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Study and modelling of the effect of herbicides pulse exposure on freshwater microalgae

Copin Pierre-Jean

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Faculté des géosciences
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Institut des dynamiques de la surface terrestre

Study and modelling of the effect of herbicides pulse exposure on freshwater microalgae

Thèse de doctorat

Présentée à la
Faculté des geosciences et de l'environnement de l'Université de Lausanne
par

Pierre-Jean Copin

Master of Science Msc Sciences et ingénierie de l'environnement
De l'Ecole Polytechnique Fédérale de Lausanne

Jury

Professeur Suren Erkman, Université de Lausanne, Président du jury.
Docteur Nathalie Chèvre, Université de Lausanne, Directeur de thèse.
Professeur Torsten Vennemann, Université de Lausanne, Rapporteur de thèse.
Docteur Nathalie Vallotton, Dow Chemicals, Experte externe.
Docteur Annette Aldrich, Agroscope, Experte externe.

Lausanne, Aout 2015

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CH-1015 Lausanne

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Directrice de thèse :	Mme Privat Docent Nathalie Chèvre
Experte externe :	Mme le Docteur Annette Aldrich
Experte externe :	Mme le Docteur Nathalie Vallotton
Expert interne :	M. le Professeur Torsten Vennemann

Le Doyen de la Faculté des géosciences et de l'environnement autorise l'impression de la thèse de

Monsieur Pierre-Jean COPIN

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intitulée

STUDY AND MODELLING OF THE EFFECT OF HERBICIDES PULSE EXPOSURE ON FRESHWATER MICROALGAE

Lausanne, le 13 novembre 2015

Pour le Doyen de la Faculté des géosciences et de l'environnement

Professeur Suren Erkman, Vice-doyen

« Quand le dernier arbre sera abattu, la dernière rivière empoisonnée, le dernier poisson capturé, alors seulement vous vous apercevrez que l'argent ne se mange pas. »
Prophétie des indiens Cree

« Et mon pouvoir est redoutable tant que je puis opposer la force de mes mots à celle du monde, car celui qui construit des prisons s'exprime moins bien que celui qui bâtit la liberté. »
Stig Dagerman

« N'oubliez pas qu'il faut prendre la vie avec sérénité et joie, quoiqu'il arrive. »
Rosa Luxembourg

A mes parents qui m'ont toujours soutenu dans mes choix

A ma booooty, que j'aime tellement fort

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Résumé grand public

De plus en plus de substances chimiques sont émises et détectées dans l'environnement. Parmi ces substances, on trouve les herbicides qui sont utilisés en agriculture pour lutter contre la présence des mauvaises herbes. Après leur application sur les sols, les herbicides peuvent être entrainés par les eaux de pluie jusque dans les ruisseaux et les rivières. Les concentrations de ces substances varient donc de manière importante dans les systèmes aquatiques en période de pluie ou en période de temps sec. Des pics élevés de concentrations sont suivis de période de concentrations très faibles ou nulles. Les herbicides présents dans les cours d'eau peuvent engendrer des effets toxiques sur les algues et les plantes aquatiques. Or les tests classiques d'écotoxicologie effectués en laboratoire sont réalisés en exposant les espèces vivantes à des polluants de manière continue. Ils ne permettent donc pas d'évaluer les effets des concentrations fluctuantes comme celles des herbicides. Le but de cette thèse est d'étudier et de modéliser les effets des concentrations fluctuantes d'herbicide sur les espèces de microalgues vertes *Scenedesmus vacuolatus* et *Pseudokirchneriella subcapitata*. Des expériences en laboratoire ont également été effectuées dans le but de valider le modèle. Quatre herbicides ont été testés. Il s'agit de l'atrazine (utilisé jusqu'à récemment pour le maïs), du diuron (utilisé dans la vigne), de l'isoproturon (utilisé pour les céréales) et du S-métolachlore (utilisé pour le maïs). Les résultats de ce travail de thèse indiquent que les effets des concentrations fluctuantes d'herbicide peuvent être modélisés sur des algues d'eau douce. Le modèle est relativement simple pour les inhibiteurs de la photosynthèse tels que l'atrazine, le diuron ou l'isoproturon. Il nécessite la connaissance de deux paramètres, le taux de croissance de l'algue sans polluant et la courbe dose-réponse pour chaque substance. Cependant, des expériences supplémentaires doivent être réalisées si la substance étudiée induit un délai de l'effet et du rétablissement ou si une algue est cultivée avec une autre algue dans le même milieu de croissance. Le modèle pourrait également être adapté pour tenir compte des mélanges de substances. Appliqué pour prédire les effets sur les algues de scénarios réels, le modèle montre que les longs pics de concentrations jouent le rôle le plus important. Il est donc crucial de les mesurer lors du monitoring des cours d'eau. D'autre part, une évaluation du risque effectuée avec ce modèle montre que l'impact des pics de concentrations sur les espèces les plus sensibles est total. Cela met en évidence, une fois de plus, l'importance de tenir compte de ces concentrations fluctuantes dans l'évaluation du risque environnemental des herbicides, mais également des autres polluants.

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Glossary and Abbreviation

AA-EQS Annual Average Environmental Quality Standards. Defined in the EU Water Framework Directive. It aims in protecting the aquatic species against the occurrence of chronic effects.

Dose-response curve It is a statistical curve determined for a given organism and a given substance. It describes the relationship between increasing the dose (or concentration) of the substance and the change in response that results from this increase in concentration. It is obtained from laboratory experiments. It allows to predict, for a species, the effect intensity (usually expressed in a percentage between o and 100) for a given concentration of the substance.

EC_{50}, EC_x The 50% or x% Effect Concentration. It refers to the concentration of a substance where 50 % or x% of its maximal effect is observed for a specified endpoint (for example growth inhibition) in a population. This value is statistically determined based on the dose-response curve for a given species and a given substance.

Endpoint It is a variable that can be quantitatively measured and used to evaluate the effects of a toxic agent on a given individual, population, or community. The endpoint can be, for example, mortality, reproduction or growth inhibition. A measurement endpoint designta calculated values such as NOEC or EC_{50}.

HC_5-NOEC, HC_5-EC_{50} Hazardous concentration for 5 % of species. It refers to the predicted concentration of a substance that will affect 5 % of species to a specific toxic threshold. These values are determined from the SSD curve of the substance obtained with NOEC or EC_{50} data.

Mode of action It is defined as a common set of physiological and behavioral signs that characterize the action, i.e., a type of adverse biological response, of a chemical substance on an organism. Substances can have a specific mode of action on organisms having the particular receptors sensitive to the compound, like, for example, the phenylureas herbicides that have a mode of action of “photosystem II inhibition” on algae.

NOEC No Observed Effect Concentration. It is the highest tested concentration of a toxicant that causes no statistically discernable effect on the organisms exposed.

OECD Organisation for Economic Cooperation and Development. The mission of the OECD is to promote policies that will improve the economic and social well-being of people around the world.

SV The unicellular freshwater microalga *Scenedesmus vacuolatus*.

P The unicellular freshwater microalga *Pseudokirchneriella subcapitata*.

μ It is defined as the average growth rates of several successive batch cultures of the algae studied in growth media. It is obtained with a control charter. In the model, it corresponds to the growth rate of the control and the culture treated during the recovery periods.

$\mu_{inh\ x}$ It is the growth rate of the algae studied at concentration *x* obtained from the growth response curve of the standard ecotoxicity test. In the model, it corresponds to the growth rate of the culture treated during the pulse periods.

SSD Species Sensitivity Distribution. It is a statistical distribution describing the variation among a set of species in toxicity of a certain compound or mixture.

Summary

For several years, scientists study the effects of chemicals on the environment. Ecotoxicological tests are performed in laboratory on aquatic or terrestrial species such as microalgae, microcrustacea, fish, bees, birds and earthworms, to determine the toxicity of these substances. In classical laboratory experiments, the exposure of these species to these chemicals is most of the time continuous. However, in the environment, after agricultural application on crops and during and after rain events, chemicals such as herbicides do not contaminate streams and creeks continuously but rather in pulses. The problematic of these pulses is that they often exceed the Annual Average Environmental Quality Standards (AA-EQS), defined in the EU Water Framework Directive, which aims in protecting the aquatic species against the occurrence of chronic effects. Two pulses are often separated by a period of recovery during which low to no concentrations of herbicides is detected in surface water. Recovery periods are mostly not taken into account in traditional ecotoxicological tests while they are crucial to characterize the effect of the pulse exposure. Another weakness of the standard testing procedure is that they do no consider interactions between organisms and focus only on single species.

The aim of this thesis is to investigate and to model the effects of sequential herbicides pulses on unicellular freshwater microalgae. The model proposed is a population dynamics model taking into account the concentration and the duration of pulse exposure, the mode of action of the herbicides and the competition between several algae in co-culture. The model proposed is based on two parameters: i) the typical growth rate of the algae, obtained by monitoring growth rates of several successive batch cultures in growth media, characterizing both the growth of the control and the culture treated during the recovery periods; ii) the growth rate of the algae exposed to pulses, determined from a dose-response curve obtained with a standard toxicity test. Laboratory experiments are conducted in parallel to validate the predictions of the model. The microalgae used in this study are the green algae *Scenedesmus vacuolatus* and *Pseudokirchneriella subcapitata*. Four herbicides were studied: the photosystem II inhibitors atrazine, diuron and isoproturon and the very long chain fatty acids inhibitor S-metolachlor.

Our results show that for the photosystem II inhibitors the model is suitable to predict the cell density inhibition on the two algae species. The recovery of the algae after being exposed to

pulse concentrations of these herbicides is direct and complete. The application of pulse exposure scenarios of photosystem II inhibitors demonstrated that the two algae studied are mostly affected by the longest pulses. It is therefore crucial to detect such characteristic pulses when monitoring herbicide concentrations in streams. The model was also validated for the S-metolachlor on the alga *S. vacuolatus*. Three specificities of this substance were integrated in the model. They concern i) the effect of the herbicide that begins only after 20 hours of exposure based on the optical density and algal cells size measurements; ii) the delay of recovery that also last 20 hours and that is not dependent on the pulse exposure duration or the height of the peak concentration; iii) the modification of the algae sensitivity that happens when algae have been previously exposed to a pulse of S-metolachlor. Finally, a study of the algae *S. vacuolatus* in co-culture with the algae *P. subcapitata* was performed to evaluate the changes this implicates for the modelling. Our results showed that the alga *S. vacuolatus* seemed to negatively influence the growth of the alga *P. subcapitata*. Allelopathy could be a possible explanation of this growth inhibition. Despite the supplementary stress for the algae in co-culture competing for nutrients, the toxicity of the herbicide was lower, for the two algae, when they were in co-culture than in separated culture. The comparisons between the laboratory and the modelled effects showed a good agreement despite minor differences due to the cells count method used to determine the parameters of the model.

To conclude, our study showed the effects of pulse exposure of herbicides can be modeled on algae, but requires several experiments if the compound has a mode of action that induces a delay in the effect and recovery or if the algae is in co-culture with another algae. Some preliminary results showed that the effects of mixture in pulses were well predicted. Indeed not considering mixture is also a weakness of the standard testing procedure. The application of a typical environmental exposure scenario showed that the cell density inhibition is total with S-metolachlor due to the delay in the recovery this substance induces. It is interesting as it has the lowest toxicity, compared to the other herbicides tested, when it is evaluated with classical testing. This point highlights the importance of taking into account pulse exposure in the registration procedure of the pesticide. Further research should be performed to integrate the pulse exposure effect with other organisms such as *Daphnies* species or fish and other substances such as biocides or pharmaceuticals. Therefore, the registration procedure of pesticides, biocides and pharmaceuticals will be greatly improved. Finally, the risk assessment showed that pulse exposure could engender up to 100% effects on the most sensitive species. Considering pulse exposure in risk assessment is therefore crucial.

Résumé

Depuis plusieurs années, les scientifiques étudient les effets des produits chimiques sur l'environnement. En écotoxicologie, des tests sont menés en laboratoire sur des espèces aquatiques et terrestres comme les microalgues, les microcrustacés, les poissons, les abeilles et les vers de terre pour déterminer la toxicité de ces substances. Pour les tests standardisés de laboratoire, une exposition continue des substances chimiques sur ces espèces est considérée la plupart du temps. Cependant, dans l'environnement, après une application sur les cultures agricoles et durant et après des précipitations, certains produits chimiques tels que les herbicides ne contaminent pas les ruisseaux continuellement mais plutôt sous forme de pics d'exposition. La problématique engendrée par ces pics d'exposition d'herbicide est qu'ils dépassent souvent les Normes de Qualité Environnementale exprimées en valeur Moyenne Annuelle (NQE-MA), définies dans la Directive Cadre sur l'Eau de l'Union Européenne et qui visent à protéger les espèces aquatiques des effets chroniques. Deux pics de concentrations d'herbicide se distinguent souvent entre eux par une période de rétablissement durant laquelle de faible voire aucune concentrations d'herbicides ne sont détectées dans les eaux de surface. Ces périodes de rétablissement ne sont la plupart du temps pas prises en compte dans les tests écotoxicologiques standardisés alors qu'ils sont essentiels pour caractériser l'effet des pics d'exposition. Une autre lacune de ces tests standardisés est qu'ils se focalisent uniquement sur une espèce et qu'ils ne considèrent donc pas les interactions entre plusieurs organismes croissant dans un même milieu.

Le but de cette thèse est d'étudier et de modéliser les effets de plusieurs pics d'exposition répétés de concentration d'herbicides sur des microalgues unicellulaires d'eau douce. Le modèle proposé est un modèle de dynamique des populations prenant en compte la concentration et la durée de ces pics d'exposition, le mode d'action des herbicides et la compétition entre plusieurs algues cultivées dans le même milieu de croissance. Le modèle est basé sur deux paramètres : i) la croissance caractéristique des algues, obtenue en mesurant les taux de croissance de plusieurs cultures successives d'algues dans un milieu de croissance dépourvu d'herbicides. Ce paramètre caractérise la croissance du contrôle et de la culture exposée aux pics de concentration durant les périodes de rétablissement ; ii) les taux de croissance de la culture d'algue durant les pics d'exposition, déterminés à partir d'une courbe dose-réponse obtenu en appliquant un test de toxicité standard sur l'algue. Des tests avec pics d'herbicides successifs sont réalisés au laboratoire pour valider les prédictions du modèle. Les

microalgues utilisées dans cette étude sont les algues vertes *Scenedesmus vacuolatus* et *Pseudokirchneriella subcapitata*. Quatre herbicides ont été utilisés. Il s'agit de l'atrazine, du diuron et de l'isoproturon qui sont des inhibiteurs du photosystème II et du S-métolachlore qui est un inhibiteur de la synthèse d'acides gras à très longues chaines (AGTLC).

Nos résultats indiquent que pour les inhibiteurs du photosystème II, le modèle est approprié pour prédire l'inhibition de la densité cellulaire sur les deux espèces d'algues étudiées. Le rétablissement des algues après avoir été exposé aux pics de concentration de ces herbicides est direct et total. L'application de scénarios de pics d'exposition d'inhibiteurs du photosystème II a démontré que les deux algues étudiées sont principalement affectées par les pulses les plus longs. Il est donc crucial de détecter de telles caractéristiques décrivant les pics d'exposition quand des mesures de concentrations d'herbicides sont effectuées dans des ruisseaux. Le modèle était également validé pour le S-métolachlore sur l'algue *S. vacuolatus*. Trois caractéristiques de cette substance ont été intégrées dans le modèle. Il s'agit i) de l'effet de l'herbicide qui se déclenche uniquement après 20 heures d'exposition. Ce résultat est basé sur les mesures de densité optique et de taille des cellules algales au laboratoire ; ii) du délai de rétablissement dont la durée est également de 20 heures et qui n'est pas dépendant de la durée ou de la concentration du pic d'exposition ; iii) de la modification de la sensibilité de l'algue qui peut se produire lorsque cette dernière a été précédemment exposée à un pic de concentration de S-métolachlore. Finalement, une étude sur les deux algues dans le même milieu de croissance (*S. vacuolatus* et *P. subcapitata*) a été effectuée pour évaluer les changements que cela implique pour la détermination des deux paramètres du modèle. Nos résultats ont indiqué que l'algue *S. vacuolatus* semblait influencer négativement la croissance de l'algue *P. subcapitata*. L'allélopathie pourrait être une explication à cette inhibition de la croissance. Malgré le stress supplémentaire induit par la culture de ces deux algues dans le même milieu de croissance luttant pour les mêmes nutriments, la toxicité de l'herbicide était plus faible, pour les deux algues, quand elles étaient cultivées ensemble que séparément. Les comparaisons entre les effets mesurés au laboratoire et les effets modélisés dévoilaient une bonne concordance malgré certaines petites différences causées par la méthode de comptage utilisée pour déterminer les deux paramètres du modèle.

En conclusion, notre étude a montré que les effets des pics d'herbicides peuvent être modélisés sur des algues d'eau douce. Cependant, plusieurs expériences supplémentaires doivent être menées si le composé a un mode d'action qui induit un délai de l'effet et du

rétablissement ou si une algue est cultivée avec une autre algue dans le même milieu de croissance. Certains résultats préliminaires ont montré que les effets des pics de mélange d'herbicides, qui ne sont également pas pris en comptes dans les tests standardisés, étaient bien prédits par le modèle. L'application d'un scénario d'exposition qu'on peut détecter dans l'environnement a montré que l'inhibition de la densité cellulaire était totale avec le S-métolachlore dû au délai dans le rétablissement que cette substance induit. Ce résultat est surprenant car le S-métolachlore a la plus faible toxicité, comparé aux autres herbicides étudiés, quand il est évalué à l'aide d'un test standardisé. Ce résultat met en avant l'importance de prendre en compte les concentrations fluctuantes dans les procédures d'enregistrement des pesticides. D'autres recherches devront être menées pour évaluer l'effet des pics sur d'autres organismes aquatiques comme les microcrustacés ou les poissons mais également avec d'autres substances telles que les biocides ou les médicaments. Ainsi, la procédure d'enregistrement de toutes ces substances serait grandement améliorée. Finalement, l'évaluation du risque a révélé que les pics d'exposition pouvaient engendrer 100% d'effets sur les espèces les plus sensibles. Par conséquent, la prise en compte des pics d'exposition dans l'évaluation du risque est nécessaire.

Chapter 1

Introduction

1.1 General introduction

Pesticides are used worldwide since 50's in increasing quantities. If their key role in the development of agriculture is undoubted, the side effect engendered by this use is of increasing concern. The term « pesticide » is often used interchangeably with the term « plant protection product ». However, the term « pesticide » includes also the substances used in non-agricultural applications to kill pests and called biocides (EU 2009a). Indeed, a plant protection product is defined as a substance or a mixture of substances used to protect plants and plant-based products against pests and their action (EU 1991) whereas a pesticide is a substance or a mixture of substances used to kill pests (Spellman 2009). Several types of pesticides exist according to the target species they focus on: herbicides, insecticides, fongicides, rodenticides and other compounds (nematicides, molluscicides, algicides...) (Gauthier-Clerc and Thomas 2010). In this thesis, we use the term « pesticide » rather than « plant protection product ».

A pesticide, in its commercial form, called also pesticide formulation, is constituted of a mixture of two parts (Chèvre and Erkman 2011):

- The active ingredient that contains the toxic property.
- The inert or other ingredients as the carriers or the adjuvants. The carriers, such as an organic solvent or mineral clay, allow a pesticide to be dispersed effectively. They improve application effectiveness, safety, handling and storage. The adjuvants are used to improve mixing, application or to enhance the performance of the active ingredient. The adjuvants can compose more than 50% of the final product. They include surfactants, compatibility agents, antifoaming agents, spray colorants and drift control agents (Fishel 2013).

Some studies showed that pesticide formulations can be more toxic than the active ingredient. Indeed, the glyphosate formulation, a famous herbicide called also in its commercial form Roundup, is more toxic than the glyphosate active ingredient. This is caused by the important toxicity of the polyoxyethylene tallowamine surfactant composing the glyphosate formulation (Daouk et al. 2013).

1.1.1 A history of pesticides

The first pesticides used were inorganic compounds such as minerals and metals. Some information was recorded about the use of arsenic sulfides to control garden insects in China in AD 900. The earliest known record of arsenic use as an insecticide in the western world is 1669; it was applied in the honey as an ant bait. Copper compounds were used since 1880 in the famous Bordeaux mixture to control fungi. Sulfur was applied as fungicide at the beginning of the 19th century and remained very popular in agriculture because of its low toxicity for the humans and the environment. These inorganic compounds were very employed until the mid 1930's to control pests (Costa et al. 1987). From the 1930's, first synthetic organic pesticides, in particular insecticides, were proposed for public use (Costa et al. 1987). Between 1935 and 1950, the development of DDT (Dichlorodiphenyltrichloroethane) and other organochlorine insecticides enabled the survival of many soldiers who were exposed to insect vectors of malaria and yellow fever in tropical countries during the world war II (Cockerham and Shane 1994) but they also served to eradicate the epidemics as typhus in Naples in 1943 (Perry 1998). Organochlorines were also used extensively in all aspects of agriculture and forestry after the World War II (Schmidt and Rodrick 2003). During the second war, organophosphates were developed as potential chemical warfare agent (Delfino et al. 2009; Munro et al. 1994). From the 1970 years, organophosphates and carbamates were more commercialized in agriculture (Regnault-Roger 2014). They were promoted in agriculture because their persistence in the environment is low and they can be used in different application such as foliar treatment, seed treatment, storage places treatment or livestock protection (Pogacnik and Franko 2001; Regnault-Roger 2014). New molecules were developed in the 80s, such as pyrethroids and neonicotinoids (Gauthier-Clerc and Thomas 2010; Regnault-Roger 2014). Currently pyrethroids insecticides account for 25% of the worldwide insecticide market (Shafer et al. 2005). The first herbicides, the families of triazines, phenylureas or phenoxys, were developed during the years 50's for agriculture (Regnault-Roger 2014). In general, the use of pesticides in agriculture increased during the Green Revolution at the late 1950's, revolution that aimed in improving the food production (Pimentel 1996).

Although the productivity of cereals in the world increased, the use of pesticides has caused serious public health and environmental problems (Pimentel 1996). First, Rachel Carson highlighted the deleterious effects of insecticides, specifically DDT, on environmental

ecosystems in 1962 in her book “Silent Spring”. She described the bioaccumulation of DDT and its effects on the reproduction of birds (Carson et al. 1962). From this date, several studies on these organochlorine insecticides showed that they are persistent in the environment, bioaccumulative, slowly degradable and that they may affect the aquatic and terrestrial species (Hoffman 2003). Consequently, the production and use of organochlorine insecticides, i.e. DDT and its metabolites, Aldrin, Dieldrin, Endrine, Chlordane, Heptachlor, Hexachlorocyclohexane (HCH) and Toxaphene, were forbidden by several countries, such as USA and Sweden, in years 1970 (Costa et al. 1987) and more generally in 2001 with the adoption of the Stockholm Convention by 151 countries (Chèvre and Erkman 2011). However, in 2011, DDT was again authorized for the treatment of Malaria in several countries (WHO 2011). The effects of organophosphates and carbamates were also studies and it was showed that they have high acute toxicity (Pogacnik and Franko 2001; Regnault-Roger 2014). Pyrethroids and neonicotinoids were less toxic and with the same efficiency at lower doses than organophosphates and carbamates (Gauthier-Clerc and Thomas 2010; Regnault-Roger 2014). However, for pyrethroids, some possible chronic effects of exposure to low levels are not excluded for humans. Furthermore, acute neurotoxicity to adults has been well characterized (Kolaczinski and Curtis 2004; Shafer et al. 2005). Some effects on environmental non-target organisms were also highlighted for pyrethroids (Smith and Stratton 1986). Concerning the neonicotinoids, they are suspected to be the cause of the decline of bee populations (Blacquiere et al. 2012; Cresswell 2011; Whitehorn et al. 2012). As a consequence, the European Commission decided in 2013 to limit the use, during 2 years, of three neonicotinoids (clothianidin, imidacloprid and thiamethoxam) to improve the risk assessment of these substances (Regnault-Roger 2014). The effects of several herbicides were also highlighted during the 1990 years. Indeed, the effects of atrazine, a famous triazine substance, were well studied at the human and environmental levels (Sass and Colangelo 2006; Solomon et al. 1996). Atrazine is considered, in Europe, as an endocrine disrupter and as a carcinogenic compound (Gauthier-Clerc and Thomas 2010). It was prohibited in 2003 in the European Union and replaced by the triazine herbicide terbuthylazine (LeBaron et al. 2008). Note that higher concentrations than the non health-based limit (0.1 µg/L for any pesticide) were detected in European groundwater up to 2003. However, it is still authorized in the North America because the Environmental Protection Agency of USA (EPA) judged that the results of the available studies concerning its potential endocrine disrupter do not allowed concluding to a potential cancer risk for humans. (Sass and Colangelo 2006).

Following the highlighting of the pesticide effects, an awareness of worldwide populations occurred and pressure were applied at the political level for decisions to be taken to limit the impact of these substances. Therefore, authorization procedures were defined in several countries in the world such as in the European Union with the regulation concerning the placing of pesticides (EU 2009b). These authorization procedures allowed the commercialization of pesticides less toxic and the creation of toxicological and ecotoxicological data. However, despite the creation of these authorisation procedures, many pesticides are again marketed today. Indeed, in Switzerland, between 2005 and 2011, an average of 340 active substances of pesticides were accredited each year (Wittmer et al. 2014). Despite the establishment of pesticides authorization procedures and the number of scientific reports about the ecotoxicology of pesticides, many unknowns remain concerning ecotoxicological data of pesticides. Indeed, some substances are poorly assessed, the number of studies concerning the chronic effects of pesticides is still low and the effects induced by repeated exposition to pesticides in streams and rivers are very poorly available.

1.1.2 Sources and transfer process of pesticides in the environment

Pesticides are applied in agriculture according to three methods (Matthews et al. 2014; Salyani and Cromwell 1992):

- by aerial spray drift with aircrafts;
- by terrestrial spray drift with different kinds of ground-base equipment;
- by direct incorporation of the pesticide in the seed.

Most of the pesticides are applied by terrestrial spreading (Pimentel 1995).

Following an application for pest control on a culture, different authors showed that, for many pesticides, less than 0.1% of pesticides can reach their target pests (Pimentel and Levitan 1986; Pimentel 1995). Furthermore, Miller (2004) concluded that 98% of the sprayed insecticides and 95% of the herbicides reach a destination other than their target species, including non-target species, air, water, bottom sediments and food. Therefore, an important amount of the pesticides applied can be transferred by several processes in the different compartments of the environment, i.e water, soil and air, where they can be degraded or affect

the biota. The several pesticide transfer and degradation processes in the environment are represented and characterized on the Fig. 1.1.

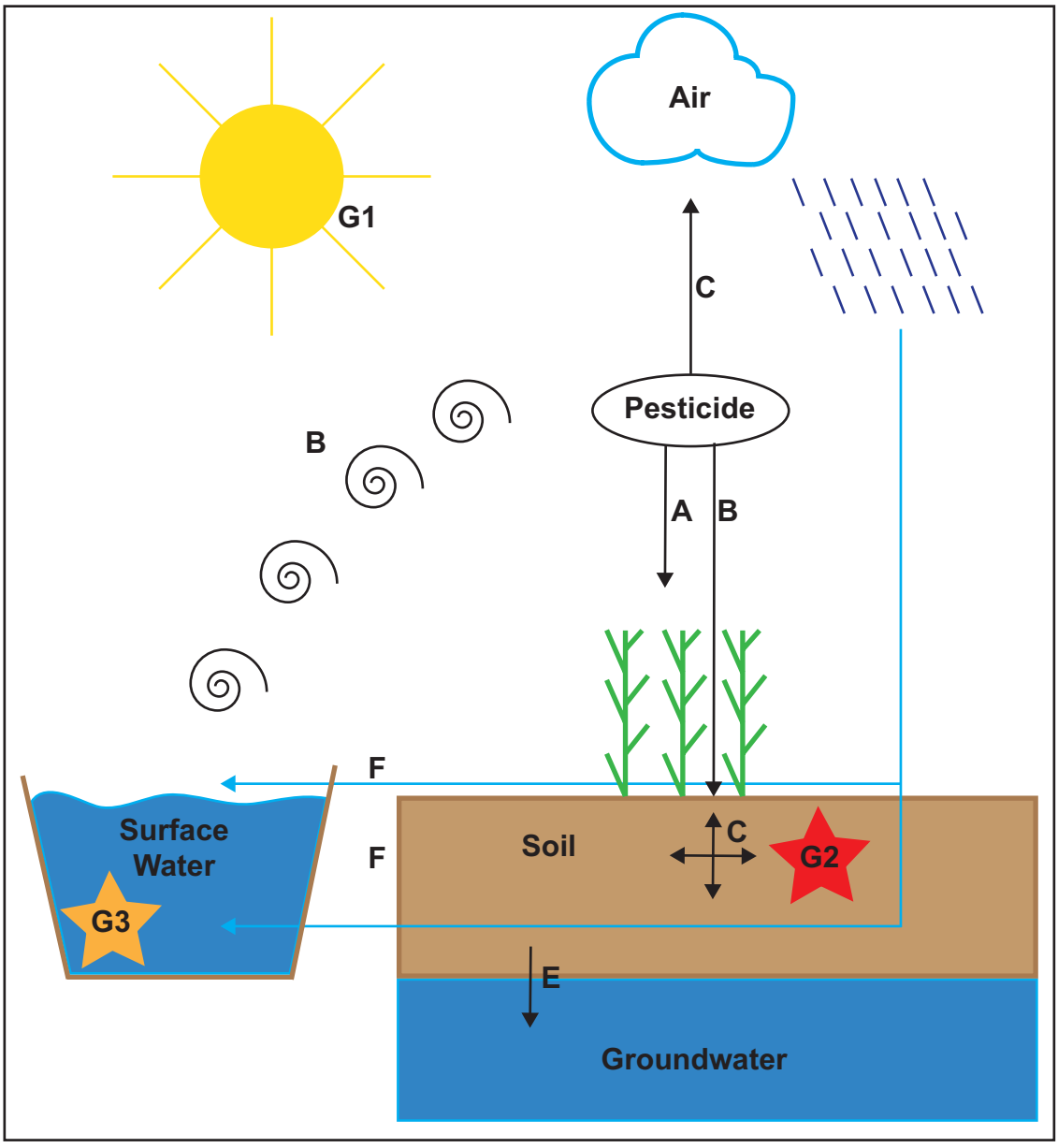


Fig. 1.1. Schematic fate of a pesticide in the environment.

A. The pesticides are absorbed by the grassy and the broadleaved weeds to treat.

B. The pesticides can be transferred directly to the compartments of soils or water by spray drift (Pimentel 1995).

C. Consequently, the pesticides can be found in the soil. According to their physicochemical properties, they can move in this compartment (Bailey and White 1970; Wauchope et al. 2002).

D. The pesticides can be transferred to the air compartment by volatilization. (Bedos et al. 2002; Bedos et al. 2010; Spencer et al. 1973).
E. The pesticides can reach the groundwater by percolation or leaching (Flury 1996; Beckert et al. 2011).
F. The pesticides can be transported to surface waters by surface runoff or subsurface runoff via drainage pipes. As it is a key process in the formation of pulse exposure in streams and rivers, this type of transfer is described in detail afterwards.
G. The residues of pesticide can be degraded in the compartments of environment either by 1) the decomposition of the microorganisms (soils), 2) the sun photodecomposition (air, water, soil) or 3) chemical processes such as hydrolysis (water).

Surface and subsurface runoff are dependent from the molecule properties, the weather conditions, the application practices, the soils types and the land uses (Brown and van Beinum 2009; Daouk et al. 2013; Freitas et al. 2008; Rabiet et al. 2010). If the water solubility of the pesticide is high and according to the soil characteristics, the surface runoff can be an important source of pesticides in streams. Indeed, 1 to 3.5% of the applied pesticide mass can be lost in a single-runoff event (Flury 1996; Leu 2003; Rabiet et al. 2010). The surface runoffs are induced by the precipitations intensity and their capacity to saturate rapidly the first centimeter of the soil. These surface runoffs reach the outlets of catchments, which can be streams, rivers or lakes. The exposure pattern of herbicides in surface waters is then dependent of several factors as the characteristic of the catchment and waterway, the intensity and timing of the rainfall, and the amounts of herbicides used (Vallotton 2007). In the case where the outlet is a stream or a river, i.e. a small or medium sized receptacle, and where the precipitations are irregular, surface runoffs are thus also irregular and therefore the concentrations of pesticides in streams and rivers fluctuate. In contrast, even if the precipitations are irregular, the concentrations of pesticides in large waterways or lakes are generally low with a slight elevation of levels following the application of the pesticide because of the receptacle size, which is bigger than streams or rivers (Vallotton 2007). Consequently, for streams and rivers, the application of a pesticide on a crop often results in pulse of medium to high concentration during and just after a rain event as illustrated in the Fig. 1.2. It is followed, a certain time after the precipitation, by an interval called “recovery” during which the concentrations of pesticide may dramatically decreased below detectable levels due to hydrologic dilution, runoff events, or degradation in soil, water and sediments (Fig. 1.2) (Reinert et al. 2002). Peak concentration or pulse can be nonrepeating or repeating

dependent on the occurrence of rainfalls and the characteristics of soil (Reinert et al. 2002). Such exposure patterns are observed in different streams in the world during and after rain events and following a period of application (Freitas et al. 2008; Garmouma et al. 1998; Leu 2003; Munz et al. 2013; Solomon et al. 1996). This kind of exposure is characteristic of streams and rivers parts located in catchments with a high agricultural activity (House et al. 1997; Muller et al. 2002). However, pulse exposure scenarios can also be observed in streams and rivers located in urban areas where the pesticides are used in non-agricultural application and named biocides. Indeed, the herbicides diuron and glyphosate are often used as weed control at the railroad tracks and at other traffic grounds in industrial and residential areas. Therefore, these non-agricultural applications can significantly contribute during and after precipitations to the irregular pesticide load in receiving water (Muller et al. 2002; Skark et al. 2004). Pulse is thus a characteristic exposure in streams and rivers. Solomon et al. (1996) showed that, for the herbicide atrazine, the biota could be exposed to pulses exceeding 20 µg/L in North American lower-order streams. Thus, they highlighted the requirement to characterize the response of aquatic organisms to the pulse exposure of substances at environmentally relevant concentrations and during the recovery periods. However, a few studies analyzed the effects of pulse exposure on aquatic organisms in ecotoxicology. The effects of this type of exposure highlighted by these studies can be problematic for aquatic organisms.

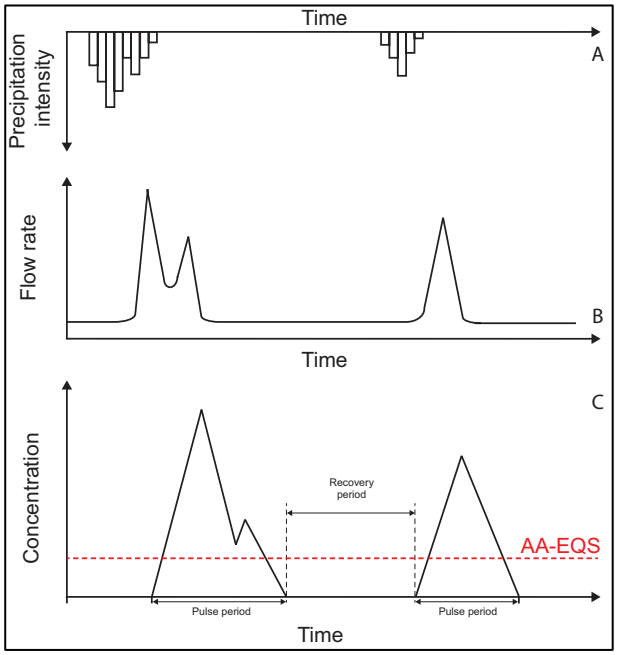


Fig. 1.2. Schematic representation of **A:** precipitation intensity. **B:** flow rate. **C:** repeating pulse exposure with the terminology used (adapted from Reinert et al. 2002).

1.1.3 Effects of herbicide pulse exposure on aquatic ecosystems

The herbicides pulse concentrations detected in streams or rivers are often well above the Annual Average Environmental Quality Standards (AA-EQS), defined in the EU Water Framework Directive, and aiming in protecting the aquatic environment against the occurrence of chronic effects (EU 2008; Lepper 2005) as shown in the Fig. 1.2. As an example, the concentrations of atrazine measured by Solomon et al. (1996) are well above the AA-EQS defined for atrazine (0.6 µg/L). The herbicide pulses can affect the density and the composition of phytoplankton, benthic and epiphytic microalgae, and macroalgae living in these streams. This is critical for the whole aquatic ecosystem, as they are energy sources for many species (Hoffman 2003). In the laboratory, microalgae and aquatic plants are generally exposed continuously to herbicides to test their toxicities. Indeed, the duration of a standard test for freshwater algae is fixed to 72 hours (OECD 2011) and to 7 days for freshwater aquatic plants (OECD 2006). Therefore, these types of tests do not allow assessing the effect of pulsed exposure, such as that occurring in streams. It is thus crucial to simulate pulse exposure scenarios in the laboratory to improve the environmental risk assessment of pulses.

1.1.3.1 Herbicide pulse exposure on algae and aquatic plants

Effects

Some authors have tried to depict the effects the herbicide pulses generate on saltwater and freshwater algae. The effect of the frequency of pulses on the response of microalgae was analysed. For example, Macinnis-Ng et al. (2004) highlighted that multiple-pulses of the herbicide formulation Irgarol 1051 had a greater impact than a single pulse on the seagrass *Zostera capricorni* despite a recovery period of 4-day between the pulses. A study showed that pulses exposure of the herbicide isoproturon inhibited systematically the growth and the effective quantum yield of the alga *Scenedesmus vacuolatus* and that the effects were reversible. The growth inhibition during pulses exposure resulted also in a loss in total biomass production (Vallotton et al. 2009). The effect of the duration of a pulse exposure was compared for 2 herbicides, atrazine and isoproturon, on the alga *S. vacuolatus*. Thus, if the growth rate of the alga was assessed, atrazine was less toxic than isoproturon after 10 hours of exposure whereas the toxicity for the 2 substances was similar after 48 hours (Vallotton et al. 2008). The effects of S-metolachlor, an herbicide with a mode of action different of

photosystem II inhibitors, were also investigated on the alga *S. vacuolatus*. No effect was detected during the first 10 hours of a 48-hour S-metolachlor exposure, even at the highest concentration studied. Furthermore, a clear physiological effect of this substance during exposure was the increase in cell diameter of exposed algae cells (Vallotton et al. 2008b).

Other studies investigated the effects of pulses on several aquatic plants, such as the floating macrophytes *Lemna minor*. Cedergreen et al. (2005) showed that the effects of herbicides pulse exposure for *L. minor* were dependent on the herbicide K_{ow} because it affects the uptake in the algae cells. This was confirmed by the study of Boxall et al (2013). They showed that the consequence of the high K_{ow} of the herbicide pentachlorophenol (log K_{ow}=5.05) was a rapid uptake across cellular membranes resulting in greater internal exposure. Consequently, the toxicity was greater under high pulse exposure of pentachlorophenol relative to lower continuous exposure scenarios (Boxall et al. 2013).

Recovery

An analysis of the recovery periods was also conducted on different algae. For the algae *S. vacuolatus, Pseudokirchneriella subcapitata*, *Desmodesmus subspicatus*, the recoveries were fast and direct after they were exposed to pulse concentrations of atrazine and isoproturon (Klaine et al. 1997; Vallotton et al. 2009; Vallotton et al. 2008a; Weber et al. 2012). Even if the alga *P. subcapitata* was exposed to a peak at 10 times the maximum predicted environmental concentration of isoproturon, the effects were only transient (Weber et al. 2012). Similarly, the recovery from exposure up to 50 µg/L of atrazine was nearly instantaneous for the alga *P. subcapitata* (Klaine et al. 1997). However, this fast and direct recovery characteristic is valid only for photosystem II inhibitors. Indeed, for other herbicides with a different mode of action as chloroacetanilides (S-metolachlor), a delay in the recovery was observed (Vallotton et al. 2008b).

The recovery was also analysed for several aquatic plants, such as the floating macrophytes *L. minor*, previously exposed to pulses. The study of Cedergreen et al. (2005) showed also that the effects of herbicides pulse exposure for *L. minor* were dependent on the mode of action of the herbicides used. Indeed, according to the herbicides mode of action, the capacity of the plant to recover after the damage induced by the herbicide pulse exposure can be extremely

variable. Boxall et al (2013) obtained the same conclusions. Indeed, they showed that the photosystem II inhibitor isoproturon has a lower impact on the macrophyte *L. minor* when it was applied in pulses than in continuous exposure, due to the typically rapid recovery of photosystem II inhibiting herbicides. However, for the metsulfuron-methyl, which is a sulfonylurea herbicide with a different mode of action than isoproturon, the toxicity is similar when it was applied in pulses and continuously. This similar toxicity between pulse and continuous exposure was caused by a lag-phase during the recovery period following a pulse exposure resulting from arrested cellular division. Other studies on aquatic plants highlighted also the importance of the herbicides mode of action in the recovery phase of *Lemna* species, which can be:

- Moderate or rapid with, for example, photosystem II inhibitors as triazines and phenylureas. Therefore, the effect was reversible in the nature (Brain et al. 2012; Mohammad et al. 2008; Mohammad et al. 2010; Teodorovic et al. 2012).
- Delayed with, for example, acetyl CoA carboxylase inhibitors as sulfonylureas or very-long-chain fatty acid biosynthesis inhibitors as chloroacetanilids. Therefore, the effect was reversible in the nature but with a delay (Mohammad et al. 2008; Mohammad et al. 2010; Rosenkrantz et al. 2012).

Sensitivity

The sensitivity of freshwater algae was also studied after being exposed to pulse concentrations of herbicides. The sensitivity of the alga *P. subcapitata* was not altered from a previous atrazine pulse exposure. Similarly, the alga *D. subspicatus* was not modified from a previous isoproturon or atrazine pulse exposure (Baxter et al. 2013; Weber et al. 2012). However, there was a slight shift in the sensitivity of the alga *S. vacuolatus* after being exposed to repeated long pulses of isoproturon with short recovery periods (Vallotton et al. 2009).

1.1.3.2 Pulse exposure on periphyton communities

The effects of pulse exposure scenarios were also assessed for more complex systems such as periphyton communities, which are complex assemblage dominated by algae, cyanobacteria and diatoms (Graham et al. 2009). Gustavson et al. (2003) showed that the effect on

periphyton of 3 herbicides pulse exposure (isoproturon, metribuzin and hexazinon) was greater than a continuous exposure for standard single-species growth-tests with phytoplankton species. Furthermore, the effects and the recovery were dependent, respectively, of the pulse duration and of the composition of the periphyton. Indeed, different periphyton groups responded differently to metribuzin exposure, i.e. either they did not recover as chlorophytes, or they recovered well as diatoms and cyanobacteria (Gustavson et al. 2003). Taking into account two fluorescence parameters, the effective and the optimal quantum yields of PSII photochemistry, it was showed that the recovery of a periphyton community exposed to herbicide pulse exposure was not direct after the chemical was removed but delayed. Indeed, the recovery of a periphyton community exposed to two photosystem II inhibitors, isoproturon and atrazine, were complete 12 hours after the pulse, except for the highest concentration of isoproturon (Laviale et al. 2011). Tlili et al. (2008; 2011) studied also the impacts on periphyton communities of the herbicide diuron or a mixture of the herbicide diuron and the fungicide tebuconazole applied in pulse exposure. In the two cases, they showed that the effects of pulsed exposure on biofilms depend on whether the biofilms had previously been exposed to the same stressors or not.

Some studies therefore investigated the effects of herbicides pulse exposure on algae, aquatic plants or periphyton community. However, the laboratory procedure to test herbicides pulse exposure is complicated to organize. Therefore, the development of models can be a useful tool to evaluate the effects of these pulse exposures.

1.1.4 Effects modeling of pesticides pulse exposure on aquatic ecosystems

As a certain number of studies were performed to analyse the effects of pesticide pulse exposure on aquatic organisms in laboratory, different models were developed simultaneously to predict these effects (Ashauer et al. 2006; Ashauer et al. 2007; Ashauer et al. 2010; Ashauer et al. 2011; Nagai 2014; Weber et al. 2012). For microcrustacea and fish, the most appropriate models consider toxicokinetics (i.e., the time course of uptake, biotransformation, and elimination of toxicants in the organism) and toxicodynamics (i.e., the dynamics of injury and recovery in the organism) (Ashauer et al. 2006; Ashauer et al. 2007; Ashauer et al. 2010; Ashauer et al. 2011; Rozman and Doull 2000). For algae, either toxicokinetic-toxycodynamic

models or toxycodynamics models considering only the injury and the recovery were suitable to simulate effects of time-variable exposure to algae (Ashauer and Brown 2013). Indeed, Nagai et al. (2014) developed a model based on toxicokinetics and toxycodynamics processes to calculate the effects of the herbicides pretilachlor, bensulfuron-methyl, pentoxazone and quinoclamine, characterized by their different mode of action, on the green alga *P. subcapitata*. However, the structure of this toxicokinetic-toxycodynamic model was complex with a difficult parameterization due to the many parameters that must be defined. Furthermore, Vallotton et al. (2008) showed that the effect of isoproturon pulse exposure, i.e. the inhibition of the PSII effective quantum yield measured with a fluorometer, was very fast for the alga *S. vacuolatus*. Moreover, the effects of atrazine and isoproturon on natural periphyton were shown to be significant rapidly, i.e. within 1h (Laviale et al. 2011). The effects of time-variable exposure are therefore conducted principally by toxicoydynamics for algae. Consequently, population dynamics models, i.e. where toxicokinetics were not taking into account, were developed. For example, Weber et al. (2012) developed a population dynamic model to assess the effect of the herbicide isoproturon on the algae *D. subspicatus* and *P. subcapitata*. In that study, the authors modelled the population fluctuations as a function of four parameters: temperature, light intensity, nutrient availability and chemical concentration. But the model proposed is mainly descriptive and therefore difficult to use for effects predictions due to the lack of information on the different variables.

Consequently, few simple models, i.e with parameters easily determinate with classical experiments (standard OECD test), were developed to predict the effect on algae exposed to sequential pulses of herbicides.

1.2 Objective of the study

The objective of this thesis is to investigate the effects of sequential herbicides pulse exposure on unicellular freshwater microalgae. The study of these pulses is very important because they can exceed the Annual Average Environmental Quality Standards (AA-EQS) (EC 2008). The laboratory results will allow us to develop a population dynamics model predicting the effects of herbicides pulse exposure on freshwater algae. This model should take into account:

i. The concentration and the duration of pulse exposure.
ii. The mode of action of the herbicides.
iii. The competition between several algae growing together.

For this purpose, the thesis is divided into 4 chapters with different goals:

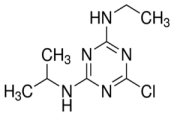
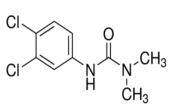
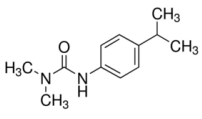
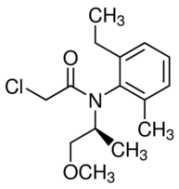
1. The development and the validation in laboratory of a model to assess the effects of the herbicide isoproturon pulse exposure on the freshwater algae species *S. vacuolatus*.
2. The application of the model to other photosystem II inhibitors and other algae, with its validation in laboratory.
3. The extension of the model to other herbicides with a different chemical mode of action than photosystem II inhibitors.
4. The extrapolation of the pulse exposure model to two algae growing in competition.

The results will serve to apply the model in real cases of multiple pulse exposure in streams and rivers. A risk assessment taking account this model will be therefore proposed in the synthesis of this thesis. This risk assessment should be used as a decision-making tool to limit or authorize the application of several herbicides in agriculture to improve the protection of aquatic environments. To reach the development of this population dynamics model, herbicides with different modes of action and several freshwater algae were selected. They are described below.

1.3 Tested herbicides

In our study, only the active ingredients of 4 herbicides were used for laboratory tests and for the validation of the model. The physicochemical characteristics of these herbicide active ingredients are presented in the table 1.1 and described below. In this thesis, we did not investigate the modification of the effect according to the K_{ow} (Cedergreen et al., 2005; Boxall et al., 2013). Indeed, the K_{ow} of the 4 substances used in this study was similar. We rather investigated if the effect and the recovery during and after an herbicide pulse exposure on an alga were direct or not. Furthermore, we analyzed if the sensitivity of the algae after being exposed to herbicide pulses was modified.

Table 1.1. Physicochemical properties of atrazine, diuron, isoproturon and S-metolachlor

Common name	**Atrazine**	**Diuron**	**Isoproturon**	**S-metolachlor**
Chemical form [1]	$C_8H_{14}ClN_5$	$C_9H_{10}Cl_2N_2O$	$C_{12}H_{18}N_2O$	$C_{15}H_{22}ClNO_2$
CAS Number [1]	1912-24-9	330-54-1	34123-59-6	87392-12-9
Application in agriculture [2]	Forbidden in Switzerland and in the European Union	Fruits, vine	Wheat, barley, rye, spelt, triticale, oat	Corn, sorghum, soya, sunflower, beet, beans, squash, chicory, fallow, miscanthus
Herbicide Family [1]	Triazine	Phenylurea	Phenylurea	Chloroacetanilide
Structure [3]				
Log K_{ow} [1]	2.7	2.87	2.5	3.05
Molar weight (g) [1]	215.68	233.09	206.28	283.79
Water solubility (mg/l) [4]	35	35.6	70.2	480
K_{oc} (mL/g) [4]	100	920	122	226
DT50 (j) [4]	29	78	22.5	21

[1] **http://sitem.herts.ac.uk/aeru/iupac/**
[2] **http://www.blw.admin.ch/psm/wirkstoffe/index.html?lang=fr**
[3] **http://www.sigmaaldrich.com/switzerland-suisse.html**
[4] **http://www.ineris.fr/siris-pesticides/bdd_siris_pesticides**

1.3.1 Atrazine

Atrazine belongs to the triazines group. It was banned in 2003 in the European Union but it is again authorized in other countries like USA. It is used in selective weed and broad-leaf control programs as pre- and postemergent herbicide (Graymore et al. 2001). In USA, it is applied principally on corn cultures (85%), the rest for sugarcane and sorghum cultures (Sass and Colangelo 2006). It is moderately soluble (35 mg/L) and has a low octanol-water partition coefficients (log Kow=2.7). It has thus low to moderate tendencies to accumulate in biota. As it inhibits the photosynthesis by interrupting electron transport through photosystem II (PSII) (Knauert 2008; Vallotton et al. 2008a), atrazine affects mostly algae and macrophytes (Solomon et al. 1996). Atrazine is one of the most intensively studied herbicide. Algal responses to atrazine vary widely according to the concentration used, the duration of exposure and the algae species tested (DeLorenzo et al. 2001). Perturbation of photosynthesis was observed on phytoplankton and periphyton communities at concentrations from 1 to 10 µg/L. From 10 to 20 µg/L, death of non-resistant species and succession with resistant species of phytoplankton occurred. At concentration up to 500 µg/L, photosynthesis, carbon-14 uptake and biomass were reduced by 95% within 2 days. Atrazine also affected the growth of periphytic algae (Graymore et al. 2001). The Annual Average Environmental Quality Standards (AA-EQS) was fixed for atrazine to 0.6 µg/L (EC 2008). Atrazine concentration peaks higher than the AA-EQS of atrazine often characterize the concentrations of atrazine in worldwide streams and rivers. Indeed, Richards and Baker (1993) obtained a representative annual hydrograph and chemograph for atrazine in the Sandusky River, a tributary to lake Erie, Ohio, USA, in 1990. The first runoff following application was characterized by high atrazine concentrations, around 20 µg/L. Similarly, pulse exposure were observed in a stream of Zurich Oberland, Switzerland, in 1999. The maximum concentration was measured at 31 µg/L (Leu et al. 2005). In France, Garmouma et al. (Garmouma et al. 1997) measured atrazine concentrations during the years 1991, 1992 and 1993 in a small stream in an agricultural basin. The concentrations pattern was also represented as pulses and the maximum concentration during these 3 years was measured to 2.45 µg/L.

1.3.2 Diuron

Diuron belongs to the phenylureas group. Diuron is principally used for pre- or post-emergence weed control in cotton, fruit and cereal crops worldwide (Sorensen et al. 2003). It can also be used also for non-agricultural applications. Indeed, occurrence of diuron in surface waters can be caused by its use in residential settlements and industrial areas as well as from weed control on railway tracks (Skark et al. 2004). It is moderately soluble (35.6 mg/L) and has a low octanol-water partition coefficients (log K_{ow}=2.87). Consequently, it has low to moderate tendencies to accumulate in biota. It inhibits photosynthesis by interrupting electron transport through photosystem II (PSII) (Knauert 2008; Vallotton et al. 2008a). Ecotoxic tests on four algae species, *Chlorella vulgaris*, *Rhaphidocelis subcapitata* (former name of *P. subcapitata*), *Chlorella pyrenoidosa* and *Scenedesmus quadricauda* showed that diuron was one of the most toxic substances among the herbicides (Ma et al. 2001; Ma et al. 2002; Ma et al. 2003; Ma et al. 2006) . Diuron is often considered as the most toxic substance within the phenylureas (DeLorenzo et al. 2001). Some studies on periphyton community showed that low concentrations caused acute and chronic effects. Indeed, short-term effects of diuron arise from 4-9 µg/L as EC_{50}. Furthermore, diuron concentrations down to 0.08 µg/L induced chronic effects (McClellan et al. 2008). Realistic concentrations of diuron (from 0.07 to 7 µg/L) caused a chain of effects in a biofilm, which included inhibitory effects on algae but also indirect effects on the relationships between biofilm components (Ricart et al. 2009). The Annual Average Environmental Quality Standards (AA-EQS) for diuron was fixed to 0.2 µg/L (EC 2008). The concentrations of diuron in worldwide streams have a pulse exposure pattern and the maximum concentrations of these peak exposures are often higher than the AA-EQS of diuron. Indeed, Field et al. (2003) obtained a pulse exposure pattern for diuron, from November 1995 to June 1997, in the Lake Creek stream, Oregon, USA. The maximum pulse concentration during this period was measured to 28 µg/L. In Switzerland, concentrations of diuron were measured between January 2008 and November 2010 in the Charmilles stream, which is located in a vineyard catchment of the Geneva region, and between April 2007 and October 2009 in the Furtbach stream, which is located in an urban catchment of the Zurich region. The dynamic of the diuron concentrations in the two streams was characterized by several pulses during the studied period. These concentrations were lower in the Furtbach stream. Indeed, the maximum concentration in the Charmilles stream was 18 µg/L whereas it was 0.3 µg/L in the Furtbach stream. In the 2 cases, the concentrations were above the AA-EQS of diuron and, consequently, the aquatic organisms

could be impacted. The concentrations of diuron in the Furtbach stream were induced by non-agricultural applications. Indeed, diuron can be used in private gardens and industrial areas (Munz et al. 2013). Concentrations of diuron during the complete years 1992 and 1993 were measured in a French stream. The pattern of concentrations was represented by pulses. The highest concentration of diuron followed an intense rainfall period and it was measured to 0.845 µg/L (Garmouma et al. 1997).

1.3.3 Isoproturon

Isoproturon belongs to the phenylureas group. Isoproturon is principally used for pre- or post-emergence weed control in cotton, fruit and cereal crops worldwide (Sorensen et al. 2003). It is moderately soluble (70.2 mg/L) and has low octanol-water partition coefficients (log K_{ow}=2.5). Therefore, it has low to moderate tendencies to accumulate in biota. As atrazine and diuron, it inhibits photosynthesis by interrupting electron transport through photosystem II (PSII) (Knauert 2008; Vallotton et al. 2008a). As the primary producers are affected principally by herbicides, ecotoxic tests were performed on several algae or macrophytes species in several studies. It was showed that the toxicity of isoproturon was two times more toxic to the alga *Scenedesmus subspicatus* compared to the macrophyte *L. minor* (Kirby and Sheahan 1994). In single species tests, isoproturon affected the growth rate of algae (Traunspurger et al. 1996). Some studies highlighted the strong toxicity of photosystem II inhibitors such as isoproturon on algae species (Ma et al. 2001; Ma et al. 2002; Ma et al. 2003; Ma et al. 2006). Isoproturon could also induce an effect on periphyton communities. Indeed, Schmitt-Jansen and Altenburger (2005) showed that isoproturon changed the community structure and primary production of microphytobenthos even when the contamination does not exceed levels of acute toxicity. The Annual Average Environmental Quality Standards (AA-EQS) for isoproturon was fixed to 0.3 µg/L (EC 2008). This AA-EQS was often exceeded by isoproturon environmental concentrations. Indeed, in worldwide streams, isoproturon reached surface waters through runoff and drainage mostly during and after rain events resulting in highly fluctuating river concentrations. Therefore, in the river Zwester Ohm, Germany, the contamination showed a seasonal pattern following the pesticide application times. The highest concentration measured was 23.2 µg/L, well above the AA-EQS (Muller et al. 2002). Similarly, the isoproturon concentrations dynamic was represented

as pulses in the Mélarchez stream from January 1992 to December 1993, with a maximum concentration measured at 1.8 µg/L (Garmouma et al. 1997).

1.3.4 S-metolachlor

S-metolachlor is a chloroacetanilide herbicide used to control pre-emergent and early post-emergent annual grassy and broadleaved weeds (Vallotton 2007). It is applied principally in cornfields but also on soybeans, sorghum, cotton and sunflower crops (Peterson et al. 2013). S-metolachlor replaced atrazine, which was banned in 2003 by the European Commission because of its endocrine disruptor potential (Joly et al. 2013; Sass and Colangelo 2006). Consequently, S-metolachlor was a part of the 10 active ingredients most used in the European Union between 1992-2003 (Eurostat 2007). As S-metolachlor is very soluble in water (480 mg/l), it has consequently the potential to leach the ground and surface waters. It has also a medium octanol-water partition coefficient (log Kow=3.05). It has then a moderate tendency to accumulate in the biota. The S-metolachlor belongs to the family of seedling growth inhibitors. This herbicide family avoids the growth of the new plant, reducing the ability of seedlings to develop normally in the soil. The herbicide is absorbed by the developing roots and shoots and can move via the xylem to areas of new growth (Gunsolus and Curran 1991) . At the molecular level, S-metolachlor inhibits the formation of very long chain fatty acids (VLCFA), causing an imbalance in the fatty acid composition of cell membranes. The rigidity and the permeability of cells are reduced and thus the cell division is inhibited (Vallotton et al. 2008b). Very few ecotoxicological studies were performed with S-metolachlor. Indeed, before 1997, in European countries, a racemic product, named metolachlor, containing two R-isomers and two S-isomers in an even ratio (50% each one), was applied. The S-isomers are characterized by their very high herbicidal activity (Moser et al. 1983). Consequently, the most ecotoxicological studies on algae were performed with metolachlor (Junghans et al. 2003; Kotrikla et al. 1999; Ma et al. 2002; Ma et al. 2003; Ma et al. 2006). A study compared the toxicity of racemic metolachlor and S-metolachlor on the alga *C. pyrenoidosa* (Liu and Xiong 2009) and showed a high toxicity of S-metolachlor. A study assessed the effects of the active ingredient S-metolachlor on periphytic diatoms. Live cell density of periphytic diatoms was reduced compared to the control for 6-day exposure to concentrations of 5 and 30 µg/l of S-metolachlor. Some diatoms species of the periphyton

were not affected by the S-metolachlor exposure whereas other ones were more sensitive (Debenest et al. 2009). The Annual Average Environmental Quality Standards (AA-EQS) is lacking for S-metolachlor at the European Union level (Caquet et al. 2013; EC 2008). However, the Ecotox Centre of Eawag-EPFL proposed an AA-EQS equivalent to 1.2 µg/L (http://www.oekotoxzentrum.ch). Similarly to the effect assessment, few data are available on S-metolachlor concentrations in streams contrary to metolachlor. For metolachlor, the concentrations in streams were fluctuating and could be represented by pulse exposure (Hall et al. 1999; Phillips et al. 1999). However, as S-metolachlor, no AA-EQS was defined for metolachlor at the European Union level (EC 2008).

1.4 Choice of species tested: the algae *S. vacuolatus* and *P. subcapitata*

1.4.1 Algae description

Microalgae are microscopic algae, which are important constituents of many ecosystems. They are found in freshwater or marine environments, but also in terrestrial ecosystems. They play a crucial role in the food chain worldwide because they represent more than half total primary production (Guschina and Harwood 2006). They are prokaryotic or eukaryotic photosynthetic microorganisms that can grow rapidly and live in difficult conditions due to their unicellular or simple multicellular structure (Mata et al. 2010). Prokaryotic algae could be for example Cyanobacteria (Cyanophyceae) (Mata et al. 2010). Most eukaryotic algae located in the freshwater ecosystems are blue-green algae (Cyanophyta), green algae (Chlorophyta), diatoms (Bacillariophyta) and red algae (Rhodophyta) (Sheehan 1998; Stevenson et al. 1996). These microalgae are energy sources critical to most aquatic ecosystems. Indeed, they transform the solar energy into chemical forms though photosynthesis. They can thus produce polysaccharides (sugars) and triaglycerides (fats), which are common sources of energy. They produce also proteins that can be used as food for the aquatic animals (Slade and Bauen 2013). As microalgae are sources of energy, they are more and more cultured for the production of biofuels. The advantages of their use as a source of energy is that they are easy to cultivate, they can grow with little attention, using non-drinking water for their growth and easy to obtain nutrients (Alam et al. 2012 ; Mata et al. 2010 ; Slade and Bauen 2013). A contamination by toxicants can affect the composition or the density of algae species involving an impact on the food chain in aquatic ecosystems

(Hoffman 2003). Indeed, microalgae can be non-target species of herbicides in the aquatic environments. Furthermore, they are also sensitive to herbicides that the weeds treated by this kind of substances because they exhibit a similar cell structure and/or processes, i.e. photosynthesis, cell membranes and walls Microalgae can therefore be used in ecotoxicological tests to determine the effect of several chemicals, particularly herbicides and heavy metal as zinc, cadmium or nickel (Hoffman 2003).

Microlagae used all over the study for laboratory tests were chlorophytes (green algae) *S. vacuolatus* (strain 211-15) and *P. subcapitata* (strain 61.81). They were chosen because they are unicellular, easily cultivated in the laboratory, representative of freshwater environments and display a higher sensitivity to a variety of hazardous substances (Backhaus et al. 2004; Blaise et al. 1986 ; Faust et al. 2001; Junghans et al. 2006; Machado and Soares 2014). The algae species *S. vacuolatus* have a circular form whereas the algae species *P. subcapitata* have a curved and twisted appearance like a sickle (Fig. 1.3). The algae species *S. vacuolatus* was obtained from the Department of Bioanalytical Ecotoxicology at the Helmholtz Centre for Environmental Research - UFZ, Leipzig, Germany. The algae species *P. subcapitata* were obtained from the Culture Collection of Algae at Göttingen University (SAG), Germany. The SAG is one of the worldwide largest service algae culture collections. It includes cyanobacteria and microalgae from aquatic and terrestrial habitats, but also from marine environments. It has a huge diversity of microalgae and cyanobacteria, i.e. 2291 strains corresponding to 538 genera and 1424 named species of algae with 370 additional strains that are still unidentified at the species level (Friedl and Lorenz 2012). It exists different strains/ isolates for a same species differing by their genomic variations that can correspond to differences in physiological and biochemical properties. 280 named species of microalgae are represented by multiple strains (Friedl and Lorenz 2012; Muller et al. 2005). For *C. vulgaris*, an alga of great value for applied research, some genomic variation was highlighted in a sample of 29 strains. (Muller et al. 2005). N. Vallotton et al. (2008a; 2008b; 2009) used the same algae species *S. vacuolatus* than us, but another strain (211-8b). The nomenclature of these 2 strains is different. Indeed, the strain 211-15 is currently designated as *Scenedesmus vacuolatus var. vacuolata.* The strain 211-8b is currently called *Scenedesmus vacuolatus* and its former name was *Chlorella fusca var. vacuolata*. They are from two distinct locations. Indeed, the strain 211-15 was isolated from material from bean in Melbourne (Australia) whereas the strain 211-8b was isolated from bark in Philadelphia (USA). Consequently, they may have different physiological behavior. Furthermore, the strain 211-8b is described as

authentic, i.e. the strain has been derived from the type material on which the formal taxonomic description of an algal species has been based. 20% of strains in the SAG database are authentic (Friedl and Lorenz 2012). As the strain 211-15 is not authentic, the molecular signature, i.e. DNA sequences and genomic fragments, is not yet available. Therefore, it is not possible to know if the 2 algae have the same genomic properties. Concerning the alga *P. subcapitata* (strain 61.81), several former names characterized it in the past: *Selenastrum capricornutum*, *Ankistrodesmus subcapitata*, *Raphidocelis subcapitata*, *Ankistrodesmus bibraianus*. It is not an authentic alga and it was discovered in the river Nitelva, in Akershus (Norway).

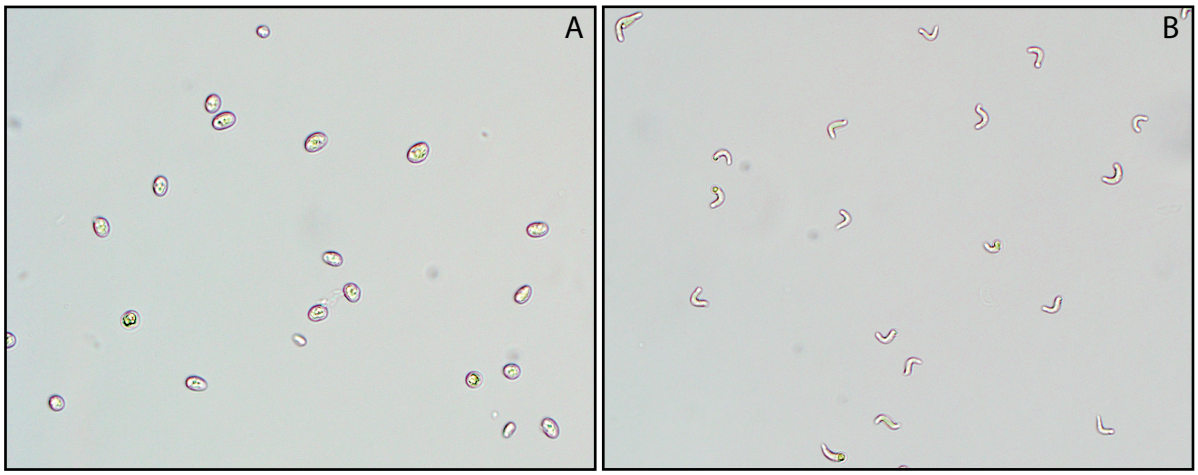


Fig. 1.3. A: Algae *S. vacuolatus* **B:** Algae *P. subcapitata* observed to the microscope.

1.4.2 Algae reproduction

The algae species *S. vacuolatus* multiply as the *Chlorella* species, i.e. by an asexual reproductive life cycle called the autosporulation (Fujishima and Steinbuchel 2009; Huss et al. 1999). The autosporulation is the formation of daughter cells with their own cell walls (Yamamoto et al. 2004). The daughter cell-wall synthesis is characterized by two clearly distinguishable stages:

1) a cell-growth process (Fig. 1.4a-c).
2) a cell-division stage (Fig. 1.4d-f).

The mother cell is constituted of a chloroplast and a nucleus. Chloroplasts are organelles of algae cells conducting the photosynthesis (Campbell et al. 2004; Shi and Theg 2013). At the beginning of the process, the daughter cell wall is formed inside the mother cell and

completely enveloped the outer surface of its plasma membrane (Fig. 1.4a). Due to the photosynthesis, the size of the chloroplast increases until its division. The size of the nucleus also increases during this period. Therefore, the size of the mother cell and the thickness of the daughter cell wall increase (Fig. 1.4b-c). During the division of the mother cell, the daughter cell wall expands by invagination as the plasma membrane (Fig. 1.4d-e). Then, the thickness of the daughter cell wall increases radically and 4 daughter cells were formed. This number of daughter cells can vary between 2 and 32 cells per mother cell for the alga *C. Vulgaris* (Fig 1.4f). Finally, the mother cell wall bursts and the daughter cells are released (Fig. 1.4g) (Yamamoto et al. 2004).

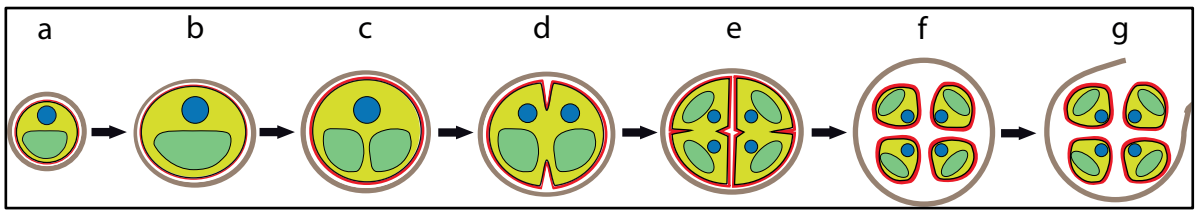


Fig. 1.4a-g. Diagrammatic representation of the algae *C. vulgaris* daughter cells formation from a mother cell by growth and division. **a** Early cell-growth phase. **b** Late cell-growth phase. **c** Chloroplast-dividing phase. **d** First protoplast-dividing phase. **e** Second-protoplast dividing phase. **f** Autospore maturing phase. **g** Hatching phase. Grey line: mother cell wall. Red line: daughter cell wall. Black line: plasma membrane. Green ellipse: chloroplast. Blue sphere: Nucleus (Yamamoto et al. 2004).

The algae species *P. subcapitata* reproduces also asexually by autosporulation. 3 phases characterizes this reproduction (Fig. 1.5). The first phase is the growth of the mother cell (stage 1 to stage 2). The second phase is the cell division, which includes two divisions of the nucleus (stage 2 to stage 4). Finally, the last phase is the release of four daughter cells. When the algae species *P. subcapitata* were exposed to metals stress, their growth were arrested before the first division of the nucleus for Cr(VI) and Cu (II) or before the release of the daughter cells for Cd(II) (Machado and Soares 2014).

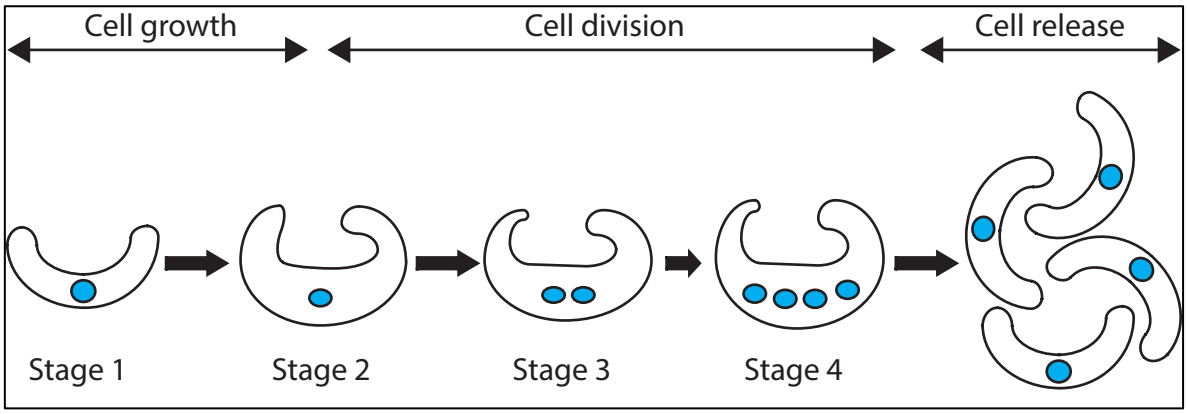


Fig. 1.5. Diagrammatic representation of the algae *P. subcapitata* reproduction. Blue sphere: nucleus of the alga (adapted from Machado and Soares 2014).

1.5 Outline of this thesis

Chapter 2: Modelling the effect of fluctuating herbicide concentrations on algae growth.

In this chapter, we aimed describing and validating with laboratory experiments a model developed to determine the effect of fluctuating concentrations of the herbicide isoproturon on the unicellular freshwater alga *S. vacuolatus*. With this model, we predicted and measured in laboratory the effect of 5 different scenarios of pulse exposure differing by their pulse and recovery durations, and also by the level of the peak concentrations. The differences between predicted and measured effects were discussed. In particular, we analyzed if the effect of each pulse of same duration were equivalent or different when the same peaks concentration was applied on the alga during a scenario. We also studied the recovery of the alga after having been exposed to a pulse exposure, i.e. if the recovery was complete or not. We investigated the sensitivity of the algae after having been exposed to pulse exposure scenario. Finally, a real case of isoproturon pulse exposure in a stream was applied to determine the effect on the alga *S. vacuolatus*. With this real case, we explored the contribution of the several types of pulse exposure on the cell density inhibition, i.e. which types of pulses (short or long, high or low) affect principally the cell density inhibition of the alga.

Chapter 3: Modelling the effects of pulse exposure of several PSII inhibitors on two algae.

The goal of this chapter was to investigate if the model developed previously was suitable for predicting the effects of photosystem II inhibitors, in particular triazines and phenylureas, on the unicellular freshwater algae. We tested pulse exposure scenarios in the laboratory with atrazine and diuron on the alga *S. vacuolatus*. The pulse exposure scenario was also tested with the herbicide isoproturon on another algae species: *P. subcapitata*. The pulse and recovery times of these scenarios were identical. They differed only by the level of the pulse concentrations tested. The effect measured in laboratory was compared with the effect predicted by the model. In each case, we also tested if the recovery of the 2 algae was complete after a peak exposure. Based on the results, we determined if the toxicity classification obtained with the dose-response curves of atrazine, diuron and isoproturon for the alga *S. vacuolatus* was conserved for the pulse exposure modeled with the same substances and for the same alga. Finally, we investigated which kind of pulse exposure, i.e. short but high in term of concentration or long but low, principally affected the algae species.

Chapter 4: Modelling the effect of exposing algae to pulses of S-metolachlor: how to include a delay to the onset of the effect and in the recovery.

In this section, we aimed in adapting the previously developed model to herbicides with other mode of action than PSII inhibitors. To do so, we assessed in laboratory the toxicity of S-metolachlor, a substance that inhibits the very long-chain fatty acid formation, on the alga *S. vacuolatus*. The toxicity of S-metolachlor was compared to that of the other herbicides studied in the two previous chapters, i.e. atrazine, diuron and isoproturon. We investigated in laboratory the differences in the effect and in the recovery between inhibitors of very long-chain fatty acid formation and inhibitors of photosystem II inhibitors. From previous studies (Vallotton 2007), we knew that the effect of S-metolachlor was not direct on the algae species *S. vacuolatus*. We studied this point in the laboratory. We also observed with a microscope the evolution of the algae cells *S. vacuolatus* during a standard test with S-metolachlor. The goal was to detect or not a modification of the algae cells morphology when they are exposed to S-metolachlor. We studied the recovery phase of the alga *S. vacuolatus* following different time of S-metolachlor exposure. We wanted to highlight if the recovery was complete after a pulse exposure of S-metolachlor or if a delay in the recovery phase was observed as in Vallotton et al. (2007). All these information observed in laboratory allowed the adaptation of the pulse exposure model developed in the two previous chapters. The predicted effects were compared with experimental pulse exposure test in laboratory.

Chapter 5: Modelling the effects of PSII inhibitor pulse exposure on two algae in co-culture.

The aim of this part was to extrapolate the pulse exposure model to the alga *S. vacuolatus* in co-culture with the alga *P. subcapitata*. To reach our goal we first investigated in laboratory the growths of the algae species *S. vacuolatus* and *P. subcapitata* in the same culture medium. These growths were compared with the growth of each alga cultured alone to observe if there was stimulation or an inhibition of the algae development when they grew together. Thereafter, the toxicity of isoproturon was tested on these two algae growing together. Therefore, we could evaluate if the substance isoproturon was more or less toxic for the alga *S. vacuolatus* when it was cultured alone or with the alga *P. subcapitata*. With these results, the model developed in the previous chapters was adapted to determine the effect on the alga

S. vacuolatus growing in co-culture with the alga *P. subcapitata* at the end of a pulse exposure scenario. The model was again validated with laboratory experiments.

Chapter 6: Synthesis and outlook.

In this chapter, we synthetized the results obtained from chapter 2 to chapter 5. To go further, a real environmental pulse exposure scenario was applied with the substances studied in this thesis, i.e., atrazine, diuron, isoproturon and S-metolachlor on the algae *S. vacuolatus* and *P. subcapitata* cultured alone but also on the alga *S. vacuolatus* in co-culture with the alga *P. subcapitata*. Second, an approach is proposed to adapt the model to assess the effects of mixture of herbicides, what is common in the environment. Some preliminary experiments are presented. Thereafter, an approach is proposed for assessing the risk of pulse exposure scenario.

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Chapter 2

Is it possible to model the effects of isoproturon pulse exposure?

In this chapter, we developed a model to assess the effects of herbicide pulse exposure on freshwater microalgae. The goal was to validate this model with laboratory experiments. The freshwater microalgae *Scenedesmus vacuolatus* is used for this study. The pulse exposure tests are performed with the herbicide isoproturon. This substance is used because it is a photosystem II inhibitor and its recovery after pulse exposure is direct and complete. Furthermore, the AA-EQS of isoproturon is often exceeded in worldwide streams. Control chart of the alga and standard toxicity tests are realized to determine the two main parameters of the model. The endpoint calculated by the model is the cell density inhibition of the algae. The uncertainty of the model is calculated using the variability of these two parameters and applying a Monte-Carlo simulation. Finally, a typical environmental pulse exposure scenario is applied with the model to explore the contribution of the several types of pulse exposure on the cell density inhibition.

Modelling the effect of fluctuating herbicide concentrations on algae growth.

Pierre-Jean Copin, Sylvain Coutu, Nathalie Chèvre

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Abstract

Herbicide concentrations fluctuate widely in watercourses after crop applications and rain events. The level of concentrations in pulses can exceed the water chronic quality criteria. In the present study, we proposed modelling the effects of successive pulse exposure on algae. The deterministic model proposed is based on two parameters: i) the typical growth rate of the algae, obtained by monitoring growth rates of several successive batch cultures in growth media, characterizing both the growth of the control and during the recovery periods; ii) the growth rate of the algae exposed to pulses, determined from a dose-response curve obtained with a standard toxicity test. We focused on the herbicide isoproturon and on the freshwater alga *Scenedesmus vacuolatus,* and we validated the model prediction based on effect measured during five sequential pulse exposures in laboratory. The comparison between the laboratory and the modelled effects illustrated that the results yielded were consistent, making the model suitable for effect prediction of the herbicide photosystem II inhibitor isoproturon on the alga *S. vacuolatus*. More generally, modelling showed that both pulse duration and level of concentration play a crucial role. The application of the model to a real case demonstrated that both the highest peaks and the low peaks with a long duration affect principally the cell density inhibition of the alga *S. vacuolatus*. It is therefore essential to detect these characteristic pulses when monitoring of herbicide concentrations are conducted in rivers.

Keywords: *Isoproturon, Pulse, Recovery, Scenedesmus vacuolatus, Modelling, Cell density inhibition*

2.1 Introduction

Herbicides are frequently detected in watercourses (Konstantinou et al., 2006; Muller et al., 2002; Skark et al., 2004). Indeed, they can reach surface waters during rain events by surface transport or drainage (Brown and van Beinum, 2009; Freitas et al., 2008). Thus, they don't contaminate the aquatic environment continuously but rather in pulses. Several authors have described this non-continuous pattern of herbicide concentrations in rivers that is mainly linked with the rain events following application periods. They are characterised by successive short pulses of high concentrations followed by period of low concentrations of various durations (House et al., 1997; Muller et al., 2002; Reinert et al., 2002). The concentrations during pulsed exposures are often above chronic water quality criteria, and even the acute quality criteria, defined to protect aquatic life from the deleterious effects of chemicals such as herbicides (Vallotton, 2007). The effects, and thus the risk of such pulses, are therefore crucial to determine (Boxall et al., 2013).

The effects of pulsed exposures to herbicides on non-target aquatic species, i.e mainly algae and macrophytes, have been subject to question for more than a decade (Reinert et al., 2002). Some authors have tried to depict the effects these pulses may generate. In general, the impact of pulsed exposures on algae and macrophytes seem to be substance dependent (Cedergreen et al., 2005). For example, isoproturon, a photosystem II inhibitor commonly applied on cereal fields, has a lower impact in pulses than in continuous exposure (Boxall et al., 2013). Indeed, photosystem II inhibitors such as triazines and phenylureas induce toxicity during the pulse exposure, but the algae recover totally, i.e. the growth rate is similar to that of non-exposed algae, after the chemical is removed (Reinert et al., 2002; Vallotton et al., 2009). Consequently, the effect of successive pulses is lower than continuous exposure at the same concentration. Similarly, water plants exposed to a 24h pulse of sulfonylureas seem to recover, usually reaching the same biomass as the control 6 days after the exposure (Rosenkrantz et al., 2012). But this can be different for other compounds and mechanisms of action. For example, Vallotton et al. (2008b) showed that a pulse of the herbicide S-metolachlor induces a delay in recovery after exposure to algae. Along the same lines, the growth of macrophytes seems to be significantly reduced after a 48h and a 96h pulse of pentachlorophenol (Boxall et al., 2013). Compound-specific uptake, degradation or dissipation rates in plants, and the potential of recovery between pulses can explain these differences of effects (Boxall et al., 2013; Cedergreen et al., 2005).

The effects of pulse exposure scenarios were also assessed for more complex systems such as periphyton communities. Gustavson et al. (2003) showed that photosynthetic activity of natural periphyton communities can be strongly disturbed by low and environmentally realistic pulse concentrations of isoproturon. Laviale et al. (2011) also showed that a 1, 3 or 7-hour peak exposure to isoproturon induces an inhibition of two fluorescence parameters, the effective and the optimal quantum yields of PSII photochemistry, on the periphyton community at environmentally relevant concentrations; however, 12 hours after the pulse, the periphyton recovery is complete at these concentrations.

Although the effects of sequential pulses of herbicides on non-target organisms are partially depicted, very few models have been developed to predict these effects (Nagai, 2014). Such latter models, however, are of particular importance due to the large varieties of pulse scenarios. It would also be a first step for risk assessment of pulsed exposures. Recently, Weber et al. (2012) simulated the effects of successive pulse exposure to isoproturon on algae populations in a flow-through system. In that study, the authors modelled the population fluctuations as a function of four parameters: temperature, light intensity, nutrient availability and chemical concentration. But the model proposed is mainly descriptive and therefore difficult to use for effects predictions due to the lack of information on the different variables.

The aim of this study was to develop a simple model, i.e with parameters easily determinate with classical experiments (standard OECD test), able to predict the cell density inhibition of algae exposed to sequential pulses of herbicides. The model was developed to simulate the effects of photosystem II inhibitors, which are widely used in European countries. In Switzerland, they are the most common herbicides found in surface waters such as lakes (Gregorio et al., 2012). Furthermore, as mentioned above, they have the advantage of not inducing a delay in the recovery phase of algae. The model will be validated by comparing the predictions with laboratory measurements obtained with 5 typical scenarios. For the experiments, we chose to test the herbicide isoproturon, which is regularly detected in rivers up to several µg/l in pulses (Garmouma et al., 1998; IFEN, 2007; Muller et al., 2002). The alga selected was *S. vacuolatus*, which has already been tested successfully with pulses (Vallotton et al., 2009). As an illustration, the model will also be used to predict the cell density inhibition for a realistic pulse scenario in a river.

2.2 Materials and methods

2.2.1 Chemical

Isoproturon, 3-(4-isopropylphenyl)-1,1-dimethylurea, (99% purity) was purchased from Ehrenstorfer GmbH. A stock solution of 3200 µg/l was prepared in an algae medium, in axenic conditions, for pulse exposure testing. This stock solution was kept in the fridge at 6.4°C. The concentration was checked analytically and the measured concentrations were in the same range as the nominal concentrations (results not shown).

2.2.2 Algae cultures

Permanent agar culture tubes of green unicellular microalga *S. vacuolatus* (Chlorophyceae ; strain 211-15, Shihira and Krauss, Melbourne, Australia) was obtained from the Department of Bioanalytical Ecotoxicology at the Helmholtz Centre for Environmental Research – UFZ, Leipzig, Germany. Microalga was cultured in a growth media described in the OECD guideline (OECD, 2011). Microalgae were cultured in the OECD medium by successive transfers in order to maintain exponential growth conditions and to possibly identify signs of abnormal growth (Le Faucheur et al., 2005). The method involves transferring regularly, i.e. every 48h, a specific volume of algae culture, defined by a calibration curve, into a new 50 ml OECD medium. 50 ml of algal suspension were placed in erlenmeyer flasks of a capacity of 250 ml on a HT Infors shaker table (90 rpm) (Le Faucheur et al., 2005; Vallotton et al., 2008a) at 25 °C and under continuous illumination at a light intensity of 70 µmol/m^2/s provided by cool-white fluorescent lamps.

Algae were inoculated in a new culture medium with an initial optical density of 0.056 at 690 nm ($OD_{\lambda 690}$), which corresponds to a density of 650,000 cells/ml. The optical density was measured with a microplate reader (ELx800™, BioTek® Instruments, Winooski, Vermont) at a wavelength of 690 nm. The cell density (cells/ml) was determined by cell counting using the improved Neubauer Haemacytometer (Optik Labor, Lancing, United Kingdom). The calibration curve was obtained by plotting the cell density as a function of the measured optical density.

A control charter was established to monitor algae growth in growth media. In our laboratory, the average growth rate of the algae was 0.027 h^{-1} with a standard deviation of 0.002 h^{-1} (average of successive 47 cultures).

2.2.3 Dose-response curve of isoproturon

The dose-response curve of isoproturon, required to parameterise the model and to defined the tested concentrations, was established following a method adapted from the standard OECD procedure (OECD, 2011). The tests were performed in the same conditions as algae cultures (see section 2.2.2). Five concentrations ranging from 4.6 to 256 µg/l and a control were tested in octoplicates. The optical density measured at the beginning and at the end of the test was used to evaluate the average specific growth rate for each concentration and for the control. Growth inhibition is the ratio between the growth rates of the different concentrations and that of the control (Eq. 2.1; see section 2.2.5.1).

2.2.4 Pulse exposure tests

Five pulse exposure scenarios were tested in the laboratory (Fig. 2.1; Table 2.1). They differed in the duration and concentration levels of the pulses, and in the duration of the recovery periods. Two cases were considered: short pulse duration and long recovery period, and long pulse duration and short recovery period. These cases can be considered as representative of two extreme scenarios that can be found in rivers. Pulse exposure scenarios differed also in the pulses concentration tested as shown in table 2.1. Algae exposed to scenarios, as well as the controls, were tested in triplicates.

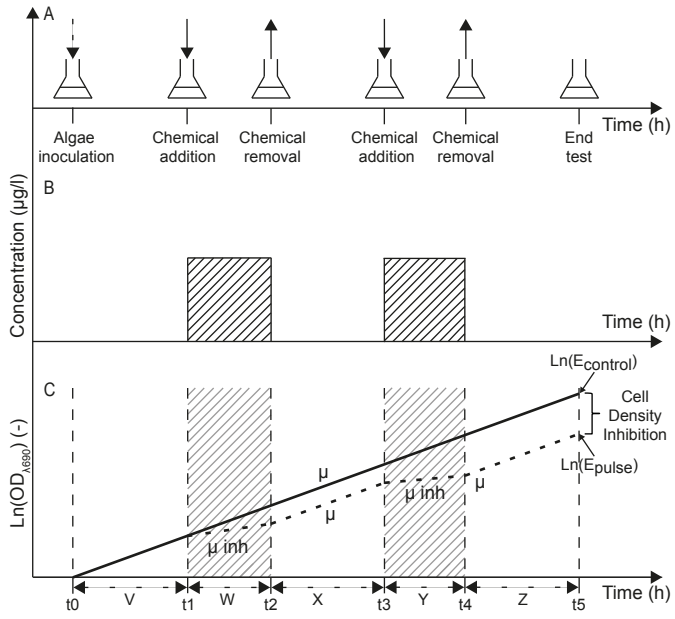

Fig. 2.1. A: Procedure of pulse exposure scenarios tested in laboratory. **B:** Pulses representation over time. **C:** Modelling representation. μ is the growth rate obtained from the control charter. μ_{inh} is the growth rate at concentration x obtained from the growth response curve. $E_{control}$ is the predicted final optical density for the model's control. E_{pulse} is the final optical density of the model for the alga exposed to pulse concentration. V corresponds to the growth duration before the first pulse (h); W and Y are the pulse durations (h); X and Z are the recovery durations (h); V is around 24 h. W, X, Y and Z are defined in Table 2.1.

Table 2.1. Parameters of duration for each pulse and recovery period with the concentration tested during the scenarios.

Scenario number	Pulse duration (h)	Recovery duration (h)	Concentration tested
1	W=5.75 Y=6	X=Z=38	EC_{50}[a]
2	W=4.25 Y=4.50	X=38, Z=0	EC_{70}[b]
3	W=Y=5	X=Z=40	EC_{80}[c]
4	W=Y=24	X=2, Z=0	EC_{10}[d]
5	W=Y=24	X=Z=3	EC_{30}[e]

[a] Effect Concentration 50%. [b] Effect Concentration 70%. [c] Effect Concentration 80%. [d] Effect Concentration 10%. [e] Effect Concentration 30%.

The test was started the same way the algae were cultured (initial cell density 650,000 cells/ml, 50 ml in 250 ml flask) and in the same conditions (see section 2.2.2). Algae grew for a short period (Fig. 2.1; V is around 24 h) at the beginning before being exposed to the first pulse. At the end of each pulse exposure, the algae were centrifuged twice for 7 min at 1046 x g and 25°C. The supernate was removed and the algae were re-suspended in growth media (Vallotton et al., 2008a). These two centrifugations allowed 99.985% of the herbicide to be removed and did not impair algal growth, as shown by Vallotton et al. (2008a). The recovery period began directly after the algae were re-suspended in the fresh medium at an initial cell density of 650,000 cells/ml to allow a new exponential growth phase. This operation was repeated one more time after the second pulse exposure. Controls were treated the same manner that the algae exposed to pulses. The optical density was measured regularly during exposure and recovery periods.

After scenarios 3 and 5, standard toxicity tests were performed with algae having undergone pulse exposure tests. The goal was to determine if algae become more tolerant towards the herbicide or, on the contrary, become more sensitive to the chemical stressor.

2.2.5 Data analysis

2.2.5.1 Growth inhibition in standard toxicity test on algae

The endpoint of this standard test, i.e. the growth inhibition (I_t) at a given concentration, is determined using the response variables, i.e. the average specific growth rate of the control (μ_C) and the average specific growth rate at this concentration (μ_T) (OECD, 2011) (Eq. 2.1):

$$I_t = \frac{\mu_C - \mu_T}{\mu_C} \times 100 \qquad (2.1)$$

2.2.5.2 Dose-response curves for standard acute toxicity test

The dose-response curve of isoproturon is obtained by plotting the growth inhibition I_t as a function of the tested concentrations C. The relationship is expressed by using a four parameter log-logistic dose response model (Vallotton et al., 2008a) (Eq. 2.2):

$$I_t = min + \frac{(max - min)}{1 + 10^{((\log EC_{50} - \log C) \times Hillslope)}} \quad (2.2)$$

where EC_{50}, the concentration inhibiting 50% of the growth ; *Hillslope*, the slope of the dose-response curve; *max* and *min* parameters are the maximum and minimum of the sigmoidal curve. The *min* parameter is fixed at 0. The *max* parameter is fixed at 100 because it was assumed that the growth inhibition is total (100 %) at high concentrations. Indeed, for the highest concentration of isoproturon tested in laboratory (256 µg/l) and used to establish the dose-response curve for *S. vacuolatus*, the inhibition was almost complete. This curve is calculated by using the statistics software Prism (GraphPad, San Diego, CA, USA).

2.2.5.3 Tolerance evaluation

The potential increase in tolerance of algae after sequential pulse exposure was tested. To do so we compared the EC_{50}s obtained with a standard test with isoproturon at the beginning and at the end of the scenario. This was done with an ANCOVA analysis with the statistic software Prism (GraphPad software, Inc). Increase or decrease of EC_{50} values would indicate a modification of the algae sensitivity (Vallotton et al., 2009).

2.2.5.4 Cell density inhibition at the end of the pulse exposure scenario

The endpoint of these pulse exposure experiments, i.e. the overall algae cell density inhibition of each scenario ($Inh_{pulse\text{-}laboratory}$), was obtained by calculating the average of the algae cell density inhibition of each replicate ($Inh_{pulse\text{-}laboratory\text{-}replicate\ i}$). The $Inh_{pulse\text{-}laboratory\text{-}replicate\ i}$ was calculated as (Eq. 2.3):

$$Inh_{pulse-laboratory-replicate\ i} = 100 \times \frac{OD_{control} - OD_{pulse,replicate\ i}}{OD_{control}} \quad (2.3)$$

with $OD_{control}$, the average optical density for the three controls at the end of the experiment and $OD_{pulse,replicate\ i}$, the final optical density for the replicate *i* of alga exposed to pulses concentration. Indeed, as a reminder, the cell density or biomass is measured as the optical density. The variables $OD_{control}$ and $OD_{pulse,replicate\ i}$ are determined in two steps. First, the optical density data measured were summed up, i.e. after each pulse exposure period, the following recovery period and the following pulse exposure period were summed to the last optical density value of the previous pulse exposure period. Second, the alga *S. vacuolatus*

grew exponentially during the test. The growth was measured by optical densities, transformed in natural logarithms, as a function of time. Therefore, each section of the control and of the replicate *i* of alga exposed to pulses concentration, corresponding to the different phases of growth (i.e. the growth before the first pulse, the pulses, the recoveries), was fitted with a linear regression (Fig. 2.2). Finally, $OD_{control}$ and $OD_{pulse,replicate\ i}$ were calculated following eq. 2.4 and 2.5:

$$OD_{control} = \exp\left[\ln\left(OD_{algae-initial}\right) + \mu_{growth-control} * t_v + \sum_{k=1}^{n} \mu_{pulse\ k-control} * \Delta t_k + \sum_{j=1}^{m} \mu_{recovery\ j-control} * \Delta t_j\right] \quad (2.4)$$

$$OD_{pulse,replicate\ i} = \exp\left[\ln\left(OD_{algae-initial}\right) + \mu_{growth-replicate\ i} * t_v + \sum_{k=1}^{n} \mu_{pulse\ k-replicate\ i} * \Delta t_k + \sum_{j=1}^{m} \mu_{recovery\ j-replicate\ i} * \Delta t_j\right] \quad (2.5)$$

with $\mu_{growth\text{-}control}$ and $\mu_{growth\text{-}replicate\ i}$, the growth rates of the linear regression curves of the controls' average and of the alga's replicate *i* before the first pulse; t_v, the duration of the laboratory phase before the first pulse; $\mu_{pulse\ k\text{-}control}$ and $\mu_{pulse\ k\text{-}replicate\ i}$, the growth rates of the linear regression curves of the controls' average and of the alga's replicate *i* during the pulse *k*. These values were fixed to 0 when the growth rates of the linear regressions during the pulse were negative; Δt_k, the duration of the laboratory pulse period *k*; *n*, the number of pulses during the experiment in laboratory; $\mu_{recovery\ j\text{-}control}$ and $\mu_{recovery\ j\text{-}replicate\ i}$, the growth rates of the linear regression curves of the controls' average and of the alga's replicate *i* during the recovery *j*; Δt_j, the duration of the laboratory recovery period *j*; *m*, the number of recovery periods during the experiment in laboratory; $OD_{algae\text{-}initial}$ is the optical density corresponding to the initial cell density of the algae used for the pulse exposure test determined with the calibration curve of the corresponding alga. For *S. vacuolatus*, the initial cell density was fixed to 650,000 cells/ml. The $OD_{algae\text{-}initial}$ was then 0.056.

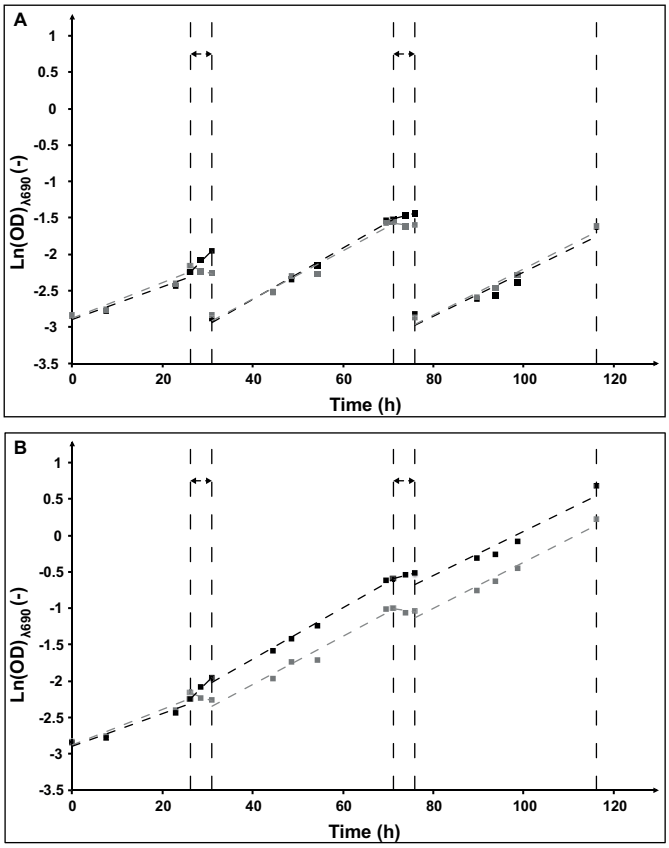

Fig. 2.2. Optical density values of the scenario 3. A: non-summed values. After each pulse, algae were re-suspended in the fresh medium at an initial cell density of 650,000 cells/ml to allow a new exponential growth. B: summed values. Black squares: controls. Grey squares: algae exposed to pulses. Black dotted lines: linear regressions fitted on data for each section of the control. Grey dotted lines: linear regressions fitted on data for each section of the replicate of the culture exposed to pulses. The lengths of pulses are indicated by arrows on the top of each graph.

2.2.5.5 Modelling

The cell density inhibition at the end of the experiment can also be expressed as (Eq. 2.6):

$$Inh_{pulse-modelling} = 100 \times \frac{E_{control} - E_{pulse}}{E_{control}} \quad (2.6)$$

where E_{pulse} is the final optical density of the model for the alga exposed to pulse concentration; $E_{control}$ is the predicted final optical density for the model's control.

$E_{control}$ can be expressed as (Eq. 2.7a and 2.7b):

$$E_{control} = \exp[\ln(OD_{algae-initial}) + \mu \times t_{2n+1}] \quad (2.7a)$$

or

$$E_{control} = \exp[\ln(OD_{algae-initial}) + \mu \times t_{2n}] \quad (2.7b)$$

where t_{2n+1} or t_{2n} is the total test duration. t_{2n+1} is used if there is a recovery phase before the end of the scenario (Eq. 2.7a). t_{2n} is used if there is a pulse period before the end of the scenario (Eq. 2.7b); n is the number of pulses during the test; $OD_{algae\text{-}initial}$ is the optical density corresponding to the initial cell density of algae determined with the calibration curve of the alga. It was fixed to 650,000 cells/ml for the alga *S. vacuolatus*. The corresponding optical density was then 0.056. μ is the growth rate of the control determined as the average growth rates of several successive batch cultures in growth media (control charter). This growth rate is assumed to be constant for the control and during recovery periods (hypothesis 1). Indeed, Vallotton et al. (2008a) showed that the alga *S. vacuolatus* exposed to isoproturon, and more generally to photosystem II inhibitors, recover directly after exposure.

E_{pulse} can also be expressed as (Eq. 2.8a and 2.8b):

$$E_{pulse} = \exp\{\ln(OD_{algae-initial}) + \mu \times [\sum_{i=0}^{n}(t_{2i+1} - t_{2i})] + \mu_{inh\,x} \times [\sum_{i=1}^{n}(t_{2i} - t_{2i-1})]\} \quad (2.8a)$$

or

$$E_{pulse} = \exp\{\ln(OD_{algae-initial}) + \mu \times [\sum_{i=0}^{n-1}(t_{2i+1} - t_{2i})] + \mu_{inh\,x} \times [\sum_{i=1}^{n}(t_{2i} - t_{2i-1})]\} \quad (2.8b)$$

where t_{2i} can be either the beginning of a recovery period or the end of a pulse exposure period; t_{2i+1} is the end of a recovery period; t_{2i-1} is the beginning of a pulse exposure period; $\mu_{inh\,x}$ is the growth rate at concentration x determined from the growth response curve of the standard ecotoxicity test; the second term of the equations corresponds to the growth of the alga at the beginning of the pulse exposure test and to the recovery periods of the alga whereas the third term corresponds to the pulse periods. The first equation 2.8a is used when the recovery phase corresponds to the end of the scenario whereas the second one (Eq. 2.8b) is used when the pulse exposure period corresponds to the end of the scenario.

In this model, the growth rate at a given concentration *x* of isoproturon ($\mu_{inh\,x}$) and for the alga *S. vacuolatus* is assumed to be similar and constant during each pulse exposure (hypothesis 2). Vallotton et al. (2009) showed that this assumption is valid by comparing the inhibition of the photosynthesis during successive pulses of isoproturon at a similar concentration for *S. vacuolatus*.

The final $Inh_{pulse\text{-}modelling}$ is expressed as (Eq. 2.9a and 2.9b):

$$Inh_{pulse-modelling} = 100\times\frac{\exp[\ln(OD_{algae-initial})+\mu\times t_{2n+1}]-\exp\{\ln(OD_{algae-initial})+\mu\times[\sum_{i=0}^{n}(t_{2i+1}-t_{2i})]+\mu_{inh\,x}\times[\sum_{i=1}^{n}(t_{2i}-t_{2i-1})]\}}{\exp[\ln(OD_{algae-initial})+\mu\times t_{2n+1}]} \quad (2.9a)$$

or

$$Inh_{pulse-modelling} = 100\times\frac{\exp[\ln(OD_{algae-initial})+\mu\times t_{2n}]-\exp\{\ln(OD_{algae-initial})+\mu\times[\sum_{i=0}^{n-1}(t_{2i+1}-t_{2i})]+\mu_{inh\,x}\times[\sum_{i=1}^{n}(t_{2i}-t_{2i-1})]\}}{\exp[\ln(OD_{algae-initial})+\mu\times t_{2n}]} \quad (2.9b)$$

The first equation 2.9a is used when the recovery phase corresponds to the end of the scenario whereas the second one (Eq. 2.9b) is used when the pulse exposure period corresponds to the end of the scenario.

As the true values of the variables μ and $\mu_{inh\,x}$ of equation 9a or 9b are unknown, we took into account their uncertainties in the simulation. The distribution of the parameter μ can be obtained from the control charter. According to the Shapiro and Wilk test, the parameter μ is assumed to follow a normal distribution. For $\mu_{inh\,x}$, the distribution can be established from the dose response curve and is also assumed to be normal (but not enough data were available to test it). The distribution of the $Inh_{pulse\text{-}modelling}$ is estimated based on a Monte Carlo simulation. To do so, 10,000 growth rates μ and $\mu_{inh\,x}$ are selected randomly from their respective distributions. Only values from the distribution of μ and $\mu_{inh\,x}$, located between "average-2 x standard deviation" and "average+2 x standard deviation", were chosen in order to consider 95% of possible data from μ and $\mu_{inh\,x}$ (Motulsky, 1995). By repeating the calculation (Eq. 2.9a or 2.9b) many times (between 9,000 and 10,000) and assuming that values of μ must be higher than $\mu_{inh\,x}$ (hypothesis 3) in order to have all the values of $Inh_{pulse\text{-}modelling}$ positives, the mean of $Inh_{pulse\text{-}modelling}$ is calculated for each scenario, along with minimum and maximum values. The model is applied using Matlab software (MATLAB R2011b, The Mathworks

Inc.). The predictive results of these Monte Carlo simulations were used to compare with the laboratory observations in order to validate the model.

2.2.5.6 Illustrative case study

Finally, the model is used to predict the effects on the cell density of the alga *S. vacuolatus* of a typical scenario that can be observed in rivers. The illustration of the scenario is available in the supplementary information (SI) (Fig. 2.1S). As long-term measurements of isoproturon concentrations in rivers are not found, a typical pattern of herbicide pollution in creeks is used (Leu, 2003) and this scenario is adapted to isoproturon.

The scenario was composed of 54 pulses. Twenty-three pulses of short duration and low concentrations, i.e. low compared to usual measured concentrations in Switzerland (< 2.5 µg/l) (Leu, 2003), were identified. 9 pulses of long duration (> 10 hours) and low concentrations, 14 pulses of short duration (< 10 hours) and high concentrations (> 2.5 µg/l) and, finally, 8 pulses of long duration and high concentration were also observed. The maximum concentration reached is fixed to 42,000 ng/l, a concentration measured by IFEN for isoproturon (2007). Using Matlab software (MATLAB R2011b, The Mathworks Inc.), the concentrations between two measured concentrations, for the same pulse, were interpolated. As a result, the concentrations are available for each minute over the entire duration of the scenario. A histogram was created to characterise the exposure between two minutes and therefore a pulse is described by several histograms with a duration of one minute each and a height corresponding to the concentration interpolated. For further information, see the SI (Fig. 2.1S). This solution was chosen to be applicable for the model developed in this study. (Fig. 2.1B and C).

2.3 Results and discussion

2.3.1 Laboratory experiments

The dose-response curve established for isoproturon provided an EC_{50} of 67 µg/l with a 95% confidence interval (61; 73). The other effect concentrations (EC_{10}, EC_{30}, EC_{70}, EC_{80}) used for pulse experiments (Table 2.1) were 17 µg/l, 39 µg/l, 114 µg/l and 265 µg/l respectively.

The results for the 5 scenarios of repeated pulsed herbicide exposures are presented on figure 2.3. The overall cell density of the algae was inhibited with each tested scenario. For the scenarios with short pulses and long recovery periods (scenarios 1, 2 and 3; fig. 2.3A, B and

C), the overall cell density inhibition is 15, 24 and 23 %, respectively. For the scenarios with long pulses and short recovery periods (scenarios 4 and 5; fig. 2.3D and E), the cell density inhibition is 17 and 44 %, respectively. As expected, for the two scenarios types, i.e. for pulse scenario with short or long durations (<6 hours or >24 hours), the cell density inhibition increases with increasing concentration. This is not surprising as the cell density is inhibited during each pulse, as indicated by a difference between the growth curve of the control (solid line) and the growth curve of the exposed algae (dotted line). For scenario 3, the cell density inhibition is close to that of scenario 2, even if the concentration tested is higher. However, the difference in the concentration is not high (Table 2.1) that can explain this low difference. Even if the recovery is instantaneous, isoproturon has a cumulative effect on the algae production. For this reason, the pulse duration plays an important role on the overall effect. A long pulse at a low concentration will have a higher effect than a very short pulse at a high concentration. In this study, scenario 5 (EC_{30} tested) therefore had a greater effect than scenarios 1, 2 and 3 (EC_{50}, EC_{70} and EC_{80} tested). Even with a very low concentration tested during pulses (scenario 4, EC_{10} tested), the cell density inhibition remained substantial.

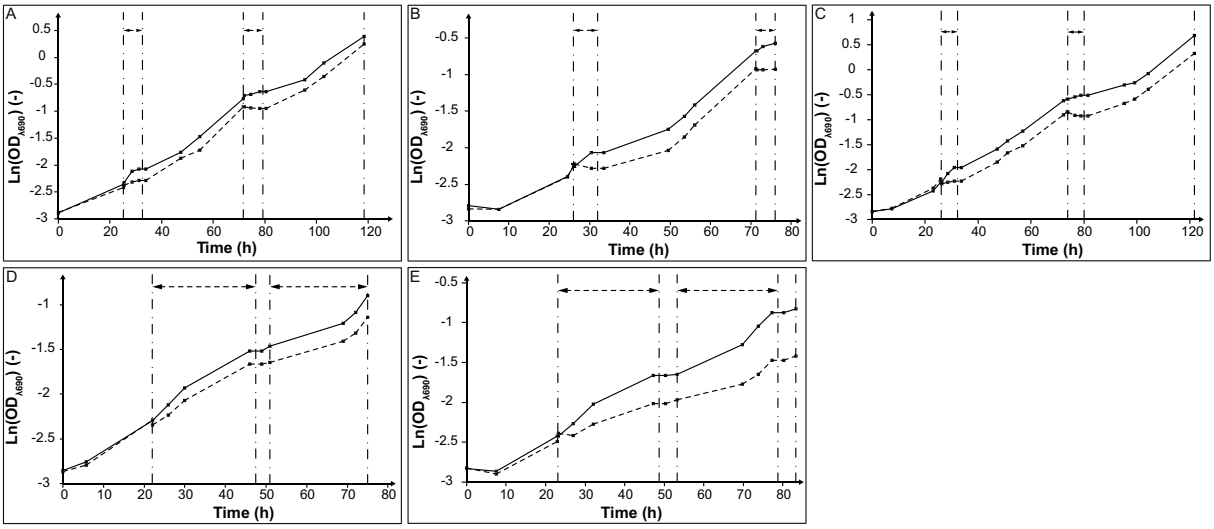

Fig. 2.3. Growth curves for 5 scenarios of pulse exposure tests in the laboratory. Black curves: controls. Black dotted curves: algae exposed to pulses. **A:** Scenario 1. **B:** Scenario 2. **C:** Scenario 3. **D:** Scenario 4. **E:** Scenario 5. Scenarios 1, 2 and 3 were for short pulses and long recovery periods. Scenarios 4 and 5 were for long pulses and short recovery periods. The lengths of pulses are indicated by arrows on the top of each graph.

No increase in algae tolerance was observed at the end of the sequential pulse exposure. Indeed, standard toxicity tests were performed at the end of scenarios 3 and 5. EC_{50}s with corresponding 95% confidence intervals were 63.27 µg/l (56.91; 70.34) and 61.96 µg/l (54.06; 71.02), respectively. These values are not statistically different from the EC_{50} obtained from the standard test (see beginning of section 2.3.1; ANCOVA analysis, p-value >0.05). We therefore do not observe a shift in sensitivity of the alga *S. vacuolatus*. This is in agreement with Weber et al. (2012), i.e. that the sensitivity or the growth wasn't modified after a repeated exposure to isoproturon. However, Vallotton et al. (2009) showed that for long pulses exposures with short recovery, the EC_{50} estimated from a 72-hour acute toxicity test with algae that had been repeatedly exposed was greater than the EC_{50} estimated for the control algae. In this case, there was a slight shift in sensitivity of algae.

As this is a critical assumption from our model, we tested, in laboratory, the hypothesis 2 that "the slopes during pulse exposure are not statistically different for a scenario if the same concentration is tested" (see section 2.2.5.5). The hypothesis was not rejected for long pulses scenarios (scenarios 4 and 5; ANCOVA analysis, p-values>0.05). The p-value was 0.10 and 0.52 for the scenarios 4 and 5 respectively. For scenarios with short pulses (scenarios 1, 2 and 3), the number of optical density measurements was too small (maximum of 3) to calculate any statistic. We also tested the assumption, in laboratory, that "the recovery is complete just after the pulse exposure" (hypothesis 1; see section 2.2.5.5). To do so, the slopes of recovery periods were compared with the slopes of the control. The results support the hypothesis 1 because the assumption was not rejected for scenarios with long recovery periods (scenarios 1, 2 and 3; ANCOVA analysis, p-values>0.05). For scenarios with short recovery periods (scenarios 4 and 5), this assumption couldn't be controlled because there were not enough optical density measurements.

2.3.2 Model application

Figure 2.4 presents the cell density inhibitions predicted with the 5 scenarios in the form of boxplots; those correspond on average to 14 %, 16 % and 19 % for scenarios 1, 2 and 3 i.e. with short pulses and long recovery periods. For long pulses and short recovery periods (scenarios 4 and 5), the cell density inhibitions are 10 % and 26 %, respectively. As already observed with the experimental results, the cell density inhibition is greater for scenario 5 than for scenarios 1, 2 and 3 despite the low concentration selected for the scenario 5 (EC_{30})

compared to the others (EC_{50}, EC_{70} and EC_{80}). The same observation can be made for scenario 4. Even if the concentration chosen is very low (EC_{10}), the cell density inhibition is very close to cell density inhibitions of scenarios 1, 2 and 3. Therefore, the longer the pulse, the lower the concentration needed to get an inhibition similar to a short but high concentration pulse.

Figure 2.4 also presents the minimum and maximum values predicted for each scenario. For scenarios 1, 2 and 3, cell density inhibition values range, respectively, between 1.3 and 24 %, 6.2 and 24 % and, 9.3 and 27 %. For scenarios 4 and 5, these values range between 0 and 33 % and, 0 and 58 %. For long pulses and short recovery periods (scenarios 4 and 5), the variation of the cell density inhibition is therefore higher than for short pulses and long recovery periods (scenarios 1, 2 and 3). This observation results from the variability given to the different parameters of the model. Indeed, the variability is higher for the parameter $\mu_{inh\,x}$, characterizing the growth during the pulse (standard deviation around 0.004) compared to the variability of the parameter μ, characterizing recovery periods, obtained from the chart control (standard deviation: 0.002). The greater variability of the parameter $\mu_{inh\,x}$ compared to the parameter μ is likely due to the size of the group, parameterising a single experiment versus a higher number of pre-cultures. Therefore, the longer the pulse exposure duration, the larger the variation predicted by the model.

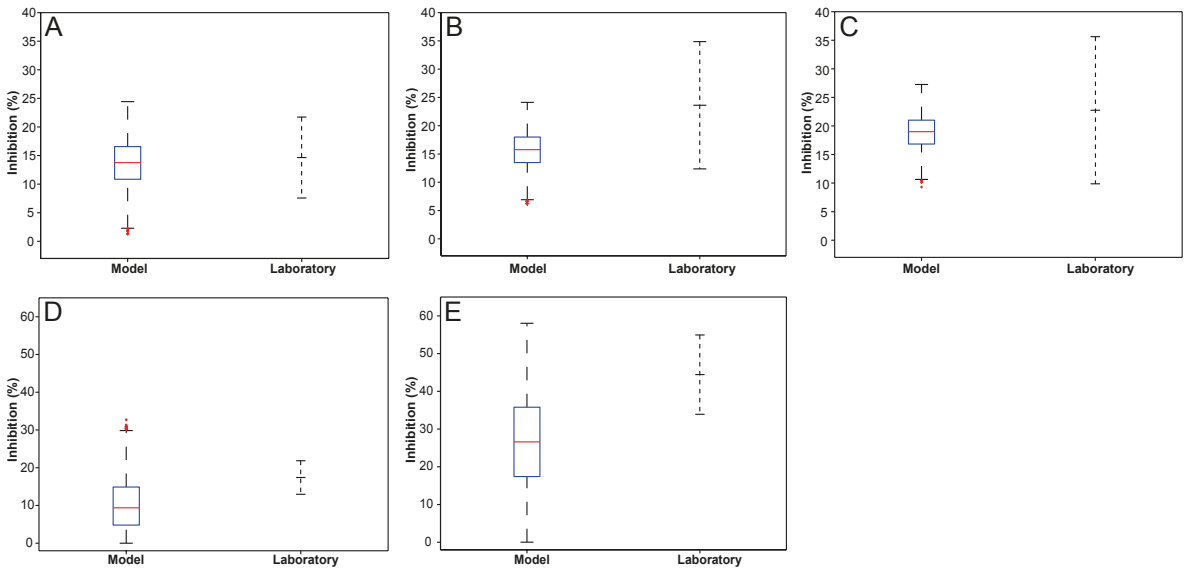

Fig. 2.4. Boxplots of each scenario for the modelling. Average and standard deviation for laboratory (three replicates). **A:** Scenario 1. **B:** Scenario 2. **C:** Scenario 3. **D:** Scenario 4. **E:**

Scenario 5. Note the difference in the scales of the y axes between figures A, B, C and figures D, E.

2.3.3 Comparison between measured and predicted results

The model predicts the observed experimental inhibitions relatively well (Fig. 2.4). For all the scenarios, the average measured cell density inhibition is included between the minimum and maximum values of the model. Furthermore, for scenario 1 (Fig. 2.4A), the experimental average cell density inhibition is very close to the average cell density inhibition given by the model (15% and 14%, respectively). For all the other scenarios, the predicted average cell density inhibition is slightly lower than the experimental average, at most 1.7 times lower. Furthermore, for three scenarios (scenarios 1, 4 and 5; fig. 2.4A, D and E), the standards deviations of the experimental results are situated within the min-max values given by the model. For the other two scenarios (scenarios 2 and 3; fig. 2.4B and C), the experimental average cell density inhibition is also situated below the maximum value given by the model. But the average cell density inhibition predicted is at maximum 1.5 lower than the measured cell density inhibition, as mentioned above. This is in the same order of magnitude as for the other scenarios. Furthermore, for these two scenarios, the variability of the predicted results is lower than that of the others, as discussed above.

If the overall comparison shows that the model could be considered as suitable to predict the effects of the pulse exposure of the different scenarios, one has to note that the predictions seems to slightly underestimate the average cell density inhibition. One reason may be that the different slopes used for modelling were defined as constant. But these slopes may differ slightly during the experiment (see fig. 2.5), particularly during the latency phases at the beginning of the test and at the beginning of the recovery parts. However, it is difficult to discuss these differences more deeply as the statistics are based on a low number of laboratory experiments (3) compared to the model predictions, that accounts for 10’000 results.

The comparison between the experimental and the predicted growth during the whole test is illustrated for scenarios 3 and 5, respectively, in figure 2.5. For scenario 3 (Fig. 2.5A and B), the majority of the points, representing laboratory control and cultures exposed to pulses respectively, are located within the bands of model predictions, showing a good agreement

between predicted and measured inhibition. For scenario 5 (Fig. 2.5C and D), the points obtained with laboratory experiments representing cultures exposed to pulses, are situated close to the lower band of the model. Consequently, the difference between predicted and measured inhibition is larger than for the scenario 3.

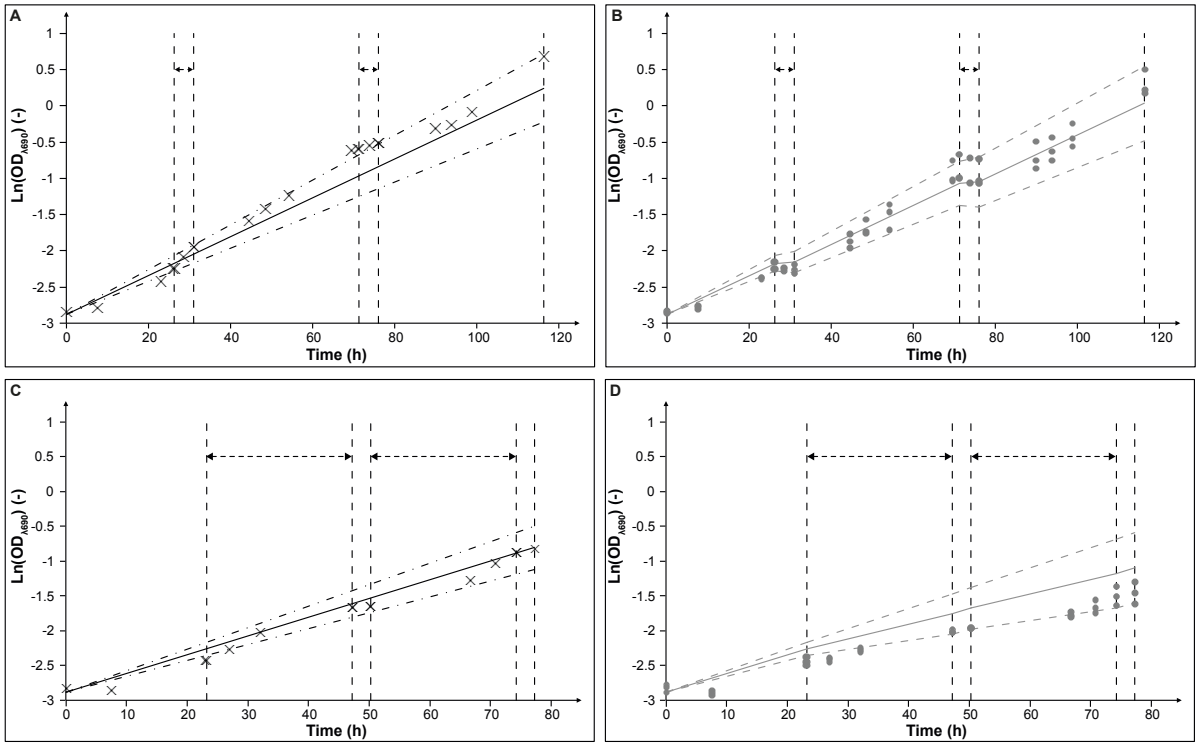

Fig. 2.5. Comparison between measured and predicted results. **A** and **B:** scenario 3. **C** and **D:** scenario 5. **A** and **C** show controls. **B** and **D** show culture exposed to pulses. Curves: results from model. Solid curves: average of the model. Dotted curves: representation of "average-2 x standard deviation" and "average+2 x standard deviation". Black crosses: average of the control measured in laboratory. Grey circles: triplicates of cultures exposed to pulses measured in laboratory. The optical density value at the beginning of the test was fixed to Ln(0.056). The lengths of pulses are indicated by arrows on the top of each graph.

As described in the material and method section, μ is assumed to be higher than $\mu_{inh\ x}$ (hypothesis 3; see section 2.2.5.5) in order to have predicted inhibition values ($Inh_{pulse\text{-}modelling}$) positives. This means that the pair of parameters μ and $\mu_{inh\ x}$, with values of $\mu_{inh\ x}$ higher than values of μ, are not taken into account in the model calculation. In this study, we tested both methods, i.e. with and without the hypothesis 3. For scenario 1, 2 and 3, no differences

existed as all μ values are higher than $\mu_{inh\,x}$ values. For scenario 4 and 5, we obtained better adequacy between predictions and measurements when the assumption of μ higher than values of $\mu_{inh\,x}$ was made. All the data are given in the SI (Table 2.1S). For example, for scenario 4, we predicted 10% inhibition and observed 17%. Without any assumption of μ higher than $\mu_{inh\,x}$, we predicted 4 %. We therefore decided to make this hypothesis in model calculations.

2.3.4 Application of the modelling in a real case

The model was applied to simulate the effect of a typical environmental exposure scenario of the herbicide isoproturon on the alga *S. vacuolatus* (see SI, Fig. 2.1S). The total predicted cell density inhibition, with its minimum and maximum values, is 19 (0; 36) %. This is high considering that the scenario was tested only on one alga. The peak concentrations are often low and short (23 pulses, each one with a duration less than 1 hour) and induce together less than 1% of effect. This percentage effect corresponds to 3% of the total cell density inhibition of the entire scenario. However, as expected, the low peaks with a long duration (9 pulses) induce, together, the principal effect (11%) corresponding to 55% of the total cell density inhibition of the entire scenario. Similarly, in the experiments, the low peaks with a long duration induce an important cell density inhibition (as illustrated by scenarios 4 and 5 in the model application; section 2.3.2). The whole inhibition of the scenario is therefore mainly driven by the set of the longest peaks. But it is also influenced by the set of high peaks (22 pulses). Indeed, for these highest peaks, the cell density inhibition corresponds to 42% of the total cell density inhibition of the entire scenario with 54 pulses. Furthermore, considering only the 4 highest pulses (corresponding to maximum concentrations of 42,000; 40,000; 35,000 and 32,000 ng/l respectively, see SI, Fig. 2.1S), the cell density inhibition corresponds to 27% of the total cell density inhibition of the entire scenario. Moreover, this set of highest peaks can also be considered as long pulses because their duration is higher than 10 hours. Consequently, the long high pulses of the entire scenario induce an important part of the whole cell density inhibition on the alga *S. vacuolatus*.

Furthermore, for the highest pulses, the steeper the increase or decrease (in term of concentration at the beginning or respectively at the end of the pulse), the less effect they induce. Thus, in the case study, the pulse with a high concentration of 42,000 ng/l is narrow, i.e it has a steep increase and decrease, and provokes a cell density inhibition of 1.11% i.e. 6

% of the total cell density inhibition of the entire scenario. In contrast, the same duration pulse that reachs only 35,000 ng/l, is larger and provokes a higher cell density inhibition (1.48 % i.e. 8% of the total cell density inhibition of the entire scenario). Consequently, to evaluate the effects of the photosystem II inhibitor isoproturon on the alga *S. vacuolatus*, it is important to be able to capture the highest peak concentrations and also the peaks with a long duration. This is applicable for small creeks, for which high dynamic of concentrations of isoproturon are observed but also in rivers where the peak duration is longer.

2.4 Conclusion

Our model allows any kind of scenario for isoproturon to be predicted on the alga *S. vacuolatus* based on a few parameters that are easily determined with classical ecotoxicity experiments. Indeed, the only condition for applying this model is the knowledge of the dose-response curve for isoproturon on the alga *S. vacuolatus*. In particular, the parameter $\mu_{inh\,x}$, measured for isoproturon with a standard toxicity test, is crucial for predicting the average cell density inhibition. More generally, further research are needed to determine if the model is suitable for predicting the effect of pulses of other photosystem II inhibitors and for other substances with a different mode of action but also with other algae. Pulse testing with multispecies cultures should also be conducted to improve the model. In conclusion, the model can be considered as suitable to assess effects of pulse exposure scenarios for the photosystem II inhibitor isoproturon on the alga *S. vacuolatus* in exponential growth. The differences between laboratory and model can be considered minor. However, it is important to have complete information about growth rates of control and exposed cultures.

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Supplementary information (SI)

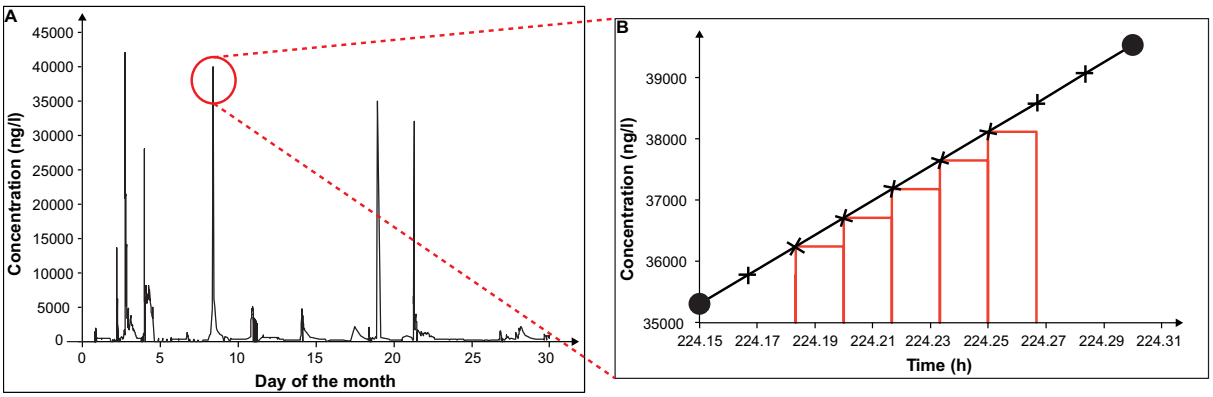

Fig. 2.1S. A: Isoproturon concentrations (ng/l) in a river during a month following the application. **B:** Zoom on a pulse part of the scenario. Black circles are measurements of isoproturon concentrations. Crosses are concentrations interpolated using Matlab software. Histograms, with a duration of one minute, are used to apply the model.

Table 2.1S. Comparison of predicted results ($Inh_{pulse\text{-}modelling}$) under different conditions with measured results ($Inh_{pulse\text{-}laboratory}$) for scenarios 4 and 5.

	Concentration tested	$Inh_{pulse\text{-}modelling}$ (%) No conditions for μ and $\mu_{inh\,x}$	$Inh_{pulse\text{-}modelling}$ (%) $\mu > \mu_{inh\,x}$	$Inh_{pulse\text{-}laboratory}$ (%)
Scenario 4	EC_{10}	4	10	17
Scenario 5	EC_{30}	24	26	44

Chapter 3

The model can be extrapolated to other photosystem II inhibitors?

The goal of this chapter was to check if the model could be extrapolated to other photosystem II inhibitors. Laboratory experiments are conducted to validate the results of the model. The pulse exposure tests are performed with the herbicides atrazine and diuron on the freshwater microalgae *Scenedesmus vacuolatus* and with the herbicide isoproturon on the freshwater microalgae *Pseudokirchneriella subcapitata*. These substances are used because they are photosystem II inhibitors and their recovery after pulse exposure is direct and complete. Furthermore, the several AA-EQS of these three substances are often exceeded in worldwide streams. The contribution of the different characteristic pulses, i.e. short or long, high or low, is studied.

Modelling the effects of pulse exposure of several PSII inhibitors on two algae

Pierre-Jean Copin, Nathalie Chèvre

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Abstract

Subsequent to crop application and during precipitation events, herbicides can reach surface waters in pulses of high concentrations. These pulses can exceed the Annual Average Environmental Quality Standards (AA-EQS), defined in the EU Water Framework Directive, which aims to protect the aquatic environment. A model was developed in a previous study to evaluate the effects of pulse exposure for the herbicide isoproturon on the alga *S. vacuolatus*. In this study, the model was extended to other substances acting as photosystem II inhibitors and to other algae. The measured and predicted effects were equivalent when pulse exposure of atrazine and diuron were tested on *Scenedesmus vacuolatus*. The results were consistent for isoproturon on the alga *Pseudokirchneriella subcapitata*. The model is thus suitable for the effect prediction of phenylureas and triazines and for the algae used: *S. vacuolatus* and *P. subcapitata*. The toxicity classification obtained from the dose-response curves (diuron > atrazine > isoproturon) was conserved for the pulse exposure scenarios modelled for *S. vacuolatus*. Toxicity was identical for isoproturon on the two algae when the dose-response curves were compared and also for the pulse exposure scenarios. Modelling the effects of any pulse scenario of photosystem II inhibitors on algae is therefore feasible and only requires the determination of the dose-response curves of the substance and growth rate of unexposed algae. It is crucial to detect the longest pulses when measurements of herbicide concentrations are performed in streams because the model showed that they principally affect the cell density inhibition of algae.

Keywords: *Pulse exposure, Recovery, Modelling, Algae, Phenylureas, Triazines*

3.1 Introduction

Numerous herbicides are regularly detected in surface water worldwide (Kalkhoff et al. 2003; Rabiet et al. 2010; Skark et al. 2004; Stangroom et al. 1998). Many of these herbicides belong to the triazines group and the phenylureas group (Gilliom 2006; Gregorio et al. 2012), which act as photosystem II inhibitors (Knauert 2008; Vallotton et al. 2008a). These types of herbicides are often measured at high concentrations because they are among the most important classes of herbicides used in the world (LeBaron et al. 2008; Sorensen et al. 2003; Stangroom et al. 1998). In Switzerland, numerous triazines and phenylureas are also regularly detected at high concentrations in surface waters, such as lakes (Chèvre et al. 2008; Gregorio et al. 2012) and streams (Chèvre et al., 2006; Munz et al. 2013).

After agricultural application and during and after rain events, herbicides are transported by surface runoff or drainage from the site of application to the surface water (Daouk et al. 2013; Rabiet et al. 2010). Consequently, in streams located in small agriculture catchments, herbicides usually occur as pulses in connection with the flux of runoff water (Boxall et al. 2013; Petersen et al. 2012). The pulses of herbicides occurring in creeks can affect the density and composition of phytoplankton, benthic and epiphytic microalgae, and macroalgae living in these streams. This is critical for the whole aquatic ecosystem, as they are energy sources for many species (Hoffman 2003). In the laboratory, freshwater algae are generally submitted continuously to herbicides to test their toxicities (OECD 2011). However, this type of test does not allow for an evaluation of the effect of pulsed exposure, such as that occurring in streams. Indeed, in the environment, the exposure duration of herbicides can be either long with low concentrations or short with high concentrations (Leu 2003). It is therefore crucial to simulate these types of pulse exposure scenarios in the laboratory to improve the environmental risk assessment of pulse exposure.

Some laboratory experiments were already performed to simulate pulse exposure to herbicides with several types of algae, such as *S. vacuolatus*, but mostly with photosystem II inhibitors (Baxter et al. 2013; Copin et al., 2015; Prosser et al., 2013; Vallotton et al. 2008a; Vallotton et al. 2008b; Vallotton et al. 2009). Other studies investigated the effects of pulses on several types of floating macrophytes, such as *Lemna minor* (Boxall et al. 2013; Brain et al., 2012; Cedergreen et al. 2005; Mohammad et al., 2010; Teodorovic et al. 2012), or on periphyton communities (Laviale et al. 2011; Tlili et al. 2008). All of these studies showed

that pulse exposures affect organisms differently than continuous exposures. They also highlighted that the recovery following a pulse exposure of a photosystem II inhibitor were rapid and complete.

Different models were developed to estimate the effects of pulse exposures on aquatic organisms (Ashauer et al. 2006; Nagai 2014; Weber et al. 2012). For microcrustacea and fish, the most appropriate models consider toxicokinetics (i.e., the time course of uptake, biotransformation, and elimination of toxicants in the organism) and the toxicodynamics (i.e., the dynamics of injury and recovery in the organism) (Ashauer et al. 2006; Ashauer et al. 2010). Recently, we developed a simple model, including a few parameters, to assess the effect of pulse exposure on algae. It was successfully validated for the photosystem II inhibitor isoproturon on the algae species *S. vacuolatus* (Copin et al., 2015). This model assumes that the external and the internal concentrations are similar for algae and therefore mainly includes toxicodynamic factors, i.e., it is a function of injury and recovery (Rozman and Doull 2000).

The aims of this study are two-fold. First, we propose to extrapolate and validate the model of pulse exposure for other photosystem II inhibitors, such as diuron, a substance belonging to the same herbicide group as isoproturon (phenylureas), and atrazine, belonging to the triazine group. Indeed, atrazine is one of the most famous triazines. Although it was banned in the European Union in 2004, it is still widely used in selective weed control programs for corn and sorghum cultures in other countries, such as the USA (Hoffman 2003; Loos and Niessner 1999; Sass and Colangelo 2006). During the late 1990s, it was the most often-used pesticide in the USA. Indeed, it was used on more than two-thirds of U.S. acreage (Fishel 2006; Hoffman 2003). Atrazine is therefore largely found in surface waters in the USA (Solomon et al. 1996). Phenylureas, such as diuron and isoproturon, are principally used for pre- or post-emergence weed control in cotton, fruit and cereal crops worldwide (Sorensen et al. 2003). They are also found in surface waters (Gilliom 2006). The second goal is to extrapolate and validate the model with another alga, *P. subcapitata*, with the herbicide isoproturon.

3.2 Materials and methods

3.2.1 Chemicals

Atrazine (Atrazine Pestanal® 99.1%, $C_{18}H_{14}CIN_5$), diuron (Diuron Pestanal® 99%, $C_9H_{10}CL_2N_2O$) and isoproturon (Isoproturon Pestanal® 99.9%, $C_{12}H_{18}N_2O$) were purchased from Sigma-Aldrich. Atrazine belongs to the triazines group. Diuron and isoproturon belong to the phenylureas group. The 3 herbicides are moderately soluble (respectively 35, 35.6 and 70.2 mg/L) and have low octanol-water partition coefficients (respectively 2.7, 2.87 and 2.5). They thus have low to moderate tendencies to accumulate in biota. These 3 herbicides inhibit photosynthesis by interrupting electron transport through photosystem II (PSII) (Knauert 2008; Vallotton et al. 2008a). Stock solutions of 10,000 µg/L (atrazine), 3200 µg/L (diuron) and 3200 µg/L (isoproturon) were prepared in an algae medium, under axenic conditions, for pulse exposure testing (nominal concentrations). These stock solutions were stored at 6.4°C. The concentrations were checked analytically at the beginning of the several experiments in laboratory, and the measured concentrations were in the same range as the nominal concentrations. Indeed, the concentrations measured for atrazine, diuron and isoproturon were respectively 9930, 3150 and 3100 µg/L.

3.2.2 Algal culture experiments

3.2.2.1 Culture condition

Permanent agar culture tubes of the green unicellular microalgae *S. vacuolatus* (Chlorophyceae; strain 211-15, Shihira and Krauss, Melbourne, Australia) and *P. subcapitata* (Chlorophyceae; strain 61.81, Nygaard, Komárek, J.Kristiansen and Skulberg, Akershus, Norway) were obtained, respectively, from the Department of Bioanalytical Ecotoxicology at the Helmholtz Centre for Environmental Research – UFZ, Leipzig, Germany and from the Institute for Plant Physiology of the University of Göttingen, Germany. Microalgae were cultured as described by Copin et al. (2015), i.e., in 250-mL Erlenmeyer flasks with 50 mL of OECD medium (OECD 2011; Van der Vliet et al. 2007) and maintained in exponential growth on an HT Infors shaker table (90 rpm) at 25°C under continuous illumination at a light intensity of 30 µmol/m^2/s provided by cool-white fluorescent lamps.

The optical density was measured with a microplate reader (ELx800™, BioTek® Instruments, Winooski, Vermont) at a wavelength of 690 nm. For *S. vacuolatus* and *P. subcapitata*, algae were inoculated in a new culture medium with, respectively, an initial optical density of 0.056 and 0.040 at 690 nm ($OD_{\lambda 690}$), which correspond to densities of 650,000 and 200,000 cells/mL. These initial algae densities were chosen to have enough algal biomass after the procedure of centrifugation used in the pulse exposure tests (see 3.2.2.3). With these initial algae densities, the growth was exponential during almost 48 hours. A similar procedure was followed by Vallotton et al. (2009).

A control charter was established to monitor algae growth in the growth media. In our laboratory, the average growth rate of *S. vacuolatus*, based on optical density values, was 0.023 h^{-1}, with a standard deviation of 0.002 h^{-1} (average of successive 45 cultures). It was 0.028 h^{-1}, with a standard deviation of 0.002 h^{-1} (average of successive 51 cultures), for *P. subcapitata*. These values corresponded to 0.035 h^{-1} and 0.059 h^{-1} if cell density values are used to determine the growth rates.

3.2.2.2 Standard acute toxicity tests

The dose-response curves of isoproturon, atrazine and diuron required us to parameterize the model and to define the tested concentrations, established following a method adapted from the standard OECD procedure (OECD 2011). The tests were performed in the same conditions as the algae cultures (see section 3.2.2.1). A 48-h duration was chosen for testing (1) because the growth of the algae *S. vacuolatus* and *P. subcapitata*, with their initial cell densities fixed respectively to 650,000 cells/mL and 200,000 cells/mL, was exponential during almost 48 hours (2) to reduce the possibility of a decrease of the tested concentration caused by the sorption of the toxicant to the rapidly increasing algal biomass (Hoffman 2003). However, the sorption of atrazine, diuron and isoproturon was assumed to be limited on the algae cell surface. Indeed, these substances have low octanol-water partition coefficients (respectively 2.7, 2.87 and 2.9) and therefore these herbicides are rather hydrophilic (3) to diminish the pH change in the test water (Hoffman 2003). For atrazine and diuron, 6 concentrations ranging from, respectively, 20 to 150 µg/L and 5 to 75 µg/L, and a control were tested in triplicate on *S. vacuolatus*. For isoproturon, 7 concentrations ranging from 10 to 200 µg/L and a control were tested in triplicates on *P. subcapitata*. The optical density measured at the beginning and end of the test was used to evaluate the average specific

growth rate for each concentration and for the control. Growth inhibition is the ratio between the growth rates of the different concentrations and that of the control (Eq. 3.1; see section 3.2.3.1).

3.2.2.3 Pulse exposure tests

Three pulse exposure scenarios were tested in the laboratory for each herbicide and algae (Fig. 3.1; Table 3.1). They differed in terms of the herbicide and alga used. The design of each scenario included two cases: a short pulse duration (W=5.25 h) and a long recovery period (X=24 h) for the first part of the scenario following by a long pulse duration (Y=20 h) and a long recovery period (Z=48 h) in the second part of the scenario. These cases in the same scenario can be considered as representative of two extreme scenarios that can be found in rivers. Concentration levels of the pulses for each herbicide used are summarized in Table 3.1. Different number of replicates was used for the three pulse exposure scenarios. The algae *S. vacuolatus* exposed to atrazine were tested with 6 replicates. The algae *S. vacuolatus* exposed to diuron were tested with 5 replicates. Finally, the algae *P. subcapitata* exposed to isoproturon were tested with 3 replicates.

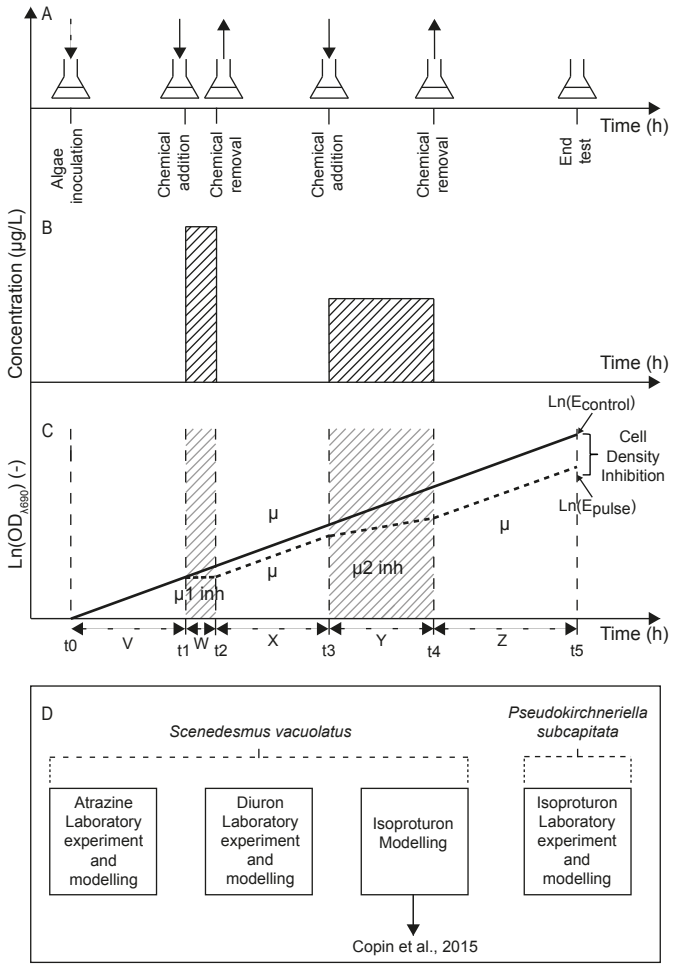

Fig. 3.1. A: Procedure of pulse exposure scenarios tested in the laboratory. **B:** Pulse representation over time. **C:** Modelling representation. μ is the growth rate obtained from the control charter of one alga. $\mu 1_{inh}$ and $\mu 2_{inh}$ are the growth rate at concentration *x1 and x2* obtained from the growth response curve of a substance on one alga. $E_{control}$ is the predicted final optical density for the model's control. E_{pulse} is the final optical density of the model for the alga exposed to the pulse concentration. V corresponds to the growth duration before the first pulse (h); W and Y are the pulse durations (h); X and Z are the recovery durations (h); V is approximately 40 h; W=5.25 h, X=24 h, Y=20 h, and Z=48 h. **D:** Diagram of the substances tested and algae used for the pulse exposure experiments and/or modelling. Adapted from Fig. 1 in Copin et al. (2015).

Table 3.1. Herbicide and algae used for each pulse exposure test in the laboratory and for the modelling.

Scenario abbreviation	Herbicide	Alga	Concentration	
			First pulse	Second pulse
Atrazine-SV	Atrazine	*S. vacuolatus*	EC_{81}	EC_{37}
Diuron-SV	Diuron	*S. vacuolatus*	EC_{75}	EC_{21}
Isoproturon-P	Isoproturon	*P. subcapitata*	EC_{80}	EC_{28}

The test was initiated the same way that the algae were cultured (initial cell density of 650,000 cells/mL for *S. vacuolatus* and 200,000 cells/mL for *P. subcapitata*, 50 mL in a 250-mL flask) and under the same conditions (see section 3.2.2.1). Algae grew for a long period (44 h for scenario Atrazine-SV and 43 h for scenarios Diuron-SV and Isoproturon-P) at the beginning before being exposed to the first pulse. At the end of each pulse exposure, the algae were centrifuged twice for 7 min at 1046 g and 25°C. For the algae *S. vacuolatus*, as the algae adhered to the inner tube-surface, the supernatant could be removed. Thereafter, the algae were re-suspended in growth media with an ultra-wave bath (Copin et al. 2015; Vallotton et al. 2008a). For the algae *P. subcapitata*, after the centrifugations, as the algae remained concentrate in the bottom of the centrifugation tube, the supernatant could be removed. These two centrifugations allowed, for the 2 algae, 99 % of the herbicide to be removed and did not impair algal growth, as shown by Vallotton et al. (2009). The loss of the algae *S. vacuolatus* and *P. subcapitata* were, respectively, 18% and 28% during the two centrifugations. The recovery period began directly after the algae were re-suspended in the fresh medium at the same initial cell densities fixed at the beginning of the test, i.e. 650,000 cells/mL for *S. vacuolatus* and 200,000 cells/mL for *P. subcapitata*. This choice of cell density was the consequence of 1) the low number of algae of culture exposed to pulses and 2) the loss of algae during the period of centrifugation. Indeed, we had the obligation to have enough algae to re-suspend the algae in the new fresh medium after the procedure of centrifugation. The optical density was measured regularly during the exposure and recovery periods.

3.2.3 Data analysis

3.2.3.1 Growth inhibition in a standard toxicity test on algae

The endpoint of this standard test, i.e., the growth inhibition (I_t) at a given concentration, is determined using the response variables, i.e., the average specific growth rate of the control (μ_C) and the average specific growth rate at this concentration (μ_T) (OECD 2011) (Eq. 3.1):

$$I_t = \frac{\mu C - \mu T}{\mu C} \quad (3.1)$$

3.2.3.2 Dose-response curves for the standard acute toxicity test

Dose-response curves are obtained by plotting the growth inhibition I_t as a function of the tested nominal concentrations C. The relationship is expressed by using a four parameter log-logistic dose response model (Vallotton et al. 2008a) (Eq. 3.2):

$$I_t = min + \frac{(max - min)}{1 + 10^{((log\ EC_{50} - log\ C) \times Hillslope)}} \quad (3.2)$$

where EC_{50} is the concentration inhibiting 50% of the growth; *Hillslope* is the slope of the dose-response curve; and the *max* and *min* parameters are the maximum and minimum of the sigmoidal curve. The *min* parameter is fixed at 0. The *max* parameter is fixed at 100 because it was assumed that the growth inhibition is 100% at high concentrations. Consequently, the relationship is now expressed as a two parameter log-logistic model.

This curve is calculated by using the statistics software Prism (GraphPad, San Diego, CA, USA). A toxicity ranking is established comparing the EC_{50} obtained in this study for all substances (atrazine, diuron and isoproturon) and algae (*S. vacuolatus* and *P. subcapitata*) with the EC_{50} obtained in Copin et al. (2015) for isoproturon and *S. vacuolatus*.

3.2.3.3 Cell density inhibition at the end of the pulse exposure scenario

The endpoint of these pulse exposure experiments, i.e., the overall algae cell density inhibition of each scenario ($Inh_{pulse\text{-}laboratory}$) with its 95% confidence interval was obtained by calculating the average and the standard deviation algae cell density inhibition of each replicate ($Inh_{pulse\text{-}laboratory\text{-}replicate\ i}$). The $Inh_{pulse\text{-}laboratory\text{-}replicate\ i}$ was calculated as (Eq. 3.3):

$$Inh_{pulse-laboratory-replicate\ i} = 100 \times \frac{OD_{control} - OD_{pulse,replicate\ i}}{OD_{control}} \quad (3.3)$$

with $OD_{control}$, the average optical density for the replicates of the controls at the end of the experiment and $OD_{pulse,replicate\ i}$, the final optical density for the replicate *i* of alga exposed to pulses concentration. For the control and for each replicate exposed to pulses, to better visualize the effect of pulses on algae, the optical density data measured were summed up, i.e. after each pulse exposure period, the following recovery period and the following pulse exposure period were summed to the last optical density value of the previous pulse exposure period. Thereafter, $OD_{control}$ and $OD_{pulse,replicate\ i}$ were determined using growth rates of the linear regressions fitted on the summed optical density values of the different parts (pulses, recovery) of the control and of the replicate *i* of the alga exposed to pulses as described in Copin et al. (2015).

3.2.3.4 Modelling

The cell density inhibition at the end of a pulse exposure scenario can also be expressed as described in Copin et al. (2015), (Eq. 3.4a or 3.4b):

$$Inh_{pulse-modelling} = 100 \times \frac{exp[ln(OD_{algae-t0}) + \mu \times t_{2n+1}] - exp\{ln(OD_{algae-t0}) + \mu \times [\sum_{i=0}^{n} (t_{2i+1} - t_{2i})] + \mu_{inh\,x} \times [\sum_{i=1}^{n} (t_{2i} - t_{2i-1})]\}}{exp[ln(OD_{algae-t0}) + \mu \times t_{2n+1}]}$$

(3.4a)

or

$$Inh_{pulse-modelling} = 100 \times \frac{exp[ln(OD_{algae-t0}) + \mu \times t_{2n}] - exp\{ln(OD_{algae-t0}) + \mu \times [\sum_{i=0}^{n-1} (t_{2i+1} - t_{2i})] + \mu_{inh\,x} \times [\sum_{i=1}^{n} (t_{2i} - t_{2i-1})]\}}{exp[ln(OD_{algae-t0}) + \mu \times t_{2n}]}$$

(3.4b)

where t_{2n+1} or t_{2n} is the total test duration; *n* is the number of pulses during the test; $OD_{algae\text{-}t0}$ is the optical density corresponding to the initial cell density of algae determined with the calibration curve of the alga. It was fixed at 650,000 cells/mL for *S. vacuolatus* and at 200,000 cells/mL for *P. subcapitata*. The corresponding optical density was then, respectively, 0.056 and 0.040. μ is the growth rate of the control determined as the average growth rate of several successive batch cultures in growth media (control charter). t_{2i} can

either be the beginning of a recovery period or the end of a pulse exposure period; t_{2i+1} is the end of a recovery period; t_{2i-1} is the beginning of a pulse exposure period; and $\mu_{inh\,x}$ is the growth rate at concentration x determined from the growth response curve of the standard ecotoxicity test. For real cases of pulse exposure scenarios, equation 3.4a is used when the recovery phase corresponds to the end of the scenario, whereas equation 3.4b is used when the pulse exposure period corresponds to the end of the scenario.

3.2.3.5 Uncertainty analysis

The variables μ and $\mu_{inh\,x}$ of equation 3.4a or 3.4b, with their uncertainties, are obtained respectively from the control charter and from the dose response curve. These uncertainties are taken into account in the model simulation. According to the Shapiro and Wilk test, the parameter μ is assumed to follow a normal distribution. For $\mu_{inh\,x}$, the distribution is also assumed to be normal (but not enough data were available to test it). The distribution of $Inh_{pulse\text{-}modelling}$ is estimated based on a Monte Carlo simulation. To do so, 10,000 growth rates μ and $\mu_{inh\,x}$ are selected randomly from their respective distributions. Only values from the distribution of μ and $\mu_{inh\,x}$, located between "average-2 x standard deviation" and "average+2 x standard deviation", were chosen in order to consider 95% of possible data from μ and $\mu_{inh\,x}$ (Motulsky, 1995). By repeating the calculation (Eq. 3.4a or 3.4b) between 9000 and 10,000 times (depending on the number of excluded values of the interval defined above) and assuming that values of μ must be higher than $\mu_{inh\,x}$ in order to have all the values of $Inh_{pulse\text{-}modelling}$ positives, the mean of $Inh_{pulse\text{-}modelling}$ is calculated for each scenario, along with minimum and maximum values. The model is applied using Matlab software (MATLAB R2011b, The Mathworks Inc.) and the same parameters of time and concentrations used in the experiments in laboratory (Table 3.1). The predictive results of these Monte Carlo simulations were used to compare with the laboratory observations to validate the model.

3.2.3.6 Comparison of predicted cell density inhibitions within the herbicide and algae

In this section, we investigated whether the toxicity ranking observed based on the dose-response curves (see section 3.2.3.2) remained similar considering the effects of pulse exposure. For this investigation, we predicted the cell density inhibition of a similar scenario for all of the herbicides and algae (Fig. 3.1). The concentrations chosen were equivalent, i.e., 28.0 µg/L for the first pulse and 10.6 µg/L for the second pulse. The concentrations of 28.0

and 10.6 µg/L were selected because, in the case study presented in Copin et al. (2015), these latter were lower than the maximum concentration tested (42 µg/L corresponding to EC_{32}). They induced respectively 20% and 5% of effect (EC_{20} and EC_5) on the alga *S. vacuolatus*. For isoproturon, the concentrations chosen were thus therefore consistent with environmental concentrations. All the other concentrations were identical to these concentrations but the effect concentrations were different according to the different dose-response curves. Therefore, according to the OECD standard tests, the concentrations tested during the first pulse for Atrazine-SV, Diuron-SV and Isoproturon-P corresponded, respectively, to EC_{33}, EC_{79} and EC_{19}. For the second pulse, it was, respectively EC_{13}, EC_{36} and EC_6. For diuron, the concentrations were also consistent with environmental concentrations. Indeed, concentrations of this herbicide was already measured up to 28.0 µg/L in streams in France (IFEN 2007). For atrazine, the concentrations were not consistent with actual environmental concentrations in Europe. Indeed, the concentrations of this herbicide were not anymore measured up to 28 µg/L (Munz et al. 2013) because it was banned in European Union in 2004 and in Switzerland in 2003. However, in case where the atrazine was used, concentrations were consistent with environmental concentrations (Leu 2003). The parameters of the model (the growth rates μ and μ_{inh}) were the same as those used in this study (Atrazine-SV, Diuron-SV and Isoproturon P) and in Copin et al. (2015) (Isoproturon-SV).

Furthermore, a pulse exposure is characterised by two parameters, the height and the width, i.e., respectively, the peak concentration and the duration (Reinert et al. 2002). The effects of each pulse for each scenario were compared to highlight which of these two parameters characterizing the pulse had the most influence on the cell density inhibition, i.e., whether the peak duration or the pulse concentration principally affected the predicted cell density inhibition. To this end, the cell density inhibition of each substance (atrazine, diuron, isoproturon) for *S. vacuolatus* and *P. subcapitata* was calculated with the model at the end of each pulse exposure.

3.3 Results and discussion

3.3.1 Laboratory experiments

3.3.1.1 Dose-response curves

The dose-response curves obtained for a 48-h duration of the photosystem II inhibitors diuron, atrazine (*S. vacuolatus*) and isoproturon (*S. vacuolatus* and *P. subcapitata*) are presented in Fig. 3.2. The data for isoproturon on the alga *S. vacuolatus* were obtained from Copin et al. (2015). For *S. vacuolatus*, the diuron, atrazine and isoproturon dose-response curves provided, respectively, the following EC_{50}s with 95% confidence intervals: 14.3 (11.9; 17.2) µg/L, 49.7 (42.2; 58.6) µg/L and 66.9 (61.2; 73.2) µg/L.

The EC_{50} of *S. vacuolatus* was found to range between 47.12 µg/L (Backhaus et al. 2004) and 100-110 µg/L (Vallotton et al. 2008a) when the algae was exposed to isoproturon (respectively, for 24 h and 48 h of exposure), which is consistent with our findings. For atrazine, the EC_{50} ranged between 38.82 µg/L (24 h of exposure) (Junghans et al. 2006) and 126-128 µg/L (48 h of exposure) (Vallotton et al. 2008a), which is also consistent with our results.

For *P. subcapitata* treated with isoproturon, the EC_{50} with a 95% confidence interval and an exposure duration of 48 h (initial cellular density of 200,000 cells/mL) is 80.4 (70.2; 92.2) µg/L. For an exposure duration of 72 h tested in the laboratory (initial cellular density of 50,000 cells/mL), the EC_{50} is 72.92 (64.6; 82.4) µg/L. These two values are not significantly different (ANCOVA analysis, p-value > 0.05). The comparison of EC_{50} calculated for an exposure of 48 h can thus be compared with EC_{50}s from the literature obtained for exposures of 72 h.

Based on these EC_{50}s, the toxicity ranking for the alga *S. vacuolatus* was: diuron < atrazine < isoproturon. Diuron was the most toxic substance and isoproturon was the least toxic. This toxicity ranking is consistent with the study of Backhaus et al. (2004), which showed that diuron was the most toxic phenylureas (6.60 µg/L) for the reproduction of *S. vacuolatus*. Faust et al. (1999) also found the same classification as this study while basing their ranking on the EC_{50} of the growth inhibition of *S. vacuolatus*. Similarly, it was demonstrated that

diuron was the most toxic substance to photosynthetic activity of lake phytoplankton (Pesce et al. 2011).

Diuron was the most toxic compound followed by atrazine and isoproturon. Furthermore, the toxic ratio between the substances was conserved between isoproturon and diuron when EC_{10} was used instead of EC_{50}, but it was different between isoproturon and atrazine. Indeed, it was equivalent to 3.5 between atrazine and diuron and to 1.3 between isoproturon and atrazine based on the EC_{50} and to 3.6 and 2.0 based on the EC_{10}. As EC_{10} were closer to relevant environmental concentrations (Van der Hoeven et al. 1997; Warne and Van Dam 2008), the difference observed between isoproturon and atrazine might be important when evaluating the risk of the substances.

The toxicity of isoproturon to *S. vacuolatus* was similar to that of *P. subcapitata:* the EC_{50} of isoproturon was almost identical for *S. vacuolatus* (66.9 µg/L) and for *P. subcapitata* (80.4 µg/L). Furthermore, the toxicity relation of EC_{50} and EC_{10} for the algae *P. subcapitata* and *S. vacuolatus* were, respectively, 1.2 and 0.9. Therefore, for environmental concentrations (EC_{10}), the difference of toxicity for isoproturon was insignificant when *P. subcapitata* and *S. vacuolatus* were used. This similarity of toxicity was confirmed in Fig. 3.2, because the curves crossed at a concentration of 24.3 µg/L, corresponding to EC_{16}, a concentration very closed to EC_{10}. The EC_{50}-48h of *P.subcapitata* is consistent with the results found in the literature. Indeed, this value ranges between 16 µg/L (Pesce et al. 2011) and 128 µg/L (Weber et al. 2012) for isoproturon exposure (72 h of exposure).

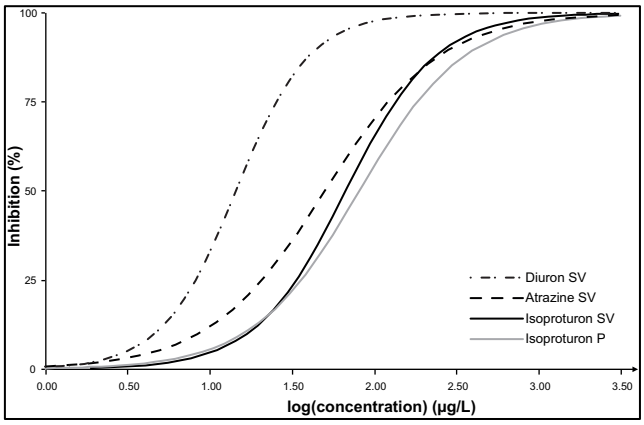

Fig. 3.2. Dose-response curves for several algae and several photosystem II inhibitors. Black dotted curve with points: Diuron, *S. vacuolatus*. Black dotted curve: Atrazine, *S. vacuolatus*.

Black curve: isoproturon, *S. vacuolatus*. The values for this curve were obtained from Copin et al. (2015). Grey curve: isoproturon, *P. subcapitata*.

3.3.1.2 Pulse exposure tests

The results for pulse exposure tests are illustrated for the Isoproturon-P scenario in Fig. 3.3. For this scenario, the cell density was inhibited during each pulse, as indicated by the difference between the growth curve of the control (black curve) and the growth curve of the exposed algae (grey curve) at the end of the experiment. For the scenarios Atrazine-SV and Diuron-SV, inhibition of the cell density was also observed comparing the control and the exposed algae. For the Atrazine-SV, Diuron-SV and Isoproturon-P scenarios, the cell density inhibition in the laboratory with a 95% confidence interval was, respectively, 23.7 (16.4; 30.9) %, 18.8 (7.2; 30.4) % and 24.8 (10.7; 39) %.

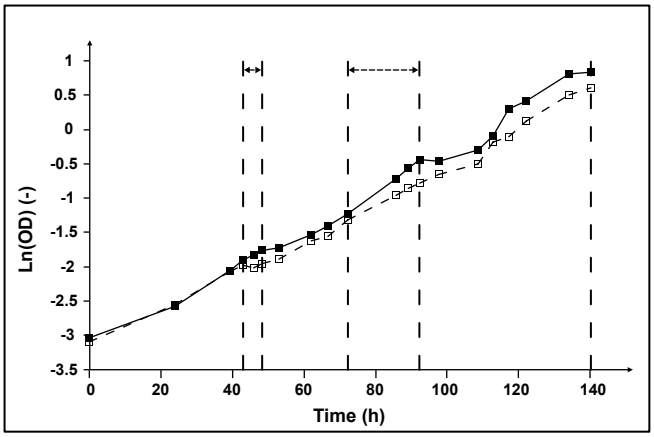

Fig. 3.3. Growth curves for the Isoproturon-P scenario in the laboratory. Black curve: control. Black squares: measurements for the control. Black dotted curve: algae exposed to pulses. White squares: measurements for algae exposed to pulses. The lengths of the pulses are indicated by arrows on the top of each graph.

For the three scenarios, we checked the hypothesis that "the recovery is complete just after the pulse exposure in the case the parameter growth rate is considered". To apply the model developed in Copin et al. (2015), we hypothesized that the growth rate μ of the control is identical to the growth rate of the recovery periods. This hypothesis was already controlled in Copin et al. (2015) for *S. vacuolatus* exposed to sequential pulses of isoproturon (ANCOVA analysis). For the Atrazine-SV, Diuron-SV and Isoproturon-P scenarios, the results also

support this hypothesis because the assumption was not rejected. Indeed, for *S. vacuolatus* exposed to pulses of atrazine or diuron, the p-values were, respectively, 0.17 and 0.43 (ANCOVA analysis, p-values>0.05). The results for atrazine confirm the conclusion of Vallotton et al. (2008a). In this study, we showed that this conclusion could also be extended to diuron. Indeed, it was shown that the recovery was complete following exposure of atrazine to *S. vacuolatus*. For *P. subcapitata*, the p-value was 0.6 (ANCOVA analysis, p-value>0.05). This is consistent for *P. subcapitata* according to Reinert et al. (2002), which showed that the recovery from an atrazine pulse exposure up to 50 µg/L was nearly instantaneous when the herbicide was removed (Reinert et al. 2002). Weber at al. (2012) also showed that a peak of isoproturon 10 times the maximum predicted environmental concentration had only transient effects on *P. subcapitata*. Furthermore, no reduced growth was observed after repeated exposure. Finally, Baxter et al. (2013) showed that the recovery of *P. subcapitata* was rapid following a 24-h pulse of high concentrations of atrazine.

3.3.2 Comparison between the measured and predicted results

The cell density inhibitions predicted by the model for the Atrazine-SV, Diuron-SV and Isoproturon-P scenarios are illustrated in the form of boxplots in Fig. 3.4. For these 3 scenarios, the experimental average cell density inhibition was very close to the average cell density inhibition given by the model (Fig. 3.4). The similarity between the measured and the predicted cell density inhibition was better achieved for these scenarios than for the Isoproturon-SV scenarios (Copin et al. 2015). In the previous publication, the comparison showed that the model slightly underestimated the average measured cell density inhibition when isoproturon was tested in pulses on *S. vacuolatus*. Furthermore, for the Atrazine-SV, Diuron-SV and Isoproturon-P scenarios, the variability of the observed experimental inhibitions was located between the minimum and maximum of the model (Fig. 3.4). This was not always the case in the Isoproturon-SV scenarios. The variability of the measured cell density inhibition in the laboratory sometimes exceeded the maximum value given by the model. Finally, for the 3 scenarios, the average measured cell density inhibition was included between the first and the third quartile of the model (Fig. 3.4). For the Isoproturon-SV scenarios, the average cell density inhibition obtained in the laboratory was often included only between the minimum and maximum of the model and not between the first and third quartile of the model. Therefore, the model predicts more precisely the observed experimental inhibitions. Consequently, the model developed in Copin et al. (2015) can be considered to be

suitable for substances belonging to the phenylurea and triazine groups when they are applied on *S. vacuolatus* and *P. subcapitata*.

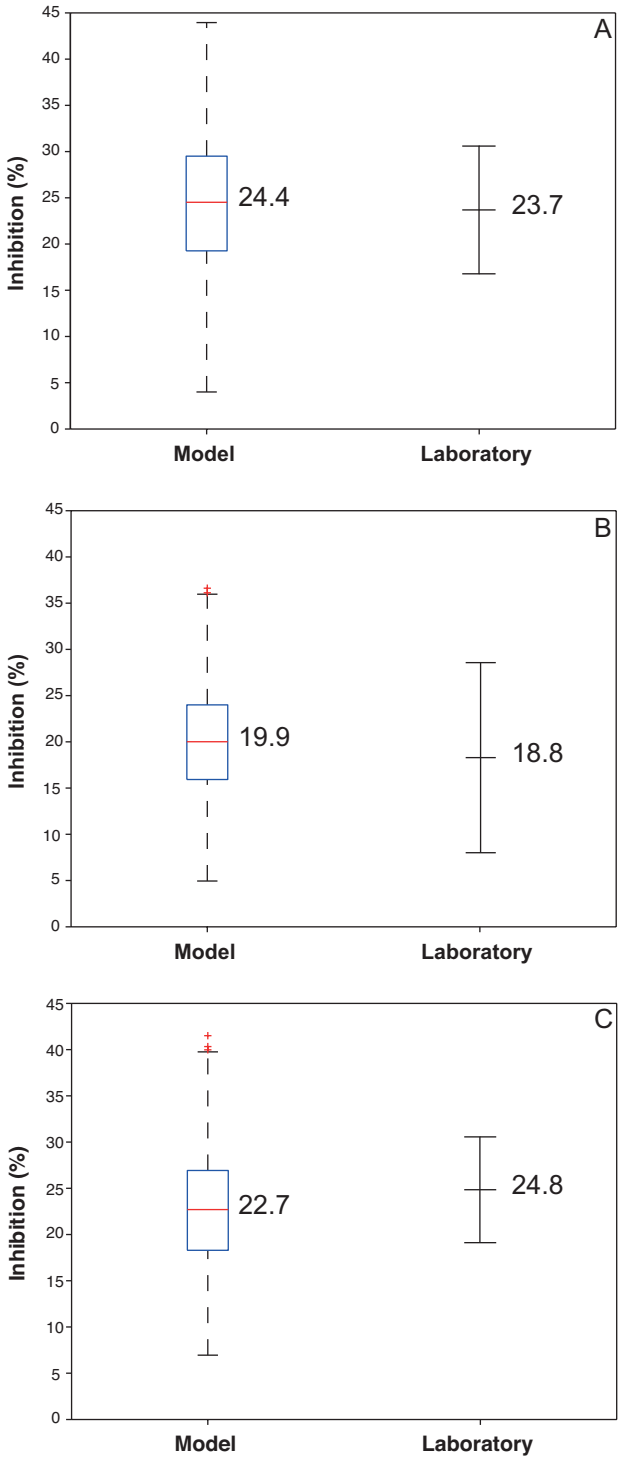

Fig. 3.4. Boxplots of modelled Atrazine-SV, Diuron-SV and Isoproturon-P scenarios. Average and standard deviation for laboratory results (six replicates of Atrazine-SV, five replicates of Diuron-SV, three replicates of Isoproturon P). The average cell density

inhibitions predicted by the model and obtained in the laboratory were noted for each scenario. **A:** Atrazine-SV scenario. **B:** Diuron-SV scenario. **C:** Isoproturon-P scenario.

3.3.3 Comparison of predicted cell density inhibitions within the herbicide and algae

The cell density inhibition averages predicted for identical concentrations of isoproturon, atrazine and diuron at each pulse exposure for *S. vacuolatus* were, respectively 6%, 13% and 25% with the different scenarios. Diuron was therefore the most toxic substance and isoproturon was the substance that least inhibited the cell density of *S. vacuolatus*. This toxicity ranking is identical to the classification established with the dose-response curves. Indeed, with the pulses concentrations chosen, where were identical for each substance, diuron caused more damage than isoproturon according to the dose-response curves (Fig. 3.2).

The cell density inhibition predicted average was 8% for *P subcapitata*. This result was very close to the average cell density inhibition predicted for the same substance, and at the same concentrations, on *S. vacuolatus* (6%). Indeed, the two concentrations tested during the two pulses experienced similar effects according to the dose-response curves of isoproturon on the 2 algae (EC_{20} and EC_5 for *S. vacuolatus*; EC_{19} and EC_6 for *P subcapitata*). Furthermore, the growth rates during the recovery phases were similar because the algae growth rates of the control charters were similar (average μ equal to 0.027 (0.002) h^{-1} for *S. vacuolatus* in Copin et al. (2015) and 0.028 (0.002) h^{-1} for *P subcapitata*, respectively). Therefore, the dose response curves and the control charters of several algae were necessary to compare the toxicity of pulse exposure of herbicides (phenylureas and triazines) between 2 algae.

For the Atrazine-SV, Diuron-SV, Isoproturon-SV and Isoproturon-P scenarios, the first pulse inhibited, respectively 4%, 9%, 2% and 2.5% of the cell density, whereas the second pulse provoked an inhibition of 9%, 16%, 4% and 5.5%. Consequently, for each substance and for each alga, a long pulse at a low concentration had a stronger effect than a short pulse at a high concentration. Even if the concentration tested for the first pulse of the Diuron-SV scenario was very high (EC_{79}), the long peaks with a lower concentration had a stronger effect. The effects were thus less caused by the pulse concentrations than by the pulse duration. For all of the scenarios, the results were therefore consistent with the conclusions of the application of

the modelling in a real case in Copin et al. (2015). Indeed, in the study of Copin et al. (2015), it was shown that the low peaks of isoproturon on *S. vacuolatus* with a long duration induced the principal effect and the highest peaks influenced also the cell density inhibition.

None of these two parameters, i.e. the concentration and the duration of pulses, had a consequential impact on the recovery potential of the algae, in the case where the parameter growth rates is used, and this even if high concentrations pulses or long pulse durations were applied. Indeed, as shown in the section 3.3.1.2, after a high peak of exposure to atrazine on *S. vacuolatus* (EC_{81}), diuron on *S. vacuolatus* (EC_{75}) or isoproturon on *P. subcapitata* (EC_{80}), the growth rates of the several controls were not statistically different to the growth rates of the treatment during the first recovery phase (ANCOVA analysis, p-values>0.05). Similarly, further to a long exposure duration, i.e. 24 hours, of atrazine on *S. vacuolatus* (EC_{37}), diuron on *S. vacuolatus* (EC_{21}) or isoproturon on *P. subcapitata* (EC_{28}), the growth rates of the several controls were not statistically different to the growth rates of the treatment during the second recovery phase (ANCOVA analysis, p-values>0.05).

3.4 Conclusions

The model developed was previously shown to be suitable to predict the cell density inhibition of any type of pulse exposure scenario on *S. vacuolatus* for herbicides without a delay in the recovery and with substances belonging to the triazine and phenylurea. This model also allows for predicting the cell density inhibition of *P subcapitata* exposed to isoproturon. To establish the toxicity classification of pulse exposure scenarios between several substances or between different algae, the dose-response curves of each substance for each alga as well as the growth rate of each alga obtained with a charter control are necessary. Indeed, the toxicity classification obtained for *S. vacuolatus* exposed to several triazine and phenylurea herbicides, with dose-response curves, is also preserved for pulse exposure scenarios on the same alga with the same substances. This toxicity ranking is also conserved for isoproturon on *S. vacuolatus* and *P subcapitata* because their growth rates during the recovery periods are identical. However, further research must be conducted to adapt this model and to validate it with laboratory experiments of herbicides with delays in the recovery phase such as the S-metolachlor. Pulse testing with multispecies in the same culture should also be conducted to improve the model. In conclusion, the model can be considered to be

suitable to assess the effects of pulse exposure scenarios for photosystem II inhibitors, such as triazines and phenylureas, on *S. vacuolatus* and *P subcapitata* in exponential growth.

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Chapter 4

The model can be used with herbicides with another mode of action that the photosystem II inhibitors?

In this chapter, we aimed to extend the model to other herbicides with a mode of action different of the photosystem II inhibitors. The freshwater microalgae *Scenedesmus vacuolatus* is used for this study. The pulse exposure tests are performed with the herbicide S-metolachlor. This substance is used because its effect is not direct and its recovery induces a delay. Furthermore, the AA-EQS of S-metolachlor is often exceeded in worldwide streams. The analysis of the trigger effect is conducted applying standard toxicity tests and measuring regularly the optical densities and the cells size of the culture treated. The delay of the recovery is established after the algae are exposed to pulses differing by their time and their concentration. The sensitivity of the algae is also studied applying thereafter a standard toxicity test on the algae previously exposed to a pulse exposure. All these parameters are then integrated in the model. A pulse exposure scenario is tested in laboratory to validate the model.

Modelling the effect of exposing algae to pulses of S-metolachlor: how to include a delay to the onset of the effect and in the recovery.

Pierre-Jean Copin, Léa Perronet, Nathalie Chèvre

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Abstract

In agriculture, herbicides are applied to improve crop productivity. During and after rain event, herbicides can be transported by surface runoff in streams and rivers. As a result, the exposure pattern in creeks is time-varying, i.e., a repeated pollution of aquatic system. In previous studies, we developed a model to assess the effects of pulse exposure patterns on algae. This model was validated for triazines and phenylureas, which are substances that induce effects directly after exposure with no delay in recovery. However, other herbicides display a mode of action characterized by a time-dependency effect and a delay in recovery. In this study, we therefore investigate whether this previous model could be used to assess the effects of pulse exposure by herbicides with time delay in effect and recovery. The current study focuses on the herbicide S-metolachlor. We showed that the effect of the herbicide begins only after 20 hours of exposure for the alga *Scenedesmus vacuolatus* based on both the optical density and algal cells size measurements. Furthermore, the duration of delay of the recovery for algae previously exposed to S-metolachlor was 20 hours and did not depend on the pulse exposure duration or the height of the peak concentration. By accounting for these specific effects, the measured and predicted effects were similar when pulse exposure of S-metolachlor is tested on the alga *S. vacuolatus*. However, the sensitivity of the alga is greatly modified after being previously exposed to a pulse of S-metolachlor. In the case of scenarios composed of several pulses, this sensitivity should be considered in the modelling. Therefore, modelling the effects of any pulse scenario of S-metolachlor on an alga is feasible but requires the determination of the effect trigger, the delay in recovery and the possible change in the sensitivity of the alga to the substance.

Keywords: *Herbicides, Algae, Modelling, Time-dependence, Recovery.*

4.1 Introduction

Herbicides are often used in agriculture to control weeds and thus to improve crop productivity. During and after precipitations, these herbicides can reach streams and rivers through surface runoff. As a result, the exposure to herbicides in the aquatic environment often occurs at a time-varying scale, i.e., as repeated herbicide pulses. Therefore, in streams located in agricultural catchments, aquatic organisms are exposed to fluctuating concentrations of pesticides related to the flux of runoff water (Boxall et al. 2013; Petersen et al. 2012). This type of exposure is characterised by periods of pulse exposure followed by periods of recovery but such exposure type is not considered in classical ecotoxicology studies (Reinert et al. 2002). A challenge is thus to assess the effects of such exposure profiles either by laboratory experiments or by modelling. The advantage of effect modelling is the possibility to predict a wide range of pulse exposure scenarios (Ashauer and Brown 2013).

Diverse models were thus developed to predict the effects of this type of exposure on aquatic organisms (Ashauer et al. 2006; Nagai 2014; Weber et al. 2012). For algae, the effects of time-variable exposure are determined mainly by toxicodynamics, i.e., the description of the chemical effect and recovery or repair mechanisms that occur in the organisms (Ashauer et al. 2007; Ashauer and Brown 2013). In previous studies, we developed a model based on toxicodynamics to predict the effect of pulse exposures on algae. This model was successfully validated for the photosynthesis inhibitors in the chemical families of triazines and phenylureas on the algae species *S. vacuolatus* and *Pseudokirchneriella subcapitata* (Copin and Chèvre 2015; Copin et al. 2015). These families of herbicides were selected because their effect is immediate when the algae species are in contact with the substances and because no delay is noted in the recovery of the algae (Copin and Chèvre 2015; Copin et al. 2015; Vallotton et al. 2008a; Vallotton et al. 2009).

However, other groups differing in their mode of action compared to triazines and phenylureas can be detected in creeks and streams such as S-metolachlor. S-metolachlor is a chloroacetanilide herbicide used to control pre-emergent and early post-emergent annual grassy and broadleaved weeds (Vallotton 2007; Waxman 1998). S-metolachlor is applied principally on cornfields but also on soybeans, sorghum, peanuts, potatoes, pod crops and cotton (Eurostat 2007; O'connell et al. 1998; Thakkar et al. 2013). S-metolachlor is composed of 88 % of S-isomers and 12 % of R-isomers (O'connell et al. 1998; Shaner et al. 2006; Xu et

al. 2010). The S-isomers are characterized by a high herbicidal activity (Moser et al. 1983). S-metolachlor belongs to the family of seedling shoot growth inhibitors. This herbicide family minimizes the growth of new plants by reducing the ability of seedlings to develop normally in the soil. The herbicide is absorbed by the developing roots and shoots and can be transferred via the xylem to areas of new growth (Gunsolus and Curran 1991). At the molecular level inside the plant, S-metolachlor inhibits the enzyme VLCFA-FAE1 synthase (Gotz and Boger 2004). This enzyme is required for the elongation of C16 and C18 to very long chain fatty acids (VLCFA) (Boger 2003). These VLCFAs are elements constituting the membrane lipids of the eukaryotic algae *S. vacuolatus* (Siegenthaler and Murata 1998). Consequently, S-metolachlor inhibits the formation of VLCFAs, causing an imbalance in the fatty acid composition of cell membranes. The rigidity and the permeability of cells are reduced, and thus, cell division is inhibited (Vallotton et al. 2008b).

Because of its relatively high water solubility (488 mg/L at T=20°C) (Weber et al. 2007; Zemolin et al. 2014), metolachlor (a mixture of 50 % of two S-isomers and 50 % of two R-isomers) was one of the most frequently herbicides detected in US surface water and groundwater between 1992 and 2001 (Gilliom 2006). Between 1989 and 1998, the concentration of metolachlor in Midwestern streams fluctuated between 2.50 and 1.44 µg/L (Scribner et al. 2000). From 2003 to 2004, the maximal concentrations of metolachlor increased from 1.56 to 5.29 µg/L in Ontario streams (Kurt-Karakus et al. 2008). The concentration of metolachlor in the Great Lakes were also detected and measured to range over 0.28 to 14 ng/L in 2005-2006 (Kurt-Karakus et al. 2010). Similarly, metolachlor was regularly detected in streams and rivers in Europe (Konstantinou et al. 2006; Munz et al. 2013; IFEN 2007; Poiger et al. 2002; Wittmer et al. 2014). This high frequency of metolachlor in European surface water resulted from the substitution of atrazine by metolachlor. Atrazine was banned in 2003 by the European Union Commission because of its endocrine disruption potential on several organisms (Joly et al. 2013; Sass and Colangelo 2006). The use of S-metolachlor reduces the load of herbicides applied on the field (Shaner et al. 2006), Switzerland, other European countries and the United States decided to switch from metolachlor to S-metolachlor in 1997 (Liu and Xiong 2009; Poiger et al. 2002). Consequently, S-metolachlor became one of the ten active ingredients the most used in the European Union in 2003 (Eurostat 2007). Metolachlor was then banned in the European Union in 2002 (EC 2002). However, in North America, both metolachlor and S-metolachlor are currently authorized for use (Vallotton et al. 2008b).

Some studies have investigated the toxicity of metolachlor and S-metolachlor on freshwater species. More ecotoxicological studies are available for metolachlor on non-target species such as algae because metolachlor was commercialized before S-metolachlor. In these studies, metolachlor was less toxic than herbicides with a photosynthesis inhibition mode of action (Junghans et al. 2003; Kotrikla et al. 1999; Ma et al. 2003; Ma et al. 2006; Ma et al. 2002). A comparative study showed that the growth rate of the alga *Chlorella pyrenoidosa* was inhibited more drastically with S-metolachlor than by metolachlor after 24, 48, 72 and 96-h of exposure (Liu and Xiong 2009). The effects of S-metolachlor were also assessed on periphytic diatoms. The live cell densities of periphytic diatoms were reduced when compared to the control for 6-day exposures of 5 and 30 µg/l of S-metolachlor. However, select diatoms species of the periphyton were not affected by S-metolachlor exposure whereas other species were more sensitive (Debenest et al. 2009).

By contrast, few S-metolachlor pulse exposure studies have been performed. Debenest et al. (2009) compared the 3-day recovery following a 3-day exposure of 30 µg/l of S-metolachlor and isoproturon on periphytic diatoms. They showed that the growth rates of periphyton were higher for S-metolachlor than for isoproturon during the recovery periods. Vallotton et al. (2008b) assessed the time-to-recovery of the alga *S. vacuolatus* following a pulse exposure of 24 hours. When the alga *S. vacuolatus* is exposed to a strongly inhibiting concentration of S-metolachlor, the recovery is delayed by 29 hours. Furthermore, no effect was observed on the algae growth below 10 hours of exposure. This effect was named the time-dependency effect.

The aim of this study was to adapt and validate the previously developed model for predicting the effects of pulse exposure for a substance with a different mode of action than photosynthesis inhibition on the alga *S. vacuolatus*. To achieve our goal, we tested S-metolachlor, a substance with a delayed effect and recovery (Vallotton et al. 2008b). Consequently, we investigated the time-dependency effects, the time-to-recovery and the change in the sensitivity of the alga *S. vacuolatus* after exposure to the compound. These observations were used to adapt the previously defined model.

4.2 Materials and Methods

4.2.1 Chemicals

S-metolachlor (S-metolachlor Pestanal® 98.4 %, $C_{15}H_{22}ClNO_2$) was purchased from Sigma-Aldrich. A stock solution of 100,000 µg/L was prepared in an algal OECD medium under axenic conditions. This stock solution was refrigerated at 6.4°C. The concentration was determined analytically by LCMS, and the measured concentration was in the same range as the nominal concentrations. The measured concentration for S-metolachlor was 97,619 µg/L.

4.2.2 Algae cultures

A permanent agar culture tube of freshwater green microalga *S. vacuolatus* (Chlorophyceae; strain 211-15, Shihira and Krauss, Melbourne, Australia) was obtained from the Department of Bioanalytical Ecotoxicology at the Helmholtz Centre for Environmental Research – UFZ, Leipzig, Germany. The algae species *S. vacuolatus* multiply, similar to the *Chlorella* species, by an asexual reproduction called autosporulation (Fujishima and Steinbuchel 2009; Huss et al. 1999; Yamamoto et al. 2004). Microalgae were cultured as described by Copin et al. (2015), i.e., with a continuous illumination at a light intensity of 30 µmol/m^2/s provided by cool-white fluorescent lamps and with a temperature of 25 °C on a HT Infors shaker table (90 rpm). The optical density was measured with a microplate reader (ELx800™, BioTek® Instruments, Winooski, Vermont) at a wavelength of 690 nm. In total, 650,000 algae cells/mL were inoculated in 250-mL Erlenmeyer flasks with 50 mL of OECD medium (OECD 2011). With this initial alga density, the growth was exponential over approximately 48 hours. The algae species *S. vacuolatus* was selected because it is unicellular, easily cultivated in the laboratory, representative of freshwater environments in which pulse exposures can be detected and measured and displays a higher sensitivity to a variety of hazardous substances (Backhaus et al. 2004; Blaise et al. 1986 ; Faust et al. 2001; Junghans et al. 2006; Machado and Soares 2014). Furthermore, the algae species *S. vacuolatus* are resistant to centrifugation, a process performed during pulse exposure experiments (Vallotton et al. 2009). A control charter was established to monitor this alga growth in OECD media. In the laboratory, the average growth rate of the algae *S. vacuolatus* based on optical density values was 0.023 h^{-1} with a standard deviation of 0.002 h^{-1} (average of 45 cultures).

4.2.3 Dose-response curves

The dose-response curve of S-Metolachlor was required for parameterising the model and for defining the tested concentrations during pulse exposure tests following a method adapted from the standard OECD procedure (OECD 2011). The experiment was performed under the identical conditions as the algae cultures. The concentrations tested ranged from 13 to 30,000 µg/L. Each control and each concentration were tested in triplicate. The optical densities measured at the beginning and at the end of the experiment (after 48 hours of exposure) were used to evaluate the average specific growth rate for each concentration and for each control. The growth inhibition was defined as the ratio between the growth rates of the different concentrations and that of the control (Eq.4.1; see section 4.2.6.1). Such testing is considered in this study as a "standard" testing.

4.2.4 Determination of the new parameters for the model

The following experiments were performed under identical conditions as the algae cultures.

4.2.4.1 Time-dependency effects

The duration of exposure required for the first effects to occur were determined by measuring the optical densities at several times during two 48-hour exposure standard tests (t=17.25, 20, 24, 28, 31, 41.5 and 48 hours for the first test and t=13.75, 17.5, 20, 24, 28, 38, 43 and 48 hours for the second test) (Fig. 4.1A). Different controls were used for each test. The concentrations tested were 900 (test 1), 3000 (tests 1 and 2), 17,000 (test 2) and 30,000 (test 2) µg/l, corresponding respectively to EC_{35}, EC_{50}, EC_{70} and EC_{76} (Effect Concentration; EC). The control and the culture exposed to these concentrations were tested in duplicate. During these tests, the maximum diameters of 20 algae cells from each replicate of the controls and of the concentrations tested were measured simultaneously with the optical densities. These values were averaged and used as a cell size indicator. These values were calculated for each exposure time. The algae cell diameters were measured by photographing algae cells with an Olympus BX53 microscope equipped with an Olympus DP26 camera. The cell size distribution of the microalgae in relation with the exposure time was established. Vallotton et al. (2008b) previously noted that the cell diameter of exposed cells increased with exposure time.

4.2.4.2 Recovery after pulse exposure

An experiment (Fig. 4.1B) was conducted 1) to observe whether a delay occurs in the recovery phase and 2) to establish the duration of the delay before the alga recovers. Each control and each concentration was tested in triplicate. During the experiment, three exposure durations were considered in three separate tests. They were fixed to 25, 30 and 48 hours (parameter x in Fig. 4.1B). The concentration tested in the experiment was 3000 µg/L, corresponding to EC_{50}. After the pulse exposure, the algae were centrifuged twice for 7 min at 1046 x g and 25°C. The supernatant was removed, and the algae were re-suspended in growth media. These two centrifugations eliminated the herbicide (Copin and Chèvre 2015). The recovery period began directly after the algae were re-suspended in the fresh medium. The duration of the observation of the recovery was fixed to 48 h. The optical density was measured regularly during the exposure and recovery periods. A second experiment was performed to determine whether the delay defined previously was dependent on the pulse concentration. The general design is similar to the previous experiment (Fig. 4.1B). Two pulse concentrations, fixed at 525 and 24,500 µg/L and corresponding to EC_{30} and EC_{75}, respectively, were considered in two separate tests. The recovery initiated after the centrifugation procedure (48-hour duration). Each control and each concentration were tested in triplicate. To better visualize the effect of S-metolachlor on algae and the delay during the recovery, the optical densities data measured were summed, i.e., after the exposure period, the following recovery period was summed to the last optical density value of the previous exposure period. This summation was performed for both experiments, for the control and for the culture exposed, following the methodology of previous publications (Copin and Chèvre 2015).

4.2.4.3 Sensitivity after pulse exposure

A test was performed to investigate whether the sensitivity of the alga *S. vacuolatus* to S-metolachlor increased after being previously exposed to the compound in pulses (Fig. 4.1C). The alga was first exposed to a 28-hour peak concentration of 3000 µg/L, corresponding to EC_{50}. The chemical was then removed by the procedure centrifugation described above. A standard test was then performed on the alga. Each control and each concentration was tested in duplicate during the standard toxicity test.

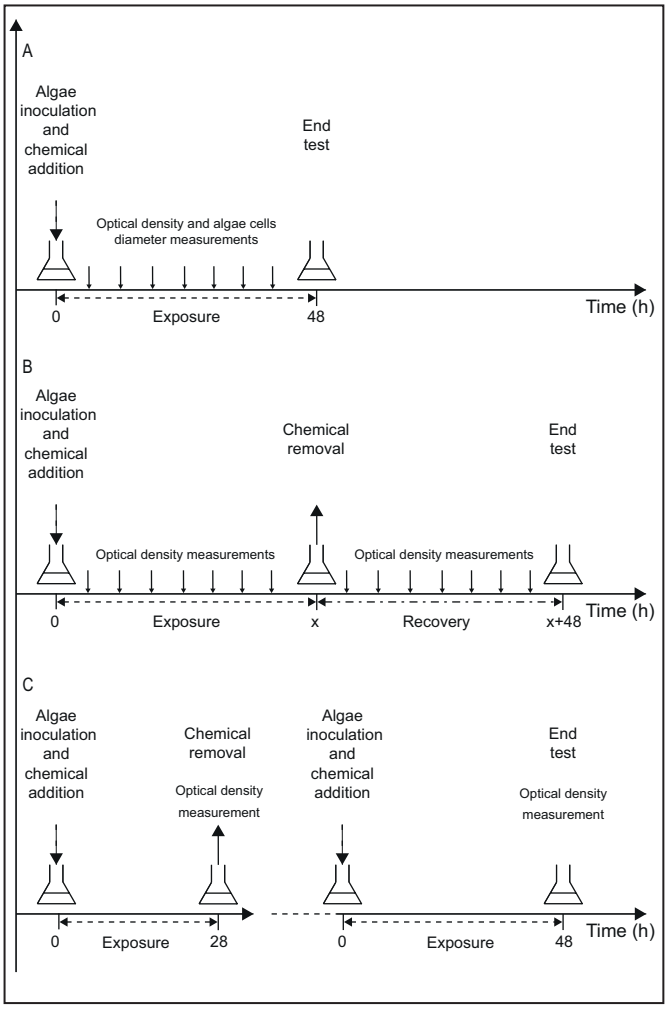

Fig. 4.1. Diagrams of the different experiments conducted to determine the specific parameters for the model. **A** Procedure to determine the time-varying effects. Two tests were conducted for this experiment. **B** Procedure to establish the delay in recovery: x=25, 30 and 48 hours. For x=25 and 30 hours, the concentration tested during the exposure corresponded to EC_{50}. For x=48 hours, different concentrations were tested during the exposure, corresponding to EC_{30}, EC_{50} and EC_{75}. Five tests were conducted for this experiment. **C** Procedure to analyse the sensitivity of the alga previously exposed to a pulse exposure. One test was conducted for this experiment.

4.2.5 Pulse exposure testing

One pulse exposure scenario of S-metolachlor was tested on the alga *S. vacuolatus* in the laboratory. This scenario was composed of two pulse-exposure periods and two recovery periods. The duration of the two pulse-exposure periods was fixed to 30 hours. The two recovery periods were fixed to 14 hours and 43 hours. The concentrations of the two pulses of

S-metolachlor were fixed to 3000 µg/L, corresponding to EC_{50}. Algae exposed to pulses of S-metolachlor and the controls were tested in triplicate. The pulse exposure test was applied following Copin and Chèvre (2015). In total, 650,000 cells/mL of algae cells were inoculated in 50 mL of OECD medium and placed on the HT Infors shaker table under the identical conditions defined in section 4.2.2. Algae grew initially for a short period of 18 h before being exposed to the first pulse. At the end of each pulse exposure, the algae were centrifuged as explained in section 4.2.4.2. The optical density was measured regularly during the exposure and recovery periods.

4.2.6 Data analysis

4.2.6.1 Growth inhibition in the standard toxicity test on algae

The growth inhibition (I_t) at a given concentration is calculated using the response variables, i.e., the average specific growth rate of the control (μ_C) and the average specific growth rate at the given concentration (μ_T) (OECD 2011) (Eq. 4.1):

$$I_t = \frac{\mu_C - \mu_T}{\mu_C} \times 100 \quad (4.1)$$

4.2.6.2 Dose-response curves for the standard acute toxicity test

The dose-response curves are obtained by plotting the growth inhibition I_t as a function of the tested concentrations C. The relationship is expressed using a four parameter log-logistic dose response model (Vallotton et al. 2008a) (Eq. 4.2):

$$I_t = min + \frac{(max - min)}{1 + 10^{\left((\log EC_{50} - \log C) \times Hillslope\right)}} \quad (4.2)$$

where EC_{50}, the concentration inhibiting 50 % of the growth; *Hillslope* is the slope of the dose-response curve; and *max* and *min* parameters are the maximum and minimum of the sigmoidal curve, respectively. The *min* parameter is fixed at 0. The *max* parameter is fixed at 100 because it was assumed that the growth inhibition is complete (100 %) at high concentrations. This curve is calculated using the statistical software Prism (GraphPad, San Diego, CA, USA).

4.2.6.3 Cell density inhibition measured at the end of the pulse exposure scenario

The overall algae cell density inhibition of the pulse exposure experiment ($Inh_{pulse\text{-}laboratory}$), was obtained by calculating the average of the algae cell density inhibition of each replicate ($Inh_{pulse\text{-}laboratory\text{-}replicate\ i}$). The $Inh_{pulse\text{-}laboratory\text{-}replicate\ i}$ was calculated as follows (Eq. 4.3):

$$Inh_{pulse-laboratory-replicate\ i} = 100 \times \frac{OD_{control} - OD_{pulse,replicate\ i}}{OD_{control}} \quad (4.3)$$

where $OD_{control}$ is the average optical density for the replicates of the controls at the end of the experiment, and $OD_{pulse,\ replicate\ i}$ is the final optical density for the replicate *i* of alga exposed to the pulse concentration. As explained above, to better visualize the effect of pulses on algae, the optical density data measured were summed for the control and for each replicate exposed to the pulses, i.e., after each pulse exposure period, the following recovery period and the following pulse exposure period were summed with the last optical density value of the previous pulse exposure period. Thereafter, $OD_{control}$ and $OD_{pulse,\ replicate\ i}$ were determined using the growth rates of the linear regressions fitted on the summed optical density values of the different parts (pulses, recovery) of the control and of the replicate *i* of the alga exposed to pulses as described in Copin et al. (2015). However, in the case of substances with a time-dependency effect, two linear regressions were considered during the pulse exposure: a portion in which the effect was not detected and a portion in which the effect was observed. Similarly, in the case of substances with a delay in the recovery period, two linear regressions were considered during the recovery periods: one regression before the delay and one regression after the algae began to recover.

4.2.6.4 Modelling

The cell density inhibition model for S-metolachlor at the end of the experiment was adapted from the equations described in Copin et al. (2015), (Eq. 4.4a and 4.4b):

$$Inh_{pulse-modelling} = 100 \times \frac{\exp[\ln(OD_{algae-t0}) + \mu \times t_{2n+1}] - \exp\{\ln(OD_{algae-t0}) + \mu \times t_1 + \sum_{i=1}^{n} X_i + \sum_{i=1}^{n} Y_i\}}{\exp[\ln(OD_{algae-initial}) + \mu \times t_{2n+1}]} \quad (4.4a)$$

or

$$Inh_{pulse-modelling} = 100 \times \frac{\exp[\ln(OD_{algae-t0}) + \mu \times t_{2n}] - \exp\{\ln(OD_{algae-t0}) + \mu \times t_1 + \sum_{i=1}^{n} X_i + \sum_{i=1}^{n-1} Y_i\}}{\exp[\ln(OD_{algae-t0}) + \mu \times t_{2n}]} \quad (4.4b)$$

The two equations refer to the two kinds of scenarios, i.e., equation 4.4a is used when the recovery phase corresponds to the end of the scenario, whereas equation 4.4b is used when the pulse exposure period corresponds to the end of the scenario.

t_{2n+1} or t_{2n} is the total test duration; n is the number of pulses during the test; and $OD_{algae\text{-}t0}$ is the optical density corresponding to the initial cell density of algae determined with the calibration curve of the alga. $OD_{algae\text{-}t0}$ is fixed at 650,000 cells/mL for the alga *S. vacuolatus*. The corresponding optical density, read with the calibration curve determined for the alga *S. vacuolatus*, is then 0.056. μ is the growth rate of the control determined as the average growth rates of several successive batch cultures in growth media (control charter). The first exponential part in the numerator and the part in the denominator describe the growth of the control. The second exponential part in the numerator describes the growth of the culture exposed to pulses. The term $\mu \times t_1$ describes the culture growth before being exposed to pulses. t_1 is the time of the beginning of the first pulse. X_i corresponds to the i^{th} pulse exposure period, and Y_i corresponds to the i^{th} recovery period.

X_i and Y_i are dependent on the exposure time and the time allowed for recovery, respectively. For X_i, the parameter t_{effect} is defined, and this time corresponds to the duration before the S-metolachlor effects begins (time-varying effect). This time is obtained with the experiment defined in section 4.2.4.1. Consequently, two cases are possible for the parameter X_i (Eq. 4.5a and 4.5b):

$$\text{If } t \leq t_{effect} : X_i = \mu \times (t_{2i} - t_{2i-1}) \quad (4.5a)$$

or

$$\text{If } t > t_{effect} : X_i = \mu \times \left(t_{effect} - t_{2i-1}\right) + \mu_{inh\,x} \times (t_{2i} - t_{effect}) \quad (4.5b)$$

where t_{2i} is the end of a pulse exposure period; $t_{2i\text{-}1}$ is the beginning of a pulse exposure period; $\mu_{inh\,x}$ is the growth rate at concentration x determined from a growth response curve. When the sensitivity of the alga is modified after a pulse exposure and when the recoveries between pulses are short, the $\mu_{inh\,x}$ during the first pulse is obtained from the growth response curve of the standard ecotoxicity test defined in section 4.2.3, and the $\mu_{inh\,x}$ during the other

pulses is defined from the growth response curve of the standard ecotoxicity test defined in section 4.2.4.3. When the sensitivity of the alga is not modified after a pulse exposure or when the recoveries between pulses are long, all $\mu_{inh\,x}$ during the several pulses are determined from the growth response curve of the standard ecotoxicity test defined in section 4.2.3.

For Y_i, the parameter t_{delay} is defined, and this time corresponds to the delay before a complete recovery of the culture previously exposed to a pulse of S-metolachlor. This time is obtained from the experiment defined in section 4.2.4.2. Therefore, two cases are defined for the parameter Y_i (Eq. 4.6a and 4.6b):

$$\text{If } t \leq t_{delay} : Y_i = 0 \quad (4.6a)$$

or

$$\text{If } t > t_{delay} : Y_i = 0 \times (t_{delay} - t_{2i}) + \mu \times (t_{2i+1} - t_{delay}) \quad (4.6b)$$

where t_{2i} is the beginning of a recovery period, and t_{2i+1} is the end of a recovery period.

As explained in Copin et al. (2015), the distribution of the $Inh_{pulse\text{-}modelling}$ is estimated based on a Monte Carlo simulation. The model is applied using Matlab software (MATLAB R2011b, The Mathworks Inc., see Appendix 6). The predictive results of these Monte Carlo simulations were used in the comparison with the laboratory observations to validate the model.

4.3 Results and Discussion

4.3.1 Laboratory experiments

4.3.1.1 Dose-response curve

The dose-response curve of S-metolachlor for the alga *S. vacuolatus* is presented in Fig. 4.2. The dose-response curves of atrazine, diuron and isoproturon obtained with the same alga (Copin and Chèvre 2015; Copin et al. 2015) are also represented in the Fig. 4.2. The EC_{50} for S-metolachlor is 2942 (1835; 4717) µg/L (95 % confidence interval). This toxicity value is consistent with Vallotton et al. (2008b), which obtained EC_{50}s of 2300 and 3000 µg/L for S-

metolachlor on the alga *S. vacuolatus*. No other EC_{50} data were available in the literature for this substance and this alga. The toxicity of S-metolachlor was compared with that of diuron, atrazine and isoproturon (Fig. 4.2). The corresponding EC_{50}s of diuron, atrazine and isoproturon for the alga *S. vacuolatus* with 95 % confidence intervals were, respectively, 14.3 (11.9; 17.2), 49.7 (42.2; 58.6) and 66.9 (61.2; 73.2) µg/l. Therefore, S-metolachlor is less toxic whereas diuron is the most toxic chemical for the alga *S. vacuolatus*. The EC_{50} toxic ratio of S-metolachlor (the EC_{50} of S-metolachlor divided by the EC_{50} of the compound) with diuron, atrazine and isoproturon is 206, 63 and 44, respectively.

This comparison does not change when using the EC_{10} of the compounds, which is closer to environmental concentrations (Van der Hoeven et al. 1997; Warne and Van Dam 2008). The toxic ratio of EC_{10} for S-metolachlor and diuron, atrazine and isoproturon were 7.3, 4.0 and 2.0, respectively. Similarly to the EC_{50}s, diuron, atrazine and isoproturon are more toxic than S-metolachlor on the alga *S. vacuolatus* when environmental concentrations were considered.

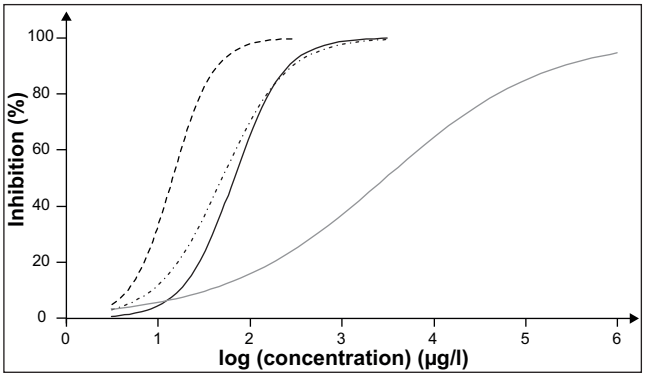

Fig. 4.2. Dose-response curves of diuron, atrazine, isoproturon and S-metolachlor for the alga *S. vacuolatus*. Black large-dotted curve: diuron. Black small-dotted curve: atrazine. Black curve: isoproturon. Grey curve: S-metolachlor.

4.3.1.2 Time varying effects

The optical densities of the alga *S. vacuolatus* were measured regularly during two standard tests (see section 4.2.4.1). The growth of the controls and the cultures exposed to S-metolachlor are represented in Fig. 4.3.

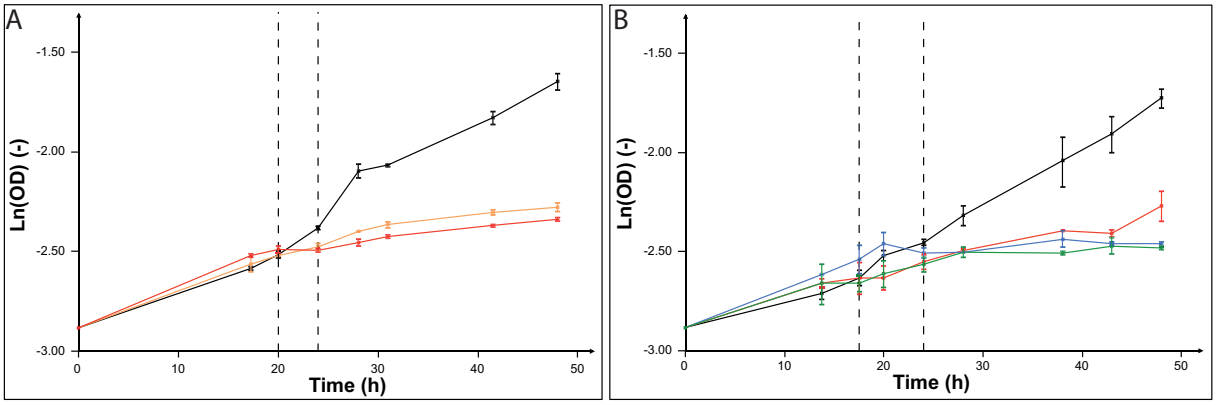

Fig. 4.3. Growth of the alga *S. vacuolatus* during two different tests (Fig. 4.3A and 4.3B). The OD is the optical density. The averages of the optical densities measured during the tests at different times are represented by squares with bars indicating standard deviation. The test duration is fixed to 48 hours. Black vertical dashed lines illustrate the period when the herbicide S-metolachlor affected the growth of the alga **A.** Black curve: controls. Orange curve: culture exposed to EC_{35}. Red curve: culture exposed to EC_{50}. **B.** Black curve: controls. Red curve: culture exposed to EC_{50}. Blue curve: culture exposed to EC_{70}. Green curve: culture exposed to EC_{76}.

The effects of S-metolachlor are observable after 20 hours in the majority of the tests (Fig 4.3A and B). A difference between the black curve (controls) and the orange and red curves (cultures exposed to, respectively, EC_{35} and EC_{50}) is noted at 20 hours (Fig 4.3A). Similarly, the blue curve (cultures exposed to EC_{70}; Fig 4.3B) is above the black curve until the growth rate decreases starting at 20 hours. However, in one test, the effects seem to initiate after 17.5 hours for two cultures exposed to S-metolachlor (Fig 4.3B). We observe a difference between the black curve (controls) and the red and green curves (cultures exposed to EC_{50} and EC_{76}, respectively) from 17.5 hours. As the effect begins after 20 hours in the most culture treated, we decided to fix the start of the effects of S-metolachlor to 20 hours for low and high concentrations. This start time is different from the photosystem II inhibitors such as diuron, atrazine and isoproturon. For these compounds, the effects are directly observable at the beginning of the exposure (Copin and Chèvre 2015; Copin et al. 2015).

4.3.1.3 Cells evolution during testing

During the two previous tests, the cell diameter (µm) of the alga *S. vacuolatus* was measured several times during the 48-hour exposure (see section 4.2.4.1). The results are presented in Table 4.1.

Table 4.1. Size of the algae cells (µm) at different times during 48 hours of exposure to EC35 (Test 1), EC50 (Test 1), EC70 (Test 2) and EC76 (Test 2) of S-metolachlor.

		Size (µm) [a]		
	Time (h)	Control	EC_{35} [b]	EC_{50} [c]
Test 1	17.25	8.88±1.59	9.23±1.27	9.47±1.08
	20	8.41±1.60	9.30±1.34 *	9.60±1.04 *
	24	7.48±1.57	8.65±1.88 *	9.35±1.18 *
	28	7.78±1.13	8.79±2.13 *	9.59±1.13 *
	31	7.31±0.90	9.40±1.74 *	9.96±1.10 *
	41.5	7.78±0.78	9.01±1.45 *	9.90±0.94 *
	48	8.20±1.30	9.76±1.97 *	10.28±1.32 *
	Time (h)	Control	EC_{70} [d]	EC_{76} [e]
Test 2	13.75	9.34±0.98	9.01±1.48	9.25±1.29
	17.5	9.66±1.12	9.50±0.76	10.07±0.93
	20	9.00±1.32	9.77±0.98 *	9.55±1.01 *
	24	7.78±1.58	10.35±0.87 *	10.06±0.91 *
	28	7.51±1.42	10.11±1.04 *	10.22±0.83 *
	38	7.79±0.69	10.53±0.86 *	10.70±1.09 *
	43	7.94±0.92	10.19±1.07 *	10.59±1.03 *
	48	8.16±0.84	10.22±0.99 *	10.61±1.02 *

[a] Data are represented as the mean ±standard deviation of the means. [b] Effect Concentration 35 %. [c] Effect Concentration 50 %. [d] Effect Concentration 70 %. [e] Effect Concentration 76 %
* The size of algae cells exposed to concentrations was significantly different to the size of the control algae cells using a Z-test (p-value > 0.05)

The effects of S-metolachlor are observable starting from 20 hours in the two tests (Table 4.1). For the cultures exposed to different concentrations of S-metolachlor (EC_{35}, EC_{50}, EC_{70} and EC_{76}), the size of their cells is statistically equivalent to that of the controls until approximately 17 hours of exposure. However, the size of cells is significantly different from the controls from 20 hours for the remainder of the two tests. After 20 hours of the experiment duration, the size of algae cells decreases for the two controls whereas it remains constant, slightly decreases or even increases for the cultures exposed to all concentrations of the herbicide. In the culture exposed to the EC35 and EC50 of S-metolachlor the size decreases after 20 hours, but to a smaller extent than the control. However, the size of algae continues to increase for the cultures exposed to EC70 and EC76 of S-metolachlor. Thereafter, the algae cell size increases again until the end of the tests for the controls. For the cultures exposed to the herbicide, the algae cell size increases during test one and remains constant for the cultures exposed to S-metolachlor in test two.

This increase in the cell size when exposed to the herbicide S-metolachlor agrees with Vallotton et al. (2008b). The increase of the algae cells size for the culture exposed to S-metolachlor results from the formation of new spores in the mother cell that are not released when the substance is present. This accumulation of new spores in the mother cell occurs because of the loss of the rigidity and permeability of cells exposed to S-metolachlor induced by the formation of VLCFAs, causing an imbalance in the fatty acid composition of cell membranes (Vallotton et al. 2008b). Furthermore, a fraction of the spores were potentially dead before the parent cell wall splintered (Liu and Xiong 2009), diminishing the growth and the development of new algae. For the concentrations EC_{35} and EC_{50}, cell division occurs after 20 hours, but few new algae are observed. However, for the high concentrations EC_{70} and EC_{76}, cell division completely stops after 20 hours as shown by the increase in and stability of the size of algae cells. Consequently, the conclusions obtained with the algae cell size confirm the results obtained with the optical densities measured regularly during the standard tests, i.e., that the beginning of the effect occurs at 20 hours.

This cell size increase was also observed for the alga *P. subcapitata* with the chemical pretilachlor, which has the identical mode of action as S-metolachlor, i.e., an inhibitor of very long-chain fatty acid formation (Nagai et al. 2011). The size of cells also increased for other chemicals with other modes of action. A diameter increase of cells of *Chlamydomonas reinhardtii* was observed with increasing paraquat concentrations, a photosystem I inhibitor (Jamers and De Coen 2010). The decrease of the algae cell size for the control after 20 hours for all concentrations corresponded to the moment when fresh spores, called autospores produced from the cell division of the mother cell, were released into the water (Yamamoto et al. 2004). After the release of these autospores between 24 and 48 h, the size of the new cells increased again until the end of the experiment.

4.3.1.4 Recovery after the pulse exposure

Fig. 4.4 presents the delay of recovery observed for different exposure durations and for several of the concentrations tested.

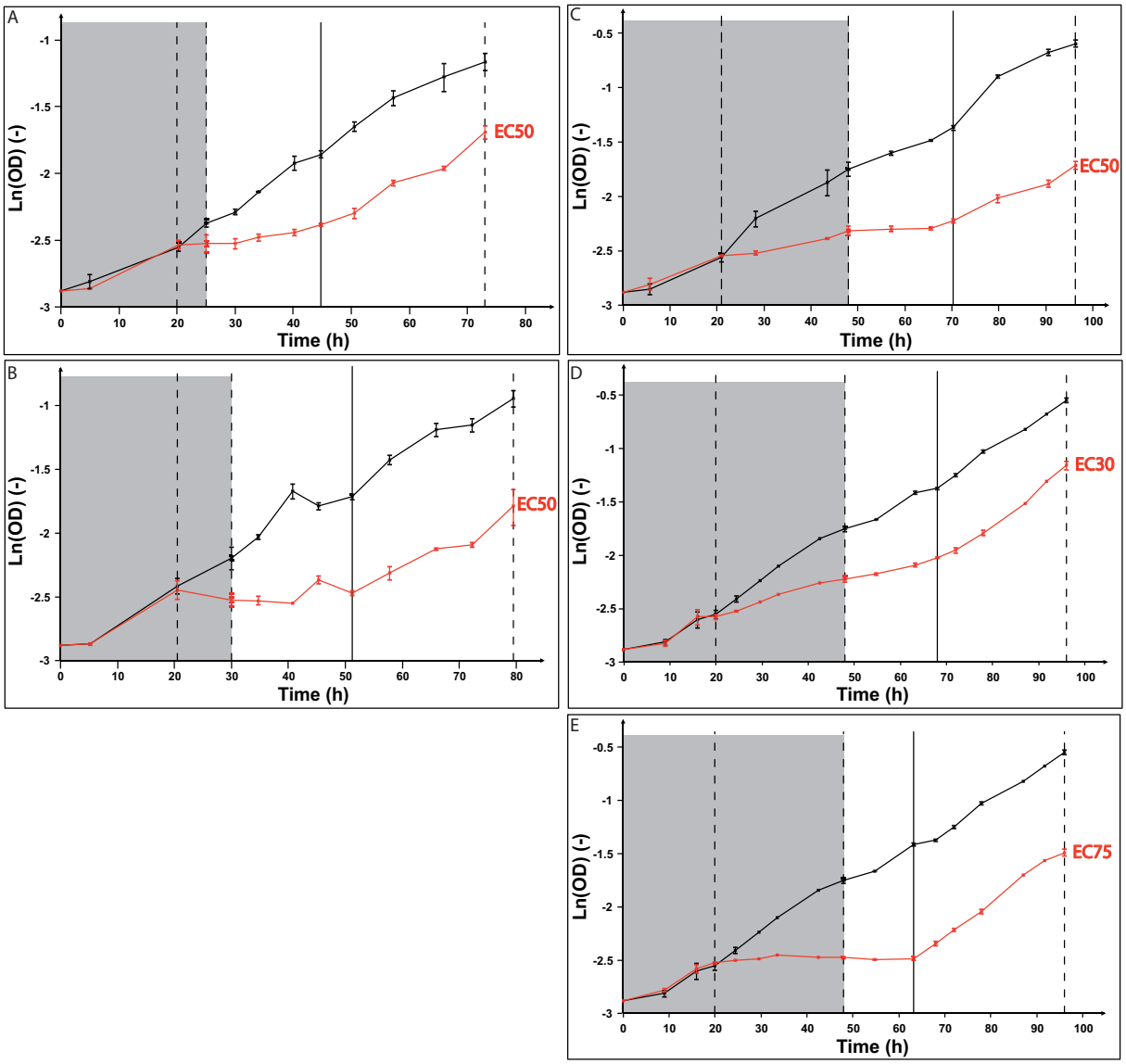

Fig. 4.4. Growth of the alga *S. vacuolatus* during five different tests characterized by their different exposure durations to S-metolachlor or their different exposure concentrations of S-metolachlor. **A:** Exposure for 25 hours and EC_{50}. **B:** Exposure for 30 hours and EC_{50}. **C:** Exposure for 48 hours and EC_{50}. **D:** Exposure for 48 hours and EC_{30}. **E:** Exposure for 48 hours and EC_{75}. The exposure duration to S-metolachlor was characterized by the grey surface. OD is the optical density. The averages of optical densities measured during the tests at different times are represented by squares. The averages of optical densities with their standard deviation at different times are represented by small dashes. The black vertical dashed lines illustrate the period during when the effect of the herbicide S-metolachlor was effective on the growth of alga *S. vacuolatus* and at the end of the experiment. The black line illustrates the time when the recovery of the culture exposed to S-metolachlor was complete after a delay. Black curve: control. Red curve: culture exposed to EC_{50}.

As mentioned in sections 4.3.1.2 and 4.3.1.3, the effect started after 20 hours of exposure for each test. A difference between the black curve (controls) and the red curve (cultures exposed to S-metolachlor) was observed visually after 20 hours of exposure (Fig. 4.4).

For each test, the analysis of the recovery shows that the growth rate of the culture exposed to S-metolachlor remains inhibited after the chemical is removed. For the cultures exposed to the EC_{50} of S-metolachlor during 25, 30 and 48 hours (Fig. 4.4A, 4.4B and 4.4C), no algal growth is observed during the first 20 hours of the recovery. The growth rate of the culture exposed to the herbicide is significantly different from the growth rate of the control (ANCOVA analysis, p-value=0.0002, 0.0029 and 0.02, respectively). However, after 20 hours, the culture begins to grow similarly to the control. The growth rate of the culture exposed is therefore no more significantly different from the growth rate of the control during this period (ANCOVA analysis, p-value=0.92, 0.14 and 0.48, respectively). Similarly, in Fig. 4.4D and 4.4E, the algae exposed to EC_{30} and EC_{75} of S-metolachlor during 48 hours do not grow during the first 20 and 15.25 hours, respectively, after the chemical was removed. The growth rate of the culture exposed to the herbicide is significantly different from the growth rate of the control (ANCOVA analysis, p-value=0.0196 and 0.0409, respectively). However, after 20 and 15.25 hours, respectively, the culture begins to grow similarly to the control. The growth rate of the culture exposed is not significantly different from the growth rate of the control during this period (ANCOVA analysis, p-value=0.1902 and 0.0956).

In summary, the recovery of the algae previously exposed to a range of concentrations of S-metolachlor during different exposure periods is complete 20 hours after the herbicide is removed from the cultures. Vallotton et al. (2008b) found a higher delay of 29 hours for the recovery of the identical algae exposed for 24-hours to a single strongly inhibiting S-metolachlor concentration. The difference between the duration of the delay in these two studies could be explained by the different test designs. In Vallotton et al. (2008b), algae grew synchronously under a dark and light illumination during the 24-hour exposure to S-metolachlor, whereas in this study, the light was continuous during the entire experiment.

Contrary to photosystem II inhibitors that are characterized by a complete and fast recovery from a pulse exposure (Copin and Chèvre 2015; Copin et al. 2015; Vallotton et al. 2008a), other substances with the identical mode of action as the herbicide S-metolachlor can also affect the recovery of aquatic species after a pulse exposure. A delay in the recovery is

observed after a 72-hour exposure of pretilachlor on the alga *P. subcapitata* (Nagai et al. 2011). However, contrary to our study, the duration of the delay induced by the exposure of pretilachlor is linked to the concentration tested. For low concentrations, a delay is not noted before the recovery, contrary to the high concentrations tested (Nagai et al. 2011). The recovery was also studied for alachlor and metazachlor, two other substances belonging to the identical chemical family as S-metolachlor (Weisshaar and Boger 1987). The complete recovery in the growth alga *Scenedesmus acutus* occurs 24 hours after a 24-hour exposure of concentrations between 50 and 100 µM of alachlor and metazachlor. Consequently, this duration of the delay in the recovery is on the identical order of magnitude as the delay of the recovery in this study. Note that for algae and macrophytes exposed to other herbicides with a mode of action different to S-metolachlor such as inhibitors of acetolactate synthase or microtubule assembly inhibitors, a delay in the recovery is also observed (Cedergreen et al. 2005; Mohammad et al. 2006; Nagai et al. 2011).

4.3.1.5 Change in the sensitivity

The toxicity of S-metolachlor to the algae was compared before and after the algae were exposed to a 28 hours pulse at a concentration corresponding to EC_{50}. The sensitivity of the alga increased significantly when previously exposed to the chemical (ANCOVA analysis, p-value <0.05). The EC_{50} of an acute toxicity test is equivalent to 2942 (1835; 4717) µg/L (95 % confidence interval). However, the toxicity test performed on the alga previously exposed to a pulse exposure provides an EC_{50} of 149 (108; 203) µg/L (95 % confidence interval). This modification of sensitivity can be explained by the delay of the recovery period induced after the pulse exposure of S-metolachlor. Prior to centrifugation, the alga begins its recovery period at the identical time as when it is exposed to several concentrations of S-metolachlor from the acute toxicity test. The period of recovery is characterized by a delay during which no algal growth is noted. Consequently, when the alga is exposed to several concentrations of S-metolachlor during this delay period, it grows with increasing difficulty. Therefore, the algae were more sensitive to additional exposure of S-metolachlor.

4.3.1.6 Pulse exposure tests

The results for pulse exposure tests are illustrated for S-metolachlor in Fig. 4.5. For this scenario, the cell density was inhibited during each pulse, as indicated by the difference between the growth curve of the control (black curve) and the growth curve of the exposed

algae (grey curve) at the end of the experiment. The effect was noted after 20.5 hours of exposure during the first pulse, agreeing with the time of the beginning of the effect at 20 hours found in sections 4.3.1.2 and 4.3.1.3. No growth is observed in the exposed algae during the first recovery period. The delay in the recovery was identified to be 20 hours for the alga exposed to S-metolachlor, as explained in the section 4.3.1.4. The first recovery period required 14 hours, and the algae do not recover during this period. During the second pulse exposure, according to sections 4.3.1.2 and 4.3.1.3, the growth of the algae is affected by the pulse concentration only 20 hours after being in contact with the alga. Thus, the alga during the second pulse exposure can begin recovering. However, the growth of the alga during the beginning of the second pulse is low because the delay period is not finished after the end of the recovery phase. Six additional hours are required to reach the 20 hours necessary for the beginning of alga recovery. Consequently, the growth of the alga during the second pulse is affected firstly by the delay phase and then by the pulse concentration when it was effective. Therefore, the effect during the second pulse occurred before 20 hours after the beginning of the second pulse. During the second recovery, a delay was observed during the first few hours. Thereafter, the alga treated by S-metolachlor grew similar to the control. For the S-metolachlor pulse exposure scenario, the cell density inhibition in the laboratory with its 95 % confidence interval was 72.3 (69.3; 75.3) %.

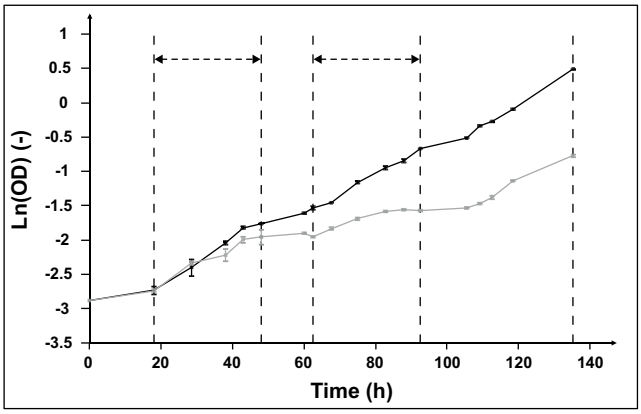

Fig. 4.5. Growth curves of the alga *S. vacuolatus* for the S-metolachlor pulse exposure scenario in the laboratory. Black curve: control. Black squares: measurements of the control. Grey curve: algae exposed to pulses. Grey squares: measurements of the algae exposed to pulses. Small black and grey dashes: averages of optical densities with their standard deviation at different times. The lengths of the pulses are indicated by the arrows on the top of each graph.

4.3.2 Comparison between the measured and predicted results

The parameters of the model, t_{effect} and t_{delay}, were fixed to 20 hours in each case. The effect is noted after 20 hours of exposure on the alga *S. vacuolatus* (see sections 4.3.1.2 and 4.3.1.3) and the delay is complete after 20 hours (see section 4.3.1.4). The recovery period between the two pulse exposure periods is 14 hours. Consequently, as the period of recovery was lower than the delay phase, the parameter $\mu_{inh,\,x}$ of the model for the second pulse was obtained from the growth response curve with an EC_{50} equivalent to 149 µg/L. The change of algae sensitivity described in section 4.3.1.5 could explain how the alga reacted to a scenario composed of two pulses separated by a short recovery period. We showed in section 4.3.1.5 that when the recovery between two pulses was lower than the delay period, the growth of the alga exposed to a second pulse was affected first by the delay phase and then by the pulse concentration when it was effective. Therefore, when the recovery separating two pulses was lower than the delay period, the parameter $\mu_{inh,\,x}$ of the model (Eq. 4.5b) during the second pulse was determined from the growth response curve with an EC_{50} equivalent to 149 µg/L.

The cell density inhibition predicted by the model is illustrated in the boxplot in Fig. 4.6. For this pulse exposure scenario, the experimental average cell density inhibition (72.3 %) was near the average cell density inhibition given by the model (67.5 %). The variability of the observed experimental inhibitions was located between the minimum and maximum of the model (Fig. 4.6) as observed in Copin et Chèvre (2015). Therefore, the model predicts precisely the observed experimental inhibitions. Consequently, the model developed in Copin et al. (Copin and Chèvre 2015; Copin et al. 2015) can be considered as suitable for substances with time-dependency effects and a delay of recovery as long-chain fatty acid inhibitors when they are applied on the alga *S. vacuolatus*.

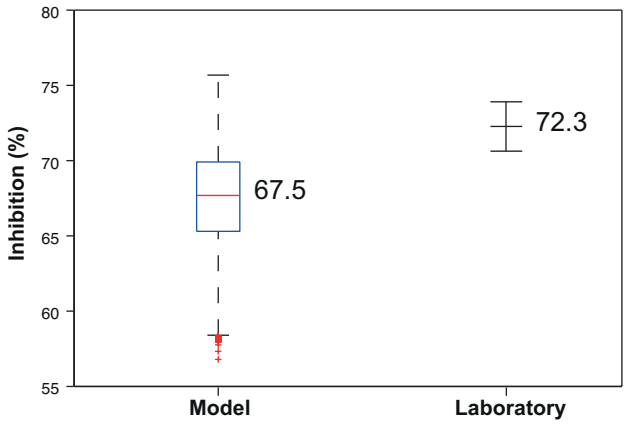

Fig. 4.6. Boxplot of the modelled scenario with S-metolachlor on the alga *S. vacuolatus*. Average and standard deviation for laboratory results (three replicates). The average cell density inhibitions predicted by the model and obtained in the laboratory were noted for each scenario. The beginning of the Y-axis is not fixed to 0 % of inhibition.

4.4 Conclusions

In this study, we adapted a previously developed model for predicting the cell density inhibition of a pulse exposure scenario on the alga *S. vacuolatus* for herbicides with a time-dependency effect and a delay in the recovery such as S-metolachlor. Therefore, the analysis of the time-dependency effect was first considered. Considering the optical density values and algal cell size, we highlighted that the effect of S-metolachlor on the alga *S. vacuolatus* is not direct and begins only after 20 hours of exposure. A delay in the recovery is also highlighted. This delay is identical regardless of the time and the concentration of the pulse exposure preceding and is fixed at 20 hours. Finally, the sensitivity of the alga increased after being previously exposed to a peak concentration. Thus, the reaction of the algae during a scenario composed of several pulses with short recovery periods can be ascertained. These parameters should be determined to apply this model to any substances with a similar mode of action as S-metolachlor such as alachlor and metazachlor. However, for substances with other mode of actions, other parameters might be of interest.

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Chapter 5

The model can be applied to an alga in co-culture with another alga?

The goal of this chapter was to adapt the model to an alga in co-culture with another alga. The freshwater microalgae *Scenedesmus vacuolatus* and *Pseudokirchneriella subcapitata* are used for this study. The herbicide isoproturon is used in this part because its toxicity on the two algae cultured alone has already been studied in the Chapters 2 and 3. The attempts of exponential growth of the two algae in co-culture are first performed. The comparison with the growth of the two algae cultured alone is made. Secondly, the toxicity of the herbicide isoproturon is tested on the two algae in co-culture and the results are compared with the toxicity of isoproturon on the two algae cultured alone. These different characteristics of the two algae in co-culture are integrated in the model to calculate the cell density inhibition of the alga *S. vacuolatus* in co-culture with the alga *P. subcapitata*. Several pulse exposure tests are performed in laboratory to validate the model.

Modelling the effects of PSII inhibitor pulse exposure on two algae in co-culture

Pierre-Jean Copin, Nathalie Chèvre

Will be submitted to *Ecotoxicology*

Abstract

A weakness of standard testing procedures is that they do not consider interactions between organisms, and they focus on single species. Furthermore, these procedures do not take into account pulse exposure. However, pulse exposure is of particular importance because in streams, after crop application and during and after precipitation, herbicide concentrations fluctuate widely and can exceed the Annual Average Environmental Quality Standards (AA-EQS), which aim to protect the aquatic environment. The growth of the algae *Scenedesmus vacuolatus* and *Pseudokirchneriella subcapitata* in a co-culture is thus analysed in this study. For initial cell densities fixed, respectively, to 100,000 and 50,000 cells/mL, the growth of each alga is exponential over at least 48 hours. *S. vacuolatus* seems to influence the growth of *P. subcapitata* negatively. Allelopathy is a possible explanation for this growth inhibition. The toxicity of the photosystem II inhibitor isoproturon is later tested on the algae *S. vacuolatus* and *P. subcapitata* cultured alone and in the co-culture. Despite the supplementary stress on the algae in the co-culture competing for nutrients, the toxicity of the herbicide is lower for the two algae when they are in the co-culture than when they are in the separated culture. A model is adapted and used to predict the cell-density inhibition on the alga *S. vacuolatus* in the co-culture with the alga *P. subcapitata* exposed to a pulse concentration of isoproturon. Four laboratory experiments are performed to validate the model. The comparison between the laboratory and the modelled effects shows good agreement. The differences can be considered minor and are principally due to the high uncertainty of the cell count used to determine the parameters of the model and to the cell number of the alga *P. subcapitata* re-suspended in a new OECD medium after the centrifugation process.

Keywords: *Algae, Co-culture, Allelopathy, Pulse, Recovery, Model.*

5.1 Introduction

Herbicides have been widely used since the Green Revolution, which aimed to improve food production in the late 1950s (Pimentel 1996). Starting at this time, the use of herbicides increased dramatically worldwide, reaching 2.5 million tons used in the 1990s (Pimentel 1996). In Switzerland, 650 tons of herbicides were applied in 2000, and 870 tons were applied in 2011 (http://faostat.fao.org). Even if they are applied to treat a weed on a field, herbicides can reach surface waters via different mechanisms of transport during or following rain events. The mechanisms of transport are primarily surface runoff but also include subsurface runoff via drainage pipes. These types of runoff depend on molecule properties, weather conditions, application practices, soil types and land uses (Brown and van Beinum 2009; Daouk et al. 2013; Freitas et al. 2008; Petersen et al. 2012; Rabiet et al. 2010). Surface runoff is induced by the precipitation's intensity and capacity to saturate rapidly the first centimetre of the soil. It carries herbicides to streams, rivers or lakes. Herbicides are thus frequently detected in surface waters worldwide (De Geronimo et al. 2014; Du Preez et al. 2005; Konstantinou et al. 2006; Kruawal et al. 2005; Solomon et al. 1996). Note that herbicides are the most important group of phytochemical compounds detected in Swiss streams (Munz et al. 2013).

The exposure pattern of herbicides in surface waters is function of several factors, such as the characteristics of the catchment and of the waterways, the intensity and timing of the rainfall, and the amounts of herbicides used (Vallotton 2007). Classically, following the application of an herbicide on a culture during and after rain events, the concentrations of herbicides in creeks and streams occur in short pulses of high concentrations or long pulses of low concentrations (Copin et al. 2015; Daouk et al. 2013; Garmouma et al. 1998; Leu 2003). These pulse concentrations are separated by time intervals during which concentrations of the herbicide can be under detectable levels. This is called the recovery period (Reinert et al. 2002).

Pulse-exposure concentrations are often well above the Annual Average Environmental Quality Standards (AA-EQS) defined in the EU Water Framework Directive, which aim to protect the aquatic environment against the occurrence of chronic effects (EU 2008; Lepper 2005). Herbicide pulses may therefore affect the density and composition of the phytoplankton, benthic and epiphytic microalgae, and macroalgae living in these streams,

which are critical for the whole aquatic ecosystem because they are energy sources for many species (Hoffman 2003). Studying the effects of pulse exposure is thus crucial. However, the standard toxicity tests for algae and aquatic plants (such as OECD guidelines) do not adequately simulate this type of exposure because they focus on continuous exposure. Indeed, the duration of a standard toxicity test for freshwater algae is generally approximately 72 hours (OECD 2011) and 7 days for freshwater aquatic plants (OECD 2006), whereas the duration of pulses can be shorter than an hour, as shown by Leu et al. (2003). Some studies have evaluated the effects of pulses of herbicides on different aquatic non-target organisms living in streams, such as microalgae (Baxter et al. 2013; Copin and Chèvre 2015; Copin et al. 2015; Klaine et al. 1997; Vallotton et al. 2009; Vallotton et al. 2008; Weber et al. 2012), aquatic macrophytes (Boxall et al. 2013; Cedergreen et al. 2005; Mohammad et al. 2008; Mohammad et al. 2010; Rosenkrantz et al. 2012; Teodorovic et al. 2012) or periphyton communities (Graham et al. 2009; Gustavson et al. 2003; Laviale et al. 2011; Tlili et al. 2008; Tlili et al. 2011). Some studies have also focused on the effect of the pulse exposure of insecticides for populations of invertebrates (Naddy et al. 2000; Naddy and Klaine 2001; Pieters 2007) or for native fish (Holdway et al. 1994; Jarvinen et al. 1988; Raymond et al. 2006). All these studies highlight the importance of the pulse and recovery periods concerning the effects on aquatic organisms. Indeed, these studies illustrate that pulse exposures can affect organisms differently than continuous exposures. Furthermore, they show that recovery following a pulse exposure can be rapid or induce a delay, influencing the final effect on aquatic organisms.

Thereafter, models were developed to assess the effects of this type of exposure on aquatic organisms (Ashauer et al. 2006; Ashauer et al. 2007; Ashauer et al. 2010; Ashauer et al. 2011; Nagai 2014; Weber et al. 2012). Consequently, a model based only on toxicodynamic factors was recently developed to assess the effect, i.e., the cell-density inhibition, of photosystem II inhibitor pulse-exposure scenarios on the algae species *S. vacuolatus* and *P. subcapitata* (Copin and Chèvre 2015; Copin et al. 2015).

Another weakness of standard testing procedures is that they do not consider interactions between organisms, and they focus on single species (OECD 2011; Reinert et al. 2002). The possible interspecific biological interactions between the species, such as algae species, i.e., competition and mortality, are thus not taken into account in these toxicity tests (De Morais et al. 2014a). The few studies that investigate these types of interactions show that one species

can affect another species when the two are in co-cultures without the addition of any substance. Indeed, blue-green algae isolated from a freshwater lake inhibits the growth of *Diatoms,* inducing an acceleration of the eutrophication (Keating 1978). Allelopathy is an explanation of this eutrophication acceleration. Allelopathy corresponds to any direct or indirect harmful or beneficial effects of one plant on other plants in its vicinity and through its secreted chemicals that escape in the environment (Inderjit and Dakshini 1994). Similarly, some macrophytes, such as *Myriophyllum spicatum* and *Cabomba caroliniana,* have an inhibitory effect on several types of blue-green algae. This effect is also induced by the release of allelopathic compounds (Nakai et al. 1999).

Some studies have analysed the effect of the continuous or pulse exposure of herbicides on aquatic organisms living in co-cultures, such as periphyton communities (Gustavson et al. 2003; Laviale et al. 2011; Tlili et al. 2008; Tlili et al. 2011), which are complex assemblages dominated by algae, cyanobacteria and diatoms (Graham et al. 2009). However, very few studies have focused on the interspecific biological interactions between aquatic organisms living in co-cultures and exposed to the continuous or pulse concentration of pesticides. De Morais et al. (2014a) assessed the effects of pentachlorophenol, a broad-spectrum pesticide, on a mixture of the cyanobacterium species *Mycrocystis aeruginosa* and the microalgae species *Chlorella vulgaris*. They show that the effects of pentachlorophenol are dependent on the interaction between the 2 species. Indeed, *M. aeruginosa* inhibits the growth of algae species *C. vulgaris* without pentachlorophenol. Therefore, when high concentrations of pentachlorophenol are applied, an effect on *M. aeruginosa* is induced, and consequently, an increase in the cell number of *C. vulgaris* is measured. Another study addressed the competition between invertebrate species exposed to pulses of the insecticide pirimicarb. Dolciotti et al. (2014) show that a multigenerational culmination effect is induced when pirimicarb pulses are tested on the species *Daphnia magna* in interaction with the pirimicarb-insensitive species *Culex pipiens*. Thereafter, the recovery of the pirimicarb pulses on the species *D. magna* is impeded due to the competition with *C. pipiens*.

Currently, no procedure exists for the culture of two algae in the same growth medium. Therefore, the first goal of this study is to define the conditions to cultivate 2 algae species, *S. vacuolatus* and *P. subcapitata*, in exponential growth, in the same OECD medium, and in a reproducible manner (OECD 2011). To observe the interspecific biological interactions between these two algae species in a co-culture, their respective growth rates are compared

with the growth rates of these same algae cultured alone. The second goal is to assess the effect of the herbicide isoproturon on these two algae in a co-culture. The results of the standard toxicity test on algae in the co-culture are compared with the results of the standard toxicity tests for each alga cultured alone to characterise the evolution of the toxicity. Finally, the model developed by Copin et al. (Copin and Chèvre 2015; Copin et al. 2015) for assessing the effects of the pulse exposure of photosystem II inhibitors is adapted to address algae competition.

5.2 Materials and Methods

5.2.1 Chemical

The pollutant tested is the herbicide isoproturon (3-(4-isopropylphenyl)-1,1-dimethylurea; CAS N° 34123-59-6; purity 99.0 %; Ehrenstorfer GmbH). Isoproturon is used for pre- or post-emergent weed control in cotton, fruit and cereal crops worldwide (Sorensen et al. 2003). It belongs to the phenylureas group, and it acts as photosynthesis inhibitor by interrupting electron transport through photosystem II (PSII; Knauert 2008; Vallotton et al. 2008). For pulse-exposure scenarios of isoproturon on algae species cultured alone, the effect is direct, and no delay during the recovery periods is observed (Copin and Chèvre 2015; Copin et al. 2015; Vallotton et al. 2009). Two stock solutions of 3200 µg/L are prepared in an algae medium in axenic conditions and are maintained in the refrigerator at 6.4°C. The concentrations are checked analytically at the beginning of the several experiments in the laboratory, and the measured concentrations are in the same range as the nominal concentrations. The concentrations measured for isoproturon are 3100 and 3300 µg/L.

5.2.2 Algae culture experiments

5.2.2.1 Culture and test conditions

Permanent agar culture tubes of the green unicellular microalgae *S. vacuolatus* (chlorophyceae; strain 211-15, Shihira and Krauss, Melbourne, Australia) and *P. subcapitata* (chlorophyceae; strain 61.81, Nygaard, Komárek, J.Kristiansen and Skulberg, Akershus, Norway) are obtained, respectively, from the Department of Bioanalytical Ecotoxicology at the Helmholtz Centre for Environmental Research – UFZ, Leipzig, Germany and from the Institute for Plant Physiology of the University of Göttingen, Germany. Microalgae are cultured alone and in a co-culture in 250-mL Erlenmeyer flasks with 50 mL of OECD

medium (OECD 2011; Van der Vliet et al. 2007) and maintained in exponential growth on an HT Infors shaker table (90 rpm) at 25°C under continuous illumination at a light intensity of 30 µmol/m^2/s provided by cool-white fluorescent lamps. Standard toxicity and pulse tests are realised in these conditions.

5.2.2.2 Separated cultures of algae

Two conditions should be fulfilled for algae cultured alone: 1) The initial cell densities of each alga cultured alone must correspond to the initial cell densities chosen for the same algae in the co-culture. Only in these conditions is a comparison of algae growth rates cultured separately and in co-culture possible. 2) The choice of the initial cell densities for each alga cultured alone should allow for an exponential growth during at least 48 hours (Fig. 5.1A). Indeed, a test duration of 48 hours is accepted in the OECD procedure (OECD 2011). Furthermore, 48 hours is the minimum of observation duration to identify signs of abnormal growth during toxicity tests (Le Faucheur et al. 2005).

The optical densities are measured with a microplate reader (ELx800™, BioTek® Instruments, Winooski, Vermont) at a wavelength of 690 nm ($OD_{\lambda 690}$) for single algae cultures. Two control charters are established for each alga cultured separately to monitor their growth and, thus, to compare them with the growth of the algae in the co-culture. With this purpose, optical densities are measured after 48 hours of exposure for several cultures. These optical densities (OD; -) are then transformed into cell densities (CD; cells.mL^{-1}) with the calibration curves determined for each alga (Eq. 5.1 and 5.2). Thus, the growth rate of each alga can be calculated.

CD=28,481,666.73*OD – 940,376.79 for the alga *S. vacuolatus* (5.1)

and

CD=25,550,159.23*OD-891,662.57 for the alga *P. subcapitata*. (5.2)

5.2.2.3 Co-culture of algae

A new procedure for the growth of the algae *S. vacuolatus* and *P. subcapitata* in the same OECD media is developed in this study. Because it is not possible to differentiate the growth of each alga in co-culture by optical density measurement, a cell-counting method with an improved Neubauer Haemacytometer (Brand, Wertheim, Germany) is used to characterise

each alga's growth (Slade and Bauen 2013). Pictures of the Neubauer slides are obtained for counting with an Olympus BX53 microscope equipped with an Olympus DP26 camera. The counting is facilitated by the fact that the two algae have different shapes. The algae species *S. vacuolatus* has a circular form, whereas the algae species *P. subcapitata* has a curved and twisted appearance, similar to a sickle. Therefore, it is possible to differentiate them during the counting.

The 2 algae in the co-culture are inoculated in a same Erlenmeyer flask with OECD media using the 2 algae previously cultured separately (Fig. 5.1A). The counting is performed between 5 and 10 times over 48 h. The initial algae cell densities tested to search for an exponential growth of each alga in the co-culture over 48 hours are summarised in Table 5.1. After the determination of the optimal initial algae cell densities, control charters are established for each alga in the co-culture.

Table 5.1. Initial cell densities of algae *S. vacuolatus* and *P. subcapitata* chosen in successive assays to search for exponential growth over at least 48 hours when they are in co-cultures.

Assay number	SV [a]	P [b]
1	325,000	325,000
2	280,000	370,000
3	400,000	200,000
4	200,000	450,000
5	200,000	50,000
6	200,000	100,000
7	200,000	200,000
8	200,000	25,000
9	10,000	25,000
10	50,000	50,000
11	100,000	50,000

[a] *S. vacuolatus.* [b] *P. subcapitata*.

5.2.2.4 Acute toxicity tests

The dose-response curves of isoproturon for the two algae cultured alone and in the co-culture are established following a method adapted from the standard OECD procedure (OECD 2011; Fig 5.1B). The tests are performed in the same conditions as the algae cultures and with the initial cell densities found for the algae *S. vacuolatus* and *P. subcapitata* (see sections 5.2.2.2 and 5.2.2.3). The isoproturon is tested on the 2 algae separately. For the alga *S. vacuolatus*, 5 concentrations of isoproturon ranging from 4.9 and 242.9 µg/L and one control are tested in triplicate. For the alga *P. subcapitata*, 5 concentrations of isoproturon ranging from 9.7 and 233.2 µg/L and one control are tested in triplicate. For the algae in the co-culture, 6 concentrations ranging from 10 to 300 µg/L and a control are tested with 6 replicates.

The algae of each species in co-culture are counted after 48 hours of exposure with the Neubauer slides under the microscope. For the algae cultured alone, the optical density measurements obtained after 48 hours of exposure are transformed into cell density measurements with the calibration curves of each alga. Thus, the growth rates of the separated culture of algae and the algae co-culture are in the same unit. For all tests, i.e., the tests on algae cultured separately or on algae in co-culture, the specific growth rate is calculated for each replicate of the concentration tested and for the control. The growth inhibition can be deducted from these growth rates (Eq. 5.3, see section 5.2.3.1).

5.2.2.5 Pulse-exposure tests on algae in co-culture

Four isoproturon pulse-exposure scenarios are tested in the laboratory on the 2 algae in the co-culture. The scenario is composed of one pulse exposure followed by a recovery period. The pulse exposure differs in the duration and concentration tested for the 4 scenarios (Fig. 5.1C; Table 5.2). The duration of the recovery period is fixed at approximately 48 hours. The algae exposed to the scenarios and the controls are tested in triplicate.

The test is started the same way the algae are cultured and under the same conditions (see section 5.2.2.1). The initial cell densities of each alga in the co-culture correspond to those fixed to obtain an exponential growth over at least 48 hours (see section 5.2.2.3). Algae grow for a certain period at the beginning (V on the Fig. 5.1C) before being exposed to the pulse concentration. At the end of each pulse exposure, the algae are centrifuged twice for 7 min at 1046 x g and 25°C. The supernate is removed, and the algae are re-suspended in growth

media. These two centrifugations allow 99.985 % of the herbicide to be removed and do not impair algal growth, as shown by Copin et al. (2015). The recovery period begins directly after the alga *S. vacuolatus* is re-suspended in the fresh medium at an initial cell density corresponding to the same cell density fixed at the beginning of the experiment. A new exponential growth phase is thus possible for this alga. As the alga *P. subcapitata* is in co-culture with the alga *S. vacuolatus*, an unknown cell density is thus also re-suspended in the same fresh OECD media. Controls are treated in the same manner as the algae exposed to pulses. The cell densities of each alga are counted regularly during the exposure and recovery periods.

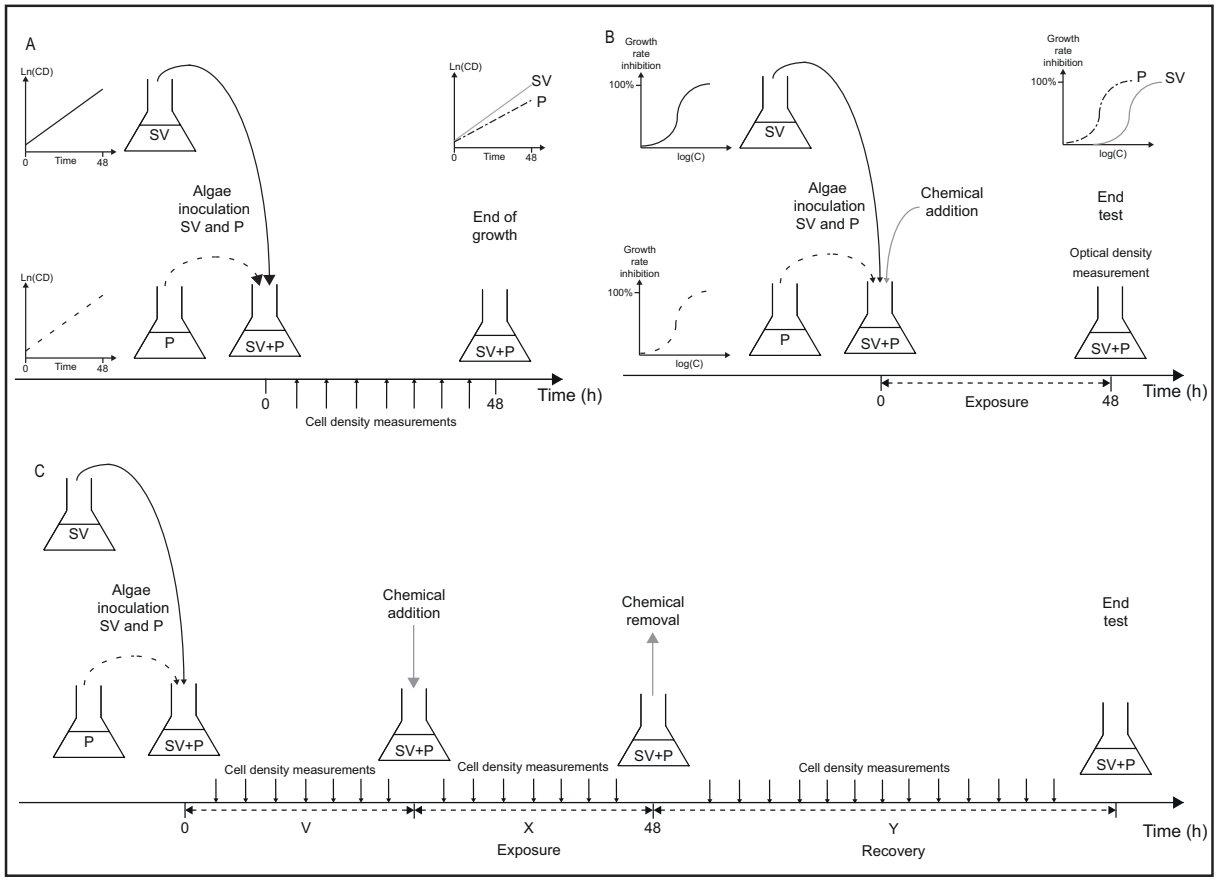

Fig. 5.1. Diagrams of the different experiments conducted in the laboratory. SV: *S. vacuolatus* cultured alone. P: *P. subcapitata* cultured alone. SV+P: *S. vacuolatus* and *P. subcapitata* in the co-culture. **A:** Procedure for the algae co-culture. The theoretical exponential growths of algae alone and in co-culture are represented. **B:** Procedure for the acute toxicity test on algae in the co-culture. The theoretical dose-response curves are illustrated for the algae alone and in the co-culture. **C:** Procedure for the pulse-exposure test. V corresponds to the growth

duration before the first pulse (h); X is the pulse duration (h); Y is the recovery duration (h); and V, X and Y are defined in Table 5.2.

Table 5.2. Parameter duration of the pulse and recovery periods in the several scenarios tested.

Scenario number	V (h)	Pulse duration (h)	Recovery duration (h)	Concentration of isoproturon tested (µg/L)
1	V=39.5	X=8.75	Y=45	64
2	V=39	X=9	Y=48	137
3	V=24	X=24	Y=49	141
4	V=40	X=8	Y=48	281

5.2.3 Data analysis

5.2.3.1 Growth inhibition in acute toxicity tests on algae cultured separately or in co-culture

The growth inhibition (I_t) at a given concentration is calculated using the response variables, i.e., the average specific growth rate of the control (μ_C) and the average specific growth rate at this concentration (μ_T) of the algae cultured separately and of each alga in the co-culture (OECD 2011; Eq. 5.3):

$$I_t = \frac{\mu_C - \mu_T}{\mu_C} \times 100 \quad (5.3)$$

5.2.3.2 Dose-response curves for standard acute toxicity test

The dose-response curves for the algae cultured separately and for each alga in the co-culture are obtained by plotting growth inhibition I_t as a function of tested concentrations C. The relationship is expressed using a four-parameter log-logistic dose response model (Vallotton et al. 2008; Eq. 5.4):

$$I_t = min + \frac{(max - min)}{1 + 10^{\left((\log EC_{50} - \log C) \times Hillslope\right)}} \quad (5.4)$$

where EC_{50} is the concentration inhibiting 50 % of the growth, *Hillslope* is the slope of the dose-response curve, and the *max* and *min* parameters are the maximum and minimum of the sigmoidal curve, respectively. The *min* parameter is fixed at 0. The *max* parameter is fixed at 100 because it is assumed that the growth inhibition is complete (100 %) at high concentrations. This curve is calculated using the statistics software Prism (GraphPad, San Diego, CA, USA).

5.2.3.3 Cell-density inhibition measured at the end of the pulse-exposure scenario

The overall cell-density inhibition of the pulse-exposure experiment is calculated for only one of the algae species in the co-culture. The algae species selected is the one with the cell density known after the centrifugation process, i.e., the alga *S. vacuolatus*. Indeed, after the centrifugation process, the cell density of the alga *S. vacuolatus* is fixed to the same cell density as that the beginning of the experiment. The overall cell-density inhibition ($Inh_{pulse\text{-}laboratory}$) is obtained by calculating the average of the algae cell-density inhibition of each replicate ($Inh_{pulse\text{-}laboratory\text{-}replicate\ i}$). The $Inh_{pulse\text{-}laboratory\text{-}replicate\ i}$ is calculated as follows (Eq. 5.5):

$$Inh_{pulse-laboratory-replicate\ i} = 100 \times \frac{CD_{control} - CD_{pulse,replicate\ i}}{CD_{control}} \quad (5.5)$$

where $CD_{control}$ is the average cell density for the replicates of the controls at the end of the experiment for the alga *S. vacuolatus* and $CD_{pulse,\ replicate\ i}$ is the final cell density for replicate *i* of the alga *S. vacuolatus* exposed to the pulse concentration. For the control and for each replicate exposed to pulses, to better visualise the effect of pulses on the algae species *S. vacuolatus*, the cell density data measured are summed up for the control and for each replicate exposed to the pulses, i.e., after each pulse-exposure period, the following recovery period and the following pulse-exposure period are summed with the last cell-density value of the previous pulse-exposure period. Thereafter, $CD_{control}$ and $CD_{pulse,\ replicate\ i}$ are determined using the growth rates of the linear regressions fitted on the summed cell-density values of the different parts (pulses, recovery) of the control and of replicate *i* of the alga *S. vacuolatus* exposed to pulses, as described in Copin et al. (2015).

5.2.3.4 Modelling

The cell-density inhibition is also calculated only for one of the algae species in the co-culture. The algae species selected is the one with the cell density known after the centrifugation process in the laboratory experiment, i.e., the alga *S. vacuolatus*. The cell-density inhibition at the end of a pulse-exposure scenario of an alga in the co-culture with another alga is adapted from the equation described by Copin and Chèvre (2015; Eq. 5.6a or 5.6b):

$$Inh_{pulse-modelling} = 100\times \frac{exp[ln(CD_{algaeCC-t0})+\mu_{CC}\times t_{2n+1}]-exp\{ln(CD_{algaeCC-t0})+\mu_{CC}\times[\sum_{i=0}^{n}(t_{2i+1}-t_{2i})]+\mu_{inh\,x-CC}\times[\sum_{i=1}^{n}(t_{2i}-t_{2i-1})]\}}{exp[ln(CD_{algaeCC-t0})+\mu_{CC}\times t_{2n+1}]}$$

(5.6a)

or

$$Inh_{pulse-modelling} = 100\times \frac{exp[ln(CD_{algaeCC-t0})+\mu_{CC}\times t_{2n}]-exp\{ln(CD_{algaeCC-t0})+\mu_{CC}\times[\sum_{i=0}^{n-1}(t_{2i+1}-t_{2i})]+\mu_{inh\,x-CC}\times[\sum_{i=1}^{n}(t_{2i}-t_{2i-1})]\}}{exp[ln(CD_{algaeCC-t0})+\mu_{CC}\times t_{2n}]}$$

(5.6b)

where t_{2n+1} or t_{2n} is the total test duration; t_{2i} can be either the beginning of a recovery period or the end of a pulse-exposure period; t_{2i+1} is the end of a recovery period; t_{2i-1} is the beginning of a pulse-exposure period; n is the number of pulses during the test; parameters $CD_{algaeCC-t0}$, μ_{CC} and $\mu_{inh\ x-CC}$ are determined for the alga *S. vacuolatus* in the co-culture with the alga *P. subcapitata*. $CD_{algaeCC-t0}$ is the initial cell density of the algae species in the co-culture; μ_{CC} is the growth rate of the control determined as the average growth rate of several successive batch cultures of the algae species *S. vacuolatus* in the co-culture with the algae species *P. subcapitata* in growth media (control charter); and $\mu_{inh\ x}$ is the growth rate at concentration x determined from the growth-response curve of the standard ecotoxicity test on the alga *S. vacuolatus* in the co-culture with the alga *P. subcapitata*. For real cases of pulse-exposure scenarios, equation 5.6a is used when the recovery phase corresponds to the end of the scenario, whereas equation 5.6b is used when the pulse-exposure period corresponds to the end of the scenario.

As explained by Copin et al. (2015), the distribution of the $Inh_{pulse\text{-}modelling}$ is estimated based on a Monte Carlo simulation. The model is applied using Matlab software (MATLAB

R2011b, The Mathworks Inc.). The predictive results of these Monte Carlo simulations are used in the comparison with the laboratory observations to validate the model.

5.3 Results and Discussion

5.3.1 Algae growth without herbicide

5.3.1.1 In co-culture

Several initial algae-cell densities are tested to achieve an exponential growth of each alga in the co-culture over 48 hours (Table 5.1). Two cases of different initial cell densities of algae in the co-culture are illustrated in Fig. 5.2. The first case (Fig. 5.2A) corresponds to an initial cell density of 280,000 cells/mL for the alga *S. vacuolatus* and 370,000 cells/mL for the alga *P. subcapitata*. The exponential growth for the alga *P. subcapitata* ends after 24 hours, which is the time when the growth of the alga *S. vacuolatus* begins. The duration of the exponential growth of *S. vacuolatus* is 48 hours. Therefore, these algae in the co-culture do not grow exponentially together over 48 hours with these initial cell densities.

The second case (Fig. 5.2B) corresponds to initial cell densities of, respectively, 100,000 cells/mL and 50,000 cells/mL for the alga *P. subcapitata*. In this assay, the two algae grow exponentially over at least 48 hours. Therefore, these initial cell densities are chosen to test the herbicide on the algae in the co-culture. Note that it is the only case listed in Table 5.1 that allows for a simultaneous exponential growth rate.

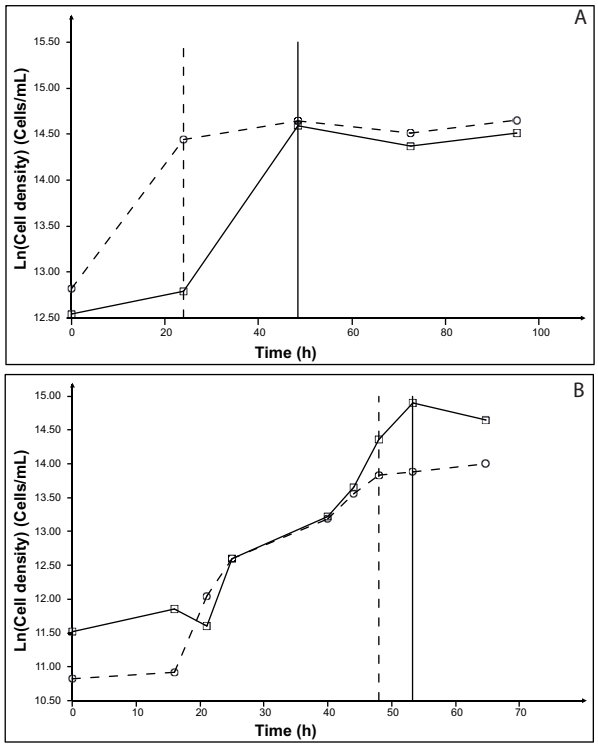

Fig. 5.2: Growth curves for the algae *S. vacuolatus* (SV) and *P. subcapitata* (P) in co-culture with different initial cell densities. Black curves and squares: growth curves and measurements for SV. Black dotted curves and circles: growth curves and measurements for *P. subcapitata*. The black vertical dashed line indicates the end of the exponential growth of *P. subcapitata*. The black vertical line indicates the end of the exponential growth of *S. vacuolatus*. **A:** initial cell densities of 280,000 cells/mL and 370,000 cells/mL for, respectively SV and P. **B:** initial cell densities of 100,000 cells/mL and 50,000 cells/mL for, respectively, SV and P.

5.3.1.2 Cultures separated

Based on the above results, the initial cell densities of the algae *S. vacuolatus* and *P. subcapitata* are fixed, respectively, to 100,000 and 50,000 cells/mL when they are cultured separately. Two control charters are established to monitor algae growth in the growth media. In the laboratory, the average growth rate of *S. vacuolatus* is 0.0579 cells.mL^{-1}.h^{-1}, with a standard deviation of 0.0054 cells.mL^{-1}.h^{-1} (an average of successive 21 cultures). The average growth rate is 0.0746 cells.mL^{-1}.h^{-1}, with a standard deviation of 0.0017 cells.mL^{-1}.h^{-1} (average of successive 12 cultures), for *P. subcapitata*. For initial cell densities fixed to 650,000 cells/mL for the alga *S. vacuolatus* and 200,000 cells/mL for the alga *P. subcapitata*, Copin and Chèvre (2015) showed that the growth rates are, respectively, 0.035 cells.mL^{-1}.h^{-1}

and 0.059 cells.mL^{-1}.h^{-1}. Therefore, the growth of an alga measured for a same duration of exponential growth is higher for the lowest initial cell density chosen. This finding is not surprising because with low initial cell densities fixed, the growth medium is less limiting for the development of the algae than with high initial cell densities used.

5.3.1.3 Interaction between S. vacuolatus and P. subcapitata

Eleven co-cultures of algae *S. vacuolatus* and *P. subcapitata* are performed with initial cell densities fixed to 100,000 and 50,000 cells/mL, respectively. The average growth rate of each alga in the co-culture is measured. For *S. vacuolatus* and *P. subcapitata*, these growth rates and their standard deviation are, respectively, 0.0600 (0.0059) and 0.0614 (0.0088) cells.mL^{-1}.h^{-1}. The growth of the algae *S. vacuolatus* is statistically (t-test) not different when it is cultured alone or with the algae *P. subcapitata*. However, the growth of the algae *P. subcapitata* is statistically lower when it is in the co-culture and when it is cultured alone. *S. vacuolatus* therefore seems to negatively influence the growth of *P. subcapitata,* whereas *P. subcapitata* seems to have no influence on the growth of *S. vacuolatus*.

Such interactions between algae species have been highlighted in microcosms. Burell et al. (1985) showed that the algae species *C. vulgaris* inhibited the growth of the microalga *Ankistrodemus braunii*. The allelopathy process is a possible explanation of this inhibition. According to Inderjit and Dakshini (1994), allelopathy can be operative in different ways. One possible way is the production of chemicals from one alga affecting the growth of the other alga, as observed in this study. Therefore, we can hypothesise that *S. vacuolatus* produces some allelopathic substances that react negatively with the alga *P. subcapitata*. Furthermore, De Morais et al. (2014a) showed that this allelopathy is a possible explanation of the negative effect on the growth of the alga *C. vulgaris* by *M. aeruginosa*. Another way the allelopathy can be active is for the production of algal toxins affecting the growth of other organisms, such as higher plants (Inderjit and Dakshini 1994). Thus, this type of allelopathy is observed in several studies highlighting the inhibitory effect between algae and aquatic plants (Keating 1978; Nakai et al. 1999).

5.3.2 Laboratory tests

5.3.2.1 Standard acute toxicity tests

For the 3 tests performed with the herbicide isoproturon during the 48 hours of exposure on each alga cultured alone and in the co-culture, the initial cell densities are fixed to 100,000 cells/mL and 50,000 cells/mL for, respectively, the algae *S. vacuolatus* and *P. subcapitata*. The dose-response curves are represented in Fig. 5.3. The EC_{50}s for isoproturon on the algae *S. vacuolatus* and *P. subcapitata* cultured alone are, respectively, 37.3 (27.6; 50.3) µg/L and 45.6 (32.9; 63.3) µg/L (95 % confidence interval). For initial cell densities fixed to 650,000 cells/mL for the alga *S. vacuolatus* and 200,000 cells/mL for the alga *P. subcapitata*, Copin and Chèvre (2015) showed that the EC_{50}s are, respectively, 66.9 (61.2; 73.2) µg/L and 80.4 (70.2; 92.2) µg/L. For each alga, the EC_{50}s corresponding to two initial cell densities are significantly different (t-test). Consequently, for the two algae, as the initial cell density decreases, the toxicity increases. Similar results were observed by Stauber (1995), who showed that as the initial cell-density increased, the test became less sensitive to toxic compounds due to the increased binding of toxicants with an increasing number of cells.

The EC_{50} for isoproturon on the alga *S. vacuolatus* in the co-culture is 92.0 (77.9; 108.7) µg/L (95 % confidence interval). The EC_{50} of the isoproturon on the alga *P. subcapitata* in the co-culture is 117.8 (105.1; 132.1) µg/L (95 % confidence interval). These EC_{50} are significantly different from the EC_{50} obtained when the algae *S. vacuolatus* or *P. subcapitata* are cultured alone (t-test). Consequently, the toxicity of the herbicide is lower for the two algae when they are in the co-culture than in a separate culture. This lower toxicity for the algae in the co-culture is surprising. Indeed, we expected the herbicide to induce supplementary stress for the algae already competing for nutrients, and therefore, we expected a higher effect in the co-culture. However, this difference in toxicity is not confirmed for any of the concentrations tested for the alga *S. vacuolatus*. Indeed, as observed in the Fig. 5.3A, for the lowest concentrations measured in the laboratory corresponding to environmental concentrations, the growth inhibition of the alga *S. vacuolatus* is higher when it is in the co-culture than when it is cultured alone.

Similar results are highlighted in two studies on the toxicity of pentachlorophenol in the cyanobacterium *M. aeruginosa* cultured separately and in a co-culture with the microalga *C. vulgaris* (De Morais et al. 2014a; De Morais et al. 2014b). The EC_{50} value for *M. aeruginosa*

cultured alone (0.117 mg/L) is at least one order of magnitude lower than the EC_{50} value found for the co-culture test of the same strain of *M. aeruginosa* (5.775 mg/L). However, it must be taken into consideration that these studies focus on a mixture of a prokaryotic alga (*M. aeruginosa*) and a eukaryotic alga (*C. Vulgaris*), whereas the current study analyses the effects of a substance on two eukaryotic algae (*S. vacuolatus* and *P. subcapitata*; Stevenson et al. 1996). Future research is thus needed to explain this difference in toxicity between a co-culture and a culture alone exposed to isoproturon concentration.

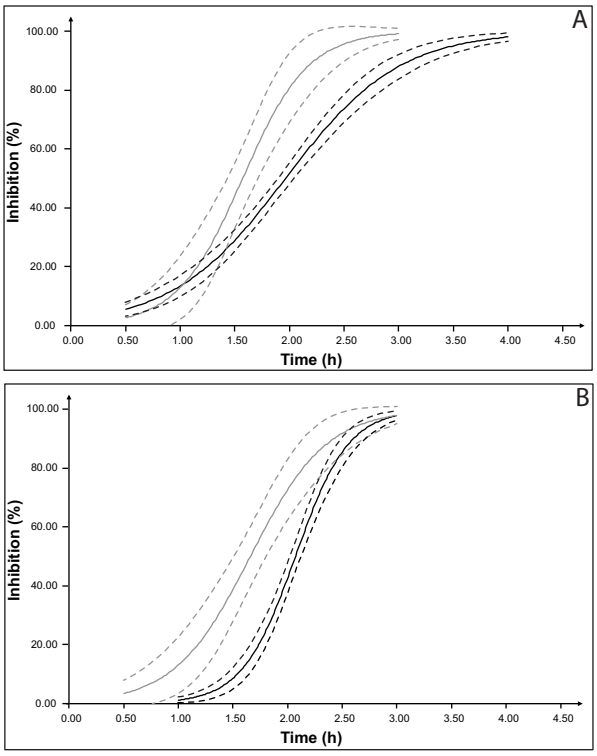

Fig. 5.3: Dose-response curves for **A:** *S. vacuolatus*. **B:** *P. subcapitata*. Black curves: algae in co-culture. Black dotted curves: 95 % confidence interval for algae in co-culture. Grey curves: algae cultured alone. Grey dotted curves: 95 % confidence interval for algae cultured alone.

5.3.2.2 Pulse-exposure tests

The concentrations tested for pulse-exposure tests on each alga in the co-culture are presented in Table 5.3. In the first scenario, the concentration tested induces a theoretically higher effect on the alga *S. vacuolatus* than on the alga *P. subcapitata*. The contrary is true for scenario 4. For scenarios 2 and 3, the effect levels are identical for each alga.

Table 5.3. Parameters of the corresponding effect concentration tested in the scenarios for each alga.

Scenario number	Concentration tested	
	SV [a]	P [b]
1	EC_{42} [c]	EC_{25} [d]
2	EC_{58} [e]	EC_{57} [f]
3	EC_{59} [g]	EC_{58} [h]
4	EC_{72} [i]	EC_{83} [j]

[a] SV: *S. vacuolatus*. [b] *P. subcapitata*. [c] Effect Concentration 42 %. [d] Effect Concentration 25 %. [e] Effect Concentration 58 %. [f] Effect Concentration 57 %. [g] Effect Concentration 59 %. [h] Effect Concentration 58 %. [i] Effect Concentration 72 %. [j] Effect Concentration 83 %.

For these 4 scenarios, the growth rate of each alga in the co-culture is inhibited during each pulse. The average (and 95 % confidence interval) cell-density inhibition of the alga *S. vacuolatus* in the co-culture is, respectively, 69 % (5; 133), 24 % (-37; 85), 60 % (40; 81) and -10 (-59; 40). The variability in the response for each scenario is high, as revealed by the confidence interval. This high variability is certainly a result of the methodology used to measure the cell density of the controls and of the culture exposed to pulse concentration, i.e., the count of algae cells with an improved Neubauer Haemacytometer. This method induces a high variability. Indeed, Willem et al (1976) showed that the error is generally equal to or less than ±26 % when phytoplankton species are counted in different counting chambers.

A hypothesis that underlines the use of the model with photosystem II inhibitors is that "the recovery is complete just after the pulse exposure in the case the parameter growth rate is considered". This hypothesis was validated by Copin et al. (Copin and Chèvre 2015; Copin et al. 2015) with an ANCOVA analysis of single-alga testing. For the four scenarios of pulse exposure, we check this hypothesis with algae in the co-culture. In other words, we control whether the growth rate μ of the control of alga *S. vacuolatus* is identical to its growth rate during recovery periods in the co-culture. The hypothesis is accepted for scenarios 2 and 4 (p-values 0.74 and 0.90, respectively; ANCOVA analysis, p-values>0.05). For scenarios 1 and 3, the hypothesis is rejected. However, the p-values are very close to the chosen level of statistical significance (alpha=0.05). Indeed, for scenarios 1 and 3, the p-values are, respectively, 0.04 and 0.03 (ANCOVA analysis). Furthermore, the high uncertainty of the

count method can again be an explanation of this significant difference. Other studies with more replicates must be led to reduce the uncertainty of measures in laboratory.

5.3.3 Comparison between the measured and predicted results

5.3.3.1 Analysis of all scenarios

The cell-density inhibitions predicted by the model for the 4 scenarios and for the alga *S. vacuolatus* in the co-culture are illustrated by boxplots in Fig. 5.4. For two scenarios (Figs. 5.4B and 5.4C), the experimental average cell-density inhibition is very close to the average cell-density inhibition given by the model. The average measured cell-density inhibition is included between the first and third quartiles of the model, which illustrates the good prediction of the model. Indeed, for the pulse-exposure scenarios of isoproturon tested on the alga *S. vacuolatus* cultured separately (Copin et al. 2015), the average cell-density inhibition obtained in the laboratory is often included only between the minimum and maximum of the model and not between the first and third quartiles of the model. Therefore, for these 2 cases, the model predicts more precisely the observed experimental inhibitions.

For scenario 3, this agreement between predicted and modelled cell density is surprising. Indeed, the growth rates of the control and the culture treated during the recovery period are significantly different in the laboratory, whereas they are supposed statistically equivalent in the model. However, we showed in section 5.3.2.2 that the p-value is very close to the chosen level of significance. Consequently, an agreement between the predicted and modelled cell density is possible.

However, for these two scenarios, the variability of the observed experimental inhibition is not located between the minimum and maximum values of the model, in contrast to pulse-exposure scenarios tested on separated cultures, such as the alga *S. vacuolatus* with atrazine and diuron and the alga *P. subcapitata* with isoproturon (Copin and Chèvre 2015). This high variability in the laboratory is certainly due to the cell count, which induces more uncertainty than the optical density values used to measure the cell-density inhibition in Copin et al. (Copin and Chèvre 2015).

For the two other scenarios (Figs. 5.4A and 5.4D), the experimental average cell-density inhibition is not correctly estimated by the average cell-density inhibition given by the model. In the first case, the average cell-density inhibition predicted is underestimated, whereas in the second case, it is overestimated compared to laboratory measurements. These 2 cases are discussed in the following sections.

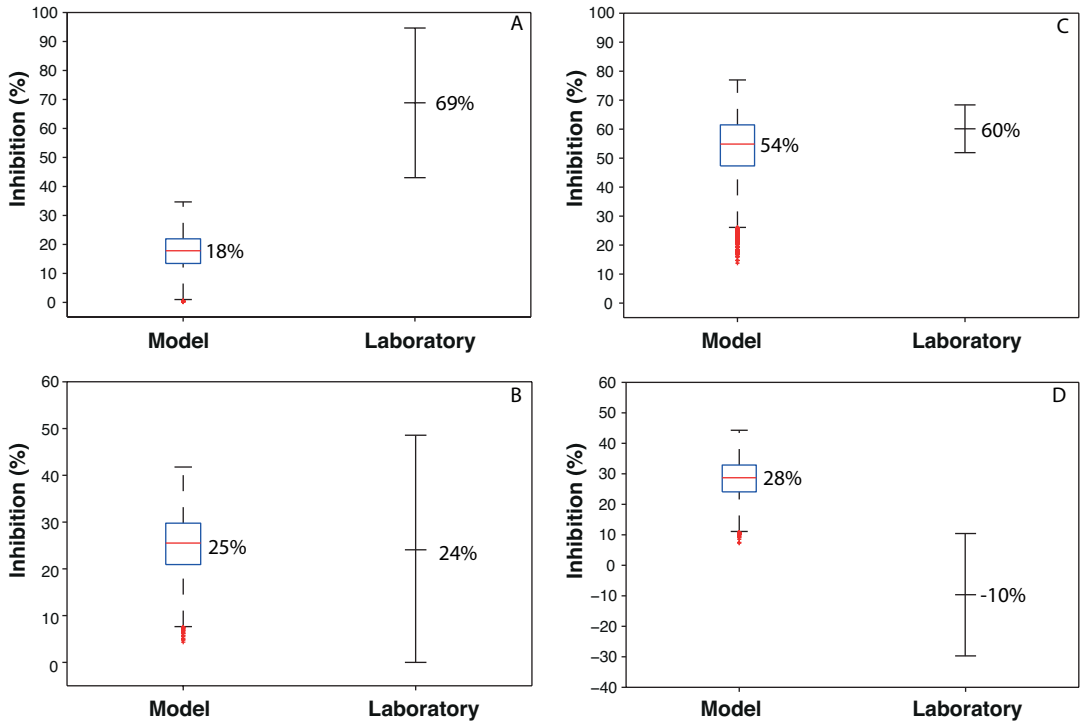

Fig. 5.4. Boxplots of the modelled scenarios for the alga *S. vacuolatus* in the co-culture with the alga *P. subcapitata* exposed to a pulse of isoproturon concentrations. Average and standard deviation for laboratory results (three replicates). The average cell-density inhibitions predicted by the model and obtained in the laboratory are noted for each scenario. **A:** Scenario 1. **B:** Scenario 2. **C:** Scenario 3. **D:** Scenario 4.

5.3.3.2 Overestimation of the predicted cell-density inhibition

For scenario 4, the cell-density inhibition is overestimated compared to the laboratory measurements. This overestimation is due to the negative cell-density inhibition obtained in the laboratory. However, the study of the growth rates of the control and the replicates of the culture treated during the recovery period in the laboratory show that there is no significant difference between these growth rates (see section 5.3.2.2). The negative cell-density inhibition in the laboratory can be thus explained by the cell densities measured for the

control and the culture treated at the end of the time fixed for the recovery part of this test (96 h, Table 5.2). In Fig. 5.5A, at 96 hours, the cell density measured for the control is located under the culture treated. Before and after this time, the control is located above the culture treated. Again, this result could be attributed to the counting method that induces a high uncertainty (Willén 1976). More replicates would be necessary to decrease this uncertainty. If the end of time of the pulse-exposure test is fixed to 90 hours instead of 96 hours, we obtain new results for the laboratory and for the model, illustrated in Fig. 5.5B. In this case, the experimental average cell-density inhibition (32 %) is very close to the average cell-density inhibition given by the model (28 %). Furthermore, the variability of the laboratory measurements is included between the minimum and maximum values predicted by the model, and the average measured cell-density inhibition is included between the first and third quartiles of the model. Moreover, the duration and concentration parameters of scenario 4 are very similar to those of scenario 2 (Table 5.2, Table 5.3). Consequently, the results predicted and measured for these 2 scenarios should be close, which is the case for the model but not for laboratory measurements when the end of scenario 4 is fixed to 96 hours. Indeed, the cell-density inhibition predicted for scenarios 2 and 4 is, respectively 25 % and 28 %, whereas the results measured in the laboratory are 24 % and -10 %, respectively. However, if the end of scenario 4 is fixed to 90 hours, the results measured in the laboratory are more similar, i.e., 24 % and 32 %. These results thus show the good agreement between the experimental and predicted measurements.

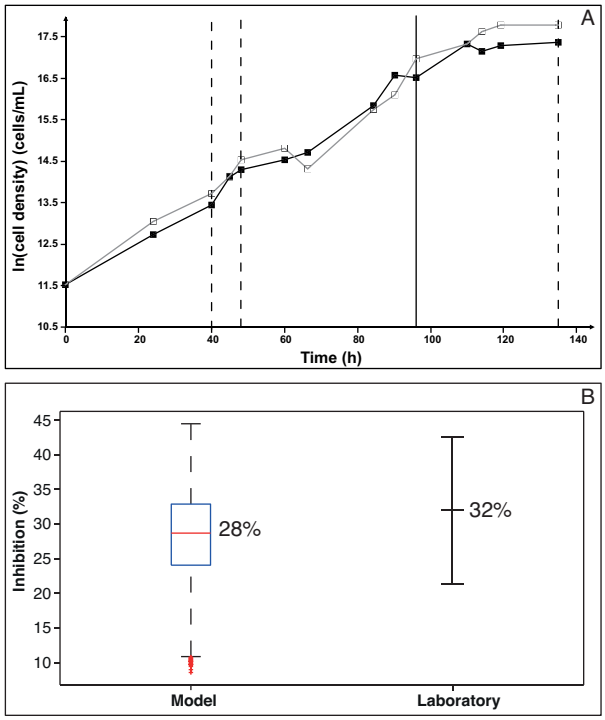

Fig. 5.5. A: Growth curves for scenario 4 in the laboratory. Black curve and squares: growth and measurements of the control. Grey curve and white squares: growth and measurements of the alga *S. vacuolatus* in the co-culture with the alga *P. subcapitata* exposed to one pulse concentration. The length of the pulse and the end of the measurements during the test are delimited by black, vertical dashed lines. The end of the test taken into account for the calculation and the measurement of the cell-density inhibition is indicated by a black line. **B:** Boxplots of modelled scenario 4 for isoproturon on the alga *S. vacuolatus* in the co-culture with the alga *P. subcapitata*. Average and standard deviation for the laboratory results (three replicates). The end of the test is fixed to 90 hours. The average cell-density inhibitions predicted by the model and obtained in the laboratory are noted for each scenario.

5.3.3.3 Underestimation of the predicted cell-density inhibition

For scenario 1, the cell-density inhibition is underestimated compared to the laboratory measurements. The statistical analysis of the growth rates of the control and the replicates of the culture treated during the recovery period in the laboratory shows that there is a significant difference between these growth rates (see section 5.3.2.2). This significant difference can be observed in Figs. 5.6B and 5.6C during the recovery period but not in Fig. 5.6A. This finding may have resulted from the number of cells of the alga *P. subcapitata* re-suspended with the alga *S. vacuolatus* just after the centrifugation process. Although the cell

density of the alga *S. vacuolatus* re-suspended in the fresh medium is known and corresponds to 100,000 cells/mL, the cell density of the alga *P. subcapitata* is not fixed when it is also re-suspended in the same fresh OECD media. For the 4 scenarios, the cell density of the alga *P. subcapitata* after the centrifugation process for each replicate of the control and the culture treated is summarised in Table 5.4. For scenarios 2, 3 and 4, the cell densities of the alga *P. subcapitata* in the co-culture with *S. vacuolatus* for each replicate of the culture treated are very close. For replicate 1 of the culture treated in scenario 1, the cell density of the alga *P. subcapitata* is similar to other scenarios, which is the reason why during the recovery, replicate 1 of the culture treated grows as the control. However, for replicates 2 and 3 of scenario 1, the cell density of the alga *P. subcapitata* is not similar to the cell densities measured in the other scenarios. Consequently, because these cell densities of *P. subcapitata* are higher for these 2 replicates, it is possible that the alga *S. vacuolatus* in the co-culture with the alga *P. subcapitata* grows with more difficulty for these 2 replicates.

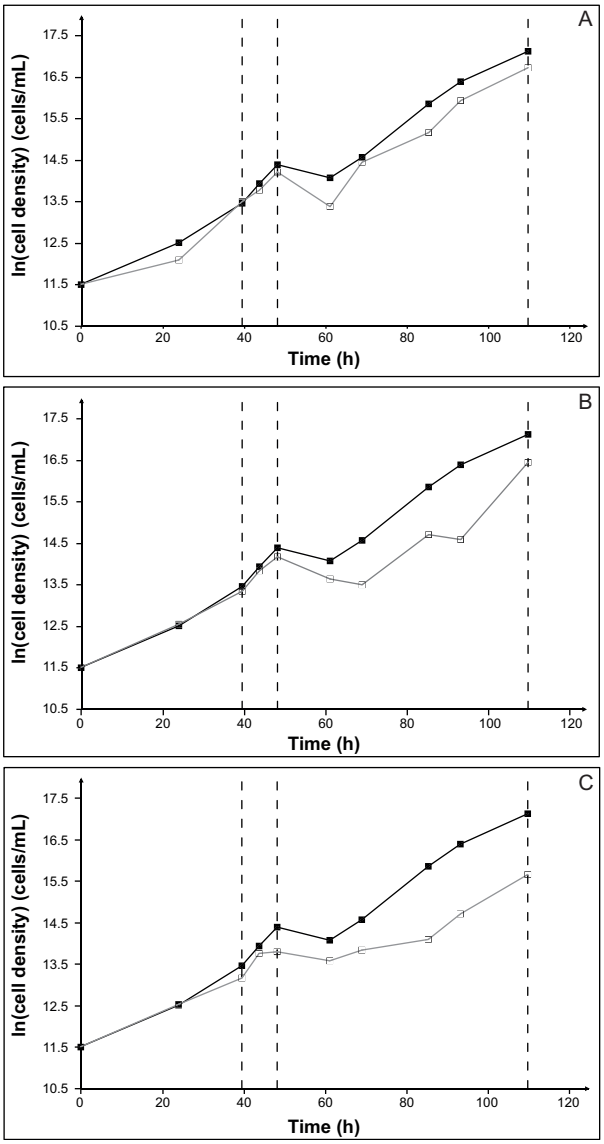

Fig. 5.6. Growth curves for scenario 1 in the laboratory. Black curve and squares: growth and measurements of the control. The length of the pulse and the end of the measurements during the test are delimited by black, vertical dashed lines. Grey curve and white squares: growth and measurements of **A:** replicate 1, **B:** replicate 2 and **C:** replicate 3 of the algae *S. vacuolatus* in the co-culture with algae *P. subcapitata* exposed to one pulse concentration.

Table 5.4. Cell densities of the alga *P. subcapitata* measured after the centrifugation process and at the beginning of the recovery period for each replicate of the control and the culture treated. The alga *P. subcapitata* is re-suspended in a fresh OECD medium with the alga *S. vacuolatus*. The cell density of the alga *S. vacuolatus* re-suspended in the OECD medium is fixed to 100,000 cells/mL.

	Cell density of the alga *P. subcapitata*			
	Scenario 1	Scenario 2	Scenario 3	Scenario 4
Control replicate 1	55,000	45,000	20,000	45,000
Control replicate 2	30,000	15,000	60,000	15,000
Control replicate 3	30,000	15,000	95,000	15,000
Culture treated replicate 1	15,000	20,000	15,000	20,000
Culture treated replicate 2	70,000	30,000	25,000	30,000
Culture treated replicate 3	45,000	20,000	15,000	20,000

5.4 Conclusions

The growth of the algae *S. vacuolatus* and *P. subcapitata* in a co-culture is studied for the first time. For initial cell densities fixed to 100,000 and 50,000 cells/mL for, respectively, the algae *S. vacuolatus* and *P. subcapitata*, the growth of each alga is exponential over at least 48 hours. *S. vacuolatus* seems to negatively influence the growth of *P. subcapitata*. Allelopathy is a possible explanation of this growth inhibition. The toxicity of the photosystem II inhibitor isoproturon is then tested on the algae *S. vacuolatus* and *P. subcapitata* cultured alone and in a co-culture. Despite the supplementary stress for the algae in the co-culture competing for nutrients, the toxicity of the herbicide is lower for the two algae when they are in a co-culture than when they are in a separated culture. Finally, a model previously developed for calculating the cell-density inhibition of the algae *S. vacuolatus* or *P. subcapitata* exposed to pulse concentrations of isoproturon is adapted to be applied on the alga *S. vacuolatus* in a co-culture with the alga *P. subcapitata*. The model predicts the laboratory measurements. The differences can be considered minor and are due principally to the high uncertainty of the cell count used to determine the parameters of the model and the cell number of the alga *P. subcapitata* re-suspended in a new OECD medium after the centrifugation process. Further research is needed to determine whether the model is suitable for predicting the effect of pulses on more complex systems, such as periphyton communities, to improve the environmental realism of the standard testing procedure in the laboratory.

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Chapter 6

Synthesis and outlooks

The goals of this last chapter were i) to summarize all the studies performed in laboratory concerning the pulse exposure tests; ii) to present the predicted results obtained with the model; iii) to conclude on the suitability of the model using herbicides inducing a delay or not of the effect and of the recovery on several freshwater microalgae cultured alone or in co-culture. An additional analysis is conducted applying the model to a typical environmental pulse exposure scenario with the four herbicides used in this thesis on the algae *Scenedesmus vacuolatus* and *Pseudokirchneriella subcapitata* cultured alone and in co-culture. Further experiments are performed to introduce the problematic of pulse exposure of mixture substances. The integration of other chemicals and other organisms used for pulse exposure tests are also addressed. Finally, a risk analysis is proposed to improve the protection of the aquatic species against the occurrence of pulse exposure.

6.1 Synthesis

The general aim of this thesis was to investigate the effects of sequential pulse exposure of herbicides on unicellular freshwater microalgae. After agricultural application and during and after rain events, herbicides are transported by surface runoff or drainage from the site of application to the surface water (Brown and van Beinum 2009; Daouk et al. 2013; Freitas et al. 2008; Rabiet et al. 2010). In streams located in small agriculture catchments, the concentrations of pesticides fluctuate greatly in connection with the flux of runoff water and correspond therefore to "pulses" (Vallotton 2007). These pulses can exceed the Annual Average Environmental Quality Standards (AA-EQS), which aims in protecting the aquatic species against the occurrence of chronic effects (EU 2008; Lepper 2005). Consequently, the study of the effects of pulse exposure in creeks and streams is of particular importance. A model was thus developed to assess the effects of this kind of exposure. It is presented in the four chapters of this thesis. Indeed, these four chapters aimed in defining the parameters that should be integrated into the model, i.e., the concentration and the duration of pulse exposure, the mode of action of the herbicides and the competition between several algae growing together. In this synthesis, we will highlight and discuss the major findings from chapter 2 to chapter 5 of the thesis. Thereafter, some preliminary experiments are presented and some new research approaches are discussed to allow future investigations. The three last parts of the synthesis therefore focus on the prediction of the effects of realistic environmental scenarios, on mixture effects of pulse exposure, and on the integration of the pulses in risk assessment

6.1.1 Model for photosystem II inhibitors (Chapter 2 and 3)

A pulse exposure model was first developed and validated with laboratory measurements for herbicides acting as photosystem II inhibitors, and this on several types of freshwater algae species. The photosystem II inhibitors used are atrazine, diuron and isoproturon. The freshwater algae species on which the pulse exposure was tested are *Scenedesmus vacuolatus* and *Pseudokirchneriella subcapitata*. The effect of pulse exposure measured during the tests was the cell density inhibition of the algae species. The model has two parameters, which can be determined based on laboratory measurements. The first parameter is the growth rate of the algae characterizing the growth of the control. As the recovery is direct after pulse exposure of photosystem II inhibitors (Vallotton et al. 2009), this growth rate is also used to describe

the growth of the culture treated during the recovery periods. During our studies, it was verified that the growth rates of the control and the culture treated during the recovery periods are statistically equivalent. This parameter, called μ, is determined using control charters for each alga studied. The second parameter of the model is the growth rate of the algae exposed to several pulses and is called μ_{inh}. This parameter is obtained from a growth-response curve obtained with a standard toxicity test, i.e. following the OECD procedure (OECD 2011). For pulses of similar concentrations, it was showed that the growth rate during each pulse was equivalent. Furthermore, the sensitivity of the algae *S. vacuolatus* was studied. This sensitivity was not modified after a repeated pulse exposure of isoproturon. It was therefore not integrated in the model for photosystem II inhibitors. The uncertainty of the model was taken into account by the variability of the parameter μ, which was obtained from the control charter and by the variability of the parameter μ_{inh}, which was determined with the replicates used to define the different growth response curves in laboratory. The measured and predicted effects were consistent when pulse exposure of atrazine, diuron and isoproturon were tested on the alga *S. vacuolatus*. For isoproturon, some differences were highlighted between measured and predicted cell density inhibition but they can be considered minor. The results were equivalent for isoproturon on the alga *P. subcapitata*. The model was thus suitable to predict the effect of photosystem II inhibitors on freshwater microalgae. The toxicity classification obtained from the dose-response curves (diuron>atrazine>isoproturon) was conserved for the pulse exposure scenarios modeled for the alga *S. vacuolatus*. The study of a scenario adapted from a real case study to the herbicide isoproturon showed that the highest peaks and the low peaks with a long duration affect principally the cell density inhibition. Consequently, when a measurement campaign of photosystem II inhibitors concentrations is planned in creeks or streams, it is important to catch these specific pulses.

6.1.2 Model for herbicides with a mode of action inducing a delay in the effect and in the recovery (Chapter 4)

The model was then extended to S-metolachlor, i.e., an herbicide with a different mode of action than photosystem II inhibitors. Metolachlor and S-metolachlor replaced the herbicide atrazine banned in 2003 in the European Union and were thus frequently detected in creeks and streams since this period (Konstantinou et al. 2006; Munz et al. 2013; IFEN 2007; Poiger et al. 2002; Wittmer et al. 2014). This substance acts differently on the alga *S. vacuolatus*

compared to photosystem II inhibitors. The effect is not direct and begins only after 20 hours of exposure based on optical density and algal cells size measurements. After this time period, fresh autospores are not released from the mother cell, and the size of the algae exposed to S-metolachlor continues to increase. Concerning the recovery after a pulse exposure, it is not instantaneous and occurred after a delay of 20 hours. This recovery does not depend on the pulse exposure duration or on the level of concentration of the pulse exposure. For scenarios composed of several pulses, the sensitivity of the alga is modified during the different pulses. Indeed, this sensitivity depends of the recovery time. If the recovery time is lower than the delay (20h), the sensitivity increases. These three characteristics of the herbicide S-metolachlor, i.e. the delays of the effect, the delay of the recovery and the modification of the sensitivity, were integrated in the model. The uncertainties were also defined for the two parameters μ and μ_{inh} defined previously. A scenario of S-metolachlor pulse exposure was tested in laboratory. The predicted values determined by the model were consistent with the measured cell density inhibitions. Therefore, the model was suitable to predict the cell density inhibition for the herbicide S-metolachlor on the alga *S. vacuolatus*. This model may be used for substances with a similar mode of action than S-metolachlor such as alachlor and metazachlor. But to extend this model to other substances with different modes of action, it will be crucial to analyse the effect and the recovery in relation with the time of each substance, but also the sensitivity of the substance after a pulse exposure.

6.1.3 Model for algae in competition (Chapter 5)

In the environment, an herbicide reaching surface water affects not only one alga but also several algae growing together. The model was thus extrapolated to the algae species *S. vacuolatus* in competition with the algae species *P. subcapitata*. The growth of these 2 algae in co-culture was first investigated. To obtain an exponential growth of the two algae in co-culture, their initial cell densities are fixed to 100,000 and 50,000 cells/mL respectively for the algae *S. vacuolatus* and *P. subcapitata*. The study of the respective growth of each alga showed that the alga *S. vacuolatus* seems to negatively influence the growth of *P. subcapitata* whereas *P. subcapitata* seems to have no influence on the growth of *S. vacuolatus*. It has been supposed that the allelopathy process could be at the origin of the negative effect of the alga *S. vacuolatus* on the alga *P. subcapitata* (Inderjit and Dakshini 1994). Second, a standard

toxicity test was applied to assess the effect of the herbicide isoproturon on these two algae in co-culture. Surprisingly, the effect on the two algae in co-culture was lower than the effect on each alga cultured alone. Further research should be conducted on algae in co-culture to better understand the possible interactions between the algae submitted to an herbicide stress. Finally, a pulse exposure test was simulated in the laboratory to determine the cell density inhibition of the alga *S. vacuolatus* in co-culture with the alga *P. subcapitata*. The results were compared with the cell density inhibition predicted by the adapted model. Indeed, the two parameters of the model μ and μ_{inh} described above (see section 6.1.1) were taken equivalent to the ones obtained for the algae *S. vacuolatus* in co-culture. Therefore, the parameter μ corresponds to the growth rate of the alga *S. vacuolatus* in co-culture with the alga *P. subcapitata* and obtained from several batch cultures used to determine a control charter. The parameter μ_{inh} is the growth rate of the algae *S. vacuolatus* in co-culture with the alga *P. subcapitata* obtained from the growth-response curve determined with the standard toxicity test. The uncertainty was also integrated in the model taking into account the variability of the control charter and of the growth response curve. The comparisons between the measured and predicted cell density inhibition were consistent for two of the four pulse exposure scenarios tested. The differences for two other pulse exposure scenarios can be explained by the high uncertainty of the measurement method used to determine these 2 parameters, i.e., the cells count (Willén 1976), The cell density of the alga *P. subcapitata* re-suspended after the centrifugation process is also a parameter that can explain these differences. Further research should be conducted on this concept of interaction between the species studying for example the effect of pulse exposure on periphyton communities.

6.2 Outlooks

6.2.1 Application of environmental realistic pulse exposure scenario

A typical pattern of herbicide pollution in creeks was used in the Chapter 2 and adapted to isoproturon (Leu 2003). The predicted cell density inhibition of this environmental scenario, with its minimum and maximum values, on the alga *S. vacuolatus* was 19 (0, 36) %. This scenario can be also applied on the alga *P. subcapitata* and on the alga *S. vacuolatus* in co-culture with the alga *P. subcapitata* because the model was suitable for these two different cases. Predicted cell density inhibitions, with their minimum and maximum values, were

respectively 22.5 (0, 42) % and 67 (1, 94) %. For the two algae cultured alone, the results are very similar. Consequently, the algae species *S. vacuolatus* and *P. subcapitata* react equivalently to pulse exposure scenarios. It is not surprising because the two algae have equivalent growth rates characterizing the control and the culture treated during the recovery periods (μ), i.e., respectively 0.023 h^{-1} and 0.028 h^{-1}. Furthermore, the toxicity of isoproturon on the two algae allowing the determination of the growth rates during pulse periods (μ_{inh}) are also similar, i.e., respectively EC_{50}s with 95% confidence intervals of 66.9 (61.2; 73.2) and 80.4 (70.2; 92.2) μg/L. Concerning the algae species *S. vacuolatus* in co-culture, it induced a higher predicted cell density inhibition than the algae *S. vacuolatus* cultured alone. This large difference can be due to the initial cell densities chosen for the experiment, i.e., respectively 650,000 cells/mL and 100,000 cells/mL. Indeed, the growth rates μ of the control and the culture treated during the recovery periods are higher for the algae *S. vacuolatus* in co-culture compared to the algae *S. vacuolatus* cultured alone, i.e. respectively 0.06 $cells.mL^{-1}.h^{-1}$ and 0.035 $cells.mL^{-1}.h^{-1}$. Furthermore, this difference of inhibition can be also due to the toxicity of isoproturon on the algae *S. vacuolatus* in co-culture and cultured alone that characterizes the growth of the culture treated during the pulse periods. The toxicity of isoproturon is lower for the algae *S. vacuolatus* in co-culture with initial cell density of 100,000 cells/mL compared when it is cultured alone with initial cell density of 650,000 cells/mL according to the EC_{50}s with its 95% confidence interval measured in laboratory, i.e., respectively 92.0 (77.9; 108.7) and 66.9 (61.2; 73.2) μg/L. However, for the lowest concentrations tested in laboratory corresponding to environmental concentrations (Van Der Hoeven et al. 1997; Warne and Van Dam 2008), the growth inhibition of the alga *S. vacuolatus* is higher when it is in co-culture than when it is cultured alone. Indeed, until 47 μg/l, isoproturon is more toxic on the algae *S. vacuolatus* in co-culture than when it is cultured alone. Consequently, as the maximum concentration in the environmental real scenario is 42 μg/L, the growth inhibition is higher for the algae *S. vacuolatus* in co-culture during all the pulses. Thus, the cell density inhibition predicted by the model is much higher when the algae *S. vacuolatus* is in co-culture.

The "real" scenario can also be used to predict the effects of the other substances studied in this thesis such as diuron, atrazine and S-metolachlor on the alga *S. vacuolatus*. Indeed, the maximum pulse concentration of the real scenario is 42 μg/L. Some equivalent concentrations or very close to this maximum concentration were already measured in streams in the world for atrazine (Gilliom 2006; Leu 2003; Pope et al. 1997; Richards and Baker 1993; Wu et al.

2010), diuron (IFEN 2007; Field et al. 2003; Munz et al. 2013) or S-metolachlor (Agence de l'eau Adour-Garonne 2012). For atrazine and diuron, with this scenario, the total predicted cell density inhibition of the alga *S. vacuolatus* with their minimum and maximum values is respectively 30 (0, 55) % and 26 (0, 45) %. These results are slightly higher than the results obtained with isoproturon. Therefore, the cell density inhibitions determined with this real scenario conserve the toxicity classification obtained from the dose-response curves (diuron > atrazine > isoproturon) on the alga *S. vacuolatus*. Concerning the S-metolachlor, the real scenario predicts an inhibition of 100%. This is not surprising because the pulse is long enough to generate a delay and the recovery period not long enough to allow a recovery (see section 6.1.2). Consequently, while the control continues growing, the culture treated does not grow during the first 20 hours of the recovery periods. Although S-metolachlor is the less toxic substance studied for the algae *S. vacuolatus* according to the EC_{50} measured in laboratory, it seems therefore to be the most problematic substance for the algae *S. vacuolatus* when it is exposed to pulse exposure due to this delay of recovery. This result highlights the importance to integrate the study of pulse exposure effects on aquatic organisms in the registration procedure of the pesticide. Furthermore, it would be essential to focus on the chronic effects of this substance on the freshwater algae when pulses exceed the AA-EQS.

6.2.2 Integration of the substances mixture in the model

Another weakness of the standard testing procedures is that they do not consider mixture of substances (OECD 2011). Indeed, in the aquatic environment, pesticides occur as mixtures, not as single contaminants (Faust et al. 2001). Therefore, mixture could be integrated in the model developed in this thesis. For mixture of photosystem II inhibitors, the model developed in the chapter 2 can be used. Only the determination of the growth rate μ_{inh} of the alga during the pulse periods of herbicide mixture changes. To calculate this growth rate, several steps should be followed: 1) the growth-response curves of the different substances used in the mixture should be established for the alga studied; 2) assuming different proportion of mixture, several growth response curves should be calculated with the following equation (Eq. 6.1) (Backhaus et al. 2003, Altenburger et al. 2000; Faust et al. 2001):

$$ECx_{mix} = \left(\sum_{i=1}^{n} \frac{p_i}{EC_{x_i}}\right)^{-1} \quad (6.1)$$

with $EC_{x\ mix}$, the total concentration of the mixture provoking x% effect; p_i, the fraction of component i in the mixture; EC_{xi}, the individual effect concentration of each substance i provoking x% effect. Thus, if the fraction of each component i in the mixture is known during the pulse period and as the growth response curves of each substance is known, the parameter μ_{inh} can be calculated. However, no uncertainty can be considered for the parameter μ_{inh} because the growth response curves are determined only by calculation. It can be noted that in real environmental pulse exposure scenarios, these fractions of the components are known as most of the time multiple substances are analyzed simultaneously in a sample The growth rates of the control and the culture treated during the recovery periods are supposed similar for the model as previously illustrated in Copin et al. (Copin and Chèvre 2015; Copin et al. 2015) and are equivalent to the parameter μ obtained with the control charter of the algae studied.

Preliminary experiments were conducted during this thesis to observe if laboratory measurements are in agreement with model prediction when mixtures are considered. Three pulse exposure tests with mixture of herbicides were performed. Each test was composed of two pulses and of two recovery periods that have different durations. Two substances were tested in mixture on the alga *S. vacuolatus*: isoproturon and terbuthylazine, which have a similar mode of action, i.e., the photosynthesis inhibition. Growth response curves of isoproturon and terbuthylazine were determined in laboratory for the alga *S. vacuolatus*. The growth response curves of four different mixtures with four proportions of isoproturon and terbuthylazine are represented in the Fig. 6.1. The proportion of 0.85 of isoproturon and 0.15 of terbuthylazine corresponds to two of the three pulse exposure tests performed in the laboratory. The concentrations of isoproturon and terbuthylazine correspond to the EC_{50}s of the two substances. The proportion of 0.91 and 0.09 corresponds to the third pulse exposure test assessed in the laboratory. The concentrations of isoproturon and terbuthylazine correspond to the EC_{80}s of the two substances. For the three scenarios, we checked the hypothesis that the growth rate of the control is equivalent to the growth rates of the culture treated during the recovery periods. The hypothesis was accepted for the three scenarios (ANCOVA analysis, p-values>0.05). The comparison between the measured and predicted cell density inhibition are represented in the Fig. 6.2. For the 3 scenarios, the experimental average cell density inhibition is very close to the average cell density inhibition given by the model. The uncertainty of the model is very low as illustrated by the low variability of the boxplots. This is due to the fact that we only consider one source of uncertainty, i.e., that of

the parameter μ. To obtain the uncertainty of the parameter μ_{inh}, some standard tests should be performed in laboratory with several proportions, i.e., for example for p_i=0.20, 0.40, 0.60, 0.80 and respectively for p_t=0.80, 0.60, 0.40 and 0.20. Furthermore, the pulse periods are short for the 3 tests. Consequently, some additional pulse exposure tests should be performed with longer pulse periods and shorter recovery periods to confirm the validation of the model for these 2 substances in mixture. The model should be also validated for other mixture of two and more than two substances with the same mode of action.

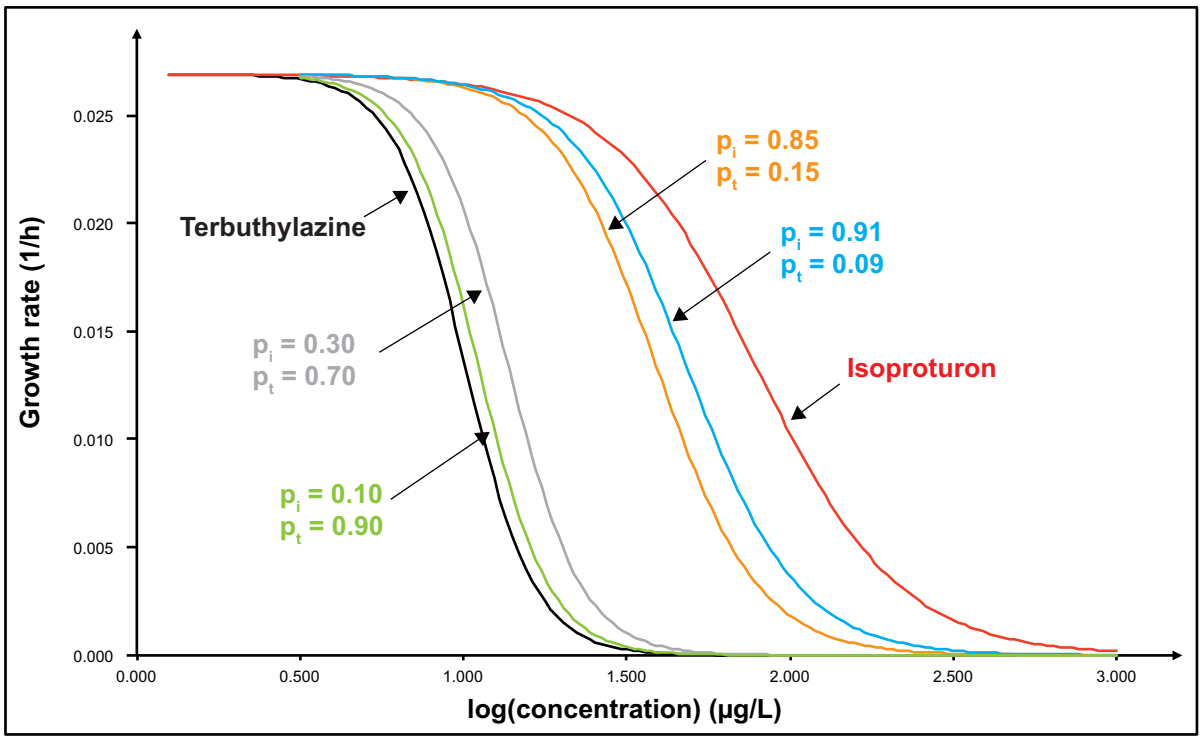

Fig. 6.1: Growth-response curves of terbuthylazine (black curve) and isoproturon (red curve). Growth response curves of different proportion in the mixture of isoproturon and terbuthylazine (green, grey, orange and blue curves).

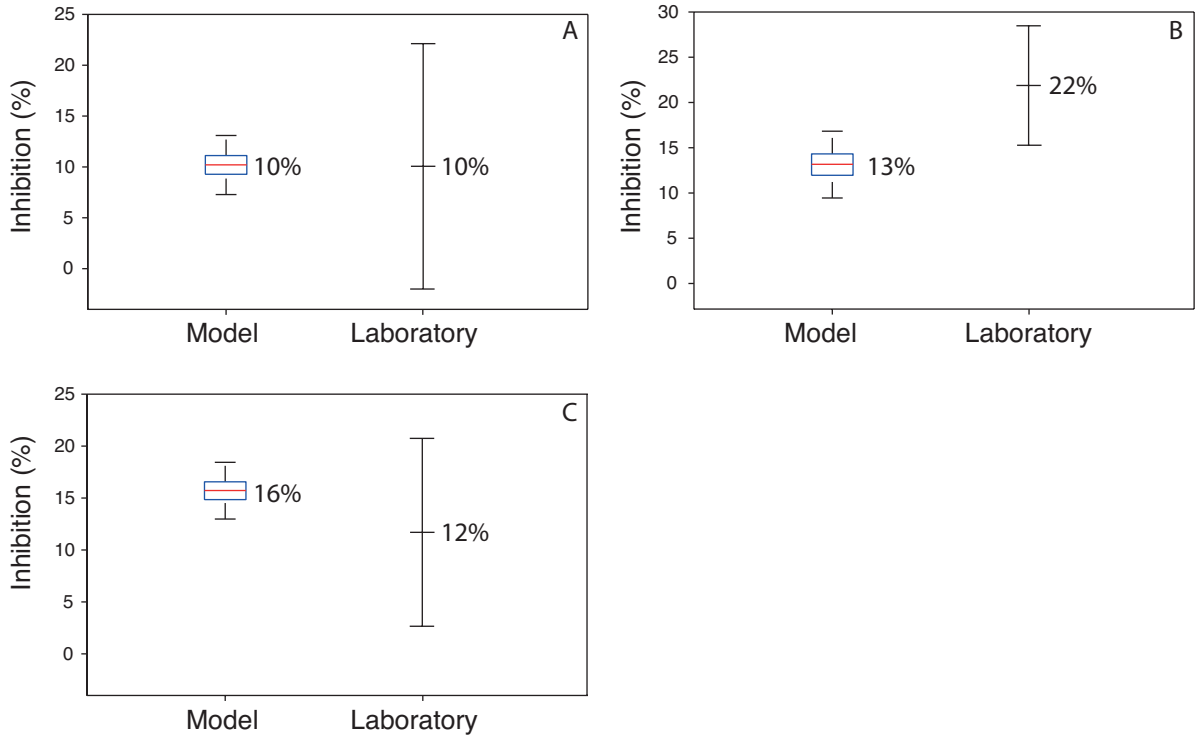

Fig. 6.2: Boxplots of modelled scenarios for the alga *S. vacuolatus* exposed to pulses of isoproturon and terbuthylazine mixture. Average and standard deviation for laboratory results (three replicates). The average cell density inhibitions predicted by the model and obtained in the laboratory are noted for each scenario. **A:** Scenario 1. EC_{50} of the mixture tested (p_i=0.85 and p_t=0.15). Time of pulse periods: 4 h. Time of recovery periods: 38 and 48 h **B:** Scenario 2. EC_{50} of the mixture tested (p_i=0.91 and p_t=0.09). Time of pulse periods: 5.25 h. Time of recovery periods: 38 h **C**: Scenario 3. EC_{80} of the mixture tested (p_i=0.85 and p_t=0.15). Time of pulse periods: 4 h. Time of recovery periods: 38 and 48 h.

6.2.3 Risk of pulse exposure

One idea to extrapolate our results to risk assessment would be to extrapolate the parameter μ_{inh} to the most sensitive species. For herbicides, the most sensitive species are often photosynthetic organisms such as cyanobacteria and algae (Naylor 1996). To do so, we propose the following approach. First we can define the most sensitive algae based on Species Sensitivity Distribution (SSD) curves. Indeed, the SSD is a statistical function describing the variation in toxicity of a certain compound among a set of species. The species set can be composed of a species from a specific taxon, a selected species assemblage, or a natural community (Posthuma et al. 2001). The 5th percentile of a chronic toxicity distribution (HC_5-

NOEC), i.e., obtained with NOEC data, was proposed as a concentration that is protective for most species in a community (Posthuma et al. 2001). In this study, we can therefore use the HC_5 as representative of the most sensitive species to protect. For predicting a μ_{inh} for the most sensitive species, we need to define the dose response curve for this species, i.e to know the EC_{50} and the slope of the curve. The EC_{50} is easily obtained from SSD-EC_{50}. We assume that the HC_5-EC_{50} is the EC_{50} value for the most sensitive species to be protected. For example, Chèvre et al. (2006) determines different HC_5-EC_{50} for triazines and phenylureas. For the substances used in this thesis, i.e., atrazine, diuron and isoproturon, the HC_5-EC_{50} are respectively 2.1, 0.12 and 0.35µg/L. To obtain the slope, we can assume that the dose response slopes of substances with a similar mode of action are identical. Indeed, this assumption was done by Smit et al (2001) even if no evidence has been found to confirm this assumption. In this study, the slope is therefore fixed to -1.1836. The parameter μ_{inh} of the model can be therefore determined for all pulse concentrations. However, the parameter μ, also needed for modeling is not known for the most sensitive species. Consequently, we compared the μ obtained with the most sensitive algae cultured in this thesis, i.e. *S. vacuolatus* and *P. subcapitata*, with the growth rates of several algae in the OECD guideline (*P. subcapitata*, *Desmodesmus subcapitata*, *Naviculla pelliculosa*, *Aphanizomenon flos-aquae*, *Synechococcus leopoliensis*) (OECD 2011). The algae *S. vacuolatus* has the lowest growth rate compared to the other algae. This minimum growth rate chosen is then integrated in the model and corresponds to the parameter μ. However, a literature review on algae growths should be conducted to estimate the value of this parameter.

The effects of the real pulse exposure scenario used previously (see section 6.2.1) were assessed using the two parameters described above, i.e for the most sensitive algae species, and this for isoproturon, atrazine and diuron. The real pulse exposure scenario of S-metolachlor is not simulated with the most sensitive algae species because no data were available for the HC_5. The cell density inhibition predicted by the model is 100 % in all cases. This result is not surprising based on the mathematic. Indeed, the EC_{50}s of atrazine, diuron and isoproturon on the algae *S. vacuolatus* and *P. subcapitata* used to determine the parameter μ_{inh} are 10, 100, 190 and 200 higher respectively to the EC_{50}s of the most sensitive algae obtained with the SSD. As a consequence, we might assume that the risk of such pulses scenarios is very high and that the algae species are undoubtedly affected by the pulse exposure scenario. Going further, we might say that the exceedance of the AA-EQS by very high peaks of herbicide concentration or very long pulses should be limited to protect the

aquatic species (EU 2008; Lepper 2005). However, the described approach is a proposal and based on the results above, the procedure to extrapolate a risk from the results of pulse exposure on one alga should be refined. Furthermore, research should be conducted to investigate the long-term effects of pulse exposure and therefore the risk they present.

6.2.4 Pulse effects of pesticides on microcrustacea and fish

The effects of metals and pesticides pulse exposure were also analyzed for other organisms such as microcrustacea and fish (Handy 1994; Reinert et al. 2002). For example, the mortality, the growth and the reproduction of the microcrustacea *Daphnia magna* exposed to pulse exposure of arsenic were assessed. The mortality of *D. magna* increased with exposure concentration and duration of arsenic. The mortality was also influenced by the recovery time. Indeed, for concentrations of arsenic lower than 5000 µg/L, the longer the recovery time between two pulses of arsenic, the more important the mortality was. However, for the surviving *D. magna* species, their growth was not affected by pulse exposure The growth of *D. magna* was not affected by arsenic pulse exposure. Finally, the reproduction was only disrupted by pulse exposure of high arsenic concentration and long durations (Hoang et al. 2007). The responses of *D. magna* were also studied for pulse exposure of widely used insecticide as chlorpyrifos. One study of Naddy et al. (2000) showed that the exposure-response (i.e., EC_{50}) curves from pulse exposure of chlorpyrifos described a curvilinear response. Furthermore, another study of Naddy and Klaine (2001) highlighted the importance of the recovery time between pulses. However, contrary to Hoang et al. (2007), the microcrustacea *D. magna* could withstand an acutely lethal insecticide chlorpyrifos exposure if there is adequate recovery time between exposures. Ashauer et al. (2010) analyzed the toxicity of repeated pulses of the insecticide diazinon on the freshwater amphipod *Gammarus pulex* and highlighted the importance of the species recovery on the total effect. Indeed, stronger effects were observed after pulse exposure on *G. pulex*. It was due to the slow recovery of this organism, which was observed after only 28 days. Some authors also described the effect of metals and pesticides pulse exposure on early life stages of fish. Two hours pulse-exposed metals cadmium and zinc at moderate concentrations affected significantly the early life stages of the rainbow fish Melanotaenia fluviatilis. Indeed, the percentage of hatching of embryos exposed to pulse decreased, spinal deformities were

induced and toxic effects on larvae at relatively low concentrations were observed. However, the 2h pulse exposure of these metals was less toxic than a continuous exposure of 96h (Williams and Holdway 2000). The effects of three insecticides (chlorpyrifos, endrin, fenvalerate) applied in pulses on the fathead minnow larvae (Pimephales promelas) were assessed. A chlorpyrifos pulse of 5 hours at a concentration of 96h LC50 involved deformities and a reduction of growth. However, for endrin and fenvalerate, at a concentration for each substance corresponding to their 96h LC50, the pulse time necessary to cause, respectively, a reduction of growth and a reduction of survival and growth was higher. Indeed, it was determined to 48h for endrin and 96h for fenvalerate (Jarvinen et al. 1988). Recently, some studies even tried to combine the concept of pulse exposure with the notion of predation. Indeed, Janssens and Stoks (2012) analyzed the responses of a 24h exposure of the insecticide endosulfan and the herbicide Roundup on the major antipredator traits and the resulting mortality by predation in larvae of the damselfly Enallagma cyathigerum. Further research could be conducted to model the effects of pesticides pulse exposure on other organisms such as microcrustaceas and fish to improve the risk assessment of fluctuating concentrations in streams.

6.2.5 Pulse exposure effect assessment with other substances

400 pesticides were registered in Switzerland in 2009 (Chèvre and Erkman 2011). This number of pesticide is very low considering the number of synthetic chemicals used in consumer products in European Union, i.e. more than 100,000 substances (Hartung and Rovida 2009). Concerned by the proliferation of these chemicals that can be toxic, the European Union decided, in 2000's years, to improve their regulations and particularly their homologation process. To succeed to this goal, the European Commission developed the REACH regulation (Registration, Evaluation, Authorisation and Restriction of Chemicals) that was accepted by the European Parliament in 2007. This REACH regulation required manufacturers and importers to assess the risk to human health and the environment of chemicals, already or soon marketed, produced in annual quantities exceeding one ton. Pesticides, biocides and pharmaceuticals are not concerned by the REACH regulation. Indeed, they are regulated by other European legislation (Chèvre and Erkman 2011; Penman et al. 2015 ; Petry et al. 2006; Tarazona et al. 2014). In most ecotoxicological studies, the pulse

exposure process of chemicals is not addressed. However, some synthetic substances can reach the surface water not continuously but rather in pulses. It is the case of the biocides used in the paints or to control weeds at the railroad tracks and at other traffic grounds in industrial and residential areas. They can be transported until streams or creeks during and after rain events and contribute thus to the formation of pulses (Muller et al. 2002; Skark et al. 2004). Pharmaceuticals can also be detected as pulse concentration in surface waters. Indeed, the wastewater network is often constituted of different combined sewer overflows. During storms, a part of the wastewater can reach directly the surface water without being treated to the water treatment plant. Consequently, pharmaceuticals can be detected in streams or creeks as pulse concentrations. Pulse exposure effects should therefore be studied for other substances that herbicides.

6.3 Conclusion

Our study showed the effects of pulse exposure of herbicides can be modeled on algae, but requires several experiments if the compound has a mode of action that induces a delay to the onset of effect and in the recovery. The predicted effects of real scenarios show that pulse exposure induce important effects even with compounds allowing rapid recovery. These effects can even be total when a delay in the recovery is observed. However, to be used as risk assessment, some more investigations should be conducted regarding the mixture toxicity and the influence of algae competition. But preliminary assessment seems to indicate that exceedance of the chronic quality criteria in surface waters are of concern and should be investigate more precisely.

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Appendix

Appendix 1

In this appendix, the absorption spectrum of the alga *Scenedesmus vacuolatus* is represented for wavelength included between 300 and 700 nm. A peak can be detected at the wavelength of 690 nm. It is the reason why we measured the optical densities of the alga *S. vacuolatus* at the wavelength of 690 nm.

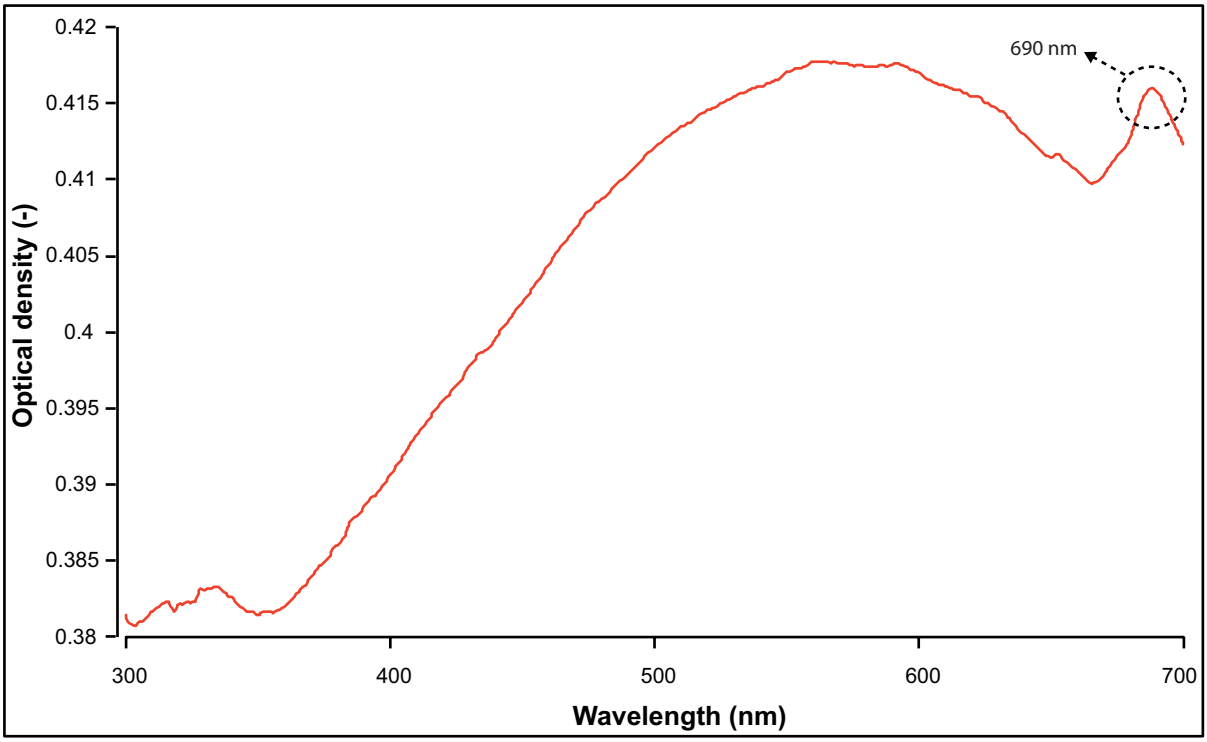

Fig. A1.1: Absorption spectrum of the alga *S. vacuolatus* (No dilution).

Appendix 2

In this appendix, the calibration curves of each alga studied, i.e., *S. vacuolatus* and *Pseudokirchneriella subcapitata*, are illustrated. The red squares in the figures correspond to the measurements of cell density with an improved Neubauer Haemacytometer (Brand, Wertheim, Germany) and of optical density with a microplate reader (ELx800™, BioTek® Instruments, Winooski, Vermont) at a wavelength of 690 nm ($OD_{\lambda 690}$). The black line is the linear regression of the measurements. The equation of the linear regression corresponds to the equation of the calibration curve.

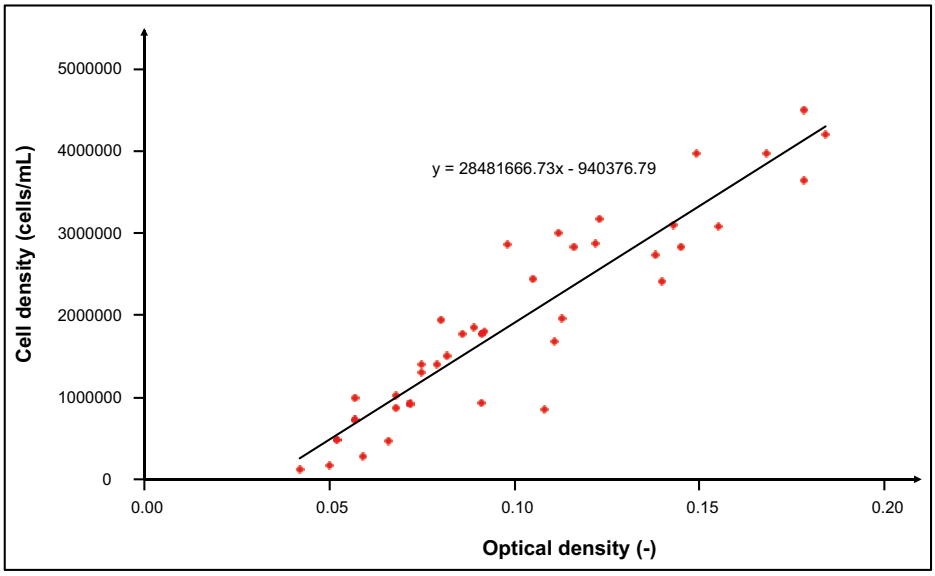

Fig. A2.1: Calibration curve of the alga *S. vacuolatus*.

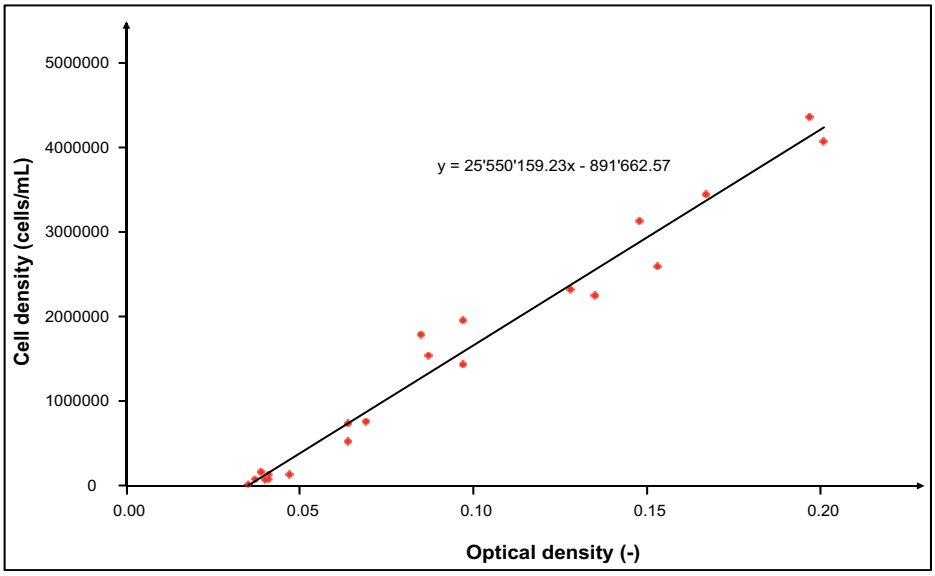

Fig. A2.2: Calibration curve of the alga *P. subcapitata*.

Appendix 3

The control charts of the algae *S. vacuolatus* and *P. subcapitata* for, respectively, 650,000 cells/mL and 200,000 cells/mL are represented in this appendix. For the alga *S. vacuolatus*, two control charts are established for this initial cell density due to a difference of growth between the algae cultures after a certain period of time. This growth difference is due to the decrease of the light intensity the HT Infors shaker table with the time. For the three figures, the black line indicates the average growth rate. Black dotted lines indicate the 95% confidence interval. Red lines indicate the minimum and maximum values. Red circles correspond to the growth rate of algae measured in laboratory.

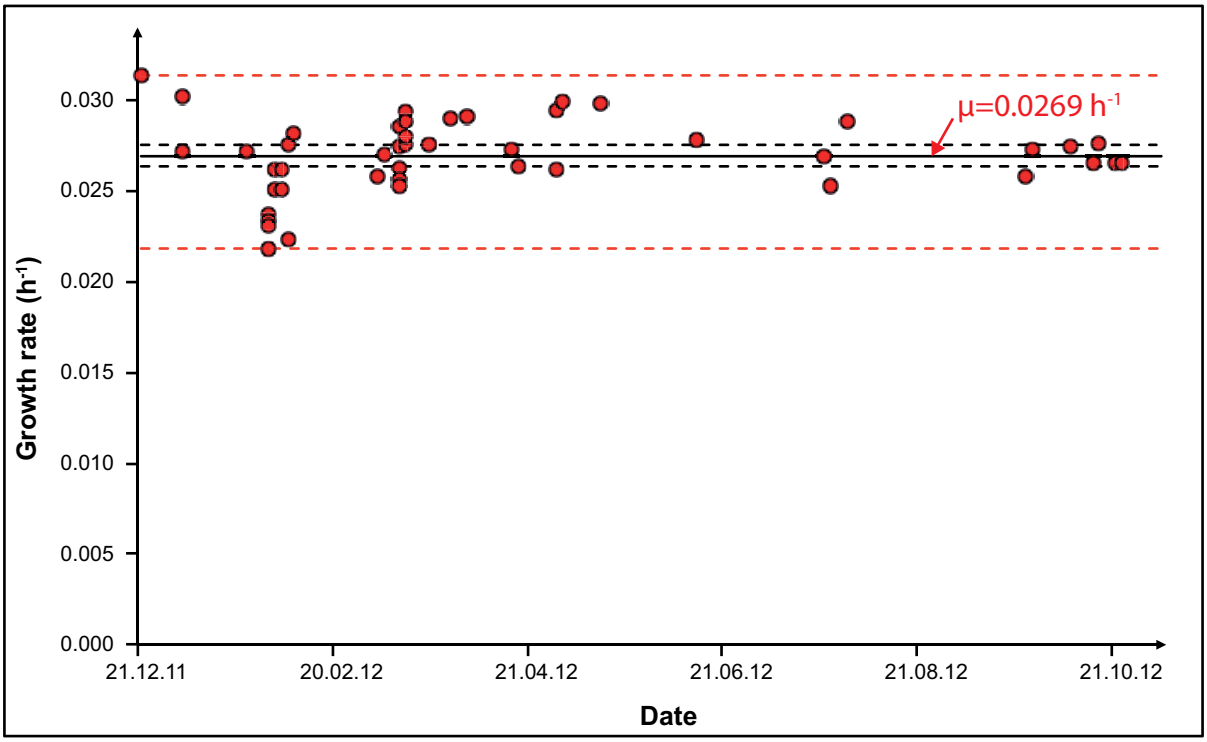

Fig. A3.1: First chart control of the alga *S. vacuolatus* with an initial cell density of 650,000 cells/mL. Used by the model for isoproturon.

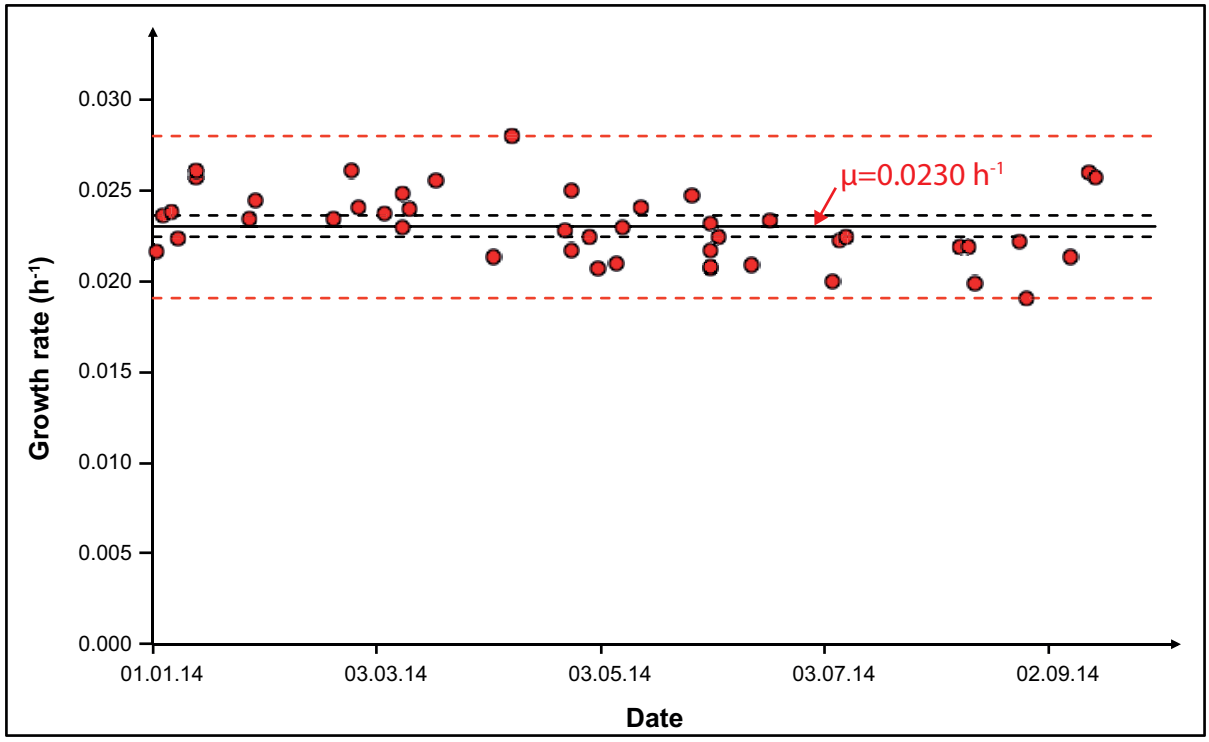

Fig. A3.2: Second chart control of the alga *S. vacuolatus* with an initial cell density of 650,000 cells/mL. Used by the model for atrazine, diuron and S-metolachlor.

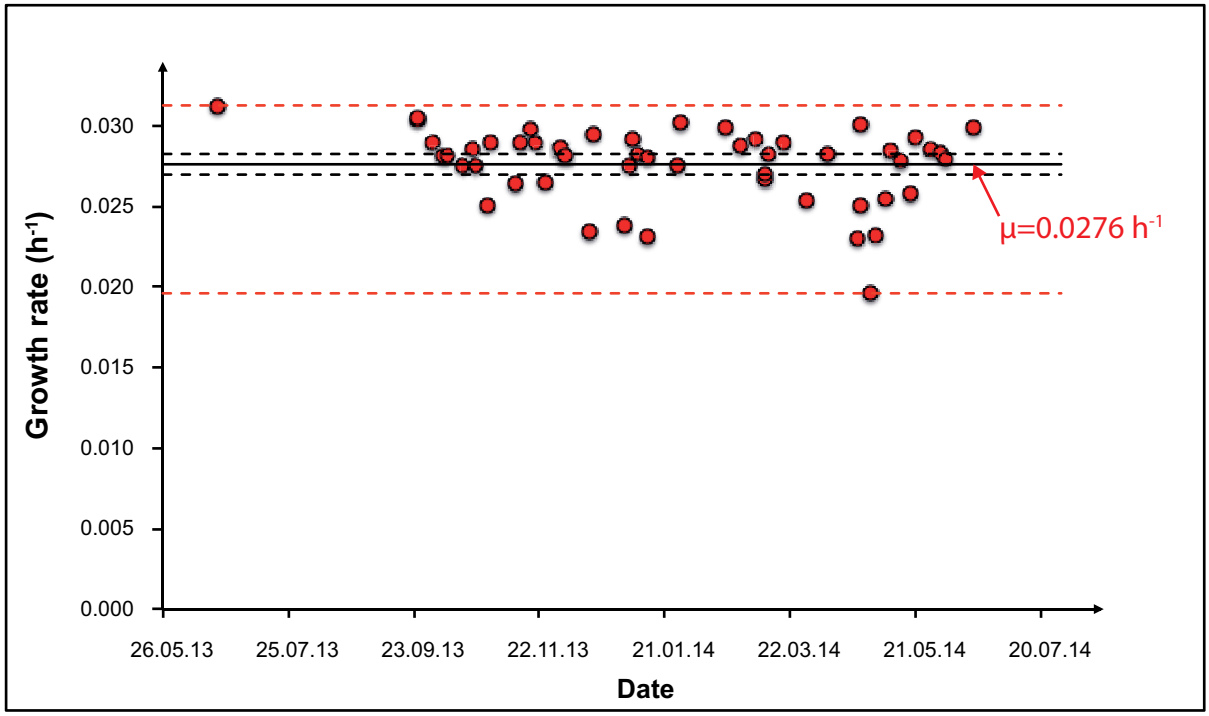

Fig. A3.3: Chart control of the alga *P. subcapitata* with an initial cell density of 200,000 cells/mL. Used by the model for isoproturon.

Appendix 4

In this appendix, the distribution of the growth rates is represented for the algae *S. vacuolatus* and *P. subcapitata*. The measured growth rates for each alga correspond to black squares. The parameter μ for the two algae follow therefore a normal distribution as illustrated in the two figures.

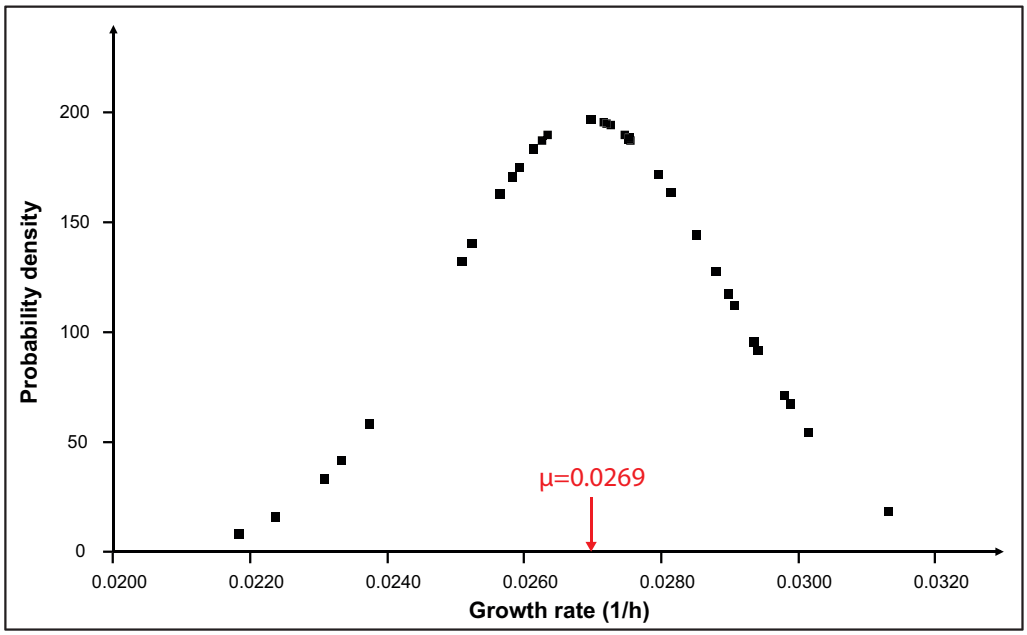

Fig. A3.1: Distribution of the growth rates of the alga *S. vacuolatus*.

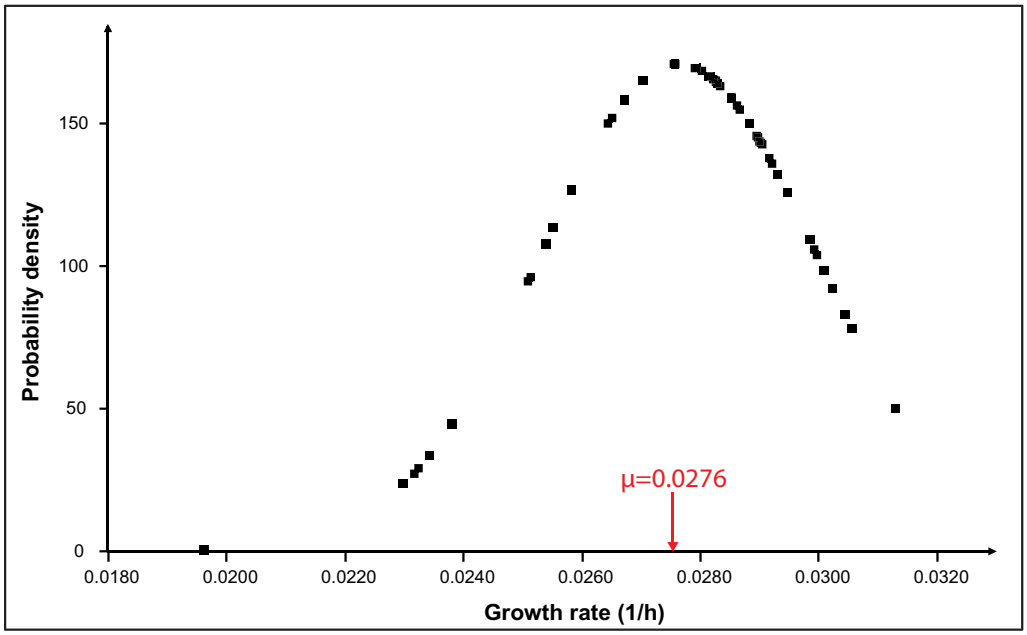

Fig. A3.2: Distribution of the growth rates of the alga *P. subcapitata*.

Appendix 5

In this appendix, dose-response curves and growth-response curves are represented for the four herbicides used in this thesis, i.e., atrazine, diuron, isoproturon and S-metolachlor on the alga *S. vacuolatus*. These curves are also represented for isoproturon on the alga *P. subcapitata*. Characteristic parameters of these curves such as EC_{50}, hillslope and top value are noted in the different figures. For all figures, the black line represents the log-logistic curve. Black dotted lines represent the 95% confidence interval of the log-logistic curve. Black squares correspond to the measure of inhibition in laboratory. They are represented with their error bars.

Atrazine

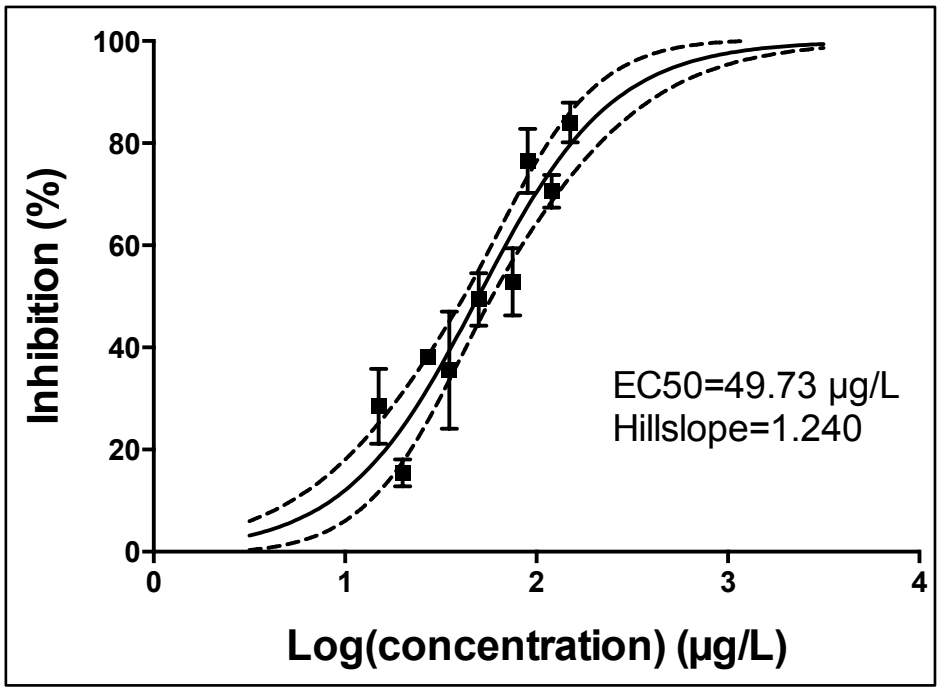

Fig. A5.1: Dose-response curve for atrazine on the alga *S. vacuolatus*.

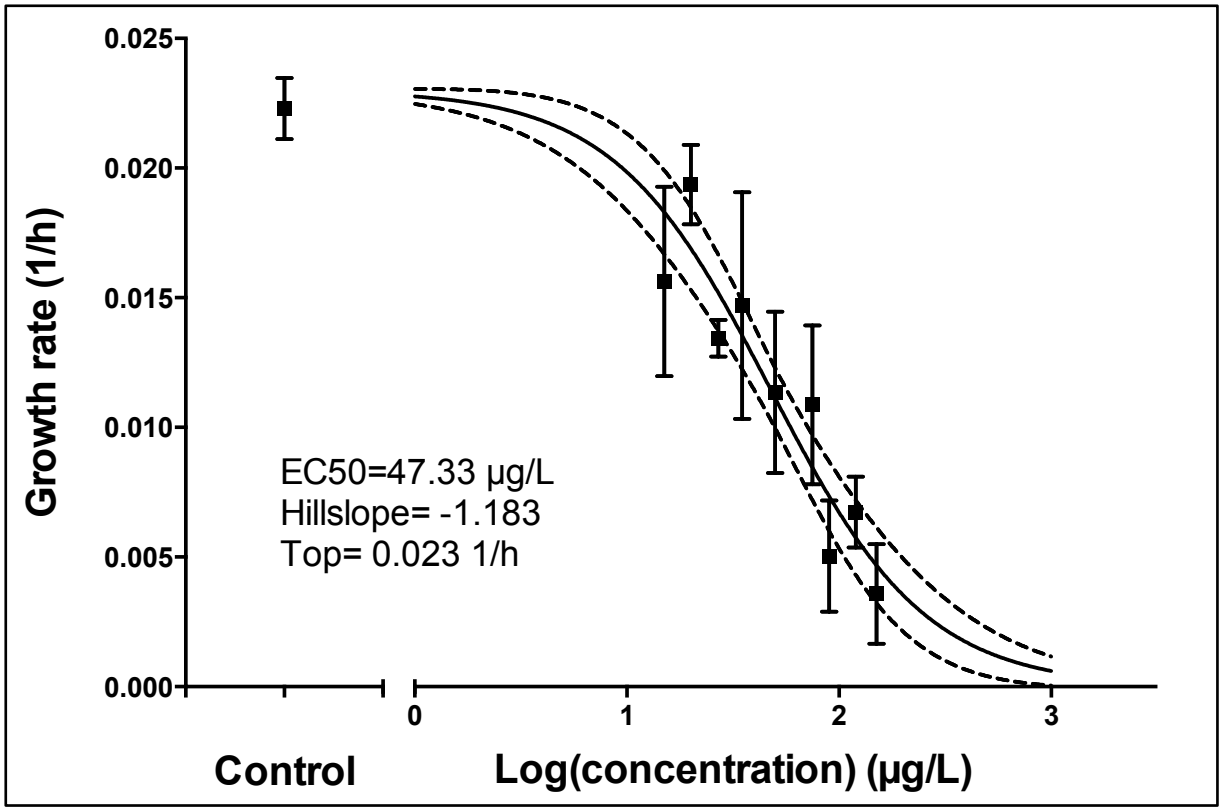

Fig. A5.2: Growth-response curve for atrazine on the alga *S. vacuolatus*.

Diuron

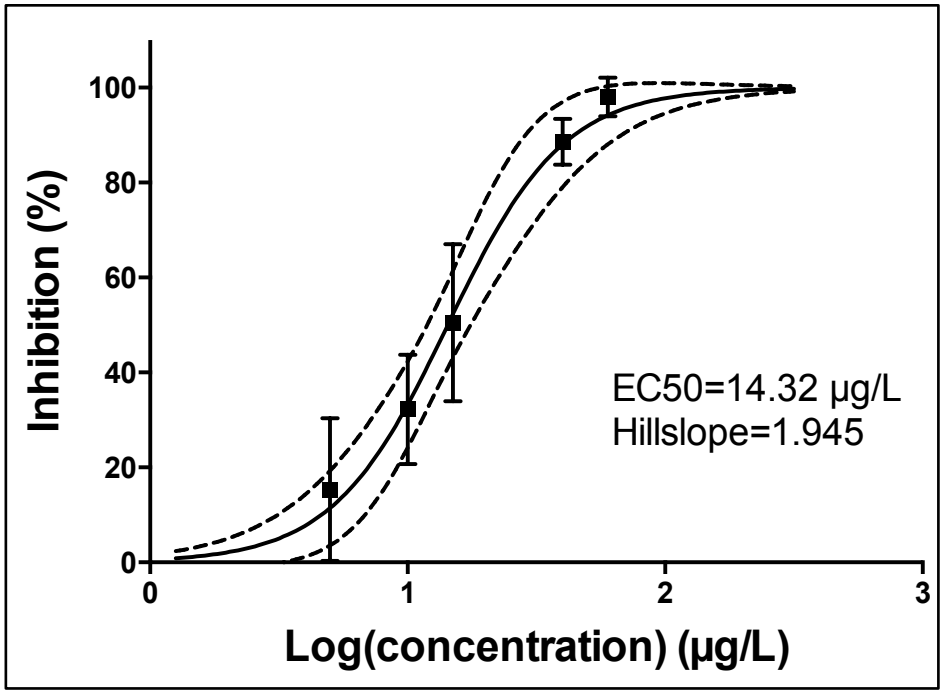

Fig. A5.3: Dose-response curve for diuron on the alga *S. vacuolatus*.

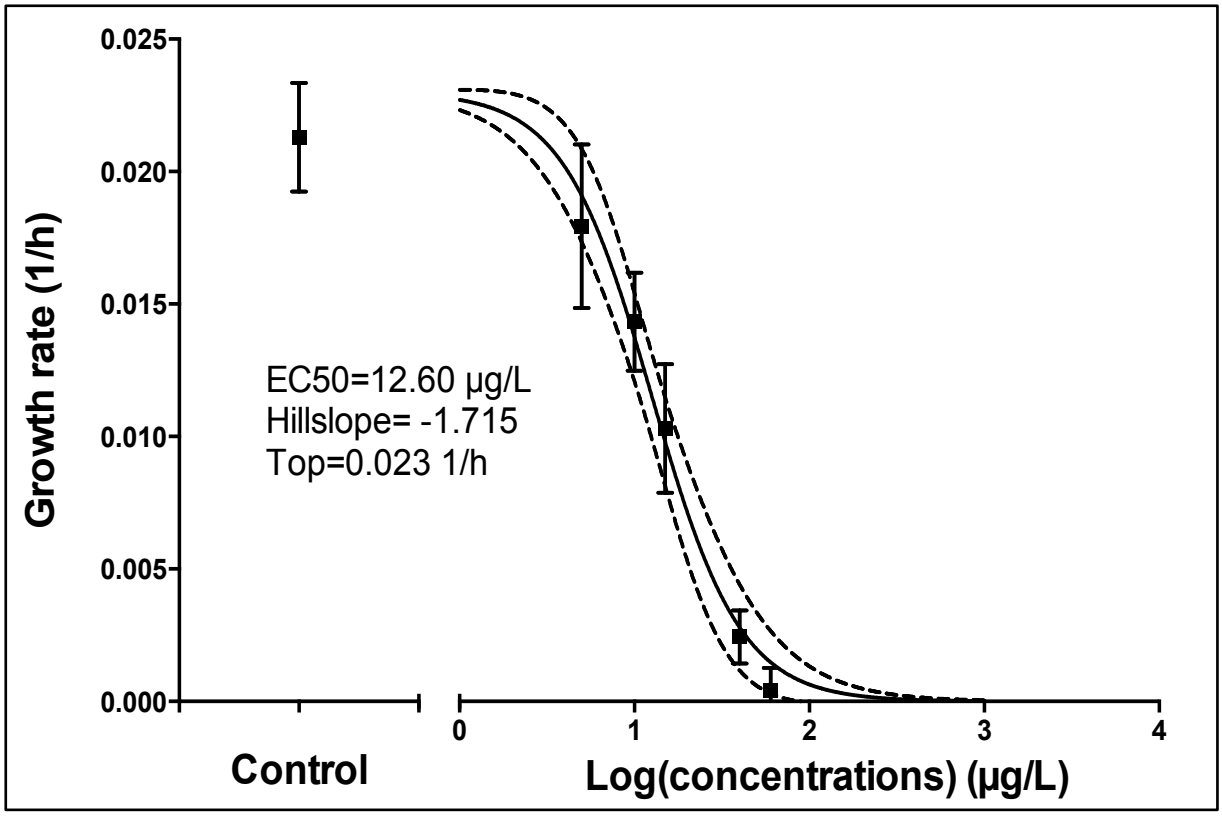

Fig. A5.4: Growth-response curve for diuron on the alga *S. vacuolatus*.

Isoproturon (S. vacuolatus)

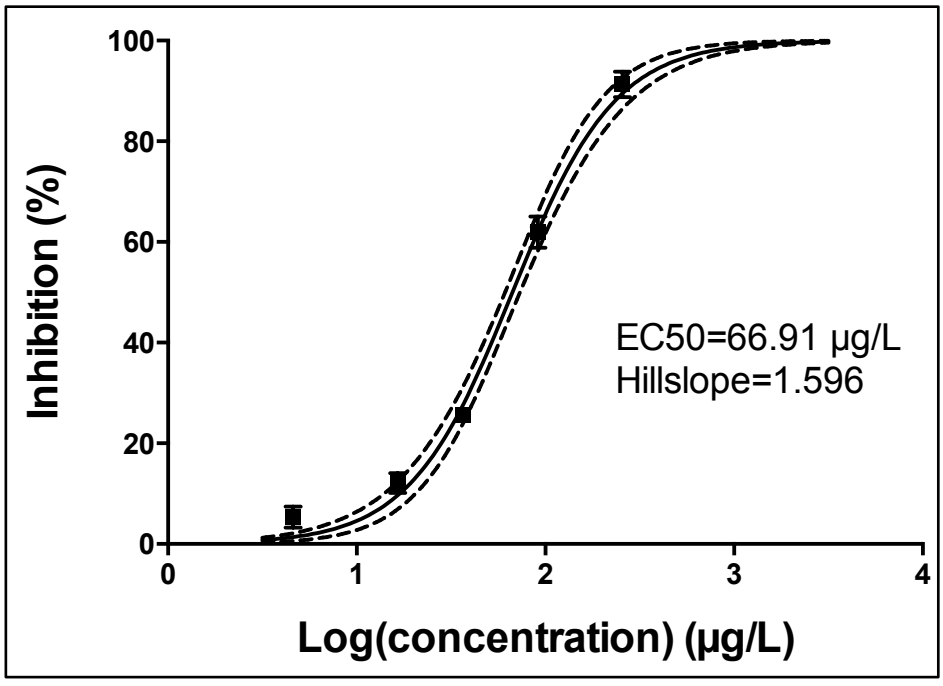

Fig. A5.5: Dose-response curve for isoproturon on the alga *S. vacuolatus*.

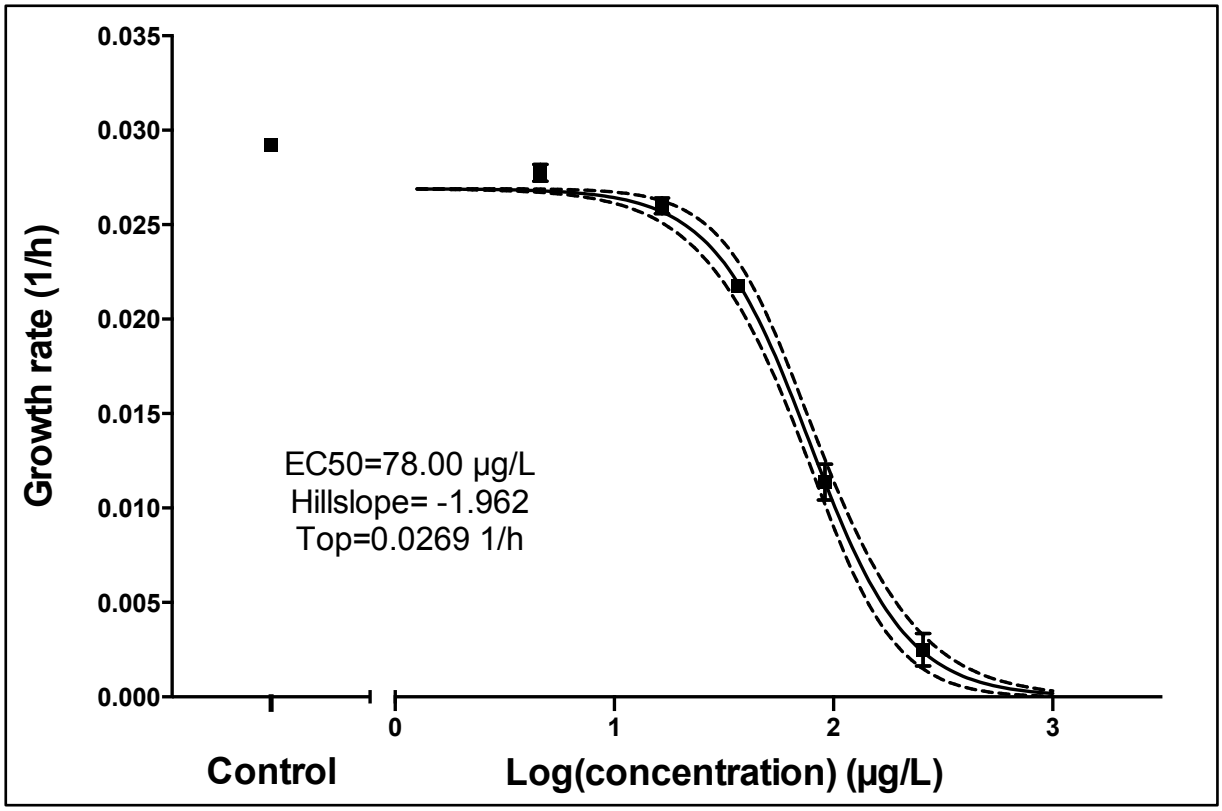

Fig. A5.6: Growth-response curve for isoproturon on the alga *S. vacuolatus*.

Isoproturon (P. subcapitata)

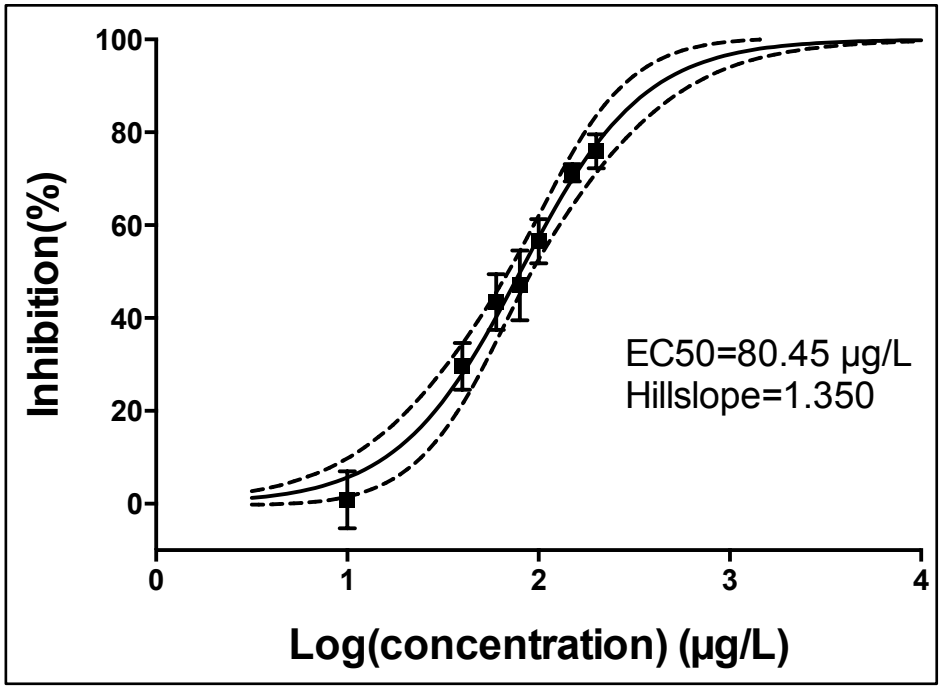

Fig. A5.7: Dose-response curve for isoproturon on the alga *P. subcapitata*.

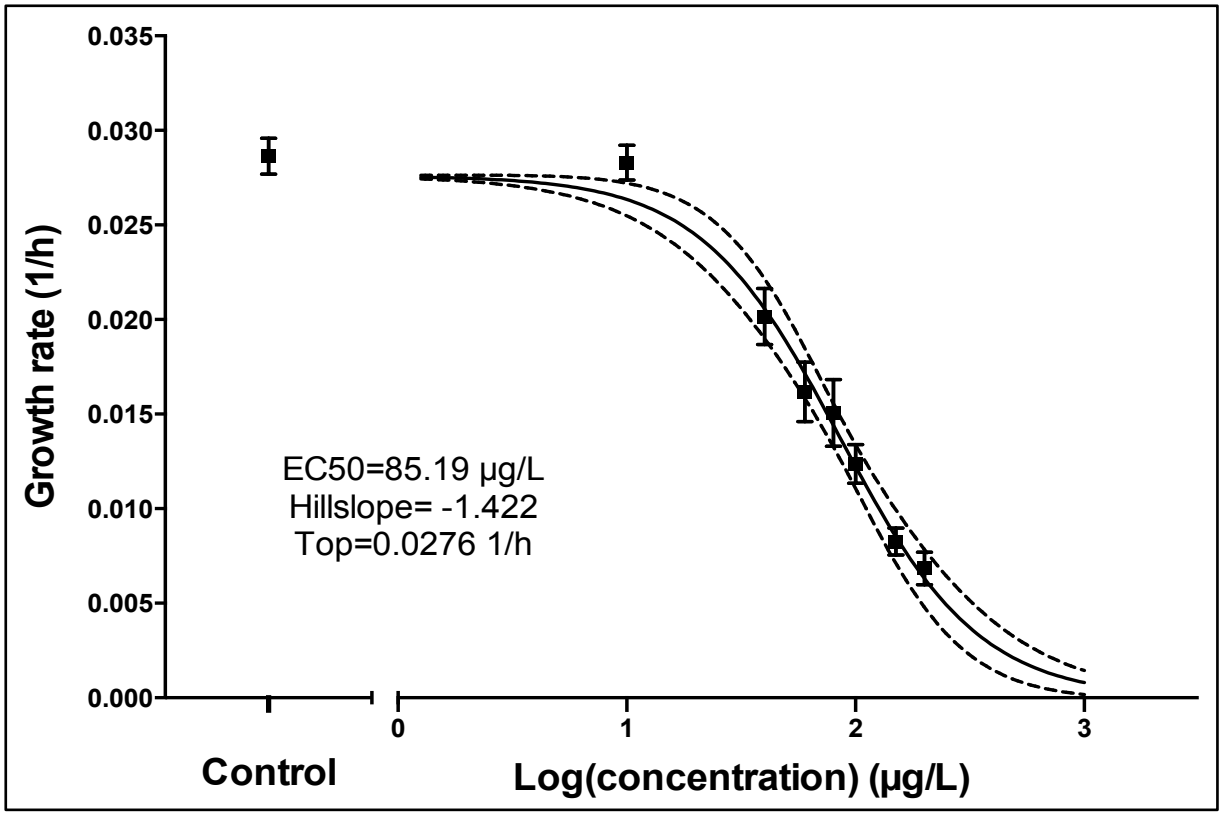

Fig. A5.8: Growth-response curve for isoproturon on the alga *P. subcapitata*.

S-metolachlor

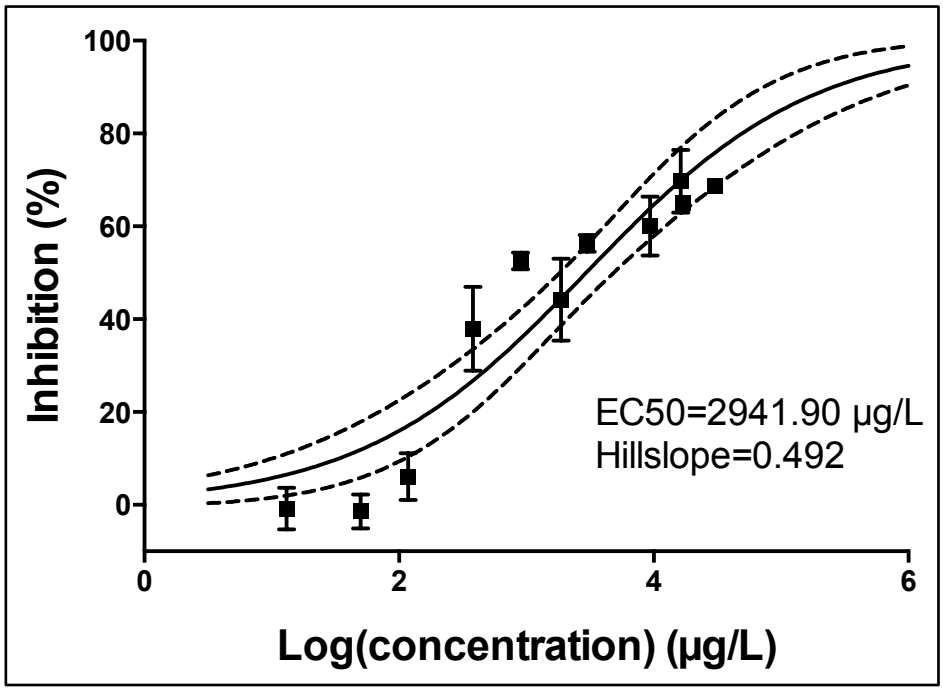

Fig. A5.9: Dose-response curve for S-metolachlor on the alga *S. vacuolatus*.

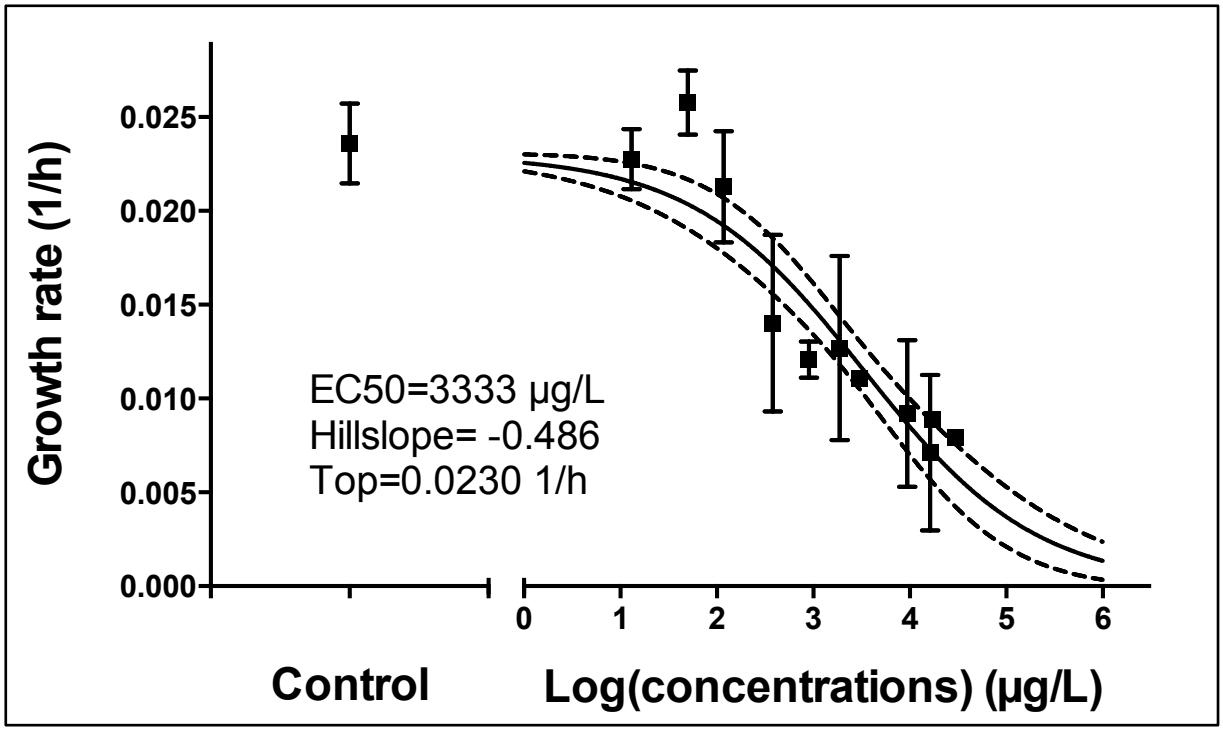

Fig. A5.10: Growth-response curve for S-metolachlor on the alga *S. vacuolatus*.

Appendix 6

In this appendix, the Matlab scripts are presented for the photosystem II inhibitors, for S-metolachlor, for the two algae in co-culture and for the typical environmental exposure scenario. For the photosystem II inhibitors and the typical environmental exposure scenario, the scripts are given for isoproturon on the alga *P. subcapitata*.

Photosystem II inhibitors

```
% Scenario composed of two pulses of different concentrations
and two recovery periods.

% Definition of the parameter μ more or less 2 times its
standard deviation for the alga P. subcapitata. This parameter
changes if another alga is used.
mu=[0.0230,0.0323];

% Definition of the parameters μinh of the 2 pulses more or
less 2 times their standard deviations for the alga P.
subcapitata. These parameters change for other concentrations
and for other photosystem II inhibitors.
mu1=[-0.0014,0.0125];
mu2=[0.0135,0.0279];

% Random definition of 10,000 values for the 3 parameters.
These 3 parameters follow a normal distribution.
r=randn(1,10000);
s=randn(1,10000);
t=randn(1,10000);
munorm=0.0276+0.0023*r;
mu1norm=0.0055+0.0035*s;
mu2norm=0.0207+0.0036*t;

% Definition of the duration of pulses and recoveries.
% Value t1=43.00
t1=input('entrez t1:');
% Value t2=48.25
t2=input('entrez t2:');
% Value t3=72.25
t3=input('entrez t3:');
% Value t4=92.25
t4=input('entrez t4:');
% Value t5=140.25
t5=input('entrez t5:');
i=numel(munorm);
j=numel(mu1norm);
jj=numel(mu2norm);
```

```
% Only the random values of the 3 parameters included between
their averages more or less 2 times their standard deviations
are taken into account.
k=1;
u=1;
while (u<=i)
     if (munorm(u)>=mu(1)) && (munorm(u)<=mu(2))
          munormvecteur(k)=munorm(u);
          u=u+1;
          k=k+1;
     else munorm(u)=[];
          i=i-1;
     end;
end;

l=1;
m=1;
while (m<=j)
     if (mu1norm(m)>=mu1(1)) && (mu1norm(m)<=mu1(2))
          mu1normvecteur(l)=mu1norm(m);
          m=m+1;
          l=l+1;
     else mu1norm(m)=[];
          j=j-1;
     end;
end;

ll=1;
mm=1;
while (mm<=jj)
     if (mu2norm(mm)>=mu2(1)) && (mu2norm(mm)<=mu2(2))
          mu2normvecteur(ll)=mu2norm(mm);
          mm=mm+1;
          ll=ll+1;
     else mu2norm(mm)=[];
          jj=jj-1;
     end;
end;

% Random redistribution of the 3 parameters.
n=numel(munormvecteur);
o=numel(mu1normvecteur);
oo=numel(mu2normvecteur);
p1=min(n,o);
p2=min(n,oo);
p=min(p1,p2);

S=size(munormvecteur);
D=size(mu1normvecteur);
DD=size(mu2normvecteur);

ind1=randperm(S(2),p);
```

```
ind2=randperm(D(2),p);
ind3=randperm(DD(2),p);

for I=1:p
     f(I)=munormvecteur(ind1(I));
end;

for J=1:p
     g(J)=mu1normvecteur(ind2(J));
end;

for JJ=1:p
     gg(JJ)=mu2normvecteur(ind3(JJ));
end;

% Calculation of the cell density inhibition for the different
values of the parameters μ and μinh. The values μinh>μ are
excluded to avoid negative cell density inhibition. 0.040
corresponds to the initial optical density of the alga P.
subcapitata.
a=1;
b=1;
while (a<=p)
     if f(a)>=gg(a)
          ans1(a)=(log(0.040)+f(a)*t5);
          ans2(a)=(log(0.040)+f(a)*t1+g(a)*(t2-t1)+f(a)*(t3-
          t2)+gg(a)*(t4-t3)+f(a)*(t5-t4));
          delta(b)=exp(ans1(a))-exp(ans2(a));
          a=a+1;
          b=b+1;
     else g(a)=[];
          f(a)=[];
          gg(a)=[];
          p=p-1;
     end;
end;

e=numel(delta);

for h=1:e
     ans3(h)=exp(log(0.040)+f(h)*t5);
     inh(h)=(delta(h)/ans3(h))*100;
end;

moyenne=mean(inh);
sigma=std(inh);
```

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S-metolachlor

```
% Scenario composed of two pulses of same concentration and
two recovery periods.

% Definition of the parameter μ more or less 2 times its
standard deviation for the alga S. vacuolatus. This parameter
changes if another alga is used.
mu=[0.0191,0.0269] ;

% Definition of the parameters μinh of the 2 pulses more or
less 2 times their standard deviations for the alga S.
vacuolatus. Although the concentrations tested of the 2 pulses
are equivalent, the modification of sensitivity of the alga
with S-metolachlor induces the definition of a different μinh
(mu3) if the recovery duration is lower than 20 hours.
mu1=[0.0022,0.0215];
mu2=[0.0022,0.0215];
mu3=[-0.0020,0.0031];

% Random definition of 10,000 values for the 4 parameters.
These 4 parameters follow a normal distribution.
r=randn(1,10000);
s=randn(1,10000);
t=randn(1,10000);
tt=randn(1,10000);
munorm=0.0230+0.0019*r;
mu1norm=0.011849+0.0048*s;
mu2norm=0.011849+0.0048*t;
mu3norm=0.000562+0.0013*tt;

% Definition of the duration of pulses and recoveries.
% Value t1=18
t1=input('entrez t1:');
% Value t2=48
t2=input('entrez t2:');
% Value t3=62.5
t3=input('entrez t3:');
% Value t4=92.5
t4=input('entrez t4:');
% Value t5=135.25
t5=input('entrez t5:');
i=numel(munorm);
j=numel(mu1norm);
jj=numel(mu2norm);
jjj=numel(mu3norm);

% Only the random values of the 4 parameters included between
their averages more or less 2 times their standard deviations
are taken into account.
k=1;
u=1;
while (u<=i)
```

```
     if (munorm(u)>=mu(1)) && (munorm(u)<=mu(2))
          munormvecteur(k)=munorm(u);
          u=u+1;
          k=k+1;
     else munorm(u)=[];
          i=i-1;
     end;
end;

l=1;
m=1;
while (m<=j)
     if (mu1norm(m)>=mu1(1)) && (mu1norm(m)<=mu1(2))
          mu1normvecteur(l)=mu1norm(m);
          m=m+1;
          l=l+1;
     else mu1norm(m)=[];
          j=j-1;
     end;
end;

ll=1;
mm=1;
while (mm<=jj)
     if (mu2norm(mm)>=mu2(1)) && (mu2norm(mm)<=mu2(2))
          mu2normvecteur(ll)=mu2norm(mm);
          mm=mm+1;
          ll=ll+1;
     else mu2norm(mm)=[];
          jj=jj-1;
     end;
end;

lll=1;
mmm=1;
while (mmm<=jjj)
     if (mu3norm(mmm)>=mu3(1)) && (mu3norm(mmm)<=mu3(2))
          mu3normvecteur(lll)=mu3norm(mmm);
          mmm=mmm+1;
          lll=lll+1;
     else mu3norm(mmm)=[];
          jjj=jjj-1;
     end;
end;

% Random redistribution of the 4 parameters.
n=numel(munormvecteur);
o=numel(mu1normvecteur);
oo=numel(mu2normvecteur);
ooo=numel(mu3normvecteur);
p1=min(n,o);
p2=min(n,oo);
```

```
p3=min(n,ooo);
pp=min(p1,p2);
ppp=min(p1,p3);
p=min(pp,ppp);
S=size(munormvecteur);
D=size(mu1normvecteur);
DD=size(mu2normvecteur);
DDD=size(mu3normvecteur);
ind1=randperm(S(2),p);
ind2=randperm(D(2),p);
ind3=randperm(DD(2),p);
ind4=randperm(DDD(2),p);


for I=1:p
     f(I)=munormvecteur(ind1(I));
end;

for J=1:p
     g(J)=mu1normvecteur(ind2(J));
end;

for JJ=1:p
     gg(JJ)=mu2normvecteur(ind3(JJ));
end;

for JJJ=1:p
     ggg(JJJ)=mu3normvecteur(ind4(JJJ));
end;

% Calculation of the cell density inhibition for the different
values of the parameters μ and μinh. The characteristics of S-
metolachlor, i.e., the beginning of the effect after 20 hours,
the delay of 20 hours for the recovery and the modification of
sensitivity are taken into account in the calculation. The
values μinh>μ are excluded to avoid negative cell density
inhibition. 0.056 corresponds to the initial optical density
of the alga S. vacuolatus.
a=1;
b=1;
t6=t2-t1; % Duration of pulse 1.
t7=t3-t2; % Duration of recovery 1.
t8=t4-t3; % Duration of pulse 2.
t9=t5-t4; % Duration of recovery 2.
t10=t6-20; % Duration of effect during pulse 1.
t11=t7-20; % Duration of recovery 1 after delay.
t12=t8-20; % Duration of effect during pulse 2.
t13=t9-20; % Duration of recovery 2 after delay.


while (a<=p)
     if  t6<=20  &&  t8<=20  %  We  supposed  that  if  the  pulse
```

```
duration is less than 20 hours, there is no effect and
therefore no delay during the recovery.
          ans1(a)=(log(0.056)+f(a)*t5);
          ans2(a)=(log(0.056)+f(a)*t5);
          delta(b)=exp(ans1(a))-exp(ans2(a));
          a=a+1;
          b=b+1;

     elseif t6<=20
          if f(a)>=gg(a)
               if t9>20
                    ans1(a)=(log(0.056)+f(a)*t5);
                    ans2(a)=(log(0.056)+f(a)*t1+f(a)*t6+f(a)*t7
                    +f(a)*20+gg(a)*t12+0*20+f(a)*t13);
                    delta(b)=exp(ans1(a))-exp(ans2(a));
                    a=a+1;
                    b=b+1;
               else
                    ans1(a)=(log(0.056)+f(a)*t5);
                    ans2(a)=(log(0.056)+f(a)*t1+f(a)*t6+f(a)*t7
                    +f(a)*20+gg(a)*t12+0*t9);
                    delta(b)=exp(ans1(a))-exp(ans2(a));
                    a=a+1;
                    b=b+1;
               end;
          else g(a)=[];
               f(a)=[];
               gg(a)=[];
               p=p-1;
          end;

     elseif t8<=20
          if f(a)>=g(a)
               if t7>20
                    ans1(a)=(log(0.056)+f(a)*t5);
                    ans2(a)=(log(0.056)+f(a)*t1+f(a)*20+g(a)*t1
                    0+0*20+f(a)*t11+f(a)*t8+f(a)*t9);
                    delta(b)=exp(ans1(a))-exp(ans2(a));
                    a=a+1;
                    b=b+1;
               else
                    ans1(a)=(log(0.056)+f(a)*t5);
                    ans2(a)=(log(0.056)+f(a)*t1+f(a)*20+g(a)*t1
                    0+0*t7+f(a)*t8+f(a)*t9);
                    delta(b)=exp(ans1(a))-exp(ans2(a));
                    a=a+1;
                    b=b+1;
               end;
          else g(a)=[];
               f(a)=[];
               gg(a)=[];
               p=p-1;
```

```
          end;

    else if f(a)>=gg(a) && f(a)>=g(a)
              if t7>20 && t9>20
                   ans1(a)=(log(0.056)+f(a)*t5);
                   ans2(a)=(log(0.056)+f(a)*t1+f(a)*20+g(a)*t1
                   0+0*20+f(a)*t11+f(a)*20+gg(a)*t12+0*20+f(a)
                   *t13);
                   delta(b)=exp(ans1(a))-exp(ans2(a));
                   a=a+1;
                   b=b+1;
              elseif t7>20 && t9<=20
                   ans1(a)=(log(0.056)+f(a)*t5);
                   ans2(a)=(log(0.056)+f(a)*t1+f(a)*20+g(a)*t1
                   0+0*20+f(a)*t11+f(a)*20+gg(a)*t12+0*t9);
                   delta(b)=exp(ans1(a))-exp(ans2(a));
                   a=a+1;
                   b=b+1;
              elseif t7<=20 && t9>20
                   ans1(a)=(log(0.056)+f(a)*t5);
                   ans2(a)=(log(0.056)+f(a)*t1+f(a)*20+g(a)*t1
                   0+0*t7+f(a)*20+ggg(a)*t12+0*20+f(a)*t13);
                   delta(b)=exp(ans1(a))-exp(ans2(a));
                   a=a+1;
                   b=b+1;
              else
                   ans1(a)=(log(0.056)+f(a)*t5);
                   ans2(a)=(log(0.056)+f(a)*t1+f(a)*20+g(a)*t1
                   0+0*t7+f(a)*20+ggg(a)*t12+0*t9);
                   delta(b)=exp(ans1(a))-exp(ans2(a));
                   a=a+1;
                   b=b+1;
              end;
         else g(a)=[];
              f(a)=[];
              gg(a)=[];
              ggg(a)=[];
              p=p-1;
         end;
     end;
end;

e=numel(delta);

for h=1:e
     ans3(h)=exp(log(0.056)+f(h)*t5);
     inh(h)=(delta(h)/ans3(h))*100;
end;

moyenne=mean(inh);
sigma=std(inh);
```

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Isoproturon for the alga S. vacuolatus in co-culture with the alga P. subcapitata

```
% Scenario composed of one pulse and one recovery period. The
parameters μ and μinh were obtained with the method count.

% Definition of the parameter μ more or less 2 times its
standard deviation for the alga S. vacuolatus. This parameter
changes if another alga is used.
mu=[0.0482,0.0717] ;

% Definition of the parameter μinh more or less 2 times its
standard deviation for the alga S. vacuolatus.
mu1=[0.0224,0.0529];

% Random definition of 10,000 values for the 2 parameters.
These 2 parameters follow a normal distribution.
r=randn(1,10000);
s=randn(1,10000);
munorm=0.0600+0.0059*r;
mu1norm=0.0376+0.0076*s;

% Definition of the pulse and recovery duration.
% Value t1=39.5
t1=input('entrez t1:');
% Value t2=48.25
t2=input('entrez t2:');
% Value t3=93.25
t3=input('entrez t3:');
i=numel(munorm);
j=numel(mu1norm);

% Only the random values of the 2 parameters included between
their average more or less 2 times their standard deviations
are taken into account.
k=1;
u=1;
while (u<=i)
     if (munorm(u)>=mu(1)) && (munorm(u)<=mu(2))
          munormvecteur(k)=munorm(u);
          u=u+1;
          k=k+1;
     else munorm(u)=[];
          i=i-1;
     end;
end;

l=1;
m=1;
while (m<=j)
     if (mu1norm(m)>=mu1(1)) && (mu1norm(m)<=mu1(2))
          mu1normvecteur(l)=mu1norm(m);
```

```
          m=m+1;
          l=l+1;
     else mu1norm(m)=[];
          j=j-1;
     end;
end;

% Random redistribution of the 2 parameters.
n=numel(munormvecteur);
o=numel(mu1normvecteur);
p=min(n,o);
S=size(munormvecteur);
D=size(mu1normvecteur);
ind1=randperm(S(2),p);
ind2=randperm(D(2),p);

for I=1:p
     f(I)=munormvecteur(ind1(I));
end;

for J=1:p
     g(J)=mu1normvecteur(ind2(J));
end;

% Calculation of the cell density inhibition for the different
values of the parameters μ and μinh. The values μinh>μ are
excluded to avoid negative cell density inhibition. 100,000
correspond to the initial cell density of the alga S.
vacuolatus.
a=1;
b=1;
while (a<=p)
     if f(a)>=g(a)
     ans1(a)=(log(100000)+f(a)*t3);
     ans2(a)=(log(100000)+f(a)*t1+g(a)*(t2-t1)+f(a)*(t3-t2));
     delta(b)=exp(ans1(a))-exp(ans2(a));
     a=a+1;
     b=b+1;
     else g(a)=[];
          f(a)=[];
          p=p-1;
     end;
end;

e=numel(delta);

for h=1:e
     ans3(h)=exp(log(100000)+f(h)*t3);
     inh(h)=(delta(h)/ans3(h))*100;
end;

moyenne=mean(inh);
```

```
sigma=std(inh);
```

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Typical environmental exposure scenario of Isoproturon

```
% Import of the concentration and time data of the typical
environmental exposure scenario of isoproturon. Interpolation
for each minute of the concentration data.
temps=xlsread('Scenarioreel_temps.xls');
concentration=xlsread('Scenarioreel_Concentration_isoproturon'
);
xi=[1.42:1/60:743.83];
concentration1=interp1(temps,concentration,xi);
concentration2=concentration1';

% Definition of the parameter μ for the alga P. subcapitata.
mu=0.0276;

% For each minute, definition of the parameter μinh for the
alga P. subcapitata.
nbconc=length(concentration2);
for i=1:nbconc
     concentration3(i)=log10(concentration2(i));
end;

for j=1:nbconc
     if concentration3(j)==-Inf
          concentration4(j)=0;
     else
          concentration4(j)=concentration3(j);
     end;
end;

% Substitution of the growth rate μinh by μ when the
concentration tested is 0.
nbconc1=nbconc-1;
for a=1:nbconc1
     if concentration4(a)==0
          muinh(a)=mu;
     else
          muinh(a)=mu/(1+10^((1.93-concentration4(a))*(-
          1.422)));
     end;
end;

% Random definition of 1000 values for the parameter μ. This
parameter follows a normal distribution.
muvecteur=mu*ones(1,1000);
rr=randn(1,1000);
muint=[0.0270;0.0283];
munorm=muvecteur+0.0023*rr;

% Only the random values of the parameter μ included between
its average more or less 2 times its standard deviation are
taken into account.
t=numel(munorm);
```

```
u=1;
v=1;
while (v<=t)
     if (munorm(v)>=muint(1)) && (munorm(v)<=muint(2))
          munormvecteur(u)=munorm(v);
          v=v+1;
          u=u+1;
     else munorm(v)=[];
          t=t-1;
     end;
end;

% Random redistribution of the parameter μ.
w=numel(munormvecteur);
x=size(munormvecteur);
ind5=randperm(x(2),w);

for y=1:w
     f(y)=munormvecteur(ind5(y));
end;

% Definition of the uncertainty of the parameter μinh
depending of the concentration tested.
kk=0;
ans2=log(0.040)*ones(1,1000);
dt=1/60;

for b=1:nbconc1
     if concentration4(b)==0
          kk=kk+1;
     elseif (concentration4(b)~=0 && concentration4(b)<=0.8735
     && concentration4(b)>2.9775)
          mu1inf(b)=muinh(b)-0.000663;
          mu1sup(b)=muinh(b)+0.000663;
          mu1=[mu1inf(b);mu1sup(b)];
          s=randn(1,1000);
          mu1norm=muinh(b)+0.001*s;

     elseif              (concentration4(b)>0.8735              &&
     concentration4(b)<=1.136  &&  concentration4(b)>2.7085  &&
     concentration4(b)<=2.9775)
          mu1inf(b)=muinh(b)-0.001105;
          mu1sup(b)=muinh(b)+0.001105;
          mu1=[mu1inf(b);mu1sup(b)];
          s=randn(1,1000);
          mu1norm=muinh(b)+0.002*s;

     elseif              (concentration4(b)>1.136              &&
     concentration4(b)<=1.416  &&  concentration4(b)>1.636  &&
     concentration4(b)<=2.7085)
          mu1inf(b)=muinh(b)-0.001548;
          mu1sup(b)=muinh(b)+0.001548;
```

```
            mu1=[mu1inf(b);mu1sup(b)];
            s=randn(1,1000);
            mu1norm=muinh(b)+0.003*s;

      else
            mu1inf(b)=muinh(b)-0.001990;
            mu1sup(b)=muinh(b)+0.001990;
            mu1=[mu1inf(b);mu1sup(b)];
            s=randn(1,1000);
            mu1norm=muinh(b)+0.004*s;
      end;

% Only the random values of the parameter μinh included
between its average more or less 2 times its standard
deviation are taken into account.
      if concentration4(b)~=0
            c=numel(mu1norm);
            d=1;
            e=1;
            while (e<=c)
                  if (mu1norm(e)>=mu1(1)) && (mu1norm(e)<=mu1(2))
                        mu1normvecteur(d)=mu1norm(e);
                        e=e+1;
                        d=d+1;
                  else mu1norm(e)=[];
                        c=c-1;
                  end;
            end;
      end;

% Random redistribution of the parameter μinh.
      if concentration4(b)~=0
            h=numel(mu1normvecteur);
            k=size(mu1normvecteur);
            ind2=randperm(k(2),h);

            for l=1:h
                  g(l)=mu1normvecteur(ind2(l));
            end;

            m=numel(g);
            n=numel(ans2);

% Random exclusion of several values of parameters μinh at the
different times of the scenario to allow the calculation.
            if m>n
                  mm=size(g);
                  ind3=randperm(mm(2),m);
                  dif=m-n;
                  for o=1:dif
                        g(ind3(o))=0;
                  end;
```

```
                g(g==0)=[];

            else
                nn=size(ans2);
                ind4=randperm(nn(2),n);
                dif=n-m;
                for p=1:dif
                     ans2(ind4(p))=0;
                end;
                ans2(ans2==0)=[];
            end;

% Calculation taking into account only the pulses.
            q=min(m,n);
            for r=1:q
                ans1(r)=ans2(r)+g(r)*dt;
            end;
            ans2=ans1;
      end;
end;

% Random exclusion of several values of the parameters μ or
μinh to allow the calculation.
if w>q
      qq=size(f);
      qqq=numel(f);
      difference=w-q;
      ind7=randperm(qq(2),qqq);
      for pp=1:difference
           f(ind7(pp))=0;
      end;
      f(f==0)=[];
      www=numel(f);

else
      rr=size(ans2);
      rrr=numel(ans2);
      difference=q-w;
      ind8=randperm(rr(2),rrr);
      for ss=1:difference
      ans2(ind8(ss))=0;
      end;
      ans2(ans2==0)=[];
      www=numel(ans2);
end;

% Calculation taking into account the pulses (ans2) and the
recovery periods (terme1).
time=dt*nbconc1;
for zz=1:www
      terme1(zz)=f(zz)*(kk*dt);
      ans4(zz)=ans2(zz)+terme1(zz);
```

```
end;

% Calculation of the cell density inhibition for the different
values of the parameters μ and μinh. The values μinh>μ are
excluded to avoid negative cell density inhibition. 0.040
corresponds to the initial optical density of the alga P.
subcapitata.
ans3=log(0.040)*ones(1,www);
for zz=1:www
     ans5(zz)=ans3(zz)+f(zz)*time;
end;

ii=numel(ans4);
jj=numel(ans5);
ee=min(ii,jj);
var1=1;
var2=1;
while (var1<=ee)
     if ans5(var1)>=ans4(var1)
          delta(var2)=exp(ans5(var1))-exp(ans4(var1));
          var1=var1+1;
          var2= var2+1;
     else
          ans5(var1)=[];
          ans4(var1)=[];
          ee=ee-1;
     end;
end;

hh=numel(delta);

for gg=1:hh
     inh(gg)=(delta(gg)/(exp(ans5(gg))))*100;
end;

moyenne=mean(inh);
```

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Conferences

SETAC Europe 22nd Annual meeting / World 6th World Congress, Berlin, Germany.
SETAC : Society of Environmental Toxicology and Chemistry.
May 20-24, 2012.
Session: Linking exposure to effects in environmental risk assessment.
Poster corner presentation.

Effect modelling of intermittent discharge of herbicides in watercourses.
P.J. Copin, N. Chèvre
University of Lausanne, Lausanne, Switzerland

Abstract:
In ecotoxicology, effects of agricultural herbicides on non-target organisms, like algae, are often simulated referring to a continuous exposure. But this kind of exposure is very far from the reality. Indeed, exposure models of pesticides in aquatic systems after agricultural applications and after rain events are generally irregular i.e. characterized by short pulses exposure and recovery periods. These pulses exposure, even if they are short, can impair the growth of algae or modify their chlorophyll content. Very little is known about the effects on primary producers of short but high peaks of herbicides fluxes, and on how the algae respond during the recovery period between pulses. The main goal of this study is to develop a model for estimating the effects, and even the risk, on algae population to a repeated herbicide stress. This model will be validated by laboratory experiments applying three typical scenarios of pulses exposure. The scenarios differ from each other by the concentration of the different peaks, the exposure duration and, finally, the recovery period between the pulses. The first one corresponds to three pulses of high herbicides concentrations (EC80) and long recovery periods (around 2/3 of the pulse duration). The second scenario corresponds to three pulses of low herbicides concentrations (EC20) and short recovery periods (around 1/3 of the pulse duration). And the last one is a mix of the 2 previous scenarios. These scenarios are tested on the green algae *Scenedesmus vacuolatus*, which is characteristic of primary producers found in rivers. Two photosynthetic inhibitors herbicides, isoproturon and therbuthylazine, are used as test compounds. As endpoint, the growth of algae will be regularly measured during pulses exposure and recovery periods. The model as well as its validation will be discussed.

SETAC Europe 23rd Annual meeting, Glasgow, Scotland.
SETAC : Society of Environmental Toxicology and Chemistry.
May 12-16, 2013.
Session: Spatio-temporal configuration of toxicants and organisms in the environment-consequences for ERA.
Poster.

Effect modelling of intermittent discharge of herbicides accounting for mixture and competition between species.

P.J. Copin, University of Lausanne / Faculty of Geosciences and Environment.
N. Chèvre, University of Lausanne / Faculty of Geosciences and Environment.

Abstract:
Toxicity of herbicides towards freshwater aquatic species is usually tested for a continuous exposure in laboratory. But, in real environmental conditions, especially after agricultural applications and rain events, aquatic species are typically exposed to non-continuous concentrations, i.e. pulses, of herbicides in streams. These scenarii are characterized by periods of high or low exposure followed by periods of recovery. The effect on aquatic species will therefore depend on the duration of these two periods and on the herbicide concentrations during the pulse exposure. In general, even if the exposure or the concentration is short, the growth of algae can be affected by such events. In a previous study, pulse exposure scenarii of Isoproturon were tested on the alga *Scenedesmus vacuolatus*, in laboratory. A model was developed and validated to predict the algae growth inhibition after exposure. However, in the field, algae are not only exposed to one chemical, but to multiple stressors such as mixture toxicity, physico-chemical changes, nutrient changes, etc. Among others, the competition between species may play a role in the response to a chemical stress. Therefore, for this study, we chose to make more complex the system and we tested i) pulse exposure on one alga with a mixture of herbicides and ii) pulse exposure of one herbicide on two algae in competition. Concretely, for the first kind of exposure, pulses of a mixture of isoproturon and terbuthylazine were applied on the alga *Scenedesmus vacuolatus*. For the second kind of exposure, a methodology had to be implemented to allow the development of two algae in the same growth medium in laboratory. To allow an easy counting, two algae with a different shape were chosen: a circular one (*Scenedesmus vacuolatus*) and a rangy one (*Pseudokirchneriella subcapitata*). This study highlighted the accuracy of the model used.

SGM 11th Annual meeting, Lausanne, Switzerland.
SGM : Swiss Geoscience Meeting.
November 15-16, 2013.
Session: Limnological and hydro(geo)logical advances for mid-sized lakes as a water resource for the next century.
Poster corner presentation.

Enhancement of the representativeness of herbicide effect modelling in watercourses.

P.J. Copin, N. Chèvre.
University of Lausanne, Lausanne, Switzerland.

Abstract:
These last years, many herbicides were detected in Swiss watercourses. The concentrations of some of these herbicides were higher than the criteria of 0.1 µg/l defined in the Swiss legislation. Moreover, these herbicides are discharged non-continuously in watercourses or streams after crop applications and during rain events. It is therefore important to determine the effects of these non-continuous exposures on aquatic species. Algae species are specifically interesting as they are commonly very sensitive to herbicides. Furthermore, they are at the base of the food chain. Consequently, if they are damaged, the whole fauna may be affected. In this context, a model was developed to assess the growth inhibition of the green alga Scenedesmus vacuolatus caused by non-continuous exposure scenarios to the herbicide isoproturon. The uncertainties of the predictions were also estimated. This model was validated with laboratory experiments. Non-continuous exposure is characterized by periods of exposure (with the herbicide) and recovery (without the herbicide). The effects on algae species will therefore depend on the length of these 2 types of periods but also on the herbicide concentration during the pulse exposure. Indeed, even if the pulse duration is short, a high concentration can inhibit the growth of the algae. To improve the environmental representativeness of this model, it was adapted to predict the effects of non-continuous exposure to mixture of herbicides. Finally, the model was also evaluated by testing pulse exposure scenario of isoproturon on two algae species growing in the same medium: a circular one (*Scenedesmus vacuolatus*) and a rangy one (*Pseudokirchneriella subcapitata*). The model validity is discussed for each case studied.

SEFS 9th Annual meeting, Geneva, Switzerland.
SEFS : Symposium for European Freshwater Sciences.
July 5-10, 2015.
Session: Freshwater toxicology at the community and ecosystem level.
Oral presentation.

Modelling the effects of pulse exposure for several PSII inhibitors and algae.

P.J. Copin, N. Chèvre.
University of Lausanne, Lausanne, Switzerland.

Abstract:
After crop application and precipitations, herbicides can fluctuate widely in watercourses. These pulses can exceed water chronic quality criteria, which aims to protect aquatic environment. A model was developed to evaluate the effects of successive pulse exposure on algae. The model proposed is based on two parameters: i) the typical growth rate of the algae, obtained by monitoring growth rates of several successive batch cultures in growth media, characterizing both the growth of the control and during the recovery periods; ii) the growth rate of the algae exposed to pulses, determined from a dose-response curve. We focused on herbicides photosystem II inhibitors atrazine, diuron and isoproturon and on the freshwater algae S. vacuolatus and P. subcapitata. We validated the model prediction based on effect measured in laboratory. The comparison between the laboratory and the modelled effects illustrated that the results yielded were consistent, making the model suitable for effect prediction of the photosystem II inhibitors on the algae S. vacuolatus and P. subcapitata. The application of the model proves that the longest peaks affect the cell density inhibition of algae the most. It is therefore crucial to capture these high fluctuations when monitoring of herbicide concentrations are conducted in streams.

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Modelling the effect of fluctuating herbicide concentrations on algae growth

Pierre-Jean Copin [a,*], Sylvain Coutu [b,1], Nathalie Chèvre [a]

[a] Institut des dynamiques de la surface terrestre (IDYST), Faculté des Géosciences et de l'Environment, Université de Lausanne, Géopolis, Quartier Mouline, CH-1015 Lausanne, Switzerland

[b] Laboratoire de technologie écologique, Institut d'ingénierie de l'environnement, Faculté de l'environnement naturel, architectural et construit (ENAC), Ecole Polytechnique Fédérale de Lausanne (EPFL), 1015 Lausanne, Switzerland

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ABSTRACT

Herbicide concentrations fluctuate widely in watercourses after crop applications and rain events. The level of concentrations in pulses can exceed the water chronic quality criteria. In the present study, we proposed modelling the effects of successive pulse exposure on algae. The deterministic model proposed is based on two parameters: (i) the typical growth rate of the algae, obtained by monitoring growth rates of several successive batch cultures in growth media, characterizing both the growth of the control and during the recovery periods; (ii) the growth rate of the algae exposed to pulses, determined from a dose–response curve obtained with a standard toxicity test. We focused on the herbicide isoproturon and on the freshwater alga *Scenedesmus vacuolatus*, and we validated the model prediction based on effect measured during five sequential pulse exposures in laboratory. The comparison between the laboratory and the modelled effects illustrated that the results yielded were consistent, making the model suitable for effect prediction of the herbicide photosystem II inhibitor isoproturon on the alga *S. vacuolatus*. More generally, modelling showed that both pulse duration and level of concentration play a crucial role. The application of the model to a real case demonstrated that both the highest peaks and the low peaks with a long duration affect principally the cell density inhibition of the alga *S. vacuolatus*. It is therefore essential to detect these characteristic pulses when monitoring of herbicide concentrations are conducted in rivers.

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1. Introduction

Herbicides are frequently detected in watercourses (Konstantinou et al., 2006; Muller et al., 2002; Skark et al., 2004). Indeed, they can reach surface waters during rain events by surface transport or drainage (Brown and van Beinum, 2009; Freitas et al., 2008). Thus, they do not contaminate the aquatic environment continuously but rather in pulses. Several authors have described this non-continuous pattern of herbicide concentrations in rivers that is mainly linked with the rain events following application periods. They are characterised by successive short pulses of high concentrations followed by period of low concentrations of various durations (House et al., 1997; Muller et al., 2002; Reinert et al., 2002). The concentrations during pulsed exposures are often above chronic water quality criteria, and even the acute quality criteria, defined to protect aquatic life from the deleterious effects of chemicals such as herbicides (Vallotton, 2007). The effects, and thus the risk of such pulses, are therefore crucial to determine (Boxall et al., 2013).

The effects of pulsed exposures to herbicides on non-target aquatic species, i.e mainly algae and macrophytes, have been subject to question for more than a decade (Reinert et al., 2002). Some authors have tried to depict the effects these pulses may generate. In general, the impact of pulsed exposures on algae and macrophytes seem to be substance dependent (Cedergreen et al., 2005). For example, isoproturon, a photosystem II inhibitor commonly applied on cereal fields, has a lower impact in pulses than in continuous exposure (Boxall et al., 2013). Indeed, photosystem II inhibitors such as triazines and phenylureas induce toxicity during the pulse exposure, but the algae recover totally, i.e. the growth rate is similar to that of non-exposed algae, after the chemical is removed (Reinert et al., 2002; Vallotton et al., 2009). Consequently, the effect of successive pulses is lower than continuous exposure at the same concentration. Similarly, water plants exposed to a 24 h pulse of sulfonylureas seem to recover, usually reaching the same biomass as the control 6 days after the exposure

* Corresponding author. Fax: +41 21 692 3545.
E-mail addresses: pierre-jean.copin@unil.ch (P.-J. Copin), sylvain.coutu@epfl.ch, scoutu@stanford.edu (S. Coutu), nathalie.chevre@unil.ch (N. Chèvre).
[1] Permanent address: Department of Environmental Earth System Science, Stanford University, Stanford, CA 94305, USA.

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(Rosenkrantz et al., 2012). But this can be different for other compounds and mechanisms of action. For example, Vallotton et al. (2008b) showed that a pulse of the herbicide S-metolachlor induces a delay in recovery after exposure to algae. Along the same lines, the growth of macrophytes seems to be significantly reduced after a 48 h and a 96 h pulse of pentachlorophenol (Boxall et al., 2013). Compound-specific uptake, degradation or dissipation rates in plants, and the potential of recovery between pulses can explain these differences of effects (Boxall et al., 2013; Cedergreen et al., 2005).

The effects of pulse exposure scenarios were also assessed for more complex systems such as periphyton communities. Gustavson et al. (2003) showed that photosynthetic activity of natural periphyton communities can be strongly disturbed by low and environmentally realistic pulse concentrations of isoproturon. Laviale et al. (2011) also showed that a 1, 3 or 7-h peak exposure to isoproturon induces an inhibition of two fluorescence parameters, the effective and the optimal quantum yields of PSII photochemistry, on the periphyton community at environmentally relevant concentrations; however, 12 h after the pulse, the periphyton recovery is complete at these concentrations.

Although the effects of sequential pulses of herbicides on non-target organisms are partially depicted, very few models have been developed to predict these effects (Nagai, 2014). Such latter models, however, are of particular importance due to the large varieties of pulse scenarios. It would also be a first step for risk assessment of pulsed exposures. Recently, Weber et al. (2012) simulated the effects of successive pulse exposure to isoproturon on algae populations in a flow-through system. In that study, the authors modelled the population fluctuations as a function of four parameters: temperature, light intensity, nutrient availability and chemical concentration. But the model proposed is mainly descriptive and therefore difficult to use for effects predictions due to the lack of information on the different variables.

The aim of this study was to develop a simple model, i.e with parameters easily determinate with classical experiments (standard OECD test), able to predict the cell density inhibition of algae exposed to sequential pulses of herbicides. The model was developed to simulate the effects of photosystem II inhibitors, which are widely used in European countries. In Switzerland, they are the most common herbicides found in surface waters such as lakes (Gregorio et al., 2012). Furthermore, as mentioned above, they have the advantage of not inducing a delay in the recovery phase of algae. The model will be validated by comparing the predictions with laboratory measurements obtained with 5 typical scenarios. For the experiments, we chose to test the herbicide isoproturon, which is regularly detected in rivers up to several μg/l in pulses (Garmouma et al., 1998; IFEN, 2007; Muller et al., 2002). The alga selected was *Scenedesmus vacuolatus*, which has already been tested successfully with pulses (Vallotton et al., 2009). As an illustration, the model will also be used to predict the cell density inhibition for a realistic pulse scenario in a river.

2. Materials and methods

2.1. Chemical

Isoproturon, 3-(4-isopropylphenyl)-1,1-dimethylurea, (99% purity) was purchased from Ehrenstorfer GmbH. A stock solution of 3200 μg/l was prepared in an algae medium, in axenic conditions, for pulse exposure testing. This stock solution was kept in the fridge at 6.4 °C. The concentration was checked analytically and the measured concentrations were in the same range as the nominal concentrations (results not shown).

2.2. Algae cultures

Permanent agar culture tubes of green unicellular microalga *S. vacuolatus* (Chlorophyceae; strain 211-15, Shihira and Krauss, Melbourne, Australia) was obtained from the Department of Bioanalytical Ecotoxicology at the Helmholtz Centre for Environmental Research – UFZ, Leipzig, Germany. Microalga was cultured in a growth media described in the OECD guideline (OECD, 2011). Microalgae were cultured in the OECD medium by successive transfers in order to maintain exponential growth conditions and to possibly identify signs of abnormal growth (Le Faucheur et al., 2005). The method involves transferring regularly, i.e. every 48 h, a specific volume of algae culture, defined by a calibration curve, into a new 50 ml OECD medium. 50 ml of algal suspension were placed in erlenmeyer flasks of a capacity of 250 ml on a HT Infors shaker table (90 rpm) (Le Faucheur et al., 2005; Vallotton et al., 2008a) at 25 °C and under continuous illumination at a light intensity of 70 μmol/m^2/s provided by cool-white fluorescent lamps.

Algae were inoculated in a new culture medium with an initial optical density of 0.056 at 690 nm ($OD_{\lambda 690}$), which corresponds to a density of 650,000 cells/ml. The optical density was measured with a microplate reader (ELx800™, BioTek® Instruments, Winooski, Vermont) at a wavelength of 690 nm. The cell density (cells/ml) was determined by cell counting using the improved Neubauer Haemacytometer (Optik Labor, Lancing, United Kingdom). The calibration curve was obtained by plotting the cell density as a function of the measured optical density.

A control charter was established to monitor algae growth in growth media. In our laboratory, the average growth rate of the algae was 0.027 h^{-1} with a standard deviation of 0.002 h^{-1} (average of successive 47 cultures).

2.3. Dose–response curve of isoproturon

The dose–response curve of isoproturon, required to parameterise the model and to defined the tested concentrations, was established following a method adapted from the standard OECD procedure (OECD, 2011). The tests were performed in the same conditions as algae cultures (see Section 2.2). Five concentrations ranging from 4.6 to 256 μg/l and a control were tested in octoplicates. The optical density measured at the beginning and at the end of the test was used to evaluate the average specific growth rate for each concentration and for the control. Growth inhibition is the ratio between the growth rates of the different concentrations and that of the control (Eq. (1); see Section 2.5.1).

2.4. Pulse exposure tests

Five pulse exposure scenarios were tested in the laboratory (Fig. 1; Table 1). They differed in the duration and concentration levels of the pulses, and in the duration of the recovery periods. Two cases were considered: short pulse duration and long recovery period, and long pulse duration and short recovery period. These cases can be considered as representative of two extreme scenarios that can be found in rivers. Pulse exposure scenarios differed also in the pulses concentration tested as shown in Table 1. Algae exposed to scenarios, as well as the controls, were tested in triplicates.

The test was started the same way the algae were cultured (initial cell density 650,000 cells/ml, 50 ml in 250 ml flask) and in the same conditions (see Section 2.2). Algae grew for a short period (Fig. 1; V is around 24 h) at the beginning before being exposed to the first pulse. At the end of each pulse exposure, the algae were centrifuged twice for 7 min at 1046*g* and 25 °C. The supernate was removed and the algae were re-suspended in growth media (Vallotton et al., 2008a). These two centrifugations

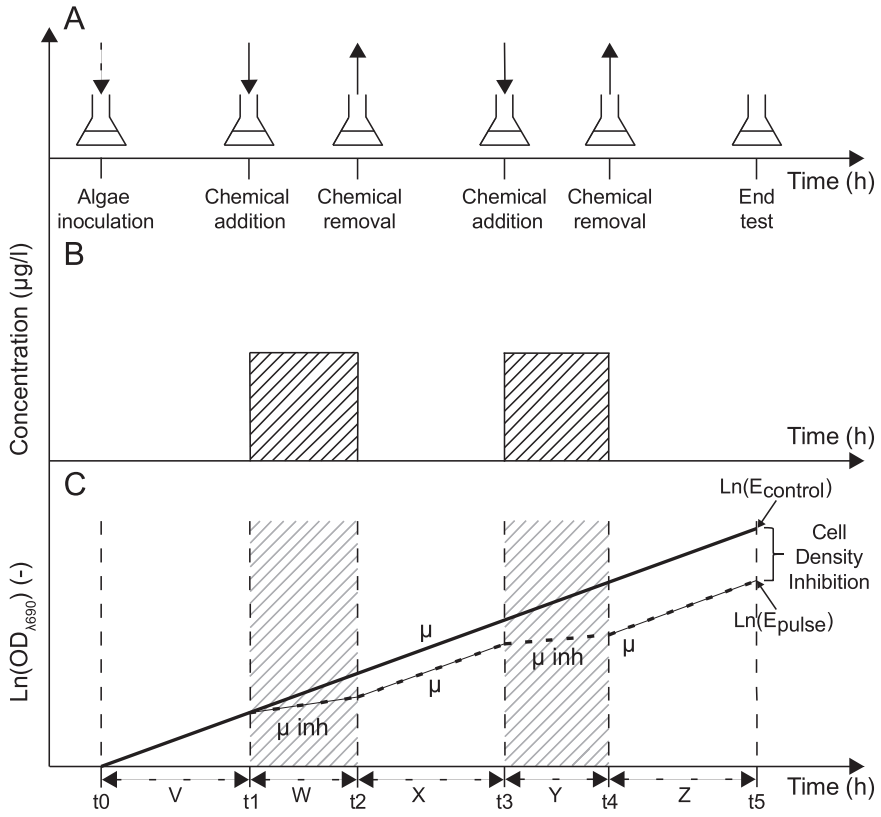

Fig. 1. (A) Procedure of pulse exposure scenarios tested in laboratory. (B) Pulses representation over time. (C) Modelling representation. μ is the growth rate obtained from the control charter. μ_{inh} is the growth rate at concentration x obtained from the growth response curve. $E_{control}$ is the predicted final optical density for the model's control. E_{pulse} is the final optical density of the model for the alga exposed to pulse concentration. V corresponds to the growth duration before the first pulse (h); W and Y are the pulse durations (h); X and Z are the recovery durations (h); V is around 24 h. W, X, Y and Z are defined in Table 1.

Table 1
Parameters of duration for each pulse and recovery period with the concentration tested during the scenarios.

Scenario number	Pulse duration (h)	Recovery duration (h)	Concentration tested
1	W=5.75 Y=6	X=Z=38	EC50[a]
2	W=4.25 Y=4.50	X=38, Z=0	EC70[b]
3	W=Y=5	X=Z=40	EC80[c]
4	W=Y=24	X=2, Z=0	EC10[d]
5	W=Y=24	X=Z=3	EC30[e]

[a] Effect concentration 50%.
[b] Effect concentration 70%.
[c] Effect concentration 80%.
[d] Effect concentration 10%.
[e] Effect concentration 30%.

allowed 99.985% of the herbicide to be removed and did not impair algal growth, as shown by Vallotton et al. (2008a). The recovery period began directly after the algae were re-suspended in the fresh medium at an initial cell density of 650,000 cells/ml to allow a new exponential growth phase. This operation was repeated one more time after the second pulse exposure. Controls were treated the same manner that the algae exposed to pulses. The optical density was measured regularly during exposure and recovery periods.

After scenarios 3 and 5, standard toxicity tests were performed with algae having undergone pulse exposure tests. The goal was to determine if algae become more tolerant towards the herbicide or, on the contrary, become more sensitive to the chemical stressor.

2.5. Data analysis

2.5.1. Growth inhibition in standard toxicity test on algae

The endpoint of this standard test, i.e. the growth inhibition (I_t) at a given concentration, is determined using the response variables, i.e. the average specific growth rate of the control (μ_C) and the average specific growth rate at this concentration (μ_T) (OECD, 2011) (Eq. (1)):

$$I_t = \frac{\mu_C - \mu_T}{\mu_C} \times 100 \quad (1)$$

2.5.2. Dose–response curves for standard acute toxicity test

The dose–response curve of isoproturon is obtained by plotting the growth inhibition I_t as a function of the tested concentrations C. The relationship is expressed by using a four parameter log–logistic dose response model (Vallotton et al., 2008a) (Eq. (2)):

$$I_t = min + \frac{(max - min)}{1 + 10^{((\log EC50 - \log C) \times Hillslope)}} \quad (2)$$

where EC_{50}, the concentration inhibiting 50% of the growth; *Hillslope*, the slope of the dose–response curve; *max* and *min* parameters are the maximum and minimum of the sigmoidal curve. The *min* parameter is fixed at 0. The *max* parameter is fixed at 100 because it was assumed that the growth inhibition is total (100%) at high concentrations. Indeed, for the highest concentration of isoproturon tested in laboratory (256 µg/l) and used to establish the dose–response curve for *S. vacuolatus*, the inhibition was almost complete.

This curve is calculated by using the statistics software Prism (GraphPad, San Diego, CA, USA).

2.5.3. Tolerance evaluation

The potential increase in tolerance of algae after sequential pulse exposure was tested. To do so we compared the EC50s obtained with a standard test with isoproturon at the beginning and at the end of the scenario. This was done with an ANCOVA analysis with the statistic software Prism (GraphPad software, Inc). Increase or decrease of EC50 values would indicate a modification of the algae sensitivity (Vallotton et al., 2009).

2.5.4. Cell density inhibition at the end of the pulse exposure scenario

The endpoint of these pulse exposure experiments, i.e. the overall algae cell density inhibition of each scenario ($Inh_{pulse\text{-}laboratory}$), was obtained by calculating the average of the algae cell density inhibition of each replicate ($Inh_{pulse\text{-}laboratory\text{-}replicate\ i}$). The $Inh_{pulse\text{-}laboratory\text{-}replicate\ i}$ was calculated as (Eq. (3)):

$$Inh_{pulse-laboratory-replicate\ i} = 100 \times \frac{OD_{control} - OD_{pulse,\ replicate\ i}}{OD_{control}} \quad (3)$$

with $OD_{control}$, the average optical density for the three controls at the end of the experiment and $OD_{pulse,replicate\ i}$, the final optical density for the replicate i of alga exposed to pulses concentration. Indeed, as a reminder, the cell density or biomass is measured as the optical density. The variables $OD_{control}$ and $OD_{pulse,replicate\ i}$ are determined in two steps. First, the optical density data measured were summed up, i.e. after each pulse exposure period, the following recovery period and the following pulse exposure period were summed to the last optical density value of the previous pulse exposure period. Second, the alga *S. vacuolatus* grew exponentially during the test. The growth was measured by optical densities, transformed in natural logarithms, as a function of time. Therefore, each section of the control and of the replicate i of alga exposed to pulses concentration, corresponding to the different phases of growth (i.e. the growth before the first pulse, the pulses, the recoveries), was fitted with a linear regression (Fig. 2). Finally, $OD_{control}$ and $OD_{pulse,replicate\ i}$ were calculated as following Eqs. (4) and (5):

$$OD_{control} = \exp[\ \ln\left(OD_{algae-initial}\right) + \mu_{growth-control} \times t_v + \sum_{k=1}^{n} \mu_{pulse\ k-control} \times \Delta t_k + \sum_{j=1}^{m} \mu_{recovery\ j-control} \times \Delta t_j\ ] \quad (4)$$

$$OD_{pulse,replicate\ i} = \exp[\ \ln\left(OD_{algae-initial}\right) + \mu_{growth-replicate\ i} \times t_v + \sum_{k=1}^{n} \mu_{pulse\ k-replicate\ i} \times \Delta t_k + \sum_{j=1}^{m} \mu_{recovery\ j-replicate\ i} \times \Delta t_j\ ] \quad (5)$$

with $\mu_{growth\text{-}control}$ and $\mu_{growth\text{-}replicate\ i}$, the growth rates of the linear regression curves of the controls' average and of the alga's replicate i before the first pulse; t_v, the duration of the laboratory phase before the first pulse; $\mu_{pulse\ k\text{-}control}$ and $\mu_{pulse\ k\text{-}replicate\ i}$, the growth rates of the linear regression curves of the controls' average and of the alga's replicate i during the pulse k. These values were fixed to 0 when the growth rates of the linear regressions during the pulse were negative; Δt_k, the duration of the laboratory pulse period k; n, the number of pulses during the experiment in laboratory; $\mu_{recovery\ j\text{-}control}$ and $\mu_{recovery\ j\text{-}replicate\ i}$, the growth rates of the linear regression curves of the controls' average and of the alga's replicate i during the recovery j; Δt_j, the duration of the laboratory recovery period j; m, the number of recovery periods during the experiment in laboratory; $OD_{algae\text{-}initial}$ is the optical density corresponding to the initial cell density of the algae used for the pulse exposure test determined with the calibration curve of the corresponding alga. For *S. vacuolatus*, the initial cell density was fixed to 650,000 cells/ml. The $OD_{algae\text{-}initial}$ was then 0.056.

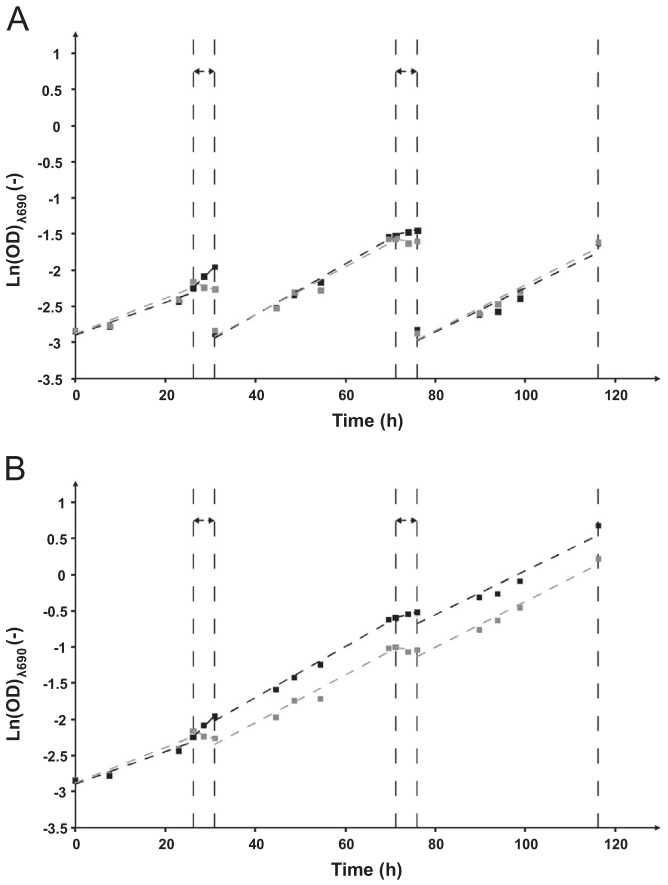

Fig. 2. Optical density values of the scenario 3. (A) non-summed values. After each pulse, algae were re-suspended in the fresh medium at an initial cell density of 650,000 cells/ml to allow a new exponential growth. (B) summed values. Black squares: controls. Grey squares: algae exposed to pulses. Black dotted lines: linear regressions fitted on data for each section of the control. Grey dotted lines: linear regressions fitted on data for each section of the replicate of the culture exposed to pulses. The lengths of pulses are indicated by arrows on the top of each graph.

2.5.5. Modelling

The cell density inhibition at the end of the experiment can also be expressed as (Eq. (6)):

$$Inh_{pulse-modelling} = 100 \times \frac{E_{control} - E_{pulse}}{E_{control}} \quad (6)$$

where E_{pulse} is the final optical density of the model for the alga exposed to pulse concentration; $E_{control}$ is the predicted final optical density for the model's control.

$E_{control}$ can be expressed as (Eqs. (7a) and (7b)):

$$E_{control} = \exp[\ \ln\left(OD_{algae-initial}\right) + \mu \times t_{2n+1}] \quad (7a)$$

or

$$E_{control} = \exp[\ \ln\left(OD_{algae-initial}\right) + \mu \times t_{2n}] \quad (7b)$$

where t_{2n+1} or t_{2n} is the total test duration. t_{2n+1} is used if there is a recovery phase before the end of the scenario (Eq. (7a)). t_{2n} is

used if there is a pulse period before the end of the scenario (Eq. (7b)); n is the number of pulses during the test; $OD_{algae\text{-}initial}$ is the optical density corresponding to the initial cell density of algae

The final $Inh_{pulse\text{-}modelling}$ is expressed as (Eqs. (9a) and (9b)):

$$Inh_{pulse-modelling} = 100 \times \frac{\exp[\ln(OD_{algae-initial}) + \mu \times t_{2n+1}] - \exp\left\{\ln(OD_{algae-initial}) + \mu \times \left[\sum_{i=0}^{n}(t_{2i+1} - t_{2i})\right] + \mu_{inh\,x} \times \left[\sum_{i=1}^{n}(t_{2i} - t_{2i-1})\right]\right\}}{\exp[\ln(OD_{algae-initial}) + \mu \times t_{2n+1}]} \quad (9a)$$

or

$$Inh_{pulse-modelling} = 100 \times \frac{\exp[\ln(OD_{algae-initial}) + \mu \times t_{2n}] - \exp\left\{\ln(OD_{algae-initial}) + \mu \times \left[\sum_{i=0}^{n-1}(t_{2i+1} - t_{2i})\right] + \mu_{inh\,x} \times \left[\sum_{i=1}^{n}(t_{2i} - t_{2i-1})\right]\right\}}{\exp[\ln(OD_{algae-initial}) + \mu \times t_{2n}]} \quad (9b)$$

determined with the calibration curve of the alga. It was fixed to 650,000 cells/ml for the alga *S. vacuolatus*. The corresponding optical density was then 0.056. μ is the growth rate of the control determined as the average growth rates of several successive batch cultures in growth media (control charter). This growth rate is assumed to be constant for the control and during recovery periods (hypothesis 1). Indeed, Vallotton et al. (2008a) showed that the alga *S. vacuolatus* exposed to isoproturon, and more generally to photosystem II inhibitors, recover directly after exposure.

E_{pulse} can also be expressed as (Eqs. (8a) and (8b)):

$$E_{pulse} = \exp\left\{\ln(OD_{algae-initial}) + \mu \times \left[\sum_{i=0}^{n}(t_{2i+1} - t_{2i})\right] + \mu_{inh\,x} \times \left[\sum_{i=1}^{n}(t_{2i} - t_{2i-1})\right]\right\} \quad (8a)$$

or

$$E_{pulse} = \exp\left\{\ln(OD_{algae-initial}) + \mu \times \left[\sum_{i=0}^{n-1}(t_{2i+1} - t_{2i})\right] + \mu_{inh\,x} \times \left[\sum_{i=1}^{n}(t_{2i} - t_{2i-1})\right]\right\} \quad (8b)$$

where t_{2i} can be either the beginning of a recovery period or the end of a pulse exposure period; t_{2i+1} is the end of a recovery period; t_{2i-1} is the beginning of a pulse exposure period; $\mu_{inh\,x}$ is the growth rate at concentration x determined from the growth response curve of the standard ecotoxicity test; the second term of the equations corresponds to the growth of the alga at the beginning of the pulse exposure test and to the recovery periods of the alga whereas the third term corresponds to the pulse periods. The first Eq. (8a) is used when the recovery phase corresponds to the end of the scenario whereas the second one (Eq. (8b)) is used when the pulse exposure period corresponds to the end of the scenario.

In this model, the growth rate at a given concentration x of isoproturon ($\mu_{inh\,x}$) and for the alga *S. vacuolatus* is assumed to be similar and constant during each pulse exposure (hypothesis 2). Vallotton et al. (2009) showed that this assumption is valid by comparing the inhibition of the photosynthesis during successive pulses of isoproturon at a similar concentration for *S. vacuolatus*.

The first Eq. (9a) is used when the recovery phase corresponds to the end of the scenario whereas the second one (Eq. (9b)) is used when the pulse exposure period corresponds to the end of the scenario.

As the true values of the variables μ and $\mu_{inh\,x}$ of Eqs. (9a) and (9b) are unknown, we took into account their uncertainties in the simulation. The distribution of the parameter μ can be obtained from the control charter. According to the Shapiro and Wilk test, the parameter μ is assumed to follow a normal distribution. For $\mu_{inh\,x}$, the distribution can be established from the dose response curve and is also assumed to be normal (but not enough data were available to test it). The distribution of the $Inh_{pulse\text{-}modelling}$ is estimated based on a Monte Carlo simulation. To do so, 10,000 growth rates μ and $\mu_{inh\,x}$ are selected randomly from their respective distributions. Only values from the distribution of μ and $\mu_{inh\,x}$, located between "average − 2 × standard deviation" and "average + 2 × standard deviation", were chosen in order to consider 95% of possible data from μ and $\mu_{inh\,x}$ (Motulsky, 1995). By repeating the calculation (Eqs. (9a) and (9b)) many times (between 9000 and 10,000) and assuming that values of μ must be higher than $\mu_{inh\,x}$ (hypothesis 3) in order to have all the values of $Inh_{pulse\text{-}modelling}$ positives, the mean of $Inh_{pulse\text{-}modelling}$ is calculated for each scenario, along with minimum and maximum values. The model is applied using Matlab software (MATLAB R2011b, The Mathworks Inc.). The predictive results of these Monte Carlo simulations were used to compare with the laboratory observations in order to validate the model.

2.6. Illustrative case study

Finally, the model is used to predict the effects on the cell density of the alga *S. vacuolatus* of a typical scenario that can be observed in rivers. The illustration of the scenario is available in the supplementary information (SI) (Fig. 1S). As long-term measurements of isoproturon concentrations in rivers are not found, a typical pattern of herbicide pollution in creeks is used (Leu, 2003) and this scenario is adapted to isoproturon.

The scenario was composed of 54 pulses. Twenty-three pulses of short duration and low concentrations, i.e. low compared to usual measured concentrations in Switzerland (< 2.5 µg/l) (Leu, 2003), were identified. 9 pulses of long duration (> 10 h) and low concentrations, 14 pulses of short duration (< 10 h) and high concentrations (> 2.5 µg/l) and, finally, 8 pulses of long duration and high concentration were also observed. The maximum concentration reached is fixed to 42,000 ng/l, a concentration measured by IFEN for isoproturon (2007). Using Matlab software

(MATLAB R2011b, The Mathworks Inc.), the concentrations between two measured concentrations, for the same pulse, were interpolated. As a result, the concentrations are available for each minute over the entire duration of the scenario. A histogram was created to characterise the exposure between two minutes and therefore a pulse is described by several histograms with a duration of one minute each and a height corresponding to the concentration interpolated. For further information, see the SI (Fig. 1S). This solution was chosen to be applicable for the model developed in this study (Fig. 1B and C).

3. Results and discussion

3.1. Laboratory experiments

The dose–response curve established for isoproturon provided an EC50 of 67 μg/l with a 95% confidence interval (61; 73). The other effect concentrations (EC10, EC30, EC70, EC80) used for pulse experiments (Table 1) were 17 μg/l, 39 μg/l, 114 μg/l and 265 μg/l respectively.

The results for the 5 scenarios of repeated pulsed herbicide exposures are presented on Fig. 3. The overall cell density of the algae was inhibited with each tested scenario. For the scenarios with short pulses and long recovery periods (scenarios 1, 2 and 3; Fig. 3A, B and C), the overall cell density inhibition is 15%, 24% and 23%, respectively. For the scenarios with long pulses and short recovery periods (scenarios 4 and 5; Fig. 3D and E), the cell density inhibition is 17% and 44%, respectively. As expected, for the two scenarios types, i.e. for pulse scenario with short or long durations (<6 h or >24 h), the cell density inhibition increases with increasing concentration. This is not surprising as the cell density is inhibited during each pulse, as indicated by a difference between the growth curve of the control (solid line) and the growth curve of the exposed algae (dotted line). For scenario 3, the cell density inhibition is close to that of scenario 2, even if the concentration tested is higher. However, the difference in the concentration is not high (Table 1) that can explain this low difference. Even if the recovery is instantaneous, isoproturon has a cumulative effect on the algae production. For this reason, the pulse duration plays an important role on the overall effect. A long pulse at a low concentration will have a higher effect than a very short pulse at a high concentration. In this study, scenario 5 (EC30 tested) therefore had a greater effect than scenarios 1, 2 and 3 (EC50, EC70 and EC80 tested). Even with a very low concentration tested during pulses (scenario 4, EC10 tested), the cell density inhibition remained substantial.

No increase in algae tolerance was observed at the end of the sequential pulse exposure. Indeed, standard toxicity tests were performed at the end of scenarios 3 and 5. EC50s with corresponding 95% confidence intervals were 63.27 μg/l (56.91; 70.34) and 61.96 μg/l (54.06; 71.02), respectively. These values are not statistically different from the EC50 obtained from the standard test (see beginning of Section 3.1; ANCOVA analysis, p-value >0.05). We therefore do not observe a shift in sensitivity of the alga *S. vacuolatus*. This is in agreement with Weber et al. (2012), i.e. that the sensitivity or the growth was not modified after a repeated exposure to isoproturon. However, Vallotton et al. (2009) showed that for long pulses exposures with short recovery, the EC50 estimated from a 72-h acute toxicity test with algae that had been repeatedly exposed was greater than the EC50 estimated for the control algae. In this case, there was a slight shift in sensitivity of algae.

As this is a critical assumption from our model, we tested, in laboratory, the hypothesis 2 that "the slopes during pulse exposure are not statistically different for a scenario if the same concentration is tested" (see Section 2.5.5). The hypothesis was not rejected for long pulses scenarios (scenarios 4 and 5; ANCOVA analysis, p-values >0.05). The p-value was 0.10 and 0.52 for the scenarios 4 and 5 respectively. For scenarios with short pulses (scenarios 1, 2 and 3), the number of optical density measurements was too small (maximum of 3) to calculate any statistic. We also tested the assumption, in laboratory, that "the recovery is complete just after the pulse exposure" (hypothesis 1; see Section 2.5.5). To do so, the slopes of recovery periods were compared with the slopes of the control. The results support the hypothesis

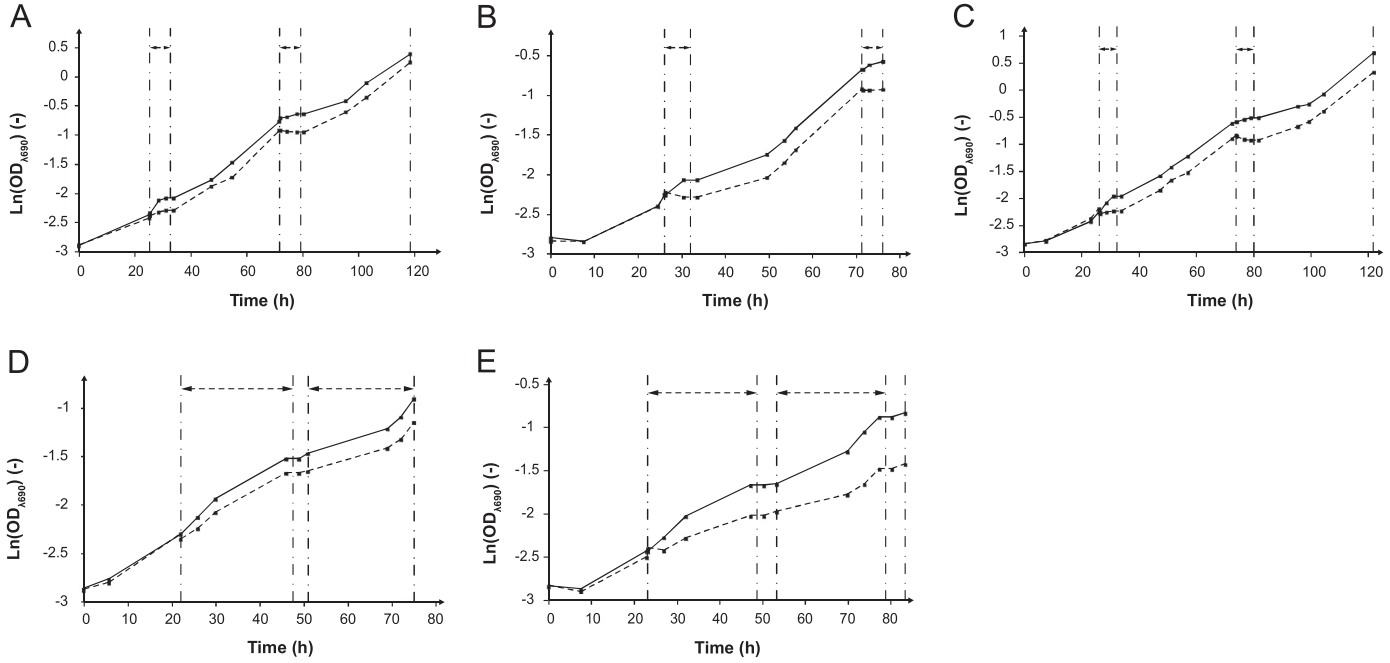

Fig. 3. Growth curves for 5 scenarios of pulse exposure tests in the laboratory. Black curves: controls. Black dotted curves: algae exposed to pulses. (A) Scenario 1. (B) Scenario 2. (C) Scenario 3. (D) Scenario 4. (E) Scenario 5. Scenarios 1, 2 and 3 were for short pulses and long recovery periods. Scenarios 4 and 5 were for long pulses and short recovery periods. The lengths of pulses are indicated by arrows on the top of each graph.

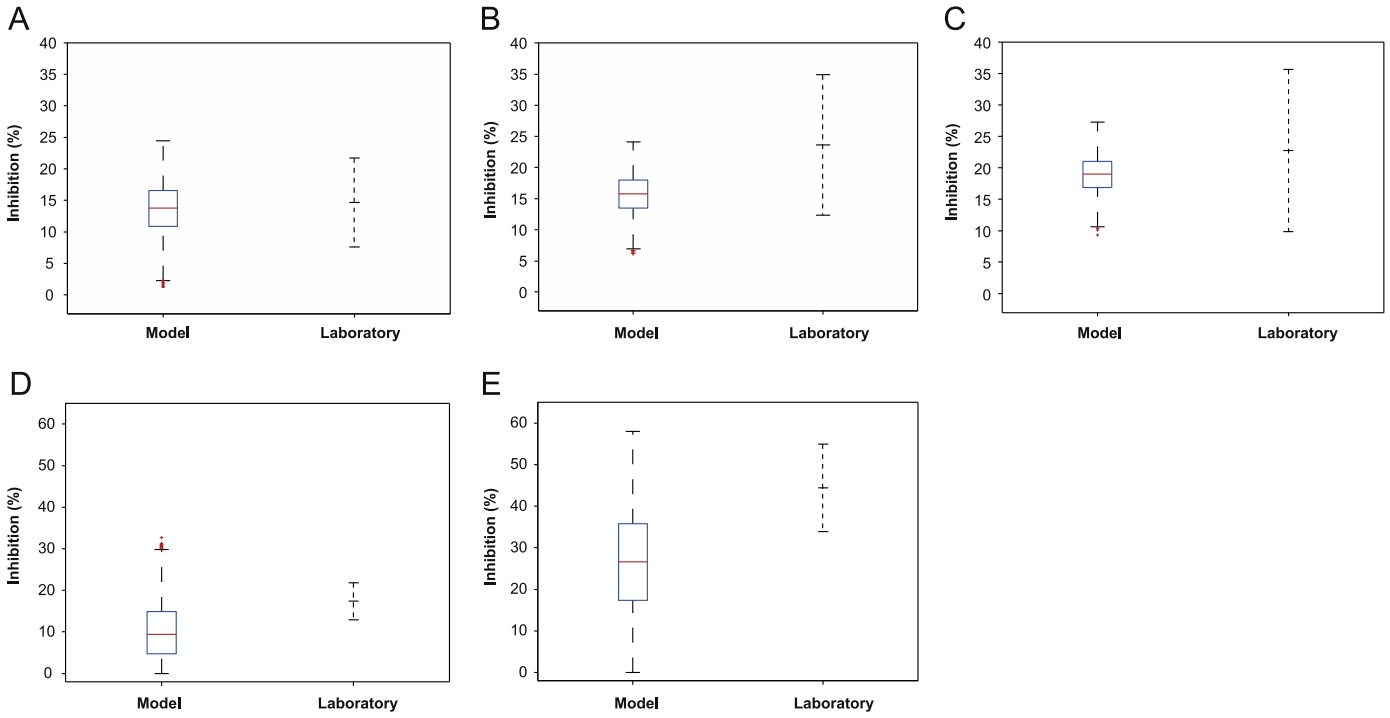

Fig. 4. Boxplots of each scenario for the modelling. Average and standard deviation for laboratory (three replicates). (A) Scenario 1. (B) Scenario 2. (C) Scenario 3. (D) Scenario 4. (E) Scenario 5. Note the difference in the scales of the y axis between figures A, B, C and figures D, E.

1 because the assumption was not rejected for scenarios with long recovery periods (scenarios 1, 2 and 3; ANCOVA analysis, *p*-values > 0.05). For scenarios with short recovery periods (scenarios 4 and 5), this assumption could not be controlled because there were not enough optical density measurements.

3.2. Model application

Fig. 4 presents the cell density inhibitions predicted with the 5 scenarios in the form of boxplots; those correspond on average to 14%, 16% and 19% for scenarios 1, 2 and 3 i.e. with short pulses and long recovery periods. For long pulses and short recovery periods (scenarios 4 and 5), the cell density inhibitions are 10% and 26%, respectively. As already observed with the experimental results, the cell density inhibition is greater for scenario 5 than for scenarios 1, 2 and 3 despite the low concentration selected for the scenario 5 (EC30) compared to the others (EC50, EC70 and EC80). The same observation can be made for scenario 4. Even if the concentration chosen is very low (EC10), the cell density inhibition is very close to cell density inhibitions of scenarios 1, 2 and 3. Therefore, the longer the pulse, the lower the concentration needed to get an inhibition similar to a short but high concentration pulse.

Fig. 4 also presents the minimum and maximum values predicted for each scenario. For scenarios 1, 2 and 3, cell density inhibition values range, respectively, between 1.3% and 24%, 6.2% and 24% and, 9.3% and 27%. For scenarios 4 and 5, these values range between 0% and 33% and, 0% and 58%. For long pulses and short recovery periods (scenarios 4 and 5), the variation of the cell density inhibition is therefore higher than for short pulses and long recovery periods (scenarios 1, 2 and 3). This observation results from the variability given to the different parameters of the model. Indeed, the variability is higher for the parameter $\mu_{inh\ x}$, characterizing the growth during the pulse (standard deviation around 0.004) compared to the variability of the parameter μ, characterizing recovery periods, obtained from the chart control (standard deviation: 0.002). The greater variability of the parameter $\mu_{inh\ x}$ compared to the parameter μ is likely due to the size of the group, parameterising a single experiment versus a higher number of pre-cultures. Therefore, the longer the pulse exposure duration, the larger the variation predicted by the model.

3.3. Comparison between measured and predicted results

The model predicts the observed experimental inhibitions relatively well (Fig. 4). For all the scenarios, the average measured cell density inhibition is included between the minimum and maximum values of the model. Furthermore, for scenario 1 (Fig. 4A), the experimental average cell density inhibition is very close to the average cell density inhibition given by the model (15% and 14%, respectively). For all the other scenarios, the predicted average cell density inhibition is slightly lower than the experimental average, at most 1.7 times lower. Furthermore, for three scenarios (scenarios 1, 4 and 5; Fig. 4A, D and E), the standards deviations of the experimental results are situated within the min–max values given by the model. For the other two scenarios (scenarios 2 and 3; Fig. 4B and C), the experimental average cell density inhibition is also situated below the maximum value given by the model. But the average cell density inhibition predicted is at maximum 1.5 lower than the measured cell density inhibition, as mentioned above. This is in the same order of magnitude as for the other scenarios. Furthermore, for these two scenarios, the variability of the predicted results is lower than that of the others, as discussed above.

If the overall comparison shows that the model could be considered as suitable to predict the effects of the pulse exposure of the different scenarios, one has to note that the predictions seems to slightly underestimate the average cell density inhibition. One reason may be that the different slopes used for modelling were defined as constant. But these slopes may differ slightly during the experiment (see Fig. 5), particularly during the latency phases at the beginning of the test and at the beginning of the recovery parts. However, it is difficult to discuss these differences more deeply as the statistics are based on a low number of laboratory

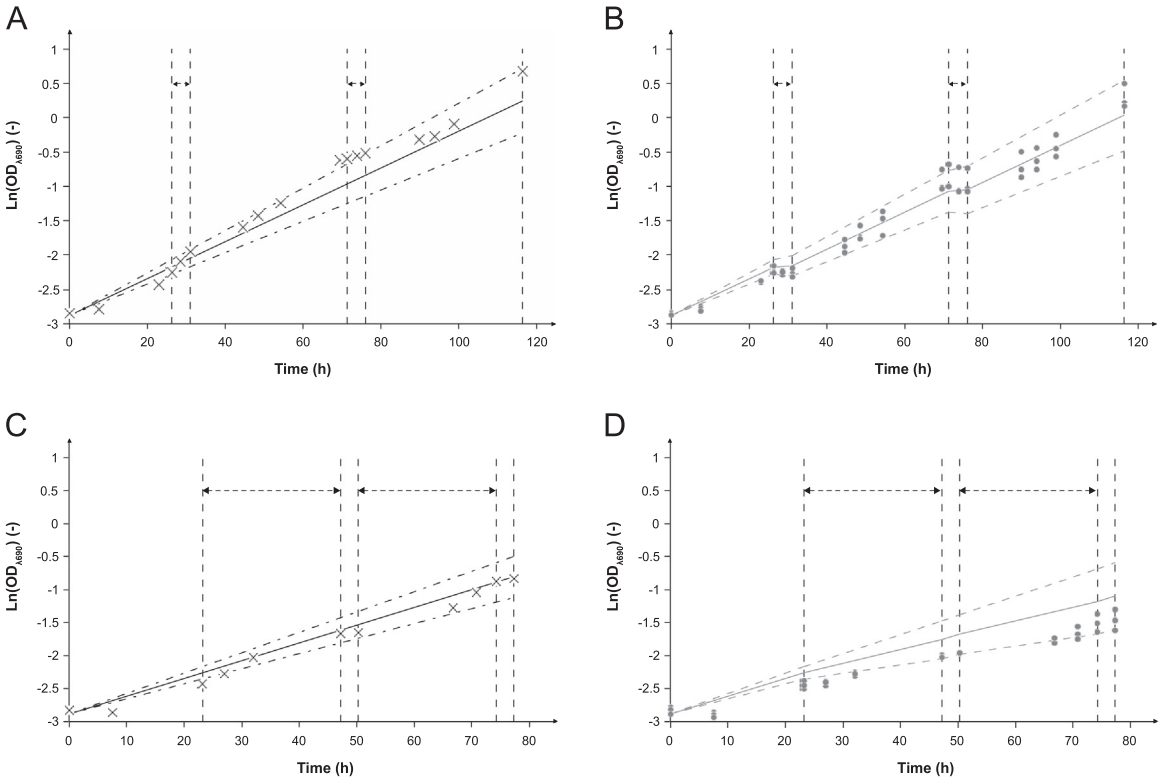

Fig. 5. Comparison between measured and predicted results. (A and B) scenario 3. (C and D) scenario 5. A and C show controls. B and D show culture exposed to pulses. Curves: results from model. Solid curves: average of the model. Dotted curves: representation of "average − 2 × standard deviation" and "average + 2 × standard deviation". Black crosses: average of the control measured in laboratory. Grey circles: triplicates of cultures exposed to pulses measured in laboratory. The optical density value at the beginning of the test was fixed to Ln(0.056). The lengths of pulses are indicated by arrows on the top of each graph.

experiments (3) compared to the model predictions, that accounts for 10,000 results.

The comparison between the experimental and the predicted growth during the whole test is illustrated for scenarios 3 and 5, respectively, in Fig. 5. For scenario 3 (Fig. 5A and B), the majority of the points, representing laboratory control and cultures exposed to pulses respectively, are located within the bands of model predictions, showing a good agreement between predicted and measured inhibition. For scenario 5 (Fig. 5C and D), the points obtained with laboratory experiments representing cultures exposed to pulses, are situated close to the lower band of the model. Consequently, the difference between predicted and measured inhibition is larger than for the scenario 3.

As described in Section 2, μ is assumed to be higher than $\mu_{inh\ x}$ (hypothesis 3; see Section 2.5.5) in order to have predicted inhibition values ($Inh_{pulse\text{-}modelling}$) positives. This means that the pair of parameters μ and $\mu_{inh\ x}$, with values of $\mu_{inh\ x}$ higher than values of μ, are not taken into account in the model calculation. In this study, we tested both methods, i.e. with and without the hypothesis 3. For scenario 1, 2 and 3, no differences existed as all μ values are higher than $\mu_{inh\ x}$ values. For scenario 4 and 5, we obtained better adequacy between predictions and measurements when the assumption of μ higher than values of $\mu_{inh\ x}$ was made. All the data are given in the SI (Table. 1S). For example, for scenario 4, we predicted 10% inhibition and observed 17%. Without any assumption of μ higher than $\mu_{inh\ x}$, we predicted 4%. We therefore decided to make this hypothesis in model calculations.

3.4. Application of the modelling in a real case

The model was applied to simulate the effect of a typical environmental exposure scenario of the herbicide isoproturon on the alga *S. vacuolatus* (see SI, Fig. 1S). The total predicted cell density inhibition, with its minimum and maximum values, is 19 (0; 36)%. This is high considering that the scenario was tested only on one alga. The peak concentrations are often low and short (23 pulses, each one with a duration less than 1 h) and induce together less than 1% of effect. This percentage effect corresponds to 3% of the total cell density inhibition of the entire scenario. However, as expected, the low peaks with a long duration (9 pulses) induce, together, the principal effect (11%) corresponding to 55% of the total cell density inhibition of the entire scenario. Similarly, in the experiments, the low peaks with a long duration induce an important cell density inhibition (as illustrated by scenarios 4 and 5 in the model application; Section 3.2). The whole inhibition of the scenario is therefore mainly driven by the set of the longest peaks. But it is also influenced by the set of high peaks (22 pulses). Indeed, for these highest peaks, the cell density inhibition corresponds to 42% of the total cell density inhibition of the entire scenario with 54 pulses. Furthermore, considering only the 4 highest pulses (corresponding to maximum concentrations of 42,000; 40,000; 35,000 and 32,000 ng/l respectively, see SI, Fig. 1S), the cell density inhibition corresponds to 27% of the total cell density inhibition of the entire scenario. Moreover, this set of highest peaks can also be considered as long pulses because their duration is higher than 10 h. Consequently, the long high pulses of the entire scenario induce an important part of the whole cell density inhibition on the alga *S. vacuolatus*.

Furthermore, for the highest pulses, the steeper the increase or decrease (in term of concentration at the beginning or respectively at the end of the pulse), the less effect they induce. Thus, in the case study, the pulse with a high concentration of 42,000 ng/l is narrow, i.e it has a steep increase and decrease, and provokes a cell density inhibition of 1.11% i.e. 6% of the total cell density inhibition of the entire scenario. In contrast, the same duration pulse that reachs only 35,000 ng/l, is larger and provokes a higher cell

density inhibition (1.48% i.e. 8% of the total cell density inhibition of the entire scenario). Consequently, to evaluate the effects of the photosystem II inhibitor isoproturon on the alga *S. vacuolatus*, it is important to be able to capture the highest peak concentrations and also the peaks with a long duration. This is applicable for small creeks, for which high dynamic of concentrations of isoproturon are observed but also in rivers where the peak duration is longer.

4. Conclusion

Our model allows any kind of scenario for isoproturon to be predicted on the alga *S. vacuolatus* based on a few parameters that are easily determined with classical ecotoxicity experiments. Indeed, the only condition for applying this model is the knowledge of the dose–response curve for isoproturon on the alga *S. vacuolatus*. In particular, the parameter $\mu_{inh\ x}$, measured for isoproturon with a standard toxicity test, is crucial for predicting the average cell density inhibition. More generally, further research are needed to determine if the model is suitable for predicting the effect of pulses of other photosystem II inhibitors and for other substances with a different mode of action but also with other algae. Pulse testing with multispecies cultures should also be conducted to improve the model. In conclusion, the model can be considered as suitable to assess effects of pulse exposure scenarios for the photosystem II inhibitor isoproturon on the alga *S. vacuolatus* in exponential growth. The differences between laboratory and model can be considered minor. However, it is important to have complete information about growth rates of control and exposed cultures.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2014.12.010.

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Modelling the effects of pulse exposure of several PSII inhibitors on two algae

Pierre-Jean Copin *, Nathalie Chèvre

Institut des dynamiques de la surface terrestre (IDYST), Faculté des Géosciences et de l'Environment, Université de Lausanne, Géopolis, Quartier Mouline, CH-1015 Lausanne, Switzerland

HIGHLIGHTS

- Pulse exposure is characteristic of herbicide concentrations in streams.
- Effects of photosystem II inhibitors pulse exposure on several algae are modelled.
- Predicted results are consistent with experimental observations in laboratory.
- Toxicity ranking obtained with standard test is conserved for pulse exposure test.
- The longest peaks mainly affect the predicted cell density inhibition of algae.

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ABSTRACT

Subsequent to crop application and during precipitation events, herbicides can reach surface waters in pulses of high concentrations. These pulses can exceed the Annual Average Environmental Quality Standards (AA-EQS), defined in the EU Water Framework Directive, which aims to protect the aquatic environment. A model was developed in a previous study to evaluate the effects of pulse exposure for the herbicide isoproturon on the alga *Scenedesmus vacuolatus*. In this study, the model was extended to other substances acting as photosystem II inhibitors and to other algae. The measured and predicted effects were equivalent when pulse exposure of atrazine and diuron were tested on *S. vacuolatus*. The results were consistent for isoproturon on the alga *Pseudokirchneriella subcapitata*. The model is thus suitable for the effect prediction of phenylureas and triazines and for the algae used: *S. vacuolatus* and *P. subcapitata*. The toxicity classification obtained from the dose–response curves (diuron > atrazine > isoproturon) was conserved for the pulse exposure scenarios modelled for *S. vacuolatus*. Toxicity was identical for isoproturon on the two algae when the dose–response curves were compared and also for the pulse exposure scenarios. Modelling the effects of any pulse scenario of photosystem II inhibitors on algae is therefore feasible and only requires the determination of the dose–response curves of the substance and growth rate of unexposed algae. It is crucial to detect the longest pulses when measurements of herbicide concentrations are performed in streams because the model showed that they principally affect the cell density inhibition of algae.

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1. Introduction

Numerous herbicides are regularly detected in surface water worldwide (Kalkhoff et al., 2003; Rabiet et al., 2010; Skark et al., 2004; Stangroom et al., 1998). Many of these herbicides belong to the triazines group and the phenylureas group (Gilliom, 2006; Gregorio et al., 2012), which act as photosystem II inhibitors (Knauert, 2008; Vallotton et al., 2008a). These types of herbicides are often measured at high concentrations because they are among the most important classes of herbicides used in the world (LeBaron et al., 2008; Sorensen et al., 2003; Stangroom et al., 1998). In Switzerland, numerous triazines and phenylureas are also regularly detected at high concentrations in surface waters, such as lakes (Chevre et al., 2008; Gregorio et al., 2012) and streams (Chevre et al., 2006; Munz et al., 2013).

After agricultural application and during and after rain events, herbicides are transported by surface runoff or drainage from the

* Corresponding author.
E-mail addresses: pierre-jean.copin@unil.ch (P.-J. Copin), nathalie.chevre@unil.ch (N. Chèvre).

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site of application to the surface water (Daouk et al., 2013; Rabiet et al., 2010). Consequently, in streams located in small agriculture catchments, herbicides usually occur as pulses in connection with the flux of runoff water (Boxall et al., 2013; Petersen et al., 2012). The pulses of herbicides occurring in creeks can affect the density and composition of phytoplankton, benthic and epiphytic microalgae, and macroalgae living in these streams. This is critical for the whole aquatic ecosystem, as they are energy sources for many species (Hoffman, 2003). In the laboratory, freshwater algae are generally submitted continuously to herbicides to test their toxicities (OECD, 2011). However, this type of test does not allow for an evaluation of the effect of pulsed exposure, such as that occurring in streams. Indeed, in the environment, the exposure duration of herbicides can be either long with low concentrations or short with high concentrations (Leu, 2003). It is therefore crucial to simulate these types of pulse exposure scenarios in the laboratory to improve the environmental risk assessment of pulse exposure.

Some laboratory experiments were already performed to simulate pulse exposure to herbicides with several types of algae, such as *Scenedesmus vacuolatus*, but mostly with photosystem II inhibitors (Baxter et al., 2013; Copin et al., 2015; Prosser et al., 2013; Vallotton et al., 2008a,b; Vallotton et al., 2009). Other studies investigated the effects of pulses on several types of floating macrophytes, such as *Lemna minor* (Boxall et al., 2013; Brain et al., 2012; Cedergreen et al., 2005; Mohammad et al., 2010; Teodorovic et al., 2012), or on periphyton communities (Laviale et al., 2011; Tlili et al., 2008). All of these studies showed that pulse exposures affect organisms differently than continuous exposures. They also highlighted that the recovery following a pulse exposure of a photosystem II inhibitor were rapid and complete.

Different models were developed to estimate the effects of pulse exposures on aquatic organisms (Ashauer et al., 2006; Nagai, 2014; Weber et al., 2012). For microcrustacea and fish, the most appropriate models consider toxicokinetics (i.e., the time course of uptake, biotransformation, and elimination of toxicants in the organism) and the toxicodynamics (i.e., the dynamics of injury and recovery in the organism) (Ashauer et al., 2006, 2010). Recently, we developed a simple model, including a few parameters, to assess the effect of pulse exposure on algae. It was successfully validated for the photosystem II inhibitor isoproturon on the algae species *S. vacuolatus* (Copin et al., 2015). This model assumes that the external and the internal concentrations are similar for algae and therefore mainly includes toxicodynamic factors, i.e., it is a function of injury and recovery (Rozman and Doull, 2000).

The aims of this study are two-fold. First, we propose to extrapolate and validate the model of pulse exposure for other photosystem II inhibitors, such as diuron, a substance belonging to the same herbicide group as isoproturon (phenylureas), and atrazine, belonging to the triazine group. Indeed, atrazine is one of the most famous triazines. Although it was banned in the European Union in 2004, it is still widely used in selective weed control programs for corn and sorghum cultures in other countries, such as the USA (Hoffman, 2003; Loos and Niessner, 1999; Sass and Colangelo, 2006). During the late 1990s, it was the most often-used pesticide in the USA. Indeed, it was used on more than two-thirds of U.S. acreage (Fishel, 2006; Hoffman, 2003). Atrazine is therefore largely found in surface waters in the USA (Solomon et al., 1996). Phenylureas, such as diuron and isoproturon, are principally used for pre- or post-emergence weed control in cotton, fruit and cereal crops worldwide (Sorensen et al., 2003). They are also found in surface waters (Gilliom, 2006). The second goal is to extrapolate and validate the model with another alga, *Pseudokirchneriella subcapitata*, with the herbicide isoproturon.

2. Materials and methods

2.1. Chemicals

Atrazine (Atrazine Pestanal® 99.1%, $C_{18}H_{14}ClN_5$), diuron (Diuron Pestanal® 99%, $C_9H_{10}CL_2N_2O$) and isoproturon (Isoproturon Pestanal®99.9%, $C_{12}H_{18}N_2O$) were purchased from Sigma–Aldrich. Atrazine belongs to the triazines group. Diuron and isoproturon belong to the phenylureas group. The 3 herbicides are moderately soluble (respectively 35, 35.6 and 70.2 mg/L) and have low octanol–water partition coefficients (respectively 2.7, 2.87 and 2.5). They thus have low to moderate tendencies to accumulate in biota. These 3 herbicides inhibit photosynthesis by interrupting electron transport through photosystem II (PSII) (Knauert, 2008; Vallotton et al., 2008a). Stock solutions of 10,000 μg/L (atrazine), 3200 μg/L (diuron) and 3200 μg/L (isoproturon) were prepared in an algae medium, under axenic conditions, for pulse exposure testing (nominal concentrations). These stock solutions were stored at 6.4 °C. The concentrations were checked analytically at the beginning of the several experiments in laboratory, and the measured concentrations were in the same range as the nominal concentrations. Indeed, the concentrations measured for atrazine, diuron and isoproturon were respectively 9930, 3150 and 3100 μg/L.

2.2. Algal culture experiments

2.2.1. Culture condition

Permanent agar culture tubes of the green unicellular microalgae *S. vacuolatus* (Chlorophyceae; strain 211-15, Shihira and Krauss, Melbourne, Australia) and *P. subcapitata* (Chlorophyceae; strain 61.81, Nygaard, Komárek, J. Kristiansen and Skulberg, Akershus, Norway) were obtained, respectively, from the Department of Bioanalytical Ecotoxicology at the Helmholtz Centre for Environmental Research – UFZ, Leipzig, Germany and from the Institute for Plant Physiology of the University of Göttingen, Germany. Microalgae were cultured as described by Copin et al. (2015), i.e., in 250-mL Erlenmeyer flasks with 50 mL of OECD medium (OECD, 2011; Van der Vliet, 2007) and maintained in exponential growth on an HT Infors shaker table (90 rpm) at 25 °C under continuous illumination at a light intensity of 30 μmol/m^2/s provided by cool-white fluorescent lamps.

The optical density was measured with a microplate reader (ELx800™, BioTek® Instruments, Winooski, Vermont) at a wavelength of 690 nm. For *S. vacuolatus* and *P. subcapitata*, algae were inoculated in a new culture medium with, respectively, an initial optical density of 0.056 and 0.040 at 690 nm ($OD_{\lambda 690}$), which correspond to densities of 650,000 and 200,000 cells/mL. These initial algae densities were chosen to have enough algal biomass after the procedure of centrifugation used in the pulse exposure tests (see Section 2.2.3). With these initial algae densities, the growth was exponential during almost 48 h. A similar procedure was followed by Vallotton et al. (2009).

A control charter was established to monitor algae growth in the growth media. In our laboratory, the average growth rate of *S. vacuolatus*, based on optical density values, was 0.023 h^{-1}, with a standard deviation of 0.002 h^{-1} (average of successive 45 cultures). It was 0.028 h^{-1}, with a standard deviation of 0.002 h^{-1} (average of successive 51 cultures), for *P. subcapitata*. These values corresponded to 0.035 h^{-1} and 0.059 h^{-1} if cell density values are used to determine the growth rates.

2.2.2. Standard acute toxicity tests

The dose–response curves of isoproturon, atrazine and diuron required us to parameterize the model and to define the tested concentrations, established following a method adapted from the standard OECD procedure (OECD, 2011). The tests were performed

in the same conditions as the algae cultures (see Section 2.2.1). A 48-h duration was chosen for testing (1) because the growth of the algae *S. vacuolatus* and *P. subcapitata*, with their initial cell densities fixed respectively to 650,000 cells/mL and 200,000 cells/mL, was exponential during almost 48 h (2) to reduce the possibility of a decrease of the tested concentration caused by the sorption of the toxicant to the rapidly increasing algal biomass (Hoffman, 2003). However, the sorption of atrazine, diuron and isoproturon was assumed to be limited on the algae cell surface. Indeed, these substances have low octanol–water partition coefficients (respectively 2.7, 2.87 and 2.9) and therefore these herbicides are rather hydrophilic (3) to diminish the pH change in the test water (Hoffman, 2003). For atrazine and diuron, 6 concentrations ranging from, respectively, 20 to 150 µg/L and 5 to 75 µg/L, and a control were tested in triplicate on *S. vacuolatus*. For isoproturon, 7 concentrations ranging from 10 to 200 µg/L and a control were tested in triplicates on *P. subcapitata*. The optical density measured at the beginning and end of the test was used to evaluate the average specific growth rate for each concentration and for the control. Growth inhibition is the ratio between the growth rates of the different concentrations and that of the control (Eq. (1); see Section 2.3.1).

2.2.3. Pulse exposure tests

Three pulse exposure scenarios were tested in the laboratory for each herbicide and algae (Fig. 1; Table 1). They differed in terms of the herbicide and alga used. The design of each scenario included two cases: a short pulse duration (W = 5.25 h) and a long recovery period (X = 24 h) for the first part of the scenario following by a long pulse duration (Y = 20 h) and a long recovery period (Z = 48 h) in the second part of the scenario. These cases in the same scenario can be considered as representative of two extreme scenarios that can be found in rivers. Concentration levels of the pulses for each herbicide used are summarized in Table 1. Different number of replicates was used for the three pulse exposure scenarios. The algae *S. vacuolatus* exposed to atrazine were tested with 6 replicates. The algae *S. vacuolatus* exposed to diuron were tested with 5 replicates. Finally, the algae *P. subcapitata* exposed to isoproturon were tested with 3 replicates.

The test was initiated the same way that the algae were cultured (initial cell density of 650,000 cells/mL for *S. vacuolatus* and 200,000 cells/mL for *P. subcapitata*, 50 mL in a 250-mL flask) and under the same conditions (see Section 2.2.1). Algae grew for a long period (44 h for scenario Atrazine-SV and 43 h for scenarios Diuron-SV and Isoproturon-P) at the beginning before being exposed to the first pulse. At the end of each pulse exposure, the algae were centrifuged twice for 7 min at 1046g and 25 °C. For the algae *S. vacuolatus*, as the algae adhered to the inner tube-surface, the supernatant could be removed. Thereafter, the algae were re-suspended in growth media with an ultra-wave bath (Copin et al., 2015; Vallotton et al., 2008a). For the algae *P. subcapitata*, after the centrifugations, as the algae remained concentrate in the bottom of the centrifugation tube, the supernatant could be removed. These two centrifugations allowed, for the 2 algae, 99% of the herbicide to be removed and did not impair algal growth, as shown by Vallotton et al. (2009). The loss of the algae *S. vacuolatus* and *P. subcapitata* were, respectively, 18% and 28% during the two centrifugations. The recovery period began directly after the algae were re-suspended in the fresh medium at the same initial cell densities fixed at the beginning of the test, i.e. 650,000 cells/mL for *S. vacuolatus* and 200,000 cells/mL for *P. subcapitata*. This choice of cell density was the consequence of (1) the low number of algae of culture exposed to pulses and (2) the loss of algae during the period of centrifugation. Indeed, we had the obligation to have enough algae to re-suspend the algae in the new fresh medium after the procedure of centrifugation. The optical density was measured regularly during the exposure and recovery periods.

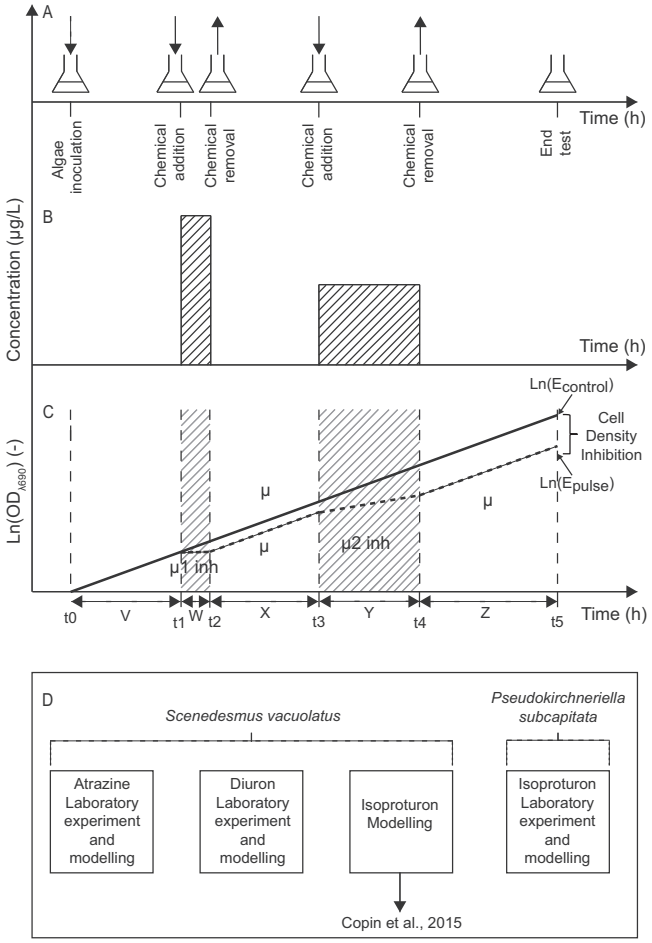

Fig. 1. (A) Procedure of pulse exposure scenarios tested in the laboratory. (B) Pulse representation over time. (C) Modelling representation. μ is the growth rate obtained from the control charter of one alga. $\mu 1_{inh}$ and $\mu 2_{inh}$ are the growth rate at concentration $x1$ and $x2$ obtained from the growth response curve of a substance on one alga. $E_{control}$ is the predicted final optical density for the model's control. E_{pulse} is the final optical density of the model for the alga exposed to the pulse concentration. V corresponds to the growth duration before the first pulse (h); W and Y are the pulse durations (h); X and Z are the recovery durations (h); V is approximately 40 h; W = 5.25 h, X = 24 h, Y = 20 h, and Z = 48 h. (D) Diagram of the substances tested and algae used for the pulse exposure experiments and/or modelling. Adapted from Fig. 1 in Copin et al. (2015).

Table 1
Herbicide and algae used for each pulse exposure test in the laboratory and for the modelling.

Scenario abbreviation	Herbicide	Alga	Concentration	
			First pulse	Second pulse
Atrazine-SV	Atrazine	*S. vacuolatus*	EC_{81}	EC_{37}
Diuron-SV	Diuron	*S. vacuolatus*	EC_{75}	EC_{21}
Isoproturon-P	Isoproturon	*P. subcapitata*	EC_{80}	EC_{28}

2.3. Data analysis

2.3.1. Growth inhibition in a standard toxicity test on algae

The endpoint of this standard test, i.e., the growth inhibition (I_t) at a given concentration, is determined using the response variables, i.e., the average specific growth rate of the control (μ_C) and the average specific growth rate at this concentration (μ_T) (OECD, 2011) (Eq. (1)):

$$I_t = \frac{\mu C - \mu T}{\mu C} \quad (1)$$

2.3.2. Dose–response curves for the standard acute toxicity test

Dose–response curves are obtained by plotting the growth inhibition I_t as a function of the tested nominal concentrations C. The relationship is expressed by using a four parameter log–logistic dose response model (Vallotton et al., 2008a) (Eq. (2)):

$$I_t = min + \frac{(max - min)}{1 + 10^{((\log EC_{50} - \log C) \times Hillslope)}} \quad (2)$$

where EC_{50} is the concentration inhibiting 50% of the growth; *Hillslope* is the slope of the dose–response curve; and the *max* and *min* parameters are the maximum and minimum of the sigmoidal curve. The *min* parameter is fixed at 0. The *max* parameter is fixed at 100 because it was assumed that the growth inhibition is 100% at high concentrations. Consequently, the relationship is now expressed as a two parameter log–logistic model.

This curve is calculated by using the statistics software Prism (GraphPad, San Diego, CA, USA). A toxicity ranking is established comparing the EC_{50} obtained in this study for all substances (atrazine, diuron and isoproturon) and algae (*S. vacuolatus* and *P. subcapitata*) with the EC_{50} obtained in Copin et al. (2015) for isoproturon and *S. vacuolatus*.

2.3.3. Cell density inhibition at the end of the pulse exposure scenario

The endpoint of these pulse exposure experiments, i.e., the overall algae cell density inhibition of each scenario ($Inh_{pulse\text{-}laboratory}$) with its 95% confidence interval was obtained by calculating the average and the standard deviation algae cell density inhibition of each replicate ($Inh_{pulse\text{-}laboratory\text{-}replicate\ i}$). The $Inh_{pulse\text{-}laboratory\text{-}replicate\ i}$ was calculated as (Eq. (3)):

$$Inh_{pulse\text{-}laboratory\text{-}replicate\ i} = 100 \times \frac{OD_{control} - OD_{pulse,replicatei}}{OD_{control}} \quad (3)$$

with $OD_{control}$, the average optical density for the replicates of the controls at the end of the experiment and $OD_{pulse,replicate\ i}$, the final optical density for the replicate i of alga exposed to pulses concentration. For the control and for each replicate exposed to pulses, to better visualize the effect of pulses on algae, the optical density data measured were summed up, i.e. after each pulse exposure period, the following recovery period and the following pulse exposure period were summed to the last optical density value of the previous pulse exposure period. Thereafter, $OD_{control}$ and $OD_{pulse,replicate\ i}$ were determined using growth rates of the linear regressions fitted on the summed optical density values of the different parts (pulses, recovery) of the control and of the replicate i of the alga exposed to pulses as described in Copin et al. (2015).

2.3.4. Modelling

The cell density inhibition at the end of a pulse exposure scenario can also be expressed as described in Copin et al. (2015), (Eqs. (4a) or (4b)):

$$Inh_{pulse\text{-}modelling=} 100 \times \frac{\exp\left[\ln(OD_{algae\text{-}t0}) + \mu \times t_{2n+1}\right] - \exp\left\{\ln(OD_{algae\text{-}t0}) + \mu \times \left[\sum_{i=0}^{n}(t_{2i+1} - t_{2i})\right] + \mu_{inhx} \times \left[\sum_{i=1}^{n}(t_{2i} - t_{2i-1})\right]\right\}}{\exp\left[\ln(OD_{algae\text{-}t0}) + \mu \times t_{2n+1}\right]} \quad (4a)$$

or

$$Inh_{pulse\text{-}modelling=} 100 \times \frac{\exp\left[\ln(OD_{algae\text{-}t0}) + \mu \times t_{2n}\right] - \exp\left\{\ln(OD_{algae\text{-}t0}) + \mu \times \left[\sum_{i=0}^{n-1}(t_{2i+1} - t_{2i})\right] + \mu_{inhx} \times \left[\sum_{i=1}^{n}(t_{2i} - t_{2i-1})\right]\right\}}{\exp[\ln(OD_{algae\text{-}t0}) + \mu \times t_{2n}]} \quad (4b)$$

where t_{2n+1} or t_{2n} is the total test duration; n is the number of pulses during the test; $OD_{algae\text{-}t0}$ is the optical density corresponding to the initial cell density of algae determined with the calibration curve of the alga. It was fixed at 650,000 cells/mL for *S. vacuolatus* and at 200,000 cells/mL for *P. subcapitata*. The corresponding optical density was then, respectively, 0.056 and 0.040. μ is the growth rate of the control determined as the average growth rate of several successive batch cultures in growth media (control charter). t_{2i} can either be the beginning of a recovery period or the end of a pulse exposure period; t_{2i+1} is the end of a recovery period; t_{2i-1} is the beginning of a pulse exposure period; and $\mu_{inh\ x}$ is the growth rate at concentration x determined from the growth response curve of the standard ecotoxicity test. For real cases of pulse exposure scenarios, Eq. (4a) is used when the recovery phase corresponds to the end of the scenario, whereas Eq. (4b) is used when the pulse exposure period corresponds to the end of the scenario.

2.3.5. Uncertainty analysis

The variables μ and $\mu_{inh\ x}$ of Eqs. (4a) or (4b), with their uncertainties, are obtained respectively from the control charter and from the dose response curve. These uncertainties are taken into account in the model simulation. According to the Shapiro and Wilk test, the parameter μ is assumed to follow a normal distribution. For $\mu_{inh\ x}$, the distribution is also assumed to be normal (but not enough data were available to test it). The distribution of $Inh_{pulse\text{-}modelling}$ is estimated based on a Monte Carlo simulation. To do so, 10,000 growth rates μ and $\mu_{inh\ x}$ are selected randomly from their respective distributions. Only values from the distribution of μ and $\mu_{inh\ x}$, located between "average − 2 × standard deviation" and "average + 2 × standard deviation", were chosen in order to consider 95% of possible data from μ and $\mu_{inh\ x}$ (Motulsky, 1995). By repeating the calculation (Eqs. (4a) or (4b)) between 9000 and 10,000 times (depending on the number of excluded values of the interval defined above) and assuming that values of μ must be higher than $\mu_{inh\ x}$ in order to have all the values of $Inh_{pulse\text{-}modelling}$ positives, the mean of $Inh_{pulse\text{-}modelling}$ is calculated for each scenario, along with minimum and maximum values. The model is applied using Matlab software (MATLAB R2011b, The Mathworks Inc.) and the same parameters of time and concentrations used in the experiments in laboratory (Table 1). The predictive results of these Monte Carlo simulations were used to compare with the laboratory observations to validate the model.

2.3.6. Comparison of predicted cell density inhibitions within the herbicide and algae

In this section, we investigated whether the toxicity ranking observed based on the dose–response curves (see Section 2.3.2) remained similar considering the effects of pulse exposure. For this investigation, we predicted the cell density inhibition of a similar scenario for all of the herbicides and algae (Fig. 1). The concentrations chosen were equivalent, i.e., 28.0 µg/L for the first pulse and 10.6 µg/L for the second pulse. The concentrations of 28.0 and 10.6 µg/L were selected because, in the case study presented in Copin et al. (2015), these latter were lower than the maximum concentration tested (42 µg/L corresponding to EC_{32}). They

induced respectively 20% and 5% of effect (EC_{20} and EC_5) on the alga *S. vacuolatus*. For isoproturon, the concentrations chosen were thus therefore consistent with environmental concentrations. All the other concentrations were identical to these concentrations but the effect concentrations were different according to the different dose–response curves. Therefore, according to the OECD standard tests, the concentrations tested during the first pulse for Atrazine-SV, Diuron-SV and Isoproturon-P corresponded, respectively, to EC_{33}, EC_{79} and EC_{19}. For the second pulse, it was, respectively EC_{13}, EC_{36} and EC_6. For diuron, the concentrations were also consistent with environmental concentrations. Indeed, concentrations of this herbicide was already measured up to 28.0 μg/L in streams in France (IFEN, 2007). For atrazine, the concentrations were not consistent with actual environmental concentrations in Europe. Indeed, the concentrations of this herbicide were not anymore measured up to 28 μg/L (Munz et al., 2013) because it was banned in European Union in 2004 and in Switzerland in 2003. However, in case where the atrazine was used, concentrations were consistent with environmental concentrations (Leu, 2003). The parameters of the model (the growth rates μ and μ_{inh}) were the same as those used in this study (Atrazine-SV, Diuron-SV and Isoproturon P) and in Copin et al. (2015) (Isoproturon-SV).

Furthermore, a pulse exposure is characterised by two parameters, the height and the width, i.e., respectively, the peak concentration and the duration (Reinert et al., 2002). The effects of each pulse for each scenario were compared to highlight which of these two parameters characterizing the pulse had the most influence on the cell density inhibition, i.e., whether the peak duration or the pulse concentration principally affected the predicted cell density inhibition. To this end, the cell density inhibition of each substance (atrazine, diuron, isoproturon) for *S. vacuolatus* and *P. subcapitata* was calculated with the model at the end of each pulse exposure.

3. Results and discussion

3.1. Laboratory experiments

3.1.1. Dose–response curves

The dose–response curves obtained for a 48-h duration of the photosystem II inhibitors diuron, atrazine (*S. vacuolatus*) and isoproturon (*S. vacuolatus* and *P. subcapitata*) are presented in Fig. 2. The data for isoproturon on the alga *S. vacuolatus* were obtained from Copin et al. (2015). For *S. vacuolatus*, the diuron, atrazine and isoproturon dose–response curves provided, respectively, the following EC_{50}s with 95% confidence intervals: 14.3 (11.9; 17.2) μg/L, 49.7 (42.2; 58.6) μg/L and 66.9 (61.2; 73.2) μg/L.

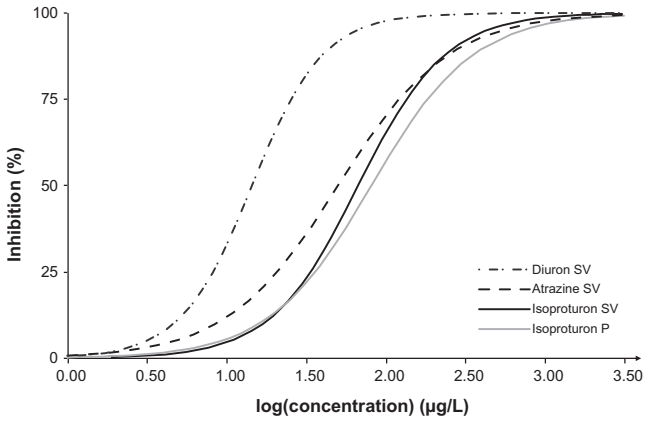

Fig. 2. Dose–response curves for several algae and several photosystem II inhibitors. Black dotted curve with points: Diuron, *S. vacuolatus*. Black dotted curve: Atrazine, *S. vacuolatus*. Black curve: isoproturon, *S. vacuolatus*. The values for this curve were obtained from Copin et al. (2015). Grey curve: isoproturon, *P. subcapitata*.

The EC_{50} of *S. vacuolatus* was found to range between 47.12 μg/L (Backhaus et al., 2004) and 100–110 μg/L (Vallotton et al., 2008a) when the algae was exposed to isoproturon (respectively, for 24 h and 48 h of exposure), which is consistent with our findings. For atrazine, the EC_{50} ranged between 38.82 μg/L (24 h of exposure) (Junghans et al., 2006) and 126–128 μg/L (48 h of exposure) (Vallotton et al., 2008a), which is also consistent with our results.

For *P. subcapitata* treated with isoproturon, the EC_{50} with a 95% confidence interval and an exposure duration of 48 h (initial cellular density of 200,000 cells/mL) is 80.4 (70.2; 92.2) μg/L. For an exposure duration of 72 h tested in the laboratory (initial cellular density of 50,000 cells/mL), the EC_{50} is 72.92 (64.6; 82.4) μg/L. These two values are not significantly different (ANCOVA analysis, p-value > 0.05). The comparison of EC_{50} calculated for an exposure of 48 h can thus be compared with EC_{50}s from the literature obtained for exposures of 72 h.

Based on these EC_{50}s, the toxicity ranking for the alga *S. vacuolatus* was: diuron < atrazine < isoproturon. Diuron was the most toxic substance and isoproturon was the least toxic. This toxicity ranking is consistent with the study of Backhaus et al. (2004), which showed that diuron was the most toxic phenylureas (6.60 μg/L) for the reproduction of *S. vacuolatus*. Faust et al. (1999) also found the same classification as this study while basing their ranking on the EC_{50} of the growth inhibition of *S. vacuolatus*. Similarly, it was demonstrated that diuron was the most toxic substance to photosynthetic activity of lake phytoplankton (Pesce et al., 2011).

Diuron was the most toxic compound followed by atrazine and isoproturon. Furthermore, the toxic ratio between the substances was conserved between isoproturon and diuron when EC_{10} was used instead of EC_{50}, but it was different between isoproturon and atrazine. Indeed, it was equivalent to 3.5 between atrazine and diuron and to 1.3 between isoproturon and atrazine based on the EC_{50} and to 3.6 and 2.0 based on the EC_{10}. As EC_{10} were closer to relevant environmental concentrations (Van der Hoeven et al., 1997; Warne and Van Dam, 2008), the difference observed between isoproturon and atrazine might be important when evaluating the risk of the substances.

The toxicity of isoproturon to *S. vacuolatus* was similar to that of *P. subcapitata:* the EC_{50} of isoproturon was almost identical for *S. vacuolatus* (66.9 μg/L) and for *P. subcapitata* (80.4 μg/L). Furthermore, the toxicity relation of EC_{50} and EC_{10} for the algae *P. subcapitata* and *S. vacuolatus* were, respectively, 1.2 and 0.9. Therefore, for environmental concentrations (EC_{10}), the difference of toxicity for isoproturon was insignificant when *P. subcapitata* and *S. vacuolatus* were used. This similarity of toxicity was confirmed in Fig. 2, because the curves crossed at a concentration of 24.3 μg/L, corresponding to EC_{16}, a concentration very closed to EC_{10}.

The EC_{50}-48 h of *P. subcapitata* is consistent with the results found in the literature. Indeed, this value ranges between 16 μg/L (Pesce et al., 2011) and 128 μg/L (Weber et al., 2012) for isoproturon exposure (72 h of exposure).

3.1.2. Pulse exposure tests

The results for pulse exposure tests are illustrated for the Isoproturon-P scenario in Fig. 3. For this scenario, the cell density was inhibited during each pulse, as indicated by the difference between the growth curve of the control (black curve) and the growth curve of the exposed algae (grey curve) at the end of the experiment. For the scenarios Atrazine-SV and Diuron-SV, inhibition of the cell density was also observed comparing the control and the exposed algae. For the Atrazine-SV, Diuron-SV and Isoproturon-P scenarios, the cell density inhibition in the

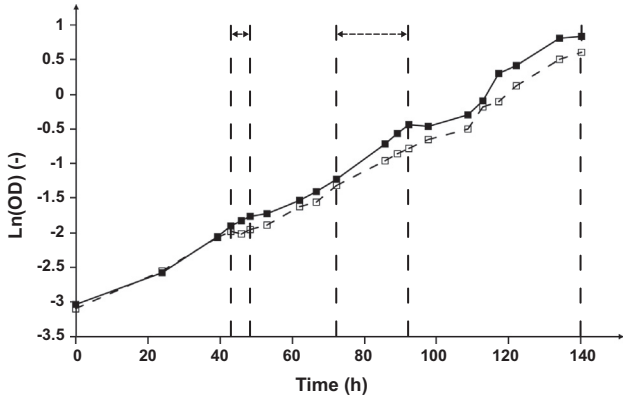

Fig. 3. Growth curves for the Isoproturon-P scenario in the laboratory. Black curve: control. Black squares: measurements for the control. Black dotted curve: algae exposed to pulses. White squares: measurements for algae exposed to pulses. The lengths of the pulses are indicated by arrows on the top of each graph.

laboratory with a 95% confidence interval was, respectively, 23.7 (16.4; 30.9)%, 18.8 (7.2; 30.4)% and 24.8 (10.7; 39)%.

For the three scenarios, we checked the hypothesis that "the recovery is complete just after the pulse exposure in the case the parameter growth rate is considered". To apply the model developed in Copin et al. (2015), we hypothesized that the growth rate μ of the control is identical to the growth rate of the recovery periods. This hypothesis was already controlled in Copin et al. (2015) for *S. vacuolatus* exposed to sequential pulses of isoproturon (ANCOVA analysis). For the Atrazine-SV, Diuron-SV and Isoproturon-P scenarios, the results also support this hypothesis because the assumption was not rejected. Indeed, for *S. vacuolatus* exposed to pulses of atrazine or diuron, the *p*-values were, respectively, 0.17 and 0.43 (ANCOVA analysis, *p*-values > 0.05). The results for atrazine confirm the conclusion of Vallotton et al. (2008a). In this study, we showed that this conclusion could also be extended to diuron. Indeed, it was shown that the recovery was complete following exposure of atrazine to *S. vacuolatus*. For *P. subcapitata*, the *p*-value was 0.6 (ANCOVA analysis, *p*-value > 0.05). This is consistent for *P. subcapitata* according to Reinert et al. (2002), which showed that the recovery from an atrazine pulse exposure up to 50 µg/L was nearly instantaneous when the herbicide was removed (Reinert et al., 2002). Weber et al. (2012) also showed that a peak of isoproturon 10 times the maximum predicted environmental concentration had only transient effects on *P. subcapitata*. Furthermore, no reduced growth was observed after repeated exposure. Finally, Baxter et al. (2013) showed that the recovery of *P. subcapitata* was rapid following a 24-h pulse of high concentrations of atrazine.

3.2. Comparison between the measured and predicted results

The cell density inhibitions predicted by the model for the Atrazine-SV, Diuron-SV and Isoproturon-P scenarios are illustrated in the form of boxplots in Fig. 4. For these 3 scenarios, the experimental average cell density inhibition was very close to the average cell density inhibition given by the model (Fig. 4). The similarity between the measured and the predicted cell density inhibition was better achieved for these scenarios than for the Isoproturon-SV scenarios (Copin et al., 2015). In the previous publication, the comparison showed that the model slightly underestimated the average measured cell density inhibition when isoproturon was tested in pulses on *S. vacuolatus*. Furthermore, for the Atrazine-SV, Diuron-SV and Isoproturon-P scenarios, the variability of the observed experimental inhibitions was located

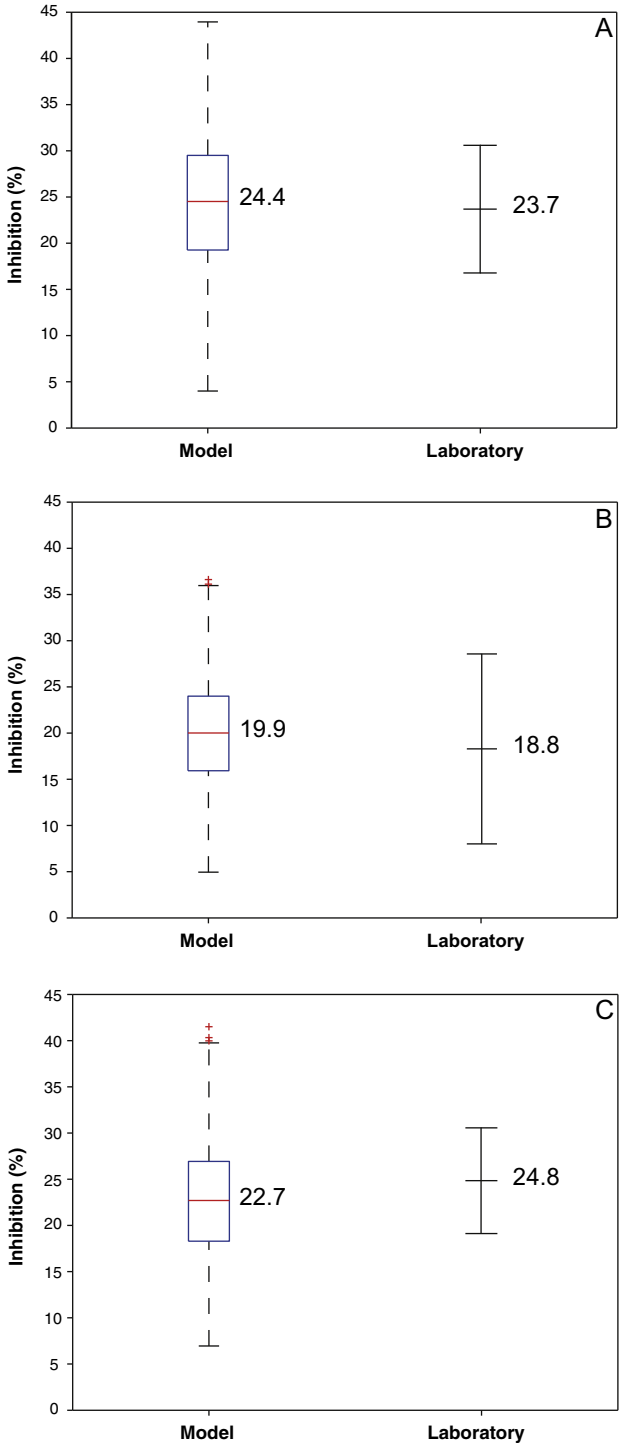

Fig. 4. Boxplots of modelled Atrazine-SV, Diuron-SV and Isoproturon-P scenarios. Average and standard deviation for laboratory results (six replicates of Atrazine-SV, five replicates of Diuron-SV, three replicates of Isoproturon P). The average cell density inhibitions predicted by the model and obtained in the laboratory were noted for each scenario. (A) Atrazine-SV scenario. (B) Diuron-SV scenario. (C) Isoproturon-P scenario.

between the minimum and maximum of the model (Fig. 4). This was not always the case in the Isoproturon-SV scenarios. The variability of the measured cell density inhibition in the laboratory sometimes exceeded the maximum value given by the model. Finally, for the 3 scenarios, the average measured cell density inhibition was included between the first and the third quartile of the model (Fig. 4). For the Isoproturon-SV scenarios, the average cell

density inhibition obtained in the laboratory was often included only between the minimum and maximum of the model and not between the first and third quartile of the model. Therefore, the model predicts more precisely the observed experimental inhibitions. Consequently, the model developed in Copin et al. (2015) can be considered to be suitable for substances belonging to the phenylurea and triazine groups when they are applied on *S. vacuolatus* and *P. subcapitata*.

3.3. Comparison of predicted cell density inhibitions within the herbicide and algae

The cell density inhibition averages predicted for identical concentrations of isoproturon, atrazine and diuron at each pulse exposure for *S. vacuolatus* were, respectively 6%, 13% and 25% with the different scenarios. Diuron was therefore the most toxic substance and isoproturon was the substance that least inhibited the cell density of *S. vacuolatus*. This toxicity ranking is identical to the classification established with the dose–response curves. Indeed, with the pulses concentrations chosen, where were identical for each substance, diuron caused more damage than isoproturon according to the dose–response curves (Fig. 2).

The cell density inhibition predicted average was 8% for *P subcapitata*. This result was very close to the average cell density inhibition predicted for the same substance, and at the same concentrations, on *S. vacuolatus* (6%). Indeed, the two concentrations tested during the two pulses experienced similar effects according to the dose–response curves of isoproturon on the 2 algae (EC_{20} and EC_5 for *S. vacuolatus*; EC_{19} and EC_6 for *P subcapitata*). Furthermore, the growth rates during the recovery phases were similar because the algae growth rates of the control charters were similar (average μ equal to 0.027 (0.002) h^{-1} for *S. vacuolatus* in Copin et al. (2015) and 0.028 (0.002) h^{-1} for *P subcapitata*, respectively). Therefore, the dose response curves and the control charters of several algae were necessary to compare the toxicity of pulse exposure of herbicides (phenylureas and triazines) between 2 algae.

For the Atrazine-SV, Diuron-SV, Isoproturon-SV and Isoproturon-P scenarios, the first pulse inhibited, respectively 4%, 9%, 2% and 2.5% of the cell density, whereas the second pulse provoked an inhibition of 9%, 16%, 4% and 5.5%. Consequently, for each substance and for each alga, a long pulse at a low concentration had a stronger effect than a short pulse at a high concentration. Even if the concentration tested for the first pulse of the Diuron-SV scenario was very high (EC_{79}), the long peaks with a lower concentration had a stronger effect. The effects were thus less caused by the pulse concentrations than by the pulse duration. For all of the scenarios, the results were therefore consistent with the conclusions of the application of the modelling in a real case in Copin et al. (2015). Indeed, in the study of Copin et al. (2015), it was shown that the low peaks of isoproturon on *S. vacuolatus* with a long duration induced the principal effect and the highest peaks influenced also the cell density inhibition.

None of these two parameters, i.e. the concentration and the duration of pulses, had a consequential impact on the recovery potential of the algae, in the case where the parameter growth rates is used, and this even if high concentrations pulses or long pulse durations were applied. Indeed, as shown in Section 3.1.2, after a high peak of exposure to atrazine on *S. vacuolatus* (EC_{81}), diuron on *S. vacuolatus* (EC_{75}) or isoproturon on *P. subcapitata* (EC_{80}), the growth rates of the several controls were not statistically different to the growth rates of the treatment during the first recovery phase (ANCOVA analysis, p-values > 0.05). Similarly, further to a long exposure duration, i.e. 24 h, of atrazine on *S. vacuolatus* (EC_{37}), diuron on *S. vacuolatus* (EC_{21}) or isoproturon on *P. subcapitata* (EC_{28}), the growth rates of the several controls were not statistically different to the growth rates of the treatment during the second recovery phase (ANCOVA analysis, p-values > 0.05).

4. Conclusions

The model developed was previously shown to be suitable to predict the cell density inhibition of any type of pulse exposure scenario on *S. vacuolatus* for herbicides without a delay in the recovery and with substances belonging to the triazine and phenylurea. This model also allows for predicting the cell density inhibition of *P subcapitata* exposed to isoproturon. To establish the toxicity classification of pulse exposure scenarios between several substances or between different algae, the dose–response curves of each substance for each alga as well as the growth rate of each alga obtained with a charter control are necessary. Indeed, the toxicity classification obtained for *S. vacuolatus* exposed to several triazine and phenylurea herbicides, with dose–response curves, is also preserved for pulse exposure scenarios on the same alga with the same substances. This toxicity ranking is also conserved for isoproturon on *S. vacuolatus* and *P subcapitata* because their growth rates during the recovery periods are identical. However, further research must be conducted to adapt this model and to validate it with laboratory experiments of herbicides with delays in the recovery phase such as the S-metolachlor. Pulse testing with multispecies in the same culture should also be conducted to improve the model. In conclusion, the model can be considered to be suitable to assess the effects of pulse exposure scenarios for photosystem II inhibitors, such as triazines and phenylureas, on *S. vacuolatus* and *P subcapitata* in exponential growth.

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Modelling the effect of exposing algae to pulses of S-metolachlor: How to include a delay to the onset of the effect and in the recovery

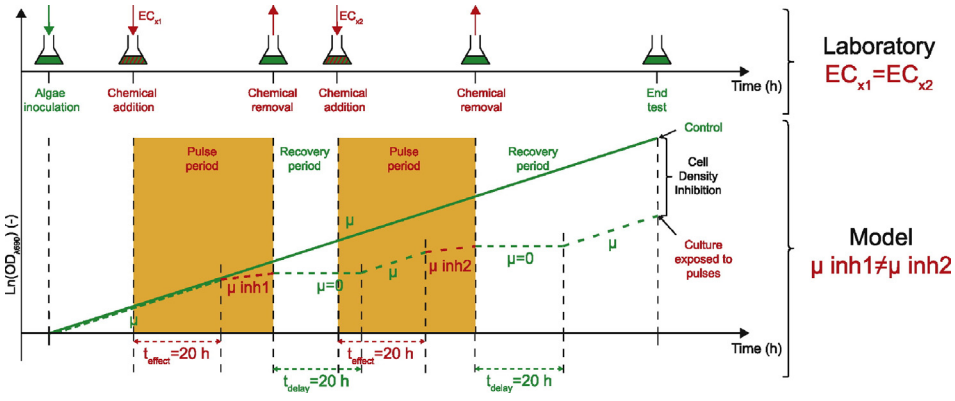
Pierre-Jean Copin [a,*], Léa Perronet [b], Nathalie Chèvre [a]

[a] Institut des dynamiques de la surface terrestre (IDYST), Faculté des Géosciences et de l'Environment, Université de Lausanne, Géopolis, Quartier Mouline, CH 1015 Lausanne, Switzerland
[b] Ecole des Mines d'Alès, 30100 Alès, France

HIGHLIGHTS

- Effect of S-metolachlor pulse exposure on the alga *S. vacuolatus* is modelled.
- Time-dependency of S-metolachlor was incorporated in the model.
- Delay in effect and in recovery was recorded for S-metolachlor on *S. vacuolatus*.
- The sensitivity of the alga increases after being previously exposed to a pulse.
- The model was found to be effective for S-metolachlor and for alga *S. vacuolatus*.

GRAPHICAL ABSTRACT



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ABSTRACT

In agriculture, herbicides are applied to improve crop productivity. During and after rain event, herbicides can be transported by surface runoff in streams and rivers. As a result, the exposure pattern in creeks is time-varying, i.e., a repeated pollution of aquatic system. In previous studies, we developed a model to assess the effects of pulse exposure patterns on algae. This model was validated for triazines and phenylureas, which are substances that induce effects directly after exposure with no delay in recovery. However, other herbicides display a mode of action characterized by a time-dependency effect and a delay in recovery. In this study, we therefore investigate whether this previous model could be used to assess the effects of pulse exposure by herbicides with time delay in effect and recovery. The current study focuses on the herbicide S-metolachlor. We showed that the effect of the herbicide begins only after 20 h of exposure for the alga *Scenedesmus vacuolatus* based on both the optical density and algal cells size measurements. Furthermore, the duration of delay of the recovery for algae previously exposed to S-metolachlor was 20 h and did not depend on the pulse exposure duration or the height of the peak concentration. By accounting for these specific effects, the measured and predicted effects were similar when pulse exposure of S-metolachlor is tested on the alga *S. vacuolatus*. However, the sensitivity of the alga is greatly modified after being previously exposed to a pulse of S-metolachlor. In the case of scenarios composed of several pulses, this sensitivity should be considered in the modelling. Therefore, modelling the effects of any pulse

* Corresponding author at: Institut des dynamiques de la surface terrestre (IDYST), Faculté des Géosciences et de l'Environment, Université de Lausanne, Géopolis, Quartier Mouline, CH 1015 Lausanne, Switzerland.
E-mail addresses: pierre-jean.copin@unil.ch (P.-J. Copin), lea.perronet@mines-ales.org (L. Perronet), nathalie.chevre@unil.ch (N. Chèvre).

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scenario of S-metolachlor on an alga is feasible but requires the determination of the effect trigger, the delay in recovery and the possible change in the sensitivity of the alga to the substance.

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1. Introduction

Herbicides are often used in agriculture to control weeds and thus to improve crop productivity. During and after precipitations, these herbicides can reach streams and rivers through surface runoff. As a result, the exposure to herbicides in the aquatic environment often occurs at a time-varying scale, i.e., as repeated herbicide pulses. Therefore, in streams located in agricultural catchments, aquatic organisms are exposed to fluctuating concentrations of pesticides related to the flux of runoff water (Boxall et al., 2013; Petersen et al., 2012). This type of exposure is characterized by periods of pulse exposure followed by periods of recovery but such exposure type is not considered in classical ecotoxicology studies (Reinert et al., 2002). A challenge is thus to assess the effects of such exposure profiles either by laboratory experiments or by modelling. The advantage of effect modelling is the possibility to predict a wide range of pulse exposure scenarios (Ashauer and Brown, 2013).

Diverse models were thus developed to predict the effects of this type of exposure on aquatic organisms (Ashauer et al., 2006; Nagai, 2014; Weber et al., 2012). For algae, the effects of time-variable exposure are determined mainly by toxicodynamics, i.e., the description of the chemical effect and recovery or repair mechanisms that occur in the organisms (Ashauer et al., 2007; Ashauer and Brown, 2013). In previous studies, we developed a model based on toxicodynamics to predict the effect of pulse exposures on algae. This model was successfully validated for the photosynthesis inhibitors in the chemical families of triazines and phenylureas on the algae species *Scenedesmus vacuolatus* and *Pseudokirchneriella subcapitata* (Copin and Chèvre, 2015; Copin et al., 2015). These families of herbicides were selected because their effect is immediate when the algae species are in contact with the substances and because no delay is noted in the recovery of the algae (Copin and Chèvre, 2015; Copin et al., 2015; Vallotton et al., 2008a; Vallotton et al., 2009).

However, other groups differing in their mode of action compared to triazines and phenylureas can be detected in creeks and streams such as S-metolachlor. S-metolachlor is a chloroacetanilide herbicide used to control pre-emergent and early post-emergent annual grassy and broadleaved weeds (Vallotton, 2007; Waxman, 1998). S-metolachlor is applied principally on cornfields but also on soybeans, sorghum, peanuts, potatoes, pod crops and cotton (Eurostat, 2007; O'connell et al., 1998; Thakkar et al., 2013). S-metolachlor is composed of 88% of S-isomers and 12% of R-isomers (O'connell et al., 1998; Shaner et al., 2006; Xu et al., 2010). The S-isomers are characterized by a high herbicidal activity (Moser et al., 1983). S-metolachlor belongs to the family of seedling shoot growth inhibitors. This herbicide family minimizes the growth of new plants by reducing the ability of seedlings to develop normally in the soil. The herbicide is absorbed by the developing roots and shoots and can be transferred via the xylem to areas of new growth (Gunsolus and Curran, 1991). At the molecular level inside the plant, S-metolachlor inhibits the enzyme VLCFA-FAE1 synthase (Gotz and Boger, 2004). This enzyme is required for the elongation of C16 and C18 to very long-chain fatty acids (VLCFA) (Boger, 2003). These VLCFAs are elements constituting the membrane lipids of the eukaryotic algae *S. vacuolatus* (Siegenthaler and Murata, 1998). Consequently, S-metolachlor inhibits the formation of VLCFAs, causing an imbalance in the fatty acid composition of cell membranes. The rigidity and the permeability of cells are reduced, and thus, cell division is inhibited (Vallotton et al., 2008b).

Because of its relatively high water solubility (488 mg/L at T = 20 °C) (Weber et al., 2007; Zemolin et al., 2014), metolachlor (a mixture of 50% of two S-isomers and 50% of two R-isomers) was one of the most frequently herbicides detected in US surface water and groundwater between 1992 and 2001 (Gilliom, 2006). Between 1989 and 1998, the concentration of metolachlor in Midwestern streams fluctuated between 2.50 and 1.44 μg/L (Scribner et al., 2000). From 2003 to 2004, the maximal concentrations of metolachlor increased from 1.56 to 5.29 μg/L in Ontario streams (Kurt-Karakus et al., 2008). The concentration of metolachlor in the Great Lakes were also detected and measured to range over 0.28 to 14 ng/L in 2005–2006 (Kurt-Karakus et al., 2010). Similarly, metolachlor was regularly detected in streams and rivers in Europe (Konstantinou et al., 2006; Munz et al., 2013; IFEN (Institut français de l'environnement), 2007; Poiger et al., 2002; Wittmer et al., 2014). This high frequency of metolachlor in European surface water resulted from the substitution of atrazine by metolachlor. Atrazine was banned in 2003 by the European Union Commission because of its endocrine disruption potential on several organisms (Joly et al., 2013; Sass and Colangelo, 2006). The use of S-metolachlor reduces the load of herbicides applied on the field (Shaner et al., 2006), Switzerland, other European countries and the United States decided to switch from metolachlor to S-metolachlor in 1997 (Liu and Xiong, 2009; Poiger et al., 2002). Consequently, S-metolachlor became one of the ten active ingredients the most used in the European Union in 2003 (Eurostat, 2007). Metolachlor was then banned in the European Union in 2002 (EC CotEC, 2002). However, in North America, both metolachlor and S-metolachlor are currently authorized for use (Vallotton et al., 2008b).

Some studies have investigated the toxicity of metolachlor and S-metolachlor on freshwater species. More ecotoxicological studies are available for metolachlor on non-target species such as algae because metolachlor was commercialized before S-metolachlor. In these studies, metolachlor was less toxic than herbicides with a photosynthesis inhibition mode of action (Junghans et al., 2003; Kotrikla et al., 1999; Ma et al., 2003; Ma et al., 2006; Ma et al., 2002). A comparative study showed that the growth rate of the alga *Chlorella pyrenoidosa* was inhibited more drastically with S-metolachlor than by metolachlor after 24, 48, 72 and 96 h of exposure (Liu and Xiong, 2009). The effects of S-metolachlor were also assessed on periphytic diatoms. The live cell densities of periphytic diatoms were reduced when compared to the control for 6-day exposures of 5 and 30 μg/L of S-metolachlor. However, select diatoms species of the periphyton were not affected by S-metolachlor exposure whereas other species were more sensitive (Debenest et al., 2009).

By contrast, few S-metolachlor pulse exposure studies have been performed. Debenest et al. (2009) compared the 3-day recovery following a 3-day exposure of 30 μg/L of S-metolachlor and isoproturon on periphytic diatoms. They showed that the growth rates of periphyton were higher for S-metolachlor than for isoproturon during the recovery periods. Vallotton et al. (2008b) assessed the time-to-recovery of the alga *S. vacuolatus* following a pulse exposure of 24 h. When the alga *S. vacuolatus* is exposed to a strongly inhibiting concentration of S-metolachlor, the recovery is delayed by 29 h. Furthermore, no effect was observed on the algae growth below 10 h of exposure. This effect was named the time-dependency effect.

The aim of this study was to adapt and validate the previously developed model for predicting the effects of pulse exposure for a substance with a different mode of action than photosynthesis inhibition on the alga *S. vacuolatus*. To achieve our goal, we tested S-metolachlor, a substance with a delayed effect and recovery (Vallotton et al., 2008b). Consequently, we investigated the time-dependency effects, the time-to-recovery and the change in the sensitivity of the alga *S. vacuolatus* after exposure to the compound. These observations were used to adapt the previously defined model.

2. Materials and methods

2.1. Chemicals

S-metolachlor (S-metolachlor Pestanal® 98.4%, $C_{15}H_{22}ClNO_2$) was purchased from Sigma-Aldrich. A stock solution of 100,000 μg/L was prepared in an algal OECD medium under axenic conditions. This stock solution was refrigerated at 6.4 °C. The concentration was determined analytically by LCMS, and the measured concentration was in the same range as the nominal concentrations. The measured concentration for S-metolachlor was 97,619 μg/L.

2.2. Algae cultures

A permanent agar culture tube of freshwater green microalga *S. vacuolatus* (Chlorophyceae; strain 211–15, Shihira and Krauss, Melbourne, Australia) was obtained from the Department of Bioanalytical Ecotoxicology at the Helmholtz Centre for Environmental Research — UFZ, Leipzig, Germany. The algae species *S. vacuolatus* multiply, similar to the *Chlorella* species, by an asexual reproduction called autosporulation (Fujishima and Steinbuchel, 2009; Huss et al., 1999; Yamamoto et al., 2004). Microalgae were cultured as described by Copin et al. (2015), i.e., with a continuous illumination at a light intensity of 30 μmol/m^2/s provided by cool-white fluorescent lamps and with a temperature of 25 °C on a HT Infors shaker table (90 rpm). The optical density was measured with a microplate reader (ELx800™, BioTek® Instruments, Winooski, Vermont) at a wavelength of 690 nm. In total, 650,000 algae cells/mL were inoculated in 250-mL Erlenmeyer flasks with 50 mL of OECD medium (OECD, 2011). With this initial alga density, the growth was exponential over approximately 48 h. The algae species *S. vacuolatus* was selected because it is unicellular, easily cultivated in the laboratory, representative of freshwater environments in which pulse exposures can be detected and measured and displays a higher sensitivity to a variety of hazardous substances (Backhaus et al., 2004; Blaise et al., 1986; Faust et al., 2001; Junghans et al., 2006; Machado and Soares, 2014). Furthermore, the algae species *S. vacuolatus* are resistant to centrifugation, a process performed during pulse exposure experiments (Vallotton et al., 2009). A control charter was established to monitor this alga growth in OECD media. In the laboratory, the average growth rate of the algae *S. vacuolatus* based on optical density values was 0.023 h^{-1} with a standard deviation of 0.002 h^{-1} (average of 45 cultures).

2.3. Dose–response curves

The dose–response curve of S-metolachlor was required for parameterising the model and for defining the tested concentrations during pulse exposure tests following a method adapted from the standard OECD procedure (OECD, 2011). The experiment was performed under the identical conditions as the algae cultures. The concentrations tested ranged from 13 to 30,000 μg/L. Each control and each concentration were tested in triplicate. The optical densities measured at the beginning and at the end of the experiment (after 48 h of exposure) were used to evaluate the average specific growth rate for each concentration and for each control. The growth inhibition was defined as the ratio between the growth rates of the different concentrations and that of the control (Eq. (1); see Section 2.6.1). Such testing is considered in this study as a "standard" testing.

2.4. Determination of the new parameters for the model

The following experiments were performed under identical conditions as the algae cultures.

2.4.1. Time-dependency effects

The duration of exposure required for the first effects to occur were determined by measuring the optical densities at several times during two 48-hour exposure standard tests (t = 17.25, 20, 24, 28, 31, 41.5 and 48 h for the first test and t = 13.75, 17.5, 20, 24, 28, 38, 43 and 48 h for the second test) (Fig. 1A). Different controls were used for each test. The concentrations tested were 900 (test 1), 3000 (tests 1 and 2), 17,000 (test 2) and 30,000 (test 2) μg/L, corresponding respectively to EC_{35}, EC_{50}, EC_{70} and EC_{76} (Effect Concentration; EC). The control and the culture exposed to these concentrations were tested in duplicate. During these tests, the maximum diameters of 20 algae cells from each replicate of the controls and of the concentrations tested were measured simultaneously with the optical densities. These values were averaged and used as a cell size indicator. These values were calculated for each exposure time. The algae cell diameters were measured by photographing algae cells with an Olympus BX53 microscope equipped with an Olympus DP26 camera. The cell size distribution of the microalgae in relation with the exposure time was established. Vallotton et al. (2008b) previously noted that the cell diameter of exposed cells increased with exposure time.

2.4.2. Recovery after pulse exposure

An experiment (Fig. 1B) was conducted 1) to observe whether a delay occurs in the recovery phase and 2) to establish the duration of the delay before the alga recovers. Each control and each concentration was tested in triplicate. During the experiment, three exposure durations were considered in three separate tests. They were fixed to 25, 30 and 48 h (parameter x in Fig. 1B). The concentration tested in the experiment was 3000 μg/L, corresponding to EC_{50}. After the pulse exposure, the algae were centrifuged twice for 7 min at 1046 × *g* and 25 °C. The supernatant was removed, and the algae were re-suspended in growth media. These two centrifugations eliminated the herbicide (Copin and Chèvre, 2015). The recovery period began directly after the algae were re-suspended in the fresh medium. The duration of the observation of the recovery was fixed to 48 h. The optical density was measured regularly during the exposure and recovery periods. A second experiment was performed to determine whether the delay defined previously was dependent on the pulse concentration. The general design is similar to the previous experiment (Fig. 1B). Two pulse concentrations, fixed at 525 and 24,500 μg/L and corresponding to EC_{30} and EC_{75}, respectively, were considered in two separate tests. The recovery initiated after the centrifugation procedure (48-hour duration). Each control and each concentration were tested in triplicate. To better visualize the effect of S-metolachlor on algae and the delay during the recovery, the optical densities data measured were summed, i.e., after the exposure period, the following recovery period was summed to the last optical density value of the previous exposure period. This summation was performed for both experiments, for the control and for the culture exposed, following the methodology of previous publications (Copin and Chèvre, 2015).

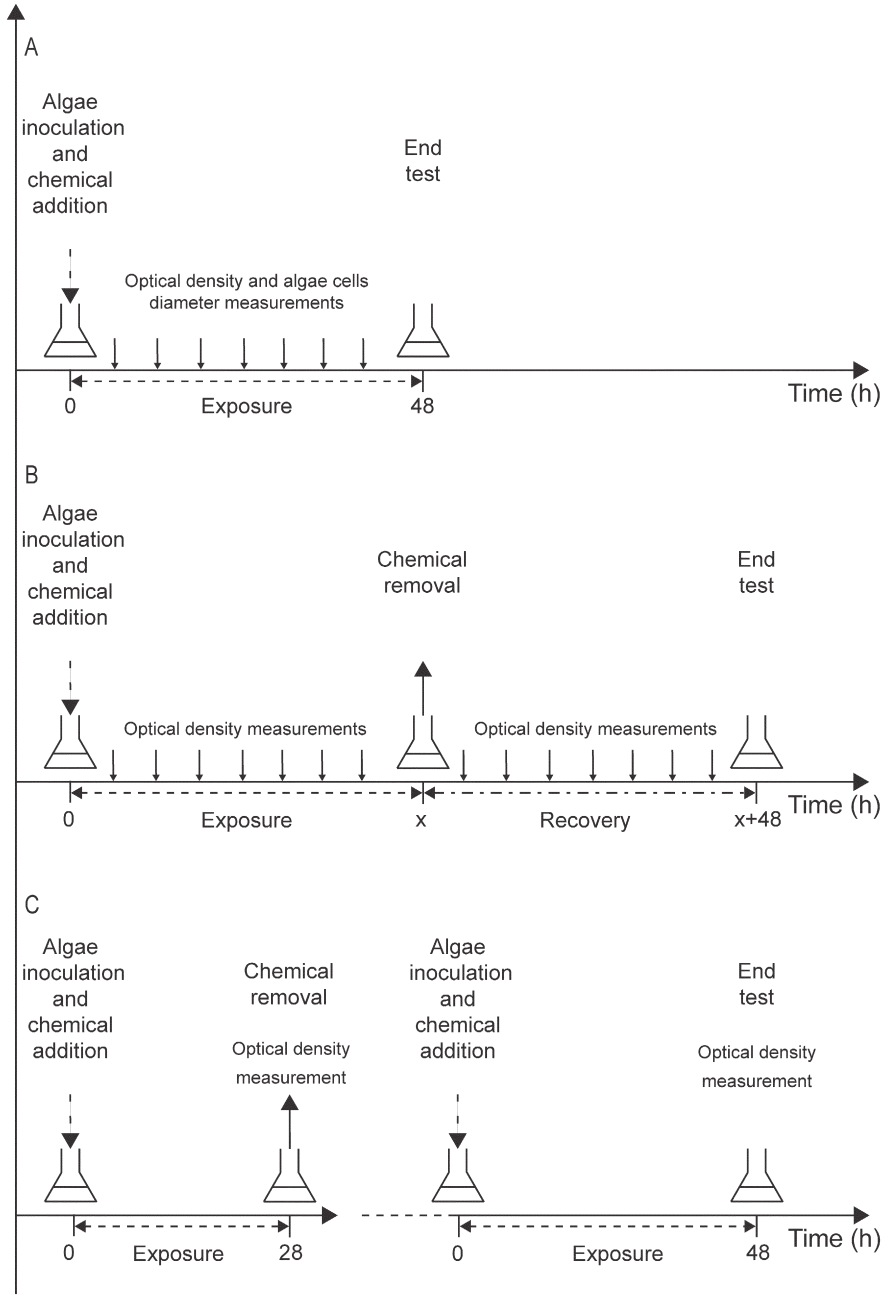

Fig. 1. Diagrams of the different experiments conducted to determine the specific parameters for the model. **A** Procedure to determine the time-varying effects. Two tests were conducted for this experiment. **B** Procedure to establish the delay in recovery: x = 25, 30 and 48 h. For x = 25 and 30 h, the concentration tested during the exposure corresponded to EC_{50}. For x = 48 h, different concentrations were tested during the exposure, corresponding to EC_{30}, EC_{50} and EC_{75}. Five tests were conducted for this experiment. **C** Procedure to analyse the sensitivity of the alga previously exposed to a pulse exposure. One test was conducted for this experiment.

2.4.3. Sensitivity after pulse exposure

A test was performed to investigate whether the sensitivity of the alga *S. vacuolatus* to S-metolachlor increased after being previously exposed to the compound in pulses (Fig. 1C). The alga was first exposed to a 28-h peak concentration of 3000 μg/L, corresponding to EC_{50}. The chemical was then removed by the procedure centrifugation described above. A standard test was then performed on the alga. Each control and each concentration was tested in duplicate during the standard toxicity test.

2.5. Pulse exposure testing

One pulse exposure scenario of S-metolachlor was tested on the alga *S. vacuolatus* in the laboratory. This scenario was composed of two pulse-exposure periods and two recovery periods. The duration of the two pulse-exposure periods was fixed to 30 h. The two recovery periods were fixed to 14 h and 43 h. The concentrations of the two pulses of S-metolachlor were fixed to 3000 μg/L, corresponding to EC_{50}. Algae exposed to pulses of S-metolachlor and the controls were tested in triplicate. The pulse exposure test was applied following Copin and Chèvre (2015). In total, 650,000 cells/mL of algae cells were inoculated in 50 mL of OECD medium and placed on the HT Infors shaker table under the identical conditions defined in Section 2.2. Algae grew initially for a short period of 18 h before being exposed to the first pulse.

At the end of each pulse exposure, the algae were centrifuged as explained in Section 2.4.2. The optical density was measured regularly during the exposure and recovery periods.

2.6. Data analysis

2.6.1. Growth inhibition in the standard toxicity test on algae

The growth inhibition (I_t) at a given concentration is calculated using the response variables, i.e., the average specific growth rate of the control (μ_C) and the average specific growth rate at the given concentration (μ_T) (OECD, 2011) (Eq. (1)):

$$I_t = \frac{\mu_C - \mu_T}{\mu_C} \times 100 \tag{1}$$

2.6.2. Dose–response curves for the standard acute toxicity test

The dose–response curves are obtained by plotting the growth inhibition I_t as a function of the tested concentrations C. The relationship is expressed using a four parameter log-logistic dose–response model (Vallotton et al., 2008a) (Eq. (2)):

$$I_t = min + \frac{(max - min)}{1 + 10^{((\log EC_{50} - \log C) \times Hillslope)}} \tag{2}$$

where EC_{50}, the concentration inhibiting 50% of the growth; *Hillslope* is the slope of the dose–response curve; and *max* and *min* parameters are the maximum and minimum of the sigmoidal curve, respectively. The *min* parameter is fixed at 0. The *max* parameter is fixed at 100 because it was assumed that the growth inhibition is complete (100%) at high concentrations. This curve is calculated using the statistical software Prism (GraphPad, San Diego, CA, USA).

2.6.3. Cell density inhibition measured at the end of the pulse exposure scenario

The overall algae cell density inhibition of the pulse exposure experiment ($Inh_{pulse\text{-}laboratory}$), was obtained by calculating the average of the algae cell density inhibition of each replicate ($Inh_{pulse\text{-}laboratory\text{-}replicate\ i}$). The $Inh_{pulse\text{-}laboratory\text{-}replicate\ i}$ was calculated as follows (Eq. (3)):

$$Inh_{pulse\text{-}laboratory\text{-}replicate\ i} = 100 \times \frac{OD_{control} - OD_{pulse,\text{replicate } i}}{OD_{control}} \tag{3}$$

where $OD_{control}$ is the average optical density for the replicates of the controls at the end of the experiment, and $OD_{pulse,\ replicate\ i}$ is the final optical density for the replicate i of alga exposed to the pulse concentration. As explained above, to better visualize the effect of pulses on algae, the optical density data measured were summed for the control and for each replicate exposed to the pulses, i.e., after each pulse exposure period, the following recovery period and the following pulse exposure period were summed with the last optical density value of the previous pulse exposure period. Thereafter, $OD_{control}$ and $OD_{pulse,\ replicate\ i}$ were determined using the growth rates of the linear regressions fitted on the summed optical density values of the different parts (pulses, recovery) of the control and of the replicate i of the alga exposed to pulses as described in Copin et al. (2015). However, in the case of substances with a time-dependency effect, two linear regressions were considered during the pulse exposure: a portion in which the effect was not detected and a portion in which the effect was observed. Similarly, in the case of substances with a delay in the recovery period, two linear regressions were considered during the recovery periods: one regression before the delay and one regression after the algae began to recover.

2.7. Modelling

The cell density inhibition model for S-metolachlor at the end of the experiment was adapted from the equations described in Copin et al. (2015), (Eqs. (4a) and (4b)):

$$Inh_{pulse\text{-}modelling} = 100 \times \frac{\exp\left[\ln\left(OD_{algae-t0}\right) + \mu \times t_{2i+1}\right] - \exp\left\{\ln\left(OD_{algae-t0}\right) + \mu \times t_1 + \sum_{i=1}^{n} X_i + \sum_{i=1}^{n} Y_i\right\}}{\exp\left[\ln\left(0D_{algae\text{-}initial}\right) + \mu \times t_{2n+1}\right]} \tag{4a}$$

Or

$$Inh_{pulse\text{-}modelling} = 100 \times \frac{\exp\left[\ln\left(OD_{algae-t0}\right) + \mu \times t_{2n}\right] - \exp\left\{\ln\left(OD_{algae-t0}\right) + \mu \times t_1 + \sum_{i=1}^{n} X_i + \sum_{i=1}^{n-1} Y_i\right\}}{\exp\left[\ln\left(OD_{algae-t0}\right) + \mu \times t_{2n}\right]} \tag{4b}$$

The two equations refer to the two kinds of scenarios, i.e., Eq. (4a) is used when the recovery phase corresponds to the end of the scenario, whereas Eq. (4b) is used when the pulse exposure period corresponds to the end of the scenario.

t_{2n+1} or t_{2n} is the total test duration; n is the number of pulses during the test; and $OD_{algae\ -\ t0}$ is the optical density corresponding to the initial cell density of algae determined with the calibration curve of the alga. $OD_{algae\ -\ t0}$ is fixed at 650,000 cells/mL for the alga *S. vacuolatus*. The corresponding optical density, read with the calibration curve determined for the alga *S. vacuolatus*, is then 0.056. μ is the growth rate of the control determined as the average growth rates of several successive batch cultures in growth media (control charter). The first exponential part in the numerator and the part in the denominator describe the growth of the control. The second exponential part in the numerator describes the growth of the culture exposed to pulses. The term $\mu \times t_1$ describes the culture growth before being exposed to pulses. t_1 is the time of the beginning of the first pulse. X_i corresponds to the ith pulse exposure period, and Y_i corresponds to the ith recovery period.

X_i and Y_i are dependent on the exposure time and the time allowed for recovery, respectively. For X_i, the parameter t_{effect} is defined, and this time corresponds to the duration before the S-metolachlor effects begins (time-varying effect). This time is obtained with the experiment defined in

Section 2.4.1. Consequently, two cases are possible for the parameter X_i (Eqs. (5a) and (5b)):

$$\text{If } t \le t_{effect} : X_i = \mu \times (t_{2i} - t_{2i-1}) \quad (5a)$$

or

$$\text{If } t > t_{effect} : X_i = \mu \times (t_{effect} - t_{2i-1}) + \mu_{inh\ x} \times (t_{2i} - t_{effect}) \quad (5b)$$

where t_{2i} is the end of a pulse exposure period; t_{2i-1} is the beginning of a pulse exposure period; $\mu_{inh\ x}$ is the growth rate at concentration x determined from a growth response curve. When the sensitivity of the alga is modified after a pulse exposure and when the recoveries between pulses are short, the $\mu_{inh\ x}$ during the first pulse is obtained from the growth response curve of the standard ecotoxicity test defined in Section 2.3, and the $\mu_{inh\ x}$ during the other pulses is defined from the growth response curve of the standard ecotoxicity test defined in Section 2.4.3. When the sensitivity of the alga is not modified after a pulse exposure or when the recoveries between pulses are long, all $\mu_{inh\ x}$ during the several pulses are determined from the growth response curve of the standard ecotoxicity test defined in Section 2.3.

For Y_i, the parameter t_{delay} is defined, and this time corresponds to the delay before a complete recovery of the culture previously exposed to a pulse of S-metolachlor. This time is obtained from the experiment defined in Section 2.4.2. Therefore, two cases are defined for the parameter Y_i (Eq. (6a) and (6b)):

$$\text{If } t \le t_{delay} : Y_i = 0 \quad (6a)$$

or.

$$\text{If } t > t_{deley} : Y_i = 0 \times (t_{deley} - t_{2i}) + \mu \times (t_{2i+1} - t_{delay}) \quad (6b)$$

where t_{2i} is the beginning of a recovery period, and t_{2i+1} is the end of a recovery period.

As explained in Copin et al. (2015), the distribution of the $Inh_{pulse\text{-}modelling}$ is estimated based on a Monte Carlo simulation. The model is applied using Matlab software (MATLAB R2011b, The Mathworks Inc., see SI). The predictive results of these Monte Carlo simulations were used in the comparison with the laboratory observations to validate the model.

3. Results and discussion

3.1. Laboratory experiments

3.1.1. Dose–response curve

The dose–response curve of S-metolachlor for the alga *S. vacuolatus* is presented in Fig. 2. The dose–response curves of atrazine, diuron and isoproturon obtained with the same alga (Copin and Chèvre, 2015; Copin et al., 2015) are also represented in the Fig. 2. The EC_{50} for S-metolachlor is 2942 (1835; 4717) μg/L (95% confidence interval). This toxicity value is consistent with Vallotton et al. (2008b), which obtained EC_{50}s of 2300 and 3000 μg/L for S-metolachlor on the alga *S. vacuolatus*. No other EC_{50} data were available in the literature for this substance and this alga. The toxicity of S-metolachlor was compared with that of diuron, atrazine and isoproturon (Fig. 2). The corresponding EC_{50}s of diuron, atrazine and isoproturon for the alga *S. vacuolatus* with 95% confidence intervals were, respectively, 14.3 (11.9; 17.2), 49.7 (42.2; 58.6) and 66.9 (61.2; 73.2) μg/L. Therefore, S-metolachlor is less toxic whereas diuron is the most toxic chemical for the alga *S. vacuolatus*. The EC_{50} toxic ratio of S-metolachlor (the EC_{50} of S-metolachlor divided by the EC_{50} of the compound) with diuron, atrazine and isoproturon is 206, 63 and 44, respectively.

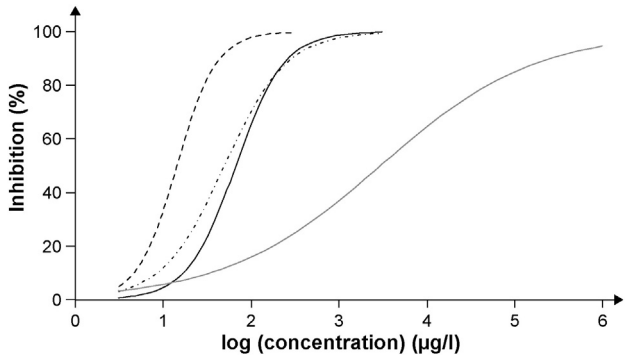

Fig. 2. Dose–response curves of diuron, atrazine, isoproturon and S-metolachlor for the alga *S. vacuolatus*. Black large-dotted curve: diuron. Black small-dotted curve: atrazine. Black curve: isoproturon. Grey curve: S-metolachlor.

This comparison does not change when using the EC_{10} of the compounds, which is closer to environmental concentrations (Van der Hoeven et al., 1997; Warne and Van Dam, 2008). The toxic ratio of EC_{10} for S-metolachlor and diuron, atrazine and isoproturon were 7.3, 4.0 and 2.0, respectively. Similarly to the EC_{50}s, diuron, atrazine and isoproturon are more toxic than S-metolachlor on the alga *S. vacuolatus* when environmental concentrations were considered.

3.1.2. Time-varying effects

The optical densities of the alga *S. vacuolatus* were measured regularly during two standard tests (see Section 2.4.1). The growth of the controls and the cultures exposed to S-metolachlor are represented in Fig. 3.

The effects of S-metolachlor are observable after 20 h in the majority of the tests (Fig. 3A and B). A difference between the black curve (controls) and the orange and red curves (cultures exposed to, respectively, EC_{35} and EC_{50}) is noted at 20 h (Fig. 3A). Similarly, the blue curve (cultures exposed to EC_{70}; Fig. 3B) is above the black curve until the growth rate decreases starting at 20 h. However, in one test, the effects seem to initiate after 17.5 h for two cultures exposed to S-metolachlor (Fig. 3B). We observe a difference between the black curve (controls) and the red and green curves (cultures exposed to EC_{50} and EC_{76}, respectively) from 17.5 h. As the effect begins after 20 h in the most culture treated, we decided to fix the start of the effects of S-metolachlor to 20 h for low and high concentrations. This start time is different from the photosystem II inhibitors such as diuron, atrazine and isoproturon. For these compounds, the effects are directly observable at the beginning of the exposure (Copin and Chèvre, 2015; Copin et al., 2015).

3.1.3. Cell evolution during testing

During the two previous tests, the cell diameter (μm) of the alga *S. vacuolatus* was measured several times during the 48-hour exposure (see Section 2.4.1). The results are presented in Table 1.

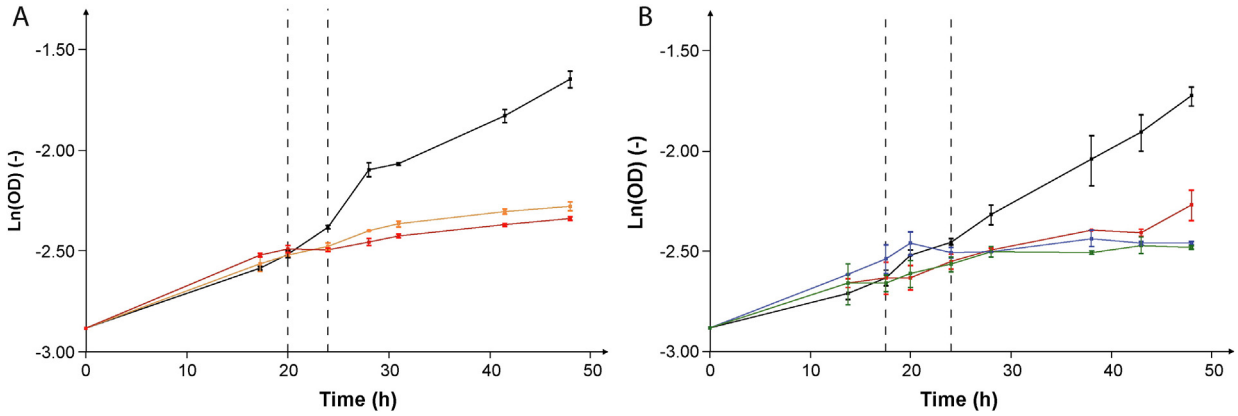

Fig. 3. Growth of the alga *S. vacuolatus* during two different tests (Fig. 3A and B). The OD is the optical density. The averages of the optical densities measured during the tests at different times are represented by squares with bars indicating standard deviation. The test duration is fixed to 48 h. Black vertical dashed lines illustrate the period when the herbicide S-metolachlor affected the growth of the alga **A.** Black curve: controls. Orange curve: culture exposed to EC_{35}. Red curve: culture exposed to EC_{50}. **B.** Black curve: controls. Red curve: culture exposed to EC_{50}. Blue curve: culture exposed to EC_{70}. Green curve: culture exposed to EC_{76}.

The effects of S-metolachlor are observable starting from 20 h in the two tests (Table 1). For the cultures exposed to different concentrations of S-metolachlor (EC_{35}, EC_{50}, EC_{70} and EC_{76}), the size of their cells is statistically equivalent to that of the controls until approximately 17 h of exposure. However, the size of cells is significantly different from the controls from 20 h for the remainder of the two tests. After 20 h of the experiment duration, the size of algae cells decreases for the two controls whereas it remains constant, slightly decreases or even increases for the cultures exposed to all concentrations of the herbicide. In the culture exposed to the EC35 and EC50 of S-metolachlor the size decreases after 20 h, but to a smaller extent than the control. However, the size of algae continues to increase for the cultures exposed to EC70 and EC76 of S-metolachlor. Thereafter, the algae cell size increases again until the end of the tests for the controls. For the cultures exposed to the herbicide, the algae cell size increases during test one and remains constant for the cultures exposed to S-metolachlor in test two.

This increase in the cell size when exposed to the herbicide S-metolachlor agrees with Vallotton et al. (2008b). The increase of the algae cells size for the culture exposed to S-metolachlor results from the formation of new spores in the mother cell that are not released when the substance is present. This accumulation of new spores in the mother cell occurs because of the loss of the rigidity and permeability of cells exposed to S-metolachlor induced by the formation of VLCFAs, causing an imbalance in the fatty acid composition of cell membranes (Vallotton et al., 2008b). Furthermore, a fraction of the spores were potentially dead before the parent cell wall splintered (Liu and Xiong, 2009), diminishing the growth and the development of new algae. For the concentrations EC_{35} and EC_{50}, cell division occurs after 20 h, but few new algae are observed. However, for the high concentrations EC_{70} and EC_{76}, cell division completely stops after 20 h as shown by the increase in and stability of the size of algae cells. Consequently, the conclusions obtained with the algae cell size confirm the results obtained with the optical densities measured regularly during the standard tests, i.e., that the beginning of the effect occurs at 20 h.

This cell size increase was also observed for the alga *P. subcapitata* with the chemical pretilachlor, which has the identical mode of action as S-metolachlor, i.e., an inhibitor of very long-chain fatty acid formation (Nagai et al., 2011). The size of cells also increased for other chemicals with other modes of action. A diameter increase of cells of *Chlamydomonas reinhardtii* was observed with increasing paraquat concentrations, a photosystem I inhibitor (Jamers and De Coen, 2010). The decrease of the algae cell size for the control after 20 h for all concentrations corresponded to the moment when fresh spores, called autospores produced from the cell division of the mother cell, were released into the water (Yamamoto et al., 2004). After the release of these autospores between 24 and 48 h, the size of the new cells increased again until the end of the experiment.

Table 1
Size of the algae cells (μm) at different times during 48 h of exposure to EC_{35} (Test 1), EC_{50} (Test 1), EC_{70} (Test 2) and EC_{76} (Test 2) of S-metolachlor.

	Time (h)	Size (μm)[a]		
		Control	EC_{35}[b]	EC_{50}[c]
Test 1	17.25	8.88 ± 1.59	9.23 ± 1.27	9.47 ± 1.08
	20	8.41 ± 1.60	9.30 ± 1.34*	9.60 ± 1.04*
	24	7.48 ± 1.57	8.65 ± 1.88*	9.35 ± 1.18*
	28	7.78 ± 1.13	8.79 ± 2.13*	9.59 ± 1.13*
	31	7.31 ± 0.90	9.40 ± 1.74*	9.96 ± 1.10*
	41.5	7.78 ± 0.78	9.01 ± 1.45*	9.90 ± 0.94*
	48	8.20 ± 1.30	9.76 ± 1.97*	10.28 ± 1.32*
	Time (h)	Control	EC_{70}[d]	EC_{76}[e]
Test 2	13.75	9.34 ± 0.98	9.01 ± 1.48	9.25 ± 1.29
	17.5	9.66 ± 1.12	9.50 ± 0.76	10.07 ± 0.93
	20	9.00 ± 1.32	9.77 ± 0.98*	9.55 ± 1.01*
	24	7.78 ± 1.58	10.35 ± 0.87*	10.06 ± 0.91*
	28	7.51 ± 1.42	10.11 ± 1.04*	10.22 ± 0.83*
	38	7.79 ± 0.69	10.53 ± 0.86*	10.70 ± 1.09*
	43	7.94 ± 0.92	10.19 ± 1.07*	10.59 ± 1.03*
	48	8.16 ± 0.84	10.22 ± 0.99*	10.61 ± 1.02*

[a] Data are represented as the mean ± standard deviation of the means.
[b] Effect concentration 35%.
[c] Effect concentration 50%.
[d] Effect concentration 70%.
[e] Effect concentration 76%
* The size of algae cells exposed to concentrations was significantly different to the size of the control algae cells using a Z-test (p-value > 0.05).

3.1.4. Recovery after the pulse exposure

Fig. 4 presents the delay of recovery observed for different exposure durations and for several of the concentrations tested.

As mentioned in Sections 3.1.2 and 3.1.3, the effect started after 20 h of exposure for each test. A difference between the black curve (controls) and the red curve (cultures exposed to S-metolachlor) was observed visually after 20 h of exposure (Fig. 4).

For each test, the analysis of the recovery shows that the growth rate of the culture exposed to S-metolachlor remains inhibited after the chemical is removed. For the cultures exposed to the EC_{50} of S-metolachlor during 25, 30 and 48 h (Fig. 4A, B and C), no algal growth is observed during the first 20 h of the recovery. The growth rate of the culture exposed to the herbicide is significantly different from the growth rate of the control (ANCOVA analysis, p-value = 0.0002, 0.0029 and 0.02, respectively). However, after 20 h, the culture begins to grow similarly to the control. The growth rate of the culture exposed

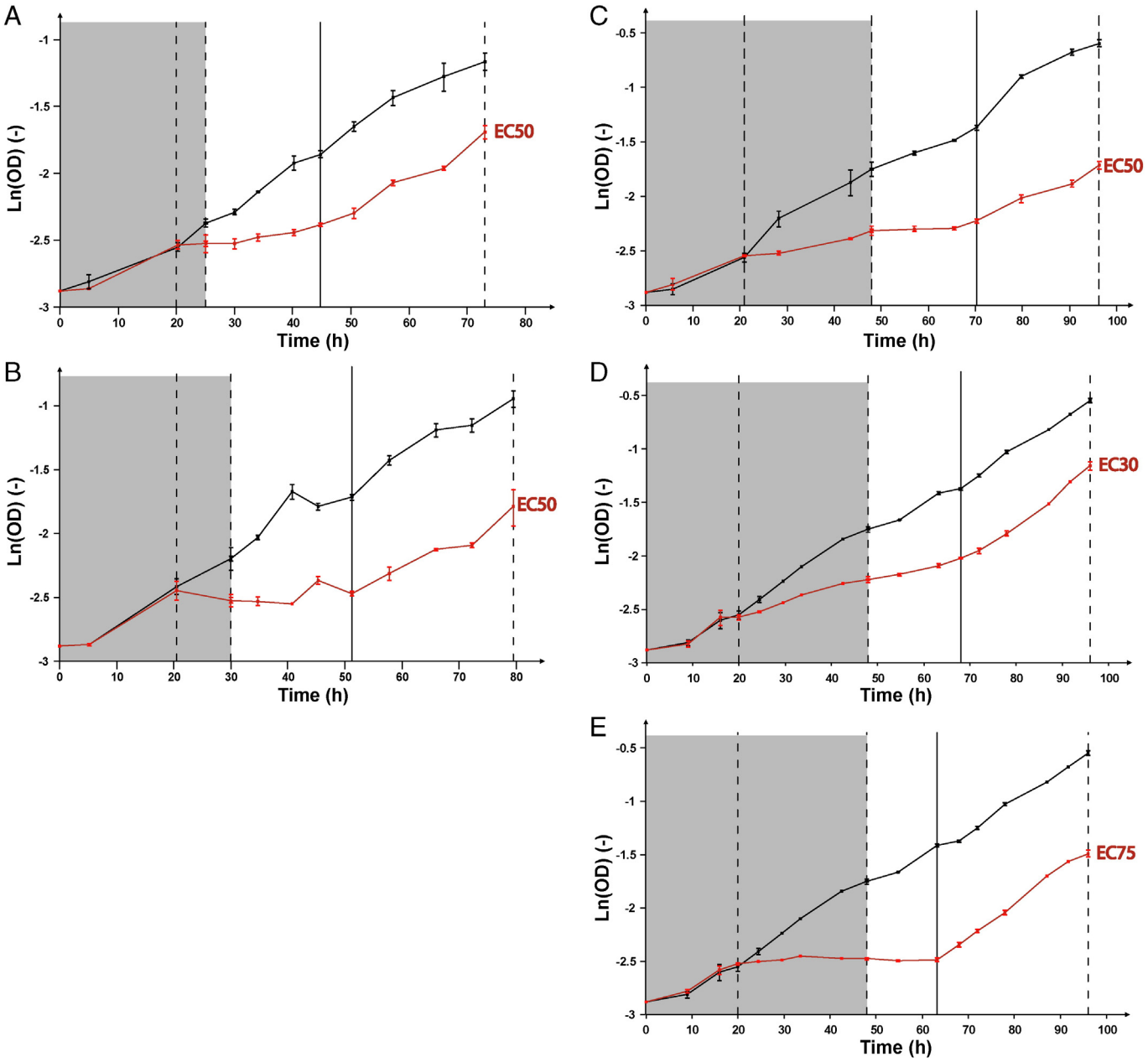

Fig. 4. Growth of the alga *S. vacuolatus* during five different tests characterized by their different exposure durations to S-metolachlor or their different exposure concentrations of S-metolachlor. **A**: Exposure for 25 h and EC_{50}. **B**: Exposure for 30 h and EC_{50}. **C**: Exposure for 48 h and EC_{50}. **D**: Exposure for 48 h and EC_{30}. **E**: Exposure for 48 h and EC_{75}. The exposure duration to S-metolachlor was characterized by the grey surface. OD is the optical density. The averages of optical densities measured during the tests at different times are represented by squares. The averages of optical densities with their standard deviation at different times are represented by small dashes. The black vertical dashed lines illustrate the period during when the effect of the herbicide S-metolachlor was effective on the growth of alga *S. vacuolatus* and at the end of the experiment. The black line illustrates the time when the recovery of the culture exposed to S-metolachlor was complete after a delay. Black curve: control. Red curve: culture exposed to EC_{50}.

is therefore no more significantly different from the growth rate of the control during this period (ANCOVA analysis, p-value = 0.92, 0.14 and 0.48, respectively). Similarly, in Fig. 4D and E, the algae exposed to EC_{30} and EC_{75} of S-metolachlor during 48 h do not grow during the first 20 and 15.25 h, respectively, after the chemical was removed. The growth rate of the culture exposed to the herbicide is significantly different from the growth rate of the control (ANCOVA analysis, p-value = 0.0196 and 0.0409, respectively). However, after 20 and 15.25 h, respectively, the culture begins to grow similarly to the control. The growth rate of the culture exposed is not significantly different from the growth rate of the control during this period (ANCOVA analysis, p-value = 0.1902 and 0.0956).

In summary, the recovery of the algae previously exposed to a range of concentrations of S-metolachlor during different exposure periods is complete 20 h after the herbicide is removed from the cultures. Vallotton et al. (2008b) found a higher delay of 29 h for the recovery of the identical algae exposed for 24-hour to a single strongly inhibiting S-metolachlor concentration. The difference between the duration of the delay in these two studies could be explained by the different test designs. In Vallotton et al. (2008b), algae grew synchronously under a dark and light illumination during the 24-hour exposure to S-metolachlor, whereas in this study, the light was continuous during the entire experiment.

Contrary to photosystem II inhibitors that are characterized by a complete and fast recovery from a pulse exposure (Copin and Chèvre, 2015; Copin et al., 2015; Vallotton et al., 2008a), other substances with the identical mode of action as the herbicide S-metolachlor can also affect the recovery of aquatic species after a pulse exposure.

A delay in the recovery is observed after a 72-hour exposure of pretilachlor on the alga *P. subcapitata* (Nagai et al., 2011). However, contrary to our study, the duration of the delay induced by the exposure of pretilachlor is linked to the concentration tested. For low concentrations, a delay is not noted before the recovery, contrary to the high concentrations tested (Nagai et al., 2011). The recovery was also studied for alachlor and metazachlor, two other substances belonging to the identical chemical family as S-metolachlor (Weisshaar and Boger, 1987). The complete recovery in the growth alga *Scenedesmus acutus* occurs 24 hour after a 24-hour exposure of concentrations between 50 and 100 μM of alachlor and metazachlor. Consequently, this duration of the delay in the recovery is on the identical order of magnitude as the delay of the recovery in this study. Note that for algae and macrophytes exposed to other herbicides with a mode of action different to S-metolachlor such as inhibitors of acetolactate synthase or microtubule assembly inhibitors, a delay in the recovery is also observed (Cedergreen et al., 2005; Mohammad et al., 2006; Nagai et al., 2011).

3.1.5. Change in the sensitivity

The toxicity of S-metolachlor to the algae was compared before and after the algae were exposed to a 28-hour pulse at a concentration corresponding to EC_{50}. The sensitivity of the alga increased significantly when previously exposed to the chemical (ANCOVA analysis, p-value < 0.05). The EC_{50} of an acute toxicity test is equivalent to 2942 (1835; 4717) μg/L (95% confidence interval). However, the toxicity test performed on the alga previously exposed to a pulse exposure provides an EC_{50} of 149 (108; 203) μg/L (95% confidence interval). This modification of sensitivity can be explained by the delay of the recovery period induced after the pulse exposure of S-metolachlor. Prior to centrifugation, the alga begins its recovery period at the identical time as when it is exposed to several concentrations of S-metolachlor from the acute toxicity test. The period of recovery is characterized by a delay during which no algal growth is noted. Consequently, when the alga is exposed to several concentrations of S-metolachlor during this delay period, it grows with increasing difficulty. Therefore, the algae were more sensitive to additional exposure of S-metolachlor.

3.1.6. Pulse exposure tests

The results for pulse exposure tests are illustrated for S-metolachlor in Fig. 5. For this scenario, the cell density was inhibited during each pulse, as indicated by the difference between the growth curve of the control (black curve) and the growth curve of the exposed algae (grey curve) at the end of the experiment. The effect was noted after 20.5 h of exposure during the first pulse, agreeing with the time of the beginning of the effect at 20 h found in Sections 3.1.2 and 3.1.3. No growth is observed in the exposed algae during the first recovery period. The delay in the recovery was identified to be 20 h for the alga exposed to S-metolachlor, as explained in the section 3.1.4. The first recovery period required 14 h, and the algae do not recover during this period. During the second pulse exposure, according to Sections 3.1.2 and 3.1.3, the growth of the algae is affected by the pulse concentration only 20 h after being in contact with the alga. Thus, the alga during the second pulse exposure can begin recovering. However, the growth of the alga during the beginning of the second pulse is low because the delay period is not finished after the end of the recovery phase. Six additional hours are required to reach the 20 h necessary for the beginning of alga recovery. Consequently, the growth of the alga during the second pulse is affected firstly by the delay phase and then by the pulse concentration when it was effective. Therefore, the effect during the second pulse occurred before 20 h after the beginning of the second pulse. During the second recovery, a delay was observed during the first few hours. Thereafter, the alga treated by S-metolachlor grew similar to the control. For the S-metolachlor pulse exposure scenario, the cell density inhibition in the laboratory with its 95% confidence interval was 72.3 (69.3; 75.3) %.

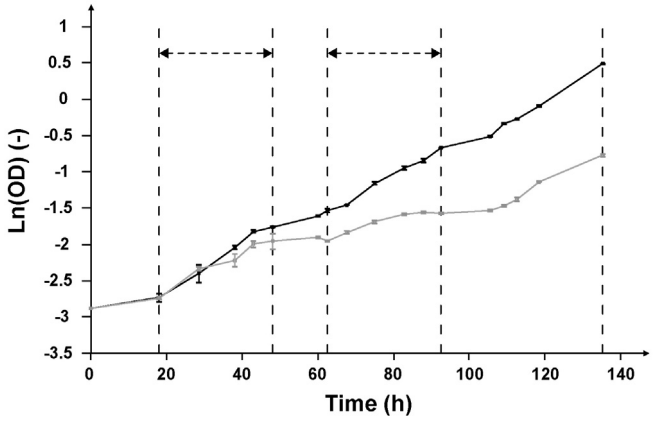

Fig. 5. Growth curves of the alga *S. vacuolatus* for the S-metolachlor pulse exposure scenario in the laboratory. Black curve: control. Black squares: measurements of the control. Grey curve: algae exposed to pulses. Grey squares: measurements of the algae exposed to pulses. Small black and grey dashes: averages of optical densities with their standard deviation at different times. The lengths of the pulses are indicated by the arrows on the top of each graph.

3.2. Comparison between the measured and predicted results

The parameters of the model, t_{effect} and t_{delay}, were fixed to 20 h in each case. The effect is noted after 20 h of exposure on the alga *S. vacuolatus* (see Sections 3.1.2 and 3.1.3) and the delay is complete after 20 h (see Section 3.1.4). The recovery period between the two pulse exposure periods is 14 h. Consequently, as the period of recovery was lower than the delay phase, the parameter $\mu_{inh,\,x}$ of the model for the second pulse was obtained from the growth response curve with an EC_{50} equivalent to 149 μg/L. The change of algae sensitivity described in Section 3.1.5 could explain how the alga reacted to a scenario composed of two pulses separated by a short recovery period. We showed in Section 3.1.5 that when the recovery between two pulses was lower than the delay period, the growth of the alga exposed to a second pulse was affected first by the delay phase and then by the pulse concentration when it was effective. Therefore, when the recovery separating two pulses was lower than the delay period, the parameter $\mu_{inh,\,x}$ of the model (Eq. (5b)) during the second pulse was determined from the growth response curve with an EC_{50} equivalent to 149 μg/L.

The cell density inhibition predicted by the model is illustrated in the boxplot in Fig. 6. For this pulse exposure scenario, the experimental average cell density inhibition (72.3%) was near the average cell density inhibition given by the model (67.5%). The variability of the observed

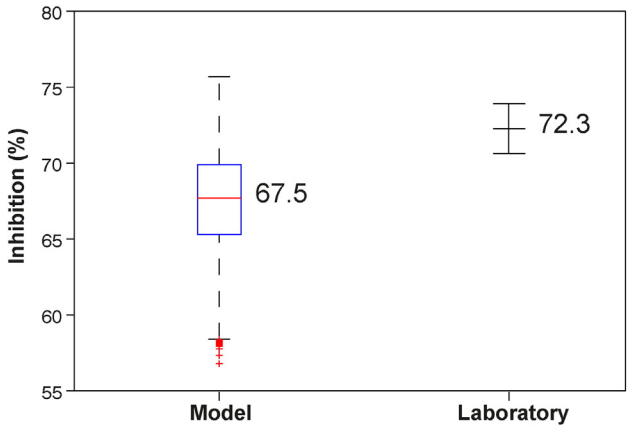

Fig. 6. Boxplot of the modelled scenario with S-metolachlor on the alga *S. vacuolatus*. Average and standard deviation for laboratory results (three replicates). The average cell density inhibitions predicted by the model and obtained in the laboratory were noted for each scenario. The beginning of the Y-axis is not fixed to 0% of inhibition.

experimental inhibitions was located between the minimum and maximum of the model (Fig. 6) as observed in Copin et Chèvre (2015). Therefore, the model predicts precisely the observed experimental inhibitions. Consequently, the model developed in Copin et al. (Copin and Chèvre, 2015; Copin et al., 2015) can be considered as suitable for substances with time-dependency effects and a delay of recovery as long-chain fatty acid inhibitors when they are applied on the alga *S. vacuolatus*.

4. Conclusions

In this study, we adapted a previously developed model for predicting the cell density inhibition of a pulse exposure scenario on the alga *S. vacuolatus* for herbicides with a time-dependency effect and a delay in the recovery such as S-metolachlor. Therefore, the analysis of the time-dependency effect was first considered. Considering the optical density values and algal cell size, we highlighted that the effect of S-metolachlor on the alga *S. vacuolatus* is not direct and begins only after 20 h of exposure. A delay in the recovery is also highlighted. This delay is identical regardless of the time and the concentration of the pulse exposure preceding and is fixed at 20 h. Finally, the sensitivity of the alga increased after being previously exposed to a peak concentration. Thus, the reaction of the algae during a scenario composed of several pulses with short recovery periods can be ascertained. These parameters should be determined to apply this model to any substances with a similar mode of action as S-metolachlor such as alachlor and metazachlor. However, for substances with other mode of actions, other parameters might be of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2015.08.154.

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DYNAMICS AND ENVIRONMENTAL RISK ASSESSMENT OF THE HERBICIDE GLYPHOSATE AND ITS METABOLITE AMPA IN A SMALL VINEYARD RIVER OF THE LAKE GENEVA CATCHMENT

SILWAN DAOUK,*† PIERRE-JEAN COPIN,† LUCA ROSSI,‡ NATHALIE CHÈVRE,† and HANS-RUDOLF PFEIFER†
†Institute of Earth Sciences, Faculty of Geosciences and Environment, University of Lausanne, Lausanne, Switzerland
‡Ecological Engineering Laboratory, School of Architecture, Civil and Environment Engineering, École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland

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Abstract: The use of pesticides may lead to environmental problems, such as surface water pollution, with a risk for aquatic organisms. In the present study, a typical vineyard river of western Switzerland was first monitored to measure discharged loads, identify sources, and assess the dynamic of the herbicide glyphosate and its metabolite aminomethylphosphonic acid (AMPA). Second, based on river concentrations, an associated environmental risk was calculated using laboratory tests and ecotoxicity data from the literature. Measured concentrations confirmed the mobility of these molecules with elevated peaks during flood events, up to 4970 ng/L. From April 2011 to September 2011, a total load of 7.1 kg was calculated, with 85% coming from vineyards and minor urban sources and 15% from arable crops. Compared with the existing literature, this load represents an important fraction (6–12%) of the estimated amount applied because of the steep vineyard slopes (~10%). The associated risk of these compounds toward aquatic species was found to be negligible in the present study, as well as for other rivers in Switzerland. A growth stimulation was nevertheless observed for the algae *Scenedesmus vacuolatus* with low concentrations of glyphosate, which could indicate a risk of perturbation in aquatic ecosystems, such as eutrophication. The combination of field and ecotoxicity data allowed the performance of a realistic risk assessment for glyphosate and AMPA, which should be applied to other pesticide molecules. *Environ Toxicol Chem* 2013;32:2035–2044. © 2013 SETAC

Keywords: Glyphosate Aminomethylphosphonic acid Vineyard river Species sensitivity distribution Risk assessment

INTRODUCTION

Contamination of surface water by pesticide molecules represents an unwanted side effect of their use and a potential risk for aquatic organisms, especially in agricultural areas. Their transfer from fields to surface water happens mainly by rainfall-induced surface runoff, but may also occur in part by subsurface runoff and via drainage pipes according to landscape configuration [1,2]. This transfer depends largely on the intrinsic properties of molecules, but weather conditions and application practices, as well as soil types and land use, are also important parameters to consider [3,4].

The widely used herbicide glyphosate (*N*-[phosphonomethyl]glycine) and its metabolite aminomethylphosphonic acid (AMPA) are among the main pesticide molecules found in surface waters worldwide [5]. The main properties of these two molecules are given in Table 1. In 2010 in France, 46% of more than a thousand surface water samples contained AMPA and 25% contained glyphosate, the 2 most frequent pesticide molecules detected (www.statistiques.developpement-durable.gouv.fr). In Switzerland, data on their occurrence in surface waters are scarce, despite extensive use of glyphosate in both urban and agricultural areas. Hanke et al. [6] analyzed several water samples and found low concentrations of glyphosate and AMPA in the Rhine River and 2 lakes of eastern Switzerland (up to 75 ng/L). However, tributaries of the 2 lakes showed higher concentrations, up to 390 ng/L.

In agriculture, the expansion of minimum and no-till practices to avoid soil erosion and organic matter mineralization—as well as the recent banning of other molecules such as atrazine—led to an increased application of nonselective herbicides, such as glyphosate [7,8]. In the Swiss watershed of Lake Geneva, glyphosate is among the 10 most used pesticides and is applied in many different types of culture [9]. For instance, in grapevine, a crop mainly located in western Switzerland, glyphosate is nearly the only herbicide used [10], which may result in a delicate situation in terms of weed management with an elevated risk to develop resistance [7,11]. The few grab samples taken in Swiss rivers showed elevated concentrations, often above the legal threshold of 100 ng/L for each molecule of pesticide, defined in the Swiss federal ordinance on water protection (OEaux) [6,12]. It is therefore important to further monitor glyphosate and AMPA in Swiss streams and assess the related risk for aquatic ecosystems.

Theoretically, glyphosate has a low toxicity toward animals, such as mammals, birds, fish, and invertebrates, which do not possess the targeted metabolic pathway; thus, glyphosate would affect mainly plants, bacteria, and fungi [9]. Indeed, its median lethal dose ranges from 800 mg/kg to 1340 mg/kg in mammals, and from 1170 mg/kg to >2000 mg/kg in amphibians [13]. Nevertheless, some studies have revealed that glyphosate accumulated and reduced the growth of earthworm species [14,15] or had long-term effects on the development and fertility of the predator insect *Chrysoperla externa* [16]. Several studies showed that the glyphosate formulation (i.e., the commercial form of glyphosate) is more toxic [17] and ecotoxic [15,18] than the acid or isopropylamine salt forms, or both. The polyoxyethylene tallowamine surfactant has indeed

* Address correspondence to Silwan.Daouk@unil.ch.
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Table 1. Main properties of glyphosate and aminomethylphosphonic acid (AMPA)[a]

Properties	Glyphosate	AMPA
Formula	$C_3H_8NO_5P$	CH_6NO_3P
Molecular weight (g/mol)	169.07	111.04
Water solubility (g/L)	10.5	5.8
Log K_{OW}	−3.2	−1.63
Half-degradation time $(DT50)_{field}$	3–174[b]	76–240[b]
Sorption coefficient K_d (L/kg)	222	–

[a]Source: www.eu-footprint.org or as otherwise noted.
[b]Source: Laitinen, 2009 [44].

been found to be largely responsible for the toxicity of glyphosate formulations [19,20].

In freshwater ecosystems, glyphosate generally shows a low toxicity, but seems to be more toxic in alkaline than in acidic waters [21,22]. Exposures to short, but high, concentrations of glyphosate formulation perturb the composition and the growth of phytoplankton communities, but also, in some cases, stimulate primary production [23]. This latter effect is attributed either to the increase of nitrogen and phosphorus by glyphosate degradation, which stimulates the growth of periphyton organisms such as cyanobacteria [24], or to the pressure on herbivorous organisms because of the herbicide. Growth stimulation because of glyphosate exposure was also observed in single algae tests [25] and with ameba [26]. For algae, this stimulation—called the hormesis effect—also involves photosynthesis and chlorophyll-a synthesis.

The objectives of the present study were 2-fold: first, to gain a more precise understanding of the sources and dynamics of the herbicide glyphosate and its metabolite AMPA in a typical vineyard catchment, and second, to assess the environmental risk of the measured concentrations of glyphosate and AMPA in the river draining this catchment. To our knowledge, the present study is the first to combine transport dynamics and global risk assessment of this widely used herbicide in a real situation.

MATERIALS AND METHODS

Study area and sampling

The Lutrive is a small river to the east of the city of Lausanne, Switzerland, with its spring located on the Swiss plateau at an altitude of 842 m, and its mouth on Lake Geneva at 372 m, with a mean slope of 7.3% (Figure 1). Its small watershed (6.4 km^2) is characterized by the following different land uses: agricultural fields (45%), urban and impervious surfaces (31%), and forests (24%). Vineyards account for 4.1% of the agricultural surface and are mainly located in its lower southern subcatchment (1.2 km^2). This latter shows a steeper slope (∼10%) and also comprises some urban and impervious surfaces (Lutry village),

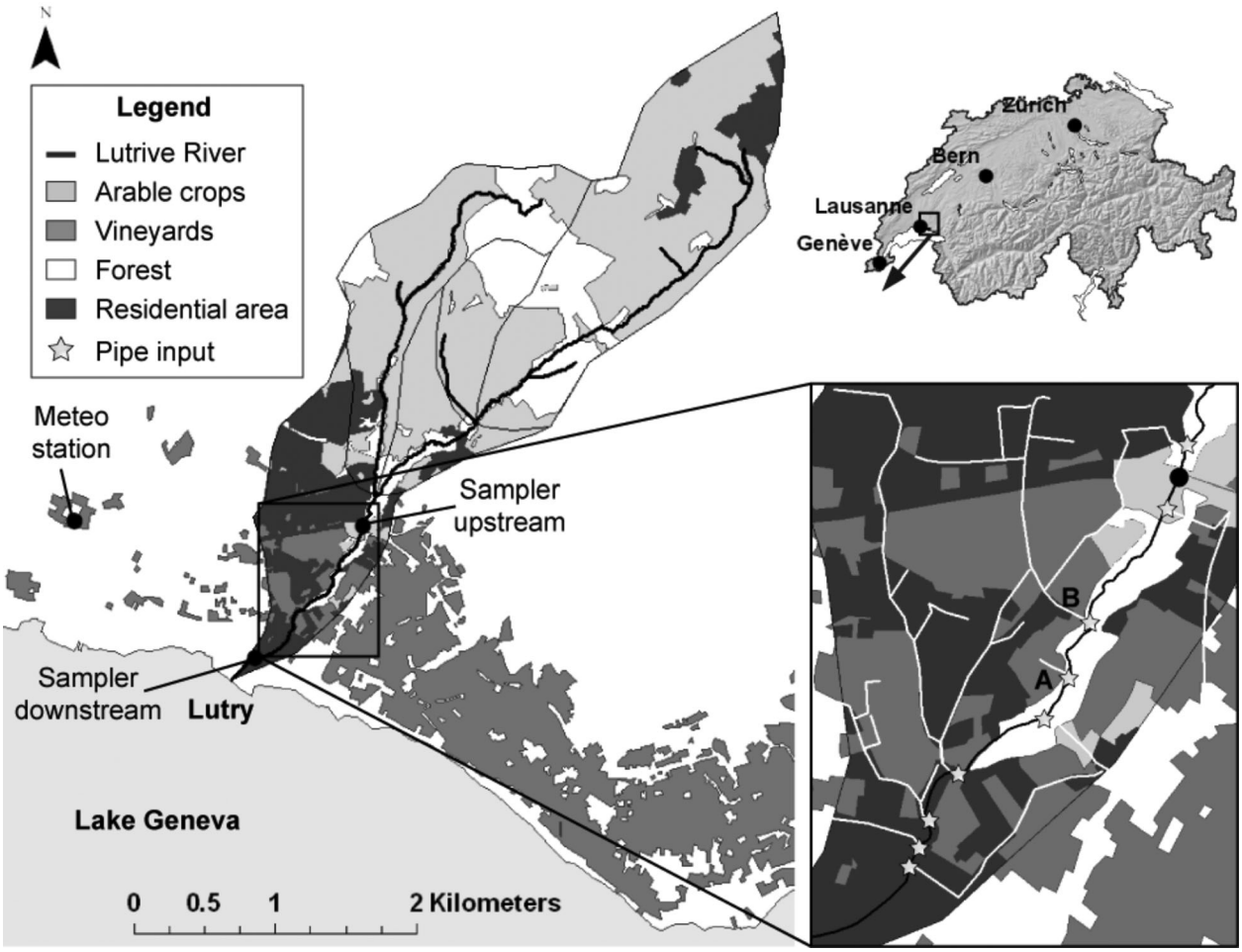

Figure 1. Map of the Lutrive River watershed in western Switzerland with locations of the 2 automatic samplers, upstream and downstream from the vineyard area above the Lutry village, and of the meteorological station of Pully (MeteoSwiss); the zoomed area shows the clear water network in the vineyard area with the drainage pipe **(A)** and the storm water channel **(B)** sampled in May and June 2011.

whereas land use in the upper part of the catchment (5.2 km^2) is mainly characterized by crop fields and forests. The Lutrive River is rain dominated, with only a small contribution from winter snowmelt (December–February). Hourly precipitation data from the meteorological station of Pully, located 2 km from the Lutrive River, were obtained from MeteoSwiss, the official Swiss meteorological network (Figure 1).

In vineyards, glyphosate is generally applied from April to August [10]. In the catchment studied, glyphosate application data were extrapolated from interviews with a few wine-growers ($n = 6$; 3.5% of the vineyard area). Typical application rates ranged from 3 L/ha to 5 L/ha of formulated products containing 360 g/L of active ingredient. The application is made manually because of the important slope of the plots, and generally only under vine rows, leaving a grass cover in between them. Glyphosate is applied once in spring (mid-April), with possible other sporadic and local application(s) later in the season if weed development is considerable. For arable crops (e.g., seed bed preparation for new crops), glyphosate is mainly applied in August and September [27].

The Lutrive River was sampled during the year 2011 upstream and downstream of the vineyard area (Figure 1). Two automatic samplers (ISCO 6712, Teledyne) equipped with polyethylene bottles and ultrasonic flow modules to measure the water level were implemented. These 2 locations were chosen to discriminate the sources of glyphosate (vineyard + urban vs agricultural) and to study the concentration dynamics at different time scales. The water level (h) was monitored every 5 min, and the rating curve for water discharge (Q) was estimated on the basis of NaCl dilution gauging experiments.

The automatic samplers were programmed for 2 types of sampling: a regular and an event-based sampling. One-third of the bottles were dedicated to the regular sampling and the other two-thirds to the event-based sampling. The first was done every 4 d and the second when the water level reached a predefined level, indicating the start of a rain event, and then every 2 h. Fifty samples for the downstream site and 20 for the upstream site were selected from April 2011 to October 2011 and analyzed. Twenty of them—15 downstream and 5 upstream—were regularly collected during dry periods to estimate background levels of glyphosate and AMPA.

Many incoming pipes were observed along the river (Figure 1, stars), discharging potentially polluted waters, coming from either agricultural fields or residential areas: 13 grab samples were collected from one agricultural drainage pipe (Figure 1A), situated approximately at an average depth of 60 cm, and 7 from 1 storm water channel (Figure 1B) in the vineyard area in May 2011 and June 2011 for pesticide analysis.

Analytical methods

Electrical conductivity and pH were measured in the field; samples were then stored in high-density polyethylene bottles at −20 °C until analysis. Dissolved organic and inorganic carbon concentrations were measured with a C-analyzer (Liquitoc Elementar). Major ion concentrations (Na^+, Ca^{2+}, K^+, Mg^{2+}, Cl^-, NO_3^-, SO_4^{2-}) were obtained with an ion chromatography system (ICS-1100/2100, Dionex Thermo Fisher). The herbicide glyphosate and its metabolite AMPA were quantified by ultra-performance liquid chromatography coupled to tandem mass spectrometry after their derivatization with 9-fluorenylmethyl chloroformate followed by solid-phase extraction. Previously, the method was adapted from Hanke et al. [6] and validated with a limit of quantitation of 10 ng/L and a good reproducibility [28]. To evaluate the relative proportion of AMPA compared with glyphosate, its molar ratio was calculated according to Coupe et al. [29]

$$\%\text{AMPA} = [\text{AMPA}]/([\text{glyphosate}] + [\text{AMPA}]) \times 100 \quad (1)$$

where [AMPA] and [glyphosate] are their respective molar concentrations in water.

Glyphosate and AMPA loads were estimated with a basic numeric integration [30]

$$\text{Load} = k \ \Sigma \ c_i \ q_i \ t_i \quad (2)$$

where k is a unit conversion factor, c_i the concentration of the sample i, q_i the discharge at its sampling time (t_i), and $t_i = 0.5\,(t_{i+1} - t_{i-1})$. Then AMPA loads were transformed proportional to glyphosate loads by multiplication with their molecular weight ratio (1.52) [29].

Risk assessment

The aquatic risk factor (RF) for a given pesticide can be evaluated using Equation 3 [31]

$$\text{RF} = \text{MEC}/\text{PNEC} \quad (3)$$

where MEC is the measured environmental concentration of the pesticide and PNEC is the predicted no effect concentration. This latter is equal to HC_5/AF [31], where the HC_5 is the hazardous concentration for 5% of the species derived from the species sensitivity distribution (SSD), which is based on no-observed-effect concentration (NOEC) data [32], and AF is the appropriate assessment factor between 1 and 5 depending on the available ecotoxicological data [31]. In our case, a factor of 5 was used, as only 15 NOEC data were used covering just 3 taxonomic groups.

The NOEC data for glyphosate were obtained from the US Environmental Protection Agency ECOTOX Database (www.epa.gov/ecotox) and from the literature. Note that the geometric mean was calculated for species with 2 or more NOEC values found in the literature. Classically, only the most sensitive species are considered to construct the SSD curve for pesticides. However, for glyphosate they were difficult to determine. According to Solomon and Thompson [23], diatoms seem to be the most sensitive species, whereas Relyea [33] considered amphibians the most sensitive species. For this reason, all available data were therefore considered to build the SSD curve. However, only data expressed in concentration of active ingredient (μg a.i./L) were considered to build the SSD. Thus, when the formulation was tested and expressed in μg/L but not according to the active substance (μg a.i./L), data were not considered. Furthermore, a difference was made between the tests with the active ingredient (more than 95% of purity, glyphosate acid and isopropylamine salt of glyphosate) and the formulation, expressed in active ingredient (Table 2). Indeed, the formulation is assumed to have a greater effect than the active ingredient, which could influence the risk analysis [22].

No NOEC data were found for AMPA. Therefore, the SSD for AMPA was predicted, based on the SSD of glyphosate, following a methodology proposed by Chèvre et al. [34]. This requires determining the toxic ratio (TR) between glyphosate and AMPA, which was done by conducting an acute toxicity test on the alga *Scenedesmus vacuolatus*. The TR was determined using Equation 4 [34]

Table 2. No-observed-effect concentration (NOEC) data for the active ingredients glyphosate acid and IPA salt

Species scientific name	Species group	Exposure duration (d)	NOEC (μg/L)	Reference
Chlorella pyrenoidosa	Algae, moss, fungi	4	108 000	[45]
Scenedesmus acutus	Algae, moss, fungi	4	2000[a]	[46]
Scenedesmus acutus	Algae, moss, fungi	4	3200	[46]
Scenedesmus quadricauda	Algae, moss, fungi	4	770[a]	[46]
Scenedesmus quadricauda	Algae, moss, fungi	4	1250	[46]
Bufo americanus	Amphibians	16	1000	[33]
Hyla versicolor	Amphibians	16	1000	[33]
Lithobates clamitans clamitans	Amphibians	16	1000	[33]
Lithobates pipiens	Amphibians	16	1000	[33]
Lithobates sylvaticus	Amphibians	16	100	[33]
Rana catesbeiana	Amphibians	16	1000	[33]
Carassius auratus	Fish	4	2 880 000	[47]
Carassius auratus	Fish	4	3 431 000	[47]
Oncorhynchus mykiss	Fish	4	823 500	[47]
Cyprinus carpio	Fish	4	1700[a]	[48]
Oreochromis niloticus	Fish	4	310[a]	[48]
Utterbackia imbecillis	Molluscs	1	10 040	[49]

[a] NOECs obtained from tests using only the active ingredient (more than 95% of purity) as the tested substance.

$$TR = EC50_{glyphosate}/EC50_{AMPA} \quad (4)$$

Median effective concentrations (EC50s) were obtained from the dose–response curve fitted with a log–logistic model. This method assumes that glyphosate and AMPA have a similar mode of toxic action, which has not yet been proved. This approach therefore allows a first risk evaluation, but more ecotoxicity data will be needed to assess a reliable risk for AMPA.

Acute toxicity test with algae

The toxic ratio (Equation 4) was determined through laboratory bioassays testing glyphosate and AMPA toxicity with the algae *S. vacuolatus* (Chlorophyceae; strain 211-8b, Shihira and Krauss). Glyphosate (Pestanal, 99.2%) and AMPA (aminomethylphosphonic acid, 99%) were purchased from Sigma-Aldrich, and the algae *S. vacuolatus* were from the Department of Bioanalytical Ecotoxicology at the Helmholtz Centre for Environmental Research in Leipzig, Germany. Algae were cultured in 50 mL of a sterile inorganic medium [35], in 250-mL sterile Erlenmeyer flasks. They were maintained in an HT Infors shaker at 25 °C and shaken at 0.94 g [36], with continuous illumination of 70 μmol/m^2/s from by cool-white fluorescent lamps to provide optimal conditions of exponential growth.

Standard acute toxicity tests for glyphosate and AMPA were performed according to the Organization for Economic Cooperation and Development procedure [35]. The exposure concentrations varied from 2 mg/L to 80 mg/L for glyphosate and from 60 mg/L to 160 mg/L for AMPA. Each concentration and control was done in triplicate. No adsorption or degradation was assumed during the test, and thus tested concentrations were the nominal concentrations. Algae growth was determined by measuring the absorbance at a wavelength of 690 nm with a microplate reader (ELx800, BioTek Instruments) at the beginning and at the end of the test. The effect (i.e., the growth inhibition) was then assessed with a linear regression of the natural logarithm of optical density over time. Growth rates for the different nominal concentrations (GR_E) and the control (GR_C) were measured simultaneously [36]. The growth inhibition (Inh_{growth}) for each tested concentration was then deduced from these growth rates

$$Inh_{growth} = 100 \times (GR_C - GR_E)/GR_C \quad (5)$$

The NOEC was determined using a *t* test, comparing the growth of a given concentration with the growth of the control.

RESULTS AND DISCUSSION

Precipitations and discharge

During 2011, the total annual precipitation in the study area was 916 mm, with a daily maximum of 48 mm (17 July 2011). This is less than the mean annual rainfall of 1101 mm/yr calculated for 30 yr (1961–1991). This is certainly due to the occurrence of 2 significant dry periods from January to February and during November (Figure 2). Mean discharges were 0.163 m^3/s and 0.198 m^3/s at the upstream and downstream stations, respectively. Discharge was in general lower than 1 m^3/s, except during several flood events, which mainly occur during intense summer rainfall (May–July). A discharge of 1.59 m^3/s was reached on 22 May 2011 after 9.3 mm of rain had fallen in 1 h, whereas discharges of more than 3 m^3/s were reached in June to July, with rainfall of more than 20 mm/d. The maximum of 3.47 m^3/s upstream and 5 m^3/s downstream was reached on 5 September 2011 at 5 AM after 17 mm of rain had fallen in 1 h.

River samples

The chemical composition of the river water was dominated by calcium (Ca^{2+}) and bicarbonate (HCO_3^-) ions and showed a mean pH of 8.2 and a mean electrical conductivity of 300 μS/cm, with minimum and maximum values of 90 μS/cm and 430 μS/cm, respectively (data not shown). These alkaline conditions are known to enhance the potential toxicity of glyphosate, as already mentioned [21,22].

For glyphosate and AMPA, several concentration peaks were found largely above the 100-ng/L threshold for each molecule of pesticide (Figure 3). These peaks related well to the main rain events (>10–20 mm/d), revealing the transfer of these compounds from fields to surface water mainly by surface runoff. Concentration ranges of glyphosate and AMPA are in agreement with previous results of other rivers in Switzerland (25–3940 ng/L) [6,12], but are lower than in other countries (2–700 μg/L) [37,38]. Concentrations were much higher downstream than upstream, as the main vineyard area is located in the

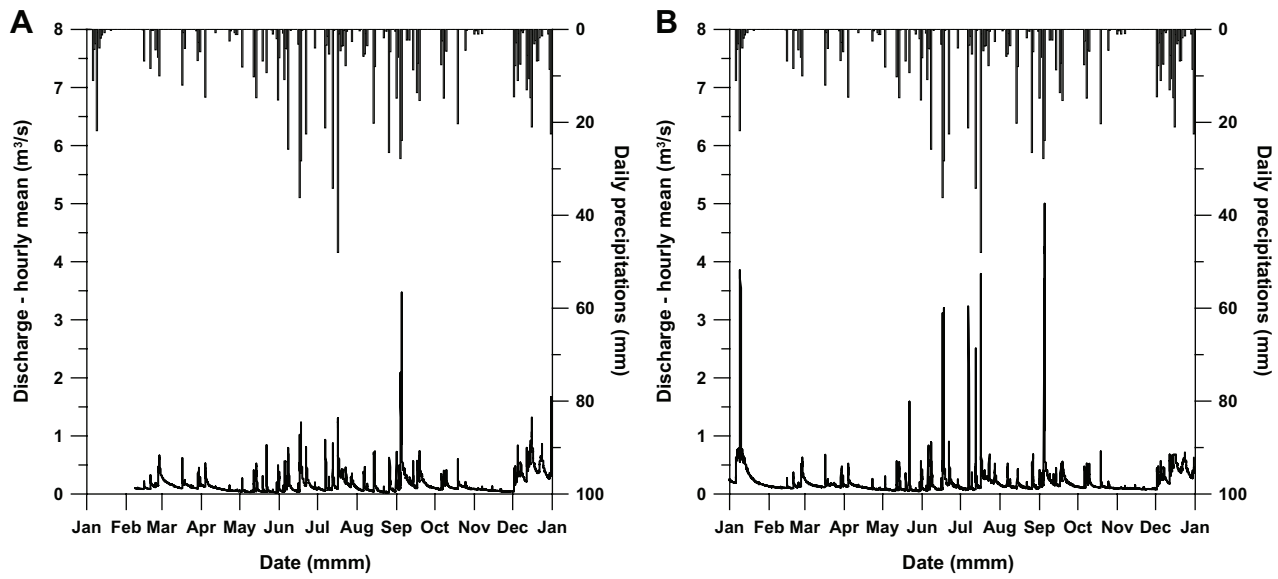

Figure 2. Hourly discharge of the Lutrive River measured at the (**A**) upstream and (**B**) downstream station for 2011. Daily precipitations from the meteorological station of Pully are shown on the right axis (Source: MeteoSwiss).

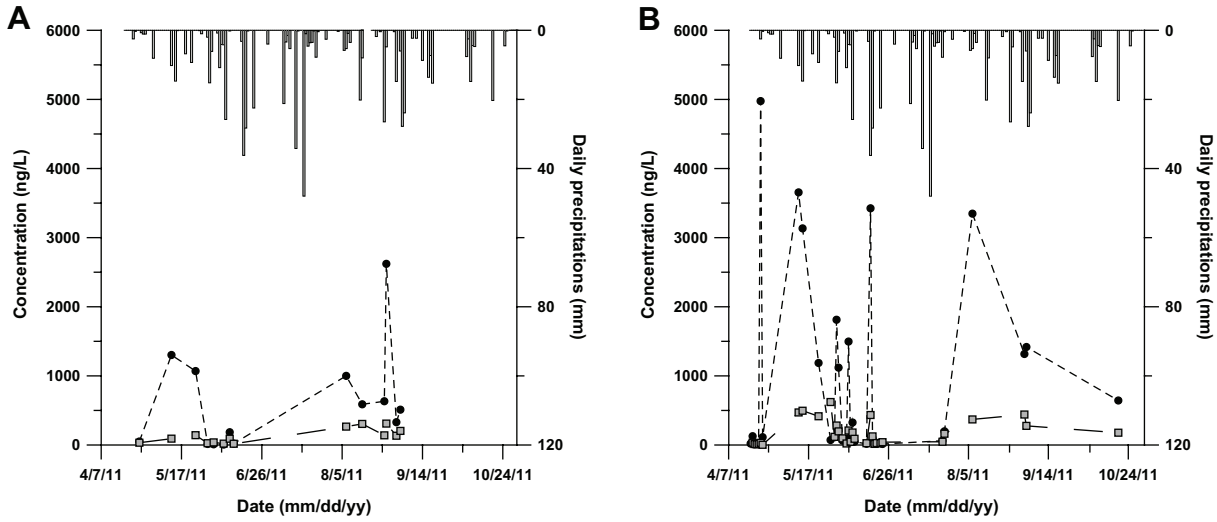

Figure 3. Concentrations of glyphosate (●) and aminomethylphosphonic acid (AMPA; ▒) (**A**) upstream and (**B**) downstream of the vineyard subcatchment for the period April to October 2011. Daily precipitations from the meteorological station of Pully are shown on the right axis (Source: MeteoSwiss). Dashed lines between samples were added for a better dynamic visibility, although they are totally hypothetical.

southern subcatchment (Figure 1). This may also be explained by urban sources occurring in the same subcatchment, such as private gardening and weed management along railways, which are known to represent sources for surface water pollution by glyphosate [27,39].

The highest glyphosate concentration (4970 ng/L) was observed at the outlet during the first rain after the application, 15 April 2011, despite its low intensity (2.5 mm) and a period of 8 d of dry weather between (Figure 3B). Then glyphosate concentrations decreased down to 3000 ng/L to 3500 ng/L in mid-May and then from 1000 ng/L to 2000 ng/L in early June, although rainfall events became more important (>10 mm/d). This decrease certainly reflects the degradation of glyphosate into AMPA in vineyard soils. However, the important rainfall of 17 June 2011 (36 mm) gave rise to a higher concentration (3420 ng/L), suggesting either a higher transfer rate or other applications in the vineyard. This peak was equivalent to the one observed in early August with much lower rain (11 mm in 2 d), which could indicate a late application in the vineyards, but also the beginning of the contribution of glyphosate applied on arable crops in the upper part of the catchment (Figure 3A; 1000 ng/L). Indeed, the highest contamination peak upstream resulting mainly from application on crops was observed in late August (2620 ng/L).

Concentrations of AMPA did not vary much, with a mean concentration of 170 ng/L, a median concentration of 160 ng/L at the outlet, and a maximum of 620 ng/L observed on 28 May 2011. The downstream molar ratio of AMPA (Equation 1) increased from 10% to 30% 1 mo after glyphosate application up to 60% to 90% after 2 mo, revealing glyphosate biodegradation in soils. The ratio decreased to 20% after every important rain event, revealing new inputs of glyphosate in the river from surface runoff. Nevertheless, the median value for AMPA was 55%, suggesting a slightly higher proportion relative to

glyphosate in most samples. This is in contrast to previous results for a French vineyard river [29], where a median value of 31% was observed. This discrepancy may be explained by the bigger size of the catchment and the larger proportion of urban area in the present study, with the possible associated release of AMPA resulting from the degradation of phosphonate detergent molecules [39], or from glyphosate applications in private gardens, paved forecourts, and streets, or from both factors [27]. Although these 2 latter types of application are forbidden in Switzerland by the chemical risk reduction ordinance (ORRChem), they were proved to be not negligible [27]. Thus, the urban environment can also be an important contributor to glyphosate and AMPA in surface waters in some cases [27,39], as already mentioned. Nevertheless, in the present study, urban sources are likely to be minor compared with the vineyard contribution.

During rain events, glyphosate and AMPA concentrations increased rapidly with river discharge, reaching a maximum almost at the same time, and also decreased rapidly afterward (Figure 4A). A lag time of only 1 h was observed between the rainfall and the discharge peak, reflecting the small size of the catchment. This finding suggests a fast transfer of glyphosate and AMPA to the river at the catchment scale, such as from runoff on impervious surfaces, as previously suggested by Hanke et al. [27], and also the concentration of this runoff water in channels of different types (see *Drainage and storm water* and Figure 1). In general, glyphosate and AMPA concentrations increased with river discharge, with the exception of concentrations above 3000 ng/L with relatively small discharge (<0.6 m^3/s), which are representative of sampling just after application (Figure 4B). Although sources cannot be clearly differentiated between vineyard and residential areas, because of the mixed land use in the subcatchment considered, the steepness of the vineyards ($\sim$10%) likely gives rise to a fast transport of the herbicide by surface runoff to the river, after spring applications because of the presence of surrounding impervious surfaces.

Total loads

When glyphosate and AMPA loads were cumulated (Equation 2), a total amount of 7.1 kg was found downstream from mid-April to early September, which is 3 times higher than previously found in other areas of Switzerland [27]. When one assumes an application of glyphosate on 50% to 100% of the total agricultural area and at the recommended rate of 1080 g/ha, this total load represents, respectively, 12% to 6%, which is much higher than the 2% or less found in previous studies [29,40]. This discrepancy can be attributed to the steepness of the vineyard area and also, in part, to the uncertainties of the extrapolated applied amount. During the same period, a total amount of 1.04 kg was found upstream of the vineyard area, which represents 15% of the load found downstream. Note that this upper part corresponds to 80% of the total watershed area. Thus, the relative contribution of vineyards (85%) and the minor contribution of applications in residential areas are more important than the contribution from arable crops, for which other herbicide molecules may be in use at the same time. These results highlight the importance of vineyards, even with relatively small surfaces (20%), to the potential pollution of receiving waters.

Drainage and storm water

Concentrations of glyphosate and AMPA observed in grab samples, taken in the drainage pipe and the storm water channel, were highly variable (Figure 5). Glyphosate and AMPA contamination peaks of 2050 ng/L and 350 ng/L, respectively, were observed in the drainage pipe (Figure 5A) in early May, after 8 mm of rain. The second peaks of 940 ng/L and 590 ng/L of glyphosate and AMPA, respectively, were observed on 20 June 2011, after 68 mm of rain in 2 d. These latter peaks apparently did not contribute to the peaks observed in the Lutrive River outlet (3420 ng/L of glyphosate), which occurred at the beginning of the rain event (17 June 2011). Indeed, percolation water, carrying glyphosate and AMPA molecules, obviously took 2 d to reach the depth of the drainage pipe and was then carried away toward the river. In fact, the drainage waters of this pipe are not directly discharged into the river, but in the forest strip along it, where retention of glyphosate and AMPA could occur. In any event, these results confirm the leaching of the strongly sorbed herbicide glyphosate (mean $K_d = 222$; www.eu-footprint.org/ppdb) and the importance of drainage pipes in its transfer to surface water in agricultural areas, as previously observed [41].

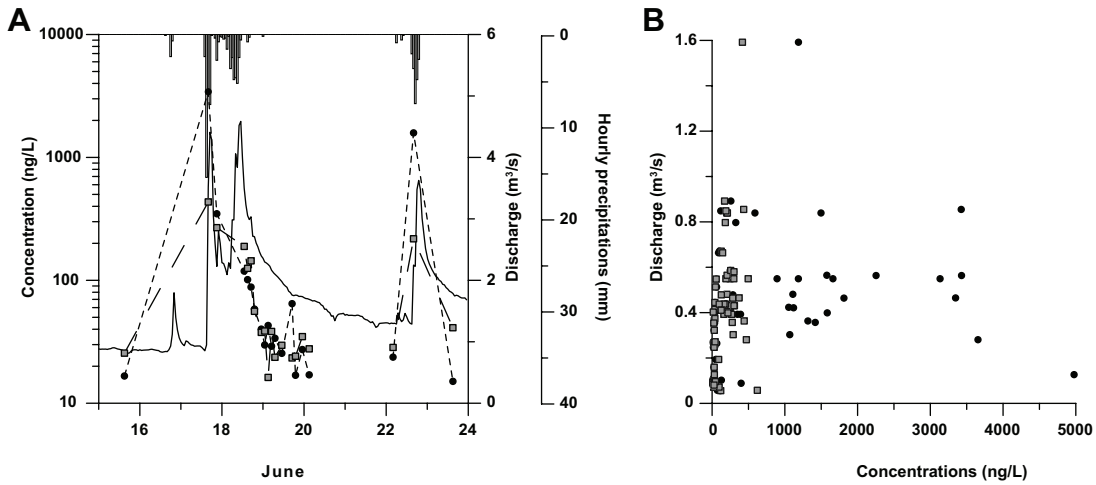

Figure 4. (**A**) Concentration dynamics of glyphosate (●) and aminomethylphosphonic acid (AMPA; ■) at the downstream station during the rain events occurring between 15 June and 23 June 2011, with discharge and hourly precipitations (Source: MeteoSwiss). Note the logarithmic scale; dashed lines between samples were added for a better dynamic visibility, although they are totally hypothetical. (**B**) Discharge of the Lutrive River (m^3/s) versus glyphosate (●) and AMPA (■) concentrations at the downstream station for the period April to October 2011.

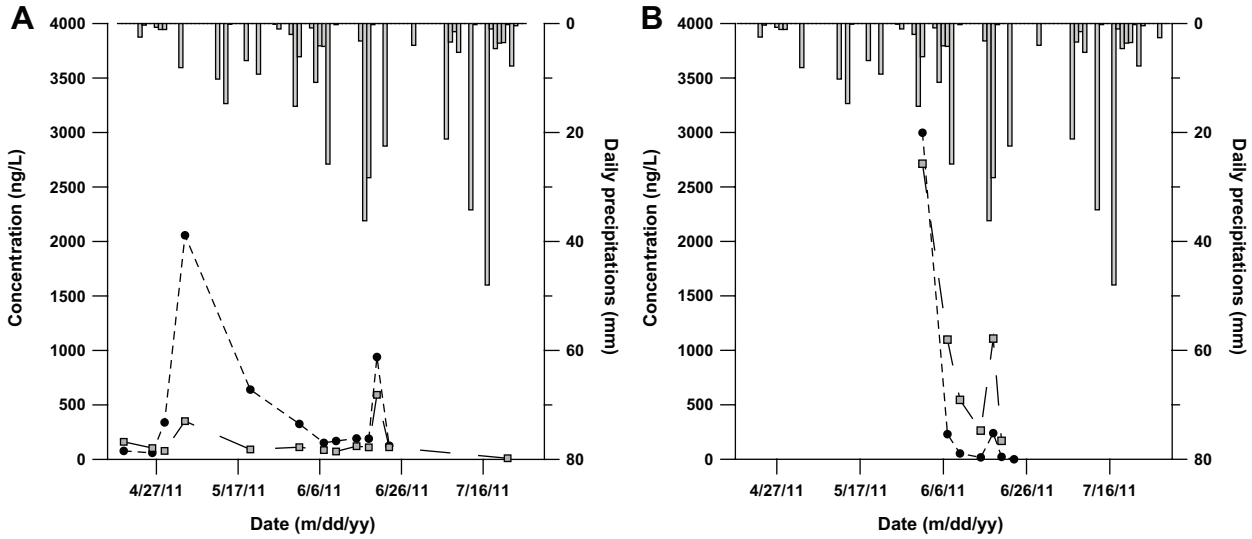

Figure 5. Concentrations of glyphosate (●) and aminomethylphosphonic acid (AMPA; ■) observed in sporadic grab samples of (**A**) a drainage pipe in a vineyard parcel and (**B**) a storm water channel in the vicinity of the vineyards, from April to June 2011. Daily precipitations are shown on the right (Source: MeteoSwiss). Dashed lines between samples were added for a better dynamic visibility, although they are totally hypothetical.

The storm water samples taken in the vicinity of vineyard parcels (Figure 5B) showed higher concentrations than those taken in the drainage pipe (Figure 5A). This channel carried runoff waters coming from many vineyard parcels, located above a road, as well as a residential area, whereas the drainage pipe carried percolation waters from only a few vineyard parcels (cf. Figure 1). Except for the first sample, AMPA concentrations were up to 4 times higher than for glyphosate, suggesting either another urban source or its slower degradation in soils (half-degradation time $[DT50]_{field} = 151$ d for AMPA and 12 d for glyphosate; www.eu-footprint.org/ppdb).

Risk assessment

The results of the risk assessment approach are based on the SSD curves for glyphosate and AMPA (Figure 6). The SSD of glyphosate was obtained from the available NOEC data (Table 2), whereas the SSD of AMPA was extrapolated from the SSD of glyphosate, using the TR (Equation 4). This latter is based on EC50 values, resulting from the standard acute toxicity test, for the algae *S. vacuolatus*. These EC50s are presented in Table 3, as are values found in the literature for several algae in freshwater, and for the active ingredient with a purity higher than 95%. Thus, according to Equation 4, $TR \leq 43\,361/160\,000 \leq 0.27$. Interestingly, the NOEC data in the literature obtained for tests conducted with glyphosate and tests conducted with the formulation (recalculated in glyphosate) do not differ. This is surprising, as the glyphosate formulation is known to be more ecotoxic than the active ingredient, due to the important toxicity of the polyoxyethylene tallowamine surfactant [19,20,22]. However, this may be because of the few available ecotoxicity data (15 NOEC values used in our calculation, from Table 2).

The minimum, maximum, and mean concentrations of glyphosate and AMPA measured in the Lutrive River were lower than those of the 2 SSD–NOEC curves of glyphosate and AMPA (Figure 6), and thus are expected to have a very low

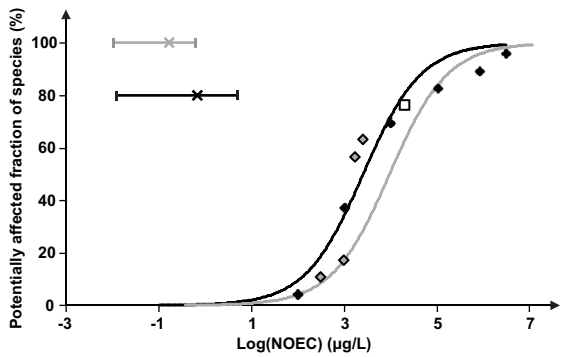

Figure 6. Species sensitivity distribution (SSD) curve for glyphosate (black line) obtained from literature data (black and gray diamonds) and predicted SSD for aminomethylphosphonic acid (AMPA; gray line). The gray diamonds correspond to literature no-observed-effect concentration (NOEC) values obtained with the active ingredient (more than 95% of purity, glyphosate acid and IPA salt of glyphosate), and the black ones with the formulated products, expressed in active ingredient. The white square corresponds to the NOEC of glyphosate derived from the laboratory test. The minimum, maximum, and mean concentrations of glyphosate (black line) and AMPA (gray line) measured in the Lutrive River are represented on the top left of the figure.

Table 3. Median effective concentration (EC50) values in µg/L for several algae found in the literature with their confidence intervals when available

Species scientific name	EC50 (µg/L)	References
Scenedesmus vacuolatus (glyphosate)	43 361 (40 190;46 783)[a]	Present study
Scenedesmus vacuolatus (AMPA)	>160 000[a]	Present study
Pseudokirchneriella subcapitata	5555	[50]
Scenedesmus quadricauda	7200 (4400;8900)	[46]
Scenedesmus acutus	10 200 (10 400;11 200)	[46]
Pseudokirchneriella subcapitata	24 700 (22 800;26 700)	[19]
Scenedesmus quadricauda	70 500	[51]
Pseudokirchneriella subcapitata	129 000 (108 000;158 000)	[52]
Pseudokirchneriella subcapitata	270 000 (224 400;315 600)	[20]

[a] Values obtained in the laboratory; for aminomethylphosphonic acid (AMPA), no published data were found.

Table 4. Minimum and maximum concentrations in μg/L and maximum risk factors in several rivers and streams of the Lake Geneva catchment and of other countries in the world, for glyphosate and aminomethylphosphonic acid (AMPA)

	Glyphosate		AMPA	
HC_5/5 [μg/L]	7.5		27.5	
Rivers and streams [reference]	Concentrations (min–max) (μg/L)	Maximum risk factors	Concentrations (min–max) (μg/L)	Maximum risk factors
Lutrive [present study]	(0.015–4.97)	0.67	(0.01–0.62)	0.023
Venoge River [12] [a]	0.12	0.016	0.12	0.004
Charmilles stream [12] [a]	2.8	0.38	3.94	0.143
Rhine river (Switzerland) [6]	(0.025–0.055)	0.007	(0.055–0.065)	0.002
Streams in Buenos Aires Province (Argentina) [38]	(100–700)	95[b]	–	–
Rivers in France [37]	(2–165)	22[b]	(2.1–48.1)	1.74[b]

[a]For the Venoge River and Charmilles streams, only one measure was available.
[b]Maximum risk factors exceeding the norm of 1.

effect on aquatic species. Indeed, for the glyphosate maximum concentration of 4970 ng/L measured in the Lutrive River, the fraction of affected species is 1.28%, whereas for a minimum concentration of 10 ng/L the same fraction decreases to 0.02%. For AMPA, the maximum concentration of 620 ng/L leads to 0.12% of affected species, and less than 0.01% for the minimum concentration of 10 ng/L.

For glyphosate, the HC_5 was found to be 37 μg/L and approximately 138 μg/L for AMPA. The risk factor is therefore equivalent to 0.67 and 0.023 for glyphosate and AMPA, respectively. As these values are much less than 1 [31], the risk can be considered small for the Lutrive River. However, for AMPA, the results should be considered a preliminary evaluation. Indeed, the SSD was predicted from the SSD of glyphosate (Figure 6). Accordingly, the risk to aquatic species because of glyphosate and AMPA maximum concentrations measured in other rivers or streams of the Lake Geneva catchment was also considered negligible (Table 4). Indeed, the risk factor calculated for glyphosate and AMPA concentrations found in the Venoge River and in the small Charmilles stream in the Geneva area was also very low (Table 4). However, the risk factor probably depends on the river size, as it is attenuated with highly diluted concentrations in larger rivers, such as the Venoge or the Rhine River, as compared with smaller ones, such as the Charmilles or the Lutrive River in the present study. It may be different worldwide, as the risk of glyphosate and AMPA is high in some parts of France or in Argentina, as illustrated in Table 4. The much higher concentrations found in Argentina can be linked to the more intensive nature of current agriculture practices, such as direct sowing in glyphosate-tolerant transgenic soybean cultivation, combined with a soil that is highly susceptible to hydric erosion [38]. Thus, applications are higher than in Swiss vineyards, and lead to a large transfer of glyphosate, partly attached to particles [42], from the near fields to adjacent streams with precipitations, because of the low retention of the bare soils associated with this practice.

Although glyphosate seems not to present a great risk for the aquatic ecosystem, the stimulation effects at low concentrations represent an interesting result. In the algae test conducted, growth stimulation was observed with the three first concentrations, 2000 μg/L, 10 000 μg/L, and 20 000 μg/L. Corresponding inhibition values, with 95% confidence interval, were −0.015 (−0.043; 0.013), 0.006 (−0.008; 0.019), and −0.015 (−0.055; 0.025). This is because of the hormesis effect, which was also observed with other algae or species [24–26]. A possible explanation could be the N or P content of glyphosate [23]. If such an effect does not constitute a risk (no inhibition is observed), one could argue that stimulation is also an effect. It can then be supposed that low concentrations of glyphosate, in rivers or lakes, could promote the development of algae. This may be considered a risk, because such changes can affect the ecosystem, as already demonstrated with phosphorus pollution and eutrophication [43].

CONCLUSIONS

Our results confirm the mobility of the herbicide glyphosate and its metabolite AMPA, which are leached and/or washed from vineyard and urban areas and enter surface waters along different pathways. Small vineyard surfaces could generate high concentrations of herbicides and contribute considerably to the total load calculated at the outlet. The results also reveal interesting details on herbicide dynamics in urbanized landscapes, such as the Lavaux vineyard. Indeed, in addition to their physicochemical properties, they strongly depend on application rates, precipitation regime, land use, and the presence of drains or constructed channels in the catchment.

With the methodology used in the present study, the ecotoxicological risk, linked to the maximum concentrations of glyphosate and AMPA observed in the Lutrive River, was found to be negligible (risk factor < 1). However, the risk factor was assessed for the active ingredient, and surfactants are known to participate actively in glyphosate-formulated product toxicity toward aquatic organisms. In addition, growth stimulation on the algae *S. vacuolatus* in the laboratory was observed, illustrating a potential hormesis effect for this active ingredient. Furthermore, to come up with a realistic risk assessment, other pesticide molecules applied in vineyards should be taken into account and mixture toxicity tests performed. Another improvement in risk assessment could be the use of pulse exposure tests in the laboratory. Indeed, the input of herbicides into the aquatic environments often occurs in pulses of a few hours, rather than as continuous flows, resulting in fluctuating exposure of aquatic organisms to these pollutants. Future integration of the dynamic of concentrations in risk assessment will help to better characterize the potential risk of glyphosate.

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Sulfamethoxazole and isoproturon degradation and detoxification by a laccase-mediator system: Influence of treatment conditions and mechanistic aspects

Jonas Margot[a,*], Pierre-Jean Copin[b], Urs von Gunten[a,c], D.A. Barry[a], Christof Holliger[a]

[a] School of Architecture, Civil and Environmental Engineering (ENAC), Ecole Polytechnique Fédérale de Lausanne (EPFL), Station 2, 1015 Lausanne, Switzerland
[b] Faculty of Geosciences and the Environment, University of Lausanne, 1015 Lausanne, Switzerland
[c] Eawag, Swiss Federal Institute of Aquatic Science and Technology, Überlandstrasse 133, 8600 Dübendorf, Switzerland

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ABSTRACT

The potential of laccase-mediator systems (LMS) for the removal and detoxification of two wastewater micropollutants, the antibiotic sulfamethoxazole (SMX) and the herbicide isoproturon (IPN), was assessed. The influence of various parameters on micropollutant oxidation rates, such as pH, mediator, enzyme and pollutant concentrations, was investigated with three mediators: 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), syringaldehyde (SA) and acetosyringone (AS). Both pollutants were completely transformed within a few hours in presence of laccase and ABTS, as well as, for SMX, in presence of AS or SA. The three mediators were consumed during the reaction (no catalytic reactions observed), at a ratio mediator/pollutant between 1.1 and 16 mol/mol. Faster oxidation kinetics were observed at lower pH values, but also higher mediator/pollutant ratios were required. Several transformation products were formed, including cross-coupled products. Product mixtures were always less toxic to algae than untreated pollutants. Finally, a kinetic model that could explain the experimental observations was established. Based on the findings in this study LMS appears to be a promising option to treat concentrated and potentially toxic industrial effluents.

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1. Introduction

The presence of polar organic micropollutants such as pharmaceuticals and pesticides in wastewater, their poor removal in municipal wastewater treatment plants (WWTPs), and their potential impact on aquatic organisms motivate development of new treatment processes able to deal with these substances [1–4]. Bio-oxidation of these pollutants, catalyzed by oxidative enzymes such as laccase, is a potentially attractive treatment option [5]. Laccases, multi-copper oxidases produced by many fungi and bacteria, can oxidize a wide range of pollutants containing phenol and aniline moieties, including several pharmaceuticals and pesticides, requiring only oxygen as a co-substrate [6].

Many pollutants are recalcitrant to laccase oxidation. One way to increase their range of action is to use redox mediators [7]. Mediators are organic compounds that can be oxidized by laccase to free radicals. As they are less specific, these radicals can oxidize other pollutants and thus increase the spectrum of compounds potentially degraded by these enzymes. Mediators are often described as "electron shuttles" that, once oxidized to radicals by laccase, may be reduced back to their parent compound during the oxidation of a pollutant [8]. This ideal catalytic cycle (Scheme 1), where only oxygen is consumed during pollutant oxidation, is a means to increase the range of action of laccase. The mediator recycling does, however, not always happen and consumption of mediators during the reaction is possible. In this case, the term "laccase enhancer" is a better descriptor [9]. Nevertheless, mediators or enhancers notably widen the substrate range of laccases. Despite several studies on laccase-mediator systems (LMS) for micropollutant removal in wastewater [10–14], the mechanistic aspects of the kinetics of pollutant oxidation, the fate of the mediator during the reaction, and the conditions required for an optimal pollutant oxidation are not completely understood.

* Corresponding author at: EPFL, ENAC, IIE, ECOL, Station 2, 1015 Lausanne, Switzerland. Fax: +41 21 693 8035.
E-mail addresses: jonas.margot@epfl.ch (J. Margot), pierre-jean.copin@unil.ch (P.-J. Copin), urs.vongunten@epfl.ch (U. von Gunten), andrew.barry@epfl.ch (D.A. Barry), christof.holliger@epfl.ch (C. Holliger).

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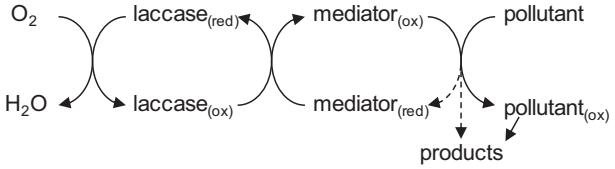


Scheme 1. Ideal laccase-mediator reaction model.

A variety of organic compounds may act as mediators, as long as they can be oxidized by laccase and the free radical formed is stable enough to diffuse away from the enzymatic pocket. Furthermore, the reduction potential has to be high enough to oxidize a target compound [15]. One of the most commonly used synthetic mediators is 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) [9]. This compound is oxidized by laccases to a stable radical cation $ABTS^{\bullet+}$ (Fig. S1, Supporting information (SI)) [8]. Natural mediators have also been identified, mostly lignin-derived phenolic compounds, the most effective of which are syringaldehyde (SA) and acetosyringone (AS) [15]. SA and AS have similar structures and are oxidized by laccase to an unstable phenoxy radical (Figs. S1, SI) [16].

To assess the potential of LMS for micropollutant removal in wastewater, two pollutants of environmental concern are investigated in this study: the sulfonamide antibiotic sulfamethoxazole (SMX) and the herbicide isoproturon (IPN) (Figs. S1, SI). Due to its wide consumption and only poor removal in WWTPs, SMX is ubiquitous in municipal wastewater effluents (in average around 200 ng l^{-1} in Switzerland) [17] and may generate risks for sensitive aquatic organisms in the receiving waters [18]. IPN is a herbicide commonly used in urban areas (parks, gardens, cemeteries) and therefore also frequently detected in municipal WWTP effluents [17]. Due to its toxicity at very low concentrations, it was selected as a priority substance by the European Union [19].

The aims of this study were (i) to assess the potential of LMS for SMX and IPN degradation and detoxification with three mediators: ABTS, AS and SA, and (ii) to determine the influence of the operational parameters (pH, laccase, mediator and pollutant concentrations) on LMS-based oxidation kinetics. Based on these experiments (a) optimal conditions for pollutant degradation were identified, (b) mechanistic aspects of LMS-based systems were elucidated, and (c) the potential of LMS for the treatment of micropollutants in wastewater was assessed.

2. Materials and methods

2.1. Chemicals and enzyme

SMX and IPN (purity > 99%), laccase powder from Trametes versicolor (ref. 38.429, Sigma), ABTS diammonium salt, SA, AS, and all other chemicals were purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland). Stock solutions of SMX (3.95 mM, 1 g l^{-1}), IPN (4.85 mM, 1 g l^{-1}), AS (10 mM, 1.96 g l^{-1}) and SA (10 mM, 1.82 g l^{-1}), were prepared in pure methanol and stored at −18 °C. Stock solutions of ABTS (10 mM, 5.14 g l^{-1}) and laccase (2 g l^{-1}) were prepared in pure water, stored at 4 °C and replaced for any new set of experiments. A stock solution of aqueous chlorine (OCl^-, 8.16 mM) was prepared by diluting a sodium hypochlorite solution (around 5%, Sigma) 100 times. The final OCl^- concentration was measured at pH 10.9 by spectrophotometry at 292 nm (extinction coefficient $\epsilon_{292\,nm}$ of 362 M^{-1} cm^{-1}) [20].

A stock solution of the ABTS radical cation (at 5 mM) was produced by chemical oxidation of ABTS (6.9 mM in pure water) with aqueous chlorine (2.5 mM), in acidic conditions (pH < 5), at a slightly under-stoichiometric ratio to avoid potential residual chlorine in the solution [21]. Another solution of ABTS radical cations was produced by ultrafiltration (Vivaspin 20 centrifugation devices, PES, MWCO: 3 kDa, from Startorius AG, Göttingen, Germany) of a solution of ABTS (500 μM) oxidized by laccase, in order to remove (most of) the enzyme (>60 kDa). The exact $ABTS^{\bullet+}$ concentration was determined by spectrophotometry at 420 nm ($\epsilon_{420\,nm}$ of 36,000 M^{-1} cm^{-1}) [22].

2.2. Laccase activity test

The laccase activity was determined using a colorimetric assay by measuring the oxidation of 0.5 mM ABTS in an oxygen-saturated acetate buffer (0.1 M) at pH 4.5 and 25 °C as described by Margot et al. [23]. A sample aliquot containing laccase was added to the solution and the increase of absorbance at 420 nm was monitored with a temperature-controlled spectrophotometer (U-3010, Hitachi, Tokyo, Japan). One unit of activity (U) was defined by the oxidation of one μmol of ABTS per min.

2.3. Micropollutant, mediator and transformation product analyses

Determinations of SMX, IPN, AS, and SA concentrations were carried out by reverse phase liquid chromatography with a diode-array detector (HPLC-DAD) (LC-2000plus, Jasco, Tokyo, Japan, equipped with Bondapack-C18 column, 15–20 μm, 3.9 mm × 300 mm, Waters™, Milford, USA). Aliquots of 50 μl were injected. Separation of the compounds and the transformation products was conducted with a 30-min gradient, at 1 ml min^{-1}, of pure H_2O containing 0.1% acetic acid (pH 3.23) and increasing concentration of methanol (with 0.1% acetic acid) from 5 to 52% (v/v) or 15 to 60%, for SMX, AS and SA, or IPN determination, respectively. The compounds were detected at 268, 305, 305 and 242 nm for SMX, AS, SA, and IPN, respectively. The limit of quantification (LOQ) was around 0.1 mg l^{-1} (around 0.5 μM), and the accuracy of the measurements (coefficient of variation of 10 injections) was around 1–2%.

Characterization of the transformation products formed during the laccase-mediated reaction was carried out by HPLC coupled to a mass spectrometer (UPLC-MS). Aliquots of 10 μl of each sample were injected in the column (Acquity UPLC system, with a HSS T3 (C_{18}) column, 2.1 × 100 mm, 1.8 μm, Waters), which was eluted at 30 °C in 20 min at 0.4 ml min^{-1} with a mobile phase composed of pure water and methanol, in a gradient mode, from 2 to 95% methanol. Transformation products were characterized (m/z ratio and retention time) and quantified (signal intensity) by MS (Xevo TQ MS, Waters) in scan mode (40–2000 m/z, scan time 0.4 s) and a positive electrospray ionization mode (ES+, cone voltage 30 V). Based on the retention times, some of the transformation products characterized by MS could be related to transformation products observed by HPLC-DAD, which gave further information about their UV/visible absorption spectrum.

2.4. Micropollutant oxidation assay in laccase-mediator systems under various conditions

Micropollutant oxidation assays were performed at different pH values (3–9) in citrate or phosphate buffers (30–40 mM) containing the pollutant at around 100 μM (20–25 mg l^{-1}) and variable concentrations of mediator (10–1000 μM). Batch reactions were conducted in 2-ml glass vials containing 1 ml of an oxygen-saturated reaction mixture. Reactions were initiated by adding laccase to obtain an initial activity between 100 and 650 U l^{-1}. Vials were incubated in the dark at 25 °C under static conditions for several hours (usually around 20 h). After defined reaction times (every 40–160 min), aliquots (50 μl) were withdrawn from each vial and directly injected into the HPLC column to

analyze micropollutant and mediator concentrations. Controls without laccase or without mediators were performed to assess micropollutant degradation by mediators or by laccase alone, respectively. Experiments were typically carried out in duplicate.

Several experiments were performed under various conditions to better understand laccase-mediated reactions: (i) three mediators were tested (namely ABTS, AS and SA) with either SMX or IPN; (ii) for each mediator, degradation kinetics were studied at various pH values (from 3 to 9), diverse mediator concentrations (from 10 to 1000 μM), various laccase activities (from 100 to 650 U l^{-1}), and various pollutants concentrations (from 50 to 150 μM).

For the transformation product analyses by UPLC-MS, lower micropollutants concentrations were used: (i) IPN at 20 μM with 500 μM ABTS and 560 U l^{-1} laccase at pH 5, and (ii) SMX at 10 μM with 50 μM mediator (ABTS, AS or SA) and 560 U l^{-1} laccase at pH 6. After defined reaction times (around every hour), 10 μl were withdrawn from each vial and directly injected into the UPLC-MS to follow the kinetics of transformation product formation. Controls with laccase and mediators without pollutants were also performed.

Dissolved oxygen consumption experiments were conducted in a closed (airtight) cell containing an oxygen probe and 3 ml of reactive solution. The cell was closed just after addition of laccase in an air–oxygen saturated solution, without any headspace.

2.5. Evaluation of the role of the ABTS radical cation

The role of the ABTS radical cation ($ABTS^{\bullet+}$) in the oxidation reaction was assessed by comparing degradation kinetics in solutions containing micropollutants (100 μM) and (i) only ABTS (550 μM) chemically oxidized by HOCl, (ii) chemically oxidized ABTS (550 μM) and laccase (280 U l^{-1}), (iii) ABTS oxidized by laccase (160–200 μM, ultrafiltered) with very low laccase activity (<10 U l^{-1}), and (iv) ABTS oxidized by laccase (160–200 μM, ultrafiltered) with further addition of laccase (200–250 U l^{-1}). These experiments were conducted in duplicate, at pH 5 with IPN and pH 5 or 6 with SMX.

2.6. Ecotoxicity test

A growth inhibition assay on the green alga *Pseudokirchneriella subcapitata* was selected to evaluate the toxicity of the micropollutants before and after treatment with the laccase-mediator system, as green algae are among the most sensitive organisms to the herbicide IPN but also to the antibiotic SMX (Ecotoxicity database AiiDA: www.aiida.tools4env.com).

For the toxicity test, mediators (AS and SA) and SMX stock solutions were prepared in pure water, as methanol is toxic to the green algae at the level present in the solutions (50% growth inhibition at 1 g l^{-1} (0.125% v/v), data not shown).

The samples tested consisted of IPN (100 μM) or SMX (150 μM) in a citrate-phosphate (20–40 mM) buffer at pH 5 or 6, respectively, with or without reaction during 40 h (with IPN) or 88 h (with SMX) at 25 °C with a mediator concentration of 500 μM (either ABTS, AS or SA with SMX and only ABTS with IPN) and laccase (540 U l^{-1}). Controls were performed with each mediator and laccase incubated without pollutant, at the same concentrations. Long reaction times were used to assure complete reaction of the pollutants and stabilisation of the transformation products. These solutions were then diluted 200 times (with IPN) or 20 times (with SMX) in the algae growth medium prior to the toxicity tests to achieve concentrations of pollutants (0.5 and 7.5 μM for IPN and SMX, respectively) in the algae medium that still allow them to grow [24,25].

The algae growth inhibition test was performed according to the OECD guideline 201 [26] with the green algae *Pseudokirchneriella subcapitata* (Chlorophyceae ; strain SAG 61.81, from the Culture Collection of Algae, Göttingen, Germany), maintained as described by Valloton et al. [27]. Briefly, exponentially growing algae (initially around 50,000 cells ml^{-1} in sterile mineral AAP growth medium, pH 7.5) were exposed to the diluted samples over a period of 72 h, in an incubation shaker (Infors HT, Bottmingen, Switzerland) at 25 °C and 90 rpm, with continuous illumination (70 μmol m^{-2} s^{-1}) by cool-white fluorescent lamps. Algae growth was determined as described by Daouk et al. [28] by measuring the optical density at a wavelength of 690 nm (linearly correlated to the cell density) with a microplate reader (ELx800™, BioTek® Instruments, Winooski, Vermont) at the beginning and at the end of the test. The growth rate was calculated as the natural logarithmic increase in the optical density over time, and the growth inhibition was then determined by relative comparison of the growth rate of algae exposed to the sample to the one of algae growing in pure mineral media (controls), according to the OECD 201 guideline. Tests were carried out in triplicate, with a coefficient of variation for the growth rates below 16% and 2.5% for the samples and the controls, respectively, meeting the validity criteria of the OECD guideline. SMX and IPN were analysed before and after 72 h of incubation in the samples not treated by laccase-mediator. Less than 3–6% loss was observed for both compounds, confirming a constant exposure during the test.

2.7. Reaction modelling of laccase-mediator systems

Based on the results of the experiments, a kinetic model of laccase-mediator reactions was established, considering Michaelis–Menten type kinetics for laccase/mediator reactions and second-order rate kinetics for mediator/pollutant reactions (see Section 13, SI). The various differential equations were solved numerically with the ode45 solver (variable step Runge-Kutta method) within MATLAB (MathWorks, USA).

3. Results and discussion

3.1. Oxidation of IPN and SMX with various mediators

IPN or SMX did not react with the enzyme or the mediators alone during the time of incubation (up to 72 h) (Fig. S2, SI). Recalcitrance of SMX to laccase oxidation was recently reported [29], although this compound was oxidized by crude *Phanerochaete chrysosporium* laccase extract in another study [30], possibly due to the presence of other oxidative enzymes or mediators in the extracted solution.

With LMS, IPN was completely oxidized in the presence of ABTS in less than 20 h at pH 4–6 (Fig. 1 A). The two other natural mediators (AS and SA) were on the contrary not able to mediate the oxidation of IPN, even at high concentrations (500 μM, pH 5) and for long reaction times (up to 96 h) (Fig. S2 A, SI).

SMX appeared to be much more reactive to LMS oxidation, with reasonable oxidation rates in presence of ABTS and very fast oxidation (almost complete removal in less than 1 h) in the presence of both AS or SA at pH 6 (Fig. 1B–D). Very few studies have been published on SMX oxidation by LMS. Recently, a few studies showed that SMX could be oxidized by laccase in presence of several mediators (1-hydroxybenzotriazole (HTB), syringic acid, AS and SA) [29,31,32]. Oxidation by LMS of other sulfonamides (sulfadimethoxine and sulfamonomethoxine) was also reported [33]. The mechanisms and the conditions for the oxidation of these pollutants by LMS are, however, still largely unknown.

3.2. pH influence on the oxidation kinetics

As shown in previous studies [23,34], pH has a strong influence on the laccase activity, with higher activities (for *T. versicolor*

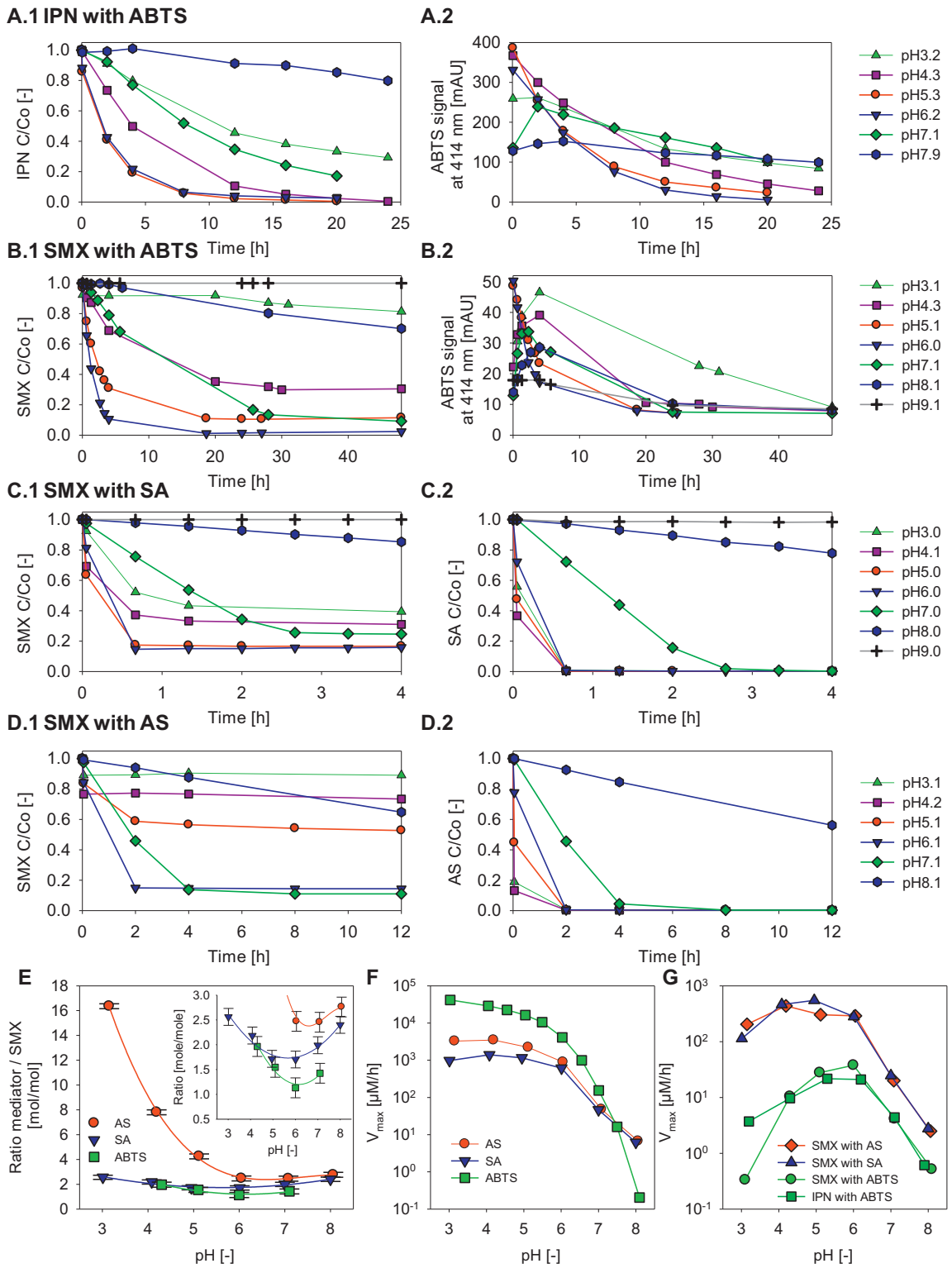


Fig. 1. pH effect on laccase-mediated reactions. (A) Isoproturon (IPN, A.1) in presence of ABTS (A.2: oxidized ABTS HPLC signal). (B) Sulfamethoxazole (SMX, B.1) in presence of ABTS (B.2). (C) SMX (C.1) in presence of syringaldehyde (SA, C.2). (D) SMX (D.1) in presence of acetosyringone (AS, D.2). (E) Stoichiometric ratio of mediator consumed per mole of SMX oxidized in presence of AS (●), SA (▼) and ABTS (■). Insert: zoom into the low ratio range. Error bars: standard deviation. (F) Initial maximum oxidation rates (Vmax, in µM/h) of the mediators AS (●), SA (▼) and ABTS (■) as a function of the pH. (G) Initial maximum oxidation rates of SMX in presence of AS (◊), SA (▲) and ABTS (●), and initial maximum oxidation rates of IPN in presence of ABTS (■) as a function of the pH. Experimental conditions: (A) 100 µM IPN, 500 µM ABTS, 560 U l^{-1} laccase (lac). (B) 73 µM SMX, 100 µM ABTS, 315 U 1^{-1} lac. (C) 75 µM SMX, 110 µM SA, 320 U l^{-1} lac. (D) 93 µM SMX, 201 µM AS, 560 U l^{-1} lac. (F) 201 µM AS with 560 U l^{-1} lac, 110 µM SA with 320 U l^{-1} lac, and 500 µM ABTS with 370 U l^{-1} lac. (G) 73–93 µM SMX with 201 µM AS (560 U l^{-1} lac), 110 µM SA (320 U l^{-1} lac) and 100 µM ABTS (315 U l^{-1} lac), and 100 µM IPN with 500 µM ABTS (560 U l^{-1} lac).

laccase) under acidic conditions (pH 4–6) and almost no activity in alkaline solutions (pH > 7.5–8). It was therefore expected that LMS oxidation would also be strongly influenced by pH, especially during the first stage of the mediator oxidation by laccase. To further elucidate the effect of the pH, experiments were performed in the pH range from 3 to 9. As presented in Fig. 1, kinetics of SMX and IPN abatement varied significantly as a function of pH. Fast oxidation of both compounds was observed at pH 5-6 for all three mediators, with decreasing rates at lower (3–4) or higher (7–8) pH-values. No significant oxidation was observed at pH 9. The optimal pH range for IPN and SMX oxidation was around 5–6 (Fig. 1G or Fig. S3, SI), significantly higher than the optimal pH for mediator oxidation (pH < 3–4) (Fig. 1F). This difference could be related to a higher self-reaction of mediator radicals at lower pH (produced locally at high concentrations), leading to a decreasing pollutant exposure with reactive species (cf. Section 3.7.5).

3.3. Mediator consumption and effect of pH on the ratio mediator/pollutant

Mediators are often described as electron-shuttles between laccase and the substrate, with catalytic action of the mediator (Scheme 1) [8]. However, it appears from our results that neither ABTS, SA nor AS acted as catalysts. These three mediators were consumed during the reaction, with a mediator/pollutant molar ratio in excess of unity. For AS and SA, a clear decrease in their concentration was observed as the reaction progressed, up to their complete disappearance (Figs. 1 C.2 and D.2). This consumption was independent of the pollutant concentration (similar decay rates were observed with or without the presence of SMX, Fig. S5, SI), suggesting that the pollutant was not involved in the disappearance of the mediators. The removal of SA and AS was thus probably caused by an irreversible oxidation by laccase. Although ABTS has been described as a catalytic mediator, with a constant recycling of its radical cation $ABTS^{\bullet+}$ during the oxidation of various substrates [35], no catalytic reactions were observed in this study.

The disappearances of the mediators AS and SA (Figs. 1 C.2 and D.2) were proportional to the disappearance of SMX (linear correlation $r > 0.99$) (Fig. S4, SI). This allowed determining the stoichiometry of the mediator-SMX reaction (molar ratio of the SMX oxidized relative to the mediator consumed). These ratios were pH-dependent, varying from 1.7 (pH 5–6) to 2.4–2.5 (pH 3 and 8), and from 2.4 (pH 6–7) to 16 (pH 3), for SA and AS, respectively (Fig. 1E).

ABTS was not quantified during the reaction, but the radical cation $ABTS^{\bullet+}$ could be determined semi-quantitatively (HPLC-DAD signal intensity at 414 nm). The disappearance of the $ABTS^{\bullet+}$ signal (Figs. 1 A.2 and B.2) was closely linked to the removal of SMX and IPN (Figs. 1 A.1 and B.1), suggesting that the radical cation was involved and consumed during the reaction. The possibility that $ABTS^{\bullet+}$ was reduced back to ABTS was ruled out as laccase activity stayed relatively constant until the end of the experiments, and thus ABTS was oxidized back to $ABTS^{\bullet+}$ at a much higher rate (around 300 μM per min) than its observed consumption (less than 1 μM per min). The molar ratio of ABTS consumed per SMX oxidized was also pH-dependent, with 1.1 at pH 6 and up to 2 mol/mol at pH 4 (Fig. 1E), which is lower than for SA and AS. With IPN, this ratio could not be determined as a function of pH, but at pH 5, a molar ratio ABTS/IPN of 2.28 (±0.3) was found (based on Fig. 2B). IPN required thus 1.5 times more mediator than SMX for a similar extent of oxidation.

The increase in the mediator/pollutant ratio observed at low pH, most pronounced for AS, was probably related to the very fast oxidation of the mediators at low pH, leading to the rapid production of a high level of reactive mediator species. These reactive species reacted then probably more easily with each other than with the pollutants (present in lower concentrations), increasing therefore the mediator/pollutant ratio required at low pH (see Section 3.9 for further discussion).

3.4. Effect of the concentrations of enzyme, mediators and pollutants on the pollutant oxidation rate

To better understand the reactions involved in laccase-mediator systems, several experiments were performed in which either the mediator or the enzyme concentration was varied.

3.4.1. Effect of the enzyme concentration

It was observed that, at pH 5 (Fig. S6 A, SI), the oxidation of IPN with ABTS was not significantly influenced by a variation of the laccase activity by a factor of five (from 120 to 600 U l^{-1}), while a strong influence on the rates was observed when reducing the mediator concentration by a factor of five (from 500 to 100 μM). Therefore, it can be assumed that at pH 5 the rate-limiting step was not the oxidation of the mediator by laccase but the reaction of the oxidized mediator with the pollutant. However, at higher pH, when the mediator oxidation by laccase becomes limiting, higher laccase activity is expected to increase the pollutant oxidation rate.

3.4.2. Effect of the mediator and pollutant concentration

A strong influence of the mediator concentration on the pollutant oxidation rates under acidic conditions was observed for both IPN and SMX (Figs. 2 A and B). As shown in Fig. 2C, the oxidation rates increased proportionally with increasing mediator concentrations, reaching a plateau at high mediator levels. The assumption behind this saturation effect (plateau) was that, at high mediator concentrations (and sufficient laccase activity), high levels of reactive radicals are quickly produced, with a tendency to react with each other (possibly already in the enzymatic pocket) rather than with the pollutants. However, this saturation effect was not observed when, instead of keeping the pollutant concentration constant, the pollutant level was varied in proportion to the mediator concentration so as to keep the mediator/pollutant ratio constant (Fig. 2C and Fig. S6 B, SI). In this case, a linear increase in the oxidation rate was observed as a function of the mediator (and pollutant) concentration, which was expected since the radical/pollutant ratio stayed constant.

In contrast to the results under acidic conditions, at pH 7, no effect of mediator concentrations on the rate of SMX oxidation by the three mediators (Figs. 2 D–F) was observed. At the three mediator concentrations tested (100, 200 and 500 μM), SMX was oxidised at the same constant rate (zero-order reaction) until all the mediator was consumed (Fig. S 5, SI). As laccase is several orders of magnitude less active in neutral-alkaline than in acidic conditions (Fig. 1F), this observation points towards a rate limitation of the oxidation of the mediator by laccase. Enzymatic reactions follow Michaelis-Menten kinetics. Therefore, the rate of the reaction does not vary with the mediator concentration when it is present at sufficiently high levels to saturate all the reactive sites of the enzymes. The radicals constantly produced are thus expected to react directly with SMX, leading to zero-order kinetics as long as sufficient mediator is present.

At a constant mediator/pollutant ratio (at pH 5) and with a sufficiently high mediator concentration, the relative pollutant removal rate appeared to be independent of the initial pollutant concentration (similar to first-order kinetics) (Fig. S 6 B, SI). Although this was not tested with low mediator/pollutant concentrations, it is expected that this independence would not be valid once the mediator concentration becomes lower than a certain threshold, related to the affinity constant of the enzyme for the mediator (Michaelis constant K_m).

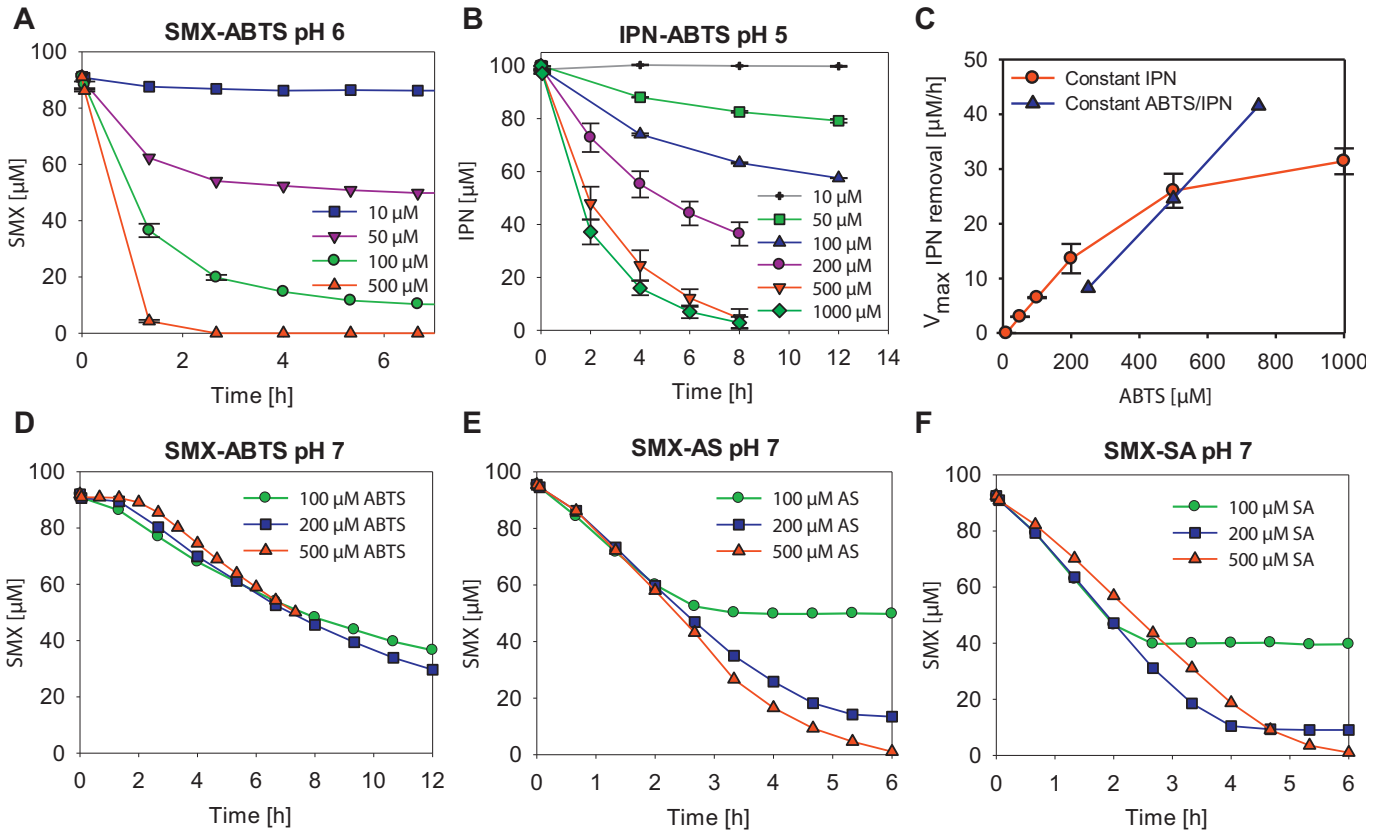


Fig. 2. Influence of mediator concentrations on sulfamethoxazole (SMX) and isoproturon (IPN) oxidation in presence of laccase. (A) Residual SMX concentrations at pH 6 with various concentrations of ABTS (450 U l^{-1} laccase). (B) Residual IPN concentrations at pH 5 with various concentrations of ABTS (560 U l^{-1} laccase). (C) Initial maximum IPN oxidation rates (V_{max}, in µM h^{-1}) as a function of the ABTS concentration with constant initial IPN concentration (100 µM, ●), and variable IPN concentrations (50, 100 and 150 µM, ▲) to maintain a ratio ABTS/IPN of five (pH 5, 560 U l^{-1} laccase). (D-F) Residual SMX concentrations at pH 7 with various concentrations of (D) ABTS (450 U l^{-1} laccase), (E) acetosyringone (AS) (520 U l^{-1} laccase), and (F) syringaldehyde (SA) (455 U l^{-1} laccase). Error bars: range of values of duplicates.

3.5. Oxygen consumption during laccase-mediated reactions

In the laccase-mediated reaction systems, around 0.25 mole of oxygen (one mole of electrons transferred) was consumed per mole of mediator (ABTS, AS and SA) oxidized during the initial phase of the reaction (when the mediator was still present at high concentration). Oxygen consumption stopped once SA was oxidized while, in presence of AS and ABTS, almost complete oxygen depletion was observed (Fig. S7, SI). This further oxygen consumption suggests that additional oxidation of the transformation products of AS and ABTS occurred slowly. In all cases, complete oxygen depletion was observed only after complete pollutant oxidation, suggesting no oxygen limitation in the reaction.

3.6. Role of the ABTS radical cation for the oxidation of SMX or IPN

For AS and SA, it is expected that the unstable and reactive phenoxy radicals generated during their oxidation by laccase (Fig. S1, SI) are the reactive species causing the pollutant oxidation. For ABTS, it is not clear which reactive species are involved in pollutant oxidation. It is reported that $ABTS^{\bullet+}$ can oxidize several compounds, mainly polyphenols, phenols or anilines [35,36]. To elucidate the fate of the selected target compounds, we investigated if $ABTS^{\bullet+}$ alone (oxidized chemically) could also oxidize SMX and IPN. As presented in Figs. S8 C and S9 C (SI), no SMX or IPN removal was observed with $ABTS^{\bullet+}$ alone, while addition of laccase (280 U l^{-1}) in the same solution led to complete removal of both compounds in a few hours. Low laccase activity (7–9 U l^{-1}) enabled pollutant oxidation but when laccase was inhibited no degradation was observed with $ABTS^{\bullet+}$ (Figs. S8 A and S9 A, SI). This demonstrates that $ABTS^{\bullet+}$ is not directly responsible for SMX or IPN oxidation and that laccase is necessary to catalyze this reaction.

To investigate if the reactive product responsible for pollutant degradation was formed during ABTS or $ABTS^{\bullet+}$ oxidation by laccase, ABTS was chemically oxidized by chlorine to form a solution containing only $ABTS^{\bullet+}$, which was then used to treat the pollutants by addition of laccase. A comparison between the removal efficiencies of IPN and SMX by laccase with oxidized $ABTS^{\bullet+}$ or laccase with ABTS showed almost identical results (Fig. S10, SI), suggesting that the reactive species were formed from the reaction of $ABTS^{\bullet+}$ with laccase. Moreover, $ABTS^{\bullet+}$, which is quite stable in pure solution (half-life of 47 h at 20–23 °C) [21], was degraded in presence of the pollutants and laccase, at a rate strongly correlated with the removal rates of the micropollutants (Figs. 1 A.2 and B.2 and Fig. S8 B, SI). This suggests that laccase reacts with $ABTS^{\bullet+}$ producing reactive species, which in turn react with the pollutants.

A transformation product with a UV/vis spectrum similar to a degradation product of the ABTS di-cation ($ABTS^{2+}$) was detected in samples incubated with ABTS and laccase, suggesting an $ABTS^{2+}$ formation (see Section 3.7). Although the direct oxidation of $ABTS^{\bullet+}$ (reduction potential $E^0 = 0.6$ V) to the stronger oxidant $ABTS^{2+}$ ($E^0 = 1.1$ V) by laccase (E^0 around 0.8 V) is thermodynamically unfavourable, it was suggested that this reaction could slowly happen inside of the enzymatic pocket (the electrostatic interaction in the binding site may lower the reduction potential of the ABTS di-cation) [37]. $ABTS^{2+}$ is reported to oxidize several compounds such

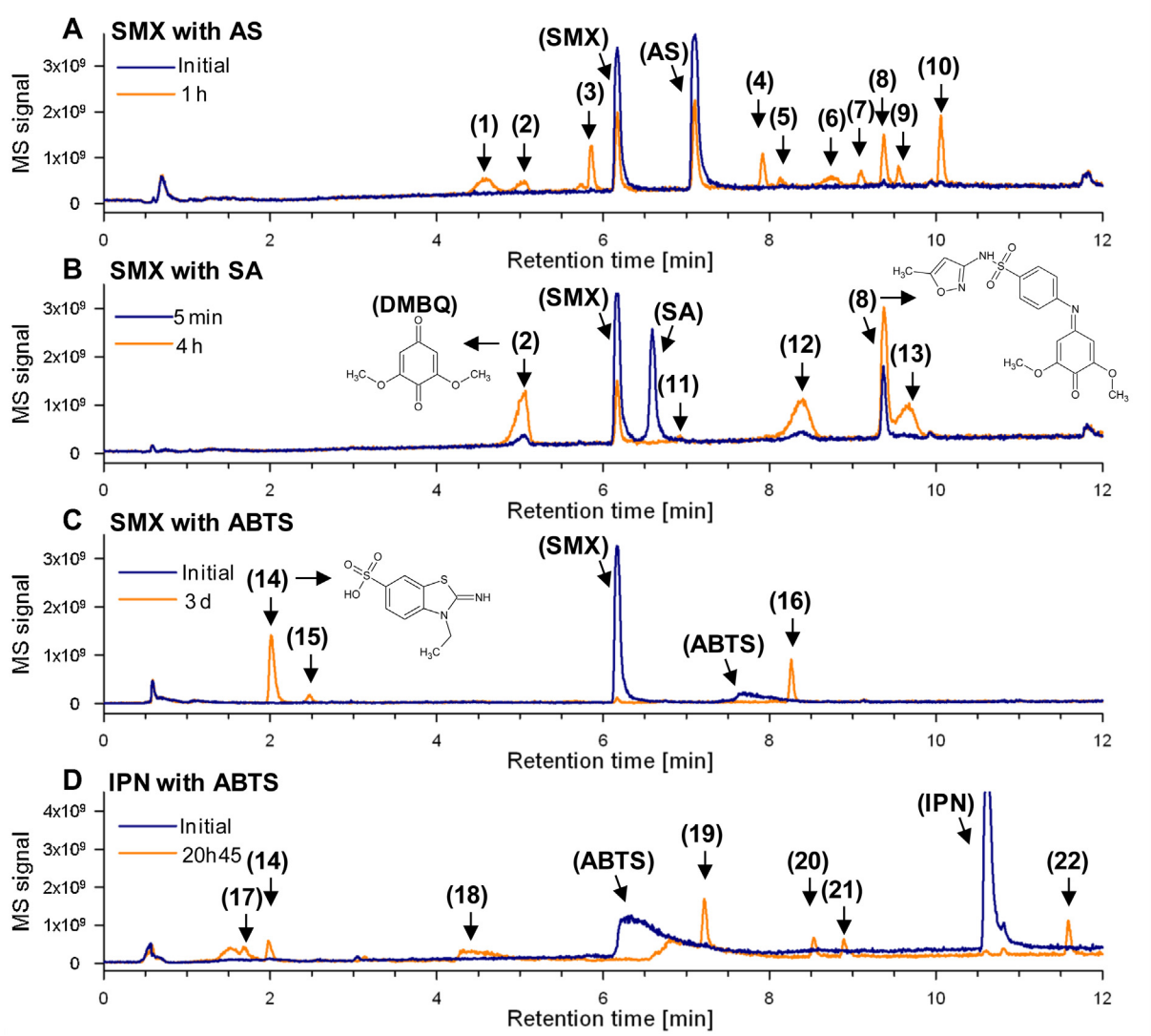


Fig. 3. UPLC-MS chromatograms before and during laccase-mediated reactions. (A) sulfamethoxazole (SMX) in presence of acetosyringone (AS), (B) SMX in presence of syringaldehyde (SA), (C) SMX in presence of ABTS, and (D) isoproturon (IPN) in presence of ABTS. Numbers in bracket: ID of the main transformation products (TPs). The structures of TPs 2, 8 and 14 (confirmed by other studies) is presented as well. Suggested structures for some of the other transformation products are shown in Fig. S11 C, SI.

as aromatic alcohols that cannot be oxidized by $ABTS^{\bullet+}$ [38,39]. $ABTS^{2+}$ (low solubility and very low stability in water) or one of its degradation products could therefore be the reactive species responsible for pollutant oxidation in the combined laccase/ABTS system [37,39]. A slow production of $ABTS^{2+}$ may explain why the pollutant oxidation in the laccase/ABTS system takes several hours while the enzyme oxidizes ABTS completely to $ABTS^{\bullet+}$ within a few minutes. These results indicate that in the present case, the real mediator is not ABTS but its radical cation $ABTS^{\bullet+}$, which is oxidized by laccase to a reactive species with higher reduction potential (possibly $ABTS^{2+}$) which, in turn, reacts with IPN or SMX.

3.7. Characterization of the transformation products

Oxidation by LMS does not lead to complete pollutant mineralization because laccase and the oxidized mediators react mainly with some specific (electron donating) moieties of organic compounds. As shown in the chromatograms in Fig. 3, several transformation products were detected by UPLC-MS for the reaction of SMX in presence of the three mediators and for the reaction of IPN in presence of ABTS.

3.7.1. Transformation products formed in the laccase-AS-SMX system

During SMX oxidation by laccase in presence of AS, 10 main transformation products were detected (ID number 1–10, Fig. 3A). Six of them had a molar mass higher than SMX or AS suggesting that they were coupling products (Table 1). Several products (1–3 and 6) were also generated during the oxidation of AS by laccase. Product 2, with a mass of 168 g mol^{-1} and a maximum UV/vis absorbance at 290 nm (Table S1, SI), was identified as 2,6-dimethoxy-1,4-benzoquinone (DMBQ) (Fig. 3B), as observed in other studies [33,40]. Product 6, with a mass of 332 g mol^{-1}, is likely (but exact structure not confirmed) a dimeric product of AS, as proposed in Fig. S11, SI. Similar dimeric products were observed by Ibrahim et al. [40]. Products 4–5 and 7–10 were observed only when SMX was present. Product 8, with a mass of 403 g mol^{-1} and UV–vis absorption spectrum maxima at 200, 314 and 405 nm, was identified based on similar studies [29,33] as a coupling product between DMBQ and SMX (Fig. 3B). The masses of products 5, 7, 9 and 10 (Table 1) suggest that they were also coupling products between AS radicals and SMX (structure suggestion in Fig. S11, SI), as also proposed by Shi et al. [29]. All these transformation products appeared rapidly during the first hour of reaction, linked

Table 1
Retention time, molar mass and m/z of the parent compounds and the transformation products (TPs) detected by UPLC-MS during the laccase-mediated transformation of sulfamethoxazole (SMX) in presence of either acetosyringone (AS), syringaldehyde (SA) or ABTS, or isoproturon (IPN) in presence of ABTS. The numbers of the transformation products correspond to Fig. 3. Structures of compounds with similar masses are suggested for some transformation products and presented in Fig. S11, SI.

ID	Retention time [min]	Molar mass [g/mol][a]	*m/z* of adducts[b]	Types of product[c]	Structure propositon[d]
Parents compounds					
(AS)	7.11	196	197 + 219	Acetosyringone	(AS)
(SA)	6.6	182	183 + 205	Syringaldehyde	(SA)
(ABTS)	6.44–7.69	514	514 + 515 + 536 + 558	ABTS	(ABTS)
(SMX)	6.19	253	254 + 276	Sulfamethoxazole	(SMX)
(IPN)	10.62	206	207 + 229 + 435	Isoproturon	(IPN)
Transformation products AS + SMX					
(1)	4.58	180	181 + 203	AS TP	–
(2)	5.05	168	169 + 191	2,6-Dimethoxy-1,4-benzoquinone (DMBQ)	(2)
(3)	5.85	182	183 + 205	AS TP	(V)
(4)	7.91	248	249 + 271 + 519	–	–
(5)	8.11	447	448 + 470	*Coupling AS–SMX*	(III)
(6)	8.77	332	333 + 355 + 687	Dimeric AS TP	(I)
(7)	9.1	415	416 + 438 + 454	*Coupling AS–SMX*	–
(8)	9.37	403	404 + 426	Coupling SMX-DMBQ	(8)
(9)	9.56	417	418 + 440	*Coupling AS-SMX*	(IV)
(10)	10.06	445	446 + 468	*Coupling AS-SMX*	–
Transformation products SA + SMX					
(2)	5.05	168	169 + 191	2,6-Dimethoxy-1,4-benzoquinone (DMBQ)	(2)
(11)	6.93	281	282 + 304	–	–
(12)	8.38	318	319 + 341 + 659 + 351 + 373	Dimeric SA TP	(II)
(8)	9.37	403	404 + 426 + 829	Coupling SMX–DMBQ	(8)
(13)	9.66	348	349 + 371 + 367 + 381 + 719 + 403	–	–
Transformation products ABT + SMX					
(14)	2.01	258	259 + 281	ABTS TP	(14)
(15)	2.48	98	99	*SMX fragment*	(VI)
(16)	8.26	238	239 + 261	*SMX fragment*	(VII)
Transformation products ABTS + IPN					
(17)	1.69	273	274 + 296	ABTS TP	(VIII)
(18)	1.99	258	259 + 281	ABTS TP	(14)
(19)	4.72	546	547 + 569	ABTS TP	–
(20)	7.23	222	223 + 245	*Hydroxy-isoproturon*	(IX)
(21)	8.56	445	446 + 468	*Coupling IPN + fragments ABTS*	–
(22)	8.9	447	448 + 470	*Coupling IPN + fragments ABTS*	–
(23)	11.61	232	233 + 255	–	–

[a] Molar mass M deduced from the *m/z* of the adducts.
[b] *m/z* of ESI MS products with positive ion mode: M + H: [M + 1]; M + Na: [M + 23]; 2M + Na: [2M + 23]; M + CH_3OH: [M + 32]; M + NH_4: [M + 18].
[c] Transformation products may come from the mediator degradation by laccase (also observed without pollutant), or by reaction with the pollutant. In italics : suggestion based on the mass of the by-product. (-): no suggestion.
[d] Refers to the structures proposed in Supporting information, Fig. S 11, based on the mass and the relation log Kow/retention time of the proposed molecule. (-): no suggestion.

to the degradation of SMX and AS (Fig. S12, SI). Concentrations of products 2, 4, 6, 7 and 8 were stable for more than 72 h. In contrast, products 1, 3, 5, 9 and 10 vanished within this time (Fig. S12, SI) suggesting that these products were either not stable or further oxidized by laccase, which is consistent with the additional oxygen consumption observed (cf. Section 3.5). After 72 h, the two main (in signal intensity) products still present in solution were 2 and 8 (Fig. S12, SI).

3.7.2. Transformation products formed in the laccase-SA-SMX system

During SMX oxidation by laccase in presence of SA, only five main transformation products were detected (Table 1). Two of them were the same as found with AS, namely products 2 and 8, which is not surprising because product 2 (DMBQ) is a typical product of SA oxidation [40]. These two dominant products were much more abundant with SA than with AS, with a 3.5–3.8 times higher signal intensity (Figs. 3A and B). The third most abundant transformation product was 12, which is very likely a dimeric product of SA, with a structure similar to the dimeric AS (product 6) (Fig. S11, SI). Similar to AS, the five transformation products appeared during the first hour of reaction together with SMX and SA removal, and were then stable for more than 72 h (Fig. S12, SI).

3.7.3. Transformation products formed in the laccase-ABTS-SMX system

During SMX oxidation by laccase in presence of ABTS, only three transformation products were clearly visible (Fig. 3C, Table 1). Product 14 was also observed during ABTS oxidation by laccase without any pollutant and was identified as 3-ethyl-6-sulfonate benzothiazolinone imine, an ABTS fragment. Its chemical structure (Fig. 3C) was elucidated in other studies [41,42]. Products 15 and 16 were possibly, based on their mass and retention time (relative to their log K_{ow}), degradation products of SMX (Fig. S11, SI). No coupling products with a mass higher than the parent compounds were detected. The three products appeared gradually during 72 h in parallel to the disappearance of SMX and ABTS (sum of ABTS and $ABTS^{\bullet+}$). The highest signal intensity was observed for the ABTS fragment 14, followed by SMX fragment 16.

3.7.4. Transformation products formed in the laccase-ABTS-IPN system

During IPN oxidation by laccase in presence of ABTS, 7 transformation products were detected (Fig. 3D, Table 1). Three of them (14, 17 and 18) were ABTS degradation products, also observed during ABTS oxidation without pollutant, and (at very low concentrations for 17 and 18) with SMX. Product 14 was the same

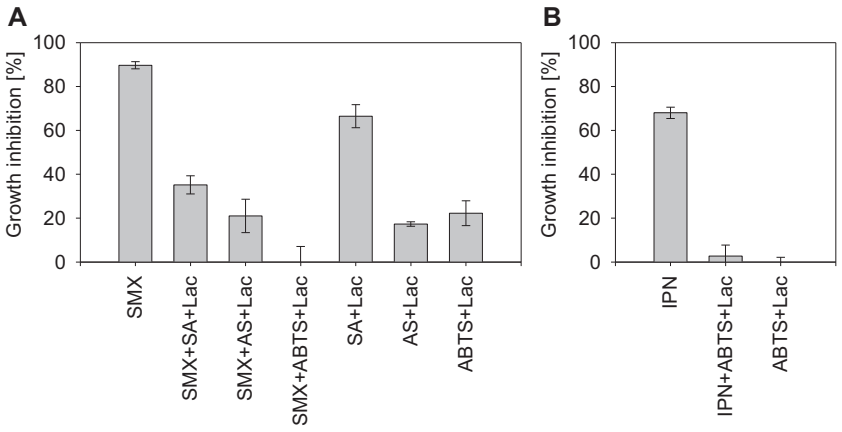


Fig. 4. Growth inhibition of the green algae *Pseudokirchneriella subcapitata*. After 72 h exposition to (A) sulfamethoxazole (SMX) (7.5 μM); SMX treated with a laccase (Lac)-mediator (SA, AS or ABTS) system (mixture of transformation products, TPs); or laccase with mediators in absence of SMX (mediators at 25 μM. SA: syringaldehyde, AS: acetosyringone); and (B) isoproturon (IPN) (0.5 μM); IPN treated with a laccase-mediator (ABTS) system (mixture of TPs); or laccase with ABTS (at 2.5 μM) in absence of IPN. Average and standard deviation of triplicates.

ABTS fragment as detected in the ABTS-SMX system and product 17 was probably also an ABTS fragment (Fig. S11, SI). Product 18 had a higher mass (546 g mol^{-1}) than ABTS (514 g mol^{-1}), but could not be identified. Products 14 and 17 had UV/vis absorption spectra with maxima at 200, 258, 286, 294 and 218, 258, 284, 292 nm respectively (Table S1, SI). Similar absorption spectra (220, 254, 284, 292 nm) were found for a decomposition product of $ABTS^{\bullet+}$ in neutral-alkaline solutions ($ABTS^{\bullet+}$ is unstable under alkaline conditions) [39], suggesting that products 14 and 17 were related to $ABTS^{\bullet+}$ degradation. Product 18 had an absorption spectrum with maxima at 222, 264, 292, 300 nm, which corresponds to the absorption spectrum of a (not clearly identified) decomposition product of $ABTS^{2+}$ observed in other studies [39]. As proposed by Majcherczyk et al. [39] and as discussed before, this result may suggest that $ABTS^{2+}$ was involved in the laccase-mediated reaction. Apart from product 17, which appeared rapidly (within 3 h) and then disappeared slowly, both products 14 and 18 appeared gradually at a rate proportional to ABTS (and $ABTS^{\bullet+}$) degradation (Fig. S12, SI).

The four other detected transformation products (19–22) had all a mass higher than IPN, suggesting the potential formation of coupling products. None of them could be identified but, according to its mass and retention time, product 19 could possibly be a hydroxylated IPN (Fig. S11, SI).

3.7.5. *Influence of pH on the type of transformation products*

Several transformation products detected by UPLC-MS were related (by retention time comparisons) to transformation products observed by HPLC-UV/vis (chromatograms presented in Figs. S13–15, SI). Their relative abundance after complete reaction of the mediators with SMX could thus be determined at various pH values (Fig. S16, SI).

For SMX in the laccase-SA system (Fig. S16 A, SI), the abundance of the dimeric SA (product 12) observed at low pH (pH 3) was a factor two higher compared to pH 4–7, and almost no dimeric SA was detected at pH 8. In contrast, the coupling product SMX-DMBQ (product 8) was much more abundant (10 times) at pH 6–8 than at pH 3. The production of DMBQ (product 2) during SA oxidation was the highest at pH 6–7.

Similar observations were made in the laccase-AS system in presence of SMX (Fig. S16 B, SI) where the coupling product SMX-DMBQ (8) was observed in high abundance at pH 7–8 but almost not detected at pH 3–4. The dimeric AS (product 6) was also almost absent at pH 8 but present in high abundance at pH 3–6. The production of DMBQ (product 2) during AS oxidation was highest at pH 6–7.

These results support the assumption that, at low pH, the reactive mediator radicals are rapidly formed, favouring their coupling to form dimeric SA and AS rather than reacting with SMX. Under neutral to alkaline conditions, most of the reactive SA or AS radicals (slowly produced) react directly with SMX to form the coupling product SMX-DMBQ (product 8) or decompose to DMBQ (product 2).

The pH influenced also the relative product distribution in the laccase-ABTS system in the presence of SMX (Fig. S16 C, SI). Product 17, an ABTS fragment, was more abundant at low pH while the ABTS fragment 14 was present at higher levels at a higher pH. This suggests that ABTS decomposes into different products depending on the pH. The abundance of the SMX fragment 16 was correlated with the percentage SMX removal observed at the different pH values.

3.8. *Toxicity of transformation products*

The evolution of the toxicity of the transformation products formed from IPN and SMX in laccase-mediated systems was assessed by ecotoxicity tests with green algae. After the treatment, both pollutants were not detected in the solutions (>99% abatement). The toxicity of the solutions containing mixtures of transformation products (Section 3.7) was compared to solutions containing the parent compounds, or the mediator oxidized by laccase in the absence of the pollutant (Fig. 4).

SMX at 7.5 μM (1.9 mg l^{-1}) inhibited 90% of algae growth compared to the control. Similar SMX toxicity to *Pseudokirchneriella subcapitata* were observed in other studies, with EC_{50} (concentration inhibiting 50% of the growth) reported in the range of 0.15–0.5 mg l^{-1} [43] or at 1.9 mg l^{-1} [24]. Laccase-mediated treatments reduced this toxicity by 61% in presence of SA, 77% in presence of AS and 100% in presence of ABTS (Fig. 4A), demonstrating the much lower algal toxicity of the mixture of transformation products compared to the non-treated SMX.

To evaluate if the residual toxicity observed in presence of SA and AS was due to SMX transformation products or to mediator transformation products, the same bioassays were conducted in absence of SMX. High algae growth inhibition (66%) was observed in the solution with SA (25 μM), and lower but significant inhibition was observed with laccase-induced oxidation of AS and ABTS (17 and 22%, respectively). Especially with SA, the residual toxicity was higher in absence than in presence of SMX transformation

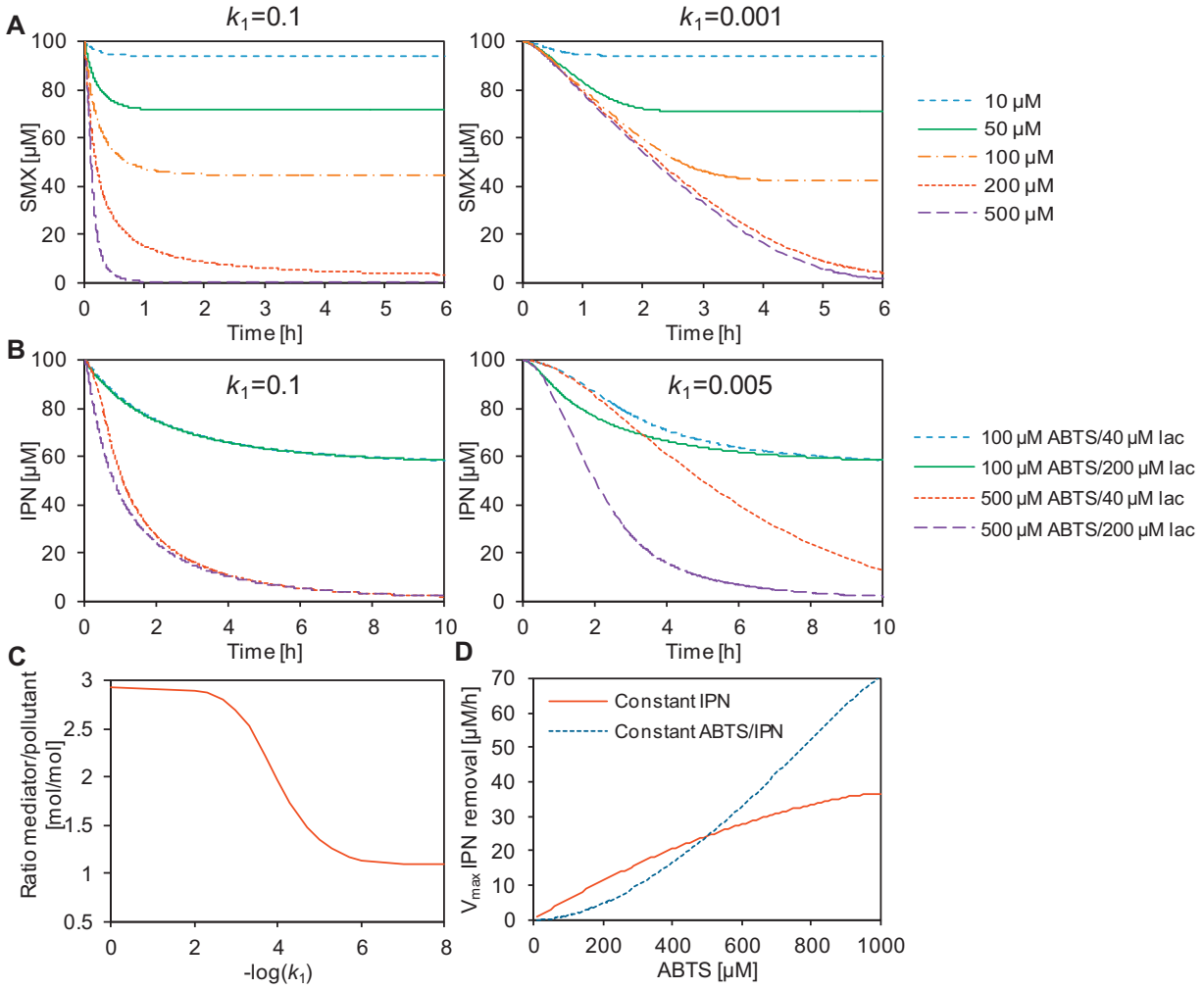


Fig. 5. Results of the kinetic model. (A) Influence of the mediator concentration (from 10 to 500 µM) on the pollutant (SMX) oxidation kinetics, with either the pollutant oxidation as the limiting step (k_1 = 0.1 µM 1 h^{-1}, similar to what is observed at pH 5–6, Fig 5A, left) or mediator oxidation limiting step (k_1 = 0.001 μM^{-1} h^{-1}, similar to what is observed at pH 7, Fig 5A, right). (B) Influence of ABTS and laccase (lac) concentrations on the pollutant (IPN) oxidation kinetics, with either the pollutant oxidation as the rate limiting step (k_1 = 0.1 μM^{-1} h^{-1}, similar to what is observed at pH 5–6, Fig. 5B, left) or mediator oxidation limiting step (k_1 = 0.005 μM^{-1} h^{-1}, similar to what is observed at pH 7, Fig. 5B, right). (C) Stoichiometric ratio between the mediator consumed per mole of pollutant oxidized, as a function of the reaction rate constant k_1 (in μM^{-1} h^{-1}). (D) Initial maximal pollutant (IPN) oxidation rates (V_{max} in µM h^{-1}) as a function of the mediator concentration (ABTS), with either constant IPN concentration (100 µM) or constant ABTS/IPN ratio (5 mol mol^{-1}). The modeling parameters are presented in Table S2, SI.

products. The product 2 (DMBQ) was produced in lower quantities in presence of SMX (Fig. S14, SI) (due to the reaction of the SA phenoxy radical with the pollutant), and also present in lower concentrations (2–8 times) in the oxidized AS solution (which was less toxic). This suggests that DMBQ might be responsible for a part of the toxicity observed with SA and AS. The toxicity of the oxidized SA was also reported in other studies (20% bacterial inhibition at 0.25 µM) [32]. Mediator transformation products were thus probably the reason for the residual toxicity observed after complete SMX oxidation. As the pH influences the relative abundance of each product (Section 3.7.5), different toxicity may thus also be observed at various pH values, with possibly lower toxicity at alkaline pH (lower concentrations of DMBQ).

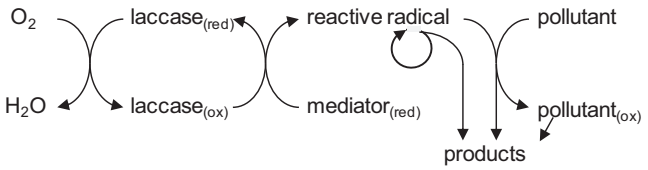
IPN at 0.5 µM (103 µg l^{-1}) inhibited 68% (±3%) of algae growth (Fig. 4B), which is similar to what was reported by Pavlić et al. [25] (70% inhibition at 100 µg l^{-1}). After the treatment in the laccase-ABTS system, more than 95% of this toxicity disappeared, showing the very low toxicity of the transformation product mixture compared to IPN.

These results show that laccase-mediated reactions can significantly reduce toxicity of SMX and IPN to algae (among the most sensitive organisms for these pollutants) despite the formation of several transformation products. The synthetic mediator ABTS was most efficient with almost complete IPN and SMX toxicity removal, while residual toxicity was still observed with the natural mediators AS and SA. Laccase-mediated systems appear thus to be an interesting way to decontaminate effluents, which are toxic to sensitive aquatic organisms. However, precautions must be taken when treating effluents with low toxicity because oxidized mediators (especially SA) may generate significant residual toxicity at low concentrations (<25 µM).

3.9. Mechanistic aspects of laccase-mediated reactions

The ideal scheme of laccase-mediated reactions where the mediator is continuously recycled during the redox process (Scheme 1) does not correspond to the observations in this study. Based on our results, an alternative laccase-mediated oxidation model is proposed (Scheme 2).

As illustrated in Scheme 2 and described in Eqs. (1)–(4), our results suggest that the mediator (med) is oxidized by laccase (lac) to reactive radicals ($R^{\bullet}$) (Eq. (1)) that will either react by a



Scheme 2. Proposition of a laccase-mediator reaction model.

radical–radical coupling reaction, producing products P_1 (such as dimeric AS and SA) (Eq. (2)), further react to more stable products (P_2) (e.g., 2,6 DMBQ for AS and SA) (Eq. (3)), or react with other compounds present in the solution, such as the pollutants (poll), at a stoichiometric ratio a (number of moles of radical needed to oxidize one mole of pollutant) to produce products P_3 (e.g., oxidation products of SMX or coupling products SMX-DMBQ) (Eq. (4)). $k_1 - k_4$ are the rate constants for Reactions (1)–(4), respectively. This mechanistic description is coherent with the nature of the transformation products detected (Section 3.7) (illustration for SA and SMX in Fig. S17, SI).

$$\text{lac} + \text{med} + \frac{1}{4}O_2 \xrightarrow{k_1} \text{lac} + R^{\bullet} + \frac{1}{2}H_2O \quad (1)$$

$$R^{\bullet} + R^{\bullet} \xrightarrow{k_2} P_1 \quad (2)$$

$$R^{\bullet} \xrightarrow{k_3} P_2 \quad (3)$$

$$R^{\bullet} + \frac{1}{a}\text{poll} \xrightarrow{k_4} P_3 \quad (4)$$

Based on this reaction model, a kinetic model was established (Section 13, SI) and used to simulate the behavior of the laccase-mediated reactions under various conditions. The results of selected simulations are presented in Fig. 5.

Both the effect of the mediator concentrations on the oxidation rates of SMX under acidic conditions and the absence of such an effect under neutral conditions (Fig. 2) could be correctly reproduced by the model by varying the rate constant k_1 (Eq. (1)) (Fig. 5A).

As discussed before, under acidic conditions, increasing the mediator concentration increased the pollutant oxidation rate, reaching progressively a plateau (a maximum) at high mediator concentrations (Fig. 2C). This saturation effect was attributed to significant cross-reactions between the radicals produced in high quantities (reactions of Eq. (2) favoured over Eq. (4)), and were correctly reproduced by the model (Fig. 5D). This saturation effect was not observed with constant mediator/pollutant ratios (Fig. 2C), a phenomenon confirmed with the model (Fig. 5D). Indeed, reaction rates of Eq. (2) and Eq. (4) were enhanced in the same way with the parallel increase in both mediator and pollutant concentrations.

As discussed before, under acidic conditions a strong increase in laccase activity (up to a factor 5) did not significantly increase the pollutant oxidation rates, while an increase in the mediator concentration strongly enhanced the reaction (Fig. S6 A, SI), suggesting that the oxidation of the mediator by laccase was not the limiting step. This phenomenon was reproduced with the model by choosing a relatively high (non-limiting) reaction rate k_1 (Fig. 5B). At higher pH (lower k_1), when the mediator oxidation by laccase becomes rate-limiting, the model shows that a higher laccase activity is necessary to increase the pollutant oxidation rate (Fig. 5B).

The increase in the required mediator/pollutant ratio observed at low pH (Fig. 1E) was reproduced with the model by increasing the reaction rate constant k_1 (as observed at low pH) (Fig. 5C). Indeed, at low k_1 values ($-\log(k_1) > 5$), the reaction is limited by the oxidation of the mediator by laccase (similar to the observations at neutral-alkaline pH) and the ratio mediator/pollutant is close to the minimum set for this simulation (ratio of 1). As k_1 increases ($-\log(k_1) < 5$), corresponding to a decrease in pH, the reaction becomes more and more limited by the oxidation of the pollutant by the radical (k_4). The radicals, rapidly produced, tend to accumulate in the solution and to react with each other or be further transformed (k_2 and k_3) rather than reacting with the pollutant. This leads to an increase in the mediator/pollutant ratio.

The proposed model was able to qualitatively reproduce all the different scenarios observed in the experiments, confirming that the mechanistic description proposed is adequate to describe laccase-mediated reactions.

3.10. Practical implications

This study highlights several points regarding the potential application of laccase-mediated systems for the treatment of micropollutants in contaminated waters. In particular, it is possible to assess the feasibility of treating very low pollutant concentrations in wastewater and how to enhance oxidation rates.

3.10.1. Treatment of very low pollutant concentrations

The model developed allowed assessing the oxidation kinetics for very low concentrations of pollutants. The time required to remove 90% of a (fictive) pollutant was modelled as a function of the pollutant concentration using two scenarios: (i) constant ratio mediator/pollutant (ratio of five), and (ii) constant mediator concentration (at 500 μM) (Fig. 6).

In the first scenario, when the pollutant, and therefore the mediator, were present at high concentrations (>100 and 500 μM,

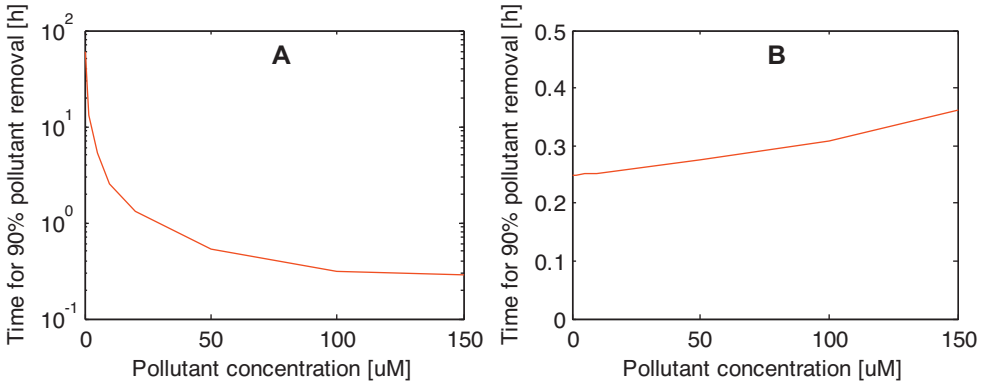


Fig. 6. Modeling of the time needed to remove 90% of the pollutant as a function of the pollutant concentration. (A) With a constant mediator/pollutant ratio of five (log scale for the y-axis). (B) With a constant mediator concentration of 500 μM. The modeling parameters are presented in Table S2, SI.

respectively), the removal time was independent of the pollutant concentration (similar to the experimental results, Fig. S6 B). But, as shown in Fig. 6A, this was valid only for pollutant concentrations >100 μM, corresponding to mediator concentrations >50 times the affinity constant of the enzyme for the mediator (Michaelis constant K_m, chosen at 10 μM). At lower pollutant (and therefore mediator) concentrations, the time required to remove 90% of the pollutant was predicted to increase by a factor around 10 every time the mediator concentration was divided by 10 (18 min at 500 μM up to 60 h at 2.5 μM). The relatively low affinity of the enzyme for the mediator implies that adding mediator at concentrations lower than the K_m of the enzyme (which is for instance around 20 μM for ABTS with this laccase, Fig. S18, SI) will require excessively long reaction times (>50 times the minimum).

When the mediator concentration was kept constant (at a value far above the K_m, scenario 2), only a limited effect of the pollutant concentration on the removal time was predicted (Fig. 6B), showing the possibility, with high mediator doses, to treat pollutants rapidly even at very low pollutant concentrations.

Treatment of low pollutant concentrations (e.g., 1 μg l^{-1} or 0.005 μM) will thus require very high mediator/pollutant ratios to avoid too long reaction times (e.g., a ratio above 4000 to keep a mediator concentration above 20 μM), which may lead to increase the toxicity of the water due to the release of high quantities of mediator transformation products.

3.10.2. Strategy to improve oxidation rates

The results presented above show that, depending on the pH, different strategies have to be applied to optimize the rate of pollutant degradation in laccase-mediated processes. If the reaction is limited by the oxidation of a pollutant by the radical (pH < 6), increasing the laccase activity has almost no effect and the best strategy is to increase the mediator concentration. However, this increase should stay below a certain threshold (mediator/pollutant ratio < 10) because at higher mediator concentrations reaction rates will reach a plateau due to high losses of the radicals formed from the laccase-mediator reaction (self-reactions). If the reaction is limited by the mediator oxidation by laccase (pH > 7), increasing the mediator concentration will not affect the oxidation rate of the target compound, as long as the mediator concentration is 50 times the K_m value of the enzyme. The strategy in this case is thus to increase the laccase activity. When both reactions are limiting (pH 6–7), an increase in both laccase and mediator concentrations should be considered.

3.10.3. Limitations of laccase-mediator systems for municipal wastewater

Despite fast oxidation of SMX and IPN in laccase-mediated systems and their related toxicity removal, addition of laccase and mediators in real treatment systems to increase micropollutant removal faces many limitations in terms of its feasibility: (i) The high concentration of mediator required (>10 μM) due to mediator consumption during the reaction, the relatively low affinity of laccase for the mediator and the possible loss of radicals by reaction with other matrix components. (ii) The potential formation of toxic transformation products due to the oxidation of the mediator. (iii) The formation of several mediator transformation products at concentrations possibly much higher than the target pollutant. Therefore, an application of LMS to treat municipal wastewater with very low micropollutant concentrations appears to be unrealistic. However, LMS may be an option for treatment of industrial wastewater that contains concentrated and toxic pollutants, such as effluents of pharmaceutical or pesticide industries. Indeed, in contrast to biological water treatment, LMS are not subject to intoxication and may be used to reduce the toxicity of highly polluted effluents, prior to further biological treatment of the transformation products.

4. Conclusions

The use of laccase-mediator systems effectively transformed IPN (with ABTS) and SMX (with ABTS, AS ad SA) to less toxic transformation products, consisting mostly of coupling products. The pH had a strong influence on the oxidation kinetics (faster at low pH) and on the required mediator/pollutant ratio (higher at low pH). Indeed, the three mediators tested did not act as catalysts and were therefore consumed in the process. Our results suggest that laccase oxidizes mediators to reactive radicals, which either spontaneously degrade into more stable products, react with each other (coupling reactions between radicals) or with the pollutants. Despite the requirement of high amount of mediators, LMS appears to be a potentially promising technology to treat concentrated and toxic effluents.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bej.2015.06.008

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