Norwegian University
of Life Sciences

Master's Thesis 2017 60 ECTS
Faculty of Environmental Sciences and Natural Resource Management

Characterization of pesticide hazard and risk to freshwater algae under ecologically relevant exposure conditions in Norway

Linn Anette Haug
Master of Science in Environment and Natural Resources –
Specialisation Environmental pollutants and ecotoxicology

Acknowledgements

This is the final project at the Master of Science in Environment and natural resources – Specialisation in Environmental Pollutants and Ecotoxicology at the Norwegian University of Life Sciences. This project was conducted in collaboration with and funded by The Norwegian Institute for Water Research (NIVA).

The water sampling and chemical analyses was conducted by Geir Tveiti, Hans Ragnar Norli and Sven Roar Odenmarck (NIBIO), and the solid phase extractions (SPE) was performed by Kine Bæk (NIVA).

First and foremost, I would like to thank my main supervisor; Senior researcher Knut Erik Tollefsen, and my co-supervisor; Research scientist Tânia Gomes, for their excellent guidance and support during this process. I also wish to thank Lene Fredriksen, Li Xie, You Song and Ana Catarina Almeida for valuable support in the laboratory, and everyone I got to know at NIVA, especially Natasa, Stefanie and David. You made every day a wonderful day.

I would also like to thank my friends and family for all the valuable help with reading and discussing this thesis. Finally, a huge thank you to Bjørn-Erik, for all the motivation and great company during these stressful times, I couldn't have done it without you <3

Sammendrag

Balansen i det akvatiske økosystemet er under konstant press fra menneskelig påvirkning, og store mengder forurensende stoffer slippes ut i vannforekomster i hele verden. Blant disse er pesticider en stor kilde til utslipp, og dette skyldes i stor grad avrenning fra jordbruk og skogbruk. Et pesticid er ethvert stoff eller blandinger av stoffer produsert med det formål å forhindre, ødelegge eller begrense en skade eller sykdom på levende organismer.

I Norge er bruken av sprøytemidler hovedsakelig nedadgående, bortsett fra når det gjelder herbicider, som har hatt en svak økning de siste årene. Herbicider utgjør den største andelen av sprøytemiddelbruk i Norge, og utgjorde 69,5% av bruken i 2014. Individuell risikovurdering er gjort for stoffene som er godkjent for bruk i Norge, men det er en økende bekymring omkring blandinger av disse stoffene og de mulige negative effektene disse blandingene kan ha på det akvatiske miljøet rundt spesielt jordbruksområdene.

I løpet av sommeren 2015 (juni-august) ble det tatt vannprøver fra Heiabekken, Råde kommune, i forbindelse med overvåkningsprogrammet JOVA. I et samarbeid mellom Norsk institutt for vannforskning (NIVA) og NIBIO, ble det gjennomført ekstraksjon og kjemiske analyser av disse vannprøvene for blant annet å undersøke tilstedeværelsen av pesticider. Ved hjelp av en kumulativ toksisitetsvurdering basert på innsamlet litteratur, ble de største bidragsyterne for toksisitet i blandingen identifisert. Det ble gjennomført toksisitetstesting (72-timers veksthemming og 5-timers PSII-aktivitet) av en miljørelevant syntetisk pesticidblanding og de to viktigste herbicidene i blandingen, metribuzin og metamitron, på ferskvannsalgen *Chlamydomonas reinhardtii*.

Eksperimentene viste at den syntetiske blandingen måtte oppkonsentreres ~100 ganger i forhold til de målte miljøkonsentrasjonene for å gi en 50% veksthemming, men at både blandingen og enkeltstoffene hadde en tydelig redusert dose-responskurve ved økende konsentrasjoner. PSII-aktiviteten ble også tydelig påvirket, spesielt av metribuzin som er regnet som den mest potente risikodriveren for alger i blandingen.

Den kumulative toksisitetsvurderingen basert på CA-prediksjonsmodellen ble sammenliknet med de eksperimentelle toksisitetsstudiene gjort på *C. reinhardtii*. Giftigheten av blandingen ble relativt godt estimert, innenfor en faktor på 2, i forhold til de eksperimentelle studiene.

Abstract

The aquatic ecosystem balance is under constant pressure from human influence, and substantial amounts of pollutants are released to water bodies all over the world. Among these, pesticides are an important group, mainly due to runoff from agriculture and forestry. A pesticide is any compound or combinations of compounds produced with the aim to prevent, destroy or mitigate a pest.

In Norway, the use of pesticides is in general decrease, except for herbicide use, which has had a weak increase in recent years. Herbicides make up the largest proportion of pesticide use in Norway, constituting 69.5% of the use in 2014. Individual risk assessment is performed for the compounds approved for use in Norway, but there is an increasing concern regarding mixtures of these compounds and the possible adverse effects these mixtures may have on the aquatic environment around agricultural areas.

During the summer 2015 (June- August) water samples were collected from Heiabekken, Råde municipality, in conjunction with the monitoring programme JOVA. In a collaboration between NIVA and NIBIO, extractions and chemical analyses of these water samples were performed to investigate the presence of pesticides. By a cumulative toxicity assessment based on collected literature, the main contributors to toxicity in the mixture was identified. Toxicological testing (72 h growth inhibition and 5 h PSII-efficiency) was conducted on an environmentally relevant synthetic mixture of pesticides, and the two main herbicides in the mixture, metribuzin and metamitron, to the freshwater alga *Chlamydomonas reinhardtii.*

The experiments showed that the synthetic mixture had to be concentrated up to a factor of ~100 compared to the measured environmental concentrations, before a 50% growth inhibition could be seen. Still, both the synthetic mixture and the single compounds had a clear dose-response reduction with increasing concentrations. PSII-efficiency was also clearly affected, especially by metribuzin, which is regarded as the most potent risk driver for algae in the mixture.

The cumulative toxicity assessment with the CA prediction model was compared to the experimental toxicity studies conducted on *C. reinhardtii*. The toxicity of the mixture was well estimated, within a factor of 2, compared to the experimental studies.

Contents

Introduction

Background

There are several anthropogenic activities that affect the balance of ecosystems, and emission of toxic metals and organic chemicals (POPs, PCBs, PPPs) is one of them. These emissions can be either deliberate, as in agricultural context, or by leaching, and accidental spills (Hartgers et al. 1999).

Around 300 million tons of synthetic compounds are used in industry and consumer products every year. In addition, a lot of pollution comes from diffuse sources of agricultural activities, where 140 million tons of manure and several million tons of pesticides are added yearly (FAO statistical database 2006, according to Schwarzenbach et al. 2006).

A pesticide is any substance or mixture of substances produced to prevent, destroy or mitigate a pest. Pesticides are divided into herbicides, insecticides, fungicides, depending on the target pest species. Pest species includes any living organism that may cause damage and economic loss, or transmits or produces disease. Examples of pest species are insects, birds, rodents, unwanted plants, or microorganisms.

In Norway, the use of plant protection products is in general decrease each year, except for herbicide use, which has had a weak increase of 2,2 % in use from 2005 to 2014. Herbicides are the main type of pesticide used on crops in Norway, and constituted 69,5% of the pesticide use in Norway in 2014 (Statistics Norway)

Several pesticides are used during the growth season in Norway. Individual risk assessment for these pesticides are, and has been, performed to identify potential environmental effects, but the possible effects caused by combinations of these compounds are often unknown. Therefore, focus has increased both nationally and internationally on developing methods to predict the combined effects of complex pollutant mixtures (Petersen et al. 2015; Cedergreen et al. 2008).

The aim of this thesis is to characterize and evaluate hazard and risk of pesticides to algae as non-target organisms under Norwegian conditions with the use of prediction tools and experimental studies.

Theory

Pesticides

Surface waters are being subjected to increasing pressure because of contamination by various mineral and organic pollutants. Most of the organic pollutants are pesticides, which are not only used in agriculture and forestry, but also for weed clearance in road verges and railway tracks (Villeneuve et al. 2011). The term pesticides mainly include herbicides, fungicides and insecticides, all organic compounds designed to prevent attacks from, and/or kill, unwanted organisms. The Mode of Action (MoA) of main classes of pesticides are presented in table 1 below.

Table 1. MoA of main classes of pesticides on target organisms (according to DeLorenzo et al. 2001)

Main class	Functional groups	General mode of action	Site of action
Organophosphates	Carbamates	Nervous system inhibition	Acetylcholinesterase
Organochlorides	Cyclodienes	Nervous system inhibition	GABA-receptor
Herbicides	Acylanilides, ureas, cyclic ureas, phenylcarbamates, triazines, triazinones	Photosysnthesis inhibition	Photosystem II (PSII), Hill reaction in the electron transport
	Bipyridiniums	Photosynthesis inhibition	Photosystem I (PSI), light reaction
	Pyridazinones	Biosynthesis inhibition	Carotene accumulation
	Chloroacetamide	Biosynthesis inhibition	Fatty acid synthesis
	Dinitroanilines, phosphoric amides, chlorthadimethyl, propyzamide, cholchicine, terbutol	Biosynthesis inhibition	Microtubule formation
Broad-spectrum biocides	Chlorophenols	Multiple inhibiting actions	Phosphorylation, protein synthesis, lipid biosynthesis
	Tributyl tins, trialkyl tins	Respiratory system inhibition	Mitochondrial ATPase

There are a vast number of pesticides in use, with major differences in properties and effects, as seen in the table above. Organophosphates and organochlorides are mainly insecticides, acting by disrupting the nervous system, whereas herbicides predominantly act on various pathways of the photosynthesis (Table 1).

However, the MoA of pesticides might not be the same in target and non-target organisms (DeLorenzo et al. 2001). Some pesticides may actually be much more toxic to non-target organisms than the organisms they were meant to defeat. The pesticides often end up in nearby water bodies, where the major concern is on how this might affect the non-target aquatic organisms (Almeida 2015).

Herbicides constitute an important class of pesticides, especially in forestry and agriculture, where they are used to control weeds and unwanted vegetation. It can also be used to defoliate trees, as the so-called Agent Orange used by the U.S. military during the Vietnam war. Many of the herbicides in use, such as ureas and triazinones, act by inhibiting the Hill reaction of electron transport, thereby affecting the Photosystem II (Almeida 2015; DeLorenzo et al. 2001).

Monitoring of exposure in agricultural environments globally and in Norway

The available information on herbicide contamination of surface freshwater ecosystems varies considerably between countries. There is a lot of information available in Europe and North America, less in Asia and South America, and almost no information regarding herbicides in surface waters of Africa. Villeneuve et al. (2011) explains this with the fact that there has not been a heavy use of herbicides in Africa, Asia and South America compared to the other regions, even though this has changed in the last decades.

Even though the quantity of herbicides in surface waters varies between regions, all surface freshwater ecosystems now seems to be contaminated by herbicides to some extent. However, the herbicide concentrations depend on the size and land use of the watersheds, in addition to seasonal differences in both weather and agricultural activities (Villeneuve et al. 2011; Neumann et al. 2003). When it comes to agricultural areas, the amount of

pesticides contaminating water bodies also depends on the methods, intensity and agricultural practice (Huber et al. 2000).

Photosynthesis-inhibiting herbicides are deliberately released into the environment by agricultural activities, mainly during the spraying season and in periods of high precipitation. The presence of herbicides in the environment may however also be caused by leaching and accidental spills. Either way, the herbicides released may affect non-target aquatic organisms (Lürling & Roessink 2006).

The Norwegian Agricultural Environmental Monitoring Programme (JOVA) is a national programme responsible for monitoring the soil and water in agriculture dominated catchments in Norway. This programme was initiated in 1992 with the aim to document the effects of agricultural practices on runoff and water quality to the nearby watersheds. Today there are 13 catchments being monitored, and JOVA has established a database with long time-series of data for soil erosion, nutrient runoff, and pesticide loss in the context of agricultural practices. The catchment areas being monitored are representative for various agricultural locations in Norway, especially focusing on regions with intensively cropped areas and areas with a high density of livestock, which has a higher risk of polluting the water bodies. The Norwegian Food Safety Authority (Mattilsynet) is responsible for the approval of pesticides in Norway, and use the results from JOVA in this work (www.nibio.no/jova). JOVA is the best developed programme for monitoring agricultural activities in Norway, and their monitoring results will be used as a basis for this study.

In the water sampling from Heiabekken catchment in 2014, all samples contained pesticides; six herbicides, six fungicides and two insecticides, in total detected 52 times through the period April- November. Herbicides were detected 16 times through the water sampling season (Hauken & Stenrød 2016).

Adversity and Mode of Action (MoA)

Standardized procedures and guidelines for toxicity tests have been developed in the last decades. Results from such tests are required by authorities to be able to allow or ban various chemicals. The objective of these tests is to rank chemicals by their environmental toxicity, to be able to use these results in general environmental hazard evaluations (Nyholm & Källqvist 1989).

Toxicological principles

Endpoints

To be able to measure toxicity, one needs to have an endpoint. The endpoint is what we look for in the test organism. An endpoint can be stochastic, an all-or-none endpoint, or it could be deterministic. Deterministic endpoints are endpoints all individuals reach to a varying degree, but they are dose-dependent. The main endpoints in ecotoxicology are lethality, reduction in reproduction, growth inhibition and behavioural change. Stochastic endpoints are described as a response, while deterministic endpoints are described as effect (Stenersen 2004).

Dose- response/effect

The amount of reaction products and the velocity of a chemical reaction increase with the reactant concentrations. This is explained by the law of mass action, and means that there will always be a positive relation between the dose of a chemical and the degree of poisoning This is often illustrated by the famous words of Paracelsus (1493-1541), “All substances are poisons; there is none which is not a poison. The right dose differentiates a poison from a remedy” (Walker et al. 2012, p.97).

The figure below (figure 1) illustrates a hypothetical example of various S-shaped curves that are often found when testing several groups of species, each with a high number of individuals, for a response or an effect of a given stressor. The response or effect is defined as the number of individuals getting symptoms higher than the toxicity threshold (Stenersen 2004, p.21). The toxicity threshold marks the lowest concentration of a substance that consistently results in symptoms of toxicity, and should be found in the area between the No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC).

These two values may be determined if a higher dose gave an effect in the same toxicity test (Walker et al. 2012).

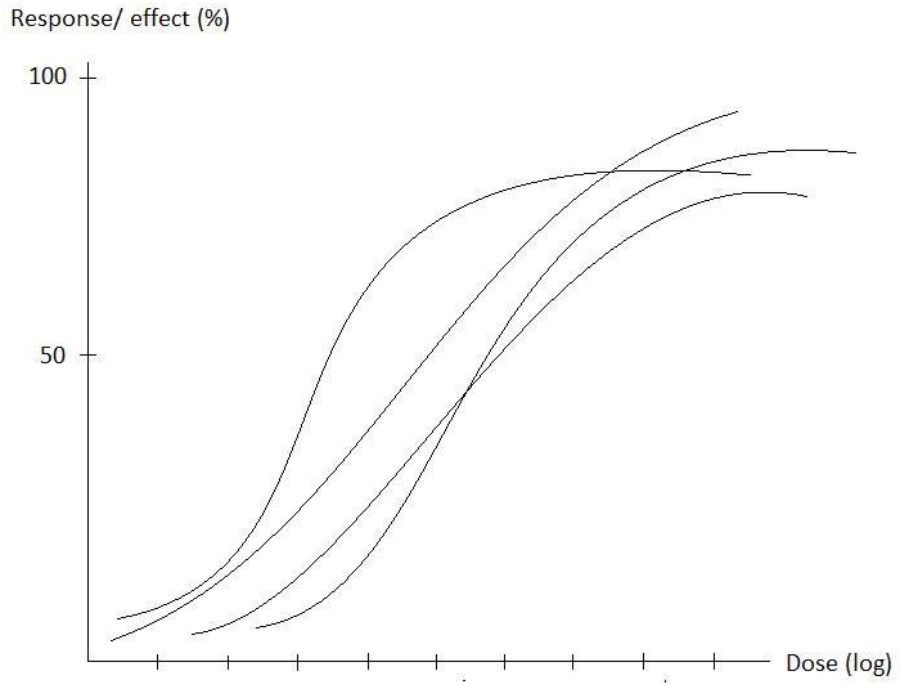


Figure 1. A hypothetical example of the effects (%) on four species of algae of a chemical stressor at different doses (log).

Effect values

The traditional method used in the evaluation of toxicity is to determine the lethal concentration where 50 % of the individuals in the test die (LC_{50}). However, this approach is not regarded as a fully adequate parameter anymore (Walker et al. 2012). This is because all test species experience a certain degree of adverse effects, often long before they are exposed to a lethal concentration of the current stressor (Juneau et al. 2002), and these adverse effects should be accounted for in the toxicological assessment.

When measuring the endpoints, the effect values that are used are often the No Observed Effect Concentration (NOEC) and various forms of Effect concentration x (EC_x), where x represents the percentage of individuals experiencing the measured effect. Most often, the EC_{50} are used, which means the concentration in which 50% of the test population are

experiencing the measured effect. This is an important concentration level, but there has been an increasing focus on also including the EC_5 and/or EC_{10} in the evaluation, to make sure to protect the most sensitive species as well as the more robust, in addition to account for the possibility of combined effects (Connon et al. 2012).

The adversity caused by a certain stressor is measured and described by the specific endpoints, i.e. growth inhibition, while Mode of Action (MoA) explains the reason for the adversity shown by endpoint analyses. The term Mode of Action can be defined as the series of key processes starting with the interaction between a chemical contaminant and a target site (e.g. receptor). It continues through functional and/or anatomical changes in an organism, leading to sub-lethal or lethal effects (US EPA 2000, according to Beyer et al. 2014).

One of the main approaches used to fill the need for a greater understanding of the toxicity of chemicals is the endpoint assay of growth inhibition (GI). The GI-assay makes it possible to determine at which concentration of the current stressor, the adverse effects begin.

The photosystem II (PSII)-efficiency assay is shown to be a good approach to determine the MoA of especially herbicide stressors, which eventually leads to adverse effects on growth. Herbicides are the most widely used type of pest control, and many commercial herbicides act by binding to photosystem II, which is a pigment-protein membrane complex, thereby resulting in photosynthesis inhibition (Esperanza et al. 2015; Schuler & Rand 2008; Eullaffroy & Vernet 2003). The PSII-efficiency assay can give insight to whether the stressors affect the photosynthesis, or more accurately the photosystem II, and which part of the photosystem II is affected. The PSII-efficiency assay is generally used on plants, but is increasingly regarded as a method also applicable for green algae in the laboratory (Juneau et al. 2005).

Growth inhibition (GI) assay

The Organization for Economic Cooperation and Development (OECD) recommend a base set of ecotoxicological tests, and a growth-inhibition test with freshwater algae is one of these. This test is also legally demanded in the European Common Market countries for chemicals produced in quantities over 100 tons annually (Nyholm & Källqvist 1989).

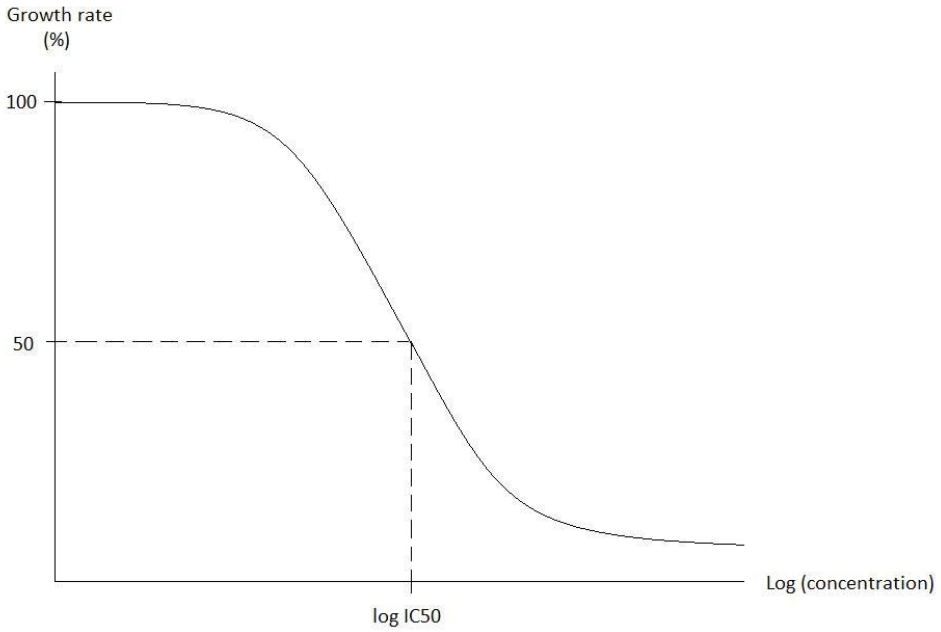


Figure 2: Example of a growth inhibition (GI) curve, showing the relationship between growth rate (%) of the test organism and the concentration of given stressor.

The GI-assay is a relatively easy method for evaluating the potential impact of certain stressors, i.e. chemicals, on the algal growth rate compared to the control algae not exposed to the given chemical. The graph showing the relationship between growth rate and concentration (see example above in fig. 2), give a good indication of which concentration of a given stressor is needed before the growth of the test organism is affected.

Before the implementation of the GI-test as a standard test, there had been a debate on which of the two endpoints; growth rate or biomass, was the more favourable one. Biomass (or the biomass integral) as a parameter is argued to be more sensitive than growth rate, as well as taking into account the changes in the growth pattern induced by the chemical(s) (Weyers & Vollmer 2000).

However, Weyers & Vollmer (2000) have also pointed out three reasons to support the use of growth rate as an endpoint; the ecological, practical, and the theoretical reasons. The ecological reason is that growth rate is more ecologically relevant, since the algal population is more influenced by rates than by absolute numbers (i.e. sedimentation, grazing).

The practical reason is that growth rate is more robust against deviations in the test conditions. Since the biomass results depend to a large degree on e.g. control growth rate,

nutrients and light (Nyholm & Källqvist 1989), it is only possible to compare rate values, and not biomass values, from different studies with slightly different test designs.

The theoretical reason for choosing growth rate as an endpoint in the growth inhibition study, is the seeming sensitivity of the biomass values to be attributed to the exponential growth during the test. This depends entirely on the duration of the test. Because of this, only results from tests with identical duration can be compared.

In their study, Weyers and Vollmer (2000) concluded that the growth rate endpoint is only slightly less sensitive than the biomass endpoint. Because of the reasons given above, and since both endpoints can be easily calculated from the same data, the growth rate is regarded as an excellent parameter.

PSII-efficiency assay

The photosynthesis is a complex and tightly integrated series of redox reactions and enzymatic processes regulating the survival of photosynthetic organisms like microalgae. The light energy, which is absorbed by chlorophyll molecules (e.g. photosystem antennae), can be used in three competing processes. It can be 1) transferred through the electron transport chain to fix carbon. This is called photochemical quenching, 2) dissipate as heat, which is called non-photochemical quenching, or 3) become reemitted by a slightly longer wavelength as light, which is called chlorophyll fluorescence. By monitoring the chlorophyll *a* (Chl *a*) fluorescence, it is possible to assess the photosynthetic apparatus response to exposure to environmental stressors compared to the activity under normal conditions (Gomes et al. 2017; Ralph et al. 2007; Maxwell & Johnson 2000). Chlorophyll fluorometers were originally developed to assess processes associated with primary photochemistry and electron transport in the photosynthesis. This makes fluorometry a great tool for ecotoxicological research, to examine the inhibiting effect herbicides have on the photosynthetic electron transport chain (Juneau et al. 2007).

Since herbicides are developed to kill non-desirable plants mainly by inhibiting the photosynthesis (Tomlin CDS 2000), the PSII-efficiency assay may be used to determine the toxicity of compounds.

In the last decades, the use of PAM-fluorometry for assessment of herbicide effects on the photosynthesis has increased. This method has the possibility to look at the change in energy dissipation pathways in plants and other primary producers when they are exposed to herbicide toxic effects. Different fluorescence parameters can be measured and used as indicators of toxicity. Juneau et al. (2007) concludes that because most herbicides has a direct inhibition on PSII/PSI- electron transport in both plants and algae, changes in the chlorophyll fluorescence caused by herbicides, can be related to PSII-activity. Also, the fluorescence parameters may be used as reliable indicators of herbicides affecting other metabolic processes in the cell that is indirectly connected to the photosynthetic electron transport (e.g. carbon assimilation, nitrogen metabolism, and lipid, pigment or protein biosynthesis) (Juneau et al. 2007).

Fv/Fm is one of the parameters most commonly used, but because of certain uncertainties with only using one parameter, it is recommended to use a combination of several fluorescence parameters to achieve complementary information on the herbicide mode of action (Juneau et al. 2007). The dark-acclimation period required to measure Fv/Fm removes the light stress from the PSII reaction centres, reducing the influence of non-photochemical quenching. Therefore, Fv/Fm measures the optimal photosynthetic efficiency and does not reflect the true nature of PSII-activity under normal light conditions. For this reason, $^{\Phi}$PSII or Y(II) is regarded as a more sensitive indicator than Fv/Fm, despite being more difficult to interpret correctly (Ralph et al. 2007).

The use of non-photochemical fluorescence quenching offer a highly sensitive indicator of toxicity, but this parameter is seldom used for herbicide toxicity research. Juneau et al. (2007) suggests combining the studies of fluorescence parameters with other well-known toxicity indicators, e.g. EC_{50}-values. When a dark-adapted sample is exposed to light, it can take several minutes for the photosynthetic apparatus to optimise all its integrated enzymatic pathways. While the photosynthetic apparatus adjusts to the light, the energy proportions diverted to various processes fluctuates, and this phenomenon is known as the Kautsky effect (Ralph et al. 2007).

Most quenching coefficients must be interpreted carefully and separated from each other. The photochemical quenching coefficients and the non-photochemical quenching

coefficients describe the same fluorescence signal in separate ways. It is only the relative quenching coefficients qP (rel) and qN (rel) that match together, and can be used to demonstrate an energy de-excitation shift from the photochemical to the non-photochemical route (Buschmann 1999). The unquenched fluorescence parameter (UQF(rel)) considers the fraction of the unquenched fluorescence yield which is related to closed PSII-reaction centers. When these are closed, they do not participate in electron transport. This parameter is complementary to qP(rel) and qN(rel) and may facilitate the use of PAM fluorometry as a diagnostic tool in environmental and ecotoxicological studies (Juneau et al. 2005). Also, there is a linear relationship between the UQF(rel) parameter and cell density (Dewez et al. 2008). The PSII fluorescence parameters are presented with equations in table 2.

Table 2. Fluorescence parameters calculated from PAM fluorometry measurements in *C. reinhardtii* exposed to herbicides (Adjusted from Gomes et al., 2017)

Parameter	**Definition**	**Equation**	**Reference**
Fv/Fm	Maximum quantum efficiency of PSII	(Fm-Fo)/(Fm)	Schreiber, 2004
OEC	Efficiency of the oxygen evolving complex	Fo/(Fm-Fo)	Kriedemann et al., 1985
$^{\Phi}$PSII	Effective quantum efficiency of PSII	(F'm-Ft)/F'm	Genty et al., 1989
qP	Coefficient of photochemical quenching	(F'm-Ft)/(F'm-F'o)	Schreiber et al., 1986; Juneau & Popovic, 1999
qN	Coefficient of non-photochemical quenching	1-(F'm-F'o)/(Fm-Fo)	Schreiber et al., 1986; Juneau & Popovic, 1999
NPQ	Non-photochemical quenching	(Fm-F'm)/F'm	Bilger & Bjorkman, 1990
ETR	Relative photosynthetic electron transport rate	0,5 x $^{\Phi}$PSII x PAR x I_A*	Genty et al., 1989
qP(rel)	Relative photochemical quenching	(F'm-Ft)/(Fm-F'o)	Buschmann, 1995
qN(rel)	Relative non-photochemical quenching	(Fm-F'm)/(Fm-F'o)	Buschmann, 1995
*Where 0,5 is a factor that assumes equal distribution of energy between PSII and PSI, PAR is the actinic photosynthetically active radiation generated by Diving-PAM, and I_A is the assumed absorbance by the photosynthetic organism (0,84).			

Combined toxicity assessment (CTA)

Organisms in polluted environments are often exposed to a complex combination of chemical contaminants and this exposure can sometimes lead to toxic effects even though each individual stressor is present in concentrations lower than the No Observable Effect Concentration (NOEC) (Kortenkamp 2008; Brian et al. 2007; Silva et al. 2002). This phenomenon is often called combined toxicity or cocktail effect.

The toxicity of a chemical could possibly be affected by the presence of another chemical. It can either be enhanced (synergism), reduced (antagonism) or unaffected (no interaction). Synergistic effects mean that the two compounds combined results in a greater effect than the sum of each individual compounds' effect (1+2=4). Antagonistic effects mean that the combined effects are lower than the sum of each individual compound's effect (2+2=3), which is explained by one compound reducing the effect of the other. Interactions may occur in the uptake, distribution, metabolism or excretion of chemicals, which is called the toxicokinetic phase, or by acting on a receptor, cellular target or organ, which is called the toxicodynamic phase (Almeida 2015; Fernández & Beiras 2001).

When two chemicals have no effect on the toxicity of each other, the combined effect is what we call additive. Then the combined effect of the mixture of these two compounds can be predicted simply by the summation of the toxic effect caused by each individual chemical, after adjusting for the differences in toxic potencies, or toxic units (TU). (Fernández & Beiras 2001; Groten et al. 2001). The additive effects can be divided in two types: concentration addition (CA) or independent action (IA). CA refers to chemicals that act by similar MOA on the same biological site, affecting the same endpoint, and are therefore considered as dilutions of the same compound. Here, all chemicals have a similar MoA which contributes to the adverse effect. IA refers to chemicals that act by dissimilar MoA, acting on the same physiological system with different function, or acting on different systems. Then the effects will only occur when the individual compounds exceed their threshold level of effect (Almeida 2015; Altenburger et al. 2003; Groten et al. 2001).

Because the research on chemical toxicity traditionally has been performed with single compounds, potential mixture effects have not been detected. It is therefore possible that the adverse effects of combined toxicity in ecotoxicological compounds have been

underestimated. Contaminants with either similar or different Mode of Action (MoA) can influence each other's toxicity, and lead to an almost unlimited number of additive, synergistic or antagonistic effects (Kortenkamp 2008; Silva et al. 2002).

Combined toxicity may be studied by a bottom-up or a top-down approach. In the top-down approach, the models can be built based mainly on the observed data, while in the bottom-up approach, they can be based on our broader understanding of the biology of the organisms, and the compounds mechanisms of action in the organisms of interest. A bottom-up approach is often used to study simple mixtures of ≤10 chemicals, but is almost impossible to use for complex mixtures, involving tens, hundreds or thousands of chemicals with a nearly unidentified composition. The concentration addition (CA) and independent action (IA) models are prediction models normally used to predict the effect of a mixture containing many chemicals (Altenburger et al. 2003; Groten et al. 2001).

Mixture deviation ratio (MDR) may be used to determine type of combined toxicity, and model accuracy. MDR is defined as $MDR = \frac{Expected}{Observed}$ where Expected is the effective concentration of the mixture predicted by one of the models and Observed is the effective concentration for the mixture gained from experimental toxicity testing. MDR ranging from 0.5 to 2.0 could imply possible synergistic or antagonistic interaction between substances (Wieczerzak et al. 2016).

Cumulative risk assessment (CRA)

One of the main goals of aquatic ecotoxicology is to predict the effects of contaminants in ecosystems. Prediction models require that existing observations, mainly from experimental toxicity studies, may be used to generate possible scenarios present in the environment (Almeida 2015).

In risk assessment, the terms hazard and risk are important. While hazard is defined as the potential to cause harm, risk is defined as the probability that harm will be caused. Risk assessment involves a comparison of the following two factors; the toxicity of a compound towards one or more species, and the anticipated exposure of an organism to this compound (Walker et al. 2012).

Single chemical risk assessment is conducted by comparing measured environmental concentrations (MEC) with the risk threshold for each individual compound. The risk thresholds are usually derived from ecotoxicological laboratory testing (Walker et al. 2012; Almeida 2015). From toxicity tests, the NOEC and the EC_{50} can be estimated, and be compared with the MECs to see if this chemical pose a risk. If the toxicity is low (low hazard), or the environmental concentrations are low, the compound is regarded as a low-risk chemical (Walker et al. 2012).

Indicators such as risk quotients (RQs) and toxic units (TUs) are normally applied. These compare the measured environmental concentrations (MEC) of the compounds to the concentrations originating a certain effect.

The values NOEC or the Predicted No Effect Concentrations (PNEC), are used to calculate the RQs. The PNEC is estimated by dividing LC50 or EC_{50} for the most sensitive species tested in the laboratory by an assessment factor (AF) related to the endpoint and data support. This factor accounts for the great uncertainty in extrapolating data from laboratory toxicity for one species to expected field toxicity to other species (Almeida 2015; Walker et al. 2012).

The requirements for ecotoxicological risk assessment are limited to single active PPPs and formulations of these. There is currently a low level of consensus for implementation of large scale studies to address combined toxicity and cumulative risk of ecologically relevant environmental mixtures. The scientific methodology and evaluations for cumulative effect assessment of PPPs in the environment is mostly found within the research community, and has not yet been included in regulatory frameworks and guidelines (Tollefsen et al. 2016).

Because of the vast number of contaminants, and possible mixtures of these, a modelling approach might be suitable to determine possible mixture effects. The modelling approach requires the exact composition of the mixture of concern, both the number and concentration ratio of components, and the nature of these components must be known. For all the compounds, there must be available toxicity data (Altenburger et al. 2013).

There are mainly two types of prediction models to consider for CRA, Concentration Addition (CA) and Independent action (IA). Both models require that there is no evidence of synergistic effects, and that the mixtures may be encountered in the environment. The data

requirements are considerably different for the CA and IA models. While the CA model requires only the effect concentrations of single substances present in the mixture, the IA model's data requirements increases with decreasing concentration shares of the components, hence also with an increasing number of components in the mixture (Altenburger et al. 2013, Cedergreen et al. 2008).

The theoretical principle of CA is that chemicals that do not interact only varies in potency, and therefore can be regarded as dilutions of one another. Different dilutions of the same chemical will of course always have an additive effect and they will operate by the same mechanism of action (Berenbaum 1989). It is therefore often assumed that mixtures of different chemicals with the same molecular target also will have an additive toxic effect, as is the principle of the CA model. To calculate the joint effect of chemicals using the CA prediction model, it is necessary to account for the degree of dilution, according to each chemical's single-substance toxicity, and calculate the toxic units (TU) and then the sum of toxic units (STU) for all the chemicals in the mixture (Petersen et al. 2015; Cedergreen et al. 2008). When data on acute toxicity to species are the only data available, assessment factors (AF), or safety factors (normally 100-1000), are applied to the lowest lethal concentration (LC50) from three representative taxonomic groups. The taxonomic groups are usually crustaceans, fish and algae. The safety factors are supposed to protect the non-target species from adverse effects caused by the compounds (Malaj et al. 2014).

If multiple stressors are acting, a CRA approach should be used to characterize and quantify all the combined risks (EPA 2007). For the CRA, indicators such as sum of the toxic units (STU) or sum of the risk quotients (SRQ) must be applied, involving the summation of all the TUs or RQs of all the chemicals present (Backhaus & Faust 2012), The overall risk quotient for a mixture (RQ_{STU}) can also be calculated for the most sensitive taxa to the mixture, using an appropriate AF (Backhaus & Faust 2012; EU 2009). For establishing the size of this AF, the uncertainties of extrapolating from single-species laboratory data to a multi-species ecosystem must be taken into account. Uncertainties to consider are the intra- and inter-laboratory variation of toxicity data, biological variance between species and individuals, the short-term or long-term toxicity extrapolation, and the laboratory data to field impact (EC 2003).

Test organisms

Algae are a large and varied group of photosynthetic organisms. They are at the bottom of the food chain (phytoplankton) and serve as an important food source for other organisms (Lindeman 1942). These organisms' production lays the foundation for all the organisms at higher trophic levels (Wetzel 2001; Lindeman 1942).

As primary producers, microalgae play an important role in aquatic ecosystems, where they produce oxygen and energy while at the same time recycling nutrients many other organisms depend on (Gomes et al. 2017).

Because of the important ecological function of algae in fresh water ecosystems, this study aims to determine the risk of relevant pesticides on algae as a group. Because of the wide variety in algal species, there is also variety in sensitivity towards toxicity of chemicals. Rojicková-Padrtová & Marsálek (1999) found that for oxyfluorphene the variability in sensitivity was as high as five orders of magnitude. The green algae (Chlorophyta) test species in their experiment could be divided in two groups depending on their tolerance to herbicides. *Chlamydomonas reinhardtii* was regarded as one of the sensitive species, placed in the second group. However, the differences in sensitivity between the species in this group was not significant, and the authors concluded that any of these species could be recommended as test organisms in single algal assays (Rojicková-Padrtová & Marsálek 1999)

The microalga *C. reinhardtii* was chosen as a representative organism in the experimental study of this project, and will be compared to other species of algae regarding sensitivity towards the chosen pesticides.

C. reinhardtii is a unicellular green alga often used in ecotoxicity studies because of its rapid growth rate and other characteristics making it suitable for laboratory culturing and testing. The alga is sensitive to many contaminants, and its complete biology has been thoroughly described. Its entire genome has also been sequenced, so that several molecular and genomic tools are available. (Gomes et al. 2017; Harris 2009; Merchant et al. 2007). This microalga can therefore be very suitable for studying adaptive responses on a cellular and molecular level after exposure to organic contaminants.

Effects of pesticides in algae

Any adverse impact on algae is likely to affect organisms at higher trophic levels, both by direct toxicity, but also by reducing the occurrence of algae in the ecosystem, leading to a change in the species composition, and thereby resulting in negative consequences for the entire aquatic ecosystem (Franklin et al. 2000)

Several studies have indicated that exposure of algal communities consisting mainly of green algae and cyanobacteria to typical concentrations of the triazinone herbicides metamitron and metribuzin, could alter the species composition in favour of the cyanobacteria (i.e. Lürling & Roessink 2006; Brock et al. 2004).

Despite extensive regulation and technological advances, pesticides continue to threaten non-target species, especially those groups with similar physiology to pest species. Herbicides, as expected, accounted for most of the exceedances in algae (Malaj et al. 2014).

Malaj et al. (2014) performed a continental scale chemical risk assessment encompassing fish, invertebrates and algae in freshwater ecosystems. They compared the measured concentrations of 223 chemicals in 91 European river basins with available toxicity information for the chosen organism groups to determine the spatial distribution of chemical risk on a continental scale. They found that pesticides were responsible for 96% of the exceedances of acute risk threshold related to algae (Malaj et al. 2014).

Objectives

This thesis aims to characterize the hazard and risk of pesticides to freshwater algae as non-target organisms under Norwegian conditions, with the use of experimental studies and prediction tools.

Based on the findings of fifteen pesticides in water samples collected from Heiabekken, I will make a synthetic mixture to determine the combined toxic effect of these pesticides. This will be performed by 72 h growth inhibition and 5 h Photosystem II-efficiency tests on the green algae *C. reinhardtii*. The results will be compared to the cumulative risk assessment of the same compounds performed based on the NIVA risk assessment database (NIVA Radb).

Then I will perform 72 h growth inhibition assessment and 5h Photosystem II-efficiency assessment for the two main herbicide stressors in the mixture, metribuzin and metamitron, to evaluate *C.* reinhardtii's sensitivity towards these two compounds, to determine if *C. reinhardtii* is a suitable test species for these compounds and mixtures of these. In addition, the experimental effort aims to further determine the cause of pesticide toxicity to green algae, by assessing the chlorophyll *a* (Chl *a*) fluorescence and corresponding parameters in the photosystem II.

Materials and methods

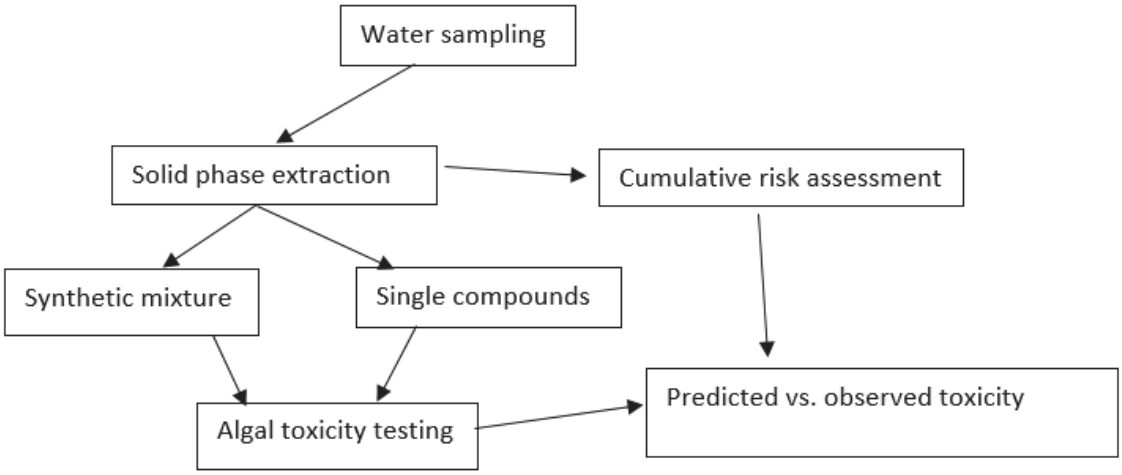


Figure 3: Overview of the different activities and the correlation between them.

Figure 3 illustrates the correlation between the various parts of this project. The water samples from Heiabekken undergo solid phase extraction (SPE). These extracts are subject to chemical analysis, to be able to identify the components and their percentage of the total mixture. This will further be used to produce a synthetic mixture for experimental studies, and to assess the cumulative risk of the components present in Heiabekken. The identification of main herbicide risk drivers will form the basis for the selection of single compounds to test in the algal toxicity experiments, in addition to the synthetic mixture.

The algal toxicity testing and the cumulative risk assessment will then provide the basis for determination of the predicted vs. the observed toxicity, hence determine if the modelling approach successfully predicts the toxicity of the environmental mixture of pesticides.

Field sampling

Surface water sample collection

Water samples were collected from Heiabekken in Råde municipality (figure 4). This is a stream in Heia catchment classified to be in a very poor ecological condition (Vann-nett 2016), and has therefore been included in the Norwegian Agricultural Environmental

Monitoring Programme (JOVA), which is a national programme for soil and water monitoring in agriculture dominated catchments in Norway (www.nibio.no/jova). Heiabekken has outlet to Kurefjorden, an important wetland and nature reserve (Ramsar convention).

Water sampling for this project were conducted regularly through the summer of 2015 by NIBIO and stored until use. Detailed information regarding water sampling can be found in Appendix 1.

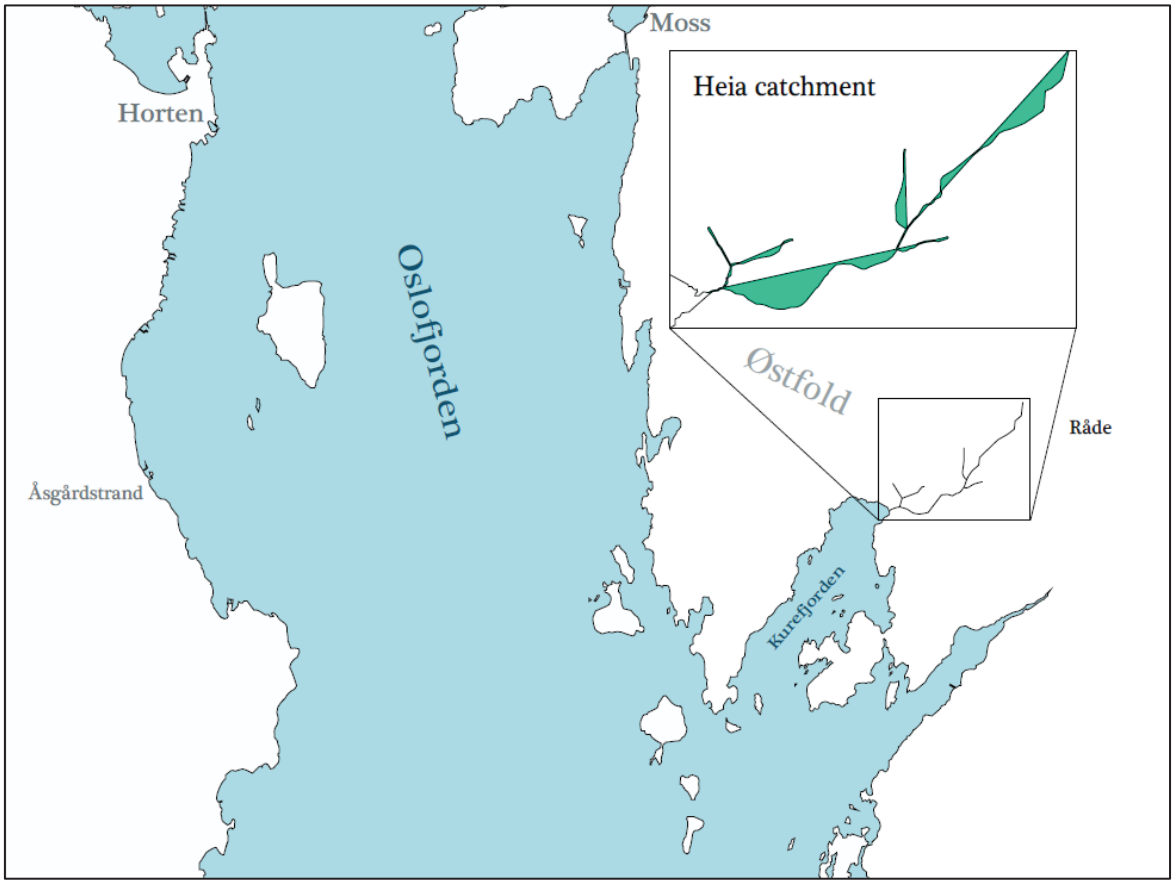


Figure 4. Heia catchment, Råde municipality, with outlet to Kurefjorden, Østfold. Made in Adobe™ Illustrator.

Extraction and analysis of water samples

The active PPP substances in the water samples were extracted by solid-phase extraction (SPE) using 1 gram Strata-X-CW 33 µm columns (Phenomenex, Torrance, CA, USA).

SPE is commonly used 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 and properties of SPE and whole water samples is given in appendix 2.

The thawed water samples (5,5 L) were decanted equally to five glass bottles. The five water samples were loaded individually to five separate SPE columns (3 mL/min) that had been activated and prewashed with 40 mL acetonitrile and 40 mL MilliQ water.

After drying of the SPE columns, they were eluted with 20 mL acetonitrile containing 5% methanoic acid and the eluates were pooled and evaporated to dryness under nitrogen (40°C). The samples were resolved in 5 mL methanol and evaporated to 1 mL before being filtered through a 0,2 µm Nylon Spin-X Centrifuge Tube filter (Costar, Corning, NY, USA).

The filters were then washed with 0,5 mL methanol to ensure collection of all sample material, before the samples were evaporated to 110 µL total volume. The resulting eluates were then merged and subjected to chemical analysis by LC-MS/MS and GC-MS/MS (detailed method description in Tollefsen et al. 2016).

The solid phase extractions (SPE) were performed by NIVA, and the analyses of the extracts were performed by NIBIO.

Cumulative risk assessment (CRA)

Ecotoxicity data for algae, aquatic plants, crustaceans and fish were collected from various databases, as well as data from European Food Safety Authority (EFSA) and NIBIO, to develop a NIVA risk assessment database (NIVA Radb). These data were then used to calculate predicted no effect concentrations (PNEC) using prediction models assuming Concentration Addition (CA). Measured environmental concentrations (MEC) of the active pesticides at the monitoring site of Heiabekken catchment were obtained in collaboration with the Norwegian Agricultural Environmental Monitoring (JOVA) programme. The observed and the modelled mixture are compared, as shown in figure 5 below, to determine the model's suitability for predicting the toxicity.

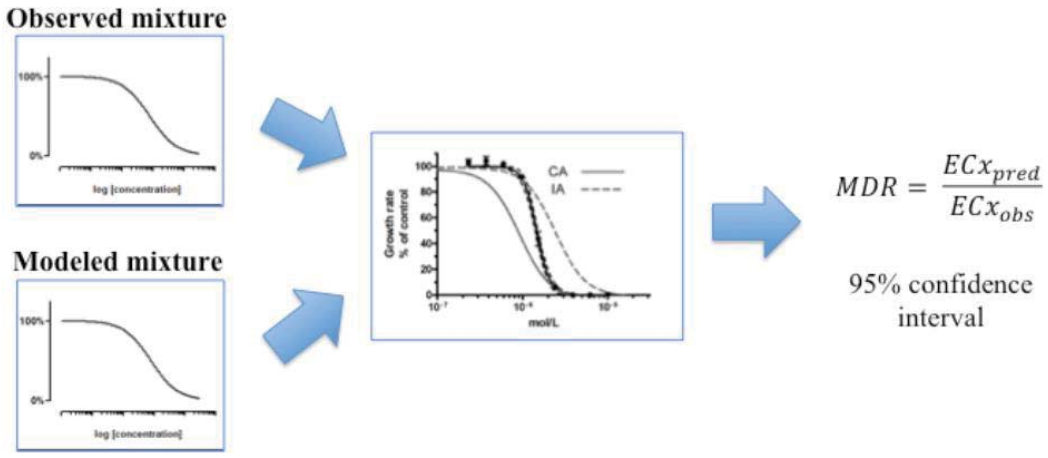


Figure 5. Schematic representation of the cumulative risk assessment (Reproduced with permission from Almeida 2015).

The CA model is expressed by $ECX(mix) = (\sum_{i=1}^{n} \frac{pi}{ECxi})^{-1}$ where ECXmix is the total predicted effect concentration of the mixture that induces an effect X, pi is the relative fraction of component i in the mixture and ECxi is the concentration of substance i inducing an effect when acting alone (Almeida 2015; Altenburger et al. 2013).

If the CA curve is not significantly different from the observed effects, the model is considered to explain the combined effects.

The model deviation ratios (MDRs) are calculated and used as an indicator of the combined toxicity (Belden & Lydy 2006), according to this equation, $MDR = \frac{ECXpred}{ECXobs}$ where EC_{Xpred} and ECXobs are the predicted and the observed effect concentrations, respectively. Additivity is assumed if MDR values are within a factor of 2 (Belden et al. 2007).

The Risk Quotient (RQ) for each compound are calculated according to the EPA guidelines (2004, according to Almeida 2015), $RQ = \frac{MEC}{NOEC}$ where MEC is the measured environmental concentration and NOEC is the No Observed Effect Concentration for each compound. The calculation of the RQ of mixtures are performed by the equation for the Sum of RQ (SRQ): $SRQ = \sum_{i=1}^{n} RQi$ to determine the potential cumulative risk. If RQ or SRQ ≥1 indicated a potential environmental risk (Backhaus & Karlsson 2014; Backhaus & Faust 2012).

The Toxic Unit (TU) for each compound was calculated according to: $TU = \frac{MEC}{EC50}$, and the Sum of Toxic Unit (STU) were then calculated according to the equation: $STU = \sum_{i=1}^{n} TUi$, and used to describe the toxicity of the mixture. A TU or STU value ≥1 are interpreted as a potential risk of the mixture to the environment (Backhaus & Karlsson 2014; Backhaus & Faust 2012).

Finally, the RQ and STU will be combined by the following equation, to determine the overall risk quotient for the mixture (RQ_{STU}) to algae: $RQ_{STU} = \max(STU_{algae}) \times AF$, where AF is the assessment factor accounting for the uncertainties in the experimental data.

Experimental testing

Chemicals

The pesticides detected in the water sample from Heiabekken included fungicides (Fenhexamid, Pencycuron, Iprodione, boscalid, metalaxyl, mandipropamide, propamocarb and prothioconazole-desthio), herbicides (Clopyralid, MCPA, metamitron, metribuzin, 2,6-dichlorobenzamide and prosulfocarb), and one insecticide (imidacloprid). The main properties of these pesticides are presented in table 3 below.

Table 3. Main properties of pesticide compounds detected in the water sample from Heiabekken (2015).

Compound	Pesticide class	Functional group	CAS-number	Target organism	Mode of Action
Clopyralid	Herbicide	Picolinic acid	1702-17-6	Broadleaf weeds, especially thistles and clovers	-
MCPA	Herbicide	Phenoxy	94-74-6	Broadleaf weeds including thistle and dock	Systemic
Fenhexamid	Fungicide	Carboxamide	126833-17-8	-	-
Imidacloprid	Insecticide	Neonicotinoids	138261-41-3	Insects	CNS: blocking the nicotinic acetylcholine receptors
Metamitron	Herbicide	Triazinone	41394-05-2	Grass, broad-leaved weeds (beet and strawberry)	Photosystem II
Metribuzin	Herbicide	Triazinone	21087-64-9	Weeds (potato and carrot)	Photosystem II
Pencycuron	Fungicide	Phenylurea	66063-05-6	Rhizoctonia solani (plant pathogen)	
Iprodione	Fungicide and nematicide	Hydantoin		Several fungal diseases Nematodes	Blocks mycelium growth
Boscalid	Fungicide	Nicotinamide	188425-85-6	Several types of fungal diseases	Blocks germination
Metalaxyl	Fungicide	Acylalanine	57837-19-1	Pythium and Phytophthora	Systemic
2,6-dichloro-benzamide	Broad-spectrum herbicide	Benzoic acid amide	2008-58-4	Seedlings of monocot and dicot species	Reproduction/ development effects
Mandi-propamide	Fungicide	Mandelamide	374726-62-2	Oomycete pathogens on grapes, potatoes	Inhibits spore germination and cellulose synthesis
Propamocarb	Fungicide	-	24579-73-5	Oomycetes	Systemic
Prosulfocarb	Herbicide	Thiocarbamate	52888-80-9	Grass, broad-leaved weeds	Cholinesterase inhibitor
Prothioconazole-desthio	Fungicide	Metabolite of prothioconazole, which is a triazolinthione	120983-64-4	-	-

Synthetic mixture

The fifteen pesticide compounds detected in the water sample analyses were combined to make a synthetic mixture equivalent to that of the sample from Heiabekken. Each compound was first dissolved in 1 mL dimethyl sulfoxide (DMSO), before they were combined according to the table in appendix 3, resulting in the synthetic mixture stock solution with concentration factor of 300.000 compared to the environmental concentrations (T15). An illustration of the process is shown in figure 6.

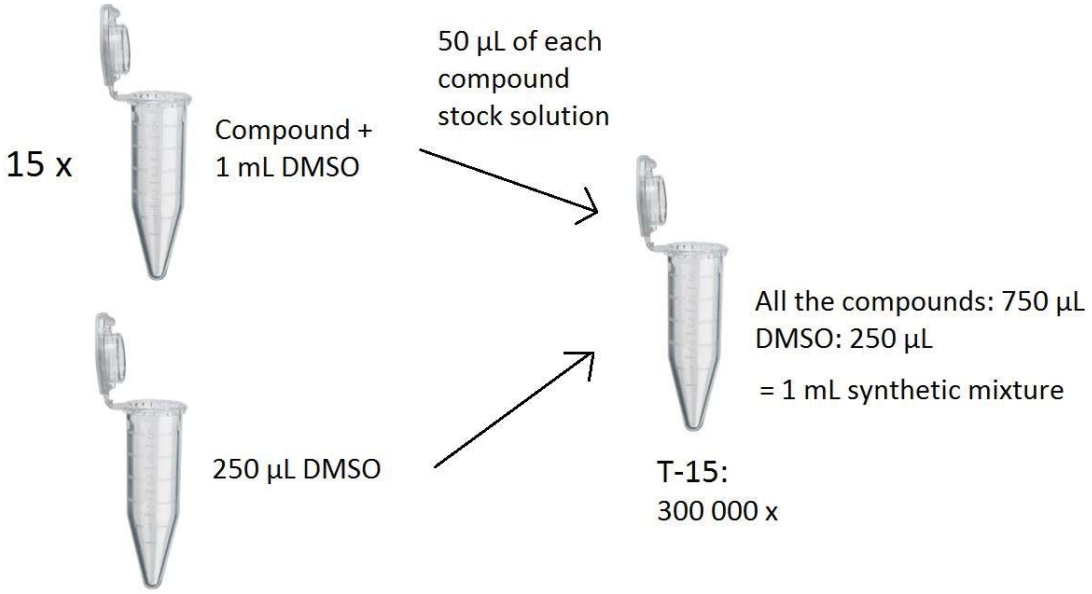


Figure 6. Illustration of the synthetic mixture making. 50 µL stock solutions of each compound was combined with 250 µL DMSO.

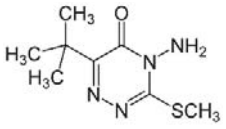
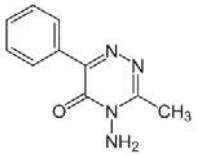
The synthetic mixture, with a concentration factor of 300.000 to the Heiabekken sample was diluted further to gain the concentration series from concentration factor 300.000 to 100. When these were added to the wells together with algae and high-salt media (HSM), they were diluted further, gaining a dilution series with concentration factors from 3000 to 1 compared to the environmental concentration (see Appendix 3 and 4 for more details).

The properties of all compounds present in this mixture are described in table 3 above. The two single compounds used in experimental testing are described further in the next section.

Single compounds

The two main herbicide stressors present in the environmental water sample were used for experimental testing as single compounds, to compare the effects of the synthetic mixture to the effects from the single compounds regarded as being the main risk drivers. The properties of the two herbicides, metribuzin and metamitron are described in table 4.

Table 4. Properties of the two main herbicides present in the mixture used for experimental testing of single compounds

Properties	Herbicides	
Functional group	Triazinones	
Compound	Metribuzin	Metamitron
CAS number	21087-64-9	41394-05-2
IUPAC name	4-amino-6-tert-butyl-3-(methylthio)-as-triazin-5(4H)-one	4-Amino-3-methyl-6-phenyl-1,2,4-triazin-5-one
Structural formula		
Target organism	Used both pre- and post-emergence in crops including soy bean, potatoes, tomatoes and sugar cane	Grass and broad-leaved weeds in beet and strawberry crops
Mode of Action (MoA)	Inhibiting photosynthesis by disrupting photosystem II	Inhibiting photosynthesis by disrupting photosystem II

Both metribuzin and metamitron are triazinone herbicides widely used in agriculture and regularly detected in surface waters. Metribuzin is through its inhibition of the electron transport in PSII (Eullaffroy and Vernet 2003), regarded as extremely toxic to non-target aquatic plants. Metamitron also inhibits photosystem II, but the toxicity of metamitron is lower than metribuzin (Fairchild et al. 1998).

Algae culturing

Exposure experiments were performed using the unicellular freshwater alga C. reinhardtii (NIVA-CHL153; Norwegian Institute for Water Research, Oslo, Norway), grown in high-salt growth medium (HSM; Harris 2009, p. 242) with an initial number of 107 cells/L. The C. reinhardtii cultures were kept for 3 days in 1 L of HSM at 20±2°C, with orbital shaking at 90 rpm under continuous illumination (93±6 µmol/m^2/s) provided by cool-white fluorescence lamps (TLD 36W/950, Philips, London, UK) in an Infors Multritron 2 incubator (Infors AG, Bottmingen, Switzerland) to ensure that cultures were in the exponential growth phase before ready for experimental studies.

All glass material used for the preparation of the media and experiments was appropriately washed and autoclaved prior to use to avoid microbial contamination. Culture samples were regularly observed under the microscope to detect microbial contamination.

Growth inhibition assay

Three days prior to testing, an algae inoculum must be prepared, by transferring 200 µL from the previous algae inoculum to a new 100 mL Erlenmeyer flask containing approximately 50 mL high-salt-medium (HSM). This inoculum should cultivate under the same conditions as during the testing (20±0,5°C, continous light, 100 µmol/m^2/s, 90 rpm).

The estimation of algae density of the inoculum was performed with the Coulter Multisizer Counter. The estimated value was then used to determine the amount of algae inoculum needed for the tests, and how much HSM needed for the dilution.

The concentration of algae needed for each well in the 96-wells microplate was 10x10^3 cells/mL, and therefore an inoculum with 20 x 10^3 cells/mL was made according to the formulas 1) and 2).

1) $$\frac{20 \times 10^3\ cells\ /mL}{number\ of\ cells\ in\ inoculum} \times 10\ mL = \ x\ mL\ inoculum\ to\ add$$

2) $$10\ mL - x\ mL\ inoculum = y\ mL\ HSM\ to\ add$$

The algae solution was added to the 96-wells microplates (Falcon™, Oslo, Norway), together with positive and negative controls, and blank wells. Each concentration in the dilution series was tested with 4 replicates.

After 0, 24, 48 and 72 hrs, the density of algae in the microplates was determined by fluorescence measurements in the Cytofluor (Fluorescence multi-well plate reader, ThermoFisher Scientific, Applied Biosystems, USA). In this programme, the cells will be excited at wavelength 485 nm, and the emission will be determined at wavelength 685 nm.

To achieve the algae growth rate, the blank values were subtracted for each measurement, and the growth was calculated by formula 3).

3) $$\mu/_d = \ln(T1) - \ln(T0)\,\frac{\ln(T1)-\ln(T0)}{hours} \times 24hrs \qquad for\ 24{,}48{,}72hrs \qquad |\times 10^3$$

Photosystem II-efficiency

PSII efficiency of *C.reinhardtii* exposed to various herbicides was determined following Herlory et al. (2013) and Juneau et al. (2002) with minor adjustments in the experimental conditions used in these studies. Algal cells were exposed to a dilution series of metribuzin, metamitron and a synthetic mixture of the PPPs found in Heiabekken water sample, for 5hrs. The final algal concentration was 3 million cells/200 µL of HSM in black 96-wells microplates (Corning Costar, Cambridge, MA, USA) under ambient light at room temperature (20±1°C).

Immediately before each exposure experiment, algal cells were collected by centrifugation at 7000 rpm for 15 minutes (Avanti J-265 XP Centrifuge, Beckman Coulter, California, USA) for removal of growth medium. The HSM was then decanted and cells washed with 50 mL deficient HSM (medium lacking EDTA and trace metals) before a new round of centrifugation at 7000 rpm for 10 minutes. Washed cells were re-suspended in 10 mL of deficient HSM and centrifuged at 3000 rpm for 10 minutes and the resulting pellet was resuspended in 2 mL of deficient HSM. Cell number was counted with a Multisizer counter (Beckman Coulter Counter Multisizer 3, Miami, USA) and adjusted to an initial cell density of 18 x 107 cells/mL in 6 mL of deficient HSM.

Chlorophyll *a* fluorescence was determined by a PAM fluorometer (Diving-PAM, Heinz Walz GmbH, Effeltrich, Germany) and fluorescence parameters were calculated according to the formulas expressed in Table 2.

Upon reading of the plates, C. reinhardtii cells were dark-adapted for 20 min to allow complete oxidation of PSII reaction centres before the minimum and maximum fluorescence yields of PSII in the dark-adapted state (Fo and Fm) were determined for each concentration and corresponding control. Both yields were used to calculate the maximum quantum yield (Fv/Fm) and the efficiency of the oxygen-evolving complex (OEC) of PSII. Subsequently, dark-acclimated cells were illuminated by actinic light at an intensity equivalent to the incubation light (~100 µmol/m^2/s and the current fluorescence yield Ft, the minimum and maximum fluorescence yield in light (F'o and F'm, respectively) were recorded. With the light-adapted yields, the effective quantum yield (ΦPSII), the coefficients of photochemical (qP) and non-photochemical (qN) quenching, non-photochemical quenching (NPQ), relative photosynthetic electron transport rate (ETR), as well as relative photochemical (qP(rel)) and non-photochemical (qN(rel)) quenching, were calculated.

0.01–300 µM atrazine (CAS number: 1912- 24-9, purity ≥97%) obtained from Sigma-Aldrich (United Kingdom) were dissolved in dimethyl sulfoxide (Sigma-Aldrich, UK, purity ≥99%) and used as positive controls in the assay.

Calculations, statistics and graphical treatments

The calculations of the results from the experimental studies were performed in Excel, before the results were transferred to GraphPad Prism 7, for statistical analysis and graphical treatments. For the Growth inhibition results, the growth rate was calculated as the % of control algae, and the background noise was minimized by subtracting the DMSO control values. The PSII parameters were calculated with the help of the PSII parameter formulas presented in table 2 in the Adversity and Mode of Action chapter in the Introduction.

In GraphPad Prism 7, the values were transformed to log values (x) before non-linear regression analyses were performed by dose-response inhibition and log (inhibitor) vs. response (variable slope, four parameters).

Results

JOVA summary

The measured environmental concentrations (MECs) determined during the sampling period of 2015 varied considerably, from low ng/L to low µg/L. Both the number of detected pesticides and their concentrations were in general increasing with time, leading to the highest concentrations at the end of the period (10.07-27.08). However, the herbicides metribuzin and MCPA were detected at higher concentrations in the first part of the sampling period (09.06-26.06). The MECs for pesticides and the observed patterns for nutrient concentrations and water flow measured at the JOVA monitoring station matched quite well. Higher concentrations of PPPs was detected during the period 10.07 to 14.08, which was a period of frequent spraying, followed by leaching due to periods with more rain and hence more leaching from crops to water bodies. The analysed samples included in this risk assessment covered the main spraying season in the studied catchment, and were selected to represent the pesticide exposure conditions with the highest risk to aquatic organisms (Detailed results presented in Tollefsen et al. 2016).

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). Overall, the MECs in Heiabekken during 2015 are reasonably comparable to the last 5-year period, but in the upper range regarding total number of detections and total number of different pesticides detected (Detailed results presented in Tollefsen et al. 2016).

Experimental effort

The results from the 72h growth inhibition tests and the 5h PSII-efficiency tests of exposure of *C.reinhardtii* are presented below in figures and tables. Both the GI test and the PSII test were performed for the synthetic mixture and the two single compounds metribuzin and metamitron. For each of these stressors, three independent, parallel tests were performed.

The table for GI (table 5) show the exact values of EC_{50} and correlation coefficient (R^2) of the regression line for both the synthetic mixture and the two single compounds. The table for

PSII-efficiency (table 6) compare the different PSII-parameters and their EC_{50} and correlation coefficient (R^2).

Growth inhibition studies

The graphs in each figure below each represent one of the three independent, parallel experiments, and shows the relationship between concentration of synthetic mixture, metribuzin and metamitron, respectively, and *C.reinhardtii* growth rate (% of control). The three graphs in each figure represents each of three independent, parallel experiments.

Synthetic mixture:

The GI test for the synthetic mixture after 48 h (fig.7) and 72h (fig.8) showed EC_{50}-values of a concentration factor 194.6 and 218.2, respectively. These concentrations are 194.6 and 218.2 times higher than the measured environmental concentration in Heiabekken.

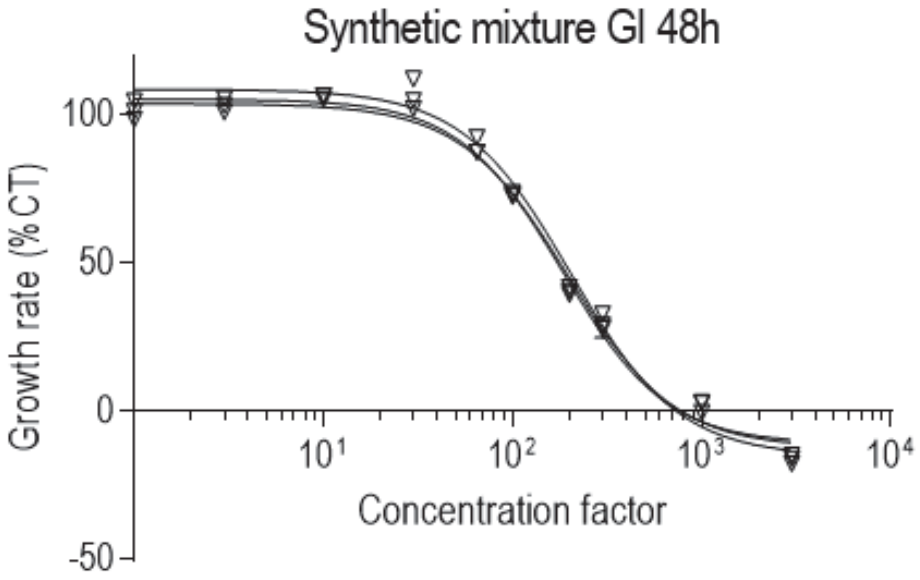


Figure 7. *C.reinhardii* growth inhibition after exposure to synthetic mixture measured after 48h. Based on three independent, parallel experiments.

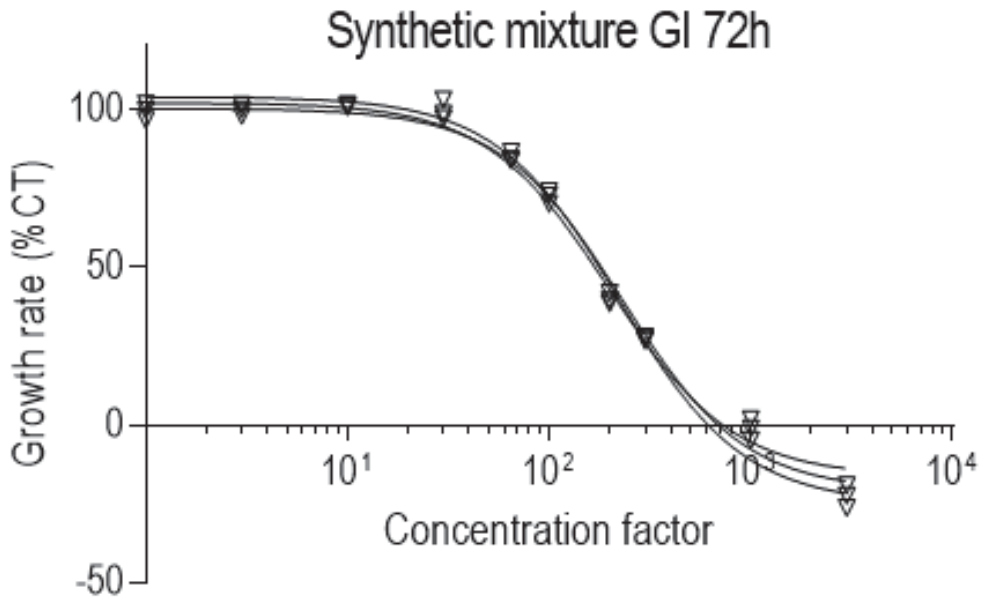


Figure 8. *C. reinhardtii* growth inhibition test of synthetic mixture measured after 72h. Based on three independent, parallel experiments.

Metribuzin:

A dose- response decrease was seen in the GI test for metribuzin after 48h (fig.9) and 72h (fig.10), both showing an EC_{50}-value of 41.5 µg/L.

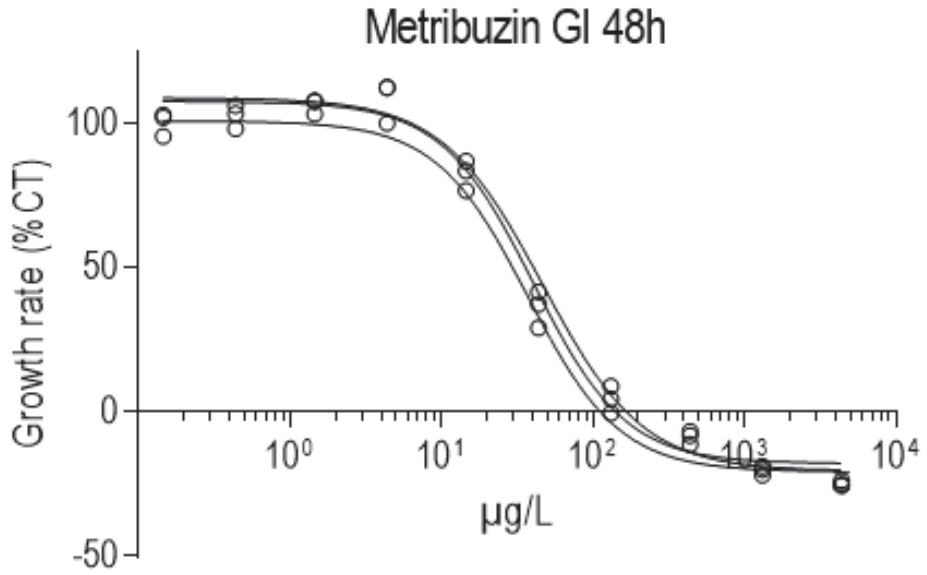


Figure 9. *C. reinhardtii* growth inhibition of metribuzin measured after 48h. Based on three independent, parallel experiments.

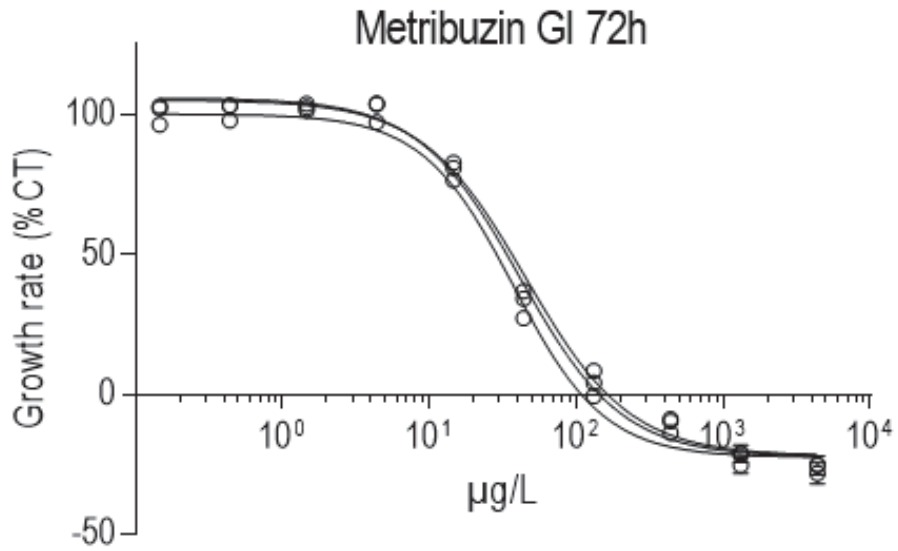


Figure 10. *C.reinhardtii* growth inhibition of metribuzin measured after 72h. Based on three independent, parallel experiments.

Metamitron:

The GI test for metamitron after 48h (fig.11) and 72h (fig.12) showed EC_{50}-values of 14297.3 µg/L and 21993.7 µg/L, respectively. This is a much higher value than the EC_{50} for metribuzin, and the growth inhibition curve is also much steeper, compared to metribuzin which had a more gradual decrease in growth rate.

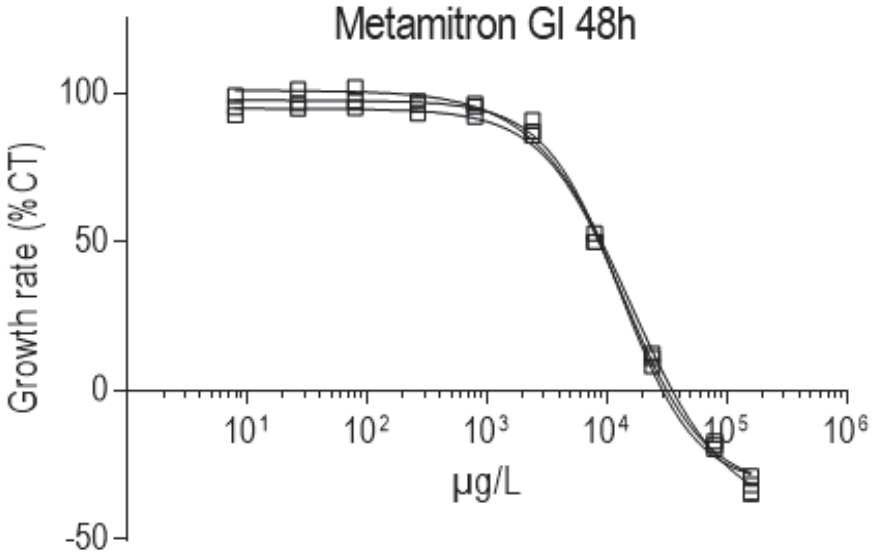


Figure 11. *C.reinhardtii* growth inhibition of metamitron measured after 48h. Based on three independent, parallel experiments.

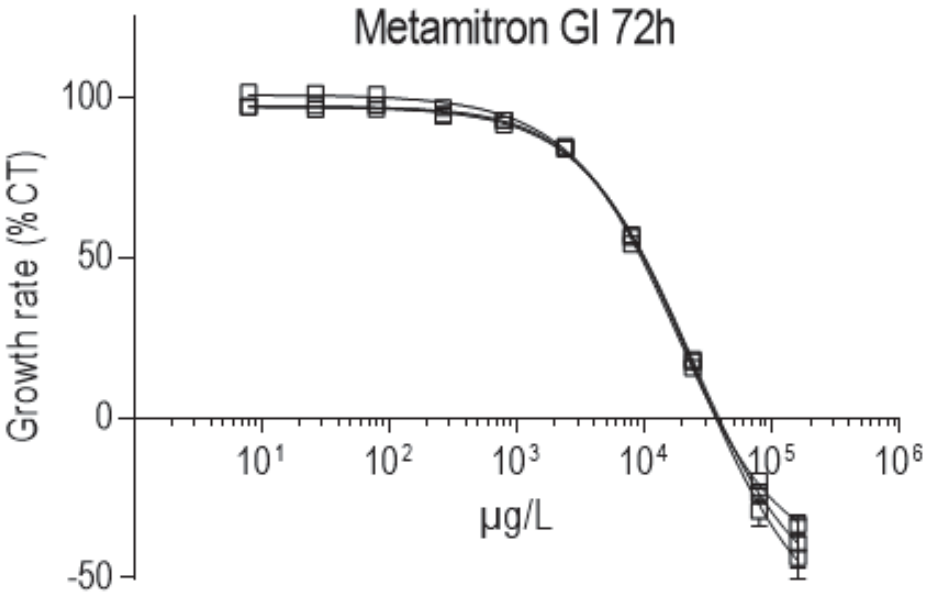


Figure 12. *C.reinhardtii* growth inhibition of metamitron measured after 72h. Based on three independent, parallel experiments.

Table 5 shows a summary of the statistical parameters R^2 and EC_{50} for the regression analyses of growth inhibition results of *C.reinhardtii* exposed to synthetic mixture, metribuzin and metamitron, respectively, for 72 hours.

Table 5. Statistical parameters in the analyses of growth inhibition 72h for synthetic mixture, metribuzin and metamitron.

Growth inhibition, average values	**48h**		**72h**	
	R^2	**EC_{50}**	**R^2**	**EC_{50}**
Synthetic mixture	0.987	194.57x	0.991	218.17x
Metribuzin	0.991	41.52 µg/L	0.992	41.47 µg/L
Metamitron	0.996	14297.3 µg/L	0.994	21993.7 µg/L

PSII-efficiency studies

The following figures shows the different parameters of PSII-efficiency measured in *C.reinhardtii* after 5 hours of exposure to synthetic mixture, metribuzin and metamitron, respectively. The parameters shown for each stressor is the effective quantum efficiency (Y(II)), maximum quantum efficiency (Fv/Fm), oxygen evolving complex efficiency (OEC), and the relative photochemical quenching (qP(rel)), relative non-photochemical quenching (qN(rel)), and finally the unquenched fluorescence yield (UQF(rel)). Each figure show one graph, based on values from three independent, parallel studies with four replicates.

Synthetic mixture

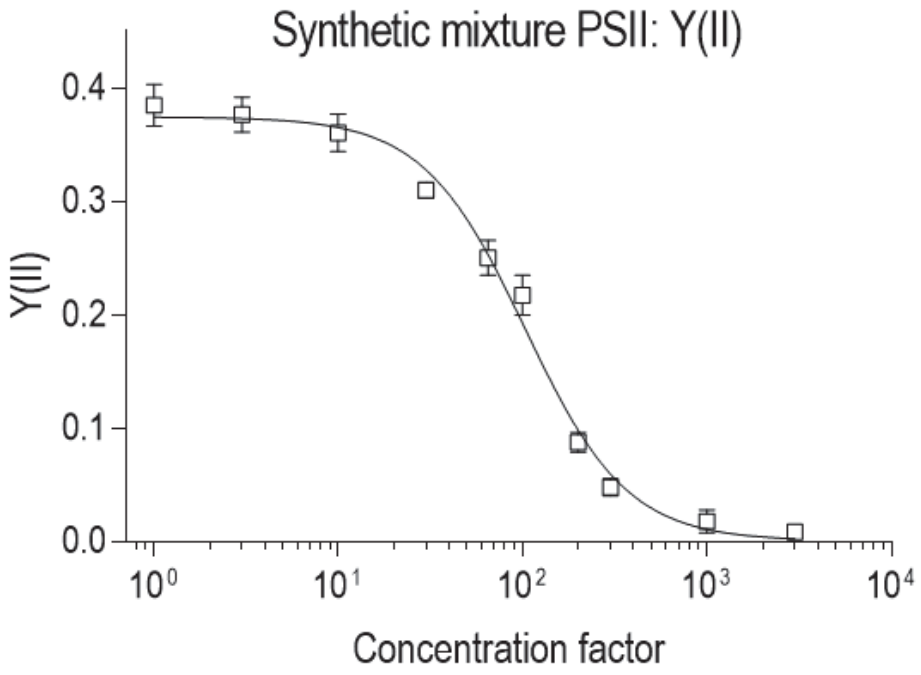


Figure 13. The PSII effective quantum efficiency measured in *C.reinhardtii* after 5h exposure to the synthetic mixture.

Fig 13 shows that the effective quantum efficiency of PSII clearly decreases with increasing concentration factor of the synthetic mixture, showing an EC_{50} at a concentration factor of 103.4 compared to MEC from the Heiabekken water sample.

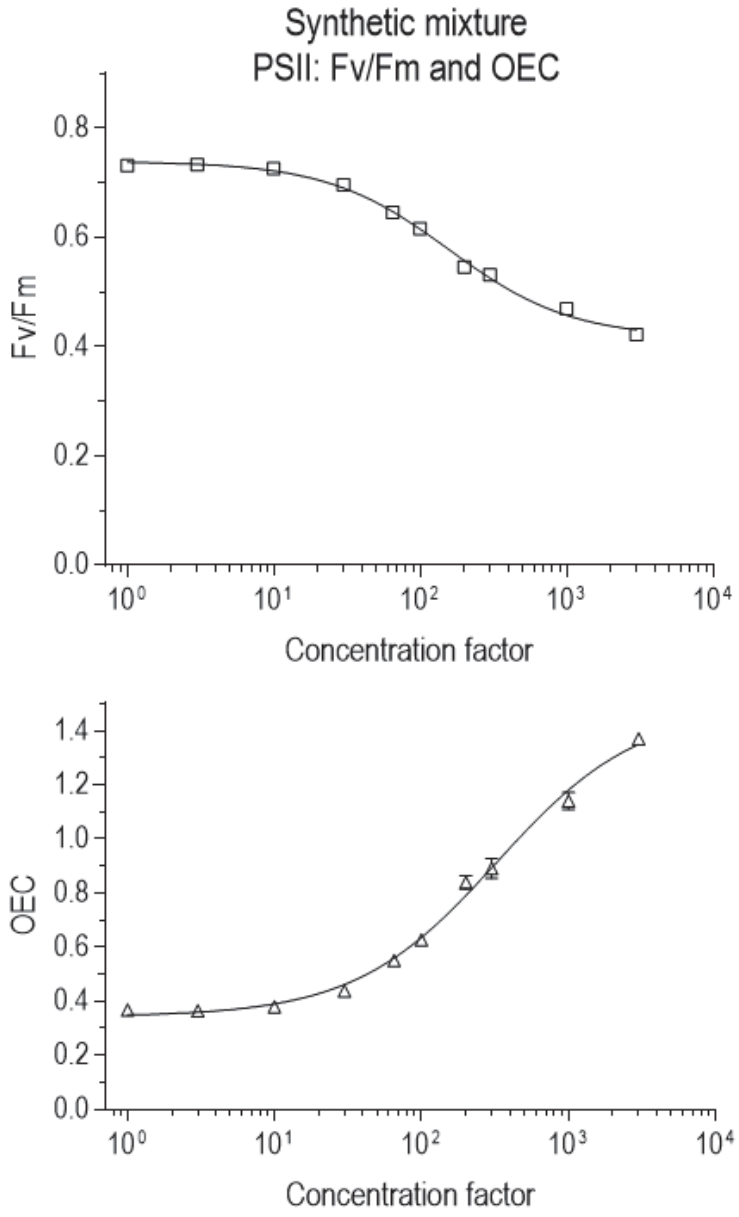


Figure 14. The PSII maximum quantum efficiency and the oxygen evolving complex efficiency measured *in C. reinhardtii* after 5h exposure to the synthetic mixture.

The oxygen evolving complex efficiency (OEC) increases significantly with increasing concentration factor of the synthetic mixture (EC_{50}= 326.2 times higher concentration than MEC). Fv/Fm decreases slightly in the same concentration increase, however not reaching 50% inhibition in the concentration factors studied (fig.14).

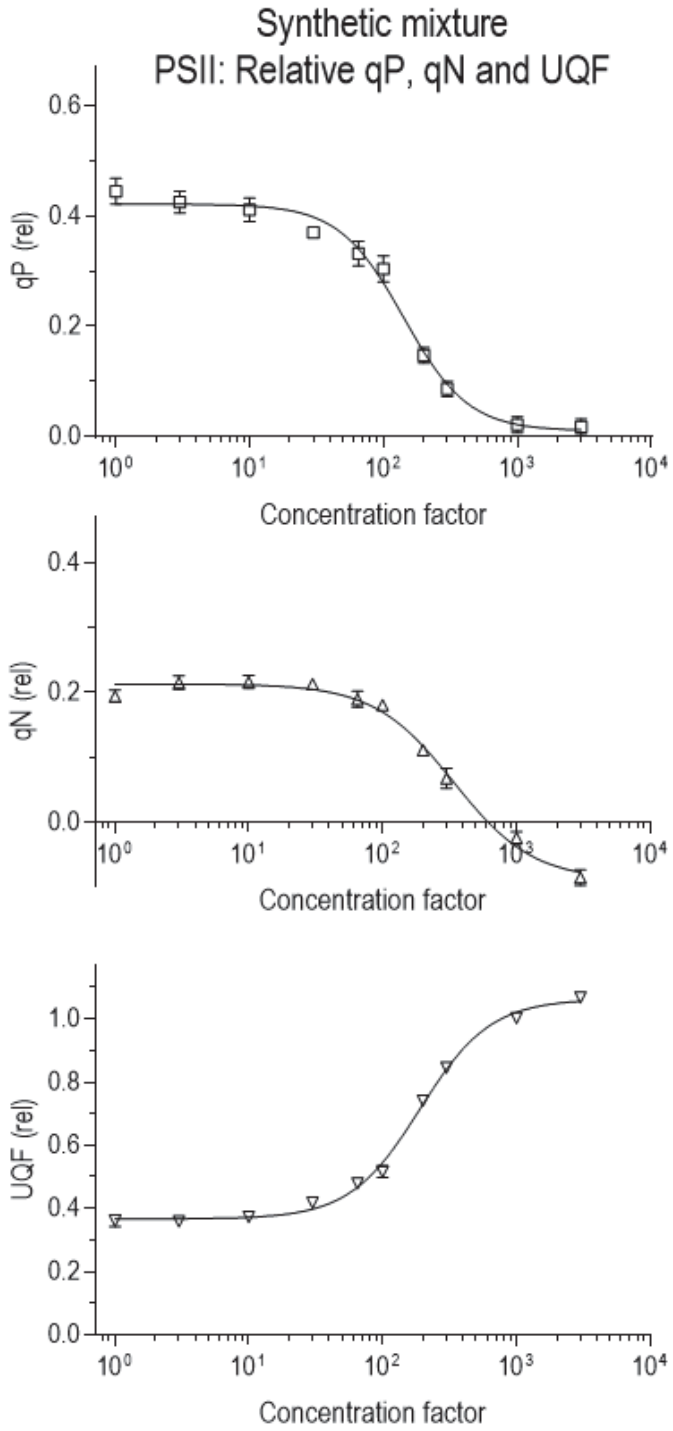


Figure 15. The relative qP, qN and UQF measured in *C. reinhardtii* after 5h exposure to the synthetic mixture.

There is a clear relationship between the relative quenching parameters and the relative unquenched fluorescence parameter (UQF(rel)). When the photochemical and non-photochemical quenching is reduced due to higher concentration factor of the synthetic

mixture, the UQF(rel) increases. At the same time, the relative non-photochemical quenching (qN(rel)) decreases gradually under the same conditions (fig.15).

Metribuzin

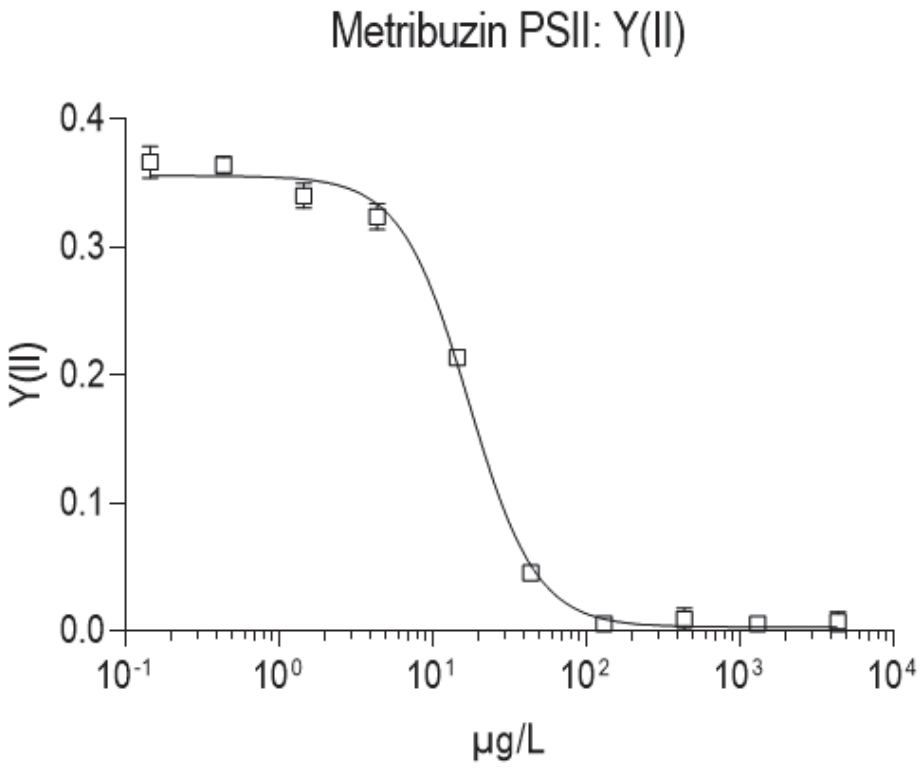


Figure 16. PSII effective quantum efficiency measured in *C. reinhardtii* after 5h exposure to the herbicide metribuzin.

The Y(II) measured after 5 h exposure to the herbicide metribuzin (fig.16), rapidly decreases with increasing concentrations after ~5 µg metribuzin/L, showing an EC_{50} of 17.3 µg/L.

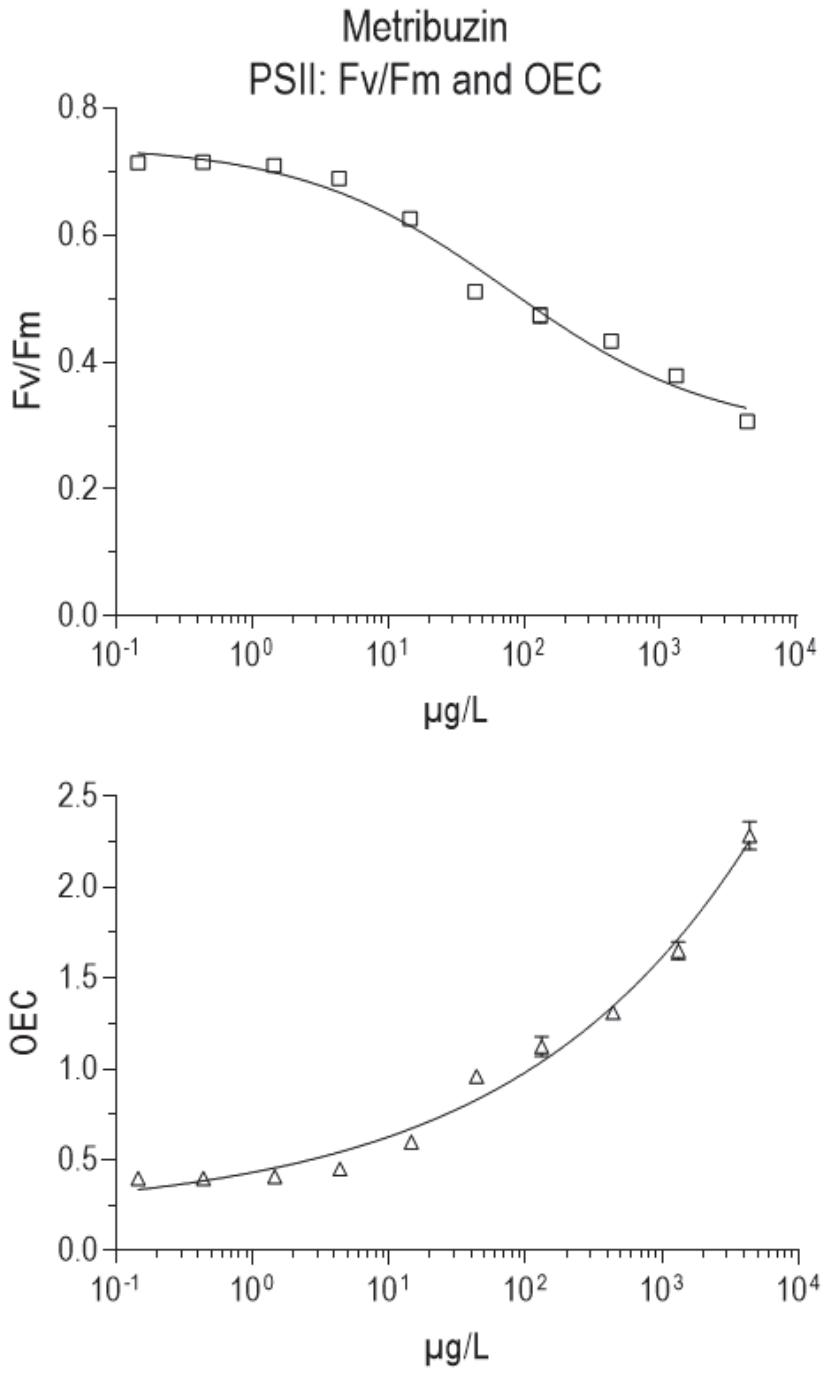


Figure 17. The PSII maximum quantum efficiency and the oxygen evolving complex efficiency measured *in C. reinhardtii* after 5h exposure to the herbicide metribuzin.

The Fv/Fm decreases slightly with increasing concentrations of metribuzin, showing an EC_{50} of 77.13 µg/L, however not reaching a 50% inhibition within the concentration factors analysed in this study. The OEC increases significantly with increasing concentrations of metribuzin, reaching a level of over 2.5 in the highest concentration of metribuzin (fig.17).

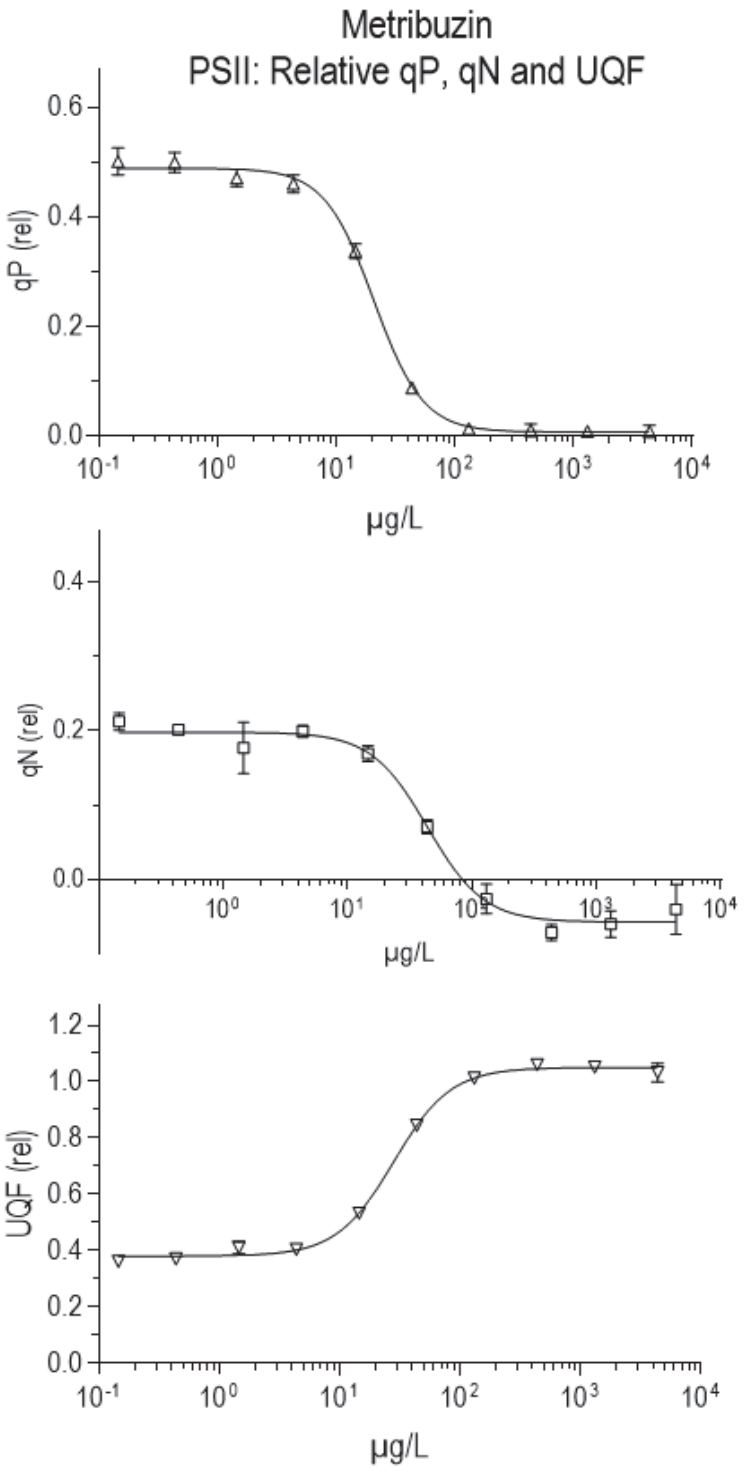


Figure 18. The relative qP, qN and UQF measured in *C. reinhardtii* after 5h exposure to metribuzin.

As seen in fig. 18, the UQF (rel) are negatively related to the qP (rel) and qN (rel), in that the UQF (rel) increases, and the qP (rel) and qN(rel) decreases, when the concentration of

metribuzin increases. qN (rel) is gradually decreasing after a concentration of ~10 µg metribuzin/L.

Metamitron

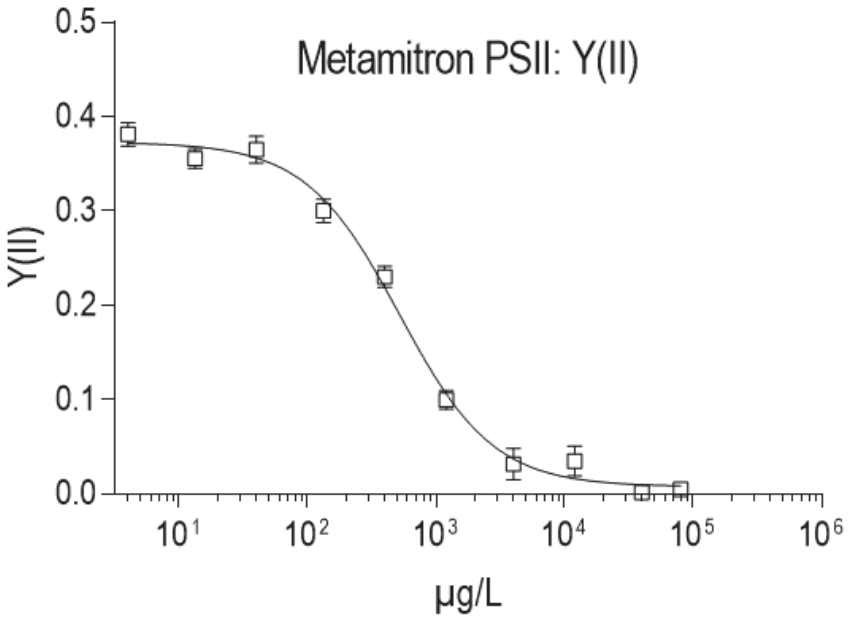


Figure 19. PSII-efficiency parameters analysed in *C. reinhardtii* after 5h exposure to the herbicide metamitron.

A dose-response decrease was also seen for metamitron for the parameters Y(II) and Fv/Fm (fig. 19 and 20, respectively), although not as rapidly as for the synthetic mixture and metribuzin, which is a more potent herbicide. The OEC (fig.20) also increases more gradually in the algae exposed to metamitron compared to the OEC in algae exposed to synthetic mixture or metribuzin.

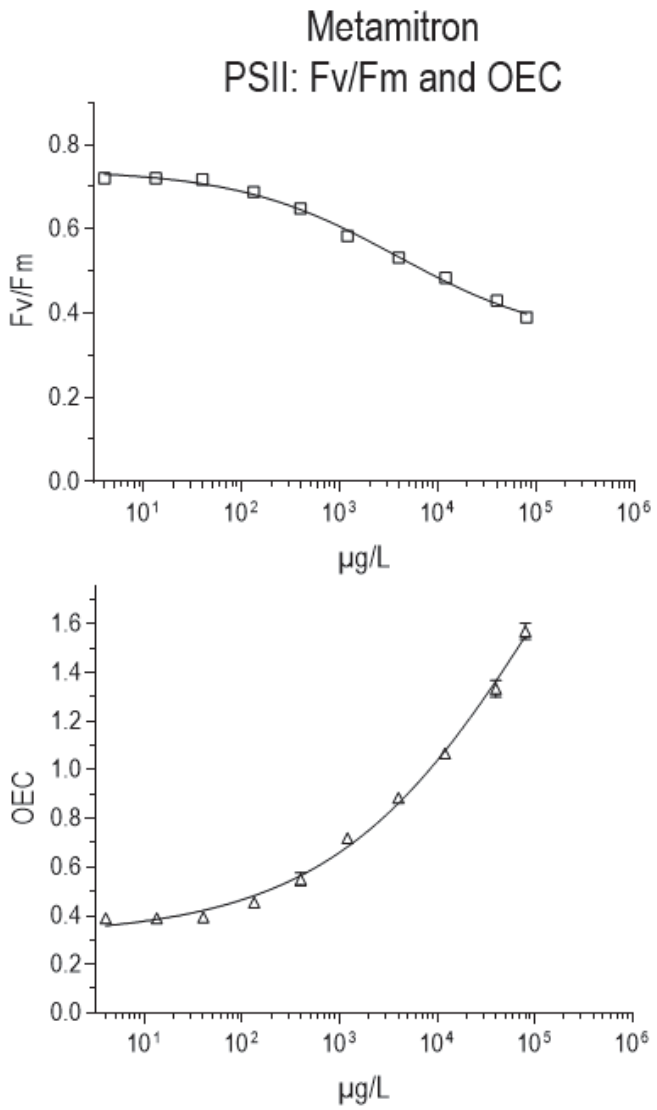


Figure 20. The PSII maximum quantum efficiency and the oxygen evolving complex efficiency measured *in C. reinhardtii* after 5h exposure to the herbicide metamitron.

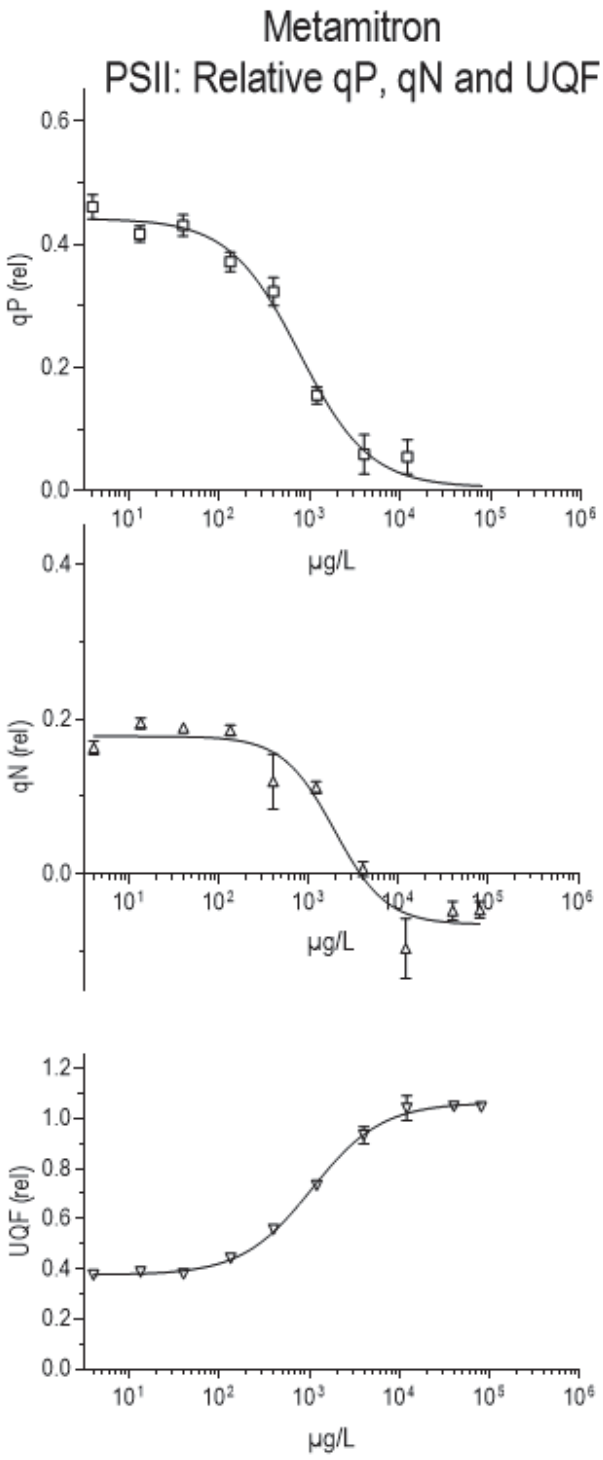


Figure 21. The relative qP, qN and UQF measured in *C. reinhardtii* after 5h exposure to metribuzin.

As seen in fig. 21, the parameter qP (rel) decreases, and UQF (rel) increases, at increasing metamitron concentrations (EC_{50}: 770.6 and 1063 µg/L, respectively). qN (rel) also decreases (EC_{50}: 1928 µg/L), although not as rapidly as qP (rel), which has a steeper curve.

Table 6 shows a summary of statistical parameters for the analyses of PSII-efficiency in *C. reinhardtii* after 5h exposure to synthetic mixture of pesticides, metribuzin or metamitron.

Table 6: Statistical parameters for the analyses of PSII-efficiency 5h in synthetic mixture, metribuzin and metamitron.

PSII parameters		R^2	EC_{50}
Fv/Fm	**Synthetic mixture**	0.967	157.4x
	Metribuzin	0.963	77.13 µg/L
	Metamitron	0.968	3852 µg/L
OEC	**Synthetic mixture**	0.960	326.2x
	Metribuzin	0.950	Not determined
	Metamitron	0.969	144599 µg/L
qP (rel)	**Synthetic mixture**	0.867	141.6x
	Metribuzin	0.764	44.17 µg/L
	Metamitron	0.882	770.6 µg/L
qN (rel)	**Synthetic mixture**	0.889	338.9x
	Metribuzin	0.952	20.88 µg/L
	Metamitron	0.719	1928 µg/L
UQF (rel)	**Synthetic mixture**	0.959	190.1x
	Metribuzin	0.960	28.25 µg/L
	Metamitron	0.930	1063 µg/L
Y(II)	**Synthetic mixture**	0.905	103.4x
	Metribuzin	0.973	17.3 µg/L
	Metamitron	0.927	519.6 µg/L

Cumulative risk assessment

Laboratory results showed that the toxicity of the Heiabekken environmental mixture could be well estimated (within a factor of 2) for the alga *C. reinhardtii*, based on already existing toxicity data (detailed in Tollefsen et al. 2016). The variation by a factor of 2 could possibly be explained by the variation in species sensitivity towards the mixture.

Discussion

Microalgae are important as test organisms in ecotoxicological studies mainly for two reasons. First, they have low requirements for thriving in laboratory settings and are sensitive to contaminants. Secondly, and most important, they are responsible for the main source of biomass production, and therefore have a key role in the aquatic ecosystem. Negative effects caused by pesticides, especially herbicides, will therefore not only impact algae themselves, but may also affect the entire aquatic community to some degree. For instance, previous research (i.e. Lürling & Roessink 2006) has demonstrated that green algae and diatoms are more sensitive towards herbicides than i.e. cyanobacteria, which suggests that high concentrations of herbicides might affect the distribution of these species in the ecosystem, and possibly cause cyanobacterial blooms.

Experimental studies

Growth inhibition studies

The 72 h growth inhibition studies on the test species *C. reinhardtii* showed a clear dose-response curve for both the single compounds. Metribuzin was, with an EC_{50} of 41.5 µg/L, a much more potent stressor than metamitron, which showed an EC_{50} of 14297.3 and 21993.7 µg/L after 48h and 72h, respectively (fig.9, 10, 11 and 12). These results are consistent with other studies of the same compounds (i.e. Almeida 2015; Lürling & Roessink 2006).

The toxic effect of the synthetic mixture (fig.7 and 8) may partly be explained by the presence of metribuzin as one of the components in the mixture. Metribuzin was considered the main herbicide risk driver identified in the combined toxicity assessment. Metamitron was regarded as the second herbicide risk driver, however with a much lower toxicity than metribuzin, which the results from this study also confirmed.

One of the fungicides present in the mixture, pencycuron, was also regarded as one of the main risk driver of overall toxicity to non-target species. However, since this compound is regarded as a fungicide, with a known MoA affecting fungi, and this study focused on microalgae as the non-target organism group, it is less likely that this compound was responsible for the toxicity seen in *C. reinhardtii.*

Photosystem II- efficiency

The use of pulse amplitude modulated (PAM) fluorometry to measure chlorophyll *a* fluorescence is more and more regarded as a simple, rapid and sensitive tool in ecotoxicological testing on effects in photosynthetic activity of the green microalga *C. reinhardtii* (i.e. Gomes et al. 2017; Herlory et al. 2013; Juneau et al. 2005; Juneau & Popovic 1999). Their general site of action is also shown to be the same in *C. reinhardtii* as in the target organisms (table 1).

All in all, all the determined parameters of the PSII- fluorescence were affected in this study of PSII-efficiency after 5 hours exposure to synthetic mixture, metribuzin and metamitron, suggesting that the tested compounds have several specific sites of action within the photosystem II in *C. reinhardtii.*

Maximum quantum efficiency (Fv/Fm), measure the maximal photochemical efficiency of PSII after a period of dark-adaption. This parameter is often used, as it has been shown to be strongly affected by environmental and toxicological stressors (Ralph et al. 2007).

The Fv/Fm results for the synthetic mixture in this study, however, shows that this parameter only decreases slightly, and not reaching 50% inhibition in the concentration factors used (fig. 14). This is also the case for metamitron, while metribuzin results in 50% inhibition of Fv/Fm at 77.13 µg/L (fig. 17 and 20).

A decrease in the Fv/Fm ratio may be a result of either an increase in minimum fluorescence (F^o) or a decrease in maximum fluorescence (F_m), or a decrease in both parameters (Ralph et al. 2007; Maxwell & Johnson 2000).

Effective quantum yield of PSII (Y(II) or ΦPSII) is based on F'm (maximum fluorescence) and F (fluorescence yield) which is measured in light, hence requiring active photosystems (Genty et al. 1989). Interpretation of Y(II) is quite complex as this parameter is influenced by several activities in the photosynthesis. Still, Y(II) provides a good indication of the amount of energy used in photochemistry (Juneau et al. 2002; Maxwell & Johnson 2000). In controlled laboratory experiments with consistent light conditions, Y(II) is regarded as a more sensitive indicator of toxic impacts than Fv/Fm, especially for PSII herbicides (Ralph et al. 2007).

The Y(II) parameter measured in *C. reinhardtii* after exposure to the synthetic mixture showed a clear dose-response curve (fig. 13) with an EC_{50} of a concentration factor of 103.4 compared to the environmental sample from Heiabekken. A clear dose-response decrease was also seen for metribuzin and metamitron (fig. 16 and 19), showing an EC_{50} of 17.3 and 519.6 µg/L, respectively.

The efficiency of the oxygen evolving complex (OEC) reflects the state of the water photo-oxidation process in photosynthetic organisms and is regarded as one of the most sensitive components of the electron transport chain in the photosynthesis (Gomes et al. 2017; Kriedemann et al. 1985). *C. reinhardtii* exposed to the synthetic pesticide mixture and the two single herbicides metribuzin and metamitron, clearly showed an impact in the water-splitting apparatus in the doses tested. In this parameter, the parameter activity clearly increased with increasing concentrations of the stressors. The synthetic mixture resulted in an EC_{50} of concentration factor 326.2 compared to the measured concentrations in Heiabekken, while metamitron resulted in an EC_{50} of 144599 µg/L. For metribuzin, this parameter could not be determined to a satisfying degree, however the graph shows that all the stressors resulted in an increase in OEC activity for the doses tested in this study (fig. 14, 17 and 20, table 6).

Quenching analysis can monitor the energy used by the electron transport chain leading to carbon fixation. This is known as photochemical quenching. Energy dissipated as heat to protect the photosynthetic apparatus is known as non-photochemical quenching (NPQ) (Ralph et al. 2007). Photosynthetic organisms have evolved NPQ mechanisms to protect the photosynthesis apparatus from oxidative stress (Gomes et al. 2017), but several studies have shown that it is not always appropriate to use this parameter (Juneau et al. 2005; Buschmann 1999; Genty et al. 1989). These parameters also gave inconclusive results in this study (not presented). For this reason, the relative photochemical (qP(rel)) and non-photochemical quenching (qN(rel)) coefficients were suggested by Buschmann (1999). Both qP(rel) and qN(rel) are normalized to the same reference signal, and thereby expressing the relative amount of photochemical and non-photochemical quenching as a fraction of the total quenching, when going from a dark-adapted to a light-adapted state (Gomes et al. 2017; Juneau et al. 2005).

Both qP (rel) and qN (rel) were affected by the synthetic mixture (fig. 15), showing a decrease in both parameters with an EC_{50} of a concentration factor of 141.6 and 338.9, respectively. Metribuzin and metamitron showed comparable results (fig. 18 and 21), however, metamitron resulted in a greater decrease with dose than metamitron, as was expected due to the potency differences of the compounds. The EC_{50} of metribuzin was calculated to 44.17 µg/L for qP(rel) and 20.88 µg/L for qN (rel), while for metamitron, the EC_{50} of qP(rel) showed 770.6 µg/L and 1928 µg/L for qN(rel). This suggests that the parameter qN(rel) is more sensitive to metribuzin than qP(rel) is, while the opposite is the case for metamitron, with a greater effect caused in the qP(rel) than in the qN(rel). Another interesting finding is that for these herbicides, both parameters were decreased, while in the same species of algae exposed to gamma radiation (Gomes et al. 2017), the qN(rel) increased while qP(rel) decreased with increasing doses. Gamma radiation and herbicides are very different stressors, affecting various parts of the PSII, however this finding could be an incentive to also check other herbicides, to see if this is the normal effect seen in *C. reinhardtii* exposed to various herbicides, or if this varies from herbicide to herbicide.

UQF (rel) is a parameter complementary to qP (rel) and qN (rel) showing the unquenched fluorescence, and naturally this fluorescence will increase when the quenching parameters decrease, as seen in *C. reinhardtii* exposed to synthetic mixture, metribuzin and metamitron, showing EC_{50} of concentration factor 190,1 in synthetic mixture compared to MEC, and 28,25 µg/L and 1063 µg/L for metribuzin and metamitron, respectively (fig.15, 18 and 21).

Adversity vs. Mode of Action

The PSII assay, which investigates the Mode of Action of the synthetic mixture and the individual chemicals, are much more sensitive than the growth inhibition assay which is an adversity, or endpoint, assay. Knowing the Mode of Action of pesticides, and in this case, herbicides in particular, on non-target organisms as green algae, helps us understand some of the complex nature of the surrounding environments. By knowing that the non-target organisms are susceptible to adverse effects at an earlier stage than is shown by growth inhibition studies, one can take this into account when deciding on spraying limits in agriculture and forestry.

Predicted vs observed toxicity

The assumptions behind the CA model regarding the similarity of site of action and similarity of slope has been debated ever since the model was introduced. Some argue that CA is also valid for compounds with just a slightly similar mode of action on a higher biochemical level. This makes the principle behind CA somewhat questionable, in that if this is true, one could argue that if affecting the same effect measure would be enough, all chemicals could be reduced to having the same mode of action if they all affected the growth of the test organism. However, although the CA model is shown to be a precise reference model of mixtures of chemicals with similar target sites, the current results for mixtures of chemicals with a similar overall mode of action has been inconclusive (Altenburger et al. 2013; Cedergreen et al. 2008).

The CA model provided a realistic prediction of the combined toxicity of pesticides, and is regarded as a predictive for compounds present in complex mixtures from Heiabekken (Tollefsen et al. 2016; Almeida 2015). Laboratory results showed that the toxicity of the environmental mixture could be estimated quite well for the algae *C. reinhardtii* (within a factor of 2) based on existing toxicity data. The variation of a factor of 2 may be due to variations in species sensitivity towards these pesticides. The model based the prediction on a variety of species from different taxa and their sensitivity to each individual compound, and *C. reinhardtii* could be more or less sensitive than these species.

Ecological relevance

The response of phytoplankton to photosynthesis-inhibiting herbicides depends on the sensitivity of the species, and in general green algae and diatoms are more sensitive than cyanobacteria (Fairchild et al. 1998; Peterson et al.1997). Because of the difference in sensitivity, competitive interactions between species could lead to replacement of the susceptible algae by more resistant ones, and in this way affecting the algal community structure (Bérard et al. 1999; Kasai & Hanazato 1995;). The achievement of information regarding the pesticide hazard and risk to non-target microalgae present in the aquatic environments surrounding agricultural areas, may help understand more of the complex interactions in the aquatic environment to prevent adverse effects to these systems.

Conclusion

Because of the vast number of potentially hazardous chemicals being released into the environment, and the large complexity in natural systems, it is very expensive and time-consuming to perform toxicity studies for every possible potential mixture. The use of prediction models may be an appropriate alternative, and for Heiabekken the CA prediction model provided a good toxicity estimate for the pesticides detected in water samples from this site, within a factor of 2 compared to the experimental studies perform on *C. reinhardtii.*

The measured concentrations of pesticides in Heiabekken was relatively low, and to be able to show any significant adverse effects (EC_{50}) of the synthetic mixture to *C. reinhardtii*, the mixture had to be upconcentrated to a concentration factor of ~100 compared to the MECs. This suggests that the concentrations of pesticides in Heia catchment do not pose a real risk to the non-target organisms, here shown for microalgae represented by *C. reinhardtii*, which is regarded as one of the more sensitive species of microalgae. However, there is always a slight chance that some of the compounds were lost during the Solid phase enrichment process, hence leading to lower concentrations measured in the chemical analyses than were originally present in the water sample.

The results from the experimental effort of this study, however, show that if the same compounds would be present in the aquatic environment at higher concentrations than that found in the water samples from 2015, it could have adverse impacts on the non-target organisms living there. The PSII-efficiency assay showed that the tested compounds had an impact on all the parameters, which suggests that these compounds have several specific sites of action within the photosystem II. This is a good reason to continue monitoring watersheds close to agricultural areas, and to make sure that the allowed doses of pesticide spraying do not increase significantly in the future.

Future prospects

Further studies on this subject could include the analysis of RNA expression after exposure to these compounds, to be able to compare the PSII-efficiency results with genes or other parts of the DNA being transcribed in algae in the presence of chemical stressors.

References

Almeida, A.C.G. 2015. Toxicity of single biocides and their mixtures in the algae Chlamydomonas reinhardtii. PhD thesis, Norwegian University of Life Sciences

Altenburger, R., Arrhenius, Å., Backhaus, T., Coors, A., Faust, M. & Zitzkat, D. 2013. Ecotoxicological combined effects from chemical mixtures. Environmental research of the federal ministry of the environment, nature conservation and nuclear safety, Report No. (UBA-FB) 001789/1, 203 p.

Altenburger, R., Nendza, M. & Schuurmann, G., 2003. Mixture toxicity and its modelling by quantitative structure-activity relationships. Environmental toxicology and chemistry, 22, pp. 1900-1915

Backhaus, T. & Faust, M. 2012. Predictive environmental risk assessment of chemical mixtures: a conceptual framework. Environmental Science & Technology, 46, pp. 2564-2573.

Backhaus, T. & Karlsson, M. 2014. Screening level mixture risk assessment of pharmaceuticals in STP effluents. Water Research, 49, pp. 157-165.

Belden, J.B., Gilliom, R.J. & Lydy, M.J. 2007. How well can we predict the toxicity of pesticide mixtures to aquatic life? Integrated Environmental Asssessment and Management, 3(3), pp. 364-372.

Belden, J.B. & Lydy, M.J. 2006. Joint toxicity of chlorpyrifos and esfenvalerate to fathead minnows and midge larvae. Environmental toxicology and chemistry, 25, pp. 623-629.

Berenbaum, M.C. 1989. What is synergy? Pharmacological reviews, 41, pp. 93-129.

Bérard, A., Leboulanger, C. & Pelte, T. 1999. Tolerance of Oscillatoria limnetica Lemmermann to Atrazine in Natural Phytoplankton Populations and in Pure Culture: Influence of Season and Temperature. Archives of Environmental Contamination and Toxicology, 37, pp. 472–479.

Beyer, J., Petersen, K., Song, Y., Ruus, A., Grung, M., Bakke, T. & Tollefsen, K.E. 2014. Environmental risk assessment of combined effects in aquatic ecotoxicology: A discussion paper. Marine Environmental Research, 96, pp. 81-91.

Brian, J.V., Harris, C.A., Scholze, M., Kortenkamp, A., Booy, P., Lamoree, M., Pojana, G., Jonkers, N., Marcomini, A. & Sumpter, J.P. 2007. Evidence of estrogenic mixture effects on the reproductive performance of fish. Environmental Science & Technology, 41, pp. 337-344.

Brock, T.C.M., Crum, S.J.H., Deneer, J.W., Heimbach, F., Roijackers, R.M.M. & Sinkeldam, J.A. 2004. Comparing aquatic risk assessment methods for the photosynthesis-inhibiting herbicides metribuzin and metamitron. Environmental pollution, 130, pp. 403-426.

Buschmann, C. 1999. Photochemical and non-photochemical quenching coefficients of the chlorophyll fluorescence: comparison of variation and limits. Photosynthetica, 37(2), pp. 217-224.

Cedergreen, N., Christensen, A.M., Kamper, A., Kudsk, P., Mathiassen, S.K., Streibig, J.C. & Sørensen, H. 2008. Environmental toxicology and chemistry, 27(7), pp. 1621-1632.

Connon, R.E., Geist, J. & Werner, I. 2012. Effect-based tools for monitoring and predicting the ecotoxicological effects of chemicals in the aquatic environment. Sensors, 12, pp. 12741-12771.

DeLorenzo, M.E., Scott, G.I. & Ross, P.E. 2001. Toxicity of pesticides to aquatic microorganisms: A review. Environmental toxicology and chemistry, 20(1), pp. 84-98.

Dewez, D., Didur, O., Vincent-Héroux, J. Popovic, R. 2008. Validation of photosynthetic-fluorescence parameters as biomarkers for isoproturon toxic effect on alga Scenedesmus obliquus. Environmental pollution, 151, pp.93-100.

EC, 2003. Technical guidance document on risk assessment. Institute for Health and Consumer Protection, European Chemicals Bureau. TGD Part II. Office for Official Publications of the European Communities, 328 p.

EPA, 2007. Concepts, methods and data sources for cumulative health risk assessment of multiple chemicals, exposures and effects: a resource document. U.S. Environmental Protection Agency, National centre for Environmental Assessment, 412 p.

Esperanza, M., Seoane, M., Rioboo, C. Herrero, C. & Cid, Á. 2015. Chlamydomonas reinhardtii cells adjust the metabolism to maintain viability in response to atrazine stress. Aquatic toxicology, 165, pp. 64-72.

EU, 2009. Council of the European Union: Council conclusion on combination effects of chemicals, 2988th Environmental council meeting, Brussels.

Eullaffroy, P. & Vernet, G. 2003. The F684/F735 chlorophyll fluorescence ratio: a potential tool for rapid detection and determination of herbicide phytotoxicity in algae. Water research, 37, pp. 1983-1990.

Fairchild, J.F., Ruessler, D.S. & Carlson, A.R. 1998. Comparative sensitivity of five species of macrophytes and six species of algae to atrazine, metribuzin, alachlor, and metolachlor. Environmental toxicology and chemistry, 17 (9), pp. 1830-1834.

Fernández, N. & Beiras, R. 2001. Combined toxicity of dissolved mercury with copper, lead and cadmium on embryogenesis and early larval growth of the Paracentotus lividus Sea-Urchin. Ecotoxicology, 10, pp. 263-271.

Franklin, N.M., Stauber, J.L., Markich, S.J. & Lim, R.P. 2000. pH-dependent toxicity of copper and uranium to a tropical freshwater alga (Chlorella sp.). Aquatic Toxicology, 48, pp. 275–289

Genty, B., Briantais, J.-M. & Baker, N.R. 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochimica et biophysica Acta, 990, pp. 87-92.

Gomes, T., Xie, L., Brede, D.A., Lind, O.-C., Solhaug, K.A., Salbu, B. & Tollefsen, K.E. 2017. Sensitivity of the green algae Chlamydomonas reinhardtii to gamma radiation: photosynthetic performance and ROS formation. Aquatic toxicology, 183, pp. 1-10.

Groten, J.P., Feron, V.J. and Suhnel, J., 2001. Comparison of an array of in vitro assays for the assessment of the estrogenic potential of natural and synthetic estrogens, phytoestrogens and xenoestrogens. Toxicology 166,

Harris, E. 2009. The Chlamydomonas sourcebook: Introduction to Chlamydomonas and its laboratory use, 2nd edition. Elsevier Inc. 292 p.

Hartgers, E.M., Heugens, E.H.W. & Deneer, J.W. 1999. Effect of lindane on the clearance rate of Daphnia magna. Archives of environmental contamination and toxicology, 36, pp. 399-404.

Hauken, M. & Stenrød, M. 2016. Jord- og vannovervåkning i landbruket (JOVA). Feltrapporter fra programmet i 2014. NIBIO RAPPORT, 2 (85), pp. 47-50.

Herlory, O., Bonzom, J.-M. & Gilbin, R. 2013. Sensitivity evaluation of the green alga Chlamydomonas reinhardtii to uranium by pulse amplitude modulated (PAM) fluorometry. Aquatic toxicology, 140-141, pp. 288-294.

Huber, A., Bach, M. & Frede, H.G. 2000. Pollution of surface waters with pesticides in Germany: modelling non-point source inputs. Agriculture, ecosystems & environment, 80, pp. 191-204.

Juneau, P., Berdey, A.E. & Popovic, R. 2002. PAM fluorometry in the determination of the sensitivity of Chlorella vulgaris, Selenastrum capricornutum, and Chlamydomonas reinhardtii to copper. Archives of environmental contamination and toxicology, 42, pp. 155-164.

Juneau, P., Green, B.R. & Harrison, P.J. 2005. Simulation of Pulse-Amplitude-Modulated (PAM) fluorescence: Limitations of some PAM-parameters in studying environmental stress effects. Photosynthetica, 43(1), pp. 75-83.

Juneau, P. & Popovic, R. 1999. Evidence for the rapid phytotoxicity and environmental stress evaluation using the PAM fluorometrix method: Importance and future application. Ecotoxicology, 8, pp. 449-455.

Juneau, P., Qiu, B. & Deblois, C.P. 2007. Use of chlorophyll fluorescence as a tool for determination of herbicide toxic effect: Review. Toxicological & Environmental Chemistry, 89(4), pp. 609-625.

Kasai, F. & Hanazato, T. 1995. Effects of the triazine herbicide, simetryn, on freshwater plankton communities in experimental ponds. Environmental pollution, 89(2), pp. 197-202.

Kortenkamp, A. 2008. Low dose mixture effects of endocrine disrupters: implications for risk assessment and epidemiology. International journal of andrology, 31(2), pp. 233-240.

Kriedemann, P.E., Graham, R.D. & Wiskich, J.T. 1985. Photosynthetic dysfunction and in vivo changes in chlorophyll a fluorescence from manganese-deficient wheat leaves. Australian Journal of Agricultural Research, 36, pp. 157-169.

Lindeman, R.L. 1942. The trophic-dynamic aspect of ecology. Ecology, 23 (4), pp. 399-417.

Lürling, M. & Roessink, I. 2006. On the way to cyanobacterial blooms: Impact of the herbicide metribuzin on the competition between a green alga (Scenedesmus) and a cyanobacterium (Microcystis). Chemosphere, 65, pp. 618-626.

Malaj, E., von der Ohe, P.C., Grote, M., Kühne, R., Mondy, C.P., Usseglio-Polatera, P., Brack, W., and Schäfer, R.B. 2014. Organic chemicals jeopardize the health of freshwater ecosystems on the continental scale. PNAS, 111(26), pp. 9549-9554.

Maxwell, K. & Johnson, G.N. 2000. Chlorophyll fluorescence – a practical guide. Journal of experimental botany, 51 (345), pp.659-668.

Merchant, S.S., Prochnik, S.E., Vallon, O., Harris, E.H., Karpowicz, S.J., Witman, G.B., Terry, A., Salamov, A., Fritz-Laylin, L.K. et al. 2007. The Chlamydomonas genome reveals the evolution of key animal and plant functions. Science, 318(5848), pp. 245-250.

Neumann, M., Liess, M., & Schultz, R. 2003. A qualitative sampling method for monitoring water quality in temporary channels or point sources and its application to pesticide contamination. Chemosphere, 51, pp. 509-513.

NIBIO 2016. JOVA programme. Available at: www.nibio.no/jova

Nyholm, N. & Källqvist, T. 1989. Methods fo growth inhibition toxicity tests with freshwater algae. Environmental toxicology and chemistry, 8, pp. 689-703.

Petersen, K., Stenrød, M., Odenmarck, S.R., Fredriksen, L., Gomes, T., Backhaus, T. & Tollefsen, K.E. 2015. Exposure and toxicity of mixtures of plant protection products (PPPs) in

the environment under Norwegian conditions. Evaluation of a cumulative environmental risk assessment of PPPs. Norwegian Institute for water research, Report SNO. 6830-2015.

Peterson, H.G., Boutin, C., Freemark, K.E. & Martin, P.A. 1997. Toxicity of hexazinone and diquat to green algae, diatoms, cyanobacteria and duckweed. Aquatic toxicology, 39(2), pp.111-134.

Ralph, P.J., Smith, R.A., Macinnis-Ng, C.M.O. & Seery, C.R. 2007. Use of fluorescence-based ecotoxicological bioassays in monitoring toxicants and pollution in aquatic systems: Review. Toxicological & Environmental chemistry, 89(4), pp. 589-607.

Rojicková-Padrtová, R. & Marsálek, B. 1999. Selection and sensitivity comparisons of algal species for toxicity testing. Chemosphere, 38(14), pp. 3329-3338.

Schuler, L.J. & Rand, G.M. 2008. Aquatic risk assessment of herbicides in freshwater ecosystems of south Florida. Archives of Environmental contamination and toxicology, 54, pp. 571-583.

Schwarzenbach, R.P., Escher, B.I., Fenner, K., Hofstetter, T.B., Johnson, C.A., von Gunten, U. and Wehrli, B. 2006. The challenge of micropollutants in aquatic systems (review). Science, 313, pp. 1072-1077.

Silva, E., Rajapakse, N. & Kortenkamp, A. 2002. Something from «nothing» - Eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. Environmental Science and Technology, 36, pp. 1751-1756.

Statistics Norway. 2015. Bruk av plantevernmidler på friland, 2001-2014. Available at: https://www.ssb.no/jord-skog-jakt-og-fiskeri/statistikker/jordmil/friland

Stenersen, J. 2004. How to measure toxicity, p.18-27; Pesticides that disturb energy production, p. 35-45; Herbicides that inhibit photosynthesis, p. 45-54, in Chemical pesticides: Mode of action and toxicology. 2004. CRC Press LLC. 247 p.

Tollefsen, K.E., Bæk, K., Almeida, A.C., Haug, L.A., Norli, H.R., Odenmarck, S.R. and Stenrød, M. 2016. Evaluation of combined toxicity assessment and cumulative risk assessment of ecologically relevant mixtures of plant protection products (PPPs) under Norwegian conditions.

Tomlin CDS, 2000. The pesticide manual. 12th edition. Boston, Farnham, UK: The British Crop Protection Council, 1250 p.

Vann-nett, 2016. Heiabekken, faktaark for vannforekomst. Available at: http://vann-nett.no/portal/Water?WaterbodyID=003-19-R

Villeneuve, D.L. & Garcia-Reyero, N. 2011. Vision & Strategy: Predictive ecotoxicology in the 21st century. Environmental toxicology and chemistry, 30(1), pp.1-8.

Walker, C.H., Sibly, R.M., Hopkin, S.P., Peakall, D.B. 2012. Testing for Ecotoxicity, p.97-124, in Principles of ecotoxicology, 4th edition. CRC Press Taylor & Francis Group. 247 p.

Wetzel, R.G. 2001. Limnology: Lake and river ecosystems. 3rd edition. Academic press, 1006 p.

Weyers, A. & Vollmer, G. 2000. Algal growth inhibition: effect of the choice of growth rate or biomass as endpoint on the classification and labelling of new substances notified in the EU. Chemosphere 41, pp. 1007-1010.

Wieczerzak, M., Kudlak, B., Yotova, G., Nedyalkova, M. & Tsakovski, S. 2016. Modeling of pharmaceuticals mixture toxicity with deviation ratio and best-fit function models. Science of the Total Environment, 571, pp. 259-268.

Appendices

Appendix 1: Water sample collection

The long-term Norwegian Agricultural Environmental Monitoring Programme (JOVA) document 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) The water level and velocity in the Heiabekken stream is measured by an ultrasonic sensor (Hydrovision, Tyskland). These parameters, together with the water pipe diameter are used to determine the water flow, in the program Q-vision (Hydrovision, Tyskland).

The water flow value is used to determine when to collect water samples, by the use of a Q-eye M-11 logger (Hydrovision, Tyskland) sends a signal to the water sampler (ISCO GLS) when the water flow is sufficiently high. Then subsamples of 100 mL are collected in a 20 L tank. The number of subsamples in a given period depends on the water flow.

Four composite stream water samples were obtained in summer of 2015 (dates: 09.06-26.06, 26.06-10.07, 10.07-27.07, and 27.07-14.08).

Appendix 2: Summary of the differences in properties of SPE and whole water samples

Table A2a: Composition and properties of the SPE method 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

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%). 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. The MCPA was eluted with the other compounds, while clopyralid remained bound to the Strata X-CW column. However, as clopyralid were only found at very low concentrations in Heiabekken, 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 A2b: 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.

Appendix 3: Pesticide compound in the synthetic mixture

Table A3: Pesticide compounds in the synthetic mixture

Compound	C1 (mg/mL)	V1 (µL)	C2 (mg/mL)	V2 (µL)
Clopyralid	1,14	50	0,057	1000
MCPA	0,102	50	0.0051	1000
Fenhexamide	0,15	50	0,0075	1000
Imidacloprid	11,4	50	0,57	1000
Metamitron	1,5	50	0.075	1000
Metribuzin	0,54	50	0,027	1000
Pencycuron	1,14	50	0,057	1000
Iprodione	1,26	50	0,063	1000
Boscalid	0,348	50	0,0174	1000
Metalaxyl	0,498	50	0,0249	1000
2,6-dichlorobenzamide	0,072	50	0,0036	1000
Mandipropamide	0,072	50	0,0036	1000
Propamocarb	1,14	50	0,057	1000
Prosulfocarb	0,36	50	0,018	1000
Prothioconazole-desthio	0,078	50	0,0039	1000
Total		750	Add 250 µL DMSO = T15 (300.000x)	

Appendix 4: Stock solutions dilution of synthetic mixture, and final concentrations

Table A4a: The making of stock solution dilutions of the synthetic mixture was performed by this scheme, starting with the highest concentration.

C1 (x)	V1 (µL)	C2 (x)	V2 (µL)	Volume of DMSO
300000	150	100000	450	300
100000	135	30000	450	315
30000	150	10000	450	300
10000	135	3000	450	315
3000	150	1000	450	300
1000	135	300	450	315
300	150	100	450	300
100	135	30	450	315
30000	30	20000	450	420
30000	9,967	6500	460	450,033

Table A4b: Final test concentrations of the synthetic mixture

	C1 (concentration factor)	V1 (µL)	**C2 (concentration factor)**	V2 (µL)	HSM to add (µL)	Algae ($20*10^3$)
T15	300000	2	**3000**	200	98	100
T14	100000	2	**1000**	200	98	100
T13	30000	2	**300**	200	98	100
T12	10000	2	**100**	200	98	100
T11	3000	2	**30**	200	98	100
T10	1000	2	**10**	200	98	100
T09	300	2	**3**	200	98	100
T08	100	2	**1**	200	98	100
E2	20000	2	**200**	200	98	100
E1	6500	2	**65**	200	98	100

Norges miljø- og biovitenskapelig universitet
Noregs miljø- og biovitskapelege universitet
Norwegian University of Life Sciences

Postboks 5003
NO-1432 Ås
Norway