Toxicity of single biocides and their mixtures in the algae *Chlamydomonas reinhardtii*

Giftighet av biocider og blandinger av disse for algen *Chlamydomonas reinhardtii*

Philosophiae Doctor (PhD) Thesis

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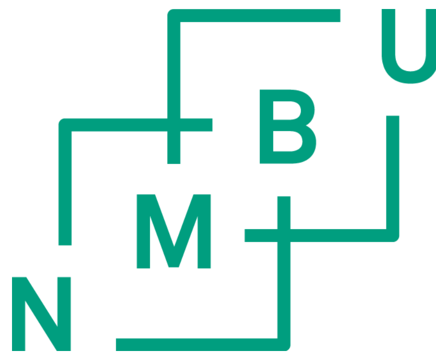

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Ana Catarina Almeida, Oslo 2015

“The love for all living creatures is the most noble attribute of man.”

Charles Darwin

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Summary

Aquatic organisms are exposed to several organic compounds including biocides. These compounds are widely used, for instance as disinfectants, in antifouling paints, or as material preservatives. Biocides can originate from different sources such as agricultural, urban and industrial runoff. Their presence in the aquatic environment is cause of concern, as they can be highly toxic, not only to target, but also to non-target organisms. Each type of biocide has specific effects according to its mode of action (MoA). Additionally, they may exist in complex mixtures and affect organisms through combined toxicity. This study intended to characterise the single and combined effects of five environmentally relevant biocides, aclonifen, bifenox, dichlofluanid, metribuzin and triclosan on the unicellular algae *Chlamydomonas reinhardtii*.

Biocide toxicity was examined by analysing their effects in the freshwater microalgae through three different toxic endpoints: inhibition of growth, Photosystem II (PSII) efficiency and formation of Reactive Oxygen Species (ROS). The combined toxicity assessment was conducted using the concentration (CA) and independent action (IA) prediction models to analyse if the compounds in a mixture caused toxicity by similar or dissimilar MoA, respectively. For the compounds/mixtures which MoA and adverse outcomes were understood, preliminary Adverse Outcome Pathways (AOPs) were developed to collect, organize and evaluate all the relevant information. The results were also used to assess the potential environmental risk of the biocides to algae when present as single chemicals and in mixtures, by using a Risk Quotients (RQs) and Toxic Units (TUs) approach.

The growth inhibition test allowed verifying the general toxicity of each biocide and of the mixture with all the compounds. The order of toxic potency was: bifenox> metribuzin> dichlofluanid> aclonifen> triclosan. The IA model best predicted the mixture involving all the biocides at 48 h and 72 h, thus suggesting that the compounds had different MoA. A potential antagonism was observed particularly at 24 h for low to median effect levels, possible due to the fact that the different compounds required longer

time to propagate the effects to the apical level (growth). In this study, metribuzin, dichlofluanid, bifenox and triclosan showed a potential risk to algae, although the risk by dichlofluanid might be overestimated due to lack of adequate exposure information. The mixture with all the compounds presented a potential environmental risk for algae.

From the 5 tested compounds (aclonifen, bifenox, dichlofluanid, metribuzin and triclosan), only aclonifen and metribuzin showed effects on the PSII efficiency, with the first being the most toxic. This effect was correlated with the inhibition of growth, showing that the inhibition of PSII was the main toxic MoA for these compounds. The effects of the binary mixture were best described by the IA model, consistent with these herbicides displaying additive effects by dissimilar MoA. For the growth, IA best fitted the data in the beginning of exposure, whereas the data was best predicted by CA at longer exposures. A concentration-dependent deviation from additivity, interpreted as synergy, was observed for medium to high concentrations of this mixture. While the single compounds did not present a risk at environmentally relevant concentrations, the effects of the binary mixture were higher than expected and a potential environmental risk was identified.

The formation of ROS was a potential MoA for aclonifen and metribuzin; therefore, a high-throughput assay for ROS detection was used to analyse the 5 compounds (aclonifen, bifenox, dichlofluanid, metribuzin and triclosan). Among these, only aclonifen, metribuzin and bifenox induced ROS in a significant and concentration-dependent manner. The combined effects of the three herbicides were also studied in binary and ternary mixtures. The best predictions were achieved by the CA model when testing the ternary mixture and the binary mixture of aclonifen and bifenox at low to median effect levels, whereas synergism was observed at higher effects levels. The binary mixture of aclonifen and metribuzin was best predicted by the IA model, while the binary mixture of bifenox and metribuzin was equally well predicted by the two models. The combination of ROS formation and inhibition of photosynthesis was proposed to explain the observed combined effects.

The present work demonstrated that *C. reinhardtii* is a suitable model organism to evaluate the toxicity of biocides and their mixtures. The applied methods were able to determine both sublethal and lethal effects of the studied compounds and provided a better understanding on their MoA. The CA and IA models provided good predictions for the observed effects of the mixtures of biocides with similar and dissimilar MoA. The cumulative risk assessment using TUs and RQs based approaches were shown to be an applicable way for predicting the risk of the biocides mixtures to algae.

The present work has contributed to advance the field of ecotoxicology by providing a better understanding of the MoA of commonly used biocides, deciphering the combined toxicity of simple mixtures of these and identifying whether these biocides and their mixtures represent a risk to algae under ecological relevant exposure scenarios. Given the limited data available on the studied biocides, the knowledge gathered in the present work contributed to the characterization of their MoA and ecotoxicological effects in *C. reinhardtii*. This information can be integrated to further develop risk assessment tools for a better understanding and protection of the aquatic environment.

Sammendrag

Vannlevende organismer er utsatt for en rekke organiske forbindelser, inkludert biocider. Disse forbindelsene er mye brukt blant annet som desinfeksjonsmidler, i bunnstoff, eller som konserveringsmidler. Biocider kan stamme fra ulike kilder som landbruk, urban og industriell avrenning. Deres tilstedeværelse i det akvatiske miljøet er bekymringsverdig da de kan være svært giftige til målorganismer, men også arter de ikke er utviklet for å påvirke. Hver type biocid har spesifikke effekter i henhold til sin virkningsmekanisme (MoA). I tillegg eksisterer disse stoffene i komplekse blandinger og påvirke organismer gjennom kombinasjonsgiftighet. Dette studiet hadde til hensikt å karakterisere effekten av enkeltstoffer og blandinger av de fem miljørelevante biocidene aclonifen, bifenox, diklofluanid, metribuzin og triklosan på den encellede algen *Chlamydomonas reinhardtii*.

Giftigheten av biocidene ble undersøkt ved å analysere deres effekter på ferskvannsalgen gjennom tre forskjellige giftighetsmekanismer: hemming av vekst, hemming av fotosystem II (PSII) effektivitet og dannelse av reaktive oksygenarter (ROS). Vurderingen av kombinasjonseffekter ble utført ved bruk av prediksjonsmodeller basert på konsentrasjonaddisjon (CA) og uavhengig samvirkeinteraksjon (IA) for å analysere om forbindelsene i en blanding skyldes effekten av samme eller ulik virkningsmekanisme. For forbindelsene/blandinger der MoA og skadeeffekter ble kartlagt, ble såkalte Adverse Outcome Pathways (AOP) utviklet for å samle, organisere og vurdere relevant informasjon. Resultatene ble også brukt til å vurdere den potensielle risikoen av biocidene på alger når tilstede som enkeltstoffer og i blandinger ved hjelp av beregning av risikokvotienter (RQ) og toksiske enheter (TU).

Testen for veksthemming i alger verifiserte den generelle toksisitet av hvert biocid og blanding av disse. Giftighetspotensialet til de ulike stoffene var: bifenox> metribuzin> diklofluanid> aclonifen> triklosan. Modellen for uavhengig samvirkeinteraksjon predikerte effekten av alle biocidene ved 48 og 72 timer, og antydet at forbindelsene hadde ulike MoA. En potensiell antagonisme ble observert etter 24 timer eksponering for lave til intermediære effektnivåer, trolig på grunn av at forbindelsene hadde ulik evne til

å gi effekter på organismenivå (vekst hemming). I dette studiet ble det påvist at metribuzin, diklofluanid, bifenox og triklosan hadde en potensiell risiko i forhold til alger, selv om risikoen av diklofluanid muligens var overestimert pga. mangel på tilstrekkelig eksponerings-informasjon. Blandingen med alle forbindelser viste seg å representere en potensiell miljørisiko for alger.

Av de 5 testede forbindelsene (aclonifen, bifenox, diklofluanid, metribuzin og triklosan) var det bare aclonifen og metribuzin som ga effekter på PSII effektivitet, der aclonifen var den mest giftige. Denne effekten viste godt samsvar med veksthemming, som viser at inhiberingen av PSII var den viktigste MoA for disse forbindelsene. Virkningene av den binære blandingen ble best beskrevet av IA, noe som var i samsvar med at disse herbicidene ga additive effekter og hadde ulik MoA. For veksthemming ga IA best tilpasning til de eksperimentelle data i begynnelsen av eksponeringen, mens CA ga best tilpasning til effektdataene ved lengre eksponering. Et konsentrasjonsavhengig avvik fra additivitet, tolket som synergi, ble observert for middels til høye konsentrasjoner av denne blandingen. Mens de enkelte forbindelser ikke utgjorde en risiko for alger ved miljørelevante konsentrasjoner, var effekten av den binære blanding høyere enn forventet og representerte en potensiell miljørisiko for algene.

Dannelsen av ROS er en potensiell MoA for aclonifen og metribuzin og medførte at et høy-kapasitetsassay for deteksjon av ROS ble brukt for å analysere effekten av de 5 forbindelsene (aclonifen, bifenox, diklofluanid, metribuzin og triklosan). Av disse biocidene var det bare aclonifen, bifenox og metribuzin som indusert ROS på en signifikant og konsentrasjonsavhengig måte. Samvirkeeffekten av de tre herbicidene ble også undersøkt i binære og ternære blandinger. De beste prediksjonene ble oppnådd ved bruk av CA modellen for den ternære (alle tre stoffene) og binære blandingen av aclonifen og bifenox med lav til intermediære effektnivåer, mens synergisme ble observert ved høyere effekternivåer. Den binære blanding av aclonifen og metribuzin ble beste predikert av IA-modellen, mens den binære blanding av bifenox og metribuzin var predikert like godt av de to modellene. Ble foreslått at en kombinasjon av ROS dannelse og inhibering av fotosyntesen kunne forklare de observerte blandingseffektene.

Dette arbeidet viste at *C. reinhardtii* er en velegnet modellorganisme for å vurdere giftigheten av biocider og deres blandinger. De anvendte metodene var i stand til å bestemme både subletale og letale effekter av forbindelsene testet og ga en bedre forståelse av deres MoA. CA og IA-modellene ga gode prediksjoner av de observerte blendingseffektene med lik og ulik MoA. Den kumulative risikovurdering ved bruk av TUs og RQs baserte tilnærminger viste seg å være en relevant måte å forutsi miljørisikovurdering av biocidblandinger.

Dette arbeidet har bidratt til å utvikle økotoksikologisk forskning ved å gi en bedre innsikt i MoA til vanlig anvendte biocider, avdekke sammenhengen om kombinasjonseffekter av enkle blandinger av disse, og identifisere hvorvidt disse biocidene og deres blandinger utgjør en risiko for alger under økologisk relevante eksponeringssituasjoner. Gitt den begrensede datatilgjengeligheten for disse biocidene har kunnskap samlet i dette arbeidet bidratt til karakterisere deres MoA og undersøke økotoksikologiske effekter i *C. reinhardtii*. Denne informasjonen kan samlet benyttes til å videreutvikle risikovurderingsverktøy og dermed bedre både kunnskapen om risiko og vern av organismer i det akvatiske miljøet.

List of papers

This PhD thesis is based on the following papers, which will be referred in the text by their roman numerals (I-III):

Paper I

Almeida, A.C., Petersen, K., Langford, K., Thomas, K.V., Tollefsen, K.E., in prep. Combined toxicity of five priority biocides on the growth of *Chlamydomonas reinhardtii*.

Paper II

Almeida, A.C., Langford, K., Thomas, K.V., Tollefsen, K.E., in prep. Photosystem II (PSII) efficiency in *Chlamydomonas reinhardtii* exposed to environmentally occurring biocides.

Paper III

Almeida, A.C., Gomes, T., Thomas, K.V., Tollefsen, K.E., in prep. Induction of reactive oxygen species (ROS) in *Chlamydomonas reinhardtii* after exposure to single biocides and their simple mixtures

Abbreviations

3,5-DCP – 3,5-dichlorophenol

AF – assessment factor

AO – adverse outcome

AOP – Adverse Outcome Pathway

CA – concentration addition

carboxy-H_2DFFDA - 5-(and-6)-carboxy-2′,7′-difluorodihydrofluorescein diacetate

CAT - catalase

CRC – concentrations response curve

DCMU - 3-(3,4-dichlorophenyl)-1,1-dimethylurea, diuron

DDT – dichlorodiphenyltrichloroethane

DEET - N,N-diethyl-meta-toluamide

DFFDA - difluorodihydrofluorescein diacetate

DPE - diphenylether

DPEI - 5-[2-chloro-4-(trifluoro-methyl)phenoxy]-2-nitroacetophenone oxime-*O*-(acetic acid, methyl ester)

DMS - *N,N*-dimethylsulfamide

DMSO – dimethyl sulfoxide

EDA – effect-directed analysis

EC – European Commission

EC_{50} – concentration of a compound that gives half-maximal response

EPSP - 5-enolpyruvylshikimate 3-phosphate synthase

EU – European Union

F_v/F_m - maximum quantum yield

G6PDH - glucose-6-phosphate dehydrogenase

GPX - glutathione peroxidase

GR – glutathione reductase

GSH – glutathione

GST - glutathione-*S*-transferase

GSSG - glutathione disulphide

GR - glutathione reductase

H_2O_2 - hydrogen peroxide

IA – independent action

KE – key event

KER - key event relationship

LC_{50} – lethal concentration required to kill 50% of the population

LOEC - Lowest Observed Effect Concentration

MDR – model deviation ratio

MiE – molecular initiating event

MEC – Measured Environmental Concentration

MoA – mode of action

MOA – mechanism of action

MOPs - 3-(N-Morpholino)propanesulfonic acid

m/z - mass-to-charge ratio

NOEC – No Observed Effect Concentration

NOED - no observed effect dose

$^1O_2$ - singlet oxygen

O_2^- - superoxide anion radical

OECD – Organisation for Economic Co-operation and Development

OH^- - hydroxyl radical

PBPK - pharmacokinetic physiologically based modelling

PCP - personal care products

PEC – predicted effect concentration

PNEC – predicted no effect concentration

PRX – peroxiredoxin

PSI – photosystem I

PSII – photosystem II

ROS – reactive oxygen species

RQ – risk quotient

RQ_{STU} - overall risk quotient for a mixture

-SH – sulfhydryl group

SOD – superoxide dismutase

TBT – tributyltin

TU – toxic unit

γ-GCS - γ-glutamylcysteine synthetase

1. Introduction

The presence of a large number of different organic contaminants such as biocides in the aquatic environment is cause for concern. Organic compounds are continuously being produced for industrial, domestic, or agricultural use. Some of these compounds enter in the wastewater as part of the influent and, unless specifically transformed by the wastewater treatment processes, may be emitted in the effluent and released into receiving waters (Lishman et al., 2006). Freshwater basins like lakes and rivers, particularly those in lowland regions, are especially affected as may be the receptors for several water sources such as treated and non-treated sewage effluents, urban and rural run-off, and industrial effluents. Some parts of these effluents will runoff into surface water bodies, while other will infiltrate and contaminate the groundwater system. This contamination can restrict the use and re-use of water, an extremely important natural resource that needs to be protected (Bedding et al., 1982; Murray et al., 2010).

Organic compounds are normally present at low concentrations (in ng or μg/L) in the aquatic environment. However, long-term exposure to low concentrations of certain organic contaminants may have deleterious effects on organisms (Bedding et al., 1982). The number and quantities of organic compounds in use increases every year. Therefore their risk of to aquatic organisms constantly increases (Bedding et al., 1982). Although the individual environmental concentration of each compound is generally low, compounds may affect organisms through combined toxicity. Their combinations can produce effects different from those originated by the single compounds, such as additivity, synergism (greater than additivity), or antagonism (less than additivity) (Altenburger et al., 2003; Groten et al., 2001).

1.1. Biocides

Biocides are a relevant group of organic contaminants from an ecotoxicological point of view (la Farré et al., 2008). These are widely used products to control organisms that are harmful to human or animal health or that can damage natural or manufactured materials.

Examples of these harmful organisms include pests and germs, such as fungus and bacteria. These biocidal products are for instance disinfectants, industrial chemicals as antifouling paints, and material preservatives. However, due to their intrinsic chemical characteristics and constant use, these products can also pose a risk to non-target organisms and to the overall environment (EU, 2012).

Biocides can be divided in 22 product types and distributed under four main categories of usage (Table 1 in supplementary data): disinfectants, preservatives, pest control and other biocidal products (EU, 2012). Before being commercialized, biocidal active substances need the approval of regulatory entities and to be in accordance with specific regulations, such as the Regulation No 528/2012 of the European Parliament and of the Council concerning their availability on the market and use. This regulation takes into account the precautionary principle, in order to guarantee the protection of not only human and animal health, but also the environment. It came to replace the Directive 98/8/EC for a better applicability of the imposed rules (EU, 2012). Although biocides are well-regulated products, their hazardous effects should still be the object of scientific and regulatory scrutiny to safeguard against any unforeseen effects in the aquatic environment. Some of these biocides are considered of emerging concern with insufficient toxicity information, and in certain cases also highly toxic to organisms, and in many cases to photosynthetic primary producers (EU, 2013; USEPA, 2008).

1.2. Ecotoxicological testing

Ecotoxicology can be defined as the study of harmful effects of chemicals on ecosystems, including the effects not only in individual organisms, but also the consequent effects at the population level and above. The term was introduced by Truhaut in 1969, derived from the words of “ecology” and “toxicology”, being a discipline within the wider field of environmental toxicology. In ecotoxicology, the ecosystem response is studied at all levels (Fig. 1), concerned with the wide variety of effects on individual organisms at the different organizational levels: molecular, cellular and whole organism. The final objective is to understand how these consequences at the organism level can then affect

populations, community compositions, and ultimately the whole ecosystem (Walker et al., 2001).

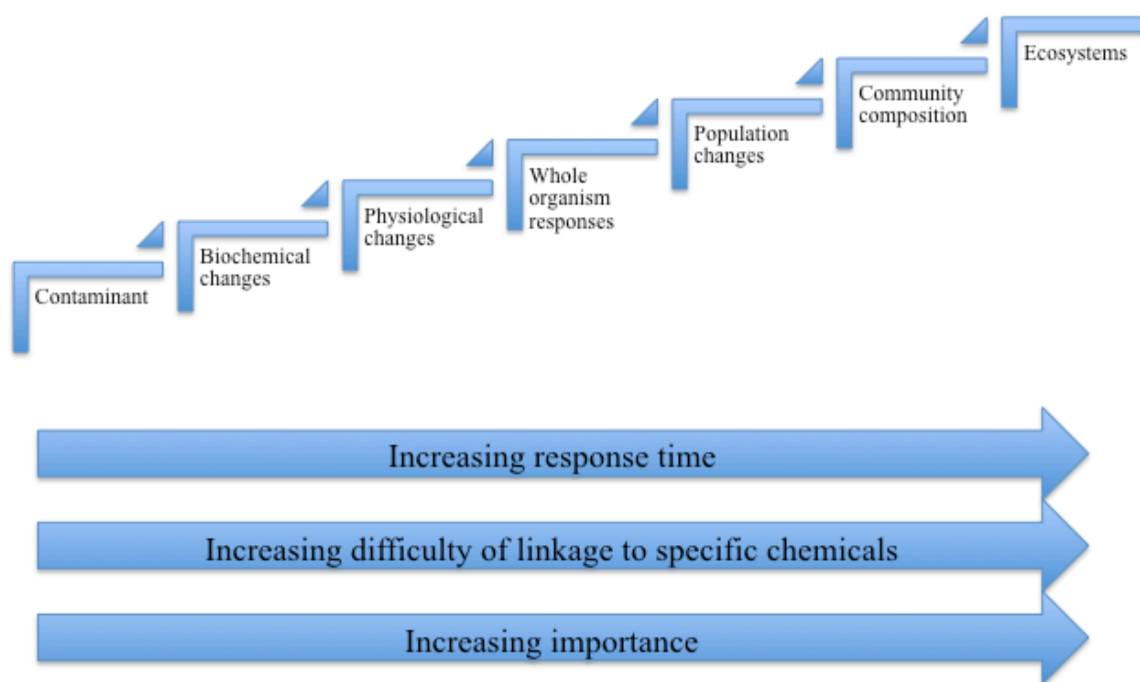

Fig. 1. Schematic relationship of linkages between responses at different organizational levels (adapted from Walker et al., 2001).

1.2.1. Toxicokinetics and toxicodynamics

A contaminant present in the environment can enter in an organism by one or more routes of uptake. Depending on the chemical, the species and the environmental conditions, one route of uptake may be dominant or more than one may be significant. Both the efficiency of uptake and the degree of toxic effect differ between these routes. With aquatic organisms, direct uptake from water is a route of major importance. Uptake can also occur from food during its passage through the alimentary system. The relative importance of these routes of uptake differs between organisms and between chemicals and depends on environmental conditions. In some cases, all of these routes may operate in one organism at one time. Much of the toxicity testing carried out with aquatic organisms (e.g.: fish, daphnia, algae) is concerned with direct absorption of chemicals from water (*i.e.*, the bioconcentration). The chemicals may be in solution, in suspension or both (Walker et al., 2001).

The bioavailability, uptake, metabolism, storage, and excretion of chemicals establish toxicokinetics, which is related with the fate of chemicals in organisms. Toxicokinetics has relevance to ecotoxicology as it aids to understand and predict the behaviour of organic pollutants within living organisms. Bioavailability is the potential for uptake of a substance by an organism. It is can be expressed as the fraction taken up by the organism in relation to the total amount of the substance available. Factors affecting the bioavailability of a chemical depend on the route of uptake, and if the chemical is in the sediment, dissolved in water, or in organisms. For water-soluble substances, the primary source of toxicant is water, and the bioavailability depends on complex formation. Lipid-soluble substances are taken up especially from sediment or from other organisms. The bioavailability from water decreases with increasing lipophilicity and with increasing amount of dissolved organic carbon or colloids in the aquatic phase. Regarding the sediment, both its properties (such as grain size) and the amount of organic material affect bioavailability. The main abiotic factors affecting bioavailability are oxygenation and pH (Nikinmaa, 2014; Walker et al., 2001).

Toxicodynamics focus on the interactions between chemicals and their site of action, regarding their harmful effects on organisms (Walker et al., 2001). It describes the time course of toxic action at the target site, following physiological impairment of the organism and the effect of any compensating mechanisms and lastly the occurrence of toxic effects at the organism level as mortality (Ashauer and Escher, 2010).

With the use of the information gathered from both toxicokinetics and toxicodynamics, models can be developed to simulate and clarify the processes that lead to toxicity and the quantification of effects (Fig. 2).

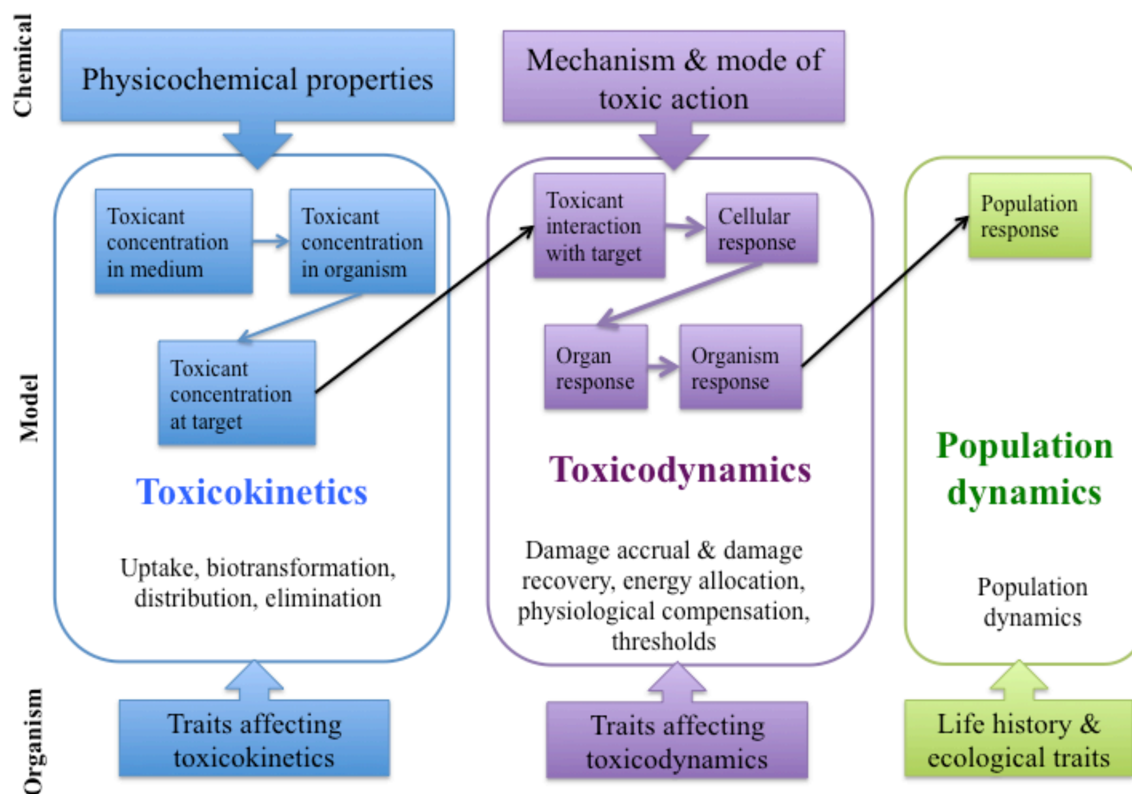

Fig. 2. Mechanistic effect models for ecotoxicology (adapted from Ashauer, 2015).

1.2.2. Adverse effects

In ecotoxicology, the relationship between the quantity of chemical to which an organism is exposed and the nature and degree of consequent toxic effects is of main importance. Dose-response relationships provide the basis for assessment of hazards and risks presented by chemicals. There are many different ways in which toxicity can be measured. The most common measured endpoint is mortality, although there is a growing interest in the use of more sophisticated indices. Biochemical, physiological, reproductive and behavioural effects can also provide measures of toxicity. Most of the toxicity tests provide an estimate of the dose that will cause a toxic response at the 50% level (EC_{50}), or the median lethal dose is the dose that will kill 50% of a population (LC_{50}). It is also possible to establish the highest concentration or dose that will not cause an effect (NOEC/NOED). These values can only be determined in situations where a higher dose

or concentration has produced an effect in the same toxicity test (Fig. 3; Walker et al., 2001).

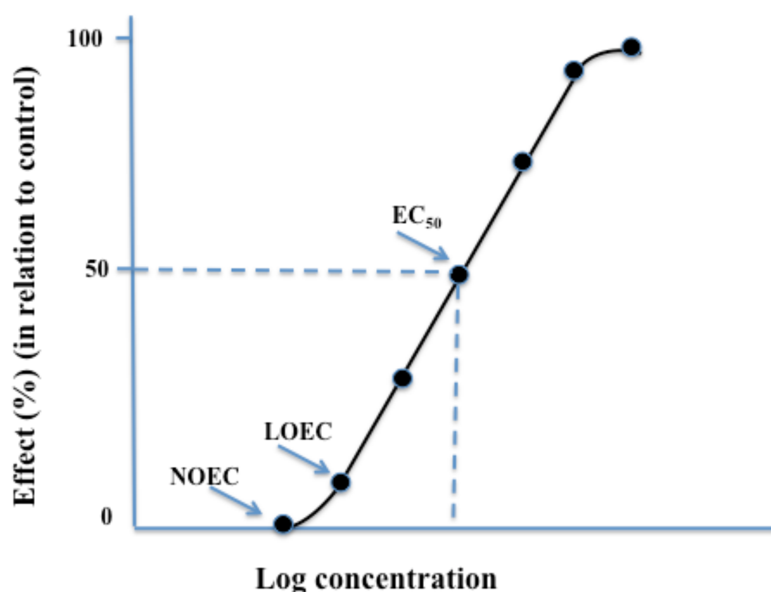

Fig. 3. Toxicity of a compound after 72 h exposure in an aquatic toxicity test (adapted from Walker et al., 2001). NOEC – no observed effect concentration; LOEC - lowest observed effect concentration; EC_{50} – median effect concentration.

The toxic effects of chemicals can be divided in acute or chronic. Acute effects are those occurring rapidly as a result of a short-term exposure to a chemical. In aquatic organisms, these normally occur within few hours, days or weeks. These effects are normally severe, leading to lethality or mortality. Chronic or subchronic toxic effects may occur when the chemical produces deleterious effects, often due to repeated or long-term exposures to low levels of persistent chemicals (Rand, 1995).

Effects may also be divided in lethal or sublethal. The latter does not require the absence of mortality, but indicates that death is not the main primary toxic endpoint being examined. The most common sublethal effects are behavioural (swimming, feeding, attraction-avoidance, prey-predator interactions), physiological (growth, reproduction, development), biochemical (blood enzyme, ion levels), or histological changes (Rand, 1995).

The observed effects can help to understand the toxicological mechanisms, *i.e.*, how chemicals produce biological effects in the organisms. Two different terms are used to described different aspects of toxicological mechanisms, MoA (mode of action) and MOA (mechanism of action). A MoA describes a functional or anatomical alteration at the cellular levels due to the exposure of an organism to a substance. It can be defined as "a common set of physiological and behavioural signs that characterize a type of adverse biological response" (Rand, 1995). MOA, on the other hand, involves the full understanding of the occurring events, describing all the changes at the molecular level, and referring to the specific biochemical interactions that a substance causes. It is usually defined as "the molecular sequences of events leading from the absorption of an effective dose of a chemical to the production of a specific biological response" (Buttherworth et al., 1995; Schlosser and Bogdanffy, 1999). The integrated information gathered from these approaches can for instance be used to develop Adverse Outcome Pathways (AOPs).

1.2.3. Adverse Outcome Pathways (AOPs)

The use of AOPs provides an improved form of organizing ecotoxicological data. It aims to "collect, organize and evaluate relevant information on a chemical, biological and toxicological effect of chemicals", enabling to make links between responses and effects occurring at different levels of organisation (OECD, 2013). It can be described as the sequential progression of events starting from the molecular initiating event (MIE) to the *in vivo* adverse outcome (AO), showing the interactions between the involved pathways. It starts with the MIEs where the chemical interacts with a biological target (e.g.: protein oxidation, DNA biding, etc.), proceeding to a sequential series of biological activities (e.g.: gene activation, altered tissue development, etc.), and finishing in the final adverse effect relevant for risk assessment (e.g.: mortality, disrupted reproduction, etc.; Fig. 4). If well developed, it can provide valuable information for both scientific and regulatory purposes, as it aims to assemble, describe and evaluate available information relevant for an adverse outcome of regulatory importance. The information is divided into 3 main events: the MIE, key events (KE) and the AO where each is interlinked with key event

relationships (KERs) that assess the weight of evidence between the different events that is occurring (OECD, 2013).

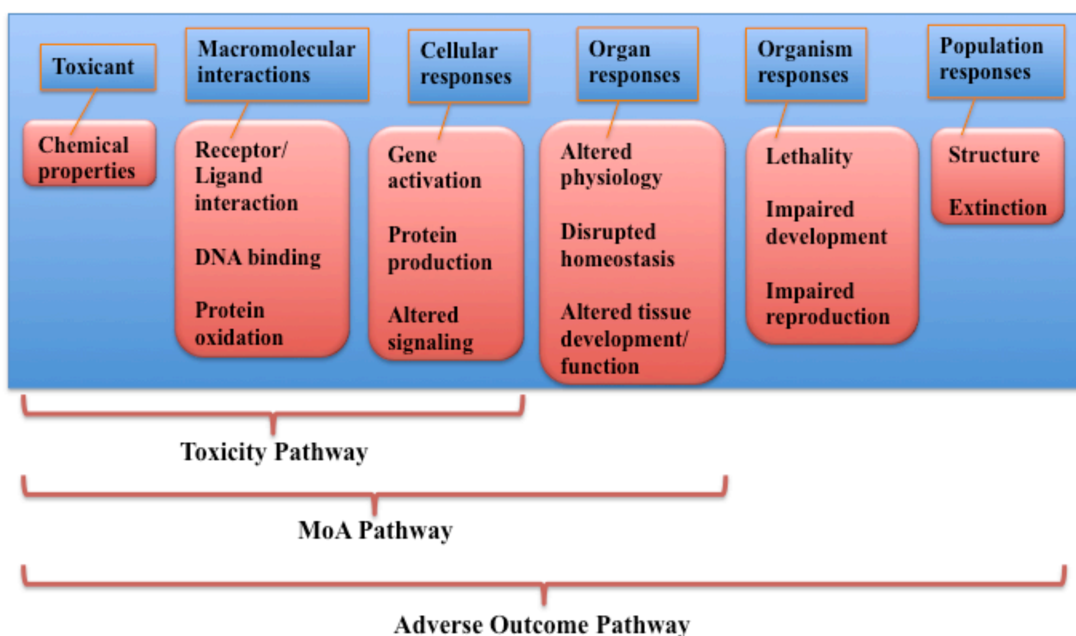

Fig. 4. A schematic representation of an AOP (adapted from OECD, 2011).

The AOP concept represents an innovative form of interpretation and use of both qualitative and quantitative data. These have been increasingly used to build biologically robust linkages between MIE and AO, providing causal links (e.g. the MoA and MOA) for a given chemical or group of chemicals and adversity at the individual, population and sometimes also extrapolation to higher levels of organisation (Kramer et al., 2011).

1.3. Effects of biocides

The number of biocides in use is large and includes a high number of different compounds with very different characteristics and specific effects according to their MoA. For instance, organochlorine, organophosphate, and carbamate insecticides act predominantly by disrupting the nervous system function, whereas herbicides predominantly target different photosynthesis pathways (Table 1). However, the MoA of biocides in target and non-target organisms might not be the same (DeLorenzo et al., 2001).

Table 1. Main biocide MoA on target organisms (adapted from DeLorenzo et al., 2001).

Main class	Group	General toxic effect	Site of action
Organophosphates	Carbamates	Nervous system inhibition	Acetylcholinesterase
Organochlorines	Cyclodienes	Nervous system inhibition	GABA receptor
Herbicides	Ureas, cyclic ureas, triazines, acylanilides, phenylcarbamates, triazinones	Photosynthesis inhibition	Photosystem II (PSII), Hill reaction in the electron transport
	Bipyridiniums	Photosynthesis inhibition (light reaction)	Photosystem I (PSI)
	Pyridazinones	Biosynthesis inhibition	Carotene accumulation
	Chloroacetamide	Biosynthesis inhibition	Fatty acid synthesis
	Dinitroanilines, phosphoric amides, chlorthaldimethyl, propyzamide, cholchinine, 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

Some biocides are much more toxic to non-target organisms than to those which they are used against. They also often leach into water bodies, where their toxicity to aquatic organism represents the major concern. Their toxicity to a given organism is dependent on its uptake and metabolism, and also on the affinity of the site of action of the chemical in that specific organism (Nikinmaa, 2014). In Table 2 are examples of effects of the main biocides classes in non-target organisms.

Table 2. Known MoA of some biocides classes in non-target organisms (adapted from Nikinmaa, 2014).

General group	Chemical group	MoA
Insecticides	Carbamates	Inhibition of cholinesterase, nerve conduction and synapse function.
	Organophosphates	Inhibition of cholinesterase.
	Organochlorines	Affect mainly synaptic transmission.
	Neonicotinoids	Inhibition of cholinesterase.
Herbicides	Atrazine	PSII inhibition
	Diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea)	Inhibitor of photosynthesis.
	Glyphosate	It inhibits EPSP enzyme that catalyzes a step in tryptophan, phenylalanine, and tyrosine production.
	Linurin	Non-specific inhibitor of photosynthesis.
Fungicides	Benzimidazoles	Inhibit mitotic division of fungal cells.
	Dithiocarbamates	Inhibit fungal growth.
	Famoxadones	Inhibit mitochondrial energy production.
	Fenamidones	Inhibit mitochondrial energy production.
	Chloronitriles	Inhibit fungal growth.
	Copper	Inhibit fungal growth.
	Sulfur	Inhibit fungal growth.
	Strobilurines	Inhibit mitochondrial energy production.
	Triazoles	Inhibit C14-demethylase.

Herbicides are an important class of biocides specifically used to handle or control unwanted vegetation such as weeds or defoliate trees. Over half of the existing herbicides act primarily on the light reactions in photosynthesis pathway. Many group of herbicides act by inhibiting the Hill reaction of electron transport, affecting the Photosystem II (PSII), such as ureas, cyclic ureas, triazines, acylanilides, phenylcarbamates, and triazinones. Others act by intercepting electrons from the reducing side of Photosystem I (PSI), such as the bipyridinium herbicides diquat and paraquat (Corbett et al., 1984; DeLorenzo et al., 2001; Nikinmaa, 2014).

For many other biocides, although deleterious effects have been documented, the mechanism of toxicity to non-target organisms often remains unknown (DeLorenzo et al., 2001; Nikinmaa, 2014).

1.4. Algal toxicity

Algae contribute approximately 40 to 50% of the oxygen in the atmosphere (Andersen, 2005). Therefore, changes in their density and composition can affect the chemical and biological quality of the environment. The evaluation of the phytotoxicity of a contaminant is an essential component of any ecological risk assessment. Freshwater microalgae are used more frequently in phytotoxicity tests than any other type of freshwater or marine plant. Algae have also been found to be more sensitive than animal species to several potential contaminants such as organic contaminants, including biocides (Hoffman et al., 2003).

One of the most used tests, namely for regulatory purposes, is the inhibition of algal growth. There are however other ways of evaluating the toxic properties of chemicals, most of them involving the chemicals MoA. For example, determining the inhibition of PSII in photosynthetic organisms exposed to herbicides, or the formation of Reactive Oxygen Species (ROS) in cells exposed to certain chemicals. These approaches become even more viable if the molecular mechanisms of toxicity are known, leading to a better understanding of how the chemicals negatively interact with cellular macromolecules (Walker et al., 2001).

1.4.1. Inhibition of algal growth

The Organization for Economic Co-operation and Development (OECD) published guidelines for testing chemicals using the “Freshwater Alga and Cyanobacteria, Growth Inhibition Test” (guideline 201). In this test, exponentially growing test organisms are exposed to the test substance in batch cultures normally for 72 h. The measured response is the reduction of growth in a series of algal cultures exposed to various concentrations

of a substance. The response is evaluated as a function of the exposure concentrations in comparison with the average growth of controls (organisms not exposed to the test substance). To measure the reduction on the growth rate, the exposed cultures should have unrestricted exponential growth with sufficient nutrients and continuous light for a sufficient period of time (OECD, 2011).

1.4.2. Photosystem II (PSII) efficiency

Biocides can also affect the photosynthetic capacity of algae and plants. Photosynthesis is an extremely complex and highly integrated series of redox and enzymatic processes, which are critical to the survival of phototrophs. As photosynthesis involves several delicate processes that can be affected by contaminants, it has the potential to be used as an ecotoxicological endpoint to assess the impact of toxicants (Falkowski and Raven, 2007; Nestler et al., 2012a; Ralph et al., 2007). Chlorophyll *a* fluorescence is a convenient method for assessing the condition of the photosynthetic apparatus. This is a feasible toxic endpoint that can be adapted to test different toxicant types, and able to provide results at environmentally relevant concentrations of toxicants. Chlorophyll *a* has been used for over 20 years as an indicator of stress responses for both terrestrial and aquatic phototrophs, and can provide an understanding of the MoA of contaminants and (Ralph et al., 2007).

Chlorophyll *a* fluorescence is a simple method for measuring the amount of absorbed energy used in the photochemical processes. It enables the monitoring of several photochemical processes linked with photosynthesis, providing an insight into the organism's overall health. When a photon of light is captured by a chloroplast, it excites chlorophyll *a* to an elevated state. This excitation energy can be used in 3 ways: be transferred down through the electron transport chain to ultimately fix carbon (photochemical quenching), be dissipated as heat (non-photochemical quenching), or be re-emitted at a slightly longer wavelength in the form of fluorescence (55% of absorbed energy). Changes in chlorophyll *a* fluorescence due to toxicant exposure can be linked to their impact on photosynthetic processes, such as binding to the plastoquinone or

blocking the electron transport. Fluorometers can be used to measure the relative changes in the amount of fluorescence emission from chlorophyll *a* molecules (Ralph et al., 2007).

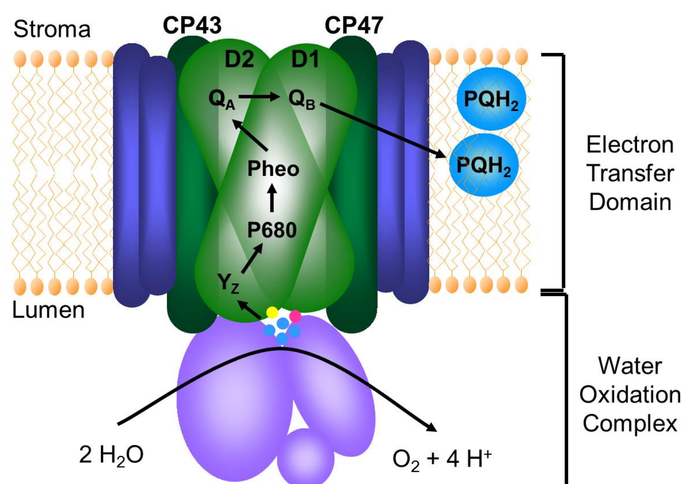

Fig. 5. Photosystem II (PSII) structure (https://newunderthesunblog.wordpress.com/the-basics/the-light-reactions/photosystem-ii/photosystem-ii-structure/).

Most of the studies analysing the PSII efficiency have been made for herbicides that specifically target the photosynthetic apparatus. If a toxicant interrupts the process of electron transport, the fluorescence emission will change. This is the case of PSII inhibitors that act by competing with plastoquinone at the Q_B binding site of the D1 protein in PSII reaction centre, inhibiting energy transfer and affecting algae growth (Fig. 5). PSII is formed by more than 25 polypeptides and surrounded by a variety of chlorophylls *a* and *b* binding proteins. At the enzymatic heart of the complex are two polypeptides, the D1 and D2, being the PSII primary donor P680 between these proteins. The pheophytin and Q_A are on the D2 protein, and the redox-active tyrosine (Y_Z) and the Q_B plastoquinone on the D1 protein. In the D2 protein, the Q_A plastoquinone remains relatively fixed while Q_B plastoquinone can move freely to in and out of the D1 protein at the "Q_B site". Inhibitors of PSII electron transport generally bind at the "Q_B site", preventing the reduction and binding of the Q_B plastoquinone. The inhibition of PSII activity is particularly a well-characterised MoA for many herbicides, for instance for triazines, triazinones and ureas (Table 2; Falkowski and Raven, 2007).

For example, the phenylurea herbicide diuron (DCMU) reversibly inhibits photosynthetic electron flow to the plastoquinone in PSII by blocking the electron transport chain just after the primary electron acceptor (Q_A). This process causes a simultaneous decrease in photochemical and non-photochemical quenching. Atrazine and metribuzin are also triazine herbicides that interfere with photosynthesis by binding to the Q_B-binding niche on the D1 protein of the photosystem II complex (Fairchild et al., 1998). Although the mechanisms of action and the impact sites remain unconfirmed for some contaminant classes, the PSII (which can effectively be monitored using chlorophyll *a* fluorescence) is generally the most sensitive target site for many herbicides (Cedergreen, 2014; Falkowski and Raven, 2007; Magnusson et al., 2008).

1.4.3. Reactive Oxygen Species (ROS)

The exposure of organisms to biocides can potentially induce the production of ROS. Although aerobic organisms have significant energetic advantages by using molecular oxygen as a terminal oxidant in respiration, its presence in the cellular environment poses a constant oxidative threat to cellular structure and processes (Alscher et al., 1997). Though O_2 is a completely harmless molecule, it has the potential to be partially reduced and form toxic ROS. In photosynthetic organisms, ROS are always formed by the inevitable leakage of reactive electrons from the electron transport activities of chloroplasts, mitochondria and the plasmamembrane (Foyer et al., 1997). However, ROS production can also be stimulated by several environmental stresses such as exposure to herbicides (Tanaka, 1994), heavy metals (Weckx and Clijsters, 1996), high levels of light (photoinhibition, photooxidation; Foyer et al., 1997), drought (Loggini et al., 1999), high salt concentration (Meneguzzo et al., 1999), extremes of temperature (Rao et al., 1995), UV irradiation (Murphy and Huerta, 1990), air pollutants including ozone (Sharma et al., 1996), water stress (Boo and Jung, 1999), mechanical and physical stress (Legendre et al., 1993), and also in response to biotic stresses such as invasion by various pathogens (Low and Merida, 1996).

The partial reduction of O_2 in endogenous reactions gives rise to the formation of both radical and non-radical ROS, which are highly toxic (Livingstone, 2001). The major ROS are indicated in Table 2 in the supplementary data.

As oxygen is required for the life of all aerobic organisms, these have developed effective mechanisms to reduce oxidative stress. Oxidative stress can be defined as a disturbance in the pro-oxidant–antioxidant balance in favour of the former, leading to potential damage. The damage may be no only the direct oxidative damage, but also the indirect failure of any repair or replacement systems necessary to repair any cellular damage. An increase may either be caused by increased ROS formation or by decreased efficiency of their removal, due to either decreased amounts of ROS scavengers (redox buffers such as glutathione and ascorbate) or decreased antioxidant enzyme activity. These ROS molecules react very quickly with existing biomolecules and can disturb their function. The different ROS species have significantly different stability and reactivity (Table 4; Livingstone, 2001; Nikinmaa, 2014).

Although ROS are usually considered as molecules associated with oxidative stress, several studies exist showing that they take part in normal cellular signalling (Foyer and Noctor, 2003). This increases the possibilities of ROS to be toxicologically important. Even at concentrations that do not cause measurable structural alterations, cellular signaling may still be disturbed (Nikinmaa, 2014).

In Figure 6 the redox cycle is briefly described along with the generation of ROS by the presence of a contaminant, together with some antioxidant defences and of their known toxic consequences. Antioxidants are any substance that can significantly delay or prevent the oxidation of a substrate in an organism. These can be enzymes that directly remove free radicals or molecules that decrease the formation of radicals, like proteins that minimize the availability of pro-oxidants (Livingstone, 2001; Nikinmaa, 2014).

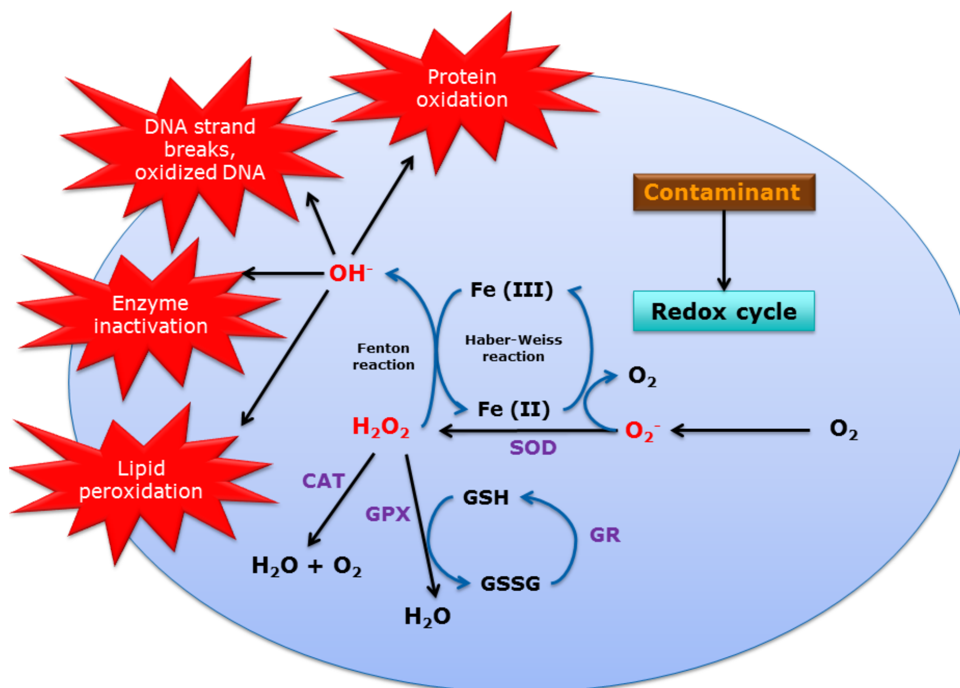

Fig. 6. ROS production, defence mechanisms and effects of free radicals on cells exposed to organic compounds. $^1O_2$ - singlet oxygen, O_2^- - superoxide anion radical, OH^- - hydroxyl radical, SOD - superoxide dismutase, H_2O_2 - hydrogen peroxide, CAT - catalase, GPX - glutathione peroxidase, GSH - glutathione, GSSG - glutathione disulphide (oxidized form of GSH), GR - glutathione reductase (adapted from Unfried et al., 2007).

The enzymes involved in antioxidant defence can be divided into enzymes involved in free radical or redox metabolism, and enzymes indirectly associated with redox changes. There are enzymes involved in the synthesis of the major small antioxidant molecules such as glutathione (γ-glutamylcysteine synthetase (γ-GCS), glutathione synthetase) and ascorbate (the rate-limiting enzyme in synthesis), enzymes involved in the formation of pro-oxidants, and enzymes regulating the equilibrium of redox couples (in addition to GSH/GSSG, the major ones are NAD^+/NADH + H^+ and $NADP^+$/NADPH +H^+; a major enzyme affecting redox-couple balance is a rate-limiting enzyme of the pentose phosphate pathway, glucose-6-phosphate dehydrogenase (G6PDH) (Nikinmaa, 2014).

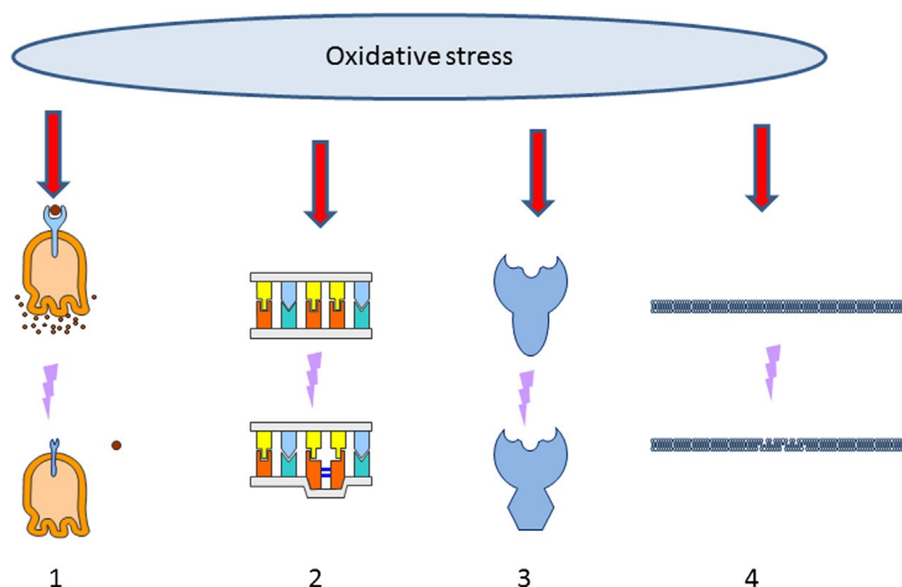

Fig. 7. Different levels of oxidative stress effects (Nikinmaa, 2014).

There are different levels of oxidative stress (Fig. 7). Before any structural changes occur, oxidative stress can affect signaling, as ROS seem to be involved in cellular signaling (1 in Fig.7). If the oxidative stress exceeds a certain threshold, where the oxidant defences are no more able to reduce the stress, effects at different levels can be observed. Oxidative stress can cause effects on DNA (2 in Fig. 7), for instance the increased formation of DNA adducts. If DNA damage overwhelms the repair capacity, an increased mutation rate is observed. Oxidative stress can affect the three-dimensional structure of proteins (3 in Fig. 7), with consequences in their activity. Oxidative stress can also influence lipids (4 in Fig. 7), causing for example lipid peroxidation. Such changes can cause alteration in the permeability of cell membranes (Nikinmaa, 2014).

Oxidative damage in aquatic animals has often been examined by measuring protein carbonylation and oxidation (as indicators of changes in protein structure), and lipid peroxidation using the TBARS (thiobarbituric-acid-reactive species) assay, and using the comet assay (as an indicator of damage to DNA structure). Oxidative damage can thus be observed in all major biomolecules. More recently fluorescent probes have been specifically designed to detect ROS *in vivo* or to label ROS-induced cellular damage (Gomes et al., 2005).

1.5. Combined toxicity

In the aquatic environment, organisms are exposed to mixtures of contaminants. Although the concentration of each individual contaminant is normally low, combined effects can occur even if the compounds are present at concentrations below their NOEC. Because of this, additive and interactive (synergism and antagonism) effects between chemicals have always to be considered (Altenburger al., 2003).

In the case of interaction, a chemical influences the biological action of the other. Interactions can occur either in the toxicokinetic (uptake, distribution, metabolism or excretion of chemicals), or in the toxicodynamic phase (effects of the chemicals on a receptor, cellular target or organ; Groten et al., 2001). If there are no interactions among the compounds, the effects are additive. These additive effects can be of 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 toxic endpoint and can be considered as dilutions of the same compounds. IA refers to chemicals that act by dissimilar MoA, acting on different physiological systems or functionally different when acting on the same system. While for CA all the chemicals having a similar MoA contribute to the effect, for IA the effects will only occur when the individual compounds exceed their threshold of effect (Altenburger al., 2003; Groten et al., 2001).

CA and IA do not make any assumption on the targeted biological system, not either considers any specific properties of mixture components beyond the similarity or dissimilarity of their toxic action. This simplicity allows establishing general assumptions for mixture toxicity assessment, essential for considering the joint action of chemicals in regulatory guidelines. However, these concepts do not describe all the biological possibilities. Both concepts provide a structure of reference, with IA describing the extreme situation of completely independently acting chemicals and CA describing the opposite extreme of completely interchangeable chemicals (Backhaus et al., 2010).

1.5.1. Assessment of combined toxicity

Different strategies can be used for studying mixtures, like for instance isobolographic schemes, effect/response surfaces, effect summation, and the use of prediction models (Fig. 8).

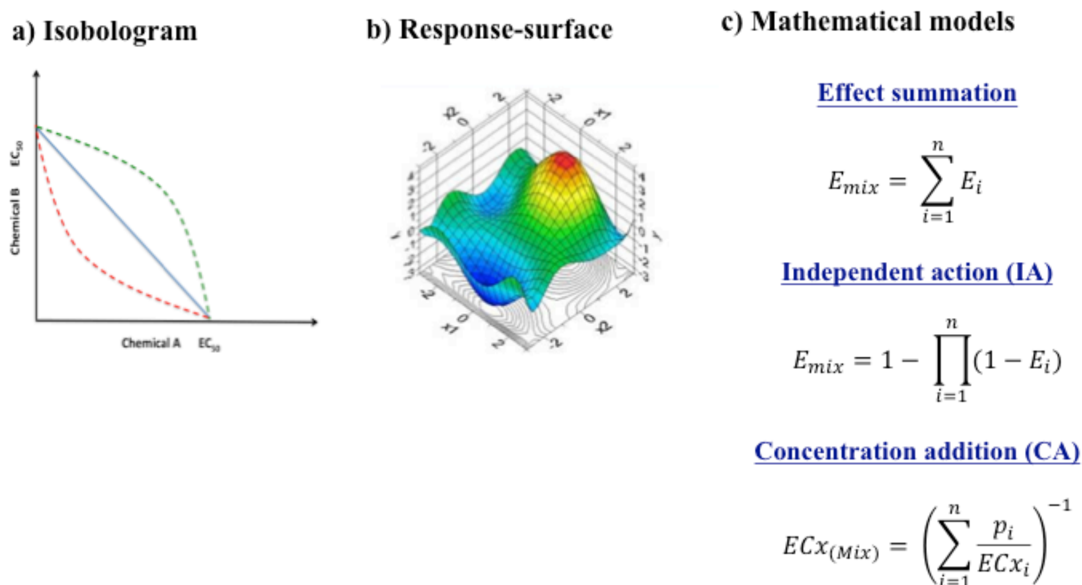

Fig. 8. Different strategies for studying mixtures (adapted from Groten et al., 2001).

The selected approach depends not only on the number of chemicals in the study, but also on the objectives of the study. A simple mixture consists of a relatively small number of chemicals (ten or less), which composition is qualitatively and quantitatively known. A complex mixture includes tens, hundreds or thousands of chemicals, which composition is qualitatively and quantitatively not fully identified (Altenburger et al., 2003; Groten et al., 2001).

A bottom-up approach is often used to study simple mixtures for instance in exposure studies with designed mixtures, but is virtually impossible to use for complex mixtures. The concentration addition (CA) and independent action (IA) and prediction models are the most used, allowing to predict the effect of a mixture containing a larger number of

chemicals, results not easily achieved when using isoboles or response-surfaces (Altenburger et al., 2003; Groten et al., 2001).

1.6. Risk assessment

A main goal of aquatic toxicology is to predict the effects of contaminants in ecosystems. Predictions require that existing observations can be used to generate scenarios, with laboratory studies normally used to obtain data on toxicity of contaminants and their mixtures. Thus, possible contaminants can be studied before their appearance in the environment. However, many studies do not include all the contaminants, neither their interactions, nor interactions with natural abiotic and biotic factors (Walker et al., 2001).

1.6.1. Risk assessment of single chemicals

Risk assessment of single chemicals depends on making a comparison between the toxicity of a compound expressed as a concentration (EC_{50}, LC_{50} or NOEC) and the anticipated exposure of an organism to the same chemical, expressed in the same units (the concentration in water, food or soil to which the organism is exposed). From toxicity tests, the NOEC (no observed effect concentration) and an EC_{50} can be estimated. Then, these values can be compared with a putative “high” environmental concentration to decide whether a risk exists (Walker et al., 2001).

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. These concentrations can be the No Observed Effect Concentration (NOEC), or the predicted no effect concentration (PNEC), which are used to calculate the RQs. The PNEC is estimated by dividing LC_{50} 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 allows accounting for the great uncertainty in extrapolating data from laboratory toxicity for one species to expected field toxicity to other species (Walker et al., 2001). The RQ is calculated using the most

sensitive data and is species-specific. The TUs are calculated using the EC_{50} values, being also species-specific (Backhaus and Karlsson, 2014).

1.6.2. Cumulative risk assessment

If multiple agents or stressors are acting, a cumulative risk assessment approach (Fig. 9) has to be used to used, characterising and quantifying all the combined risks (EPA, 2007). The risks posed by multiple chemicals are examined and the population exposure is evaluated through multiple routes of exposure over time, with different exposure periods and intensity for different chemicals and analysed relatively to each other. It has also to be determined if the exposures to multiple chemicals can lead to toxicokinetic or toxicodynamic interactions (EPA, 2007).

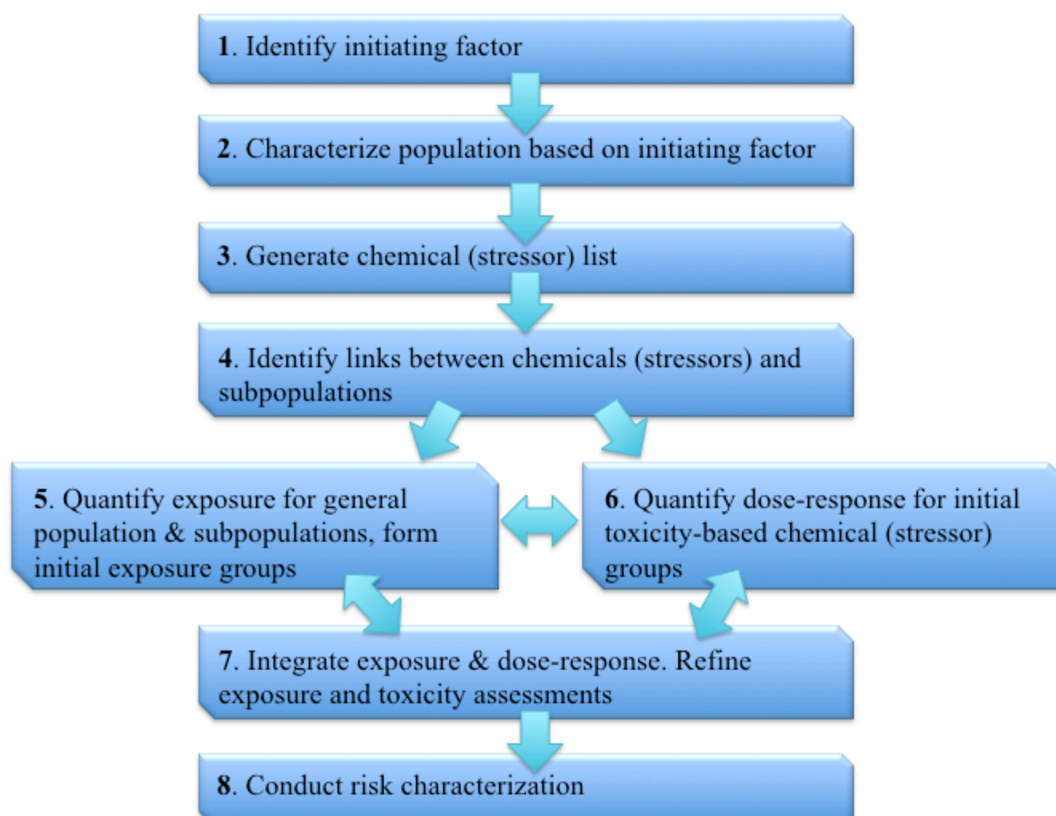

Fig. 9. Key Steps in a Cumulative Risk Assessment (adapted from EPA, 2007).

For the cumulative risk assessment, indicators such as sum of the toxic units (STU) or sum of the risk quotients (SRQ) have to be applied, involving the summation of all the TUs or RQs of all the chemicals present, respectively (Backhaus and 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 and Faust, 2012; EU, 2009). For establishing the size of this AF, a number of uncertainties must be taken into account to extrapolate from single-species laboratory data to a multi-species ecosystem, These uncertainties are for instance the intra- and inter-laboratory variation of toxicity data, intra- and inter-species variations (biological variance), the short-term to long-term toxicity extrapolation, and the laboratory data to field impact extrapolation (e.g., additive, synergistic and antagonistic effects) (EC, 2003).

1.7. Objectives

Biocides are products extensively used for the protection of humans, animals, or physical objects against harmful organisms. However, they can also pose a risk to non-target species due to their intrinsic characteristics. Some are of higher concern due to high toxicity to primary producers, wide use and distribution in surface waters. Moreover, many biocides have limited toxicity data to support ecological hazard and risk assessment, thus being considered chemicals of emerging concern. These are also compounds normally used in combination to potentiate results, and interactions have already been observed. Therefore, their co-occurrence can lead to combined toxicity that cannot be predicted on basis of effect data from the individual compounds alone. So, there is a clear need for understanding their MoA and how these may lead to combined toxicity

Therefore, the objectives of the present study were to assess the toxicity of biocides in the unicellular algae *Chlamydomonas reinhardtii* to:

- Characterize the toxicity of single biocides at the adverse and mechanistic levels. This was achieved by analysing their effects on the growth, PSII efficiency and formation of ROS in *C. reinhardtii*;
- Assess the combined toxicity using the CA and IA prediction models to differentiate between additivity and interactions;
- Identify the MoA of the mixture of biocides producing effects on the analysed toxicological endpoints;
- Assess if single or mixtures of biocides represent a risk under ecologically relevant exposure scenarios, using the Risk Quotients (RQs) and Toxic Units (TUs) approaches.

2. Methodology

2.1. Test compounds

For the present study the test compounds were selected according to their environmental relevance, taking into account their presence in the freshwater environment, high toxicity to algae, and also for having different MoAs. A list of 39 biocides was assembled from literature (Table 3 in supplementary data) and five chemicals considered of high priority were selected for the study (Table 3): aclonifen, bifenox, dichlofluanid, metribuzin and triclosan.

Most of the selected chemicals are herbicides, as these are normally more toxic to algae, due to their MoA. Herbicides such as aclonifen and bifenox have been proposed as priority aquatic substances by the European Union (EU, 2013). Both are diphenylether (DPE) compounds, known to affect the photosystem function in primary producers. Aclonifen inhibits carotenoid and chlorophyll biosynthesis (Kilinc et al., 2011). Bifenox is known to inhibit specific enzymes in the chloroplasts (Grossman, 2005). Other herbicides such as metribuzin are known to cause toxicity to primary producers specifically by interfering with the electron transport in the photosynthesis pathway (Fairchild et al., 1998). Metribuzin is a triazinone herbicide that inhibits electron transport by binding to the Dl protein in PS II (Buman et al., 1992).

Dichlofluanid is chemical can be used as an antifoulant, fungicide, acaricide, wood preservative, etc. (Cima et al., 2008). Its known MoA included the inhibition of cellular enzymes and disruption of mitochondrial function in various organisms (Cima et al., 2008).

Triclosan is another chemical product with a biocidal function, a ubiquitous contaminant not only widely used in personal care products (PCPs), but also as a wood preservative, bactericide and fungicide (USEPA, 2008). It is known to affect multiple target sites in a cell and thus reported to be toxic to a number of organisms (von der Ohe et al., 2012).

Table 3. List of the test compounds. Note: Information gathered from: Pesticide Properties DataBase, University of Herfordshire, 2013[a]; EU pesticides database, 2015[b].

Compound	Use	Emergent	Toxicity (µg/L)					MoA
			LOEC	NOEC	EC_{50}	PNEC	PEC	
Aclonifen	Herbicide	Proposed new priority substance (EU)		4.9[a]	28[a]	0.25[b]		Systemic and selective. Inhibition of carotenoid biosynthesis.
Bifenox	Herbicide	Proposed new priority substance (EU)	3[a]	0.175[a]	1.5[a]		6.93[b]	Selective, absorbed by foliage, new shoots and roots to inhibit protoporphyrinogen oxidase (Protox).
Dichlofluanid	Wood preservative, antifoulants, fungicide, acaricide	Yes (NORMAN)	50[a]	1000[a]	133[a]	0.00001[b]	0.0014[b]	Inhibits thiol-containing enzymes by forming disulfide bridges; stimulates Ca^{2+} Efflux from mitochondria.
Metribuzin	Herbicide			19[a]	23[a]			Selective, systemic with contact and residual activity. Inhibits photosynthesis (PS II).
Triclosan	PCP, antiseptic, disinfectant, preservative, bacterioscide, fungicide	Forgotten priority substance	0.015[b]	0.69[b]	1.4-19[b]	0.05[b]	0.41[b]	Multiple target sites.

2.2. Test organism

Chlamydomonas reinhardtii was selected as the model organism for the present study. This is a eukaryotic unicellular green alga, commonly found in freshwater ecosystems. It is approximately 10 µm in length and 3 µm in with, and moves by means of two flagella (Fig. 10; Merchant et al., 2007). This is one of the most commonly used algal species in different types of studies. It has been identified as one of the most sensitive algal species to a number of contaminants including biocides (Chalew and Halden, 2009). This algae species grows rapidly and attains logarithmic growth in 3 days, is easily maintained in controlled laboratory conditions, allowing a rapid assessment of toxicological endpoints (Harris, 2009). Due to its sensitivity to PSII inhibitors, it has also been used in several ecotoxicological studies (e.g.: Alric et al., 2010; Fischer et al., 2006; Guenther et al., 1990; Juneau et al., 2007).

Due to its well-known biology and genome, ecological role in CO_2 fixation, and a key species in the aquatic ecosystems, *C. reinhardtii* has also used as a model organism for research on several biological processes such as protein synthesis, stress responses, flagella motility (Harris, 2009), circadian rhythms (Mittag et al., 2005), flagellar function and assembly (Pazour et al., 2005). Its capability of growing photosynthetically, heterotrophically and mixotrophically also makes it possible to control its life cycle by nitrogen and light (Lien and Knutsen, 1979). It has also been used in several biomonitoring studies due to its capacity to accumulate contaminants (Torres et al., 2008). It is an ideal organism for elucidating the function, biosynthesis, and regulation of the photosynthetic apparatus (Harris, 2009). All experiments were performed using cultures of the strain NIVA-CHL153, from the Norwegian Institute for Water Research, Oslo, Norway.

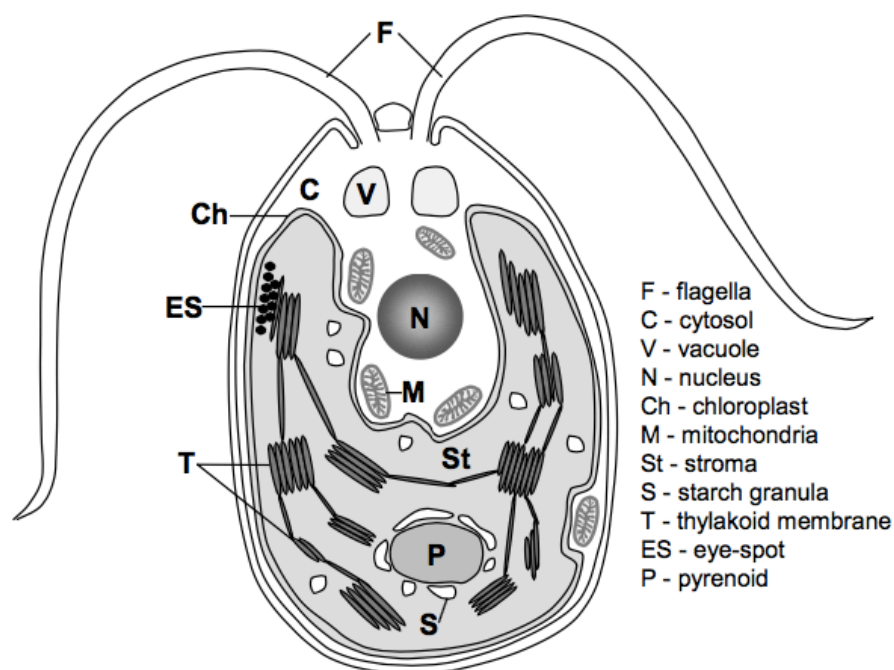

Fig. 10. Schematic representation of *Chlamydomonas reinhardtii*. It is formed by: a glycoprotein cell wall; an individualized nucleus; a large cup-shaped chloroplast, with a single large pyrenoid where the starch formed from photosynthetic products is stored; two small contractile vacuoles, which have an excretory function and are located near the flagella; a red pigment "eyespot" that is light-sensitive and allows the cells to sense light direction and intensity, and respond to it by swimming either towards or away from the light to help finding an environment with optimal light conditions for photosynthesis (adapted from Turkina, 2008).

2.3. Bioassays and endpoints

Different levels of organisations were characterised to analyse the single and combined toxicity of the selected biocides, with the inhibition of algal growth being indicative of the general adverse outcome (ecologically highly relevant and normally usually used for risk assessment), the PSII efficiency and ROS for assessing the MoA (Table 4).

For each test, at least three independent experiments with triplicates were made for each chemical and mixture. As the selected test compounds were organic, Talaquil media was used, and prepared at least 24 h prior to usage to allow equilibrium of all components

(Sziválk et al., 2009). All flasks and glassware used for media preparation and experiments were autoclaved before usage to avoid any microbial contamination. Culture samples were checked microscopically to detect the presence of any microbial contamination.

Table 4. Bioassays and toxic endpoints studied for the selected biocides and positive controls.

Compound	CAS number	Type	Toxic endpoints		
			Growth inhibition	**PSII efficiency**	**ROS**
Aclonifen	74070-46-5	Diphenyl ether	X	X	X
Bifenox	42576-02-3	Diphenyl ether	X	X	X
Dichlofluanid	1085-98-9	Organochloride	X	X	X
Metribuzin	21087-64-9	Triazinone	X	X	X
Triclosan	3380-34-5	Chlorophenol	X	X	X
3,5-dichlorophenol (3,5-DCP)	591-35-5	Chlorophenol	X (positive control)		
Atrazine	1912-24-9	Triazine		X (positive control)	X (additional control)
H_2O_2	7722-84-1	Peroxide			X (positive control)
Paraquat dichloride hydrate	75365-73-0	Bipyridyl			X (additional control)

2.3.1. Toxicity assessment

2.3.1.1. Inhibition of growth

Inhibition of growth was quantified from the measurements of algal density as a function of time, being expressed as the logarithmic increase in biomass (average specific growth rate) during each period of exposure (OECD, 2011). Tests were made according to the OECD Guideline 201 (OECD, 2011). The schematic representation of the method is in Fig. 11.

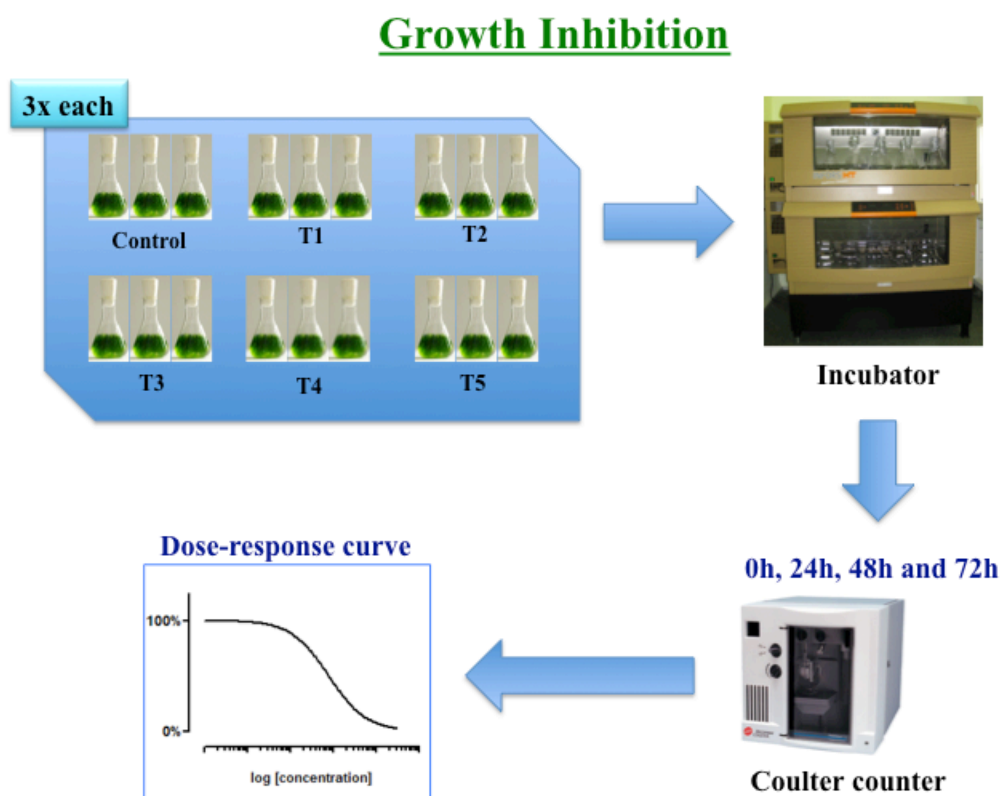

Fig. 11. Schematic representation of the algal growth inhibition test.

In essence, algal cells were cultured in glass flasks with 50 ml of Talaquil media (Szivák et al., 2009). Flasks with 10^4 cells/ml as the initial number of cells were incubated at 20±2°C in continuous light (83±6 μmol/m^2/s^1, Philips TLD 36W/950) with orbital shaking (90 rpm) in an Infors Multitron 2 incubator shaker (Infors AG, Bottmingen, Switzerland). After attaining an exponential growth at 72 h, algae cells from the cultures

were used to inoculate sub-cultures that were exposed to the control (0.01% v/v DMSO), positive control (3,5-DCP), test compounds and mixtures. The exposed cultures were maintained in the same conditions as the algal stock cultures.

The exposed algae were incubated for 72 h and growth was monitored at 24 h, 48 h and 72 h by a multisizer counter (Beckman-Coulter Multisizer 3 Coulter Counter; Miami, FL, USA) for determine the cell density. The average growth rate (μ) for each test concentration was calculated from initial cell concentration and cell concentration at the time of the last cell count using the equation (OECD, 2011):

$$\mu_{n-0} = \frac{\ln(N_n) - \ln(\mathrm{N}_0)}{\mathrm{t}_n - t_0} \times 24 \ (day^{-1}) \qquad \textbf{Eq. 1}$$

Where μ_{n-0} is the average specific growth rate from time *0* to *n*, N_n is the cell density at time *n* and N_0 is the cell density at time *0*.

The inhibition of growth rate was calculated as a percentage of control (%CT):

$$Inhibition\ of\ growth\ (\%\ CT) = \frac{\mu_x}{\mu_C} \times 100 \qquad \textbf{Eq. 2}$$

Where μ_x is the average specific growth rate (μ) for concentration *x*, and μ_c is the mean value for average specific growth rate in the control.

A sigmoidal dose-response curve with variable slope was made using the equation:

$$\gamma = Bottom + \frac{Top - Bottom}{1 + 10^{(\log EC_{50} - \ logX) \times Slope)}} \qquad \textbf{Eq. 3}$$

Where Y is the effect, X is the concentration, Bottom is the baseline effect (control), top is the maximal effect plateau (full growth inhibition), and log EC_{50} is the concentration causing 50% effect.

The lowest observed effect concentration (LOEC) and the no observed effect concentration (NOEC) were determined statistically (OECD, 2011).

2.3.1.2. PSII efficiency

Several parameters have been developed to specifically identify impacts on particular components of the photosynthetic pathways. Differences in fluorescence can be analysed by maximum quantum yield. Quantum yield is the ratio of moles of product formed or substrate consumed to the moles of photons absorbed in a photochemical reaction. Maximum quantum yield (F_v/F_m) is one of the most commonly used parameters to indicate the maximal PSII photochemical efficiency, and based on chlorophyll *a* fluorescence. It is a measure of the largest quantity of product formed or substrate consumed to the smallest number of photons absorbed (Falkowski and Raven, 2007; Nestler et al., 2012a; Ralph et al., 2007).

C. reinhardtii was cultured has previously described. The exposed algae where grown for 72 h, and the PSII efficiency was monitored at 0h, 24 h, 48 h and 72 h. Maximum quantum yield (F_v/F_m) was used to indicate the maximal PSII photochemical efficiency, as described by Kitajima and Butler (1975) and adapted to a 96-well microplate. The schematic representation of this method is in Fig. 12. PSII was monitored using chlorophyll *a* fluorescence, recorded on a Cytofluor 2300 (Millipore; Billerica, MA, USA) with excitation/emission at 485/685 nm. In brief, 200 µl of exposed algae were transferred into NUNC MicroWell™ 96-Well microplates (NUNC, Thermo Scientific, Roskilde, Denmark), chlorophyll *a* fluorescence measurement was made after 20 min adaption to dark to determine the fluorescence yield of PSII in a dark adapted state (F_o). Then, 5 µl of diuron (DCMU) at a final concentration of 10 µM were added to block the electron transport in the PSII. A second fluorescence measurement was made immediately after to determine the maximal fluorescence yield in a light adapted state (F_m). The fluorescence of variable yield (F_v) was calculated as F_m-F_o, and F_v/F_m was used to express PSII primary photochemical efficiency, expressed as percentage of control (% CT):

$F_v/F_m = [F_m\text{-}F_o]/F_m$ **Eq. 4**

Where F_v is the fluorescence of variable yield, F_m the maximal fluorescence yield in a light adapted state and F_o the fluorescence yield of PSII in a dark adapted state.

Data was then normalized using the minimum and maximum values recorded for the positive control atrazine, to allow the fitting of curves to a sigmoidal dose-response curve (Eq. 3).

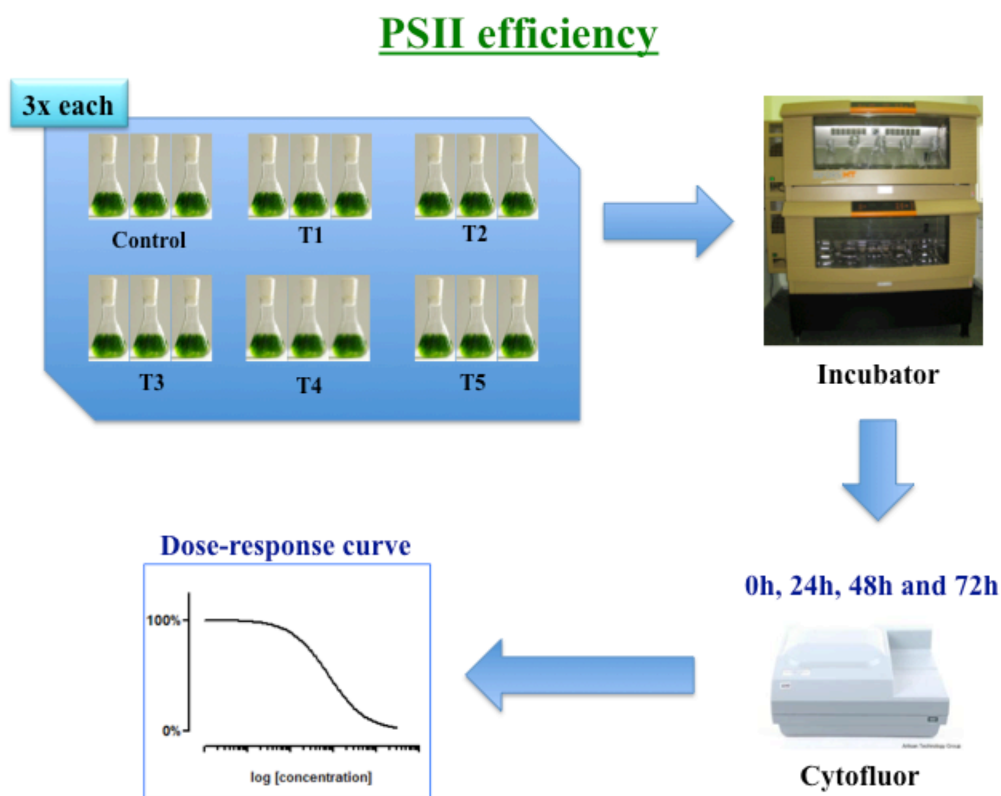

Fig. 12. Schematic representation of the toxicity assessment by the PSII efficiency.

2.3.1.3. ROS

Fluorescent probes are excellent sensors of ROS due to their high sensitivity, simplicity in data collection, and high spatial resolution in microscopic imaging techniques. Among the several fluorescent probes available for the investigation of oxidative stress in living

cells, those based on dihydrofluoresceine diacetate have been the most applied (Gomes et al., 2005).

Algal cells were initially cultured in glass flasks, with an initial number of 10^7 cells in 1L Talaquil media (Szivák et al., 2009). Flasks were incubated as previously described (subsection 2.3.1.1.). Immediately before each test, algal cells were collected by centrifugation, washed and resuspended in MOPS 0.01 M (3-(N-Morpholino)propanesulfonic acid) buffered at pH 7.45.

The ROS production was determined essentially as described by Szivák et al. (2009) and Stoiber et al. (2011) after optimization of algae density, choice of microplates, concentration of probe and exposure time for *C. reinhardtii*. Stock solutions of 50 mM 5-(and-6)-carboxy-2′,7′-difluorodihydrofluorescein diacetate (carboxy-H_2DFFDA; Invitrogen, Molecular Probes Inc., Eugene, OR, USA) were prepared in anhydrous DMSO and stored in aliquots at -20°C until use. In brief, a final concentration of $3x10^5$ cells in 100 µl of MOPs was added to each well in a 96-well microplate (Falcon™, Oslo, Norway). 100 µl of assay working solution were prepared by diluting the probe in assay buffer (final concentration 5 µM) with the different concentrations of test compounds (final concentration of DMSO 0.05% v/v) and then added to the microplate. The schematic representation of this method is in Fig. 13.

Microalgae cells were incubated under ambient light for 6 h. As H_2DFFDA is transformed to fluorescent difluorodihydrofluorescein diacetate (DFFDA) after oxidation, the resulting fluorescent product was directly quantified by fluorescence using the microplate reader 1400 Multilabel Counter, Victor 3 (Perkin Elmer) at 488 nm excitation and 520 nm emission (Szivák et al., 2009). Readings were made hourly to monitor the ROS formation for a maximum of 6 h in the dark. At the end of exposure, microalgae cells were observed under the microscope, to verify their survival, and only concentrations with live cells were taken into account. If the compound had any interference with the fluorescence reading, the background fluorescence was subtracted, and the formation of ROS was determined as fold induction compared to the control

(Mean±SEM). Data was then normalized according to minimum and maximum values of positive control H_2O_2, to allow the fitting of curves to a sigmoidal dose-response curve (Eq. 3).

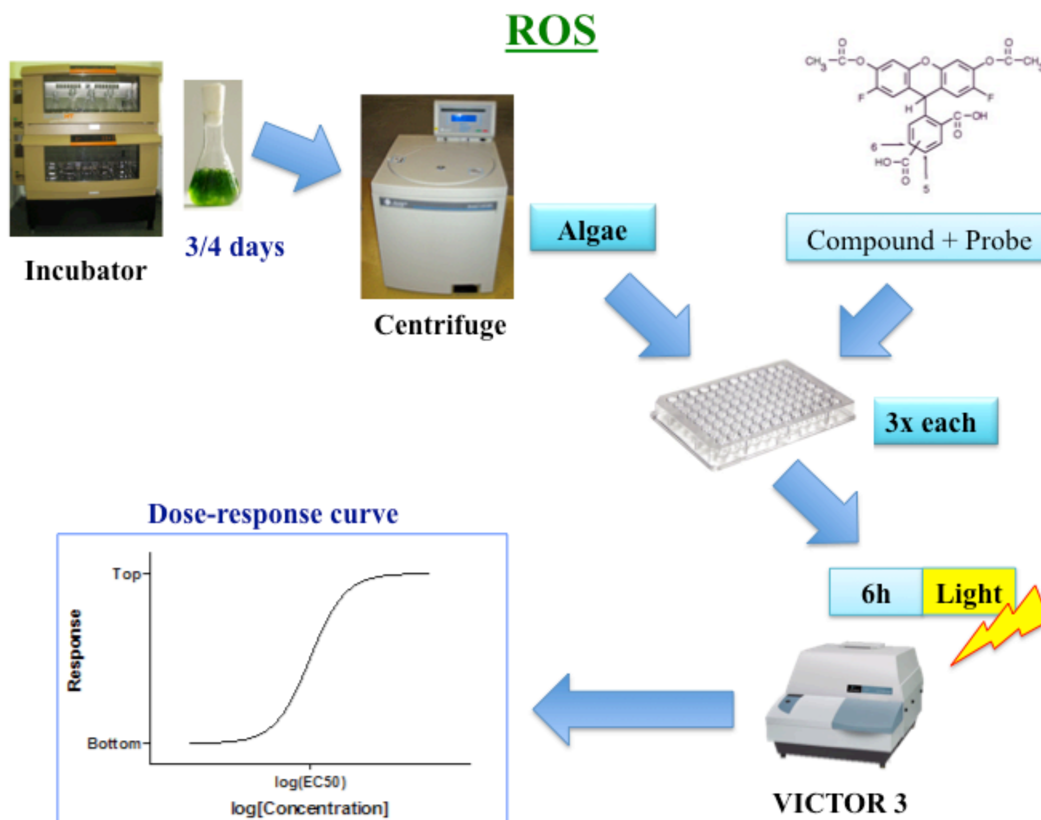

Fig. 13. Schematic representation of the toxicity assessment by the ROS assay.

2.3.2. Chemical analysis

Stock standard solutions were diluted in dichloromethane for confirmatory analysis of bifenox, dichlofluanid, triclosan and metribuzin by gas chromatography-high resolution mass spectrometry (GCT-Premier, Waters Corp, Milford MA, USA). The analytes were separated on a 30 m × 0.25 mm, 0.25 µm film thickness DB-5MS column (Agilent Technologies) with helium carrier gas. Splitless injection at 250 °C was used. The initial temperature of 60 °C was held for 2 min, followed by an increase of 5 °C/min to 310 °C

and held for 5 min. The m/z (mass-to-charge ratio) used for quantification are shown in Table 7.

Stock solutions of aclonifen were diluted into methanol/water for confirmatory analysis by liquid chromatography-high resolution mass spectrometry (Acquity UPLC system with a Xevo G2-S QTOF mass spectrometer, Waters Corp, Milford MA, USA). Chromatography was performed on a Waters Acquity BEH C18 column (2.1 × 50 mm) with acetonitrile and 0.1% formic acid in water as mobile phases. Aclonifen was eluted over a 10 min gradient from 10% methanol to 98% methanol. The m/z used for quantification are shown in Table 5.

Table 5. m/z for the quantification of analytes by gas and liquid chromatography.

Liquid chromatography	
Analyte	m/z
Aclonifen	265.04
Gas Chromatography	
Analyte	m/z
Dichlofluanid	123.0142+223.9219
Metribuzin	214.088
Bifenox	340.986
Triclosan	287.951+218.0145

2.3.3. Combined toxicity

When organisms are exposed to a mixture of contaminants, the effects might be different than when they are exposed to the same compounds separately. Therefore, additive and interactive effects between chemicals have to be properly analysed (Altenburger al., 2003). A mixture with the biocides affecting each endpoint was designed based on the CA prediction model (Backhaus et al., 2010). A schematic representation of the combined toxicity assessment is in Fig. 14.

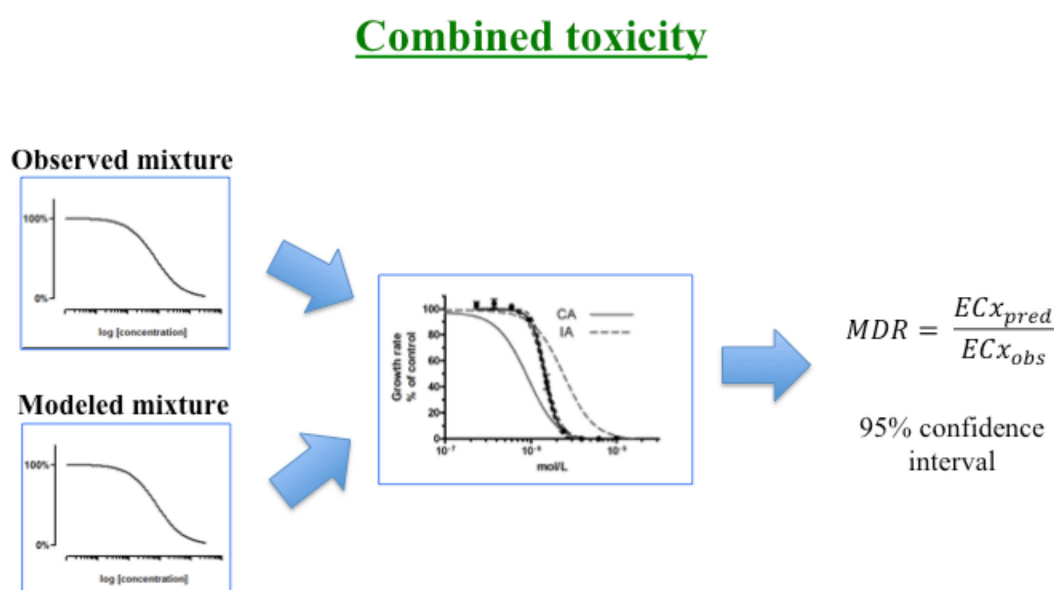

Fig. 14. Schematic representation of the combined toxicity assessment.

The CA model is expressed by:

$$ECx_{(Mix)} = \left(\sum_{i=1}^{n} \frac{p_i}{ECx_i}\right)^{-1} \quad \textbf{Eq. 5}$$

Where the ECx_{mix} is is the total predicted effect concentration of the mixture that induces an effect x, p_i is the relative fraction of component i in the mixture and ECx_i is the concentration of substance i inducing an effect x when exposed alone.

The IA model is expressed by:

$$E_{mix} = 1 - \prod_{i=1}^{n}(1 - E_i) \qquad \textbf{Eq. 6}$$

Where E_{mix} is the effect of a mixture of n compounds and E_i is the effect of substance I when exposed alone (Altenburger et al., 2003; Bliss, 1939).

The resulting CRC for the experimental data was compared to those of CA and IA models. The non-linear regression calculated for observed data and for each model (CA and IA) was used to construct CRCs for each mixture. The effect levels obtained for the observed data were compared to those of CA and IA, and additive effects were assumed to occur if no significant differences were detected between the observed effect concentrations and those predicted by the models (description of statistical approaches in subsection 2.4.). If the curves were not significantly different, the model was considered to explain the combined effects. In addition, the model deviation ratios (MDRs) were also calculated as an indicator of the combined toxicity (Belden and Lydy, 2006):

$$MDR = \frac{ECx_{pred}}{ECx_{obs}} \qquad \textbf{Eq. 7}$$

Where ECx_{pred} is the predicted effect concentrations and ECx_{obs} the observed effect concentrations. Additivity was mainly assumed if MDR values were within a factor of 2 ($0.5 \leq MDR \leq 2$; Belden et al., 2007).

2.3.4. Cumulative risk assessment

The well-established mixture toxicity concepts provided a good-tiered framework for environmental hazard and risk assessment of the tested compounds and subsequent mixtures. A representation of how the combined toxicity assessment was made is in Fig. 15.

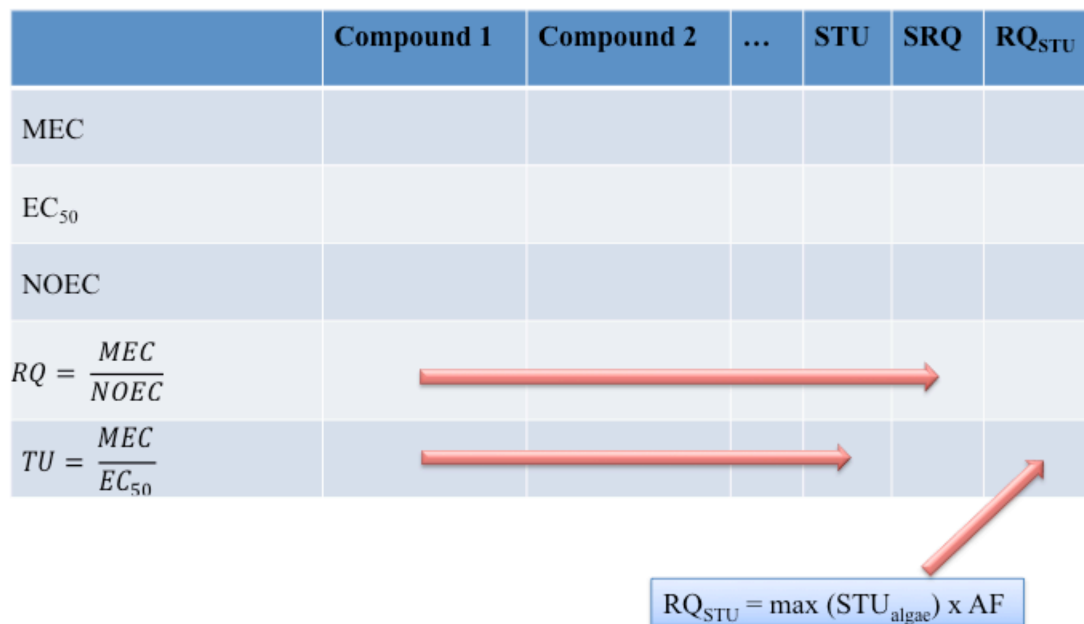

Fig. 15. Schematic representation of the combined toxicity assessment (adapted from Backhaus and Faust, 2012).

The Risk Quotient (RQ) for each compound (the ratio between the expected exposure and the effect (hazard) of the compound) was calculated according to the EPA guidelines (EPA, 2004):

$$RQ = \frac{MEC}{NOEC} \qquad \textbf{Eq. 8}$$

Where MEC is the Measured Environmental Concentrations and NOEC the No Observed Effect Concentrations.

The calculation of the RQ for mixtures was extrapolated from single substances to chemical mixtures by means of CA. The sum of RQ (SRQ) was calculated to evaluate the

potential cumulative hazard as (Backhaus and Faust, 2012; Backhaus and Karlsson, 2014):

$$SRQ = \sum_{i=1}^{n} RQ_i$$ **Eq. 9**

A value larger or equal to 1 of RQ or SQR was interpreted as a potential environmental risk (Backhaus and Faust, 2012; Backhaus and Karlsson, 2014).

The toxic unit (TU) for each compound was calculated as (Backhaus and Faust, 2012; Backhaus and Karlsson, 2014; EU, 2003):

$$TU = \frac{MEC}{EC_{50}}$$ **Eq. 10**

A TU of 1 indicates that the MEC is expected to cause a 50% effect on the growth inhibition in the respective species used for derivation of the EC_{50}.

The toxicity of the mixtures was then described by the sum of all individual TUs of the present compounds (Backhaus and Faust, 2012; EU, 2003):

$$STU = \sum_{i=1}^{n} TU_i$$ **Eq. 11**

A value larger or equal to 1 of TU or STU was interpreted as a potential environmental risk for the analysed mixture (Backhaus and Faust, 2012).

The overall risk quotient for the mixture (RQ_{STU}) to algae (the organism group that is regarded as the most sensitive to the mixture) was calculated according to Backhaus and Faust (2012):

$$RQ_{STU} = \max(STU_{algae}) \times AF$$ **Eq. 12**

Being max(STU algae) the sum of the TU for algae and AF the assessment factor of 100

(EU, 2009). Currently there is no guideline for how to determine the AF for calculating RQ_{STU}, so this values was chosen according to literature (EC, 2003; Petersen et al., 2003).

2.4. Statistical and graphical treatment

The non-linear regressions using a sigmoidal dose-response curve with variable slope (Eq. 3) were modelled in GraphPad Prim 6 software (GraphPad Software Inc., La Jolla, CA, USA). The same software was used to determine the significant differences between concentrations for each compound and mixture, between compounds and mixtures, and to compare the effect concentrations obtained from the experimental data with those calculated by the CA and IA prediction models (*i.e.*, to determine which model best describes the combined toxicity of a mixture; Motulsky, 1998; EPA, 2006). For data non-normally distributed and/or variance not homogeneous, the non-parametric tests Kruskal-Wallis and Dunn's were applied. For data normally distributed and with homogeneous variance, the parametric one-way ANOVA was used, along with the Tukey test for multiple comparisons. Correlation analysis between effects was performed with the non-parametric Spearman correlation (one-tailed) test, as data was not normally distributed. A *p*-value <0.05 was considered as statistically significant for all the tests.

3. Main findings

3.1. Combined toxicity of five priority biocides on the growth of *Chlamydomonas reinhardtii* (Paper I)

Algal toxicity tests such as the growth inhibition test, which are highly ecologically relevant for risk assessment, have been extensively used for testing emerging compounds including herbicides, pesticides and antifoulants (Cedergreen and Streibig, 2005). The objective of this study was to characterise the single and combined effects of the five environmentally occurring biocides, aclonifen, bifenox, dichlofluanid, metribuzin, and triclosan, on the growth of *C. reinhardtii*. Combined toxicity assessment was conducted by the CA and IA prediction models to differentiate between additivity and interactions that can lead to antagonism or synergy when present in mixtures. The obtained results were also used to determine the potential environmental risk of the tested biocides using a species-specific risk assessment for the single compounds and their mixture.

3.1.1. Single toxicity

All of the compounds had EC_{50} levels in the nM range, except for aclonifen (24 h) and triclosan (at all time-points), which caused effects at concentrations one order of magnitude higher than the others (Fig. 16). Bifenox and metribuzin were the most toxic chemicals and affected the growth of *C. reinhardtii* at fairly similar concentrations. Bifenox acts by cellular membrane disruption and inhibition of photosynthesis (EFSA, 2007), while metribuzin inhibits the photosynthetic electron transport at the photosystem II receptor site (EFSA, 2010). Dichlofluanid was the 3rd most toxic, known to inhibit thiol-containing enzymes by forming disulphide bridges, and to stimulate Ca^{2+} efflux from mitochondria (Johansson et al., 2012). Aclonifen was less toxic, although also affecting photosynthesis by inhibiting the biosynthesis of carotenoids, and as bifenox, specifically targeting protoporphyrinogen oxidase (Protox) synthesis (Kilinc et al., 2011). Triclosan, with multiple toxic MoAs (Franz et al., 2008) was the least toxic. While triclosan is a multi-purpose personal care product commonly used as an antibacterial

agent and preservative, all the others are specifically acting herbicides or fungicides (von der Ohe et al., 2012).

The results of the chemical analysis on the stock standard solutions confirmed the concentrations of the stock solutions used for the exposures, except for aclonifen. The method was not sufficiently robust for the analysis of this compound and data was not reproducible. As the measured concentrations did not surpass ±20% of the nominal concentrations, the nominal concentrations were used throughout.

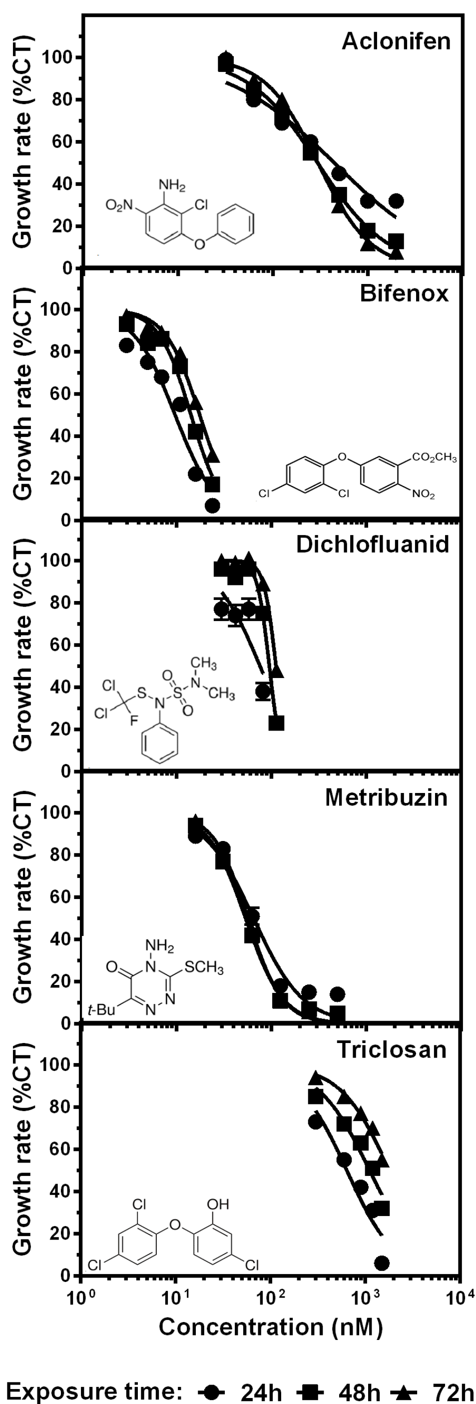

Fig. 16. Growth rate (% of control, CT - control) of *Chlamydomonas reinhardtii* exposed to the biocides aclonifen, bifenox, dichlofluanid, metribuzin, and triclosan for 24 h, 48 h and 72 h (solid symbols). The data (Mean±SEM) represent experimental results from 3 independent experiments and their corresponding concentration-response curves (solid line).

3.1.2. Combined toxicity

The study on the combined effects of the five biocides showed that the mixture predominantly caused additive effects (Fig. 17). At 48 h and 72 h, the IA model best estimated the mixture effect at almost all effect levels. At lower effect levels, deviations from additivity and indicative of antagonism were observed, especially at 24 h. The best fit of the IA model indicates that the compounds display dissimilar MoA, following the principles of independent action.

The chemical analysis also confirmed the nominal exposure concentrations of each compound on the equipotent mixture (measured concentrations within 20% of the nominal concentrations).

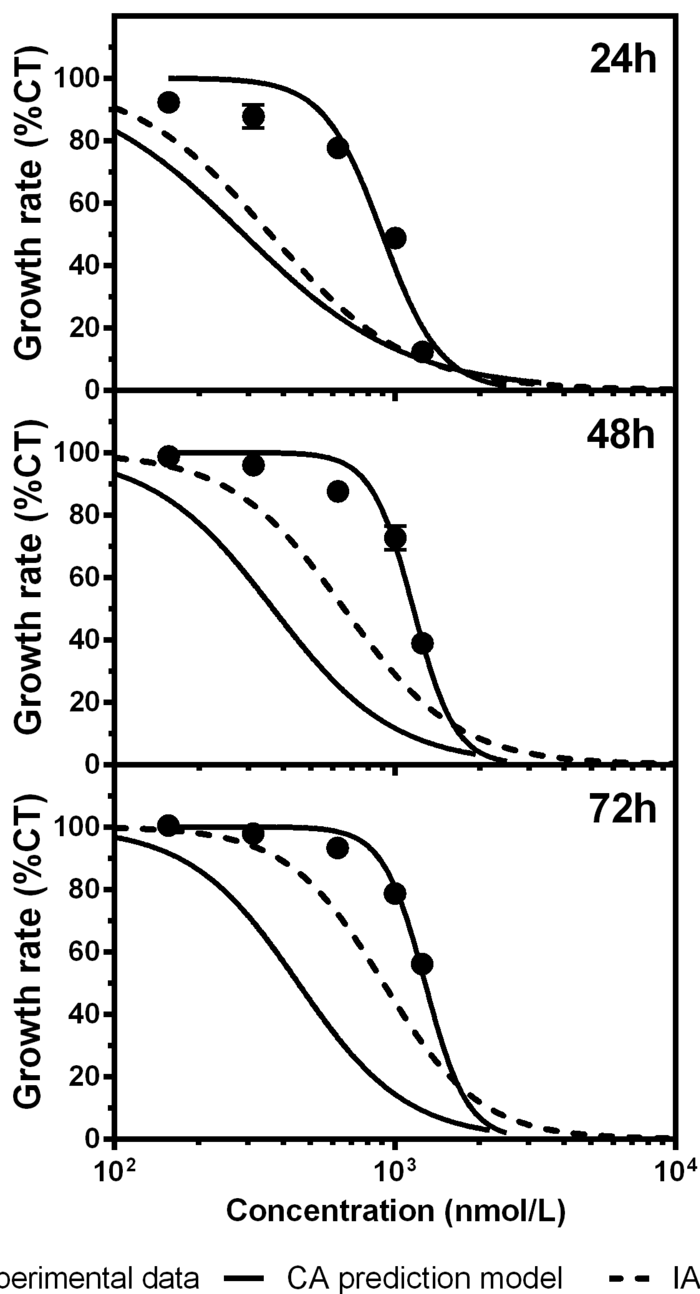

Fig. 17. Growth rate (% of control, CT - control) for *Chlamydomonas reinhardtii* exposed to an equipotent mixture of aclonifen, bifenox, dichlofluanid, metribuzin and triclosan for 24 h, 48 h and 72 h (solid circles). The data (Mean±SEM) represent experimental results from 3 independent experiments and their corresponding concentration-response curves (dotted line). Prediction of combined toxicity by CA (solid line) and IA (broken line) of the mixture is displayed to indicate assumptions of additivity.

3.1.3. Environmental relevance

For this study, minimum, median, 95 quartile and maximum MEC values were calculated from values found in literature for freshwater environments. As no studies were found for dichlofluanid, concentrations for *N,N*-dimethylsulfamide (DMS), a common degradation product of dichlofluanid and tolylfluanid, were used instead. Results indicate a potential environmental risk for metribuzin, triclosan, bifenox, and possibly dichlofluanid towards *C. reinhardtii*, mostly at high MECs (Table 6). The order of potential environmental risk for *C. reinhardtii* was the same when considering either RQ or TU: metribuzin> dichlofluanid> triclosan> bifenox> aclonifen. The SRQ, STU and RQ_{STU} indicate that the analysed mixture with the five biocides can represent a potential environmental risk for *C. reinhardtii* (Table 6).

Table 6. Risk assessment for *Chlamydomonas reinhardii* based on the risk quotient (RQ) and toxic unit (TU) for the single biocides, aclonifen, bifenox, dichlofluanid, metribuzin and triclosan and based on the sum of the risk quotients (SRQ), sum of the toxic units (STU), and on the overall risk quotient (RQ_{STU}) for the mixture. EC_{50} - concentration of a compound that gives half-maximal response; NOEC - No Observed Effect Concentration (NOEC); MEC - Measured Environmental Concentrations. Note: *Measured concentration of a common degradation product of dichlofluanid and tolylfluanid, *N,N*-dimethylsulfamide (DMS); bold text indicates a potential risk.

		Aclonifen	**Bifenox**	**DMS***	**Metribuzin**	**Triclosan**	**SRQ**	**STU**	**RQ_{STU}**
EC_{50} (nM)		298	18	113	57	1804			
NOEC (nM)		32	3	30	16	299			
MEC (nM)	**Min**	0.01	0.0005	1331	0.004	62			
	Median	0.7	1	5836	0.5	224			
	95	4.2	8	9308	9000	6042			
	Max	5.1	8	9907	10000	7944			
RQ	**MEC_{min}**	0.0004	0.0002	**44**	0.0003	0.2	**45**		
	MEC_{median}	0.02	0.4	**195**	0.03	0.8	**196**		
	MEC_{95}	0.1	**3**	**310**	**563**	**20**	**896**		
	MEC_{max}	0.2	**3**	**330**	**625**	**27**	**985**		
TU	**MEC_{min}**	0.00004	0.000002	**4**	0.00001	0.2		**5**	**5000**
	MEC_{median}	0.002	0.004	**20**	0.002	0.8		**20**	**20000**
	MEC_{95}	0.01	0.03	**31**	**30**	**20**		**82**	**82000**
	MEC_{max}	0.02	0.03	**33**	**34**	**27**		**94**	**94000**

3.2. Photosystem II (PSII) efficiency in *Chlamydomonas reinhardtii* exposed to environmentally occurring biocides (Paper II)

Interference with photosynthesis has been identified as one of the major targets for many herbicides in algae (Ralph et al., 2007). The objective of this study was to characterise the single and combined effects the five studied biocides on the photosynthesis, measured as the efficiency of PSII in *C. reinhardtii*. Maximum quantum yield (F_v/F_m) was used to indicate the maximal PSII photochemical efficiency. The toxicity of the biocides was also assessed as inhibition of algal growth to verify if the effects on the PSII also affected regulatory-relevant toxicity endpoints. The CA and IA models were used to assess the combined effects of the biocides affecting the PSII, to determine if combinations of these could cause additivity, antagonism or synergy when present in a mixture. Moreover, prediction of cumulative risk by RQs and TUs was conducted to determine if the overall impact of simple mixtures of biocides could represent a risk under ecologically relevant exposure scenarios.

3.2.1. Single compound toxicity

From the 5 studied biocides, only aclonifen and metribuzin showed a significant effect on the PSII efficiency, with metribuzin being 5 times more potent than aclonifen (Fig. 18). Both are known to affect photosynthesis, although by different MoA. While metribuzin reduces photosynthetic activity by inhibiting the electron transport in the PSII (Buman et al., 1992), aclonifen inhibits chlorophyll and carotenoid biosynthesis (Killinc et al., 2009). Both compounds had EC_{50} values in the nM range for PSII efficiency and growth rate, and a significant correlation was observed between both parameters at all exposure durations tested. The same stock solutions with confirmed concentrations from the first study were used for this study (measured concentrations within 20% of the nominal concentrations).

The main MoA of metribuzin was photosynthesis inhibition leading to growth inhibition in *C. reinhardtii* and consistent with other studies (Oettmeier et al., 1982). For aclonifen,

the reduction in PSII efficiency was contributing to the overall inhibition of algal growth, but also other MoAs seemed to be occurring.

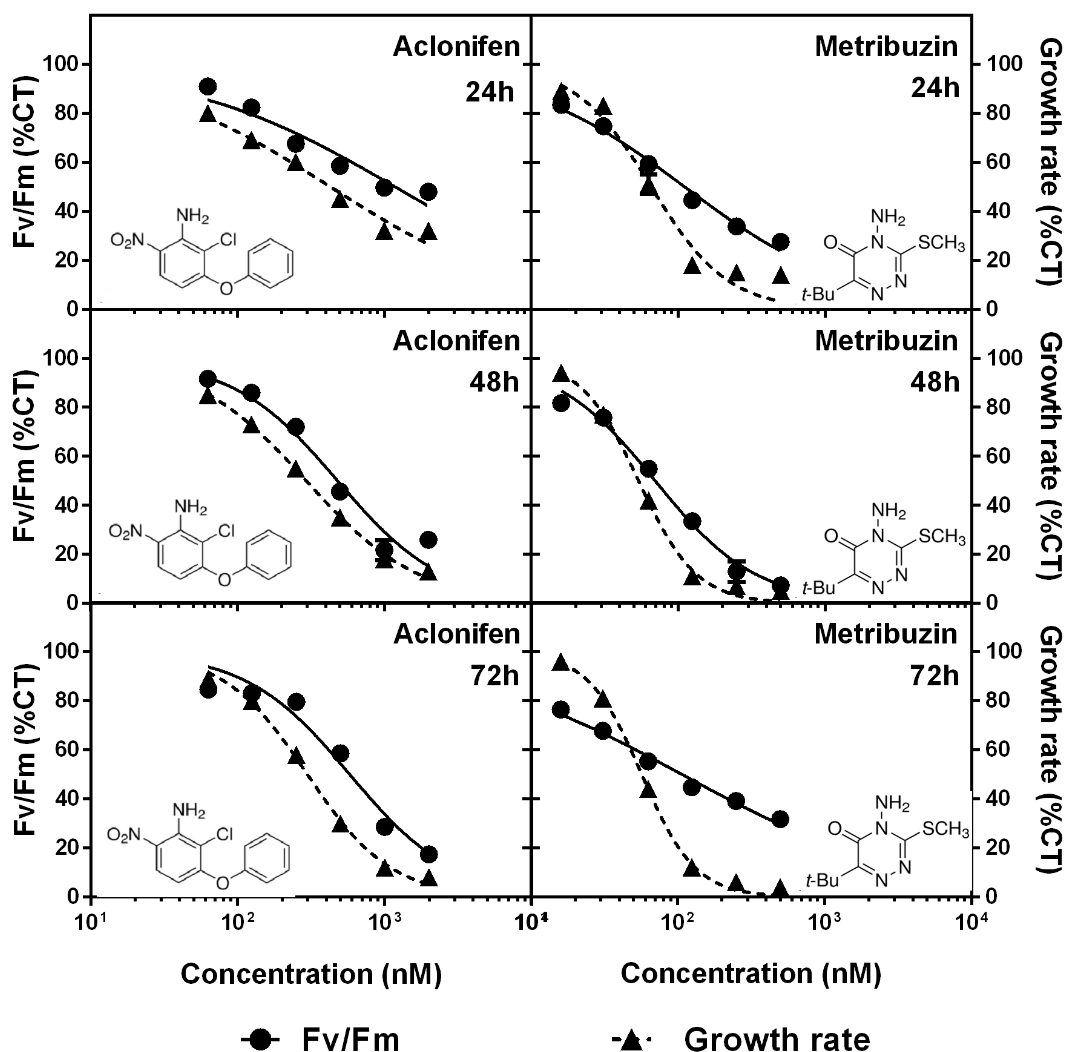

Fig. 18. Photosystem II (PSII) primary photochemical efficiency expressed as normalized F_v/F_m (% of control, CT; closed circles) and growth rate (% of control, CT; closed triangles) in *Chlamydomonas reinhardtii* exposed for 24 h, 48 h and 72 h to aclonifen and metribuzin. The data (Mean±SEM) represent experimental results from 3 independent experiments and their corresponding concentration-response curves (solid line for F_v/F_m and broken line for growth rate).

3.2.2. Combined toxicity

The combined effects of aclonifen and metribuzin on the PSII efficiency were well predicted by the IA model (Fig. 19), indicating that the compounds had an additive effect but caused toxicity through different MoAs. For growth inhibition (Fig. 20), a temporal and concentration-dependent variance in the combined effects of the studied compounds was observed. While at the beginning of exposure (24 h) the IA model best predicted the effects of the mixture, at both 48 h and 72 h the CA model best predicted the effects for low to medium effect levels. A potential synergism between both compounds was observed for both toxic endpoints at medium to high effect levels, especially on the inhibition of growth (Fig. 20).

Chemical analysis confirmed the concentrations of metribuzin in the mixture (measured concentration within 20% of the nominal concentration). However, as already mention in subsection 3.1.1., the method was not sufficiently robust for the analysis of aclonifen.

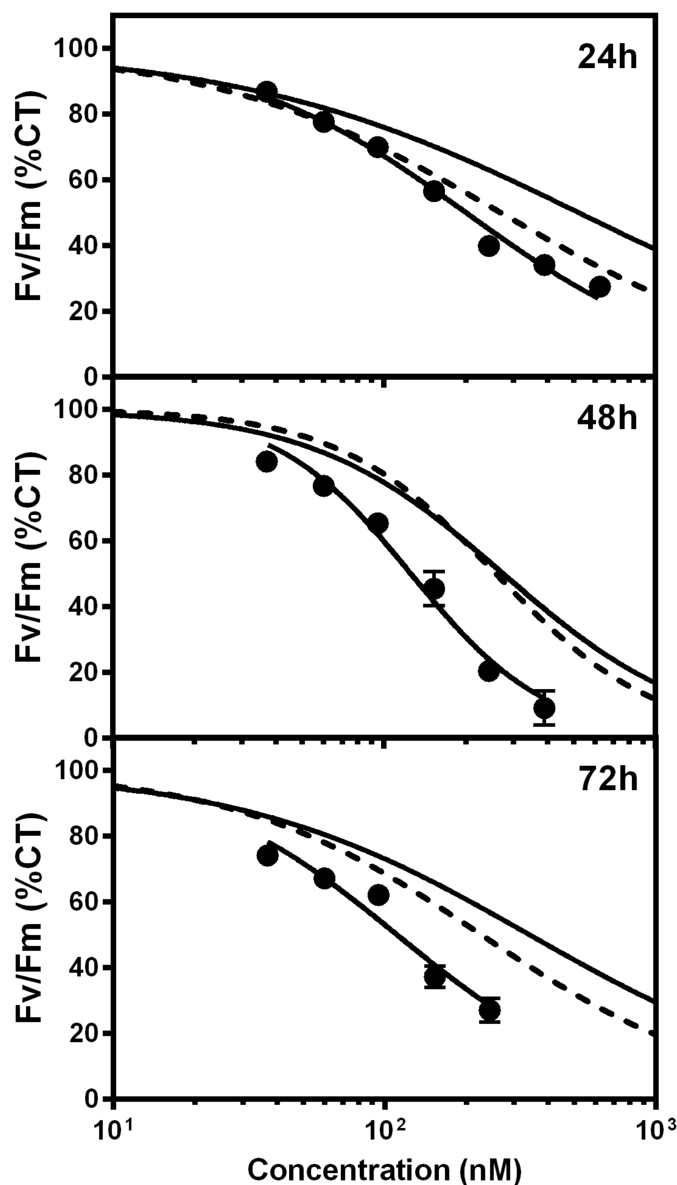

Fig. 19. Photosystem II (PSII) primary photochemical efficiency (F_v/F_m) in *Chlamydomonas reinhardtii* exposed for 24 h, 48 h and 72 h to the equipotent binary mixture of aclonifen and metribuzin, along with the curves obtained for the mixture models CA and IA (normalized data) for each time-point. The data (Mean±SEM) represent experimental results from 3 independent experiments and their corresponding concentration-response curves (dotted line). Prediction of combined toxicity by CA (solid line) and IA (broken line) of the mixture is displayed to indicate assumptions of additivity.

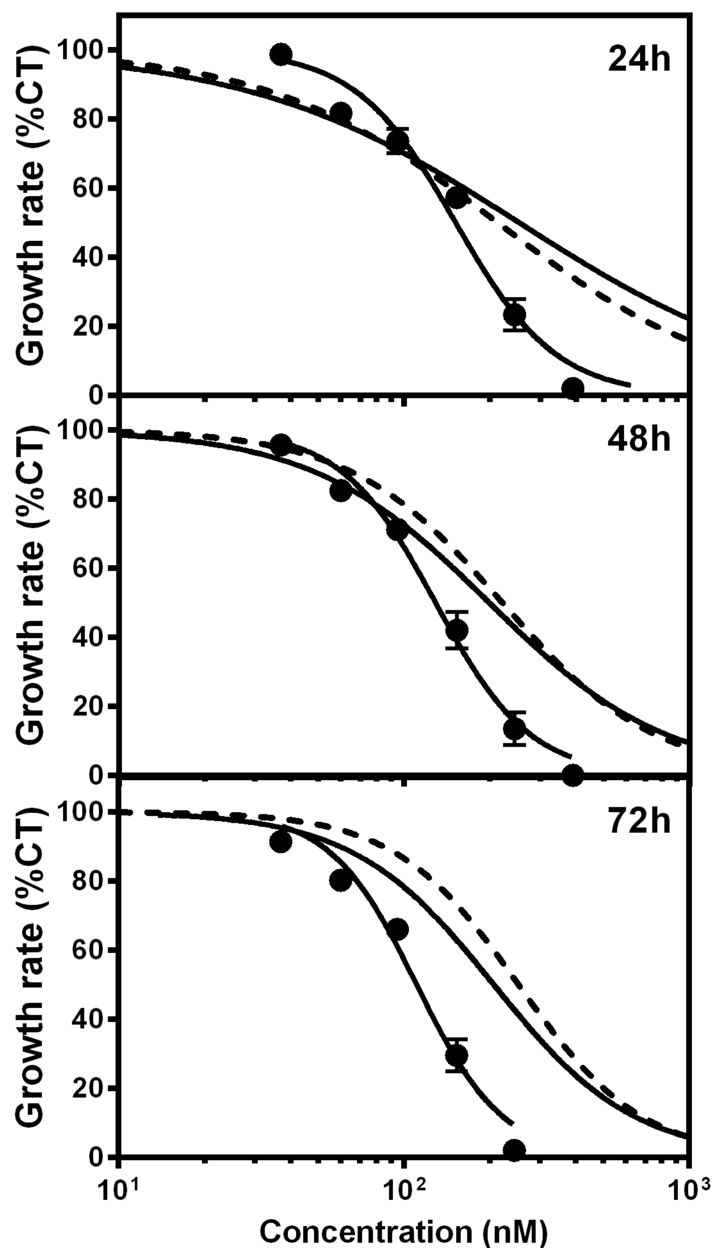

Fig. 20. Growth rate (% of control, CT) for *Chlamydomonas reinhardtii* exposed for 24 h, 48 h and 72 h to the equipotent binary mixture of aclonifen and metribuzin (solid circles). The data (Mean±SEM) represent experimental results from 3 independent experiments and their corresponding concentration-response curves (dotted line). Prediction of combined toxicity by CA (solid line) and IA (broken line) of the mixture is displayed to indicate assumptions of additivity.

3.2.3. Environmental relevance

The RQs and TUs calculated for the biocides affecting the PSII in *C. reinhardtii* are presented in Table 9. No risk scenarios were identified for any of the single compounds or mixture. However, when considering the overall risk quotient (RQ_{STU}) for the binary mixture, a potential environmental risk was identified for median MEC values. Therefore, assuming the additivity principles, this binary mixture showed a potential risk at environmentally relevant concentrations (Table 7).

Table 7. Risk assessment for *Chlamydomonas reinhardtii* based on the PSII efficiency data using the risk quotient (RQ) and toxic unit (TU) for the single compounds and the sum of the risk quotients (SRQ), sum of the toxic units (STU), and on the overall risk quotient (RQ_{STU}) for the binary mixture. EC_{50} - concentration of a compound that gives half-maximal response; NOEC - No Observed Effect Concentration (NOEC); MEC - Measured Environmental Concentrations. Note: bold text indicates a potential risk.

		Aclonifen	**Metribuzin**	**SRQ**	**STU**	**RQ_{STU}**
EC_{50} (nM)		578	107			
NOEC (nM)		125	31			
MEC (nM)	**Min**	0.01	0.0005			
	Median	0.7	1			
	95	4	8			
	Max	5	8			
RQ	**MEC_{min}**	0.0001	0.00002	0.0001		
	MEC_{median}	0.01	0.04	0.05		
	MEC_{95}	0.03	0.3	0.3		
	MEC_{max}	0.04	0.3	0.3		
TU	**MEC_{min}**	0.00003	0.00001		0.00003	0.003
	MEC_{median}	0.001	0.02		0.02	**2**
	MEC_{95}	0.01	0.1		0.1	**13**
	MEC_{max}	0.01	0.1		0.1	**13**

3.3. Induction of reactive oxygen species (ROS) in *Chlamydomonas reinhardtii* after exposure to single biocides and their mixtures (Paper III)

Biocide toxicity can also be related to the formation of ROS, as some of these compounds are known to widely affect the photosynthetic apparatus of algae and plants (Jamers and Coen, 2010; Nestler et al., 2012b; Ramel et al., 2009; Szivák et al., 2009). This study intended to investigate the production of ROS in *C. reinhardtii* exposed to the five studied biocides and their mixtures, using the specific and sensitive fluorescence probe carboxy-H_2DFFDA. A combined toxicity assessment of the ROS-generating biocides was conducted to characterise how these produced ROS when present in simple mixtures.

3.3.1. Single toxicity

Only the herbicides aclonifen, bifenox and metribuzin induced the formation of ROS in *C. reinhardtii* (Fig. 21). Aclonifen was the most toxic compound. It is known to inhibit Protox, leading to the accumulation of protoporphyrin IX inside the cells. This reacts with oxygen in the presence of light and lead to the formation of singlet oxygen ($^1O_2$) and superoxide anion (O_2-). Aclonifen also inhibits carotenoid biosynthesis, leading to an increase in membrane sensitivity to ROS (Kilinc et al., 2009).

Metribuzin was the second most toxic compound (Fig. 21), a PSII herbicide analogue to plastoquinone that is known to inhibit photosynthesis. Reactive singlet oxygen ($^1O_2$) is formed due to the formation of a chlorophyll triplet state capable of reacting with triplet oxygen. The $^1O_2$ can then damage adjacent chlorophyll-bearing proteins, separate the chlorophylls from their energy transfer systems and from protective pigments, causing further photogeneration of singlet oxygen (Jones, 2005; Rutherford and Krieger-Liskay, 2001).

Bifenox was the least toxic (Fig. 21), although with the steepest slope and NOEC. It is also a DPE herbicide like aclonifen and is known to instigate membrane disruption and

inhibition of photosynthesis through the inhibition of Protox, thus causing light-dependent oxygen radical formation (EFSA, 2007; Grossman, 2005).

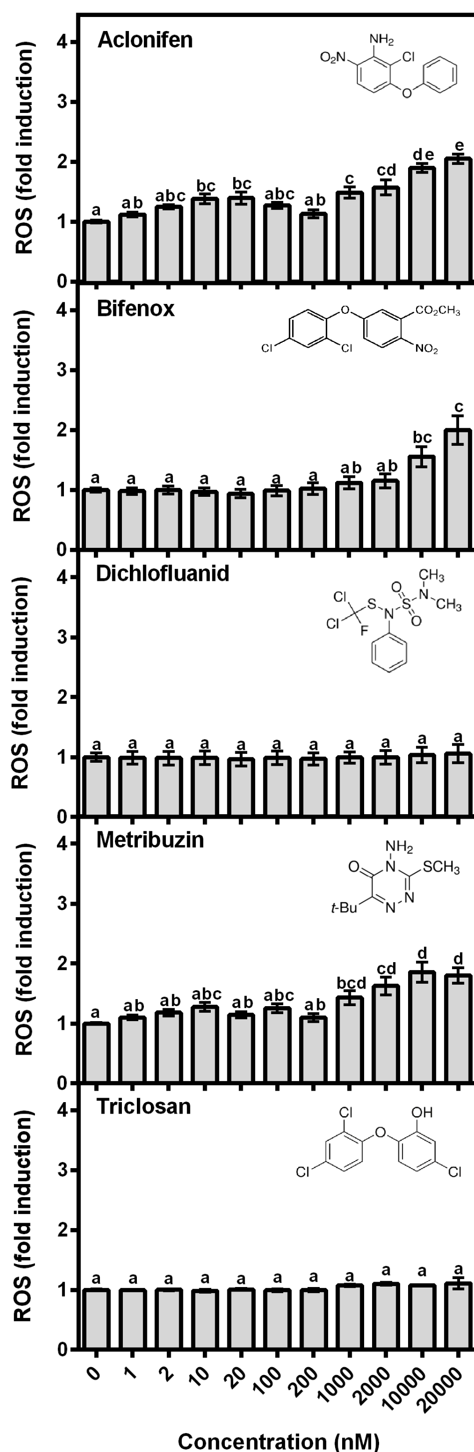

Fig. 21. Reactive oxygen species (ROS) formation in *Chlamydomonas reinhardtii* exposed to the biocides aclonifen, bifenox, dichlofluanid, metribuzin and triclosan for 6 h. The data (Mean±SEM) represent 3 independent studies. Letters indicate significant differences between concentrations (p<0.05).

3.3.2. Combined toxicity

The CA model best predicted the ternary mixture (aclonifen, bifenox and metribuzin), indicating that compounds acted by the same MoA (Fig. 22). The binary mixture of aclonifen and bifenox was also best predicted by the CA model at lower to median mixture concentrations (Fig. 22), while at higher effect levels more than additive effects were observed (*i.e.*, synergism). The IA model best predicted the binary mixture of aclonifen and metribuzin (Fig. 22).

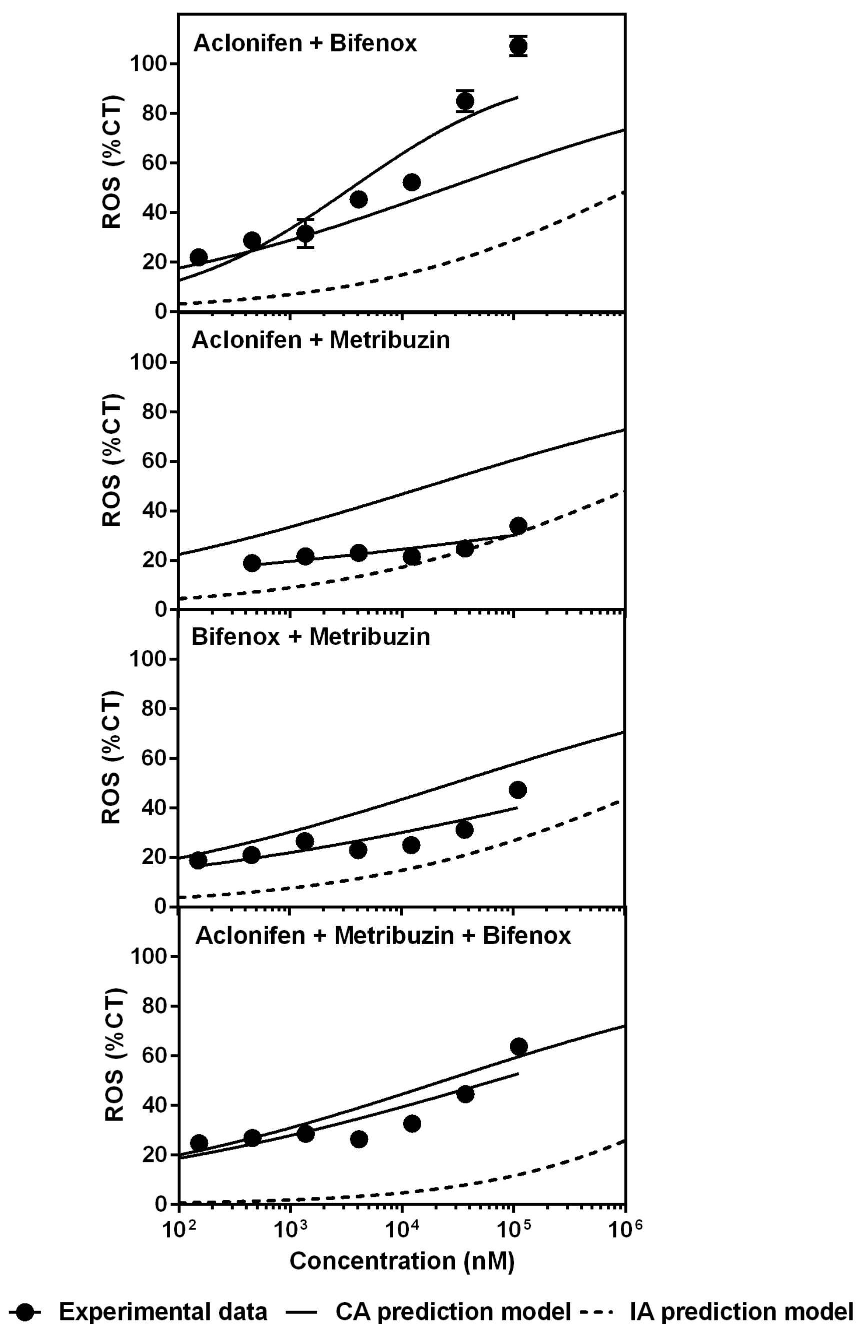

Fig. 22. Reactive oxygen species (ROS) formation (% of control, %CT) in *Chlamydomonas reinhardtii* exposed to equipotent mixtures of different biocides in combination with the combined toxicity predictions obtained from CA and IA mixture models. The data (Mean±SEM) represent experimental results from 3 independent experiments and their corresponding concentration-response curves (dotted line). Prediction of combined toxicity by CA (solid line) and IA (broken line) of the mixture is displayed to indicate assumptions of additivity.

4. Discussion

Biocides are products extensively used for the protection of humans, animals, or objects against harmful organisms. Although well regulated, many have limited toxicity data to support environmental hazard and risk assessment, thus being considered chemicals of emerging concern. Although most of the compounds in the aquatic environment are at concentration below their NOEC, they may still contribute to substantial effects when present in mixtures (Altenburger et al., 2003). CA and IA prediction models have been frequently employed to analyse the combined effects of mixtures of biocides (Belden et al., 2007; Cedergreen, 2014; Faust et al., 2003), but only a few studies have investigated the effects of environmentally relevant mixtures of this type of compounds with different MoA. Therefore, more studies are needed to gather information on emergent and priority compounds as the ones studied herein. Further information is also required to use this knowledge for regulatory purposes.

4.1. Ecological role of unicellular green algae

Chlamydomonas reinhardtii was used as a model organism. This single cell green alga has already been used for decades in biological and photosynthesis research (Harris, 2009). Recently it has also been gaining importance in ecotoxicology to analyse the effects of several stressors including biocides (Fischer et al., 2010; Jamers and Coen, 2010; Nestler et al., 2012a,b; Reboud, 2002). Due to its rapid growth, easy maintenance, and sensitivity to biocides (Harris, 2009), *C. reinhardtii* represents a good tests species in ecotoxicological studies.

Moreover, this alga has several cellular components similar to those in plants that are also targeted by many biocides. Algae are an important component of the phytoplankton, accounting for a considerable part of both oxygen and biomass production in the aquatic ecosystems. As primary producers, they are at the basis of the aquatic food web and thus being of high ecological relevance. Adverse effects on these organisms can also negatively impact higher trophic levels including zooplankton and fish, thus potentially

disturbing the whole ecosystem. Hence, it is important to thoroughly assess the effects of biocides on algae (Chalew and Halden, 2009; Nestler et al., 2012 a,b).

4.2. Algal ecotoxicological bioassays

Three bioassays were used in the present work to assess the toxicity of the target compounds: algal growth inhibition, PSII efficiency and formation of ROS. As done in the first study of the present work (Paper I), the results on growth inhibition are normally reported as concentration values where specific thresholds for effect on the monitored endpoint are achieved. These include for instance the effective concentration causing 50% inhibition (EC_{50}), as well as the no observable effect concentration (NOEC). These values can then be used as measurements of the toxic potential of contaminants and to predict the ecological effects that they can potentially induce when introduced into the environment, as made in this work and explained in subsection 4.5.

The five biocides showed to be highly toxic to algae, presenting EC_{50} values mostly in the nM range (Table 8). Aclonifen and metribuzin showed effects on the three analysed toxic endpoints. A clear relationship between the tested endpoints, reported as decrease in PSII efficiency and formation of ROS leading to inhibition of growth, was clearly established for these two compounds, showing that PSII were relevant for more risk assessment-relevant considerations (Fig. 23). ROS formation occurred at higher concentrations than the two other endpoints, and was thus considered more informative for MoA assessment.

Table 8. EC_{50} (nM) of single chemicals and analysed mixtures obtained in the three used assays. Note: - not detected

Compound	Growth inhibition	PSII efficiency	ROS
Aclonifen	294 – 429	481 - 1178	1.15×10^4
Bifenox	10 – 18	-	4.06×10^4
Dichlofluanid	76 – 113	-	-
Metribuzin	54 – 66	70 - 110	2.56×10^4
Triclosan	638 - 1804	-	-

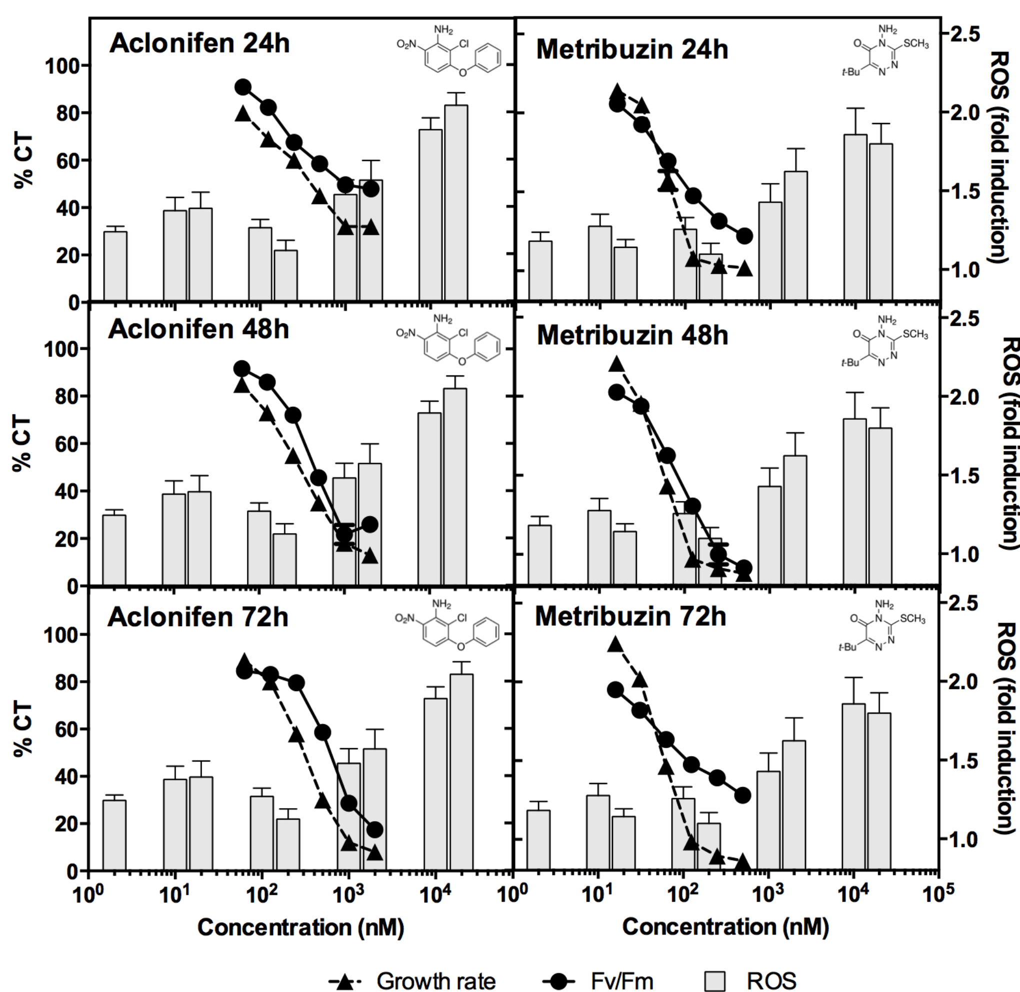

Fig. 23. Growth rate (% of control, CT; closed triangles) and PSII primary photochemical efficiency (normalized F_v/F_m; % of control, CT; closed circles) in *Chlamydomonas reinhardtii* exposed for 24 h, 48 h 72 h to aclonifen and metribuzin, along with the ROS formation (fold induction; columns) at 6 h exposure. The data (Mean±SEM) represent experimental results from 3 independent experiments and their corresponding concentration-response curves for growth rate (broken line) and F_v/F_m (solid line).

The growth inhibition was the most sensitive parameter tested, and leading to the lowest effect concentrations (*i.e.*, EC_{50}). However, although ecologically significant, this toxicity endpoint is integrative of multiple MoA, and thus not providing in-depth information about the underlying toxicity mechanisms of the compounds. One of the main aspects of ecotoxicological research in the 21st century is the understanding of the MoA by which contaminants affect organisms, ultimately affecting the whole-organism survival or

health (Eggen et al., 2004). To get this information, the effects on organisms should be investigated below the whole cell/organism level targeting specific cell components, such as the analysed PSII efficiency and formation of ROS. These processes also tend to respond earlier and at lower effect concentrations than effects occurring at the organism level such as the inhibition of growth (Nestler et al., 2012a).

One of the most important cellular systems in green algae is the photosynthetic apparatus. If the processes involved in photosynthesis are impaired by contaminants, these can be used as an ecotoxicological endpoint to assess their impact in organisms (Ralph et al., 2007). The Maximum quantum yield (F_v/F_m) used in the second study (Paper II) is one of the most commonly used parameters to indicate the maximal PSII photochemical efficiency, and is based on measurement of chlorophyll *a* fluorescence (Falkowski and Raven, 2007; Nestler et al., 2012a; Ralph et al., 2007). The analysis of chlorophyll fluorescence signal of PSII proved to be a highly sensitive parameter for the compounds affecting this cellular component (aclonifen and metribuzin). The effects on this endpoint clearly affected the algal growth. However, most of the studies found in literature lacked a summary statistic values such as EC_{50} and NOEC (Ralph et al., 2007) for PSII inhibition, thus making the comparison between the present data with published studies difficult.

As it was proposed in Paper II that aclonifen and metribuzin could cause a decrease in the PSII efficiency by inducing the formation of ROS, this was investigated in more detail in Paper III. For this study, the use of a probe (carboxy-H_2DFFDA) was chosen to directly measure the general oxidative stress produced (Sziválk et al., 2009). This assay was found to accommodate rapid high-throughput screening for ROS formation, where algae cells were exposed to contaminants in a 96-well plate for up to 6 h in the presence of ambient light. Among the five studied biocides, the assay identified aclonifen, bifenox and metribuzin as ROS inducers. The assay was also adequately sensitive to demonstrate different ROS patterns for the studied compounds. Aclonifen and metribuzin showed a biphasic increase in fold induction, indicating that at low concentrations the formed free radicals are locally detoxified as verified in other studies (Ledford and Niyogi, 2005;

Nestler et al., 2012b). Bifenox presented a constant increase, which might produce less reactive radicals able to travel further along cell compartments spreading the induced oxidative stress (Nestler et al., 2012a,b). Interestingly, the reduction in the ROS formation observed for aclonifen and metribuzin occurred at concentrations starting to cause both PSII and growth inhibition (Fig. 23), potentially suggesting that ROS formation is contributing to the toxicity observed for these compounds.

4.3. Single toxicity

The five biocides showed to be highly toxic to algae. As found in literature and verified in this work, these biocides showed to have apparent different, not only showed by the different patterns and effects presented in each assay but also by the prediction models. They can also be considered as compounds of emerging concern, as little information on their toxicity and effects were found in literature. The limited data available for these compounds make this study important for contributing to the characterisation of their ecotoxicological effects. Aclonifen, bifenox and metribuzin are all herbicides, frequently applied not only in agricultural but also in suburban and urban areas (Todd and Suter, 2012). Dichlofluanid is mostly used as an antifoulant and wood preservative (Cima et al., 2008). Triclosan biocidal function is due to its cosmetic function, being the first considered as a secondary function (compound regulated under Regulation No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products; EC, 2009).

With the information obtained in these assays along with that gathered from literature regarding the MoAs for each compound, preliminary AOPs were proposed for aclonifen (Fig. 24), bifenox (Fig. 25) and metribuzin (Fig. 26) These AOPs allowed to build biological relationships starting from the MIE, to KEs and concluding in the AOs, and thus providing causal links between the MoA of contaminants and their adverse effects. By doing so, assembly of data from different levels of organisation are extrapolated to higher organizational levels based on thorough mechanistic understanding (OECD, 2013). However, these conceptual AOPs still have to be further developed and better

evaluated, and the present assembly is to our knowledge the first effort to do so for invertebrates such as algae.

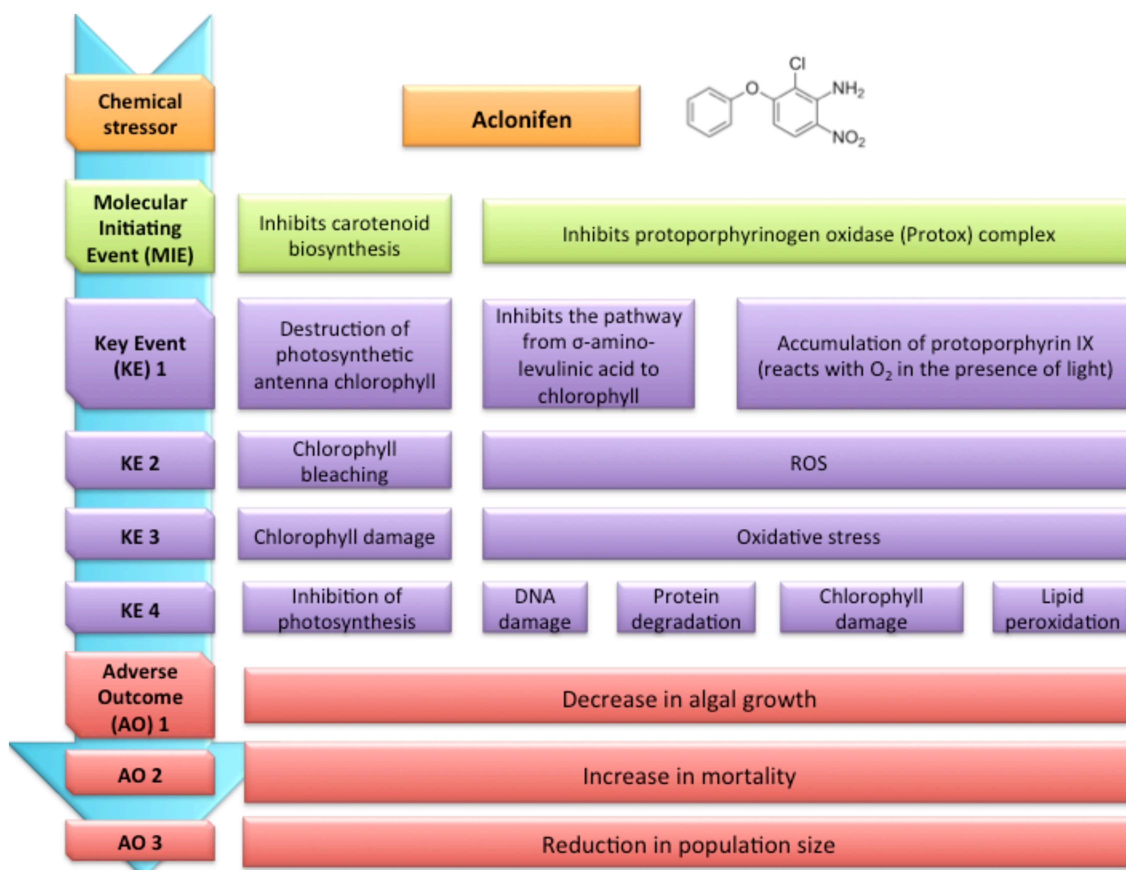

Fig. 24. Proposed AOPs for aclonifen.

Aclonifen (Fig. 24) inhibits the Protox complex, an enzyme involved in the pathway for the formation of chlorophyll from σ-amino-levulinic acid (Ensminger and Hess, 1985). This inhibition leads to the accumulation of protoporphyrin IX in cells, that then reacts with oxygen in the presence of light, causing the formation of ROS (as observed in Paper III), namely singlet oxygen ($^1O_2$) and superoxide anion (O_2-). The ROS formed clearly affected the PSII efficiency as shown in Paper II, and may potentially lead to oxidative stress and damage to cellular macromolecules like DNA (DNA damage and repair), proteins (protein degradation), chlorophyll, and membranes (lipid peroxidation) (Killinc et al., 2009; Ledford and Niyogi, 2005). Such perturbations can ultimately lead to apoptosis or necrosis (Ledford and Niyogi, 2005) with subsequent changes to more apical

endpoints such as algae survival and growth. Aclonifen is also known to inhibit carotenoid biosynthesis, which is an effective protector of chlorophyll scavenging ROS and dissipating the excess of absorbed energy. The inhibition of carotenoids can originate the destruction of photosynthetic antenna chlorophyll and bleaching of chlorophyll (Guseinova et al., 2005; Killinc et al., 2009).

Bifenox (Fig. 25) is known to instigate membrane disruption and inhibition of photosynthesis through the inhibition of Protox, thus causing light-dependent oxygen radical formation (EFSA, 2007; Grossman, 2005). However, the specific MoA for this compound was not found on literature. Bifenox presented a similar ROS pattern to that observed for paraquat on Paper III, with a monotonic concentration-dependent induction. Paraquat diverts electrons from PSI to molecular oxygen, producing superoxide radicals including H_2O_2 radicals (Hess, 2000). These, contrary to the radicals produced by aclonifen and metribuzin, are less reactive and can travel further into the cells, thus leading to more widespread of damage (Ledford and Niyogi, 2005). For paraquat, oxidative stress is not only observed in the chloroplasts but also in mitochondria and nucleus, with a depletion of antioxidant capacity and potentially making algae more susceptible to ROS (Nestler et al., 2012a,b). However, as already mention, no effects on the PSII efficiency were observed for this compound (Paper II).

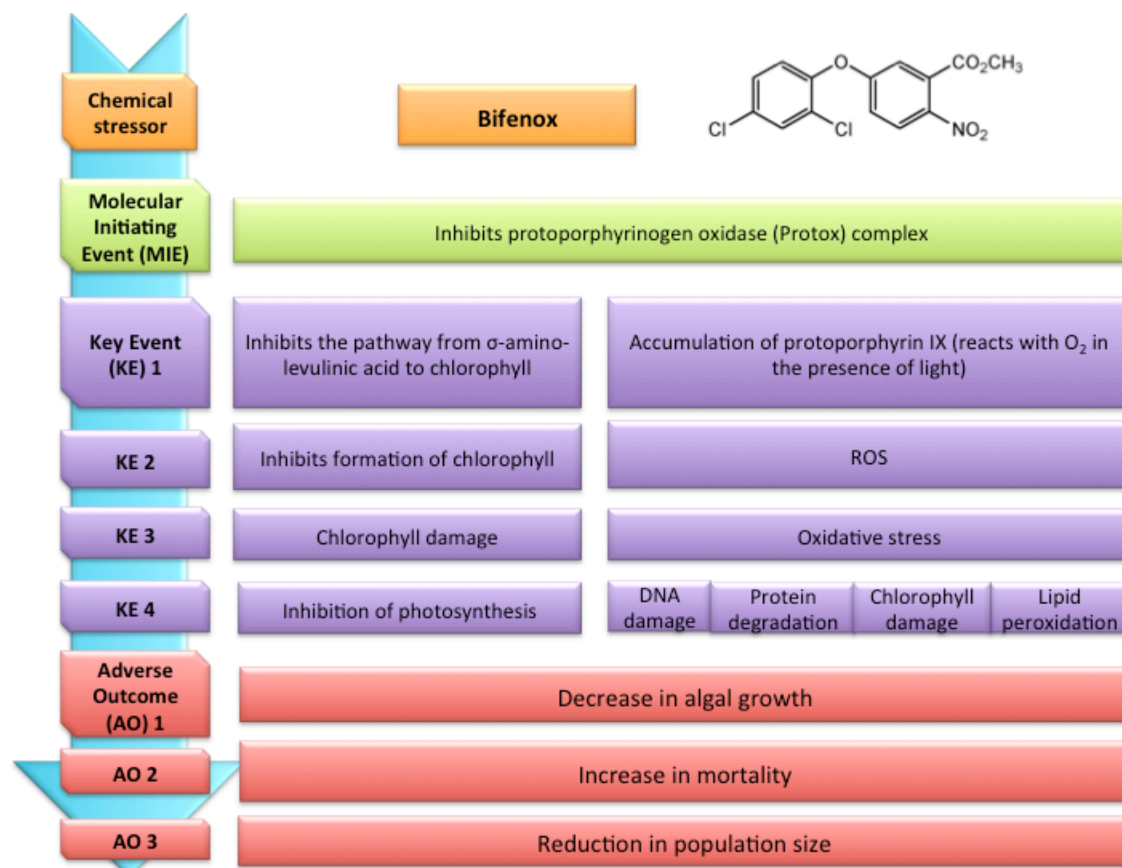

Fig. 25. Proposed AOPs for bifenox.

Metribuzin (Fig. 26) blocks the electron transport in the Hill reaction inhibiting the PSII by binding to the Dl protein, a membrane polypeptide containing the plastoquinone-binding site (Buman et al., 1992). This binding prevents the reduction of $NADP^+$ required for CO_2 fixation (Eullaffroy and Vernet, 2003), originating the formation of ROS as observed in Paper III. It originates the formation of a chlorophyll triplet state (^{3}Chl) in the reaction centre capable of reacting with triplet oxygen ($^3O_2$), and forming singlet oxygen ($^1O_2$). These reactive species can then cause damage to chlorophylls, separating them from their energy transfer systems and from protective pigments (carotenoids), causing further photogeneration of singlet oxygen (Jones, 2005; Rutherford and Krieger-Liskay, 2001). The formed ROS might also affect the PSII efficiency as proposed in Paper II.

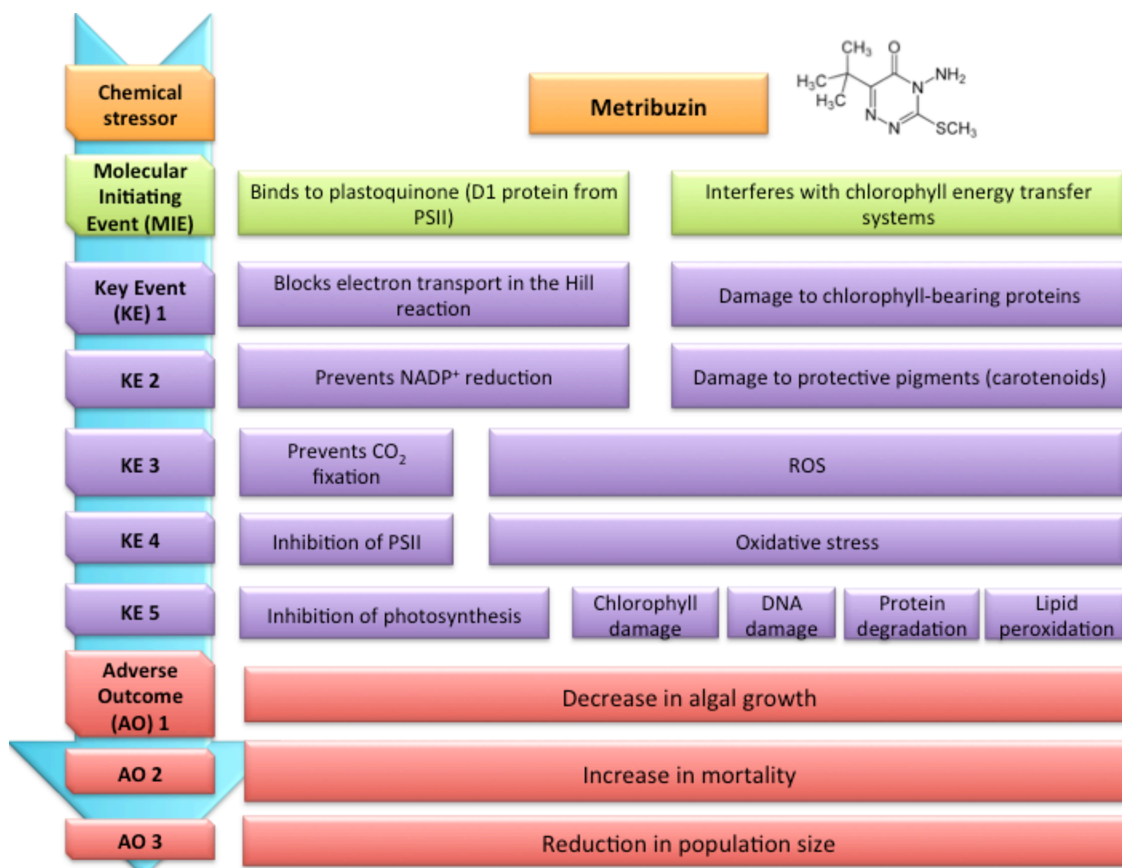

Fig. 26. Proposed AOPs for metribuzin.

For dichlofluanid and triclosan, information on their MoA was based on published studies, as these did not cause PSII inhibition or ROS formation. Both are known for having multiple toxic MoAs. Dichlofluanid inhibits thiol-containing enzymes by forming disulphide bridges, and to stimulate Ca^{2+} efflux from mitochondria (Johansson et al., 2012). Triclosan originates the uncoupling of oxidative phosphorylation and inhibition of non-photochemical quenching, a mechanism that is used to dispose of excess energy when the light energy absorption exceeds the capacity for photosynthesis (Franz et al., 2008). Although these mechanisms would also lead to inhibition of growth, development of similar AOPs were not undertaken, as the MoA was not investigated in detail.

4.4. Combined toxicity

CA and IA prediction models were used to study the combined toxicity of the biocides on *C. reinhardtii*. Overall, these gave a good prediction of the observed effects. Two approaches were used to verify which of the models best predicted the observed data, the MDRs values (that only take into account the median) and the statistical differences (significant differences accounting also with the variance of data). The use of the models was straightforward for the growth inhibition data, as this could be modelled straightforward without using any data manipulations/normalisations (*i.e.*, already following a classical CRC with effects from 0 to 100%). On the other hand, for PSII efficiency and ROS, data had to be normalized according to the minimum and maximum values obtained for the positive controls for each test (atrazine and H_2O_2, respectively). After this transformation, the PSII efficiency data became adequate for use in the prediction models, while for the ROS data some constrains were still present. This was due to the small initial slopes obtained for some of the individual compounds and mixtures (which had to be removed), along with the fact that the maximal formation of ROS (100%) was not possible to achieve, as already showed in other studies measuring ROS (Nestler et al., 2012a). Nevertheless, these constrains were reduced by normalisation and the predictions were effectively applied.

In the first study (Paper I), the growth inhibition test exhibited the different MoAs of the five studied compounds, which was verified by the IA model best predicting the effects. This was in agreement with previous studies on combined toxicity of biocides to aquatic organisms (Belden et al., 2007), including algae (Faust et al., 2003), where the IA model also best estimated the mixtures of compounds with dissimilar MoA. Most of the tested compounds, especially the herbicides, are known to ultimately affect photosynthesis but through different MoA. Generally, while herbicides are known to interact specifically with molecular targets in target organisms (such as undesirable algae and plants), antifoulants such as dichlofluanid and fungicides as triclosan normally display a more general toxicity to a wide range of organisms (Cedergreen, 2014). A potential antagonism was observed particularly at 24 h for low to median effect levels. Although this might be

due to chemical interactions that reduce the activity of certain compounds (Richter and Escher, 2005; Wehtje et al., 1991), there may be a temporal variation in the time each MoA takes to propagate their perturbations to the apical level (*i.e.*, algal growth), a general toxic endpoint indicative of the overall health status of the exposed organisms (Nestler et al., 2012a).

On the second study (Paper II) only the binary mixture of aclonifen and metribuzin was studied. Here, the PSII efficiency was well estimated by the IA model, indicating that the compounds have additive effects mediated by different MoAs, and in agreement with the specificity of this endpoint (Nestler et al., 2012a). On the other hand, a temporal and concentration-dependent variation in the combined effects was observed for the growth inhibition. At the beginning of exposure (24 h) the IA model best predicted the effects on the growth, revealing the different MoAs of the compounds. After 48 h and 72 h, the CA model best predicted the effects for low to medium effect levels, possibly due to the contribution of more biological targets and pathways to the overall toxicity affecting growth. At higher concentrations and for both endpoints, additivity underestimated the effects, indicating a possible synergy between the herbicides. Synergistic effects for mixtures of PSII inhibitors and other herbicides have already been reported in algae, attributed to interactions occurring in steps leading to ROS formation (Cedergreen, 2014). At high concentrations, while both herbicides induced the formation of ROS, aclonifen might also have prevented the repair of ROS-induced damages in the PSII complexes, a process that is continuously occurring during photosynthesis in natural conditions (Cedergreen, 2014). These processes do not seem to occur in algae when exposed to the compounds separately, and represent a potential explanation for why apparent synergistic interactions were occurring for this binary mixture. This information was used to propose an initial AOP for the studied mixture (Fig. 27).

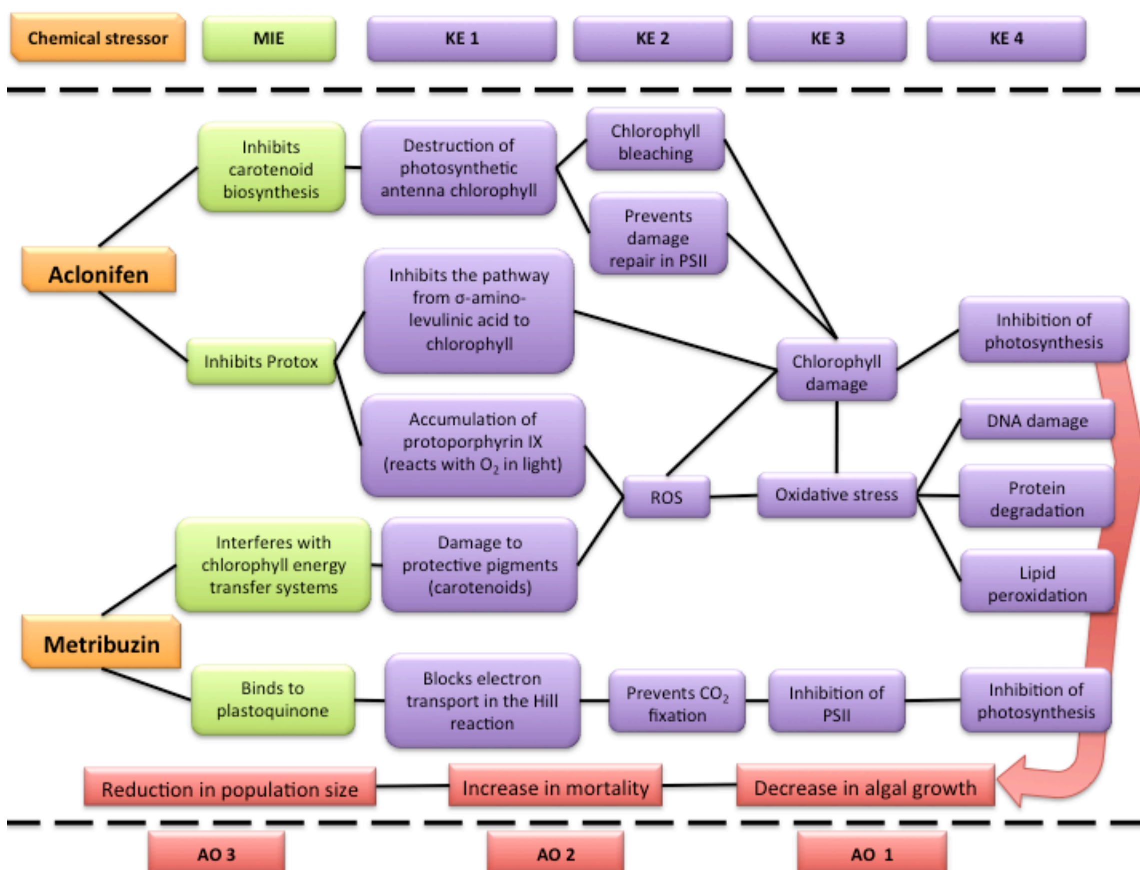

Fig. 27. Proposed AOPs for the mixture of aclonifen and metribuzin.

In the third study (Paper III), the best predictions were made by the CA model for the ternary mixture of aclonifen, bifenox and metribuzin and for the binary mixture of aclonifen and bifenox. CA normally provides good to excellent predictions for the mixture toxicity of biocides when analysing toxic endpoints such as algal growth (Backhaus and Faust, 2012: Cedergreen et al., 2008). However, at higher effect levels, the mixture of aclonifen and bifenox seemed to induce more than additive effects (*i.e.*, synergism). Both compounds have common MoA involving the formation of singlet oxygen and superoxide anion by the reaction of the accumulated protoporphyrin IX with oxygen in presence of light (Grossman, 2005; Kilinc et al., 2009). Aclonifen also inhibits carotenoid biosynthesis, and thus reduce the detoxification capacity of cells (Kilinc et al., 2009). The mixture of both compounds seems to induce not only an overall decline in the antioxidant defence mechanisms by producing high ROS levels, but also potentially reduce the carotenoid-mediated detoxification capacity of microalgae cells, thus making

them more susceptible to ROS and oxidative stress. The different type of ROS formed by the two compounds can also enhance the effects seen in the mixture. The ROS species formed due to the presence of aclonifen are possibly readily detoxified locally, while those produced by bifenox might be less reactive and able to pass through cell compartments and exert damage elsewhere (Grossman, 2005; Ledford and Niyogi, 2005). With a potential impairment of the antioxidant defence system by the combined action of both compounds, the inefficient removal of ROS may prevent the protection of target molecules within the range of their generation site. As a consequence, less reactive species can diffuse further, spreading oxidative stress and damage to other subcellular compartments (Ledford and Niyogi, 2005). A preliminary AOP was also proposed for this mixture (Fig. 28).

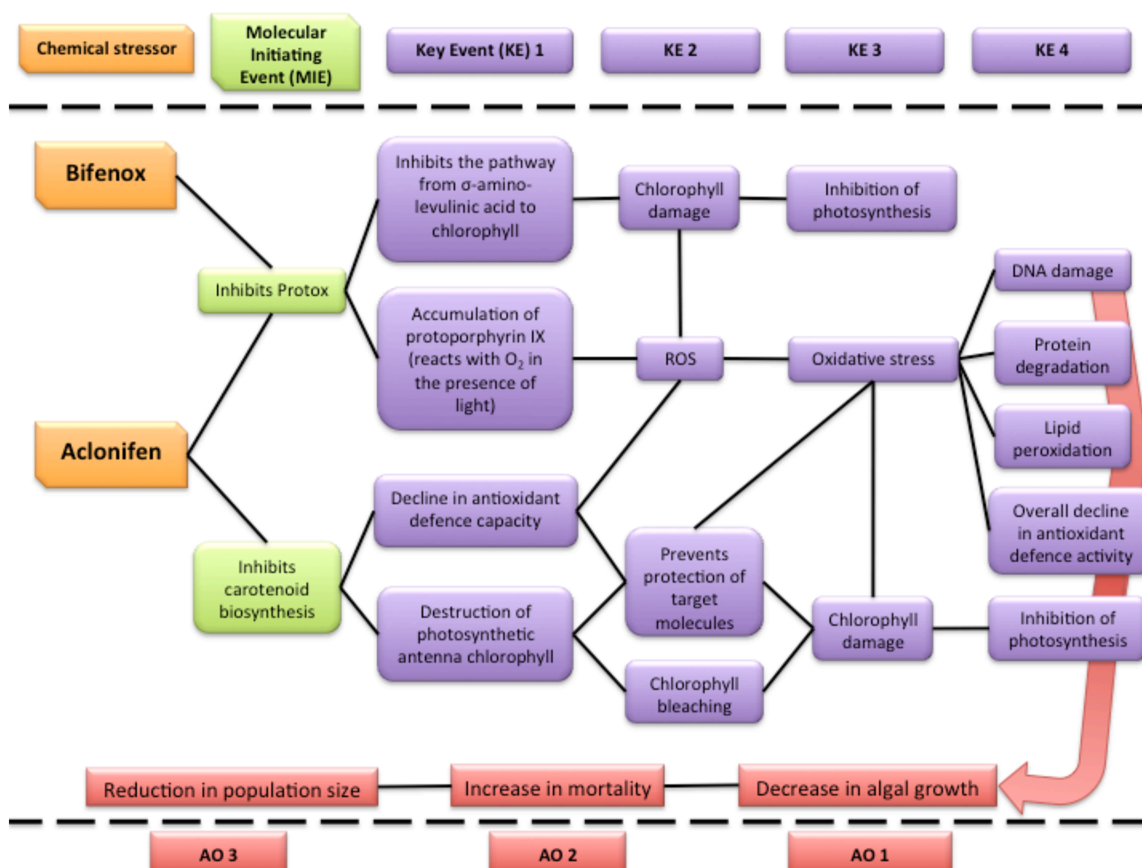

Fig. 28. Proposed AOPs for the mixture of aclonifen and bifenox.

Regarding the other two binary mixtures the observed combined effects were additive, with the binary mixture of bifenox and metribuzin well predicted by the two models. The mixture of aclonifen and metribuzin was best predicted by the IA model, indicating that ROS are produced by different MoA. While aclonifen is known to induce ROS through the oxidation of protoporphyrin IX (Kilinc et al., 2009), metribuzin likely produces ROS due to the formation of a ^{3}Chl state (Jones, 2005).

4.5. Environmental implications

In the present work, a conceptual basis for cumulative (mixture) risk assessment (Backhaus and Faust, 2012) was applied on Papers I (section 3.1.3.), and II (section 3.2.3.). A component based-approach was used, based on the fact that the toxicity of the mixture is a function of the toxicity of the individual compounds (Backhaus et al., 2013)

The risk assessment of each biocide and mixtures were based on the precautionary principle of CA, where the RQs and/or TUs concepts were used. The SRQs and STUs for the mixtures were inferred from single substances, and the RQ_{STU} calculated based on the sum of toxic units for the most sensitive trophic level (algae in this case) (Backhaus and Faust, 2012; Backhaus and Karlsson, 2014). An assessment factor (AF) of 100 was chosen to correctly extrapolate the biological variance, short-term to long-term exposure and laboratory to field exposure (Backhaus and Faust, 2012; Backhaus et al., 2013; EU, 2003, 2009). For the ROS data in Paper III this approach was not used, as ROS formation was considered being more informative of the MoA than predicting actual risk. The finding that the major ROS production occurred at higher concentrations than those causing PSII and growth inhibition confirm that the ROS data may not adequately assess the direct potential for effects at regulatory-relevant endpoints associated with risk assessment.

The test compounds were chosen based on their environmental relevance, so they are very likely to occur in freshwater environments. The proportion of each compound in the mixtures was made according to a fixed ratio ray design, to avoid that only one or two

contaminants would account for the predicted effect. So, although they may not exactly reflect any environmental state, the observed results show that the studied compounds may potentially affect algae (and probably other organisms) in the aquatic environment through combined toxicity. The MEC values for each studied biocide were calculated based on published literature for freshwater environments and minimum, median, 95 percentile and maximum values were calculated and used for the initial risk assessment.

The first study (Paper I) showed that a potential environmental risk was identified for metribuzin, triclosan, bifenox and possibly dichlofluanid, mostly at high environmental values ($\geq MEC_{95}$). However, as the values used for dichlofluanid were based on the metabolite DMS, the actual environmental concentration of this compound is probably lower than that of DMS due to rapid degradation in the aquatic environment (EPA, 2012). The highest risk was identified for metribuzin, in accordance with published studies where this biocide was identified as a main risk driver for algae in agricultural streams (Petersen et al., 2013). Triclosan, despite its relatively low toxicity to *C. reinhardtii*, was still identified as having a potential environmental hazard due to the high MECs reported. The combined risk assessment indicated a potential cumulative risk for the studied mixture. As these five biocides showed to have mostly additive effects, their co-occurrence in the environment would increase the potential environmental risk according to the principles of additivity. These compounds are likely to co-occur in surface waters with emissions from agricultural runoff (aclonifen, bifenox, metribuzin), municipal wastewater effluents (triclosan), and runoff waters from recreational boating activity and house painting (dichlofluanid). Moreover, not only these biocides but also other biocides and chemical compounds can occur in the same recipient, indicating a potential for combined effects.

In Paper II, the initial risk assessment performed did not identify a risk for any of the individual compounds when considering the effects on the PSII efficiency. The RQ_{STU}, a conservative estimate for cumulative risk assessment by assuming additivity (Backhaus and Faust, 2012), verified that the combination of aclonifen and metribuzin represented an environmental risk at median MEC values. Therefore, while the risk of these

chemicals alone did not seem to be sufficient to cause effects, their existence as a mixture can possibly lead to combined toxicity. As compounds co-exist in the environment, it might be expected that the overall risk of ecologically relevant mixtures of PSII inhibitors may even be higher than that predicted, as the observed synergism.

CA usually provides good to excellent predictions of the observed mixture toxicities (Belden et al., 2007; Backhaus and Faust, 2012). According to Belden et al. (2007) for pesticide mixtures, predictions by CA fall within a factor of 2 from the observation on 88% of the cases, independently of the mixture components present similarity or dissimilarity MoA. This is also in agreement with Cedergreen et al., (2008) observing that substantial deviations from CA only occurred for 6% of the investigated 158 data sets (more than a factor of 2 between predictions and observations) for mixtures of compounds with different molecular target sites. The application of IA for the prediction of combined toxicity is still scarce (Backhaus and Faust, 2012). Some strategies have been proposed, such as the use of species sensitivity distributions (SSDs) to calculate the IA-expected species sensitivity distribution using standard EC_{50} and/or NOEC values. However, this approach assumes that data for a sufficient number of taxa is available for each mixture component (Backhaus and Faust, 2012).

Synergistic interactions were observed in the present work and as in several examples found in the literature, normally restricted to mixtures with a few (frequently two) compounds. However, this is a situation relevant within the context of biocidal product authorization (Backhaus et al., 2013). The use of an additional assessment factor such as the one suggested by Backhaus et al. (2013) named "IF" (Interaction Factor) can be adopted, particularly if no toxicity data is available for the chemicals. This factor accounts for the possibility of synergistic interactions (higher mixture toxicity than predicted due to chemical, toxicokinetic and/or -dynamic interactions). This IF does not account for any of the other potential error sources (Backhaus et al., 2013). However, it is still challenging to assess the cumulative risk of compounds likely to cause synergy (Cedergreen, 2014), as the ones observed in this study. New and improved approaches have still to be further developed for accounting with the occurrence of interactions

between compounds for suitable a risk assessment.

4.6. Future studies

During the execution of this work some thoughts for further knowledge and new studies have arose. More information on the toxic MoA of some of the studied chemicals should be obtained, especially for dichlofluanid and triclosan. It would also be important to improve knowledge on the occurrences of the studied mixtures in the environment and the levels of additional contaminants in the environment. This is particularly important for risk assessment in order to detect and prevent undesired effects on non-target organisms. It would be also important to evaluate if sub-lethal exposure concentrations of these biocides can affect population in longer and more ecologically relevant studies.

Future work could involve a more comprehensive investigation with multi-endpoint assays for MoA characterisation in *C. reinhardtii*. This would provide a more detailed description of toxic mechanisms underlying the responses of specific endpoints of interest, for instance analysing the effects of ROS in specific cellular complexes or the presence of particular antioxidant defence mechanisms. This kind of investigations would, however, require more complex experimental setups and was not relevant in the present work.

Future studies should aim to clarify the underlying toxic mechanisms involved in the observed deviations from the predicted additive effects of the mixtures, applying for instance transcriptional studies to identify KEs involved in the propagation of effects from the MiE to the adverse outcome in a more through initiative to develop AOPs. Therefore, the “omics” tools now available for measuring for instance gene expression, protein interactions and metabolite flux could provide further experimental platforms for examining cellular responses with high resolution and coverage.

Initial AOPs were proposed for some of the single compounds, as well as for their mixtures. This strategy could be further developed for the studied compounds and their

mixtures to better clarify their MoA. This has shown to be a valuable approach to assemble, describe and evaluate all the available information for each contaminant that is relevant for both scientific and regulatory purposes and its application onto combined toxicity is a natural continuation of this work. Future AOP developments may hopefully provide better links between responses occurring at the molecular level with effects occurring at the higher organisational levels (e.g., population dynamics), and by doing so provide a feasible way to exploit toxicity assessment both in terms of a bottom-up and top-down approach. Whereas the bottom-up approach often make use of single chemical information from well-characterised mixtures to assess combined toxicity as performed in the present study, top-down approaches exploit the power of a combination of bioassay testing, fractionation of complex environmental samples with that of high-resolution chemical analysis to identify and quantify the most toxic compounds in a ecologically-relevant mixture.

5. Conclusions

In the present work, the single and combined effects of five environmentally relevant biocides, aclonifen, bifenox, dichlofluanid, metribuzin and triclosan, on the unicellular algae *C. reinhardtii* were studied. This alga demonstrated to be a suitable model organism to evaluate the toxicity of biocides and their mixtures. The five selected compounds showed to be highly toxic to algae, can be detected in the freshwater environment, present different MoAs and are considered as compounds of emerging concern. This study provided a more insight understanding on their toxic effects to algae.

The applied methods screened the effects of these biocides on the growth, PSII efficiency and formation of ROS. These three endpoints studied helped to characterise the toxic MoA of the single compounds and their mixtures on *C. reinhardtii.* The growth inhibition test allowed discriminating the general toxicity of each biocide and of the mixture as a whole. This study allowed obtaining data relevant for risk assessment as was monitoring growth inhibition that is considered a feasible proxy for population effects.

The PSII efficiency study enabled to discriminate that the herbicides aclonifen and metribuzin affected the PSII as a toxic MoA and correlated with those of growth inhibition. As the formation of ROS was a possible toxic mechanism affecting the PSII, this endpoint was also analysed and confirmed for aclonifen, bifenox and metribuzin.

The prediction models used in this study proved to be a good evaluating tool for the toxicity of the analysed mixtures. For the growth inhibition, IA best predicted the toxicity of the mixture with all the 5 compounds. For the PSII efficiency, only the binary of aclonifen and metribuzin (the compounds producing effects) was analysed and was also best predicted by IA, albeit the synergistic effects for high concentrations. As aclonifen, bifenox and metribuzin produced ROS, the combined toxicity of their simple mixtures was tested and best predicted by CA, except that of aclonifen and metribuzin best predicted by IA. For this endpoint, synergistic interactions were also observed for high concentrations of the binary mixture of aclonifen and bifenox

The MoA of the single compounds aclonifen, bifenox and metribuzin was partially characterized and the obtained information along with published knowledge was used to develop preliminary AOPs to assemble all the available information. The same approach was used for two mixtures showing synergistic interactions, aclonifen and metribuzin and aclonifen and bifenox. The observed interactions seemed to be possibly promoted by the overproduction of ROS and to the overall decline in the antioxidant defence mechanisms.

Based in the principles of additivity, the use of RQs and/or TUs approach was applied in Papers II, giving this work a potential value to be used for regulatory purposes. When considering algal growth inhibition, all the single compounds presented a risk to algae for median to high MEC values, with their mixture showing a potential risk at all concentrations. Considering the PSII efficiency data, only the binary mixture of aclonifen and metribuzin presented a risk for algae, with the single compounds not showing risk. This study also highlighted the need to further develop the already available tools for risk assessment at ecologically relevant exposure scenarios, especially when addressing less known compounds as these biocides and especially when synergistic interactions occur.

Concluding, the present PhD work contributed to advance the field of ecotoxicology by developing knowledge on the MoA of commonly used biocides. The combined toxicity of their simple mixtures was also established for identifying if these biocides and their mixture represent a risk to algae under environmentally relevant concentrations.

6. References

Alric, J., Lavergne, J., Rapport, F., 2010. Redox and ATP control photosynthetic cyclic electron flow in *Chlamydomonas reinhardtii* (I) aerobic conditions. Biochem. Biophys. Acta 1797, 44-51.

Alscher, R.G., Donahue, J.L., Cramer, C.L., 1997. Reactive oxygen species and antioxidants: relationships in green algae. Physiol. Plantarum 100, 224-233.

Altenburger, R., Nendza, M., Schuurmann, G., 2003. Mixture toxicity and its modelling by quantitative structure–activity relationships. Environ. Toxicol. Chem. 22, 1900–1915.

Andersen, 2005. Algal culturing techniques. Elsevier, London, 589p.

Ashauer and Escher, 2010. Advantages of toxicokinetics and toxicodynamics modelling in aquatic ecotoxicology and risk assessment. J. Environ. Monit. 12, 2056-2061.

Ashauer, 2015. http://www.ecotoxmodels.org/toxicokinetic-toxicodynamic-models/

Backhaus, T., Blanck, H., Faust, M., 2010. Hazard and risk assessment of chemical mixtures under REACH. State of the art, gaps and options for improvement. Swedish Chemicals Agency, Sundbyberg.

Backhaus, T., Faust, M., 2012. Predictive environmental risk assessment of chemical mixtures: a conceptual framework. Environ. Sci. Technol. 46, 2564-2573. Swedish Chemicals Agency, 75p.

Backhaus, T., Altenburger, R., Faust, M., Frein, D., Frische, T., Johansson, P., Kehrer, A., Porsbring, T., 2013. Proposal for environmental mixture risk assessment in the context of the biocidal product authorization in the EU. Env. Sci. Eur. 25:4.

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

Barek, J., Cabalková, D., Fischer, J., Navrátil, T., Pecková, K., Yosypchuk, B., 2011. Voltammetric determination of the herbicide Bifenox in drinking and river water using a silver solid amalgam electrode. Environ. Chem. Lett 9, 83-86.

Bedding, N.D., McIntyre, A.E., Perry, R., Lester, J.N., 1982. Organic contaminants in the aquatic environment. I. Sources and occurrence. Sci. Total Environ. 25, 143-167.

Belden, J.B., Lydy, M.J., 2006. Joint toxicity of chlorpyrifos and esfenvalerate to fathead minnows and midge larvae. Environ. Toxicol. Chem. SETAC 25, 623–629.

Belden, J.B., Gilliom, R.J., Lydy, M.J., 2007. How well can we predict the toxicity of pesticide mixtures to aquatic life? Int. Environ. Assess. Manage 3, 364–372.

Bliss, C.I., 1939. The toxicity of poisons applied jointly. Ann. J. Appl. Biol. 26, 585–615.

Bogert, C.J., Quill, T.F., McCarty, L.S., Mason, A.M., 2004. Can mode of action predict mixture toxicity for risk assessment? Toxicol. Appl. Pharmacol 201, 85-96.

Boo, Y.C., Jung, J., 1999. Water deficit-induced oxidative stress and antioxidant defenses in rice plants. J. Plant Physiol. 155, 255-261.

Brack, W., 2003. Effect-directed analysis: a promising tool for the identification of organic toxicants in complex mixture? Anal. Bioanal. Chem. 377, 397-407.

Brack, W., Schmitt-Jansen, M., Machala, M., Brix, R., Barceló, D., Schymanski, E., Streck, G., Schulze, T., 2008. How to confirm identified toxicants in effect-directed analysis. Anal. Bioanal. Chem. 390, 1959-1973.

Buman, R.A., gealy, D.R., Fuerst, E.P., 1992. Relationship between temperature and triazinone herbicide activity. Pestic. Biochem. Phys. 43, 22-28.

Butterworth, B.E., Conolly, R.B., Morgan, K.T., 1995. A strategy for establishing mode of action of chemical carcinogens as a guide for approaches to risk assessments. Cancer Lett. 93, 129-146.

Cedergreen, N., Streibig, J.C., 2005. The toxicity of herbicides to non-target aquatic plants and algae: assessment of predictive factors and hazard. Pest. Manag. Sci. 61, 1152–1160.

Cedergreen, N., Christensen, A.M., Kamper, A., Kudsk, P., Matthiasen, S., Streibig, J.C., Sørensen, H., 2008. A review of independent action as a reference model for binary mixtures of compounds with different molecular target sites. Environ. Toxicol. Chem. 27, 1621–1632.

Cedergreen, N., 2014. Quantifying synergy: a systematic review of mixture toxicity studies within environmental toxicology. PLOS ONE 9 (5), e96580.

Chalew, T.E.A., Halden, R.U., 2009. Environmental exposure of aquatic and terrestrial biota to triclosan and triclocarban. JAWRA 45 (1), 4-13.

Cima, F., Bragadin, M., Ballarin, L., 2008. Toxic effects of new antifouling compounds on tunicate haemocytes I. Sea-Nine 211™ and chlorothalonil. Aquat. Toxicol. 86, 299-312.

Corbett, J.R., Wright, K., Baillie, A.C., 1984. The Biochemical Mode of Action of Pesticides. Academic, London, UK.

DeLorenzo, M.E., Scott, G.I., Ross, P.E., 2001. Toxicity of pesticides to aquatic microorganisms: a review. Environ. Toxicol. Chem. 20, 84-98.

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 L-2985 Luxembourg, 328p.

EC, 2009. Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products. Official Journal of the European Union L 342/59.

EFSA, 2007. Conclusion on the peer review of bifenox. European Food Safety Authority Scientific Report 119, 1-84.

EFSA, 2010. Conclusion regarding the peer review of the pesticide risk assessment of the active substance metribuzin. European Food Safety Authority Scientific Report 88, 1-74.

Eggen, R.I.L., Behra, R., Burkhardt-Holm, P., Escher, B.I., Schweigert, N., 2004. Viewpoint: challenges in ecotoxicology – mechanistic understanding will help overcome the newest challenges. Environ. Sci. Technol. February 1, 58A-64A.

Ensminger, M.P., Hess, F.D., 1985. Action spectrum of the activity of acifluorfen-methyl, a diphenyl ether herbicide, in *Chlamydomonas eugametos*. Plant Physiol. 77, 503-505.

EPA, 2000. Science Policy Council handbook: risk characterization. U.S. Environmental Protection Agency, Washington, DC.

EPA, 2004. Overview of the ecological risk assessment process in the office of pesticide programs: endangered and threatened species effects determinations. U.S. Environmental Protection Agency. Office of Prevention, Pesticide, and Toxic Substances, 92p.

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 Center for Environmental Assessment, Cincinnati, 412p.

EU pesticides database, 2015. http://ec.europa.eu/sanco_pesticides

EU, 2008. Directive 2008/105/EC of the European Parliament and of the Council of 16 December 2008 on environmental quality standards in the field of water policy, amending and subsequently repealing Council Directive 82/176/EEC, 83/5223/EEC, 84/156/EEC, 84/491/EEC, 86/280/EEC and amending Directive 2000/60/EC of the European Parliament and of the Council.

EU, 2009. Council of the European Union: Council conclusion on combination effects of chemicals, 2988th Environment Council Meeting, Brussels, 22 Dec. 2009.

EU, 2012. Regulation (EU) No 528/2012 of The European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products. Official Journal of the European Union L 167/1-L 167/123.

EU, 2013. Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy. Official Journal of the European Union L 226/1-L 226/16.

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 Res. 37, 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. Environ. Toxicol. Chem. 17, 1830–1834.

Falkowski, P.G., Raven, J.A., 2007. Aquatic Photosynthesis, second edition. Princeton University Press, New Jersey, USA.

Faust, M., Altenburger, R., Backhaus, T., Blanck, H., Boedeker, W., Gramatica, P.,Hamer, V., Scholze, M., Vighi, M., Grimme, L.H., 2003. Joint algal toxicity of 16 dissimilarly acting chemicals is predictable by the concept of independentaction. Aquat. Toxicol. 63 (1), 43–63.

Fischer, B.B., Wiesendanger, M., Eggen, R.I.L., 2006. Growth condition-dependent sensisivity, photodamage and stress response of *Chlamydomonas reinhardtii* exposed to high light conditions. Plant Cell Physiol. 47(8), 1135-1145.

Fischer, B.B., Eggen, R.I.L., Niyogi, K.K., 2010. Characterization of singlet oxygen-accumulating mutants isolated in a screen for altered oxidative stress response in *Chlamydomonas reinhardtii*. BMC Plant Biol. 10, 279.

Foyer, C.H., Lopez-Delgado, H., Oat, J.F., Scott, I.M., 1997. Hydrogen peroxide and glutathione-associated mechanisms of acclimatory stress tolerance and signalling. Physiol. Plant 100, 241-254.

Foyer, C.H., Noctor, G., 2005. Oxidant and antioxidant signalling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. Plant. Cell. Environ. 28, 1056–71.

Franz, S., Altenburger, R., Heilmeier, H., Schmitt-Jansen, M., 2008. What contributes to the sensivity of microalgae to triclosan? Aquat. Toxicol. 90, 102–108.

Gomes, A., Fernandes, E., Lima, J.L.F.C., 2005. Fluorescence probes used for detection of reactive oxygen species. J. Biochem. Biophys. Methods 65, 45-80.

Grossman, K., 2005. What it takes to get a herbicide's mode of action. Physionomics, a classical approach in a new complexion. Pest. Manag. Sci. 61 (5), 423-431.

Groten, J.P., Feron, V.J., 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, 79-89.

Guenther, J.E., Nemson, J.A., Melis, A., 1990. Development of photosystem II in dark grow *Chlamydomonas reinhardtii*. A light-dependent conversion of PS II_{β}, Q_B-nonreducing centers to the PS II_{α}, Q_B-reducing form. Photosynth. Res. 24, 35-46.

Guseinova, I.M., Suleimanov, S.Y., Aliyev, J.A., 2005. The effect of norflurazon on protein composition and chlorophyll organization in pigment-protein complex of photosystem II. Photosynth. Res. 84, 71-76.

Harris, E.H., 2009. The Chlamydomonas Sourcebook. Introduction to Chlamydomonas and its laboratory use. Volume 1, 435 p.

Hess, F.D., 2000. Light-dependent herbicides: an overview. Weed Sci. 48, 160–170.

Hoffman, D.J., Rattner, B.A., Burton, G.A.Jr., Cairns, J.Jr., 2003. Handbook of ecotoxicology. Lewis Publishers, London, second edition, 1289p.

https://newunderthesunblog.wordpress.com/the-basics/the-light-reactions/photosystem-ii/photosystem-ii-structure/ (Fig. 5)

Jamers, A., Coen, W.D., 2010. Effect assessment of the herbicide paraquat on a green alga using differential gene expression and biochemical biomarkers. Environ. Toxicol. Chem. 29, 893-901.

Johansson, P., Eriksson, K.M., Axelsson, L., Blanck, H., 2012. Effects of seven antifouling compounds on photosynthesis and inorganic carbon use in sugar kelp *Saccharina latissima* (Linnaeus). Arch. Environ. Contam. Toxicol. 63 (3), 365-377.

Jones, R., 2005. The ecotoxicological effects of photosystem II herbicides on corals. Mar. Pollut. Bull. 51, 495-506.

Juneau, P., Qiu, B., Deblois, C.P., 2007. Use of chlorophyll fluorescence as a tool for determination of herbicide toxic effect: review. Toxicol. Environ. Chem. 89(4), 609-625.

Kilinc, Ö., Grasset, R., Reynaud, S., 2009. The herbicide aclonifen: the complex theoretical bases of sunflower tolerance. Pestic. Biochem. Physiol. 100, 193-198.

Kilinc, Ö., Grasset, R., Reynaud, S., 2011. The herbicide aclonifen: the complex theoretical bases of sunflower tolerance. Pestic. Biochem. Physiol. 100, 193-198.

Kitajima, M., Butler, W.L., 1975. Quenching of chlorophyll fluorescence and primary photochemistry in chloroplast by dibromothymoquinone. Biochim. Biophys. Acta 326, 105-115.

Kowal, S., P. Balsaa, F. Werres and T. C. Schmidt, 2009. Determination of the polar pesticide degradation product N,N-dimethylsulfamide in aqueous matrices by UPLC–MS/MS. Anal. Bioanal. Chem. 395, 1787-1794.

Kramer, V.J., Etterson, M.A., Hecker, M., Murphy, C.A., Roesijadi, G., Spade, D.J., Spromberg, J.A., Wang, M., Ankley, G.T., 2011. Adverse outcome pathways and ecological risk assessment: bridging to population-level effects. Environ. Toxicol. Chem. 30, 64-76.

la Farré, M., Pérez, S., Kantiani, L., Barceló, D., 2008. Fate and toxicity of emerging pollutants, their metabolites and transformation products in the aquatic environment. Trend. Anal. Chem. 27, 991-1007

Langford, K., 2012. Screening of selected alkylphenolic compounds, biocides, rodenticides and current use pesticides. Statlig program for forurensningsovervåking, Klima- og Forurensnings- Direktoratet. Norwegian Institute for Water Research, Rapportnr. 1116/2012, TA 2899, p. 69.

Ledford, H.K., Niuogi, K.K., 2005. Singlet oxygen and photo-oxidative stress management in plants and algae. Plant Cell. Environ. 28, 1037-1045.

Legendre, L., Yueh, Y.G., Crain, R., Haddock, N., Heinstein, P.F., Low, P.S., 1993. Phospholipase-C activation during elicitation of the oxidative burst in cultured plant cells. J. Bioi. Chem. 268, 24559-24563.

Lien, T., Knutsen, G., 1979. Synchronous growth of *Chlamydomonas reinhardtii (Chlorophyceae)* - Review of optimal conditions. J. Phycol. 15, 191-200.

Lishman, L., S.A. Smyth, K. Sarafin, S. Kleywegt, J. Toito, T. Peart, B. Lee, M. Servos, M. Beland, and P. Seto, 2006. Occurrence and reductions of pharmaceuticals and personal care products and estrogens by municipal wastewater treatment plants in Ontario, Canada. Sci. Total Environ. 367, 544-558.

Livingstone, D.R., 2001. Contaminant–stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Mar. Pol. Bull. 42, 656–666.

Loggini, B., Scartazza, A., Brugnoli, E., Navari-Izzo, F., 1999. Antioxidative defense system, pigment composition and photosynthetic efficiency in two wheat cultivars subjected to drought. Plant Physiol. 199, 1091-1099.

Low, P.S., Merida, J.R., 1996. The oxidative burst in plant defense: function and signal transduction. Physiol. Plant 96, 533-542.

Magnusson, M., Heimann, K., Negri, A.P., 2008. Comparative effects of herbicides on photosynthesis and growth of tropical estuarine microalgae. Mar. Pollut. Bull. 56, 1545-1552.

Martínez, K., Ferrer, I., Hernando, M.D., Fernández-Alba, A.R., Marcé, R.M., Borrull, F., Barceló, D., 2001. Occurrence of antifouling biocides in the Spanish Mediterranean marine environment. Environ Technol. 22 (5), 543-52.

Meneguzzo, S., Navari-Izzo, F., Izzo, R., 1999. Antioxidative responses of shoots and roots of wheat to increasing NaCI concentrations. J. Plant. Physiol1. 55, 274-280.

Merchant, S.S., Prochnik, S.E., Vallon, O., 2007. The Chlamydomonas genome reveals the evolution of key animal and plant functions. Science 12, 318, 245-50.

Mittag, M., Kiaulehn, S., Johnson, C.H., 2005. The circadian clock *in Chlamydomonas reinhardtii*. What is it for? What is it similar to? Plant. Physiol. 137, 399–409.

Murphy, T.M., Huerta, A.J., 2006. Hydrogen peroxide formation in cultures rose cells in response to UV-C radiation. Physiol. Pantarum 78, 247-253.

Murray, K.E., Thomas, S.M., Bodour, A.A., 2010. Prioritizing research for trace pollutants and emerging contaminants in the freshwater environment. Environ. Pollut. 158, 3462-3471.

Nestler, H., Groh, K.J., Schönenberg, R., Behra, R., Schirmer, K., Eggen, R.I.L., Suter, H.J.-F., 2012a. Multiple-endpoint assay provides a detailed mechanistic view of responses to herbicide exposure in *Chlamydomonas reinhardtii*. Aquat. Toxicol. 110-111, 214-224.

Nestler, H., Groh, K.J., Schönenberg, R., Eggen, R.I.L., Suter, H.J-F., 2012b. Linking proteome responses with physiological and biochemical effects in herbicide-exposed *Chlamydomonas reinhardtii*. J. Proteomics75, 5370-5385.

Nikinmaa, M., 2014. An Introduction to Aquatic Toxicology. Academic Press, USA, 253p.

OECD, 2011. OECD Guidelines for the Testing of Chemicals, Section 2: Effects on Biotic Systems Test No. 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test. OECD Publishing, Business & Economics, 1-25.

OECD, 2013. Guidance document on developing and assessing adverse outcome pathways. Series on testing and assessment No 184. Paris, 45p.

Oettmeier, W., Masson, K., Fedtke, C., Konze, J., Schmidt, R.R., 1982. Effect of different Photosystem II inhibitors on chloroplasts isolated from species either susceptible or resistant toward s-triazine herbicides. Pestic. Biochem. Physiol. 18, 357–367.

Pazour, G. J., N. Agrin, J. Leszyk and G. B. Witman, 2005 Proteomic analysis of a eukaryotic cilium. J. Cell Biol. 170, 103–113.

Pedersen, R., Bechmann, M., Deelstra, J., Eggestad, H.O., Greipsland, I., Stenrød, M., Fystro, G., Selnes, S., Riley, H., Stubhaug, E., 2014. Jord- og vannovervåking i landbruket (JOVA). Feltrapport fra programmet i 2012 (Bioforsk).

Pesticide Properties DataBase, University of Herfordshire, 2013. http://sitem.herts.ac.uk/aeru/ppdb/en/index.htm

Petersen, K., Stenrød, M., Tollefsen, K.E., 2013. Initial environmental risk assessment of combined effects of plant protection products in six different areas in Norway. Norwegian Institute for Water Research, REPORT SNO. 6588-2013

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. Toxicol. Environ. Chem. 89, 589–607.

Ramel, F., Sulmon, C., Bogard, M., Couée, I., Gouesbet, G., 2009. Differential patterns of reactive oxygen species and antioxidative mechanisms during atrazine injury and sucrose-induced tolerance in *Arabidopsis thaliana* plantlets. BMC Plant Biol. 9, 28.

Rand, G.M., Wells, P.G., MacCarty, L.S., 1995. Introduction to aquatic toxicology, in: Rand G.M. (Ed.), Fundamentals of Aquatic Toxicology II: Effects, Environmental Fate, and Risk Assessment. Taylor and Francis, Bristol, PA.

Rao, G.U., Shivanna, K.R., Sawhney, V.K.,1995. High-temperature tolerance of Petunia and Nicotiana pollen. Current Science 69,351–355.

Reboud, X., 2002. Response of *Chlamydomonas reinhardtii* to herbicides: negative relationship between toxicity and water solubility across several herbicide familes. Bull. Environ. Contam. Toxicol. 69, 554-561.

Richter, M., Escher, B.I., 2005. Mixture toxicity of reactive chemicals by using two bacterial growth assays as indicators of protein and DNA damage. Environ. Sci. Technol. 39, 8753 – 8761.

Rutherford, A.W., Krieger-Liskay, A., 2001. Herbicide-induced oxidative stress in photosystem II. Trends Biochem. Sci. 26, 648–653.

SCCS, 2010. Opinion on triclosan, antimicrobial resistance. Scientific Committee on Consumer Safety (SCCP/1251/09), 56 p.

Schlosser, P.M., Bogdanffy, M.S., 1999. Determining modes of action for biologically based risk assessments. Regulatory toxicology and pharmacology RTP 30, 75-79.

Sharma, Y.K., León, J., Raskin, I., Davis, K.R., 1996. Ozone-induced responses is *Arabidopsis thaliana*: the role of salicylic acid in the accumulation of defense-related transcripts and induced resistance. Proc. Natl. Acad. Sci. USA 93, 5099-5104.

Stoiber, T.L., Shafer, M.M., Perkins, D.A.K., Hemming, J.D.C., Armstrong, D.E., 2007. Analysis of glutathione endpoints for measuring copper stress in *Chlamydomonas reinhardtii*. Environ. Toxicol. Chem. 26, 1563-1571.

Stoiber, T.L., Shafer, M.M., Armstrong, D.E., 2011. Induction of reactive oxygen species in *Chlamydomonas reinhardtii* in response to contrasting trace metal exposures. Environ. Toxicol. 28, 516-523.

Szívak, I., Behra, R., Sigg, L., 2009. Metal-indiced reactive oxygen species production in *Chlamydomonas reinhardtii* (Chlorophyceae). J. Phycol. 45, 427–435.

Tanaka, K., 1994. Tolerance to herbicides and air pollutants. In: Foyer, C.H., Mullineaux P.M. (Eds.) Causes of Photooxidative stress and amelioration by defense systems in plants, 365-378.

Todd, B., Suter, G.W. 2012. Herbicides. Environmental Protection Agency. http://www.epa.gov/caddis/ssr_herb_int.html.

Torres, M.A., Barros, M.P., Campos, S.C.G., Pinto, E., Rajamani, S., Sayre, R.T., Colepicolo, P., 2008. Biochemical biomarkers in algae and marine pollution: a review. Ecotoxicol. Environ. Saf. 71 (1), 1-12.

Turkina, M., 2008. Functional proteomics of protein phosphorylation in algal photosynthetic membranes. Linköping Univesity Medical Dissertions No 1038.

Unfried, K., Albrecht, C., Klotz, L., Mikecz, A.V., Grether–Beck, S., Schins, R.P.F., 2007. Cellular responses to nanoparticles: target structures and mechanisms. Nanotoxicology 1, 52–71.

USEPA, 2002. Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms (4th ed.). EPA-821-R-02-013.

USEPA, 2008. Ecological hazard and environmental revised risk assessment chapter triclosan (pc code: 054901; case no.: 2340). United States Environmental Protection Agency. Washington, d.c. 20460. Office of prevention, pesticides and toxic substances, 33 p.

von der Ohe, P.C., Schmitt-Jansen, M., Slobodnik, J., Brack, W., 2012. Triclosan – the forgotten priority substance? Environ. Sci. Pollut. Res. 19, 585–591.

Walker, C.H., Hopkin, S.P., Sibly, R.M., Peakall, D.B., 2001. Principles of Ecotoxicology, Second Edition. Taylor & Francis, New York, 326p.

Weckx, J.E.J., Clijsters, H., 1996. Oxidative damage and defense mechanisms in primary leaves of *Phaseolus vulgaris* as a result of root assimilation of toxic amounts of copper. Physiol. Plant 96, 506-512.

Wehtje, G.R, Wilcut, J.W., Dylewski, D.P., McGuire, J.A., Hicks, T.V., 1991. Antagonism of paraquat phytotoxicity in peanuts (*Arachis-Hypogaea*) and selected weed species by naptalam. Weed Sci. 39, 634–639.

Supplementary data

Table 1. Biocidal product-types and their descriptions (adapted from EU, 2012).

Main group	Product-type	Description
1. Disinfectants	1. Human hygiene	Products used for human hygiene, applied on or in contact with human skin or scalps for the primary purpose of disinfection.
	2. Disinfectants and algaecides not intended for direct application to humans or animals	Products used for the disinfection of surfaces, air and water; products used as algaecides for water treatment; products used to be incorporated in materials with disinfecting properties.
	3. Veterinary hygiene	Products used for the disinfection of surfaces and materials used for veterinary purposes.
	4. Food and feed area	Products used for the disinfection of materials in contact with food (including water) for humans and animals.
	5. Drinking water	Products used for the disinfection of drinking water for humans and animals.
2. Preservatives	6. Preservatives for products during storage	Products used for the preservation of manufactures products, for the storage or uses as rodenticide, insecticide, etc.
	7. Film preservatives	Products used for the preservation of films or coatings by the control of microbial deterioration or algal growth.
	8. Wood preservatives	Products used for the preservation of wood, including preventive and curative products.
	9. Fibre, leather, rubber and polymerized materials preservatives	Products used for the preservation of fibrous or polymerized materials by the control of microbiological deterioration.
	10. Construction material preservatives	Products used for the preservation of masonry,

		composite materials, or other construction materials by the control of microbiological and algal attack.
	11. Preservatives for liquid-cooling and processing systems	Products used for the preservation of water or other liquids used in cooling and processing systems by the control of harmful organisms such as microbes, algae and mussels.
	12. Slimicides	Products used for the prevention or control of slime growth on materials, equipment and structures, used in industrial processes.
	13. Working or cutting fluid preservatives	Products to control microbial deterioration in fluids used for working or cutting metal, glass or other materials.
3. Pest control	14. Rodenticides	Products used for the control of mice, rats or other rodents.
	15. Avicides	Products used for the control of birds.
	16. Molluscicides, vermicides and products to control other invertebrates	Products used for the control of molluscs, worms and invertebrates not covered by other product-types.
	17. Piscicides	Products used for the control of fish.
	18. Insecticides, acaricides and products to controls other arthropods	Products used for the control of arthropods (e.g. insects, arachnids and crustaceans).
	19. Repellents and attractants	Products used to control harmful organisms by repelling or attracting
	20. Control of other vertebrates	Products used for the control of vertebrates other than those already covered.
4. Other biocidal products	21. Antifouling products	Products used to control the growth and settlement of fouling organisms on vessels, aquaculture equipment or other

		structures used in water.
	22. Embalming and taxidermist fluids	Products used for the disinfection and preservation of human or animal corpses.

Table 2. The major reactive oxygen species (ROS; adapted from Nikinmaa, 2014).

Compound	Chemical equation	Remarks
Superoxide	$O_2^{\bullet -}$	Produced for instance in the electron transport chain of mitochondria. Half-life of the molecule is in the microsecond order, being selective in its reactivity. It is the substrate of superoxide dismutase (SOD).
Hydroperoxyl radical	$HO_2^{\bullet}$	Formed in the dismutation of superoxide, and the reaction is normally continued to form hydrogen peroxide and oxygen molecules.
Hydroxyl radical	$OH^{\bullet}$	Very reactive, but short half-life (1 nanosecond) restricting its movement to a few nanometers in the cell. May take part in cellular signaling. It is formed in the Fenton or Harber-Weiss reactions involving iron and copper.
Peroxyl radical	$RO_2^{\bullet}$	Half-life of several milliseconds. Plays an important role in lipid peroxidation.
Alkoxyl radical	$RO^{\bullet}$	Plays an important role in lipid peroxidation.
Carbonate radical	$CO_3^{\bullet -}$	Formed when hydroxyl radicals react with carbonate or bicarbonate ions. It is a potent oxidant.
Carbon dioxide radical	$CO_2^{\bullet -}$	
Singlet oxygen	$^1O_2^{\bullet}$	There are two types of singlet oxygen, being the radical species $^1\Sigma g^+$ rapidly converted to the non-radical $^1\Delta g$. The species is involved in photo-oxidation (photosensitization), which is inhibited by vitamin E.
Hydrogen peroxide	H_2O_2	Causes cell senescence and apoptosis at concentrations > 10 µM (apoptosis is changed to necrotic cell death at > 100 µM). At low concentrations, the molecule may be involved in cellular signaling, as it for instance promotes cell proliferation. Several enzymes, e.g. SOD, produce hydrogen peroxide. The molecule, which is quite stable and very membrane permeant, is only weakly reactive, but its reactions with iron (and copper)

		produce the highly reactive hydroxyl radical.
Peroxynitrite	ONOO-	Attacks mainly tyrosines of proteins, inactivating several enzymes like SOD.
Peroxynitrous acid	ONOOH	Strong oxidant and nitrant in aqueous solution, dissociating to peroxynitrite.
Nitrosoperoxycarbonate	$ONOOCO_2^-$	Less reactive than peroxynitrite, most of which is converted to nitrosoperoxycarbonate in the presence of carbon dioxide.
Hypochlorous acid	HOCl	Instead of being included in ROS, could also be called a reactive halogen-containing compound.
Hypobromous acid	HOBr	Instead of being included in ROS, could also be called a reactive halogen-containing compound.
Ozone	O_3	Produced when an oxygen molecule photodissociates to oxygen atoms, which further react with oxygen molecules. Causes inflammation and oxidizes lipids.

Table 3. Compiled list of priority biocides. Note: the selected biocides are in bold; information gathered from: Pesticide Properties DataBase, University of Herfordshire, 2013; Petersen et al., 2013; EU pesticides database, 2015.

Compound	Use	Emergent	Toxicity (µg/L)					MoA
			LOEC	NOEC	EC_{50}	PNEC	PEC	
2,4-D	Herbicide			100000	24200		58	Selective, systemic, absorbed through roots and increases biosynthesis and production of ethylene causing uncontrolled cell division and vascular tissue damage.
Aclonifen	Herbicide	Proposed new priority substance (EU)		4.9	28	0.25		Systemic and selective. Inhibition of carotenoid biosynthesis.
Azoxysrtobin	Fungicide			800	360	4.4		Respiration inhibitor.
Bentazone	Herbicide			25700	10100	540		Selective action, absorbed by foliage with very little translocation. Inhibits photosynthesis (PS II).
Bifenox	Herbicide	Proposed new priority substance (EU)	3	0.175	1.5		6.93	Selective, absorbed by foliage, new shoots and roots to inhibit protoporphyrinogen oxidase (Protox).
Boscalide	Fungicide				3750	2.6		Protectant, foliar absorption, translocates, inhibits spore germination and germ tube elongation.
Carbendazim	Fungicide				>7700	0.03		Systemic with curative and protectant activity. Inhibition of mitosis and cell division.
Clopyralid	Herbicide			17000	30500	1080		Selective, systemic, absorbed through leaves and roots.
Cyazofamid	Fungicide				25	0.5		Foliar and soil preventative action with some residual activity. Respiration inhibitor.
Cyprodinil	Fungicide				2600	0.176		Systemic, absorbed through foliage. Inhibits protein synthesis.
DEET	Insecticide	Yes (NORMAN)			41000			Inhibits the activity of acetylcholinesterase.
Dicamba	Herbicide			25000	1800	45		Selective, systemic, absorbed through

								leaves and translocates throughout plant.
Dichlofluanid	Wood preservative, antifoulants, fungicide, acaricide	Yes (NORMAN)	50	1000	133	0.00001	0.0014	Inhibits thiol-containing enzymes by forming disulfide bridges; stimulates Ca^{2+} Efflux from mitochondria.
Dichlorprop	Herbicide			180000	1000000	0.5		Selective, systemic, absorbed through leaves and translocates to roots. Synthetic auxin causing stem and leaf malformations leading to death.
Dimethoate	Insecticide			32000	90400	4		Systemic with contact and stomach action. Acetylcholinesterase inhibitor.
Fenamidone	Fungicide				3840	0.25		Protective and curative action. Respiration inhibitor.
Fenhexamid	Fungicide			5360	>26100	10.1		Foliar applied with protective action. Disrupts membrane function. Inhibits spore germination.
Fluroxypyr	Herbicide			56000	49800	1230		Foliar uptake causing auxin-type response.
Imazalil	Fungicide, veterinary treatment				870	0.43		Systemic with curative and protective properties. Disrupts membrane function.
Imidacloprid	Insecticide, veterinary treatment			10000	>10000	180		Systemic with contact and stomach action. Acetylcholine receptor agonist.
Iprodione	Fungicide			3200	1800	17		Contact action with protectant and some eradicant activity. Signal transduction inhibitor.
Irgarol	Algicide, antifouling	Proposed new priority substance (EU)		0.146	1.452	1.46		Inhibition of the photosynthetic activity in photo-system II (PSII). Incorporation of CO_2 in organic molecules is inhibited leading to an inhibition in growth.
Kresoxim	Metabolite of kresoxim-methyl, a fungicide and bacteriacide				>500000	100		Kresoxim-methyl: Protective, curative, eradicative action and long residual effects. Acts by binding to Quinone outer site blocking electron transfer and respiration of the fungi.
Mandipropamid	Fungicide				19800	10		Inhibits spore germination with preventative action.
MCPA	Herbicide			60000	79800	15.2		Selective, systemic with translocation.

								Synthetic auxin.
Mecoprop	Herbicide			56000	237000	2200		Selective, systemic, absorbed by leaves with translocation.
Metalaxyl	Fungicide			10000	33000	10		Systemic with curative and protective action, acts by suppressing sporangial formation, mycelial growth and the establishment of new infections.
Metamitron	Herbicide			100	400	10		Selective, systemic, absorbed mainly by roots and translocated. Inhibits photosynthesis (PS II).
Metribuzin	Herbicide			19	23			Selective, systemic with contact and residual activity. Inhibits photosynthesis (PS II).
Pencycuron	Fungicide			100	>300	1		Non-systemic with protective action. Inhibition of mitosis and cell division.
Phenmedipham	Herbicide				86	1.22		Selective, systemic, absorbed through leaves and translocated. Inhibits photosynthesis (PS II).
Pinoxaden	Herbicide				910	0.91		Systemic. Acetyl coenzyme A carboxylase inhibitor, inhibiting fatty acid synthesis.
Propiconazole	Fungicide	Yes (NORMAN)		460	9000			Adverse effects on CYP mediated processes, disruption of membrane function: sterol biosynthesis inhibitors.
Prothioconazole	Fungicide			2920	1100			Systemic with protective, curative and eradicative action. Long lasting activity.
Pyrimethanil	Fungicide				1200	18.8		Protective action with some curative properties.
Quinoxyfen	Fungicide	Proposed new priority substance (EU)			27			Multiple target sites. Systemic with protective properties, translocates and inhibits appressoria development stopping infections.
Thiabendazole	Fungicide	Yes (NORMAN)			460000			Adverse effects on CYP mediated processes. Central nervous system side effects and hepatototoxic potential.
Tolylfluanid	Fungicide. Insecticide (phenylsulfamide)	Yes (NORMAN)		100	1500			Inhibits thiol-containing enzymes by forming disulfide bridges. Same class of chemicals as dichlofluanid.

Triclosan	PCP, antiseptic, disinfectant, preservative, bacterioscide, fungicide	Forgotten priority substance	0.015	0.69	1.4-19	0.05	0.41	Multiple target sites.

Paper I

Combined toxicity of five priority biocides on the growth of *Chlamydomonas reinhardtii*

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Abstract

Biocides are products extensively used for the protection of humans, animals, or physical objects against harmful organisms. Many biocides have limited toxicity data to support ecological risk assessment, thus being considered chemicals of emerging concern. As these chemicals often co-occur in the aquatic environment as mixtures, they may also lead to combined toxicity that cannot be predicted on basis of effect data from the individual compounds alone. This study intended to characterise the single and combined effects of five environmentally relevant biocides: aclonifen, bifenox, dichlofluanid, metribuzin, and triclosan in a standardised growth inhibition test with the unicellular algae *Chlamydomonas reinhardtii*. Concentration Addition (CA) and Independent Action (IA) prediction models were used to predict the combined effects of these biocides, and determine whether synthetic mixtures of these caused additive, antagonistic or synergistic effects. Algal growth inhibition was studied at 3 different exposure durations (24 h, 48 h and 72 h) to evaluate the effect of exposure time on single and combined toxicity of these chemicals. The photosynthesis disruptors bifenox (EC_{50}= 10-18 nM) and metribuzin (EC_{50}= 54-66 nM) were the most toxic biocides to *C. reinhardtii*. While bifenox is known for inhibiting chlorophyll synthesis, metribuzin inhibits the photosystem II (PSII). Dichlofluanid, an inhibitor of thiol-containing enzymes and a mitochondrial disruptor, was the 3rd most toxic compound (EC_{50}= 76-113 nM). The carotenoids biosynthesis disruptor aclonifen was less toxic (EC_{50}= 294-429 nM), whereas the more generally acting triclosan (EC_{50}= 638-1804 nM) was the least toxic compound of the ones tested. The IA model best described the combined effects of these compounds, demonstrating that the combined toxicity occurred by additivity but mediated through

dissimilar Modes of Action (MoA). Potential antagonism was observed for low to median effect levels after short exposure duration (typically 24h), although the reason for this temporal variance in combined toxicity was not identified. Initial risk assessment on basis of the present and reported effect data suggest that metribuzin, bifenox, dichlofluanid and triclosan represent a risk to algae when exposed alone, and that the combined toxicity may enhance the risk under ecologically relevant exposure scenarios.

Keywords: Biocides; Microalgae; Growth inhibition; Mixtures; Prediction models; Environmental risk assessment.

1. Introduction

Biocides are products extensively used to protect humans, animals, or physical objects against harmful organisms by the toxic action of their active substances (ECHA, 2014). Many are contaminants of emerging concern with few published health standards, guidelines, and insufficient toxicity information (Brack, 2012). These compounds are regulated in the European Union, namely by the Regulation N° 528/2012 of the European Parliament and of the Council related with their availability on the market and use (EU, 2012). Low concentrations of several biocides (often in the ng per litre range) have already been detected in aquatic environments in the vicinity of agriculture and urban areas (Wittmer et al., 2010). These compounds are normally applied in combination to potentiate the effect of each other, and synergistic interactions of pesticides (e.g., for organophosphate and carbamate insecticides, azole fungicides, triazine herbicides and pyrethroid insecticides) have already been observed in organisms such as microalgae, phytoplankton, bacteria and crustaceans (Férnandez-Alba et al., 2002; Gatidou and Thomaidis, 2007; Cedergreen, 2014). The environmental risks of biocide mixtures are still poorly understood, as well as how the mixtures interact with the biological targets and cause effects at the organism level. This applies in particular to several biocides that already exist as mixture formulations in market products and also due to their co-occurrence in agricultural, marine and freshwater recipients as complex mixtures (Belden et al., 2007; Cedergreen, 2014; Pedersen et al., 2014). A new EFSA guidance document (EFSA, 2013) which provides information of how to perform a tiered risk assessment for active ingredients in formulations has been developed (EFSA, 2013), but no regulatory framework is effectively in place to assess the risk of environmental mixtures of active ingredients from different products and formulations.

It is already well documented that compounds present in a complex mixture can act either by additivity, synergism (more than additivity), or antagonism (less than additivity). The combined effect of contaminants can be characterized by prediction models such as concentration addition (CA; Loewe, 1927) for compounds with similar mode of action (MoA) and independent action (IA; Bliss, 1939) for compounds with dissimilar MoA. Both of these models are based on the assumption that compounds affect the same toxicity endpoint in the same manner (*i.e.*, the same trend), although the MoA of the chemicals may either be similar (CA model) or dissimilar (IA model). Deviations from these additivity predictions indicate interactions causing potentiation (synergy) or suppression (antagonism) of the response

monitored (Altenburger et al., 2003). Such deviations from additivity predictions (CA and IA) can be assessed by several methods including the use of model deviation ratio (MDR) that determine the ratio between the predicted and observed exposure concentration for a given effect level. A ratio within a factor of two ($0.5 \leq$ MDR≤ 2) is considered to be indicative of additivity as this is within the expected interlaboratory/inter-experimental variation for most species (Belden and Lydy, 2006).

Combined toxicity of biocides has frequently been demonstrated to occur by additivity when effects have been assessed at the whole organism level using endpoints such as inhibition of growth (Altenburger et al., 2003; Petersen et al., 2014). However, other studies have also demonstrated synergistic effects after exposure to pesticide mixtures in algae monitoring growth (Belden et al., 2007; Cedergreen, 2014). Although a high number of biocides exist, some have been identified as being of high priority as they display high toxicity to primary producers, are considered contaminants of emerging concern, and have an ubiquitous presence in surface waters (EU, 2013; USEPA, 2008). These chemicals display a number of different MoAs due to their function as pesticides but also due to previously non-characterised MoA in non-target species. In-depth knowledge of the MoA of biocides is thus often considered key to understand how these chemicals interact with their biological targets and how these interactions may give rise to combined toxicity. Herbicides such as aclonifen and bifenox, which have been proposed as priority aquatic substances by the European Union (EU, 2013) both affect the photosystem function in primary producers. Whereas aclonifen inhibits carotenoid and chlorophyll biosynthesis (Kilinc et al., 2011), bifenox is known to inhibit specific enzymes in the chloroplasts causing inhibition of chlorophyll synthesis (Grossman, 2005). Other biocides such as dichlofluanid (antifoulant, fungicide, acaricide, wood preservative, etc.) display multiple MoAs, including the inhibition of thiol-containing enzymes and disruption of mitochondrial function in various organisms (Cima et al., 2008). Other herbicides such as metribuzin are known to cause toxicity to primary producers by specifically interfering with the electron transport in the photosynthesis pathway (Fairchild et al., 1998). Triclosan, an ubiquitous contaminant widely used in Personal Care Products (PCPs) and as a wood preservative, bactericide and fungicide (USEPA, 2008), is known to affect multiple target sites in a cell and thus reported to be toxic to a number of organisms (von der Ohe et al., 2012).

The well-established mixture toxicity concept can provide a tiered framework for environmental risk assessment of mixtures. According to Backhaus and Faust (2012), CA should be applied as a precautious first tier regardless of the MoA of the mixture components. If a mixture behaves by additivity and the sized of the concerned toxic unit is constant, any mixture component can be exchanged by another chemical without changing the overall mixture toxicity. Therefore, the calculation of a risk quotient (RQ) and toxic unit (TU) for mixtures can be extrapolated from single substances to chemical mixtures by means of CA. Then, the risk quotient for the mixture (RQ_{STU}) can be calculated based on the sum of toxic units for the most sensitive trophic level (*i.e.*, with the highest STU). This approach is considered a solid conceptual basis for mixture risk assessment (Backhaus and Faust, 2012; Backhaus and Karlsson, 2014).

Algal toxicity tests have been extensively used for assessing the toxicity of a number of emerging compounds including biocides such as herbicides, pesticides and antifoulants (Cedergreen and Streibig, 2005). Among the most commonly used algae species, the unicellular green algae *Chlamydomonas reinhardtii* has been identified as one of the most sensitive species to biocide exposures (Chalew and Halden, 2009). This algae species grows rapidly and attains logarithmic growth in 3 days, is easily maintained in controlled laboratory conditions and is sensitive to a number of contaminants. This organism is about 10 µm in diameter, swims by means of two flagella, is easy to collect and identify (Harris, 2009). It has a specific carbon-concentrating mechanism and has been used in various mechanistic studies due to a well-known biology and sequenced genome (Merchant et al., 2007). It has already been used to assess the toxicity of numerous single herbicides, complex mixtures and is also used in biomonitoring studies due to its capacity to accumulate contaminants (Prado et al., 2009; Torres et al., 2008).

The objective of this study was to characterise the single and combined effects of the five ecologically relevant biocides, aclonifen, bifenox, dichlofluanid, metribuzin, and triclosan, on the growth of *C. reinhardtii*. Combined toxicity assessment was conducted by the CA and IA prediction models to differentiate between additivity and interactions that can lead to antagonism or synergy when present in mixtures. The obtained results were also used to determine the potential environmental risk of the tested biocides using classical risk assessment for single compounds and mixtures.

2. Material and Methods

2.1. Test compounds and standards

The test compounds aclonifen (CAS number: 74070-46-5), bifenox (CAS number: 42576-02-3), dichlofluanid (CAS number: 1085-98-9), metribuzin (CAS number: 21087-64-9), triclosan (CAS number: 3380-34-5) and 3,5-dichlorophenol (3,5-DCP; positive control; CAS number: 591-35-5) were all purchased from Sigma-Aldrich (United Kingdom) with ≥ 97.0% purity. Dimethylsulphoxide (DMSO, Sigma-Aldrich, United Kingdom, purity ≥ 99%) was used as solvent for all compounds. The compounds were stored in DMSO at -20 °C until use.

2.2. Algae growth inhibition test

Experiments were performed using cultures of freshwater green algae *C. reinhardtii* (NIVA-CHL153; Norwegian Institute for Water Research, Oslo, Norway). The algal growth inhibition tests were made according to the OECD Guideline 201 (OECD, 2011). In summary, algal cells were cultured in glass flasks with 50 ml of Talaquil media (Szivák et al., 2009), prepared at least 24 h prior usage to allow equilibrium of components. With 10^4 cells/ml as the initial number of cells, the flasks were incubated at 20±2°C in continuous light (83±6 μmol/m^2/s^1, Philips TLD 36W/950, London, UK) with orbital shaking (90 rpm) in an Infors Multitron 2 incubator shaker (Infors AG, Bottmingen, Switzerland). After achieving an exponential growth (72 h), algae cells from the cultures were used to inoculate sub-cultures that were exposed to the control (0.01% v/v DMSO), positive control (3,5-DCP), test compounds and mixtures (consult Table 1 on supplementary data for details on used concentrations). The exposed cultures were also made in 50 ml of Talaquil and maintained in the same conditions as the algal stock cultures. The exposed algae where incubated for 72 h and growth was monitored at 24 h, 48 h and 72 h by a multisizer counter (Beckman-Coulter Multisizer 3 Coulter Counter; Miami, FL, USA). The average growth rate (μ) for each test concentration was calculated from the initial cell concentration and cell concentration at the time of the last cell count using the equation:

$$\mu_{n-0} = \frac{\ln(N_n) - \ln(N_0)}{t_n - t_0} \times 24 \ (day^{-1}) \qquad \textbf{Eq. 1}$$

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

At least three independent experiments with triplicates were made for each chemical and mixture (exposure concentrations in Table 1 from supplementary data). All flasks and glassware used for media preparation and experiments were autoclaved before usage to avoid any microbial contamination. Culture samples were checked microscopically to detect the presence of any microbial contamination.

2.3. Single toxicity assessment

The results were modelled using a sigmoidal concentration-response curve (CRC) with variable slope:

$$\gamma = Bottom + \frac{Top - Bottom}{1 + 10^{(\log EC_{50} - \log X) \times Slope)}} \qquad \textbf{Eq. 2}$$

Where Y is the effect, X is the concentration, Bottom is the baseline effect (control), top is the maximal effect plateau (full growth inhibition), and log EC_{50} is the concentration causing 50% effect.

Concentration-response analyses were made in the same way for all individual toxicants and for the mixture, and the EC_{50}, Hill slope and goodness of fit (R^2) where calculated for each.

2.4. Combined toxicity assessment

A mixture of the five compounds was designed based on the CA prediction model (Eq. 3). The resulting effective concentrations of the mixtures were predicted as:

$$ECx_{mix} = \left(\sum_{i=1}^{n} \frac{p_i}{ECx_i}\right)^{-1} \qquad \textbf{Eq. 3}$$

Where the ECx_{mix} is the total predicted effect concentration of the mixture that induces an effect x, p_i is the relative fraction of component *i* in the mixture and ECx_x is the concentration of substance *i* inducing an effect *x* when exposed alone (Backhaus et al., 2010).

A fixed ratio ray design based on the ratios at the EC_{50} concentrations from the individual CRCs after 72 h exposure was used. An equitoxic mixture was chosen to avoid that only one or two of the compounds were dominating the response (consult Table 1 in supplementary data for information on the concentration of each compound in the mixture). The observed effects were modelled using a sigmoidal dose-response curve with variable slope (Eq. 2). The resulting CRC for the mixture was compared to both the CA prediction and the IA prediction model (Eq. 4).

The effect of a mixture of dissimilarly acting compounds were predicted by the IA model:

$$E_{mix} = 1 - \prod_{i=1}^{n}(1 - E_i) \qquad \textbf{Eq. 4}$$

Where E_{mix} is the effect of a mixture of *n* compounds and E_i is the effect of substance *I* when exposed alone (Bliss, 1939; Altenburger et al., 2003).

Additive effects were assumed to occur if no significant differences were detected between the observed effect concentrations and those predicted by the CA and IA models, being the MoA of the compounds considered as similar or dissimilar, respectively (see statistical methods for details). Model deviation ratios (MDRs) were also used to help in this detection, being the effects of the compounds considered as additive when MDRs were within a factor of 2 (0.5≤ MDR≤ 2; Belden et al., 2007). The MDR values were calculated by:

$$MDR = \frac{ECx_{pred}}{ECx_{obs}} \qquad \textbf{Eq. 5}$$

Where ECx_{pred} is the predicted effect concentrations and ECx_{obs} the observed effect concentrations (Belden and Lydy, 2006).

2.5. Initial environmental risk assessment

The potential environmental risk of each biocide was based on calculation of the risk quotient (RQ) for each compound, *i.e.*, the ratio between the expected exposures and the risk of the compound as described by EPA guidelines (EPA, 2004):

$$RQ = \frac{MEC}{NOEC} \qquad \textbf{Eq. 6}$$

Being MEC the maximum Measured Environmental Concentrations reported in literature, and NOEC the No Observed Effect Concentrations (values from the 72 h growth inhibition test in *C. reinhardtii*).

The RQ for the mixture was extrapolated from single substances by means of CA. The sum of RQ (SRQ) was calculated to evaluate the potential cumulative risk as (Backhaus and Faust, 2012; Backhaus and Karlsson, 2014):

$$SRQ = \sum_{i=1}^{n} RQ_i \qquad \textbf{Eq. 7}$$

A value larger or equal to 1 of RQ or SQR was interpreted as a potential environmental risk (Backhaus and Faust, 2012; Backhaus and Karlsson, 2014).

The potential environmental risk of the five individual biocides was based on calculation of toxic units (TU) of the biocides for *C. reinhardtii* (Backhaus and Faust, 2012; Backhaus and Karlsson, 2014; EU, 2009):

$$TU = \frac{MEC}{EC_{50}} \qquad \textbf{Eq.8}$$

Using the Measured Environmental Concentrations (MEC) reported in the literature, and the EC_{50} values for 72 h growth inhibition in *C. reinhardtii*. A TU of 1 indicated that the MEC was expected to cause a 50% effect on the growth inhibition on the growth inhibition in the respective species used for derivation of the EC_{50}.

The toxicity of the mixture was then described by the sum of all individual TUs of the present compounds (Backhaus and Faust, 2012; EU, 2009):

$$STU = \sum_{i=1}^{n} TU_i \qquad \textbf{Eq. 9}$$

A value larger or equal to 1 of TU or STU was interpreted as a potential environmental risk for the analysed mixture (Backhaus and Faust, 2012).

The overall risk quotient for the mixture (RQ_{STU}) to algae (the organism group that is regarded as the most sensitive to the mixture) was calculated according to Backhaus and Faust (2012):

$RQ_{STU} = max\ (STU_{algae})\ x\ AF$ **Eq. 10**

Being max (STU algae) the sum of the TU for algae and AF the assessment factor of 100 (EU, 2009). Currently there is no guideline for how to determine the AF for calculating RQ_{STU}, so this values was chosen according to literature (EU, 2003; Petersen et al., 2013).

2.6. Chemical analysis

Stock standard solutions were diluted in dichloromethane for the confirmatory analysis of bifenox, dichlofluanid, triclosan and metribuzin by gas chromatography-high resolution mass spectrometry (GCT-Premier, Waters Corp, Milford MA, USA). The analytes were separated on a 30 m × 0.25 mm, 0.25 ìm film thickness DB-5MS column (Agilent Technologies) with helium carrier gas. Splitless injection at 250 °C was used. The initial temperature of 60 °C was held for 2 min, followed by an increase of 5 °C/min to 310 °C and held for 5 min. The m/z used for quantification were dichlofluanid, 123.0142+223.9219; metribuzin, 214.088; bifenox, 340.986, and triclosan, 287.951+218.0145. The results of the chemical analysis on the stock standard solutions (Table 2 in supplementary data) confirmed the concentrations of the stock solutions used for the exposures, except for aclonifen. The method was not sufficiently robust for the analysis of this compound and data was not reproducible. The nominal exposure concentrations of each compound on the equipotent mixture were also confirmed using the same method (Table 3 in supplementary data). As the measured concentrations did not surpass a ±20% of the nominal concentrations, these concentrations were used herein.

2.7. Statistical analysis

The non-linear regressions using a sigmoidal dose-response curve with variable slope were modelled in GraphPad Prim 6 software (GraphPad Software Inc., La Jolla, CA, USA). The same software was used to compare the significant differences between the effect levels for the experimental data with those calculated by the CA and IA prediction models (Motulsky, 1998; EPA, 2006). As data was normally distributed and displaying a homogeneous variance, a parametric one-way ANOVA was used in combination with the Tukey test for multiple comparisons. A *p*-value of 0.05 was considered significant.

3. Results

3.1. Single biocide exposure

The tested biocides reduced the growth of *C. reinhardtii* in a concentration-dependent manner to less than 50% of the control (Table 1; Fig. 1). The solvent DMSO did not cause any effects on its own at the concentrations used (results not shown). The positive control 3,5-DCP displayed a high-quality CRC ($R^2 \geq 0.95$) with EC_{50} values in the µM range (supplementary data Fig. 1; Table 1).

The applied non-linear regression fitted well the observed growth inhibition data for all the tested compounds (Table 1). At 24 h exposure, R^2 values were $\geq$ 0.89 for all compounds except dichlofluanid ($R^2 = 0.68$). At 48 h exposure, R^2 values were high for all compounds ($R^2 \geq 0.94$), whereas the highest R^2 values were obtained at 72 h ($R^2 \geq 0.97$). The order of potency was: bifenox> metribuzin> dichlofluanid> aclonifen> triclosan. Bifenox displayed the highest toxicity at 24 h exposure (EC_{50}= 10 nM), while triclosan was the least toxic after 72 h exposure (EC_{50}= 1804 nM). The toxicity of dichlofluanid, bifenox and triclosan decreased with exposure time, from 24 h to 72 h exposure. The toxicity of metribuzin and aclonifen increased until 48 h, achieving stability after this time-point until the end of exposure. The slopes of the CRCs were also different between biocides (Table 1). The shallowest slopes were obtained for aclonifen at all time-points, while dichlofluanid showed the steepest at 48 h and 72 h.

3.2. Combined effects

The data obtained for the growth inhibition with mixture of the five biocides was well described by the applied non-linear regression analysis ($R^2 \geq 0.94$) for all time points (Fig. 2; Table 2). At 24 h exposure, the effect of the mixture was less than additive at low to median effect levels (growth rate >50%; Table 2). For higher effect levels (≥50% inhibition of growth, corresponding to a growth rate ≤50%), the mixture was well predicted by the IA prediction model (Table 2). At concentrations causing ≥60% growth inhibition (Table 2), the combined toxicity was also well-predicted by the CA model. At 48 h the IA model described the obtained data best except at the two lowest effect levels (5% and 10% growth inhibition), where lower than additive effects were observed (Table 2). The mixture was also well predicted by the CA model at concentrations causing a ≥80% growth inhibition at 48h (Table 2). At 72 h the tested mixture was best predicted by the IA model at all effects levels (Table

2), with CA only predicting the effects for the three highest effect levels (≥80% inhibition of growth). Overall, the IA model best predicted the effects of the biocide mixture on the growth of *C. reinhardtii*, and in particular at longer exposure durations (≥48 h). Deviations from additivity, being indicative of antagonism, were mainly occurring at low (24 h and 48 h exposure) to medium (24 h exposure) concentrations. The two different approaches to identify deviations from additivity predictions (*i.e.*, statistical differences and MDRs) gave fairly similar results (Table 2). Details about the statistical analysis have been provided in supplementary Table 4.

3.3. Environmental risk assessment

The RQs and TUs calculated for the five biocides are on Table 3. The MEC values were based on published data for freshwater environments (Table 5 in supplementary data). Bifenox presented a risk for *C. reinhardtii* only when present at high environmental concentrations (MEC_{95} and MEC_{max}) and just when considering the RQ. Dichlofluanid presented a risk for *C. reinhardtii* at all concentrations for both RQ and TU. However, for this biocide the MEC values are for a common degradation product of both dichlofluanid and tolylfluanid, *N,N*-dimethylsulfamide (DMS), adding uncertainty to the calculated risk. Metribuzin and triclosan showed a risk when present at high environmental concentrations (MEC_{95} and MEC_{max}), when considering both TU and RQ. Triclosan presented a risk for *C. reinhardtii* only when present at high environmental concentrations (MEC_{95} and MEC_{max}) for both RQ and TU. Therefore, results indicate a potential environmental risk for bifenox, metribuzin, triclosan and possibly dichlofluanid towards *C. reinhardtii*, mostly at high MECs. The order of potential environmental risk for *C. reinhardtii* was the same when considering either RQ or TU: metribuzin> dichlofluanid> triclosan> bifenox> aclonifen.

The SRQ, STU and RQ_{STU} indicate that the analysed mixture with the five biocides can represent a potential environmental risk for *C. reinhardtii* (Table 3).

4. Discussion

4.1. Effects of single compounds

The five tested biocides reduced the growth of *C. reinhardtii* in a concentration-dependent manner. All compounds had EC_{50} levels in the nM range, except aclonifen at 24 h and triclosan at all time-points of the study, which were toxic at 1 to 2 orders of magnitude higher

than the others. Bifenox and metribuzin were the most toxic chemicals and affected the growth of *C. reinhardtii* in the same order of magnitude. The MoA of the different studied compounds and their role as biocides (such as the fact that herbicides are the most potent towards primary producers as algae) may explain the differences in potency.

Bifenox acts by cellular membrane disruption and inhibition of photosynthesis (EFSA, 2007). It is known to inhibit the protoporphyrinogen oxidase (Protox), an enzyme that catalyses the last common step in chlorophyll and heme synthesis, causing the formation of oxygen radicals in the presence of light (Grossman, 2005). Based on the available acute toxicity data, the European Food Safety Authority has classified bifenox as very toxic to aquatic organisms (EFSA, 2007). A 96h EC_{50} (static exposure) of 0.5 nM has been reported for *Desmodesmus subspicatus* when measured as reduction of algal biomass and 0.6 nM when assessed as changes to the growth rate (EFSA, 2007). This is an order of magnitude lower than the EC_{50} values observed in this study (EC_{50}= 10–18 nM), indicating that bifenox may display species-specific toxicity. Bifenox' toxicity decreased slightly over time, possible due to the fact that it is readily degradable by phototransformation into less toxic metabolites such as 2,4-dichlorophenol (EFSA, 2007).

Metribuzin is toxic to plants by the inhibition of the photosynthetic electron transport at the photosystem II receptor site (EFSA, 2010). Based on the available acute toxicity data, metribuzin is classified as very toxic to aquatic organisms, including to green algae (EFSA, 2010). An EC_{50} of 93 nM has been reported for *Scenedesmus subspicatus*, slightly higher but in the same order of magnitude as that obtained in the present study for *C. reinhardtii* (EC_{50}= 54-66 nM). The toxicity of this compound remained fairly stable over the 72 h exposure period, likely due to its high stability the lack of degradation (EFSA, 2010).

Dichlofluanid is considered to be to be very toxic to aquatic organisms (EPA, 2012). Among the tested biocides, dichlofluanid was the 3rd most toxic compound to *C. reinhardtii*. This biocide is known to inhibit thiol-containing enzymes by forming disulphide bridges, and to stimulate Ca^{2+} efflux from mitochondria in primary producers (Johansson et al., 2012). Previous studies have reported a 72 h EC_{50} of 390 nM for *Selenastrum capricornutum* (Fernández-Alba et al., 2002), which is up to 3 times less toxic than the toxicity observed in the present study for *C. reinhardtii* (EC_{50}= 76-113 nM). Although not studied in detail, *C. reinhardtii* seems to be one of the most sensitive algae to this biocide. As seen for bifenox, the

toxicity of dichlofluanid decreased over time, a finding that agrees with reports of rapid degradation in the aquatic environment (EPA, 2012). Its main hydrolysis metabolite, dimercaptosuccinic acid (DMSA), is also biodegradable and has low toxicity to aquatic organisms (EPA, 2012).

Aclonifen is considered to be very persistent in aquatic environment, displaying a high potential for bioaccumulation (ECHA, 2011) and may therefore be of environmental concern. The present data show that this biocide was less toxic than bifenox, metribuzin and dichlofluanid with an EC_{50} in the high nanomolar range (EC_{50}= 294-429 nM). This compound also affects photosynthesis by inhibiting the biosynthesis of carotenoids, and as bifenox, specifically target protoporphyrinogen oxidase synthesis that is a key for successful conversion of α-amino-levulinic acid to chlorophyll (Kilinc et al., 2011).

Triclosan was the least toxic compound of the ones tested. The EC_{50} was an order of magnitude higher than those observed for the other compounds. This is not surprising as all the other compounds are specifically acting herbicides or fungicides, while triclosan is a multi-purpose personal care product commonly used as an antibacterial agent and preservative (von der Ohe et al., 2012). Triclosan is known to exhibit multiple toxic MoAs, including uncoupling of oxidative phosphorylation and inhibition of non-photochemical quenching, a mechanism that is used to dispose of excess energy when the light energy absorption exceeds the capacity for photosynthesis (Franz et al., 2008). Lack of energy disposal by chemicals such as triclosan can lead to damage in the pigments where non-photochemical quenching occurs (Franz et al., 2008). The observation that triclosan toxicity decreased over time in the present study is in agreement with reports that triclosan biodegrade in water (Singer et al., 2002) and is susceptible to biotransformation in algae (Orvos et al., 2002). Freshwater unicellular algae are generally more sensitive to triclosan than other taxonomic groups of unicellular green algae (Orvos et al., 2002). For example, the algae *S. subspicatus* and *S. capricornutum* showed high susceptibility to triclosan, with EC_{50} values in the range of 5–66 nM (Orvos et al., 2002), a toxic potency about one order of magnitude higher than that found for *C. reinhardtii* in the present study (EC_{50}= 638–1804 nM). Other studies have also reported as much as two orders of magnitude differences in the sensitivity of microalgae to triclosan (Orvos et al., 2002; Franz et al., 2008). This discrepancy is probably due to interspecies differences in target site susceptibility and differential toxicokinetics in different algae species (Franz et al., 2008).

4.2. Combined effects

The study on the combined effects of the five biocides showed that the mixture predominantly caused additive effects on the growth inhibition of *C. reinhardtii*. At 48 h and 72 h, the IA model best estimated the mixture effect at almost all effect levels. The best fit of IA indicates that the selected compounds likely displayed dissimilar MoA, following the principles of independent action (Bliss, 1939; Altenburger et al., 2003). Therefore, the biocides present in the studied mixture were expected to interact with different molecular targets and display different MoAs. The results are in agreement with previous studies on combined toxicity of biocides to aquatic organisms (Belden et al., 2007), including algae (Faust et al., 2003) where the IA model best estimated the mixtures of compounds with different MoA. Most of the tested compounds, especially the herbicides, are known to ultimately affect photosynthesis therefore expected to be highly toxic also to algae. While herbicides are known to interact specifically with key molecular targets in primary producers such as algae, antifoulants such as dichlofluanid and fungicides as triclosan normally display a more general toxic MoA and may affect a wider range of organisms (Cedergreen, 2014).

Although the potential for synergistic effects is often considered the greatest concern for complex mixtures, the potential for additivity and antagonism is of equal importance to decipher how ecological relevant mixtures cause combined toxicity. A recent review of combined effects of pesticides report that additive effects were obtained for 88 % of the investigated mixtures, while 5% were synergistic and 5% antagonistic. In some of these studies, the CA model also tended to over-predict toxicity (Belden et al., 2007; Cedergreen, 2014) as observed in the present study. For those studies, as it seems to be the case in the analysed mixture, the effects of the compounds were additive leading to the inhibition of growth, but by independent ways. Their specific toxic effects in the exposed algae were not the same, meaning that each biocides particular effect (*i.e.*, MoA) did not affect the other (Altenburger et al., 2003), but they all ultimately contributed for the same general apical effect, the inhibition of algal growth.

At low to median effect levels for the 24 h exposure, effects lower than additive indicating antagonism were verified. Antagonism is frequently observed for mixtures of herbicides and although not studied in detail herein, some general causes have been suggested (Cedergreen et al., 2007). Chemical interactions between the chemicals themselves may reduce the activity of the single compounds in a mixture (Wehtje et al., 1991; Richter and Escher, 2005), as the

presence of certain biocides has been reported to affect the uptake and metabolism rates of other compounds, affecting for instance the activity of certain enzymes (Ottis et al, 2005; Wehtje et al., 1992). Also, more "universal" biocides (such as dichlofluanid and triclosan) can affect the transport of more specific and quickly acting biocides (such as herbicides aclonifen, bifenox and metribuzin), decreasing the metabolisation rate and slowing the transport processes of the more active compounds (Scherder et al., 2005). However, the ultimate outcome of a given mixture is the sum of all the possible interactions that propagate their effects on growth and further studies are needed to clarify which interaction(s) are causing antagonism in this particular case. It is also important to recognize that the antagonism observed in the present study was only occurring after short exposure times (≤48h) and at low to medium effect concentrations. Therefore, this might also be due to the interval of time needed for all the toxic effects to combine and ultimately affect the growth of algae, a general toxic endpoint indicative of the overall health status of the exposed organisms.

4.3. Potential environmental risk

Algal growth is a chronic endpoint reflecting successful reproduction and normal population recruitment. Pollutants inducing inhibition of algal growth, either by exposure to single chemicals or complex mixtures, are considered ecologically relevant and a trigger for environmental concern as changes may interfere with normal population trajectories. Algae, which are central in ecosystem functions by being the basis of the aquatic food web and important in carbon fixation (Harris, 2009), may play an important role also in propagation of toxic effects to higher organisational levels.

The prediction of environmental risk is normally based upon comparison between ecologically-relevant exposure concentrations and the concentrations required to cause toxicity to one or more species (Backhaus and Faust, 2012; Backhaus and Karlsson, 2014). The MEC values (minimum, median, 95 quartile and maximum) selected for this study were calculated from values found in literature for freshwater. No studies were found regarding the concentrations of dichlofluanid in freshwater, as this compound has very low solubility and is instable in water. However, DMS, a common degradation product of dichlofluanid and tolylfluanid, has been frequently detected in quantities above the European water quality standards (Loos et al., 2010; Langford et al., 2012), and was used as instead of dichlofluanid.

Using these MEC values, two approaches were used to analyse the environmental risk of the studied compounds, calculating both RQs and TUs for single compounds and mixture. Both methods provided similar conclusions. A potential environmental risk was identified for metribuzin, triclosan, bifenox and possibly dichlofluanid towards *C. reinhardtii*, mostly at high environmental values. However, as the MECs used for dichlofluanid were based on the metabolite DMS, the actual environmental concentration of this compound is probably lower due to rapid degradation in the aquatic environment (EPA, 2012). Moreover, dichlofluanid is known to accumulate in sediments and to be moderately bioavailable (Sakkas et al., 2006), thus indicating that the predicted environmental risk may be overestimated. The highest risk was identified for metribuzin, in accordance with published studies where this biocide was identified as main risk drivers for algae in agricultural streams (Petersen et al., 2013). Triclosan, despite its relatively low toxicity to *C. reinhardtii*, was still identified as having a potential environmental risk due to the high MECs reported.

The combined risk assessment indicated a potential cumulative risk for the studied mixture. As these five biocides showed to have mostly additive effects, their co-occurrence in the environment would increase the potential environmental risk according to the principles of additivity. The MECs used in the present study were measured in freshwater but in different water bodies and limited information is available regarding the co-occurrence of these biocides in the same location. However, in theory these compounds are likely to co-occur in surface waters with emissions from agricultural runoff (aclonifen, bifenox, metribuzin), municipal wastewater effluents (triclosan), and runoff waters from recreational boating activity and house painting (dichlofluanid). Moreover, not only these biocides but also other biocides and chemical compounds can occur in the same recipient, thus indicating a potential for combined effects beyond that demonstrated herein. More research and improved knowledge on co-occurrence of compounds with the potential to cause combined toxicity to algae is particularly important for risk assessment in order to identify undesired effects on non-target organisms (Backhaus and Faust, 2012; Cedergreen, 2014). In this respect, improved knowledge of how exposure to sub-lethal concentrations of these biocides affects freshwater population and communities in longer and more ecologically relevant studies are urgently needed.

5. Conclusions

The algal toxicity test with *Chlamydomonas reinhardtii* was used to assess the single and combined toxicity of five environmentally relevant biocides. Bifenox and metribuzin were the most toxic biocides, both affecting photosynthesis in different ways. While bifenox is known to inhibit chlorophyll synthesis, metribuzin inhibits the PSII. Dichlofluanid, known affect mitochondria function, was the third most toxic biocide of the ones tested. Aclonifen, reported to affect photosynthesis by inhibiting carotenoids biosynthesis, was less toxic. Triclosan, known to have a broad MoA with multiple molecular targets, was the least toxic compound. The combined effect on the growth of C. *reinhardtii* was characterised by applying CA and IA prediction models and identified that toxicity predominantly occurred by additivity. The combined effects were best estimated by the IA model, thus demonstrating that the different biocides caused toxicity in the algae by dissimilar MoAs. A potential antagonism was also identified after short-term exposure and at low to median effect concentrations, albeit the basis for this effect was not further evaluated. A potential environmental risk was identified metribuzin, triclosan, bifenox and possibly dichlofluanid. The combined risk assessment also indicated a potential cumulative risk for the studied mixture.

Conflict of interest

The authors declare that no conflicts of interest exist.

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References

Altenburger, R., Nendza, M., Schuurmann, G., 2003. Mixture toxicity and its modelling by quantitative structure–activity relationships. Environ. Toxicol. Chem. 22, 1900–1915.

Backhaus, T., Blanck, H., Faust, M., 2010. Risk and risk assessment of chemical mixtures under REACH. State of the art, gaps and options for improvement. Swedish Chemicals Agency, Sundbyberg.

Backhaus, T., Faust, M., 2012. Predictive environmental risk assessment of chemical mixtures: a conceptual framework. Environ. Sci. Technol. 46, 2564-2573.

Backhaus, T., Karlsson, M., 2014. Screening level mixture risk assessment of pharmaceticals in STP effluents. Water Res. 49, 157-165.

Barek, J., Cabalková, D., Fischer, J., Navrátil, T., Pecková, K., Yosypchuk, B., 2011. Voltammetric determination of the herbicide Bifenox in drinking and river water using a silver solid amalgam electrode. Environ. Chem. Lett 9, 83-86.

Battaglin, W.A., Furlong, E.T., Burkhardt, M.R., 2001. Concentrations of selected sulfonylurea, sulfonamide, and imidazolinone herbicides, other pesticides, and nutrients in 71 streams, 5 reservoir outflows, and 25 wells in the Midwestern Unites States, 1998. Water-Resources Investigations Resport 00-4225. U.S. Department of the Interior, U.S. Geological Survey, 131 p.

Belden, J.B., Lydy, M.J., 2006. Joint toxicity of chlorpyrifos and esfenvalerate to fathead minnows and midge larvae. Environ. Toxicol. Chem. SETAC 25, 623–629.

Belden, J.B., Gilliom, R.J., Lydy, M.J., 2007. How well can we predict the toxicity of pesticide mixtures to aquatic life? Int. Environ. Assess. Manage 3, 364–372.

Bliss, C.I., 1939. The toxicity of poisons applied jointly. Ann. J. Appl. Biol. 26, 585– 615.

Brack, 2012. Emerging substances of toxicological concern in a world full of chemicals – the NORMAN way to find the needles in the haystack. Norman Bulletin 3, 1-2.

Cedergreen, N., Streibig, J.C., 2005. The toxicity of herbicides to non-target aquatic plants and algae: assessment of predictive factors and risk. Pest. Manag. Sci. 61, 1152–1160.

Cedergreen, N., Kudsk, P., Matthiasen, S., Streibig, J.C., 2007. Combination effects of herbicides: Do species and test system matter? Pest. Manage. Sci. 63, 282–295.

Cedergreen, N., 2014. Quantifying Synergy: A Systematic Review of Mixture Toxicity Studies within Environmental Toxicology. PLOS ONE 9 (5), e96580.

Chalew, T.E.A., Halden, R.U., 2009. Environmental exposure of aquatic and terrestrial biota to triclosan and triclocarban. JAWRA 45 (1), 4-13.

Cima, F., Bragadin, M., Ballarin, L., 2008. Toxic effects of new antifouling compounds on tunicate haemocytes I. Sea-Nine 211^{TM} and chlorothalonil. Aquat. Toxicol. 86, 299-312.

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 L-2985 Luxembourg, 328p.

EC, 2006. Draft Assessment Report (DAR) - public version. Initial risk assessment by the rapporteur Member States Germany for the existing active substance aclonifen in the third stage (part A) of the review programme referred to in Article 8(2) of Council Directive 91/414/EEC.

EC, 2011. European Commission (DG Environment). Technical support for the impact assessment of the review of priority substances under Directive 2000/60/EC Substance assessment: aclonifen. Entec UK Limited.

ECHA, 2011. Annex 1 – Background document to the opinion proposing harmonised classification and labelling at Community level of Benzenamine, 2-chloro-6-nitro-3-phenoxy-

(Aclonifen). Committee for Risk Assessment. European Chemicals Agency. ECHA/RAC/ CLH-O-0000001543-79-03/A1, 51 p.

ECHA, 2014. Guidance on the biocidal products regulation. Volume II: efficacy. Part A: information requirements. European Chemicals Agency, Version 1.1, November 2014, 35 p.

EFSA, 2007. Conclusion on the peer review of bifenox. European Food Safety Authority Scientific Report 119, 1-84.

EFSA, 2010. Conclusion regarding the peer review of the pesticide risk assessment of the active substance metribuzin. European Food Safety Authority Scientific Report 88, 1-74.

EFSA, 2013. Guidance on the tiered risk assessment for plant protection products for aquatic organisms in edge-of-field surface waters. European Food Safety Authority Scientific Report 911(7) 3290, 1-268.

EPA, 2004. Overview of the Ecological Risk Assessment Process in the Office of Pesticide Programs: Endangered and Threatened Species Effects Determinations. U.S. Environmental Protection Agency. Office of Prevention, Pesticide, and Toxic Substances. 92 p.

EPA, 2006. Data quality assessment: statistical methods for practitioners. U.S. Environmental Protection Agency. EPA QA/G-9S, Office of Environmental Information, Washington DC, 198 p.

EPA, 2012. Antifouling paints reassessment. Preliminary risk assessment. Environmental Protection Agency. New Zealand Government, 71 p.

EU, 2009. Council of the European Union: Council conclusion on combination effects of chemicals, 2988th Environment Council Meeting, Brussels, 22 Dec. 2009.

EU, 2012. Regulation (EU) No 528/2012 of The European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products. Official Journal of the European Union L 167/1-L 167/123.

EU, 2013. Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy. Official Journal of the European Union L 226/1-L 226/16.

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. Environ. Toxicol. Chem. 17, 1830–1834.

Faust, M., Altenburger, R., Backhaus, T., Blanck, H., Boedeker, W., Gramatica, P.,Hamer, V., Scholze, M., Vighi, M., Grimme, L.H., 2003. Joint algal toxicity of 16 dissimilarly acting chemicals is predictable by the concept of independentaction. Aquat. Toxicol. 63 (1), 43–63.

Fernández-Alba, A.R., Hernando, M.D., Piedra, L., Christi, Y., 2002. Toxicity evaluation of single and mixed antifouling biocides measured with acute toxicity bioassays. Anal. Chim. Acta 456, 303-312.

Franz, S., Altenburger, R., Heilmeier, H., Schmitt-Jansen, M., 2008. What contributes to the sensivity of microalgae to triclosan? Aquat. Toxicol. 90, 102–108.

Gatidou, G., Thomaidis, N.S., 2007. Evaluation of single and joint toxic effects of two antifouling biocides, their main metabolites and copper using phytoplankton bioassays. Aquat. Toxicol. 85, 184–191.

Grossman, K., 2005. What it takes to get a herbicide's mode of action. Physionomics, a classical approach in a new complexion. Pest. Manag. Sci. 61 (5), 423-431.

Harris, E.H., 2009. The Chlamydomonas Sourcebook. Introduction to Chlamydomonas and its laboratory use. Volume 1, 435 p.

Hostovsky, M., Blahova, J., Plhalova, L., Kopriva, V., Svobodova, Z., 2014. Effects of the exposure of fish to triazine herbicides. Neuroendocrinol. Lett. 35, 3-25.

James, A., Bonnomet, V., Morin, A., Fribourg-Blanc, B., 2009. Implementation of requirements on Priority substances within the Context of the Water Framework Directive.

Contract N° 07010401/2008/508122/ADA/D2. Prioritisation process: Monitoring-based ranking. INERIS / IOW: 58.

Johansson, P., Eriksson, K.M., Axelsson, L., Blanck, H., 2012. Effects of seven antifouling compounds on photosynthesis and inorganic carbon use in sugar kelp *Saccharina latissima* (Linnaeus). Arch. Environ. Contam. Toxicol. 63 (3), 365-377.

Kilinc, Ö., Grasset, R., Reynaud, S., 2011. The herbicide aclonifen: the complex theoretical bases of sunflower tolerance. Pestic. Biochem. Physiol. 100, 193-198.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M, Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams 1999-2000: A national reconnaissance. Environ. Sci. Technol. 36, 1202-1211.

Kumar, Y.B., Singh, N., Singh, S.B., 2013. Removal of atrazine, metribuzin, metochlor and alachlor by granular carbon. J. Environ. Anal. Toxicol. 3, 7.

Langford, K.H., Beylich, B.A., Bæk, K., Fjeld, E., Kringstad, A., Høyfeldt, A., Øxnevad, S., Thomas, K.V., 2012. Screening of selected alkylphenolic compounds, biocides, rodenticides and current use pesticides. Statlig program for forurensningsovervåking, Klima- og Forurensnings- Direktoratet. Norwegian Institute for Water Research, Rapportnr. 1116/2012, TA 2899, p. 69.

Loewe, S., 1927. Die Mischarznei. Versuch einer allgemeinen pharmakologie der arzneikombinationen. Klin. Wochenschr 6, 1077-1085.

Loos, R., Locoro, G., Comero, S., Contini, S., Schwesig, D., Werres, F., Balsaa, P., Gans, O., Weiss, S., Blaha, L., Bolchi, M., Gawlik, B.M., 2010. Pan-European survey on the occurrence of selected polar organic persistent pollutants in ground water. Water Research 44, 4115-4126.

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., Maréchal-Drouard, L., Marshall, W.F., Qu, L.-H., Nelson, D.R., Sanderfoot, A.A., Spalding, M.H., Kapitonov, V.V., Ren, Q., Ferris, P.,

Lindquist, E., Shapiro, H., Lucas, S.M., Grimwood, J., Schumtz, J., Chlamydomonas annotation team, JGI Annottation Team, Grigoriev, I.V., Rokhsar, D.S., Grossman, A.R., 2007. The Chlamydomonas genome reveals the evolution of key animal and plant functions. Science 318, 245-251.

Motulsky, H., 1998. Comparing dose-response or kinetic curves with GraphPad Prism. In HMS Beagle: The BioMedNet Magazine, Issue 34, July 10, 13 p.

OECD, 2011. OECD Guidelines for the Testing of Chemicals, Section 2: Effects on Biotic Systems Test No. 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test. OECD Publishing, Business & Economics, 1-25.

Orvos, D.R., Versteg, D.J., Inauen, J., Capdevielle, M., Rothenstein, A., Cunningham, V., 2002. Aquatic toxicity of Triclosan. Environ. Toxicol. Chem., 21, 1338-1349.

Ottis, B.V., Mattice, J.D., Talbert, R.E., 2005. Determination of antagonism between cyhalofop-butyl and other rice (*Oryza sativa*) herbicides in barnyardgrass (*Echinochloa crus-galli*). J. Agric. Food Chem. 53, 4064–4068.

Pedersen, R., Bechmann, M., Deelstra, J., Eggestad, H.O., Greipsland, I., Stenrød, M., Fystro, G., Selnes, S., Riley, H., Stubhaug, E., 2014. Jord- og vannovervåking i landbruket (JOVA). Feltrapport fra programmet i 2012 (Bioforsk).

Petersen, K., Stenrød, M., Tollefsen, K.E., 2013. Initial environmental risk assessment of combined effects of plant protection products in six different areas in Norway. Norwegian Institute for Water Research, REPORT SNO. 6588-2013

Petersen, K., Heiaas, H.H., Tollefsen, K.E., 2014. Combined effects of pharmaceuticals, personal care products, biocides and organic contaminants on the growth of *Skeletonema pseudocostatum*. Aquat. Toxicol. 150, 45-54.

Prado, R., García, R., Rioboo, C., Herrero, C., Abalde, J., Cid, A., 2009. Comparison of the sensitivity of different toxicity test endpoints in a microalga exposed to the herbicide paraquat. Environ. Int. 35, 240-247.

Richter, M., Escher, B.I., 2005. Mixture toxicity of reactive chemicals by using two bacterial growth assays as indicators of protein and DNA damage. Environ. Sci. Technol. 39, 8753 – 8761.

Sakkas, V.A., Konstantinou, I.K., Albanis, T.A., 2006. Photochemical fate of organic booster biocides in the aquatic environment, in: Konstantinou, I. (Ed.), Antifouling paint biocides. 5. The handbook of environmental chemistry. Springer, New York, 171–200.

Scherder, E.E., Talbert, R.E., Lovelace, M.L., 2005. Antagonism of cyhalofop grass activity by halosulfuron, triclopyr, and propanil. Weed Technol. 19, 934–941.

Singer, H., Muller, S., Tixier, C., Pillonel, L., 2002. Triclosan: occurrence and fate of a widely used biocide in the aquatic environment. Field measurements in wastewater treatment plants, surface waters, and lake sediments. Environ. Sci. Technol. 36, 4998–5004.

Szívak, I., Behra, R., Sigg, L., 2009. Metal-indiced reactive oxygen species production in *Chlamydomonas reinhardtii* (Chlorophyceae). J. Phycol. 45, 427–435.

Torres, M.A., Barros, M.P., Campos, S.C.G., Pinto, E., Rajamani, S., Sayre, R.T., Colepicolo, P., 2008. Biochemical biomarkers in algae and marine pollution: a review. Ecotoxicol. Environ. Saf. 71 (1), 1-12.

USEPA, 2008. Ecological risk and environmental revised risk assessment chapter triclosan (pc code: 054901; case no.: 2340). United States Environmental Protection Agency. Washington, d.c. 20460. Office of prevention, pesticides and toxic substances, 33 p.

von der Ohe, P.C., Schmitt-Jansen, M., Slobodnik, J., Brack, W., 2012. Triclosan – the forgotten priority substance? Environ. Sci. Pollut. Res. 19, 585–591.

Wehtje, G.R, Wilcut, J.W., Dylewski, D.P., McGuire, J.A., Hicks, T.V., 1991. Antagonism of paraquat phytotoxicity in peanuts (*Arachis-Hypogaea*) and selected weed species by naptalam. Weed Sci. 39, 634–639.

Wehtje, G., Wilcut, J.W., McGuire, J.A., 1992. Paraquat phytotoxicity, absorption and

translocation in peanut and selected weeds as influenced by cloramben. Weed Sci. 40, 471–476.

Wind, T., Werner, U., Jacob, M., Hauk, A., 2004. Environmental concentrations of boron, LAS, EDTA, NTA and Triclosan simulated with GREAT-ER in the River Utter. Chemosphere 54, 1135-1144.

Wittmer, I.K., Bader, H.-P., Scheidegger, R., Singer, H., Lück, A., Hanke, I., Carlsson, C., Stamm, C., 2010. Significance or urban and agricultural land use for biocide and pesticide dynamics in surface waters. Water Res. 44 (9), 2850-2862.

Figure Captions

Fig. 1. Growth rate (% of control, CT - control) of *Chlamydomonas reinhardtii* exposed to the biocides aclonifen, bifenox, dichlofluanid, metribuzin, and triclosan for 24 h, 48 h and 72 h (solid symbols). The data (Mean±SEM) represent experimental results from 3 independent experiments and their corresponding concentration-response curves (solid line).

Fig. 2. Growth rate (% of control, CT - control) for *Chlamydomonas reinhardtii* exposed to a equipotent mixture of aclonifen, bifenox, dichlofluanid, metribuzin and triclosan for 24 h, 48 h and 72 h (solid circles). The data (Mean±SEM) represent experimental results from 3 independent experiments and their corresponding concentration-response curves (dotted line). Prediction of combined toxicity by CA (solid line) and IA (broken line) of the mixture is displayed to indicate assumptions of additivity.

Table 1. Inhibition of *Chlamydomonas reinhardtii* growth after exposure to the 5 biocides aclonifen, bifenox, dichlofluanid, metribuzin, triclosan and to the assay positive control 3,5-diclorophenol. The data show the EC_{50} (nM; 95% confidence intervals in parentheses), Hill slope and goodness of fit (R^2) of the growth inhibition concentration-response curves at each time point (24 h, 48 h and 72 h) for each compound.

Compound	**EC_{50} (nM)**			**Hill slope**			**Goodness of fit (R^2)**		
	24 h	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**
Aclonifen	429 (316 to 582)	294 (268 to 324)	298 (268 to 331)	-0.7	-1.1	-1.5	0.97	1.00	1.00
Bifenox	10 (7 to 13)	14 (12 to 17)	18 (16 to 19)	-2.0	-2.6	-2.4	0.94	0.96	0.99
Dichlofluanid	76 (29 to 203)	96 (88 to 105)	113 (120 to 115)	-1.9	-6.8	-6.8	0.68	0.98	1.00
Metribuzin	66 (47 to 92)	54 (48 to 61)	57 (52 to 62)	-1.7	-2.2	-2.4	0.96	0.99	1.00
Triclosan	638 (420 to 970)	1111 (868 to 1421)	1804 (1418 to 2295)	-1.7	-1.6	-1.7	0.89	0.94	0.97
3,5-Dichlorophenol*	9386 (8764 to 10053)	7515 (7358 to 7675)	8577 (8445 to 8711)	-2.9	-3.9	-4.5	0.95	1.00	1.00

*Positive control

Table 2. Inhibition of *Chlamydomonas reinhardtii* growth after exposure to the mixture of the five biocides. The data show the EC_{50} (nM; 95% confidence intervals of EC_{50} between parentheses), Hill slope and goodness of fit (R^2) for the experimental data and the corresponding CA and IA models, and model deviation ratios (MDRs) for each model at each time-point. The different effect levels (growth rate) for the mixture are shown for each model at each time point (24 h, 48 h and 72 h). Note: bold text indicates that MDRs were within a factor of two and * indicates that the model predictions were not significantly different from the observed data.

		24 h		**48 h**		**72 h**	
Experiment	**EC_{50} (nM)**	900 (701 to 1156)		1161 (1040 to 1295)		1282 (1141 to 1441)	
	Hill slope	-4.2		-5.8		-5.8	
	Goodness of fit (R^2)	0.94		0.98		0.98	
CA model	**EC_{50} (nM)**	289 (284 to 294)		367 (363 to 371)		452 (449 to 455)	
	Hill slope	-1.5		-2.0		-2.3	
	Goodness of fit (R^2)	1.00		1.00		1.00	
IA model	**EC_{50} (nM)**	359 (340 to 379)		658 (629 to 688)		911 (879 to 943)	
	Hill slope	-1.8		-2.2		-2.5	
	Goodness of fit (R^2)	1.00		1.00		1.00	
	Growth rate (% CT)	**MDR CA**	**MDR IA**	**MDR CA**	**MDR IA**	**MDR CA**	**MDR IA**
	95	0.1	0.2	0.1	0.2	0.2	0.4*
	90	0.1	0.2	0.2	0.3	0.2	0.4*
	80	0.2	0.3	0.2	0.4*	0.2	**0.5***
	70	0.2	0.3	0.2	0.4*	0.3	**0.6***
	60	0.3	0.3	0.3	**0.5***	0.3	**0.6***

50	0.3	0.4*	0.3	**0.6***	0.4	**0.7***
40	0.4*	**0.5***	0.4	**0.6***	0.4	**0.8***
30	**0.5***	**0.5***	0.4	**0.7***	0.4	**0.9***
20	**0.6***	**0.6***	**0.5***	**0.9***	**0.5***	**1.0***
10	**0.8***	**0.8***	**0.6***	**1.1***	**0.6***	**1.2***
5	**1.1***	**1.0***	**0.8***	**1.3***	**0.8***	**1.4***

Table 3. Risk assessment for *Chlamydomonas reinhardtii* based on the risk quotient (RQ) and toxic unit (TU) for the single biocides, aclonifen, bifenox, dichlofluanid, metribuzin and triclosan, and based on the sum of the risk quotients (SRQ), sum of the toxic units (STU), and on the overall risk quotient (RQ_{STU}) for the mixture. EC_{50} - concentration of a compound that gives half-maximal response; NOEC - No Observed Effect Concentration (NOEC); MEC - Measured Environmental Concentrations. Note: *Measured concentration of a common degradation product of dichlofluanid and tolylfluanid, *N,N*-dimethylsulfamide (DMS); bold text indicates a potential risk.

		Aclonifen	Bifenox	DMS*	Metribuzin	Triclosan	SRQ	STU	RQ_{STU}
EC_{50} (nM)		298	18	113	57	1804			
NOEC (nM)		32	3	30	16	299			
MEC (nM)	**Min**	0.01	0.0005	1331	0.004	62			
	Median	0.7	1	5836	0.5	224			
	95	4.2	8	9308	9000	6042			
	Max	5.1	8	9907	10000	7944			
RQ	**MEC_{min}**	0.0004	0.0002	**44**	0.0003	0.2	**45**		
	MEC_{median}	0.02	0.4	**195**	0.03	0.8	**196**		
	MEC_{95}	0.1	**3**	**310**	**563**	**20**	**896**		
	MEC_{max}	0.2	**3**	**330**	**625**	**27**	**985**		
TU	**MEC_{min}**	0.00004	0.000002	**4**	0.00001	0.2		**5**	**5000**
	MEC_{median}	0.002	0.004	**20**	0.002	0.8		**20**	**20000**
	MEC_{95}	0.01	0.03	**31**	**30**	**20**		**82**	**82000**
	MEC_{max}	0.02	0.03	**33**	**34**	**27**		**94**	**94000**

Figure 1

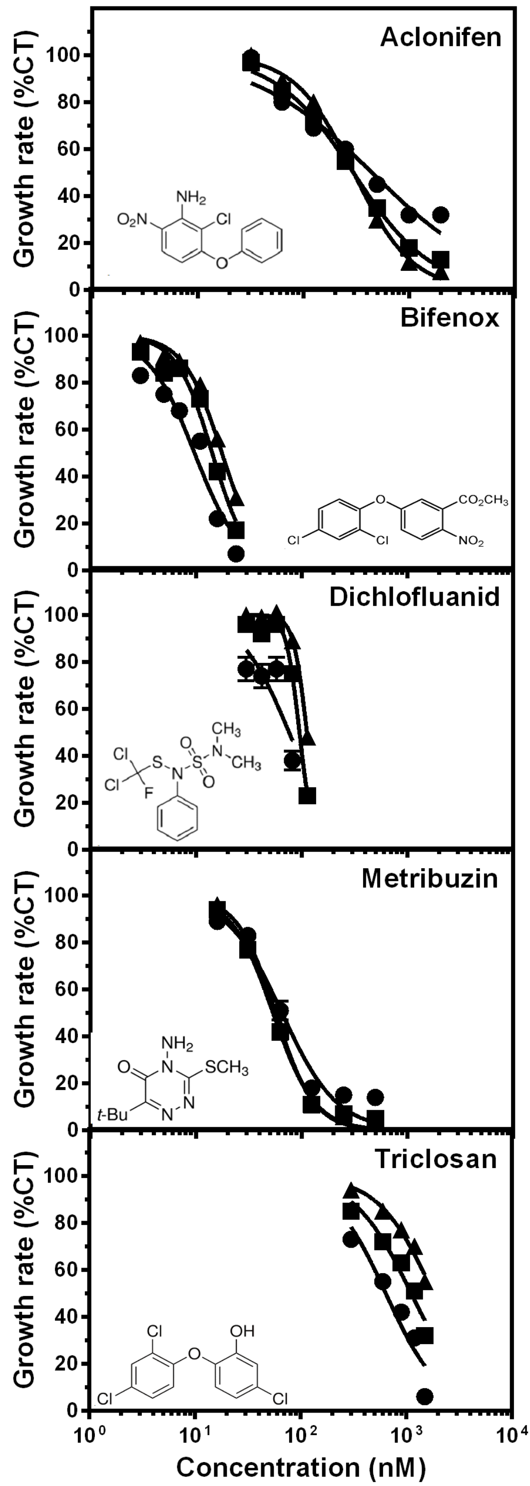
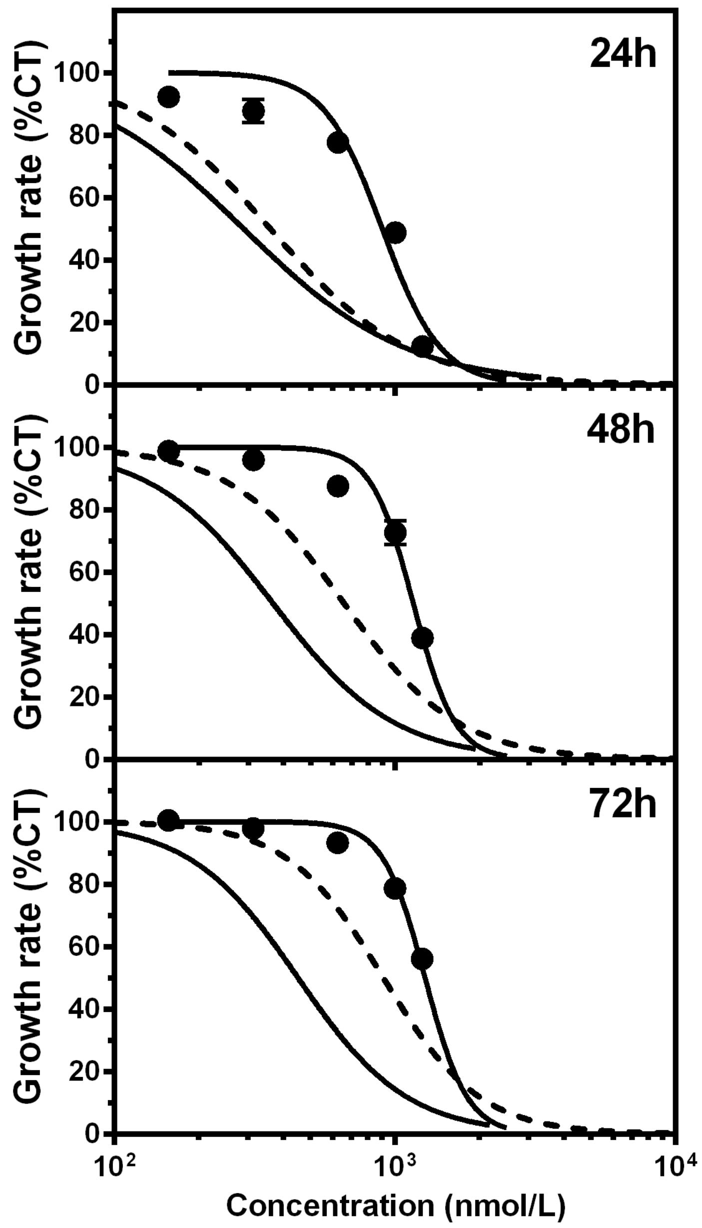


Figure 2



Experimental data — CA prediction model — IA prediction model

Combined toxicity of five priority biocides on the growth of *Chlamydomonas reinhardtii*

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Appendix A. Supplementary data

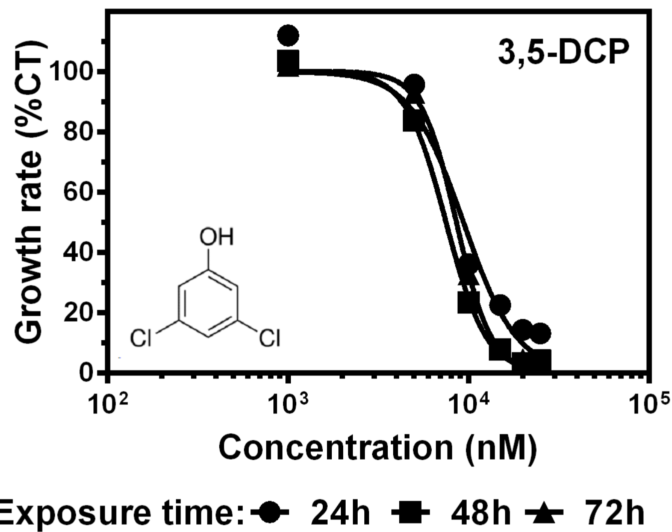

Fig 1. Growth rate (% of control, CT - control) of *Chlamydomonas reinhardtii* exposed to 3,5-dichlorophenol (3,5-DCP, positive control) for 24 h, 48 h and 72h. The data (Mean±SEM) represent experimental results from 3 independent experiments and their corresponding concentration-response curves (solid line).

Table 1. Nominal exposure concentrations for each compound and the 5-compound equipotent mixture.

Compound/mixture	nM
Aclonifen	63 125 250 500 1000 2000
Bifenox	3 5 7 11 16 24
Dichlofluanid	30 42 58 82 114 160
Metribuzin	16 31 63 125 250 500
Triclosan	300 600 900 1200 1500 1800
3.5-DCP	0 1000 5000 10000 15000 20000
Mixture 5 compounds	78 156 313 625 1250

	2500 5000 10000

Table 2. Concentrations (M) of the stock standard solutions used for the exposures and the respective value obtained from chemical analysis. Note: - method not sufficiently robust for aclonifen.

Compounds	Stock solutions (M)	Chemical analysis (M)
Aclonifen	0.01	-
Bifenox	0.0014	0.0012
Dichlofluanid	0.0006	0.0005
Metribuzin	0.023	0.025
Triclosan	0.023	0.019

Table 3. Nominal exposure concentrations of each compound on the equipotent mixture and the respective value obtained from chemical analysis. Note: - method not sufficiently robust for aclonifen.

Compounds	Nominal exposure concentration (M)	Chemical analysis (M)
Aclonifen	0.01	-
Bifenox	0.0009	0.001
Dichlofluanid	0.005	0.005
Metribuzin	0.0009	0.0008
Triclosan	0.08	0.09

Table 4. Significant differences between the effect concentrations for the observed data and for those calculated from each prediction model (Concentration addition (CA) and Independent Action (IA)) after one-way ANOVA analysis in combination with the Tukey post hoc test. Only *p-values* lower than 0.05 were considered significant. Note: SS – sum of the squares; DF – degrees of freedom; MS – mean squares; F – ratio between the two mean square values; DFn – degrees of freedom for the numerator; DFd - degrees of freedom for the denominator.

Time-point (h)	**Effect concentration (EC)**	**ANOVA**	**SS**	**DF**	**MS**	**F (DFn, DFd)**	***P-value***
24	$\leq EC_{45}$	**Treatment (between columns)**	0.353	2	0.177	F (2, 24) = 4.296	P = 0.0254
		Residual (within columns)	0.986	24	0.0411		
		Total	1.340	26			
		Tukey's multiple comparisons test	**Mean Diff.**	**95% CI of diff.**	**Significant**		
		Observed vs. CA	0.260	0.02174 to 0.4991	Yes		
		Observed vs. IA	0.220	-0.01903 to 0.4583	No		
		CA vs. IA	-0.0408	-0.2794 to 0.1979	No		
48	$\leq EC_{75}$	**Treatment (between columns)**	1.353	2	0.6763	F (2, 42) = 16.23	P < 0.0001
		Residual (within columns)	1.750	42	0.04166		
		Total	3.103	44			
		Tukey's multiple comparisons test	**Mean Diff.**	**95% CI of diff.**	**Significant**		
		Observed vs. CA	0.423	0.242 to 0.604	Yes		
		Observed vs. IA	0.177	-0.00457 to 0.358	No		
		CA vs. IA	-0.246	-0.427 to -0.065	Yes		

72	**≤ EC_{95}**	**Treatment (between columns)**	5785000	2	2893000	$F_{(2, 54)} = 12.08$	$P < 0.0001$
		Residual (within columns)	12930000	54	239393		
		Total	18710000	56			
		Tukey's multiple comparisons test	**Mean Diff.**	**95% CI of diff.**	**Significant**		
		Observed vs. CA	764.6	382.0 to 1147	Yes		
		Observed vs. IA	247	-135.6 to 629.5	No		
		CA vs. IA	-517.6	-900.2 to -135.0	Yes		

Table 5. Environmental concentrations of aclonifen, bifenox, dichlofluanid, metribuzin and triclosan found in literature.

Compound	Concentration (nM)	Place	References
Aclonifen	0.01	France	EC, 2006
	0.6	France	EC, 2006
	2.3	France	EC, 2011
	5.2	France	EC, 2011
	2.6	France	EC, 2011
	0.2	Sweden	EC, 2011
	0.1	Sweden	EC, 2011
	0.9	Norway	Pedersen et al., 2014
Bifenox	0.004	Europe	James et al., 2009
	0.6	Europe	James et al., 2009
	10000	Czech Republic	Barek et al., 2011
DMS*	4249	Europe	Loos et al., 2010
	5836	Norway	Langford et al., 2012
	9907	Norway	Langford et al., 2012
	1331	Norway	Langford et al., 2012
	6912	Norway	Langford et al., 2012
Metribuzin	0.6	Norway	Pedersen et al., 2014
	0.9	US	Battaglin et al., 2001
	1.5	US	Battaglin et al., 2001
	8.2	US	Battaglin et al., 2001
	1.8	US	EPA, 2004
	0.2	US	EPA, 2004
	0.5	US	Hostovsky et al., 2014
	8.2	US	Hostovsky et al., 2014
	0.0005	Canada	Kumar et al. 2013
	0.6	Brazil	Hostovsky et al., 2014
	1.6	Brazil	Hostovsky et al., 2014
Triclosan	62	Switzerland	Singer et al., 2002
	338	Switzerland	Singer et al., 2002
	104	Germany	Wind et al., 2004
	311	Germany	Wind et al., 2004
	138	US	Kolpin et al., 2002
	7944	US	Kolpin et al., 2002

*MEC values from a common degradation product of dichlofluanid and tolylfluanid, *N,N*-dimethylsulfamide (DMS);

Paper II

Photosystem II (PSII) efficiency in *Chlamydomonas reinhardtii* exposed to environmentally occurring biocides

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Abstract

Biocides are chemicals used to control hazardous organisms, but they can also pose a risk to non-target species. Some of these compounds are widely used, with ubiquitous distribution in different surface waters, and are highly toxicity to primary producers such as algae. Interference of photosynthesis through the specific inhibition of photosystem II (PSII) is a well-known Mode of action (MoA) for many biocides. This study intended to characterise the single and combined effects of the five environmentally relevant biocides aclonifen, bifenox, dichlofluanid, metribuzin, and triclosan, on the PSII of *Chlamydomonas reinhardtii*. PSII efficiency measured as the reduction in the maximum quantum yield (F_v/F_m) was determined after 24 h, 48 h and 72 h exposure to each biocide to establish concentration response relationships. Growth inhibition, measured as the number of cells *per* ml, was determined for those chemicals causing PSII inhibition to verify that inhibition of photosynthesis in algae was also causing whole organism responses consistent with regulatory-relevant toxicity endpoints. Concentration Addition (CA) and Independent Action (IA) prediction models were used to assess the combined effects. Only aclonifen and metribuzin showed a significant effect on PSII efficiency and strongly correlated with a reduction in growth. The effects of this binary mixture on the PSII activity was best described by the IA model, consistent with these herbicides displaying additive effects by dissimilar MoA. For growth inhibition, the IA model provided the best predictions for short exposure durations (<24 h), whereas the CA model provided better predictions for longer exposures (48-72 h). A concentration-dependent deviation from additivity, interpreted as synergy, was observed for medium to high concentrations for both the endpoints studied. Initial risk assessment using the data obtained herein suggest that median to high environmental concentrations of these contaminants can

pose a risk to algae if present in combination.

Keywords: PSII efficiency; Maximum quantum yield; Biocides; Mixture; Prediction models; Synergy; Microalgae; Microplate.

1. Introduction

Biocides are compounds used to control organisms that pose a threat to human or animal health, or that cause damage to natural or manufactured materials, such as pests and bacteria. Examples of this type of compounds are disinfectants, insect repellents, and anti-fouling paints for ships and material preservatives. However, due to their intrinsic characteristics, they can also pose a risk to the environment (EU, 2013). Primary producers such as algae may be affected by exposure to these chemicals, and thus introduce a serious threat to organisms forming the basis of several aquatic food webs, and therefore fundamental to aquatic ecosystems (Cedergreen and Streibig, 2005). Some biocides are considered of higher concern than others due to their high toxicity to primary producers, their widespread use and broad distribution in surface waters (USEPA, 2008; EU, 2013). Many of these biocides display multiple Modes of Action (MoAs) including inhibition of cellular enzymes involved in photosynthesis and disruption of mitochondrial functions as demonstrated by dichlofluanid (Cima et al., 2008). Triclosan, a wood preservative, bactericide and fungicide widely used in Personal Care Products (PCPs) and often considered a forgotten priority substance, is known to affect multiple target sites in different organisms (USEPA, 2008; von der Ohe et al., 2012). Herbicides, on the other hand, often display specific Mode of action (MoA) associated with interference with photosynthesis, including inhibition of carotenoid and chlorophyll biosynthesis (e.g., aclonifen), inhibition of chloroplast activity (e.g., bifenox) and interference with the electron transport chain (e.g., metribuzin) (Grossman, 2005; Fairchild et al., 1998, Killinc et al., 2009).

The ecotoxicological risk assessment of contaminants is normally performed using data from standardized tests like the algal growth inhibition test (USEPA, 2002; OECD, 2011). Although ecologically highly relevant, the toxicity endpoint measured is *per se* integrative in nature, and does not providing detailed information about the underlying toxicity mechanisms or MoA. Combining the use of relevant endpoints for population evaluation such as growth inhibition with more specific physiological endpoints can contribute to a better understanding of how certain chemicals cause toxicity (Nestler et al., 2012). Photosynthesis, a particularly complex series of redox and enzymatic processes, is a vital process for the survival of photosynthetic organisms. If the involved processes are damaged by contaminants, photosynthesis activity can be used as an ecotoxicological endpoint to assess the impact of

these compounds. Interference with photosynthesis has been identified as one of the major targets for many contaminants such as herbicides in algae (Ralph et al., 2007).

Microalgae have been commonly used for testing the toxicity of several compounds including herbicides, pesticides and antifoulants (Cedergreen and Streibig, 2005). *Chlamydomonas reinhardtii* in particular is one of the most commonly used algal species and identified as one of the most sensitive to biocides (Chalew and Halden, 2009). This alga is easily grown and subjected to exposure studies, been reported to be sensitive to several contaminants, and has a short generation time and thus allowing a rapid assessment of toxicological endpoints. This is a small organism, easy to collect and identify (Harris, 2009), and already used in many ecotoxicological studies with herbicides, either as single compounds (Prado et al., 2009) or multi-compound mixtures (Knauert et al., 2008). Due to its sensitivity to photosystem II (PSII) inhibitors, it has also been used in several ecotoxicological studies, namely the measurement of PSII efficiency (e.g. Guenther et al., 1990; Fischer et al., 2006; Juneau et al., 2007; Alric et al., 2010).

The measurement of chlorophyll *a* fluorescence has proven to be a powerful tool to assess the condition of the photosynthetic apparatus. It is a simple method for measuring the quantity of absorbed energy used in photochemical processes (*i.e.*, photosynthesis) that also provide an indication of the organism's overall health (Ralph et al., 2007). Changes in chlorophyll *a* fluorescence due to exposure to a contaminant can be due to its impact on photosynthetic processes like binding to the plastoquinone or blocking electron transport (Falkowski and Raven, 2007). This is the case of PSII inhibitors, that act by competing with plastoquinone at the Q_B binding site of the D1 protein in PSII reaction centre, inhibiting energy transfer and affecting algae growth (Falkowski and Raven, 2007; Magnusson et al., 2008). The inhibition of PSII activity is a well-characterised MoA for many herbicides (Falkowski and Raven, 2007; Cedergreen, 2014). Several bioassays have been developed to specifically identify impacts on particular components of the photosynthetic pathways. Maximum quantum yield (F_v/F_m) is one of the most commonly used parameters to indicate the maximal PSII photochemical efficiency, based on chlorophyll *a* fluorescence (Falkowski and Raven, 2007; Nestler et al., 2012; Ralph et al., 2007).

Biocides are normally used in combination to potentiate their effects, and interactions have already been observed in several organisms like microalgae, phytoplankton, and crustaceans

(Férnandez-Alba et al., 2002; Backhaus et al., 2004; Gatidou and Thomaidis, 2007; Cedergreen, 2014). As there are still many uncertainties regarding the assessment of the environmental risk posed by these types of compounds, and how they interact with biological targets as complex mixtures, experimental evaluation of their combined toxicity is urgently needed (Bellas, 2006; Ruedel, 2012). Even if the concentration of a biocide in the environment is below its threshold level to exert an effect, the occurrence of numerous compounds in mixtures may give rise to combined toxicity that cannot be explained by the presence of the single chemicals alone. Compounds in a mixture can act either by additivity, synergism (more than additivity), or antagonism (less than additivity) and can be predicted by mathematical models based on the principles of concentration addition (CA; Loewe, 1927) and independent action (IA; Bliss, 1939). The CA model is normally used for compounds with similar MoA, whereas the IA model is applied for compounds with dissimilar MoA. The CA model is based on the assumption that compounds affecting the same endpoint or biological target with the same trend will do this in an additive manner. The IA model assumes also assumes additivity, but here the compounds display different MoA and affecting different targets, although the interaction of the two affect the same apical toxicity endpoint. Deviations from additivity may give rise to potentiation (synergy) or suppression (antagonism) of the measured response (Altenburger et al., 2003, 2004). These deviations from additivity can be assessed by methods such as model deviation ratio (MDR), which determines the ratio between the predicted and observed exposure concentration for a given effect level (Belden and Lydy, 2006). The CA and IA prediction models have already been successfully used to predict the inhibition of photosynthetic activity by mixtures of several PSII inhibitors and in most cases predictions are in good agreement with the CA model (Vighi et al., 2003). Therefore, if these compounds follow the concept of additivity, cumulative effects is likely to occur if these compounds co-occur with other PSII inhibitors (Vighi et al., 2003).

The well-established mixture toxicity concepts CA and IA can provide a tiered framework for environmental risk and risk assessment of mixtures. According to Backhaus and Faust (2012), principles of CA can be applied as a precautionary approach to assess cumulative risk regardless if whether compounds in the mixtures act by similar or dissimilar MoA. The calculation of a risk quotient (RQ) for single compounds (*i.e.*, the ratio between the expected exposure and the effect of a given compound) can be extrapolated to chemical mixtures based on the CA assumptions. First, the cumulative risk that the mixture represents is calculated by

the sum of the toxic units (TU) for each compound based on data for the most sensitive species (Backhaus and Faust, 2012). If a risk (RQ>1), a species-specific risk quotient for the mixture (RQ_{STU}) can be calculated based on the sum of toxic units for the most sensitive species group or trophic level (*i.e.*, the species group with the highest STU). This approach is considered a solid conceptual basis for cumulative risk assessment (Backhaus and Faust, 2012; Backhaus and Karlsson, 2014).

The objective of this study was to characterise the single and combined effects of the five environmentally relevant biocides aclonifen, bifenox, dichlofluanid, metribuzin, and triclosan, on photosynthesis measured as inhibition of the PSII in *C. reinhardtii*. Maximum quantum yield (F_v/F_m) was used to indicate the maximal PSII photochemical efficiency, in an optimized format using a 96-well microplate. The toxicity of the biocides was also assessed as inhibition of algal growth to verify that the effects on the PSII also affect regulatory-relevant toxicity endpoints. Moreover, the CA and IA models were used to assess the combined effects of the biocides affecting the PSII and growth and determine if combinations of these caused additivity, antagonism or synergy when present in a mixture.. Finally, prediction of cumulative risk by deriving Risk Quotients (RQs) and Toxic Units (TUs) were conducted to determine if the overall impact of simple mixtures of biocides can represent a risk under environmentally-relevant exposure scenarios.

2. Material and Methods

2.1. Test compounds and standards

The test compounds aclonifen (CAS number: 74070-46-5), bifenox (CAS number: 42576-02-3), dichlofluanid (CAS number: 1085-98-9), metribuzin (CAS number: 21087-64-9), triclosan (CAS number: 3380-34-5) and atrazine (positive control; CAS number: 1912-24-9) were purchased from Sigma-Aldrich (United Kingdom) with ≥97.0% purity. Dimethylsulphoxide (DMSO, Sigma-Aldrich, United Kingdom, purity ≥99%) was used as solvent for all compounds.

2.2. PSII efficiency

Freshwater green algae *C. reinhardtii* (NIVA-CHL153; Norwegian Institute for Water Research, Oslo, Norway) was cultured in glass flasks with Talaquil media (Szivák et al., 2009), prepared at least 24 h prior usage to allow the equilibrium of compounds. Glass flasks

with 50ml of media were inoculated with 10^4 cells/ml (stock cultures), and incubated in an Infors Multitron 2 incubator shaker (Infors AG, Bottmingen, Switzerland) at 20±2°C for 72 h, under continuous light 83±6 $\mu mol/m^2/s^1$ provided by cool–white fluorescence lamps (Philips TLD 36W/950, London, UK), and with orbital shaking at 90 rpm. After achieving an exponential growth (72 h), algae cells were inoculate into new sub-cultures exposed to control (0.01% v/v DMSO), positive control (atrazine), test compounds and defined mixtures of these (see supplementary data Table 1 for details on used concentrations). The exposed cultures were maintained in the same conditions as the stock cultures.

The exposed algae where grown for 72 h, and the PSII efficiency was monitored at 0 h, 24 h, 48 h and 72 h. Maximum quantum yield (F_v/F_m) was used to indicate the maximal PSII photochemical efficiency, as described by Kitajima and Butler (1975) and adapted to a 96-well microplate. PSII was monitored using chlorophyll *a* fluorescence, recorded on a Cytofluor 2300 (Millipore; Billerica, MA, USA) with excitation/emission at 485/685 nm. In brief, 200 µl of exposed algae were transferred into NUNC MicroWell™ 96-Well microplates (NUNC, Thermo Scientific, Roskilde, Denmark), chlorophyll *a* fluorescence measurement was made after 20 min adaption to dark to determine the fluorescence yield of PSII in a dark adapted state (F_o). Then, 5 µl of diuron (DCMU, ≥98%, Sigma-Aldrich, UK) at a final concentration of 10 µM were added to block the electron transport in the PSII. A second fluorescence measurement was made immediately to determine the maximal fluorescence yield in a light adapted state (F_m). The fluorescence of variable yield (F_v) was calculated as F_m-F_o, and F_v/F_m was used to express PSII primary photochemical efficiency, expressed as percentage of control (% CT) (Eq. 1).

$$F_v/F_m = [F_m - F_o]/F_m \quad \textbf{Eq. 1}$$

Where F_v is the fluorescence of variable yield, F_m the maximal fluorescence yield in a light adapted state and F_o the fluorescence yield of PSII in a dark adapted state.

Data was then normalized using the minimum and maximum values recorded for the positive control (atrazine), to allow the fitting of sigmoidal concentration-response curves.

2.3. Algal growth inhibition

For the compounds affecting the PSII, the algal growth was also monitored in the same exposed cultures. A multisizer counter (Beckman-Coulter Multisizer 3 Coulter Counter; Miami, FL, USA) was used to determine the number of algae cells after 0 h, 24 h, 48 h and 72 h. The average growth rate (μ) for each test concentration was calculated from initial cell concentration and cell concentration at the time of the last cell count using the equation:

$$\mu_{n-0} = \frac{\ln(N_n) - \ln(N_0)}{t_n - t_0} \times 24\ (day^{-1}) \qquad \textbf{Eq. 2}$$

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

At least three independent experiments with triplicates were prepared for each chemical and mixture for the two endpoints. All used flasks and glassware were autoclaved before usage to avoid any microbial contamination. Culture samples were checked microscopically to detect the occurrence of any microbial contamination.

2.4. Single toxicity assessment

The results were modelled to obtain a sigmoidal dose-response curve with variable slope, using the equation:

$$\gamma = Bottom + \frac{top - bottom}{1 + 10^{(\log EC_{50} - \, logX) \times slope)}} \qquad \textbf{Eq. 3}$$

Bottom is the Y value at the bottom plateau, top is the Y value at the top plateau and log EC_{50} is the logarithm of the concentration causing 50% effect.

Concentration-response curves were made in the same way for all toxicants and for the mixture. The EC_{50}, Hill slope and goodness of fit (R^2) where also calculated.

2.5. Combine toxicity assessment

A mixture with the compounds affecting the PSII was established based on the CA prediction model (Eq. 4). The effective concentrations of the mixtures were predicted as:

$$ECx_{mix} = \left(\sum_{i=1}^{n} \frac{p_i}{ECx_i}\right)^{-1}$$ **Eq. 4**

The ECx_{mix} is the total predicted effect concentration of the mixture that indices an effect x, p_i is the relative fraction of component i in the mixture and ECx_i is the concentration of substance i inducing an effect x when exposed alone.

A fixed ratio design was used for the mixture, based on the ratios at the EC_{50} concentrations from the individual CRCs after 48 h exposure (maximum assay response), avoiding that one toxicant would dominate the predicted effect. As for the single compounds, the observed effects were modelled using a sigmoidal CRC with variable slope (Eq. 3). The CRC for the mixture was compared to both the CA predictions (Eq. 4) and the IA predictions (Eq. 5).

$$E_{mix} = 1 - \prod_{i=1}^{n}(1 - E_i)$$ **Eq. 5**

Where E_{mix} the effect of a mixture of n compounds and E_i the effect of substance I when exposed alone.

The EC_{50}, Hill slope and goodness of fit (R^2) values were calculated for all curves. The non-linear regressions for observed data and each model (CA and IA) were used to calculate the corresponding effect levels. Then, the effect levels obtained for the observed data was compared to those of CA and IA, and additive effects were assumed to occur if no significant differences were detected between the observed effect concentrations and those predicted by the models. If the curves were not significantly different, the model was considered to explain the combined effects, being the compounds acting by similar (CA) or dissimilar (IA) MoA. Model deviation ratios (MDRs) were also applied to verify the occurrence of additive effects, estimated to occur when these were within a factor of 2 ($0.5 \leq MDR \leq 2$; Belden et al., 2007), calculated by:

$$MDR = \frac{ECx_{pred}}{ECx_{obs}}$$ **Eq. 6**

ECx_{pred} is the predicted effect concentrations and ECx_{obs} the observed effect concentrations. Synergy was defined as more than two fold deviations from the predictions, while antagonism was assumed when the MDR was less than 0.5 (Belden and Lydy, 2006).

2.6. Initial environmental risk assessment

The potential environmental risk of each biocide was based on calculation of the risk quotient (RQ) for each compound, *i.e.*, the ratio between the expected exposures and the risk of the compound as defined by EPA guidelines (EPA, 2004):

$$RQ = \frac{MEC}{NOEC} \quad \textbf{Eq. 7}$$

Being MEC the maximum Measured Environmental Concentrations reported in literature, and NOEC the No Observed Effect Concentrations on the PSII inhibition after 48 h exposure in *C. reinhardtii*.

The RQ for the mixture was extrapolated from single substances having effects on the PSII by means of CA. The sum of RQ (SRQ) was calculated to evaluate the potential cumulative risk as (EPA, 2004; Backhaus and Faust, 2012; Backhaus and Karlsson, 2014):

$$SRQ = \sum_{i=1}^{n} RQ_i \quad \textbf{Eq. 8}$$

A value larger or equal to 1 of RQ or SQR was interpreted as a potential environmental risk (Backhaus and Faust, 2012; Backhaus and Karlsson, 2014; EPA, 2004).

The potential environmental risk of the individual biocides inducing inhibition of PSII was also based on calculation of toxic units (TU) for *C. reinhardtii* (Backhaus and Faust, 2012; Backhaus and Karlsson, 2014; EU, 2009):

$$TU = \frac{MEC}{EC_{50}} \quad \textbf{Eq. 9}$$

Using the Measured Environmental Concentrations (MEC) reported in the literature, and the EC_{50} the concentration that gives half-maximal response in PSII inhibition in algae after 48 h

exposure. A TU of 1 indicated that the MEC was expected to cause a 50% effect on the PSII inhibition in the respective species used for derivation of the PSII efficiency.

The toxicity of the mixture was then described by the sum of all individual TUs of the present compounds (Backhaus and Faust, 2012; EU, 2009):

$$STU = \sum_{i=1}^{n} TU_i \qquad \textbf{Eq. 10}$$

A value larger or equal to 1 of TU or STU was interpreted as a potential environmental risk for the analysed mixture (Backhaus and Faust, 2012).

The overall risk quotient for the mixture (RQ_{STU}) to algae (the organism group that is regarded as the most sensitive to the mixture) was calculated according to Backhaus and Faust (2012):

$$RQ_{STU} = \max(STU_{algae}) \times AF \qquad \textbf{Eq. 11}$$

Being max(STU algae) the sum of the TU for algae and AF the assessment factor of 100 (EU, 2009). Currently there is no guideline for how to determine the AF for calculating RQ_{STU}, so this values was chosen according to literature (EU, 2003; Petersen et al., 2013).

2.7. Chemical analysis

Stock standard solutions were diluted in dichloromethane for the confirmatory analysis of bifenox, dichlofluanid, triclosan and metribuzin by gas chromatography-high resolution mass spectrometry (GCT-Premier, Waters Corp, Milford MA, USA). The analytes were separated on a 30 m × 0.25 mm, 0.25 ìm film thickness DB-5MS column (Agilent Technologies) with helium carrier gas. Splitless injection at 250 °C was used. The initial temperature of 60 °C was held for 2 min, followed by an increase of 5 °C/min to 310 °C and held for 5 min. The m/z used for quantification were dichlofluanid, 123.0142+223.9219; metribuzin, 214.088; bifenox, 340.986, and triclosan, 287.951+218.0145. The results of the chemical analysis on the stock standard solutions (Table 2 in supplementary data; Almeida et al., *in prep*) confirmed the concentrations of the stock solutions used for the exposures, except for aclonifen. The method was not sufficiently robust for the analysis of this compound and data was not reproducible. The nominal exposure concentrations of each compound on the equipotent mixture were also confirmed using the same method (Table 3 in supplementary

data). As the measured concentrations did not surpass a ±20% of the nominal concentrations, these concentrations were used throughout.

2.8. Statistical analysis

Statistical analysis was performed in GraphPad Prim 6 (GraphPad Software Inc., La Jolla, CA, USA). Non-linear regressions were used to obtain a sigmoidal dose-response curve with variable slope for each chemical and mixture. Correlation analysis between the effects of single compounds and mixture on the PSII efficiency and inhibition of growth were performed with the non-parametric Spearman correlation (one-tailed) test. Significant differences in the PSII efficiency between exposure groups were calculated for each compound by Kruskal-Wallis non-parametric test followed by Dunn's post hoc test to identify the NOEC. The significant differences between the effect levels obtained for the experimental data with those calculated by the CA and IA prediction models were calculated by the parametric one-way ANOVA was used in combination with the Tukey test for multiple comparisons, as data was normally distributed and with homogeneous variance (Motulsky, 1998; EPA, 2006). A *p*-value of 0.05 was considered significant.

3. Results

3.1. Single biocide exposure

The effect of the positive control atrazine on the PSII efficiency of *C. reinhardtii* was analysed prior to testing the target compounds (Fig. 1). Atrazine showed a high-quality (R^2= 0.96) concentration curve (CRC), with an EC_{50} of $6x10^5$ nM and Hill slope of -0.6 (Tables 1). DMSO (0.01% v/v used as solvent did not cause any effects compared to algae kept in pure growth media (results not shown).

Out of the five biocides (aclonifen, bifenox, dichlofluanid, metribuzin and triclosan) tested for inhibition of PSII efficiency in *C. reinhardtii*, only aclonifen and metribuzin showed a significant effect (Fig. 2; consult Table 1 in supplementary data for information about the exposure concentrations). The normalisation of the CRCs was performed using the minimum and maximum values recorded for the positive control (atrazine). Following normalization of the data to adjust for negative values in the Fv/Fm ratio, both compounds affected the PSII in a clear concentration-dependent manner and reduced the growth of the algae to less than 50% of the control (Fig. 3; Table 1). The responses for these two compounds were well-fitted by

the non-linear regression, with R^2≥0.93 for aclonifen and R^2≥0.98 for metribuzin at all exposure durations and endpoints (Table 1). Both compounds showed highest effect on PSII after 48 h exposure, with metribuzin (NOEC= 107 nM; EC_{50}= 70 nM) being more potent than aclonifen (NOEC= 578 nM; EC_{50}= 481 nM), and having steeper CRC slopes at 24 h and 48 h exposure (Table 1). The inhibition of growth observed for these two compounds were also well-fitted by the non-linear regression, with R^2≥0.97 for aclonifen and R^2≥0.96 for metribuzin (Fig. 3, Table 1). Also for the growth inhibition, the two compounds were most toxic at 48 h (Table 1), with metribuzin (NOEC= 16 nM; EC_{50}= 54 nM) displaying the steepest slope and being more toxic than aclonifen (NOEC= 32 nM; EC_{50}= 294 nM).

The correlations between the effects of aclonifen and metribuzin on the PSII efficiency and inhibition of growth were all significant (p<0.05; Table 2), indicating a strong coherence between the two endpoints. For both compounds, the NOEC and EC_{50} values were higher for the PSII efficiency than for the growth inhibition, although this difference was less pronounced at 48 h (Fig. 3; Table 1).

3.2. Combined effects

The combined effects of aclonifen and metribuzin on PSII efficiency were well described by the non-linear regression analysis (R^2≥0.95 at all exposure durations; Table 2 and Fig. 1 in supplementary data). For this endpoint, the 48 h exposure concentrations were chosen as data input to the prediction models, as these showed less variance, and thus introducing the lowest errors in the predictions. At 24 h, 48 h and 72 h the effects of the mixture were best predicted by the IA model, with 0.5≤ MDR≤ 2 at almost all effect levels (Fig. 4; Table 3). However, considering the MDR values, more than additive effects were observed at high effects levels, especially at 48 h exposure, as indicated by the MDR >2 (Table 3).

The effects of the mixture on algal growth were well described by the applied non-linear regression analysis, as demonstrated by R^2≥0.97 at all time points (Fig. 5; Table 4). For the growth inhibition test, the 72 h exposure concentrations were chosen as an input to the prediction models (Table 1), using the chemical-specific CRC parameters for the individual compounds (EC_{50} and Hill slope). At 24 h the IA model best predicted the effects of the mixture. At 48 h the CA model provided the best fit to growth rates higher than 50%, while at growth rates lower than 40% the CA underestimated the effects, thus indicating that the

components of the mixture caused synergy in combination (MDR>2). At 72h the CA model provided the best fit to the three highest growth rates (80%-95%), and as seen at 48h potential synergism (MDR>2) were identified at all other effect levels (Table 4).

The exposure duration and effect levels affected differently the combined effects of the studied compounds on the two studied endpoints (Tables 3 and 4; for more information on the statistical analysis results please consult Tables 4 and 5 on the supplementary data for PSII efficiency and growth inhibition, respectively).

The correlation between the PSII efficiency and inhibition of growth for the mixture also showed a high and significant strength of association at all time exposure durations (Table 2; Fig. 1 in supplementary data)). For the mixture, the EC_{50} values were similar for both PSII efficiency and growth inhibition (Tables 2 and 3 in supplementary data, respectively), especially at 48 h (PSII efficiency: EC_{50}= 125.8 nM; Growth rate: EC_{50}= 128.8 nM) and 72 h (PSII efficiency: EC_{50}= 110.9 nM; Growth rate: EC_{50}= 110.8 nM). The toxicity of the mixture increased with time and led to 1.8 and 1.4 times higher toxicity at 72 h for PSII efficiency and growth inhibition, respectively (Tables 2 and 3 Fig. 1 in supplementary data).

3.3. Potential environmental risks

The RQs and TUs calculated for the biocides affecting the PSII in *C. reinhardtii* are in Table 5. No risk scenarios were identified for any of the single compounds or mixture (Table 5). However, when considering the RQ_{STU} from MEC_{median} (RQ_{STU}= 2), the results indicated a potential environmental risk.

4. Discussion

4.1. Effects of single compounds

Atrazine, the positive compound used, showed a high-quality CRC and the assay was highly reproducible for all the analysed compounds. However, although maximum quantum yield (F_v/F_m) is one of the most used fluorescence parameters to detect effects on the PSII, microalgae are the most widely used tested organism, and herbicides the most commonly tested chemicals, most of the studies found in literature lacked effect values such as EC_{50} and NOEC (Ralph et al., 2007). Therefore, the obtained data for atrazine could not be directly compared with published data.

From the 5 studied biocides, only aclonifen and metribuzin showed a significant effect on the PSII efficiency, with metribuzin being 5 times more potent than aclonifen. Both are known to affect photosynthesis, although by different MoA. While metribuzin reduces photosynthesis activity by inhibiting the electron transport in the PSII (Buman et al., 1992), aclonifen inhibits chlorophyll and carotenoid biosynthesis (Killinc et al., 2009). Both compounds had EC_{50} values in the nM range for both inhibition of PSII efficiency and growth, and a significant correlation was observed between both parameters at all exposure periods tested. The largest effects occurred at 48 h exposure for both chemicals and for the two studied endpoints, and were consistent with reports for algae toxicity studies elsewhere (Lürling, 2011).

Metribuzin is a potent herbicide inhibiting algal growth (Lürling, 2011) and classified as very toxic to aquatic organisms (EFSA, 2006). In the present study its toxicity increased slightly with time, in agreement with its high stability to abiotic hydrolysis and lack of biodegradability (EFSA, 2006). Studies with other algae such as *S. obliquus* (Lürling and Roessink, 2006) and *Pseudokirchneriella subcapitata* (Choi et al., 2012) also showed a significant reduction in the efficiency of PSII after exposure to this compound. Due to the development of metribuzin as an herbicide, the MoA towards photosynthetic organisms is well established. Metribuzin is a triazinone herbicide that inhibits the electron transport through binding to the Dl protein in PSII (Buman et al., 1992). The Dl protein, also named as 32-kDa protein or QB binding protein, is a membrane-spanning polypeptide containing the plastoquinone-binding site. This type of herbicides inhibits PSII specifically by interfering with the binding of plastoquinone, and thus blocking the electron transport in the Hill reaction (Buman et al., 1992). This binding prevents the $NADP^+$ reduction required for CO_2 fixation (Eullaffroy and Vernet, 2003) and is believed to cause chlorophyll photodamage by reactive oxygen species (ROS; Jones, 2005). This type of herbicides can also damage adjacent chlorophyll-bearing proteins by interfering with the chlorophyll energy transfer systems and damaging protective pigments (carotenoids), which is believed to cause additional ROS generation (Jones, 2005).

In the present study, the reduction in PSII efficiency (EC_{50}= 70–110 nM) and inhibition of growth (EC_{50}= 54-65 nM) occurred at similar concentrations of metribuzin, with high correlation between endpoints. This suggests that the main MoA of metribuzin was photosynthesis inhibition leading to growth inhibition in *C. reinhardtii* and consistent with suggestions elsewhere (Oettmeier et al., 1982). Similar consistency between EC values was

also obtained for PSII efficiency (EC_{50}= 75nM) and growth inhibition (EC_{50}= 70nM) in *S. obliquus* (Lürling, 2011).

Aclonifen is also considered to be very toxic to aquatic organisms (ECHA, 2011). In the present study, the toxicity of aclonifen increased with time as seen for metribuzin, and were found to be in agreement the fact that it is not readily biodegradable in water and considered persistent in the aquatic environment (ECHA, 2011). Aclonifen has a diphenylether (DPE) nucleus, being a potent herbicide in the presence of light. The target of most of the DPE compounds is the protoporphyrinogen oxidase (Protox) complex in the pathway leading from σ-amino-levulinic acid to chlorophyll (Ensminger and Hess, 1985). The inhibition of this oxidase leads to the accumulation of protoporphyrin IX in cells, due to the translocation of protoporphyrinogen IX from the chloroplasts to the cytoplasm. The accumulated protoporphyrin IX then reacts with oxygen in the presence of light, causing the formation of ROS, which can then lead to oxidative stress on cellular macromolecules like DNA (DNA damage and repair), proteins (protein degradation), chlorophyll and membrane damage (lipid peroxidation) (Ledford and Niyogi, 2005; Killinc et al., 2009). These impairments can ultimately lead to either programmed cell death (apoptosis) or acute cellular injury by autolysis (necrosis; Ledford and Niyogi, 2005).

Aclonifen is also known to inhibit carotenoid biosynthesis, which is an effective protector of chlorophyll scavenging ROS and dissipating the excess of absorbed energy. This leads to the destruction of photosynthetic antenna chlorophyll and to a rapid bleaching of the chlorophyll (Guseinova et al., 2005; Killinc et al., 2009). The present study is to our knowledge the first to report PSII inhibition of this compound in algae.

For aclonifen, the reduction in PSII efficiency (EC_{50}= 481-1178 nM) and inhibition of growth (EC_{50}= 294-429 nM) also occurred at concentrations in the same order of magnitude. The correlation between CRCs for both endpoints suggests that the reduction in PSII efficiency was contributing to the overall inhibition of algal growth, albeit the apparent lower sensitivity of the former indicates that PSII inhibition may not be the only MoA for the observed reduction in growth. In comparison to other species, higher sensitivity has been reported for growth inhibition *in Selenastrum capricornutum* (EC_{50}= 26-110 nM) than the toxicity reported herein for aclonifen (Andersson and Andersson, 1994). The reason for this large discrepancy in sensitivity was not investigated in the present study, but it is well established

that differences in toxicodynamics and toxicokinetics contribute to interspecies susceptibility, especially for contaminants with more specific MoA (Vaal et al., 2000; Nyman et al., 2014).

4.2. Combined effects

The PSII efficiency was well estimated at all time-points and effect levels by the IA model, indicating that the compounds have additive effects, but likely mediated by different MoAs. While metribuzin inhibits the PSII (Jones, 2005), aclonifen inhibits chlorophyll and carotenoid biosynthesis (Killinc et al., 2009), with both contributing for the inhibition of algal growth, a more generalized toxic endpoint. For this, a temporal and concentration-dependent variance in the combined effects of the studied compounds was observed. While at the beginning of exposure (at 24h) the IA model best predicted the effects of the mixture on the growth, the CA provided the best fit to the experimental data at low to medium effect levels at longer exposure durations (48h and 72h). Therefore, for the growth inhibition it seems that in the beginning of the exposure the different MoAs of the compounds were the predominant drivers for toxicity, whereas toxicity seemed to be mediated through more similar MoA at longer durations of exposure. This is probably due to the contribution of more biological targets and toxicity pathways to the overall toxicity affecting growth and the overall heath status of algae (Petersen et al. 2014).

A potential synergism between the two compounds was observed for both toxic endpoints at medium to high effect levels, especially on the growth inhibition. Other mixture toxicity studies with biocides in algae (Faust et al., 2003) and herbicides in *Lemna minor* (Cedergreen and Streibig, 2005) also reported synergistic effects obtained for higher concentrations (Faust et al., 2003). Synergistic effects for mixtures of PSII inhibitors and other herbicides have also been previously reported in algae and plants (Cedergreen, 2014), and the suggested causes are interactions occurring in steps leading to ROS formation. The other compound (aclonifen) may not only induce ROS, but can also prevent the repair of damages in the PSII complexes, a process that is continuously occurring during photosynthesis in natural conditions (Cedergreen, 2014). Therefore, at high effect levels may these two herbicides seem to trigger an increase in the inflicted damage and prevention of repair that is not present when the two chemicals are exposed separately. This information was used to develop a schematic representation on the MoA for the studied mixture (Fig. 6). The similar effect concentrations (*i.e.*, EC_{50}) for both endpoints indicate that inhibition of photosynthesis was likely the main MoA of the mixture of aclonifen and metribuzin (Oettmeier et al., 1982).

4.3. Environmental implications

Contaminants affecting the normal development and growth of algae should be a cause of concern. As the studied herbicides affect the photosynthetic capacity of these essential aquatic organisms, changes in the grazing community structure or starvation of consumers may occur when these contaminants are present in the environment. Moreover, as aclonifen and metribuzin both affect photosynthesis, they not only directly affect the fixation of carbon necessary for growth, but also indirectly disturb the transference of energy to higher levels of the food chain. This loss of efficiency costs energy, having also implications on the capacity of algae to cope with additional stresses if levels in the environment are sufficiently high (Raph et al., 2007). The initial risk assessment performed did not identify a risk for any of the individual compounds, even though the RQ and TU values for metribuzin were up to 10-fold higher than those found for aclonifen. Aclonifen is commonly used in many European countries, especially France (main market of this compound in Europe; EC, 2006, 2011), but also in Nordic countries such as Sweden (EC, 2011) and Norway (Pedersen et al., 2014). Metribuzin seems to be more commonly used in the US (Battaglin et al., 2001; EPA, 2004; Hostovsky et al., 2014), Canada (Kumar et al., 2013), Brazil (Hostovsky et al., 2014), and Norway (Pedersen et al., 2014). Although the compounds are not likely to cause effects on the PSII at environmentally realistic concentrations, they may still cause toxicity and represent risk scenarios through other MoAs (Almeida et al, *in prep*).

While the risk of these chemicals alone might not be sufficient to cause effects, their existence as a mixture can lead to combined toxicity, that in the present study was interpreted as additivity and even synergism. The calculation of a RQ_{STU} for mixtures, which represent a conservative estimate for cumulative risk assessment by assuming additivity (Backhaus and Faust, 2012), verified that the combination of aclonifen and metribuzin represented an environmental risk at MEC_{median}. As environmental mixtures contain more complex mixtures that those studied herein, it may be expected that the overall risk of ecologically relevant mixtures of PSII inhibitors may even be higher than that predicted. As the likelihood that combined effects occur by additivity and follow CA increase with the number of compounds in the mixture (Warne and Hawker, 1995), the current approach may represent a suitable first tier approach to assess the cumulative risk of these and other PSII inhibitors. This also confirms the findings that more complex mixtures of biocides such as those studied can represent a risk to algae under ecologically relevant exposure scenarios (Almeida et al., *in prep*). It still remains a challenge to assess the cumulative risk of compounds in which simple

mixtures are likely to cause synergy, such as that observed for aclonifen and metribuzin (Cedergreen, 2014). Additional effort to identify compounds and the MoA that cause synergy is clearly warranted to improve the ability to accurately predict both combined toxicity and cumulative risk.

Conclusions

A high-throughput assay was used in the present study to assess the effects of five biocides aclonifen, bifenox, dichlofluanid, metribuzin and triclosan on the PSII efficiency of *C. reinhardtii*. Among these, only aclonifen and metribuzin showed a significant effect, with the latter being the most potent. Both affected the photosynthesis in algae in a concentration-dependent manner and with the largest potency at 48h exposure. Although these biocides likely caused effects by different MoA, large coherence were seen between the inhibition of PSII and effects on the growth of the algae. Their combined effects were analysed in an equipotent mixture by the use of CA and IA prediction models. CA provided the best predictions at low to intermediate concentrations when monitoring growth inhibition, indicative of a similar MoA. The disturbance on the PSII efficiency was best predicted by the IA model, indicating that the compounds affect the PSII by dissimilar MoA. Although additivity was identified at low effect concentrations of the 2 compounds, synergy was identified at medium to high effect concentrations when monitoring the two endpoints. This possible synergism might be due to simultaneous inhibition of PSII and increase in oxidative stress causing toxicity, and potentiation by interference with damage repair in the PSII complexes. Risk assessment based on environmental concentrations of the two chemicals along with the data obtained in the present study suggest that even though the single compounds did not represent a risk, it may be enhanced when considering their combined effects under ecologically relevant scenarios.

Conflict of interest

The authors declare the inexistence of any conflicts of interest.

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References

Almeida, A.C., Petersen, K., Langford, K., Thomas, K.V., Tollefsen, K.E., *in prep*. Combined toxicity of five priority biocides on the growth of *Chlamydomonas reinhardtii*.

Alric, J., Lavergne, J., Rapport, F., 2010. Redox and ATP control photosynthetic cyclic electron flow in *Chlamydomonas reinhardtii* (I) aerobic conditions. Biochim. Biophys. Acta 1797, 44-51.

Altenburger, R., Nendza, M., Schuurmann, G., 2003. Mixture toxicity and its modelling by quantitative structure–activity relationships. Environ. Toxicol. Chem. 22, 1900–1915.

Altenburger, R., Walter, H., Grote, M., 2004. What contributes to the combined effect of a complex mixture? Environ. Sci. Technol. 38, 6353-6362.

Andersson, L., Andersson, Y., 1994. Ecotocicological evaluation of the herbicide aclonifen. National Chemical Inspectorate, Solna, Sweden.

Backhaus, T., Faust, M., Scholze, M., Gramatica, P., Vighi, M., Grimme, L.H., 2004. Joint algal toxicity of phenylurea herbicides is equally predictable by concentration and independent action. Environ. Toxicol. Chem. 23, 258–264.

Backhaus, T., Faust, M., 2012. Predictive environmental risk assessment of chemical mixtures: a conceptual framework. Environ. Sci. Technol. 46, 2564-2573.

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

Battaglin, W.A., Furlong, E.T., Burkhardt, M.R., Peter, C.J., 2000. Occurrence of sulfonurea, sulfonamide, imidazolinone, and other herbicides in rivers, reservoirs and ground water in the Midwestern Unites States, 1998. Sci. Total Environ. 248, 123–133.

Bechmann M, Stenrød M, Pengerud A, Grønsten HA, Deelstra J, Eggestad HO, Hauken M., 2014. Erosjon og tap av næringsstoffer og plantevernmidler fra jordbruksdominerte nedbørfelt. Bioforsk Rapport 9 (84). Ås (Norway): Bioforsk. ISBN 978-82-17-01284-9.

Belden, J.B., Lydy, M.J., 2006. Joint toxicity of chlorpyrifos and esfenvalerate to fathead minnows and midge larvae. Environ. Toxicol. Chem. SETAC 25, 623–629.

Bellas, J., 2006. Comparative toxicity of alternative antifouling biocides on embryos and larvae of marine invertebrates. Sci. Total Environ. 367, 573-585.

Bliss, C.I., 1939. The toxicity of poisons applied jointly. Ann. J. Appl. Biol. 26, 585– 615.

Buman, R.A., gealy, D.R., Fuerst, E.P., 1992. Relationship between temperature and triazinone herbicide activity. Pestic. Biochem. Phys. 43, 22-28.

Cedergreen, N., Streibig, J.C., 2005. The toxicity of herbicides to non-target aquatic plants and algae: assessment of predictive factors and risk. Pest. Manag. Sci. 61, 1152–1160.

Cedergreen, N., 2014. Quantifying Synergy: A Systematic Review of Mixture Toxicity Studies within Environmental Toxicology. PLOS ONE 9 (5), e96580.

Chalew, T.E.A., Halden, R.U., 2009. Environmental exposure of aquatic and terrestrial biota to triclosan and triclocarban. JAWRA 45 (1), 4-13.

Choi, C.J., Berges, J.A., Young, E.B., 2012. Rapid effects of diverse toxic water pollutants on chlorophyll *a* fluorescence: variable responses among freshwater microalgae. Water Res. 46, 2615-2626.

Cima, F., Bragadin, M., Ballarin, L., 2008. Toxic effects of new antifouling compounds on tunicate haemocytes I. Sea-Nine 211™ and chlorothalonil. Aquat. Toxicol. 86, 299-312.

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 L-2985 Luxembourg, 328p.

ECHA, 2011. Annex 1 – Background document to the opinion proposing harmonised classification and labelling at Community level of Benzenamine, 2-chloro-6-nitro-3-phenoxy- (Aclonifen). Committee for Risk Assessment. European Chemicals Agency. ECHA/RAC/ CLH-O-0000001543-79-03/A1, 51 p.

EFSA, 2006. Conclusion regarding the peer review of the pesticide risk assessment of the active substance metribuzin. EFSA Scientific Report 88, 1-74.

Ensminger, M.P., Hess, F.D., 1985. Action spectrum of the activity of acifluorfen-methyl, a diphenyl ether herbicide, in *Chlamydomonas eugametos*. Plant Physiol. 77, 503-505.

EPA, 2004. Overview of the Ecological Risk Assessment Process in the Office of Pesticide Programs: Endangered and Threatened Species Effects Determinations. U.S. Environmental Protection Agency. Office of Prevention, Pesticide, and Toxic Substances. 92 p.

EU, 2009. Council of the European Union: Council conclusion on combination effects of chemicals, 2988th Environment Council Meeting, Brussels, 22 Dec. 2009.

EU, 2013. Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy. Official Journal of the European Union L 226/1-L 226/16.

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 Res. 37, 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. Environ. Toxicol. Chem. 17, 1830–1834.

Falkowski, P.G., Raven, J.A., 2007. Aquatic Photosynthesis, second ed. Princeton University Press, New Jersey, USA.

Faust, M., Altenburger, R., Backhaus, T., Blanck, H., Boedeker, W., Gramatica, P.,Hamer, V., Scholze, M., Vighi, M., Grimme, L.H., 2003. Joint algal toxicity of 16 dissimilarly acting chemicals is predictable by the concept of independentaction. Aquat. Toxicol. 63 (1), 43–63.

Fernández-Alba, A.R., Hernando, M.D., Piedra, L., Christi, Y., 2002. Toxicity evaluation of single and mixed antifouling biocides measured with acute toxicity bioassays. Anal. Chim. Acta 456, 303-312.

Fischer, B.B., Wiesendanger, M., Eggen, R.I.L., 2006. Growth condition-dependent sensisivity, photodamage and stress response of Chlamydomonas reinhardtii exposed to high light conditions. Plant Cell Physiol. 47(8), 1135-1145.

Gatidou, G., Thomaidis, N.S., 2007. Evaluation of single and joint toxic effects of two antifouling biocides, their main metabolites and copper using phytoplankton bioassays. Aquat. Toxicol. 85, 184–191.

Grossman, K., 2005. What it takes to get a herbicide's mode of action. Physionomics, a classical approach in a new complexion. Pest. Manag. Sci. 61 (5), 423-431.

Guenther, J.E., Nemson, J.A., Melis, A., 1990. Development of photosystem II in dark grow *Chlamydomonas reinhardtii*. A light-dependent conversion of PS II_{β}, Q_B-nonreducing centers to the PS II_{α}, Q_B-reducing form. Photosynth. Res. 24, 35-46.

Guseinova, I.M., Suleimanov, S.Y., Aliyev, J.A., 2005. The effect of norflurazon on protein composition and chlorophyll organization in pigment-protein complex of photosystem II. Photosynth. Res. 84, 71-76.

Harris, E.H., 2009. The Chlamydomonas Sourcebook. Introduction to Chlamydomonas and its laboratory use. Volume 1, 435 p.

Jones, R., 2005. The ecotoxicological effects of photosystem II herbicides on corals. Mar. Pollut. Bull. 51, 495-506.

Juneau, P., Qiu, B., Deblois, C.P., 2007. Use of chlorophyll fluorescence as a tool for determination of herbicide toxic effect: review. Toxicol. Environ. Chem. 89(4), 609-625.

Kilinc, Ö., Grasset, R., Reynaud, S., 2009. The herbicide aclonifen: the complex theoretical bases of sunflower tolerance. Pestic. Biochem. Physiol. 100, 193-198.

Kitajima, M., Butler, W.L., 1975. Quenching of chlorophyll fluorescence and primaty photochemistry in chloroplast by dibromothymoquinone. Biochim Biophys Acta 326, 105-115.

Ledford, H.K., Niuogi, K.K., 2005. Singlet oxygen and photo-oxidative stress management in plants and algae. Plant Cell. Environ. 28, 1037-1045.

Loewe, S., 1927. Die Mischarznei. Versuch einer allgemeinen pharmakologie der arzneikombinationen. Klin. Wochenschr 6, 1077-1085.

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 cyanibacteruium (*Microcystis*). Chemosphere 65, 618-626.

Lürling, M., 2011. Metribuzin impairs the unicell-colony transformation in the green alga *Scenedesmus obliquus*. Chemosphere 82, 411-417.

Magnusson, M., Heimann, K., Negri, A.P., 2008. Comparative effects of herbicides on photosynthesis and growth of tropical estuarine microalgae. Mar. Pollut. Bull. 56, 1545-1552.

McCarty, L.S., Mackay, D., 1993. Enhancing ecotoxicological modeling and assessment. Environ. Sci. Technol. 27 (9), 1719− 1728.

Moschet, C., Wittmer, I., Simovic, J., Junghans, M., Piazzoli, A., Singer, H., Stamm, C., Leu, C., Hollender, J., 2014. How a Complete Pesticide Screening Changes the Assessment of Surface Water Quality. Environ. Sci. Technol. 48, 5423–5432.

Nestler, H., Groh, K.J., Schönenberg, R., Behra, R., Schirmer, K., Eggen, R.I.L., Suter, H.J.-F., 2012. Multiple-endpoint assay provides a detailed mechanistic view of responses to herbicide exposure in *Chlamydomonas reinhardtii*. Aquat. Toxicol. 110-111, 214-224.

Nyman, A.-M., Schirmer, K., Ashauer, R., 2014. Importance of toxicokinetics for interspecies variation in sensitivity to chemicals. Environ. Sci. Technol. 48, 5946-5954.

OECD, 2011. OECD Guidelines for the Testing of Chemicals, Section 2: Effects on Biotic Systems Test No. 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test. OECD Publishing, Business & Economics, 1-25.

Oettmeier, W., Masson, K., Fedtke, C., Konze, J., Schmidt, R.R., 1982. Effect of different Photosystem II inhibitors on chloroplasts isolated from species either susceptible or resistant toward s-triazine herbicides. Pestic. Biochem. Physiol. 18, 357–367.

Pedersen, R., Bechmann, M., Deelstra, J., Eggestad, H.O., Greipsland, I., Stenrød, M., Fystro, G., Selnes, S., Riley, H., Stubhaug, E., 2014. Jord- og vannovervåking i landbruket (JOVA). Feltrapport fra programmet i 2012 (Bioforsk).

Petersen, K., Stenrød, M., Tollefsen, K.E., 2013. Initial environmental risk assessment of combined effects of plant protection products in six different areas in Norway. Norwegian Institute for Water Research, REPORT SNO. 6588-2013.

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. Toxicol. Environ. Chem. 89, 589–607.

Richards, P.R., Baker, D.B., 1993. Pesticide concentration patterns in agricultural drainage networks in the Lake Erie basin. Environ. Toxicol. Chem. 12, 13–26.

Ruedel, H., 2012. Environmental monitoring of biocides: an emerging issue? NORMAN bulletion 3, 3-4.

Schuler, L.J., Rand, G.M., 2008. Aquatic risk assessment of herbicides in freshwater

ecosystems of South Florida. Arch. Environ. Contam. Toxicol. 54, 571–583.

Scribner, E.A., Battaglin, W.A., Goolsby, D.A., Thurman, E.M., 2000. Changes in herbicide concentrations in Midwestern streams in relation to change in use, 1989–1998. Sci. Total Environ. 248, 255–263.

Shipitalo, M.J., Malone, R.W., Owens, L.B., 2008. Impact of glyphosate-tolerant soybean and glufosinate-tolerant corn production on herbicide losses in surface runoff. J. Environ. Qual. 37, 401–408.

Szívak, I., Behra, R., Sigg, L., 2009. Metal-indiced reactive oxygen species production in *Chlamydomonas reinhardtii* (Chlorophyceae). J. Phycol. 45, 427–435.

USEPA, 2002. Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms (4th ed.). EPA-821-R-02-013.

USEPA, 2008. Ecological risk and environmental revised risk assessment chapter triclosan (pc code: 054901; case no.: 2340). United States Environmental Protection Agency. Washington, d.c. 20460. Office of prevention, pesticides and toxic substances, 33 p.

Vaal, M., Van Leeuwen, C., Hoekstra, J., Hermens, J., 2000. Variation in sensitivity of aquatic species to toxicants: Practical consequences for effect assessment of chemical substances. Environ. Manage. 25 (4), 415−423.

Vighi, M., Altenburger, R., Arrhenius, Å, Backhaus, T., Bödeker, W., Blanck, H., Consolaro, F., Faust, M., Finizio, A., Froehner, K., Gramatica, P., Grimme, L.H., Grönvall, F., Hamer, V., Scholze, M., Walter, H., 2003. Water quality objectives of toxic chemicals: problems and perspectives. Ecotox. Environ. Safe. 54, 139-150.

von der Ohe, P.C., Schmitt-Jansen, M., Slobodnik, J., Brack, W., 2012. Triclosan – the forgotten priority substance? Environ. Sci. Pollut. Res. 19, 585–591.

Figure Captions

Fig. 1. Photosystem II (PSII) primary photochemical efficiency (F_v/F_m, % of control, %CT) in *Chlamydomonas reinhardtii* exposed for 24 h, 48 h and 72 h to atrazine (positive control). The data (Mean±SEM) represent experimental results from 3 independent experiments and their corresponding concentration-response curves (solid line).

Fig. 2. Photosystem II (PSII) primary photochemical efficiency (F_v/F_m, % of control, %CT) in *Chlamydomonas reinhardtii* exposed for 24 h, 48 h and 72 h to aclonifen, bifenox, dichlofluanid, metribuzin and bifenox. The data (Mean±SEM) represent experimental results from 3 independent experiments and their corresponding concentration-response curves (solid line).

Fig. 3. Photosystem II (PSII) primary photochemical efficiency expressed as normalized F_v/F_m (% of control, CT; closed circles) and growth rate (% of control, CT; closed triangles) in *Chlamydomonas reinhardtii* exposed for 24 h, 48 h and 72 h to aclonifen and metribuzin. The data (Mean±SEM) represent experimental results from 3 independent experiments and their corresponding concentration-response curves (solid line for F_v/F_m and broken line for growth rate).

Fig. 4. Photosystem II (PSII) primary photochemical efficiency (F_v/F_m) in *Chlamydomonas reinhardtii* exposed for 24 h, 48 h and 72 h to the binary mixture of aclonifen and metribuzin, along with the curves obtained for the mixture models CA and IA (normalized data) for each time-point. The data (Mean±SEM) represent experimental results from 3 independent experiments and their corresponding concentration-response curves (dotted line). Prediction of combined toxicity by CA (solid line) and IA (broken line) of the mixture is displayed to indicate assumptions of additivity.

Fig. 5. Growth rate (% of control, CT) for *Chlamydomonas reinhardtii* exposed for 24 h, 48 h and 72 h to the equipotent binary mixture of aclonifen and metribuzin (solid circles). The data (Mean±SEM) represent experimental results from 3 independent experiments and their corresponding concentration-response curves (dotted line). Prediction of combined toxicity by CA (solid line) and IA (broken line) of the mixture is displayed to indicate assumptions of additivity.

Fig. 6. Schematic representation of the Mode of Action (MoA) for the mixture of aclonifen and metribuzin. Interactions seem to occur in steps leading to the formation of ROS, with aclonifen not only inducing ROS, but can also preventing the repair of damages in the Photosystem II (PSII) complexes.

Table 1. Photosystem II (PSII) efficiency and growth inhibition in *Chlamydomonas reinhardtii* after exposure to the 2 biocides aclonifen, metribuzin and to the assay positive control atrazine. The data show the EC_{50} (nM; 95% confidence intervals in parentheses), Hill slope and goodness of fit (R^2) of the PSII efficiency and growth inhibition concentration-response curves at each time point (24 h, 48 h and 72 h) for each compound.

Test	**Compound**	**EC_{50} (nM)**			**Hill slope**			**Goodness of fit (R^2)**		
		24 h	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**
PSII efficiency	**Aclonifen**	1178 (648 to 2143)	481 (333 to 696)	578 (407 to 821)	-0.6	-1.2	-1.2	0.93	0.96	0.96
	Metribuzin	110 (90 to 136)	70 (59 to 83)	107 (86 to 133)	-0.8	-1.3	-0.6	0.99	0.99	0.98
	Atrazine*	$6x10^5$ ($5x10^5$ to $7x10^5$)	$6x10^5$ ($4x10^5$ to $9x10^5$)	$6x10^5$ ($5x10^5$ to $7x10^5$)	0.05	-1.3	-0.6	1.0	0.97	0.99
Growth inhibition	**Aclonifen**	429 (316 to 582)	294 (268 to 324)	298 (268 to 331)	-0.7	-1.1	-1.5	0.97	1.0	1.0
	Metribuzin	66 (47 to 92)	54 (48 to 61)	57 (52 to 62)	-1.7	-2.2	-2.4	0.96	0.99	1.0
	Atrazine*	$1x10^5$ ($1.03x10^5$ to $1.04 x10^5$)	$5x10^5$ ($1x10^5$ to $2x10^6$)	$1x10^5$ ($5x10^4$ to $2x10^5$)	-6.4	-0.5	-1.0	1.00	0.81	0.94

* Positive control

Table 2. Correlation (Spearman's correlation coefficient, r) between the Photosystem II (PSII) efficiency and the inhibition of growth for *Chlamydomonas reinhardtii* exposed to aclonifen and metribuzin and to their equipotent binary mixture at 24 h, 48 h and 72 h.

	24 h		**48 h**		**72 h**	
Spearman's correlation	**r**	***p*-value**	**r**	***p*-value**	**r**	***p*-value**
Aclonifen	0.991	0.0004*	0.964	0.0014*	1	0.0002*
Metribuzin	1	0.0002*	1	0.0002*	1	0.0002*
Mixture	1	< 0.0001*	1	0.0002*	1	0.0014*

*Statistically significant

Table 3. Inhibition of Photosystem II (PSII) in *Chlamydomonas reinhardtii* after exposure to the equipotent mixture of the aclonifen and metribuzin. The data show the EC_{50} (nM; 95% confidence intervals of EC_{50} between parentheses), Hill slope and goodness of fit (R^2) for the experimental data and the corresponding CA and IA models, and model deviation ratios (MDRs) for each model at each time-point. The different effect levels (PSII efficiency) for the mixture are shown for each model at each time point (24 h, 48 h and 72 h). Note: bold text indicates that MDRs were within a factor of two and * indicates that the model predictions were not significantly different from the observed data.

		24 h		**48 h**		**72 h**	
Experiment	**EC_{50} (nM)**	201 (175 to 231)		126 (107 to 147)		111 (82 to 151)	
	Hill slope	-1.0		-1.7		-1.2	
	Goodness of fit (R^2)	0.99		0.98		0.95	
CA model	**EC_{50} (nM)**	523 (520 to 526)		275 (275 to 275)		341 (328 to 355)	
	Hill slope	-0.7		-1.2		-0.8	
	Goodness of fit (R^2)	1.00		1.00		1.00	
IA model	**EC_{50} (nM)**	270 (260 to 280)		259 (254 to 264)		227 (213 to 241)	
	Hill slope	-0.8		-1.5		-1.0	
	Goodness of fit (R^2)	1.00		1.00		1.00	
	PSII efficiency (%)	**MDR CA**	**MDR IA**	**MDR CA**	**MDR IA**	**MDR CA**	**MDR IA**
	95	**0.7**	**0.6***	**1.1**	**1.5***	**1**	**1.2***
	90	**0.9**	**0.8***	**1.3**	**1.6***	**1.4**	**1.3***
	80	**1.4**	**1.0***	**1.6**	**1.8***	**1.8**	**1.6***
	70	**1.8**	**1.1***	**1.8**	**1.9***	2.2	**1.7***
	60	2.2	**1.2***	**2**	**2.0***	2.6	**1.9***
	50	2.6	**1.3***	2.2	2.1*	3.1	**2.0***
	40	3.1	**1.5***	2.4	2.1*	3.6	2.2*
	30	3.9	**1.7***	2.7	2.2*	4.2	2.4*
	20	5	**1.9***	3	2.4*	5.2	2.7*

10	7.2	2.3*	3.7	2.6*	7	3.1*
5	10.2	2.8*	4.3	2.8*	9.2	3.6*

Table 4. Inhibition of *Chlamydomonas reinhardtii* growth after exposure to the equipotent mixture of the aclonifen and metribuzin. The data show the EC_{50} (nM; 95% confidence intervals of EC_{50} between parentheses), Hill slope and goodness of fit (R^2) for the experimental data and the corresponding CA and IA models, and model deviation ratios (MDRs) for each. The different effect levels (growth rate) for the mixture are shown for each model at each time point (24 h, 48 h and 72 h). Note: bold text indicates that MDRs were within a factor of two and * indicates that the model predictions were not significantly different from the observed data.

		24 h		**48 h**		**72 h**	
Experiment	**EC_{50} (nM)**	152 (124 to 185)		129 (113 to 147)		111 (90 to 136)	
	Hill slope	-2.5		-2.6		-2.9	
	Goodness of fit (R^2)	0.97		0.99		0.98	
CA model	**EC_{50} (nM)**	253 (244 to 263)		199 (197 to 200)		207 (206 to 207)	
	Hill slope	-0.9		-1.4		-1.8	
	Goodness of fit (R^2)	1.00		1.00		1.00	
IA model	**EC_{50} (nM)**	213 (199 to 228)		221 (214 to 228)		252 (247 to 257)	
	Hill slope	-1.1		-1.6		-2.0	
	Goodness of fit (R^2)	1.00		1.00		1.00	
	Growth rate (%)	**MDR CA**	**MDR IA**	**MDR CA**	**MDR IA**	**MDR CA**	**MDR IA**
	95	0.2	0.3*	**0.6***	**0.9**	**1.0***	**1.5**
	90	0.4	0.4*	**0.7***	**1**	**1.2***	**1.6**
	80	**0.6**	**0.7***	**1.0***	**1.2**	**1.4***	**1.8**
	70	**0.9**	**0.9***	**1.2***	**1.4**	**1.5**	**2**
	60	**1.3**	**1.1***	**1.3***	**1.6**	**1.7**	2.1
	50	**1.7**	**1.4***	**1.5***	**1.7**	**1.9**	2.3
	40	2.2	**1.7***	**1.8**	**1.9**	**2**	2.4
	30	3	2.2*	**2**	2.1	2.2	2.6
	20	4.3	2.9*	2.4	2.4	2.5	2.8
	10	7.6	4.4*	3.2	2.9	3	3.2

5	12.7	6.5*	4.1	3.4	3.6	3.5

Table 5. Risk assessment for *Chlamydomonas reinhardtii* based on the PSII efficiency data, using the risk quotient (RQ) and toxic unit (TU) for the single compounds and the sum of the risk quotients (SRQ), sum of the toxic units (STU), and on the overall risk quotient (RQ_{STU}) for the binary mixture. EC_{50} - concentration of a compound that gives half-maximal response; NOEC - No Observed Effect Concentration (NOEC); MEC - Measured Environmental Concentrations. Note: bold text indicates a potential risk.

		Aclonifen	Metribuzin	SRQ	STU	RQ_{STU}
EC_{50} (nM)		578	107			
NOEC (nM)		125	31			
MEC (nM)	**Min**	0.01	0.0005			
	Median	0.7	1			
	95	4	8			
	Max	5	8			
RQ	**MEC_{min}**	0.0001	0.00002	0.0001		
	MEC_{median}	0.01	0.04	0.05		
	MEC_{95}	0.03	0.3	0.3		
	MEC_{max}	0.04	0.3	0.3		
TU	**MEC_{min}**	0.00003	0.00001		0.00003	0.003
	MEC_{median}	0.001	0.02		0.02	**2**
	MEC_{95}	0.01	0.1		0.1	**13**
	MEC_{max}	0.01	0.1		0.1	**13**

Figure 1

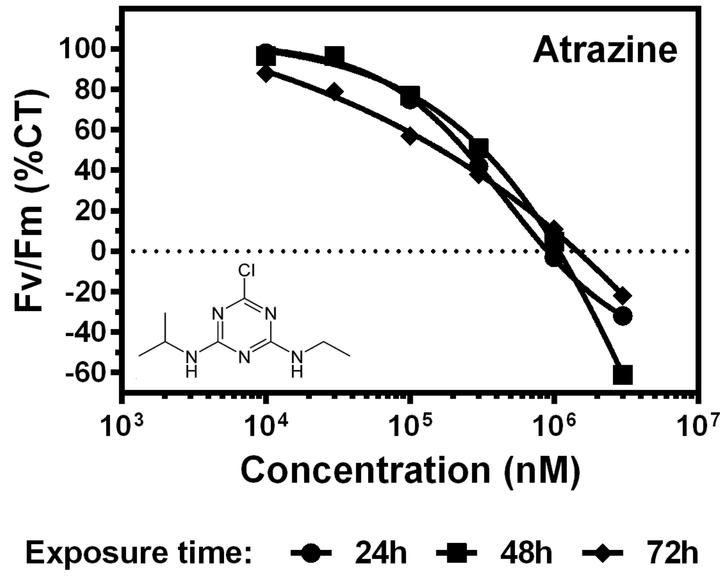


Figure 2

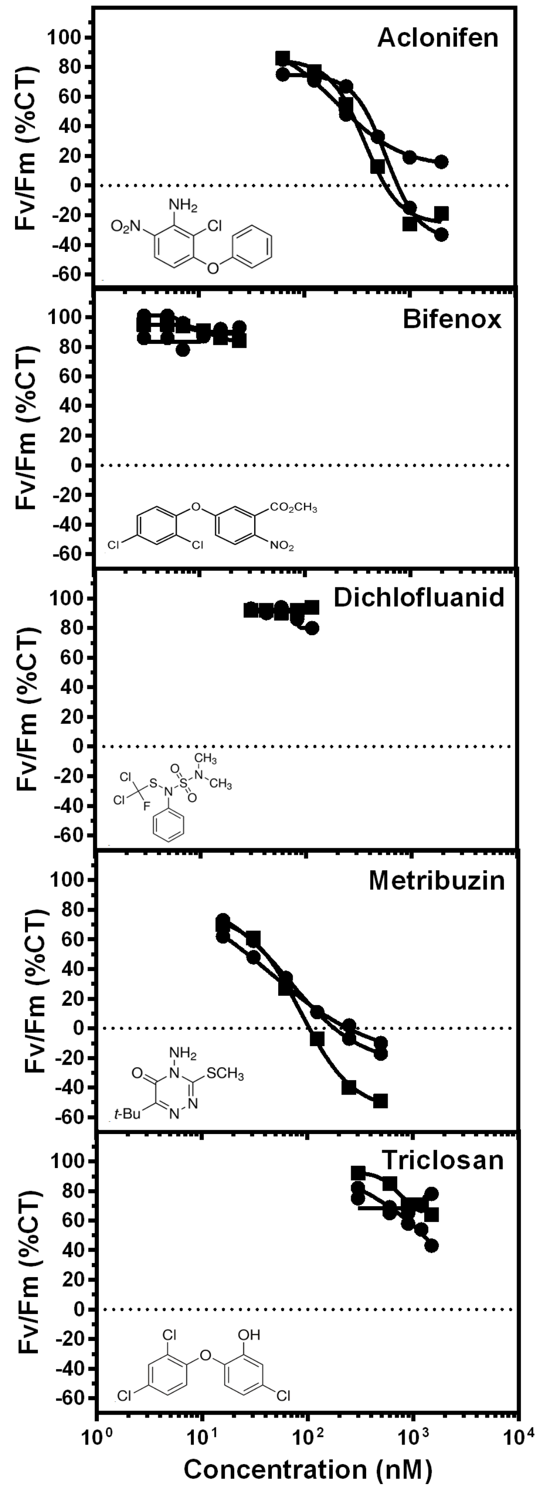


Figure 3

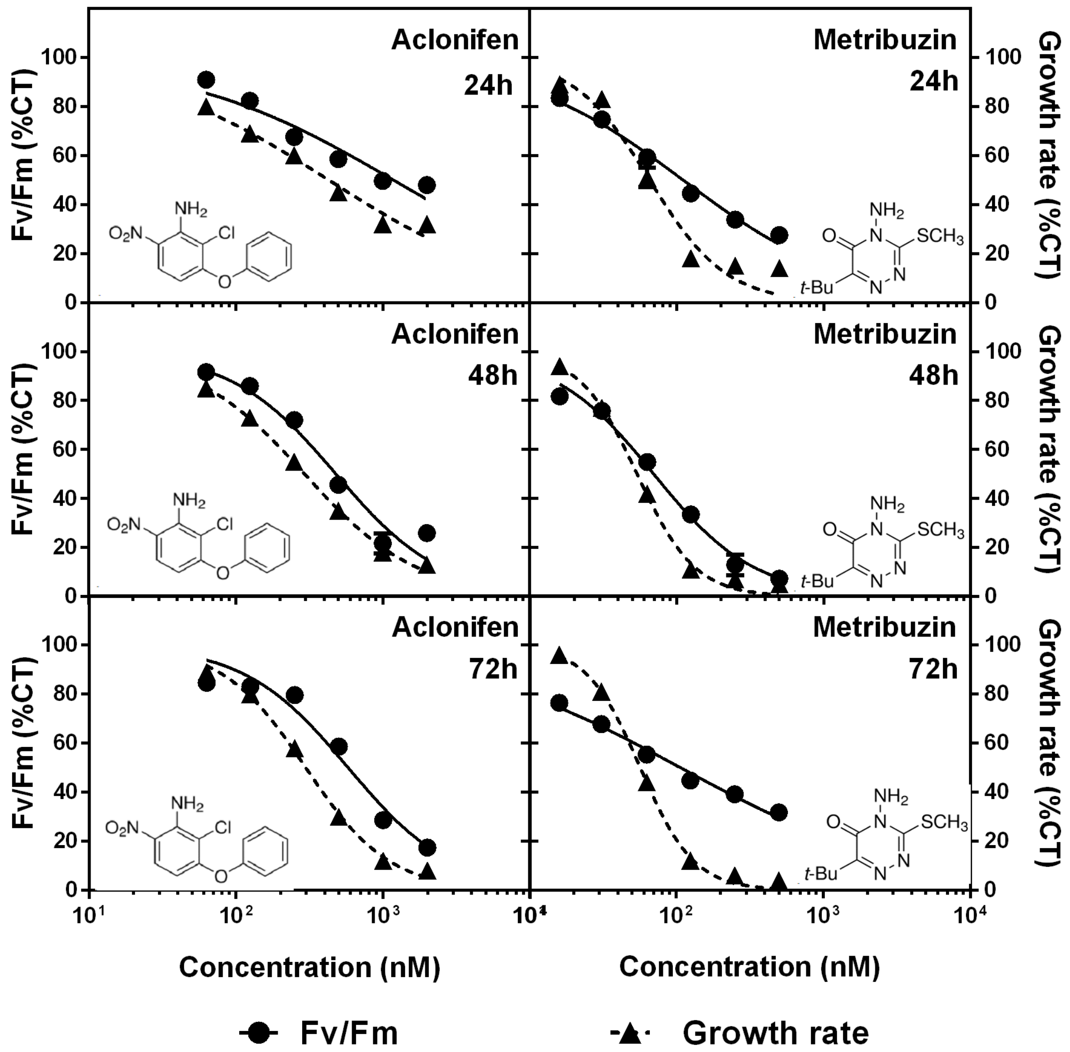


Figure 4

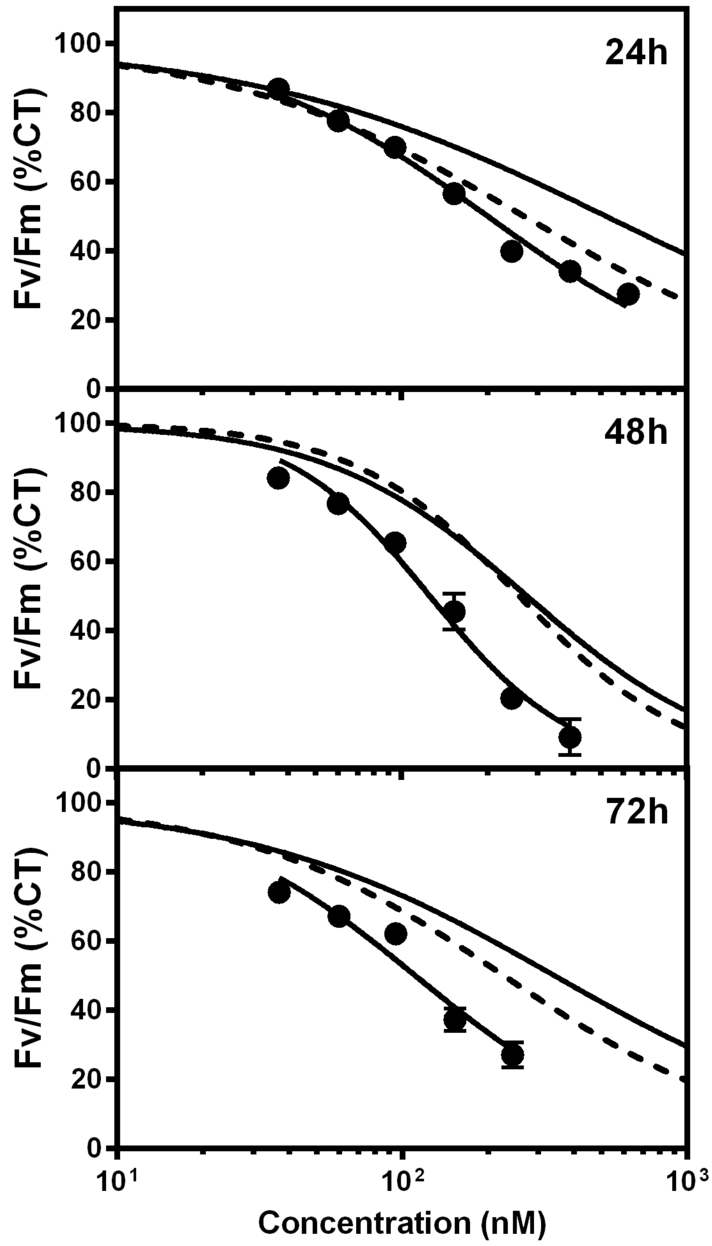


Figure 5

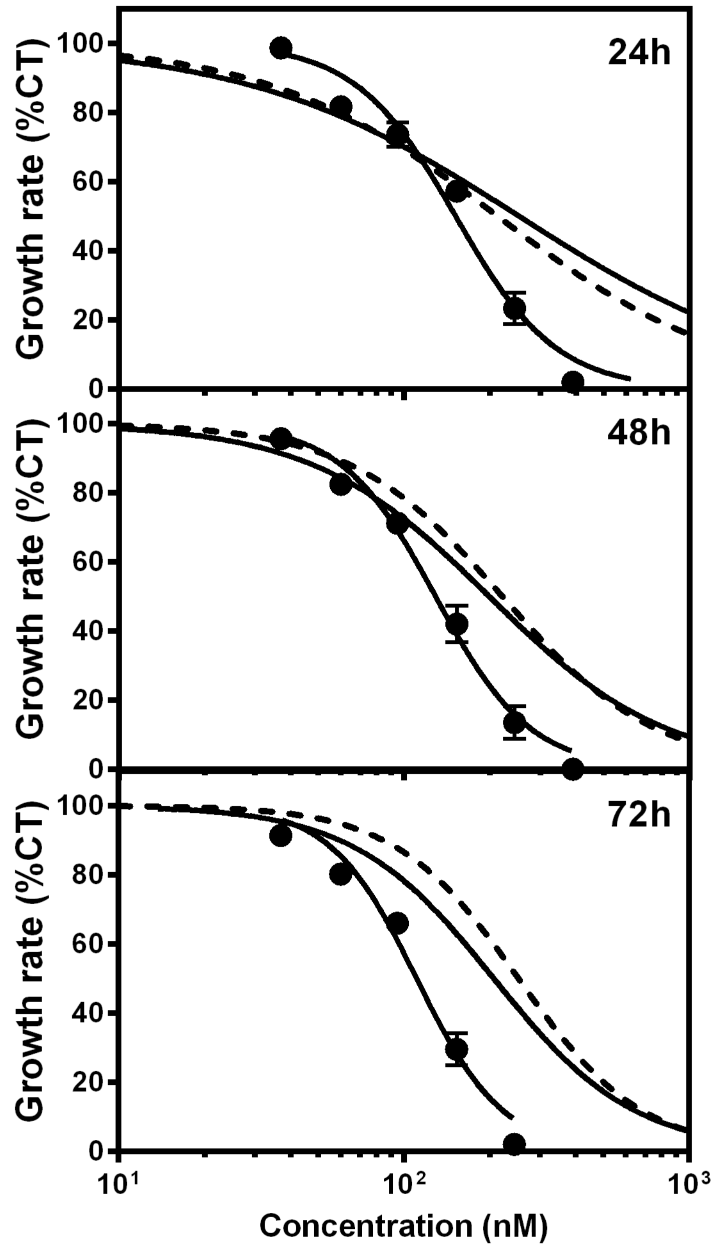
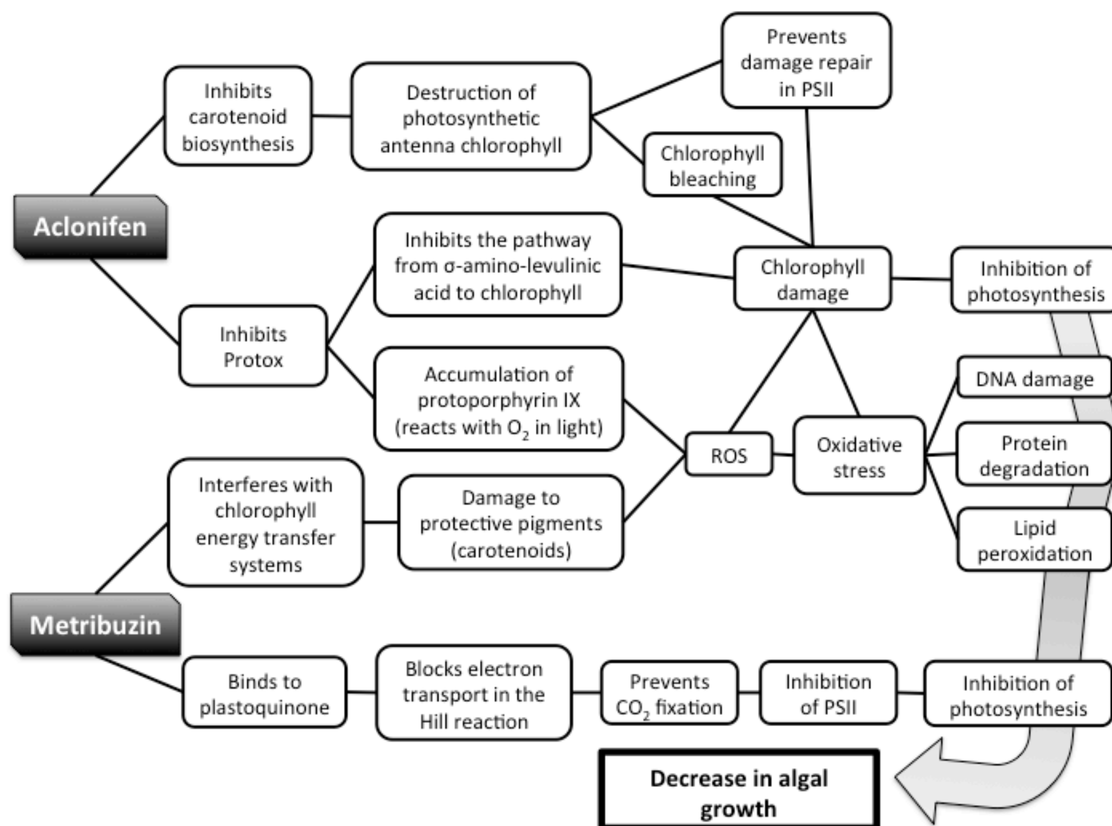


Figure 6



Photosystem II (PSII) efficiency in *Chlamydomonas reinhardtii* exposed to environmentally occurring biocides

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Appendix A. Supplementary data

Table 1. Exposure concentrations used for each compound and mixture.

Compound/mixture	nM
Aclonifen	63 125 250 500 1000 2000
Bifenox	3 5 7 11 16 24
Dichlofluanid	30 42 58 82 114 160
Metribuzin	16 31 63 125 250 500
Triclosan	300 600 900 1200 1500 1800
Mixture Total concentration: 1.0×10^4 • Aclonifen: 1.3×10^3 • Metribuzin: 2.5×10^2	37 60 95 153 244 391 625 1000

Table 2. Concentrations (M) of the stock standard solutions used for the exposures and the respective value obtained from chemical analysis (M). Note: - method not sufficiently robust for aclonifen (Almeida et al., in prep).

Compounds	Stock solutions (M)	Chemical analysis (M)
Aclonifen	0.01	-
Bifenox	0.0014	0.0012
Dichlofluanid	0.0006	0.0005
Metribuzin	0.023	0.025
Triclosan	0.023	0.019

Table 3. Nominal exposure concentrations (M) of each compound on the equipotent mixture and the respective value obtained from chemical analysis (M). Note: - method not sufficiently robust for aclonifen.

Compounds	Nominal exposure concentration (M)	Chemical analysis (M)
Aclonifen	0.01	-
Metribuzin	0.0009	0.0007

Table 4. Significant different between the effect concentrations for the observed data and for those calculated from each prediction model (Concentration addition (CA) and Independent Action (IA)) for the Photosystem II (PSII) efficiency, after one-way ANOVA analysis in combination with the Tukey post hoc test. Only *p-values* lower than 0.05 were considered significant. Note: SS – sum of the squares; DF – degrees of freedom; MS – mean squares; F – ratio between the two mean square values; DFn – degrees of freedom for the numerator; DFd - degrees of freedom for the denominator.

Time-point	**ANOVA**	**SS**	**DF**	**MS**	**F (DFn, DFd)**	***P-value***
24 h	**Treatment (between columns)**	3911000000	2	1955000000	F (2, 294) = 3.188	P = 0.0427
	Residual (within columns)	1.803E+11	294	613400000		
	Total	1.843E+11	296			
	Tukey's multiple comparisons test	**Mean Diff.**	**95% CI of diff.**	**Significant**		
	Observed vs. CA	-8312	-16605 to -19.87	Yes		
	Observed vs. IA	-1429	-9722 to 6863	No		
	CA vs. IA	6883	-1409 to 15175	No		
48 h	**Treatment (between columns)**	12880000	2	6438000	F (2, 294) = 7.026	P = 0.0010
	Residual (within columns)	269400000	294	916346		
	Total	282300000	296			
	Tukey's multiple comparisons test	**Mean Diff.**	**95% CI of diff.**	**Significant**		
	Observed vs. CA	-507	-827.5 to -186.5	Yes		
	Observed vs. IA	-301.3	-621.8 to 19.23	No		
	CA vs. IA	205.8	-114.8 to 526.3	No		
72 h	**Treatment (between columns)**	348700000	2	174400000	F (2, 294) = 3.975	P = 0.0198
	Residual (within columns)	12900000000	294	43860000		
	Total	13250000000	296			
	Tukey's multiple comparisons test	**Mean Diff.**	**95% CI of diff.**	**Significant**		
	Observed vs. CA	-2588	-4806 to -370.7	Yes		

	Observed vs. IA	-784.3	-3002 to 1433	No		
	CA vs. IA	1804	-413.6 to 4021	No		

Table 5. Significant different between the effect concentrations for the observed data and for those calculated from each prediction model (Concentration addition (CA) and Independent Action (IA)) for the growth inhibition, after one-way ANOVA analysis in combination with the Tukey post hoc test. Only *p-values* lower than 0.05 were considered significant. Note: SS – sum of the squares; DF – degrees of freedom; MS – mean squares; F – ratio between the two mean square values; DFn – degrees of freedom for the numerator; DFd - degrees of freedom for the denominator.

Time-point	**Effect concentration (EC)**	**ANOVA table**	**SS**	**DF**	**MS**	**F (DFn, DFd)**	***P-value***
24 h	$\leq EC_{99}$	**Treatment (between columns)**	73220000	2	36610000	F (2, 294) = 4.686	P = 0.0099
		Residual (within columns)	2297000000	294	7813000		
		Total	2370000000	296			
		Tukey's multiple comparisons test	**Mean Diff.**	**95% CI of diff.**	**Significant**		
		Observed vs. CA	-1214	-2150 to -278.2	Yes		
		Observed vs. IA	-543.9	-1480 to 392.0	No		
		CA vs. IA	670.1	-265.7 to 1606	No		
48 h	$> EC_{40}$	**Treatment (between columns)**	58545	2	29273	F (2, 174) = 7.814	P = 0.0006
		Residual (within columns)	651802	174	3746		
		Total	710347	176			
		Tukey's multiple comparisons test	**Mean Diff.**	**95% CI of diff.**	**Significant**		
		Observed vs. CA	-24.79	-51.42 to 1.852	No		
		Observed vs. IA	-44.45	-71.09 to -17.81	Yes		
		CA vs. IA	-19.66	-46.30 to 6.974	No		

72 h	**$> EC_{70}$**	**Treatment (between columns)**	29520	2	14760	F (2, 84) = 16.10	P < 0.0001
		Residual (within columns)	77003	84	916.7		
		Total	106523	86			
		Tukey's multiple comparisons test	**Mean Diff.**	**95% CI of diff.**	**Significant**		
		Observed vs. CA	-17	-35.97 to 1.968	No		
		Observed vs. IA	-44.7	-63.67 to -25.73	Yes		
		CA vs. IA	-27.69	-46.66 to -8.722	Yes		

Table 6. Environmental concentrations of aclonifen and metribuzin found in literature (Almeida et al., in prep).

Compound	Concentration (nM)	Country	References
Aclonifen	0.013	France	EC, 2006
	0.567	France	EC, 2006
	2.343	France	EC, 2011
	5.195	France	EC, 2011
	2.645	France	EC, 2011
	0.189	Sweden	EC, 2011
	0.068	Sweden	EC, 2011
	0.869	Norway	Pedersen et al., 2014
Metribuzin	0.560	Norway	Pedersen et al., 2014
	0.933	US	Battaglin et al., 2001
	1.535	US	Battaglin et al., 2001
	8.214	US	Battaglin et al., 2001
	1.773	US	EPA, 2004
	0.233	US	EPA, 2004
	0.467	US	Hostovsky et al., 2014
	8.214	US	Hostovsky et al., 2014
	0.0005	Canada	Kumar et al. 2013
	0.644	Brazil	Hostovsky et al., 2014
	1.638	Brazil	Hostovsky et al., 2014

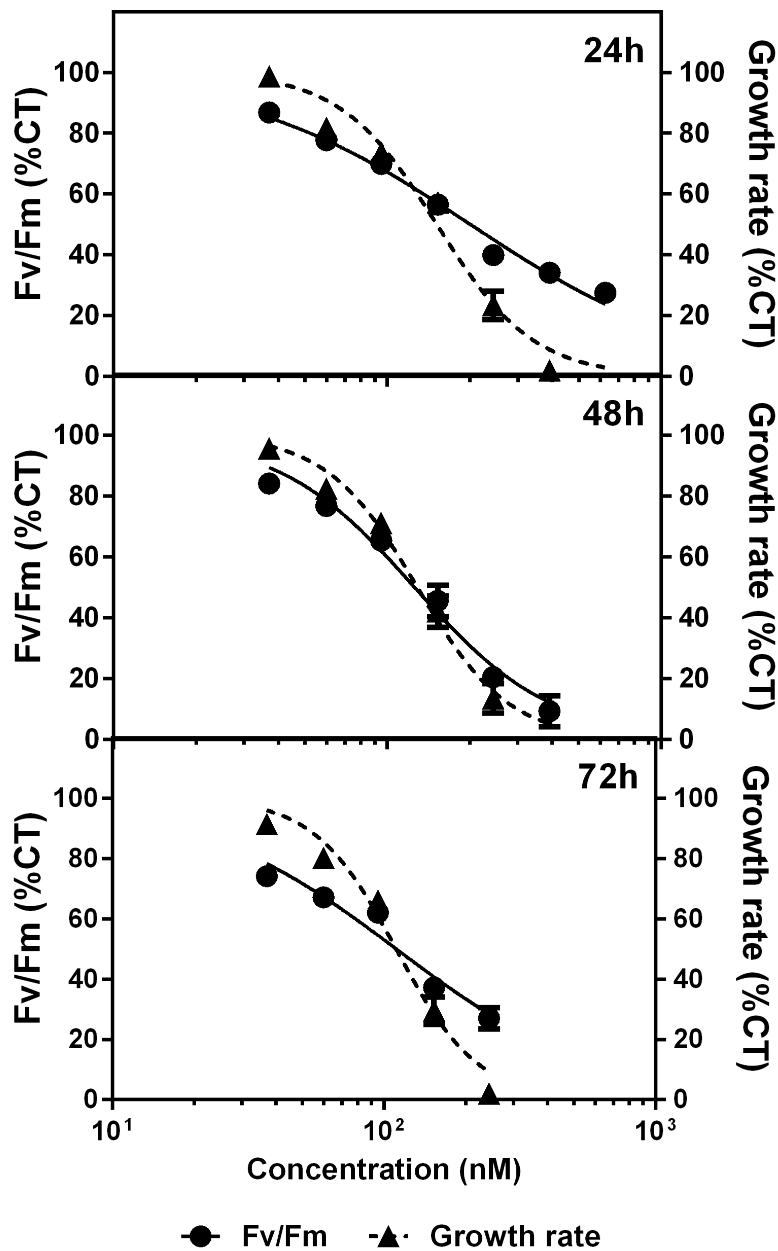

Fig. 1. PSII primary photochemical efficiency (F_v/F_m, % of control, CT; normalized data; closed circles) and growth rate (% of control, CT; closed triangles) in *Chlamydomonas reinhardtii* exposed for 24 h, 48 h and 72 h to the mixture of aclonifen and metribuzin. The data (Mean±SEM) represent experimental results from 3 independent experiments and their corresponding concentration-response curves for F_v/F_m (solid line) and for growth rate (broken line).

Paper III

Induction of reactive oxygen species (ROS) in *Chlamydomonas reinhardtii* after exposure to single biocides and their simple mixtures

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Abstract

Reactive oxygen species (ROS) are normal by-products of cellular respiration and photosynthesis in primary producers. The toxicity of biocides can be associated with the formation of ROS, as these compounds are known to interfere with the photosynthetic apparatus. This study investigated the production of ROS in the microalgae *Chlamydomonas reinhardtii* exposed to five environmentally relevant biocides aclonifen, bifenox, dichlofluanid, metribuzin, and triclosan, and their mixtures. A high-throughput method using the fluorescent probe carboxy-H_2DFFDA was used for detection of ROS. Results showed that aclonifen, bifenox and metribuzin induced ROS in a concentration-dependent manner. Aclonifen, a Protox and carotenoid inhibitor, was the most toxic and closely followed by the photosystem II (PSII) inhibitor metribuzin and the Protox inhibitor bifenox. The bactericide triclosan and the antifoulant dichlofluanid did not produce ROS at the concentrations tested. The combined effects of the three herbicides were studied in binary and ternary mixtures using the Concentration Addition (CA) and Independent Action (IA) prediction models. The best predictions were achieved by CA when testing the ternary mixture and the binary mixture of aclonifen and bifenox at low to median effect levels, whereas synergism was observed at high concentrations. IA best predicted the mixture of aclonifen and metribuzin, while both models equally predicted the mixture of bifenox and metribuzin. The production of ROS was identified as a relevant toxic mechanism for the effects of these herbicides in *C. reinhardtii* and a description of their mode of action was proposed to decipher how they act in combination to cause additivity and synergism.

Keywords: Biocides; Reactive oxygen species (ROS); Mixture toxicity; Prediction models; Freshwater microalgae.

Introduction

Biocides are products extensively used to control organisms considered dangerous to human or animal health, or that can damage natural or manufactured materials. Due to their intrinsic characteristics, these chemicals can also affect non-target organisms present in the aquatic environment including primary producers such as algae and plants. These compounds are subject to several regulations at the international level (EU, 2013), but with their continuous and increased use, thorough analysis on their toxic and hazardous effects on non-target organisms is necessary (ECHA, 2014). The toxicity of biocides can conveniently be determined in microalgae, an important group of photosynthetic organisms accounting for more than 50% of global primary production in the aquatic environment (Harris, 2009). *Chlamydomonas reinhardtii* is a well-studied microalgae that has been used as a model organism in several physiological, biochemical and genetic studies for more than a decade. It has a rapid growth, attaining a logarithmic growth in 3 days, being easily maintained in controlled laboratory conditions (Harris, 2009). It is considered particularly sensitive to biocides exposure (Chalew and Halden, 2009), and has already been used to assess the toxicity of numerous single biocides such as paraquat (Prado et al., 2009), diuron and norflurazon (Nestler et al., 2012a), as well as mixtures of several biocides (Fischer et al., 2010; Knauert et al., 2008).

Some biocides are considered of specific concern due to their high toxicity to primary photosynthetic organisms in combination with a wide distribution in surface waters and a general lack of thorough toxicity assessments (EU, 2013; USEPA, 2008). Some of these compounds such as the two herbicides aclonifen and bifenox have already been proposed as priority aquatic substances by the European Union (EU, 2013). Aclonifen is known to inhibit carotenoid biosynthesis, while bifenox inhibit specific enzymes present in the chloroplasts both leading to chlorophyll inhibition (Grossman, 2005; Kilinc et al., 2011). Metribuzin, another herbicide, is known to be highly toxic to primary producers by interfering with the electron transport during photosynthesis (Fairchild et al., 1998). Other biocides such as dichlofluanid (antifoulant, fungicide, acaricide, wood preservative, etc.) display multiple modes of action (MoAs) involving the inhibition of thiol-containing enzymes and disruption of mitochondrial function in several organisms (Cima et al., 2008). Another important priority substance with multiple MoAs is triclosan (von der Ohe et al., 2012), a widely used

compound in personal care products (PCPs), but also commonly used as a wood preservative, bactericide and fungicide (USEPA, 2008).

Knowledge on the mode of action (MoA) is essential to understand biocide toxicity and how they interact with certain biological targets. Some of the toxicity of biocides is associated with the formation of reactive oxygen species (ROS), through interference with the photosynthetic apparatus of organisms (Jamers and Coen, 2010; Ramel et al., 2009; Szivák et al., 2009; Nestler et al., 2012b). ROS are by-products of cellular respiration and light associated photosynthetic mechanisms, and normally formed due to the escape of electrons from the electron transport activities of mitochondrias, plasma membranes and chloroplasts (Foyer et al., 1997). Superoxide radicals ($O_2•^-$), hydroxyl radical (•OH) and hydrogen peroxide (H_2O_2) are some of the most common ROS that can react with biomolecules and change their biochemical activities. Exogenous stressors can also stimulate ROS production in biological systems including the exposure to trace and heavy metals (Weckx and Clijsters, 1996; Stoiber et al., 2011), herbicides (Alscher et al., 1997), high light intensities (Foyer et al., 1997), dryness (Loggini et al., 1999), extreme temperature and UV radiation (He and Häder, 2002), osmotic stress (Boo and Jung, 1999), mechanical and physical stresses (Legendre et al., 1993), and pathogens (Low and Merida, 1996). Cellular ROS generated by the electron transport processes in chloroplasts and mitochondria are usually low and strictly regulated by antioxidant mechanisms to maintain a “redox homeostasis” within cells (Apel and Hirt, 2004; Pospíšil, 2009). However, the increased production of ROS due to the presence of contaminants may overwhelm the antioxidant capacity of cells and consequently cause oxidative damage in DNA, proteins and lipids. To a further extent this increase in oxidative stress can result in adverse toxic effects as mutations, necrosis, apoptosis and mortality (Knauert and Knauer, 2008; Stoiber et al., 2007, 2011).

Few studies have directly measured the production of ROS in microalgae exposed to biocides. Most of the studies on ROS formation in microalgae have focused on metals and in particular the determination of sub-lethal responses such as modulation of the antioxidant system (levels of glutathione and expression of antioxidant enzymes) and/or increase in cellular damage including lipid peroxidation and DNA damage and repair (Stoiber et al., 2007; Žegura et al., 2004). A direct ROS production assay using non-fluorescent probes like 5-(and-6)-carboxy-2′,7′-difluorodihydrofluorescein diacetate (carboxy-H_2DFFDA) has been implemented to assess the intracellular generation of ROS in cells and simple organisms such as microalgae

(e.g., Sziválk et al., 2009). This probe is a chemically reduced acetylated form of fluorescein, more photostable and cell-permeable. After entering cells, carboxy-H_2DFFDA is hydrolysed by cellular esterases to non-fluorescent difluorodihydrofluorescein (H_2DFF), which is then oxidized by the presence of ROS producing the highly green-fluorescent difluorofluorescein, DFF (Gunawan et al., 2013). Even though this probe is normally described as a marker of general oxidative stress in cells, it has proven useful to detect ROS formation in microalgae exposed to contaminants such as the herbicides diuron, norfluzan and paraquat (Nestler et al., 2012a) and metals such as Ag, Cr, Cu, Fe, Pb and Zn (Sziválk et al., 2009).

Single toxicity assessment for biocides is not sufficient to adequately address realistic exposure scenarios, as these compounds exist as mixtures in the environment. Combined effects such as additivity, antagonism and synergy of multi-compound mixtures have already been observed in freshwater environments (Cedergreen, 2014; Gatidou and Thomaidis, 2007). The combined effects of contaminants in a mixture are often characterized by prediction models developed from concepts such as Concentration Addition (CA; Loewe, 1927) and Independent Action (IA; Bliss, 1939). These models are based on the hypothesis that all the compounds in a mixture affect the same endpoint in the same trend, acting by similar (CA) or dissimilar (IA) MoA. Deviations from these additive effects indicate that interactions of two or more compounds are occurring in the mixture and may give rise to either synergy or antagonism (Altenburger et al., 2003). Although additivity is most commonly observed for the toxicity of biocides on microalgae (Altenburger et al., 2003; Petersen et al. 2014), synergism has also been reported for certain mixtures (Belden et al., 2007; Cedergreen, 2014).

This study investigated the production of ROS in *C. reinhardtii* exposed to five ecologically relevant biocides aclonifen, bifenox, dichlofluanid, metribuzin, and triclosan. These responses were compared with those of microalgae exposed to 3 well known ROS inducers atrazine, paraquat and hydrogen peroxide (H_2O_2). A method using the carboxy-H_2DFFDA probe was used to provide a rapid (6 h) and high-throughput (96 well format) assay to screen compounds for their capacity to produce ROS in *C. reinhardtii*. In addition, combined toxicity assessment with simple mixtures of the ROS-generating biocides was conducted to characterise how these produced ROS when present in simple mixtures.

Materials and Methods

Test compounds

Hydrogen peroxide (H_2O_2, CAS number: 7722-84-1) was used as positive control. As several preliminary studies had to be performed to optimize the assay, atrazine (CAS number: 1912-24-9) and paraquat dichloride hydrate (CAS number: 75365-73-0) were also used as controls. The target biocides studied were: aclonifen (CAS number: 74070-46-5), bifenox (CAS number: 42576-02-3), dichlofluanid (CAS number: 1085-98-9), metribuzin (CAS number: 21087-64-9), and triclosan (CAS number: 3380-34-5). All were purchased from Sigma-Aldrich (United Kingdom) with ≥ 97.0% purity, except H_2O_2 with ≥ 30% purity. The test compounds were all diluted in anhydrous dimethyl sulfoxide (DMSO, Sigma-Aldrich, United Kingdom) and stored at -20°C until use. The positive controls paraquat and H_2O_2 were directly diluted in the assay buffer 0.01 M (3-(N-Morpholino)propanesulfonic acid (MOPS, CAS number: 1132-61-2; ≥ 99.5% purity) with a pH of 7.45.

Microalgae cultures

The freshwater green microalgae *Chlamydomonas reinhardtii* (NIVA-CHL153; Norwegian Institute for Water Research, Oslo, Norway) was used for testing the formation of ROS after exposure to the test compounds and their mixtures. Algal cells were initially cultured in glass flasks, with an initial number of 10^7 cells in 1L Talaquil media (Sziválk et al., 2009), prepared at least 24h before usage to allow the equilibrium of media components. Flasks were incubated for 3 to 4 days in an Infors Multitron 2 incubator shaker (Infors AG, Bottmingen, Switzerland) at 20 ± 2°C, with orbital shaking at 90 rpm and under continuous light (83 ± 6 $\mu mol/m^2/s^1$) provided by cool–white fluorescence lamps (TLD 36W/950, Philips, London, UK). Immediately before each test, algal cells were collected by centrifugation, washed and resuspended in the assay buffer.

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

ROS assay

The ROS production was determined essentially as described by Sziválk et al. (2009) and Stoiber et al. (2011) for *C. reinhardtii* after optimization of algae density, choice of

microplates, concentration of probe and exposure time. Stock solutions of 50 mM carboxy-H_2DFFDA (Invitrogen, Molecular Probes Inc., Eugene, OR, USA) were prepared in anhydrous DMSO and stored in aliquots at -20°C until use. In brief, a final concentration of $3x10^5$ cells in 100 µl of MOPs was added to each well in a 96-well microplate (Falcon™, Oslo, Norway). 100 µl of assay working solution was prepared by diluting the probe in assay buffer (final concentration 5 µM) with the test compounds at the different concentrations (final concentration of DMSO 0.05% v/v). The working solution for each biocide was added to the microplate containing the algae suspension and incubated under ambient light for 6 h at room temperature. As H_2DFFDA is transformed to fluorescent difluorodihydrofluorescein diacetate (DFFDA) by oxidation, the resulting fluorescent product was directly quantified by fluorescence using the microplate reader 1400 Multilabel Counter, Victor 3 (Perkin Elmer) at a excitation/emission wavelength of 488/520 nm (Szivák et al., 2009). Readings were recorded hourly to monitor the ROS formation for a maximum of 6 h. At the end of exposure, microalgae cells were observed under the microscope to verify their survival, and only concentrations with live cells were taken into account into the subsequent analyses. The natural fluorescence of the compounds in combination with the probe (without presence of algae) was analysed and this fluorescence was subtracted to eliminate interference of non-algal ROS production with the fluorescence readings. The formation of ROS was determined under normal light conditions and expressed as fold induction comparative to the control.

Single chemicals toxicity

Data from the compounds inducing ROS was normalized according to minimum and maximum values of H_2O_2 (positive control) and modelled with a non-linear regression using a sigmoidal dose-response curve with variable slope. The equation used was:

$$\gamma = Bottom + \frac{Top - Bottom}{1+10^{(\log EC_{50} - \, logX) \times Slope)}} \qquad \textbf{Eq. 1}$$

Where Y is the effect, X is the concentration, Bottom is the baseline effect (control), top is the maximal effect plateau (maximum ROS formation), and log EC_{50} is the concentration causing 50% effect.

Concentration-response analyses were made with the same method for all compounds, and the EC_{50}, Hill slope and goodness of fit (R^2) values were calculated.

Combined toxicity

Only compounds that caused ROS formation were taken into consideration for the mixture toxicity study. A fixed fixed-ratio (equitoxic) ray design was used, based on the ratios of the EC_{50} concentrations from the individual concentration response curves (CRCs) after 6 h exposure. Equal toxicity contribution of each component was determined on basis of the studies with single stressors to avoid that a single compound dominated the overall response (consult Table 1 in supplementary data for information on the concentration of each compound in the mixtures). The observed effects of the mixtures were modelled using a sigmoidal dose-response curve with variable slope (as for the single compounds), after normalization of data with minimum and maximum values of the most potent positive control (H_2O_2).

The resulting CRCs for the mixtures were compared to both CA (Eq. 2) and IA predictions (Eq. 3).

$$ECx_{mix} = \left(\sum_{i=1}^{n} \frac{p_i}{ECx_i}\right)^{-1} \quad \textbf{Eq. 2}$$

Being ECx_{mix} the total predicted effect concentration of the mixture that induces an effect x, p_i the relative fraction of component i in the mixture and ECx_i the concentration of substance i inducing an effect x when exposed alone.

$$E_{mix} = 1 - \prod_{i=1}^{n}(1 - E_i) \quad \textbf{Eq. 3}$$

Being E_{mix} is the effect of a mixture of n compounds and E_i is the effect of substance I when exposed alone.

The EC_{50}, Hill slope and goodness of fit (R^2) values were calculated for all curves. The non-linear regression calculated for the observed data and for each model (CA and IA) was used to calculate the effect levels for each curve. The effect levels obtained for the observed data was compared to those of CA and IA, and additive effects were assumed to occur if no significant differences were detected between the observed effect concentrations and those predicted by the models (see statistical methods for details). If the curves were not significantly different,

the model was considered to explain the combined effects. Model deviation ratios (MDRs; Eq. 4) were also calculated to help this detection:

$$MDR = \frac{ECx_{pred}}{ECx_{obs}} \qquad \textbf{Eq. 4}$$

Being ECx_{pred} the predicted effect concentrations and ECx_{obs} the observed effect concentrations (Belden and Lydy, 2006). Additive effects were assumed to occur when the MDRs were within a factor of 2 ($0.5 \leq MDR \leq 2$), as proposed by Belden et al. (2007).

Statistical analysis

The non-linear regressions using a sigmoidal dose-response curve with variable slope were modelled in GraphPad Prim 6 software (GraphPad Software Inc., La Jolla, CA, USA). The same software was used to determine the significant differences in ROS formation between concentrations for each compound and the mixtures, between compounds and mixtures, and to compare the effect levels for the experimental data with those calculated by the CA and IA prediction models (Motulsky, 1998; EPA, 2006). For data non-normally distributed and/or variance not homogeneous, the non-parametric tests Kruskal-Wallis and Dunn's post hoc test were applied. For data being normally distributed and with homogeneous variance, the parametric one-way ANOVA was used in combination with the Tukey post hoc test for multiple comparisons. A *p*-value of 0.05 was considered significant.

Results

Assay evaluation

An exposure time of 6 h provided the best response in the ROS assay as yielding the lowest intra and inter-assay variance (data not shown). The relative fluorescence units (RFU) obtained for all tested compounds increased in a concentration-dependent manner and with time (Figs. 1 and 2 in supplementary data). Unexposed microalgae (control) showed a small increase in fluorescence with time in all tests performed under normal light conditions (Fig. 1 in supplementary data), as expected from normal cellular processes. No formation of ROS was detected in microalgae exposed to the positive control paraquat in the dark, whereas a concentration-dependent increase in ROS was observed when exposing the algae under standard light conditions (Fig. 3 in supplementary data). For this reason, all the ROS

determination in this study was performed in microalgae exposed under standard light conditions. No changes in fluorescence were observed for any of the single test chemicals or their mixtures when performing the studies without the presence of microalgae (Table 2 in supplementary data). Hydrogen peroxide caused an increase in ROS when incubated in the absence of microalgae and the resulting baseline fluorescence was therefore subtracted for a more accurate estimation of ROS production caused by this compound. The exposure to the solvent (0.05% DMSO) did not cause any induction of ROS compared to incubations without the use of the solvent.

The positive control H_2O_2 induced a uniform (monotonic) concentration-dependent increase in ROS formation in *C. reinhardtii* (Fig. 1), with the 2 highest concentrations ($\geq 2 \times 10^6$ nM) significantly different from the control. The highest concentration of H_2O_2 (2.5×10^6 nM) led to a 3-fold increase in ROS production, whereas the other two controls (atrazine and paraquat) induced a slightly lower ROS production (Fig. 1). Interestingly, these compounds displayed different concentration-response curves. Atrazine showed a small, but non-significant, increase in ROS formation at the lowest concentrations (2-20 nM, ($p>0.05$), followed by a decrease to control levels at medium concentrations (100-200 nM) and a concentration-dependent increase in ROS at the highest exposure concentrations ($0.1\text{-}2 \times 10^4$ nM). This ROS induction was significantly different from control at concentrations higher than 2×10^3 nM, showing a maximum of 2-fold induction at the highest atrazine concentration (2×10^4 nM, $p<0.05$). Paraquat led to a monotonic concentration-dependent increase in ROS, as seen for H_2O_2, with a similar maximum ROS induction as that of atrazine for 2×10^4 nM ($p<0.05$).

Single biocide exposure

Of the biocides tested, only aclonifen, bifenox and metribuzin induced the formation of ROS in *C. reinhardtii* (Fig. 2). The biocides dichlofluanid and triclosan did not induce ROS at any of the tested concentrations compared to the control ($p>0.05$). Aclonifen and metribuzin showed a similar pattern to that observed for atrazine, with a small increase in ROS at the lower and higher concentrations tested. For aclonifen, a significant increase in ROS was observed already at 10 nM, whereas higher concentrations caused up to a 2-fold increase in ROS production ($p<0.05$). A similar, but not as pronounced biphasic induction was observed for metribuzin where a significant increase in ROS formation was observed at 1×10^3 nM ($p<0.05$). Bifenox caused a similar ROS induction pattern as that observed for paraquat and H_2O_2, with a significant increase in ROS formation from 1×10^4 nM (1.6-fold up to 2.0-fold

at highest concentration, $p<0.05$). No significant differences in ROS production were found between the highest concentrations of aclonifen, bifenox and metribuzin ($p>0.05$).

Normalization to the minimum and maximum response of H_2O_2 (Fig. 4 in supplementary data) enabled fitting classical CRCs to the experimental data and calculate NOEC, EC_{50}, Hill slopes and goodness of fit (Table 1). The non-linear regression fitted well to the responses at the intermediate to high concentration range for the 3 active compounds ($R^2 \geq 0.87$; Table 1). The initial increase in ROS in the lower part of the non-uniform CRC for aclonifen and metribuzin (5 first concentrations) were not considered relevant for the toxicity assessment and were therefore excluded in the construction of the CRCs. After normalisation to the maximum response of H_2O_2, aclonifen was found to be the most potent ROS-inducer (EC_{50}= 1.2 x 10^4 nM), followed by metribuzin (EC_{50}= 2.6 x 10^4 nM) and bifenox (EC_{50}= 4.1 x 10^4 nM). Nevertheless, bifenox presented the lowest NOEC value (100 nM) and the highest Hill slope (0.29).

Combined effects

Only aclonifen, bifenox and metribuzin were used for the assessment of combined toxicity as causing ROS production at the concentrations tested (Fig. 3; Table 1 in supplementary data for more information on the mixtures). All the binary and the ternary mixtures induced ROS (Fig. 3; consult Fig. 5 in supplementary data for more information on the fluorescence increment for tested mixtures), where the binary mixture of aclonifen and bifenox was the most potent displaying an induction comparable to the induction observed for the highest concentration of H_2O_2 (4-fold increase at highest concentration). The ternary mixture was the second most potent, with a significant increase from the control already at 152 nM (1.1-fold up to 2.4 fold at highest concentration). The two binary mixtures aclonifen and metribuzin and bifenox and metribuzin induced considerably lower ROS formation (1.4-fold and 1.8-fold at highest concentration, respectively) than the mixture of aclonifen and bifenox. For the mixture of aclonifen and metribuzin, only the two highest concentrations caused a significant increase in ROS formation compared to the control ($p<0.05$). The mixture of bifenox and metribuzin was slightly more toxic than the combination of aclonifen and metribuzin, with a significant increase in ROS formation at 13.7 x 10^2 nM ($p<0.05$).

The CRCs for the mixtures were calculated after normalization of data (Fig. 4; Table 2). The data for the mixtures were well described by the applied non-linear regression analysis,

indicated by a $R^2 \geq 0.75$ (Table 2). The binary mixture of aclonifen and bifenox presented the best-fitted curve ($R^2 = 0.87$). Although the MDR values were calculated for all the mixtures, only a few values covered the experimental data due to the shallow slope of the CRCs (Table 2). Therefore, the significant differences between the effect concentrations for the experimental data with those calculated by the CA and IA prediction models were chosen as a better method to evaluate the fit of the obtained data on the prediction models (for more information on the statistical results consult Table 3 in supplementary data). The CA model best predicted the mixture of aclonifen and bifenox for effect concentrations between EC_{20} and EC_{70} (Table 2). For effects higher than the EC_{70} the mixture seemed to cause more than additive effects, indicative of synergism. A shallower CRC slope was observed for the two binary mixtures of aclonifen and metribuzin, and bifenox and metribuzin, with only minor differences in the response for the different combinations tested (Fig. 4; Table 2). The mixture of aclonifen and metribuzin was best predicted by the IA model, while the mixture of bifenox and metribuzin was well predicted by the both models. The ternary mixture was best predicted by the CA model.

Discussion

Leakage of electrons from the chloroplast, mitochondrial and plasma membrane electron transport is a natural source for ROS production in algae (Apel and Hirt, 2004) and lead to the production of basal levels of ROS as part of aerobic metabolism (Foyer and Noctor, 2005). However, these pro-oxidant reactive species are also crucial for life, as being involved in several biological functions like photosynthesis, photorespiration, mitochondrial and protein oxidation in algae (Foyer and Noctor, 2005). ROS are also used as second messengers in signal transduction cascades in several processes, such as mitosis, tropisms and cell death, thus being vital to normal development and survival. This “oxidative signalling” is often considered a key for successful monitor and adjustment to changes in the environment at the molecular or functional level in various cell types (Foyer and Noctor, 2005). As excessive ROS production may be harmful to organisms and lead to free radical attack on proteins, lipids and DNA and to a further extent to disruption of cellular structures, cellular signalling and cellular death (Apel and Hirt, 2004; Foyer and Noctor, 2005), its levels are kept low. Microalgae have numerous antioxidant defence mechanisms including several enzymatic responses, as for example catalase, superoxide dismutase, glutathione-S-transferase, and non-enzymatic mechanisms as metallothionein, carotenoids, glutathione, among others (Mallick

and Mohn, 2000). Problems arise if an imbalance between the production and the detoxification of ROS occurs, with the potential for causing oxidative stress and subsequent cellular damage (Alscher et al., 1997). The measurement of oxidative stress is a common endpoint to assess and compare the toxicity of different compounds in microalgae (Cheloni and Slaveykova, 2013) and has been used with success to identify chemicals that may potentially cause oxidative damage in organisms such as algae (Nestler et al., 2012b). Several methods are currently used to analyse oxidative stress in cells including direct quantification of ROS and indirect determination of antioxidant capacity, induction of antioxidant enzyme systems (Stoiber et al., 2007) and cellular damage (Tripathi et al., 2006; Collén et al., 2003). However, as ROS are difficult to detect due to their very short lifetime, specific and highly sensitive fluorescence probes have been developed to rapid and directly detect the formation of ROS (Cheloni and Slaveykova, 2013). The fluorescent probe carboxy-H_2DFFDA has previously displayed a large potential to detect ROS production in algae (Szivák et al., 2009; Stoiber et al., 2011) due to higher photostability than its chlorinated derivative 2',7'-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA). As seen in the present study, carboxy-H_2DFFDA performed well in *C. reinhardtii* exposed to different ROS generating compounds. ROS production was strongly enhanced in the presence of light, which is consistent with the finding that ROS production in algae is closely related to photosynthetic processes (Knauert and Knauer, 2008). The major production sites of ROS in photosynthetic organisms are the reaction centres of PSI and PSII in chloroplast thylakoids, thus making ROS generation in microalgae predominantly a light dependent mechanism (Asada, 2006; Knauert and Knauer, 2008).

H_2O_2 and the two biocides atrazine and paraquat, well-recognized ROS inducers (Leisinger et al., 2001; Mendez-Alvarez et al., 1999), were used to evaluate the performance of the assay in *C. reinhardtii*. Although both compounds caused increase in ROS, their response pattern was slightly different. While atrazine demonstrated an apparent biphasic concentration-dependent increase in ROS formation, paraquat and H_2O_2 displayed a classical monotonic CRC. These are compounds with different and well-known MoAs. H_2O_2 toxicity is related with the formation of hydroxyl radicals and consequent oxidation of biomolecules (Russell, 2003). This compound is very mobile and able to pass through membranes, entering into vulnerable parts inside cells, thus becoming highly reactive and toxic. Atrazine inhibits the Hill reaction and the associated noncyclic photophosphorylation in chloroplasts, affecting PSII activity (DeLorenzo et al., 2001). It originates the generation of triplet chlorophyll (^{3}Chl), which if not

quenched by carotenoids may induce the formation of singlet oxygen that induces lipid peroxide formation (Fai et al., 2007). Damage of PSII due to photoinhibition occurs at both the donor site (water-splitting complex) and the acceptor site (QA) and/or (QB) acceptors (Jones et al., 2003). Paraquat toxic effects are also well revised. This compound is known to divert electrons from the Photosystem I (PSI) to molecular oxygen, producing radicals such as superoxide radicals, H_2O_2 radicals, hydroxyl radicals (•OH), and other reactive species (Hess, 2000; Jamers and Coen, 2010). It exerts oxidative stress not only in the chloroplasts but also in mitochondria and nucleus, resulting in a depletion of the antioxidant capacity of cells (Nestler et al., 2012b). It is also a known inducer of antioxidant enzymes such as glutathione-*S*-transferases, ascorbate peroxidases, dehydroascorbate reductase and glutamate-cysteine ligase (Hess, 2000; Jamers and Coen, 2010).

Effects of the single biocides

Among the studied compounds, only herbicides aclonifen, bifenox and metribuzin induced the formation of ROS in *C. reinhardtii*. These biocides exhibited different EC_{50} values, but within the same order of magnitude. Aclonifen, a diphenyl ether (DPE) herbicide, was the most toxic of the tested biocides, although with a biphasic response curve only obtaining an EC_{50} of 1.1 x 10^4 nM. Aclonifen is known to inhibit the enzyme Protox in the pathway leading from α-amino-levulinic acid to chlorophyll. This causes the accumulation of protoporphyrin IX inside the cells by the translocation of Protox from the chloroplast (Kilinc et al., 2009). Then the accumulated protoporphyrin IX in the plasma membrane suffers oxidation by reacting with the oxygen produced by photosynthesis, ultimately causing the formation of singlet oxygen ($^1O_2$) and superoxide anion (O_2-) that can damage cellular components. Moreover, aclonifen also inhibits carotenoid biosynthesis and thus reduce the overall carotenoid-mediated detoxification of ROS emitted by protoporphyrin IX in the light (Kilinc et al., 2009). Although singlet oxygen and superoxide anion are normally generated in PSII under normal physiological conditions, their production can be further stimulated by the presence of ROS-generating compounds (Asada, 2006; Knauert and Knauer, 2008). These ROS are too reactive to reach far from its site of origin in the chloroplast, probably only directly affecting lipids and membrane proteins near its site of production (Ledford and Niyogi, 2005). Microalgae have developed efficient protection mechanisms to prevent the formation of these ROS, such as the presence of low molecular weight antioxidants such as ascorbate and β-carotene, but also induction of antioxidant enzymes such as superoxide dismutase (Mallick and Mohn, 2000). β-carotene is a carotenoid, an important naturally occurring pigment located in the

thylakoid membranes in the chloroplasts. Carotenoids have two main functions, one being the expansion of the spectrum of the photosynthetically active radiation (PAR) and secondly the protection of light–harvesting pigments in the antenna complexes against ROS-mediated photochemical damage by dissipating energy and detoxifying ROS (Pinto et al., 2003; Fischer et al., 2010).

Metribuzin, a triazinone herbicide, was the second most toxic compound. This compound is a PSII herbicide analogue to plastoquinone, which reverse the binding to the Q_B biding site on the D1 protein of the PSII complex (Jones, 2005). A ^{3}Chl state is produced in the reaction centre that is capable of reacting with triplet oxygen ($^3O_2$), forming the reactive singlet oxygen ($^1O_2$). This oxygen species can then damage adjacent chlorophyll-bearing proteins, separate the chlorophylls from their energy transfer systems and from protective pigments (carotenoids), causing further photogeneration of singlet oxygen (Jones, 2005).

Aclonifen and metribuzin showed a similar pattern to that observed for atrazine, with a concentration-dependent biphasic increase in ROS formation. While both aclonifen and atrazine affect the PSII, aclonifen and metribuzin also affect carotenoids synthesis. Although there is an initial induction of ROS in the lower concentrations, the antioxidant protective systems in the cells were likely able to compensate for the increase in ROS observed at these concentrations (Ledford and Niyogi, 2005). These compounds cause the formation of singlet and triplet oxygen and superoxide anion that most probably have mechanisms of detoxification near the site of generation (Ledford and Niyogi, 2005). It has already been shown that PSII inhibitors as diuron leads to induction of antioxidant enzymes such as ascorbate peroxidase, peroxiredoxins and thioredoxins, which directly protect the photosynthetic apparatus from oxidative damage in microalgae (Nestler et al., 2012b). After reaching a plateau of ROS formation, the capacity of this antioxidant defence system is exceeded, ultimately leading to cell death (Ledford and Niyogi, 2005).

Bifenox was the least potent ROS inducer of the tested herbicides (NOEC=100 nM; EC_{50}= 4.1 x 10^4 nM), although with the steepest CRC slope and lowest NOEC. This result contrasts the study by Almeida et al. (*in prep*) showing that bifenox is more toxic than aclonifen and metribuzin when analysing growth inhibition in *C. reinhardtii* (EC_{50}= 18 nM, 298 nM and 57 nM, respectively). This compound is also a DPE herbicide as aclonifen that can cause membrane disruption and inhibition of photosynthesis by Protox inhibition and thus

originating light-dependent oxygen radical formation (Grossman, 2005; EFSA, 2007). However, bifenox caused a similar ROS induction pattern as that observed for paraquat and H_2O_2. In contrast to ROS production caused by aclonifen and metribuzin, H_2O_2 radicals caused by bifenox can be less reactive and travel further from its origin causing higher cellular damage compared to hydroxyl radicals (Ledford and Niyogi, 2005). However, similarly to paraquat, it seems that the antioxidant defence mechanisms present in algae were not able to deal with the ROS formed by bifenox at high concentrations. Other studies also suggest that compounds like paraquat cause an overall decline of the antioxidant defence activity and thus render the microalgae susceptible to cellular attack by ROS (Nestler et al., 2012a,b).

Dichlofluanid and triclosan showed no induction of ROS and were not considered further in the present work. As non-herbicides, dichlofluanid and triclosan display multiple and more general toxic MoAs than the other tested biocides. Dichlofluanid is used as an antifoulant, fungicide, acaricide and wood preservative, being able to inhibit thiol-containing enzymes and disrupt mitochondrial activity in various organisms (Cima et al., 2008). Triclosan, a diphenylether derivative, is a contaminant widely used as a wood preservative, bactericide and fungicide, and known to affect multiple target sites and thus toxic to a high number of organisms (USEPA, 2008; von der Ohe et al., 2012). Both compounds do not seem to promote oxidative stress or pose a significant stress condition to lead to the formation of ROS in the conditions used in this study. The short-term exposure (6 hours) of algae to both compounds could not be enough to allow a substantial accumulation of more stable reactive species (Nestler et al., 2012b). Nonetheless, the activation of antioxidant defence mechanisms to counteract ROS formation, as referred previously, cannot be excluded as a possible explanation to the lack of response seen with these compounds.

Combined effects

Little is known about the production of ROS in microalgae exposed to mixtures, as effect studies are often focused on looking at broad adverse endpoints such as algal growth (e.g. Altenburger et al., 2003; Belden et al., 2007; Cedergreen, 2014; Petersen et al. 2014). The present study showed that the simple mixtures of ROS-producing biocides tested caused combined toxicity that in general were predicted by the IA and CA prediction models. As the CA model best predicted the ternary mixture and the binary mixture of aclonifen and bifenox, their combined effects suggest that they induce the formation of ROS in *C. reinhardtii* by the

same MoA. The CA prediction model normally provides good to excellent predictions for the mixture toxicity of biocides when analysing adverse toxic endpoints such as algal growth (Cedergreen et al., 2008; Backhaus and Faust, 2012). In 88% of pesticide mixtures studies, predictions by CA have been reported to fall within a factor of 2 independently of whether compounds with similar or dissimilar MoA were tested (Belden et al., 2007). Another study also showed that only 6% of the investigated 158 data sets showed substantial deviations from CA (Cedergreen et al., 2008; Backhaus and Faust, 2012). The CA model, although slightly conservative, can be largely applicable with small probability of underestimating effects due to interactions (Belden et al., 2007). These conclusions seem also to be valid for the studies performed in the present work as the observed data often fell between the CA and IA predicted effects. The IA model best predicted the binary mixture of aclonifen and metribuzin, while both models provided good fit to the responses of the mixture of bifenox and metribuzin. In both cases the combined effect of the compounds were additive, but at least in the case of aclonifen and metribuzin the compounds seemed to induce ROS by different MoA (see effects of single biocides section).

Interestingly, the mixture of aclonifen and bifenox seemed to cause more than additive effects (*i.e.*, synergism) at higher mixture effect concentrations. It is known that synergistic interactions between chemicals can occur at higher concentrations, however this mechanism for combined toxicity has been rarely reported (Cedergreen, 2014). Apart from some mixtures of pesticides and metals, for most of the synergistic combinations found in literature the involved mechanisms are still scarce, especially when involving not well-known compounds as bifenox. Most of the observed interactions described were between PSII herbicides, metals or non-azole fungicides in antifouling mixtures, together with mixtures of metals or metals and organic pesticides (Cedergreen, 2014). As mentioned in the previous section, both aclonifen and bifenox have common MoA that involve inducing the formation of singlet oxygen and superoxide anion by the reaction of the accumulated protoporphyrin IX with oxygen in presence of light (Grossman, 2005; Kilinc et al., 2009). On the other hand, aclonifen also inhibits carotenoid biosynthesis (antioxidant in the scavenging of ROS), thus reducing the detoxification capacity of cells (Kilinc et al., 2009). Accordingly, the mixture of both compounds seems to induce not only an overall decline in the antioxidant defence mechanisms by overproducing ROS, but also potentially reduce the carotenoid-mediated detoxification capacity of microalgae cells, thus rendering them more susceptible to ROS and oxidative stress. The type of ROS formed by both compounds can also magnify the effects

seen in the mixture, as shown by the different ROS induction patterns obtained for each biocide. The ROS species formed due to the presence of aclonifen are possibly readily detoxified locally, while those produced by bifenox might be less reactive and able to pass through cell compartments disseminating damage (Grossman, 2005; Ledford and Niyogi, 2005). With the impairment of the antioxidant defence system by the combined action of the MoAs of both compounds, the inefficient removal of ROS will prevent the protection of target molecules within the range of their generation site and lead to a higher diffusion of the less reactive species formed spreading oxidative stress and damage to other subcellular compartments (Ledford and Niyogi, 2005).

To better clarify how these events are linked and affecting each other, the information gathered for explaining the observed synergism was integrated in a schematic representation to propose the MoA of this mixture in *C. reinhardtii* (Fig. 5).

Conclusions

The ROS assay used in this study successfully documented the concentration-dependent ROS production in *C. reinhardtii* exposed to biocides aclonifen, bifenox and metribuzin. On the contrary, no ROS formation was detected after exposure to dichlofluanid and triclosan. This toxicological endpoint seems to be highly sensitive in reflecting the different toxic mechanisms of biocides, supported by the different induction patterns expressed.

ROS production of simple mixtures of the herbicides was mainly additive. CA and IA prediction models suggested that the combined toxicity occurred both by similar and dissimilar MoA, respectively. Synergism was identified as a possible interaction between the combination of aclonifen and bifenox, probably associated to an interaction between different MoA. As the three herbicides showed to have additive effects in *C. reinhardtii*, their co-occurrence in the environment can potentiate their harmful effects.

Future studies should identify additional stressors causing ROS in algae species and determine how they interact (additivity, synergism or antagonism) and propagate toxicity. Propagation mechanisms from how and where ROS are produced in algae until oxidative stress occurs should also be further investigated. More ecologically relevant studies with longer exposure scenarios should also be investigated for a better assessment of how these

compounds can affect populations.

Conflict of interest

The authors declare the inexistence of any conflicts of interest.

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References

Almeida AC, Petersen K, Thomas KV, Tollefsen KE. *in prep*. Combined toxicity of five priority biocides on the growth of *Chlamydomonas reinhardtii*.

Alscher RG, Donahue JL, Cramer CL. 1997. Reactive oxygen species and antioxidants: relationships in green algae. Physiol Plantarum 100:224-233.

Altenburger R, Nendza M, Schuurmann G. 2003. Mixture toxicity and its modelling by quantitative structure–activity relationships. Environ Toxicol Chem 22:1900–1915.

Apel K, Hirt H. 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol 55:373-399.

Asada K. 2006. Production and scavenging of reactive oxygen species in chloroplasts and their functions. Plant Physiol 141:391-396.

Backhaus T, Faust M. 2012. Predictive environmental risk assessment of chemical mixtures: a conceptual framework. Environ Sci Technol 46:2564-2573.

Belden JB, Lydy MJ. 2006. Joint toxicity of chlorpyrifos and esfenvalerate to fathead minnows and midge larvae. Environ Toxicol Chem SETAC 25:623–629.

Belden JB, Gilliom RJ, Lydy MJ. 2007. How well can we predict the toxicity of pesticide mixtures to aquatic life? Int Environ Assess Manage 3:364–372.

Bliss CI. 1939. The toxicity of poisons applied jointly. Ann J Appl Biol 26:585– 615.

Boo YC, Jung J. 1999. Water deficit-induced oxidative stress and antioxidant defenses in rice plants. J Plant Physiol155:255-261.

Cedergreen N, Christensen AM, Kamper A, Kudsk P, Matthiasen S, Streibig JC. Sørensen, H., 2008. A review of independent action as a reference model for binary mixtures of compounds with different molecular target sites. Environ Toxicol Chem 27:1621–1632.

Cedergreen N. 2014. Quantifying Synergy: A Systematic Review of Mixture Toxicity Studies within Environmental Toxicology. PLOS ONE 9:e96580.

Chalew TEA, Halden RU. 2009. Environmental exposure of aquatic and terrestrial biota to triclosan and triclocarban. JAWRA 45:4-13.

Cheloni G, Slaveykova VI. 2013. Optimization of the C11-BODIPY$^{581/591}$ dye for the determination of lipid oxidation in *Chlamydomonas reinhardtii* by flow cytometry. Cytometry Part A 83A:952-961.

Cima F, Bragadin M, Ballarin L. 2008. Toxic effects of new antifouling compounds on tunicate haemocytes I. Sea-Nine 211^{TM} and chlorothalonil. Aquat Toxicol 86:299-312.

Collén J, Pinto E, Pedersen M, Colepicolo P. 2003. Induction of oxidative stress in the red macroalga Gracilaria tenuistipitata by pollutant metals. Arch Environ Contam Toxicol 45:337–42.

DeLorenzo ME, Scott GI, Ross PE. 2001. Toxicity of pesticides to aquatic microorganisms: a review. Environ Toxicol Chem 20:84-98.

ECHA. 2014. Guidance on the biocidal products regulation. Volume II: efficacy. Part A: information requirements. European Chemicals Agency, Version 1.1, November 2014:35 p.

EFSA. 2007. Conclusion on the peer review of bifenox. European Food Safety Authority Scientific Report 119:1-84.

EU, 2013. Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy. Official Journal of the European Union L 226/1-L 226/16.

Fai PB, Grant A, Reid B. 2007. Chlorophyll a fluorescence as a biomarker for rapid toxicity assessment. Environ Toxicol Chem 26:1520-1531.

Fairchild JF, Ruessler DS, Carlson AR. 1998. Comparative sensitivity of five species of macrophytes and six species of microalgae to atrazine, metribuzin, alachlor, and metolachlor. Environ Toxicol Chem 17:1830–1834.

Fischer BB, Eggen RIL, Niyogi KK. 2010. Characterization of singlet oxygen-accumulating mutants isolated in a screen for altered oxidative stress response in *Chlamydomonas reinhardtii*. Plant Biol 10:279.

Foyer C H, Noctor G. 2005. Oxidant and antioxidant signalling in plants: a re-evaluation of

the concept of oxidative stress in a physiological context. Plant Cell Environ 28:1056–71.

Foyer CH, Lopez-Delgado H, Oat JF, Scott IM. 1997. Hydrogen peroxide- and glutathione-associated mechanisms of acclimatory stress tolerance and signalling. Physiol Plant 100:241-254.

Gatidou G, Thomaidis NS. 2007. Evaluation of single and joint toxic effects of two antifouling biocides, their main metabolites and copper using phytoplankton bioassays. Aquat Toxicol 85:184–191.

Grossman K. 2005. What it takes to get a herbicide's mode of action. Physionomics, a classical approach in a new complexion. Pest Manag Sci 61:423-431.

Gunawan C, Sirimanoonphan A, Teoh WY, Marquis CP, Amal R. 2013. Submicron and nano formulations of titanium dioxide and zinc oxide stimulate unique cellular toxicological responses in the green microalga *Chlamydomonas reinhardtii*. J Hazard Mater 260:984-992.

Harris EH. 2009. The Chlamydomonas Sourcebook. Introduction to Chlamydomonas and its laboratory use. Volume 1. 435 p.

He Y-Y, Häder D-P. 2002. UV-B-induced formation of reactive oxygen species and oxidative damage of the cyanobacterium *Anabaena* sp.: protective effects of ascorbic acid and *N*-acetyl-L-cysteine. J Photochem Photobiol B 66:115-124.

Hess FD. 2000. Light-dependent herbicides: an overview. Weed Sci 48:160–170.

Jamers A, Coen WD. 2010. Effect assessment of the herbicide paraquat on a green alga using differential gene expression and biochemical biomarkers. Environ Toxicol Chem 29:893-901.

Jones RJ, Muller J, Haynes D, Schreiber U. 2003. Effects of herbicides diuron and atrazine on corals of the Great Barrier Reef, Australia. Mar Ecol Prog Ser 251:153-167.

Jones R. 2005. The ecotoxicological effects of photosystem II herbicides on corals. Mar Pollut Bull 51:495–506.

Kilinc Ö, Reynaud S, Perez L, Tissut M, Ravanel P. 2011. Physiological and biochemical modes of action of the diphenylether aclonifen. Pestic Biochem Physiol 93:65-71.

Kilinc Ö, Grasset R, Reynaud S. 2011. The herbicide aclonifen: the complex theoretical bases of sunflower tolerance. Pestic Biochem Physiol 100:193-198.

Knauert S, Knauer K. 2008. The role of reactive oxygen species in copper toxicity to two freshwater green microalgae. J Phycol 44:311–319.

Knauert S, Escher B, Singer H, Hollender J, Knauer K. 2008. Mixture toxicity of three photosystem II inhibitors (atrazine, isoproturon, and diuron) toward photosynthesis of freshwater phytoplankton studied in outdoor mesocosmos. Environ Sci Technol 42:6424–6430.

Ledford HK, Niyogi KK. 2005. Singlet oxygen and photo-oxidative stress management in plants and algae. Plant Cell Environ 28:1037–1045.

Legendre L, Yueh YG, Crain R, Haddock N, Heinstein PF, Low PS. 1993. Phospholipase-C activation during elicitation of the oxidative burst in cultured plant cells. J Bioi Chem 268:24559-24563.

Leisinger U, Rüfenacht K, Fischer B, Pesaro M, Spengler A, Zehnder AJB, Eggen RIL. 2001. The glutathione peroxidase homologous gene from *Chlamydomonas reinhardtii* is transcriptionally up-regulated by singlet oxygen. Plant Mol Biol 46:395-408.

Loewe S. 1927. Die Mischarznei. Versuch einer allgemeinen pharmakologie der arzneikombinationen. Klin. Wochenschr 6:1077-1085.

Loggini B, Scartazza A, Brugnoli E, Navari-Izzo F. 1999. Antioxidative defense system, pigment composition and photosynthetic efficiency in two wheat cultivars subjected to drought. Plant Physiol 199:1091-1099.

Low PS, Merida JR. 1996. The oxidative burst in plant defense: function and signal transduction. Physiol Plant 96:533-542.

Mallick N, Mohn FH. 2000. Reactive oxygen species: response of algal cells. J Plant Physiol 157:183-193.

Mendez-Alvarez S, Leisinger U, Eggen RI. 1999. Adaptive responses in *Chlamydomonas reinhardtii*. Int Microbiol 2:15-22.

Nestler H, Groh KJ, Schönenberg R, Behra R, Schirmer K, Eggen RIL, Suter HJ-F. 2012a. Multiple-endpoint assay provides a detailed mechanistic view of responses to herbicide exposure in *Chlamydomonas reinhardtii*. Aquat Toxicol 110-111:214-224.

Nestler H, Groh KJ, Schönenberg R, Eggen RIL, Suter HJ-F. 2012b. Linking proteome responses with physiological and biochemical effects in herbicide-exposed *Chlamydomonas reinhardtii*. J Proteomics75:5370-5385.

Petersen K, Heiaas HH, Tollefsen KE. 2014. Combined effects of pharmaceuticals, personal care products, biocides and organic contaminants on the growth of *Skeletonema pseudocostatum*. Aquat Toxicol 150:45-54.

Pinto E, Sigaud-Kutner TCS, Leitão MAS, Okamoto OK, Morse D, Colepicolo P. 2003. Heavy metal-induced oxidative stress in algae. J Phycol 39:1008–1018.

Pospíšil P. 2009. Production of reactive oxygen species by photosystem II. Biochim Biophys Acta 1787:1151-1160.

Prado R, García R, Rioboo C, Herrero C, Abalde J, Cid A. 2009. Comparison of the sensitivity of different toxicity test endpoints in a microalga exposed to the herbicide paraquat. Environ Int 35:240-247.

Ramel F, Sulmon C, Bogard M, Couée I, Gouesbet G. 2009. Differential patterns of reactive oxygen species and antioxidative mechanisms during atrazine injury and sucrose-induced tolerance in *Arabidopsis thaliana* plantlets. Plant Biol 9:28.

Russell AD. 2003. Similarities and differences in the responses of microorganisms to biocides. J Antimicrob Chemother 52:750–763.

Stoiber TL, Shafer MM, Perkins DAK, Hemming JDC, Armstrong DE. 2007. Analysis of glutathione endpoints for measuring copper stress in *Chlamydomonas reinhardtii*. Environ Toxicol Chem 26:1563-1571.

Stoiber TL, Shafer MM, Armstrong DE. 2011. Induction of reactive oxygen species in *Chlamydomonas reinhardtii* in response to contrasting trace metal exposures. Environ Toxicol 28:516-523.

Szivák I, Behra R, Sigg L. 2009. Metal-induced reactive oxygen species production in *Chlamydomonas reinhardtii* (Chlorophyceae). J Phycol 45:427–435.

Tripathi BN, Mehta SK, Amar A, Gaur JP. 2006. Oxidative stress in *Scenedesmus* sp. during short- and long-term exposure to Cu and Zn. Chemosphere 62:538–544.

USEPA. 2008. Ecological hazard and environmental revised risk assessment chapter triclosan (pc code: 054901; case no.: 2340). United States Environmental Protection Agency. Washington, d.c. 20460. Office of prevention, pesticides and toxic substances. 33 p.

von der Ohe PC, Schmitt-Jansen M, Slobodnik J, Brack W. 2012. Triclosan – the forgotten priority substance? Environ Sci Pollut Res 19:585–591.

Weckx JEJ, Clijsters H. 1996. Oxidative damage and defense mechanisms in primary leaves of *Phaseolus vulgaris* as a result of root assimilation of toxic amounts of copper. Physiol Plant 96:506-512.

Žegura B, Lah TL, Filipič M. 2004. The role of reactive oxygen species in microcysrin-LR-induced DNA damage. Toxicology 200:59-68.

Figure captions

Fig. 1. Reactive oxygen species (ROS) formation in *Chlamydomonas reinhardtii* exposed to atrazine, paraquat and hydrogen peroxide (H_2O_2) for 6 h. The data (Mean±SEM) represent 3 independent studies. Letters indicate significant differences between concentrations (p<0.05).

Fig. 2. Reactive oxygen species (ROS) formation in *Chlamydomonas reinhardtii* exposed to the biocides aclonifen, bifenox, dichlofluanid, metribuzin and triclosan for 6 h. The data (Mean±SEM) represent 3 independent studies. Letters indicate significant differences between concentrations (p<0.05).

Fig. 3. Reactive oxygen species (ROS) formation in *Chlamydomonas reinhardtii* exposed to equipotent mixtures of different biocides for 6 h. The data (Mean±SEM) represent 3 independent studies. Letters indicate significant differences between concentrations (p<0.05).

Fig. 4. Reactive oxygen species (ROS) formation (% of control, %CT) in *Chlamydomonas reinhardtii* exposed to equipotent mixtures of different biocides in combination with the combined toxicity predictions obtained from CA and IA mixture models. The data (Mean±SEM) represent experimental results from 3 independent experiments and their corresponding concentration-response curves (dotted line). Prediction of combined toxicity by CA (solid line) and IA (broken line) of the mixture is displayed to indicate assumptions of additivity.

Fig. 5. Schematic representation of the Mode of Action (MoA) for the mixture of aclonifen and bifenox. Both compounds instigate the formation of ROS by the reaction of the accumulated protoporphyrin IX with oxygen, potentiated by the presence of light. Aclonifen inhibits the carotenoid biosynthesis, reducing the detoxification capacity of cells (Grossman, 2005; EFSA, 2007; Kilinc et al., 2009). The mixture of the two compounds might induce an overall decline in the antioxidant defence mechanisms, reducing the detoxification capacity of cells, making them more susceptible to ROS.

Table 1. The No Observed Effect Concentration (NOEC), EC_{50} (nM; 95% confidence intervals of EC_{50} in parentheses), Hill slope and goodness of fit (R^2) for Reactive oxygen species (ROS) production in *Chlamydomonas reinhardtii* exposed to the biocides aclonifen, bifenox and metribuzin for 6 h.

Compound	NOEC (nM)	EC_{50} (nM)	Hill slope	Goodness of fit (R^2)
Aclonifen	200	$1x10^4$ ($9x10^3$ to $2x10^4$)	0.3	1.00
Bifenox	100	$4x10^4$ ($7x10^3$ to $2x10^5$)	0.3	0.87
Metribuzin	200	$3x10^4$ ($5x10^3$ to $1x10^5$)	0.2	0.91

Table 2. Production of ROS in *Chlamydomonas reinhardtii* after exposure to the equipotent mixtures. The data show the EC_{50} (nM; 95% confidence intervals of EC_{50} between parentheses), Hill slope and goodness of fit (R^2) for the experimental data and the corresponding CA and IA models, and model deviation ratios (MDRs) for each model. The different effect levels (% of ROS) for the mixture are shown for each model. Note: bold text indicates that MDRs were within a factor of two and * indicates that the model predictions were not significantly different from the observed data.

Mixtures		Aclonifen+Bifenox		Aclonifen+Metribuzin		Bifenox+Metribuzin		Aclonifen+Bifenox+Metribuzin	
Experiment	**EC_{50} (nM)**	3565 (1203 to 10568)		8.680×10^7 (52999 to 1.422×10^{11})		996881 (28915 to 3.437×10^7)		67673 (10167 to 450451)	
	Hill slope	0.5		0.1		0.2		0.2	
	Goodness of fit (R^2)	0.89		0.75		0.75		0.78	
CA model	**EC_{50} (nM)**	25941 (25885 to 25997)		17223 (16646 to 17820)		29714 (28292 to 31207)		23815 (22897 to 24771)	
	Hill slope	0.3		0.2		0.2		0.3	
	Goodness of fit (R^2)	1.00		1.00		1.00		1.00	
IA model	**EC_{50} (nM)**	1.2×10^6 (1.1×10^6 to 1.2×10^6)		1.3×10^6 (1.2×10^6 to 1.4×10^6)		2.3×10^6 (2.1×10^6 to 2.4×10^6)		1.2×10^7 (1.1×10^7 to 1.3×10^7)	
	Hill slope	0.4		0.3		0.3		0.4	
	Goodness of fit (R^2)	1.00		1.00		1.00		1.00	
	% of ROS	**MDR CA**	**MDR IA**	**MDR CA**	**MDR IA**	**MDR CA**	**MDR IA**	**MDR CA**	**MDR IA**
	95	1264	4482	-	-	-	-	-	-
	90	341	2316	-	-	-	-	-	-
	80	83	1131	-	-	-	-	-	-
	70	32*	703	-	-	-	-	-	-
	60	15*	476	-	-	-	-	0.3*	78

50	7.3*	332	-	-	0*	2.3*	0.4*	179
40	3.6*	232	-	-	0.1*	5.9*	0.4*	412
30	**1.6***	157	0	**1***	0.1*	16*	**0.5***	1024
20	**0.6***	98	0	14*	0.2*	58*	**0.7***	3110
10	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-

Figure 1

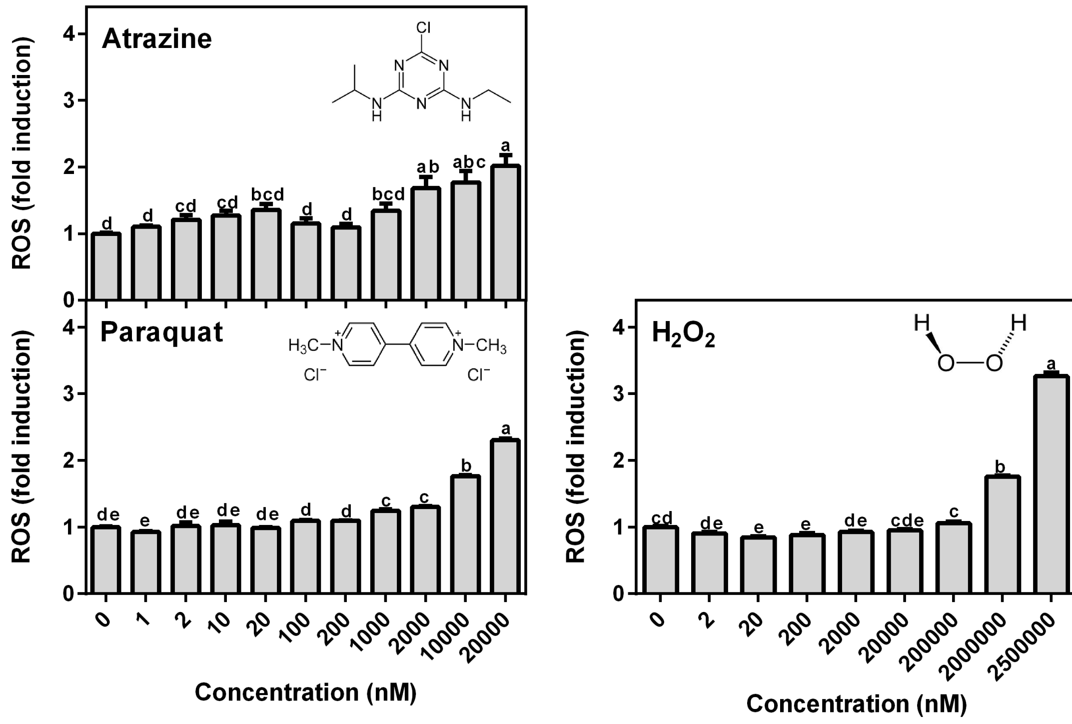


Figure 2

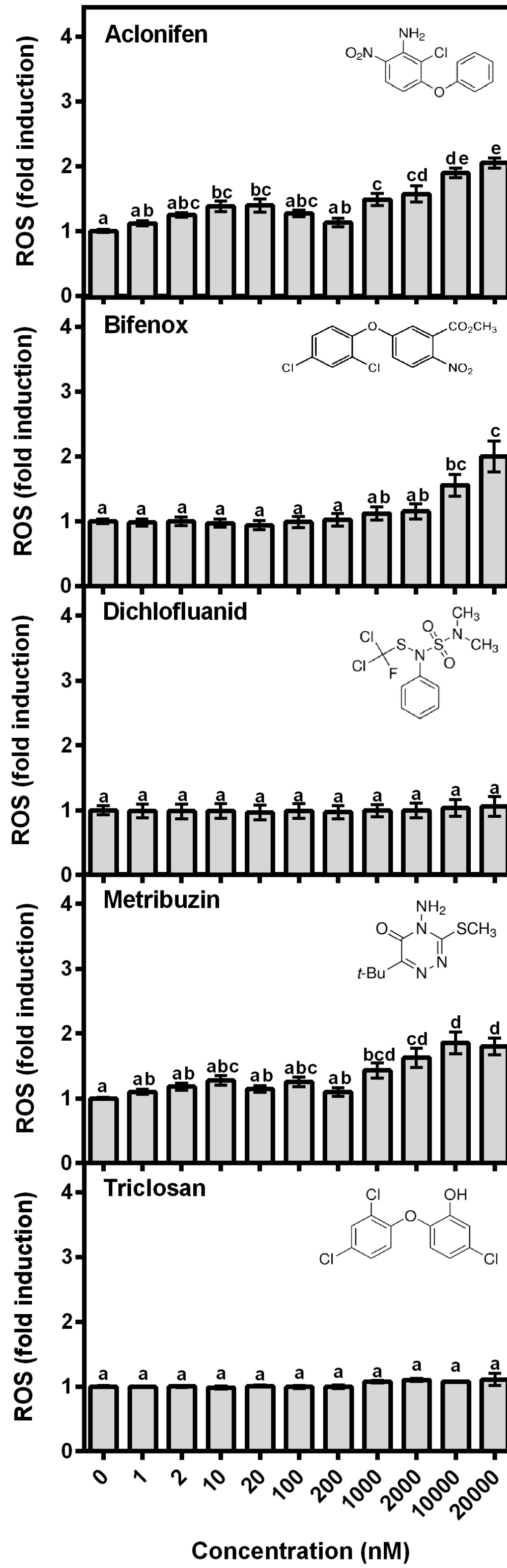


Figure 3

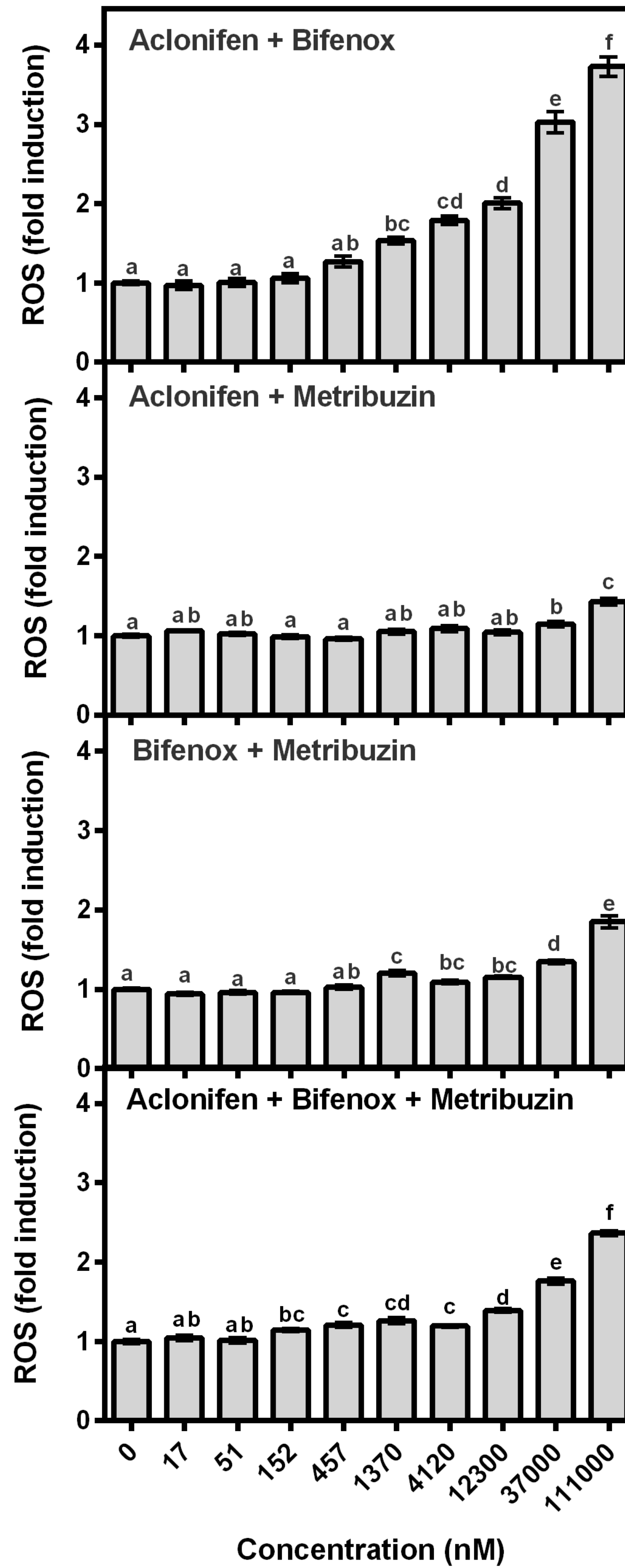


Figure 4

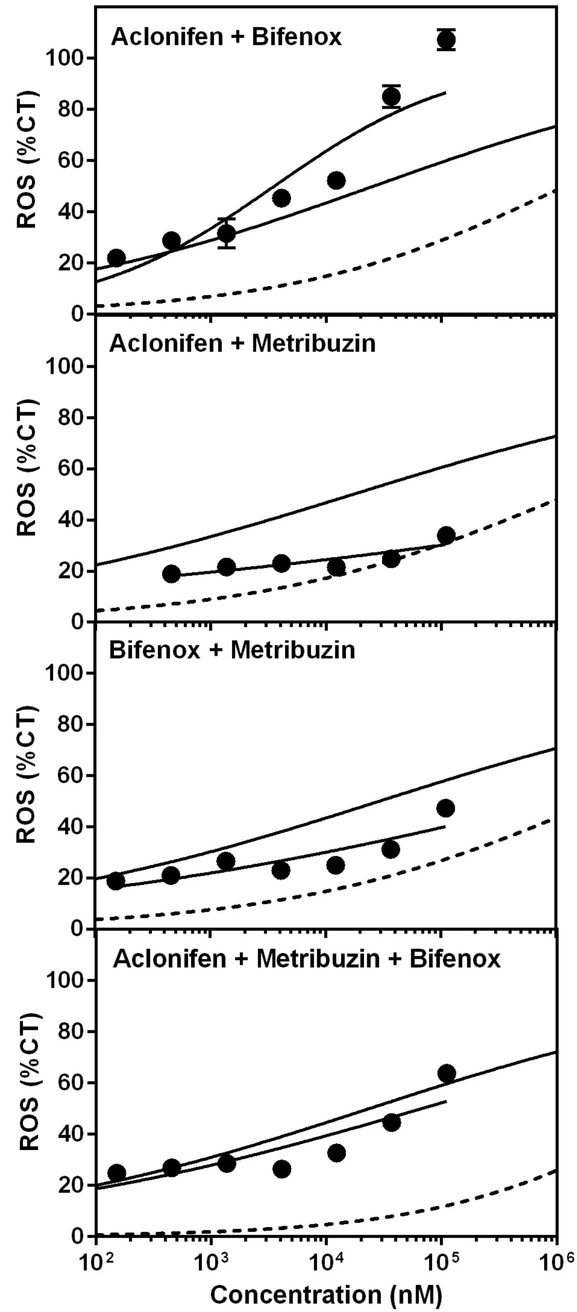
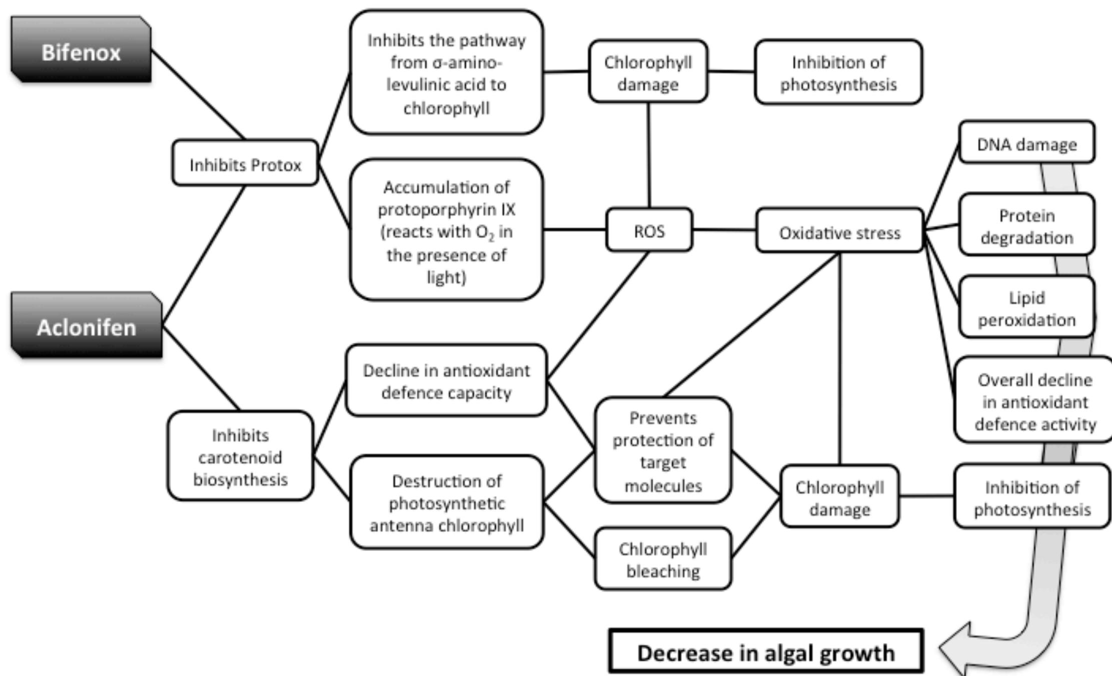


Figure 5



Induction of reactive oxygen species (ROS) in *Chlamydomonas reinhardtii* after exposure to single biocides and their simple mixtures

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Appendix A. Supplementary data

Table 1. Individual and total concentrations of the biocides aclonifen, bifenox, dichlofluanid, metribuzin and triclosan used in the mixtures.

Mixture	Concentration of each compound in the mixture (nM)			Total concentration (nM)
	Aclonifen	Bifenox	Metribuzin	
Aclonifen+bifenox	2.2×10^5	7.8×10^5	-	1×10^6
Aclonifen+metribuzin	3.1×10^5	-	6.9×10^5	1×10^6
Bifenox+metribuzin	-	6.1×10^5	3.9×10^5	1×10^6
Aclonifen+bifenox+metribuzin	1.5×10^5	5.2×10^5	3.3×10^5	1×10^6

Table 2. Production of ROS in blanks (no algae) after 6h exposure to the biocides aclonifen, bifenox, dichlofluanid, metribuzin and triclosan and their mixtures. The data represent relative fluorescence units (RFU; mean±SD).

	Mean±SEM	**CV (%)**
Aclonifen	600±1	2
Atrazine	598±1	2
Bifenox	634±1	3
Dichlofluanid	647±1	2
Metribuzin	638±1	3
Paraquat	596±1	3
Triclosan	643±1	3
Mixture aclonifen+bifenox	562±1	2
Mixture aclonifen+metribuzin	575±1	2
Mixture bifenox+metribuzin	583±1	2
Mixture aclonifen+bifenox+metribuzin	548±1	2

Table 3. Significant differences between the effect concentrations calculated for the observed and for each prediction model Concentration addition (CA) and Independent Action (IA) for each mixture obtained after one-way ANOVA analysis in combination with the Tukey post hoc test. Only *p-values* lower than 0.05 were considered significant.

Mixture	ANOVA	SS	DF	MS	F (DFn, DFd)	P value	Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant
Aclonifen + bifenox	**Treatment (between columns)**	58.0	2	29	F (2, 45) = 12.97	P < 0.0001	Observed vs. CA	-1.189	-2.470 to 0.0925	**No**
	Residual (within columns)	100.6	45	2.24			Observed vs. IA	-2.687	-3.968 to -1.405	Yes
	Total	158.6	47				CA vs. IA	-1.498	-2.779 to -0.216	Yes
Aclonifen + bifenox (≥EC$_{80}$)	**Treatment (between columns)**	130.0	2	64.98	F (2, 57) = 56.83	P < 0.0001	Observed vs. CA	-2.669	-3.482 to -1.855	Yes
	Residual (within columns)	65.2	57	1.14			Observed vs. IA	-3.433	-4.247 to -2.619	Yes
	Total	195.1	59				CA vs. IA	-0.764	-1.578 to 0.0492	**No**
Aclonifen + metribuzin	**Treatment (between columns)**	51.2	2	25.60	F (2, 45) = 75.74	P < 0.0001	Observed vs. CA	2.036	1.538 to 2.534	Yes
	Residual (within columns)	15.2	45	0.34			Observed vs. IA	-0.283	-0.781 to 0.215	**No**
	Total	66.4	47				CA vs. IA	-2.319	-2.817 to -1.820	Yes
Bifenox + metribuzin	**Treatment (between columns)**	16.2	2	8.11	F (2, 18) = 9.56	P = 0.0015	Observed vs. CA	1.134	-0.123 to 2.390	**No**
	Residual (within columns)	15.3	18	0.85			Observed vs. IA	-1.018	-2.275 to 0.238	**No**
	Total	31.5	20				CA vs. IA	-2.152	-3.409 to -0.896	Yes
Aclonifen + bifenox + metribuzin	**Treatment (between columns)**	79.8	2	39.89	F (2, 54) = 6.70	P = 0.0025	Observed vs. CA	0.384	-0.786 to 1.554	**No**
	Residual (within columns)	321.6	54	5.96			Observed vs. IA	-2.551	-3.720 to -1.381	Yes
	Total	401.4	56				CA vs. IA	-2.935	-4.104 to -1.765	Yes

SS – sum of the squares; DF – degrees of freedom; MS – mean squares; F – ratio between the two mean square values; DFn – degrees of freedom for the numerator; DFd - degrees of freedom for the denominator.

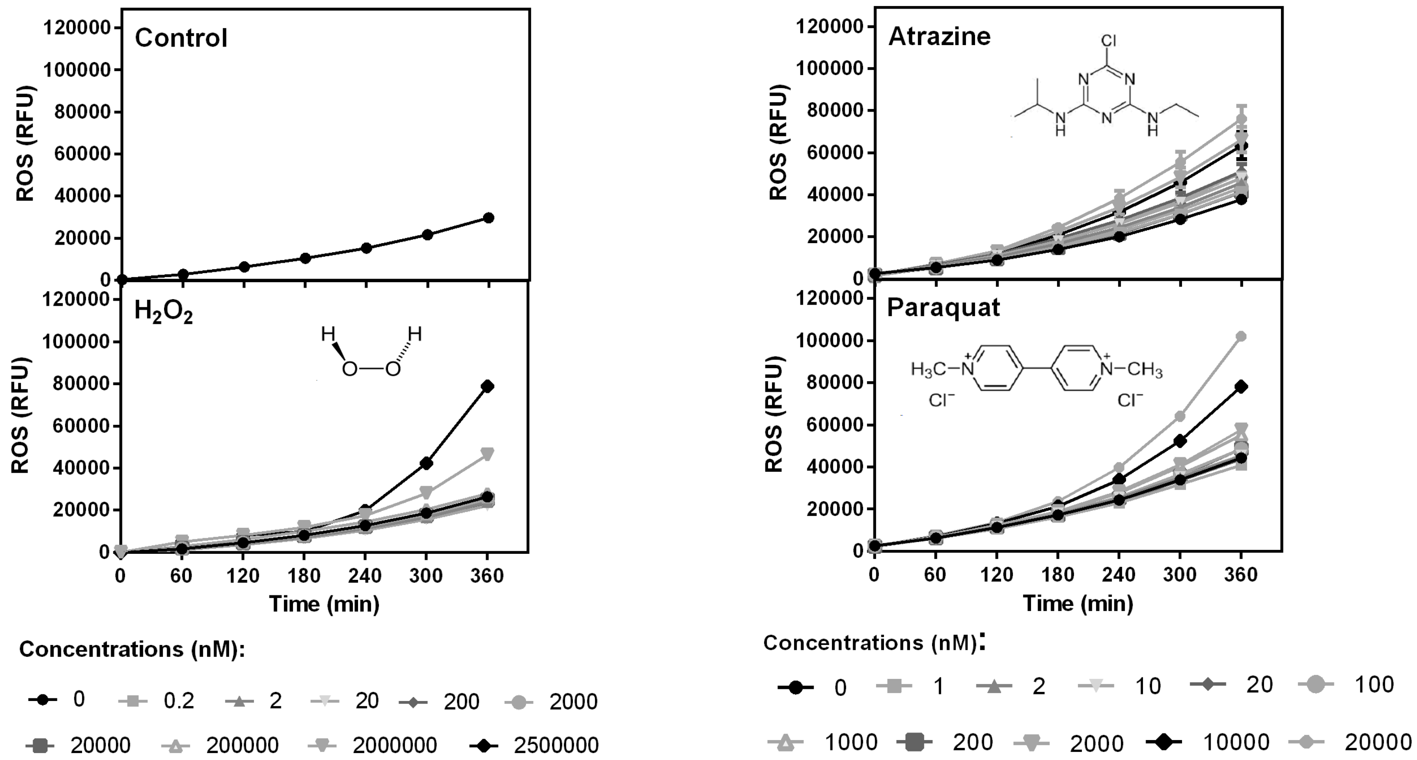

Fig. 1. Reactive oxygen species (ROS; fluorescence) as a function of time in *Chlamydomonas reinhardtii* exposed for 6 h in the light to the positive controls atrazine, paraquat and H_2O_2, along with a control (algae + dye). The experimental results (Mean±SEM) represent 3 independent studies. RFU – relative fluorescence units.

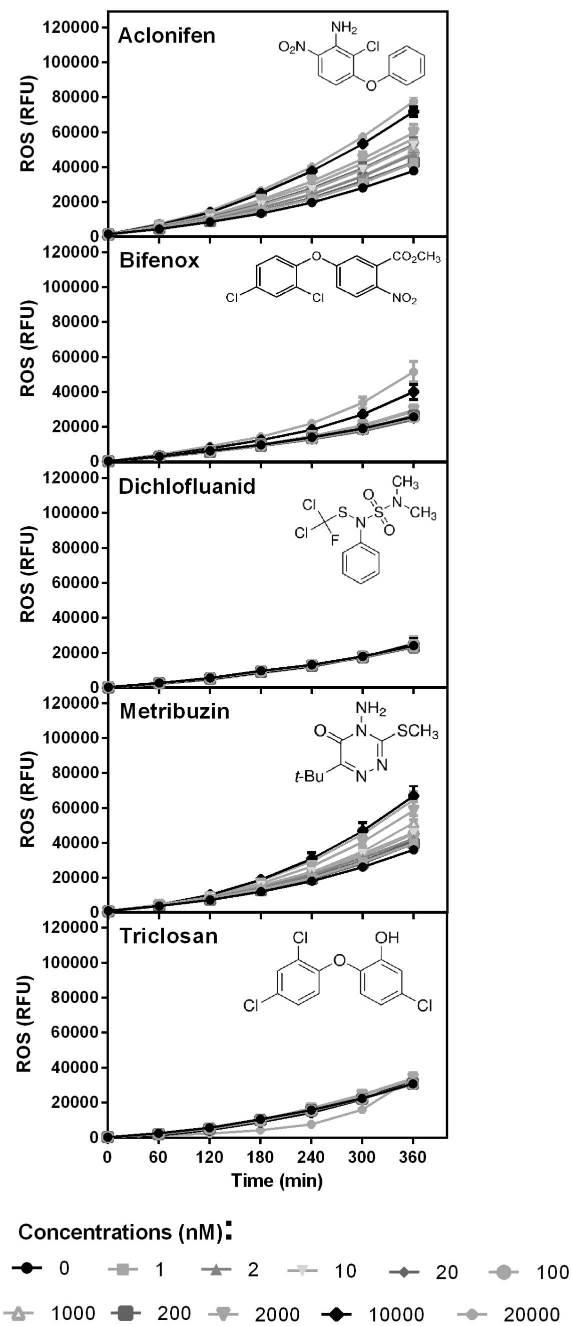

Fig. 2. Reactive oxygen species (ROS; fluorescence) as a function of time in *Chlamydomonas reinhardtii* exposed in the light to aclonifen, bifenox, dichlofluanid, metribuzin and triclosan for 6 h. The experimental results (Mean±SEM) represent 3 independent studies. RFU – relative fluorescence units.

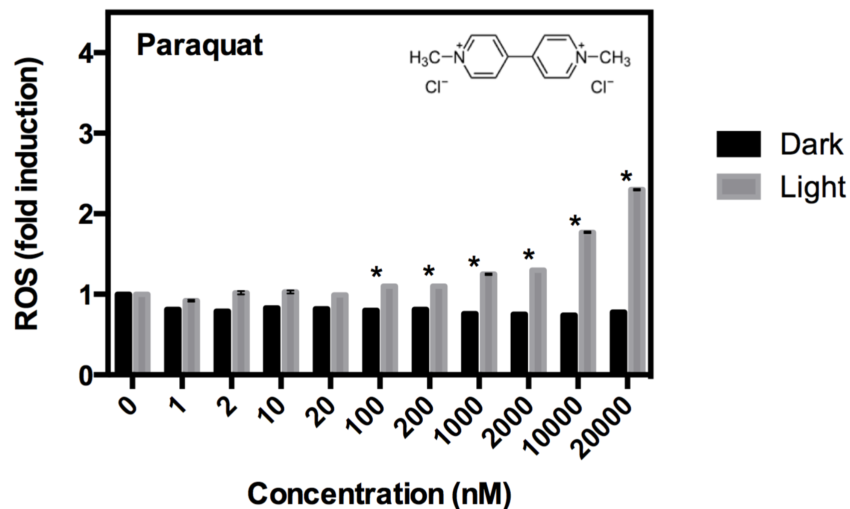

Fig. 3. Reactive oxygen species (ROS) formation in *Chlamydomonas reinhardtii* exposed to paraquat in the dark (dark columns) and in ambient light (grey columns) for 6 h. The experimental results (Mean±SEM) represent 3 independent studies. Asterisks indicate significant differences between concentrations ($p<0.05$).

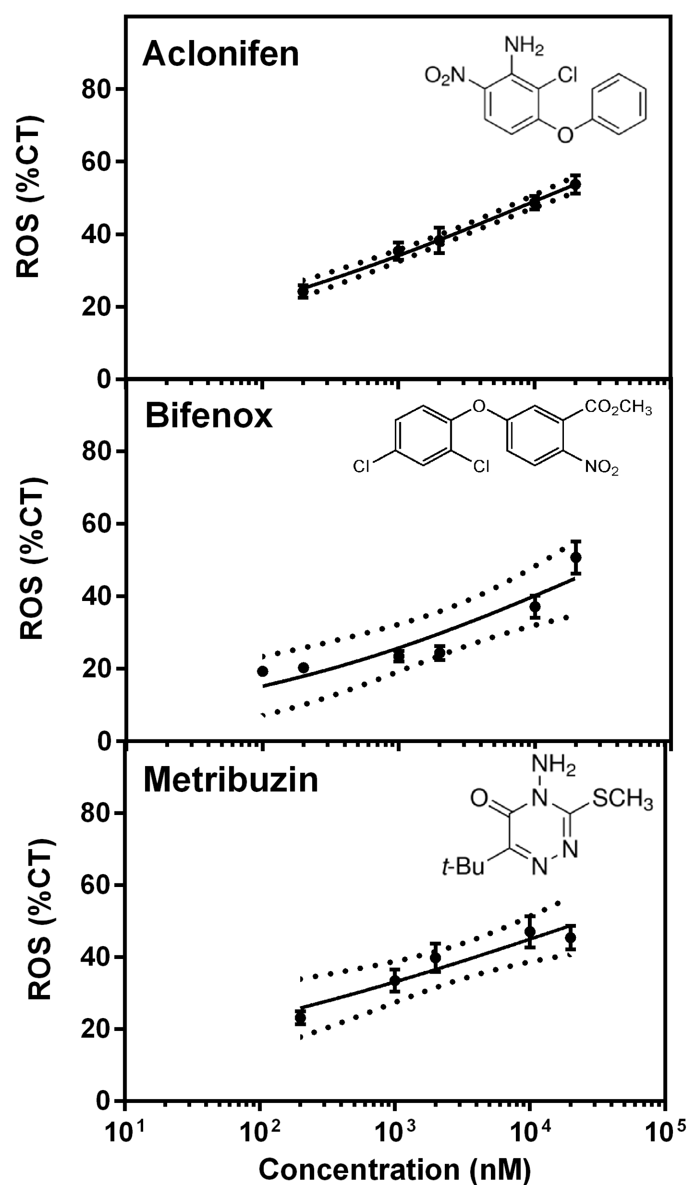

Fig. 4. Reactive oxygen species (ROS) formation (% of control, H_2O_2, %CT) in *Chlamydomonas reinhardtii* exposed to the biocides aclonifen, bifenox and metribuzin for 6 h. The concentration-response curves with 95% confidence were modelled by non-linear regression using a sigmoidal concentration-response curve with a variable slope. The experimental results (Mean±SEM) represent the upper part of the concentration response curve from 3 independent studies.

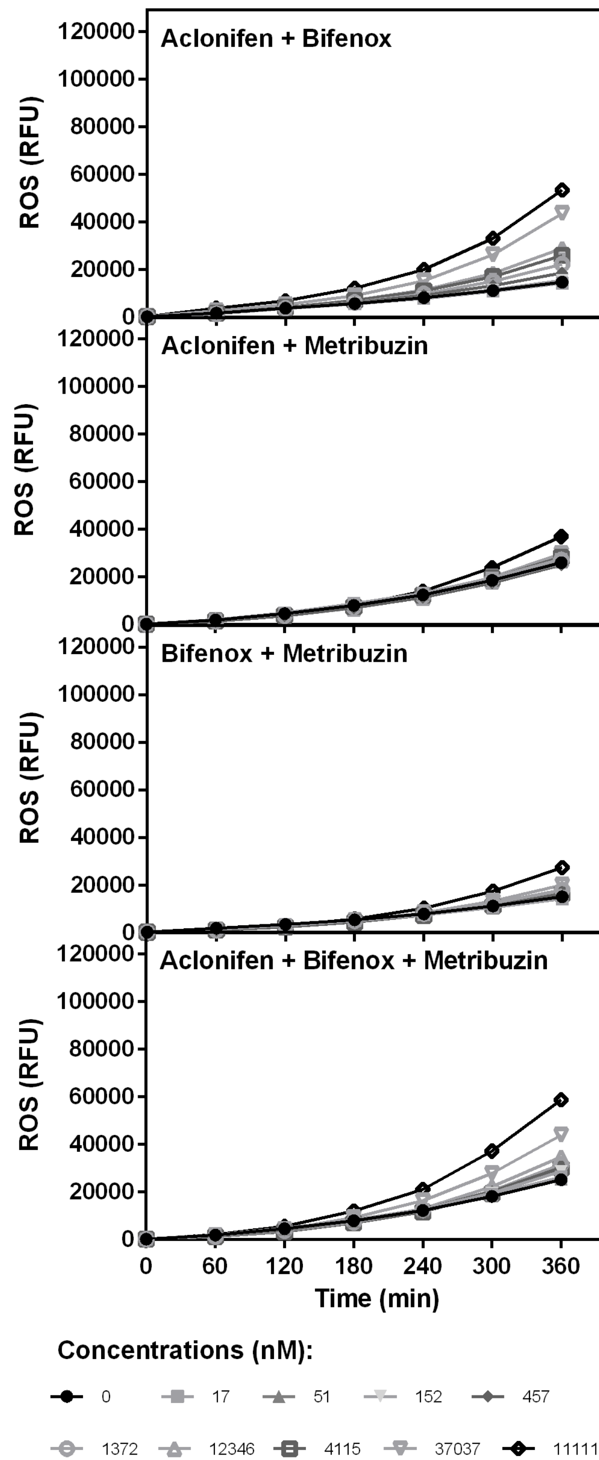

Fig. 5. Reactive oxygen species (ROS; fluorescence) as a function of time in *Chlamydomonas reinhardtii* exposed in the light to the mixtures for 6 h. The experimental results (Mean±SEM) represent 3 independent studies. RFU – relative fluorescence units.