatus* and *D. subspicatus*. However, as already mentioned, recovery is not an option in the current regulatory risk assessment and selection of species to be tested should be based on its sensitivity to the tested substance. Potentially, higher sensitivity of other species would be covered by the respective AF, however in absence of relevant ECx or NOEC values based on peak concentration in the pulsed exposure studies, derivation of relevant RAC is not possible.

The detailed evaluation of the position paper by Ranke & Eck (2018) may be found in Appendix 2 under KCP 10.2.3/14.

Overall, the part of the position paper by Ranke & Eck (2018) regarding the comparison of exposure profiles with exposure regime in the pulsed exposure studies with algae could be accepted, provided that sufficient information regarding FOCUS exposure profiles was available in the modelling report by Ranke (2018). However, the approach taken with regard to the risk assessment was not agreed as it included recovery and have not considered the

EC_{10}/NOEC values. Furthermore, neither of the pulsed-exposure studies with algae were considered sufficiently reliable to be considered in the risk assessment. It is also questionable, if testing of single not most sensitive species is sufficiently protective.

The pulsed exposure studies for aquatic macrophytes were not evaluated as being new active substance data not required for the risk assessment (acceptable risk could be concluded based on EU agreed Tier 1 data and Step 3/4 PEC_{sw} values).

Comments on additional information provided by the Applicant (31.08.2021):

The zRMS appreciates additional information provided by the Applicant, however the studies by Liedtke (2013d and 2013e) were extensively evaluated in the course of the first assessment of AG-F8-250 CS and decision of rejection was carefully taken with consideration of all available indications regarding evaluation of such studies and requirements they must fulfil to be used in the risk assessment.

Detailed information regarding reasons for rejection is provided in the comment above as well as in the zRMS evaluation of the studies provided in Appendix 2 and is not repeated here. The zRMS maintains its conclusions regarding acceptability of the peak-exposure studies performed with flurochloridone.

9.5.2 Risk assessment

The evaluation of the risk for aquatic and sediment-dwelling organisms was performed in accordance with the recommendations of the "Guidance document on tiered risk assessment for plant protection products for aquatic organisms in edge-of-field surface waters in the context of Regulation (EC) No 1107/2009", as provided by the Commission Services (SANTE-2015-00080, 15 January 2015).

The relevant global maximum FOCUS Step 1, 2, 3 and 4 (accounting for vegetated/drift buffer distances) PEC_{SW} for risk assessments covering the proposed use pattern (i.e. for the risk envelope of 2 L product/ha corresponding to 500 g a.s./ha) and the resulting PEC/RAC ratios are presented in the table below. Aquatic risk assessments based on FOCUS Step 3 and 4 are additionally presented for the reduced rate of 1.5 L product/ha (i.e. 375 g a.s./ha).

As this is accepted by the zonal Rapporteur Member State, risk assessments based on FOCUS Step 4 modelling are also presented using VFSmod for FOCUS run-off (R) scenarios. For PEC_{SW} modelling, reference is made to Section B8 of this submission.

In the following tables, the ratios between predicted environmental concentrations in surface water bodies (PEC_{SW}, PEC_{SED}) and regulatory acceptable concentrations (RAC) for aquatic organisms are given per intended use for each FOCUS scenario and each organism group.

Table 9.5-3: Aquatic organisms: acceptability of risk (PEC/RAC < 1) for flurochloridone for each organism group based on FOCUS Steps 1, 2 and 3 calculations for the use of AG-F8-250 CS in potato (pre-emergence)

Group		**Fish acute**	**Fish chronic**	**Inverteb. acute**	**Inverteb. chronic**	**Sed. dwell. chronic**	**Algae**	**Algae (geomean)**	~~Algae (SSD)~~	~~Algae (geometric mean)~~	~~Algae (recovery)~~	**Macrophytes**
Test species		***Oncorhynchus mykiss***	***Oncorhynchus mykiss***	***Daphnia magna***	***Daphnia magna***	***Chironomus riparius***	***Pseudokirchn. subcapitata***	**Algal species (n=9)**	~~Algal species (n = 8)~~	~~Algal species (n = 8)~~	~~Algal species (n = 8)~~	***Lemna gibba***
Endpoint		LC_{50}	NOEC	EC_{50}	NOEC	NOEC	72 h E_rC_{50}	Geomean E_rC_{50}	~~HC_5~~	~~Geomean E_rC_{50}~~	~~Worst-case NOAEC~~	7 d E_yC_{50}
[µg/L]		3000	360	3500	830	250	4.7	6.43	~~0.90~~	~~> 13.8~~	~~10~~	48
AF		100	10	100	10	10	10	10	~~3~~	~~10~~	~~1~~	10
RAC [µg/L]		30	36	35	83	25	0.47	0.643	~~0.30~~	~~> 1.38~~	~~10~~	4.80
FOCUS Scenario	**PEC $_{gl-max}$ (µg/L)**											
Step 1 – 500 g a.s./ha (risk envelope)												
	92.94	**3.10**	**2.58**	**2.66**	**1.12**	**3.72**	**197.7**	**144.5**	~~**309.8**~~	~~**< 67.35**~~	~~**9.29**~~	**19.4**
Step 2 – 500 g a.s./ha (risk envelope)												
N-Europe	19.60	0.653	0.544	0.560	0.236	0.784	**41.7**	**30.5**	~~**65.33**~~	~~**< 14.20**~~	~~**1.96**~~	**4.08**
S-Europe	36.44	**1.21**	**1.01**	**1.04**	0.439	**1.46**	**77.5**	**56.7**	~~**121.5**~~	~~**< 26.41**~~	~~**3.64**~~	**7.59**
Step 3 – 500 g a.s./ha (risk envelope)												
D3/ditch	2.621	0.087	0.073	0.075	0.032	0.105	**5.58**	**4.1**	~~**8.74**~~	~~**< 1.90**~~	~~0.262~~	0.546
D4/pond	0.763	0.025	0.021	0.022	0.009	0.030	**1.62**	**1.2**	~~**2.54**~~	~~**< 0.553**~~	~~0.076~~	0.159
D4/stream	2.169	0.072	0.060	0.062	0.026	0.087	**4.61**	**3.4**	~~**7.23**~~	~~**< 1.57**~~	~~0.217~~	0.452
D6/ditch	2.598	0.087	0.072	0.074	0.031	0.104	**5.53**	**4.0**	~~**8.66**~~	~~**< 1.88**~~	~~0.260~~	0.541
D6/ditch 2nd	2.641	0.088	0.073	0.076	0.032	0.106	**5.62**	**4.1**	~~**8.80**~~	~~**< 1.91**~~	~~0.264~~	0.550
R1/pond	0.400	0.013	0.011	0.011	0.005	0.016	0.851	0.62	~~**1.33**~~	~~< 0.290~~	~~0.040~~	0.083
R1/stream	3.812	0.127	0.106	0.109	0.046	0.152	**8.11**	**5.9**	~~**12.7**~~	~~**< 2.76**~~	~~0.381~~	0.794
R2/stream	2.398	0.080	0.067	0.069	0.029	0.096	**5.10**	**3.7**	~~**7.99**~~	~~**< 1.74**~~	~~0.240~~	0.500
R3/stream	8.001	0.267	0.222	0.229	0.096	0.320	**17.0**	**12.4**	~~**26.7**~~	~~**< 5.80**~~	~~0.800~~	**1.67**
Step 3 – 375 g a.s./ha (reduced rate) (as PL is the only cMS, only scenarios relevant for Poland considered)												
D3/ditch	1.965	0.066	0.055	0.056	0.024	0.079	**4.18**	**3.1**				0.41
D4/pond	0.548	0.018	0.015	0.016	0.0066	0.022	**1.17**	**0.85**				0.11
D4/stream	1.627	0.054	0.045	0.046	0.020	0.065	**3.46**	**2.5**				0.34
R1/pond	0.297	0.010	0.008	0.0085	0.004	0.012	0.632	**0.46**				0.062
R1/stream	2.813	0.094	0.078	0.080	0.034	0.11	**5.99**	**4.4**				0.59

AF: Assessment factor; PEC: Predicted environmental concentration; RAC: Regulatory acceptable concentration; PEC/RAC ratios above the relevant trigger of 1 are shown in bol

In the following tables, the risk assessments are presented considering Step 4 modelling (with and without VFSmod).

~~In deviation to the approach taken in the table above, assessments based on 'recovery' potential of algae, in a more conservative approach are also presented (see Table 9.5-4 below) applying an assessment factor of 10 for the risk envelope (Recovery-RAC = 1 µg a.s./L). Furthermore, additional assessments are presented for an adapted (weight-of-evidence) SSD-RAC of 0.9 µg a.s./L (AF = 1) as well as the geometric mean-RAC of > 1.38 µg a.s./L supporting the overall weight-of-evidence~~.

Table 9.5-4: Aquatic organisms: PEC calculation and acceptability of risk (PEC/RAC < 1) for flurochloridone based on FOCUS Step 4 calculations and toxicity data for group with mitigation of spray drift and run-off for the use of AG-F8-250 CS in potato (pre-emergence) – 500 g a.s./ha

Intended use **Active substance** **Application rate [g/ha]**		pre-emergence, potato flurochloridone 1 x 500 g/ha			
Vegetated filter strip (m)	**Step 3 (default distance)**	**10**	**20**	**10 (VFSmod)**	**20 (VFSmod)**
D3 ditch	2.621	0.455	0.237	-	-
D4 pond	0.763	0.761	0.76	-	-
D4 stream	2.169	0.857	0.857	-	-
D6 ditch	2.598	0.457	0.329	-	-
D6 ditch 2nd	2.641	2.049	2.049	-	-
R1 pond	0.400	0.183	0.099	0.076	0.050
R1 stream	3.812	1.726	0.902	0.417	0.218
R2 stream	2.398	0.964	0.505	0.550	0.287
R3 stream	8.001	3.649	1.914	0.932	0.303
RAC [µg/L] 0.47		**Algae chronic** **PEC/RAC ratio**			
Vegetated filter strip (m)	**Step 3 (default distance)**	**10**	**20**	**10 (VFSmod)**	**20 (VFSmod)**
D3 ditch	**5.58**	0.968	0.504	-	-
D4 pond	**1.62**	**1.62**	**1.62**	-	-
D4 stream	**4.61**	**1.82**	**1.82**	-	-
D6 ditch	**5.53**	0.972	-	-	-
D6 ditch 2nd	**5.62**	**4.36**	**4.36**	-	-
R1 pond	0.851	-	-	-	-
R1 stream	**8.11**	**3.67**	**1.92**	0.89	-
R2 stream	**5.10**	**2.05**	**1.07**	**1.17**	0.61
R3 stream	**17.0**	**7.76**	**4.07**	**1.98**	0.64
~~RAC [µg/L] 1.0~~		~~Algae chronic – $NOAEC_{recovery}$ (AF = 1) PEC/RAC ratio~~			
~~Vegetated filter strip (m)~~	~~Step 3 (default distance)~~	~~10~~	~~20~~	~~10 (VFSmod)~~	~~20 (VFSmod)~~
~~D3 ditch~~	~~2.62~~	~~0.46~~	~~-~~	~~-~~	~~-~~
~~D4 pond~~	~~0.76~~	~~0.76~~	~~-~~	~~-~~	~~-~~
~~D4 stream~~	~~2.17~~	~~0.86~~	~~-~~	~~-~~	~~-~~
~~D6 ditch~~	~~2.60~~	~~0.46~~	~~-~~	~~-~~	~~-~~
~~D6 ditch 2nd~~	~~2.64~~	~~2.05~~	~~2.05~~	~~-~~	~~-~~
~~R1 pond~~	~~0.40~~	~~0.19~~	~~-~~	~~-~~	~~-~~
~~R1 stream~~	~~3.81~~	~~1.73~~	~~0.90~~	~~0.42~~	~~-~~
~~R2 stream~~	~~2.40~~	~~0.96~~	~~0.52~~	~~0.55~~	~~-~~
~~R3 stream~~	~~8.00~~	~~3.65~~	~~1.91~~	~~0.93~~	~~-~~

RAC [µg/L] 0.3		Algae chronic - SSD PEC/RAC ratio			
Vegetated filter strip (m)	Step 3 (default distance)	10	20	10 (VFSmod)	20 (VFSmod)
D3 ditch	8.74	1.52	0.790	-	-
D4 pond	2.54	2.54	2.53	-	-
D4 stream	7.23	2.86	2.86	-	-
D6 ditch	8.66	1.52	1.10	-	-
D6 ditch 2nd	8.80	6.83	6.83	-	-
R1 pond	1.33	0.610	-	-	-
R1 stream	12.7	5.75	3.01	1.39	0.73
R2 stream	7.99	3.21	1.68	1.02	0.96
R3 stream	26.7	12.16	6.38	1.98	1.01
RAC [µg/L] 0.9		Algae chronic - SSD (adapted SSD-RAC) PEC/RAC ratio			
Vegetated filter strip (m)	Step 3 (default distance)	10	20	10 (VFSmod)	20 (VFSmod)
D3 ditch	2.91	0.51	-	-	-
D4 pond	0.85	-	-	-	-
D4 stream	2.41	0.95	-	-	-
D6 ditch	2.89	0.51	-	-	-
D6 ditch 2nd	2.93	2.28	2.28	-	-
R1 pond	0.44	-	-	-	-
R1 stream	4.24	1.92	1.00	0.46	-
R2 stream	2.66	1.07	0.56	0.33	-
R3 stream	8.89	4.05	2.12	1.04	0.54
RAC [µg/L] > 1.38		Algae chronic – geometric mean PEC/RAC ratio			
Vegetated filter strip (m)	Step 3 (default distance)	10	20	10 (VFSmod)	20 (VFSmod)
D3 ditch	< 1.90	< 0.330	-	-	-
D4 pond	< 0.553	-	-	-	-
D4 stream	< 1.57	< 0.621	-	-	-
D6 ditch	< 1.88	< 0.331	-	-	-
D6 ditch 2nd	< 1.91	< 1.48	< 1.48	-	-
R1 pond	< 0.290	-	-	-	-
R1 stream	< 2.76	< 1.25	< 0.654	< 0.30	-
R2 stream	< 1.74	< 0.699	-	-	-
R3 stream	< 5.80	< 2.64	< 1.39	< 0.68	-
RAC [µg/L] 4.8		**Macrophytes chronic PEC/RAC ratio**			
Vegetated filter strip (m)	**Step 3 (default distance)**	**10**	**20**	**10 (VFSmod)**	**20 (VFSmod)**
D3 ditch	0.546	-	-	-	-
D4 pond	0.159	-	-	-	-
D4 stream	0.452	-	-	-	-
D6 ditch	0.541	-	-	-	-
D6 ditch 2nd	0.550	-	-	-	-
R1 pond	0.083	-	-	-	-
R1 stream	0.794	-	-	-	-
R2 stream	0.500	-	-	-	-
R3 stream	**1.669**	0.760	-	0.19	-

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

Accordingly, in case of the risk envelope of 500 g a.s./ha, for a 20 m run-off buffer distance, an acceptable risk for aquatic organisms (driven by the risk for algae) is presented based on the Ecological Threshold

Option and accounting for the ´adapted SSD-RAC´ or the geometric mean RAC with the exception of FOCUS scenarios D6 2nd ditch and R4 stream. If accounting for the recovery RAC (Ecological Recovery Option) based on data for multiple species as additional weight-of-evidence, an acceptable risk for algae is indicated even at the default distance (AF = 1) or if accounting for a 10 m vegetated buffer (AF = 10). As argued above, it is again emphasised that the growth inhibition of the most sensitive species only marginally exceeded the 50% effect level in the study. Accordingly, an assessment factor of 10 which is intended to cover inter-species variability in sensitivities and the fact that here a No-Observed-Effect endpoint is used, renders risk assessments based on ´recovery´ overly conservative. Therefore, these assessments give confidence in the conclusion of an acceptable risk for the intended worst-case uses (risk envelope).

Table 9.5-5: Aquatic organisms: PEC calculation and acceptability of risk (PEC/RAC < 1) for flurochloridone based on FOCUS Step 4 calculations and toxicity data for group with mitigation of spray drift and run-off for the use of AG-F8-250 CS in potato (pre-emergence) – 375 g a.s./ha

Intended use **Active substance** **Application rate [g/ha]**		pre-emergence, potato flurochloridone 1 x 375 g/ha			
Vegetated filter strip (m)	**Step 3 (default distance)**	**10**	**20**	**10 (VFSmod)**	**20 (VFSmod)**
D3 ditch	1.965	0.342	0.178	-	-
D4 pond	0.548	0.547	0.546	-	-
D4 stream	1.627	0.635	0.635	-	-
D6 ditch	1.948	0.343	0.251	-	-
D6 ditch 2nd	1.980	1.487	1.487	-	-
R1 pond	0.297	0.137	0.074	0.058	0.037
R1 stream	2.813	1.274	0.666	0.313	0.164
R2 stream	1.798	0.703	0.368	0.412	0.215
R3 stream	5.831	2.659	1.395	0.686	0.227
RAC [µg/L] 0.47		**Algae chronic** **PEC/RAC ratio**			
Vegetated filter strip (m)	**Step 3 (default distance)**	**10**	**20**	**10 (VFSmod)**	**20 (VFSmod)**
D3 ditch	**4.18**	0.73	-	-	-
D4 pond	**1.17**	**1.16**	**1.16**	-	-
D4 stream	**3.46**	**1.35**	**1.35**	-	-
D6 ditch	**4.14**	0.73	-	-	-
D6 ditch 2nd	**4.21**	**3.16**	**3.16**	-	-
R1 pond	0.632	-	-	-	-
R1 stream	**5.99**	**2.71**	**1.42**	0.67	-
R2 stream	**3.83**	**1.50**	**0.78**	0.88	-
R3 stream	**12.4**	**5.66**	**2.97**	**1.46**	0.48

RAC [µg/L] 1.0		Algae chronic – $NOAEC_{recovery}$ (AF = 1) PEC/RAC-ratio			
Vegetated filter strip (m)	Step 3 (default distance)	10	20	10 (VFSmod)	20 (VFSmod)
D3 ditch	1.97	0.34			
D4 pond	0.53	0.55			
D4 stream	1.62	0.64			
D6 ditch	1.95	0.34			
D6 ditch 2nd	1.96	1.49	1.49		
R1 pond	0.20				
R1 stream	2.84	1.27	0.67	0.91	
R2 stream	1.30	0.79	0.22	0.41	
R3 stream	3.82	2.66	1.40	0.69	
RAC [µg/L] 0.2		Algae chronic – SSD PEC/RAC-ratio			
Vegetated filter strip (m)	Step 3 (default distance)	10	20	10 (VFSmod)	20 (VFSmod)
D3 ditch	6.55	1.14	0.58		
D4 pond	1.82	1.82	1.82		
D4 stream	5.42	2.12	2.12		
D6 ditch	6.40	1.14	0.84		
D6 ditch 2nd	6.60	4.96	4.96		
R1 pond	0.99				
R1 stream	9.39	4.25	2.22	1.04	0.55
R2 stream	5.09	2.34	1.23	0.57	0.72
R3 stream	10.2	8.86	4.65	2.20	0.76
RAC [µg/L] 0.9		Algae chronic – SSD (adapted SSD-RAC) PEC/RAC-ratio			
Vegetated filter strip (m)	Step 3 (default distance)	10	20	10 (VFSmod)	20 (VFSmod)
D3 ditch	2.19	0.38			
D4 pond	0.61				
D4 stream	1.81	0.71			
D6 ditch	2.13	0.38			
D6 ditch 2nd	2.20	1.65	1.65		
R1 pond	0.33				
R1 stream	3.13	1.42	0.74	0.35	
R2 stream	2.00	0.78	0.41	0.46	
R3 stream	6.48	2.95	1.55	0.76	
RAC [µg/L] ≤ 1.38		Algae chronic – geometric mean PEC/RAC-ratio			
Vegetated filter strip (m)	Step 3 (default distance)	10	20	10 (VFSmod)	20 (VFSmod)
D3 ditch	≤ 1.42	≤ 0.25			
D4 pond	≤ 0.40				
D4 stream	≤ 1.18	≤ 0.46			
D6 ditch	≤ 1.41	≤ 0.25			
D6 ditch 2nd	≤ 1.42	≤ 1.08	≤ 1.08		
R1 pond	≤ 0.29				
R1 stream	≤ 2.04	≤ 0.92		≤ 0.25	
R2 stream	≤ 1.30	≤ 0.51			
R2 stream	≤ 4.22	≤ 1.92	≤ 1.01	≤ 0.50	

RAC [µg/L] 4.8		**Macrophytes chronic PEC/RAC ratio**			
Vegetated filter strip (m)	**Step 3 (default distance)**	**10**	**20**	**10 (VFSmod)**	**20 (VFSmod)**
D3 ditch	0.41	-	-	-	-
D4 pond	0.11	-	-	-	-
D4 stream	0.34	-	-	-	-
D6 ditch	0.41	-	-	-	-
D6 ditch 2nd	0.41	-	-	-	-
R1 pond	0.062	-	-	-	-
R1 stream	0.59	-	-	-	-
R2 stream	0.37	-	-	-	-
R3 stream	**1.21**	0.55	-	0.14	-

PEC: Predicted environmental concentration; RAC: Regulatory acceptable concentration; PEC/RAC ratios above the relevant trigger of 1 are shown in **bold**
Grey shading: refinement considered to be supportive for overall weight-of-evidence

In case of the lower use rate and taking into account VFSmod modelling, likewise a 20 m vegetated buffer distance is indicated to be required for R scenarios if based on the assessments applying the Tier 1 as well as the conservative SSD-RAC. If further refining based on adapted SSD-RAC or geometric mean RAC as well as $NOAEC_{recovery}$ (with conservative AF = 10), a 10 m vegetated buffer distance is sufficiently protective and the relevant FOCUS drainage scenarios (refer to national requirements of the concerned Member State) pass the risk assessments.

Relevant FOCUS scenarios

The zonal Rapporteur Member State (Poland) is the only concerned Member State. For national registration, only the following sub-set of FOCUS scenarios are relevant in accordance with Polish requirements.

- D3 ditch
- D4 pond
- D4 stream
- R1 pond
- R1 stream

The margin of safety for these scenarios is even more comfortable than under consideration of additional and more critical FOCUS scenarios (i.e. D6, R2 and R3).

This gives even more confidence in the conclusions of an acceptable risk for national registration in Poland for both uses (i.e. 2 and 1.5 L product/ha) if a 10 m vegetated buffer distance is accounted for.

zRMS comments:

The risk assessment presented in Tables 9.5-3 and 9.5-4 above is in general agreed by the zRMS. The calculations based on geometric mean E_rC_{50} for algae were struck through, as in case the number of additional toxicity data is ≥8, the SSD approach is more appropriate. The calculation based on recovery was also struck through, as recovery is not accepted in the current regulatory risk assessment.

Based on the calculations performed with Steps 1-3 PEC_{sw}, acceptable acute and chronic risk could be concluded for fish, *Daphnia magna* and sediment-dwellers. For aquatic macrophytes acceptable risk could be concluded for most of Step 3 scenarios with exception of scenario R3.

The risk for algae was unacceptable in all FOCUS scenarios.

The risk was refined with consideration of the risk mitigation measures. Based on performed calculations acceptable risk could be concluded for aquatic macrophytes in scenario R3, provided that 10 m vegetated filter strip to surface water bodies is respected.

In most of FOCUS scenarios no acceptable risk could be concluded for algae using Tier 1 E_rC_{50} or Tier 2 HC_5. The only exceptions were scenarios D3 ditch (with acceptable risk at 10 or 20 m buffer for Tier 1 E_rC_{50} or Tier 2 HC_5, respectively), scenario D6 ditch (with acceptable risk at 10 m buffer for Tier 1 E_rC_{50} and no acceptable risk for Tier 2 HC_5) and scenario R1 pond (with acceptable risk at Step 3 or 10 m buffer for Tier 1 E_rC_{50} or Tier 2 HC_5, respectively).

Further refinement was thus deemed necessary.

During the commenting period additional Step 4 surface water modelling performed using VFSmod has been submitted by the Applicant in order to further refine the run-off in R scenarios (Weber & Jarvis, 2020). The additional simulations has been evaluated by the zRMS and considered acceptable. Details of the calculations may be found in the final Core Assessment, Part B, Section 8 (March 2021).

The additional risk assessment for algae based on obtained PEC_{sw} values is presented below. In evaluation the standard Tier 1 toxicity endpoint was considered with AF of 10. As the derived RAC of 0.47 µg a.s./L is lower than the lowest endpoint of 0.918 µg a.s./L for *A. falcatus*, the risk assessment is considered to be sufficiently protective and higher sensitivity of other taxa is accounted for in the applied AF of 10. Taking this into account, the higher tier HC_5 was not considered.

As the vegetated filter strip would not be reduced when new calculations are used, aquatic macrophytes were not taken into account in below calculations, as acceptable risk could be concluded with 10 m VFS already with results of the previous modelling.

Intended use		pre-emergence, potato	
Active substance		flurochloridone	
Application rate [g/ha]		1 x 500 g/ha	
Mitigation	**Step 3 (default buffer distance)**	**10 m (buffer + VFSmod)**	**20 m (buffer + VFSmod)**
D3 ditch	2.621	0.455	0.237
D4 pond	0.763	0.761	0.760
D4 stream	2.169	0.857	0.857
D6 ditch	2.598	0.457	0.329
D6 ditch 2nd	2.641	2.049	2.049
R1 pond	0.400	0.076	0.050
R1 stream	3.812	0.417	0.218
R2 stream	2.398	0.550	0.287
R3 stream	8.001	0.932	0.303
RAC [µg/L]		**Algae chronic**	
0.47		**PEC/RAC ratio**	
Mitigation	**Step 3 (default buffer distance)**	**10 m (buffer + VFSmod)**	**20 m (buffer + VFSmod)**
D3 ditch	**5.58**	0.968	-
D4 pond	**1.62**	**1.62**	**1.62**
D4 stream	**4.61**	**1.82**	**1.82**
D6 ditch	**5.53**	0.972	-
D6 ditch 2nd	**5.62**	**4.36**	**4.36**
R1 pond	0.851	-	-
R1 stream	**8.11**	0.89	-
R2 stream	**5.10**	**1.17**	0.61
R3 stream	**17.0**	**1.98**	0.64

The risk assessment for algae based on additional Step 4 simulation performed using VFSmod demonstrated acceptable risk in all R scenarios provided that in R1 scenario 10 m vegetated filter strip is respected, while in R2 and R3 scenarios 20 m vegetated filter strip is applied. In scenario D3 10 meters unsprayed buffer zone to surface water bodies is required.

The risk in D4 and D6 scenarios remains unresolved.

Comments on additional risk assessment provided by the Applicant (31.08.2021):

In line with zRMS comments provided in point 9.5.1.1 above, following refinement options for algae were not agreed upon:

- geometric mean E_rC_{50} calculated with consideration of *Anabaena flos-aquae*,
- SSD approach with modified assessment factor,
- refinements based on recovery,
- refinements based on results of pulsed-exposure studies.

Risk refinement based on not agreed options has been struck through in the above tables. Detailed justification of the zRMS position may be found in point 9.5.1.1.

On the other hand, the zRMS agreed with the Applicant that the HC_5 calculated in SSD approach was fully relevant as derived SSD-RAC it was even more conservative than the Tier 1-RAC. For this reason consideration of the geometric mean E_rC_{50} calculated with exclusion of endpoint for insensitive *A. flos-aquae* has been accepted. The geometric mean of 6.43 µg a.s./L was calculated resulting with RAC of 0.643 µg a.s./L. The risk assessment for algae based on this refined value is presented below. Since Poland is the only cMS for AG-F8-250 CS, only scenarios representative for Poland has been considered in the below calculations. Respective evaluation is performed for both application rates of 500 and 375 g a.s./ha. For R1 scenario only PEC_{sw} calculated using VFSmod were considered.

Intended use		pre-emergence, potato	
Active substance		flurochloridone	
Application rate [g/ha]		1 x 500 g/ha	
Mitigation	**Step 3 (default buffer distance)**	**10 m (buffer + VFSmod)**	**20 m (buffer + VFSmod)**
D3 ditch	2.621	0.455	0.237
D4 pond	0.763	0.761	0.760
D4 stream	2.169	0.857	0.857
R1 pond	0.400	0.076	0.050
R1 stream	3.812	0.417	0.218
RAC [µg/L]		**Algae geometric mean for 9 species, AF = 10**	
0.643		**PEC/RAC ratio**	
Mitigation	**Step 3 (default buffer distance)**	**10 m (buffer + VFSmod)**	**20 m (buffer + VFSmod)**
D3 ditch	**4.1**	0.71	-
D4 pond	**1.2**	**1.2**	**1.2**
D4 stream	**3.4**	**1.3**	**1.3**
R1 pond	0.62	0.12	-
R1 stream	**5.9**	0.65	-

Intended use		pre-emergence, potato	
Active substance		flurochloridone	
Application rate [g/ha]		1 x 375 g/ha	
Mitigation	**Step 3 (default buffer distance)**	**10 m (buffer + VFSmod)**	**20 m (buffer + VFSmod)**
D3 ditch	1.965	0.342	0.178
D4 pond	0.548	0.547	0.546
D4 stream	1.627	0.635	0.635
R1 pond	0.297	0.058	0.037
R1 stream	2.813	0.313	0.164
RAC [µg/L]		**Algae chronic**	
0.643 (geometric mean for 9 species, AF = 10)		**PEC/RAC ratio**	
Mitigation	**Step 3 (default buffer distance)**	**10 m (buffer + VFSmod)**	**20 m (buffer + VFSmod)**
D3 ditch	**3.1**	0.53	-
D4 pond	0.85	0.85	-
D4 stream	**2.5**	0.99	-
R1 pond	0.46	0.09	-
R1 stream	**4.4**	0.49	-

Based on performed above calculations acceptable risk to aquatic organisms from application of AG-F8-250 CS to potatoes at application rate of 375 g a.s./ha may be concluded, provided that 10 m vegetated filter strip from surface water bodies is respected.

For higher application rate of 500 g a.s./ha acceptable risk may be concluded in scenarios D3 and R1 with consideration of vegetated filter strip of 10 m. However, the risk to algae in scenario D4 remains unresolved.

Pulsed exposure studies

For final assessment of potential risk to algae, a comparison of the predicted course of exposure for a 20 m vegetated buffer distance including the standard assessment factor (Environmental Trigger Curve) with the course of exposure in the pulsed exposure studies by Liedtke (2013d and 2013e; Reference numbers: 90015421 and 90015432) was performed by Ranke and Eck (2018, KCP 10.2.3/14).

Following the respective exposures in the two studies, full recovery of algae was observed for both concentration curves accounting for the assumed assessment factors. Thus, it can be concluded that the exposure patterns constitute No Observed Adverse Exposure Patterns (NOAEP). A graphical overlay suggests that for the FOCUS R stream scenarios, the four (R1 and R3 stream) or two (R2 stream) exposure events above the threshold are considered to be sufficiently covered by the four pulses with exposure peaks which are higher, wider and more closely spaced (also refer to the summary of the statement by Ranke and Eck in the Appendix below).

By comparing the Area Under Curve (AUC) of the relevant pulsed exposure effect studies with the predicted AUC for moving time window (with the time window selected by the zonal rapporteur), TERs were calculated. The respective TERs are summarized in following table for the intended uses in potato.

Table 9.5-6: Risk assessment based on comparison of AUC for pulsed exposure data for 20 m vegetated buffer distance

Crop / Crop group	Growth stage (BBCH)	Test substance	Test species	Max. application rate [kg a.s./ha]	NOAEP pattern	FOCUS scenario		PEC_{SW} [µg a.s./L]	TER_{LT}
20 m vegetated buffer									
Potatoes	00-09	flurochloridone	*D. subspicatus*	0.5	Recovery of algae following pulsed exposure	D3	ditch	< ETC	No risk (Tier 1)
						D4	pond		Not covered
							stream		No covered
						D6	ditch		No risk (Tier 1)
							2nd ditch		Not covered
						R1	pond		No risk (Tier 1)
							stream		90.6
						R2	stream		119.8
						R3	stream		73.4

The higher tier risk analysis of the simulated exposure profiles considering a 20 m vegetated buffer strip showed that the FOCUS R scenarios that did not pass the tier 1 assessment are conservatively covered by the pulsed exposure study with the first pattern (Liedtke, 2013a) that was specifically designed for run off scenarios.

All of these runoff scenarios pass the assessment by a great margin of safety in regard to the ratio of AUC in the ecotoxicological test to the predicted AUC. Also the graphical analysis is based on the tier 1 uncertainty factor, while the available data on multiple algal species suggest that even a lower assessment factor can be considered acceptable.

The risk from exposure in the drainage scenarios is not covered by this assessment.

As was shown in the analysis by Ranke and Eck (2018), for all relevant FOCUS R stream scenarios for the zonal evaluation (i.e. R1, R2 and R3) the graphical analysis shows that the course of predicted exposure is

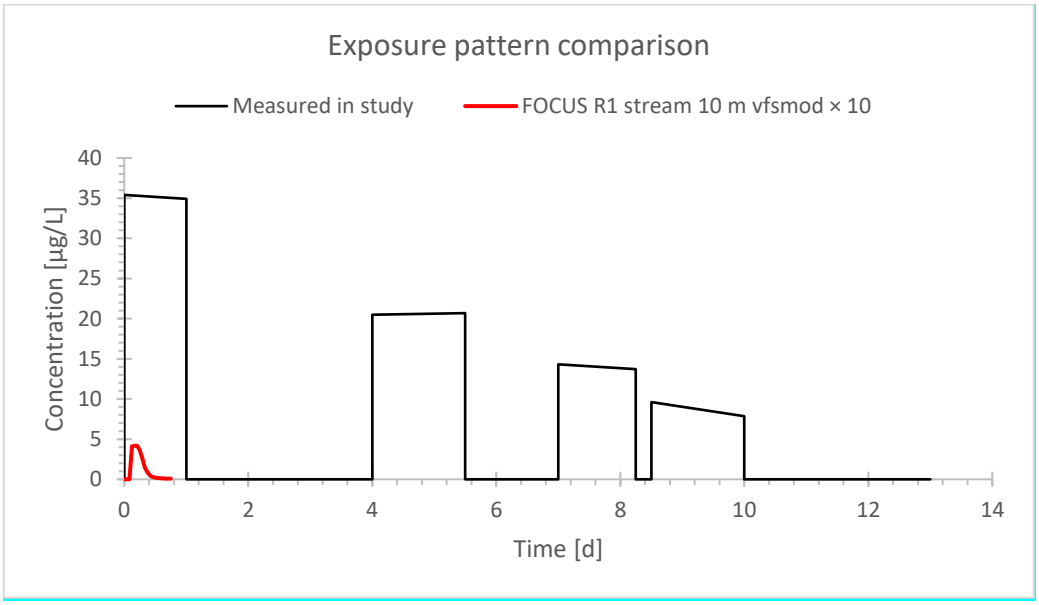
well covered by the ETC conservatively applying an Assessment Factor of 10. This approach is highly conservative for the following reasons:

- The assessment is based on the No Observed Adverse Effect Pattern instead of the peak concentration causing 50% effect levels (i.e. based on revised E_rC_{50}).
- The AF of 10, normally considered in context with median Effective Concentrations but not with No Observed Effect Concentrations, is overly conservative.
- Based on the fact that multiple-single species testing is available and as the tested species was within an order of magnitude of the sensitivity of the most sensitive species tested and as it was also similarly resilient with recovery potential comparable to that of the most sensitive species, this would justify reduced AF even in context with an E_rC_{50} and even more so in case of the NOEC or ´NOAEP´.

Therefore, and further supported by the presented comparison based on Area Under the Curve of exposure (see below), these risk assessments unequivocally indicate acceptable risk from exposure as in accordance with the FOCUS R scenarios.

FOCUS modelling with VFSmod is acceptable for national risk assessments in Poland. The respective assessments and superposition of the ETC on predicted course of exposure is presented below based on revised modelling for the relevant FOCUS R1 stream scenario.

In the following graph, the superposition of the ETC (i.e. the exposure pattern or ´NOAEP´) from the study by Liedtke (2013d) over R1 stream scenario is presented. In deviation to the original analysis by Ranke and Eck (2018) applying the Tier 1 RAC of 0.47 µg a.s./L as threshold level for critical scenario definition, in a more conservative approach, a threshold level of 0.1 µg a.s./L is selected. This threshold corresponds with the rounded RAC based on E_rC_{50} for the most sensitive test species (i.e. *A. falcatus*).



Graph 2: Superposition of the study exposure pattern by Liedtke (2013d) over the FOCUS R1 stream scenario accounting for AF = 10

Accordingly, the single maximum exposure peak of FOCUS R1 stream at 10 m vegetated buffer distance is well covered by the exposure tested in the study by Liedtke.

In conclusion, an acceptable risk for algae exposed to AG-F8-250 CS in potatoes for national registration in Poland is presented based on Tier 2C risk assessment, if a 10 m vegetated buffer distance is respected in case of the risk envelope.

Area Under the Curve of Exposure of effect data vs. field exposure

In further support of the ETC-based assessments, a comparison of AUC of the effect study with the predicted AUC of exposure from FOCUS modelling was provided.

With reference to the argumentation provided above (reference is made to Point 9.5.1.1; ´Algal recovery potential´), rating the deviation and re-establishment of algal cell densities from baseline densities within a biological context, the short-term algistatic effects, neither those of the extended standard tests with multiple species, nor of the pulsed-exposure studies are considered to represent ecologically relevant observations. The course of exposure in the revised exposure studies by Liedtke (2013d/e) are therefore considered to represent No Observed Effect Patterns. As elaborated above, the application of an AF of 10 in order to generate an Ecotoxicological Trigger Curve is overly conservative.

This overall conservatism is further supported by comparison of AUC. The AUC approach is considered to be an adequate way to set the field exposure and the exposure in the Tier 2C study into context, in this case clearly showing that the tested exposure scenario by far exceeds the cumulative exposure of the respective FOCUS R scenarios.

The respective assessment is presented in the following table for the R1 stream scenario (as relevant scenario for national registration in Poland) and for a 20 m vegetated buffer distance. For details, reference is also made to Ranke and Eck (2018).

Table 9.5-7: Risk assessment for algae based on AUC (pulsed experimental vs. predicted exposure) – 20 m vegetated buffer distance – 500 g a.s./ha (risk envelope)

FOCUS scenario	Event no.	Maximum PEC_{SW} [µg a.s./L]	Peak duration [d]	Interval to following peak [d]	AUC [µg a.s./L*d]	Cumulative AUC [µg a.s./L*d]	AUC of effect study [µg a.s./L*d] [a)]	AUC_{study}/AUC_{FOCUS}
R1 stream	1	0.653	0.375	-	0.238	1.167	96.6	82.8
	2	**0.902**	0.541	12.5	0.455			
	3	0.618	0.334	5.5	0.199			
	4	0.555	0.5	3.62	0.275			

[a)] AUC based on Liedtke (2013d); pulsed exposure study with 4 individual pulses

Accordingly, the ratio of AUC between the ETC and predicted exposure shows a safety factor of > 80; i.e. on basis of the ETC, this is an additional safety factor of 8 (on top of the standard AF of 10). In conclusion, the assessment based on the pulsed exposure study by Liedtke with a high level of certainty confirms acceptable risk for algae exposed in line with the predicted run-off exposure.

The respective assessment in the following table is presented for the revised modelling applying VFSmod for a reduced vegetated buffer distance of 10 m. The scenario exposure statistics for R1 stream were conservatively based on a revised threshold level of 0.1 µg a.s./L (see explanations above). For R1 stream, unlike from standard modelling, the revised modelling only shows a single peak exposure.

Table 9.5-8: Risk assessment for algae based on AUC (pulsed experimental vs. predicted exposure) – 10 m vegetated buffer distance (VFSmod) – 500 g a.s./ha (risk envelope)

FOCUS scenario	Event no.	Maximum PEC_{SW} [µg a.s./L]	Peak duration [d]	Interval to following peak [d]	AUC [µg a.s./L*d]	Cumulative AUC [µg a.s./L*d]	AUC of effect study [µg a.s./L*d] [a]	AUC_{study}/AUC_{FOCUS}
R1 stream	1	**0.417**	0.25	-	0.083	0.083	96.6	1164

[a] AUC based on Liedtke (2013d); pulsed exposure study with 4 individual pulses
[b] AUC = 2.0 µg a.s./L*h (refer to Weber 2020)

The single peak exposure of 0.417 µg a.s./L lasting for an exposure duration of 0.25 days (i.e. 6 hours) only gives an AUC of 0.083 µg a.s./L*d which is by a factor of about 1164 below the AUC covered in the Tier 2C study; i.e. by an additional safety factor of 116 on top of the conservative standard AF of 10.

For completeness, a scenario analysis and risk assessment is also presented in the following table based on AUC for the reduced rate corresponding to 375 g a.s./ha for the relevant FOCUS R1 stream scenario based on VFSmod modelling.

Table 9.5-9: Risk assessment for algae based on AUC (pulsed experimental vs. predicted exposure) – 10 m vegetated buffer distance (VFSmod) – 375 g a.s./ha (reduced rate)

FOCUS scenario	Event no.	Maximum PEC_{SW} [µg a.s./L]	Peak duration [d]	Interval to following peak [d]	AUC [µg a.s./L*d]	Cumulative AUC [µg a.s./L*d]	AUC of effect study [µg a.s./L*d] [a]	AUC_{study}/AUC_{FOCUS}
R1 stream	1	**0.313**	0.25	-	0.0625 [b]	0.0625	96.6	1546

[a] AUC based on Liedtke (2013d); pulsed exposure study with 4 individual pulses
[b] AUC = 1.5 µg a.s./L*h (refer to Weber 2020)

In case of the reduced rate, the safety margin based on AUC amounts to 1546 which is by a factor of 155 greater than the standard assessment factor of 10.

In conclusion, the assessment based on AUC further supports the conclusion of an acceptable risk for algae exposed in accordance with the relevant FOCUS R1 stream scenario if a 10 m vegetated buffer distance is respected in case of the risk envelope (i.e. for the intended uses corresponding to 500 g a.s./ha) and the reduced rate of 375 g a.s./ha.

zRMS comments:

Detailed evaluation of approach taken with regard to the risk assessment based on pulsed exposure studies with algae is presented in the commenting box in point 9.5.1.1 above and is not repeated here.

Overall, the part of the position paper by Ranke & Eck (2018) regarding the comparison of exposure profiles with exposure regime in the pulsed exposure studies with algae could be accepted, provided that sufficient information regarding FOCUS exposure profiles was available in the modelling report by Ranke (2018). However, the approach taken with regard to the risk assessment was not agreed as it included recovery and have not considered the EC_{10}/NOEC values, required by EFSA Supporting publication 2019:EN-1673. Furthermore, neither of the pulsed-exposure studies with algae were considered sufficiently reliable to be considered in the risk assessment. It is also questionable, if testing of single not most sensitive species is sufficiently protective.

For details of evaluation of the pulsed exposure studies, please refer to Appendix 2, KCP 10.2.3/10 and KCP 10.2.3/11. Detailed evaluation of the position paper by Ranke & Eck (2018) is presented in Appendix 2, KCP 10.2.3/14.

During the commenting period additional Step 4 surface water modelling performed using VFSmod was submitted by the Applicant in order to further mitigate the run-off in R scenarios. The risk assessment based on the derived PEC_{sw} values and standard Tier 1 toxicity data enabled to demonstrate acceptable risk, provided that 10 m vegetated filter strip is respected in scenario R1 and 20 m vegetated filter strip is applied in scenarios R2 and R3. Taking this

into account, the unacceptable risk is now concluded in D4 and D6 (2nd) scenarios only.

In order to further defend the approach in the risk assessment based on the pulsed-exposure studies, during the commenting period the position paper by Eck et al. (2020) has been provided by the Applicant. No summary of the position paper was provided, so the paper is not summarised in Appendix 2, but below the Applicants' position (text in italics) with the zRMS opinion as presented in the Reporting Table is provided.

Relevance of the recovery in the Tier 1 and Tier 2 risk assessment

Please refer to Point 2 A of the expert statement.
In an alternative and more conservative approach, the risk assessments presented in the expert statement account for an AF of 10 resulting in a worst-case RACrecovery of 1.0 µg a.s./L and additionally consider risk mitigation buffer distances. In addition to the assessments based on standard FOCUS modelling, alternative assessments are amended applying VFSmod as this is considered acceptable by the RMS for national registration.
Accordingly, even if applying an overly conservative AF of 10, an acceptable risk is indicated for algae for the relevant FOCUS scenarios for national registration in Poland if accounting for a 20 m vegetated (i.e. combined drift and run-off buffer) distance. Relevant drainage scenarios are covered by a 10 m drift-only buffer distance. Based on modelling applying VFSmod, a 10 m (vegetated) buffer distance would also be sufficiently protective for the R1 stream scenario.
In conclusion, based on the data for 'recovery' and applying a conservative approach, an acceptable risk for algae for the relevant FOCUS scenarios in Poland is indicated if a 10 m vegetated buffer distance is respected.

The additional risk assessment presented in Point 2A of the expert statement (Eck et al., 2020, Rep. No 2000626.SW0-4623) has been checked by the zRMS and it is noted that presented calculations were still based on the recovery option. In the position paper the authors argue that recovery is an option allowed by EFSA (2013), but it should be kept in mind that this option is described for Tier 3 data (i.e. mesocosm and microcosm experiments), while the Applicant would like to use recovery option for Tier 1 and Tier 2 data, which is not foreseen by the EFSA aquatic guidance. Furthermore, in EFSA Supporting publication 2019:EN-1673 (point 4.5) it is clearly indicated that ERO is not an option at Tier 2. Rules for RAC derivation with Tier 1 and 2 data are clearly described in EFSA (2013) and endpoints based on recovery are not relevant at these Tiers of the risk assessment.

It should be also noted that consideration of ERO-RAC based on meso- and microcosm experiments was a subject of further discussions at the zonal and national level. During the Central Zone harmonisation meetings in area of ecotoxicology it was concluded that for the zonal assessment the ERO option is not relevant. The same approach was agreed at the national level in Poland.

Overall, consideration of ERO option at Tier 1 and 2 is not foreseen by the EFSA aquatic guidance, while ERO option based on Tier 3 data is not agreed at the Central Zone level and in Poland. Taking this into account the risk assessment presented in Point 2A of the position paper cannot be agreed upon.

Consideration of the geometric mean approach as additional line of evidence in addition to the SSD approach

Please refer to Point 2 B of the expert statement.
The applicant had proposed to consider the geometric mean approach as an additional line of evidence. The applicant is of the opinion that the generation of sufficient data principally allowing for an SSD should not result in a bias towards more risk as compared to the geometric mean approach merely through the omittance of additional data not fitting within the distribution of a subset of data, which is a view also supported by the EFSA Guidance document.
In this context, it is stressed that by selecting only the 7 most sensitive species within the distribution and by applying the standard assessment factor of 10, the geometric mean ErC50 would calculate to > 4.41 µg a.s./L and the respective RAC to > 0.44 µg a.s./L (see Table 1, 2B of expert statement), which is still higher than the SSD-RAC of 0.30 µg a.s./L.
Assessments are additionally presented based on revised FOCUS modelling applying VFSmod as considered acceptable for assessments in the national context in Poland. In addition to the revised RAC for algae, the assessments are presented based on the originally defined SSD-RAC of 0.3 µg a.s./L.
Accordingly, if based on the adjusted SSD-RAC, an acceptable risk is indicated by risk quotients below 1 for the relevant FOCUS drainage scenarios, if a 10 m (drift) buffer distance is respected. Based on standard modelling, for R1 stream, the quotient only marginally exceeds the trigger of 1 which is considered to be acceptable (the quotient rounded to two significant figures indicates acceptable risk) if a 20 m vegetated filter strip (combined drift and run-off buffer) is accounted for.

If based on revised modelling applying VFSmod as considered acceptable for national risk assessments in Poland, an acceptable risk is also indicated for R1 stream with the risk quotient of < 1 if a vegetated buffer distance of only 10 m is accounted for.

The zRMS would like to emphasise that the EFSA aquatic guidance (2013) does not indicate at what conditions the geomean approach with the toxicity data for ≥8 algae species would supersede the SSD approach. It is only indicated that in such case geomean approach could be still applied, but SSD would be the preferred option.

The Applicant proposed to consider the geomean approach as additional line of evidence but the zRMS would like to point out that such a line of evidence might be evidence that the exposure of algae to flurochloridone under practical conditions of use would be unlikely (e.g. in case the drainage or run-off peaks occur during winter at dormancy) or evidence that decrease in algae population would not affect other taxa depending on algae.

However, consideration of the more favourable outcome of the risk assessment based on different and not preferred option cannot be considered to be the actual "line of evidence" used to conclude on acceptability of the risk.

It should be also noted that issue of consideration of the geometric mean endpoint was discussed in detail during the general ecotoxicology meeting and in EFSA Supporting publication 2019:EN-1673 it is pointed out that the geometric mean approach may be followed in case the toxicity data on additional species are insufficient to carry out species sensitivity distribution (SSD). This clearly indicates preference to SSD approach over geometric mean when sufficient data points are available, which is the case for flurochloridone in AG-F8-250 CS.

Taking this into account, the HC_5 is considered to be more relevant for purposes of the risk assessment for AG-F8-250 CS and the fact that the geometric mean value results with acceptable risk cannot be the reason to disregard HC_5.

In addition to that in the position paper it is indicated that according to EFSA (2013):

the size of the AF should ideally not result in an SSD-$RAC_{sw; ac}$ higher than the Tier 3 RAC derived from effect class 1 and 2 of micro-/ mesocosm studies, nor should it result in an SSD-$RAC_{sw; ac}$ lower than the Tier 1 $RAC_{sw; ac}$ on the basis of standard test species and/or Geomean-$RAC_{sw; ac}$ […] on the basis of the same toxicity data that were used to construct the SSD

Based on that the the statement authors propose to adjust the RAC using the assessment factor reduced to 2 or even 1.

It should be, however, noted that in the above quotation the authors of the position paper ignore the fact, that indications mentioned are relevant for aquatic invertebrates, for which range of AF is proposed, while for the primary producers EFSA (2013) is straightforward indicating that SSD-RAC should be derived using median HC_5 and AF of 3 with no further options to reduce or increase this value.

Taking all this into account it is concluded that the risk assessment for algae presented in the Core Assessment was performed fully in line with indications of EFSA (2013) as well as zonal and national requirements and indicated unacceptable risk using the option preferred by the guidance document. The AF for primary producers cannot be reduced due to the geometric mean endpoint higher than HC_5.

Discussion on applicability of the standard validity criteria to the pulsed exposure studies

Please refer to Point 2 C of the expert statement.
The studies with pulsed exposure provided are higher tier data with a design significantly deviating from standard Tier 1 studies for which the validity criteria for acceptable testing are defined. Therefore, the Applicant insists, that the criteria are not applicable to the higher tier revised exposure testing.
As argued by the zRMS, for Tier 2C refinements (i.e. higher tier effect data from revised exposure testing), the principal idea is to generate alternative ECX (i.e. ErC50) values to be used for revised risk assessments. This, of course, would require concentration-response testing.
The EFSA Guidance (2013) states that 'higher tier effect assessments do not necessarily need to be performed by simulating constant exposure normally used in standardised lower tier tests but may address the exposure regimes predicted for edge-of-field surface waters'. 'For the derivation of a chronic RAC by means of refined chronic toxicity tests it is proposed to apply an AF of 10 […] to the EC50 for plants […; note by the Applicant: this includes algae] under the conditions that the (repeated pulsed) exposure regime […] is realistic to worst case when compared with the relevant predicted (modelled) field exposure profile. […]. The duration of the test

is long enough to allow the observation of delayed effects, the refined chronic RAC is compared with the PECsw, max.´.
In deviation to this approach, the studies by Liedtke (2013d/e) were performed at individual exposure patterns which were intended to be protective for a series of predicted FOCUS patterns allowing for an additional Assessment Factor (AF). Accordingly, the tests represent a simulation of worst-case field exposure patterns. Environmental Trigger Curves (ETCs) were generated by accounting for an AF by division of the experimental exposure profile by an appropriate, or even overly conservative factor. As the course of exposure in the ecotoxicological experiments over the respective study duration reflects an NO(A)EC-pattern (or NOAEP) and as additionally AF was considered, this approach is distinctly more conservative as compared to risk assessments based on revised ErC50. It also directly links the exposure in the field with the exposure in ecotoxicological testing ideally matching the ERC concept.

First of all the zRMS would like to point out that the pulsed exposure studies are actually modified standard Tier 1 studies, so differences between the test designs are not so drastic.

Nevertheless, the zRMS acknowledges the differences and for this reason in comments to the studies by Liedtke (2013d and 2013e) it was clearly indicated that the validity criteria would not be applied in a way they are applied to the standard studies. However, the available raw data should enable to check the variability of specific growth rates in the control cultures for respective intervals, as they should not be too variable, also in the study performed under modified exposure. Being aware of the differences between the standard and modified exposure studies, the zRMS have not indicated that such a calculation is mandatory, but should be performed for illustrative purposes. The information presented in the study report was not detailed enough and for this reason no such an illustrative calculation was possible, so the variability of the specific growth rates in controls remains unknown and for this reason the results cannot be considered to be fully reliable. It is also noted that concerns regarding reliability of the obtained results were not the main reasons for the studies rejection (for details, see comments of the zRMS to the summaries of studies in Appendix 2).

With regard to the approach taken for calculation of the endpoints the zRMS would like to point out that there are certain rules set by the EFSA aquatic guidance (2013) and these should be followed when such studies are designed. In case non-standard approach being against the guidance recommendations is taken – its acceptability should be first consulted with the authorities in order to avoid rejection of the studies during their evaluation.

It should be also noted that currently extensive discussion on Tier 2C studies is carried out at the EU, zonal and national level due to high level of uncertainty. Multiple countries do not accept results of these studies at all, even if performed correctly. Taking this into account, novel design of Tier 2C studies increases the level of uncertainty, which cannot be accounted for as there are no criteria against which such studies could be checked. In addition to that, the risk assessment scheme based on results of Tier 2C studies has been developed with assumption of certain endpoints and it is not known what criteria should be taken when the required endpoints are not available or cannot be calculated due to the test design.

Overall, the zRMS would recommend to first perform the Tier 2C studies fully in line with requirements of the EFSA aquatic guidance (2013) and then consider non-standard approaches, if necessary. Otherwise, there is a high risk of rejection of studies not compliant with current requirements. As already indicated above, non-standard test design should be always consulted with the authorities before the test initiation.

As studies by Liedtke (2013d and 2013e) were not performed in line with current requirements and due to the test design it was not possible to calculate endpoints relevant for the risk assessment, the zRMS maintains the decision, that the studies are not relevant for purposes of the risk refinement.

Discussion on relevance of less sensitive species used in the pulsed-exposure studies

With reference to the argumentation provided under Point 2A, the Applicant is of the opinion that the comparable ´recovery´ is still a valid justification. However, and independent of the resilience of the test species, both species differ only by a factor of about 5.1 with regard to median effective concentrations (ErC50 of 4.7 vs. 0.918 µg a.s./L for D. subspicatus and A. falcatus, respectively) and are therefore in the same order of magnitude regarding sensitivity. Desmodesmus actually was among the most sensitive genera tested.
Therefore, and with reference to the conservative Assessment Factor applied which allows for inter-species extrapolation, Desmodesmus is considered to be an appropriate test species for revised exposure testing with sufficient protectiveness for the other test species.

With regard to the species tested, it was already explained in the commenting box in point 9.5.1.1, that zRMS would not expect the pulsed-exposure study with non-standard test species such as *A. falcatus*, for which the standard conditions of the study were not developed within OECD 201 and the prolonged study would result with too high variability of specific growth rates in the control cultures.
Nevertheless, there was a standard species more sensitive to flurochloridone (*P. subcapitata*) and in opinion of the zRMS this species should have been used in the pulsed-exposure studies as representing worst case.

It was also clearly explained in commenting box in point 9.5.1.1 that higher sensitivity of other species would be accounted for in AF applied to the endpoint. However, in absence of the required endpoints no relevant RAC could be calculated and due to the non-standard test design it is not fully clear, if the approach taken by the Applicant would be protective also for more sensitive taxa, as there are no criteria against which this non-standard approach could be validated.

Additional analysis of the exposure regime in the pulsed exposure study by Liedtke (2013d) in comparison to R1 stream exposure profile

Please refer to Point 2 E of the expert statement.
Tier 2C Risk assessments
As was shown in the analysis by Ranke and Eck (2018), for all relevant FOCUS R stream scenarios for the zonal evaluation (i.e. R1, R2 and R3) the graphical analysis shows that the course of predicted exposure is well covered by the ETC conservatively applying an Assessment Factor of 10. As noted above, this approach is highly conservative.
As indicated by the zRMS, FOCUS modelling with VFSmod is acceptable for national risk assessments, the respective assessments and superposition of the ETC on predicted course of exposure is presented based on revised modelling for the relevant FOCUS R1 stream scenario. In the graph on page 12, the superposition of the ETC (i.e. the exposure pattern or 'NOAEP') from the study by Liedtke (2013d) over R1 stream scenario is presented. In deviation to the original analysis by Ranke and Eck (2018) applying the Tier 1 RAC of 0.47 µg a.s./L as threshold level for critical scenario definition, in a more conservative approach, a threshold level of 0.1 µg a.s./L is selected. This threshold corresponds with the rounded RAC based on ErC50 for the most sensitive test species (i.e. A. falcatus).
Accordingly, the single maximum exposure peak of FOCUS R1 stream at 10 m vegetated buffer distance is well covered by the exposure tested in the study by Liedtke.
In conclusion, an acceptable risk for algae exposed to AG-F8-250 CS in potatoes for national registration in Poland is presented based on Tier 2C risk assessment, if a 10 m vegetated buffer distance is respected.

As already indicated in the zRMS comments to the analysis of Ranke & Eck (2018, KCP 10.2.3/14), the general concept of comparison of exposure profiles in FOCUS scenarios and exposure regime in the pulsed exposure studies was agreed. It was noted that the detailed assessment was not possible as the exposure profiles from the FOCUS modelling were not presented in Ranke (2018) and only general summary graph including all scenarios was available.

The detailed exposure profiles are now presented in the new surface water modelling by Weber & Jarvis (2020) submitted during the commenting period. Nevertheless, no detailed analysis was performed by the zRMS, as based on results of Step 4 modelling performed using VFSmod acceptable risk to algae could be concluded in all R scenarios based on Tier 1 toxicity data, provided that respective vegetated filter strips are respected.

The additional analysis presented in the position paper does not resolve the issue of exposure regime in the studies which have not covered the exposure profiles in D scenarios, for which unacceptable risk remains.

Discussion on AUC approach in the higher tier risk assessment

Please refer to Point 2 F of the expert statement.
With reference to Point 2A above, rating the deviation and re-establishment of algal cell densities from baseline densities within a biological context, the short-term algistatic effects, neither those of the extended standard tests with multiple species, nor of the pulsed-exposure studies are considered to represent ecologically relevant observations. The course of exposure in the revised exposure studies by Liedtke (2013d/e) are therefore considered to represent No Observed Effect Patterns. As elaborated above, the application of an AF of 10 in order to generate an Ecotoxicological Trigger Curve is overly conservative.
This overall conservatism is further supported by comparison of AUC. The AUC approach is considered to be an adequate way to set the field exposure and the exposure in the Tier 2C study into context, in this case clearly showing that the tested exposure scenario by far exceeds the cumulative exposure of the respective FOCUS R scenarios.

Assessment is presented for R1 stream scenario with 20m vegetated buffer distance and with 10 vegetated buffer distance (VFSmod). In conclusion, the assessment based on AUC further supports the conclusion of an acceptable risk for algae exposed in accordance with the relevant FOCUS R1 stream scenario if a 10 m vegetated buffer distance is respected.

The Applicant indicates that the exposure regimes in studies by Liedtke (2013d and 2013e) represent the No Observed Effects Patterns. The zRMS respectfully disagrees with this statement, as at some time points >50% effect was seen on the tested species, which was, however, not taken into account by the Applicant as this was followed by recovery. However, in opinion of the zRMS it cannot be concluded that no effects were observed in the study, even if they were followed by recovery, as actually they were quite severe.

Furthermore, recovery is not an option at Tier 1 and Tier 2, in line with the EFSA aquatic guidance (2013) and endpoints (or approaches) based on recovery cannot be accepted in refinement of the risk performed at Tier 2.

Further the zRMS would like to point out that the AUC concept is not indicated in EFSA aquatic guidance (2013) as relevant for the Tier 2C regulatory risk assessment purposes. Even though the Applicant argues that the AUC concept is even more conservative than the approach required by EFSA (2013), it is noted that no criteria enabling its validation exist and therefore no reliable conclusion may be taken by the regulators.

Furthermore, the approach proposed by the Applicant includes recovery, which is not an option at Tier 1and Tier 2 regulatory risk assessment and is also not accepted in the Central Zone and in Poland for Tier 3 assessments.

Overall, the zRMS maintains the decision that the Applicants' novel approach in the risk assessment based on comparison of the AUC in the pulsed exposure studies and FOCUS exposure profiles is not acceptable, as it includes recovery and no criteria enabling its validation exist.

Overall, the risk to algae from flurochloridone following application of AG-F8-250 CS remains unresolved in D4 and D6 scenarios.

Additional modelling performed with consideration of wider buffer zones would not address the risk in D scenarios, where the exposure is driven by drainage and currently there are no efficient mitigation measures enabling reduction of the exposure. The only option would be reduction of the application rate, which potentially may not be possible from the efficacy point of view.

Comments on additional information provided by the Applicant (31.08.2021):

The zRMS appreciates additional risk assessment provided by the Applicant, however the studies by Liedtke (2013d and 2013e) were extensively evaluated in the course of the first assessment of AG-F8-250 CS and decision of rejection was carefully taken with consideration of all available indications regarding evaluation of such studies and requirements they must fulfil to be used in the risk assessment. In addition to that, the approach taken in the risk assessment based on the pulsed-exposure studies has been evaluated in detail, which may be seen from the extent discussion provided above.

Detailed information regarding reasons for rejection of peak-exposure studies and the risk assessment based on thier results is provided in the comments above as well as in the zRMS evaluation of the studies provided in Appendix 2 and is not repeated here. The zRMS maintains its conclusions regarding acceptability of the peak-exposure studies performed with flurochloridone and their consideration in the risk assessment.

The risk to algae from flurochloridone in scenario D4 remains unresolved for higher application rate of 500 g a.s./ha.

Scenario D6 was not considered further as being not relevant for Poland.

~~Potentially, the risk in R scenarios could be further refined with STEP 4 PEC_{sw} values calculated using VFSmod, which is currently acceptable in Poland (but may be not accepted in other MS). However, this would not address the risk in D scenarios, where the exposure is driven by drainage and currently there are no efficient mitigation measures enabling reduction of the exposure. The only option would be reduction of the application rate, which potentially may not be possible from the efficacy point of view.~~

Degradation products/metabolites of flurochloridone:

The risk assessments for the relevant degradation products are presented in the following tables.

Table 9.5-7: Aquatic organisms: acceptability of risk (PEC/RAC < 1) for R42819 for each organism group based on FOCUS Steps 1, 2 calculations for the use of AG-F8-250 CS in potato (pre-emergence)

Group		**Algae**	**Aquatic macrophytes**
Test species		***S. subspicatus***	***L. gibba***
Endpoint (µg/L)		E_rC_{50} 2300	E_rC_{50} 8200
AF		10	10
RAC (µg/L)		230	820
FOCUS Scenario	**PEC $_{gl\text{-}max}$ (µg/L)**		
Step 1			
	75.00	0.33	0.091
Step 2			
S-Europe			

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

Table 9.5-8: Aquatic organisms: acceptability of risk (PEC/RAC < 1) for R406639 for each organism group based on FOCUS Steps 1, 2 calculations for the use of AG-F8-250 CS in potato (pre-emergence)

Group		**Algae**
Test species		***S. subspicatus***
Endpoint (µg/L)		E_rC_{50} 3300
AF		10
RAC (µg/L)		330
FOCUS Scenario	**PEC $_{gl\text{-}max}$ (µg/L)**	
Step 1		
	20.71	0.063
Step 2		
S-Europe		

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

zRMS comments:

The risk assessment for flurochloridone metabolites is agree by the zRMS. Based on performed calculations acceptable risk to most sensitive species may be concluded from these compounds.

9.5.3 Overall conclusions

~~An acceptable risk for aquatic organisms is indicated if a 20 m vegetated buffer distance is accounted for and if drainage entry into surface water bodies is excluded.~~

zRMS comments:

Based on the performed evaluation acceptable acute and chronic risk following application of AG-F8-250 CS at 500 g a.s./ha could be concluded for fish, *Daphnia magna*, sediment-dwellers and aquatic macrophytes with no need for risk mitigation measures, with exception of aquatic macrophytes in R3 scenario, where 10 m vegetated filter strip was required in order to demonstrate acceptable risk.
Available data were sufficient to demonstrate acceptable risk to algae in all R scenarios provided that in R1 scenario 10 m vegetated filter strip is respected, while in R2 and R3 scenarios 20 m vegetated filter strip is applied. In scenario D3 10 meters unsprayed buffer zone to surface water bodies is required.
However, available data were not sufficient to demonstrate acceptable risk to algae in D4 and D6 scenarios from application of higher rate of 500 g a.s./ha, which remains thus unresolved.

Additional modelling performed with consideration of wider buffer zones would not address the risk in D scenarios, where the exposure is driven by drainage and currently there are no efficient mitigation measures enabling reduction of the exposure. The only option would be reduction of the application rate, which potentially may not be possible

from the efficacy point of view.

Performed evaluation demonstrated acceptable risk to aquatic organisms from application of AG-F8-250 CS to potatoes at application rate of 375 g a.s./ha, provided that 10 m vegetated filter strip from surface water bodies is respected.

~~Potentially, the risk in R scenarios could be further refined with STEP 4 PEC_{sw} values calculated using VFSmod, which is currently acceptable in Poland (but may be not accepted in other MS). However, this would not address the risk in D scenarios, where the exposure is driven by drainage and currently there are no efficient mitigation measures enabling reduction of the exposure. The only option would be reduction of the application rate, which potentially may not be possible from the efficacy point of view.~~

9.6 Effects on bees (KCP 10.3.1)

9.6.1 Toxicity data

Studies on the toxicity to bees have been carried out with flurochloridone. Full details of these studies are provided in the respective EU DAR and related documents.

Effects on bees of formulation AG-F8-250 CS were evaluated based on data on the technical active substance as part of the EU assessment of flurochloridone. AG-F8-250 CS was a representative formulation in the EU review of flurochloridone. The intended use pattern for the core assessment is within the use pattern considered for EU review. Therefore appropriate assessments for bees to exposure from AG-F8-250 CS were evaluated as part of the EU review of flurochloridone, where all study references and conclusions can be found.

The selection of studies and endpoints for the risk assessment is in line with the results of the EU review process. However, additional studies are provided in line with recent data requirements.

Table 9.6-1: Endpoints and effect values relevant for the risk assessment for bees

Species	Substance	Exposure System	Results	Reference
Apis mellifera	flurochloridone	Oral	**LD_{50} > 100 µg a.s./bee**	EFSA Scientific Report 2010 (Bocksch S. 2003)
Apis mellifera	flurochloridone	Contact	**LD_{50} > 100 µg a.s./bee**	EFSA Scientific Report 2010 (Bocksch S. 2003)
Apis mellifera	Flurochloridone 250 EC	Chronic	LDD_{50} > 97.9 µg product/bee/day	KCP 10.3.1.2 (Molitor A.M. 2017a)
Apis mellifera	Flurochloridone 250 EC	Bee larvae	22d NOED = 15.4 µg product/larva per developmental period [a)]	KCP 10.3.1.3 (Molitor A.M. 2017b)

[a)] Based on the cumulative feeding volume from day 3 until day 6 of 140 µL (T1-T4, R) or from day 3 until day 4 of 50 µL (T5) diet/ larva and a density of the diet of 1.1 g/cm^3
bold: endpoints relevant for risk assessments

zRMS comments:

Data on toxicity of the active substance to bees provided in Table 9.6-1 are in line with EU agreed endpoints reported in EFSA Journal 2010;8(12):1869.

Studies on acute toxicity of the formulated product to bees were deemed not necessary as it is possible to extrapolate from the active substance data.

Additional studies on chronic effects of the formulated product to adult bees and larvae listed in Table 9.6-1 were evaluated by the zRMS and considered acceptable. The reported endpoints are confirmed. Summary of the performed studies together with zRMS evaluation may be found in Appendix 2.

It is noted that the additional studies were performed with EC formulation and not CS formulation, for which the authorisation is sought. Nevertheless, in case of chronic and larvae toxicity studies the test organisms are exposed during several consecutive days to freshly prepared doses, so the type of formulation is of lesser importance, especially EC formulations are usually most toxic. In addition to that the content of flurochloridone in the tested EC formulation and AG-F8-250 CS is the same.

9.6.1.1 Justification for new endpoints

Risk assessments are based on the EU agreed endpoints for acute effects.

In addition to the EU agreed endpoints and in order to fulfil recent data requirements, new endpoints are provided for chronic adult and larval toxicity for the formulated product AG-F8-250 EC. These studies are required according to Regulation (EC) No. 284/2013. No studies are available for the CS formulation. However, data for the EC formulation are considered to be protective. For the encapsulated product type, the active substance release is slower. As in case of chronic bee and bee larval testing continuous dosing or repeated dosing, respectively is performed, the actual exposure of bees over the relevant exposure time (10 days or 22 days, respectively) is assumed to be either comparable or less severe in case of the CS type of product.

However, in the absence of noted guidance on risk assessments based on chronic bee and bee larval toxicity data, risk assessments are presented based on acute data as available for the technical active.

zRMS comments:

No new studies with the active substance were performed so no new endpoints were considered.

Studies performed with the formulated product were required in order to fulfil the data requirements set by the Commission Regulation (EU) No 284/2013.

9.6.2 Risk assessment

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)[8] for the maximum single application rate of 0.50 kg a.s./ha.

9.6.2.1 Hazard quotients for bees

No test was conducted with the actual formulated product AG-F8-250 CS. Risk assessments were based on the data for the active substance (Bocksch, 2003).

Table 9.6-2: First-tier assessment of the risk for bees due to the use of formulation in crop (bare soil/potato)

Intended use	pre-emergence, potato		
Active substance	flurochloridone		
Application rate [g/ha]	1 × 500		
Test design	**LD_{50} (lab.) (µg/bee)**	**Single application rate (g/ha)**	**Q_{HO}, Q_{HC} criterion: $Q_H \leq 50$**
Oral toxicity	> 100	500	< 5.0
Contact toxicity	> 100	500	< 5.0

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

The Hazard Quotients for acute oral and contact toxicity based on the data for the active substance flurochloridone are below the trigger indicating a low and acceptable risk for bees from exposure to AG-F8-250 CS in accordance with the worst-case use pattern.

zRMS comments:

[8] European Commission. Health & Consumer Protection Directorat – General (2002). Draft Working Document. Guidance Document on Terrestrial Ecotoxicology Under Council Directive 91/414/EEC. SANCO/10329/2002 rev.

The acute risk assessment for bees presented in Table 9.6-2 is agreed by the zRMS.

Based on performed calculations acceptable risk to bees may be concluded from the intended uses of AF-F8-250 CS.

No study on acute toxicity of the formulation was available, however in case of the solo formulation the risk assessment based on the active substance data is considered sufficient.

Please note that the evaluation has been performed in line with SANCO/10329/2002 rev 2 final, as according to conclusions of the Central Zone Steering Committee (CZSC), recommendations of EFSA (2013) should not be considered for the zonal evaluations until the guidance is noted at the EU level. Therefore risk assessment based on indications of EFSA (2013) must be performed at the national level by cMS that do require such evaluation.

9.6.2.2 Higher-tier risk assessment for bees (tunnel test, field studies)

Not relevant.

9.6.3 Effects on bumble bees

No data available and considered necessary.

9.6.4 Effects on solitary bees

No data available and considered necessary.

9.6.5 Overall conclusions

AG-F8-250 CS was a representative formulation in the EU review of flurochloridone. The intended use pattern for the core assessment is within the use pattern considered for EU review. Therefore appropriate assessments for bees to exposure from AG-F8-250 CS were evaluated as part of the EU review of flurochloridone, where all study references and conclusions can be found.

Risk assessments with Hazard Quotients for both, acute oral and contact toxicity are below the trigger indicating a low and acceptable risk for bees from exposure to AG-F8-250 CS in accordance with the worst-case use pattern.

9.7 Effects on arthropods other than bees (KCP 10.3.2)

9.7.1 Toxicity data

Effects on non-target arthropods of formulation AG-F8-250 CS were evaluated as part of the EU assessment of flurochloridone.

The selection of studies and endpoints for the risk assessment is in line with the results of the EU review process. Full details of these studies are provided in the EU DAR.

Table 9.7-1: Endpoints and effect values relevant for the risk assessment for non-target arthropods

Species	**Substance**	**Exposure System**	**Results**	Reference
Typhlodromus pyri (protonymphs)	AG-F8-250 CS formulation containing 250 g a.s./L (nominal)	Laboratory test glass plates (2D)	LR_{50} > 1000 g a.s./ha **ER_{50} > 1000 g a.s./ha**	EFSA Scientific Report 2010 (Adelberger, I. 1999)
Aphidius rhopalosiphi (adults)	AG-F8-250 CS formulation containing 250 g a.s./L (nominal)	Laboratory test glass plates (2D)	LR_{50} > 1000 g a.s./ha **ER_{50} > 1000 g a.s./ha**	EFSA Scientific Report 2010 (Schuld, M. 1999)

bold: endpoints relevant for risk assessments

zRMS comments:

As AG-F8-250 CS was a representative formulation during the EU review, toxicity data for non-target arthropods reported in Table 9.7-1 were taken from EFSA Journal 2010;8(12):1869 and are confirmed to be correct.

The studies were performed with the representative formulation Racer 25 CS (Flurochloridone 25 CS). As already mentioned in the introductory part of this document, there are only minor differences between the composition of the representative formulation and current composition of AG-F8-250 CS (Racer 250 CS) and for this reason endpoints derived at the EU level may be used in support of evaluation of the current formulation.

9.7.1.1 Justification for new endpoints

Risk assessments are based on the EU agreed endpoints for 2D laboratory studies.

In addition to the EU agreed endpoints and in order to fulfil recent data requirements, new endpoints are provided for extended laboratory test on barley leaves for the formulated actual product AG-F8-250 CS. These studies are required according to Regulation (EC) No. 284/2013. The data for the product are considered relevant for the risk assessment.

zRMS comments:

Although additional extended laboratory study is mentioned by the Applicant in the text above, no such study has been submitted in support of this zonal evaluation or used in the risk assessment.
However, as acceptable risk could be concluded using Tier I data, no further evaluation was deemed necessary and the study mentioned was thus not requested from the Applicant.

9.7.2 Risk assessment

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[9].

Risk assessments are presented for the intended pre-emergence uses in potato at the maximum single use rate of 500 g product/ha (overall risk envelope; refer to Table 9.1 2).

9.7.2.1 Risk assessment for in-field exposure

Effects of the formulation AG-F8-250 CS were assessed with *Aphidius rhopalosiphi* and *Typhlodromus pyri* (standard laboratory test).

Table 9.7-2: First- tier assessment of the in-field risk for non-target arthropods due to the use of AG-F8-250 CS in potato (Pre-emergence)

Intended use	Pre-emergence, potato		
Active substance/product	AG-F8-250 CS		
Application rate [g/ha]	1 x 500 g/ha		
MAF	1		
Test species (Laboratory testing)	**L/ER_{50} (lab.) (g/ha)**	**$PER_{in\text{-}field}$ (g/ha)**	**$HQ_{in\text{-}field}$ criterion: HQ ≤ 2**
Typhlodromus pyri	> 1000	500	< 0.50
Aphidius rhopalosiphi	> 1000	500	< 0.50

MAF: Multiple application factor; PER: Predicted environmental rate; HQ: Hazard quotient;. Criteria values shown in bold breach the relevant trigger.

zRMS comments:

The in-field risk assessment presented in Table 9.7-2 above is agreed by the zRMS.

On the basis of the performed calculations acceptable in-field risk for non-target arthropods may be concluded from the intended uses of AG-F8-250 CS.

9.7.2.2 Risk assessment for off-field exposure

The exposure and risk assessment of non-target arthropods to AG-F8-250 CS in the off-field area presented below for the default distance (i.e. 1 m) to the field edge was calculated following ESCORT 2 guidance.

[9] Candolfi MP, Barrett KL, Campbell PJ, Forster R, Grandy N, Huet M-C, Lewis G, Oomen PA, Schmuck R, Vogt H (2000) 'Guidance Document on regulatory testing procedures for plant protection products with non-target arthropods' From the work-shop, European Standard Characteristics of Non-target Arthropod Regulatory Testing (ESCORT 2) 21-23 March 2000.

Table 9.7-3: First- and higher-tier assessment of the off-field risk for non-target arthropods due to the use of AG-F8-250 CS in potato (Pre-emergence)

Intended use		Pre-emergence, potato				
Active substance/product		AG-F8-250 CS				
Application rate [g/ha]		1 x 500 g/ha				
MAF		1				
vdf		10 (Tier 1)				
Test species Tier I	**LR_{50} (lab.) [g/ha]**	**Drift value [%]**	**Drift factor**	**$PER_{off\text{-}field}$ [g/ha]**	**CF**	**$HQ_{off\text{-}field}$ criterion: HQ ≤ 2**
Typhlodromus pyri	> 1000	2.77	0.0277	1.385	10	<0.014 ~~<0.0013~~
Aphidius rhopalosiphi	> 1000					<0.014 ~~<0.0013~~

MAF: Multiple application factor; vdf: Vegetation distribution factor; (corr.) PER: (corrected) Predicted environmental rate; CF: Correction factor; HQ: Hazard quotient. Criteria values shown in bold breach the relevant trigger.

Unacceptable effects on arthropods are not expected in the off-crop area without the consideration of risk mitigation measures; i.e. for the default distance of 1 m, as shown in the table above.

zRMS comments:

The off-field risk assessment presented in Table 9.7-3 above is agreed by the zRMS. It seems, however, that by mistake in performed calculations the correction factor was not considered and HQ values were thus amended by the zRMS accordingly.

Overall, based on the corrected calculations acceptable off-field risk for non-target arthropods may be concluded from the intended uses of AG-F8-250 CS with no need for risk mitigation measures.

9.7.2.3 Additional higher-tier risk assessment

Not relevant.

9.7.2.4 Risk mitigation measures

No risk mitigation needed.

9.7.3 Overall conclusions

The Hazard Quotients for both, in-field as well as off-field risk, are below the trigger indicating low and acceptable risk for terrestrial arthropods other than bees from exposure to AG-F8-250 CS in accordance with the worst-case use pattern.

9.8 Effects on non-target soil meso- and macrofauna (KCP 10.4)

9.8.1 Toxicity data

Studies on the toxicity to earthworms have been carried out with flurochloridone. Full details of these studies are provided in the respective EU DAR and related documents.

Effects on earthworms of formulation AG-F8-250 CS were evaluated as part of the EU assessment of flurochloridone.

In addition to the data evaluated during EU review of the active substance flurochloridone, the notifier has conducted a new 14 day acute study with the active substance (technical grade) on *Eisenia foetida* for confirmatory data purpose. Whereas acute data are no longer required, the study is nevertheless included here for information. Fulfilling recent data requirements, additional data are made available on *Hypoaspis aculeifer* and *Folsomia candida*.

The selection of studies and endpoints for the risk assessment is in line with the results of the EU review process.

Table 9.8-1: Endpoints and effect values relevant for the risk assessment for earthworms and other non-target soil organisms (meso- and macrofauna)

Species	Substance	Exposure System	Results	Reference
~~*Eisenia fetida*~~	~~AG-F8-250 CS CS formulation containing 22.61% a.s. (w/w)~~	~~Mixed into substrate 14 d, acute 10 % peat content~~	~~14 d **LC_{50} corr. = 90 mg a.s./kg d.w. soil** a)~~	~~EFSA Scientific Report 2010 (Jackson, D. and Bembridge, J. D., 1995)~~
~~*Eisenia fetida*~~	~~flurochloridone~~	~~Mixed into substrate 14 d, acute 10 % peat content~~	~~14 d **LC_{50} corr. = 227 mg a.s./kg d.w. soil** a)~~	~~EFSA Scientific Report 2010 (Ellgehausen, H., 1985)~~
Eisenia fetida	AG-F8-250 CS formulation containing 250 g a.s./L (nominal)	Overspray 56 d, chronic 5 % peat content	56 d **NOEC corr. = 5 mg a.s./kg d.w. soil** a)	EFSA Scientific Report 2010 (Bätscher, R, 2004)
Hypoaspis aculeifer	AG-F8-250 CS formulation	Mixed into substrate 14 d, chronic 5 % peat content	14 d NOEC (reproduction) = 500 mg product/kg d.w. soil i.e. 14 d **NOEC corr. = 56.2 mg a.s./kg d.w. soil** a) b)	KCP 10.4.2.1. (Geary, N. 2017b)
Folsomia candida	AG-F8-250 EC formulation	Mixed into substrate 28 d, chronic 5 % peat content	28 d NOEC (reproduction) = 125 mg product/kg d.w. soil i.e. 28 d **NOEC corr. = 15.3 mg a.s./kg d.w. soil** a) c) ~~28 d NOEC (reproduction) = 250 mg product/kg d.w. soil i.e. 28 d **NOEC corr. = 30.6 mg a.s./kg d.w. soil** a) c)~~	KCP 10.4.2.1. (Geary, N. 2017a)

a) Corrected value derived by dividing the endpoint by a factor of 2 due to reduced peat content used for testing and/or log P_{OW} <2 in accordance with SANCO/10329/2002 rev.[10]; endpoint correction in line with recent EFSA Technical Report (2015)[11] conservatively was performed regardless of peat content used for ecotoxicological testing.
b) based on an analysed active substance content of 247.1 g/L and a product density of 1.1 g/mL
c) based on an analysed active substance content of 251.6 g/L and a product density of 1.028 g/mL
bold: endpoints relevant for risk assessments

[10] European Commission. Health & Consumer Protection Directorate – General (2002). Draft Working Document. Guidance Document on Terrestrial Ecotoxicology Under Council Directive 91/414/EEC. SANCO/10329/2002 rev.
[11] 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.

zRMS comments:

The acute toxicity values for earthworms given in Table 9.8-1 are in line with EU agreed endpoints reported in EFSA Journal 2010;8(12):1869. However, as acute toxicity to earthworms is no longer a data requirement, the acute endpoints are struck through as not considered in the risk assessment.

The chronic toxicity of AG-F8-250 CS to earthworms was investigated at the EU level and endpoint provided in Table 9.8-1 is in agreement with value reported in EFSA Journal 2010;8(12):1869. The study was performed with the representative formulation Racer 25 CS (Flurochloridone 25 CS). As already mentioned in the introductory part of this document, there are only minor differences between the composition of the representative formulation and current composition of AG-F8-250 CS (Racer 250 CS) and for this reason endpoints derived at the EU level may be used in support of evaluation of the current formulation.

No endpoints for soil macro- and meso-fauna are available from the EU review for the active substance or its relevant soil metabolites, therefore in order to fulfil the data requirements the Applicant performed additional studies on toxicity of AG-F8-250 CS to *Hypoaspis aculeifer* and AG-F8-250 EC to *Folsomia candida*. The studies were evaluated by the zRMS and considered acceptable. Respective summaries of the studies may be found in Appendix 2.
It is noted that the study on toxicity to F. candida was performed with EC formulation and not CS formulation, for which the authorisation is sought. Nevertheless, in case of EC formulation the test organisms are exposed to the whole concentration of the active compound immediately after introduction, while in case of CS formulations the release of the active compound is slower. Taking this into account it is concluded that the study for EC formulation is sufficiently protective and may be used in the risk assessment for CS formulation, especially endpoints are expressed in terms of the active substance and content of flurochloridone in both formulations is the same.

All endpoints were corrected due to flurochloridone log Pow >2, in line with EFSA Supporting publication 2015:EN-924.

9.8.1.1 Justification for new endpoints

Risk assessments for earthworms are based on the EU agreed endpoints for the active substance and the formulated product.

Acute data on earthworms are no longer required in accordance with EU Regulations EC 283/2013 and EC 284/2013. In a comprehensive approach and with acute data available on earthworms, however, respective assessments are presented here.

In addition, fulfilling recent data requirements, new endpoints are provided on *Hypoaspis aculeifer* for the formulated actual product AG-F8-250 CS and on *Folsomia candida* for the formulated product AG-F8-250 EC. These studies are required according to Regulation (EC) No. 284/2013. The data for the product are considered relevant for the risk assessment. No study on *Folsomia* is available for the CS formulation. However, data for the EC formulation are considered to be protective. For the encapsulated product type, the active substance release is slower. Over the relevant exposure time (28 days) exposure is assumed to be either comparable or less severe in case of the CS type of product.

zRMS comments:

No new studies with the active substance or its metabolites were performed so no new endpoints were considered. Studies performed with the formulated product were required in order to fulfil the data requirements set by the Commission Regulation (EU) No 284/2013.

For details regarding derivation of the endpoints, please refer to point 9.8.1 above and Appendix 2.

9.8.2 Risk assessment

The evaluation of the risk for earthworms and other non-target soil organisms (meso- and macrofauna) 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).

9.8.2.1 First-tier risk assessment

The relevant PEC_{soil} for risk assessments covering the proposed use pattern are taken from Section 8 (Environmental Fate), Chapter 8.7.2, Table 8.7-3. According to the assessment of environmental-fate data, multi-annual accumulation in soil does not need to be considered for flurochloridone.

Table 9.8-2: First-tier assessment of the acute and chronic risk for earthworms and other non-target soil organisms (meso- and macrofauna) due to the use of AG-F8-250 CS in potato (pre-emergence)

Intended use	1 x 500 g/ha AG-F8-250 CS in pre-emergence potato		
~~**Acute effects on earthworms**~~			
~~**Product/active substance**~~	~~**LC_{50} [mg a.s./kg d.w. soil]**~~	~~**PEC_{soil} [mg/kg dw]**~~	~~**TER_a (criterion TER ≥ 10)**~~
~~flurochloridone~~	~~227~~	~~0.67~~	~~338.8~~
~~AG-F8-250 CS~~	~~90~~	~~0.67~~	~~134.3~~
Chronic effects on earthworms			
Product/active substance	**NOEC [mg/kg dw]**	**PEC_{soil} [mg/kg dw]**	**TER_{lt} (criterion TER ≥ 5)**
AG-F8-250 CS	5	0.6936 ~~0.67~~	7.2 ~~7.5~~
metabolite R406639	0.5	0.0069	72.5
metabolite R42819	0.5	0.0103	48.5

Chronic effects on other soil macro- and mesofauna				
Product/active substance	**Test species**	**NOEC [mg a.s./kg d.w. soil] [1)]**	**PEC_{soil} [mg/kg dw]**	**TER_{lt} (criterion TER ≥ 5)**
AG-F8-250 CS formulation	*Hypoaspis aculeifer*	56.2	0.6936 ~~0.67~~	81.0 ~~83.9~~
metabolite R406639	*Hypoaspis aculeifer*	5.62	0.0069	814.5
metabolite R42819	*Hypoaspis aculeifer*	5.62	0.0103	545.6
AG-F8-250 EC formulation	*Folsomia candida*	15.3 ~~30.6~~	0.6936 ~~0.67~~	22.1 ~~45.7~~
metabolite R406639	*Folsomia candida*	1.53	0.0069	221.7
metabolite R42819	*Folsomia candida*	1.53	0.0103	148.5

TER values shown in bold fall below the relevant trigger.
[1)] In absence of the experimental data, for metabolites 10 times toxicity of the parent was assumed

zRMS comments:

The risk assessment presented in Table 9.8-2 above has been amended with consideration of:

- higher $PEC_{soil,accu}$ calculated for flurochloridone in the course of evaluation in area of Section 8,
- lower NOEC value for *F. candida* agreed following evaluation of the study.

As no toxicity data were available for flurochloridone metabolites, the illustrative risk assessment for these compounds was performed with the worst case assumption of the toxicity being 10 times higher comparing to the parent compound. The zRMS is aware that available toxicity data were derived from studies performed with the formulated products, nevertheless consideration of endpoints expressed in terms of the active substance is considered sufficient for this illustrative risk assessment, especially no data gap in this area has been identified in the course of the EU review.

Overall, based on presented above calculations, acceptable risk to soil macro- and meso-fauna may be concluded following application of AG-F8-250 CS according to the Central Zone GAP.

The acute risk assessment presented in Table 9.8-2 above has been struck through as being no longer a data requirement.

9.8.2.2 Higher-tier risk assessment

Not relevant.

9.8.3 Overall conclusions

With TERs for chronic risk exceeding the relevant trigger values, the intended uses of AG-F8-250 CS do not pose an unacceptable risk to earthworms as well as soil meso- and macrofauna other than earthworms.

9.9 Effects on soil microbial activity (KCP 10.5)

9.9.1 Toxicity data

Studies on effects soil microorganisms have been carried out with flurochloridone. Full details of these studies are provided in the respective EU DAR and related documents.

Effects on soil microorganisms of formulation AG-F8-250 CS were evaluated based on data on the active substance as part of the EU assessment of flurochloridone.

The provision of further data on the formulation is not considered essential, because the application of AG-F8-250 CS had no significant effect on soil micro-organisms at 7.5 kg as./ha to soil nitrogen and carbon transformation (15 times higher than the maximum application rate of 0.500 kg as/ha).

The selection of studies and endpoints for the risk assessment is in line with the results of the EU review process.

Table 9.9-1: Endpoints and effect values relevant for the risk assessment for soil microorganisms

Endpoint	Substance	Exposure System	Results	Reference
N-mineralisation	flurochloridone	42 d, aerobic sand; clay	NOAER = 7500 g a.s./ha i.e. **NOAEC = 10.00 mg/kg d.w. soil** [a)] (<25 % effect at 42 days)	EFSA Scientific Report 2010 (Ellgehausen, H. 1986)
~~C-mineralisation~~	~~flurochloridone~~	~~14 d, aerobic soil type~~	~~NOAER = 7500 g a.s./ha i.e. **NOAEC = 10.00 mg/kg d.w. soil** [a)] (<25 % effect at 42 days)~~	~~EFSA Scientific Report 2010 (Ellgehausen, H. 1986)~~
N-mineralisation	AG-F8-250 CS	No study available	Not available	Not applicable
~~C-mineralisation~~	~~AG-F8-250 CS~~		~~Not available~~	~~Not applicable~~

NOAER/C: No Observed Adverse Effect Rate/Concentration; i.e. the maximum rate of concentration at which ≤ 25% effects on soil microflora functions were observed in the test

[a)] soil concentrations for 5 cm soil penetration depth and soil bulk density of 1500 kg/m^3 and for BBCH 00-09., i.e. without crop interception.

bold: endpoints relevant for risk assessments

zRMS comments:

Information regarding effects of flurochloridone on nitrogen mineralisation is in line with EU agreed data reported in EFSA Journal 2010;8(12):1869.

As AG-F8-250 CS is a solo formulation of flurochloridone separate studies with the formulated product were deemed not necessary as it is possible to extrapolate from the active substance.

Information regarding effects on carbon mineralisation is no longer a data requirement and for this reason is struck through in Table 9.9-1.

9.9.1.1 Justification for new endpoints

Risk assessments are based on the EU agreed endpoints.

9.9.2 Risk assessment

The evaluation of the risk for soil microorganisms 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). According to recent data requirements, only nitrogen transformation data are considered relevant. However, the assessments are also presented based on available soil respiration data as available.

The relevant PEC_{soil} for risk assessments covering the proposed use pattern are taken from Section 8 (Environmental Fate), Chapter 8.7.2, Table 8.7-3 and were already used in the risk assessment for earthworms and other non-target soil organisms (meso- and macrofauna) (see 9.8).

Table 9.9-2: Assessment of the risk for effects on soil micro-organisms due to the use of AG-F8-250 CS in potato (pre-emergence)

Intended use	1 x 500 g/ha AG-F8-250 CS in pre-emergence potato			
N-mineralisation				
Product/active substance	**NOAEC [mg a.s./kg d.w. soil]**	**PEC_{soil} [mg/kg dw]**	**Risk acceptable?**	**Margin of Safety**
flurochloridone	10.00 (at 42 d)	0.6936 ~~0.67~~	yes	14.4 ~~14.9~~
R406639	1.0	0.0069	yes	144.9
R42819	1.0	0.0103	yes	97.1
~~**C-mineralisation**~~				
~~**Product/active substance**~~	~~**NOAEC [mg a.s./kg d.w. soil]**~~	~~**PEC_{soil} [mg/kg dw]**~~	~~**Risk acceptable?**~~	
~~flurochloridone~~	~~10.00 (at 42 d)~~	~~0.67~~	~~yes~~	~~14.9~~

NOAER/C: No Observed Adverse Effect Rate/Concentration; i.e. the maximum rate of concentration at which ≤ 25% effects on soil microflora functions were observed in the test

zRMS comments:

The risk assessment for flurochloridone presented in Table 9.9-2 has been amended by the zRMS with consideration of PEC_{soil} values agreed in the course of evaluation in area of Section 8.

As effects of flurochloridone metabolites on soil microbial activity were not investigated in the course of the EU review, the illustrative risk assessment has been inserted by the zRMS based on worst case assumption of 10 times toxicity of the parent.

On the basis of the performed evaluation acceptable risk to soil micro-organisms from application of AG-F8-250 CS on bare soil may be concluded.

As investigation of effects on carbon mineralisation is no longer a data requirement, the evaluation performed for this parameter is struck through in Table 9.9-2.

9.9.3 Overall conclusions

An acceptable risk for soil microbial functions is indicated for the intended worst-case uses of AG-F8-250 CS by Predicted Environmental Concentrations lower than the No Observed Adverse Effect Concentrations (i.e. concentrations causing less than 25% effect on nitrogen transformation or carbon respiration after ≤ 100 days).

9.10 Effects on non-target terrestrial plants (KCP 10.6)

9.10.1 Toxicity data

Studies on the toxicity to non-target terrestrial plants have been carried out with the formulated product AG-F8-250 CS. Full details of these studies are provided in the respective EU DAR and related documents.

Effects on non-target terrestrial plants of formulation AG-F8-250 CS were evaluated as part of the EU assessment of flurochloridone.

The selection of studies and endpoints for the risk assessment is in line with the results of the EU review process. As the active substance flurochloridone is an herbicide, assessments are based on dose-response tests (Tier 2) on 6 plant species for seedling emergence and growth as well as vegetative vigour.

Table 9.10-1: Endpoints and effect values relevant for the risk assessment for non-target terrestrial plants

Species	Substance	Exposure System	Results	Reference
Most sensitive species (n= 6): *Brassica napus* d	AG-F8-250 CS flurochloridone 25 CS	21 d Vegetative vigour	**ER_{50}** (shoot fresh weight) **= 59 g a.s./ha**	EFSA Scientific Report 2010 (Fiebig 2003b)
Most sensitive species (n= 6): *Allium cepa* m	AG-F8-250 CS flurochloridone 25 CS	21 d Seedling emergence	**ER_{50}** (shoot fresh weight) **= 340 g a.s./ha**	EU DAR (Fiebig 2003a)

m: monocotyledonous; d: dicotyledonous
bold: endpoints relevant for risk assessments

zRMS comments:

Endpoints reported in Table 9.10-1 were taken from EFSA Journal 2010;8(12):1869 (vegetative vigour) and DAR of February 2006 (seedling emergence) and are confirmed to be correct.

The studies were performed with the representative formulation Racer 25 CS (Flurochloridone 25 CS). As already mentioned in the introductory part of this document, there are only minor differences between the composition of the representative formulation and current composition of AG-F8-250 CS (Racer 250 CS) and for this reason endpoints derived at the EU level may be used in support of evaluation of the current formulation.

No detailed information regarding the phytotoxicity of the tested formulation are available either in the LoEP or in the DAR. As re-evaluation of the studies already agreed at the EU level is not foreseen by the available guidance documents, issue of phytotoxicity was not considered further and endpoints as agreed at the EU level are used. Nevertheless, from the study summaries presented in the DAR it seems that phytotoxic effects were consistent with effects observed on shoot weight and height.

9.10.1.1 Justification for new endpoints

Risk assessments are based on the EU agreed endpoints.

9.10.2 Risk assessment

9.10.2.1 Tier-1 risk assessment (based on screening data)

Not relevant.

9.10.2.2 Tier-2 risk assessment (based on dose-response data)

The risk assessment is based on the "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.

The assessment is based on the intended worst-case use in pre-emergence potatoes at a single application rate of 500 g a.s./ha as well as for the reduced rate of 375 g a.s./ha (see 9.1.2). The following table shows the assessment for the default distance of 1 m.

Table 9.10-2: Assessment of the risk for non-target plants due to the use of AG-F8-250 CS in potato (pre-emergence)

Intended use	pre-emergence, potato
Active substance/product	AG-F8-250 CS
Application rate [g/ha]	1x 500
MAF	1

Test species	**ER_{50} [g/ha]**	**Drift value (%)**	**Drift factor**	**$PER_{off\text{-}field}$ (g/ha)**	**TER criterion: TER ≥ 5**
	Vegetative vigour				
Brassica napus	59	2.77 (90^{th} percentile)	0.0277	13.85	**4.3**
	Seedling emergence/growth				
Allium cepa	340	2.77 (90th percentile)	0.0277	13.85	24.6

MAF: Multiple application factor; PER: Predicted environmental rate; TER: toxicity to exposure ratio. TER values shown in bold fall below the relevant trigger.

In case of the risk envelope, potential risk is indicated at the default distance based on the assessment for vegetative vigour. Revised assessments applying risk mitigation measures are presented under Point 9.10.2.4 below.

Table 9.10-3: Assessment of the risk for non-target plants due to the use of AG-F8-250 CS in potato (pre-emergence)

Intended use	pre-emergence, potato
Active substance/product	AG-F8-250 CS
Application rate [g/ha]	1x 375
MAF	1

Test species	**ER_{50} [g/ha]**	**Drift value (%)**	**Drift factor**	**$PER_{off\text{-}field}$ (g/ha)**	**TER criterion: TER ≥ 5**
	Vegetative vigour				
Brassica napus	59	2.77 (90th percentile)	0.0277	10.39	5.7
	Seedling emergence/growth				
Allium cepa	340	2.77 (90th percentile)	0.0277	10.39	32.7

MAF: Multiple application factor; PER: Predicted environmental rate; TER: toxicity to exposure ratio. TER values shown in bold fall below the relevant trigger.

In case of the reduced rate of 1.5 L product/ha (corresponding to 375 g a.s./ha), an acceptable risk is indicated without the necessity to consider risk mitigations.

zRMS comments:

The risk assessment presented in table 9.10-2 is agreed by the zRMS.

Based on performed calculations acceptable risk may be concluded for seedling emergence from the maximum intended application rate (500 g a.s./ha) covering also lower rate of 375 g a.s./ha. The risk based on vegetative vigour endpoint is acceptable for the lower application rate of 375 g a.s./ha with no need for risk mitigation measures, while further evaluation is deemed necessary for higher application rate of 500 g a.s./ha ~~vegetative vigour~~ and is presented in point 9.10.2.4 below.

9.10.2.3 Higher-tier risk assessment

Not relevant.

9.10.2.4 Risk mitigation measures

In order to reduce the off-field exposure, risk mitigation measures can be implemented. These 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 for the intended worst-case uses using typical mitigation measures are summarised in the following table.

Table 9.10-3: Risk assessment for non-target terrestrial plants due to the use of AG-F8-250 CS in potato (pre-emergence) considering risk mitigation (in-field no-spray buffer zones, and drift-reducing nozzles)

Intended use		pre-emergence, potato		
Active substance/product		AG-F8-250 CS		
Application rate (g/ha)		1 x 500		
MAF		1		
Buffer strip [m]	**Drift value [%]**	**Drift factor**	**$PER_{off\text{-}field}$ [g/ha]**	**$PER_{off\text{-}field}$ 50 % drift red. [g/ha]**
1	2.77	0.0277	13.85	6.93
5	0.57 ~~0.59~~	0.0057 ~~0.0059~~	2.85 ~~2.95~~	1.43 ~~1.49~~
Toxicity value			**TER**	
ER_{50} = 59 g/ha			**criterion: TER ≥ 5**	
1			**4.3**	8.5
5			20.7 ~~20.0~~	-

MAF: Multiple application factor; PER: Predicted environmental rates; TER: toxicity to exposure ratio. Criteria values shown in bold breach the relevant trigger.

For the intended worst-case uses in potatoes, the risk to terrestrial non-target plants is indicated to be acceptable, provided a buffer distance of 5 m to the field edge is respected or alternatively if 50% drift reducing nozzles at the default distance are applied.

zRMS comments:

The risk assessment presented in table 9.10-3 is in general agreed by the zRMS.

It was, however, noted that by mistake not correct drift value has been considered for 5 m unsprayed buffer zone – it should be 0.57% instead of 0.59%. This mistake will have no impact on the derived conclusions, but the calculations in Table 9.10-3 were amended with consideration of the correct drift value for consistency.

Based on performed calculations acceptable risk may be concluded for non-target terrestrial plants provided that 5 m unsprayed buffer zone to non-agricultural land is respected or the spray drift is reduced by 50% using appropriate drift reducing techniques..

9.10.3 Overall conclusions

From the worst case application rate of 500 g a.s./ha an acceptable off-field risk is indicated for terrestrial non-target plants exposed towards AG-F8-250 CS in accordance with the intended worst-case use patterns in pre-emergence crops based on the data for vegetative vigour as well as seedling emergence and growth with the necessity to account for risk mitigation requirements as 5 m buffer distance or 50% drift reducing nozzles.
Acceptable risk with no need for risk mitigation measures could be concluded for lower intended application rate of 375 g a.s./ha.

9.11 Effects on other terrestrial organisms (flora and fauna) (KCP 10.7)

No further relevant effect data are available and to be considered.

9.12 Monitoring data (KCP 10.8)

No monitoring data are available and to be considered.

9.13 Classification and Labelling

The study testing toxicity of AG-F8-250 CS towards algae resulted in an endpoint < 1 mg/L (*Selenastrum capricornutum* E_rC_{50} = 0.197 mg a.s./L $_{mm}$, correct data as recalculated by the rapporteur and as presented in the DAR and in the final addendum). No chronic toxicity data for fish, crustacea and algae/aquatic plant are available. The active substance flurochloridone has a fish BCF of smaller than 500 (220 at steady-state and 292 at end of 28-day exposure); however, it is not readily biodegradable. Therefore, AG-F8-250 CS is classified by the hazard class and category "Aquatic Acute 1" and "Aquatic Chronic 1" under Regulation 1272/2008 and the hazard statements H400 and H410 apply.

zRMS comments:

The CLP classification of AG-F8-250 CS for the acute and chronic aquatic hazard is agreed by the zRMS.

It is noted that no justification for chronic aquatic hazard has been presented by the Applicant. According to information provided in the DAR of February 2006, the NOEC value of 0.019 mg a.s./L for algae was derived from study performed with the formulation AG-F8-250 CS. Although the endpoint expressed in terms of the formulation was not given, the rough estimation based on flurochloridone content of 22.7% in the formulated product indicates that the NOEC would be <0.1 mg product/L. As flurochloridone is not readily biodegradable, this endpoint is sufficient for classification of the formulation for chronic aquatic hazard in category 1 with hazard statement H410.

Based on the classification, the following phrases must be included in the label:

Hazard statement: H410
Signal word: Warning
Pictogram: GHS09
Safety phrases: P391, P501

Appendix 1 Lists of data considered in support of the evaluation

List of data submitted by the applicant and relied on

Data point	Author(s)	Year	**Title** **Company Report No.** **Source (where different from company)** **GLP or GEP status** **Published or not**	Vertebrate study Y/N	Owner*
KCP 10.2.3/01	Liedtke, A.	2013a	Flurochloridone technical: Toxicity to *Chlamydomonas reinhardtii* in a 72-Hour Algal Growth Inhibition Test Supplemented with Testing for Recovery of Growth Report No. D65727 (test facility report number); 90015442 (sponsor report number) Harlan Laboratories Ltd., Itingen, Switzerland GLP Unpublished	N	ADM
KCP 10.2.3/02	Liedtke, A.	2013b	Flurochloridone technical: Toxicity to *Chlorella vulgaris* in a 72-Hour Algal Growth Inhibition Test Report No. D65738 (test facility report number); 90015443 (sponsor report number) Harlan Laboratories Ltd., Itingen, Switzerland GLP Unpublished	N	ADM
KCP 10.2.3/03	Liedtke, A.	2013c	Flurochloridone technical: Toxicity to *Navicula pelliculosa* in a 72-Hour Algal Growth Inhibition Test Supplemented with Testing for Recovery of Growth Report No. D65740 (test facility report number); 90015444 (sponsor report number) Harlan Laboratories Ltd., Itingen, Switzerland GLP Unpublished	N	ADM
KCP 10.2.3/04	Scheerbaum, D.	2013a	Flurochloridone Technical – Alga, Growth Inhibition Test with *Pseudokirchneriella subcapitata*, 72 hours Report No. SPO15371 (test facility report number); 90015448 (sponsor report number) Dr. U. Noack-Laboratorien, Sarstedt, Germany GLP Unpublished	N	ADM
KCP 10.2.3/05	Scheerbaum, D.	2013b	Flurochloridone Technical – Alga, Growth Inhibition Test with *Nitzschia communis*, 72 hours Report No. SNC15371 (test facility report number); 90015449 (sponsor report number) Dr. U. Noack-Laboratorien, Sarstedt, Germany GLP Unpublished	N	ADM
KCP 10.2.3/06	Scheerbaum, D.	2013c	Flurochloridone Technical – Alga, Growth Inhibition Test with *Synechococcus leopoliensis*, 72 hours Report No. SSL15371 (test facility report number); 90015450 (sponsor report number) Dr. U. Noack-Laboratorien, Sarstedt, Germany GLP Unpublished	N	ADM

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner*
KCP 10.2.3/07	Scheerbaum, D.	2013d	Flurochloridone Technical – Alga, Growth Inhibition Test with *Chromulina nebulosa*, 72 hours Report No. SCN15371 (test facility report number); 90016462 (sponsor report number) Dr. U. Noack-Laboratorien, Sarstedt, Germany GLP Unpublished	N	ADM
KCP 10.2.3/08	Scheerbaum, D.	2013e	Flurochloridone Technical – Alga, Growth Inhibition Test with *Ankistrodesmus falcatus*, 72 hours Report No. SAF15371 (test facility report number); 90016463 (sponsor report number) Dr. U. Noack-Laboratorien, Sarstedt, Germany GLP Unpublished	N	ADM
KCP 10.2.3/9	Wenzel, A.	2015a	Freshwater Alga, Growth Inhibition Test Flurochloridone (technical): *Desmodesmus subspicatus* Toxicity Test - Testing for Recovery of Growth Report No. ADM-003/4-10/B (test facility report number); 90016481 (sponsor report number) Fraunhofer IME, Schmallenberg, Germany GLP Unpublished	N	ADM
10.3.1.2/01	Molitor, A.M.	2017	AG-F8-250 EC (Flurochloridone 250 EC) - Assessment of Effects on the Adult Honey Bee, *Apis mellifera* L., in a 10 Day Chronic Feeding Test under Laboratory Conditions Report No. S17-00282 (test facility report number); 90020495 (sponsor report number) Eurofins Agroscience Services EcoChem GmbH / Eurofins Agroscience Services Ecotox GmbH, Niefern-Öschelbronn, Germany GLP Unpublished	N	ADM
10.3.1.3/01	Molitor, A.M.	2018	AG-F8-250 EC (Flurochloridone 250 EC) - Honey Bee (*Apis mellifera* L.) 22 Day Larval Toxicity Test (Repeated Exposure) Report No. S17-00318 (test facility report number); 90020496 (sponsor report number) Eurofins Agroscience Services EcoChem GmbH / Eurofins Agroscience Services Ecotox GmbH, Niefern-Öschelbronn, Germany GLP Unpublished	N	ADM
10.4.2.1/01	Geary, N.	2017a	AG-F8-250 EC (Flurochloridone 250 EC) – A laboratory test to determine the effects of fresh residues on the springtail *Folsomia candida* (Collembola, Isotomidae) in an artificial soil substrate Report No. AGAN-17-26 (test facility report number); 90020545 (sponsor report number) Mambo-Tox Ltd., Southampton, UK GLP Unpublished	N	ADM

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner*
10.4.2.1/02	Geary, N.	2017b	AG-F8-250 CS (Flurochloridone 250 CS) – A laboratory test to determine the effects of fresh residues on the predatory soil mite *Hypoaspis aculeifer* (Acari, Laelapidae) Report No. AGAN-17-29 (test facility report number); 90020988 (sponsor report number) Mambo-Tox Ltd., Southampton, UK GLP Unpublished	N	ADM

*The sponsor company ADAMA Agan Ltd. (ADM) is a member of ADAMA Agricultural Solutions.
Under Article 59, Regulation 1107/2009/EC, the sponsor company claims data protection for these studies. For details on country specific data protection, refer to Part A.

List of data submitted or referred to by the applicant and relied on, but already evaluated at EU peer review

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
As all endpoints for flurochloridone and its metabolites as well as part of the studies with the representative formulation were taken from the EU review, for the list of respective studies please refer to the flurochloridone DAR.					

List of data submitted by the applicant and not relied on

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner*	Reason for rejection
KCP 10.2.2/01	Weber, B.	2012	TEST ITEM: Flurochloridone technical. Effect on Survival and Reproduction of *Daphnia magna* in a Semi-Static Test over Three Weeks Report No. D45354 (test facility report number); 90015011 (sponsor report number) Harlan Laboratories Ltd., Itingen, Switzerland GLP Unpublished	N	ADM	New active substance data, not required for the risk assessment.
KCP 10.2.3/10	Liedtke, A.	2013d	Flurochloridone (Trans isomer): Toxicity to *Desmodesmus subspicatus* in a Pulse Exposure Growth Inhibition Test Supplemented with Testing for Recovery of Growth Report No. D59890 (test facility report number); 90015421 (sponsor report number) Harlan Laboratories Ltd., Itingen, Switzerland GLP Unpublished	N	ADM	Study design not relevant to derive endpoints required by theEFSA aquatic guidance (2013).
KCP 10.2.3/11	Liedtke, A.	2013e	Flurochloridone (Trans isomer): Toxicity to *Desmodesmus subspicatus* in a Pulse Exposure Growth Inhibition Test Supplemented with Testing for Recovery of Growth Report No. D65547 (test facility report number); 90015432 (sponsor report number) Harlan Laboratories Ltd., Itingen, Switzerland GLP Unpublished	N	ADM	Study design not relevant to derive endpoints required by theEFSA aquatic guidance (2013).
KCP 10.2.3/12	Wenzel, A.	2015b	Macrophyte Pulse Exposure Growth Inhibition Test: Flurochloridone (trans-isomer): Sediment-free *Lemna minor* Toxicity Test - Testing for Recovery of Growth Report No. ADM-005/4-11/I (test facility report number); 90016482 (sponsor report number) Fraunhofer IME, Schmallenberg, Germany GLP Unpublished	N	ADM	New active substance data, not required for the risk assessment.
KCP 10.2.3/13	Wenzel, A.	2015c	Macrophyte Pulse Exposure Growth Inhibition Test: Flurochloridone (trans-isomer): Sediment-free *Myriophyllum spicatum* Toxicity Test - Testing for Recovery of Growth Report No. ADM-005/4-13/K (test facility report number); 90016483 (sponsor report number) Fraunhofer IME, Schmallenberg, Germany GLP Unpublished	N	ADM	New active substance data, not required for the risk assessment.
KCP 10.2.3/14	Ranke, J. and Eck, G.	2018	Pulsed exposure of algae following application of flurochloridone in Poland Eurofins Regulatory AG, Report No. jrwb-129; 000100958 (sponsor report number) GLP not applicable Unpublished	N	ADM	Approach in the risk assessment not in line with EFSA (2013).

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner*	Reason for rejection
KCP 10.2.3/15	Eck, G., Weber, D. and Jarvis T.	2020	Expert statement: Response to the evlauation of the Central Zone Rapporteur Member State (PL) on the algal risk assessment for AG-F8-250 CS Report No. 2000626.SW0-4623 Exponent International Ltd GLP not applicable Unpublished	N	ADM	Approach in the risk assessment not in line with EFSA (2013).

List of data relied on not submitted by the applicant but necessary for evaluation

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Owner
There were no studies relied on and not submitted by the Applicant.					

Appendix 2 Detailed evaluation of the new studies

A 2.1 KCP 10.1 Effects on birds and other terrestrial vertebrates

A 2.1.1 KCP 10.1.1 Effects on birds

A 2.1.1.1 KCP 10.1.1.1 Acute oral toxicity

No additional data submitted.

A 2.1.1.2 KCP 10.1.1.2 Higher tier data on birds

No additional data submitted.

A 2.1.2 KCP 10.1.2 Effects on terrestrial vertebrates other than birds

A 2.1.2.1 KCP 10.1.2.1 Acute oral toxicity to mammals

Please refer to Part B. Section 6 of this submission.

A 2.1.2.2 KCP 10.1.2.2 Higher tier data on mammals

No additional data submitted.

A 2.1.3 KCP 10.1.3 Effects on other terrestrial vertebrate wildlife (reptiles and amphibians)

No additional data submitted.

A 2.2 KCP 10.2 Effects on aquatic organisms

A 2.2.1 KCP 10.2.1 Acute toxicity to fish, aquatic invertebrates, or effects on aquatic algae and macrophytes

No additional data submitted.

A 2.2.2 KCP 10.2.2 Additional long-term and chronic toxicity studies on fish, aquatic invertebrates and sediment dwelling organisms

A 2.2.2.1.1 Study 1: Chronic toxicity to *Daphnia magna*

Comments of zRMS:	The reason for submission of this long-term study on toxicity of flurochloridone to Daphnia magna is unclear, as sufficient information is available in EFSA Journal 2010;8(12):1869 and the EU agreed endpoint is relevant for the risk assessment. In addition to that, results of the below study were not considered by the Applicant in the risk assessment. Therefore, in line with indications of SANCO/10326/2004, rev. 8 (2012), the study was not evaluated by the zRMS as providing new active substance data not necessary for the risk assessment. The study summary is thus struck through and shaded.

Reference:	KCP 10.2.2/01
Report	TEST ITEM: Flurochloridone technical. Effect on survival and reproduction of *Daphnia magna* in a semi-static test over three weeks, Weber, B., 2012, D45354 (report number), 90015011 (sponsor report number)
Guideline(s):	Yes, OECD 211 (2008)
Deviations:	No
GLP:	Yes
Acceptability:	Not evaluated, new active substance data not necessary for the risk assessment
Duplication (if vertebrate study)	-

~~**Materials and Methods**~~

~~**1. Test material** flurochloridone technical (=flurochloridone)~~
~~**Description** Not reported~~
~~**Lot/Batch #** 11083467~~
~~**Purity** 95.5%~~
~~**Stability of test material** Stable under storage conditions (original packaging, normal storage conditions)~~
~~Expiry date: 08/2013~~

~~**2. Vehicle and/or positive control** Vehicle control: test water~~

~~**3. Test organism**~~

~~**Species** Water flea (*Daphnia magna* Straus)~~
~~**Strain** Clone 5~~
~~**Source** Originally supplied by the University of Sheffield/UK in 1992 and since then bred successfully at the test site.~~
~~**Age** The daphnids used for the test originated from parental daphnids that were at least 14 days old but not older than four weeks and were not~~

first brood progeny. At the start of the test, the test animals were less than 24 hours old.

Acclimation period The *Daphnia* were bred in culture medium identical to the medium used for the test and under temperature and light conditions identical to those of the test.

Feeding During the test, the daphnids were fed daily with a food mixture containing a suspension of green algae of the species *Desmodesmus subspicatus* (freshly grown at the test facility) and a fish food (TETRA MIN Hauptfutter, obtained from TETRA Werke, 49304 Melle / Germany) suspension.

Test units The test was performed in 100 mL glass beakers containing 80 mL of test medium. The test vessels were covered with glass plates.

4. Environmental conditions

Test water The test was conducted in reconstituted water (Elendt M7 medium). Analytical grade salts and additives were dissolved in purified water to obtain the following nominal concentrations:

Macro nutrients:

$NaHCO_3$	65 mg/L
K_2HPO_4	0.18 mg/L
KH_2PO_4	0.14 mg/L
$MgSO_4 \cdot 7\ H_2O$	123 mg/L
$Na_2SiO_3 \cdot 9\ H_2O$	10 mg/L
$CaCl_2 \cdot 2\ H_2O$	294 mg/L
$NaNO_3$	0.27 mg/L
KCl	5.8 mg/L

Trace elements:

H_3BO_3	125 µg/L
$MnCl_2 \cdot 4\ H_2O$	25 µg/L
$ZnCl_2$	6.3 µg/L
$CoCl_2 \cdot 6\ H_2O$	2.5 µg/L
$CuCl_2 \cdot 2\ H_2O$	1.6 µg/L
$Na_2MoO_4 \cdot 2\ H_2O$	6.3 µg/L
$FeSO_4 \cdot 7\ H_2O$	50 µg/L
$Na_2EDTA \cdot 2\ H_2O$	625 µg/L
LiCl	12.5 µg/L
RbCl	12.5 µg/L
$SrCl_2 \cdot 6\ H_2O$	12.5 µg/L
NaBr	3.1 µg/L
KI	2.5 µg/L
Na_2SeO_3	1.0 µg/L
NH_4VO_3	0.3 µg/L

Vitamins:

Thiamine HCl	75 µg/L
Cyanocobalamine (B12)	1.0 µg/L
Biotin (B6)	0.75 µg/L

Hardness 2.5 mmol/L (=250 mg/L as $CaCO_3$)
Alkalinity 0.9 mmol/L
Water temperature 20-21°C

Lighting	16 hour light (light intensity: approximately 400-540 Lux) to 8 hour dark photoperiod, with a 30-minute transition period
Shaking	Before use, the test water was aerated until oxygen saturation. During the test, the test media were not aerated.

B. STUDY DESIGN AND METHODS

1. In-life dates 06.03.2012 to 11.04.2012

2. Experimental conditions

Test design

Neonates of *Daphnia magna* were exposed in a semi-static 21-day test to the test substance at five concentrations and a test water control. Toxic effects on survival and reproduction of the daphnids were assessed and the test animals were observed for visual abnormalities. The test media of all treatments were renewed on days 2, 5, 7, 9, 12, 14, 16, and 19 of the test period (except for the highest test treatment for which no test media were prepared from day 9 until day 21 due to mortality of the daphnids).

Number of animals per treatment

The study was started with ten daphnids per treatment. Each test animal was kept individually in one test unit.

Test conditions

The water temperature was maintained at 20-21°C and the test systems were illuminated at a 16-hour light to 8-hour dark photoperiod with a 30-minute transition period. The dissolved oxygen concentration in the test media and control was at least 7.9 mg/L. The pH values in the test media and control were between 7.6 and 8.2. No remarkable observations were made concerning the appearance of the test media. All test media were clear solutions throughout the test medium renewal periods.

Test concentrations

Dilutions of 1:10, 1:32, 1:100, 1:320 and 1:1000 of a filtrate with the loading rate of 100 mg test substance/L were tested. Additionally, a control was tested in parallel (test water without test substance). The selection of the test concentrations was based on the results of a range-finding test.

Treatment/Application

Due to the low water solubility of the test substance, a dispersion with the loading rate of 100 mg/L was prepared at the start of the test and at each test medium renewal by dispersing 100 mg of the test substance (dosing range: 100.0 – 100.3 mg) in 1000 mL of test water. The dispersions were subjected to ultrasonic treatment and intense stirring. Thereafter, the dispersion was filtered through a membrane filter (Schleicher & Schuell, Type NC45, pore size 0.45 µm). The undiluted filtrate was used as a stock solution and was serially diluted (1:10, 1:32, 1:100, 1:320 and 1:1000) with test water for preparation of the test media. The test media were freshly prepared just before the start of the test (= introduction of the daphnids) and before each test medium renewal. At study start and at each test medium renewal, the (surviving) test animals were carefully transferred into the fresh test medium by means of glass tubes.

Analytics

The concentration of flurochloridone in the test media and control was analysed by HPLC-MS/MS using external calibration. The test substance was separated on a column (Inertsil ODS-3; 2.1 mm x 33 mm; 3 µm; eluent A: 95 vol. water + 5 vol. methanol + 0.1 vol. formic acid + 5 mM ammonium formate; eluent B: 95 vol. methanol + 5 vol. water + 0.1 vol. formic acid + 5 mM ammonium formate; gradient: hold 0.5 min 60% A/40% B, in 1.5 min to 10% A/90% B, hold 1.0 min 10% A/90% B, in 0.1 min to 60% A/40% B, hold 0.9 min 60% A/40% B) at a flow rate of 400 µL/min and an injection volume of 5 µL. Detection was performed with a MDS Sciex API 5000 triple stage quadrupole mass spectrometer (heater gas

temperature: 450°C; spray voltage: 4800 V; ionisation mode: ESI; scan mode: MRM; ion polarity: positive; m/z 312.0 → 292.0; retention time: approximately 2 minutes). The method was validated and the LOQ was set to 0.0324 mg/L flurochloridone.

3. Sampling and measurements

The test replicates were observed for mortality (immobilisation) of adults on days 0-2 and thereafter three times per week before renewal of the test media. On the same dates, the test replicates were observed for live and dead offspring and for the presence of aborted eggs.

For determination of the test substance concentrations, duplicate samples were taken from the freshly prepared and aged test media at one treatment period of the first, second and last week (period 0-2 days, 7-9 days and 16-19 days). Since at the highest test concentration (dilution 1:10 of the undiluted filtrate with loading rate 100 mg/L), all adult daphnids had died by day 9, no test media of this treatment were prepared from day 9 until day 21 and no test medium samples were taken from this treatment at day 16. The following aged test medium samples were taken in duplicate:

Samples with food, taken from the actual test by combining the contents of all replicate test beakers at the end of the test medium renewal period.
Samples without food and test animals, incubated separately during the renewal periods under test conditions.

At the beginning and end of each test medium renewal period, pH and dissolved oxygen (one replicate of each test concentration and control) and water temperature (one control replicate) were measured. At the same time, appearance of the test media was visually inspected and recorded. The room temperature was continuously monitored.

4. Calculation of toxicity

Mean mortality was calculated for each test concentration and the control.

The reproduction rate was calculated as the total number of living offspring produced per parent female surviving until the end of the test. The mean reproduction rate was calculated for each test concentration and the control as well as the % deviation of the mean reproduction rate in the test substance treatments in relation to the control.

5. Statistics

The mean reproduction rates of the daphnids at the test concentrations were compared to the control by Dunnett t-test.

The EC_{10}, EC_{20} and EC_{50} for the inhibition of the reproduction rate after 21 days could not be calculated due to the absence of a toxic effect of the test substance on the reproduction rate up to the highest test concentration with surviving parental daphnids.

Results and Discussion

Analytical results
The concentrations of flurochloridone were measured in one of the duplicate test medium samples from the dilutions 1:100, 1:32, and 1:10 of sampling days 0, 2, 7, and 9. Since at the highest test concentration (dilution 1:10) all adult daphnids had died by day 9, at sampling days 16 and 19 samples were only analysed from the dilutions of 1:32 and 1:100. Samples of lower test substance concentrations were not analysed since they were not relevant for the interpretation of the biological results. From the control samples, one of the duplicate samples of each sampling date (Days 0, 2, 7, 9, 16 and 19) was analysed. Analysis results are presented in the following table.

Table A 2.2.2 1: Concentrations of flurochloridone in the test media

Dilution[a]	Measured concentration						Mean[b] measured concentration [mg/L]
	day 0	day 2	day 7	day 9	day 16	day 19	
	fresh [mg/L]	aged [mg/L]	fresh [mg/L]	aged [mg/L]	fresh [mg/L]	aged [mg/L]	
Control	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	n.a.
1:100	0.199	0.211	0.211	0.234	0.178	0.161	0.20
1:32	0.593	0.604	0.674	0.657	0.484	0.464	0.58
1:10	2.08	2.06	2.23	2.51	n.d.	n.d.	2.2

a) dilution of undiluted filtrate with a loading rate of 10 mg/L flurochloridone
b) arithmetic mean
LOQ 0.0324 mg/L flurochloridone
n.a. not applicable
n.d. not determined

The analytical results confirm the stability of the active substance over the test medium renewal periods. The biological results were related to the arithmetic mean measured concentrations of the test substance. In the test media of the dilutions 1:100, 1:32, and 1:10, flurochloridone concentrations of 0.161 to 0.234 mg/L, 0.464 to 0.674 mg/L, and 2.06 to 2.51 mg/L, respectively, were measured at the start and end of the test medium renewal periods. The test substance proved to be stable over the test medium renewal periods of 48 and 72 hours. Concentrations of flurochloridone at the end of the test medium renewal periods amounted to 90% to 113% of the initially measured values. Therefore, the biological results were related to the arithmetic mean measured concentrations of the test substance.

After 21 hours of exposure, mortality (immobilization) of test organisms was either 0% or 10% in the control and up to and including the mean measured concentration of 0.20 mg/L. At the two highest mean measured concentrations of 0.58 and 2.2 mg/L, mortality was 20% and 100%, respectively (see following table).

The first young offspring released from their parent animals were recorded in the control and at all test concentrations with surviving parent animals at observation on day 9. Thus, the time of the first brood was not affected by flurochloridone up to and including the mean measured concentration of 0.58 mg/L.

The mean reproduction rate of daphnids in the control was 132 ± 12 living offspring per surviving adult. In the test substance treatments with surviving parental daphnids, the mean reproduction rate ranged between 123 ± 6.6 and 141 ± 13 living offspring per surviving adult, which was not statistically significantly different from the control at any test concentration (see following table).

With the exception of the reported mortality, no visible abnormalities were observed at the test animals during the test.

Thus, the 21-day overall NOEC was determined to be the mean measured concentration of 0.58 mg/L, as mortality was 20% (being tolerated as no effect by the test guideline for the control) and no effects on reproduction occurred at this test concentration. The 21-day LOEC was 2.2 mg/L, due to 100% mortality of *Daphnia magna* observed at this test concentration. The 21-day EC_{50} for reproduction could not be determined but was clearly higher than the mean measured concentration of 0.58 mg/L.

~~**Table A 2.2.2-2: Effects of flurochloridone on survival and reproduction of *Daphnia magna***~~

~~**Treatment**~~		~~**Mean mortality after 21 days [%]**~~	~~**Mean reproduction rate**~~	
~~**Dilution[a)]**~~	~~**Mean measured test concentration [mg flurochloridone /L]**~~		~~**[living offspring per surviving adult]**~~	~~**[% of control]**~~
~~Control~~		~~0~~	~~131.6~~	~~100.0~~
~~1:1000~~	~~n.d.~~	~~10~~	~~123.0~~	~~93.5~~
~~1:320~~	~~n.d.~~	~~0~~	~~129.0~~	~~98.0~~
~~1:100~~	~~0.20~~	~~10~~	~~122.6~~	~~93.1~~
~~1:32~~	~~0.58~~	~~20~~	~~141.1~~	~~107.2~~
~~1:10~~	~~2.2~~	~~100~~	~~n.a.~~	~~n.a.~~
~~**Endpoints [mg flurochloridone /L]**~~				
~~21-day EC_{50}~~	~~> 0.58~~			
~~21-day NOEC~~	~~0.58~~			
~~21-day LOEC~~	~~2.2~~			

~~Note: There were no statistically significant differences in the mean reproduction rate between the control and the test substance treatments with surviving adults (Dunnett t-test, one-sided smaller, $\alpha = 0.05$).~~
~~a) dilution of undiluted filtrate with a loading rate of 10 mg/L flurochloridone~~
~~n.d. not determined~~
~~n.a. not applicable (all adult daphnids died during the test)~~

~~The validity criteria of the test were fulfilled: mortality in the control ≤ 20%; mean reproduction rate in the control ≥ 60 living offspring per surviving adult at the end of the test.~~

~~**Conclusion**~~

~~In this test on survival and reproduction of *Daphnia magna*, the 21-day overall NOEC and EC_{50} (reproduction) of flurochloridone were determined to be 0.58 mg/L and > 0.58 mg/L, respectively.~~

A 2.2.3 KCP 10.2.3 Further testing on aquatic organisms

A 2.2.3.1 Study 1: Toxicity to algae including recovery - *Chlamydomonas reinhardtii*

Comments of zRMS:	The study was performed in line with OECD 201 with no deviations regarding environmental conditions, exposure phase, etc.. The aim of the study was to generate additional toxicity data for algae and for this reason in the study non-standard species (*Chlamydomonas reinhardtii*) was used. This is acceptable, however it should be kept in mind that the environmental conditions or the acceptability criteria in OECD 201 were not verified for this species. Two out of three validity criteria of OECD 201 were fulfilled (increase of biomass and CV of average specific growth rate for the whole study period), but CV for section-by-section specific growth rates in controls was 39.7%, while it should not exceed 35%. Nevertheless, as the validity criteria were not verified for this species the zRMS is of the opinion that this slight deviation should not invalidate the study for non-standard species. Due to the low solubility of flurochloridone in water, no fixed test concentrations were used, but dilutions of a filtrate with the loading rate of 100 mg test substance/L. Then, the measured concentrations at test initiation and termination were determined. As the measured test item concentrations dropped <80% of initial concentration, the results were based on geometric mean measured concentrations. It is, however, noted that in Table A 2.2.3-1 the % recovery of the initial concentrations is given in relation to geometric mean measured concentrations, while this should be given as comparison of concentrations measured at test initiation and termination. For this reason Table A 2.2.3-1 has been amended accordingly. It is noted that the chemical analyses were performed only at two highest test concentrations, while according to OECD 201 at least the lowest and highest test concentration should be analysed. In the study no effects on algae were seen at lower concentrations, however it is not known if this was due to no response of algae to the exposure or due to concentrations of the test item too low to induce the effects. However, no effects were also seen at dilution

	1:1800 (second highest) and significant effects were only seen at the highest concentration, so concentrations measured in remaining test item groups would not impact the derived endpoints. Nevertheless, the derived E_bC_{50} may not be fully reliable due to steepness of the dose-response curve. Due to effects <50% the E_rC_{50} was determined to be above the highest concentration tested. The recovery part is retained for information but it was not evaluated by the zRMS, as recovery is not taken into account in the risk assessment. The part of the summary regarding recovery phase is thus presented in grey letters, to be distinguishable from the evaluated part. Overall, the study is considered acceptable rather as additional information with following endpoints (based on geometric mean measured concentrations): E_rC_{50} >25.0 µg a.s./L E_yC_{50} = 17.0 µg a.s./L NOEC = 7.7 µg a.s./L

Reference:	KCP 10.2.3/01
Report	Flurochloridone technical: Toxicity to *Chlamydomonas reinhardtii* in a 72-Hour Algal Growth Inhibition Test Supplemented with Testing for Recovery of Growth, , Liedtke, A., 2013a, D65727 (report number), 90015442 (sponsor report number)
Guideline(s):	Yes, OECD 201 (2006)
Deviations:	The mean coefficient of variation of the daily growth rates in the control (section-by-section growth rates) was slightly above the validity criterion of the guideline (39.7%; guideline ≤ 35%). Since the deviation was slight, this is not expected to have affected the quality and integrity of the study.
GLP:	Yes
Acceptability:	Acceptable rather as additional information due to deficiencies (see commenting box)
Duplication (if vertebrate study)	-

Materials and Methods

A. MATERIALS

1. Test material flurochloridone technical (= flurochloridone)
- **Description** Not reported
- **Lot/Batch #** 11083467
- **Purity** 95.5%
- **Stability of test material** Stable under storage conditions (original packaging, normal storage conditions)
  Expiry date: 08/2013

2. Vehicle and/or positive control Vehicle control: test water

3. Test organism

- **Species** Freshwater green alga *Chlamydomonas reinhardtii*
- **Strain** CCAP 11/32B
- **Source** Culture Collection of Algae and Protozoa (CCAP 11/32B, Dunstaffnage Marine Laboratory, Oban, Argyll, PA37 1QA, Scotland / UK)
- **Age** Algae cells were taken from an exponentially growing pre-culture set up four days prior to the start of the test.

Acclimation period An inoculum culture was set up four days before the start of exposure. The algae were cultivated under test conditions. The inoculum culture was diluted threefold one day before the start of the test to ensure that the algae were in the exponential growth phase when used to inoculate the test solutions.

Test units 50-mL Erlenmeyer flasks containing 15 mL of test medium. The test vessels were covered with glass dishes.

4. Environmental conditions

Test water The algae were cultivated and tested in reconstituted test water (3N-BBM+V medium; Bold Basal Medium with 3-fold nitrogen and vitamins, modified). Analytical grade salts were dissolved in sterile purified water to obtain the following nominal concentrations:

Macro-nutrients:	
$NaNO_3$	750 mg/L
KH_2PO_4	175 mg/L
$MgSO_4 \cdot 7\ H_2O$	75 mg/L
$K_2HPO_4 \cdot 3\ H_2O$	75 mg/L
$CaCl_2 \cdot 2\ H_2O$	25 mg/L
NaCl	25 mg/L
Trace elements:	
$FeCl_3 \cdot 6\ H_2O$	582 µg/L
$MnCl_2 \cdot 4\ H_2O$	246 µg/L
$ZnCl_2$	17 µg/L
$CoCl_2 \cdot 6\ H_2O$	12 µg/L
$Na_2MoO_4 \cdot 2\ H_2O$	24 µg/L
Vitamins:	
Thiaminhydrochloride	1.2 mg/L
Cyanocobalamin	10 µg/L

The pH of the test water was 6.3±0.2.

Water temperature 20-21°C

Lighting Continuous illumination at a mean light intensity (measured at the level of the test solutions) of approximately 6200 Lux (range: 5410 to 6960 Lux) at the start of exposure and 6300 Lux (range: 5350 to 7080 Lux) at the start of recovery using fluorescent tubes (Philips TLD 36W/840)

Shaking Algae suspensions were continuously stirred using magnetic stirrers.

B. STUDY DESIGN AND METHODS

1. In-life dates 18.01.2013 to 28.02.2013

2. Experimental conditions

Test design

The freshwater green alga *Chlamydomonas reinhardtii* was exposed in a static 72-hour test to the test substance at five concentrations each with three replicates and six replicates of a test water control. The recorded effect was inhibition of algal growth based on yield and growth rate. Furthermore, algal cells were investigated by microscopic evaluation.

After 72 hours of exposure, algae from the highest test concentration and the control were transferred to fresh untreated dilution water and allowed to grow for further 72 hours under test conditions to investigate the recovery potential of the algae.

Inoculum at test start

Approximately 5000 cells/mL

Test conditions

The water temperature was maintained at 20-21°C and the test systems were continuously illuminated at a mean light intensity of 6200 and 6300 Lux (measured at the start of the exposure and recovery phase, respectively). The pH of the test media was 6.6 at the start of exposure and 6.7-7.2 at the end of exposure. At the start and end of recovery, the pH of the test media was 6.3 and 7.1-7.2, respectively. No remarkable observations were made concerning the appearance of the test media. All test media were clear solutions throughout the exposure phase.

Test concentrations

Dilutions of 1:56000, 1:18000, 1:5600, 1:1800 and 1:560 of a filtrate with the loading rate of 100 mg test substance/L were tested. Additionally, a control was tested in parallel (test water without test substance). The selection of the test concentrations was based on the results of a range-finding test.

Treatment/Application

Due to the low water solubility of the test substance, a dispersion with the loading rate of 100 mg/L was prepared at the start of the test by dispersing 100.61 mg of the test substance in 1000 mL of test water. The dispersion was subjected to ultrasonic treatment and intense stirring. Thereafter, the dispersion was filtered through a membrane filter (Schleicher & Schuell, Type NC45, pore size 0.45 µm). The undiluted filtrate was used as a stock solution and was serially diluted (1:560, 1:1800, 1:5600, 1:18000 and 1:56000) with test water for preparation of the test media. The test media were freshly prepared just before the start of the test (= start of exposure period).

After 72 hours of exposure, the replicates of each treatment were pooled and algal cells were separated. The algae of the highest treatment group (the group showing toxic effects at the end of exposure) and the control were transferred into fresh untreated test water and their cell density was determined. Three replicates for the highest treatment group and six replicates for the control were set up with a nominal algal cell density of 5000 cells/mL and the recovery of algal growth was recorded for 72 hours.

Analytics

The concentration of flurochloridone in the test media and control was analysed by HPLC-MS/MS using external calibration. The test substance was separated on a column (Inertsil ODS-3; 2.1 mm x 33 mm; 3 µm; eluent A: 95 vol. water + 5 vol. methanol + 0.1 vol. formic acid + 5 mM ammonium formate; eluent B: 95 vol. methanol + 5 vol. water + 0.1 vol. formic acid + 5 mM ammonium formate; gradient: hold 0.5 min 60% A/40% B, in 1.5 min to 10% A/90% B, hold 0.5 min 10% A/90% B, in 0.1 min to 100% A, hold 0.4 min 100% A, in 0.1 min to 60% A/40% B, hold 0.9 min 60% A/40% B) at a flow rate of 400 µL/min and an injection volume of 20 µL. Detection was performed with a MDS Sciex API 4000 triple stage quadrupole mass spectrometer (heater gas temperature: 300°C; spray voltage: 4500 V; ionisation mode:

ESI; scan mode: MRM; ion polarity: positive; m/z 312.0 → 292.0; retention time: approximately 2 minutes). The method was validated and the LOQ was set to 0.185 µg/L flurochloridone.

3. Sampling and measurements

Algal biomass was determined daily during the exposure phase and after 48 and 72 hours in the recovery phase by cell counts using fluorescent measurement (BIO-TEK® Multi-Detection Microplate Reader, Model FLx800). The measurements were performed at least in duplicate. In addition, the shape and size of algal cells from the control and the highest treatment group (dilution 1:560) were inspected visually at the end of each test period.

The test media of each test concentration and the control were sampled in duplicate at the start (without algae) and at the end (containing algae) of the exposure phase for analysis of the test substance concentration.

The pH was measured in each test substance treatment and the control at the start and at the end of each test period. The water temperature was measured daily for the duration of the test and the appearance of the test media were recorded daily during the exposure phase.

4. Calculation of toxicity

Inhibition of algal growth was determined based on the cell density (yield, y) and the specific growth rate (r) for exponentially growing cultures using the equations recommended in the test guidelines.

5. Statistics

The 72-hour EC_{50} values for the inhibition of average yield and growth rate and their 95% confidence limits were calculated as far as possible by Probit Analysis using linear maximum likelihood regression. For the determination of the LOEC and NOEC, average yield and growth rate at the test concentrations were compared to the control values by Williams t-test.

For the assessment of recovery of exposed algae cultures, yield and growth rate after 72 hours were determined for the test groups used in the recovery phase. Yield and growth rate of the test group previously treated with the highest test concentration were compared with the control by a Student t-test. Based on these results, the NOAECrecovery (No Observed Ecologically Adverse Effect Concentration) for recovery of *Chlamydomonas reinhardtii* was determined.

Results and Discussion

The concentrations of flurochloridone were measured in one of the duplicate test medium samples from the dilutions 1:1800 and 1:560 and from the control. The samples from the higher dilutions were not analyzed, since these concentrations were below the NOEC determined in this test. Analysis results are presented in the following table.

Table A 2.2.3-1: Concentrations of flurochloridone in the test media

Dilution[a)]	Measured concentration			Geometric mean measured concentration	
	0 hours	72 hours			
	[µg/L]	[µg/L]	[% of initially measured]	[µg/L]	~~[% of initially measured]~~
Control	< LOQ	< LOQ	-	< LOQ	~~n.a.~~
1:1800	10.9	5.51	50.6	7.7	~~71~~
1:560	34.0	18.3	53.8	25	~~74~~

a) dilution of undiluted filtrate with a loading rate of 10 mg/L flurochloridone
LOQ 0.185 mg/L flurochloridone
n.a. not applicable

Due to the decrease of the test substance concentrations in the test media during the exposure phase, the biological results were related to the geometric mean measured concentrations.
At the start of the exposure phase, measured concentrations of flurochloridone in the test media of the test concentrations from the dilutions 1:1800 and 1:560 were 10.9 and 34.0 µg/L, respectively. The corresponding measured concentrations at the end of the exposure phase were 5.51 and 18.3 µg/L. Due to the decrease of the test substance concentrations in the test media during the exposure phase, the biological results were related to the geometric mean measured concentrations, i.e. 7.7 and 25 µg/L.

The biomass of algae during exposure to flurochloridone and during the recovery phase is presented in the table below. The effects of flurochloridone on algal growth during the exposure and recovery phase are shown in the following tables. After exposure for 72 hours, flurochloridone had a statistically significant inhibitory effect on the growth of algae (based on growth rate and yield) at the highest geometric mean measured concentration of 25 µg/L. The overall 72-hour NOEC was, therefore, determined to be 7.7 µg/L, since up to and including this test concentration the growth rate and yield of the algae were not statistically significantly lower than in the control. The 72-hour EC_{50} for yield was determined to be 17 µg/L (95% confidence limits: 15-19 µg/L) and the 72-hour EC_{50} for growth rate was > 25 µg/L (95% confidence limits not determinable).

After 72 hours of incubation in untreated dilution water during the recovery phase, algal growth (based on growth rate and yield) in the test group previously exposed to the highest test concentration was not statistically significantly different from the control. Therefore, recovery of algae during 72 hours could be demonstrated and the 72-hour $NOAEC_{recovery}$ was defined as the highest geometric mean measured concentration of 25µg/L.

Microscopic examination of algal cells at the end of the exposure and recovery phase showed no difference in shape and size between algae from the highest treatment group (geometric mean measured 25 µg/L) and the control.

Table A 2.2.3-2: Biomass of algae exposed to flurochloridone and algal biomass during recovery

Treatment		Biomass [mean[b)] ± standard deviation] (relative fluorescence units x 10^3)					
		Exposure phase[c)]			Recovery phase		
Dilution[a)]	Geom. mean measured test conc. [µg flurochloridone/L]	24 hours	48 hours	72 hours	0 hours	48 hours	72 hours
Control	-	3.6 ± 0.3	29.7 ± 4.6	207.6 ± 13.0	3.0 ± 0.2	71.9 ± 9.3	276.3 ± 20.6
1:56000	n.a.	3.5 ± 0.1	26.9 ± 6.3	181.9 ± 22.0	n.d.	n.d.	n.d.
1:18000	n.a.	3.7 ± 0.5	32.4 ± 8.9	200.5 ± 29.9	n.d.	n.d.	n.d.
1:5600	n.a.	3.4 ± 0.3	30.1 ± 7.2	212.8 ± 13.6	n.d.	n.d.	n.d.
1:1800	7.7	4.1 ± 0.4	39.7 ± 10.6	200.8 ± 25.9	n.d.	n.d.	n.d.
1:560	25	3.5 ± 0.0	16.1 ± 1.3	52.0 ± 6.2	1.8 ± 0.1	71.4 ± 6.8	287.0 ± 35.5

n.a. not analysed, n.d. not determined
a) dilution of undiluted filtrate with a loading rate of 10 mg/L flurochloridone
b) mean of three replicates for the test substance treatments and mean of six replicates for the control
c) The initial cell density was 5000 cells/mL, corresponds to 1.37 x 10^3 relative fluorescence units.

Table A 2.2.3-3: Effects of flurochloridone on algal growth during exposure and recovery phase

Treatment		Exposure phase				Recovery phase			
Dilution[a)]	Geom. mean measured test conc. [µg flurochloridone/L]	0-72 hours yield (y)		0-72 hours growth rate (r)		0-72 hours yield (y)		0-72 hours growth rate (r)	
		y ($x 10^3$)	Inhib. [%]	r [day^{-1}]	Inhib. [%]	y ($x 10^3$)	Inhib. [%]	r [day^{-1}]	Inhib. [%]
Control	-	206.2	0.0	1.664	0.0	273.2	0.0	1.503	0.0
1:56000	n.a.	180.5	12.5	1.619	2.7	n.d.	n.d.	n.d.	n.d.
1:18000	n.a.	199.1	3.4	1.650	0.8	n.d.	n.d.	n.d.	n.d.
1:5600	n.a.	211.4	-2.5	1.672	-0.5	n.d.	n.d.	n.d.	n.d.
1:1800	7.7	199.4	3.3	1.652	0.7	n.d.	n.d.	n.d.	n.d.
1:560	25	50.6*	75.5	1.202*	27.8	285.1	-4.4	1.682	-11.9

Endpoints (95% CL) [µg flurochloridone /L]		
	Yield (y)	Growth rate (r)
72-hour EC_{50}	17 (15-19)	> 25 (95% CL not determinable)
72-hour NOEC	7.7	7.7
72-hour LOEC	25	25
72-hour $NOAEC_{recovery}$	25	25

n.a. not analysed, n.d. not determined

a) dilution of undiluted filtrate with a loading rate of 10 mg/L flurochloridone

* mean value significantly lower than in the control (according to Williams t-test, one-sided smaller, $\alpha = 0.05$)

Note: During the recovery phase, mean yield and growth rate in the test group previously treated with flurochloridone was not significantly different from the control (according to Student t-test, one-sided smaller, $\alpha = 0.05$)

In the control, the biomass increased by a factor of 147 over 72 hours (according to guideline ≥ 16). The coefficient of variation of the average specific growth rates in the replicates of the control after 72 hours was 1.2% (according to guideline ≤ 10%). Thus, both validity criteria were fulfilled. The mean coefficient of variation of the daily growth rates in the control (section-by-section growth rates) during 72 hours was 39.7% and was thus slightly above the validity criterion of the guideline (according to guideline ≤ 35%).

Conclusion

The 72-hour E_yC_{50} and E_rC_{50} values of flurochloridone for the freshwater green alga *Chlamydomonas reinhardtii* were determined to be 17 and > 25 µg/L, respectively. Recovery during 72 hours has been shown for algae pre-exposed to 25 µg/L, i.e. the 72-hour $NOAEC_{recovery}$ of flurochloridone is 25 µg/L.

A 2.2.3.2 Study 2: Toxicity to algae including recovery - *Chlorella vulgaris*

Comments of zRMS:	The study was performed in line with OECD 201 with no deviations regarding environmental conditions, exposure phase, etc.. The aim of the study was to generate additional toxicity data for algae and for this reason in the study non-standard species (*Chlorella vulgaris*) was used. This is acceptable, however it should be kept in mind that the environmental conditions or the acceptability criteria in OECD 201 were not verified for this species. Two out of three validity criteria of OECD 201 were fulfilled (increase of biomass and CV of average specific growth rate for the whole study period), but CV for section-by-section specific growth rates in controls was 36.0%, while it should not exceed 35%. Nevertheless, as the validity criteria were not verified for this species the zRMS is of the opinion that this slight deviation should not invalidate the study for non-standard species. Due to the low solubility of flurochloridone in water, no fixed test concentrations were used, but dilutions of a filtrate with the loading rate of 100 mg test substance/L. Then, the measured concentrations at test initiation and termination were determined. As the measured test item concentrations dropped <80% of initial concentration, the results were based on geometric mean measured concentrations. The chemical analyses were performed in all but the lowest treatment group. It is noted that according to OECD 201 the lowest test concentration should be included in analyses. However, reliable dose-response curve could be plotted using available concentrations and

	the lowest test concentration was below the NOEC. Therefore the zRMS is of the opinion that additional chemical analysis in this lowest test concentration would not provide information necessary for determination of the endpoints. Overall, the study is considered acceptable with following endpoints (based on geometric mean measured concentrations): E_rC_{50} = 14.3 µg a.s./L E_yC_{50} = 3.9 µg a.s./L NOEC = 1.9 µg a.s./L

Reference:	KCP 10.2.3/02
Report	Flurochloridone technical: Toxicity to *Chlorella vulgaris* in a 72-Hour Algal Growth Inhibition Test, Liedtke, A., 2013b, D65738 (report number), 90015443 (sponsor report number)
Guideline(s):	Yes, OECD 201 (2006), Commission Regulation (EC) No 761/2009, C.3 (2009)
Deviations:	Yes: The mean coefficient of variation of the daily growth rates in the control (section-by-section growth rates) was slightly above the validity criterion of the guideline (36%; guideline ≤ 35%). Since the deviation was slight, this is not expected to have affected the quality and integrity of the study.
GLP:	Yes
Acceptability:	Acceptable with some deviations (see commenting box above)
Duplication (if vertebrate study)	-

Materials and Methods

A. MATERIALS

1. Test material flurochloridone technical (=flurochloridone)
Description Not reported
Lot/Batch # 11083467
Purity 95.5%
Stability of test material Stable under storage conditions (original packaging, normal storage conditions)
Expiry date: 08/2013

2. Vehicle and/or positive control Vehicle control: test water

3. Test organism

Species Freshwater green algae *Chlorella vulgaris*
Strain SAG 211-12
Source Collection of Algal Cultures (SAG, Institute for Plant Physiology, University of Göttingen, 37073 Göttingen / Germany)
Age Algae cells were taken from an exponentially growing pre-culture set up four days prior to the start of the test.
Acclimation period An inoculum culture was set up four days before the start of exposure. The algae were cultivated under test conditions. The inoculum culture was diluted threefold one day before the start of the test to ensure that the algae were in the exponential growth phase when used to inoculate the test solutions.
Test units 50-mL Erlenmeyer flasks containing 15 mL of test medium. The test vessels were covered with glass dishes.

4. Environmental conditions

Test water	The algae were cultivated and tested in reconstituted test water (AAP medium). Analytical grade salts were dissolved in sterile purified water to obtain the following nominal concentrations:

Macro-nutrients:

$NaHCO_3$	15.0 mg/L
K_2HPO_4	1.044 mg/L
$MgSO_4 \cdot 7\ H_2O$	14.6 mg/L
$MgCl_2 \cdot 6\ H_2O$	12.16 mg/L
$CaCl_2 \cdot 2\ H_2O$	4.41 mg/L
$NaNO_3$	25.5 mg/L

Trace elements:

H_3BO_3	186.0 µg/L
$MnCl_2 \cdot 4\ H_2O$	415.0 µg/L
$ZnCl_2$	3.27 µg/L
$CoCl_2 \cdot 6\ H_2O$	1.43 µg/L
$CuCl_2 \cdot 2\ H_2O$	0.012 µg/L
$Na_2MoO_4 \cdot 2\ H_2O$	7.26 µg/L
$FeCl_3 \cdot 6\ H_2O$	160.0 µg/L
$Na_2EDTA \cdot 2\ H_2O$	300.0 µg/L

The pH of the test water was 7.5.

Hardness	0.15 mmol/L (= 15 mg/L) as $CaCO_3$
Water temperature	20°C
Lighting	Continuous illumination at a mean light intensity (measured at the level of the test solutions) of approximately 5500 Lux (range: 5010 to 5870 Lux) using fluorescent tubes (Philips TLD 36W/840)
Shaking	Algae suspensions were continuously stirred using magnetic stirrers.

B. STUDY DESIGN AND METHODS

1. In-life dates 09.11.2012 to 01.03.2013

2. Experimental conditions

Test design

The freshwater green alga *Chlorella vulgaris* was exposed in a static 72-hour test to the test substance at five concentrations each with three replicates and six replicates of a test water control. The recorded effect was inhibition of algal growth based on yield and growth rate. Furthermore, algal cells were investigated by microscopic evaluation.

Inoculum at test start

Approximately 7500 cells/mL

Test conditions

The water temperature was maintained at 20°C and the test systems were continuously illuminated at a mean light intensity of 5500 Lux. The pH of the test media was 7.7-7.8 at the start of the test and 7.6-7.7 at the end of the test. No remarkable observations were made concerning the appearance of the test media. All test media were clear solutions throughout the test period.

Test concentrations

Dilutions of 1:32000, 1:10000, 1:3200, 1:1000 and 1:320 of a filtrate with the loading rate of 100 mg test substance/L were tested. Additionally, a control was tested in parallel (test water without test substance). The selection of the test concentrations was based on the results of a range-finding test.

Treatment/Application

Due to the low water solubility of the test substance, a dispersion with the loading rate of 100 mg/L was prepared at the start of the test by dispersing 100.27 mg of the test substance in 1000 mL of test water. The dispersion was subjected to ultrasonic treatment and intense stirring. Thereafter, the dispersion was filtered through a membrane filter (Schleicher & Schuell, Type NC45, pore size 0.45 µm). The undiluted filtrate was used as a stock solution and was serially diluted (1:320, 1:1000, 1:3200, 1:10000 and 1:32000) with test water for preparation of the test media. The test media were freshly prepared just before the start of the test (= start of exposure period).

Analytics

The concentration of flurochloridone in the test media and control was analysed by HPLC-MS/MS using external calibration. The test substance was separated on a column (Inertsil ODS3; 2.0 mm x 33 mm; 3 µm; eluent A: 95 vol. water + 5 vol. methanol + 0.1 vol. formic acid + 5 mM ammonium formate; eluent B: 95 vol. methanol + 5 vol. water + 0.1 vol. formic acid + 5 mM ammonium formate; gradient: in 2.0 min from 80% A/20% B to 60% A/40% B, in 0.5 min to 10% A/90% B, hold 0.2 min 10% A/90% B, in 0.1 min to 80% A/20% B, hold 2.2 min 80% A/20% B) at a flow rate of 400 µL/min and an injection volume of 20 µL. Detection was performed with a MDS Sciex API 5000 triple stage quadrupole mass spectrometer (heater gas temperature: 450°C; spray voltage: 4800 V; ionisation mode: ESI; scan mode: MRM; ion polarity: positive; m/z 312.0 → 292.0; retention time: approximately 2.2 minutes). The method was validated and the LOQ was set to 0.18 µg/L flurochloridone.

3. Sampling and measurements

Algal biomass was determined daily by cell counts using fluorescent measurement (BIO-TEK® Multi-Detection Microplate Reader, Model FLx800). The measurements were performed at least in duplicate. In addition, the shape and size of algal cells from the control and the treatment group of dilution 1:1000 were inspected visually at the end of each test period. This test concentration was chosen since the algal cell density was too low for a reliable examination at the other higher concentrations.

The test media of each test concentration and the control were sampled in duplicate (10 mL) at the start of the test (without algae) and at the end of the test (containing algae) for analysis of the test substance concentration. Additionally, at the same sampling times, duplicate samples (100 mL) were taken from the dilution 1:32000 and from the control for which additional flasks containing the test medium with algae were incubated under the test conditions.

The pH was measured in each test substance treatment and the control at the start and at the end of the test. The water temperature and the appearance of the test media were recorded daily.

4. Calculation of toxicity

Inhibition of algal growth was determined based on the cell density (yield, y) and the specific growth rate (r) for exponentially growing cultures using the equations recommended in the test guidelines.

5. Statistics

The 72-hour EC_{50} values for the inhibition of average yield and growth rate and their 95% confidence limits were calculated by Probit Analysis using linear maximum likelihood regression. For the determination of the LOEC and NOEC, average yield and growth rate at the test concentrations were compared to the control values by Williams t-test.

Results and Discussion

The concentrations of flurochloridone were measured in one of the duplicate test medium samples from the dilutions 1:10000, 1:3200, 1:1000 and 1:320 and from the control. The samples from the highest dilution (1:32000) were not analyzed, since this concentration was below the NOEC determined in this test. Analysis results are presented in the following table.

Table A 2.2.3-4: Concentrations of flurochloridone in the test media

Dilution[a)]	Measured concentration		Geometric mean measured concentration	
	0 hours	72 hours		
	[µg/L]	[µg/L]	[µg/L]	[% of initially measured]
Control	< LOQ	< LOQ	< LOQ	n.a.
1:10000	2.41	1.51	1.9	63
1:3200	5.12	4.17	4.6	82
1:1000	12.8	12.0	12.4	94
1:320	39.9	37.8	38.8	95

a) dilution of undiluted filtrate with a loading rate of 10 mg/L flurochloridone
LOQ 0.18 µg/L flurochloridone
n.a. not applicable

Due to the decrease of the test substance concentrations in the test media during the test, the biological results were related to the geometric mean measured concentrations.

At the start of the test, measured concentrations of flurochloridonein the test media of the test concentrations from the dilutions 1:10000, 1:3200, 1:1000 and 1:320 were 2.41, 5.12, 12.8 and 39.9 µg/L, respectively. The corresponding measured concentrations at the end of the test were 1.51, 4.17, 12.0 and 37.8 µg/L. Due to the decrease of the test substance concentrations in the test media during the test, the biological results were related to the geometric mean measured concentrations, i.e. 1.9, 4.6, 12.4 and 38.8 µg/L.

The biomass of algae during exposure to flurochloridoneand the effects of flurochloridone on algal growth are shown in the following tables. After exposure for 72 hours, flurochloridone had a statistically significant inhibitory effect on the growth of algae (based on growth rate and yield) at the geometric mean measured concentration of 4.6 µg/L and all higher concentrations. The overall 72-hour NOEC was, therefore, determined to be 1.9 µg/L, since up to and including this test concentration the growth rate and yield of the algae were not statistically significantly lower than in the control. The 72-hour EC_{50} for yield was determined to be 3.9 µg/L (95% confidence limits: 3.6-4.3 µg/L) and the 72-hour EC_{50} for growth rate was 14.3 µg/L (95% confidence limits: 11.7-17.8 µg/L).

Microscopic examination of algal cells at the end of the test showed no difference in shape and size between algae from the dilution 1:1000 (geometric mean measured 12.4 µg/L) and the control.

Table A 2.2.3-5: Biomass of algae exposed to flurochloridone

Treatment		Biomass[b)] [mean[c)] ± standard deviation] (relative fluorescence units x 10^3)		
Dilution [a)]	Geom. mean measured test conc. [µg flurochloridone/L]	24 hours	48 hours	72 hours
Control	-	2.1 ± 0.1	13.1 ± 0.5	49.2 ± 7.9
1:32000	n.a.	1.9 ± 0.1	13.0 ± 0.8	43.5 ± 5.1
1:10000	1.9	2.0 ± 0.1	12.7 ± 0.9	51.7 ± 10.9
1:3200	4.6	1.8 ± 0.0	7.3 ± 0.5	18.5 ± 0.9
1:1000	12.4	1.3 ± 0.0	1.6 ± 0.0	2.0 ± 0.1
1:320	38.8	0.8 ± 0.1	0.9 ± 0.1	0.8 ± 0.0

n.a. not analysed
a) dilution of undiluted filtrate with a loading rate of 10 mg/L flurochloridone
b) The initial cell density was 7500 cells/mL, corresponds to 0.14 x 10^3 relative fluorescence units.
c) mean of three replicates for the test substance treatments and mean of six replicates for the control

Table A 2.2.3-6: Effects of flurochloridone on algal growth

Treatment		Effects during exposure			
Dilution[a)]	Geom. mean measured test conc. [µg flurochloridone/L]	0-72 hours yield (y)		0-72 hours growth rate (r)	
		y (x 10^3)	Inhibition of y [%]	r [day^{-1}]	Inhibition of r [%]
Control	-	49.1	0.0	1.952	0.0
1:32000	n.a.	43.4	11.7	1.913	2.0
1:10000	1.9	51.6	-5.0	1.966	-0.7
1:3200	4.6	18.4*	62.5	1.630*	16.5
1:1000	12.4	1.9*	96.2	0.888*	54.5
1:320	38.8	0.6*	98.7	0.564*	71.1
Endpoints (95% CL) [µg flurochloridone]					
		Yield (y)		Growth rate (r)	
72-hour EC_{50}		3.9 (3.6-4.3)		14.3 (11.7-17.8)	
72-hour NOEC		1.9		1.9	
72-hour LOEC		4.6		4.6	

n.a. not analysed
a) dilution of undiluted filtrate with a loading rate of 10 mg/L flurochloridone
* mean value significantly lower than in the control (according to Williams t-test, one-sided smaller, $\alpha = 0.05$)

In the control, the biomass increased by a factor of 353 over 72 hours (according to guideline ≥ 16). The coefficient of variation of the average specific growth rates in the replicates of the control after 72 hours was 2.7% (according to guideline ≤ 10%). Thus, both validity criteria were fulfilled. The mean coefficient of variation of the daily growth rates in the control (section-by-section growth rates) during 72 hours was 36% and was thus slightly above the validity criterion of the guideline (according to guideline ≤ 35%).

Conclusion

The 72-hour E_yC_{50} and E_rC_{50} values of flurochloridone for the freshwater green alga *Chlorella vulgaris* were determined to be 3.9 and 14.3 µg/L, respectively.

A 2.2.3.3 Study 3: Toxicity to algae including recovery – *Navicula pelliculosa*

Comments of zRMS:	The study was performed in line with OECD 201 with no deviations. All validity criteria were met. Due to the low solubility of flurochloridone in water, no fixed test concentrations were used, but dilutions of a filtrate with the loading rate of 100 mg test substance/L. Then, the measured concentrations at test initiation and termination were determined. The measured test item concentrations were within 80-120% of initial concentration, but the results were based on geometric mean measured concentrations, which is acceptable. It is, however, noted that in Table A 2.2.3-7 the % recovery of the initial concentrations is given in relation to geometric mean measured concentrations, while this should be given as comparison of concentrations measured at test initiation and termination. For this reason Table A 2.2.3-7 has been amended accordingly. The recovery part is retained for information but it was not evaluated by the zRMS, as recovery is not taken into account in the risk assessment. The part of the summary regarding recovery phase is thus presented in grey letters, to be distinguishable from the evaluated part. Overall, the study is considered acceptable with following endpoints (based on geometric mean measured concentrations): E_rC_{50} = 12.0 µg a.s./L E_yC_{50} = 3.4 µg a.s./L NOEC = 1.5 µg a.s./L

Reference:	KCP 10.2.3/03

Report	Flurochloridone technical: Toxicity to *Navicula pelliculosa* in a 72-Hour Algal Growth Inhibition Test Supplemented with Testing for Recovery of Growth, Liedtke, A., 2013c, D65740 (report number), 90015444 (sponsor report number)
Guideline(s):	Yes, OECD 201 (2006)
Deviations:	No
GLP:	Yes
Acceptability:	Acceptable
Duplication (if vertebrate study)	-

Materials and Methods

A. MATERIALS

1. Test material flurochloridone technical (=flurochloridone)
Description Not reported
Lot/Batch # 11083467
Purity 95.5%
Stability of test material Stable under storage conditions (original packaging, normal storage conditions)
Expiry date: 08/2013

2. Vehicle and/or positive control Vehicle control: test water

3. Test organism

Species Freshwater green alga *Navicula pelliculosa* (Bréb.) Hilse
Strain UTEX No. 664
Source UTEX Culture Collection of Algae/MCDB, University of Texas at Austin; Austin, TX 78712-0183 / USA
Age Algae cells were taken from an exponentially growing pre-culture set up four days prior to the start of the test.
Acclimation period An inoculum culture was set up four days before the start of exposure. The algae were cultivated under test conditions. The inoculum culture was diluted threefold one day before the start of the test to ensure that the algae were in the exponential growth phase when used to inoculate the test solutions.
Test units 125-mL Erlenmeyer flasks containing 50 mL of test medium. The test vessels were covered with glass dishes.
In order to remove the adhesive *Navicula* cells from the glass wall, cell scrapers (S/TPP/240 mm) were used.

4. Environmental conditions

Test water The algae were cultivated and tested in reconstituted test water (AAP medium). Analytical grade salts were dissolved in sterile purified water to obtain the following nominal concentrations:

Macro-nutrients:

$NaHCO_3$	15.0 mg/L
K_2HPO_4	1.044 mg/L
$MgSO_4 \cdot 7\ H_2O$	14.6 mg/L
$MgCl_2 \cdot 6\ H_2O$	12.16 mg/L
$CaCl_2 \cdot 2\ H_2O$	4.41 mg/L
$NaNO_3$	25.5 mg/L

	Trace elements:
	H_3BO_3 186.0 µg/L
	$MnCl_2 \cdot 4\ H_2O$ 415.0 µg/L
	$ZnCl_2$ 3.27 µg/L
	$CoCl_2 \cdot 6\ H_2O$ 1.43 µg/L
	$CuCl_2 \cdot 2\ H_2O$ 0.012 µg/L
	$Na_2MoO_4 \cdot 2\ H_2O$ 7.26 µg/L
	$FeCl_3 \cdot 6\ H_2O$ 160.0 µg/L
	$Na_2EDTA \cdot 2\ H_2O$ 300.0 µg/L
	The pH of the test water was 7.5.
Hardness	0.15 mmol/L (= 15 mg/L) as $CaCO_3$
Water temperature	22-23°C
Lighting	Continuous illumination at a mean light intensity (measured at the level of the test solutions) of approximately 7900 Lux (range: 7080 to 8520 Lux) during exposure, 8000 Lux (range: 6900 to 6850 Lux) during the first recovery phase, 8000 Lux (range: 7110 to 8630 Lux) during the second recovery phase and 7900 Lux (range: 7140 to 8370 Lux) during the third recovery phase using fluorescent tubes (Philips TLD 36W/840)
Shaking	Algae suspensions were continuously stirred using magnetic stirrers.

B. STUDY DESIGN AND METHODS

1. In-life dates 09.11.2012 to 18.03.2013

2. Experimental conditions

Test design

The freshwater green alga *Navicula pelliculosa* was exposed in a static 72-hour test to the test substance at six concentrations each with four replicates and six replicates of a test water control. The recorded effect was inhibition of algal growth based on yield and growth rate. Furthermore, algal cells were investigated by microscopic evaluation.

After 72 hours of exposure, the recovery of algal growth was recorded for three recovery phases of 72 hours resulting in nine days of recovery in total. The first and second recovery phases were performed with the test groups pre-treated with the four highest test concentrations and the control and the third recovery phase was performed with the test groups pre-treated with the three highest test groups and the control.

Inoculum at test start

Approximately 10000 cells/mL.

Test conditions

The water temperature was maintained at 22-23°C and the test systems were continuously illuminated at a mean light intensity of 7900 Lux (exposure and third recovery phase) or 8000 Lux (first and second recovery phase). The pH of the test media was 7.6-7.7 at the start of exposure and 7.9-8.1 at the end of exposure. At the start and end of the first, second and third recovery phase, the pH of the test media was 7.6 and 7.4-7.9, 7.6 and 7.8-8.1 and 7.7 and 8.0, respectively. No remarkable observations were made concerning the appearance of the test media. All test media were clear solutions throughout the exposure phase.

Test concentrations

Dilutions of 1:32000, 1:10000, 1:3200, 1:1000, 1:320 and 1:100 of a filtrate with the loading rate of 100 mg test substance/L were tested. Additionally, a control was tested in parallel (test water without test substance). The selection of the test concentrations was based on the results of a range-finding test.

Treatment/Application

Due to the low water solubility of the test substance, a dispersion with the loading rate of 100 mg/L was prepared at the start of the test by dispersing 100.39 mg of the test substance in 1000 mL of test water. The dispersion was subjected to ultrasonic treatment and intense stirring. Thereafter, the dispersion was filtered through a membrane filter (Schleicher & Schuell, Type NC45, pore size 0.45 µm). The undiluted filtrate was used as a stock solution and was serially diluted (1:100, 1:320, 1:1000, 1:3200, 1:10000 and 1:32000) with test water for preparation of the test media. The test media were freshly prepared just before the start of the test (= start of exposure period).

After 72 hours of exposure, the replicates of each treatment were pooled and algal cells were separated. The algae of the four highest treatment groups (the groups showing toxic effects at the end of exposure) and the control were transferred into fresh untreated test water and their cell density was determined. Four replicates for each treatment group and six replicates for the control were set up with a nominal algal cell density of 10000 cells/mL and the recovery of algal growth was recorded for 72 hours. Since growth inhibition was still observed after the first recovery period, the recovery phase was prolonged for 72 hours for these treatment groups. A third recovery period of 72 hours was started with the three highest treatment groups since in this test groups, still statistically significant inhibition of growth rate was observed at the end of the second recovery period.

Analytics

The concentration of flurochloridone in the test media and control was analysed by HPLC-MS/MS using external calibration. The test substance was separated on a column (Inertsil ODS-3; 2.1 mm x 33 mm; 3 µm; eluent A: 95 vol. water + 5 vol. methanol + 0.1 vol. formic acid + 5 mM ammonium formate; eluent B: 95 vol. methanol + 5 vol. water + 0.1 vol. formic acid + 5 mM ammonium formate; gradient: hold 0.5 min 60% A/40% B, in 1.5 min to 10% A/90% B, hold 0.5 min 10% A/90% B, in 0.1 min to 100% A, hold 0.4 min 100% A, in 0.1 min to 60% A/40% B, hold 0.9 min 60% A/40% B) at a flow rate of 400 µL/min and an injection volume of 20 or 50 µL. Detection was performed with a MDS Sciex API 4000 triple stage quadrupole mass spectrometer (heater gas temperature: 300°C; spray voltage: 4500 V; ionisation mode: ESI; scan mode: MRM; ion polarity: positive; m/z 312.0 → 292.0; retention time: approximately 2 minutes). The method was validated and the LOQ was set to approximately 0.2 µg/L flurochloridone.

3. Sampling and measurements

Algal biomass was determined daily during the exposure phase and after 48 and 72 hours in the recovery phases by measurement of the algal cell density using an electronic particle counter (Coulter Counter®, Model Z2). The measurements were performed at least in duplicate. In addition, the shape and size of algal cells were inspected visually from the control and the highest treatment group (dilution 1:100) at the end of the exposure phase, from the control and the pre-treatment group of dilution 1:3200 at the end of the first and second recovery phase and from the control and the pre-treatment groups of dilutions 1:1000 to 1:100 at the end of the third recovery phase.

The test media of each test concentration and the control were sampled in duplicate at the start (without algae) and at the end (containing algae) of the exposure phase for analysis of the test substance concentration. For the stability samples, additional flasks containing the test medium with algae were incubated for each treatment under the test conditions.

The pH was measured in each test substance treatment and the control at the start and at the end of each test period. The water temperature was measured daily for the duration of the test and the appearance of the test media was recorded daily during the exposure phase.

4. Calculation of toxicity

Inhibition of algal growth was determined based on the cell density (yield, y) and the specific growth rate (r) for exponentially growing cultures using the equations recommended in the test guidelines.

5. Statistics

The 72-hour EC_{50} values for the inhibition of average yield and growth rate and their 95% confidence limits were calculated as far as possible by Probit Analysis using linear maximum likelihood regression. For the determination of the LOEC and NOEC, average yield and growth rate at the test concentrations were compared to the control values by Williams t-test or Welch t-test, where appropriate.

For the assessment of recovery of exposed algae cultures, yield and growth rate after 72 hours were determined for the test groups used in the recovery phases. Yield and growth rate of the test groups previously treated with the test substance were compared with the control by Williams t-test or Welch t-test, where appropriate. Based on these results, the $NOAEC_{recovery}$ (No Observed Ecologically Adverse Effect Concentration) for recovery of *Navicula pelliculosa* was determined.

Results and Discussions

The concentrations of flurochloridone were measured in one of the duplicate test medium samples from the dilutions 1:10000, 1:3200, 1:1000, 1:320 and 1:100 and from the control. The samples from the highest dilutions were not analyzed, since this concentration was below the NOEC determined in this test. Analysis results are presented in the following table.

Table A 2.2.3-7: Concentrations of flurochloridone in the test media

Dilution [a)]	Measured concentration			Geometric mean measured concentration	
	0 hours	72 hours			
	[µg/L]	[µg/L]	[% of initially measured]	[µg/L]	~~[% of initially measured]~~
Control	< LOQ	< LOQ	-	< LOQ	~~n.a.~~
1:10000	1.60	1.39	86.9	1.5	~~94~~
1:3200	4.75	4.71	99.2	4.7	~~99~~
1:1000	14.9	14.9	100	15	~~101~~
1:320	53.5	48.8	91.2	51	~~95~~
1:100	191	184	96.3	187	~~98~~

a) dilution of undiluted filtrate with a loading rate of 10 mg/L flurochloridone
LOQ approximately 0.2 µg/Lflurochloridone
n.a. not applicable

The biological results were related to the geometric mean measured test concentrations.

At the start of the exposure phase, measured concentrations of flurochloridone in the test media of the test concentrations from the dilutions 1:10000, 1:3200, 1:1000, 1:320 and 1:100 were 1.60, 4.75, 14.9, 53.5 and 191 µg/L, respectively. The corresponding measured concentrations at the end of the exposure phase were 1.39, 4.71, 14.9, 48.8 and 184 µg/L, equivalent to 86% to 100% of the initially measured concentrations. The biological results were related to the geometric mean measured test concentrations, i.e. 1.5, 4.7, 15, 51 and 187 µg/L.

The biomass of algae during exposure to flurochloridone and during the recovery phases is presented in the following tables. The effects of flurochloridone on algal growth during the exposure and the recovery phases are shown in Table A 2.2.3-12 and Table A 2.2.2-13, respectively. After exposure for 72 hours, flurochloridone had a statistically significant inhibitory effect on the growth of algae (based on growth rate and yield) at the test concentration of geometric mean measured 4.7 µg/L and higher. The overall 72-hour NOEC was, therefore, determined to be 1.5 µg/L, since up to and including this test concentration the growth rate and yield of the algae were not statistically significantly lower than in the control. The 72-hour EC_{50} for yield was determined to be 3.4 µg/L (95% confidence limits: 2.3-3.9 µg/L) and the 72-hour EC_{50} for growth rate was 12 µg/L (95% confidence limits: 7.3-19).

After the first 72-hour recovery phase, algal growth in the test groups previously exposed to geometric mean measured 4.7 µg/L flurochloridone and higher was still statistically significantly different from the control. After the second recovery phase, algal growth in the pre-treatment groups differed statistically significantly from the control at geometric mean measured 15 µg/L and higher for growth rate and 51µg/L and higher for yield. After the third recovery phase, no statistically significant differences between the test groups previously exposed to flurochloridone and the control were found up to the highest concentration of geometric mean measured 187 µg/L. Therefore, recovery of algae during three times 72 hours could be demonstrated and the 9-day $NOAEC_{recovery}$ was defined as 187 µg/L.

Microscopic examination of algal cells at the end of the exposure and recovery phases showed no difference in shape and size between algae from the treatment groups of dilution 1:100 (end of exposure phase), dilution 1:3200 (end of first and second recovery) and dilutions 1:1000 to 1:100 (third recovery) and the control.

Table A 2.2.3-8: Biomass of algae exposed to flurochloridone

Treatment		Density of algal cells[b] [mean[c] ± standard deviation] (10^4 cells/mL)		
Dilution[a]	Geom. mean measured test conc. [µg flurochloridone /L]	24 hours	48 hours	72 hours
Control	-	2.6 ± 0.2	7.9 ± 1.4	40.6 ± 3.9
1:32000	n.a.	2.2 ± 0.5	8.2 ± 1.5	40.7 ± 3.9
1:10000	1.5	2.0 ± 0.3	7.9 ± 1.5	42.7 ± 3.3
1:3200	4.7	2.5 ± 0.4	4.9 ± 0.5	8.1 ± 1.4
1:1000	15	2.5 ± 0.4	3.0 ± 0.4	3.6 ± 0.4
1:320	51	2.7 ± 0.6	2.8 ± 0.2	2.8 ± 0.6
1:100	187	2.2 ± 0.2	2.0 ± 0.2	2.5 ± 0.1

n.a. not analysed
[a] dilution of undiluted filtrate with a loading rate of 10 mg/L flurochloridone
[b] The initial cell density was 1.0 x 10^4 cells/mL.
[c] mean of four replicates for the test substance treatments and mean of six replicates for the control

Table A 2.2.3-9: Biomass of algae during recovery

Pre-treatment		Density of algal cells[b] [mean[c] ± standard deviation] (10^4 cells/mL)						
Dilution[a]	Geom. mean measured test conc.	Recovery phase 1[b]		Recovery phase 2			Recovery phase 3[b]	
	[µg flurochloridone /L]	48 hours	72 hours	0 hours	48 hours	72 hours	48 hours	72 hours
Control	-	12.5 ± 0.7	45.8 ± 3.5	1.5 ± 0.0	19.0 ± 2.3	52.6 ± 4.5	14.8 ± 2.3	51.3 ± 6.5
1:32000	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1:10000	1.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1:3200	4.7	8.8 ± 2.1	32.4 ± 2.1	1.3 ± 0.0	20.6 ± 1.9	57.6 ± 0.4	n.d.	n.d.
1:1000	15	2.1 ± 0.6	2.9 ± 0.2	2.7 ± 0.0	30.3 ± 2.4	66.2 ± 1.8	14.6 ± 1.6	54.4 ± 2.4
1:320	51	1.2 ± 0.4	2.1 ± 0.7	2.1 ± 0.0	12.7 ± 1.5	32.2 ± 3.7	13.6 ± 3.0	48.1 ± 6.0
1:100	187	1.0 ± 0.1	1.8 ± 0.5	2.4 ± 0.0	5.0 ± 0.6	18.2 ± 4.5	14.8 ± 1.3	46.1 ± 5.7
Endpoints (95% CL) [µg flurochloridone/L]								
9-day $NOAEC_{recovery}$		187						

n.a. not analysed,
[a] dilution of undiluted filtrate with a loading rate of 10 mg/L flurochloridone
[b] mean of four replicates for the test substance treatments and mean of six replicates for the control
[c] The cell density was nominal 1.0 x 10^4 cells/mL per replicate at the start of the first and third recovery period.

Table A 2.2.3-10: Effects of flurochloridoneon algal growth during exposure

Treatment		Effects during exposure			
Dilution [a)]	Geom. mean measured test conc. [µg flurochloridone /L]	0-72 hours yield (y)		0-72 hours growth rate (r)	
		y (x 10^3)	Inhibition of y [%]	r [day^{-1}]	Inhibition of r [%]
Control	-	39.6	0.0	1.233	0.0
1:32000	n.a.	39.7	-0.4	1.235	-0.2
1:10000	1.5	41.7	-5.4	1.251	-1.5
1:3200	4.7	7.1*	82.0	0.695*	43.6
1:1000	15	2.6*	93.5	0.423*	65.7
1:320	51	1.8*	95.4	0.0341*	72.3
1:100	187	1.5*	96.1	0.038*	75.0
Endpoints (95% CL) [µg flurochloridone/L]					
		Yield (y)		Growth rate (r)	
72-hour EC_{50}		3.4 (2.3-3.9)		12 (7.3-19)	
72-hour NOEC		1.5		1.5	
72-hour LOEC		4.7		4.7	

n.a. not analysed
a) dilution of undiluted filtrate with a loading rate of 10 mg/L flurochloridone
* mean value significantly lower than in the control (yield: according to Welch t-test, one-sided smaller, $\alpha = 0.05$; growth rate: according to Williams t-test, one-sided smaller, $\alpha = 0.05$)

Table A 2.2.3-11: Effects of flurochloridone on algal growth during the recovery phases

Pre-treatment Dilution [a)]	Recovery phase 1				Recovery phase 2				Recovery phase 3			
	0-72 hours yield (y)		0-72 hours growth rate (r)		0-72 hours yield (y)		0-72 hours growth rate (r)		0-72 hours yield (y)		0-72 hours growth rate (r)	
	y (x 10^3)	Inhib. [%]	r [day^{-1}]	Inhib. [%]	y (x 10^3)	Inhib. [%]	r [day^{-1}]	Inhib. [%]	y (x 10^3)	Inhib. [%]	r [day^{-1}]	Inhib. [%]
Control	44.8	0.0	1.274	0.0	51.1	0.0	1.185	0.0	50.3	0.0	1.310	0.0
1:32000	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1:10000	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1:3200	31.4*	29.8	1.159*	9.0	56.3	-10.3	1.264	-6.7	n.d.	n.d.	n.d.	n.d.
1:1000	1.9*	95.7	0.358*	71.9	63.5	-24.3	1.066*	10.0	53.4	-6.1	1.332	-1.6
1:320	1.1*	97.6	0.235*	81.5	30.1*	41.1	0.908*	23.4	47.1	6.4	1.290	1.6
1:100	0.8*	98.3	0.179*	85.9	15.8*	69.2	0.669*	43.5	45.1	10.3	1.276	2.6

n.d. not determined
a) dilution of undiluted filtrate with a loading rate of 10 mg/L flurochloridone
* mean value significantly lower than in the control (yield and growth rate of first recovery, yield of second recovery: according to Williams t-test, one-sided smaller, $\alpha = 0.05$; growth rate of second recovery: according to Welch t-test, one-sided smaller, $\alpha = 0.05$)
Note: After the third recovery phase, yield and growth rate in the pre-treated test groups were not significantly lower than in the control.

In the control, the biomass increased by a factor of 41 over 72 hours (according to guideline ≥ 16). The mean coefficient of variation of the daily growth rates in the control (section-by-section growth rates) during 72 hours was 32% (according to guideline ≤ 35%).The coefficient of variation of the average specific growth rates in the replicates of the control after 72 hours was 2.8% (according to guideline ≤ 10%). Thus, the validity criteria were fulfilled.

Conclusion

The 72-hour E_yC_{50} and E_rC_{50} values of flurochloridone for the freshwater green alga *Navicula pelliculosa* were determined to be 3.4 and 12 µg/L, respectively. Recovery during three times 72 hours has been shown for algae pre-exposed to 187 µg/L, i.e. the 9-day $NOAEC_{recovery}$ of flurochloridone is 187 µg/L.

A 2.2.3.4 Study 4: Toxicity to algae including recovery – *Pseudokirchneriella subcapitata*

Comments of zRMS:	The study was performed in line with OECD 201 with no deviations. All validity criteria were met. The measured test item concentrations were within 80-120% of nominal and results were thus expressed as nominal concentrations. The recovery part is retained for information but it was not evaluated by the zRMS, as recovery is not taken into account in the risk assessment. The part of the summary regarding recovery phase is thus presented in grey letters, to be distinguishable from the evaluated part. Overall, the study is considered acceptable with following endpoints (based on nominal concentrations): E_rC_{50} = 12.0 µg a.s./L E_yC_{50} = 3.4 µg a.s./L NOEC = 1.5 µg a.s./L

Reference:	KCP 10.2.3/04
Report	Flurochloridone Technical – Alga, Growth Inhibition Test with *Pseudokirchneriella subcapitata*, 72 hours, Scheerbaum, D., 2013a, SPO15371 (report number), 90015448 (sponsor report number)
Guideline(s):	OECD 201 (2006)
Deviations:	No
GLP:	Yes
Acceptability:	Acceptable
Duplication (if vertebrate study)	-

Materials and Methods

A. MATERIALS

1. Test material flurochloridone technical (=flurochloridone)
Description Brownish, waxy solid
Lot/Batch # 11083467
Purity 95.5%
Stability of test material Stable under storage conditions (original packaging, normal storage conditions)
Expiry date: 08/2013

2. Vehicle and/or positive control Vehicle control: test water
Positive control: For evaluation of the quality of the algal strain and the experimental conditions, potassium dichromate was tested as a positive control in another study in October/November 2012. The results (72-hour E_rC_{50}: 0.749 mg/L; 72-hour E_yC_{50}: 0.385 mg/L) were within the valid range following the test facility SOPs (72-hour E_rC_{50}: 0.837±0.408 mg/L; 72-hour E_yC_{50}: 0.447±0.246 mg/L).

3. Test organism

Species Freshwater green alga *Pseudokirchneriella subcapitata* HINDÁK, (formerly *Selenastrum capricornutum*)
Strain SAG 61.81

Source	Sammlung von Algenkulturen (SAG), Pflanzenphysiologisches Institut der Universität Göttingen, Nikolausberger Weg 18, D-37073 Göttingen / Germany
Age	Algae cells were taken from an exponentially growing pre-culture set up three days prior to the start of the test.
Acclimation period	The algal pre-culture was exposed to environmental conditions identical to those of the test.
Test units	Sterile 250-mL Erlenmeyer flasks containing 100 mL of test medium, sealed with cotton wool plugs

4. Environmental conditions

Test water The algae were tested in dilution water containing the following components:

Macro-nutrients:

NH_4Cl	15.0 mg/L
KH_2PO_4	1.6 mg/L
$MgSO_4 \cdot 7\ H_2O$	15.0 mg/L
$MgCl_2 \cdot 6\ H_2O$	12.0 mg/L
$CaCl_2 \cdot 2\ H_2O$	18.0 mg/L
$NaHCO_3$	50.0 mg/L

Trace elements:

H_3BO_3	185.0 µg/L
$MnCl_2 \cdot 4\ H_2O$	415.0 µg/L
$ZnCl_2$	3.0 µg/L
$CoCl_2 \cdot 6\ H_2O$	1.5 µg/L
$CuCl_2 \cdot 2\ H_2O$	0.01 µg/L
$Na_2MoO_4 \cdot 2\ H_2O$	7.0 µg/L
$FeCl_3 \cdot 6\ H_2O$	64.0 µg/L
$Na_2EDTA \cdot 2\ H_2O$	100.0 µg/L

The pH of the dilution water was 8.1±0.2

Hardness	0.24 mmol/L Ca + Mg (nominal)
Water temperature	21.5-22.0°C (mean 21.75°C)
Lighting	Continuous illumination at a mean light intensity of 67.0 $\mu E \cdot m^{-2} \cdot s^{-1}$
Shaking	Algae suspensions were continuously oscillated on a rotary shaker at approximately 70 rpm.

B. STUDY DESIGN AND METHODS

1. In-life dates 11.02.2013 to 14.02.2013 (exposure phase)

2. Experimental conditions

Test design

The freshwater green alga *Pseudokirchneriella subcapitata* was exposed in a static 72-hour test to the test substance at five concentrations each with three replicates and six replicates of a test water control. The recorded effect was inhibition of algal growth based on yield and growth rate. Furthermore, algal cells were investigated by microscopic evaluation.

After 72 hours of exposure, algae from the two highest test concentrations and the control were transferred to fresh untreated dilution water and allowed to grow for further four to seven days under test conditions to investigate the recovery potential of the algae.

Inoculum at test start

Approximately 5 x 10^3 - 10^4 cells/mL (nominal), 5089 cells/mL (actual)

Test conditions

The water temperature was maintained at 21.5-22.0°C and the test systems were continuously illuminated at a mean light intensity of 67.0 µE · m^{-2} · s^{-1}. The pH in the test substance treatments and the control was 8.03-8.08 at test start and 8.09-8.97 at test end.

Test concentrations

Nominal test substance concentrations were 0.10, 0.32, 1.0, 3.2 and 10 µg/L. In addition, a control group with untreated test water was used. The selection of the test concentrations was based on the results of a range-finding test (non-GLP).

Treatment/Application

A concentrated stock solution of nominal 10 mg/L was freshly prepared with dilution water. Adequate volumes of this stock solution were added to dilution water to prepare the test media with the desired test concentrations.

After 72 hours of exposure, algae from the two highest test concentrations of 3.2 and 10.0 µg/L and the control were transferred to fresh untreated test water. Algal suspensions of 5 mL from each test concentration replicate and of 1 mL from two control replicates were filled up with dilution water to 100 mL and allowed to grow for further four to seven days under test conditions to investigate the recovery potential of the algae.

Analytics

The content of flurochloridone in the test media and control was determined using HPLC-MS/MS analysis with external calibration. The test substance was separated on a reversed phase column (Acquity UPLC BEH C18, 1.7 µm, 50 x 2.1 mm; eluent A: HPLC-water + 1% formic acid; eluent B: acetonitrile + 1% formic acid; gradient: hold 0.2 min 90% A/10% B, in 0.8 min to 10% A/90% B, hold 1.0 min 10% A/90% B, in 0.1 min to 90% A/10% B, hold 0.9 min 90% A/10% B) at 30°C, a flow rate of 0.5 mL/min and an injection volume of 5 µL. Detection was performed with a mass selective detector (Xevo, Acquity UPLC; ionisation mode: electrospray positive; scan mode: MRM; m/z 312.05 → 292.00 (quantifier) and 145.05 (quanlifier)). The method was validated and the LOQ of the analytical method was set to 0.01 µg/L flurochloridone.

3. Sampling and measurements

Algal cell density was measured at the start of the test and every 24 hours during exposure and at distinct sampling days during recovery by chlorophyll-a-fluorescence measurement, excitation at 436 nm and emission at 685 nm. Furthermore, microscopic evaluation of the cells was performed at the start and end of exposure. Cells were checked for unusual cell shapes, colour differences, chloroplast morphology, flocculation, adherence of algae to test containers and aggregation of algal cells.

For test substance analysis, the test media were sampled at the start and end of exposure. At exposure start, samples were taken from additional replicates without algae. For the samples at exposure end after 72 hours, additional replicates with algae were set up and incubated under test conditions.

The pH was measured in each test substance treatment and the control at the start (in an additional replicate) and at the end (pooled replicates) of exposure. Room temperature was measured continuously. Light intensity was determined prior to test start.

4. Calculation of toxicity

Inhibition of algal growth was determined based on the cell density (yield, y) and the specific growth rate (r) for exponentially growing cultures using the equations recommended in the test guidelines.

5. Statistics

The 72-hour EC_{10}, EC_{20} and EC_{50} values for the inhibition of average yield and growth rate were calculated by sigmoidal dose response regression. Calculation of the confidence intervals was carried out using standard procedures.

LOEC and NOEC were determined by calculation of statistical significance of yield and growth rate by using ANOVA and Dunnett's test. When running an ANOVA, a normality test (Shapiro-Wilk test, p = 0.05) and an equal variance test (Levene median test, p = 0.05) were done first. The α value was 0.05. For calculation of statistical significance of growth rate, the transformed data ($y = y^2$) were used to pass the tests on normality and equal variance. The highest treatment was not included.

Results and Discussion

The concentrations of flurochloridone in the test media and control were determined at the start and end of exposure. Analysis results are presented in the following table.

Table A 2.2.3-12: Concentrations of flurochloridone in the test media

Nominal concentration [µg/L]	Measured concentration			
	0 hours		72 hours	
	[µg/L]	[% of nominal]	[µg/L]	[% of nominal]
Control	< LOQ	n.a.	< LOQ	n.a.
0.10	0.108	108	0.0837	84
0.32	0.346	108	0.312	98
1.0	1.08	108	1.04	104
3.2	3.31	103	3.21	100
10	10.2	102	9.66	97

LOQ of analytical method: 0.01 µg/L flurochloridone
n.a. not applicable

The analytical results confirm the correct dosing and stability of the test substance for the duration of exposure. The biological results were related to the nominal concentrations of the test substance.

At the start of exposure, measured concentrations of flurochloridone the test media were in the range of 102% to 108% of nominal. Measured concentrations of flurochloridone at the end of exposure after 72 hours ranged between 84% and 104%. Thus, the correct dosing and the stability of the test substance for the duration of exposure were confirmed. The biological results were related to the nominal concentrations of the test substance.

The biomass of algae and effects on growth during exposure to flurochloridone and during the recovery phase are presented in the following tables. After exposure for 72 hours, flurochloridone had a statistically significant inhibitory effect on the growth of algae, calculated as yield and growth rate, at the test concentrations of ≥ 1.0 and ≥ 3.2 µg/L, respectively. The overall 72-hour NOEC was, therefore, determined to be 0.32 µg/L, since up to and including this test concentration the yield of the algae was not statistically significantly lower than in the control. The 72-hour EC_{50} for yield was determined to be 1.30 µg/L (95% confidence limits: 1.09-2.18 µg/L) and the 72-hour EC_{50} for growth rate was 2.42 µg/L (95% confidence limits: 2.18-2.60 µg/L).

After four (pre-treatment group of 3.2 µg/L) and seven (pre-treatment group of 10 µg/L) days of incubation in untreated dilution water during the recovery phase, the toxic effect of flurochloridone was observed to be reversible. The growth rates in algal cultures previously treated with the test substance were similar (pre-treatment group of 10 µg/L) or even higher (pre-treatment group of 3.2 µg/L) than in the control after the respective recovery phase. Therefore, recovery of algae could be demonstrated up and including to the highest test concentration of 10 µg/L.

Microscopic evaluation of algal cells at the start and end of exposure revealed no morphological abnormalities at all test concentrations.

Table A 2.2.3-13: Biomass of algae exposed to flurochloridone and algal biomass during recovery

Nominal test concentration [µg flurochloridone /L]	Cell density [a] [cells/mL]					
	Exposure phase[b]			Recovery phase		
	24 hours	48 hours	72 hours	0 days	4 days	7 days
Control	27747	252080	1919535	19195	2102304-2355605	n.d.
0.10	30867	238103	1953756	n.d.	n.d.	n.d.
0.32	28889	261364	1975896	n.d.	n.d.	n.d.
1.0	26546	205927	1537270	n.d.	n.d.	n.d.
3.2	14527	23316	26246	1312	1159837-1943363	n.d.
10	12312	4354	3800	190	n.d.	496674-1325127

n.d. not determined

[a] Exposure phase: mean of three replicates for the test substance treatments and mean of six replicates for the control; recovery phase: cell density at 0 days calculated from the cell density at the end of exposure, cell density at days 4 and 7 given as range of two (control) or three (two highest pre-treatment groups) replicates

[b] The initial cell density was 5089 cells/mL.

Table A 2.2.3-14: Effects of flurochloridoneon algal growth during exposure and recovery phase

Nominal test concentration [µg flurochloridone /L]	Exposure phase 0-72 hours yield (y)		Exposure phase 0-72 hours growth rate (r)		Recovery phase 0-4 days [a]/0-7 days [a] growth rate (r)	
	y ($x 10^3$)	Inhibition of y [%]	r [day^{-1}]	Inhibition of r [%]	r[b] [day^{-1}]	Inhibition[b] of r [%]
Control	1914446	0.0	1.98	0.0	1.19	0.0
0.10	1948667	-1.79	1.98	-0.27	n.d.	n.d.
0.32	1970807	-2.94	1.99	-0.46	n.d.	n.d.
1.0	1532181*	20.0	1.90(*)	3.75	n.d.	n.d.
3.2	21157*	98.9	0.531*	73.2	1.78	-49.9
10	-1289*	100	-0.098*	100	1.18	0.14
Endpoints (95% CL) [µg flurochloridone /L]						
	Yield (y)		Growth rate (r)			
72-hour EC_{50}	1.30 (1.09-2.18)		2.42 (2.18-2.60)			
72-hour NOEC	0.32		1.0			
72-hour LOEC	1.0		3.2			

n.d. not determined

[a] 4 days of recovery for the control and the test group pre-treated with 3.2 µg/L and 7 days of recovery for the test group pre-treated with 10 µg/L

[b] mean values calculated from data given in the report (no statistics performed)

* mean value significantly lower than in the control (according to ANOVA followed by Dunnett's test, $\alpha = 0.05$)

(*) effect considered not biologically significant (< 10%)

In the control, the biomass increased by a factor of 377 over 72 hours (according to guideline ≥ 16). The mean coefficient of variation of the daily growth rates in the control (section-by-section growth rates) during 72 hours was 13.6% (according to guideline ≤ 35%).The coefficient of variation of the average specific growth rates in the replicates of the control after 72 hours was 0.94% (according to guideline ≤ 7%). Thus, the validity criteria were fulfilled.

Conclusion

The 72-hour E_yC_{50} and E_rC_{50} values of flurochloridone for the freshwater green alga *Pseudokirchneriella subcapitata* were determined to be 1.30 and 2.42 µg/L, respectively. During up to seven days in untreated dilution water, there is potential for algal recovery following exposure to up to and including the highest test concentration of 10 µg/L flurochloridone.

A 2.2.3.5 Study 5: Toxicity to algae including recovery – *Nitzschia communis*

Comments of zRMS:	The study was performed in line with OECD 201 with no deviations. The aim of the study was to generate additional toxicity data for algae and for this reason in the study non-standard species (*Nitzschia communis*) was used. This is acceptable, but it should be kept in mind that the environmental conditions or the acceptability criteria in OECD 201 were not verified for this species. Nevertheless, validity criteria of OECD 201 were fulfilled. The measured test item concentrations were within 80-120% of nominal and results were thus expressed as nominal concentrations. The recovery part is retained for information but it was not evaluated by the zRMS, as recovery is not taken into account in the risk assessment. The part of the summary regarding recovery phase is thus presented in grey letters, to be distinguishable from the evaluated part. Overall, the study is considered acceptable with following endpoints (based on nominal concentrations): E_rC_{50} = 4.45 µg a.s./L E_yC_{50} = 2.5 µg a.s./L NOEC = 1.0 µg a.s./L

Reference:	KCP 10.2.3/05
Report	Flurochloridone Technical – Alga, Growth Inhibition Test with *Nitzschia communis*, 72 hours, Scheerbaum, D., 2013b, SNC15371 (report number), 90015449 (sponsor report number)
Guideline(s):	OECD 201 (2006)
Deviations:	No
GLP:	Yes
Acceptability:	Acceptable
Duplication (if vertebrate study)	-

Materials and Methods

A. MATERIALS

1. Test material	flurochloridonetechnical (=flurochloridone)
Description	Brownish, waxy solid
Lot/Batch #	11083467
Purity	95.5%
Stability of test material	Stable under storage conditions (original packaging, normal storage conditions) Expiry date: 08/2013

2. Vehicle and/or positive control

Vehicle control: test water
Positive control: For evaluation of the quality of the algal strain and the experimental conditions, 3,5-dichlorophenol was tested as a positive control in March 2013. The results (72-hour E_rC_{50}: 1.35 mg/L; 72-hour E_yC_{50}: 1.14 mg/L) were within the valid range following the test facility SOPs.

3. Test organism

Species	Freshwater diatom alga *Nitzschia communis* Rabenhorst 1860
Strain	CCAC 1762 B
Source	Culture Collection of Algae at the University of Cologne (CCAC), Biozentrum Koeln, Zuelpicher Strasse 47 b, 50674 Koeln / Germany
Age	Algae cells were taken from an exponentially growing pre-culture set up three days prior to the start of the test.
Acclimation period	The algal pre-culture was exposed to environmental conditions identical to those of the test.
Test units	Sterile 250-mL Erlenmeyer flasks containing 100 mL of test medium, sealed with cotton wool plugs

4. Environmental conditions

Test water

The algae were tested in dilution water (WARIS-H medium according to the CCAC administration) containing the following components:

KNO_3	100 mg/L
$MgSO_4 \cdot 7\ H_2O$	20.0 mg/L
$(NH_4)_2HPO_4$	20.0 mg/L
$Ca(NO_3)_2 \cdot 4\ H_2O$	100 mg/L
HEPES	238.31 mg/L
Titriplex III	3.0 mg/L
H_3BO_3	1.14 mg/L
$MnCl_2 \cdot 4\ H_2O$	0.144 mg/L
$ZnSO_4 \cdot 7\ H_2O$	0.021 mg/L
$CoCl_2 \cdot 6\ H_2O$	0.004 mg/L
EDTA (Titriplex II)	5.22 mg/L
$FeSO_4 \cdot 7\ H_2O$	4980 µg/L
1 M KOH	3.03 mg/L
Vitamin B12	$2 \cdot 10^{-4}$ mg/L
Niacinamide	$1 \cdot 10^{-4}$ mg/L
Biotin	$1 \cdot 10^{-3}$ mg/L
Thiamine-HCl	0.100 mg/L
Soil extract	10 mL/L
$Na_2SiO_3 \cdot 9\ H_2O$	142.1 mg/L

Prior to testing, the pH of the dilution water was adjusted to 7.0±0.1 by addition of 1 N NaOH and 1 N HCl.

Water temperature	22.0-22.5°C (mean 22.3°C)
Lighting	Continuous illumination at a mean light intensity of 52.0 $\mu E \cdot m^{-2} \cdot s^{-1}$
Shaking	Algae suspensions were continuously oscillated on a rotary shaker at approximately 70 rpm.

B. STUDY DESIGN AND METHODS

1. In-life dates 11.03.2013 to 14.03.2013 (exposure phase)

2. Experimental conditions

Test design

The freshwater diatom alga *Nitzschia communis* was exposed in a static 72-hour test to the test substance at five concentrations each with three replicates and six replicates of a test water control. The recorded effect was inhibition of algal growth based on yield and growth rate. Furthermore, algal cells were investigated by microscopic evaluation.

After 72 hours of exposure, algae from the four highest test concentrations and the control were transferred to fresh untreated dilution water and allowed to grow for further four to eight days under test conditions to investigate the recovery potential of the algae.

Inoculum at test start

Approximately 5 x 10^3 - 10^4 cells/mL (nominal), 9569 cells/mL (actual)

Test conditions

The water temperature was maintained at 22.0-22.5°C and the test systems were continuously illuminated at a mean light intensity of 52.0 µE · m^{-2} · s^{-1}. The pH in the test substance treatments and the control was 6.97-7.01 at test start and 6.99-7.28 at test end.

Test concentrations

Nominal test substance concentrations were 1.00, 3.16, 10.0, 31.6 and 100 µg/L. In addition, a control group with untreated test water was used. The selection of the test concentrations was based on the results of a range-finding test (non-GLP).

Treatment/Application

A concentrated stock solution of nominal 10 mg/L was freshly prepared with dilution water. Adequate volumes of this stock solution were added to dilution water to prepare the test media with the desired test concentrations.

After 72 hours of exposure, algae from the four highest test concentrations of 3.16, 10.0, 31.6 and 100 µg/L and the control were transferred to fresh untreated test water. Algal suspensions of 2 to 5 mL from each test concentration replicate and from two control replicates were filled up with dilution water to 100 mL and allowed to grow for further four to eight days under test conditions to investigate the recovery potential of the algae.

Analytics

The content of flurochloridone in the test media and control was determined using HPLC-MS/MS analysis with external calibration. The test substance was separated on a reversed phase column (Acquity UPLC BEH C18, 1.7 µm, 50 x 2.1 mm; eluent A: HPLC-water + 1% formic acid; eluent B: acetonitrile + 1% formic acid; gradient: hold 0.2 min 90% A/10% B, in 0.8 min to 10% A/90% B, hold 1.0 min 10% A/90% B, in 0.1 min to 90% A/10% B, hold 0.9 min 90% A/10% B) at 30°C, a flow rate of 0.5 mL/min and an injection volume of 5 µL. Detection was performed with a mass selective detector (Xevo, Acquity UPLC; ionisation mode: electrospray positive; scan mode: MRM; m/z 312.05 → 292.00 (quantifier) and 145.05 (quanlifier)). The method was validated and the LOQ of the analytical method was set to 3.0 µg/L flurochloridone.

3. Sampling and measurements

Algal cell density was measured at the start of the test and every 24 hours during exposure and at distinct sampling days during recovery by chlorophyll-a-fluorescence measurement, excitation at 436 nm and emission at 685 nm. Furthermore, microscopic evaluation of the cells was performed at the start and end of exposure. Cells were checked for unusual cell shapes, colour differences, differences in chloroplast morphology, flocculation, adherence of algae to test containers and agglutination of algal cells.

For test substance analysis, the test media were sampled at the start and end of exposure. At exposure start, samples were taken from additional replicates without algae. For the samples at exposure end after 72 hours, additional replicates with algae were set up and incubated under test conditions.

The pH was measured in each test substance treatment and the control at the start (in an additional replicate) and at the end (pooled replicates) of exposure. Room temperature was measured continuously. Light intensity was determined prior to test start.

4. Calculation of toxicity

Inhibition of algal growth was determined based on the cell density (yield, y) and the specific growth rate (r) for exponentially growing cultures using the equations recommended in the test guidelines.

5. Statistics

The 72-hour EC_{10}, EC_{20} and EC_{50} values for the inhibition of average yield and growth rate were calculated by sigmoidal dose response regression. Calculation of the confidence intervals was carried out using standard procedures.

LOEC and NOEC were determined by calculation of statistical significance of yield and growth rate by using ANOVA and Dunnett's test. When running an ANOVA, a normality test (Shapiro-Wilk test, p = 0.05) and an equal variance test (Levene median test, p = 0.05) were done first. The α value was 0.05. Normality test failed for calculation of yield values. The two highest concentration levels with ≥ 99% inhibition were excluded to achieve normal distribution.

Results and Discussion

The concentrations of flurochloridone in the test media and control were determined at the start and end of exposure. Analysis results are presented in the following table.

Table A 2.2.3-15: Concentrations of flurochloridone in the test media

Nominal concentration [µg/L]	Measured concentration			
	0 hours		72 hours	
	[µg/L]	[% of nominal]	[µg/L]	[% of nominal]
Control	< LOQ	n.a.	< LOQ	n.a.
1.00	0.870	87	1.00	100
3.16	3.04	96	3.15	100
10.0	9.04	90	10.2	102
31.6	29.8	94	33.1	105
100	99.0	99	101	101

LOQ of analytical method: 3.0 µg/L flurochloridone
n.a. not applicable

The analytical results confirm the correct dosing and stability of the test substance for the duration of exposure. The biological results were related to the nominal concentrations of the test substance.

At the start of exposure, measured concentrations of flurochloridone in the test media were in the range of 87% to 99% of nominal. Measured concentrations of flurochloridone at the end of exposure after 72 hours ranged between 100% and 105%. Thus, the correct dosing and the stability of the test substance for the

duration of exposure were confirmed. The biological results were related to the nominal concentrations of the test substance.

The biomass of algae during exposure to flurochloridone and during the recovery phase is presented in Table A 2.2.3-18. The effects of flurochloridoneon algal growth during the exposure and recovery phase are shown in Table A 2.2.3-19. After exposure for 72 hours, flurochloridone had a statistically significant inhibitory effect on the growth of algae (yield and growth rate) at the test concentrations of ≥ 3.16 µg/L. The overall 72-hour NOEC was, therefore, determined to be 1.00 µg/L, since at this test concentration the yield and growth rate of algae were not statistically significantly lower than in the control. The 72-hour EC_{50} for yield was determined to be 2.50 µg/L (95% confidence limits: 2.08-2.71 µg/L) and the 72-hour EC_{50} for growth rate was 4.45 µg/L (95% confidence limits: 4.14-4.81 µg/L).

After four (pre-treatment groups of 3.16 and 10.0 µg/L), five (pre-treatment group of 31.6 µg/L) and eight (pre-treatment group of 100 µg/L) days of incubation in untreated dilution water during the recovery phase, the toxic effect of flurochloridone was observed to be reversible. The growth rates in algal cultures previously treated with the test substance were identical (pre-treatment group of 3.16 µg/L), slightly lower (pre-treatment group of 31.6 µg/L) or even higher (pre-treatment groups of 10 and 100 µg/L) than in the control after the respective recovery phase. Therefore, recovery of algae could be demonstrated up and including to the highest test concentration of 100 µg/L.

Microscopic evaluation of the cells at start of exposure revealed no morphological abnormalities. At the end of exposure, the chloroplasts in algal cells of the test concentrations of 10.0, 31.6 and 100 µg/L were partly lighter pigmented. Algae of the two lowest test concentrations of 1.00 and 3.16 mg/L and the control revealed no morphological abnormalities.

Table A 2.2.3-16: Biomass of algae exposed to flurochloridone and algal biomass during recovery

Nominal test concentration	Cell density [a] [cells/mL]							
	Exposure phase[b]			Recovery phase				
[µg flurochloridone /L]	24 hours	48 hours	72 hours	0 days	4 days	5 days	7 days	8 days
Control	31291	131281	343248	6865	445737-477290	n.d.	n.d.	n.d.
1.00	32729	120248	405065	n.d.	n.d.	n.d.	n.d.	n.d.
3.16	24566	43174	116477	5824	367022-409604	n.d.	n.d.	n.d.
10.0	18455	16763	16316	816	189114-219748	n.d.	n.d.	n.d.
31.6	16861	10549	12160	608	n.d.	48641-60785	n.d.	n.d.
100	16812	9549	9120	182	n.d.	6804-7912	26991-65605	107738-301586

n.d. not determined

[a] Exposure phase: mean of three replicates for the test substance treatments and mean of six replicates for the control; recovery phase: cell density at 0 days calculated from the cell density at the end of exposure, cell density at days 4, 5, 7 and 8 given as range of two (control) or three (four highest pre-treatment groups) replicates

[b] The initial cell density was 9569 cells/mL.

Table A 2.2.3-17: Effects of flurochloridoneon algal growth during exposure and recovery phase

Nominal test concentration	Exposure phase				Recovery phase	
	0-72 hours yield (y)		0-72 hours growth rate (r)		0-4 days [a]/0-5 days [a]/0-8 days [a] growth rate (r)	
[µg flurochloridone/L]	y (x 10^3)	Inhibition of y [%]	r [day^{-1}]	Inhibition of r [%]	r^b [day^{-1}]	Inhibition[b] of r [%]
Control	333679	0.0	1.19	0.0	1.05	0.0
1.00	395496[*]	-18.5	1.25	-4.76	n.d.	n.d.
3.16	106908*	68.0	0.832*	30.2	1.05	0.0
10.0	6747*	98.0	0.176*	85.3	1.39	-32.1
31.6	2591*	99.2	0.078{*}	93.4	0.903	14.0
100	-449*	100	-0.016{*}	100	1.39	-32.4
Endpoints (95% CL) [µg flurochloridone/ L]						
	Yield (y)		**Growth rate (r)**			
72-hour EC_{50}	2.50 (2.08-2.71)		4.45 (4.14-4.81)			
72-hour NOEC	1.00		1.00			
72-hour LOEC	3.16		3.16			

n.d. not determined

a) 4 days of recovery for the control and the test groups pre-treated with 3.16 and 10.0 µg/L, 5 days of recovery for the test group pre-treated with 31.6 µg/L and 8 days of recovery for the test group pre-treated with 100 µg/L

b) mean values calculated from data given in the report (no statistics performed)

* mean value significantly lower than in the control (according to ANOVA followed by Dunnett's test, $\alpha = 0.05$)

[*] significance caused by growth stimulation

{*} not statistically tested to achieve normal distribution

In the control, the biomass increased by a factor of 36 over 72 hours (according to guideline ≥ 16). The mean coefficient of variation of the daily growth rates in the control (section-by-section growth rates) during 72 hours was 20.8% (according to guideline ≤ 35%).The coefficient of variation of the average specific growth rates in the replicates of the control after 72 hours was 3.0% (according to guideline ≤ 10%). Thus, the validity criteria were fulfilled.

Conclusion

The 72-hour E_yC_{50} and E_rC_{50} values of flurochloridone for the freshwater diatom alga *Nitzschia communis* were determined to be 2.50 and 4.45 µg/L, respectively. During up to eight days in untreated dilution water, there is potential for algal recovery following exposure to up to and including the highest test concentration of 100 µg/L flurochloridone.

A 2.2.3.6 Study 6: Toxicity to algae including recovery – *Synechococcus leopoliensis*

Comments of zRMS:	The study was performed in line with OECD 201 with no deviations. The aim of the study was to generate additional toxicity data for algae and for this reason in the study non-standard species (*Synechococcus leopoliensis*) was used. This is acceptable, but it should be kept in mind that the environmental conditions or the acceptability criteria in OECD 201 were not verified for this species. Nevertheless, validity criteria of OECD 201 were fulfilled. The measured test item concentrations dropped below 80% of nominal and therefore results were expressed in terms of geometric mean measured concentrations. The recovery part is retained for information but it was not evaluated by the zRMS, as recovery is not taken into account in the risk assessment. The part of the summary regarding recovery phase is thus presented in grey letters, to be distinguishable from the evaluated part. Overall, the study is considered acceptable with following endpoints (based on nominal concentrations): E_rC_{50} = 4.07 µg a.s./L E_yC_{50} = 2.22 µg a.s./L NOEC = 0.946 µg a.s./L

Reference:	KCP 10.2.3/06
Report	Flurochloridone Technical – Alga, Growth Inhibition Test with *Synechococcus leopoliensis*, 72 hours, Scheerbaum, D., 2013c, SSL15371 (report number), 90015450 (sponsor report number)
Guideline(s):	OECD 201 (2006)
Deviations:	No
GLP:	Yes
Acceptability:	Acceptable
Duplication (if vertebrate study)	-

Materials and Methods

A. MATERIALS

1. Test material flurochloridone technical (=flurochloridone)
Description Brownish, waxy solid
Lot/Batch # 11083467
Purity 95.5%
Stability of test material Stable under storage conditions (original packaging, normal storage conditions)
Expiry date: 08/2013

2. Vehicle and/or positive control Vehicle control: test water
Positive control: For evaluation of the quality of the algal strain and the experimental conditions, 3,5-dichlorophenol was tested as a positive control in another study in January 2013. The results (72-hour E_rC_{50}: 2.76 mg/L; 72-hour E_yC_{50}: 1.82 mg/L) were within the valid range following the test facility SOPs (72-hour E_rC_{50}: 2.76±1.07 mg/L; 72-hour E_yC_{50}: 1.72±0.461 mg/L).

3. Test organism

Species	Freshwater cyanobacteria *Synechococcus leopoliensis*
Strain	SAG 1402-1
Source	Sammlung von Algenkulturen (SAG), Pflanzenphysiologisches Institut der Universität Göttingen, Nikolausberger Weg 18, D-37073 Göttingen / Germany
Age	Algae cells were taken from an exponentially growing pre-culture set up three days prior to the start of the test.
Acclimation period	The algal pre-culture was exposed to environmental conditions identical to those of the test.
Test units	Sterile 250-mL Erlenmeyer flasks containing 100 mL of test medium, sealed with cotton wool plugs

4. Environmental conditions

Test water

The algae were tested in dilution water (threefold concentrated AAP medium) containing the following components:

Macro-nutrients:

K_2HPO_4	3.132 mg/L
$MgSO_4 \cdot 7\ H_2O$	44.1 mg/L
$MgCl_2 \cdot 6\ H_2O$	36.492 mg/L
$CaCl_2 \cdot 2\ H_2O$	13.23 mg/L
$NaHCO_3$	45 mg/L
$NaNO_3$	76.5 mg/L

Trace elements:

H_3BO_3	556.5 µg/L
$MnCl_2 \cdot 4\ H_2O$	1246 µg/L
$ZnCl_2$	9.81 µg/L
$CoCl_2 \cdot 6\ H_2O$	4.29 µg/L
$CuCl_2 \cdot 2\ H_2O$	0.036 µg/L
$Na_2MoO_4 \cdot 2\ H_2O$	21.78 µg/L
$FeCl_3 \cdot 6\ H_2O$	479.4 µg/L
Na_2-EDTA	900 µg/L

MES monohydrate was added at 2665.6 mg/L to enable sufficient pH values during the test.
Prior to testing, the pH of the dilution water was adjusted to 7.5±0.2 by addition of 1 N NaOH and 1 N HCl.

Water temperature	21.0-22.0°C (mean 21.5°C)
Lighting	Continuous illumination at a mean light intensity of 27.7 µE · m^{-2} · s^{-1}
Shaking	The test containers were shaken per hand twice a day.

B. STUDY DESIGN AND METHODS

1. In-life dates 18.02.2013 to 21.02.2013 (exposure phase)

2. Experimental conditions

Test design

The freshwater cyanobacteria *Synechococcus leopoliensis* was exposed in a static 72-hour test to the test substance at five concentrations each with three replicates and six replicates of a test water control. The

recorded effect was inhibition of algal growth based on yield and growth rate. Furthermore, algal cells were investigated by microscopic evaluation.

After 72 hours of exposure, algae from the three highest test concentrations and the control were transferred to fresh untreated dilution water and allowed to grow for further four to seven days under test conditions to investigate the recovery potential of the algae.

Inoculum at test start

Approximately 5 x 10^4 – 10^5 cells/mL (nominal), 77588 cells/mL (actual)

Test conditions

The water temperature was maintained at 21.0-22.0°C and the test systems were continuously illuminated at a mean light intensity of 27.7 µE · m^{-2} · s^{-1}. The pH in the test substance treatments and the control was 7.49-7.57 at test start and 7.53-7.82 at test end.

Test concentrations

Nominal test substance concentrations were 0.50, 1.25, 3.125, 7.81 and 19.5 µg/L. In addition, a control group with untreated test water was used. The selection of the test concentrations was based on the results of a range-finding test (non-GLP).

Treatment/Application

A concentrated stock solution of nominal 10 mg/L was freshly prepared with dilution water. Adequate volumes of this stock solution were added to dilution water to prepare the test media with the desired test concentrations.

After 72 hours of exposure, algae from the three highest test concentrations of nominal 3.125, 7.81 and 19.5 µg/L and the control were transferred to fresh untreated test water. Algal suspensions of 5 mL from each test concentration replicate and from two control replicates were filled up with dilution water to 100 mL and allowed to grow for further four to seven days under test conditions to investigate the recovery potential of the algae.

Analytics

The content of flurochloridone in the test media and control was determined using HPLC-MS/MS analysis with external calibration. The test substance was separated on a reversed phase column (Acquity UPLC BEH C18, 1.7 µm, 50 x 2.1 mm; eluent A: HPLC-water + 1% formic acid; eluent B: acetonitrile + 1% formic acid; gradient: hold 0.2 min 90% A/10% B, in 0.8 min to 10% A/90% B, hold 1.0 min 10% A/90% B, in 0.1 min to 90% A/10% B, hold 0.9 min 90% A/10% B) at 30°C, a flow rate of 0.5 mL/min and an injection volume of 5 µL. Detection was performed with a mass selective detector (Xevo, Acquity UPLC; ionisation mode: electrospray positive; scan mode: MRM; m/z 312.05 → 292.00 (quantifier) and 145.05 (quanlifier)). The method was validated and the LOQ of the analytical method was set to 3.0 µg/L flurochloridone.

3. Sampling and measurements

Algal cell density was measured at the start of the test and every 24 hours during exposure and at distinct sampling days during recovery by chlorophyll-a-fluorescence measurement, excitation at 436 nm and emission at 685 nm. Furthermore, microscopic evaluation of the cells was performed at the start and end of exposure. Cells were checked for unusual cell shapes, colour differences, differences in chloroplast morphology, flocculation, adherence of algae to test containers and agglutination of algal cells.

For test substance analysis, the test media were sampled at the start and end of exposure. At exposure start, samples were taken from additional replicates without algae. For the samples at exposure end after 72 hours, additional replicates with algae were set up and incubated under test conditions.

The pH was measured in each test substance treatment and the control at the start (in an additional replicate) and at the end (pooled replicates) of exposure. Room temperature was measured continuously. Light intensity was determined prior to test start.

4. Calculation of toxicity

Inhibition of algal growth was determined based on the cell density (yield, y) and the specific growth rate (r) for exponentially growing cultures using the equations recommended in the test guidelines.

5. Statistics

The 72-hour EC_{10}, EC_{20} and EC_{50} values for the inhibition of average yield and growth rate were calculated by sigmoidal dose response regression. Calculation of the confidence intervals was carried out using standard procedures.

LOEC and NOEC were determined by calculation of statistical significance of yield and growth rate by using ANOVA and Dunnett's test. When running an ANOVA, a normality test (Shapiro-Wilk test, p = 0.05) and an equal variance test (Levene median test, p = 0.05) were done first. The α value was 0.05.

Results and Discussion

The concentrations of flurochloridone in the test media and control were determined at the start and end of exposure. Analysis results are presented in the following table.

Table A 2.2.3-18: Concentrations of flurochloridone in the test media

Nominal concentration [µg/L]	Measured concentration				Geometric mean measured concentration	
	0 hours		72 hours			
	[µg/L]	[% of nominal]	[µg/L]	[% of nominal]	[µg/L]	[% of nominal]
Control	< LOQ	n.a.	< LOQ	n.a.	< LOQ	n.a.
0.50	0.357	71	0.376	75	0.366	73
1.25	0.932	75	0.961	77	0.946	76
3.125	2.56	82	2.35	75	2.45	78
7.81	6.27	80	6.28	80	6.27	80
19.5	14.0	72	14.8	76	14.4	74

LOQ of analytical method: 3.0 µg/L flurochloridone
n.a. not applicable

Since the measured test concentrations were partly below 80%, the biological results were related to the geometric mean measured concentrations of the test substance.
At the start of exposure, measured concentrations of flurochloridone in the test media were in the range of 71% to 82% of nominal. Measured concentrations of flurochloridone at the end of exposure after 72 hours ranged between 75% and 80%. Since the measured test concentrations were partly below 80%, the biological results were related to the geometric mean measured concentrations of the test substance, i.e. 0.366, 0.946, 2.45, 6.27 and 14.4 µg/L.

The biomass of algae and effects on growth during exposure to flurochloridone and during the recovery phase are presented in the following tables. After exposure for 72 hours, flurochloridone had a statistically significant inhibitory effect on the growth of algae (yield and growth rate) at the test concentrations of ≥ 2.45 µg/L. The overall 72-hour NOEC was, therefore, determined to be 0.946 µg/L, since up to and including this test concentration the yield and growth rate of algae were not statistically significantly lower than in the control. The 72-hour EC_{50} for yield was determined to be 2.22 µg/L (95% confidence limits: 1.84-2.49 µg/L) and the 72-hour EC_{50} for growth rate was 4.07 µg/L (95% confidence limits: 3.67-4.57 µg/L).

After four (pre-treatment group of 2.45 µg/L), six (pre-treatment group of 6.27 µg/L) and seven (pre-treatment group of 14.4 µg/L) days of incubation in untreated dilution water during the recovery phase, the toxic effect of flurochloridone was observed to be reversible. The growth rates in algal cultures previously treated with the test substance were similar (pre-treatment group of 2.45 µg/L), slightly lower (pre-treatment group of 6.27 µg/L) or even higher (pre-treatment group of 14.4 µg/L) than in the control after the respective recovery phase. Therefore, recovery of algae could be demonstrated up and including to the highest test concentration of 14.4 µg/L.

Microscopic evaluation of algal cells at the start and end of exposure revealed no morphological abnormalities at all test concentrations.

Table A 2.2.3-19: Biomass of algae exposed to flurochloridone and algal biomass during recovery

Geometric mean measured test conc.	Cell density [a] [cells/mL]						
	Exposure phase[b]			Recovery phase			
[µg flurochloridone /L]	24 hours	48 hours	72 hours	0 hours	4 days	6 days	7 days
Control	174172	433754	1698782	84939	6198610-6451724	n.d.	n.d.
0.366	185843	465298	1857516	n.d.	n.d.	n.d.	n.d.
0.946	172654	451712	1766604	n.d.	n.d.	n.d.	n.d.
2.45	169944	402137	748042	37402	2406020-2474095	n.d.	n.d.
6.27	151480	241597	247992	12400	n.d.	3333924-3359509	n.d.
14.4	152745	191227	191480	9574	n.d.	1562884-2187268	5979100-6087500

n.d. not determined

[a] Exposure phase: mean of three replicates for the test substance treatments and mean of six replicates for the control; recovery phase: cell density at 0 days calculated from the cell density at the end of exposure, cell density at days 4, 6 and 7 given as range of two (control) or three (three highest pre-treatment groups) replicates

[b] The initial cell density was 77588 cells/mL.

Table A 2.2.3-20: Effects of flurochloridoneon algal growth during exposure and recovery phase

Geometric mean measured test conc. [µg flurochloridone /L]	Exposure phase				Recovery phase 0-4 days [a]/0-6 days [a]/0-7 days [a] growth rate (r)	
	0-72 hours yield (y)		0-72 hours growth rate (r)			
	y (x 10^3)	Inhibition of y [%]	r [day^{-1}]	Inhibition of r [%]	r[b] [day^{-1}]	Inhibition[b] of r [%]
Control	1621194	0.0	1.03	0.0	1.08	0.0
0.366	1779928	-9.79	1.06	-2.84	n.d.	n.d.
0.946	1689016	-4.18	1.04	-1.17	n.d.	n.d.
2.45	670454*	58.7	0.755*	26.6	1.05	2.95
6.27	170404*	89.5	0.387*	62.3	0.933	-13.2
14.4	113892	93.0	0.301*	70.7	1.20	11.3

Endpoints (95% CL) [µg flurochloridone/L]		
	Yield (y)	Growth rate (r)
72-hour EC_{50}	2.22 (1.84-2.49)	4.07 (3.67-4.57)
72-hour NOEC	0.946	0.946
72-hour LOEC	2.45	2.45

n.d. not determined

[a] 4 days of recovery for the control and the test group pre-treated with 2.45 µg/L, 6 days of recovery for the test group pre-treated with 6.27 µg/L and 7 days of recovery for the test group pre-treated with 14.4 µg/L

[b] mean values calculated from data given in the report (no statistics performed)

* mean value significantly lower than in the control (according to ANOVA followed by Dunnett's test, $\alpha = 0.05$)

In the control, the biomass increased by a factor of 22 over 72 hours (according to guideline ≥ 16). The mean coefficient of variation of the daily growth rates in the control (section-by-section growth rates) during 72 hours was 29.5% (according to guideline ≤ 35%).The coefficient of variation of the average specific growth rates in the replicates of the control after 72 hours was 2.35% (according to guideline ≤ 10%). Thus, the validity criteria were fulfilled.

Conclusion

The 72-hour E_yC_{50} and E_rC_{50} values of flurochloridone for the freshwater cyanobacteria *Synechococcus leopoliensis* were determined to be 2.22 and 4.07 µg/L, respectively. During up to seven days in untreated dilution water, there is potential for algal recovery following exposure to up to and including the highest test concentration of 14.4 µg/L flurochloridone.

A 2.2.3.7 Study 7: Toxicity to algae including recovery – *Chromulina nebulosa*

Comments of zRMS:	The study was performed in line with OECD 201 with no deviations. The aim of the study was to generate additional toxicity data for algae and for this reason in the study non-standard species (*Chromulina nebulosa*) was used. This is acceptable, but it should be kept in mind that the environmental conditions or the acceptability criteria in OECD 201 were not verified for this species. Nevertheless, validity criteria of OECD 201 were fulfilled. The measured test item concentrations were within 80-120% of nominal and results were thus expressed as nominal concentrations. The recovery part is retained for information but it was not evaluated by the zRMS, as recovery is not taken into account in the risk assessment. The part of the summary regarding recovery phase is thus presented in grey letters, to be distinguishable from the evaluated part. Overall, the study is considered acceptable with following endpoints (based on nominal concentrations): E_rC_{50} = 23.3 µg a.s./L E_yC_{50} = 18.5 µg a.s./L NOEC = 12.5 µg a.s./L

Reference:	KCP 10.2.3/07
Report	Flurochloridone Technical – Alga, Growth Inhibition Test with *Chromulina nebulosa*, 72 hours, Scheerbaum, D., 2013d, SCN15371 (report number), 90016462 (sponsor report number)
Guideline(s):	OECD 201 (2006)
Deviations:	No
GLP:	Yes
Acceptability:	Acceptable
Duplication (if vertebrate study)	-

Materials and Methods

A. MATERIALS

1. Test material flurochloridone technical (=flurochloridone)
Description Brownish, waxy solid
Lot/Batch # 11083467
Purity 95.5%
Stability of test material Stable under storage conditions (original packaging, normal storage conditions)
Expiry date: 08/2013

2. Vehicle and/or positive control

Vehicle control: test water
Positive control: For evaluation of the quality of the algal strain and the experimental conditions, 3,5-dichlorophenol was tested as a positive control in February 2013. The results (72-hour E_rC_{50}: 0.926 mg/L; 72-hour E_yC_{50}: 0.729 mg/L) were within the valid range following the test facility SOPs (72-hour E_rC_{50}: 1.03±0.433 mg/L; 72-hour E_yC_{50}: 0.691±0.163 mg/L).

3. Test organism

Species	Unicellular freshwater golden alga *Chromulina nebulosa*
Strain	UTEX LB 2642
Source	UTEX The Culture Collection of Algae, The University of Texas at Austin, 1 University Station A6700, Austin TX 78712-0183 USA
Age	Algae cells were taken from an exponentially growing pre-culture set up four days prior to the start of the test.
Acclimation period	The algal pre-culture was exposed to environmental conditions identical to those of the test.
Test units	Sterile 250-mL Erlenmeyer flasks containing 100 mL of test medium, sealed with cotton wool plugs

4. Environmental conditions

Test water

The algae were tested in dilution water (modified DYIII medium according to UTEX) containing the following components:

Macro-nutrients:

$MgSO_4 \cdot 7\ H_2O$	74.0 mg/L
$CaCl_2 \cdot 2\ H_2O$	20.0 mg/L
NH_4NO_3	10.0 mg/L
$NaNO_3$	20.0 mg/L
$Na_2SiO_3 \cdot 5\ H_2O$	11.2 mg/L
MES	1950 mg/L
Na-glycerophosphate $\cdot 5\ H_2O$	10.0 mg/L

Trace elements:

H_3BO_3	790 µg/L
$MnCl_2 \cdot 4\ H_2O$	180 µg/L
$ZnSO_4 \cdot 7\ H_2O$	20 µg/L
$CoCl_2 \cdot 6\ H_2O$	10 µg/L
$Na_2MoO_4 \cdot 2\ H_2O$	6 µg/L
$FeCl_3 \cdot 6\ H_2O$	3150 µg/L
Na_2-EDTA $\cdot 2\ H_2O$	4400 µg/L

In addition, 1.60 mg/L KH_2PO_4, 50.0 mg/L $NaHCO_3$, 0.01 µg/L $CuCl_2 \cdot 2\ H_2O$ and 3.33 mL/L peat extract were added to enable sufficient algal growth during the test.
Prior to testing, the pH of the dilution water was adjusted to 6.7±0.2 by addition of 1 N NaOH and 1 N HCl.

Water temperature	21.5-22.0°C (mean 21.8°C)
Lighting	Continuous illumination at a mean light intensity of 52.6 µE $\cdot$ m^{-2} $\cdot$ s^{-1}
Shaking	Test containers were placed on a rotary shaker and oscillated at approximately 70 rpm.

B. STUDY DESIGN AND METHODS

1. In-life dates 11.02.2013 to 14.02.2013 (exposure phase)

2. Experimental conditions

Test design

The unicellular freshwater golden alga *Chromulina nebulosa* was exposed in a static 72-hour test to the test substance at five concentrations each with three replicates and six replicates of a test water control. The recorded effect was inhibition of algal growth based on yield and growth rate. Furthermore, algal cells were investigated by microscopic evaluation.

After 72 hours of exposure, algae from the three highest test concentrations and the control were transferred to fresh untreated dilution water and allowed to grow for further four to five days under test conditions to investigate the recovery potential of the algae.

Inoculum at test start

Approximately 5 x 10^3 – 10^4 cells/mL (nominal), 9656 cells/mL (actual)

Test conditions

The water temperature was maintained at 21.5-22.0°C and the test systems were continuously illuminated at a mean light intensity of 52.6 µE · m^{-2} · s^{-1}. The pH in the test substance treatments and the control was 6.78-6.83 at test start and 6.86-6.90 at test end.

Test concentrations

Nominal test substance concentrations were 6.25, 12.5, 25.0, 50.0 and 100 µg/L. In addition, a control group with untreated test water was used. The selection of the test concentrations was based on the results of a range-finding test (non-GLP).

Treatment/Application

A concentrated stock solution of nominal 10 mg/L was freshly prepared with dilution water. Adequate volumes of this stock solution were added to dilution water to prepare the test media with the desired test concentrations.

After 72 hours of exposure, algae from the three highest test concentrations of nominal 25.0, 50.0 and 100 µg/L and the control were transferred to fresh untreated test water. Algal suspensions of 5 mL from each test concentration replicate and from two control replicates were filled up with dilution water to 100 mL and allowed to grow for further four to five days under test conditions to investigate the recovery potential of the algae.

Analytics

The content of flurochloridone in the test media and control was determined using HPLC-MS/MS analysis with external calibration. The test substance was separated on a reversed phase column (Acquity UPLC BEH C18, 1.7 µm, 50 x 2.1 mm; eluent A: HPLC-water + 1% formic acid; eluent B: acetonitrile + 1% formic acid; gradient: hold 0.2 min 90% A/10% B, in 0.8 min to 10% A/90% B, hold 1.0 min 10% A/90% B, in 0.1 min to 90% A/10% B, hold 0.9 min 90% A/10% B) at 30°C, a flow rate of 0.5 mL/min and an injection volume of 5 µL. Detection was performed with a mass selective detector (Xevo, Acquity UPLC; ionisation mode: electrospray positive; scan mode: MRM; m/z 312.05 → 292.00 (quantifier) and 145.05 (quanlifier)). The method was validated and the LOQ of the analytical method was set to 3.0 µg/L flurochloridone

3. Sampling and measurements

Algal cell density was measured at the start of the test and every 24 hours during exposure and at distinct sampling days during recovery by chlorophyll-a-fluorescence measurement, excitation at 436 nm and emission at 685 nm. Furthermore, microscopic evaluation of the cells was performed at the start and end of exposure. Cells were checked for unusual cell shapes, colour differences, differences in chloroplast morphology, flocculation, adherence of algae to test containers and agglutination of algal cells.

For test substance analysis, the test media were sampled at the start and end of exposure. At exposure start, samples were taken from additional replicates without algae. For the samples at exposure end after 72 hours, the replicates of each test group were pooled.

The pH was measured in each test substance treatment and the control at the start (in an additional replicate) and at the end (pooled replicates) of exposure. Room temperature was measured continuously. Light intensity was determined prior to test start.

4. Calculation of toxicity

Inhibition of algal growth was determined based on the cell density (yield, y) and the specific growth rate (r) for exponentially growing cultures using the equations recommended in the test guidelines.

5. Statistics

The 72-hour EC_{10}, EC_{20} and EC_{50} values for the inhibition of average yield and growth rate were calculated by sigmoidal dose response regression. Calculation of the confidence intervals was carried out using standard procedures.

LOEC and NOEC were determined by calculation of statistical significance of yield and growth rate by using ANOVA and Dunnett's test. When running an ANOVA, a normality test (Shapiro-Wilk test, p = 0.05) and an equal variance test (Levene median test, p = 0.05) were done first. The α value was 0.05.

Results and Discussion

The concentrations of flurochloridone in the test media and control were determined at the start and end of exposure. Analysis results are presented in the following table.

Table A 2.2.3-21: Concentrations of flurochloridone in the test media

Nominal concentration [µg/L]	Measured concentration			
	0 hours		72 hours	
	[µg/L]	[% of nominal]	[µg/L]	[% of nominal]
Control	< LOQ	n.a.	< LOQ	n.a.
6.25	6.10	98	5.42	87
12.5	13.4	107	12.7	102
25.0	24.6	98	27.4	110
50.0	50.4	101	55.7	111
100	101	101	99.0	99

LOQ of analytical method: 3.0 µg/L flurochloridone
n.a. not applicable

The analytical results confirm the correct dosing and stability of the test substance for the duration of exposure. The biological results were related to the nominal concentrations of the test substance.

At the start of exposure, measured concentrations of flurochloridone in the test media were in the range of 98% to 107% of nominal. Measured concentrations of flurochloridone at the end of exposure after 72 hours ranged between 87% and 111%. Thus, the correct dosing and the stability of the test substance for the duration of exposure were confirmed. The biological results were related to the nominal concentrations of the test substance.

The biomass of algae and effects on growth during exposure to flurochloridone and during the recovery phase are presented in the following tables. After exposure for 72 hours, flurochloridone had a statistically significant inhibitory effect on the growth of algae (yield and growth rate) at the test concentrations of ≥ 25.0 µg/L. The overall 72-hour NOEC was, therefore, determined to be 12.5 µg/L, since up to and including this test concentration the yield and growth rate of algae were not statistically significantly lower than in the control. The 72-hour EC_{50} for yield was determined to be 18.5 µg/L (95% confidence limits: 17.1-19.8 µg/L) and the 72-hour EC_{50} for growth rate was 23.3 µg/L (95% confidence limits: 21.7-24.3 µg/L).

After four (pre-treatment groups of 25.0 and 50.0 µg/L) and five (pre-treatment group of 100 µg/L) days of incubation in untreated dilution water during the recovery phase, the toxic effect of flurochloridone was observed to be reversible. The growth rates in algal cultures previously treated with the test substance were even higher (all test groups tested for recovery) than in the control after the respective recovery phase. Therefore, recovery of algae could be demonstrated up to and including the highest test concentration of 100 µg/L.

Microscopic evaluation of algal cells at the start and end of exposure revealed no morphological abnormalities at all test concentrations.

Table A 2.2.3-22: Biomass of algae exposed to flurochloridone and algal biomass during recovery

Nominal test concentration	Cell density [a] [cells/mL]					
	Exposure phase[b]			Recovery phase		
[µg flurochloridone /L]	24 hours	48 hours	72 hours	0 days	4 days	5 days
Control	21863	48449	194469	9723	312383-331218	n.d.
6.25	22993	51701	194366	n.d.	n.d.	n.d.
12.5	23940	56733	182493	n.d.	n.d.	n.d.
25.0	27017	32302	33618	1681	142988-150484	n.d.
50.0	28210	17586	15143	757	44960-61963	n.d.
100	21893	13929	12119	606	n.d.	67828-76265

n.d. not determined

[a] Exposure phase: mean of three replicates for the test substance treatments and mean of six replicates for the control; recovery phase: cell density at 0 days calculated from the cell density at the end of exposure, cell density at days 4 and 5 given as range of two (control) or three (three highest pre-treatment groups) replicates

[b] The initial cell density was 9656 cells/mL.

Table A 2.2.3-23: Effects of flurochloridone on algal growth during exposure and recovery phase

Nominal test concentration	Exposure phase				Recovery phase	
	0-72 hours yield (y)		0-72 hours growth rate (r)		0-4 days [a]/0-5 days [a] growth rate (r)	
[µg flurochloridone /L]	y (x 10^3)	Inhibition of y [%]	r [day^{-1}]	Inhibition of r [%]	r[b] [day^{-1}]	Inhibition[b] of r [%]
Control	184813	0.0	1.00	0.0	0.875	0.0
6.25	184710	0.06	1.00	0.01	n.d.	n.d.
12.5	172837	6.48	0.980	2.08	n.d.	n.d.
25.0	23962*	87.0	0.415*	58.6	1.11	-27.3
50.0	5487*	97.0	0.148*	85.2	1.06	-21.2
100	2463*	98.7	0.075*	92.5	0.955	-9.2
Endpoints (95% CL) [µg flurochloridone /L]						
	Yield (y)		Growth rate (r)			
72-hour EC_{50}	18.5 (17.1-19.8)		23.3 (21.7-24.3)			
72-hour NOEC	12.5		12.5			
72-hour LOEC	25.0		25.0			

n.d. not determined

[a] 4 days of recovery for the control and the test groups pre-treated with 25.0 and 50.0 µg/L and 5 days of recovery for the test group pre-treated with 100 µg/L

[b] mean values calculated from data given in the report (no statistics performed)

* mean value significantly lower than in the control (according to ANOVA followed by Dunnett's test, $\alpha = 0.05$)

In the control, the biomass increased by a factor of 20 over 72 hours (according to guideline ≥ 16). The mean coefficient of variation of the daily growth rates in the control (section-by-section growth rates) during 72 hours was 34.7% (according to guideline ≤ 35%).The coefficient of variation of the average specific growth rates in the replicates of the control after 72 hours was 2.14% (according to guideline ≤ 10%). Thus, the validity criteria were fulfilled.

Conclusion

The 72-hour E_yC_{50} and E_rC_{50} values of flurochloridone for the unicellular freshwater golden alga *Chromulina nebulosa* were determined to be 18.5 and 23.3 µg/L, respectively. During up to five days in untreated dilution water, there is potential for algal recovery following exposure to up to and including the highest test concentration of 100 µg/L flurochloridone.

A 2.2.3.8 Study 8: Toxicity to algae including recovery – *Ankistrodesmus falcatus*

Comments of zRMS:	The study was performed in line with OECD 201 with no deviations. The aim of the study was to generate additional toxicity data for algae and for this reason in the study non-standard species (*Ankistrodesmus falcatus*) was used. This is acceptable, but it should be kept in mind that the environmental conditions or the acceptability criteria in OECD 201 were not verified for this species. Nevertheless, validity criteria of OECD 201 were fulfilled. The measured test item concentrations were within 80-120% of nominal and results were thus expressed as nominal concentrations. The recovery part is retained for information but it was not evaluated by the zRMS, as recovery is not taken into account in the risk assessment. The part of the summary regarding recovery phase is thus presented in grey letters, to be distinguishable from the evaluated part. Overall, the study is considered acceptable with following endpoints (based on nominal concentrations): E_rC_{50} = 0.918 µg a.s./L E_yC_{50} = 0.516 µg a.s./L NOEC = 0.32 µg a.s./L

Reference:	KCP 10.2.3/08
Report	Flurochloridone Technical – Alga, Growth Inhibition Test with *Ankistrodesmus falcatus*, 72 hours, Scheerbaum, D., 2013e, SAF15371 (report number), 90016463 (sponsor report number)
Guideline(s):	OECD 201 (2006)
Deviations:	No
GLP:	Yes
Acceptability:	Acceptable
Duplication (if vertebrate study)	-

Materials and Methods

A. MATERIALS

1. Test material flurochloridone technical (=flurochloridone)
Description Brownish, waxy solid
Lot/Batch # 11083467

Purity	95.5%
Stability of test material	Stable under storage conditions (original packaging, normal storage conditions) Expiry date: 08/2013
2. Vehicle and/or positive control	Vehicle control: test water Positive control: For evaluation of the quality of the algal strain and the experimental conditions, potassium dichromate was tested as a positive control in February 2013. The 72-hour E_rC_{50} and E_yC_{50} were determined to be 2.35 mg/L and 1.03 mg/L, respectively.
3. Test organism	
Species	Unicellular freshwater green alga *Ankistrodesmus falcatus* CORDA
Strain	SAG 202-2
Source	Sammlung von Algenkulturen (SAG), Pflanzenphysiologisches Institut der Universität Göttingen, Nikolausberger Weg 18, D-37073 Göttingen / Germany
Age	Algae cells were taken from an exponentially growing pre-culture set up three days prior to the start of the test.
Acclimation period	The algal pre-culture was exposed to environmental conditions identical to those of the test.
Test units	Sterile 250-mL Erlenmeyer flasks containing 100 mL of test medium, sealed with cotton wool plugs
4. Environmental conditions	
Test water	The algae were tested in dilution water containing the following components:

Macro-nutrients:

NH_4Cl	15.0 mg/L
KH_2PO_4	1.6 mg/L
$MgSO_4 \cdot 7\ H_2O$	15.0 mg/L
$MgCl_2 \cdot 6\ H_2O$	12.0 mg/L
$CaCl_2 \cdot 2\ H_2O$	18.0 mg/L
$NaHCO_3$	50.0 mg/L

Trace elements:

H_3BO_3	185.0 µg/L
$MnCl_2 \cdot 4\ H_2O$	415.0 µg/L
$ZnCl_2$	3.0 µg/L
$CoCl_2 \cdot 6\ H_2O$	1.5 µg/L
$CuCl_2 \cdot 2\ H_2O$	0.01 µg/L
$Na_2MoO_4 \cdot 2\ H_2O$	7.0 µg/L
$FeCl_3 \cdot 6\ H_2O$	64.0 µg/L
$Na_2EDTA \cdot 2\ H_2O$	100.0 µg/L

	The pH of the dilution water was 8.1±0.2
Hardness	0.24 mmol/L Ca + Mg (nominal)
Water temperature	22.0-23.0°C (mean 22.5°C)
Lighting	Continuous illumination at a mean light intensity of 105 µE · m^{-2} · s^{-1}
Shaking	The test containers were shaken per hand twice a day.

B. STUDY DESIGN AND METHODS

1. In-life dates 25.02.2013 to 28.02.2013 (exposure phase)

2. Experimental conditions

Test design

The unicellular freshwater green alga *Ankistrodesmus falcatus* was exposed in a static 72-hour test to the test substance at five concentrations each with three replicates and six replicates of a test water control. The recorded effect was inhibition of algal growth based on yield and growth rate. Furthermore, algal cells were investigated by microscopic evaluation.

After 72 hours of exposure, algae from the four highest test concentrations and the control were transferred to fresh untreated dilution water and allowed to grow for further four to eight days under test conditions to investigate the recovery potential of the algae.

Inoculum at test start

Approximately 5 x 10^3 – 10^4 cells/mL (nominal), 10109 cells/mL (actual)

Test conditions

The water temperature was maintained at 22.0-23.0°C and the test systems were continuously illuminated at a mean light intensity of 105 µE · m^{-2} · s^{-1}. The pH in the test substance treatments and the control was 7.91-8.09 at test start and 8.00-8.41 at test end.

Test concentrations

Nominal test substance concentrations were 0.10, 0.32, 1.0, 3.2 and 10 µg/L. In addition, a control group with untreated test water was used. The selection of the test concentrations was based on the results of a range-finding test (non-GLP).

Treatment/Application

A concentrated stock solution of nominal 10 mg/L was freshly prepared with dilution water. Adequate volumes of this stock solution were added to dilution water to prepare the test media with the desired test concentrations.

After 72 hours of exposure, algae from the four highest test concentrations of nominal 0.32, 1.0, 3.2 and 10 µg/L and the control were transferred to fresh untreated test water. Algal suspensions of 5 mL from each replicate of test concentrations 1.0 and 3.2 µg/L and of 2 mL from each replicate of test concentrations 0.32 and 10 µg/L and from two control replicates were filled up with dilution water to 100 mL and allowed to grow for further four to eight days under test conditions to investigate the recovery potential of the algae.

Analytics

The content of flurochloridone in the test media and control was determined using HPLC-MS/MS analysis with external calibration. The test substance was separated on a reversed phase column (Acquity UPLC BEH C18, 1.7 µm, 50 x 2.1 mm; eluent A: HPLC-water + 1% formic acid; eluent B: acetonitrile + 1% formic acid; gradient: hold 0.2 min 90% A/10% B, in 0.8 min to 10% A/90% B, hold 1.0 min 10% A/90% B, in 0.1 min to 90% A/10% B, hold 0.9 min 90% A/10% B) at 30°C, a flow rate of 0.5 mL/min and an injection volume of 5 µL. Detection was performed with a mass selective detector (Xevo, Acquity UPLC; ionization mode: electrospray positive; scan mode: MRM; m/z 312.05 → 292.00 (quantifier) and 145.05 (quanlifier)). The method was validated and the LOQ of the analytical method was set to 3.0 µg/L flurochloridone.

3. Sampling and measurements

Algal cell density was measured at the start of the test and every 24 hours during exposure and at distinct sampling days during recovery by chlorophyll-a-fluorescence measurement, excitation at 436 nm and emission at 685 nm. Furthermore, microscopic evaluation of the cells was performed at the start and end of exposure. Cells were checked for unusual cell shapes, colour differences, differences in chloroplast morphology, flocculation, adherence of algae to test containers and agglutination of algal cells.

For test substance analysis, the test media were sampled at the start and end of exposure. At exposure start, samples were taken from additional replicates without algae. For the samples at exposure end after 72 hours, additional replicates with algae were set up and incubated under test conditions.

The pH was measured in each test substance treatment and the control at the start (in an additional replicate) and at the end (pooled replicates) of exposure. Room temperature was measured continuously. Light intensity was determined prior to test start.

4. Calculation of toxicity

Inhibition of algal growth was determined based on the cell density (yield, y) and the specific growth rate (r) for exponentially growing cultures using the equations recommended in the test guidelines.

5. Statistics

The 72-hour EC_{10}, EC_{20} and EC_{50} values for the inhibition of average yield and growth rate were calculated by sigmoidal dose response regression. Calculation of the confidence intervals was carried out using standard procedures.

LOEC and NOEC were determined by calculation of statistical significance of yield and growth rate by using ANOVA and Dunnett's test. When running an ANOVA, a normality test (Shapiro-Wilk test, p = 0.05) and an equal variance test (Levene median test, p = 0.05) were done first. The α value was 0.05. For calculation of statistical significance of yield and growth rate, the equal variance test failed. No transform was found to pass the equal variance test. For yield values, the highest test concentration, resulting in 100% inhibition, was excluded to achieve normally distributed data.

Results and Discussion

The concentrations of flurochloridone in the test media and control were determined at the start and end of exposure. Analysis results are presented in the following table.

Table A 2.2.3-24: Concentrations of flurochloridone in the test media

Nominal concentration [µg/L]	Measured concentration			
	0 hours		72 hours	
	[µg/L]	[% of nominal]	[µg/L]	[% of nominal]
Control	< LOQ	n.a.	< LOQ	n.a.
0.10	0.101	101	0.0935	94
0.32	0.351	110	0.328	103
1.0	1.02	102	1.01	101
3.2	3.21	100	3.06	96
10	11.6	116	9.44	94

LOQ of analytical method: 3.0 µg/L flurochloridone
n.a. not applicable

The analytical results confirm the correct dosing and stability of the test substance for the duration of exposure. The biological results were related to the nominal concentrations of the test substance.

At the start of exposure, measured concentrations of flurochloridone in the test media were in the range of 100% to 116% of nominal. Measured concentrations of flurochloridone at the end of exposure after 72

hours ranged between 94% and 103%. Thus, the correct dosing and the stability of the test substance for the duration of exposure were confirmed. The biological results were related to the nominal concentrations of the test substance.

The biomass of algae and effects on growth during exposure to flurochloridone and during the recovery phase are presented in the following tables. After exposure for 72 hours, flurochloridone had a statistically significant inhibitory effect on the growth of algae (yield and growth rate) at the test concentrations of ≥ 1.0 µg/L. The overall 72-hour NOEC was, therefore, determined to be 0.32 µg/L, since up to and including this test concentration the yield and growth rate of algae were not statistically significantly lower than in the control. The 72-hour EC_{50} for yield was determined to be 0.516 µg/L (95% confidence limits: 0.364-0.782 µg/L) and the 72-hour EC_{50} for growth rate was 0.918 µg/L (95% confidence limits: 0.782-1.07 µg/L).

After four (pre-treatment groups of 0.32 and 1.0 µg/L), seven (pre-treatment group of 3.2 µg/L) and eight (pre-treatment group of 10 µg/L and one replicate of pre-treatment group of 3.2 µg/L) days of incubation in untreated dilution water during the recovery phase, the toxic effect of flurochloridone was observed to be reversible. The growth rates in algal cultures previously treated with the test substance were only slightly lower (i.e. inhibition ≤ 12%) than in the control in all pre-treatment groups up to 10 µg/L (exception: the second highest pre-treatment group of 3.2 µg/L with about 30% inhibition. Therefore, recovery of algae could be demonstrated up and including to the highest test concentration of 10 µg/L.

Microscopic evaluation of algal cells at the start and end of exposure revealed no morphological abnormalities at all test concentrations.

Table A 2.2.3-25: Biomass of algae exposed to flurochloridone and algal biomass during recovery

Nominal test concentration	Cell density [a] [cells/mL]						
	Exposure phase[b]			Recovery phase			
[µg flurochloridone /L]	24 hours	48 hours	72 hours	0 hours	4 days	7 days	8 days
Control	29554	110229	359454	7189	1105263-1220537	n.d.	n.d.
0.10	26087	108692	343711	n.d.	n.d.	n.d.	n.d.
0.32	25489	78424	289806	5796	426095-911633	n.d.	n.d.
1.0	18960	40767	49631	2482	179112-349159	n.d.	n.d.
3.2	14291	13367	14313[c]	716	n.d.	224069-489870	562131
10	13664	< LOQ	8740[c]	175	n.d.	21870-56792	63102-167345

n.d. not determined
LOQ The limit of quantification of cell density for *Ankistrodesmus falcatus* was 7296 cells/mL.

[a] Exposure phase: mean of three replicates for the test substance treatments and mean of six replicates for the control; recovery phase: cell density at 0 days calculated from the cell density at the end of exposure, cell density at days 4, 7 and 8 given as range of two (control) or three (four highest pre-treatment groups) replicates (exception: one replicate at day 8 of pre-treatment group of 3.2 µg/L)

[b] The initial cell density was 10109 cells/mL.

[c] Cells were counted microscopically.

Table A 2.2.3-26: Effects of flurochloridone on algal growth during exposure and recovery phase

Nominal test concentration [µg flurochloridone /L]	Exposure phase 0-72 hours yield (y)		Exposure phase 0-72 hours growth rate (r)		Recovery phase 0-4 days a)/0-7 days a)/0-8 days a) growth rate (r)	
	y (x 10^3)	Inhibition of y [%]	r [day^{-1}]	Inhibition of r [%]	r b) [day^{-1}]	Inhibition b) of r [%]
Control	349345	0.0	1.19	0.0	1.27	0.0
0.10	333602	4.51	1.18	1.03	n.d.	n.d.
0.32	279697	19.9	1.09	7.80	1.18	7.1
1.0	39522*	88.7	0.529*	55.5	1.16	8.7
3.2	4204*	98.8	0.115*	90.3	0.894	29.6
10	-1369*	100	-0.049c)	100	1.11	12.3
Endpoints (95% CL) [µg flurochloridone /L]						
	Yield (y)		**Growth rate (r)**			
72-hour EC_{50}	0.516 (0.364-0.782)		0.918 (0.782-1.07)			
72-hour NOEC	0.32		0.32			
72-hour LOEC	1.0		1.0			

n.d. not determined

a) 4 days of recovery for the control and the test groups pre-treated with 0.32 and 1.0 µg/L, 7 days of recovery for the test group pre-treated with 3.2 µg/L (one replicate was kept until day 8 of recovery) and 8 days of recovery for the test group pre-treated with 10 µg/L

b) mean values calculated from data given in the report (no statistics performed)

* mean value significantly lower than in the control (according to ANOVA followed by Dunnett's test, $\alpha = 0.05$)

c) excluded to pass normality test

In the control, the biomass increased by a factor of 36 over 72 hours (according to guideline ≥ 16). The mean coefficient of variation of the daily growth rates in the control (section-by-section growth rates) during 72 hours was 13.7% (according to guideline ≤ 35%).The coefficient of variation of the average specific growth rates in the replicates of the control after 72 hours was 4.17% (according to guideline ≤ 10%). Thus, the validity criteria were fulfilled.

Conclusion

The 72-hour E_yC_{50} and E_rC_{50} values of flurochloridone for the unicellular freshwater green alga *Ankistrodesmus falcatus* were determined to be 0.516 and 0.918 µg/L, respectively. During up to eight days in untreated dilution water, there is potential for algal recovery following exposure to up to and including the highest test concentration of 10 µg/L flurochloridone.

A 2.2.3.9 Study 9: Toxicity to algae including recovery – *Desmodesmus subspicatus*

Comments of zRMS:	The study was performed in line with OECD 201 with no deviations. The test item concentrations measuredat test end were within 91-128% of nominal and within 80-120% of initial measured concentrations and results were expressed as initial measured concentrations. The recovery part is retained for information but it was not evaluated by the zRMS, as recovery is not taken into account in the risk assessment. The part of the summary regarding recovery phase is thus presented in grey letters, to be distinguishable from the evaluated part. Overall, the study is considered acceptable with following endpoints (based on nominal concentrations): E_rC_{50} = 4.96 µg a.s./L E_yC_{50} = 1.14 µg a.s./L NOEC = 0.393 µg a.s./L

Reference:	KCP 10.2.3/9
Report	Freshwater Alga, Growth Inhibition Test Flurochloridone (technical): *Desmodesmus subspicatus* Toxicity Test - Testing for Recovery of Growth, Wenzel, A. 2015a, ADM-003/4-10/B (report number), 90016481 (sponsor report number)
Guideline(s):	OECD 201 (2006)
Deviations:	No
GLP:	Yes
Acceptability:	Acceptable
Duplication (if vertebrate study)	-

Materials and Methods

A. MATERIALS

1. Test material flurochloridone (technical)
Description brown colour, waxy consistency
Lot/Batch # 1771
Purity 94.8%
Stability of test material Stable under storage conditions (room temperature, dark and dry)
Expiry date: October 2016

2. Vehicle and/or positive control Vehicle controls: control (test water without test substance) Reference substance: Sensitivity of test organisms is routinely checked using 3,5-dichlorophenol. The latest nominal E_rC_{50} value of 4.25 mg/L is in good agreement with the results of an international ring test with E_rC_{50} of 6.42 ± 2.38 mg/L.

3. Test organism

Species Freshwater green alga *Desmodesmus subspicatus*; Chlorophyta
Strain SAG 61.81
Source Collection of Algal Cultures (SAG, Institute for Plant Physiology, University of Göttingen, 37073 Göttingen / Germany)
Age Algae cells were taken from an exponentially growing pre-culture.
Acclimation period Pre-culture from stock culture set up in OECD growth medium for duration of three days before the start of exposure.
Test units 250-mL conical glass flasks covered with air-permeable silicone-sponge caps filled with 100 mL of test medium. Additional replicates filled with 120 mL medium for chemical analysis at growth test termination and start of recovery phase.

4. Environmental conditions

Test water The algae were cultivated and tested in medium prepared with purified water to obtain the following final concentrations:

$NaHCO_3$	50 mg/L
NH_4Cl	15 mg/L
K_2HPO_4	1.6 mg/L
$MgSO_4 \cdot 7\ H_2O$	15 mg/L
$MgCl_2 \cdot 6\ H_2O$	12 mg/L
$CaCl_2 \cdot 2\ H_2O$	18 mg/L
$FeCl_3 \cdot 6\ H_2O$	0.064 mg/L

H_3BO_3	0.185 mg/L
$MnCl_2 \cdot 4\ H_2O$	0.415 mg/L
$ZnCl_2$	0.003 mg/L
$CoCl_2 \cdot 6\ H_2O$	0.0015 mg/L
$CuCl_2 \cdot 2\ H_2O$	0.00001 mg/L
$Na_2MoO_4 \cdot 2\ H_2O$	0.007 mg/L
$Na_2EDTA \cdot 2\ H_2O$	0.10 mg/L

The pH of the test water was 7.5-8.0.

Hardness	Not provided
Water temperature	22 ± 2°C
Lighting	Light intensity (measured daily at test media surface level) of 60-120 µmol m^{-2} s^{-1} (4440-8880 lux)
Shaking	Continuous stirring on a laboratory shaker with 150 rpm

B. STUDY DESIGN AND METHODS

1. In-life dates 09.03.2015 to 20.03.2015

2. Experimental conditions

Test design

The freshwater green alga *Desmodesmus subspicatus* was exposed to the test substance at various concentrations under static conditions over 72 hours, followed by an exposure to test medium only to test for recovery of growth for 8 days. Growth and yield inhibition was determined daily based on cell densities.

Inoculum at test start

Approximately 5000 cells/mL

Test conditions

The water temperature was maintained at 21.0 to 22.0°C and the test systems were continuously illuminated at a measured light intensity of between 90.53 µmol m^{-2} s^{-1} and 97.12 µmol m^{-2} s^{-1} between test start and day 3. The pH of the test media was 7.75 to 7.95 at the start and 7.63 to 7.86 at the end of the growth inhibition test. During the 8-day recovery phase, test temperature was in the range of 21.5 to 22.0°C with light intensities measured in the range of 90.53 to 101.70 90.53 µmol m^{-2} s^{-1}. The pH at the end of the recovery phase was determined in the range of 7.83 to 8.05.

Test concentrations

Based on a range-finder, the algae were exposed to test item concentrations of 0.320, 1.0, 3.20, 10.0 and 32.0 µg a.s./L in four replicates per treatment. A control was tested in parallel in eight replicates. Recovery potential was determined at test item concentrations of 1.0, 3.20, 10.0 and 32.0 µg a.s./L as there was no growth inhibition at the 0.320 µg a.s./L concentration level. For recovery testing, again eight and four replicates were used for control and treatments, respectively (exception: the highest level was tested in three replicates only as initial cell density could not be obtained for four replicates).

Treatment/Application

A stock solution in test medium was prepared by dissolving 52.74 mg test item in 10 mL acetone (equivalent to 50 mg a.s./10 mL acetone). A nominal stock solution of 2.5 mg a.s./L was prepared by deposition of 500 µL acetonic solution as thin layer in a glass bottle and removal of acetone by evaporation. Following addition of 1 L growth medium, the stock solution was stirred overnight (analysed content 2.13 mg a.s./L). The stock solution was diluted with growth medium to prepare individual test concentrations in 100 mL medium.

The algal pre-culture was added (995 µL at a cell density of 5.02 x 10^5 cells/mL; 1195 µL added to additionally prepared vessels).

At end of the growth inhibition test, two controls and four treatment replicates were centrifuged, the supernatant carefully removed and the algae pellets resuspended in fresh medium (about 12 mL and 8 mL for control and test cultures, respectively) without test medium. Algal pellets per treatment level were combined and algal densities determined. Adequate aliquots were added to 100 mL growth medium without test medium to assess recovery potential.

All preparations were performed under sterile conditions.

Analytics

The concentration of flurochloridone (technical) in the test media and control was analysed by GC/MS. Samples were taken from test solutions and controls at the beginning of the exposure period and after 72 hours additional replicates were sampled. To confirm negligible concentrations at the start of the recovery phase, one additionally prepared replicate was used for sampling. Determination was performed using an internal standard following calibration. Sample preparation was done by spiking with internal standard, extraction with n-hexane, drying with Na_2SO_4 and concentration of the organic phase to 1 mL subsequently measured by GC/MS. GC/MS: Column: DB-5MS UI; 30 m, 0.25 mm ID, 0.25 µm film (Agilent). Gradient (oven): 2.2 min 80°C, then 25°C/min to 275°C for 4.0 min. Carrier gas: helium with constant flow of 0.8 mL/min, splitless inlet (280°C). MS: SIM-Mode, internal standard (4,4-DDE): target ion: 246, qualifier ion: 317.9, flurochloridone: target ion: 311, qualifier ion: 187. MS source: 250°C, MS quadrupol: 200°C, solvent delay: 9 min, Run time: 14 min.
The method was validated and the LOQ was set to 4.0 µg/L flurochloridone.

3. Sampling and measurements

Cell density was determined daily (i.e. at 24, 48 and 72 hours during exposure and also during the 8-day recovery phase) by measurements of chlorophyll fluorescence.

Test item concentrations were assessed by chemical analysis at start and end of the growth test as well as at the start of the recovery phase.

Light intensity was measured daily. Incubation temperature was measured daily during the exposure phase in an additionally prepared control vessel kept under equivalent conditions. The pH was measured in the additionally prepared replicate at beginning of the test and directly in test vessels at test end.

4. Calculation of toxicity

Mean values of cell counts for each concentration plot of the exposure test and the recovery test were used to plot growth curves. Mean average growth rates were calculated for the exposure period of day 0 to day 3 and for the recovery test, mean average growth rates for different recovery periods and sectional growth rates of individual days were calculated. Percent inhibitions of growth rate, sectional growth rate and yield and for the recovery test, percent inhibition of growth rate, sectional growth rate and cell number were calculated for the exposure period according to the guideline. Percent inhibition was plotted as a function of test item concentration for the growth test and as a function of pre-exposure concentration for the recovery test.

5. Statistics

Results of the growth inhibition test were statistically analysed to determine EC_{50}, EC_{20} and EC_{10} values together with 95% confidence intervals using Probit analysis. Individual replicate responses were used for regression analysis. NOEC was determined using appropriate statistical methods. Statistical evaluations were performed using ToxRat.

Results and Discussion

The concentrations of flurochloridone (technical) were measured in the control and the test item treatments. Analysis results are presented in the following table.

Table A 2.2.3-27: Concentrations of flurochloridone (technical) in the test media

Nominal test item concentration [µg/L]	Measured test item concentration				
	Day 0		Day 3		[%] of day 0
	[µg/L]	[%]	[µg/L]	[%]	
Growth inhibition test					
Control	< LOQ	-	< LOQ	-	-
0.320	0.393	123	0.408	128	104
1.00	1.10	110	1.10	110	100
3.20	3.28	102	3.47	108	106
10.0	8.42	84.2	9.13	91.3	108
32.0	30.93	96.7	29.9	93.3	96.6
Recovery test					
32.0	< LOQ	n.a.	Not measured, not required		

LOQ: 4.0 µg/L flurochloridone
n.a. not applicable

The correct dosing of flurochloridone (technical) was confirmed. The biological results were related to the initial measured concentrations. The flurochloridone level was below the Limit of Quantification at the highest concentration level at the start of the recovery test. Therefore, the lower concentration levels were not analysed.

At test start, measured flurochloridone concentrations were between 84.2 to 123% and after 72 hours at the end of the exposure phase between 91.3 to 128% of nominal. The evaluation was based on initial measured concentrations, since test item concentrations were stable during the test (< 20% deviation from measured initial concentrations at test termination).

The cell numbers of algae during exposure to flurochloridone and the effects of flurochloridone on algal growth are shown in the following tables. After exposure for 72 hours, flurochloridone had a statistically significant inhibitory effect on the growth of algae (based on growth rate and yield) at the initial measured concentration of 1.10 µg/L and all higher concentrations. The overall 72-hour NOEC was, therefore, determined to be 0.393 µg/L, since up to and including this test concentration the growth rate and yield of the algae were not statistically significantly lower than in the control. The 72-hour EC_{50} for yield was determined to be 1.14 µg/L and the 72-hour EC_{50} for growth rate was 4.96 µg/L.

Cell numbers of algae during the recovery phase are presented in the tables below. The delay of algal growth originating from higher treatments (i.e. ≥ 1.10 µg a.s./L initial measured) increased with increasing pre-treatment concentrations. The cell number of the control plateau phase of about 91.3 x 10^4 cells/mL was achieved after 120, 168 and 192 hours in algae cultures pre-treated with 3.28, 8.42 and 30.9 µg a.s./L, respectively. After a recovery period of 8 days (192 h), all algae pre-exposed to flurochloridone up to 30.9 µg a.s./L had fully recovered based on cell number (i.e. cell numbers were comparable to that of the controls at the beginning of the plateau phase). The yield of the control plateau phase of about 90.8 x 10^4 cells/mL was achieved after 120, 168 and 192 hours in algae cultures pre-treated with 3.28, 8.42 and 30.9 µg a.s./L, respectively. After a recovery period of 7 days (168 h), all algae pre-exposed to flurochloridone up to 30.9 µg a.s./L had fully recovered based on yield. Likewise, after a recovery period of 7 days (168 h), all algae pre-exposed to flurochloridone up to 30.9 µg a.s./L had fully recovered based on average growth rate. Also the mean sectional growth rates for the 3-day period prior to reaching the plateau phase was calculated resulting in a NOEC of ≥ 30.9 µg a.s./L for a 7-day recovery phase (as sectional growth rate of a culture in the stationary phase is zero, a comparison of sectional-growth rates of controls with test cultures per time interval is not possible).

Microscopic observations of algal cells at test start showed a normal appearance of intact cells. At test termination, a normal appearance of intact cells and very little cell debris was observed in controls as well

as the test item concentration groups of initial measured 0.393 and 1.10 µg a.s./L. In the 3.28 µg a.s./L group, little intact cells and a lot of cell debris were found, whereas at 8.42 and 30.9 µg a.s./L cell debris was predominant with very little intact cells. At the end of the recovery phase, (day 8) microscopic evaluation revealed normal appearance of intact cells and very little cell debris in all concentration levels.

Table A 2.2.3-28: Cell numbers and endpoints for algae exposed flurochloridone during the exposure phase

Treatment		Cell number [a] [mean [b] ± standard deviation] (x 10^4)		
Nominal concentration [µg/L]	**Initial measured test conc. [µg/L]**	**27 hours**	**48 hours**	**72 hours**
Control	-	3.185 ± 0.32	7.271 ± 0.87	23.933 ± 3.42
0.320	0.393	3.867 ± 0.25	7.166 ± 0.90	25.171 ± 5.23
1.00	1.10	3.463 ± 0.66	5.105 ± 0.68	12.048 ± 1.42
3.20	3.26	2.762 ± 0.16	3.363 ± 0.49	3.186 ± 0.34
10.0	8.42	2.443 ± 0.009	2.263 ± 0.08	1.828 ± 0.06
32.0	30.9	2.205 ± 0.27	1.915 ± 0.19	1.621 ± 0.18

[a] The initial cell density was 5000 cells/mL

[b] mean of four replicates for the test substance treatments and mean of eight replicates for the control

Table A 2.2.3-29: Effects of flurochloridone on algal growth during the exposure phase

Treatment		Effects during exposure			
		0-72 hours growth rate (r)		0-72 hours yield (y)	
Nominal concentration [µg/L]	**Initial measured test conc. [µg/L]**	**r**	**Inhibition of r [%]**	**y**	**Inhibition of y [%]**
Control	-	1.286	0	23.433	0
0.320	0.393	1.300	-1	24.671	-5.3
1.00	1.10	1.059	17.7*	11.548	50.7*
3.20	3.26	0.616	52.1*	2.686	88.5*
10.0	8.42	0.432	66.4*	1.328	94.3*
32.0	30.9	0.391	69.6*	1.121	95.2*
Endpoints (95% CL) [µg flurochloridone /L]					
		Yield (y)		**Growth rate (r)**	
72-hour EC_{10}		**0.403** (0.289-0.503)		**0.374** (0.311-0.442)	
72-hour EC_{50}		**1.14** (1.00-1.30)		**4.96** (4.59-5.37)	
72-hour NOEC		0.393		0.393	
72-hour LOEC		1.10		1.10	

* mean value significantly lower than in the control (according to Williams t-test (growth rate) or Welch test (yield), one-sided smaller, $\alpha = 0.05$)

Negative values (-) indicate increase in the observed parameter

Table A 2.2.3-30: Cell numbers and endpoint for algae exposed to flurochloridone during the recovery phase

Treatment		Cell number [a] [mean ± standard deviation] (x 10^4)							
Nominal concentration [µg/L]	Initial measured test conc. [µg/L]	24 hours	43 hours	67 hours	96 hours	120 hours	144 hours	168 hours	192 hours
Control	-	4.301 ± 0.3	7.811 ± 1.0	22.19 ± 2.5	91.35 ± 6.66	128.24 ± 10.9	86.68 ± 8.8	n.d.	n.d.
1.00	1.10	4.119 ± 0.2	8.607 ± 1.0	27.69 ± 3.2	102.05 ± 18.47	95.40 ± 10.7	68.87 ± 6.4	n.d.	n.d.
3.20	3.26	2.180 ± 0.2	3.584 ± 0.5	8.77 ± 1.3	45.59 ± 8.94	107.11 ± 32.4	102.95 ± 18.8	81.97 ± 26.1	n.d.
10.0	8.42	1.800 ± 0.0	1.984 ± 0.1	3.24 ± 0.1	9.381 ± 1.1	26.36 ± 4.9	54.98 ± 11.1	108.06 ± 25.7	83.42 ± 22.8
32.0	30.9	1.822 ± 0.1	1.900 ± 0.1	2.65 ± 0.2	6.425 ± 0.3	17.79 ± 2.3	36.59 ± 2.5	88.21 ± 15.9	97.88 ± 9.6
Endpoints [µg flurochloridone /L]									
$NOEC_{recovery}$ (cell number)		30.9							
$NOEC_{recovery}$ (yield)		30.9							
$NOEC_{recovery}$ (growth rate)		30.9							
$NOEC_{recovery}$ (sectional growth rate)		30.9							

[a] The initial cell density was 5000 cells/mL

n.d. cells were not counted due to decreasing cell number (plateau phase reached after 96 hours in controls and 1.10 µg/L group or after 120 hours in 3.26 µg/L group)

Validity criteria were fulfilled with a cell number increase in controls by a factor of 48 within 72 hours (> 16 required), a mean coefficient of variations in section-by-section growth rates of controls of 30% (≤ 35% required) and a coefficient of variation of average specific growth rate at test end in replicate control cultures of 4% (≤ 7% required).

Conclusion

At the end of the exposure phase, a concentration-related growth inhibition was observed for *Desmodesmus subspicatus* with 72 hour an E_rC_{50} (based on specific growth rate) and E_yC_{50} (based on algal yield) of 4.96 and 1.14 µg a.s./L, respectively. There were time-related recoveries of algal growth at flurochloridone concentrations higher than 1.10 µg a.s./L. After a recovery period of 7 days (168 h), all algae pre-exposed to flurochloridone up to 30.9 µg a.s./L had fully recovered.

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Comments of zRMS:	The study design followed recommendations of OECD 201 with exception of the exposure, which followed pulsed-exposure regime. The test item concentrations measured at test end of each exposure period were in general within 80-120% of nominal concentrations with exception of the 4th exposure phase, during which the measured concentration dropped below 80% and test results were thus expressed as geometric mean measured concentrations. In general, there were no deviations from the test guideline in terms of the environmental conditions, replication, application of the test item etc. However, the test design included only 2 test concentrations for each exposure period and was thus not suitable for calculation of ECx values, required by EFSA aquatic guidance (2013), especially during two first exposure periods effects on growth rates were greater than 50%. It should be noted that in line with EFSA (2013) the RAC for pulsed exposure study should be calculated with consideration of E_rC_{50} expressed in terms of the peak exposure, while according to EFSA Supporting publication 2019:EN-1673, EC_{10} is more relevant for primary producers in order to exclude effects of recovery and follow ETO option. None of these endpoints was (or could be) calculated due to the design of the exposure regime.

	The validity criteria were checked only for two recovery phases lasting for 72 hours. According to the study authors, this is correct, as validity criteria are applicable for 72 hours. In general, the zRMS agrees with this, however would like to point out that during the exposure and recovery phases lasting for less than 72 hours the specific growth rates of control cultures also should not be too variable and for this reason the zRMS would recommend to calculate for illustrative purposes at least CV values for shorter periods of time (e.g. 0-24 h). This would give some indication regarding variability of growth in controls. Unfortunately, such a calculation was not possible to the zRMS as in the study report only mean growth rates for particular exposure/recovery periods were given without information on the standard deviation. In addition to that, the cell number was not reported, so calculation of the specific growth rates for control cultures was not possible. Calculation of biomass increase would not be possible, as this parameter was validated for 72 hours, so is not applicable for shorter period of time. Nevertheless, information regarding validity criteria was available for the first and the last recovery phase. All validity criteria for the first recovery phase were fulfilled. For the last recovery phase, the increase in biomass and CV of average specific growth rates were fulfilled, however the CV of 85% was calculated for the daily/section by section growth rates, while according to OECD 201 it should not exceed 35%. This means that this validity criterion was not fulfilled. This together with fact that due to not sufficient reporting no illustrative CV values could be calculated for the shorter time periods during the exposure phases, makes results of the whole study not fully reliable. Taking into account uncertain reliability of the results and no ECx values calculated, the zRMS does not recommend to consider results of this study in the risk assessment.

Reference:	KCP 10.2.3/10
Report	Flurochloridone (Trans isomer): Toxicity to *Desmodesmus subspicatus* in a Pulse Exposure Growth Inhibition Test Supplemented with Testing for Recovery of Growth, Liedtke, A., 2013d, D59890 (report number), 90015421 (sponsor report number)
Guideline(s):	OECD 201 (2006), Commission Regulation (EC) No 761/2009, C.3 (2009)
Deviations:	Besides the chosen exposure and recovery pattern, there were no deviations to guideline.
GLP:	Yes
Acceptability:	In opinion of the zRMS results of this study are not fully reliable as validity criteria were not fulfilled (for the last recovery phase) or for shorter time periods the CV for daily section by section growth rates could not be calculated for illustrative purposes (for all exposure phases and recovery phases 2 and 3). Furthermore, due to selected exposure regime and effects >50% observed during two first exposure phases, calculation of ECx values required by EFSA (2013) and EFSA (2019) for the risk assessment based on refined exposure studies was not possible.
Duplication (if vertebrate study)	-

Materials and Methods

A. MATERIALS

1. Test material	flurochloridone (trans isomer)
Description	Not reported
Lot/Batch #	FLCLDN(2)-BP12-1387(V3)
Purity	Trans isomer: 99.3±0.5% Chemical purity: 99.5±0.5%
Stability of test material	Stable under storage conditions (room temperature) Expiry date: 30.04.2016

2. Vehicle and/or positive control

Vehicle controls: control (test water without test substance) and solvent control (test water without test substance but containing 0.01% DMF)
Positive control: For evaluation of the quality of the algal strain and the experimental conditions, potassium dichromate was tested as a positive control twice a year. The results of the latest positive control test performed in September 2012 (72-hour E_rC_{50}: 0.69 mg/L) were within the internal historical range (72-hour E_rC_{50}: 0.64-1.1 mg/L from 2000 to 2012).

3. Test organism

Species Freshwater green alga *Desmodesmus subspicatus* CHODAT (formerly: *Scenedesmus subspicatus*)

Strain SAG 86.81

Source Collection of Algal Cultures (SAG, Institute for Plant Physiology, University of Göttingen, 37073 Göttingen / Germany)

Age Algae cells were taken from an exponentially growing pre-culture set up three days prior to the start of the test.

Acclimation period An inoculum culture was set up three days before the start of exposure. The algae were cultivated under the test conditions and were kept in the exponential growth phase until inoculation of the test solutions.

Test units 50-mL Erlenmeyer flasks containing 15 mL of test medium. The test vessels were covered with glass dishes.

4. Environmental conditions

Test water The algae were cultivated and tested in reconstituted test water (AAP medium). Analytical grade salts were dissolved in sterile purified water to obtain the following nominal concentrations:

Macro-nutrients:

$NaHCO_3$	15.0 mg/L
K_2HPO_4	1.044 mg/L
$MgSO_4 \cdot 7\ H_2O$	14.6 mg/L
$MgCl_2 \cdot 6\ H_2O$	12.16 mg/L
$CaCl_2 \cdot 2\ H_2O$	4.41 mg/L
$NaNO_3$	25.5 mg/L

Trace elements:

H_3BO_3	186.0 µg/L
$MnCl_2 \cdot 4\ H_2O$	415.0 µg/L
$ZnCl_2$	3.27 µg/L
$CoCl_2 \cdot 6\ H_2O$	1.43 µg/L
$CuCl_2 \cdot 2\ H_2O$	0.012 µg/L
$Na_2MoO_4 \cdot 2\ H_2O$	7.26 µg/L
$FeCl_3 \cdot 6\ H_2O$	160.0 µg/L
$Na_2EDTA \cdot 2\ H_2O$	300.0 µg/L

The pH of the test water was 7.5.

Hardness 0.15 mmol/L (= 15 mg/L) as $CaCO_3$

Water temperature 21-22°C

Lighting Continuous illumination at a mean light intensity (measured at the level of the test solutions) of 6000 Lux (range: 5250 to 6790 Lux) at day 0, 6900 Lux (range: 6200 to 7500 Lux) at day 4, 6800 Lux

	(range: 6060 to 7340 Lux) at day 7 and 6300 Lux (range: 5720 to 6950 Lux) at day 10 using fluorescent tubes (Philips TLD 36W-1/840)
Shaking	During exposure and recovery phases, the test solutions were continuously stirred by magnetic stirrers.

B. STUDY DESIGN AND METHODS

1. In-life dates 07.12.2012 to 16.01.2013

2. Experimental conditions

Test design

The freshwater green alga *Desmodesmus subspicatus* was exposed to the test substance in a multiple pulse exposure test interrupted by recovery periods. The pulse exposure design included four exposure pulses with durations of 24, 36, 30 and 36 hours alternated with four recovery phases of 72, 36, 6 and 72 hours (total study duration 312 hours, i.e. 13 days). For each exposure phase, two test concentrations were tested. Additionally, a control and a solvent control group were tested in parallel. Three replicates for both test substance concentrations and the control and six replicates for the solvent control were set up. The cell density was measured at the start and end of each exposure and recovery phase (exception: no assessments during the third recovery phase, additional biomass determination at 48 hours during the first and final recovery phase).

At the end of each pulse exposure phase and each recovery phase (exception: third recovery phase), the inhibition of algal growth based on yield and growth rate compared to the solvent control was calculated. Based on the growth rate results, the NOEC for the exposure phases and the $NOEAC_{recovery}$ (No Observed Ecologically Adverse Concentration for recovery) for each recovery phase were determined. Furthermore, algal cells were investigated by microscopic evaluation after the first, second and final recovery phase. At the end of the final recovery period, the $NOAEP_{recovery}$ (No Observed Adverse Exposure Pattern) for recovery of *Desmodesmus subspicatus* was determined.

Inoculum at test start

Approximately 5000 cells/mL

Test conditions

The water temperature was maintained at 21-22°C and the test systems were continuously illuminated at a mean light intensity of 6000, 6900, 6800 and 6300 Lux measured at days 0, 4, 7 and 10, respectively. The pH of the test media was 7.3-7.9 and 7.7-8.1 at the start and end of the four exposure phases and 7.6-8.0 and 7.9-8.5 at the start and end of the four recovery phases, respectively. No remarkable observations were made concerning the appearance of the test media. All test media were clear solutions throughout the four exposure phases.

Test concentrations

The algae were exposed to the test substance according to the pulse exposure design summarised in the following table.

Table A 2.2.3-31: Pulse exposure design

Test phase		Test concentration [µg/L]	Duration
1	Exposure	10.5 and 35	24 hours
	Recovery	0	72 hours
2	Exposure	6.3 and 21	36 hours
	Recovery	0	36 hours
3	Exposure	4.5 and 15	30 hours
	Recovery	0	6 hours
4	Exposure	3.0 and 10	36 hours
	Recovery	0	72 hours

Treatment/Application

Due to the low water solubility of the test substance, the organic solvent *N,N*-dimethylformamide (DMF) was used to dose flurochloridone (trans isomer). At the start of each pulse exposure, a stock solution in DMF was prepared dissolving the respective amount of test substance in 100 mL of DMF. This stock solution was diluted with DMF to prepare the application solutions and their dilutions for the dosage of each of the two test concentrations. An aliquot of 100 µL from the respective application solution was added to 1000 mL of test water during intense stirring. For preparation of the solvent control, the same volume of DMF (without test substance) was added to the test water. The test media were freshly prepared just before the start of each of the four exposure phases.

After each exposure phase, the replicates of each treatment were pooled and algal cells were separated by filtration (membrane filter, Schleicher & Schuell, Type NC45, pore size 0.45 µm). Subsequently, the algae were transferred into test water and the algal density of each sample was determined. The recovery of algal growth was then recorded for a defined duration.

The same procedure was performed for transferring the algal cells from the recovery phase into a new exposure pulse except of the transfer of the algae into the corresponding test concentration instead of pure test water.

The number of algal cells was reduced to nominal 5000 cells/mL at the start of the first three exposure phases (days 0, 4 and 7). The number of algal cells was also reduced to nominal 5000 cells/mL at the start of the final recovery phase (day 10), in order to allow exponential growth of the algae during the whole recovery phase.

Analytics

The concentration of flurochloridone (trans isomer) in the test media and control was analysed by HPLC-MS/MS using external calibration. The test substance was separated on a column (Inertsil ODS3; 2.1 mm x 33 mm; 3 µm; eluent A: 95 vol. water + 5 vol. methanol + 0.1 vol. formic acid + 5 mM ammonium formate; eluent B: 95 vol. methanol + 5 vol. water + 0.1 vol. formic acid + 5 mM ammonium formate; gradient: hold 0.5 min 60% A/40% B, in 1.5 min to 10% A/90% B, hold 1.0 min 10% A/90% B, in 0.1 min to 60% A/40% B, hold 0.9 min 60% A/40% B) at a flow rate of 400 µL/min and an injection volume of 5 µL. Detection was performed with a MDS Sciex API 5000 triple stage quadrupole mass spectrometer (heater gas temperature: 450°C; spray voltage: 4800 V; ionisation mode: ESI; scan mode: MRM; ion polarity: positive; m/z 312.0 → 292.0; retention time: approximately 2.17 minutes). The method was validated and the LOQ was set to 2.04 µg/L flurochloridone (trans isomer).

3. Sampling and measurements

Algal biomass was determined at the start and end of each exposure and recovery phase (exception: no assessments during the third recovery phase, additional biomass determination at 48 hours during the first and final recovery phase) by cell counts using fluorescent measurement (BIO-TEK® Multi-Detection Microplate Reader, Model FLx800, wavelength: excitation 440 nm, emission 680 nm). The measurements were performed at least in duplicate.

Furthermore, algal cells from the solvent control and both test concentrations were investigated by microscopic evaluation after the first, second and final recovery phase. The shape and size of the algal cells were visually inspected.

The test media of both test concentrations and the control and solvent control were sampled in duplicate at the start (without algae) and at the end (containing algae) of the exposure phases for analysis of the test substance concentration. For the start and stability samples of each exposure phase, additional flasks containing the test medium with algae were incubated for each treatment under the test conditions.

The pH was measured in both test substance treatments and the control and solvent control at the start and at the end of each test period. The water temperature was measured and recorded at the start and end of each test period or daily for the two 72-hour recovery periods. The appearance of the test media was recorded daily during the exposure phases. The light intensity was measured at the start of the test (day 0) and on days 4, 7 and 10 in the exposure or recovery phases.

4. Calculation of toxicity

Inhibition of algal growth was determined based on the cell density (yield, y) and the specific growth rate (r) for exponentially growing cultures using the equations recommended in the test guidelines.
At the end of each pulse exposure phase and each recovery phase (exception: third recovery phase), the inhibition of algal growth based on yield and growth rate compared to the solvent control was calculated.

5. Statistics

After each exposure and recovery phase (exception: third recovery phase), average growth rate at both test concentrations was compared to the solvent control by Williams t-test (one-sided smaller, $\alpha = 0.05$) or in few cases by Welch t-test (one-sided smaller, $\alpha = 0.05$). Differences between average growth rate of the control and solvent control were tested by Student-t test (two-sided, $\alpha = 0.05$).

Based on the growth rate results for the exposure phases, the NOEC was calculated for each exposure phase. Based on the growth rate results for the recovery phases, the $NOEAC_{recovery}$ (No Observed Ecologically Adverse Concentration) for recovery was determined for each recovery phase. Yield results for the exposure and recovery phases were not used to determine the endpoints. Since different starting cell densities (only partly normalised to 5000 cells/mL) and different phase durations were used for the different exposure and recovery phases, yield results were less comparable and were not considered suitable to determine the effects on algal growth.

At the end of the final recovery period, the $NOAEP_{recovery}$ (No Observed Adverse Exposure Pattern) for recovery of *Desmodesmus subspicatus* was determined.

Results and Discussion

The concentrations of flurochloridone (trans isomer) were measured in one of the duplicate test medium samples from both test concentrations and from the solvent control. Analysis results are presented in the following table.

Table A 2.2.3-32: Concentrations of flurochloridone (trans isomer) in the test media

Test phase	Nominal concentration	Measured concentration				Geometric mean measured concentration	
		Start		End			
	[µg/L]	[µg/L]	[% of nominal]	[µg/L]	[% of nominal]	[µg/L]	[% of nominal]
Exposure 1	Solv. Control	< LOQ	n.a.	< LOQ	n.a.	< LOQ	n.a.
	10.5	10.1	97	10.0	95	10.0	95
	35	35.4	101	43.9	100	35	100
Exposure 2	Solv. Control	< LOQ	n.a.	< LOQ	n.a.	< LOQ	n.a.
	6.3	6.05	96	6.06	96	6.1	97
	21	20.5	97	20.7	99	21	100
Exposure 3	Solv. Control	< LOQ	n.a.	< LOQ	n.a.	< LOQ	n.a.
	4.5	4.55	101	4.26	95	4.4	98
	15	14.3	95	13.7	91	14	93
Exposure 4	Solv. Control	< LOQ	n.a.	< LOQ	n.a.	< LOQ	n.a.
	3.0	2.59	86	2.20	73	2.4	80
	10	9.59	96	7.87	79	8.7	87

LOQ 2.04 µg/L flurochloridone (trans isomer)
n.a. not applicable

The correct dosing of flurochloridone (trans isomer) was confirmed. The test substance was stable in the test media over the exposure periods. The biological results were related to the geometric mean measured concentrations.

At the start of the pulse exposure phases, measured concentrations of flurochloridone (trans isomer) in the test media of the test concentrations of 10.5 and 35, 6.3 and 21, 4.5 and 15 and 3.0 and 10 µg/L were between 86% and 101% of the nominal values. At the end of the first three exposure phases, 91% to 100% of the nominal values were measured. At the end of the last pulse exposure, 73% to 79% of the nominal values were found. The correct dosing of flurochloridone (trans isomer) was confirmed. The test substance was stable in the test media over the exposure periods. The biological results were related to the geometric mean measured concentrations.

The effects of flurochloridone (trans isomer) on yield and growth of algae during the exposure and the recovery phases are shown in the following tables.

After the first and second exposure phase, flurochloridone (trans isomer) had a statistically significant inhibitory effect on the growth rate of algae at both tested concentrations, i.e. 10.0 and 35 µg/L for the first exposure phase and 6.1 and 21 µg/L for the second exposure phase (geometric mean measured concentrations). Therefore, for the first and second exposure phase, no NOEC could be determined. After the third and fourth exposure phase, the growth rate of algae in both test substance treatments was not statistically significantly lower than in the solvent control. Thus, the NOEC for the third and fourth exposure phase corresponded with the higher concentrations tested, i.e. geometric mean measured 14 and 8.7 µg/L, respectively.

At the end of recovery phases 1, 2 and 4 (during recovery phase 3, no assessments were performed), pre-treatment with flurochloridone (trans isomer) had no statistically significant inhibitory effect on the growth rate of algae at all pre-treatment concentrations with exception of the test group previously treated with geometric mean measured 21 µg/L (higher test concentration) during recovery phase 2. Therefore, the $NOEAC_{recovery}$ for the first and fourth recovery phase corresponded with the higher pre-treatment concentration, i.e. 35 and 8.7 µg/L, respectively, and the $NOEAC_{recovery}$ for the second recovery phase corresponded with the lower pre-treatment concentration, i.e. 6.1 µg/L (geometric mean measured concentrations). Furthermore, it could be demonstrated, that complete recovery of algal growth after both applied exposure patterns occurred.

Microscopic examination of algal cells at the end of the first, second and final recovery phase showed no difference in shape and size between algae from the two pre-treatment groups and the solvent control.

Table A 2.2.3-33: Effects of flurochloridone on algal growth (yield) during exposure phases

Nominal test concentration [µg/L]	Geometric mean test concentration [µg/L]	Exposure phase 1 0-24 hours yield (y) y (x 10^3)	Inhib. [%]	Exposure phase 2 0-36 hours yield (y) y (x 10^3)	Inhib. [%]	Exposure phase 3 0-30 hours yield (y) y (x 10^3)	Inhib. [%]	Exposure phase 4 0-36 hours yield (y) y (x 10^3)	Inhib. [%]
Solvent control		3.3	0.0	-	-	-	-	-	-
10.5	10.0	1.5*	52.7	-	-	-	-	-	-
35	35	1.3*	59.8	-	-	-	-	-	-
Solvent control		-	-	6.7	0.0	-	-	-	-
6.3	6.1	-	-	2.8*	58.6	-	-	-	-
21	21	-	-	1.3*	80.2	-	-	-	-
Solvent control		-	-	-	-	7.6	0.0	-	-
4.5	4.4	-	-	-	-	9.6	-26.6	-	-
15	14	-	-	-	-	7.8	-3.2	-	-
Solvent control		-	-	-	-	-	-	30.2	0.0
3.0	2.4	-	-	-	-	-	-	43.7	-44.7
10	8.7	-	-	-	-	-	-	17.4*	42.3

Note: Yield results were not used to determine the study endpoints.

* mean value significantly lower than in the control (according to Williams t-test, one-sided smaller, $\alpha = 0.05$)

Table A 2.2.3-34: Effects of flurochloridone on algal growth (growth rate) during exposure phases

Nominal test concentration [µg/L]	Geometric mean test concentration [µg/L]	Exposure phase 1 0-24 hours growth rate (r) r [day^{-1}]	Inhib. [%]	Exposure phase 2 0-36 hours growth rate (r) r [day^{-1}]	Inhib. [%]	Exposure phase 3 0-30 hours growth rate (r) r [day^{-1}]	Inhib. [%]	Exposure phase 4 0-36 hours growth rate (r) r [day^{-1}]	Inhib. [%]
Solvent control		1.958	0.0	-	-	-	-	-	-
10.5	10.0	1.360*	30.5	-	-	-	-	-	-
35	35	1.239*	36.7	-	-	-	-	-	-
Solvent control		-	-	1.111	0.0	-	-	-	-
6.3	6.1	-	-	0.701*	36.9	-	-	-	-
21	21	-	-	0.430*	61.3	-	-	-	-
Solvent control		-	-	-	-	1.372	0.0	-	-
4.5	4.4	-	-	-	-	1.801	-31.3	-	-
15	14	-	-	-	-	1.378	-0.5	-	-
Solvent control		-	-	-	-	-	-	1.418	0.0
3.0	2.4	-	-	-	-	-	-	1.543	-8.9
10	8.7	-	-	-	-	-	-	1.553	-9.6
NOEC [µg/L]		n.d.		n.d.		14		8.7	

n.d. could not be determined

* mean value significantly lower than in the control (according to Williams t-test, one-sided smaller, $\alpha = 0.05$)

Table A 2.2.3-35: Effects of flurochloridone on algal growth (yield) during recovery phases

Nominal pre-treatment conc.	Geometric mean pre-treatment conc.	Recovery phase 1 0-72 hours yield (y)		Recovery phase 2 0-36 hours yield (y)		Recovery phase 3 0-6 hours yield (y)		Recovery phase 4 0-72 hours yield (y)	
[µg/L]	[µg/L]	y (x 10^3)	Inhib. [%]	y (x 10^3)	Inhib. [%]	y (x 10^3)	Inhib. [%]	y (x 10^3)	Inhib. [%]
Solvent control		66.7	0.0	-	-	-	-	-	-
10.5	10.0	33.2*	50.2	-	-	-	-	-	-
35	35	25.0*	62.4	-	-	-	-	-	-
Solvent control		-	-	36.4	0.0	-	-	-	-
6.3	6.1	-	-	15.9*	56.5	-	-	-	-
21	21	-	-	4.8*	87.0	-	-	-	-
Solvent control		-	-	-	-	n.d.	n.d.	-	-
4.5	4.4	-	-	-	-	n.d.	n.d.	-	-
15	14	-	-	-	-	n.d.	n.d.	-	-
Solvent control		-	-	-	-	-	-	56.7	0.0
3.0	2.4	-	-	-	-	-	-	55.5	2.2
10	8.7	-	-	-	-	-	-	43.0*	24.2

Note: Yield results were not used to determine the study endpoints.
n.d. not determined
* mean value significantly lower than in the control (recovery phases 1 and 2: according to Williams t-test, one-sided smaller, $\alpha = 0.05$; recovery phase 4: according to Welch t-test, one-sided smaller, $\alpha = 0.05$)

Table A 2.2.3-36: Effects of flurochloridone on algal growth (growth rate) during recovery phases

Nominal pre-treatment conc.	Geometric mean pre-treatment conc.	Recovery phase 1 0-72 hours growth rate (r)		Recovery phase 2 0-36 hours growth rate (r)		Recovery phase 3 0-6 hours growth rate (r)		Recovery phase 4 0-72 hours growth rate (r)	
[µg/L]	[µg/L]	r [day^{-1}]	Inhib. [%]	r [day^{-1}]	Inhib. [%]	r [day^{-1}]	Inhib. [%]	r [day^{-1}]	Inhib. [%]
Solvent control		1.168	0.0	-	-	-	-	-	-
10.5	10.0	1.208	-3.4	-	-	-	-	-	-
35	35	1.127	3.5	-	-	-	-	-	-
Solvent control		-	-	1.313	0.0	-	-	-	-
6.3	6.1	-	-	1.630	-24.1	-	-	-	-
21	21	-	-	1.054*	19.7	-	-	-	-
Solvent control		-	-	-	-	n.d.	n.d.	-	-
4.5	4.4	-	-	-	-	n.d.	n.d.	-	-
15	14	-	-	-	-	n.d.	n.d.	-	-
Solvent control		-	-	-	-	-	-	1.212	0.0
3.0	2.4	-	-	-	-	-	-	1.256	-3.7
10	8.7	-	-	-	-	-	-	1.237	-2.1
$NOEAC_{recovery}$ [µg/L]		35		6.1		n.d.		8.7	
$NOAEP_{recovery}$		applied exposure pattern with the higher exposure concentrations							

n.d. not determined
* mean value significantly lower than in the control (according to Williams t-test, one-sided smaller, $\alpha = 0.05$)

In the solvent control, the biomass increased over 72 hours by a factor of 33 during the first recovery phase and 38 during the final recovery phase (according to guideline ≥ 16). The mean coefficient of variation of the daily growth rates in the solvent control (section-by-section growth rates) during 72 hours was 7.7% during the first recovery phase and 85% during the final recovery phase (according to guideline ≤ 35%).The coefficient of variation of the average specific growth rates in the replicates of the solvent control after 72 hours was 3.1% during the first recovery phase and 3.0% during the final recovery phase (according to guideline ≤ 7%). Thus, the validity criteria for the test phases which were run for 72 hours were fulfilled (exception: mean coefficient of variation of section-by-section growth rate during final recovery phase).

Conclusion

At the end of the final recovery period, complete recovery of algal growth was demonstrated after both applied exposure patterns. Consequently, the applied exposure pattern with the higher exposure concentrations was determined as $NOAEP_{recovery}$ for *Desmodesmus subspicatus*.

A 2.2.3.11 Study 11: Pulsed exposure testing of trans isomer with algae– *Desmodesmus subspicatus*

Comments of zRMS:	The study design followed recommendations of OECD 201 with exception of the exposure, which simulated varying concentrations of flurochloridone over time. The test item concentrations measured at test end of some exposure periods were within 80-120% of nominal concentrations while at the end of other exposure periods they dropped below 80% of nominal and test results were thus expressed as geometric mean measured concentrations. In general, there were no deviations from the test guideline in terms of the environmental conditions, replication, application of the test item etc. At each exposure period different test item concentrations were used (6 for each exposure pattern). It should be, however, noted that in line with EFSA, 2013 the RAC for pulsed exposure study should be calculated with consideration of E_rC_{50} expressed in terms of the peak exposure, while according to EFSA Supporting publication 2019:EN-1673, EC_{10} is more relevant for primary producers in order to exclude effects of recovery and follow ETO option. None of these endpoints was (or could be) calculated due to the design of the exposure regime. The validity criteria were checked only for the recovery phases lasting for 72 hours. According to the study authors, this is correct, as validity criteria are applicable for 72 hours. In general, the zRMS agrees with this, however would like to point out that during the exposure phases lasting for less than 72 hours the specific growth rates of control cultures also should not be too variable and for this reason the zRMS would recommend to calculate for illustrative purposes at least CV values for shorter periods of time (e.g. 0-24 h, 24-48 h). This would give some indication regarding variability of growth in controls. Unfortunately, such a calculation was not possible to the zRMS as in the study report only mean growth rates for first exposure period (0-48 h) were given without information on the standard deviation. In addition to that, the cell number was not reported, so calculation of the specific growth rates for control cultures was not possible for the first exposure period. Moreover, the specific growth rates or the number of control cultures were not given for the remaining exposure phases. Calculation of biomass increase would not be possible, as this parameter was validated for 72 hours, so is not applicable for shorter period of time. The validity criteria for the recovery phase were fulfilled. Taking into account uncertain reliability of the results, lack of growth rates for exposure phases 2-6, lack of calculation of the NOEC or ECx values, the zRMS does not recommend to consider results of this study in the risk assessment.

Reference:	KCP 10.2.3/11
Report	Flurochloridone (Trans isomer): Toxicity to *Desmodesmus subspicatus* in a Pulse Exposure Growth Inhibition Test Supplemented with Testing for Recovery of Growth, Liedtke, A., 2013e, D65547 (report number), 90015432 (sponsor report number)
Guideline(s):	OECD 201 (2006), Commission Regulation (EC) No 761/2009, C.3 (2009)
Deviations:	Besides the chosen exposure and recovery pattern, there was a deviation to guideline: During exposure phases 2-6, the pH increased by more than 1.5 units. However, since the starting cell number was not normalised at the start of exposure phases 2-6 (due to short exposure phases of each 24 hours), the increase of pH can be explained by the CO2 demand of the constantly increasing number of algal cells. This is not considered to have affected the quality and integrity of the study.
GLP:	Yes
Acceptability:	In opinion of the zRMS results of this study are not fully reliable as for shorter time periods the CV for daily section by section growth rates could not be calculated for illustrative

	purposes (for all exposure phases). Furthermore, due to selected exposure regime and effects >50% observed during two firs exposure phases, calculation of ECx values required by EFSA (2013) and EFSA (2019) for the risk assessment based on refined exposure studies was not possible.
Duplication (if vertebrate study)	-

Materials and Methods

A. MATERIALS

1. Test material flurochloridone (trans isomer)

Description Not reported

Lot/Batch # FLCLDN(2)-BP12-1387(V3)

Purity Trans isomer: 99.3±0.5%
Chemical purity: 99.5±0.5%

Stability of test material Stable under storage conditions (room temperature)
Expiry date: 30.04.2016

2. Vehicle and/or positive control Vehicle controls: control (test water without test substance) and solvent control (test water without test substance but containing 0.01% DMF)
Positive control: For evaluation of the quality of the algal strain and the experimental conditions, potassium dichromate was tested as a positive control twice a year. The results of the latest positive control test performed in September 2012 (72-hour E_rC_{50}: 0.69 mg/L) were within the internal historical range (72-hour E_rC_{50}: 0.64-1.1 mg/L from 2000 to 2012).

3. Test organism

Species Freshwater green alga *Desmodesmus subspicatus* CHODAT (formerly: *Scenedesmus subspicatus*)

Strain SAG 86.81

Source Collection of Algal Cultures (SAG, Institute for Plant Physiology, University of Göttingen, 37073 Göttingen / Germany)

Age Algae cells were taken from an exponentially growing pre-culture set up three days prior to the start of the test.

Acclimation period An inoculum culture was set up three days before the start of exposure. The algae were cultivated under the test conditions and were kept in the exponential growth phase until inoculation of the test solutions.

Test units 50-mL Erlenmeyer flasks containing 15 mL of test medium. The test vessels were covered with glass dishes.

4. Environmental conditions

Test water The algae were cultivated and tested in reconstituted test water (AAP medium). Analytical grade salts were dissolved in sterile purified water to obtain the following nominal concentrations:

Macro-nutrients:

$NaHCO_3$	15.0 mg/L
K_2HPO_4	1.044 mg/L
$MgSO_4 \cdot 7\ H_2O$	14.6 mg/L
$MgCl_2 \cdot 6\ H_2O$	12.16 mg/L

$CaCl_2 \cdot 2\ H_2O$	4.41 mg/L
$NaNO_3$	25.5 mg/L
Trace elements:	
H_3BO_3	186.0 µg/L
$MnCl_2 \cdot 4\ H_2O$	415.0 µg/L
$ZnCl_2$	3.27 µg/L
$CoCl_2 \cdot 6\ H_2O$	1.43 µg/L
$CuCl_2 \cdot 2\ H_2O$	0.012 µg/L
$Na_2MoO_4 \cdot 2\ H_2O$	7.26 µg/L
$FeCl_3 \cdot 6\ H_2O$	160.0 µg/L
$Na_2EDTA \cdot 2\ H_2O$	300.0 µg/L

The pH of the test water was 7.5.

Hardness	0.15 mmol/L (= 15 mg/L) as $CaCO_3$
Water temperature	21-23°C
Lighting	Continuous illumination at a mean light intensity (measured at the level of the test solutions) of 7300 Lux (range: 6840 to 8050 Lux) at day 0 and 6800 Lux (range: 6200 to 7370 Lux) at day 10 using fluorescent tubes (Philips TLD 36W-1/840)
Shaking	During exposure and recovery phases, the test solutions were continuously stirred by magnetic stirrers.

B. STUDY DESIGN AND METHODS

1. In-life dates 15.01.2013 to 31.01.2013

2. Experimental conditions

Test design

The freshwater green alga *Desmodesmus subspicatus* was exposed to the test substance in a multiple pulse exposure test supplemented with a final recovery period. Two pulse exposure designs (A and B) were applied. Both designs included six exposure pulses: 48 hours at 1.65 µg/L, 24 hours at 1.11 µg/L, 24 hours at 0.45 µg/L, 24 hours at 0.63 µg/L, 24 hours at 1.11 µg/L and 24 hours at 0.63 µg/L for exposure design A and 48 hour at 5.5 µg/L, 24 hours at 3.7 µg/L, 24 hours at 1.5 µg/L, 24 hours at 2.1 µg/L, 24 hours at 3.7 µg/L and 24 hours at 2.1 µg/L for exposure design B (total study duration 240 hours, i.e. 10 days). Both pulse exposure designs were completed by a final recovery period of 72 hours. Additionally, a control and a solvent control group were tested in parallel. Three replicates for both exposure designs and the control and six replicates for the solvent control were set up. The cell density was measured at the start and end of each exposure phase (additionally after 24 hours during the first exposure phase) and at the start and end and after 48 hours during the final recovery phase.

Inhibition of algal growth of exposure design A and B in comparison to the solvent control was calculated based on yield and growth rate after 24 hours and at the end (48 hours) of the first pulse exposure and after 48 hours and at the end (72 hours) of the final recovery phase. Based on the growth rate results for the first exposure phase, the NOEC was determined. Based on the growth rate results for the final recovery phase, the $NOAEP_{recovery}$ (No Observed Adverse Exposure Pattern) for recovery of *Desmodesmus subspicatus* was determined.

Inoculum at test start

Approximately 5000 cells/mL

Test conditions

The water temperature was maintained at 21-23°C and the test systems were continuously illuminated at a mean light intensity of 7300 and 6800 Lux measured at the start of the first exposure (day 0) and on day 10 at the end of the recovery phase, respectively. The pH of the test media was 7.7-7.8 and 8.3-8.5 at the start and end of the first exposure phase, 8.0 and 7.7 at the start and end of the second exposure phase,7.6-7.7 and 8.4-9.8 at the start and end of the third exposure phase, 8.0-8.2 and 8.1-9.7 at the start and end of the fourth exposure phase, 7.9-8.0 and 9.2-10.6 at the start and end of the fifth exposure phase, 8.3-8.4 and 9.0-10.2 at the start and end of the sixth exposure phase and 8.0-8.2 and 7.5 at the start and end of the recovery phase, respectively. No remarkable observations were made concerning the appearance of the test media. All test media were clear solutions throughout the six exposure phases.

Test concentrations

The algae were exposed to the test substance according to the pulse exposure design summarised in the following table.

Table A 2.2.3-37: Pulse exposure design

Test phase		Test concentration [µg/L]		Duration
		Exposure design A	Exposure design B	
1	Exposure	1.65	5.5	48 hours
2	Exposure	1.11	3.7	24 hours
3	Exposure	0.45	1.5	24 hours
4	Exposure	0.63	2.1	24 hours
5	Exposure	1.11	3.7	24 hours
6	Exposure	0.63	2.1	24 hours
7	Recovery	0	0	72 hours

Treatment/Application

Due to the low water solubility of the test substance, the organic solvent *N*,*N*-dimethylformamide (DMF) was used to dose flurochloridone (trans isomer). At the start of the test (day 0), a stock solution of 275 mg/L in DMF was prepared by dissolving 27.51 mg of the test substance in 100 mL of DMF using intense stirring. This stock solution was diluted with DMF to prepare the application solutions and their dilutions for the dosage of the two test concentrations before each pulse exposure. An aliquot of 100 µL from the respective application solution was added to 1000 mL of test water during intense stirring. For preparation of the solvent control, the same volume of DMF (without test substance) was added to the test water. The test media were freshly prepared just before the start of each of the six exposure phases.

After each exposure phase, the replicates of each treatment were pooled and algal cells were separated by filtration (membrane filter, Schleicher & Schuell, Type NC45, pore size 0.45 µm). Subsequently, the algae were transferred into freshly prepared test medium and the algal density of each sample was determined.

The same procedure was performed for transferring the algal cells from the last exposure phase into the final recovery phase except for the transfer of the algae into pure test water.

The number of algal cells was reduced to nominal 5000 cells/mL at the start of the first exposure phase (days 0) and at the start of the final recovery period (day 7), in order to allow exponential growth of the algae during the test.

Analytics

The concentration of flurochloridone (trans isomer) in the test media and control was analysed by HPLC-MS/MS using external calibration. The test substance was separated on a column (Inertsil ODS-3; 2.1 mm x 33 mm; 3 µm; eluent A: 95 vol. water + 5 vol. methanol + 0.1 vol. formic acid + 5 mM ammonium formate; eluent B: 95 vol. methanol + 5 vol. water + 0.1 vol. formic acid + 5 mM ammonium formate; gradient: hold 0.5 min 60% A/40% B, in 1.5 min to 10% A/90% B, hold 0.5 min 10% A/90% B, in 0.1 min to 100% A, hold 0.4 min 100% A, in 0.1 min to 60% A/40% B, hold 0.9 min 60% A/40% B) at a flow rate of 400 µL/min and an injection volume of 50 µL. Detection was performed with a MDS Sciex API 4000 triple stage quadrupole mass spectrometer (heater gas temperature: 300°C; spray voltage: 4500 V; ionisation mode: ESI; scan mode: MRM; ion polarity: positive; m/z 312.0 → 292.0; retention time: approximately 2 minutes). The method was validated and the LOQ was set to 0.11 µg/L flurochloridone (trans isomer).

3. Sampling and measurements

Algal biomass was determined at the start and end of each exposure phase (additionally after 24 hours during the first exposure phase) and at the start and end and after 48 hours during the final recovery phase by cell counts using fluorescent measurement (BIO-TEK® Multi-Detection Microplate Reader, Model FLx800, wavelength: excitation 440 nm, emission 680 nm). The measurements were performed at least in duplicate.

Furthermore, algal cells from the solvent control and both exposure designs were investigated by microscopic evaluation after the final recovery phase. The shape and size of the algal cells were visually inspected.

The test media of all test concentrations and the control and solvent control were sampled in duplicate at the start (without algae) and at the end (containing algae) of the exposure phase for analysis of the test substance concentration. For the start and stability samples of each exposure phase, additional flasks containing the test medium with algae were incubated for each treatment under test conditions.

The pH was measured in both test substance (pre-) treatments and the control and solvent control at the start and at the end of each test period. The water temperature was measured and recorded daily during the exposure phases and at the start, at day 2 and at the end of the final recovery period. The appearance of the test media was recorded daily during the exposure phases. The light intensity was measured at the start of the first exposure (day 0) and on day 10 at the end of the recovery phase.

4. Calculation of toxicity

Inhibition of algal growth was determined based on the cell density (yield, y) and the specific growth rate (r) for exponentially growing cultures using the equations recommended in the test guidelines.
The calculation of the endpoints yield and growth rate was only useful for the first pulse exposure and the final recovery, since at the start of these two periods, the cell number in all test groups was normalised to 5000 cells/mL allowing comparable growth conditions during these two periods. Whereas, the starting cell numbers for the exposure periods 2–6 were not normalised and thus the calculation of the endpoints yield and growth rate were considered not be meaningful.

Inhibition of algal growth of exposure design A and B in comparison to the solvent control was calculated based on yield and growth rate after 24 hours and at the end (48 hours) of the first pulse exposure and after 48 hours and at the end (72 hours) of the final recovery phase.

5. Statistics

After the first exposure and the final recovery phase, average growth rate of exposure design A and B was compared to the solvent control by Williams t-test (one-sided smaller, $\alpha = 0.05$). Differences between average growth rate of the control and solvent control were tested by Student-t test (two-sided, $\alpha = 0.05$).

Based on the growth rate results for the first exposure phase, the NOEC was determined. Based on the growth rate results for the final recovery phase, the $NOAEP_{recovery}$ (No Observed Adverse Exposure Pattern) for recovery of *Desmodesmus subspicatus* was determined. Yield results for the first pulse exposure and the final recovery phase were not used to determine the endpoints since they were less comparable and were thus not considered suitable to determine the effects on algal growth.

Results and Discussion

The concentrations of flurochloridone (trans isomer) were measured in one of the duplicate test medium samples from both test concentrations and from the solvent control. Analysis results are presented in the following table.

Table A 2.2.3-38: Concentrations of flurochloridone (trans isomer) in the test media

Test phase	Nominal concentration	Measured concentration				Geometric mean measured concentration	
		Start		End			
	[µg/L]	[µg/L]	[% of nominal]	[µg/L]	[% of nominal]	[µg/L]	[% of nominal]
Exposure 1	Solv. Control	< LOQ	n.a.	< LOQ	n.a.	< LOQ	n.a.
	1.65	1.51	92	1.41	86	1.46	88
	5.5	5.17	94	4.92	90	5.0	91
Exposure 2	Solv. Control	< LOQ	n.a.	< LOQ	n.a.	< LOQ	n.a.
	1.11	1.03	93	0.950	86	0.99	90
	3.7	3.41	92	3.10	84	3.3	89
Exposure 3	Solv. Control	< LOQ	n.a.	< LOQ	n.a.	< LOQ	n.a.
	0.45	0.364	81	0.328	73	0.35	78
	1.5	1.36	91	1.30	87	1.3	87
Exposure 4	Solv. Control	< LOQ	n.a.	< LOQ	n.a.	< LOQ	n.a.
	0.63	0.543	86	0.419	67	0.48	76
	2.1	2.01	95	1.86	88	1.9	91
Exposure 5	Solv. Control	< LOQ	n.a.	< LOQ	n.a.	< LOQ	n.a.
	1.11	0.996	90	0.627	56	0.79	72
	3.7	3.19	86	2.79	75	3.0	81
Exposure 6	Solv. Control	< LOQ	n.a.	< LOQ	n.a.	< LOQ	n.a.
	0.63	0.551	87	0.274	44	0.39	62
	2.1	1.87	89	1.47	70	1.7	81

LOQ 0.11 µg/L flurochloridone (trans isomer)
n.a. not applicable

The correct dosing of flurochloridone (trans isomer) was confirmed. During the exposure periods, a decrease of test substance concentration in the test media occurred. The biological results were related to the geometric mean measured concentrations.

At the start of the pulse exposure phases, measured concentrations of flurochloridone (trans isomer) in the test media of the test concentrations of 1.65 and 5.5, 1.11 and 3.7, 0.45 and 1.5, 0.63 and 2.1, 1.11 and 3.7 and 0.63 and 2.1 µg/L were between 81 and 95% of the nominal values. At the end of the exposure phases, 44% to 90% of the nominal values were measured. The correct dosing flurochloridone (trans isomer) was confirmed. During the exposure periods, a decrease of test substance concentration in the test media occurred. The biological results were related to the geometric mean measured concentrations, i.e. 1.46, 0.99, 0.35, 0.48, 0.79 and 0.39 µg/L for exposure design A and 5.0, 3.3, 1.3, 1.9, 3.0 and 1.7 µg/L for exposure design B.

The effects of flurochloridone (trans isomer) on yield and growth of algae during the first exposure and the final recovery phase are shown in the following tables.

After the first exposure phase, flurochloridone (trans isomer) had a statistically significant inhibitory effect on the growth rate of algae at both tested concentrations, i.e. geometric mean measured 1.46 and 5.0 µg/L. Therefore, no NOEC could be determined for the first exposure phase.

The starting cell numbers for the exposure periods 2–6 were not normalised and thus the calculation of the endpoints yield and growth rate were considered not be meaningful.

At the end of the final recovery phase, pre-treatment with flurochloridone (trans isomer) had no statistically significant inhibitory effect on the growth rate of algae neither for exposure design A nor for exposure design B. Therefore, complete recovery of algal growth could be demonstrated after both applied exposure patterns.

Microscopic examination of algal cells at the end of the final recovery phase showed no difference in shape and size between algae from exposure design A and B and the solvent control.

Table A 2.2.3-39: Effects of flurochloridone on algal growth (yield), exposure phase 1 and recovery

Treatment	Exposure phase 1				Final recovery phase			
	0-24 hours yield (y)		0-48 hours yield (y)		0-48 hours yield (y)		0-72 hours yield (y)	
	y ($x 10^3$)	Inhib. [%]	y ($x 10^3$)	Inhib. [%]	y ($x 10^3$)	Inhib. [%]	y ($x 10^3$)	Inhib. [%]
Solvent control	4.4	0.0	11.2	0.0	10.1	0.0	33.0	0.0
Exposure design A	2.3*	48.6	5.9*	47.6	11.7	-15.9	36.5	-10.7
Exposure design B	1.0*	78.4	0.6*	94.5	8.7	14.2	28.9*	12.4

Note: Both designs included six exposure pulses: 48 hours at 1.65 µg/L, 24 hours at 1.11 µg/L, 24 hours at 0.45 µg/L, 24 hours at 0.63 µg/L, 24 hours at 1.11 µg/L and 24 hours at 0.63 µg/L for exposure design A and 48 hour at 5.5 µg/L, 24 hours at 3.7 µg/L, 24 hours at 1.5 µg/L, 24 hours at 2.1 µg/L, 24 hours at 3.7 µg/L and 24 hours at 2.1 µg/L for exposure design B (nominal values)

* mean value significantly lower than in the control (according to Williams t-test, one-sided smaller, $\alpha = 0.05$)

Table A 2.2.3-40: Effects of flurochloridone on algal growth (growth rate), exp. phase 1 and recovery

Treatment	Exposure phase 1				Final recovery phase			
	0-24 hours growth rate (r)		0-48 hours growth rate (r)		0-48 hours growth rate (r)		0-72 hours growth rate (r)	
	r [day^{-1}]	Inhib. [%]	r [day^{-1}]	Inhib. [%]	r [day^{-1}]	Inhib. [%]	r [day^{-1}]	Inhib. [%]
Solvent control	1.912	0.0	1.374	0.0	1.028	0.0	1.054	0.0
Exposure design A	1.377*	28.0	1.079*	21.4	1.069	-4.0	1.064	-1.0
Exposure design B	0.806*	57.8	0.292*	78.7	1.031	-0.3	1.057	-0.3
$NOAEP_{recovery}$	n.a.				both applied exposure patterns			

Note: Both designs included six exposure pulses: 48 hours at 1.65 µg/L, 24 hours at 1.11 µg/L, 24 hours at 0.45 µg/L, 24 hours at 0.63 µg/L, 24 hours at 1.11 µg/L and 24 hours at 0.63 µg/L for exposure design A and 48 hour at 5.5 µg/L, 24 hours at 3.7 µg/L, 24 hours at 1.5 µg/L, 24 hours at 2.1 µg/L, 24 hours at 3.7 µg/L and 24 hours at 2.1 µg/L for exposure design B (nominal values)

* mean value significantly lower than in the control (according to Williams t-test, one-sided smaller, $\alpha = 0.05$)

n.a. not applicable

In the solvent control during the final recovery phase, the biomass increased over 72 hours by a factor of 24 (according to guideline ≥ 16). The mean coefficient of variation of the daily growth rates in the solvent control (section-by-section growth rates) during 72 hours was 15% (according to guideline ≤ 35%).The coefficient of variation of the average specific growth rates in the replicates of the solvent control after 72 hours was 3.1% (according to guideline ≤ 7%). Thus, the validity criteria for the test phase which was run for 72 hours (final recovery phase) were fulfilled.

Conclusion

At the end of the final recovery period, complete recovery of algal growth was demonstrated after both applied exposure patterns. Consequently, the applied exposure pattern with the higher exposure concentrations (exposure design B) was determined as $NOAEP_{recovery}$ for *Desmodesmus subspicatus*.

A 2.2.3.12 Study 12: Pulsed exposure testing of trans isomer with *Lemna minor*

Comments of zRMS:	As the study provides new active substance data that are not necessary for the risk assessment, it was not evaluated by the zRMS, in line with indications of SANCO/10326/2004, rev. 8 (2012). The study summary is thus struck through and shaded.

Reference:	KCP 10.2.3/12
Report	Macrophyte Pulse Exposure Growth Inhibition Test: Flurochloridone (trans-isomer): Sediment-free *Lemna minor* Toxicity Test - Testing for Recovery of Growth, Wenzel, A., 2015b, ADM-005/4-11/I (report number), 90016482 (sponsor report number)
Guideline(s):	OECD 221 (2006)
Deviations:	No
GLP:	Yes
Acceptability:	Not evaluated, new active substance data not necessary for the risk assessment
Duplication (if vertebrate study)	-

~~**Materials and Methods**~~

~~**A. MATERIALS**~~

~~**1. Test material** flurochloridone (trans isomer)~~
~~**Description** white crystals~~
~~**Lot/Batch #** FLCLDN(2)-BP12-1387(V3)~~
~~**Purity** Trans isomer: 99.3±0.5%~~
~~Chemical purity: 99.5±0.5%~~
~~**Stability of test material** Stable under normal storage conditions (room temperature)~~
~~Expiry date: 30.04.2016~~

~~**2. Vehicle and/or positive control** Vehicle controls: control (test water without test substance)~~
~~Reference substance: Sensitivity of test organisms is routinely checked using 3,5-dichlorophenol. The nominal E_rC_{50} value of 2.7 mg/L is in good agreement with the results of an international ring test with E_rC_{50} values between 1.7 and 5.7 mg/L.~~

~~**3. Test organism**~~

~~**Species** *Lemna minor St*, Lemnaceae, Spermatophyta~~
~~**Source** Botanical Institute, University of Jena, Germany~~
~~**Acclimation period** 7-10 days before testing a pre-culture was established in test medium and conditions to obtain exponentially growing plans.~~
~~**Test units** 400 mL glass beakers (low form) covered with glass plates.~~

~~**4. Environmental conditions**~~

~~**Test water** The plants were cultivated and tested in sterilized Steinberg medium.~~

~~Macroelements:~~
~~KNO_3 350.0 mg/L~~
~~$Ca(NO_3)_2 \cdot 4\ H_2O$ 295.0 mg/L~~
~~KH_2PO_4 90.0 mg/L~~

K_2HPO_4	12.6 mg/L
$MgSO_4 \cdot 7\ H_2O$	100.0 mg/L

Microelements:

H_3BO_3	120.0 µg/L
$ZnSO_4 \cdot 7\ H_2O$	180.0 µg/L
$Na_2MoO_4 \cdot 2\ H_2O$	44.0 µg/L
$MnCl_2 \cdot 4\ H_2O$	180.0 µg/L
$FeCl_3 \cdot 6\ H_2O$	760.0 µg/L
$Na_2EDTA \cdot 2\ H_2O$	1500.0 µg/L

The pH of the test water was adjusted to 5.5 ± 0.2 by addition of HCL.

Water temperature	22.7-24.8°C
Lighting	Continuous illumination at a light intensity (measured at the distance of the fronds) of 6600 Lux (120 – 125 µgE $m^{-2}s^{-1}$)

B. STUDY DESIGN AND METHODS

1. In-life dates 02.03.2015 to 19.03.2015

2. Experimental conditions

Test design

The freshwater floating macrophyte *Lemna minor* was exposed in a sediment-free system to four pulses of test item with subsequent recovery periods achieved by transfer to fresh medium during a 10-day test period and followed by a recovery phase of 7 days. Frond numbers and total frond area were recorded at test start and at least every 2 to 3 days during the test period and subsequent recovery phase. Inhibition of growth and yield were determined in relation to control cultures.

Inoculum at test start

Colonies consisting of 2 to 4 visible fronds giving a total of 12 fronds per test vessel

Test conditions

The temperature was maintained at 22.7-24.8°C and the test systems were continuously illuminated at an intensity of 120 to 125 µgE $m^{-2}s^{-1}$. The pH of controls and freshly prepared medium was 5.89 at the start of the exposure period and ranged between 5.47 and 5.86 during exposures and was 5.97 at the end of the 10-day exposure period. Initial pH in the treatment vessels was 5.90, ranged between 5.56 and 5.80 during exposures and was 6.22 at the end of the 10-day exposure phase. During the recovery phase, the pH increased from 5.54 to 6.73.

Test concentrations

The plants were exposed to the test substance according to the pulse exposure design summarized in the following table.

Table A 2.2.3-41: Pulse exposure design

Test phase	Nominal test concentrations [µg/L]	Day	Duration
Exposure peak 1	35	1	24 hours
Recovery 1	-	1-4	72 hours
Exposure peak 2	21	4-5	36 hours
Recovery 2	-	5-7	36 hours
Exposure peak 3	15	7-8	30 hours
Recovery 3	-	8	6 hours
Exposure peak 4	10	8-10	36 hours
Recovery 4	-	10-17	7 days

6 replicates were prepared for control and treated plants, each.

Treatment/Application

A stock solution in test medium was prepared by dissolving 52.37 mg test item in 5 mL acetone (equivalent to 10.47 g/L). A nominal stock solution of about 5 mg a.s./L was prepared by deposition of 500 µL acetonic solution as thin layer in a glass bottle and removal of acetone by evaporation. Following addition of 1 L ultra-pure water, the aqueous stock solution was stirred overnight at room temperature. The stock solution was sterilized and filtered and the filtrate sent to the sub-contractor for the test phase. The concentration of the stock solution was analyzed after filtration (3.92, 3.136 and 5 mg a.s./L measured at different times). Test media were prepared by dilution of the aqueous stock solution with growth medium to prepare the individual test concentrations. Different aliquots were given to 1 L growth medium. The control consisted of Steinberg medium only.

400 mL grass beakers were served with medium and colonies of 2 to 4 fronds and 12-14 fronds in total.

At day 7, 12-14 fronds were randomly selected from control cultures and transferred to new test vessels for dilution. To avoid subjective selection out of treated plants, half of the treated cultures were transferred to new vessels, but all cultures were incubated for further 3 days (until day 10 of the exposure phase).

At day 10, again colonies of 2 to 4 fronds and 12 to 14 fronds in total were randomly selected from controls and treated cultures and transferred to new test media without test item to assess the recovery potential.

Analytics

The concentration of flurochloridone (trans-isomer) in the water phase was analysed by GC/MS. Samples were taken from representative replicates per test or pooled replicates and controls. Determination was performed using an internal standard following calibration. Sample preparation was done by spiking with internal standard and extraction with n-hexane, subsequently measured by GC/MS. GC/MS: Column: DB-5MS UI; 30 m, 0.25 mm ID, 0.25 µm film (Agilent). Gradient (oven): 2.2 min 80°C, then 25°C/min to 275°C for 4.0 min. Carrier gas: helium with constant flow of 0.8 mL/min, splitless inlet (280°C). MS: SIM-Mode, internal standard (4,4 DDE): target ion: 246, qualifier ion: 317.9, flurochloridone: target ion: 311, qualifier ion: 187, MS source: 250°C, MS quadrupol: 200°C, solvent delay: 9 min, Run time: 14 min. The method was validated and the LOQ was set to 1.5 µg/L flurochloridone.

3. Sampling and measurements

Frond numbers and total frond area were recorded during the pulse-exposure test and subsequent recovery phase for measurements at the beginning of the test and recovery period and at least every 2 to 3 days (actually on days 0, 2, 4, 7 and 10 of the exposure test phase and days 0, 2, 4 and 7 of the recovery phase). Parameters were determined using medeaLAB device for *Lemna* tests with integrated Basler Gigabit Ethernet camera and image analysis medeaLAB Count & Classify 6.8 (media AV multimedia und Software GmbH) and checked manually for missed fronds.

Furthermore, plants were visually inspected for morphological changes (e.g. frond size, deformations, appearance, indications of necrosis, chlorosis or gibbosity, discolorations, colony break-up or loss of buoyancy and in root length and appearance).

Test item concentrations were analysed at the start and end of the four exposure periods and in representative control replicates at the start and end of the 10-day growth test. Additionally, samples were taken at the end of each recovery phase. For the recovery period, test item concentrations were assessed in media samples of treated plants at the start of the recovery period and in control media samples and treated plants at the end of the recovery phase.

The temperature was recorded at least at each observation date. The pH was measured in one test vessel per test concentration and control plot during the test. The pH was checked at test initiation and end of the exposure phase as well as at start and end of the recovery phase.

4. Calculation of toxicity

Growth was expressed as logarithmic increase in measurement variables during exposure time. Additionally yield was determined. Substance-related effects were determined quantitatively by comparison to controls.

The mean value of the frond number and frond area for control and treatment were used for plotting growth curves. Due to limited surface area for plant growth, the plants were sub-cultured after 7 days for further 3 days. The relative increase during the 3-day exposure after sub-culture was used to calculate the equivalent frond number based on day 7 of the first exposure period before sub-culture. This enables plotting a continuous growth curve for the entire exposure scenario of 10 days and the evaluation over 10 days.

Percentage inhibition was calculated for average specific growth rate and yield for both frond number and frond area.

5. Statistics

Comparison of percent inhibition of treatments and controls was statistically analysed by the computer programme ToxRat® using STUDENTs t-test for homogeneous variances.

Results and Discussion

The concentrations of flurochloridone (trans isomer) were measured in samples from fresh and aged media at start and end of the exposure peaks as well as in fresh and/or aged recovery and control media. Analysis results are presented in the following table.

Table A 2.2.3-42: Concentrations of flurochloridone (trans isomer) in the test media

Test phase	Nominal concentration	Measured concentration				Geometric mean measured concentration	
		Fresh (average of 2 runs)		Aged (average of 2 runs)			
	[µg/L]	[µg/L]	[% of nominal]	[µg/L]	[% of nominal]	[µg/L]	[% of nominal]
Control day 0	n.a.	< LOQ	n.a.	n.a.		n.a.	
Exposure 1	35.0	38.3	109	44.3	127	41.2	118
Recovery 1	n.a.	n.a.		< LOQ	n.a.	n.a.	
Exposure 2	21.0	27.9	133	32.1	153	29.9	142
Recovery 2	n.a.	n.a.		< LOQ	n.a.	n.a.	
Exposure 3	15.0	15.8	105	15.7	104	15.7	105
Recovery 3	n.a.	n.a.		< LOQ	n.a.	n.a.	
Exposure 4	10.0	9.56	95.6	8.83	88.3	9.19	91.9
Control day 10	n.a.	< LOQ	n.a.	< LOQ	n.a.	n.a.	
Control day 17	n.a.	n.a.		< LOQ	n.a.	n.a.	
Recovery period	n.a.	n.a.		< LOQ	n.a.	n.a.	

LOQ = 1.5 µg/L flurochloridone (trans isomer)
n.a. = not applicable

The correct dosing of flurochloridone (trans isomer) was confirmed. Concentrations of pulses were expressed as geometric mean measured concentrations.

Measured test item concentrations were in good agreement with or slightly higher than the nominal concentrations with analytical recoveries between 95.6 and 133% and between 88.3 and 153% of nominal in fresh and aged media, respectively, during exposure pulses indicating correct or even worst-case dosing. Pulses are expressed as geometric mean measured concentrations (41.2, 29.9, 15.7 and 9.19 µg flurochloridone/L, equivalent to 118, 142, 105 and 91.9% of nominal).

After the 10-day exposure phase, the number of fronds was reduced compared to controls (by 1.9% and 6.2% for growth rate and yield, respectively). Frond area was increased (by 7.0% and 27% for growth rate and yield, respectively).

After the 7-day recovery phase, frond numbers were reduced by 2.4% (growth rate) and 4.9% (yield), whereas frond areas were increased by 3.7% (growth rate) and reduced by 2.6% (yield) compared to controls.

Frond number counts and frond areas as well as growth rates and yield inhibition are presented in the following tables.

There were no significant differences between controls and treated plants during exposure and the subsequent recovery phase.

No abnormalities were observed for controls and exposed plants during the first four days. After 7 days, plants were gibbous and after 10 days, fronds overlapped slightly in all cultures. After 10 days, frond margins showed slight chloroses. During recovery, a few overlapping fronds were observed during the last 3 days. In recovery cultures of treated plants, fronds were brighter than controls at the beginning of recovery. After the 7-day recovery phase, only margins of 6 to 8 fronds showed slight chloroses, regarded to belong to plants sub-cultured from the exposure phase. During recovery, none of the evolving plants showed chlorosis at the end of recovery.

Table A 2.2.3-43: Frond numbers and frond areas during exposure and recovery phase

Treatment group	Frond number counts (mean ± SD)									
	Exposure phase						Recovery phase			
	0 d	2 d	4 d	7 d	7 d [a)]	10 d [a)]	0 d	2 d	4 d	7 d
Control	12.0 ± 0.0	29.5 ± 1.5	38.0 ± 2.0	101.2 ± 8.6	12.5 ± 0.8	37.2 ± 1.7	13.0 ± 0.9	29.5 ± 3.5	41.2 ± 6.5	112.0 ± 20.2
Treatment	12.0 ± 0.0	28.8 ± 1.8	38.7 ± 2.9	112.3 ± 6.2	109.2 ± 5.6	276 ± 26.2	13.2 ± 0.8	31.5 ± 2.6	41.0 ± 5.2	107.3 ± 14.8

Treatment group	% of day 7		Transformed for entire exposure 0—10 days [b)]	
	7 d [a)]	10 d [a)]	10 d [a)]	
Control	100 ± 6.7	297 ± 13.8	301 ± 36.2	n.a.
Treatment	100 ± 5.1	252 ± 24.0	284 ± 33.0	

Treatment group	Frond area (mean ± SD)									
	Exposure phase						Recovery phase			
	0 d	2 d	4 d	7 d	7 d [a)]	10 d [a)]	0 d	2 d	4 d	7 d
Control	197.70 ± 8.11	310.17 ± 15.35	561.46 ± 40.47	1365.66 ± 154.94	208 ± 17.7	502 ± 57	213.0 ± 22.78	366.3 ± 60.56	683.3 ± 113.1	1624.8 ± 340.1
Treatment	206.29 ± 8.57	322.19 ± 17.01	620.82 ± 55.93	1677.68 ± 125.09	1712 ± 134	4229 ± 289	236.0 ± 13.52	414.9 ± 55.72	741.0 ± 77.43	1699.6 ± 264.9

Treatment group	% of day 7		Transformed for entire exposure 0—10 days [b)]	
	7 d [a)]	10 d [a)]	10 d [a)]	
Control	100 ± 8.5	241 ± 27.3	3313 ± 695	n.a.
Treatment	100 ± 7.8	247 ± 16.9	4161 ± 582	

[a)] after sub-culture was prepared

[b)] relative increase during 3 day exposure after sub-culture was used to calculate equivalent frond number/area based on day 7 of the first exposure period before sub-culture

SD: standard deviation; n.a. not applicable

Table A 2.2.3-44: Growth rate and yield inhibition

Treatment group	Frond numbers (mean ± SD and % reduction)							
	Exposure phase (0 – 10 days)				Recovery phase (10 – 17 days)			
	Growth rate		Yield		Growth rate		Yield	
	r	%	y	%	r	%	y	%
Control	0.322 ± 0.012	n.a.	289 ± 36.2	n.a.	0.306 ± 0.019	n.a.	99.0 ± 19.6	n.a.
Treatment	0.316 ± 0.011	1.9	271 ± 33.0	6.2	0.299 ± 0.016	2.4	94.2 ± 14.1	4.9
Treatment group	Frond areas (mean ± SD and % reduction)							
	Exposure phase (0 – 10 days)				Recovery phase (10 – 17 days)			
	Growth rate		Yield		Growth rate		Yield	
	r	%	y	%	r	%	y	%
Control	0.280 ± 0.020	n.a.	3115 ± 691.3	n.a.	0.288 ± 0.017	n.a.	1412 ± 319.8	n.a.
Treatment	0.300 ± 0.012	– 7.0	3955 ± 575.3	– 26.9	0.281 ± 0.016	2.6	1437 ± 255.8	– 3.7

Negative values indicate an increase relative to controls
SD: standard deviation; n.a. not applicable

The factor of frond number increase in the controls measured between day 0 and day 7 was 8.4 (required: ≥ 7) corresponding to an average specific growth rate of 0.304 d^{-1} (required: ≥ 0.275 d^{-1}) and to a doubling time of 2.28 days (required: < 2.5 days). Accordingly, the validity criteria are fulfilled.

Conclusion

The test item (trans-flurochloridone) did not affect growth rate and yield of frond number and frond area of *Lemna minor* following four exposure peaks of 41.2, 29.9, 15.7 and 9.19 µg flurochloridone/L (geometric mean measured), equivalent to 118, 142, 105 and 91.9% of nominal with peak durations of 24, 36, 30 and 36 hours, respectively.

A 2.2.3.13 Study 13: Pulsed exposure testing of trans isomer with *Myriophyllum spicatum*

Comments of zRMS:	As the study provides new active substance data that are not necessary for the risk assessment, it was not evaluated by the zRMS, in line with indications of SANCO/10326/2004, rev. 8 (2012). The study summary is thus struck through and shaded.

Reference:	KCP 10.2.3/13
Report	Macrophyte Pulse Exposure Growth Inhibition Test: Flurochloridone (trans-isomer): Sediment-free *Myriophyllum spicatum* Toxicity Test - Testing for Recovery of Growth, Wenzel, A., 2015c, ADM-005/4-13/K (report number), 90016483 (sponsor report number)
Guideline(s):	OECD 238 (2014)
Deviations:	None
GLP:	Yes
Acceptability:	Not evaluated, new active substance data not necessary for the risk assessment
Duplication (if vertebrate study)	-

Materials and Methods

A. MATERIALS

1. Test material flurochloridone (trans isomer)

Description	white crystals
Lot/Batch #	FLCLDN(2) BP12 1387(V3)
Purity	Trans isomer: 99.3±0.5% Chemical purity: 99.5±0.5%
Stability of test material	Stable under normal storage conditions (room temperature) Expiry date: 30.04.2016

2. Vehicle and/or positive control — Vehicle controls: control (test water without test substance)
Reference substance: Sensitivity of test organisms is routinely checked using 3,5-dichlorophenol. The nominal E_yC_{50} for fresh weight, dry weight, total shoot length and main shoot length was 6.1, 4.1, 7.7 and 2.6 mg/L, respectively. The values are in good agreement with the results of an international ring test with EC_{50} values between 3.2 and 6.9 mg/L.

3. Test organism

Species	*Myriophyllum spicatum*, Haloragaceae, Dicotyledonous
Source	Institut für Gewässerschutz, MESOCOSM GmbH, Neu-Ulrichstein, Homberg (Ohm), Germany. Sterile plants originate from German Federal Environmental Agency, Schichauweg 58, Berlin, Germany.
Acclimation period	Stock cultures grown under sterile conditions have been cultured in the test facility for about 2 years. Plants were grown under standard conditions three weeks prior to the test for pre-incubation.
Test units	glass test tubes (inner diameter approximately 20 mm, length approximately 250 mm) with aluminium caps.

4. Environmental conditions

Test water — For stock- and pre-cultures, Andrews' medium with 3% sucrose was used:

Macroelements:

KCl	0.746 mg/L
KNO_3	80.8 mg/L
$Ca(NO_3)_2 \cdot 4\ H_2O$	188.8 mg/L
$MgSO_4 \cdot 7\ H_2O$	98.6 mg/L
KH_2PO_4	27.2 mg/L
$FeSO_4 \cdot 7\ H_2O$	2.78 mg/L
$Na_2EDTA \cdot 2\ H_2O$	3.72 mg/L
Sucrose	30 g/L

Microelements:

$MnSO_4 \cdot 4\ H_2O$	223 µg/L
$ZnSO_4 \cdot 7\ H_2O$	115 µg/L
H_3BO_3	155 µg/L
$CuSO_4 \cdot 5\ H_2O$	12.5 µg/L
$(NH_4)_6Mo_7O_{24} \cdot H_2O$	3.7 µg/L

The pH of the medium was set to pH 5.8 using HCl.
For the media used in the test, a tenfold concentrated modified Andrews' medium containing 3 % sucrose (30 g/L) was prepared.
The pH of the medium was set to pH 5.8 using NaOH.
The media were sterilised by autoclaving at 121°C for 20 min.

Temperature	23 ± 2°C

Lighting 16/8 h light/dark cycle. Light intensity of 6000-8000 Lux (100 - 150 µgE $m^{-2}s^{-1}$)

B. STUDY DESIGN AND METHODS

1. In-life dates 26.02.2015 to 16.03.2015

2. Experimental conditions

Test design

The freshwater submerged and rooted macrophyte *Myriophyllum spicatum* was exposed in a sediment-free static system to four pulses of test item with subsequent recovery periods achieved by transfer to fresh medium during a 14-day test period and followed by a recovery phase of 4 days. Shoot lengths (total and main lengths), fresh and dry weights as well as number of whorls were recorded. Inhibition of growth rate and yield were determined in relation to control cultures.

Inoculum at test start

Homogeneous shoots consisting of fresh lateral branches shortened to a length of 2.5 cm from base were selected, each test tube containing one plant shoot.

Test conditions

Plants were tested in a light/dark cycle of 16 to 8 hours at a light intensity in the range of 119.52-148.90 µgE $m^{-2}s^{-1}$. The temperature was maintained at 21.5-23.5°C. The pH of the controls was between 5.20 and 5.85 in freshly prepared solutions and between 5.38 and 7.19 in aged solutions. In the treatment vessels, the pH was between 5.20 and 5.80 in fresh and between 5.15 and 7.24 in aged solutions, respectively.

Test concentrations

The plants were exposed to the test substance according to the pulse exposure design summarised in the following table.

Table A 2.2.3-45: Pulse exposure design

Test phase	Nominal test concentrations [µg/L]	Day	Duration
Exposure peak 1	35	1	24 hours
Recovery 1	-	1-4	72 hours
Exposure peak 2	21	4-5	36 hours
Recovery 2	-	5-7	36 hours
Exposure peak 3	15	7-8	30 hours
Recovery 3	-	8	6 hours
Exposure peak 4	10	8-10	36 hours
Recovery	-	10-18	8 days

20 replicates were prepared for the control and the treatment group. At the end of the pulsed exposure test, 10 replicates were used for length and weight measurements. The other 10 replicates were used for assessment of recovery potential.

Treatment/Application

An acetonic solution of flurochloridone (trans isomer) was prepared by dissolving 52.4 mg of the test item in 5 mL acetone. To achieve a nominal stock solution concentration of 5.2 mg a.s./L in ultrapure water, 500 µL of the acetonic solution were added to a glass bottle, the acetone was evaporated using an air stream and 1 L of ultrapure water was added. The stock solution was stirred overnight and sterilised by sterile filtration using a 0.2 µm PES (polyethersulfone) bottle-top filter at the next morning. For saturation of the filter material, 100 mL were filtrated first and then discarded. The subsequent filtrate was used for the test.

The flurochloridone (trans isomer) concentration of the filtered stock solution was analysed to be 3.97 mg a.s./L. The stock solution was diluted with growth medium to prepare the individual test concentrations.

Different aliquots of the stock solution (100 – 440.8 µL) were given directly to 50 mL growth medium in the test tubes to create the required test concentrations. The test solutions were carefully mixed using a sterile glass rod.

The control medium consisted of Andrew's medium with 3% sucrose only. Since the acetone was evaporated after transfer of the acetonic solution of the test item, it was not necessary to include a solvent control in the test.

All work was performed under sterile conditions.

Analytics

The concentration of flurochloridone (trans isomer) in the water phase was analysed by GC/MS. Samples were taken from representative replicates per test and controls. Determination was performed using an internal standard following calibration. Sample preparation was done by spiking with internal standard and extraction with n-hexane, subsequently measured by GC/MS. GC/MS: Column: DB-5MS UI; 30 m, 0.25 mm ID, 0.25 µm film (Agilent). Gradient (oven): 2.2 min 80°C, then 25°C/min to 275°C for 4.0 min. Carrier gas: helium with constant flow of 0.8 mL/min, splitless inlet (280°C). MS: SIM-Mode, internal standard (4,4 DDE): target ion: 246, qualifier ion: 317.9, flurochloridone: target ion: 311, qualifier ion: 187. MS source: 250°C, MS quadrupol: 200°C, solvent delay: 9 min, Run time: 14 min. The method was validated and the LOQ was set to 5 µg/L flurochloridone.

3. Sampling and measurements

Shoot length of individual plants in test tubes was determined photographically (measured in pixels on the computer screen) at the beginning of exposure and after 4 days at the end of exposure. The length is defined as length of plant from cut end to leaf apices of the main shoot. At test end, length of main shoot and lateral branches was measured with a ruler. At test end, the number of lateral branches and roots was counted.

Fresh and dry weight (after drying for 48 hours at 60°C) was determined at test end. Respective mean biomass at day 0 (test start) was obtained based on ten homogeneous additional test plants after transfer to test tubes containing tap water, photography of plants and rinsing with distilled water.

Chemical analysis was made of test media for all pulse concentrations at start and end of each pulse as well as from accomplished samples of control solutions at start and end of the pulsed-exposure test. Additionally, samples at the end of the recovery periods within the pulsed exposure test were taken for possible later analysis as well as samples from freshly prepared vessels at start of the continuous recovery phase.

Plants were inspected visually for remarkable morphological changes like chlorosis, necrosis, deformations, discolorations, etc.

The temperature was recorded daily. The pH was measured at test initiation and at the end of the test in the pooled replicate vessels per treatment. The light intensity was measured at test start and test end.

4. Calculation of toxicity

Substance-related effects (main and total shoot length, fresh weight, dry weight and number of whorles) were determined quantitatively by comparison to pooled controls. Yields and growth rate (analysed as absolute values) were calculated as endpoints.

5. Statistics

Comparison of percent inhibition of treatments and controls was statistically analysed by the computer programme ToxRat® using STUDENTs t-test. The analysis was performed for total shoot length only as no inhibition of growth rate and yield was found to exceed 5% during 10-day exposure and subsequent recovery, but with total shoot length inhibition observed during recovery phase ranging from 11.3% (yield)

~~to 7.1% (growth rate).~~

~~**Results and Discussion**~~

~~The concentrations of flurochloridone (trans isomer) were measured in samples from fresh and aged media at start and end of the exposure peaks as well as in fresh and/or aged recovery and control media. Analysis results are presented in the following table.~~

Table A 2.2.3-46: Concentrations of flurochloridone (trans isomer) in the test media

Test phase	Nominal concentration	Measured concentration				Geometric mean measured concentration	
		Fresh (average of 2 runs)		Aged (average of 2 runs)			
	[µg/L]	[µg/L]	[% of nominal]	[µg/L]	[% of nominal]	[µg/L]	[% of nominal]
Control day 0 (fresh)/1 (aged)	n.a.	< LOQ	n.a.	< LOQ	n.a.	n.a.	
Exposure 1	35.0	43.6	124.5	39.2	112.0	41.3	118
Control day 4	n.a.	n.a.		< LOQ	n.a.	n.a.	
Recovery 1	n.a.	n.a.		< LOQ	n.a.	n.a.	
Exposure 2	21.0	24.7	117.6	23.1	110.2	23.9	114
Control day 5	n.a.	n.a.		< LOQ	n.a.	n.a.	
Control day 7	n.a.	n.a.		< LOQ	n.a.	n.a.	
Recovery 2	n.a.	n.a.		< LOQ	n.a.	n.a.	
Exposure 3	15.0	22.7	151.3	19.9	132.6	21.2	142
Control day 8/1	n.a.	n.a.		< LOQ	n.a.	n.a.	
Control day 8/2	n.a.	n.a.		< LOQ	n.a.	n.a.	
Recovery 3	n.a.	n.a.		< LOQ	n.a.	n.a.	
Exposure 4	10.0	7.2	71.9	7.4	75.0	7.3	72.7
Control day 10	n.a.	n.a.		< LOQ	n.a.	n.a.	
Control day 14	n.a.	n.a.		< LOQ	n.a.	n.a.	
Recovery day 14	n.a.	n.a.		< LOQ	n.a.	n.a.	

LOQ = 5 µg/L flurochloridone (trans isomer)
n.a. = not applicable
a) additional dosing based on results of the sterile stock solution from 25th February

Concentrations of pulses were expressed as geometric mean measured concentrations.

In freshly prepared test solutions, concentrations of 43.6, 24.7, 22.7 and 7.2 µg a.s./L were measured and concentrations of 39.2, 23.1, 19.9 and 7.4 µg a.s./L in aged test solutions resulting in test item recoveries of between 71.9 and 151.3% of nominal. Pulses are expressed as geometric mean measured concentrations (41.3, 23.9, 21.2 and 7.3 µg flurochloridone/L, equivalent to 118, 114, 142 and 72.7% of nominal).

In the 14 day pulsed exposure test, no significant growth inhibition was found for any of the observed parameters. The highest inhibition found was 3.6% (growth rate) and 3.0% (yield) for number of whorls (see following table).

In the subsequent 4 day recovery period, the growth of *Myriophyllum spicatum* based on all observed parameters was not inhibited either. The highest inhibition found was 7.1% (growth rate) and 11.3% (yield) for total shoot length (see following table). However, these inhibitions were not statistically significant.

Visual observations did not show any abnormalities of plants in either controls or treatment. One control replicate was found to be unsterile, but the infestation had no influence on plant growth.

Based on these results, the growth of *Myriophyllum spicatum*, assessed by measuring the parameters main and total shoot length, fresh and dry weight and number of whorls, was not affected during the14 day pulsed exposure test with pulses of geometric mean measured concentrations of 41.3, 23.9, 21.2 and 7.3 µg flurochloridone/L, equivalent to 118, 114, 142 and 72.7% of nominal and during the subsequent 4 day recovery period.

Table A 2.2.3-47: Growth rates and yield based on main and total shoot length, fresh and dry weight and number of whorls during the test and recovery period

Treatment group	Test period (14 days)				Recovery period (4 days)			
	Growth rate		Yield		Growth rate		Yield	
	mean ± SD	% reduction	mean ± SD	% reduction	mean ± SD	% reduction	mean ± SD	% reduction
Main shoot length								
Control	0.064 ± 0.0047	-	35.52 ± 3.929	-	0.051 ± 0.0148	-	38.05 ± 15.340	-
Treatment	0.063 ± 0.0095	2.2	35.11 ± 7.132	1.2	0.057 ± 0.0131	-13.1	46.04 ± 14.384	-21.0
Total shoot length								
Control	0.087 ± 0.0146	-	59.523 ± 17.7650	-	0.085 ± 0.0110	-	89.149 ± 18.9934	-
Treatment	0.097 ± 0.0143	-11.6	72.308 ± 14.4809	-21.5	0.079 ± 0.0093	7.1 (n.s.)	79.043 ± 18.6598	11.3 (n.s.)
Fresh weight								
Control	0.103 ± 0.0129	-	180.28 ± 40.159	-	0.102 ± 0.0152	-	302.05 ± 99.374	-
Treatment	0.115 ± 0.0127	-11.5	222.72 ± 50.369	-23.5	0.102 ± 0.0196	0.3	306.83 ± 119.410	-1.6
Dry weight								
Control	0.100 ± 0.0107	-	30.64 ± 5.895	-	0.096 ± 0.0141	-	47.70 ± 14.272	-
Treatment	0.111 ± 0.0114	-11.8	38.00 ± 7.874	-24.0	0.096 ± 0.0174	0.5	48.09 ± 17.455	-0.8
Number of whorls								
Control	0.062 ± 0.0134	-	9.900 ± 2.6013	-	0.050 ± 0.0128	-	10.500 ± 3.5668	-
Treatment	0.060 ± 0.0112	3.6	9.600 ± 2.5473	3.0	0.057 ± 0.0181	-14.0	13.100 ± 4.5814	-24.8

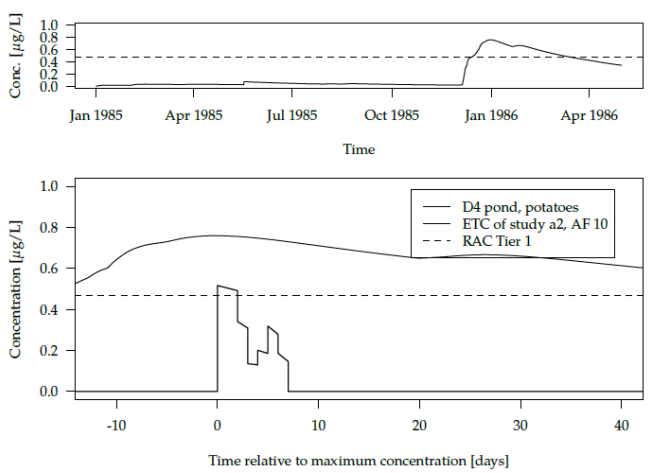
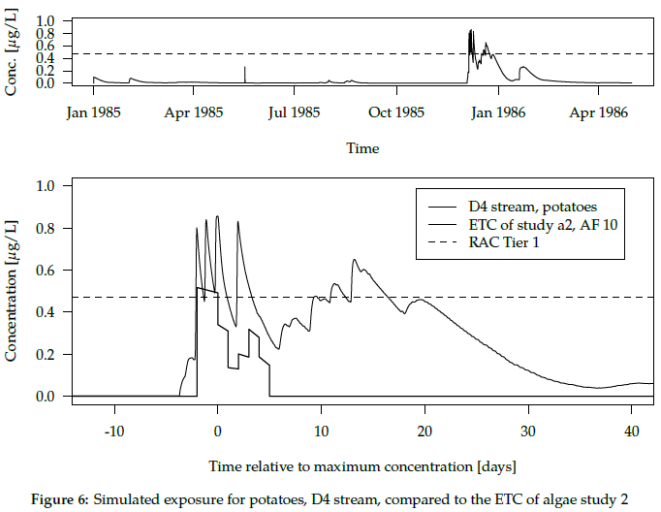
SD: standard deviation; n.s. not statistically significant (based on a STUDENTs t test)
Note: Negative % reduction values indicate an increase of the observed parameter relative to the control.

A shoot length doubling time of 10.8 days or growth rate of 0.0639/d was found (required: ≤ 14 days or 0.0495/d). The mean coefficient of variation for yield based on shoot fresh weight and additional measurement variables does not exceed 35% between replicates. More than 50% of the control replicates were kept sterile over the 14 day exposure period (actually 10 of 10 replicates). Therefore, the validity criteria are fulfilled.

Conclusion

Flurochloridone (trans isomer) did not affect the growth (calculated as growth rate and yield) of *Myriophyllum spicatum*, assessed by measuring the parameters main and total shoot length, fresh and dry weight and number of whorls, during the14 day pulsed exposure test with pulses of geometric mean measured concentrations of 41.3, 23.9, 21.2 and 7.3 µg flurochloridone/L, equivalent to 118, 114, 142 and 72.7% of nominal and during the subsequent 4-day recovery period.

A 2.2.3.14 Study 14: Expert statement – exposure scenario analysis

Comments of zRMS:	First of all it is pointed out that the analysis of the exposure pattern should have been presented in area of Section 8, as this is relevant for fate and behaviour part of assessment. The general concept of comparison of exposure profiles in FOCUS scenarios and exposure regime in the pulsed exposure studies is agreed by the zRMS. However, the respective report from FOCUS surface water modelling (Ranke, 2008) has been checked and it is noted that the exposure profiles for each relevant scenario are not presented there. The general graph presenting summary of exposure profiles in all D and R scenarios on single plots is not sufficient to aid validation, as peaks from one scenario cover peaks from another scenario making them not visible and based on the presented graph it cannot be confirmed that correct exposure profiles were considered in the position paper below. Nevertheless, in case the FOCUS exposure profiles were correct, their comparison with ETC (ecotoxicological trigger curves, obtained by division of the tested peaks by AF of 10) shows that the exposure regime in the study by Liedke (2013d) covered the exposure pattern predicted for R scenarios. The comparison of ETC from study by Liedtke (2013e) is not presented in the summary below, but in the original position paper it clearly demonstrated that the exposure pattern predicted for D scenarios was not covered by the exposure regime. Graphs are presented below.  **Figure 5:** Simulated exposure for potatoes, D4 pond, compared to the ETC of algae study 2  **Figure 6:** Simulated exposure for potatoes, D4 stream, compared to the ETC of algae study 2

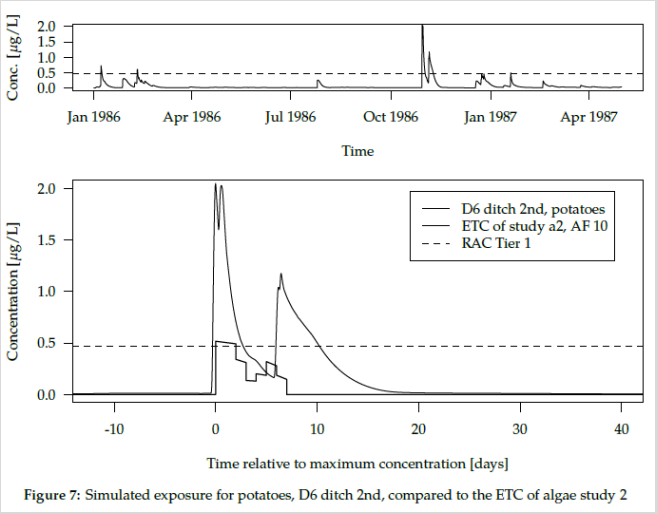


Figure 7: Simulated exposure for potatoes, D6 ditch 2nd, compared to the ETC of algae study 2

The approach taken in the risk assessment in R scenarios was based on the calculation of maximum time weighted average concentrations (TWAC) and areas under the curve (AUC) for specific time windows in the FOCUS profiles for respective scenarios. Then, the maximum AUC value for the corresponding moving windows over the simulated exposure profiles were compared with the AUC in the corresponding ecotoxicological experiment.
In this approach the recovery was taken into account, which is not correct, as ERO is not an option at Tier 2 because recovery in the field would be influenced by the relationship with other species. This is particularly important in case of short-lived species, such as algae, aquatic plants or Daphnids, for which recovery will occur between particular pulses. Taking this into account in EFSA Supporting publication 2019:EN-1673 it was indicated that the RAC for the risk assessment for primary producers based on modified exposure studies should be derived with consideration of EC_{10} values expressed in terms of the of the peak exposure (in EFSA aquatic guidance, 2013, EC_{50} was initially indicated). No such endpoints were calculated from the pulsed-exposure studies by Liedtke (2013d and 2013e). In absence of the EC_{10} values, the NOEC could be potentially considered, however in Liedtke (2013d) NOEC could be determined only for pulses 3 and 4, while for pulses 1 and 2 it could not be determined due to significant effects (>50%) seen on algae growth at considered concentrations. In study by Liedtke (2013e) the NOEC values were not calculated at all and growth rates were presented for first pulse and recovery phase at the end of exposure only. Taking this together with other deficiencies into account, neither of the studies was considered relevant for the risk assessment purposes.
In addition to that, the approach taken in the risk assessment considered also TWA concentrations from the exposure profiles, while in line with EFSA Supporting publication 2015:EN-924, exposure based on TWA concentrations should not be considered as it is considered to be not sufficiently robust to be used in the regulatory risk assessment.

Overall, the part of the position paper regarding the comparison of exposure profiles with exposure regime in the pulsed exposure studies with algae could be accepted, provided that sufficient information regarding FOCUS exposure profiles was available in the modelling report by Ranke (2018). However, the approach taken with regard to the risk assessment is not acceptable as it includes recovery and does not consider the EC_{10}/NOEC values. Furthermore, neither of the pulsed-exposure studies with algae were considered sufficiently reliable to be considered in the risk assessment.

Reference:	KCP 10.2.3/14
Report	Pulsed exposure of algae following application of flurochloridone in Poland, Ranke, J. and Eck, G., 2018, jrwb-129; 000100958
Guideline(s):	not applicable
Deviations:	not applicable
GLP:	No

Acceptability:	The principle of comparison of the FOCUS exposure profiles with exposure regime is in general acceptable. However, in report from FOCUS surface water modelling the exposure profiles for each relevant scenario are not given, so the presented evaluation could not be validated against the outcome of the results of FOCUS modelling presented in Ranke, 2018 (see Core Assessment, Part B, Section 8). Furthermore, the position paper is not relevant for the risk assessment, as neither of the pulsed-exposure studies was considered sufficiently reliable to be used in the risk assessment. In addition to that, the presented risk assessment is not in line with indications of EFSA aquatic guidance (2013) or EFSA Supporting publication 2019:EN-1673, indicating that the risk assessment based on the pulsed exposure studies should be performed with consideration of RAC derived using ECx value (in line with EFSA, 2019, preferably EC_{10}).
Duplication (if vertebrate study)	-

Summary

This statement addresses the effects of a pulsed exposure to flurochloridone on surface water algae. The exposure patterns considered in this assessment are based on FOCUS Step 4 surface water modelling accounting for a 20 m vegetated filter strip (VFS).

Two experimental pulsed exposure studies with algae are available. In a first step the concentrations measured in these experimental pulsed exposure studies are compared to the nominal exposure profiles that were designed to cover the exposure profiles simulated at FOCUS Step 4. The area under the curve is calculated for the highest patterns where no adverse effects were observed (No observed adverse effect pattern, or NOAEP).

As a second step, each scenario where the maximum PEC surface water is above the respective tier 1 threshold values is analysed graphically, showing the ecotoxicological trigger curves (ETC), based on the division of experimental exposure profile by an appropriate assessment factor. In addition the statistics for the exposure events above this conservative threshold level are generated, and the results of a moving window analysis using window sizes based on the duration of the pulsed exposure experiments are shown. The third step is based on this moving window analysis, i.e. on the calculation of maximum time weighted average concentrations (TWAC) and areas under the curve (AUC) for specific time windows.
The maximum AUC value for the corresponding moving windows over the simulated exposure profiles is compared with the AUC in the corresponding ecotoxicological experiment. In order to facilitate this comparison, toxicity exposure ratios (TERs) are calculated from these maximum moving window AUC values.

For the evaluation of these TERs, an initial assessment factor of 10 is used, although a reduced assessment factor could be considered due to the availability of multiple single species algae tests. The test species in the pulsed-exposure studies can be considered as conservatively representative for algae as the comparison of endpoints with other test species indicates. As a recent study shows (Wenzel, 2015; 90016481), *Desmodesmus* is likewise a reasonably conservative representative in regard to the potential for recovery. It should also be noted that the pulsed exposure tests for algae were conducted with the more toxic trans-isomer variant of flurochloridone.

Finally, additional recovery data from multiple single algae species tests are available indicating the potential for recovery in ecologically acceptable time frames (i.e. within 3 to 9 days) even at exaggerated concentrations [refer also to Point 9.5.1.1 for details on algal recovery potential].

For these reasons, the analysis is considered to be reasonably conservative and protective for algal communities in surface water bodies as addressed by this higher tier assessment approach.

The higher tier risk analysis of the simulated exposure profiles considering a 20 m vegetated buffer strip showed that the runoff scenarios that did not pass the tier 1 assessment (i.e. FOCUS R1, R2 and R3 stream) are conservatively covered by the pulsed exposure study with pattern 1 (refer to Liedtke 2013d; 90015421)

that was specifically designed for this purpose.

The graphical comparison of predicted exposure profiles of the relevant run-off scenarios and the TER calculations based on AUC is presented in the following.

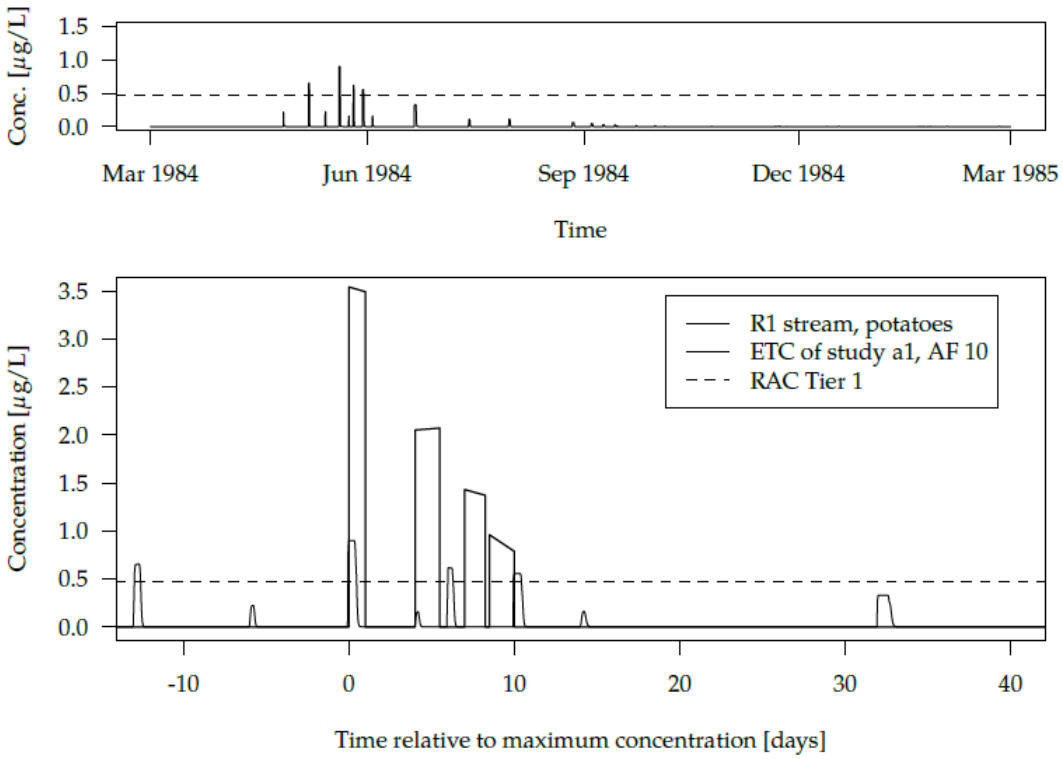


Figure 1: Simulated exposure for potatoes, R1 stream, compared to the ETC of the study by Liedtke (2013d)

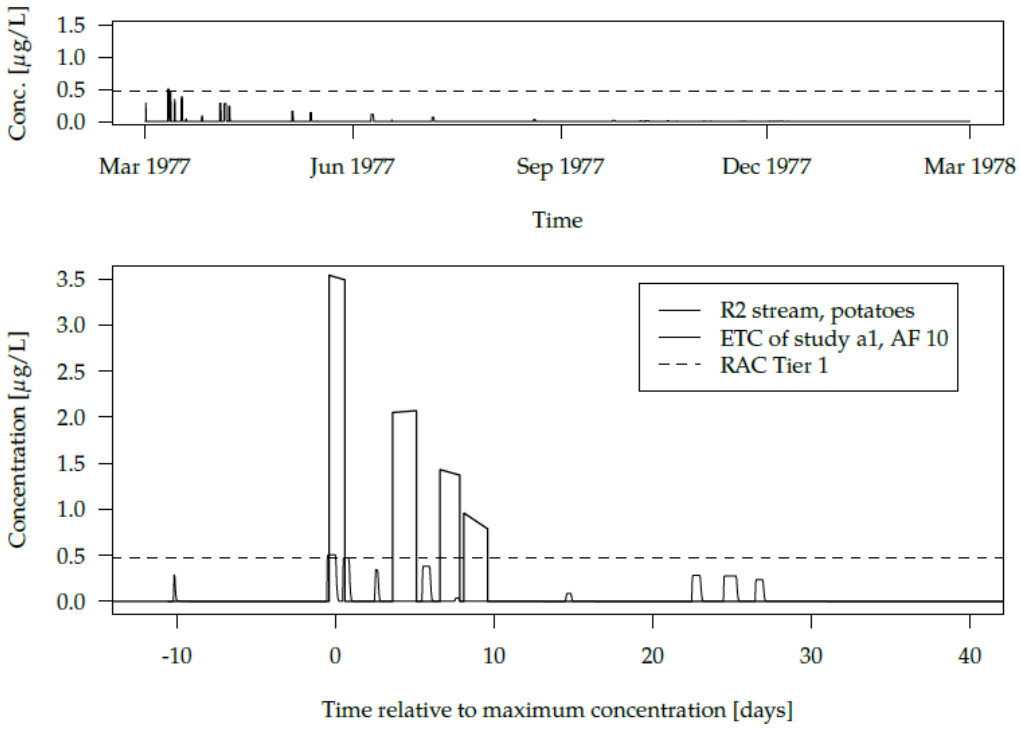


Figure 2: Simulated exposure for potatoes, R2 stream, compared to the ETC of the study by Liedtke (2013d)

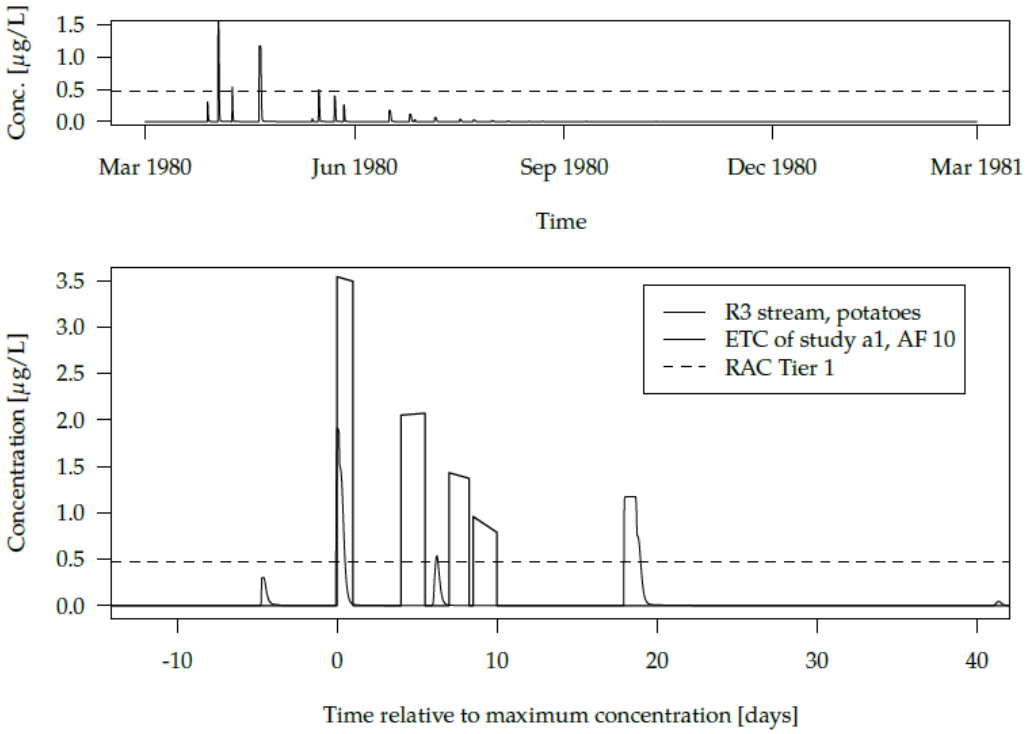


Figure 3: Simulated exposure for potatoes, R3 stream, compared to the ETC of the study by Liedtke (2013d)

The four (R1 and R3 stream) or two (R2 stream) exposure events above the threshold are considered to be sufficiently covered by the four pulses with exposure peaks which are higher, wider and more closely spaced.

Table A 2.2.3-48: TER calculation based on Areas Under the Curve (AUC) of exposure for relevant FOCUS R scenarios

AUC of exposure in Liedtke (2013d) [µg a.s./L*d]	FOCUS run-off scenario	Maximum AUC for moving window	TER ($AUC_{study}/AUC_{simulated}$)
96.6	R1 stream	1.1	90.6
	R2 stream	0.8	119.8
	R3 stream	1.3	73.4

In conclusion, all of these runoff scenarios pass the assessment by a great margin of safety in regard to the ratio of AUC in the ecotoxicological test to the predicted AUC. Also the graphical analysis is based on the tier 1 uncertainty factor, while the available data on multiple algal species suggest that a lower assessment factor can be considered acceptable.

The risk from exposure in the drainage scenarios is not covered by this assessment.

A 2.3 KCP 10.3 Effects on arthropods

A 2.3.1 KCP 10.3.1 Effects on bees

A 2.3.1.1 KCP 10.3.1.1 Acute toxicity to bees

.1 KCP 10.3.1.1.1 Acute oral toxicity to bees

No additional data submitted.

.2 KCP 10.3.1.1.2 Acute contact toxicity to bees

No additional data submitted.

A 2.3.1.2 KCP 10.3.1.2. Chronic toxicity to bees

A 2.3.1.2.1.1 Study 1: Chronic toxicity to the honeybee

Comments of zRMS:	The study was performed in line with the draft guideline on 10 days feeding toxicity but fulfilled the recommendations and criteria of OECD 245. The minimum relative humidity was below the recommended 50%, but this deviation lasted for less than 2 hours and according to OECD 245 such short-term deviations are unavoidable All validity criteria were met and the study is considered acceptable with following endpoints relevant for the risk assessment: LDD_{50} ≥97.9 µg product/bee/day NOEDD = 19.6 µg product/bee/day NOEC = 750 mg product/kg food

Reference:	KCP 10.3.1.2/01
Report	AG-F8-250 EC (Flurochloridone 250 EC) - Assessment of Effects on the Adult Honey Bee, *Apis mellifera* L., in a 10 Day Chronic Feeding Test under Laboratory Conditions, Molitor A.M., 2017, S17-00282 (report number), 90020495 (sponsor report number)
Guideline(s):	OECD Guideline Proposal (2016).
Deviations:	No
GLP:	Yes
Acceptability:	Acceptable
Duplication (if vertebrate study)	-

Materials and Methods

A. MATERIALS

1. Test material AG-F8-250 EC (Flurochloridone 250 EC)

Description liquid / clear, amber, EC (Emulsifiable concentrate)
Lot/Batch # D-8623
Purity flurochloridone: 250 g/L nominal; 250.8 g/L analysed
Density 1.028 g/cm³

Stability of test material	Stable under storage conditions (at ambient temperatures (5-30°C) under dark and dry conditions) Expiry date: 31 Aug 2018
2. Vehicle and/or positive control	Vehicle: aqueous sucrose solution (50%, w/v) Positive control: reference item
Reference item	BAS 152 11 I (dimethoate)
Description	blue liquid
Lot/Batch #	FRE-001302
Purity	400.0 g/L dimethoate (nominal content) 405.2 g/L dimethoate (analysed content) density: 1.074 g/cm³
Stability of reference item	Stable under storage conditions (at cool temperature (1-10°C), under dark and dry conditions) Expiry date: 31 Dec 2017

3. Test organism

Species	Honey bee, *Apis mellifera* L. (Hymenoptera, Apoidea)
Source	Own stock of test facility: Eutinger Straße 24, 75223 Niefern-Öschelbronn, Germany.
Age	Adult worker bees (1 to 2 days old)
Pre-treatment culturing conditions	Two days prior to test start, brood combs containing capped cells were taken out of two honey bee hives and transferred into the climatic chamber. One day prior to start of exposure, the bees were randomly collected from the outer combs of the colony, introduced into the test units and kept under test conditions.
Diet	Acclimatization: 50% (w/v) sucrose solution Exposure period: 50% (w/v) sucrose solution with either test item or reference item or pure solution (untreated control group). Syringes were replaced daily.
Test units	Stainless steel cages (8 cm x 4.0 cm x 6.0 cm); front side with transparent pane; bottom consisting of perforated steel. The cages were lined with filter paper.

4. Environmental conditions

Temperature	nominal: 33 ± 2°C, actual: acclimatisation: 31.1 – 33.0°C exposure: 31.1 – 33.4°C
Relative humidity	nominal: 50-70%, actual: acclimatisation: 49.1* – 61.1% (*short term deviations < 2 hours), exposure: 31.8* – 60.9% (*short term deviations < 2 hours)
Photoperiod	During the test, the bees were kept in constant darkness except during application and assessments.

B. STUDY DESIGN AND METHODS

1. In-life dates	15 May 2017 – 07 Jul 2017

2. Experimental conditions

Test design

In a 10-day chronic test, young adults of *Apis mellifera* L. were daily exposed to five doses of AG-F8-250 EC in 50% (w/v) aqueous sucrose solution. In parallel one control treatment group and one dose of the reference item were tested. Additionally 4 test units without bees but with full food syringes containing pure 50 % (w/v) aqueous sucrose solution were placed in the climatic chamber for the evaluation of the evaporation. Assessments of bee mortality and sub-lethal effects were done daily during the study.

Test conditions

During the whole test, the relative humidity was in the range of 31.8* – 60.9 % and the test temperature was maintained at 31.1 – 33.4 °C (*short term deviations < 2 hours). The bees were kept in darkness, except during application and assessments. The climatic chamber was ventilated.

Number of animals per treatment

Four replicates per test and reference substance treatment and untreated control were used with 10 bees per replicate.

Test doses

AG-F8-250 EC was tested at 375, 750, 1500, 3000 and 6000 mg product/kg diet. The control groups, receiving untreated 50% (w/v) aqueous sucrose solution were tested in parallel.

Reference item

The reference item, Dimethoate EC 400 was tested at a single concentration of 0.9 mg a.s./kg diet.

Treatment/Application

The application took place for a period of 10 consecutive days. Test item stock solutions were freshly prepared every day by weighing the required amount of test item on a balance. The reference item stock solution was prepared once for the whole feeding period and stored in the refrigerator. The definitive feeding solution was freshly prepared every day from the stock solution with 50 % (w/v) aqueous sucrose solution.

The bees were fed with 50 % w/v aqueous sucrose solution including the test item or the reference item. The control treatments were fed with 50% w/v untreated aqueous sucrose solution. The treated/untreated food was provided *ad libitum* in a plastic syringe, which had been weighed before application. The actual consumption was determined by reweighing the syringe containing the remaining test solution each day after removal from the test units.

The evaporation of test solutions from the feeders was investigated in additional test cages which were set up with the main test. These cages contained no bees, only pre-weighed feeders containing diet of untreated control (50 % (w/v) aqueous sucrose solution. These were placed in the test environment alongside the test units. At the daily feeder exchange the feeders were re-weighed and replaced by new feeders. This evaporation figure was reported separately.

Analytics

For verification of the exposure concentration, analytical samples and retain samples of the feeding solutions of the controls and all the test item groups were taken once directly after preparation. The determination was conducted using high performance liquid chromatography (HPLC-MS/MS). Details to the analytical method are summarized in Part B, Section 5.

3. Observations and assessments

Mortality and sub-lethal effects were assessed every 24 h ± 2 h until test end, ten days following start of exposure.

The amount of feeding solution consumed was determined daily by weighing the feeders before and after feeding. The feeding syringes were replaced daily.

4. Calculation of toxicity

The percentage of cumulative mortality was calculated for each treatment group and assessment from the number of dead individuals in relation to the number of introduced test organisms.

The consumption of feeding solution per bee per day 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 per bee per day was calculated by averaging the replicate values.

The evaporation out of the food syringes was determined by daily weighing of the syringes of the respective, additional test cages. A mean value of evaporation per day was determined over the whole test period and the daily food consumption of the control, the test item and reference item treatments was corrected by the mean value of the corresponding day.

5. Statistics

In order to determine the LC_{50} and LDD_{50} values a Weibull using linear maximum likelihood regression was used. Cochran-Armitage test (one sided greater, α= 0.05) was used to evaluate whether there are significant differences between the mortality data of the control and the test item treatment groups and to determine the NOEC and NOEDD based on mortality. Statistical calculations were made by using the statistical program ToxRat Professional 3.2.1.

Results and Discussion

The measured concentrations of flurochloridone in the larval diet of each test item group were equivalent to recoveries between 91 % and 98 % of nominal. The endpoints were therefore calculated based on nominal test item concentrations.

In the test item group the food consumption ranged between 16.3 and 27.9 mg solution per bee and day (control: on average 32.0 mg/bee/day).

Cumulative mortality in the control after 10 days was 0.0%. In the two lowest concentrations the test item AG-F8-250 EC had no statistically significant effects on honeybee mortality after 10 days compared to the control with cumulative mortalities between 0.0 and 2.5%. In the three highest concentrations of 1500, 3000 and 6000 mg product/kg diet the cumulative mortality was 12.5%, 12.5% and 50.0% respectively (see next table). The reference item showed 97.5% cumulative mortality at the end of the test.

In the two lowest concentrations no remarkable behavioural abnormalities could be observed. In the three highest concentrations of 1500, 3000 and 6000 mg product/kg diet single affected and moribund bees were observed at the three highest concentration levels on various assessment dates.

The 10-day LC_{50} was determined to be ≥ 6000 mg product/kg diet, corresponding to an LDD_{50} of ≥ 97.9 µg product/bee/day (actual consumed). The 10-day NOEC was determined to be 750 mg product/kg diet, corresponding to an NOEDD of 19.6 µg product/bee/day (actual consumed).

The validity criteria of the most current guideline (OECD 245) were met since mean mortality in the control was below 15% (actual: 0.0 %) and the average mortality in the reference item treatment was ≥ 50% at the end of the test (actual: 97.5%).

Table A 2.3.1.2-1: Results of analytical verification

Test concentration [mg product/kg diet]	Nominal Concentration of flurochloridone [mg a.s./kg diet]	Analysed Concentration of flurochloridone [mg a.s./kg diet]	Recovery [% of nominal]
Control	-	< LOD	-
375	91.5	85.7	94
750	183	167	91
1500	366	341	93
3000	732	704	96
6000	1460	1430	98

Table A 2.3.1.2-2: Mortality of bees in the chronic toxicity feeding test after 10 days

	Test concentration [mg product/kg diet]	Dose level consumed actual [µg product/bee/day]	Cumulative mortality after 10 days [%]
Control	-	-	0.0
AG-F8-250 EC	375	10.5	2.5
	750	19.6	0.0
	1500	36.4	12.5*
	3000	55.7	12.5*
	6000	97.9	50*
Reference item BAS 152 11 I	0.9 mg a.s./kg diet	0.02 µg a.s./bee/day	97.5
10-day endpoints			
LC_{50}		≥ 6000 mg product/kg food [a)]	
LDD_{50} [b)]		≥ 97.9 µg product/bee/day [a)]	
NOEC		750 mg product/kg food	
NOEDD[b)]		19.6 µg product/bee/day	

[a)] Actually calculated based on extrapolation (Weibull analysis using linear maximum likelihood regression): 6384 mg product/kg food and 102 µg product/bee/day
[b)] Based on actual doses
* Significantly different compared to the control according to Cochran-Armitage Test, one-sided greater, $\alpha = 0.05$)

Conclusion

In a 10-day chronic toxicity feeding study with AG-F8-250 EC the LDD_{50} was determined to be ≥ 97.9 µg product/bee/day (actual consumed) and the LC_{50} to be ≥ 6000 mg product/kg food. The NOEDD was determined to be 19.6 µg product/bee/day (actual consumed) and the NOEC to be 750 mg product/kg food. The validity criteria were fulfilled.

A 2.3.1.3 KCP 10.3.1.3 Effects on honey bee development and other honey bee life stages

3.1 Study 1: Toxicity to honeybee larvae

Comments of zRMS:	The study was performed in line with OECD 239. Following deviations from the test guideline were noted: 1. No emergence boxes were used from day 15, so assignment of each emerged bee to the respective replicate was not possible. However, in the study report the number of emerged bees are given per replicate of each test group (control, test item and toxic standard), so it is not fully clear what the study authors meant indicating this deviation. 2. At some time points the minimum temperature was slightly below the minimum required 34ºC (with the minimum of 33.2ºC). As this was only slight deviation lasting less than 2 hours, it is considered to have no impact on the test results. 3. The minimum relative humidity was clearly lower than recommended 95±5% (D1-D8), 80±5% (D8-D15) and 50% (D15-D22) and on some days this deviation was longer than 2 hours. Nevertheless, as all validity criteria were met, it is not expected that too low minimum humidity could have significant impact on the test results. The mean humidity over the respective time periods was at relevant level. 4. The study authors indicated that in the toxic reference group only mortality was determined, while other effects (e.g. larvae size, morphological differences or adverse effects on emergence) were not recorded. It is, however, noted that from the test report it cannot be confirmed that lack of additional assessment concerned only toxic reference groups, as in the results section of the study report it is indicated that *"During the assessments of mortality and emergence no other test item related observations such as deviating sizes, appearances and malformations of the test organisms were made"*, which could suggest that this deviation actually concerned also test item groups. It is, however, expected than any malformations or unusual behaviour would affect pupation and/or adult emergence and adults were inspected for malformations and only bees of normal appearance and size were counted as "emerged". 5. The measured concentration of flurochloridone in treatments from T1 to T4 were within ±20% of nominal, while in T5 (maximum test concentration) the analysed content of the active compound was 79%, i.e. slightly below the required minimum of 80%. However, the overall geometric mean measured concentration based on recoveries in all test groups was 87.2%, so it was at the relevant level. In addition to that, at the highest test concentration 100% larvae mortality was observed, so this slight deviation had no impact on the derived endpoints. Overall, despite deficiencies indicated above, the study is considered acceptable, as all validity criteria were met. Following endpoints are agreed: ED_{50} = 114 µg product/larva/developmental period NOED = 15.4 µg product/larva/developmental period EC_{50} = 755 µg product/kg food NOEC = 100 mg product/kg food

Reference:	KCP 10.3.1.3/01
Report	AG-F8-250 EC (Flurochloridone 250 EC) - Honey Bee (*Apis mellifera* L.) 22 Day Larval Toxicity Test (Repeated Exposure), Molitor, A.M., 2018, S17-00318 (report number), 90020496 (sponsor report number)
Guideline(s):	OECD Guidance Document 239 on Honey bee (*Apis mellifera*) Larval Toxicity Test, Repeated Exposure (2016)
Deviations:	Yes: For the toxic reference item group mortality but no other observations were assessed. No emergence boxes were used as from Day 15 to enable the assignment of each emerged

	bee to the respective replicate. Deviations (≥ 2 hours) from the recommended humidity ranges from D5 to D12 are considered unlikely to have made any discernible impact on the test performance as the validity criteria were fulfilled. The deviations are not considered to affect the integrity and validity of the study.
GLP:	Yes
Acceptability:	Acceptable with deviations (see commenting box above)
Duplication (if vertebrate study)	-

Materials and Methods

A. MATERIALS

1. Test material AG-F8-250 EC (Flurochloridone 250 EC

Description: clear, amber liquid, EC (Emulsifiable concentrate)

Lot/Batch #: D-8623

Purity: Flurochloridone: 250 g/L nominal; 250.8 g/L analysed
Density: 1.028 g/cm³

Stability of test material: Stable under storage conditions (at ambient temperatures (5-30°C) under dark and dry conditions)
Expiry date: 31 Aug 2018

2. Vehicle and/or positive control Vehicle: untreated diet (50% aqueous sucrose solution with 50% royal jelly)
Positive control: reference item

Reference item Dimethoate tech. (BAS 152 I)

Description White-grey solid
Lot/Batch # 35015A161
Purity 99.9% (w/w)
Stability of reference item Stable under storage conditions (cool (1- 10°C), dark and dry)
Expiry date: 31 Dec 2017

3. Test organism

Species Honeybee *Apis mellifera ssp. carnica* Pollmann (Hymenoptera, Apoidea)
Source Three healthy colonies maintained at test facility.
Age Synchronized first instar (L1) larvae
Pre-treatment culturing conditions The hives used for larvae collection were adequately fed, healthy, disease-free and with known history and physiological status. No pesticides had been used in the hives for at least one month.

Method of producing L1 larvae: Four days prior to the grafting of larvae, queens of several colonies were confined in their own colony in an excluder cage containing a comb with empty cells. Three days prior to the grafting, maximum 30 hours after encaging, the queens were released from the cages. The combs containing eggs were left in the excluder cages during the incubation stage until hatching on day 1. On day 1, the combs were transferred to the laboratory using an insulated container, equipped with a moist wipe, in order to avoid temperature variation. In the laboratory three combs were selected for grafting, containing the highest number of synchronized larvae. Before first application of the test item on day 3, it was assured that all larvae used were of

	similar size and alive. Therefore non-suitable larvae were replaced by individuals from the reserve plates, using larvae from the same replicate hive.
Diet	The food was composed of three different artificial diets which were adapted to the needs of the larvae at different stages of development: - Diet A (day 1): 50% royal jelly + 50% aqueous solution containing 2% yeast extract, 12% glucose and 12% fructose - Diet B (day 3): 50% royal jelly + 50% aqueous solution containing 3% yeast extract, 15% glucose and 15% fructose - Diet C (day 4-6): 50% royal jelly + 50% aqueous solution containing 4% yeast extract, 18% glucose and 18% fructose
Test units	Crystal polystyrene grafting cells (diameter 9 mm, depth 8mm) were sterilized with 70% (v/v) ethanol and placed in 48 well plates. The open plates were placed into a hermetically sealed desiccator, containing a dish filled with a saturated potassium sulphate (K_2SO_4) solution. On day 8 the plates were transferred into a second desiccator containing a dish filled with a saturated sodium chloride (NaCl) solution. The desiccators were placed in an incubator with forced air circulation. On day 15 each plate was covered by its lid and transferred from the desiccator into an incubator with automated humidity control.

4. Environmental conditions

Temperature	Nominal: 34 - 35°C (not below 23 °C or above 40 ° C); actual: D1-D8: 33.7-34.2 °C; D8-D15: 33.2-35.2 °C; D15-D22: 34.2-34.8 °C
Relative humidity	Nominal: D1-D8 95% ± 5%; D8-D15: 80 ± 5 %; D15-D22: 50 - 80 %; actual: D1-D8: 35.7-100%; D8-D15: 40.6-94.7%; D15-D22: 40.2-67.7%
Photoperiod	During the test, the bees were kept in darkness except during observations.

B. STUDY DESIGN AND METHODS

1. In-life dates 15 May 2017 – 03 Jan 2018

2. Experimental conditions

Test design

The effects of the test substance AG-F8-250 EC to honey bee larvae (*Apis mellifera* L.) were assessed in a chronic toxicity test up to day 22 of their development. From day 3 until day 6 of the test, honey bee larvae were either treated with the test item at five concentrations, the reference item dimethoate tech. at a single concentration or remained untreated (control).

Number of animals per treatment

16 larvae/replicate; 3 replicates/test and reference substance treatment and control

Test doses

The toxicity of AG-F8-250 EC was determined at 100, 300, 900, 2700 and 8100 mg product/kg diet, equivalent to cumulative doses of 15.4, 46.2, 139, 416 and 446 µg product/larva. A control group, receiving untreated artificial diet, was tested in parallel.

Reference item

The reference item, dimethoate tech. was tested at a concentration of 7.39 µg a.s./larva (equivalent to 48.0 mg dimethoate tech./kg diet).

Treatment/Application

The stock solution of the test item (equal to the highest test dose) was prepared by dissolving 1.78 g in deionized water to a final volume of 20 ml and was used to prepare the treated diets. The reference item stock solution was prepared by dissolving 0.106 or 0.107 in deionized, autoclaved water (each weight was used on two application dates). The treated diets were prepared daily by mixing the test item solutions with aqueous sucrose solution and homogenized by a laboratory rotator.

The larval diet was prepared freshly in advance and divided into aliquots using a multi stepper pipette. The aliquots were subsequently stored deep-frozen (≤ - 18 °C, with minor fluctuations) until use. On each feeding day the required amount of diet was warmed in the incubator before feeding. At test start, 20 µL of diet A was dropped into each cell, then one larva was grafted from the comb to the cell, onto the surface of the diet, using a grafting tool. All larvae were fed once a day (except at day 2). At day 3, 20 µL of diet B were administered to each larva. At day 4, 5 and 6, larvae were fed with 30, 40 and 50 µL of diet C, respectively.

3. Observations and assessments

From day 4 to day 8, dead larvae were counted and then removed. Larvae were recorded as dead, if no respiration (movement of spiracles) was observed. Additionally, assessment of mortality during pupation phase on day 15 and day 22 and assessment of adult emergence on day 22 were performed. On day 15 dead larvae, pupae and larvae that have not transformed into pupae were recorded as dead. On day 22 pupae that have not emerged were recorded as dead. Mortality was assessed with assistance of a stereo microscope, if necessary.

Assessment of adult emergence was conducted on day 22. Bees were counted as successfully emerged if they showed signs of adult eclosion. This included the presence of differentiated wings and hair or the absence of the pupal skin.
The presence of uneaten food was qualitatively recorded on day 8. Other observations and any other adverse effects were qualitatively recorded to aid in the interpretation of mortality in comparison to the control group.

Analytical dose verification was performed, samples of the test item treated larval diet of each test item group and of the larval diet of the control group were taken directly from the prepared diet. The concentrations of flurochloridone were analysed by HPLC-MS/MS. Details to the analytical method are summarized in Part B, Section 2.

4. Calculation of toxicity

For each test item group the cumulative dose per larva [µg product/larva per developmental period] was calculated based on the given test item concentration [mg product/kg diet], the cumulative feeding volume per larva and the density of the diet. Since 100 % larval mortality occurred in the test item group T5 prior to the last application on day 6, the cumulative feeding volume was adapted accordingly.

Mortality during the larval phase was evaluated on day 4, 5, 6, 7 and 8. The cumulative mortality [%] for each treatment group on day 8 was calculated from the number of dead larvae in relation to the total number of larvae per treatment group across all replicates after re-grafting on day 3.

Mortality during the pupation phase was evaluated on day 15 and on day 22. The cumulative pupal mortality [%] for each treatment group (except for the reference item group) was calculated from the number of larvae and pupae that failed to emerge until day 22 in relation to the total number of larvae that entered pupation

phase on day 8. The cumulative mortalities were corrected for control mortality according to the formula of Schneider-Orelli (1947).

The adult emergence rate [%] for each treatment group was evaluated on day 22 and was calculated from the number of adult emerged bees on day 22 in relation to the total number of larvae per treatment group after re-grafting on day 3. The inhibition of emergence was calculated compared to the corresponding control group

5. Statistics

Cochran-Armitage test (one-sided greater, $\alpha = 0.05$) was used to evaluate whether there was a significant difference between the test item groups and the control group for larval mortality on day 8, larval and pupal mortality on day 15, pupal mortality from day 8 through 22 and for adult emergence on day 22. The LOEC and NOEC were determined according to OECD Series on Testing and Assessment Number 54 (2006) for adult emergence on day 22. In addition the NOED and LOED were determined.

The EC_{10}, EC_{20} and EC_{50} as well as the corresponding ED_{10}, ED_{20} and $ED5_0$ for adult emergence on day 22 with 95 % confidence limits were calculated by Weibull analysis using linear max likelihood regression.

For the statistical evaluation the statistics program ToxRatPro, Version 3.2.1 was used.

Results and Discussion

The measured concentrations of flurochloridone in the larval diet of each test item group were equivalent to recoveries between 52 % and 123 % of nominal. The mean recoveries per treatment group in the treated larval diet were between 79 and 96 % of nominal. The mean measured concentrations of the larval diet were within ± 20 % of nominal, except for the highest treatment group, which was slightly out of the recommended range (79 % of the nominal concentration). This minimal deviation is considered to have no influence on the endpoints, since 100 % mortality was reached in both of the highest concentrated treatment groups. The results are shown in the following table.

Table A 2.3.1.3-1: Results of analytical verification

Test concentration [mg product/kg diet]	Nominal Concentration of flurochloridone [mg a.s./kg diet] [a)]	Mean recovery [% of nominal]
Control	-	-
100	24.4	96
300	73.2	85
900	220	90
2700	659	90
8100	1980	79

LOD = 3.66 mg a.s./kg diet
[a)] Analysed content of 250.8 g/L flurochloridone considered.

On day 8, larval mortality was 0.0 % in the control group. Larval mortality in the reference item group was 85.4 %. In the three lowest test item concentrations of 100, 300 and 900 mg product/kg diet, the cumulative mortality was 0.0%. At the two highest concentrations of 2700 and 8100 mg product/kg diet, the mortality was 85.4% and 100%, respectively, and was statistically significantly different from the control.

On day 22, the adult emergence rate in the control group was 89.6 %. In the lowest test item group, the emergence was not statistically significantly different from the control with 81.3%. At the concentrations of 300, 900, 2700 and 8100 mg product/kg diet the actual adult emergence was 72.9%, 45.8%, 0.0% and 0.0%, respectively, and was statistically significantly different from the control.

During the assessments of mortality and emergence no other test item related observations such as deviating sizes, appearances and malformations of the test organisms were made. On day 8, uneaten food was

observed in the test item groups containing living larvae at 900 and 2700 mg product/kg diet and in the reference item group.

Based on these results, the EC_{50} was determined to be 755 mg product/kg diet, corresponding to an ED_{50} of 114 µg product/larva. The LOED was determined to be 46.2 µg product/larva and LOEC of 300 mg product/kg diet. The NOED was determined to be 15.4 µg product/larva and the NOEC of 100 mg product/kg diet. The EC_{10} and EC_{20} were determined to be 180 and 319 mg product/kg diet, respectively, corresponding to ED_{10} and ED_{20} values of 29.4 and 50.4 µg product/ larva, respectively. The results are shown in the following table.

Table A 2.3.1.3-2: Cumulative mortality of larvae exposed to AG-F8-250 EC in a chronic toxicity test

Nominal test concentration [mg product/kg diet]	Cumulative dosage [µg product/ larva] [b]	Larval Mortality Day 8 [%]	Mortality on Day 15 [%]		Pupal Mortality from Day 8 to 22 [%]		Adult Emergence on Day 22 [%] [a]	
			Actual	M_{corr}	Actual	M_{corr}	Actual	% to control
Control		0.0	10.4	-	10.4	-	89.6	-
100	15.4	0.0	18.8	9.4	18.8	9.4	81.3	9.3
300	46.2	0.0	22.9	14.0	27.18*	18.6	72.9*	18.6
900	139	0.0	43.8*	37.3	54.2*	48.9	45.8*	48.9
2700	416	85.4*	100.0*	100.0	100.0*	100.0	0.0	100.0
8100	446	100.0*	100.0*	100.0	-	-	0.0	100.0
Reference item: Dimethoate, technical								
48	7.39 µg a.s./larva	85.4	n.d.	-	n.d.	-	n.d.	-
22-day endpoints								
		[mg product/kg diet]			[µg product/larva / developmental period] [b]			
NOEC / NOED		100			15.4			
LOEC / LOED		300			46.2			
EC_{10} / ED_{10} (95% CI)		180 (104 - 260)			29.4 (4.51 - 58.8)			
EC_{20} / ED_{20} (95% CI)		319 (213 - 421)			50.4 (12.8 - 87.7)			
EC_{50} / ED_{50} (95% CI)		755 (600 - 925)			114 (55.9 - 177)			

[a] statistical evaluation for non-emergence

[b] Based on the cumulative feeding volume from day 3 until day 6 of 140 µL (T1-T4, R) or from day 3 until day 4 of 50 µL (T5) diet/larva and a density of the diet of 1.1 g/cm^3

* Statistically significantly increased compared to control (Cochran-Armitage test, one sided greater, $\alpha = 0.05$)

n.d. not determined

The test is considered valid since the cumulative larval mortality from day 3 to day 8 was 0% (required ≤ 15%) and larval mortality in the reference item was 85.4 % (required ≥ 50%). On day 22 the adult emergence rate was 89.6 % across all replicates (required ≥ 70).

Conclusion

In this chronic larval toxicity study with AG-F8-250 EC, the NOED was determined to be 15.4 µg product /larva. The 8-day ED_{20} and ED_{10} were determined to be 50.4 (95% CI: 12.8-87.7) and 29.4 (95% CI: 4.51-58.8) µg product/larva. The validity criteria of the guideline were fulfilled.

A 2.3.1.4 KCP 10.3.1.4 Sub-lethal effects

No additional data submitted.

A 2.3.1.5 KCP 10.3.1.5 Cage and tunnel tests

No additional data submitted.

A 2.3.1.6 KCP 10.3.1.6 Field tests with honeybees

No additional data submitted.

A 2.3.2 KCP 10.3.2 Effects on arthropods other than bees

A 2.3.2.1 KCP 10.3.2.1 Standard laboratory testing

No additional data submitted.

A 2.3.2.2 KCP 10.3.2.2 Extended laboratory testing and aged residue studies

No additional data submitted.

A 2.3.2.3 KCP 10.3.2.3 Semi-field studies

No additional data submitted.

A 2.3.2.4 KCP 10.3.2.4 Field studies

No additional data submitted.

A 2.4 KCP 10.4 Effects on non-target soil meso- and macrofauna

A 2.4.1 KCP 10.4.1 Earthworms

A 2.4.1.1 KCP 10.4.1.1 Earthworms - sub-lethal effects

No additional data submitted.

A 2.4.1.2 KCP 10.4.1.2 Earthworms - field studies

No additional data submitted.

A 2.4.2 KCP 10.4.2 Effects on non-target soil meso- and macrofauna (other than earthworms)

A 2.4.2.1 KCP 10.4.2.1 Species level testing

A 2.4.2.1.1 Study 1: Toxicity to *Folsomia candida*

Comments of zRMS:	The study was performed in line with OECD 232 with no major deviations. All validity criteria were met. It is noted that the test was performed under 12 hour photoperiod, while OECD 232 indicates that light regime should preferable be 16:8 h L:D. Nevertheless, this is not indicated to be the validity criterion, so 12:12 h L:D may be also accepted, especially the validity criteria were met. It is also noted that Abbott correction for control mortality was applied, although it is not appropriate for quantile data (see OECD GD No 54). Taking this into account the corrected mortality has been struck through in the Table A 2.4.2.1-1 below. Consideration of not corrected mortality has no impact on the derived endpoints, as at concentration set as NOEC for survival mortality was the same as in controls (20%). Furthermore, according to OECD 232 the reproductive output is the main endpoint for *F. candida*. The test design was relevant for determination of NOEC but not ECx values (5 concentrations, 8 replicates for control, 4 replicates per treatment group). Nevertheless, the ECx values were calculated by the study authors and their reliability was thus checked by the zRMS in line with recommendations of EFSA Supporting publication 2019:EN-1673: • NW (normalised width) of 0.57 was calculated, which results with rating "fair" in line with Table E9 in EFSA Supporting publication 2019:EN-1673, • median EC_{10} is greater than $EC_{20,low}$, • the dose-response curve is steep with steepness of 0.68 (i.e. >0.66). Based on above indications, the calculated EC_{10} is considered to be not fully reliable. This could be expected as the test design was not sufficient to derive ECx due to too low number of concentrations tested (5 instead of 8). Since EC_{10} value is not relevant for the risk assessment purposes, the NOEC value from the study should be considered in TER calculations. It is, however, noted that at 250 mg/kg dws set as the statistical NOEC, the number of juveniles was reduced by 15%, which could be of biological relevance. Furthermore, dose-response could be observed. Therefore, in absence of the reliable EC_{10}, the zRMS is of the opinion that for precautionary reasons the NOEC should be set to the highest concentration at which no effects on reproduction were observed, i.e. 125 mg product/kg dws. Overall, the study is considered acceptable with NOEC value set to 125 mg product/kg dws (corresponding to 30.6 mg a.s./kg dws).

Reference:	KCP 10.4.2.1/01
Report	AG-F8-250 EC (Flurochloridone 250 EC) – A laboratory test to determine the effects of fresh residues on the springtail *Folsomia candida* (Collembola, Isotomidae) in an artificial soil substrate, Geary, N., 2017a, AGAN-17-26 (report number), 90020545 (sponsor report number)
Guideline(s):	Yes, OECD 232 (2016)
Deviations:	None
GLP:	Yes
Acceptability:	Acceptable with minor deviations
Duplication (if vertebrate study)	-

Material and Methods

A. MATERIALS

1. Test material AG-F8-250 EC (Flurochloridone 250 EC)

Description Brown liquid, EC (emulsifiable concentrate)
Lot/Batch # 8625
Purity Flurochloridone: 250 g/L nominal; 251.6 g/L analysed
Density: 1.028 g/cm³
Stability of test material Stable under normal conditions (at ambient temperature (30°C))
Expiry date: September 2018

2. Vehicle and/or positive control Vehicle control: Purified water
Reference item: Boric acid is routinely (once a year) tested at the test facility. The latest test determined a reproductive EC_{50} of 82.3 mg/kg soil (required in OECD 232: 50% reduction at about 100 mg/kg).

3. Test organism

Species Collembolan *Folsomia candida*
Source In-house culture at the test facility, originally purchased Bias Labs Ltd., Kirkcaldy, Fife.
Age Juvenile collembolans (9 - 11 days old)
Acclimatisation Culture was maintained in a controlled-environment room at 18.8-20.5°C and 12 hours photoperiod at 490-580 Lux.
Diet At the beginning of the test and after a period of 14 days, 6 mg of granulated dry yeast was added to each test vessel.
Test units The test arenas were glass jars (approximately 125 mL capacity and 4.5 cm in diameter), with a close-fitting lid. Each test unit was filled with 30 g wet weight of artificial soil. Test units were briefly opened for aeration every 1-4 days.

4. Environmental conditions

Soil Artificial soil was prepared with the following constituents (percentage distribution on dry weight basis):

Sphagnum peat 5%

Kaolinite clay	20%
Industrial quartz sand	74.8%
Calcium carbonate	0.2%

The dry components were mixed thoroughly. Six days prior to treatment, the dry artificial soil was partially pre-moistened by adding purified water and then during treatment the artificial soil was made up to 50% WHC.

Temperature Nominal: 20 ± 2°C; actual: 19.3 – 20.2 ºC

Photoperiod 12 hour photoperiod (light intensity: nominal: 400 - 800 Lux, actual: 580-700 Lux)

B. STUDY DESIGN AND METHODS

1. In-life dates 17 Aug 2017 to 20 Sep 2017

2. Experimental conditions

Test design

Juvenile collembolans were exposed to soil treated with the test substance at five concentrations for a period of 28 days. A water control (purified water) was tested in parallel. The reference item boric acid was tested in a separate study. After four weeks of exposure, the number of adults was counted and mortality was determined. The reproduction output was determined by counting the number of juveniles.

Number of animals per treatment

Four replicates per test substance treatment and eight replicates for the control were used with ten collembolans per replicate. An additional vessel per treatment group was set up for pH determination.

Test conditions

After application, the soil moisture content in each test vessel was adjusted to 50% of WHC by addition of water. The pH value in the test substance treatments and control was 5.60 – 5.81 at the start of the test and 5.38 – 5.60 at the end of the test. During the test period, the test temperature was 19.3-20.2°C.

Test concentrations

AG-F8-250 EC was tested at 1000, 500, 250, 125 and 62.5 mg product/kg dry soil corresponding to 244.7, 122.4, 61.2, 30.6 and 15.3 mg a.s./kg dry soil flurochloridone (based on analysed content of active substance and product density). A control (receiving purified water only) was tested in parallel. The reference item boric acid was tested in a separate study.

Treatment/Application

For the preparation of test item solutions, a stock solution (= highest test concentration) was prepared by weighing 1.113 g (1.082 mL, based on a measured product density of 1.028 g/mL) of test item and diluting it up to 100 mL purified water. The remaining test item solutions were prepared by dilution of the stock solution.18 mL of corresponding test solutions were added to each prepared amount of 227.61 g of artificial soil (equivalent to 200 g dry weight soil). For the control, 27 mL of purified water was thoroughly mixed with 341.42 g of pre-moistened artificial test soil (equivalent to 300 g dry soil). The treatment solutions were mixed with the soil and then 30 g (dry weight) of treated artificial soil was added to each test vessel and collembolans were placed in each replicate.

3. Sampling and measurements

Four weeks after introducing the test organisms the parental and juvenile collembolans in the test and control vessels were counted. The test substrate from each arena was tipped into a tray (approximately 11 cm x 17 cm in area and 6 cm in depth). Water (approx. 150-200 mL) was then added to the substrate and

stirred gently, so that the soil sank and the springtails floated to the surface. Any adult springtails floating on the water were counted and removed. To improve the contrast between the white collembolans and surrounding water surface, the water was stained dark with ink. It was assumed that any adult springtails that were recovered would have been those originally introduced and that any shortfall in the original number was an indication that they had died during the bioassay.

The efficiency of the method used to extract the springtails in this test should be > 95%. In a separate test, carried out by the Test Facility in October 2014, this was determined to be 100% for the adult springtails and 98.3% for the juvenile springtails.

The soil water content was checked at 14 DAT by reweighing the test units. Water losses were compensated for by addition of purified water if exceeding 2% of the initial water content. Water content was also determined at start and end of the study period.

At the start and end of the test, the pH of the artificial soil was measured. The test temperature and relative humidity was recorded continuously by data logger.

4. Calculation of toxicity

Mortality (number of dead adults) in % for each treatment group was calculated both before and after correction for any control treatment losses using Abbott's formula (Abbott, 1925).

The mean number of offspring produced per replicate and a measure of the standard deviation were calculated for each treatment. In addition, the percentage reduction in reproductive performance in the test-item treatment group, compared to the control group, was calculated.

5. Statistics

Probit regression analysis was performed on the data for numbers of progeny in order to determine EC_{50}, EC_{20} and EC_{10}. The data for the individual replicates in the test-item treatments were entered separately, having been first converted to positive values for the percentage decrease in reproductive success, relative to the mean control value. Any increased % values were substituted with zeros. The test item concentrations were log_{10}-transformed prior to analysis and the 95% confidence intervals for the EC_x values were also calculated. A Chi-square test for goodness of fit ($\alpha = 0.05$) was performed on the Probit line.

In order to determine LOEC and NOEC with respect to springtail survival, the 28-day mortality data for the individual test-item treatments were compared to those for the control using Fisher's Exact Test ($\alpha = 0.05$). For determine LOEC and NOEC with respect to effects on reproduction, following a check for normal distribution of the data (Shapiro-Wilk test, $\alpha = 0.05$) and for equality of variances (Levene's test, $\alpha = 0.05$), the test-item treatments were compared to the control either by one-way ANOVA and Dunnett's t-test (one-sided, $\alpha = 0.05$), or by Mann-Whitney U-test ($\alpha = 0.05$).

The statistical analysis was performed with the software SPSS, 2013.

Results and Discussion

In the control 20.0% parental mortality was observed which was not statistically significantly different from the test item concentrations up to and including 250 mg test item/kg dry soil with mortalities between 8 and 20%. Statistically significant effects on parental survival were recorded at a concentration of 500 and 1000 mg test item/kg dry soil with 95 and 100%, respectively. The NOEC for mortality was determined to be 250 mg test item/kg dry soil.

The mean number of juvenile collembolans determined at test termination was 308 in the control and 287, 317, 262, 40 and 0 at concentrations of 62.5, 125, 250, 500 and 1000 mg test item/kg dry soil, respectively. Statistically significant effects on juvenile numbers compared to controls were recorded at 500 and 1000 mg test item/kg dry soil. The NOEC for reproduction was determined to be 250 mg test item/kg dry soil.

The EC_{10}, EC_{20} and EC_{50} values for reproduction were calculated to be 245.6, 280 and 360.1 mg test item/kg dry soil, respectively.

Table A 2.4.2.1-1: Effects of AG-F8-250 EC on survival and reproduction of *Folsomia candida*

Treatment [mg product/kg dry soil]	Mortality after 28 days [%]	~~Corrected mortality [%][a)]~~	Reproduction output after 28 days	
			[mean juveniles/ test vessel ± SD]	change in numbers of juveniles, relative to control [%]
Control	20.0	-	308 ± 63.2	-
62.5	10	~~-12.5~~	287 ± 69.6	7
125	8	~~-15.6~~	317 ± 31.5	-3
250	20	~~0.0~~	262 ± 55.6	15
500	95*	~~93.8~~	40* ± 41.6	87
1000	100*	~~100~~	0* ± 0.0	100
Endpoints [mg product/kg dry soil] (95% confidence limits)				
EC_{10} (reproduction)		245.6 (159.2 - 299.7)		
EC_{20} (reproduction)		280.0 (198.4 - 333.0)		
EC_{50} (reproduction)		360.1 (293.2 - 419.8)		
NOEC (mortality, reproduction)		250		
LOEC (mortality, reproduction)		500		

***** Statistically significantly different from the control (according to Fisher's Exact Test for mortality, ($\alpha = 0.05$), and according to ANOVA and Dunnett's t-test (one-sided, $\alpha = 0.05$), or Mann-Whitney U-test ($\alpha = 0.05$) for reproduction)
~~[a)] Derived using Abbott's formula.~~
Negative values indicate an increase in reproduction compare to the control

The validity of the test was fulfilled since mean mortality of adults in the control was 20.0% (required ≤ 20%) at the end of the test, the reproduction rate was on average 308 juveniles per test vessel (required ≥ 100) and the coefficient of variation of reproduction was 20.5% in the control (required ≤ 30%).

Conclusion

In this study the NOEC of AG-F8-250 EC both for the mortality and reproduction of *Folsomia candida* was determined to be 250 mg product/kg dry soil, respectively. The EC_{10} and EC_{20} were determined at 245.6 (95% CI: 159.2-299.7) and 280 (95% CI: 198.4-333.0) mg product/kg dry soil. All validity criteria were fulfilled

A 2.4.2.1.2 Study 2: Toxicity to *Hypoaspis aculeifer*

Comments of zRMS:	The study was performed fully in line with OECD 226 with no deviations. All validity criteria were met. It is noted that Abbott correction for control mortality was applied, although it is not appropriate for quantile data (see OECD GD No 54). Taking this into account the corrected mortality has been struck through in the Table A 2.4.2.1-2 below. Consideration of not corrected mortality has no impact on the derived endpoints, as no effects of the test item on mortality were observed. Furthermore, according to OECD 226 the reproductive output is the main endpoint for *H. aculeifer*. The test design was relevant for determination of NOEC but not ECx values (5 concentrations, 8 replicates for control, 4 replicates per treatment group). Furthermore, the ECx values could not be calculated, as reduction of reproduction >10% was observed only at the highest test concentration. Overall, the study is considered acceptable with NOEC of 500 mg product/kg dws (corresponding to 112.3 mg a.s./kg dws) relevant for the risk assessment.

Reference:	KCP 10.4.2.1/02
Report	AG-F8-250 CS (Flurochloridone 250 CS) – A laboratory test to determine the effects of fresh residues on the predatory soil mite *Hypoaspis aculeifer* (Acari, Laelapidae), Geary, N., 2017b, AGAN-17-29 (report number), 90020988 (sponsor report number)
Guideline(s):	OECD 226 (2008)
Deviations:	No
GLP:	Yes
Acceptability:	Acceptable
Duplication (if vertebrate study)	-

Materials and Methods

A. MATERIALS

1. Test material AG-F8-250 CS (Flurochloridone 250 CS)

Description Brown liquid, CS (capsule suspension)
Lot/Batch # 8206
Purity Flurochloridone: 250 g/L nominal; 247.1 g/L analysed
Density: 1.1 g/cm³
Stability of test material Stable under storage conditions (at ambient temperatures (< 30°C))

Expiry date: February 2019

2. Vehicle and/or positive control Vehicle control: purified water
Positive control: The reference item dimethoate was tested in a separate study within 12 months of the present study, and resulted in an EC_{50} of 5.47 mg a.s./kg dry soil (required according to OECD 226: 3.0 – 7.0 mg a.s./kg dry soil).

3. Test organism

Species Predatory mite *Hypoaspis aculeifer*
Source From a synchronised colony, cultured at the test facility, originally obtained from Bias Labs Ltd., Kirkcaldy, UK, received November 2016.
Age Adults (30 days after starting of the egg laying for synchronisation), maximum age difference of two days
Acclimatisation Synchronised culture was maintained in a controlled-environment room at 20.3 – 21.8°C and a 16 h-photoperiod at 480-560 Lux. The culture was fed with *Tyrophagus putrescentiae* 2-3 times per week.
Diet Cheese mites and juvenile springtails were added to the soil surface of each test arena, at the beginning of the test and cheese mites *ad libitum* (2-3 times per week) throughout the test.
Test units 60 mL capacity glass jars (5.5 cm tall x 5.2 cm outer diameter, 4.4 cm inner diameter), with screw-top lids. An 8-mm-diameter hole was made in the lid for ventilation, covered with fine nylon netting. 24.56 g treated soil (20 g dry weight equivalent) was filled into each exposure unit.

4. Environmental conditions

Soil	Artificial soil was prepared with the following constituents:

Sphagnum peat	5%
Kaolin clay	20%
Industrial quartz sand	74.8%
Calcium carbonate	0.2% (to adjust pH)

The artificial soil was moistened to approximately half of the final water content six days before application. The additional water required to achieve the final nominal water content of 50% WHC was added at day 7.

Temperature	Nominal: 20 ± 2°C; actual: 20.4-21.5ºC
Photoperiod	16 hour light (light intensity: nominal 400 - 800 Lux; actual 550-650 Lux) to 8 hour dark photoperiod

B. STUDY DESIGN AND METHODS

1. In-life dates 5 Oct 2017 to 27 Oct 2017

2. Experimental conditions

Test design

Adult female mites were exposed to soil treated with the test substance at five concentrations for a period of 14 days. Purified water was used as a control treatment. The reference item dimethoate was tested in a separate study. At the end of the exposure period, the surviving individuals were extracted from the test units. The number of juveniles per test unit and additionally the number of surviving females were determined. The reproductive output and the mortality in the test item group were compared to that of the control group.

Number of animals per treatment

Ten female mites per replicate; four replicates per test substance treatment and eight replicates for the control

Two additional replicates without mites were prepared for each treatment group to determine pH.

Test conditions

After application, the soil moisture content in each test vessel was adjusted to 50% via addition of water. The pH value in the test substance treatments and control was 5.90 – 6.05 at the start of the test and 5.70-5.83 at the end of the test. During the test period, the ambient conditions were 20.4-21.5ºC, with a 16 h photoperiod of 550-650 lux.

Test concentrations

AG-F8-250 CS was tested at five concentrations of 1000, 500, 250, 125 and 62.5 mg product/kg dry soil corresponding to 224.6, 112.3, 56.16, 28.08 and 14.04 mg a.s./kg dry soil flurochloridone (based on analysed contents of active substance and product density). A control was tested in parallel. The reference item dimethoate was tested in a separate study.

Treatment/Application

The highest test item solution (stock solution) was prepared by weighing 1.112 g (1.01 mL, based on a measured product density of 1.1 g/mL) test item and dilution to 100 mL with purified water. The remaining test item solutions were prepared by dilution of the stock solution. Subsequently, 18 mL of the test solution were added to each prepared amount of artificial soil (227.61 g wet weight = 200 g soil dry weight), resulting in a final nominal water content of about 50 % of maximum WHC. An amount of 24.56 g of the treated artificial soil (= 20 g soil dry weight) were placed into each test vessel. For the control substrate, 18 mL of purified water were added to the prepared amount of artificial soil (227.61 g wet weight = 200 g soil dry weight).

3. Sampling and measurements

On day 14 after application of the test item and introduction of the test organisms, surviving mites and juveniles of *Hypoaspis aculeifer* were assessed. For this assessment, the soil from each arena was placed into individual Tullgren funnel apparatus. This consisted of a meshed container suspended over a funnel. Above the funnel was fitted a light-bulb (25 Watts, with a 24 h photoperiod). Over a two-day period, the heat of the bulbs slowly dried the soil from the top, forcing the *H. aculeifer* to move downwards until they fell from the base of the funnels into collecting vials placed beneath. These vials contained 70% v/v methyl alcohol in which the mites drowned and were preserved. Once the test soil had been removed from the arenas, the numbers of original adult and juvenile mites that remained in the test arena were counted, with the use of a binocular microscope. In addition, the number of original adult and juvenile *H. aculeifer* in the collection vial arenas were counted, following extraction from the soil. From these data the mortality of the adult females and the reproductive output were calculated.

The efficiency of the method used to extract the mites in this test should be > 95%. The extraction efficiency of the extractor was determined to be 96.2 % in a separate test.

The water content of the soil substrate in the test vessels was maintained throughout the test by weighing. For compensation of water loss additional water was added at day 7.

The pH was checked at the beginning and end of the test. Temperature was recorded continuously. In addition, the weight of each test arena was recorded at the beginning, middle and end of the test.

4. Calculation of toxicity

Mortality (number of dead adults) in % for each treatment group was calculated. Data was corrected for control mortality using Abbott's formula. The mean number of offspring produced per replicate and the standard deviation were calculated for each treatment. The percentage reduction in reproduction in the test item treatment groups compared to the control was determined.

5. Statistics

In order to determine LOEC and NOEC with respect to mortality, data for the individual test-item treatments were compared to those for the control using Fisher's Exact Test ($\alpha = 0.05$). For determination of LOEC and NOEC with respect to effects on reproduction, following a check for normal distribution of the data (Shapiro-Wilk test, $\alpha = 0.05$) and for homogeneity of variances (Levene's test, $\alpha = 0.05$), the test-item treatments were compared to the control either by one-way ANOVA and Dunnett's t-test (one-sided, $\alpha = 0.05$), or by Mann-Whitney U-test ($\alpha = 0.05$).

The mortality and reproduction data were not considered suitable for Probit regression analysis to calculate the key lethal and effect concentrations (LC_{50} and EC_{50}, EC_{20}, EC_{10}). The LC_{50} and EC_x values were therefore estimated by extrapolation from the data.

The statistical analysis was performed with the software SPSS, 2013.

Results and Discussion

Mortality of 3.0 to 8.0 % was recorded in the test item treatment groups, in the control 3.0 % parental mortality was observed. The effects in the test item treatments were not statistically significantly different from the control. The NOEC for mortality was therefore determined to be ≥ 1000 mg test item/kg dry soil.

The mean number of juvenile collembolans determined at test termination was 303 in the control and 308, 304, 303, 315 and 251 at concentrations of 62.5, 125, 250, 500 and 1000 mg test item/kg dry soil, respectively. A statistically significant effect on juvenile numbers compared to control was recorded at 1000 mg test item/kg dry soil. The NOEC and LOEC for reproduction were determined to be 500 and 1000 mg test item/kg dry soil, respectively. The EC_{20} and EC_{50} values for reproduction were calculated to be > 1000 mg test item/kg dry soil (see following table).

Table A 2.4.2.1-2: Effects of AG-F8-250 CS on survival and reproduction of *Hypoaspis aculeifer*

Treatment [mg product/kg dry soil]	Mortality after 14 days [%]	~~Corrected mortality [a)] [%]~~	Reproduction output after 14 days	
			[mean juveniles/ test vessel ± SD]	Reduction in reproduction [%]
Control	3	-	303 ± 27.3	-
62.5	3	~~0~~	308 ± 20.2	-1.5
125	5	~~3~~	304 ± 13.6	-0.1
250	3	~~0~~	303 ± 6.9	0.2
500	8	~~5~~	315 ± 17.7	-3.9
1000	5	~~3~~	251* ± 36.7	17.3
Endpoints [mg product/kg dry soil]				
LC_{50} (mortality)		> 1000		
EC_{50}, EC_{20} (reproduction)		> 1000		
NOEC (mortality)		≥ 1000		
NOEC (reproduction)		500		
LOEC (reproduction)		1000		

***** Statistically significantly different from the control (according to Fisher's Exact Test (α = 0.05) for mortality and according to ANOVA and Dunnett's t-test (one-sided, α = 0.05), or Mann-Whitney U-test (α = 0.05) for reproduction)
~~[a)] Derived using Abbott's formula.~~
Negative values indicate an increase in reproduction compared to the control

The validity of the test was fulfilled since the mortality of female adults in the control was 3% (required ≤ 20%) at the end of the test, the reproduction rate was at least 244 juveniles per control replicate (required ≥ 50) and the coefficient of variation of reproduction in the control was 9.0% (required ≤ 30%).

Conclusion

In this test on chronic toxicity to *Hypoaspis aculeifer* with AG-F8-250 CS, the NOEC was determined at 500 mg test item/kg soil dry weight. The validity criteria were fulfilled.

Remark on endpoints determined: No EC_{10} was determined in this study as effects only occurred in the highest test item concentration and therefore no dose-response curve could be established. Based on the results, however, the EC_{10} will be higher compared to the NOEC and therefore the NOEC is considered appropriate for risk assessment.

A 2.4.2.2 KCP 10.4.2.2 Higher tier testing

No additional data submitted.

A 2.5 KCP 10.5 Effects on soil nitrogen transformation

No additional data submitted.

A 2.6 KCP 10.6 Effects on terrestrial non-target higher plants

A 2.6.1 KCP 10.6.1 Summary of screening data

No additional data submitted.

A 2.6.2 KCP 10.6.2 Testing on non-target plants

No additional data submitted.

A 2.6.3 KCP 10.6.3 Extended laboratory studies on non-target plants

No additional data submitted.

A 2.7 KCP 10.7 Effects on other terrestrial organisms (flora and fauna)

No additional data submitted.

A 2.8 KCP 10.8 Monitoring data

No additional data submitted.

[i] EFSA (European Food Safety Authority), 2016. Conclusion on the peer review of the pesticide risk assessment of the active substance pendimethalin. EFSA Journal 2016;14(3):4420, 212 pp. doi:10.2903/j.efsa.2016.4420