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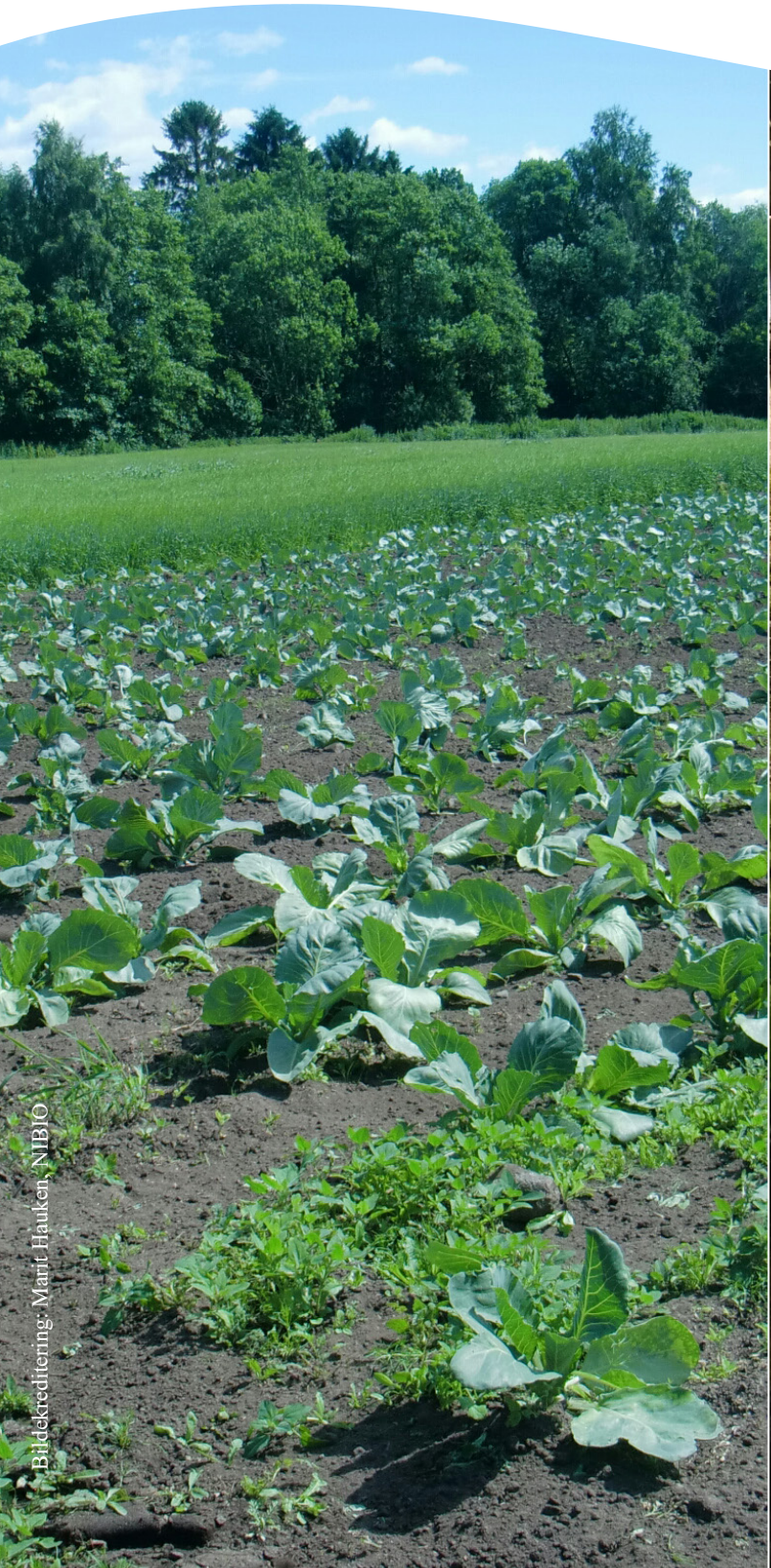
NIBIO

Evaluation of the combined toxicity assessment and cumulative risk assessment of ecologically relevant mixtures of plant protection products (PPPs) under Norwegian conditions



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REPORT

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Abstract

This project evaluated whether the principles of combined toxicity assessment (CTA) and cumulative risk assessment (CRA) can be used to predict the toxicity of ecologically-relevant mixtures of plant protection products (PPPs) in surface waters receiving run-off from Norwegian agricultural areas. A combination of testing solid phase extracts (SPE), whole surface water and a synthetic mixture in an algal bioassay and predicting combined toxicity using CTA models were conducted on selected samples from the Heia catchment (Råde, Norway). The results demonstrated that designing and testing synthetic mixtures on the basis of measured concentrations of PPPs was the best method for the accurate determination of combined toxicity due to confounding factors introduced by whole water and SPE testing. Combined toxicity models based on Concentration Addition (CA) successfully predicted the toxicity of the complex synthetic mixture and verified that a mixture of PPPs acted in an additive manner. Tiered assessment of the cumulative risk of active PPP substances and PPP formulations proposed by the European Food Safety Authority (EFSA) were considered applicable also for the CRA of complex environmental mixtures and could potentially aid the identification of relevant mixtures, risk drivers and susceptible species as input to the assessment and approval of PPPs.

4 keywords, Norwegian	4 keywords, English
1. Plantevernmidler	1. Plant protection products
2. Kombinerte effekter	2. Combined effects
3. Kumulativ risikovurdering	3. Cumulative risk assessment
4. Regulatorisk godkjenning	4. Regulatory approval

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Evaluation of the combined toxicity assessment and cumulative risk assessment of ecologically relevant mixtures of plant protection products (PPPs) under Norwegian conditions.

Preface

This project was commissioned by the Norwegian Food Safety Authority and was funded by the action plan for reduced risk from use of plant protection products. The project was performed as collaboration between NIVA and NIBIO with Knut Erik Tollefsen (NIVA) as the project leader. Knut Erik Tollefsen (NIVA) and Marianne Stenrød (NIBIO) were responsible for the planning, execution and reporting of the project. Ana Catarina Almeida and Linn Anette Haug (NIVA) were responsible for performing the ecotoxicity tests, Kine Bæk (NIVA) was responsible for solid-phase extraction and Hans Ragnar Norli and Sven Roar Odenmarck (NIBIO) were responsible for the chemical analysis. Marianne Stenrød (NIBIO) was responsible for retrieving data from the JOVA program and Knut Erik Tollefsen was responsible for performing the combined toxicity and cumulative risk assessment analysis. Employees from both partners contributed to the writing of the report.

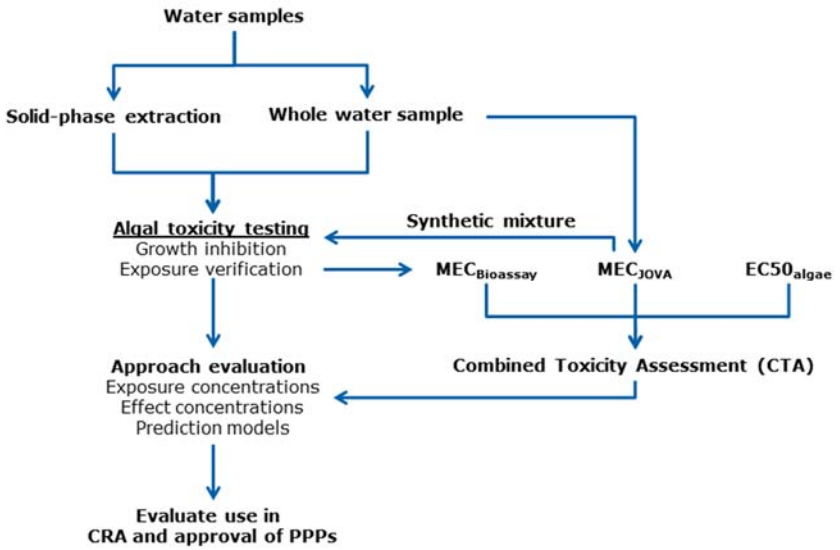
Oslo, 03.05 2016

Knut Erik Tollefsen

Contents

Summary

This project was performed to evaluate whether principles of combined toxicity assessment (CTA) and cumulative risk assessment (CRA) can be used to predict the toxicity of ecologically-relevant mixtures of plant protection products (PPPs) in selected surface waters receiving run-off from Norwegian agricultural areas. Experimental effort to assess the toxicity of surface water from the Heia catchment (Råde, Norway) during selected periods of the growing season was performed by direct testing of untreated water and solid-phase extracts (SPE) of these samples. A synthetic mixture with the composition of active substances in PPPs (i.e. pesticides) was also tested to assess the toxicity devoid of any confounding factors introduced by the sample matrix or artefacts introduced by the SPE enrichment procedure. The toxicity test was based on determination of the growth inhibiting potential of PPPs in the freshwater algae *Chlamydomonas reinhartii* as several of the main risk drivers in surface waters from Heiabekken were identified to be herbicides. Cumulative risk assessment was performed in parallel on basis of Measured Environmental Concentrations (MEC) from the JOVA monitoring program using prediction models assuming Concentration Addition (CA). The uses of CA models provide a conservative, albeit realistic prediction of the combined toxicity of PPPs and thus considered to be sufficiently protective for compounds present in complex mixtures also from Heiabekken. Experimentally-derived effect concentrations for these complex mixtures and the CA-predictions of combined toxicity were compared to assess how well the different sampling and testing strategies performed. The results from this work were finally discussed in light of how CTA principles and CRA can be used in the assessment and approval process of PPPs. The summary figure 1 display the main steps in the work performed.



Summary figure 1. Illustration of the project work-flow. Composite (14d) samples of surface water were collected in Heiabekken (Råde, Norway) and subjected to 1) chemical analysis of Plant Production Products (PPPs) to obtain Measured Environmental Concentrations (MEC_{JOVA}) and 2) toxicity testing of whole surface water and solid-phase extracts (SPE) of selected water samples taken during the growing season. The combined toxicity of the SPE sample, the surface water sample and a synthetic mixture constructed on basis of MEC_{JOVA} data were determined as growth inhibition in a bioassay with the freshwater algae Chlamydomonas reinhardtii. The individual active PPP substances in the exposure solutions from the algal bioassays were quantified ($MEC_{Bioassay}$). Combined toxicity assessment (CTA) using the sum of toxic units (STU) approach was conducted on basis of the MECs and effect concentrations for acute (EC_{50}) toxicity to algae. Measured and predicted combined toxicity were then compared to evaluate the performance of the different sampling, extraction and toxicity testing approaches. The final output of the project was the evaluation of how the approaches and data obtained could be used to assist the cumulative risk assessment (CRA) of PPPs and approval of PPPs in Norway.

The results from the experimental studies showed that whole water toxicity testing with algae was not suitable for determining the adverse effects of PPPs due to low concentrations of PPPs. In fact, the testing of whole surface waters caused a stimulation of algal growth, potentially due to the high concentrations of nutrients such as nitrogen and phosphorous. The use of SPE approaches were demonstrated to efficiently enrich PPPs to levels assumed to be toxic, and resulted in high levels of toxicity to the algae. However, the complex SPE mixtures were considerably more toxic than predicted on the basis of MEC using CA-modelling, and suggested other compounds or sample matrix factors causing at least some of the observed toxicity. The most successful approach to determine the toxicity of complex mixtures relevant for catchments such as Heia was to design and test synthetic mixtures representing the MEC of the active substances of PPPs in the recipient. Less than a factor of two difference between the experimentally determined toxicity and that predicted on the basis of CA-modelling was observed, indicating that confounding factors originating from the water itself or the SPE extraction process were minimized. The present study clearly demonstrates that concepts building on CA models seem to provide sufficiently accurate estimates of the combined toxicity of complex mixtures and confirmed that a combination of experimental and predictive approaches are likely sufficiently conservative to predict the impact of environmentally relevant PPP mixtures under Norwegian exposure scenarios. Concepts developed for the CTA of active substances in PPPs and PPP formulations were also considered applicable for environmental mixtures and could potentially aid future CRA as well as regulatory approval of PPPs. The possibilities of including a routine assessment based on the above mentioned principles in the JOVA monitoring should be explored. Furthermore, the need and possibilities for defining realistic and relevant mixture exposure scenarios from available monitoring data together with pesticide use registrations and statistics should be evaluated.

Abbreviations

AF	Assessment factor
CA	Concentration addition
CF	Concentration factor
CRA	Cumulative risk assessment
CTA	Combined toxicity assessment
EC_X	Concentration causing X % effect
EFSA	European Food Safety Authority
EQS	Environmental quality standard
EU	European Union
ETR	Exposure-Toxicity ratio
IA	Independent action
MDR	Mixture deviation ratio
MEC	Measure environmental concentration
NOEC	No observed effect concentration
PEC	Predicted environmental concentration
PNEC	Predicted no effect concentration
PPDB	Pesticide properties database
PPP	Plant protection product
RQ	Risk quotient
SPE	Solid phase extraction
STU	Sum of toxic units
TER	Toxicity exposure ratio
TU	Toxic Unit, here defined as MEC divided by EC_{50}

1. Introduction

1.1 Background

1.2 Approval and use of plant protection products

Plant protection products (PPPs) are used to control weeds (herbicides), fungal diseases (fungicides) and insect pests (insecticides) within crops and are important in plant protection practices to ensure yields of sufficient quality and quantity in conventional agriculture. PPPs are primarily used in the agricultural sector, but are also used in forestry, horticulture, amenity areas, railway areas, road areas and in private gardens. The PPPs are formulations of either single chemicals or mixtures of active chemicals (substances) that require approval by the competent authorities prior to use. Such approval is issued on basis of a thorough evaluation and peer-review of the active substances by the Member States (MS) of EU and the European Food Safety Authority (EFSA). Approval of PPPs for placing on the market or use in Norway has historically been performed by the Norwegian Food Safety Authority (NFSA) according to national regulations and from June 2015 the Norwegian national regulations are harmonized with the EU legislation (Regulation (EC) No 1107/2009). More than 100 active compounds are currently approved by the NFSA for use in Norway, and these are used for various applications (See updated list at: http://www.mattilsynet.no/plantevernmidler/godk.asp).

1.3 Occurrence

The environmental concentrations of many of active PPP substances are routinely monitored in surface water recipients in agricultural areas by the Norwegian Agricultural Environmental Monitoring Program, JOVA (www.nibio.no/jova). Over two decades the JOVA program has compiled data on the occurrence of pesticides in selected agricultural catchments covering the variety of intensive agricultural practices in Norway during the growing season. The JOVA program currently monitors 11 catchments by a combination of continuous recordings of water-flow and sampling for the analysis of nutrients, particles and pesticides (6 catchments) in agricultural streams. Emissions of PPPs are highly dependent on the pattern of use, runoff and environmental conditions such as precipitation, temperature and soil conditions and typical concentrations of PPPs in surface water range from ng/L to low µg/L (Bechmann et al., 2014; Stenrød, 2015). Although over 40 different active substances of PPPs have been detected at certain sites during the period from 1995 to 2015, between 5 and 10 active substances are normally most relevant in typical aquatic exposure scenarios during the main spraying season (Bechmann et al., 2014).

1.4 Toxicity

Many of the PPPs used to control unwanted organisms (pests) are also potentially toxic to non-target organisms such as algae, crustaceans, fish and aquatic plants inhabiting the recipients receiving run-off from agricultural areas. The toxic potency of single active PPP substances is highly species-dependent and reflects the mode of action (MoA) and their ability to affect specific biological targets in a given species. Toxicity endpoints such as the No Observed Effect Concentration (NOEC) and 50% Effect Concentrations (EC50) typically range from low µg/L to high mg/L for PPPs found in rivers and streams in agricultural dominated catchments in Norway (Petersen et al., 2013). As these runoffs typically contain multiple PPPs, such complex mixtures may give rise to combined toxicity to organisms living in the recipient. These mixtures of compounds might enhance (additivity and synergism) or decrease (antagonism) the toxicity of each other. By default, it is assumed that compounds of a mixture act additive (Kortenkamp et al., 2009). Such additive toxicity can be predicted by prediction models for Concentration Addition (CA) and Independent Action (IA), which assume that the compounds act by similar (CA) or independent (IA) MoA, respectively. Although these prediction models are based on two different

principles and assumptions, the effects of complex mixtures are predominantly predicted to occur by additivity (according to the CA model) whereas synergistic interactions are less common (Belden et al., 2007; Cedergreen, 2014; Rodney et al., 2013; Verbruggen and Brink, 2010). Studies with ecologically-relevant mixtures from two Norwegian sites monitored by JOVA confirm that the CA prediction model provide a justifiable, albeit slightly conservative estimates of combined toxicity of typical Norwegian PPP mixtures (Petersen et al., 2015).

1.5 Risk assessment

Evaluation and authorization of PPPs in the EU require that member states evaluate the potential exposure to aquatic organisms and the expected short- and long-term risks. This is typically performed by the calculation of short and long term toxicity-exposure ratios (TER) also referred to as exposure-toxicity ratios (ETR) for single PPPs in algae, daphnia and fish, and applying cut-offs considered protective for the environment (EFSA, 2013). The EFSA guidance document for the risk assessment of PPPs (EFSA, 2013) provides additional information of how to perform a tiered risk assessment for active ingredients in formulations, where a combination of measured and predicted (calculated) combined toxicity is taken into consideration. None of these documents provide detailed descriptions on how to assess the cumulative risk of mixtures of active substances or PPPs under ecologically relevant exposure scenarios (e.g. field studies). As a consequence, PPPs are assessed and regulated individually in the current Norwegian (FOR-2015-05-06-455; available at www.lovdata.no) and EU regulations (Regulation (EC) No 1107/2009) despite clear suggestions that cumulative risk assessment (CRA) should be performed for PPP mixtures (Bundschuh et al., 2014; Finizio et al., 2005; Moschet et al., 2014). A CRA performed for PPP mixtures in six different agricultural streams in Norway in 2012 confirmed that multiple active PPP substances contributed collectively to the predicted risk for aquatic organisms (Petersen et al., 2013). Of the total 56 investigated samples, as many as 8 samples were identified to represent a cumulative risk to aquatic organisms (Petersen et al., 2013). A few active PPP substances such as the herbicides aclonifen and metribuzin, as well as the fungicide metabolites prothioconazole-desthio and kresoxim-methyl, were identified to be the main risk drivers. Recent refinement of the CRA based on data complementation and experimental verification led to a reduction in uncertainty of the CRA and reduction of the number of samples above the risk threshold (Petersen et al., 2015). Albeit the use of CRA shows promise for assessing the risk of PPPs to aquatic organisms, evaluation of whether the principles of CRA can be used to predict the toxicity of PPPs under ecologically relevant exposure scenarios and samples are still required.

1.6 Objectives of the present study

The overall aim of the present study was to determine if principles of CRA can be used to predict the toxicity of ecologically-relevant PPPs in selected surface waters receiving run-off from Norwegian agricultural areas. The present work was built on previous work on cumulative risk assessment of PPPs in selected rivers and creeks in Norway (Petersen et al., 2013) and subsequent evaluation and improvement of prediction models for CRA (Petersen et al., 2015). The main objectives of the present study were to:

- Develop sampling and extraction methodology based on solid-phase extraction (SPE) capable of extracting relevant active PPP substances from surface waters.
- Perform toxicity studies with SPE, a whole water sample and a synthetic mixture from the JOVA location Heiabekken (Råde, Norway) in a standardized algal growth inhibition assay
- Assess if combined toxicity assessment based on MECs from the JOVA environmental monitoring in 2015 (July-August) could predict the toxicity of PPPs under ecologically relevant exposure scenarios.
- Evaluate how existing CRA methodology can be used to support the risk assessment in connection with the approval of PPPs in Norway.

2. Materials and methods

The overall work-flow of the project is shown in figure 1, and is further described in detail in the following chapters.

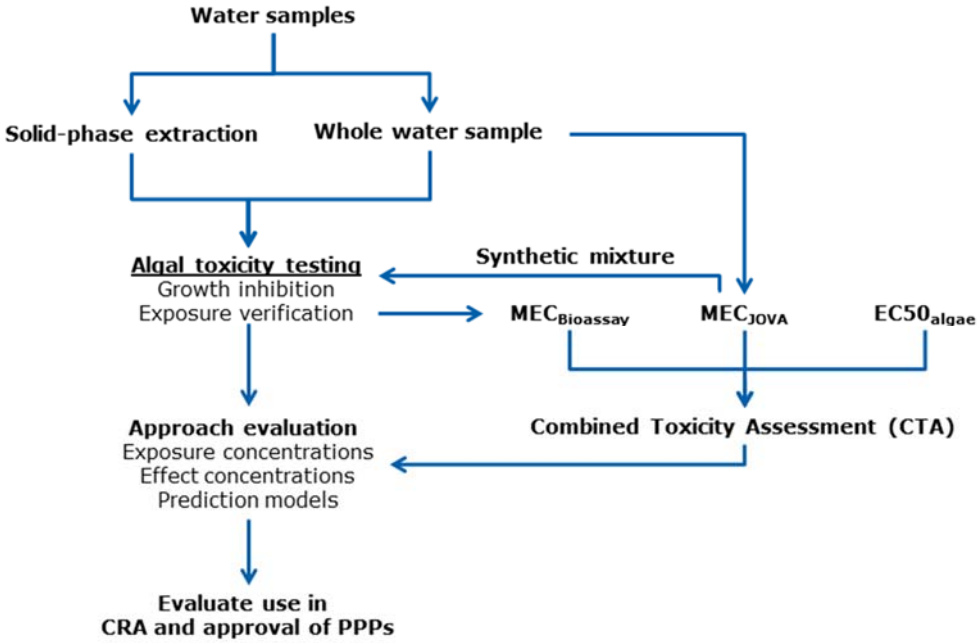


Figure 1. Illustration of the project work-flow. Composite (14d) samples of surface water were collected in Heiabekken (Råde, Norway), split into 3 aliquots and frozen for subsequent chemical analysis or bioassay testing. One aliquot was subjected to direct chemical analysis of the Measured Environmental Concentrations of active substances in Plant Production Products (PPPs) in the JOVA monitoring program (MEC_{JOVA}), one subjected to solid-phase extraction (SPE) to enrich the active PPP substances and one aliquot was kept frozen until subsequent toxicity testing. The combined toxicity of the SPE sample, the whole water sample and a synthetic mixture constructed on basis of MEC_{JOVA} data were determined as growth inhibition in a bioassay with the freshwater algae Chlamydomonas reinhardtii. The individual active PPP substances in the exposure solutions from the algal bioassays were quantified ($MEC_{Bioassay}$). Combined toxicity assessment (CTA) using the sum of toxic units (STU) approach was conducted on basis of the MECs and effect concentrations for toxicity to algae ($EC50_{algae}$). Measured and predicted combined toxicity were then compared to evaluate the performance of the different sampling, extraction and toxicity testing approaches. The final output of the project was the evaluation of how the approaches and data obtained could be used to assist the CRA of PPPs and approval of PPPs in Norway.

2.1 Sampling locations and water sampling

The long-term Norwegian Agricultural Environmental Monitoring Program (JOVA) documents the environmental consequences of current agricultural practices and changes with time, and has included monitoring of pesticide residues in agricultural streams in selected agricultural catchments since 1995 (Stenrød, 2015). A combination of continuous discharge measurements, flow proportional composite sampling over 14 days and targeted chemical analysis provides estimates of pesticide exposure concentrations in all of the JOVA monitoring catchments during the growing season (April-October) and is described in detail in Deelstra et al. (2013). Four composite stream water samples were obtained in the summer of 2015 (dates: 09.06-26.06, 26.06-10.07, 10.07-27.07 and 27.07-14.08).

The catchments included in the monitoring program are thought to represent the variety of agricultural productions in Norway but with a focus on crops with a high demand for crop protection and use of chemical pesticides (Hauken and Kværnø, 2013). The Heia catchment (Table 1) represents an area with a

broad variety of crop production types and a comparatively high frequency/need of pesticide spraying. About 60% of the agricultural area is used for cereal cropping and 20-35% of the area is for potato and vegetable production. In the dry summers the latter require irrigation that increases the risk of pesticide leaching. Previous toxicity assessments in the Heia catchment have identified risk to aquatic organisms (Petersen et al., 2015; Petersen et al., 2013).

Table 1. Selected characteristics of the monitoring catchment Heia.

Location	Drainage area (km²)	Cropped area (%)	Mean annual temperature (°C)	Mean annual precipitation (mm)	Soil type	Main crop	Start of monitoring
South East Norway	1.7	62	5.6	829	Sand, silt loam	Potatoes, grain, vegetables	1991

2.2 Preparation of test media

2.2.1 Solid phase extraction

The active PPP substances were extracted by solid-phase extraction (SPE) using 1 gram Strata-X-CW 33µm columns (Phenomenex, Torrance, CA, USA). Solid phase extraction is a commonly used method to extract selected analytes from water, to enrich these and enable up-concentrated samples to be used for chemical analysis or bioassay testing. A summary of key differences between the composition/properties of SPE and whole water samples are given in Table 2.

Table 2. Composition/properties of extracts from solid phase extraction (SPE) samples compared to whole water samples.

Composition/properties	SPE	Whole water samples
Organic compounds (incl. plant protection products)	Yes	Yes
Metals and ions*	Limited	Yes
Nutrients (nitrogen and phosphorus)*	Limited	Yes
Particles*	No	Yes
Up-concentration	Yes	No

*composition/properties that potentially may give rise to interference or confounding factors in bioassay testing.

In brief, the water samples were thawed and 5.5 L decanted equally into 5 clean glass bottles. The 5 water samples were loaded individually by a rate of approx. 3 ml/min. onto five separate SPE columns that had been activated and prewashed with 40 mL acetonitrile and 40 mL MilliQ water. The SPEs were dried, eluted with 20 mL acetonitrile containing 5% formic acid and the eluates were pooled and evaporated to dryness under nitrogen at 40 °C. The samples was resolved into 5 mL methanol and evaporated to 1 mL before being filtered through a 0.2µm Nylon Spin-X Centrifuge Tube filter (Costar, Corning, NY, USA). The filter was washed with 0.5mL methanol to ensure capturing all sample material and evaporated to 110 µL total volume. Performance evaluation of the SPE methodology was conducted with a mixture of active PPP substances in tap water prior to the extraction of the Heiabekken water samples. A recovery above 60% were achieved for all tested substances except metribuzin (40-45 %) (Table 3).

A sequential extraction to improve the retention of pesticides was also tested by passing the acidified eluate (pH3, 3M HCl) from the Strata X-CW SPE through pre-conditioned 1g EnviCarb+ (Sigma-Aldrich) SPE column, and elute the compounds by 20 ml methanol containing 5% ammonia and evaporated before subjecting the resulting eluates to chemical analysis by LC-MS/MS and GC-MS/MS (see 2.3 for details). The results from the initial tests showed that the two herbicides clopyralid and MCPA anticipated to be captured by the EnviCarb+ column were efficiently captured on the Strata X-CW column instead (results not shown). The MCPA was eluted with the other compounds, while clopyralid remained bound to the Strata X-CV column. However, as clopyralid were only found at very low concentrations in Heiabekken (Table 5) and thus considered to contribute little to toxicity, the Strata X-CW column was chosen for the extraction of PPPs from the surface water samples.

Table 3. Recovery of selected pesticides in Strata X-CW Solid phase extraction method (n=2).

Pesticide detected in monitoring campaign	Recovery (%)
Azoxystrobin	71
Boscalid	73
Clopyralid[1]	0
2,6-dichlorobenzamide (BAM)	78
Fenhexamide	101
Imidacloprid[2]	87
Iprodione	78
Mandipropamid	71
MCPA	55
Metalaxyl	58
Metamitron	83
Metribuzin[2]	43
Pencycuron	76
Propamocarb	98
Prosulfocarb	nt
Prothioconazole-desthio[2]	65
Tebuconazole	82

[1]Tested on 1 g columns. [2]Tested in 1 g and 0.2 g columns. nt: not tested. The remaining tested on 0.2 g columns.

2.2.2 **Algae exposure media**

Four ml of algal medium containing diluted SPE extracts were sampled at the start and end of the exposure studies and stored frozen at -80°C until analysis. The samples were thawed and diluted in acetonitrile and extracted with a modified QuEChERS extraction as described in Appendix 1. After filtering, the extracts were injected onto a LC-MS/MS (2µL) and GC-MS (5 µL) for analysis following the conditions outlined in Appendix 1. The geometric mean of the concentrations were calculated and used as measured concentrations of PPPs in the bioassay ($MEC_{bioassay}$).

2.3 Chemical analysis

Analysis of the water samples from the Heia catchment was performed by the JOVA monitoring program, and in accordance with the analytical methods (M101 and M15) given in Appendix 1. Analysis of the exposure media was performed only by M101 as this extraction and analysis captured most of the analytes being relevant (see 2.2.1). Although method M101 does not include the herbicides clopyralid and MCPA, these compounds were assumed to be extracted and eluted with the same efficiency as during the pre-testing phase (Table 3) and the predicted concentrations used for the subsequent calculations of MECs.

2.4 Toxicity testing

The toxicity of a serial dilution of seven SPEs comprising a procedural blank (reverse osmosis ultrapure water), reference tap water (assumed to represent natural lake water), test water from Heiabekken (taken prior to the test period) and 4 surface samples from Heiabekken (09.06-14.08) were tested in a 72h algal growth inhibition test according to ISO 8692 (ISO, 2012) and OECD Guideline for Testing of Chemicals No. 201: Freshwater alga and cyanobacteria, growth inhibition (OECD, 2011). All samples were tested for toxicity in monocultures of the freshwater species C*hlamydomonas reinhardtii* incubated at 20±1°C. Detailed description of the algal growth inhibition test can be found in Appendix 2. As the water samples were concentrated as part of the SPE procedure, their enrichment compared to the original water sample was expressed as a relative Concentration Factor (CF), where a CF value of 1 represent the original water sample (Eq. 1). A synthetic mixture with the PPP composition at Heiabekken (10.07-27.07) was tested using the same assay to determine the toxicity of the mixture of active PPP substances devoid any interfering factors from the surface water itself or the SPE matrix.

Concentration factor (CF) = extract volume in exposure media/volume of surface water extracted (1)

2.5 Data analysis

Results from the ecotoxicity tests with algae were analysed with Graphpad prism v 6 (Graphpad Software Inc., San Diego, CA, USA) using a sigmoidal concentration-response curve with variable slope curve-fitting algorithm (Eq. 2). The concentration was given as the CF.

$$Y = Bottom + \frac{(Top-Bottom)}{(1+10^{((LogEC50-X)*slope)})} \quad (2)$$

Where Y is the bioassay effect, Top is the maximum effect, Bottom is the minimum effect, EC50 is the 50% effect concentration, X is the concentration of the mixture and Slope is the slope of the concentration-response curve.

The 5% effect concentrations (EC5) were calculated by use of the graphpad calculator (http://www.graphpad.com/quickcalcs/Ecanything1) and used as a proxy for the NOEC.

2.6 Determination of toxic potency

Determination of the toxic potency of the PPP mixture in the samples from Heiabekken were performed using principles outlined by Backhaus and Faust (2012) and recently implemented in assessing cumulative risk of active PPP substances in Norwegian surface waters (Petersen et al., 2015; Petersen et al., 2013). In brief, species group-specific toxicity units (TU, Eq. 3) were determined for individual active PPP substances on basis of the reported MECs from the 2015 JOVA monitoring program and available effect concentrations for algae (Appendix 3).

$$\mathrm{TU} = \frac{\mathrm{MEC}}{\mathrm{EC50}} \quad (3)$$

The toxicity data (EC50s) were collected from the pesticide properties database, PPDB (University of Hertfordshire, 2013), EFSA (European Food Safety Authority) reports (EFSA, 2005a, b, 2010a, b, c), the EU Pesticides database (http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database), the Office of Pesticide Programs (OPP) Pesticide Ecotoxicity Database (http://www.ipmcenters.org/Ecotox/DataAccess.cfm), and unpublished reports and open literature provided by the Norwegian Food Safety Authority (Petersen et al., 2015).

The sum of toxic units (STU, Eq. 4) was then calculated on basis of the assumption that principles of Concentration Addition (CA) provided a conservative, albeit realistic prediction of combined toxicity of the PPPs (Backhaus and Faust, 2012).

$$\mathrm{STU} = \sum_{i=1}^{n} \frac{\mathrm{MEC}}{\mathrm{EC50}} \quad (4)$$

Where the EC50 can either be measured concentrations of PPPs or concentration factors (CFs) in case of SPE.

The difference between experimental (measured) and predicted effect concentrations of the active PPP substance mixtures was obtained by means of the model deviation ratio (MDR) as indicated in Eq. 5 (Belden et al., 2007). The MDR was calculated as the ratio between the predicted effect concentration ($EC_{PREDICTED}$) and the experimental (observed) effect concentration ($EC_{OBSERVED}$) at a certain effect level. This ratio is often used to discriminate between additivity ($0.2 \leq MDR \leq 5$), synergism ($MDR > 5$) and antagonism ($MDR < 0.2$) (EFSA, 2013).

$$MDR = \frac{\mathrm{ECx_{PREDICTED}}}{\mathrm{ECx_{OBSERVED}}} \quad (5)$$

3. Results and discussion

3.1 Water quality at Heiabekken

A fairly stable pH, conductivity, suspended solids, phosphorus, nitrogen and nitrate/nitrite was observed during the first 2 sampling periods in 2015 (Table 4). Elevated concentrations of suspended solids, total phosphorus, total nitrogen and nitrate/nitrite concentrations during late July/early August were associated with a period of high river flows (data not presented) and probably in connection with runoff episodes from nearby agricultural areas. In comparison, the measured high nitrogen concentrations in mid-July were occurring during a comparatively low flow period. In dry periods the potato crops in this area are usually irrigated, and this might have influenced the parameters measured.

Table 4. Water quality parameters measured in the JOVA monitoring during the sampling period.

Parameter	09.06-26.06	26.06-10.07	10.07-27.07	27.07-14.08
pH	7.1	7.2	7.3	6.9
Conductivity (mS/m)	44.3	38.3	45.1	34.0
Suspended solids – dry matter (mg/L)	15	<5	<5	240
Suspended solids – loss on ignition (mg/L)	8	<5	<5	180
Total phosphorus (µg/L)	300	390	350	1600
Dissolved phosphate (µg/L)	203	328	320	344
Total nitrogen (µg/L)	11000	11000	18000	16000
Nitrate+nitrite (µg/L N)	9300	9800	17000	14000

3.2 Environmental concentrations

The MECs determined during the JOVA monitoring campaign varied considerably, from low ng/L to low µg/L during the sampling period (Table 5). The concentrations and number of detected PPPs were generally increasing with time, leading to the highest concentrations of most PPPs at the end of the sampling period (10.07-27.08), albeit some PPPs such as the herbicides metribuzin and MCPA were detected at higher concentrations in the first part of the sampling period (09.06-26.06). The highest concentrations of PPPs were typically detected for the insecticide imidacloprid and the fungicide propamocarb in the latter part of the sampling period. The MECs for pesticides compared quite well with the observed patterns for nutrient concentrations and runoff/water flow measured at the JOVA monitoring station. Relatively high concentrations of PPPs during the period 10.07 to 14.08 were assumed to coincide with period of frequent spraying, as well as potential leaching/drainage runoff due to runoff events.

The analysed samples included in the current risk assessment covered the main spraying season in the studied catchment and were selected to represent the pesticide exposure conditions assumed to represent the highest risk to aquatic organisms. The number of pesticides detected during April-October 2015 ranged from 5 to 15, whereas 7 to 15 active PPP substances were quantified during the sampling campaigned studied herein. The four selected samples captured all pesticides detected in 2015 except the fungicide fenpropimorph (data not published). The last five years (2011-2015) pesticides were detected in 93% of all analysed stream water samples from the Heia catchment, and with detections in all analysed samples during and shortly after the growing season (April to November). For all years the maximum number of substances per sample occurred during July and early August, and reflect the period of most frequent pesticide spraying (i.e. April/May: herbicide spraying, and some potato coating with fungicide and insecticides; June-July: herbicide, fungicide and some insecticide spraying).

In all, the MECs in Heiabekken during 2015 are reasonably comparable to the last 5-year period, but somewhat in the upper range with respect to total number of detections and total number of different pesticides detected.

Table 5. Measured environmental concentrations (MEC) of active substances in plant protection products, use class, mode of action, level of quantification (LOQ) during the 2015 JOVA monitoring campaign in Heiabekken (09.06.2015–14.08.2015).

Compound (µg/L)	LOQ (µg/L)	09.06-26.06	26.06-10.07	10.07-27.07	27.07-14.08	Pesticide class	Mode of action	Fate in water[c]
Azoxystrobin	0.01	nd	nd	nd	0.01	Fungicide	Respiration inhibitor	Moderately fast photolysis (DT50 9 d)
BAM[a]	0.01	nd	nd	0.012	nd	Metabolite of dichlobenil (herbicide, banned)	Unknown	Stable
Boscalid	0.01	0.013	0.025	0.058	0.38	Fungicide	Inhibits spore germination and germ tube elongation.	Stable
Clopyralid	0.05	0.13	0.12	0.19	0.16	Herbicide	Inhibits protein synthesis	Stable
Fenhexamide	0.01	nd	0.024	0.025	nd	Fungicide	Disrupts membrane function and spore germination.	Fast photolysis (DT50 < 1d)
Imidacloprid	0.01	0.17	0.15	1.9	0.93	Insecticide	Acethylcholine receptor agonist	Fast photolysis (DT50 < 1d)
Iprodione	0.02	nd	0.029	0.21	nd	Fungicide	Signal transduction inhibitor	Fast hydrolysis (DT50 3d)
Mandipropamid	0.01	nd	nd	0.012	0.031	Fungicide	Inhibit spore germination	Moderately fast photolysis (DT50 4d)
MCPA[b]	0.01	0.029	nd	0.017	0.011	Herbicide	Synthetic auxin.	Fast photolysis (DT50 < 1d)
Metalaxyl	0.01	0.026	0.028	0.083	0.73	Fungicide	Suppress sporangial formation and mycelial growth	Stable
Metamitron	0.01	nd	0.056	0.25	0.47	Herbicide	Inhibition of photosynthesis (photosystem II).	Fast photolysis (DT50 < 1d)
Metribuzin	0.01	0.2	0.022	0.09	0.2	Herbicide	Inhibition of photosynthesis (photosystem II)	Fast photolysis (DT50 < 1d)
Pencycuron	0.01	0.029	0.56	0.19	0.2	Fungicide	Inhibition of mitosis and cell division.	Stable
Propamocarb	0.01	nd	nd	0.19	1.1	Fungicide	Lipid synthesis inhibitor.	-
Prosulfocarb	0.02	nd	nd	0.06	nd	Herbicide	Lipid synthesis inhibitor	Stable
Prothioconazole-desthio	0.01	nd	nd	0.013	0.035	Metabolite of prothioconazole, a fungicide	Sterol biosynthesis inhibitor (prothioconazole)	-
Tebuconazole	0.01	nd	0.013	nd	nd	Fungicide Herbicide	Disrupts membrane function. Sterol biosynthesis inhibitor	Stable
Number of detected PPPs		7	10	15	12			

nd: denotes compounds not detected (i.e. below LOQ) in the respective sample. [a]BAM: 2,6-dichlorobenzamide, [b]MCPA: 2-methyl-4-chlorophenoxyacetic acid. [c]Fate data is compiled from University of Hertfordshire (2016).

3.3 Toxicity testing

3.3.1 Exposure concentrations

A 20% reduction in exposure concentrations were seen on average for the different active PPP substances from the start to the end of the exposure studies with the selected extracts from Heiabekken (10.07-27.07). Fairly larger variance were observed in the stability of single PPPs in the bioassay, where pencycuron (-58%), metalaxyl (-54%), and metamitron (-43%) all displayed more than 40% reduction during the 72h exposure period (results not shown). Measured concentrations of imidicloprid (+25%), propamokarb (+13%) and fenheksamid (+10%) all apparently increased in the study, and may reflect some of the sampling and analytical uncertainty. Nevertheless, measured concentrations were on average 57% of nominal, with largest deviations for prosulfocarb (23% of nominal) and prothioconazole-desthio (115% of nominal). Several of the tested substances are known to be susceptible to fast aqueous

photolysis and hydrolysis (Table 5), and one would thus expect a reduction in the concentration for these compounds during the 72h test period. However, the reduction in prosulfocarb, pencycuron and metalaxyl could not be foreseen on basis of their photolysis and hydrolysis properties and may reflect that these compounds adsorbed to the plastic ware used in the tests.

3.3.2 SPE extracts and synthetic PPP mixture

The initial optimisation of sampling and extraction method using serial dilutions of the procedural blank, a reference tap water and test batch of surface water from Heiabekken showed that the procedural blank caused very low toxicity to the algae (48h EC50>150 (CF)), whereas the SPEs of the reference water (tap water (48h EC50=22.3 (CF)) and Heiabekken (48h EC50=3.8-13.9 (CF)) were about one order of magnitude more toxic to algae (Table 6). Most of the Heiabekken SPEs sampled in the testing period 10.07-14.08 caused slightly higher toxicity than the reference sample and the surface water sampled earlier in the season. The toxicity of the surface waters from Heiabekken displayed 2-5 times higher toxic potency than the reference water (fig. 2, fig. A2 and Table 6). All SPEs caused a concentration-dependent reduction in the growth of *C. reinhardtii*, where the 3 SPE sampled from 09.06- 27.07 were about 2.5 times more toxic than the SPE from the last sampling period (27.07-14.08). An apparent reduction in toxicity from 48h to 72h was observed (Table 6), potentially caused by reduction of individual active PPP substances during the study. As a consequence, EC values for 48h exposure were routinely used for subsequent comparisons in this report.

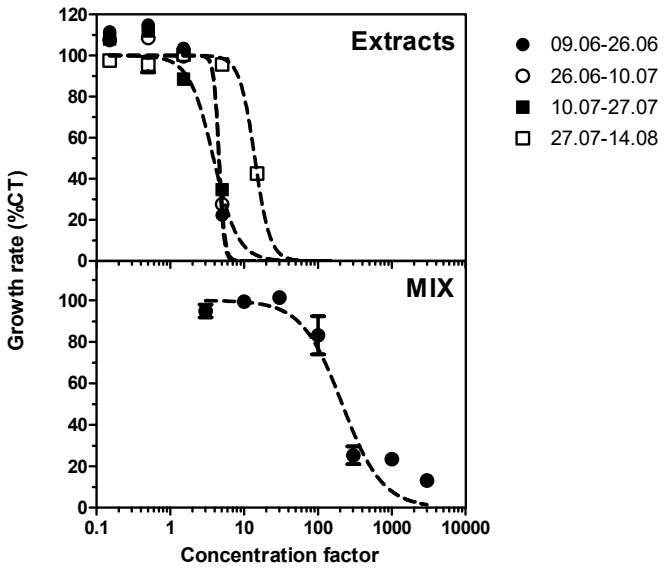


Figure 2. Inhibition of Chlamydomonas reinhardtii growth after 48h exposure to solid-phase extracts (SPE) and a synthetic mixture (MIX) of surface water from Heiabekken at different parts of the season. The data (n=3) depict the growth of C. reinhardtii compared to the control (CT).

The synthetic mixture made on basis of the composition of PPPs from Heiabekken (10.07-27.07) also caused a concentration-dependent reduction in *C. reinhardtii* growth (fig. 2), albeit with 15-50 times lower potency than the SPEs. The variation between samples was only about a factor of 3 when comparing the EC5 values and likely reflected the difference in the shape of the concentration-response curves for the different samples (Table 6).

Table 6. Summary of 50% (EC50) and 5% (EC5) effect concentrations (Concentration Factor, CF) of solid-phase extracts (SPE) of water samples from Heiabekken in the period 09.06.15 to 14.08.15, a synthetic mixture of the PPP composition at Heiabekken in the period 10.07-27.07 (MIX), tap water (Reference) and a procedural blank (Blank).

Endpoint (CF)	Exposure time (h)	09.06-26.06	26.06-10.07	10.07-27.07	27.07-14.08	MIX	Reference	Blank
EC50	24	NA	NA	NA	NA	NA	NA	>150
	48	4.5	4.6	3.8	13.9	213	22.3	>150
	72	8.1	5.1	4.6	18.9	267	20.3	>150
EC5	24	NA	NA	NA	NA	NA	NA	>150
	48	3.4	3.2	1.3	6.9	11	NA	>150
	72	3.5	3.8	3.5	15.2	6.2	NA	>150

*EC5 is a surrogate for the NOEC. NA-not applicable as the concentration-response curve was of poor quality.

3.3.3 Surface water sample

Diluted surface water from Heiabekken (10.07-27.07) caused a concentration-dependent stimulation of *C. reinhardtii* growth under the conditions tested (fig. 3). About 50% stimulation of growth occurred during the first 48h of exposure and was clearly contradicting the findings from the SPE extracts. As the whole water samples from Heiabekken contained substantial concentrations of nitrogen (N) and phosphorus (P) (Table 4), which are known to stimulate the growth of algae (Elser et al., 2007), the observed stimulation of growth were likely caused by increased availability of nutrients that counteracted any growth inhibiting potential of the PPPs. Additional experiments using control samples with similar concentrations of N and P, or by using surface water from Heiabekken stripped for PPPs (e.g. by SPE), would likely have provided a better experimental design for these studies. Despite so, the relative toxicity of the SPE extracts and PPP mixture (48h EC5=1.3-11 (CF)) indicate that concentrations of active PPP substances would likely be too low in surface water to cause any adverse effects (Table 5) unless being enriched. Pre-concentration approaches as that taken herein may provide sufficient enrichment and potentially alleviate some of the challenges introduced by co-exposure to micro-nutrients as these normally do not enrich in SPEs.

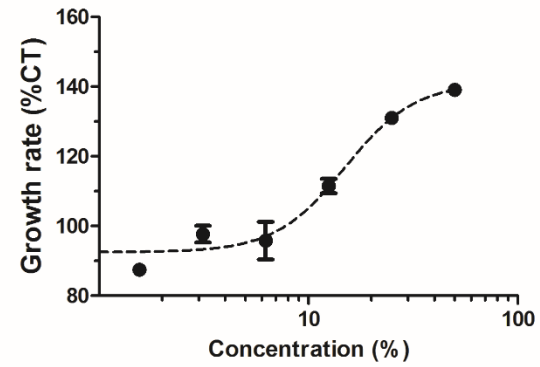


Figure 3. Stimulation of Chlamydomonas reinhardtii growth after 48h exposure to surface water from Heiabekken (10.07-27.07). The data depict the growth of C. reinhardtii compared to the control (CT).

3.4 Predicted combined toxicity

The predicted combined toxicity of samples from Heiabekken was assessed on basis of the monitoring data from the JOVA program and measured concentrations of the active PPP substances in the exposure solution from the *C. reinhardtii* bioassay. The predicted combined toxicity shows a STU_{algae} well below 1 for all samples tested (fig. 4), with the highest STU of 0.014 for EC50 after 48h of exposure. The other samples were typically displaying STUs between 0.002 and 0.014 and clearly suggested that a pre-

concentration step such as that used herein was required to concentrate the PPPs in the dilute samples to sufficient concentrations causing adverse effects (see Table A3, for details).

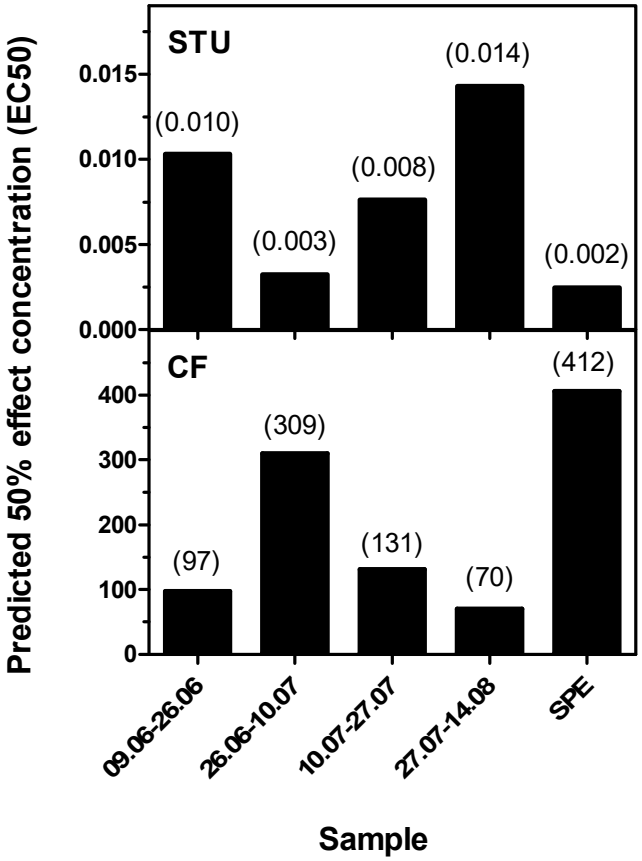


Figure 4. Predictions for combined toxicity of measured active substances of Plant Protection Products (PPPs) in surface water samples from Heiabekken (Råde, Norway) determined on basis of measured concentrations of PPPs in different water samples collected during the JOVA monitoring program (09.06-26.06, 26.06-10.07, 10.07-27.07 and 27.07-14.08) and an algal exposure solutions composed of solid phase extracts (SPE) from the water sample taken 10.07-27.07. The data depict the predicted 50% effect concentrations after 48h exposure (48h EC50) as sum of toxic units (STU) and the corresponding Concentrations factors (CF). Detailed breakdown of the data is displayed in Table A3.

The observed toxicity of the SPE extract from Heiabekken was several orders of magnitude higher than that predicted by the STU approach. Inhibition of *C. reinhardtii* growth typically occurred at slightly concentrated extracts (Table 5; 48h EC50 of 4.5-14 (CF)), whereas the combined toxicity predictions suggested that toxicity would typically occur at CFs of 70-412 (fig. 4). Although some differences between predicted and measured toxicity was expected due to lack of complete recovery of the PPPs from water in the initial extraction step (i.e. the pre-concentration for bioassay testing), this would not explain the large MDRs obtained for most SPEs (fig. 5). The fact that the SPE toxicity occurred at lower than predicted CFs, suggest that other compounds than the measured active PPP substances were contributing to the toxicity. Although it is possible that other PPPs or anthropogenic pollutants than those monitored during JOVA may have caused this higher than expected toxicity, the cause of this excess toxicity may be difficult to identify. However, the observation that the SPE of reference water, but not the procedural blank caused toxicity at fairly comparable CFs as the Heiabekken surface waters (Table 6), suggests that naturally occurring compounds in the surface waters were causing some of the toxicity observed. The present results suggest that additional clean-up steps of highly concentrated SPE or optimisation of SPE methodology to reduce the extraction of or the biological effect of non PPPs may be required to accurately determine the toxicity of PPP mixtures using SPE. Until this has been resolved, assessing the

combined toxicity of synthetic mixtures designed on basis of measured exposure data may provide an acceptable alternative to determine the toxicity and cumulative risk under ecologically relevant exposure scenarios. Previous studies with *C. reinhardtii* exposed to a synthetic mixture representing the composition of PPPs at Heiabekken verified that the predicted combined toxicity was within a factor of two of the experimental data and thus supporting that predictive modelling using CA modelling provide a realistic estimate of combined toxicity in complex samples of PPPs (Petersen et al., 2015). The fact that a MDR of less than 2 was also observed in the present study confirm that principles of additivity and use of CA modelling provide sufficient accuracy for assessing combined toxicity and cumulative risk in complex samples such as that studied herein.

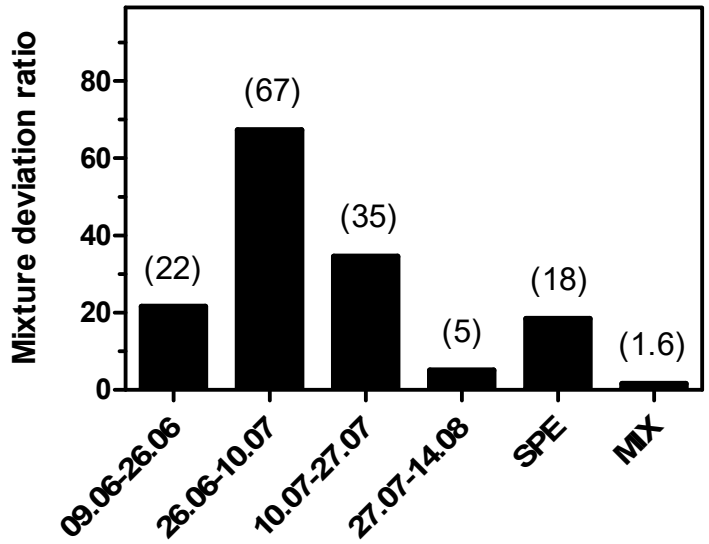


Figure 5. Mixture Deviation Ratios (MDRs) as a measure for the deviation between observed and predicted combined toxicity of active substances of Plant Protection Products (PPPs) in different samples from Heiabekken (Råde, Norway) to Chlamydomonas reinhardtii. The data depict the MDR for samples where the Measured Environmental Concentrations (MECs) were obtained by the JOVA program (09.06-26.06, 26.06-10.07, 10.07-27.07 and 27.07-14.08), an algal exposure solutions composed of solid phase extracts (SPE) from the water sample taken 10.07-27.07 and a synthetic mixture (MIX) representing the composition of PPPs in the sample taken 10.07-27.07. The MDR of the MIX was based on the observed toxicity of the mixture compared to the predicted combined toxicity of the active PPP substances in the original water sample (i.e. sample 10.07-27.07).

3.5 Use of CRA in risk assessment and regulatory approval of PPP

Current procedures for approval of pesticides include an EU-level approval process for active substances and a zonal evaluation and country-specific approval of the PPP formulations. There are regulatory risk assessment requirements covering the single active substances, safeners and synergists, as well as the formulated products containing one or more active substances as well as adjuvants and co-formulants. The regulatory requirements for combined toxicity assessment is currently limited to single active PPP substances and formulations of these and approaches to address combined toxicity and cumulative risk of ecologically relevant environmental mixtures has still not reached a level of consensus to warrant larger scale implementation in regulatory frameworks. Despite clear lack of use of CRA in risk assessment within several regulations, cumulative effects of active substances has been increasingly incorporated in the risk assessment methodology for setting of maximum residue limits (MRLs) (Regulation (EC) No 396/2005) and assessing human and animal health effects (Regulation (EC) No 1107/2009). This work including the definitions of cumulative assessment groups (CAGs, i.e. groups are formed by identifying pesticides that produce similar toxic effects in a specific organ or system) and implementation of Monte Carlo simulations in probabilistic exposure and risk assessment of large CAGs of pesticides (http://mcra.rivm.nl). Methodology and scientific evaluations for assessment of cumulative effects of PPPs in the environment are, however, at present most addressed within the research community, and

cumulative aquatic risk assessment approaches have not yet been fully standardised in relevant guidance documents.

A general consensus in research and regulatory communities seems to be that experimental or theoretical (predictive) combined toxicity assessment and CRA of complex PPP mixtures should be performed without increasing testing requirements. Using data acquired by existing regulatory assessments in combination with mixture models such as CA and IA is proposed to provide a predictive CRA when considered justified (no evidence of synergistic effects) and when being relevant (mixtures and formulations are encountered in the environment). The concepts of CA have been considered particularly useful as being easily incorporated in existing risk assessment procedures, being conservative (protective) and yielding fairly similar results as IA modelling at low levels of exposure (Kortenkamp et al., 2009). The CA model makes use of existing EC data such as NOEC and EC_X to predict combined toxicity and cumulative risk of complex mixtures, and can thus build directly upon existing regulatory testing strategies and available toxicity data. As both of the models (CA and IA) describe additivity, inclusion of MDR to differentiate between pure additivity and interactions such as antagonism (MDR<0.2) and synergism (MDR>5) have been considered useful to identify exposure scenarios of particular relevance (i.e. synergy) and to verify that prediction models are providing accurate assessment of the combined toxicity of chemicals (EFSA, 2013). Antagonism is normally of less regulatory relevance as additivity predictions (CA) are considered to be sufficiently protective (conservative) for risk assessment purposes.

A tiered approach making use of the calculated and measured combined toxicity of PPPs (EFSA, 2013) has recently been proposed for the active PPP substance and their formulations (fig. 6). This approach takes advantage of a decision tree using measured and predicted (CA) toxicity of complex mixtures (PPP formulations) and their active substances, MDR assessments and evaluation of different data used in the assessments. The final outcome of this procedure is the CRA of both single chemicals and formulations of these and identification of the toxicity drivers in the mixture.

The concepts already recommended for CRA of PPP formulations and the active substances should be further developed to also target the environmentally occurring mixtures of pesticides arising from concurrent use of many different PPPs within a single field as well as within a catchment area. The approach taken in this work, using SPE, whole water samples or synthetic mixtures, would all be applicable to such a tiered approach once methodological challenges such as that encountered for the SPE (i.e. co-extraction of natural compounds and other pollutants etc.) and testing of whole water samples (i.e. stimulation of growth due to micro-nutrients etc.) has been resolved. Toxicity testing of synthetic mixtures, based on measured concentrations in ecologically relevant water samples, is currently the most feasible approach as avoiding bias and constraints introduced by non-characterised compounds in complex samples. Although this approach will not fully account for the complex nature of environmentally relevant samples or exposure conditions, the excellent agreement between predicted and measured toxicity (Petersen et al., 2015; Petersen et al., 2013) provides confidence that CA can be used as a basis for combined toxicity and CRA is suitable to support future regulatory requirements for PPPs.

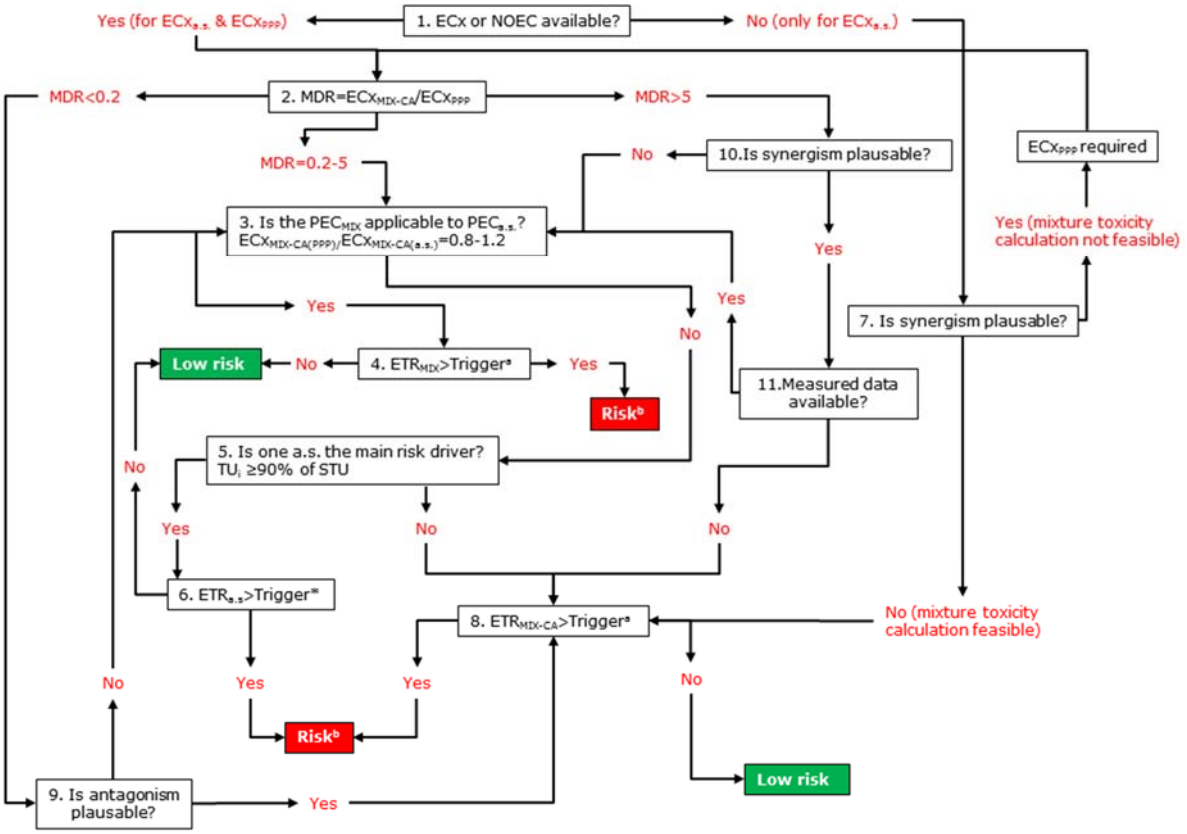


Figure 6. Decision tree for performing cumulative risk assessment of plant production products (PPP) and their active substances (a.s.) based on a combination of measured and predicted combined toxicity assessment. The procedure take advantage of available exposure data (PEC), effect data (ECx or NOEC), combined toxicity assessment using principles of Concentration Addition (MIX-CA), exposure-toxicity ratio (ETR=PEC/ECx), Mixture Deviation Ratios (MDR) and specific ETR trigger values reflecting established assessment factors (AF) to compensate for species- and endpoint-specific variance in available effect data (ETR= PEC/(ECx/AF)). Details of the procedure are described in EFSA (2013). [a]Trigger values are expected to be set to act protective for the organisms and dependent on the protective aim and available data. [b]Risk indication would in cases were methods and data refinements options has not been exhausted lead to conclusion that low risk is not demonstrated and that such refinement options should be implemented.

The scientific opinion published by EFSA in February (EFSA, 2016) gives a thorough review of important elements in regulatory environmental risk assessment (ERA) that underline the above. In agreement with our current and previous work (Petersen et al., 2015; Petersen et al., 2013), the scientific committee pointed out that chemical monitoring data and model calculations usually indicate that only a limited number of pesticides dominate the toxicity of complex mixtures of PPPs. Consequently, when addressing cumulative stress of pesticides, it appears cost-effective to focus on those pesticides that dominate the mixture using a toxic units approach as that used herein. Available monitoring data could and should be used to assess which pesticide mixtures are relevant for a CRA, which active PPP substances are the main risk drivers and identify which species are likely affected and by doing so assisting the approval or review process of PPPs and assessing their potential impact under Norwegian conditions. The monitoring data from the JOVA program provide the possibility for a retrospective risk assessment and the possibility to include toxicity assessments based on the principles described should be explored and considered implemented in future reporting from the JOVA program. Similar approaches as that taken herein can also be used to identify realistic exposure scenarios for the agroecosystem (EFSA, 2016; Schafer et al., 2013), and to identify commonly occurring mixtures that cause adverse effects. More specifically, available chemical monitoring data and pesticide use data from the JOVA program could together with crop

specific pesticide use data from national statistics (e.g. (Aarstad and Bjørlo, 2016), be used to define a set of representative and relevant mixture exposure scenarios within different productions and small catchments. A modelling (toxic unit) approach could be used to simplify the scenarios by identifying the most probable risk drivers that would require further attention and testing. This would be a good starting point to evaluate possibilities of adopting the recommendations from the recent EFSA scientific opinion (EFSA, 2016) that propose an integrated risk assessment framework combining (mesocosm) experiments, modelling and (chemical and biological) monitoring.

Much of the current research results highlight mixture toxicity effects and cumulative risk assessment for the aquatic environment, and similar effort as that should be devoted to also address combined toxicity and cumulative risk in the terrestrial environment. From an agricultural point of view the ecotoxicological effects in the soil also need to be adequately addressed as the PPPs are sprayed on the crop and soil, and one must maintain a good soil quality to ensure sufficient crop production.

4. Summary and conclusions

A combination of experimental toxicity testing and predictive combined toxicity assessment was used to assess the combined toxicity and potential impact of ecologically-relevant PPPs from the Heia catchment. In addition to developing sampling and extraction technology to enable the enrichment of surface water as a pre-concentrating step in bioassay testing, toxicity studies using SPE, whole water samples and a synthetic mixture were used to assess combined toxicity of complex samples. This study demonstrates the ability of available CRA principles (i.e. the toxic unit approach) to give a sufficiently conservative estimate of the combined toxicity of relevant environmental mixtures of pesticides. However, the current study also showed that potential bias can be introduced into toxicity testing of highly complex samples by substances other than PPPs (e.g. dissolved organic material and soluble nutrients). The results suggest that the most realistic approach to evaluate combined toxicity in such complex samples is still the testing of synthetic mixtures designed on the basis of the MECs of PPPs, albeit improvement of pre-concentration approaches such as SPE and bioassay testing may provide successful solutions in future research efforts. Whole water testing seemed to be of limited value in the present study, as co-contamination with high levels of nutrients likely caused the stimulation of growth and was thus efficiently inhibiting the efficient detection of adverse effects in such complex samples. Concepts building on CA models seem to provide sufficiently accurate estimates of the combined toxicity of complex mixtures and confirmed that a combination of experimental and predictive approaches are likely sufficiently conservative to assess the impact of environmentally relevant PPPs mixtures under Norwegian exposure scenarios. Concepts developed for the assessment of combined toxicity of active substances and PPP formulations were found to provide a good basis for the application also for environmental mixtures and potentially aid future risk assessment as well as regulatory approval of PPPs. The possibilities of including a toxicity assessment based on the above in the reporting procedure of the JOVA program should be explored. Further, the need for and possibilities in using available monitoring data and pesticide use registrations and statistics to define a set of representative and relevant mixture exposure scenarios within different productions and small catchments, should be evaluated.

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Appendix 1. Chemical analysis

Extraction of pesticides from water samples

A modified QuEChERS-extraction of water samples was used for the analysis of pesticides in the concentration range 10 – 1000 μg L^{-1}. Residues of pesticides (listed in Table 5) were extracted from 1 mL of bioassay water mixing with 0.9 mL acetonitrile (Pestiscan, LAB-SCAN POCH SA, Gliwice, Poland) and 0.1 mL ISTD (internal standard), and Citratextraction tube, Supelco 55227-U in 10 mL centrifuge tubes. The samples were extracted by end-over-end shaking (10 min; Reax2, Heidolph). After centrifugation (3000 rpm, 5 min), 1.0 mL of the supernatant was filtrated and transferred to vials for injection on LC-MS/MS (2μL) and GC-MS (5 μL).

Analysis of pesticides with LC-MS/MS

An Agilent 1200 series LC-system with binary pump, degasser and autosampler with cooling of samples at 5°C was used. The LC was equipped with an Agilent Zorbax Eclipse Plus C18 Rapid Resolution HT (2.1 mm x 100 mm, 1.8 μm particle size) for sample separation. The ionization and detection system consisted of an Agilent 6410B series triple quadrupole mass spectrometer equipped with an electrospray ionization source. Programmed injection was used with mixing 5 μL of sample with 25 μL of Milli-Q water in the injector. The mobile phase was 5 mM ammoniumformiate in Milli-Q water (A) and 5 mM ammoniumformiate in methanol (B) at a flow rate of 0.3 mL/min. A linear gradient of 10% methanol in 1 min, then linear gradient to 100 % methanol at 10 min and hold 100 % methanol for 5 min, before returning to initial conditions, was applied for the separation of the analytes on the column.

For quantification in the concentration range 10 – 1000 μg L-1, calibration standards at 0.002, 0.005, 0.02, 0.05, 0.2, and 1 μg mL^{-1} where prepared by diluting stock solutions of all pesticides (purchased from Dr. Ehrenstorfer, Augsburg, Germany) with acetonitrile. The calibrating standards contained triphenyl phosphate (TPP) as internal standard at 0.1 μg mL^{-1} equal to the concentration in acetonitril used for extraction. Samples with analyte concentrations exceeding the calibration range, were reanalyzed after dilution.

Analysis of pesticides with GC-MS

The measurements were performed on an Agilent 6890 gas chromatograph connected to an Agilent 5973 mass spectrometer using ChemStation Software version D.03.00. The gas chromatograph was equipped with a Gerstel (Mühlheim Ruhr, Germany) programmable temperature vaporizing (PTV) injector with a sintered liner. The separation was performed using a fused silica column (Chrompack CP-SIL 5CB MS, 50 m x 0.25 mm i.d., 0.40 μm film thickness, Varian Inc.). The temperature program was as follows; 80°C held for 1.0 min, 20°C min^{-1} to 160°C, held for 0 min, 5°C min^{-1} to 280°C, held for 5 min. Injection volume 5 μl. For quantification, calibration standards at 0.01, 0.05, 0.25, and 1 μg mL^{-1} where prepared by diluting stock solutions of all pesticides (purchased from Dr. Ehrenstorfer, Augsburg, Germany) with phosphate buffer. The calibrating standards contained fenoprop as internal standard at 0.2 μg mL^{-1} equal to the concentration in phosphate buffer used for extraction.

Appendix 2. Toxicity testing in *C. reinhardtii* bioassay

The growth inhibition tests were performed with *C. reinhardtii* (NIVA163 CHL153; Norwegian Institute for Water Research, Oslo, Norway) in 96-well microplates (Falcon™, Oslo, Norway). Algal cultures for inoculation were incubated in HSM growth medium (Harris, 1998) 1 to 4 days prior to the tests to ensure that the cultures were in the exponential growth phase. The tests were made in HSM modified media (Table 1).

Table A1. HSM modified media used in the exposure experiments.

Component	HSM modified media	
	Concentration (mM)	Salt
NH_4^+	0.937	NH_4NO_3
K^+	8.22	KNO_3
Na^+	0.27	$NaNO_3$
Ca^{2+}	0.07	$CaSO_4$
Mg^{2+}	0.0811	$MgSO_4$
SO_4^{2-}	0.1511	$CaSO_4$+$MgSO_4$
NO_3^-	9.427	NH_4NO_3+KNO_3+$NaNO_3$

Algae concentrations were measured with a Beckman-Coulter Multisizer 3 Coulter Counter (Miami, FL, US) and adjusted to 20 × 10^3 cells ml^{-1}. Test solutions were prepared by mixing the extracts, blank or solvent (1% v/v methanol; control) with growth medium and diluting 1:1 with algae culture (10 × 10^3 cells ml^{-1}). The final volume in each well was 200 µl. Ten concentrations plus solvent control were tested in 4 replicates per microplate for each extract. A separate microplate was made for the blank. The outer wells of the microplates were filled with 200 µl growth medium without algae to neutralize confounding bioassay factors such as edge-specific evaporation from the microplate. The microplates were incubated in an Infors Multitron 2 incubator shaker (Infors AG, Bottmingen, Switzerland) with orbital shaking at 90 rpm, continuous light intensity of 83±6 µmol m^{-2} s^{-1} and temperature of 20±2°C. Fluorescence measurements with a 530 nm excitation (bandwidth 25 nm) and a 685 nm emission (bandwidth 20 nm), were performed at the start and every 24 h with a Cytofluor 2300 (Millipore, Billerica, MA, US). The average growth rate for each sample was calculated from initial fluorescence and fluorescence after 24 h, 48 h and 72 h using Eq. 1:

$$\mu_{n-0} = \frac{\ln(Nn)-\ln(N0)}{t_n-t_0} \times 24\ (day^{-1}) \qquad \textbf{Eq. 1}$$

Being μ_{n-0} the average specific growth rate from time *0* to *n*, N_n the cell density at time *n* and N_0 the cell density at time *0*. The inhibition of growth rate was then calculated as a percentage of control (%CT).

Appendix 3. Compiled toxicity data for algae, crustaceans, fish and aquatic plants

Table A2. Acute EC_{50} and chronic NOEC toxicity data for algae, crustaceans, fish and aquatic plants and assessment factors (AF) used for calculating PNECs for compounds detected in 2012 and 2013.

	Acute effects (µg/L)				Chronic effects (µg/L)				
Compound	Algae	Crustaceans	Fish	Aquatic plants	Algae	Crustaceans	Fish	AF	Adhoc PNEC (µg/L)
Aclonifen	470	1 200	670	6	**2.5**	16	5	10	0.25
Alfa-cypermethrin	>100	0.3	28	-	-	**0.0015**	0.03	50	0.00003
Azoxystrobin	360	55	470	3 200	800	**10**	147	10	1.0
BAM*	24 200	100 000	63 400	**580**	100 000	46 200	27 200	10	58
Bentazone	10 100	64 000	100 000	**5 400**	25 700	120 000	48 000	10	540
Boscalid	3 750	5 330	2 700	-	-	1 300	**125**	50	2.5
Cyazofamid	25	190	560	33	**10**	110	130	10	1.0
Cyprodinil	2 600	220	2 410	7 710	570	**8.8**	83	10	0.88
Dicamba	1 800	41 000	100 000	**450**	25 000	97 000	180 000	10	45
Dichlorprop	26 500	100 000	109 000	**4 100**	180 000	100 000	100 000	10	410
Dimethoate	90 400	2 000	30 200	-	32 000	**40**	400	10	4
Dimethomorph	29200	7900	3400	-	9800	5	**56**	10	0.5
Fenamidone	3 840	190	740	880	1 850	**12.5**	310	10	1.25
Fenhexamid	4150	18 800	1 340	2 300	5 360	1 000	**101**	10	10.1
Phenmedipham	86	410	1 710	230	-	**61**	320	50	1.22
Fluazinam	160	220	55	53600	48	12,5	**12**	10	1.2
Fludioxonil	24	35	230	920	-	**2.5**	39	50	0.05
Fluroxypyr	49 800	100 000	14 300	**12 300**	56 000	56 000	100 000	10	1 230
Imazalil	870	3 500	1 480	-	457	<1 800	**43**	10	4.3
Imidacloprid	12000	85 000	211 000	-	10 000	**1 800**	9 020	10	180
Iprodione	1 800	250	3 700	1 000	3 200	**170**	260	10	17
Carbendazim	7 700	150	190	-	2 500	**1.5**	3.2	10	0.15
Clopyralid	5400	99 000	47500	89 000	710	7 000	**10 800**	10	71
Kresoxim	24	186	150	-	**< 3**	32		50	0.06
Mandipropamid	19 800	7 100	2 900	790	≥19 800	**76**	500	10	7.6
MCPA*	32 900	190 000	50 000	152	**4**	50 000	15 000	10	0.4
Mecoprop	16 200	91 000	100 000	**1 600**	56 000	22 200	50 000	10	160
Metalaxyl	420	5600	960	77010	7479	**1 200**	9 100	10	120
Metamitron	400	5 700	190 000	400	**100**	5700	3200	10	10
Metribuzin	20	4200	74 600	**8**	19	320	4400	10	0.8
Pencycuron	>300	>300	Z300	-	100	**49.6**	>300	10	5
Pinoxaden	5 000	8 300	10 300	13 900	**630**	-	3 200	50	12.6
Propiconazole	93	4800	4800	4828	**51**	310	95	10	5.1
Propamocarb	>85000	>100000	>92000	>18000	22000	12300	>6300	10	630
Prosulfocarb	49	510	840	690	-	**45**	310	10	4.5
Prothioconazole	1100	1300	1830	**74**	2920	560	**308**	10	7.4
Prothioconazole-desthio	73	5 500	6 630	39	-	100	**3.3**	50	0.068
Pyridate metabolite	4 930	26 100	20 000	1 800[a]	**1 700**	5 000	20 000	10	170
Pyrimethanil	1 200	2 900	10 560	7 800	1 000	**940**	1 600	10	94
Trifloxystrobin metabolite	77 100	95 300	106 000	-	15 700	**3 200**	106 000	10	320
Tebuconazole	1960	2790	4400	144	100	**10**	12	10	1

* BAM: 2,6-dichlorobenzamide, MCPA: 2-methyl-4-chlorophenoxyacetic acid

We here define acute effect data as EC_{50} values from acute tests on daphnia and fish and from the chronic tests on algae and aquatic plants. The chronic effect data are NOEC values preferably from long term studies on crustaceans and fish and chronic studies on algae. [a]NOEC value. n.f. are not found toxicity data.

Appendix 4. Verification of the algal assays performance for SPE extracts

The algal growth inhibition assays determine the chronic toxicity to *Chlamydomonas reinhardtii* over a period of 72h and are routinely run with a positive control (3,5-dichlorophenol, 3,5-DCP) to verify the performance. An 48h EC50 of 3.3 ug/L was obtained for 3,5-DCP and were found to be in accordance with the recommendations of the OECD Test guideline (OECD, 2011).

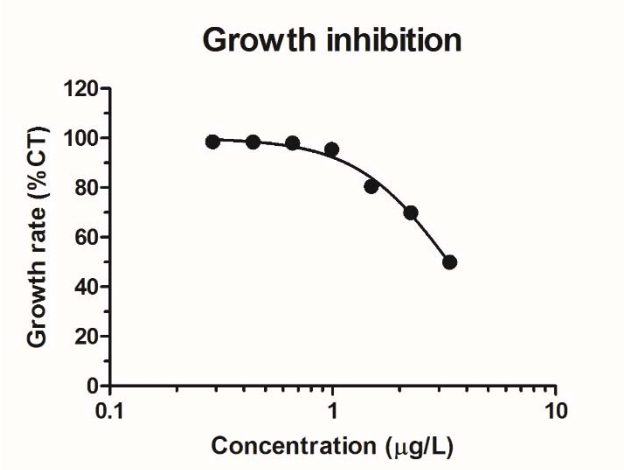

Figure A1. Toxicity measured as growth inhibition compared to control (CT) of the positive standard 3,5-dichlophenol (DCP).

Solid-phase extracts of reverse osmosis water used as a procedural blank (Blank), a reference water (tap water) and a sample from Heiabekken were tested in the algal toxicity assays. Only marginal toxicity was observed for the procedural blank after 48h exposure (48h EC50 >150 REF), whereas the reference water (48h EC50 =22.3 REF) and surface water from Heiabekken (48h EC50 =11.7 REF) were more than one order of magnitude more toxic.

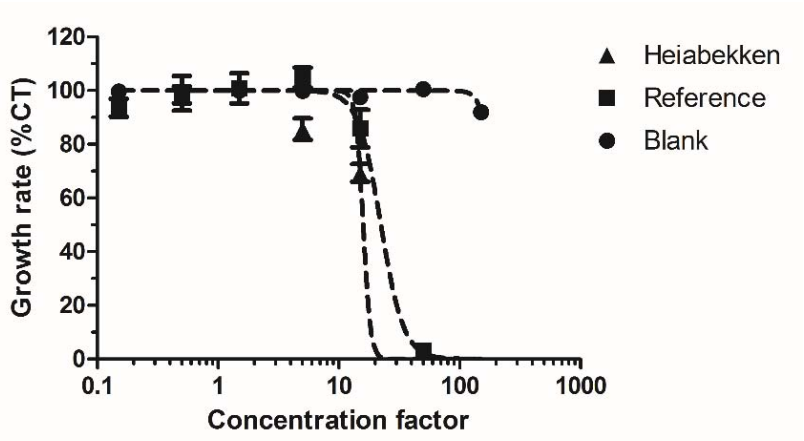

Figure A2. Inhibition of Chlamydomonas reinhardtii growth after 48h exposure to solid-phase extracts (SPE) of the procedural blank (Blank), reference tap water (Reference) and surface water from Heiabekken. The data depict growth of C. reinhardtii compared to the control (CT).

Appendix 5. Predicted combined toxicity

Table A3. Toxic Units (TU), sum of toxic units (STU) and Concentration factors (SCF) for a mixture of plant protection products (PPPs) for the composition of samples collected during the 2015 JOVA monitoring program, (09.06-26.06, 26.06-10.07, 10.07-27.07 and 27.07-14.08) and a solid phase extracts (SPE) from the water sample taken 10.07-27.07.

	Toxic Units (TU, EC50)				
Compound	**09.06-26.06**	**26.06-10.07**	**10.07-27.07**	**27.07-14.08**	**SPE**
Azoxystrobin				2.8E-05	
BAM*			5.0E-07		4.3E-07
Boscalid	3.5E-06	6.7E-06	1.5E-05	1.0E-04	1.1E-05
Clopyralid	2.4E-05	2.2E-05	3.5E-05	3.0E-05	0.0E+00
Fenhexamide		5.8E-06	6.0E-06	0.0E+00	3.7E-06
Imidacloprid	1.4E-06	1.3E-06	1.6E-05	7.8E-06	1.4E-05
Iprodione		1.6E-05	1.2E-04		6.8E-05
Mandipropamid			6.1E-07	1.6E-06	2.0E-06
MCPA[a]	1.2E-04		6.8E-05	4.4E-05	3.7E-05
Metalaxyl	6.2E-05	6.7E-05	2.0E-04	1.7E-03	1.5E-04
Metamitron		1.4E-04	6.3E-04	1.2E-03	1.6E-04
Metribuzin	1.0E-02	1.1E-03	4.5E-03	1.0E-02	1.3E-03
Pencycuron	9.7E-05	1.9E-03	6.3E-04	6.7E-04	2.5E-04
Propamocarb			2.2E-06	1.3E-05	1.8E-06
Prosulfocarb			1.2E-03		2.8E-04
Prothioconazole-desthio			1.8E-04	4.8E-04	2.1E-04
Tebuconazole		6.6E-06			
STU	1.0E-02	3.2E-03	7.6E-03	1.4E-02	2.4E-03
SCF (1/STU)	97	309	131	70	412

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