EFFECT ASSESSMENT OF FLUCTUATING EXPOSURE OF HERBICIDES WITH DIFFERENT MODES OF ACTION ON ALGAE

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Summary

Herbicides are widely applied in the environment to prevent the development of weeds in crop production. In creeks and rivers, the input of certain herbicides occurs in pulse following rain events rather than in continuous exposure. As a consequence, non-target aquatic organisms, such as algae, might be repeatedly exposed to high concentrations following field application. Indeed, these peak concentrations were shown to surpass, several times during field application season, the suggested chronic water quality criteria (CQC) for Switzerland, which is set to protect the aquatic environment on the long-term.

The scope of this thesis was to assess the effects on algae of time-varying exposure to herbicides, with a focus on the estimation of the effects caused by peak and sequential peak exposures.

A method was developed to estimate effects during herbicidal exposure on the green alga *Scenedesmus vacuolatus* as well as the subsequent effects following exposure. Three herbicides with different mode of action were selected to compare the effects induced by pulse exposure.

For atrazine and isoproturon, which are both inhibitors of photosystem II, the level of effects on the growth of alga increased with increasing exposure concentrations and duration. These herbicides exhibited different time-dependent toxicities, despite their common primary site of action on photosystem II (PSII). Indeed, the fast onset of isoproturon effects on the growth of alga indicates that short pulse to this herbicide might induce greater effects than atrazine. The growth of algae nevertheless recovered after the removal of either compounds. This may be attributed to the rapid recovery observed at the target site on PSII. Similar reversibility may be expected for other PSII inhibitors.

The effect assessment of pulse and time-dependent effects was extended to S-metolachlor, a chloroacetanilide herbicide inhibiting the biosynthesis of very long chain fatty acids (VLCFA). This herbicide induced a greater rise in toxicity than the PSII inhibitors, which suggest that the time-dependency of effects may differ to a greater extent for herbicides with different modes of action. However, effects on growth could be estimated only after 18h of
exposure to S-metolachlor. This incipient time-to-effect is related to the minimum exposure duration necessary to impair growth by the inhibition of the formation of VLCFA. Furthermore, this herbicide was shown to inhibit cell division, if the exposure coincided with a specific development stage. In contrast to the rapid recovery observed with PSII inhibitors, the algae previously exposed to S-metolachlor only recovered 29 hours following chemical removal. Similar incipient time-to-effects and delays in the recovery might be expected for other chloracetanilide herbicides that share the same primary target site.

In the last part of this study, alga was exposed sequentially to the PSII inhibitor isoproturon. For the two exposure scenarios tested, the effects on growth and on the target site were reversible following each exposure. However, growth was inhibited during each exposure resulting in reduction of biomass production. This induced a cumulative decrease of the overall biomass. After sequential exposures with short recovery periods, a slight rise in the tolerance of the alga to this herbicide was observed.

In conclusion, peak exposure to the three herbicides tested did affect the growth of algae, despite the short duration of the exposure. The effects were either limited to the exposure period or induced delayed effects following exposure, depending on the mode of action of the herbicides. Sequential exposures to isoproturon induce a cumulative reduction of the biomass over time. Based on these results the level and the number of exceedances of the CQC should be limited, with stricter limitations for herbicides that induce delayed effects.
**Résumé**

Les herbicides sont utilisés en agriculture pour lutter contre le développement de mauvaises herbes. Lors d’événements pluvieux, plusieurs d’entre eux peuvent être transportés par le ruissellement de surface, générant ainsi des pics de concentrations dans les cours d’eau récepteurs. Par conséquent, des organismes non-cibles sensibles aux herbicides, comme les algues, se trouvent potentiellement exposés à de fortes concentrations pouvant dépasser le critère de qualité chronique (CQC) proposé en Suisse et visant une protection à long-terme des organismes aquatiques.

**L’objectif de ce travail est d’évaluer les effets de fluctuations de concentrations d’herbicides sur les algues, et en particulier les effets liés à des pics d’exposition et des expositions séquencées.**

Une méthode expérimentale a été développée pour estimer les effets sur l’algue verte *Scenedesmus vacuolatus*, durant une exposition à un herbicide ainsi que durant la phase de récupération suivant l’exposition. Dans le cadre de cette étude, nous avons comparé les effets de trois herbicides ayant des modes d’action différents: l’atrazine, l’isoproturon et le S-métolachlore.

Les effets induits sur la croissance de l’algue par les inhibiteurs du photosystème II (PSII), atrazine et isoproturon, augmentent en fonction du temps et de la concentration. L’accroissement de la toxicité des deux herbicides avec le temps n’est cependant pas similaire, bien que tout deux agissent sur le même site du PS II. L’effet engendré par une courte exposition à l’isoproturon sur la croissance de *Scenedesmus vacuolatus* est supérieur à celui de l’atrazine, un pic d’exposition à cet herbicide est potentiellement plus toxique. Une rapide récupération de la croissance des algues après exposition a été observée, ce qui peut être la conséquence de la diminution rapide des effets sur le site d’action. Des effets similaires sont probables pour d’autres inhibiteurs du PSII.

L’évaluation de la toxicité en fonction de la durée d’exposition et l’évaluation des effets durant un pulse a été étendue au S-métolachlore. Cet herbicide de la famille des chloroactetanilides inhibe la synthèse d’acides gras à très longues chaînes (AGTLC). L’étude
montre que sa toxicité augmente plus fortement en fonction du temps que dans le cas des inhibiteurs du PSII. En outre il apparaît que la relation toxicité-temps du S-métolachlore diffère fortement de celles du groupe des inhibiteurs du PSII. En effet, les effets sur la croissance n’ont pu être estimés que pour des durées d’exposition supérieure à 18h. Ce temps de latence correspond à la durée nécessaire à la réduction de la croissance par inhibition de la production d’AGTLC. Nous avons également montré que cet herbicide perturbe la division cellulaire si le pic d’exposition intervient à certains stades de développement spécifiques. De plus, la récupération des algues après exposition a été observée seulement 29h après l’élimination du S-métolachlore. Ceci contrasta avec la rapide récupération observée pour les inhibiteurs du PSII. Des observations similaires sur le temps de latence pour l’induction des effets et la récupération différée des algues sont probables lors de l’exposition à d’autres chloroacetanilides ayant le même site d’action.

Dans la dernière partie de ce travail, l’algue a été exposée à l’inhibiteur du photosystème II l’isoproturon suivant deux scenarios d’exposition séquencée. Les effets sur la croissance et sur le site d’action étaient réversibles suite à chaque exposition. Durant chaque pulse, la croissance est inhibée ce qui induit une réduction de production de biomasse. Après des expositions répétées, on observe ainsi une perte cumulée de biomasse. De plus, une exposition à des pulses longs alternant avec de courtes périodes de récupération induisent une légère augmentation de la tolérance des algues.

En conclusion, un pic d’exposition aux trois herbicides induit des effets sur la croissance des algues, malgré la courte période d’exposition. Ces effets sont limités à la période d’exposition ou persistent après exposition, suivant le mode d’action de l’herbicide. De plus, une exposition séquencée à l’isoproturon induit une réduction cumulée de la biomasse. Sur la base de ces résultats, le niveau et le nombre de dépassement du CQC devraient être limité, avec des restrictions plus importantes pour des herbicides, dont la récupération n’est pas immédiate.
Chapter 1

General Introduction
Chapter 1

General Introduction

Herbicides are anthropogenic compounds applied with the goal of exerting a toxic action against unwanted plants. In agriculture, they are applied with the intention of impairing the development of weeds likely to reduce the yield of the planted crop. However, monitoring and research programmes have detected the presence of several herbicides in surface waters. These herbicides are transported from the fields or farmyards through runoff following rainfall or drainage [1]. Furthermore, some herbicides are also detected in the urban environment [2]. For herbicides used in agriculture, the pattern of herbicidal exposure varies depending on several factors including the characteristics of the watershed and waterways, the intensity and timing of the rainfall, and the amounts of herbicides used. While levels of exposure in large waterways and lakes are generally low with a slight elevation of levels following the application season of the considered herbicide [3, 4], the concentrations fluctuate to a greater extent in creeks as illustrated by the atrazine and metolachlor concentrations measured in the small creek Ror ([5]; Figure 1.1). In Switzerland, there is a concern about the water quality of brooks and small rivers that account for the largest part of natural systems following the embankment of large rivers for flood protection.

The presence of pesticides in surface waters may represent a risk for non-target species. For that reason, most countries have adopted water quality criteria (WCQ) for the protection of the aquatic environment, as well as preventing indirect effects on human health. In the European Union (EU), environmental quality standards (EQS) are devised to cover both the long-term and short-term effects from an exposure to a chemical [6]. The annual average concentration (AA-EQS) accounts for the protection against the occurrence of chronic effects. The maximum acceptable concentration (MAC-EQS) accounts for the protection against the occurrence of acute toxicity from short-term exposure [7]. Independently, a similar concept for the definition of WCQ was suggested in Switzerland [8] with the definition of a chronic (CQC) and acute quality criteria (AQC). The methods to define the criteria nevertheless differ from those applied in the EU, even if they are both based on effect values derived from standard toxicity tests.
Figure 1.1: Concentrations of atrazine (A) and metolachlor (B) measured in the creek Ror in the Greifensee watershed during the period following application of both compounds [5]. The concentrations of the detected herbicide reach peak levels following rainfall during the application season. The horizontal lines indicate the suggested chronic water quality criteria (CQC) for Switzerland: 1.8 μg/L for atrazine and 0.3 μg/L for metolachlor [8].

In creeks and rivers where aquatic organisms are exposed to fluctuating concentrations of herbicides, their concentration is likely to surpass the CQC. As shown in figure 1.1, peak concentrations of both atrazine and metolachlor measured in the creek Ror can vary in exposure duration, in the concentrations measured and the duration between peaks. The pulse durations ranged from a few hours to a day, while the recovery period also varied from a few hours to days. In the above presented example, peak concentrations surpassed the suggested...
Swiss chronic water quality criteria (CQC) many times during spring time; therefore it is important to assess the effects of fluctuation exposure.

### 1.1 Effect of pesticides fluctuating exposures

In general, peak pesticide exposure cause less toxicity on fish and macroinvertebrates than long exposure at the same exposure concentration [9-13], although few studies showed that short exposure caused higher toxicity [14, 15]. Most studies showed that the toxic effects depend both on the concentration and duration of the exposure. Nonetheless, Duquesne [16] stressed the importance of the duration of exposure to an organophosphate on daphnids, while Cold [17] showed that the concentration of exposure was the dominant factor driving the effects of a pyrethroid on Gammarus. Furthermore, short pulses can induce effects after the exposure [10] and influence the response of organisms during a second pulse. The duration of the recovery period between two exposures and the degree of reversibility of the effects are also key parameters [18-20]. Kallander [21] studied the importance of the length of recovery between 2 exposures to organophosphorus (OP) and carbamate insecticides on midges. If sufficient time (6h) was given between the carbamate pulses, the midge showed some recovery between the exposures, while the effect between OP pluses was not as reversible. The differences in recovery between pulses were related to the mode of action of the pesticide. The assessment of sequential pulse exposure can be studied with an infinite variety of exposure scenario, since the concentration of exposure, the duration of exposure and the duration of recovery following exposure can be modulated [22]. Recently, progress in the modelling of lethal effects of fluctuating exposure on invertebrates was made on groups of individuals [23]. The models, however, does not allow the estimation of the duration of recovery of a population [24, 25].

Fluctuating pesticide exposure can also result in sub-lethal effects on the exposed organisms. Observations were made on how far the enzymatic activity targeted by a pesticide was inhibited [26], on the feeding depression following exposure [27], on the reduced success in the emergence of larvae [15], and on reproductive success [16]. For example, Cold et al. [17] found that *Gammarus pulex* is most sensitive to pulse exposure during reproduction. Exposure to very low concentrations (0.05μg/l) for 1 h led to immediate disruption of reproductive pairs.
Unlike the many studies carried out on fish and invertebrates, little is known about the effects of fluctuating exposure to herbicide on algae or aquatic plants. The effects on algae and aquatic plants are typically estimated from standard toxicity tests after a defined exposure duration and does not include the assessment of the recovery [28-30]. Drost [31] observed a slight increase in the toxicity of four s-triazine (atrazine, prometon, prometryn and ametryn) on the aquatic plant *Lemna minor* between 3-6 day exposure, indicating a low time-dependent toxicity to herbicides inhibiting the photosystem II (PSII). Cedergreen [32] assessed the effect of shorter 3h exposure to six different herbicides on *L. minor*. The response after 4 days was influenced by the capacity of the plant to recover following exposure, which in turn was related to the mode of action of the herbicide. Furthermore, the recovery duration following the 3-hour pulse to PSII inhibitor was much shorter (24 hours) than that observed subsequent to a 3 day exposure by Drost [31]. This indicates that the duration of exposure may influence the rate of recovery following exposure, as observed with some insecticides. On algae, the recovery following a 96 hour exposure was again shorter, as it occurred within 12 hours [33].

Macinnis-Ng [34] assessed the effect of single and multiple pulse exposure on the photosynthesis of the seagrass *Zostera capricorni*. The recovery of the quantum yield was slower after a second exposure to irgarol indicating that sequential exposure was more harmful to *Z. capricorni*’s photosynthesis, than a single exposure. Furthermore, sequential pulse exposures to copper led to strong damage in the photosynthetic apparatus, preventing full recovery of the photosynthetic activity. As observed in the animal kingdom, these studies indicate that the time necessary to induce a toxic effects, the degree of effects during exposure and the time that the algae and aquatic plants take to recover may be species specific and influenced by duration and concentration of exposure, the recovery duration as well as the mode of action of a chemical.
1.2 Objective of the study

The objective of this thesis is to assess the effect of time-varying exposures of herbicides on algae, with a focus on the estimation of effects caused by short and sequential pulse exposures that would surpass the chronic water quality criteria. This study should provide methods for the effects assessment of fluctuating exposures on unicellular algae adapted from standardised testing methods [28, 29], which allow the determination of effects on growth after a defined 3-day exposure duration. Effects measured during and following single pulse exposure would allow a better estimation of acute effects on algae. The effects observed should relate i) to the concentration and the duration of exposures for the estimation of effects of different pulse exposures and ii) to the mode of action of the herbicides. Finally, the results obtained should support the effect assessment of more realistic exposure scenarios such as multiple pulse exposure, which in turn should support the improvement of the definition of time-dependent quality criteria.

For the purpose of this study, herbicides with different modes of action were selected and their effects assessed on an alga.

1.2.1 Tested organism: the alga Scenedesmus vacuolatus

Primary producers are of great environmental importance as they form the base of the food chain. In streams and small rivers, algae present in periphyton are usually the dominating plant representatives [35] and might be non-target organisms of herbicides. These herbicides usually target vital plant processes or structures that are shared by algae, aquatic plants and the weeds targeted in crop protection. Furthermore, algae were shown to belong to the most sensitive species of the herbicide atrazine in comparisons to water plants such as the macrophyte Lemna [36-38]. For that reason, the green alga Scenedesmus vacuolatus (Chlorophyceae; strain 211-8b, Shihira and Krauss, Philadelphia, PA, USA) was chosen for this study.

1.2.2 Tested herbicides

The herbicides chosen for this research are atrazine, isoproturon and S-metolachlor (Table 1.1). Atrazine, isoproturon and S-metolachlor are popular plant protection products applied as pre-emergent herbicides for the protection of maize, cereals and other crops. With sales
exceeding 10 tonnes per year of active ingredients, these three herbicides all belong to the 20 most largely sold chemicals on the Swiss market [39]. In the European Union, these active substances belong to the top 10 herbicides used in 2003, with quantities equal to or greater than 1800 tonnes [40].

These compounds are all regularly detected in surface waters and their concentrations fluctuate in rivers [41-45]. Monitoring programs of rivers in the Canton of Zurich in Switzerland have shown that these compounds have been present in surface waters for several years [46-48].

Table 1.1: Physico-chemical properties of atazine, isoproturon and S-metolachlor

<table>
<thead>
<tr>
<th>Common name</th>
<th>CAS Number</th>
<th>Herbicide Family</th>
<th>Structure</th>
<th>Log K&lt;sub&gt;OW&lt;/sub&gt;</th>
<th>Molar weight</th>
<th>Water solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atrazine C₈H₄ClN₅</td>
<td>1912-24-9</td>
<td>Triazine</td>
<td></td>
<td>2.61</td>
<td>215.69 g</td>
<td>35 mg/L</td>
</tr>
<tr>
<td>Isoproturon C₁₂H₁₈N₂O</td>
<td>034123-59-6</td>
<td>Phenylurea</td>
<td></td>
<td>2.87</td>
<td>206.29 g</td>
<td>65 mg/L</td>
</tr>
<tr>
<td>S-Metolachlor C₁₅H₂₂ClNO₂</td>
<td>87392-12-9</td>
<td>Chloroacetanilide</td>
<td></td>
<td>3.13</td>
<td>283.79 g</td>
<td>530 mg/L</td>
</tr>
</tbody>
</table>

Source: [www.chemfinder.com](http://www.chemfinder.com)
I. Photosystem II inhibitors

The triazine atrazine and the phenylurea isoproturon are both classified as photosystem II (PSII) inhibitors [49]. They bind at the terminal plastoquinone binding site (Q$_B$) of the D$_1$ protein, thus displacing the plastoquinone and inhibiting the electron flow in photosystem II [50]. The consequence of the inhibition of photosynthetic electron flow is an excess energy in the chloroplasts, which is reduced by emitting fluorescence, heat or producing singlet oxygen, which may induce oxidative damage [51].

**Atrazine**

This triazine herbicide is applied preemergence on corn fields and other crops against the development of annual broadleaf and grass weeds. The herbicide is registered in Switzerland and in North America. In 2004, the commission of the European Community decided not to include atrazine in Annexe 1 to the Council Directive 91/414/EEC as it could not be proved that the concentration of this compound and its breakdown products would not exceed 0.1 μg/L in groundwater. The total withdrawal of atrazine in the EU is scheduled for the end of year 2007 (Commission Decision 2004/248/EC).

**Isoproturon**

This phenyurea herbicide is applied to prevent the development of some annual monocotyledons. Isoproturon is registered in Switzerland, but its application is not authorised in S2 drinking water zones and on permeable soils. The herbicide passed the EU reevaluation in 2002 (Directive 91/414/EEC). The review report of the commission nevertheless indicates that particular attention should be given to leaching to groundwater and to the protection of aquatic organisms.

II. The chloroacetanilide

Chloroacetanilides inhibit the formation of very long chain fatty acids in plants [52]. The first step of the elongation of C18 fatty acids is inhibited as chloroacetanilide bind to the FAE1 elongase enzyme [53].
S-metolachlor

The seedling shoot and root inhibitor S-metolachlor is commonly applied on corn fields and other crops against some pre-emergent and post-emergent annual grasses and weeds [54]. In the EU and Switzerland, only the phototoxic (S)-enantiomer is registered. In the past, racemic metolachlor (R and S metolachlor) was applied. Since the application of solely S-metolachlor, a change in enantiomer composition of metolachlor in surface waters has been observed in the field [55]. The application of solely S-metolachlor is beneficial for non-target organisms, since R-metolachlor was shown to be 10 times more toxic than S-metolachlor to the non-target organism Daphnia magna [56].

1.3 Outline of this thesis

The effect assessment of fluctuation exposures were carried out in three stages

1. Effect assessment of single pulse exposure to atrazine and isoproturon (Chapter 2).

The effects of pulse exposure to the two PSII inhibitors were assessed with the goal of comparing the effects induced by herbicides that have the same primary mode of action. For this purpose, a method was developed to evaluate the effects on the growth rate of algae, as well as the subcellular effects by measuring the PSII effective quantum yield. Effects were measured during the exposure and the recovery period, which is defined at the period following exposure. Furthermore, the effects during exposure were related to the concentration and the duration of exposure by describing the time-dependency of effects on the growth rate of S. vacuolatus.

2. Effect assessment of S-metolachlor single pulse exposure (Chapter 3)

Since the effect of short exposures on other organisms has been shown to depend on the toxicant, the effect assessment of single pulse exposure was extended to a herbicide having a different mode of action, namely an inhibitor of the formation of long chain fatty acids. The method developed for the assessment of pulse and time-dependent effects on algae was applied. Furthermore, the identification of the algae development stage most sensitive to S-metolachlor, and the time-to recovery were carried out by exposing synchronously grown algae and measuring effects on algal reproduction.
3. Effect of sequential isoproturon pulse exposure (Chapter 4)

To increase the environmental relevance of the effect assessment of fluctuating exposure, algae were exposed to different scenarios of sequential exposure to isoproturon. The effects on growth and the photosynthetic effective quantum yield were measured during the exposure and the recovery periods. Furthermore, this study should indicate if the effects during repeated exposures can be considered as a series of independent or dependent exposure effects. Finally, the net decrease in biomass production throughout the sequential exposure, as compared to the control, was estimated to assess the overall effects of multiple pulses.
1.4 References


Chapter 1
Chapter 2

Effect of pulse herbicidal exposure on Scenedesmus vacuolatus: a comparison of two photosystem II inhibitors

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2.1 Abstract

Herbicide concentrations fluctuate in rivers following crop application and can reach high levels after rain events, yet the duration of these pulses is short. In the present study, we assessed the effect of atrazine and isoproturon pulse exposure on *Scenedesmus vacuolatus*, as well as the recovery in the post-exposure period. We further explored whether the time-dependent toxicity is similar for herbicides inhibiting the photosystem II (PSII). The growth rate was assessed for different exposure durations and in addition the inhibition of the effective quantum yield of PS II was measured to monitor the response at the target site. Atrazine and isoproturon did not have similar time-dependent effects on growth rate, despite their same primary mode of action on PS II. Atrazine was less toxic than isoproturon after 10 h of exposure, but the toxicity of both herbicides was similar after 48 h of exposure, showing that atrazine induced a greater increase in toxicity with time. However, both compounds inhibited the PSII effective quantum yield within one hour following exposure. Similarly, the effective quantum yield recovered completely within 4 h after removal of the toxicants, leading to rapid recovery of algal growth. The rapid onset of effects of isoproturon on the growth of the alga during exposure suggests that a single pulse to this herbicide is likely to induce greater effects than an atrazine pulse at the same concentration, even if these effects are reversible. The information gained in this study should support the effect assessment of sequential exposures as well as the risk evaluation of fluctuating herbicidal exposure.

**Keywords:** Pulse exposure, Time-dependent toxicity, Recovery, Herbicide
2.2 Introduction

Herbicides are widely used in agriculture for the control of weeds in crop production. Due to their use pattern, physical and fate properties, some herbicides have the potential to enter surface waters, and monitoring programs have demonstrated their presence in waterways [1-3]. Studies of the environmental behaviour of some herbicides have shown that they are transported from the field to water bodies by drainage and surface transport processes during and after rain events [4, 5]. A seasonal increase in the concentration of atrazine and isoproturon to the μg/L level has been regularly observed in rivers during the crop growing period [3, 6]. Atrazine is a triazine herbicide applied to pre-emergent broadleaf and grass weeds in primarily in corn but also on other crops, while isoproturon is a phenylurea herbicide applied as a pre-emergent herbicide on cereals (wheat and barley). In small agriculture catchments, the concentrations of the applied substances can increase considerably in streams during the first rain events after herbicidal application, resulting in repeated pulses [7]. This dynamic in herbicide concentrations was highlighted by measurements of atrazine levels in a small stream, in which peak concentrations reached 30 μg/L for more than one hour, exceeding 15 times the suggested chronic water quality criteria (CQC) for Switzerland [8, 9]. Isoproturon peak levels (9 μg/L) also largely surpass the suggested CQC following rain events [3]. Even if the duration of pulse exposure is short, aquatic organisms may suffer adverse effects given that exposure levels may approach concentrations where effects can be observed in laboratory studies [10].

Single pulse exposure to some pesticides has been reported to generally induce toxic effects on macroinvertebrates and fishes, even if the exposure duration is very short, i.e. a few hours. However, these effects are often lower than effects induced by continuous exposure to the same concentration over several days [11-16]. Four parameters seem to influence the effects of pulse exposures: the toxicity of the compound, the exposure level, the exposure duration and the recovery time between pulses [17, 18].

Herbicides principally impact primary producers such as algae and waterplants [19]. Nevertheless, only a few studies report the effects of herbicidal pulse exposure or the time dependence of the toxicity on primary producers. Drost et al. [20] showed that the effect of four s-triazine herbicides on the water plant *Lemna* increased insignificantly between 3 d and
6 d exposure and concluded that the toxic effect is independent of the exposure duration. The duration of exposure chosen in that study was; however, rather long compared to peak herbicidal exposures measured in the environment. Cedergreen et al. [21] reported the effects of a 3h pulse exposure to six herbicides on *Lemna minor*. For inhibitors of acetolactate synthase and of microtubule assembly, the pulse induced similar effects to a 4 d exposure at 10-fold higher concentrations, but for PSII inhibitors, 100-fold concentrations were necessary. However, the latter results reflect the overall effect on growth measured after 3 days, and therefore the capability of the plants to recover following pulse exposure greatly influenced the effect measured, which was related to the mode of action of the herbicides. The reversibility of the effects induced by the PSII inhibitor atrazine was observed on a unicellular alga following a 4 d exposure [22]. Although the exposure concentration and duration, as well as the mode of action of the herbicide, have shown to influence the degree of the effects on plants during exposure and the recovery period, the assessment of pulsed exposure on unicellular algae has not been studied systematically until now.

The objective of this study was to compare the effects of a single pulse exposure of two photosystem II (PSII) inhibitors, atrazine and isoproturon, on a unicellular alga during exposure and the subsequent recovery period. To reach our goal, we developed a new testing protocol for the observation of pulse effects on the unicellular green algae *Scenedesmus vacuolatus*. Furthermore, the inhibition of photosynthesis via PS II was measured to refine the understanding of the effects at the target site, given that both substances block the photosynthetic electron transport chain in PS II. This publication presents the developed testing method, the results that compare the time-dependent and pulse effects for both substances and, finally, discusses the use of the information gained in the effect assessment of sequential exposures, as well as the modelling of effect on algal population.
2.3 Material and Methods

2.3.1 Chemicals

Atrazine (Atrazine Pestanal®, 97.4%) and isoproturon (Isoproturon Pestanal ®, 99.8%) were purchased from Sigma-Aldrich. Stock solutions for algae toxicity testing were prepared in the alga culture medium (see below) and their concentration measured analytically by liquid chromatography–tandem mass spectrometry (LC-MS-MS) [23]. The concentration of both pesticides did not decline during a three-day exposure to algae (data not shown).

2.3.2 Algae culture conditions

The green unicellular alga, *Scenedesmus vacuolatus* (Chlorophyceae; strain 211-8b, Shihira and Krauss, Philadelphia, PA, USA) was purchased from the alga collection of the Institute for Plant Physiology of the University of Göttingen, Germany. The alga was cultured in batch cultures in a sterile inorganic medium prepared as described by Le Faucheur [24].

Batch cultures were inoculated into 50 ml medium in sterile 100-ml Erlenmeyer flasks with cells conserved on a Petri plate. Algae were maintained in exponential growth by transferring cells from a culture in the exponential growth phase (<72h) to a new culture, with an initial optical density (OD) of 0.05 at 685 nm (OD⁶₈₅), which corresponds to a density of 650,000 cells/ml. This transfer of algae into fresh media was performed up to six times to maintain the consistent sensitivity of the algae. The cell density was determined by optical measurement at 685 nm using a spectrophotometer (Uvikon 930; Kontron Instruments, Munich, Germany) in a range that exhibited a linear correlation with the cell density measurement based on microscopic Neubauer chamber cell counts (Brand, Wertheim, Germany). This relationship was shown to be similar for control algae and algae exposed to the herbicides. The cultures were maintained on a shaker at 90 rpm at 25°C with continuous illumination of 105μmol/m²/s by cool-white fluorescent lamps.
2.3.3 Experimental design

A) Standard alga toxicity test

As for the cultures, algae were inoculated in 100 ml flasks containing 50 ml of medium at an initial optical density (OD) of 0.05 at 685 nm (OD_{685}). Optical density was measured three times per day during 72 h as defined in standard protocols [25, 26]. The growth rate was calculated with a linear regression of the natural logarithm of optical density over time. The toxicity of the herbicides was determined by exposing algae to nominal concentrations ranging from 15 to 200 μg/L isoproturon (five concentrations tested in duplicates) and from 20 to 200 μg/L atrazine (five concentrations tested in triplicates) diluted from the stock solutions. Each test was repeated.

B) Time dependent toxicity (Figure 2.1a)

Algae were inoculated in the same manner as in the standard test, but the herbicides were added after 24 h. This delay in chemical addition was necessary to reach a higher algal density and thereby enable assessment of the effects during a short exposure. During the 48 h exposure duration, the algal cell density was measured directly after chemical addition, then three times within the first 10 h of exposure to enable the calculation of the growth rate during this short period. The calculation is based on the 4 cell density measurements. For the rest of the test duration, the cell density was measured three times a day as in the standard test. The assessment of the time-dependent toxicity was repeated with atrazine (exp. 1&2) and isoproturon (exp. 3&4). The algae were exposed to nominal atrazine concentrations ranging from 30 to 1000 μg/L in duplicate cultures with 2 controls. Atrazine concentration at 173 μg/L in exp.1 was tested in triplicates. The algae were exposed to nominal isoproturon concentrations from 30 to 700 μg/L tested in duplicates with 3 controls in exp. 3 and 2 controls in exp.4.
Effect of single pulse exposure: PSII inhibitors

Figure 2.1: Exposure protocol for the assessment of time-dependent effects (a), the effect of pulse exposure and the subsequent recovery (b)

C) Pulse exposure (Figure 2.1b)

Algae were exposed to a 10 to 24 h herbicidal pulse one day after the inoculation of the culture. Algae were exposed to nominal concentrations ranging from 80 to 500 µg/L atrazine and from 60 to 320 µg/L isoproturon. Cultures were handled in the same as during in the assessment of the time dependent toxicity, except that the growth rate during the 10 h pulse is based on 3 cell density measurements. The recovery period, defined as the period subsequent to exposure was studied for exposure duration not exceeding 24 h to make sure algae grew exponentially in the controls throughout the experiment. To remove the chemical, the alga cultures were centrifuged 7 min at 3000 rpm at 25°C. The supernate was discarded and the algae re-suspended in fresh media. The centrifugation was repeated a second time ensuring 99.9% isoproturon and atrazine removal (data not shown). Controls were treated in the same manner. This procedure was tested on unexposed alga culture to ensure that centrifugation did
not impair algal growth (data not shown). During recovery, the cell density was measured after chemical removal, then 3 times a day.

During the exposure and the recovery, the PSII effective quantum yield of the algae was measured using a dual channel PAM (pulse amplitude modulated) fluorometer, called ToxY-Pam (Heinz Walz GmbH, Effeltrich, Germany [27]). This instrument assesses the effective quantum yield of energy conversion at PSII reaction centers by applying pulse modulated measuring light. Light adapted algae are illuminated alternatively by measuring light (3s) for the assessment of the chlorophyll α fluorescence F and by saturation pulses (470 nm with a pulse width of 0.4s) to determine the maximum fluorescence Fm’ at λ> 640nm. F is measured shortly before the application of saturation pulses while Fm’, the maximal fluorescence, is measured during the saturation pulse [28, 29]. F and Fm’ were averaged from the three last measurements out of 5 performed that were spaced 30 s apart. Alga cultures were exposed to nominal concentration ranging from 3-500 μg/L atrazine and 3-900 μg/L isoproturon for the estimation of the EC50 on the inhibition of PSII effective quantum yield.

2.3.4 Data analysis

(A) Time dependent toxicity assessment

The natural logarithm of the optical density was plotted against time. The growth rate corresponds to the slope of the linear regression [25]. When assessing the relationship between the time and the effects, the growth rates for different exposure periods were calculated from the same data set. For example, measurements made during the first ten hours of exposure were used to calculate the ten-hour growth rate.
The growth rate EC50 values and their 95% confidence interval were calculated from the standard error (EC50 ± 1.96 * SE_{EC50}) and were estimated for different exposure durations using a four parameter log-logistic dose response model (Eq. 1), with the aid of the statistics software Prism (1992-2003 GraphPad, San Diego, CA, USA).

\[ E = \min + \frac{(\max - \min)}{1 + 10^{(\log EC50 - \log C) \times \text{Hillslope}}} \]  

Where:  
- \( C = \) Concentration; \( E = \) Endpoint, \( e.g. \) the growth rate; \( \text{Hillslope} = \) slope of the curve at EC50; \( \max, \min = \) maximum and minimum of the sigmoid curve.

The number of parameters to be estimated was reduced to two parameters, the EC50 and the Hillslope. The max parameter was set as the average of the control cultures’ growth at the exposure duration studied. The min parameter was fixed at 0, since we hypothesized that no growth occurs at high concentrations [30]. This max to min dose response curve yields a negative slope. Two quality criteria were set to select the data for the calculation of the time-dependent effect concentrations. First the growth rate of the control cultures had to range between 0.032 and 0.046 h⁻¹ during the entire test duration, which is the 95% confidence interval of algae growth based on our control data. Secondly, the correlation coefficient (\( r^2 \)) of the dose response curve was set greater than 0.5.

The time-dependent effects were fitted with a mathematical model to support the comparison of effects between the compounds. The logarithm of the EC50 was expressed as the linear function of the logarithm of time. This model has been previously used to describe time-dependent lethal effects of different chemicals [31, 32].

\[ \log EC_{50} = a \log t + b \]  
\( t = \) time; \( a, b = \) constant

The linearity of the regression, the deviation of the slope from 0, as well as the significance in differences between the slopes of two regressions were tested with an F-test (alpha 0.05), with the aid of the statistics software Prism (1992-2003 GraphPad, San Diego, CA, USA).
**Chapter 2**

**(B) Inhibition of the PSII effective quantum yield**

The effective quantum yield $Y$ is expressed as a percentage of the reduction in fluorescence $F$ to the maximal fluorescence $Fm'$ [27, 29]:

$$ Y = \frac{Fm' - F}{Fm'} $$

and the inhibition $Inh$ is calculated as follows:

$$ Inh = \frac{Y_1 - Y_2}{Y_1} $$

where $Y_1 > Y_2$, $Y_1$ being the yield of the reference and $Y_2$ that of the exposed algae.

A dose response curve can be fitted to the inhibition of the effective quantum yield on PSII using the same model as described by equation 1. Three parameters need to be defined: EC50, Hillslope and max. The latter parameter is not fixed because we observed that inhibitors of the photosynthesis do not all induce the same maximal inhibition of the effective quantum yield on PSII (data not shown). Since the lowest concentration relates to the lowest measured effects, the slope of the curve is positive. The parameter min was set at 0, because no inhibition was measured for unexposed samples.

### 2.4 Results

#### 2.4.1 Time dependency of growth inhibition

Exposure of algae to atrazine and isoproturon showed a time-dependent inhibition of the growth rate. Figure 2.2 presents the growth rate relative to the logarithm of atrazine (a) and isoproturon (b) concentrations for three exposure durations, as well as the slope of the dose response curve at the EC50 (c). At the lowest atrazine concentration, algal growth was similar at all three exposure duration. At the highest atrazine exposure concentration, the growth of algae exposed to 990 $\mu$g/L was in average 0.016 h$^{-1}$ after 10 h of exposure, but the toxicity augmented over time lowering the average growth rate to 0.004 h$^{-1}$ after 48 h. The latter corresponds to 90% growth inhibition. During the 48h exposure to isoproturon, the growth rate of the cultures exposed to the highest concentrations decrease very slightly with time. At 700 $\mu$g/L, the average growth of algae was of 0.010 h$^{-1}$ after 10 h and 0.004 h$^{-1}$ after 48 h. The growth rates of the controls during the assessments of the time-dependent toxicity were within the defined range during the entire test duration. Increasing levels of inhibition for higher
concentrations are also reflected in the shape of the curve. The parameter Hillslope (Figure 2.2c) of the dose response curve clearly decreases between a 10 and 24 h exposure to atrazine, and to a lesser extent for isoproturon. This indicates that a small change in concentration induces a greater toxic effect for the longer exposure duration.

Figure 2.2: Dose response curves of the growth rate for different exposure duration to atrazine (a) and isoproturon (b): 10 h (∆, ▲, ⋯), 24 h (▽, ▼, ⋯), 48 h (□, ■, —). Graph (c) describes the time-dependence of the slope at EC50 for atrazine and isoproturon (○, □) and it's 95% CI. The number of replicates is described in the methods section.
2.4.2 Time dependency of effect concentrations

The toxicity of atrazine and isoproturon increased for both compounds with increasing exposure duration. Figure 2.3 presents the effect concentrations estimated for different exposure durations from two experiments for each herbicide. During exposure to atrazine, the EC50 was estimated after 10 h at 423 μg/L with a 95% confidence interval (148; 1207) in experiment 1 (Exp. 1) and 618 μg/L (256; 1490) after 12 h in the replicate experiment (Exp. 2). Lengthening the exposure duration to 48 h resulted in a decrease in the EC50 to 126 μg/L (111; 144) and 128 μg/L (116; 140) in Exp. 2. Isoproturon was more toxic to S. vacuolatus than atrazine for exposure between 10-48 h. Nevertheless, its toxicity increased to a lesser degree for exposure ranging between 10-48 h. The isoproturon EC50 after 10 h of exposure was 170 μg/L (108; 269) and 142 μg/L (109;185) in the replicate experiment (Exp 3&4), lowering to 110 μg/L (100; 122) and 100 μg/L (89;113) after 48 h. The determination of isoproturon’s effect concentrations at 10 h showed lower variability in the response in comparison to atrazine. Regression lines (Eq. 2) were fitted to the four experimental sets. The slope of the regressions that describe the toxicity of atrazine as a function of time (exp. 1 and 2) did not show significant differences ($p$-value=0.24). Similarly, the slope of individual isoproturon experiments (exp. 3&4) were not significantly different ($p$-value = 0.23). The analysis of the slopes of the regression lines indicates significant time-dependent effects for isoproturon, with both slopes differing from zero ($p$-value=0.012 in exp. 3 and $p$-value=0.022 in exp. 4). The increase in toxicity over time was nevertheless significantly greater for atrazine than that for isoproturon. The differences in slopes were cross tested between each atrazine and isoproturon experiment (exp.1&3, exp. 1&4, exp. 2&3, exp. 2&4) and showed significant differences ($p$-value<0.05). The EC50 of a standard 72 h toxicity test carried out in the same experimental conditions was 66 μg/L (70; 61) for atrazine and 120 μg/L (106; 135) for isoproturon.
2.4.3 Pulse exposure: effect during exposure and recovery

Figure 2.4 presents the effects of a 24 h pulse exposure to both atrazine or isoproturon on *S. vacuolatus*’s growth and the inhibition of the PSII effective quantum yield. During the 24h preceding the pulse, similar growth was observed between the control culture and the exposed culture in both experiments. Pulse exposure to atrazine significantly reduced the growth rate of the culture exposed to 125 μg/L (*p*-value<0.001) and 510 μg/L (*p*-value<0.001). The inhibition of the growth reached 44% and 68%. Significant reduction of the growth rates during the pulse were also observed for cultures exposed at 200 μg/L (*p*-value<0.001) and 320 μg/L (*p*-value<0.001) isoproturon, inhibiting their growth rate by 51% and 64%. The recovery of growth occurred immediately following removal of atrazine, given that the growth rates of the controls and the exposed samples (125 and 510 μg/L ) did not show significant differences (*p*-value=0.75, *p*-value=0.78 ). Recovery was also complete following exposure to 200 μg/L and 320 μg/L isoproturon. Growth was not significantly different between the control and the culture previously exposed to 200 μg/L (*p*-value=0.14), but significantly higher than the control at 320 μg/L isoproturon (*p*-value<0.001). However all growth rates during recovery remained within the range defined as a quality criterion for the
growth of control cultures. Table 2.1 presents the inhibition of the growth rate of replicate alga cultures during exposure and recovery for several pulse scenarios. The response following 10h pulses showed greater variability than in the assessment of time-dependent effects, as they are based on 3 cell density measurements. Our experiments, however, indicate a systematic growth recovery of the algae during the post-exposure period which was independent of the applied concentration and duration of pulse exposure.

Figure 2.4: Atrazine (a, b) and isoproturon (c, d) 24 h pulse. The effect on the growth of *S. vacuolatus* (a, c) and the inhibition of the PSII effective quantum yield (b, d) were assessed during the exposure period and the subsequent recovery. We present the effect on single alga cultures exposed to 125 μg/L (▽, ---), 510 μg/L (○, …) atrazine, 200 μg/L (◇, ---), 320 μg/L (△, …) isoproturon, compared to a control in the respective experiments (□, —).
Table 2.1: Growth rate inhibition (Growth inh.) of replicate cultures during atrazine or isoproturon pulses and the subsequent recovery periods for varying exposure durations and concentrations. The inhibition of each replicated is given when the number of sample (n) was two. The average and standard error (SE) is presented for n>2

<table>
<thead>
<tr>
<th>Concentration [μg/L]</th>
<th>Pulse duration [h]</th>
<th>Pulse</th>
<th>Recovery</th>
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<tr>
<td></td>
<td></td>
<td>n</td>
<td>Growth inh. (SE) [%]</td>
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<tr>
<td>Atrazine</td>
<td></td>
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<tr>
<td>80</td>
<td>10</td>
<td>4</td>
<td>17.7 (14.3)</td>
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<tr>
<td>200</td>
<td>10</td>
<td>5</td>
<td>63.2 (16.4)</td>
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<tr>
<td>80</td>
<td>24</td>
<td>2</td>
<td>54.7; 34.8</td>
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<tr>
<td>125</td>
<td>24</td>
<td>4</td>
<td>36.4 (9.1)</td>
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<td>200</td>
<td>24</td>
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<td>62.7 (12.2)</td>
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<td>510</td>
<td>24</td>
<td>3</td>
<td>76.4 (14.1)</td>
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<tr>
<td>Isoproturon</td>
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<tr>
<td>60</td>
<td>10</td>
<td>2</td>
<td>46.5; 56.7</td>
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<td>120</td>
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<td>2</td>
<td>55.8; 52.4</td>
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<td>200</td>
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<td>68.4; 98.5</td>
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<td>320</td>
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<td>48.6 (4.6)</td>
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<tr>
<td>320</td>
<td>24</td>
<td>4</td>
<td>62.8 (4.0)</td>
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</table>

A rapid increase in the inhibition of the effective quantum yield (Figure 2.4b) was measured following the addition of atrazine to the algal culture. The inhibition of the effective quantum yield reached 45% at 125 μg/L and 70% at 520 μg/L by 30 min after exposure. Twenty-four hours later, the inhibition figure still lay at 51% at 125 μg/L and 63% at 520 μg/L. Atrazine removal induced a rapid reduction of the inhibition. By 45 min after its removal, inhibition decreased to 14.8% and 16%, while no inhibition was detected 5 h later. Pulse exposure to isoproturon induced the same effective quantum yield inhibition pattern (Figure 2.4d). By 30 min after exposure, the inhibition reached 47% at 200 μg/L and 55% at 320 μg/L and remained 23 h later at 49% and 52%. Similarly, a low inhibition of 9% and 12% were measured one hour following chemical removal, which further decreased in the next 2 h. The inhibition of the PSII effective quantum yield caused by isoproturon after 24 h of exposure is lower than that caused by atrazine at the same concentrations, whereas effects on growth are greater after the same exposure duration. Isoproturon induced inhibition of 50% of the effective quantum yield at 115 μg/L, while the same effect level was estimated at 46.9 μg/L for atrazine. The regression parameters max and Hillslope were estimated at 74 and 0.99 for atrazine and at 81 and 0.76 for isoproturon.
2.5 Discussion

2.5.1 Testing protocol to assess the time-dependent toxicity and pulse exposure

The assessment of the time-dependent toxicity of atrazine and isoproturon on *Scenedesmus vacuolatus* was carried out successfully. *Scenedesmus vacuolatus* proved to be a satisfactory test organism since the growth of this alga is, on the one hand, not affected by centrifugation as well as easy to handle, allowing the removal of the contaminated media. The method, however, showed some limitations. Due to biological and statistical reasons, it was difficult to assess the effect of exposures shorter than 10 h. After 10 h exposure, the effect assessment was still very variable for the following reasons. Firstly, the parameter measured, the cellular density, did not increase greatly. Therefore, effects are related to small differences in cell density. This limitation might be overcome by choosing an alga like *Chlamydomonas*, which has a greater growth rate. Secondly, the determination of the growth rate was based on only 4 cell density measurements, giving to each measurement a significant weight and inducing a greater statistical uncertainty. Finally, the slope of the concentration response curve was smaller for short exposures, leading to a greater standard error of effect concentrations. We also observed that the response of the algal population showed a greater biological variability between the replicates.

2.5.2 Comparison of atrazine and isoproturon pulse toxicity

In the present study, we have examined the toxicity of short exposures on the growth rate and the effective quantum yield of PSII of *S. vacuolatus* as a function of exposure concentration and duration. Measurements of the inhibition of the effective quantum yield on PSII showed that both substances very quickly reached their target on the binding site, the plastoquinone Q$_B$ of the D1 protein in PSII, and exerted their toxic action before any effect could be observed at the population level. This kinetics of effects on PSII is in line with the rapid atrazine uptake measured on different freshwater algae species by Tang [33] and the effect of diuron and irgarol on the maximum effective quantum yield in corals [34]. The comparison of the EC50s after 24h of exposure on both the PSII effective quantum yield and the growth showed that atrazine induced a greater inhibition of the effective quantum yield, but was less toxic on the growth rate, which reflects the general fitness of the population. For
both herbicides, the EC50 on growth after a 3 day exposure were similar to the EC50 on the inhibition of the effective quantum yield measured after a 24 h exposure, which is in accordance with observations made on Scenedesmus capricornutum by Bai Fai [35].

Pulse exposure to both atrazine and isoproturon inhibited algae growth during exposures as short as 10 h. Both tested herbicides approached the same toxicity with time, nevertheless the atrazine 10 h EC50s were more than 2.5 times higher than those of isoproturon. This indicates that the onset of effects of isoproturon on the growth of this alga was greater. Given the similar kinetics of both herbicides in algae and their same primary target site in the cells, we hypothesize that dissimilar secondary effects or metabolism in the algae induce different toxicity at the population level as well as different time-dependent effects on growth. The low temporal dynamics of effects during isoproturon exposure and its greater toxicity demonstrate that isoproturon pulses longer than 10 h are prone to induce greater effects. The concentration inhibiting the growth of Scenedesmus vacuolatus by 50% are greater than the peak concentrations measured in rivers. Nevertheless, effect concentrations determined over 24 h with synchronously cultured cells were 1.7 times lower for atrazine and 2.5 times for isoproturon [36, 37]. Furthermore, some alga species like Scenedesmus subspicatus are more sensitive to isoproturon [19]. Schmitt-Jansen and Altenburger also reported the EC50 values for different species, which ranged from 14 to 78 μg/L [38]. Therefore, it cannot be excluded that these peak concentrations could harm sensitive species.

2.5.3 Recovery following pulse exposure

The investigation further assessed the recovery of S. vacuolatus following various levels on growth inhibition that were dependent on the exposure concentration and duration. The growth of S. vacuolatus recovered within 5h following a single pulse exposure to atrazine and isoproturon, independent of the degree of growth inhibition during the short pulses. Recovery has been observed on phytoplankton following exposure to atrazine [10] and on the aquatic plant L. minor subsequent to exposure to diquat, ametryn and atrazine [20, 21]. Similarly, Klaine et al. [22] reported the almost instantaneous reversibility of effects on the growth of S. capricornutum following a 4 day exposure to lower atrazine concentrations. The fast recovery of S. vacuolatus’s growth might be a consequence of the rapid elimination of the compounds from the cells and the complete reversibility of their mode of action [39, 40]. No inhibition of the PSII effective quantum yield could be detected five hours after herbicidal removal.
independently of the exposure duration \((10 < t < 24\text{h})\) and concentration. In comparison, the recovery at the target site of corals was less systematic, since it was rapid following exposure to diuron and atrazine [41], but still incomplete 3 d after irgarol removal [34]. The measurement of the PSII effective quantum yield enabled close monitoring of the effect and recovery at the target site of PSII inhibitors in \textit{S. vacuolatus} during a pulse exposure. We were able to demonstrate the rapid reversibility of effects at the target site. This reversible binding led to a rapid recovery of algae growth and indicates that a single pulse exposure to atrazine and isoproturon induce no long lasting effect on \textit{S. vacuolatus}.

### 2.6 Conclusion and outlook

The method developed allowed the assessment of time-dependent and pulse effects of atrazine and isoproturon on a unicellular algae, which is more relevant for the risk evaluation of realistic exposures in streams than standardized tests performed over 72 h. The analysis of the time-dependent toxicity highlighted that these herbicides do not have the same dynamics of effects on growth, even if they both exert fast kinetics at their primary target site on PSII. With a low time-dependence of effects on growth, isoproturon 10 h pulses strongly inhibited growth at concentrations only 1.5 times higher than those giving the same effect after 3 d. Therefore pulse exposure could affect the most sensitive species during exposure, but atrazine and isoproturon pulses would not induce long-term effects since these are reversible.

In the future, this assessment will be extended to herbicides with other modes of action, which also occur in peak concentrations. Furthermore, the information gained in this study can be utilized in the assessment of sequential pulse exposure in view of the fact that the aquatic ecosystem is repeatedly exposed to peak concentrations. Finally, the observation made on the kinetics and the dynamics of effects could support the modelling of effects during fluctuating exposure. Existing models predicting the effects from time-dependent exposure were recently reviewed by Ashauer [42]. However, the models appraised describe lethal effects on homogenous populations of fish or invertebrates [43-45]. They are not appropriate to predict the inhibition of growth in algae populations composed of individuals at different developmental stages and should therefore be adapted.
Acknowledgements

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2.7 References


Chapter 3

S-metolachlor pulse exposure on the alga *Scenedesmus vacuolatus*: effects during exposure and the subsequent recovery

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3.1 Abstract

In streams and creeks, the aquatic flora is exposed to fluctuating concentrations of herbicides during and following their application. Peak concentrations to herbicides, like the chloroacetanilide S-metolachlor, are usually detected following rain events. In this study, we assessed the effect of S-metolachlor pulse exposure on the algae *Scenedesmus vacuolatus*. We measured the time-dependency of effects during exposure on algae population and identified the algae development stage most sensitive to S-metolachlor. Furthermore, we assessed the time-to-recovery of the algae following exposure. A 6 hour pulse exposure was sufficient to inhibit cell reproduction; however, the exposure period needed to coincide with the cell development stage most sensitive to S-metolachlor, which is the end of the cell growth phase. In algae populations composed of cells at all development stages, we initially observed an increase in the size of some algae cells, ultimately leading to an inhibition of the growth rate. In these experimental conditions, effects were observed after 18 hours of exposure and greatly increased with time. The recovery of algae following exposure to strongly inhibiting S-metolachlor concentrations was delayed and only occurred after 29 hours. These findings suggest that peak exposure to S-metolachlor may affect the growth alga in surface waters, considering that the effects extend beyond the period of exposure.

Keywords: Pulse exposure, Recovery, S-metolachlor, Algae
3.2 Introduction

Metolachlor is one of the herbicides most applied to corn and other crops to control pre-emergent and early post-emergent broadleaf and grass weeds (Muthmann, 2007). This chloroacetanilide herbicide is regularly detected in North American and European surface waters and the highest levels are usually detected during the crop growing season (Battaglin et al., 2000; Balsiger et al., 2004; Gilliom et al., 2006; Konstantinou et al., 2006). It has been shown that weather conditions play an important role, as metolachlor is primarily transported following rain events during the field application period (Ng & Clegg, 1997). The concentrations of metolachlor in creeks, like some other herbicides, are highly dynamic, with peak concentrations occurring during height flows, and low levels the rest of the time (Gilliom et al., 2006). In Switzerland, metolachlor peak exposure concentrations (>2 μg/L) have been shown to surpass its suggested chronic water quality criteria (0.3 μg/L) (Leu, 2003; Chèvre, 2006), which aim at the long term protection of the aquatic flora and fauna. It is not know whether these peak levels are toxic for the aquatic flora considering their short exposure duration.

The herbicidal effect of metolachlor has been shown to be stereospecific (Couderchet et al., 1997). Of the four metolachlor stereoisomers, just the (S)-enantiomers are phytotoxic. Only S-metolachlor is currently registered for application in the European Union1 and Switzerland2, whereas the application of the racemic metolachlor (R and S) is still authorised in the USA3. S-metolachlor is classified as an inhibitor of the formation of very long chain fatty acids (VLCFA)4. The growth of susceptible weeds is blocked following germination, as it curbs seedling shoot and root development by interfering with normal cell development, inhibiting both cell division and cell enlargement (Deal & Hess, 1980). The primary site of action of metolachlor, like that of the other chloroacetanilides, was identified as the FAE1-synthase, a starter enzyme required for the elongation of C16 and C18 to C20 fatty acids (Götz & Böger, 2004). In algae, the binding of the herbicide to the enzyme inhibits the synthesis of VLCFAs,

leading to an imbalance in the fatty acid composition of cell plasma membranes, and resulting in the loss of cell rigidity and permeability (Schmalfuss et al., 1998; Böger, 2003). Some algae species have been shown to be very sensitive to S-metolachlor with concentrations as low as 8 μg/l inhibiting the growth of *Selenastrum capricornutum* by 50% over 5 days\(^5\).

Due to this high toxicity, peak exposure to S-metolachlor may affect non-target aquatic plants and algae. In biochemical studies, the binding of the chloroacetanilide metazachlor to the VLCFA-synthetase has been observed following 30 minutes of incubation, a duration of exposure necessary to outcompete the substrate for the target domain of this enzyme (Götz & Böger, 2004). Furthermore, the binding between the herbicide and the enzyme was shown to be irreversible, which suggests that the effects of chloroacetanilides at a cellular level may not be as reversible as those induced by herbicides inhibiting photosynthesis (Vallotton et al., submitted).

The objectives of this study are to assess the effects on algae population of short S-metolachlor exposures as well as the recovery following exposure. In the first part of the study, the time-dependent toxicity to the algae *Scenedesmus vacuolatus* is assessed according to a newly developed protocol (Vallotton et al., submitted), with the goal of comparing the effects of pulse and long term exposure. Since S-metolachlor short pulses are suspected to specifically affect the cell division of *S. vacuolatus* (Fedtke, 1982), the effect assessment of pulse exposure is refined by exposing synchronously grown algae to S-metolachlor. The synchronization of the life cycles of individual algae cells provides a homogenous population of physiologically well-defined cells (Tamiya et al., 1953). This makes it possible to expose algae during specific periods of cell development and observe both physiological responses and inhibitory effects on the reproductive cycle. The purpose of these experiments is to identify if a certain cell development stage is specifically inhibited by S-metolachlor in order to support the evaluation of the effects during pulse exposure. Finally, we aim to define the time-to-recovery after exposure, which is the duration necessary to observe a recovery in cell reproduction, and evaluate if a short exposure could induce long-term effects on growth.

3.3 Material and Methods

3.3.1 Chemicals

S-metolachlor (S-metolachlor Pestanal®, 98.4%) was purchased from Sigma-Aldrich. Stock solutions were prepared in the algae culture medium (see below) at 300mg/L or 60 mg/L and their concentration controlled analytically by HPLC-MS-MS (Stoob et al., 2005). The concentrations of S-metolachlor are expressed as nominal concentrations at the beginning of the test.

3.3.2 Algae and growth media

The green unicellular algae, *Scenedesmus vacuolatus* (Chlorophyceae; strain 211-8b, Shihira and Krauss, Philadelphia, PA, USA) was purchased from the algae collection of the Institute for Plant Physiology of the University of Göttingen, Germany. The alga was cultured in batch cultures in a sterile inorganic medium prepared as described by Le Faucheur (2005), but the carbon source differed in the experimental set ups.

3.3.3 Method A: Culture conditions of algae population grown under continuous illumination

NaHCO₃ (1.2 mM) was added to the growth media, as a sole source of carbon. Batch cultures were inoculated into 50 ml of medium in sterile 100-ml Erlenmeyer flasks with an algal colony reserved an agar plate. The cultures were maintained in a shaker at 90 rpm at 25°C with continuous illumination of 105μmol/m²/s by cool-white fluorescent lamps. The algae were maintained in exponential growth by transferring cells from a culture in the exponential growth phase (<72h) to fresh medium, with an initial cellular density of 650,000 cells/ml.

A1) Experimental design for the assessment of time dependent effects

Based on the method of Vallotton et al. (submitted), algae cultures were inoculated with cultures in the second day of their exponential growth into 100 ml flasks containing 50 ml of medium at an initial cell density of 650,000 cells/mL. S-metolachlor was added 24 hours after the start of the test. Separate experiments were carried out to determine the effects of an 18 hour exposure and the time-dependency of effects between a 24 and 48 hour exposure. The cell density and the cell size distribution were measured by particle counting using a coulter...
counter (Beckman Z2 Coulter® Particle Count and Size Analyzer, Germany). During the 18 hour experiment, measurements were made four times. Exposure concentrations ranged from 3 to 90mg/l. In the assessment of effects for exposures ranging from 24 to 48 hours, algae were exposed to 1.5 to 19.5mg/l and measurements were run three times per day. All tests were carried out twice with five concentrations tested in duplicates and a minimum of two control cultures.

A2) Data analysis of time dependent effects

The growth rate was calculated with a linear regression of the natural logarithm of cell density measurements over time. EC50 values derived from the growth rate were estimated for each exposure duration, using a four parameter log-logistic dose response model (Eq. 1), with the aid of the statistics software Prism (1992-2003 GraphPad, San Diego, CA, USA). The 95% confidence interval was calculated from the standard error (EC50 ± 1.96 * SE_{EC50}):

$$E = \min + \frac{(\max - \min)}{1 + 10^{(\log \text{EC50} - \log C) \cdot \text{Hillslope}}}$$

(Equ. 1)

where E= Effect, e.g. the inhibition of the growth rate or the inhibition of reproduction; C = Concentration; Hillslope = slope of the curve at EC50; and max and min = the maximum and minimum of the sigmoid curve.

The maximum parameter was set as the average of the control cultures’ growth at the exposure duration studied, while the minimum parameter was fixed at 0 since we assumed no growth at high concentrations (Chèvre et al., 2005).

The time dependent toxicity was described with a linear regression of log EC50 values in function of the logarithm of time (Vallotton et al., submitted):

$$\log \text{EC}_{50} = a \log t + b$$

(Equ. 2)

where t = time; and a and b are constants.
3.3.4 Method B: Populations grown synchronously under a dark and light illumination cycle

Algae were inoculated in the reactor of a Sixfors fermenter (Infors AG, Bottmingen, Switzerland) into 500 ml of growth media. The carbon source was provided by continuous aeration with 1.5% CO₂, 19.7% O₂, and 78.8% N₂. Batch cultures were stirred at 200 rpm and maintained at 28°C under a cycle of 14 hours light (130 μE m⁻² s⁻¹) and 10 hours dark phase. At the start of the light phase (t₀), the population is composed of autospores just released from the rupture of the mother cell wall (Figure 3.1). During illumination (t₀-t₁₄), these cells increase their biomass for the formation of the next generation of spores and begin dividing within the cell wall (Yamamoto et al., 2004). Finally, the maturation and hatching of daughter cells occurs during the dark phase.

B1) Experimental designs

Exposure was performed in Pyrex Erlenmeyers, sealed with SVL caps. 20 mL of a suspension of newly formed cells were inoculated in each Erlenmeyer at a density of 1.5 * 10⁵ cells/ml. NaHCO₃ was added as a source of carbon at a final concentration of 1.48 mM. The flasks were placed in a water bath at 28°C at a light intensity of 86 μE m⁻² s⁻¹ throughout the test. Cultures were stirred with a Teflon-coated magnetic bar at 200 rpm.

B2) Standard test with synchronously grown algae

The effect on the cell reproduction was assessed by exposing algae during a complete reproductive cycle of 24 hours (Exp. 1; Altenburger et al., 1990). The test was run at six concentrations ranging from 95 to 850 μg/l tested in triplicate and with ten control cultures. The cell density and the cell size distribution of the algae was measured with a cell counter (Beckman Z2 Coulter® Particle Count and Size Analyzer, Germany) at the beginning of the test (t₀) and 24 hours later, at the end of the reproduction cycle (t₂₄).

B3) Short exposures during specific periods of cell development

Various exposure scenarios (Figure 3.1) were designed to assess the effect of short exposures on algal reproduction. The first strategy consisted of delayed chemical addition. Algae were exposed during the last 18 (t₆-t₂₄), 15 (t₅-t₂₄), or 10 hours (t₁₄-t₂₄) of the cell cycle (Exp. 2-4). Exposure concentrations during the 10 hour exposure (t₁₄-t₂₄) ranged from 600 to 2400 μg/l S-
metolachlor. Six concentrations ranging from 175 to 1268 µg/l S-metolachlor were tested in triplicate with ten control cultures for exposure durations of 15 (t9-t24) and 18 hours (t6-t24).

The second strategy consisted of limiting the exposure to the first part of cell cycle; therefore, the herbicide was removed before the end of the reproduction cycle at t14. The exposure window consisted of 14 hours (Exp. 5) in the light phase (t1-t14) at 90-2445 µg/l S-metolachlor with 5 control cultures. To remove S-metolachlor from the algae suspension at t14, the algae cultures were centrifuged seven minutes at 3000 rpm at 25°C in 50 ml polypropylene centrifuge tubes. The supernate was discarded and the algae re-suspended in fresh media. The
centrifugation was repeated a second time ensuring 99.9% S-metolachlor removal (data not shown). Finally, exposure was limited to a specific period of the reproductive cycle stage (Exp. 6), such as the last 6 hours of the light phase (t8-t14) at 75-2038 μg/l S-metolachlor. This set-up required delayed chemical addition at t8 as well as the chemical removal at t14. The inhibition of cell reproduction was assessed by measuring the cell density at t0 and at t24 and the cell size distribution of the algae was recorded. In the case of herbicide removal, the cell density was also measured prior to and following centrifugation to estimate the algae loss.

B4) Recovery assessment

Algae were exposed for 24 hours to 750 μg/l S-metolachlor, a concentration fully inhibiting cell division after a 24-hour exposure. The herbicide was removed by centrifugation and the algae of three cultures were pooled to ensure sufficient cell density for the rest of the experiment. Algae previously exposed to S-metolachlor were either placed once more in media supplied with S-metolachlor at 750 μg/L, leading to further inhibition of the reproduction, or in growth media. The recovery of algae growth was assessed over the next 48 hours (t24- t72) by comparing the reproduction of the cultures growing in media containing S-metolachlor versus those placed in uncontaminated media. For this purpose, the cell density was measured at t24 and then five times per day during the rest of the test duration. NaHCO3 (0.0375 mM) was added 24 hours after chemical removal to ensure carbonate saturation.

B5) Data analysis of effects on reproduction

The reproduction rate of each control (Rc) and exposed flask (RY) was calculated based on the cell density at tx (CDtx) and at t24 (CDt24). x equals 0 hours in the exposure scenarios 1 through 4 and 14 hours in the case of exposure requiring chemical removal. The number of cells during the first 14 hours of cell development was constant, since algae only build up biomass:

\[ (R_Y) = (CD_{t24} - CD_{tx}) / CD_{tx} \]  

(Equ. 3)

A quality criterion was set to ensure homogeneity between the different experiments. The average reproduction rate of the controls needed to be between 4 and 8, which are the 25th and 75th percentile of the reproduction rate of the first 15 tests performed.
The inhibition of cell reproduction (IR) was expressed as a percentage of the average reproduction rate of the controls (RC):

\[
IR \% = (1 - R_Y/R_C) \times 100\% \quad \text{(Equ. 4)}
\]

The EC\textsubscript{50} for the inhibition of reproduction and its 95% confidence interval (EC\textsubscript{50} ± 1.96* \text{SE}_{\text{EC50}}) were estimated using (Eq. 1). The maximum and minimum parameters were fixed at 100 and 0.

In the assessment of the recovery, the reproduction rate of cultures (R\textsubscript{R}) was based on the cell density measurements after chemical removal (CD\textsubscript{tR}) and at the studied time of recovery CD\textsubscript{tz}:

\[
(R_R) = (C_{D_{tz}} - C_{D_{tR}}) / C_{D_{tR}} \quad \text{(Equ. 5)}
\]

### 3.4 Results and discussion

#### 3.4.1 Time-dependent effects on the growth rate of *S. vacuolatus*

The assessment of the effects of S-metolachlor on algae during exposure showed that this herbicide caused increasing effects on algal cell growth and cell size distribution with time. The inhibition of the growth rate increased for exposure durations of 18 to 48 hours (Figure 3.2). Concentrations inhibiting the growth rate by 50% after 18 hours of exposure were estimated at 17 mg/L with a 95% CI (12.6; 23.8) and 21 mg/l (17.0; 25.7) in the replicate experiment. After 24 hours of exposure, the EC50 dropped to 5.5 (4.3; 7.2) and 9 mg/l (5.7; 13.9) and further decreased to 2.3 (1.8; 3.1) and 3 (2.2; 4.5) mg/l after 48 hours of exposure.

Surprisingly, no growth rate inhibition was observed during the first 10 hours of exposure, even at the highest concentrations tested (90mg/L). This particular aspect was further studied by exposing synchronous algae to short pulses. For longer durations of exposures, the time-dependent toxicity of S-metolachlor was greater than that of atrazine and isoproturon. The 18h-EC50/48h-EC50 ratio was 7, while the toxicities for atrazine and isoproturon in the exposure period 10-48 hours increased in average by less than a factor 5, indicating a major increase in the toxicity of S-metolachlor with time. The toxicity of S-metolachlor was, however, extremely low in comparison to that induced by PSII inhibitors. In the same
S-metolachlor single pulse exposure

Experimental set-up, the 24h-EC50 was 196 μg/L for atrazine and 130 μg/L for isoproturon (Vallotton et al., submitted). Furthermore, this strain proved to have a low sensitivity in comparison to other algae strains, such as *Selenastrum capricornutum*, (EC50= 84μg/l) and *Chlorella vulgaris* (EC50= 203μg/l) exposed to racemic metolachlor (Fairchild et al., 1998).

A clear physiological effect of S-metolachlor during exposure was the increase in the cell diameter of exposed cells (Figure 3.3). After 4.5 hours of exposure, the cell size distribution of a culture exposed to 3.2mg/l S-metolachlor remained comparable to that of the control culture, but the size of most algae cells increased with time. The average diameter of algae was 5.7 μm at 21 hours of exposure, reaching more than 8 μm after 45 hours, which is double what was observed in the controls. It seems that only a few cells were able to divide, which explains the low number of freshly released spores at 45 hours. The spores have a mean diameter of 3.6 μm, as determined in synchronized culturing of *S. vacuolatus*. As a consequence, large cells at 21 hours and 45 hours may contain sufficient biomass for the formation of seven or twelve new spores, which could be released if the cells were to recover following exposure. The inhibition of cell division during exposure is in line with Fedtke’s observations on *Chlamydomonas* cells exposed to the chloroacetanilide Alachlor (Fedtke,
After 24 hours of exposure, *Chlamydomonas* formed cell aggregates, reaching a volume ten times greater than that of the spores.

![Figure 3.3](image)

Figure 3.3: Evolution of the cell size distribution of the control (—) in comparison to that of a culture exposed to 3.2mg/l S-metolachlor over 45 hours. After 4.5 hours of exposure (---), the distribution is similar to that of the control, except that the cells enlarge with time (21 hours — —, and 45 hours ——-).

### 3.4.2 Identifying the S-metolachlor time-to-effect and the algae development stage most sensitive to this herbicide in synchronously grown cells

In the second part of this study, the effects of different S-metolachlor exposure durations on the cell reproduction of synchronously grown *S. vacuolatus* were assessed (Table 3.1 and Figure 3.4). The EC50 of a standard 24 hour toxicity test (t0-t24, exp. 1) with synchronous cells was estimated at 341 µg/l with a 95% CI (300; 389). In this experimental set-up, the sensitivity of *S. vacuolatus* was more than fifteen times greater than for cultures placed in continuous light, confirming the importance of culture conditions in toxicity testing (testing volume, light regime and intensity) (Lewis, 1995). In synchronous culturing, the strain used in this study proved to be slightly less sensitive to S-metolachlor than *S. vacuolatus* strain 211-15 exposed to a mixture of both R and S enantiomers (EC50=232µg/l) (Junghans et al., 2003).
Table 3.1: Effect of different exposure durations and periods of exposure on the reproduction rate of *S. vacuolatus*: EC50 and its upper and lower 95% confidence interval (CI).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Period of exposure</th>
<th>Exposure duration</th>
<th>EC50</th>
<th>- 95% CI</th>
<th>+ 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>[h]</td>
<td>[μg/l]</td>
<td>[μg/l]</td>
<td>[μg/l]</td>
</tr>
<tr>
<td>1</td>
<td>t0-t24</td>
<td>24</td>
<td>341</td>
<td>300</td>
<td>389</td>
</tr>
<tr>
<td>2</td>
<td>t6-t24, t9-t24</td>
<td>18</td>
<td>389</td>
<td>371</td>
<td>407</td>
</tr>
<tr>
<td>3</td>
<td>t9-t24</td>
<td>15</td>
<td>442</td>
<td>415</td>
<td>470</td>
</tr>
<tr>
<td>4</td>
<td>t14-t24</td>
<td>10</td>
<td>&gt;2400</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>t6-t14</td>
<td>14</td>
<td>697</td>
<td>504</td>
<td>964</td>
</tr>
<tr>
<td>6</td>
<td>t0-t14</td>
<td>6</td>
<td>598</td>
<td>414</td>
<td>865</td>
</tr>
</tbody>
</table>

An exposure duration of 6 hours (t8-t14, exp. 6) did inhibit the cell reproduction in a concentration dependent manner, with an EC50 estimated at 598 μg/l (414; 865), although the toxicity was lower compared to that observed after a 24 hour exposure. Additionally, all exposure scenarios (Exp. 1-3, 5) that comprised the last hours of the light phase inhibited cell reproduction in a similar concentration range. The EC50 of an 18 hour exposure (t6-t24, exp. 2) was estimated at 389 μg/L (371; 407), while that of a 15 hour exposure (t9-t24, exp. 3) was 442 μg/l (415; 470). Exposure exclusively during the light phase (t0-t14, exp. 5), also inhibited cell reproduction. The EC50 of this 14 hour exposure was 697 μg/L (504; 964). In contrast, a 10 hour exposure exclusively during the dark phase of the cell cycle (t14-t24, exp. 4) did not cause any effects, even at the highest concentration tested (2.4 mg/L). These observations indicate that the stages of cell development most sensitive to S-metolachlor occur during the light phase, thus during cell growth. Böger (2003) suggested that the inhibition of cell division is a consequence of perturbed lipid biosynthesis occurring during cell growth.
Figure 3.4: Dose-response curves for the inhibition of reproduction for different periods and durations of exposure. Three exposure periods comprise the dark phase: 24 hours (■, —), 18 hours (▲, ---) and 15 hours (▼, ——). The error bars represent the standard error of the inhibition of the reproduction rate. The dose-response curves of a 6 hour (□, —) and a 13 hour (○, ——) exposure during the light phase are flatter than in exposure scenarios that included the dark phase. The inhibition of the reproduction rate of cells exposed exclusively during the light phase was more variable, as the cultures undergo chemical removal. The inhibition of reproduction is negative when the reproduction rate of the exposed flasks is greater than that of the controls.

Exposure during the end of the light phase seems to be crucial for the inhibition of cell division. Several studies support the hypothesis that relevant processes of cell wall synthesis, such as the formation of VLCFAs (C>18), occur during this period (Atkinson et al., 1972; Couderchet et al., 1996). Furthermore, Yamamoto et al. (2004) showed that the synthesis of the cell wall of spores within the mother cell in *Chlorella vulgaris* already begins during cell growth. These results may explain why no effects were observed on the growth rate of algae population after 10 hours of exposure. In these experimental conditions, the cells present are at all stages of development, therefore a 10 hour pulse exposure might not coincide with the development stage sensitive to S-metolachlor. If the exposure duration is extended, the number of cells exposed during this sensitive stage increases and, as a consequence, causes effects on the population growth rate.
3.4.3 Recovery of algal reproduction

Figure 3.5 presents the recovery of algal reproduction following a 24 hour exposure to a strongly inhibiting S-metolachlor concentration. During day one, control cultures reached reproduction rates between 6.3 and 7.3, while cultures exposed to S-metolachlor failed to reproduce. During days 2 and 3, cultures placed back in S-metolachlor were further confronted with a strong inhibition of their reproduction rate (1.3 to 2.2). At the same time, cultures placed in uncontaminated media also did not reproduce during the first day of the recovery period. A recovery in cell division began only 24 hours following chemical removal, evidenced by the increasing reproduction rate during day 3. Reproduction rates reached 3 to 4.5 at 29 hours and leveled off at 4.6 to 6.4 at 30 hours following chemical removal. In the assessment of the effects during recovery, we could define the time-to-recovery of algal cell reproduction after a 24 hour exposure to S-metolachlor to be 29 hours. This result is in line with the inhibitory effects observed on synchronously grown *Chlamydomonas reinhardtii* following exposure to the chloroacetanilide alachlor (Fedtke, 1982). Furthermore, Weisshaar and Böger (1987) have observed that a 24 hour period is necessary to restore the growth of *Scenedesmus acutus* cultivated under continuous light, subsequent to a 24 hour exposure to metazachlor, another chloroacetanilide herbicide (Weisshaar & Böger, 1987). The delay in recovery following exposure to S-metolachlor could be the consequence of the irreversible covalent binding of the herbicide to the fatty acid elongase (Schmalfuss et al., 2000; Eckermann et al., 2003). It could be hypothesized that the time required for the replacement of inactive elongase enzymes, the synthesis of VLCFA, and the restoration of the fatty acid balance in the membranes exceeds 13 hours, because the synthesis of VLCFA is a light driven process (Kring et al., 1995). However, it cannot be excluded that other relevant cell structures or metabolic processes recovered during the dark period, allowing the recovery of cell division just one day after chemical removal.
Figure 3.5: Recovery of algae population following a 24 hour exposure to 750 µg/l S-metolachlor. The reproduction rates of three groups of algae are presented: the controls during the exposure period (+, ×, *), the cultures continuously exposed to S-metolachlor over 3 days (△, ▽, □), and algae exposed to S-metolachlor during 24 hours with a subsequent recovery period of 2 days (■, ▼, ▲).

The delay in recovery subsequent to S-metolachlor exposure contrasts with the fast recovery upon exposure to triazines and phenylureas (photosystem II inhibitors). Similar observations have been made on the duckweed *Lemna minor* following exposure to herbicides with different modes of action (Cedergreen et al., 2005). While the effects following exposure to the photosynthesis inhibitors were readily reversible, exposure to herbicides that impaired cell division induced a delayed recovery of the fronds (Cedergreen et al., 2005). These studies suggest that the mode of action of chemicals, the reversibility of their binding at the target site, and the degree of damage during exposure all influence the potential recovery following exposure. Thus, it is extremely important for risk assessment to know the mechanisms of action of herbicides.
3.5 Conclusion

A 6 hour S-metolachlor pulse inhibited the cell reproduction of algae when the exposure coincided with a specific cell growth stage of *S. vacuolatus*. The physiological effect was an inhibition of cell division made apparent by the increase in the cell volume. Furthermore, the recovery of algae cells affected by S-metolachlor was not immediate, since a delay in recovery of 29 hours was observed. The effects observed during exposure and the delayed recoveries have two implications. First, peak chloroacetanilide concentrations may be detrimental to sensitive species, since the populations are affected not only during, but also following a pulse exposure. Second, in the case of exposure to repeated pulses, delayed recovery could influence the response during a second pulse.

Acknowledgements

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3.6 References


S-metolachlor single pulse exposure


Chapter 3


Kring, F., Couderchet, M., Boger, P., 1995. Inhibition of oleic acid incorporation into a non-lipid fraction by chloroacetamide herbicides. Physiol. Plant. 95, 551-558.


Effect of sequential isoproturon pulse exposure on *Scenedesmus vacuolatus*
4.1 Abstract

Aquatic organisms are typically exposed to fluctuating concentrations of herbicides in streams. To assess the effects on algae of repeated peak exposure to the herbicide isoproturon, we subjected the alga *Scenedesmus vacuolatus* to two sequential pulse exposure scenarios. Effects on growth and on the inhibition of the effective quantum yield of photosystem II (PSII) were measured. In the first scenario, algae were exposed to short 5h pulses at high isoproturon concentrations (400 and 1000 μg/L), each followed by a recovery period of 18h, while the second scenario consisted of 22.5 h pulses at lower concentrations (60 and 120 μg/L), alternating with short recovery periods (1.5h). In addition, any changes in the sensitivity of the algae to isoproturon following sequential pulses were examined by determining the growth rate-EC50 prior to and following exposure. In both exposure scenarios, we found that algal growth and its effective quantum yield were systematically inhibited during the exposures and that these effects were reversible. Sequential pulses to isoproturon could be considered as a sequence of independent events. However, growth inhibition during each exposure also resulted in a loss in total biomass production following each subsequent exposure, which resulted in a cumulative decrease overall. Furthermore, in the second scenario, when the sequence of long pulses began to approach a scenario of continuous exposure, a slight increase in the tolerance of the algae to isoproturon was observed. These findings indicated that sequential pulses do affect algae during each pulse exposure, even if algae recover between the exposures, therefore short-term exceedances of the chronic water quality criteria, aiming at the long-term protection of the aquatic environment, should be limited.

Keywords: Sequential pulse exposure, Isoproturon, Algae.
4.2 Introduction

Isoproturon is a phenylurea herbicide applied to prevent the development of pre-emergent weeds on cereals fields (wheat and barley). It is one of the herbicides commonly detected in surface waters. Furthermore, the concentrations of isoproturon measured in rivers were shown to fluctuate. Peak concentrations are typically detected following rain events after the isoproturon field application [1-4]. In Swiss rivers, concentrations up to 8.4 μg/L were measured [5].

This herbicide is classified as an inhibitor of photosystem II (PSII), as it competes with plastoquinone for binding at the Qb-site of the D1 protein in PSII [6]. The displacement of the plastoquinone by isoproturon blocks electron transfer, which in turn inhibits photosynthesis [7]. At the cellular level, PSII inhibiting herbicides are also known to induce oxidative stress that can in turn damage proteins, lipids and other cellular components, thus inhibiting growth or leading to plant death [8].

Previous studies have shown that single pulse exposure to PSII inhibitors reduces the growth of aquatic plants and algae during exposure, but that these effects were reversible [9-12]. In creeks and rivers, the aquatic flora is, however, not solely exposed to a single pulse, but rather to fluctuating concentrations that might repeatedly exceed the suggested Swiss chronic water quality criteria (CQC) for pesticides [13]. Nevertheless, little is known about the effects of these sequential peak exposures on algae and water plants.

As summarized by Reinert [14], the long-term effects on non-target organisms of fluctuating pesticide exposure are a function of the damage sustained during exposure, the capacity of the organisms to recover and the duration of the recovery period between pulses. Sequential exposure to isoproturon could lead to an increased or a decreased effect during each subsequent exposure, or else no effect from previous exposures might be apparent in subsequent exposures [14]. If a pulse exposure does not influence the response of a subsequent pulse, the sequential exposures may be considered to be completely independent exposures events.
The aim of this study was to investigate the effects of isoproturon sequential pulse exposure on the growth of the algae *Scenedesmus vacuolatus*, and the effects at the target site of the herbicide, by exposing algae to two different exposure scenarios. In both exposure scenarios, we analysed the magnitude of effects on algae during each pulse and each recovery period. Growth and inhibition of the PSII effective quantum yield were measured to monitor population level and target site effects of isoproturon, respectively. In addition, dose-response relationships were obtained at the beginning and end of the sequential exposure experiments to assess possible changes in the sensitivity of algae to isoproturon. Finally, the net decrease in biomass production throughout the sequential exposure, as compared to the control was estimated to assess the overall effects of multiple pulses.

### 4.3 Material and Methods

#### 4.3.1 Chemicals

Isoproturon (Isoproturon Pestanal ®, 99.8%) was purchased from Sigma-Aldrich. Stock solutions for algal toxicity testing were prepared at a concentration of 30 mg/l in the algal culture medium and tested analytically using HPLC-MS-MS [15]. Experimental test concentrations are expressed as nominal concentrations.

#### 4.3.2 Algal culture conditions

The green unicellular alga *Scenedesmus vacuolatus* (Chlorophyceae; strain 211-8b, Shihira and Krauss, Philadelphia, PA, USA) was purchased from the alga collection of the Institute for Plant Physiology of the University of Göttingen, Germany. The alga was cultured in a sterile inorganic medium prepared as described by Le Faucheur [16]. Batch cultures were grown in 50 ml of medium in sterile 100-ml Erlenmeyer flasks, inoculated with cells grown and maintained on a Petri plate. The cultures were maintained on a shaker at 90 rpm at 25°C with continuous illumination of 105 μE/m²/s by cool-white fluorescent lamps.

#### 4.3.3 Experimental designs for sequential exposure

Cultures were started from algal colonies conserved on a Petri plate. Cells from this culture that had reached exponential growth were transferred to fresh media to an initial optical density (OD) of 0.05 at 685 nm (OD₆₈₅), which corresponded to a density of 650’000 cells/ml.
Cultures were exposed to the first isoproturon pulse 24 hours following inoculation. Chemical removal at the end of a pulse was achieved by centrifuging the cultures for 7 min at 3000 rpm at 25°C. The supernate was discarded and the algae were re-suspended in fresh medium. Centrifugation was repeated a second time, ensuring 99.9% removal of isoproturon. The removal of isoproturon through centrifugation did not affect algal growth. Following chemical removal, cultures were re-suspended to an OD of 0.1 in order to maintain exponential growth throughout the experiment. Controls were treated in the same manner.

The cell density and the PSII effective quantum yield were measured throughout the experiment. The cell density was determined by optical measurement at 685 nm using a spectrophotometer (Uvikon 930; Kontron Instruments, Munich, Germany) in a range in which OD exhibited a linear correlation with cell density, as determined with microscopic Neubauer chamber cell counts (Brand, Wertheim, Germany). This relationship was shown to be similar for control algal cultures and algae exposed to isoproturon.

The PSII effective quantum yield was measured using a dual channel pulse amplitude modulated (PAM) fluorometer, called ToxY-Pam (Heinz Walz GmbH, Effeltrich, Germany; [17]). This instrument assesses the effective quantum yield of energy conversion at PS II reaction centers by applying a pulse modulated measuring light. Light adapted algae are illuminated alternately by measuring light (3s) to determine the chlorophyll fluorescence, F, and by saturation pulses (470 nm with a pulse width of 0.4s) to determine the maximum fluorescence, Fm’, at λ > 640nm [18]. F is measured shortly before the application of saturation pulses, while Fm’ is measured during the saturation pulse. F and Fm’ were each averaged from the three final measurements of five total. Fluorescence measurements were spaced 30 s apart.
**A) Exposure scenario 1: short pulse with long recovery period, high isoproturon concentrations**

Algae were exposed to six 5-hour sequential isoproturon pulses with recovery periods of 19h (Figure 4.1). The exposure concentrations were 400 and 1000 μg/L isoproturon, tested in triplicate with 3 control cultures. These concentrations were chosen to induce significant effects on growth during short pulse duration. They are based on the growth response following a 10h exposure [12]. 1000 μg/L corresponds to a 10h-EC80 for the growth rate endpoint, while 400 μg/L is greater than a 10h-EC50 for the growth rate endpoint. The growth rate was calculated during each recovery period based on 4 cell density measurements, but could not be calculated during exposure because of the low number of measurements made during the short pulse. The PSII effective quantum yield was measured prior to each pulse exposure, 3 times during exposure and directly following chemical removal.

The change in sensitivity of algae was assessed by comparing the response of the algae to a 24h exposure to isoproturon at the beginning of the experiment (t=24h) and following sequential exposure (t=150h). At the beginning of an experiment, a 24-hour growth test was started, parallel to the sequential pulse experiment. At the end of the sequential pulse experiment two 24-hour tests were conducted with algae pooled from the control cultures and with algae from cultures sequentially exposed to 1000 μg/L isoproturon. In the three 24-hour tests, algae were exposed to isoproturon concentrations ranging from 30-695 μg/l.

**B) Exposure scenario 2: long pulse with short recovery time, low concentrations**

The sequential exposure consisted of 5 isoproturon pulses, each lasting 22.5 h with recovery periods of 1.5 h (Figure 4.2). Algae were exposed to 60 μg/L and 120 μg/L of isoproturon. 60 μg/L isoproturon corresponds to a 24h-EC30 for the growth rate endpoint and is less than the 24h-EC50 for the inhibition of effective quantum yield. 120 μg/L isoproturon corresponds to a 24h-EC50 for the inhibition of the effective quantum yield. The two exposure levels were tested in triplicate with 3 control cultures. The effective quantum yield was measured prior to each pulse exposure, 4 times during exposure and following chemical removal. The growth rate during the exposure periods was calculated based on 5 cell density measurements.
As in exposure scenario 1, the change in the sensitivity of algae was assessed. A 24-hour test was performed at the beginning of the experiment. Following sequential exposure to 22.5 h pulses, a 24 hour test could not be run due to the low number of cells available, therefore two 72-hour tests were run with algae from the control cultures and with algae from cultures sequentially exposed to 120 μg/L isoproturon.

4.3.4 Data analysis

(A) Algal growth

The growth rate (GR) was calculated as the slope of the linear regression of the natural logarithm of cell density measurements versus time. The inhibition of the growth rate (Inh\textsubscript{growth}) was expressed as the percent change of the experimental growth rate (GR\textsubscript{e}) from that of the controls (GR\textsubscript{c}) (Eq. 1).

\[
\text{Inh}_{\text{growth}} = 100 \times \frac{\text{GR}_{\text{e}} - \text{GR}_{\text{c}}}{\text{GR}_{\text{c}}} \tag{1}
\]

The significance of the difference between the average growth rate of the controls and the exposed alga was tested with a student test. The hypotheses were H\textsubscript{0}: μ\textsubscript{1} = μ\textsubscript{2} and H\textsubscript{1}: μ\textsubscript{1} ≠ μ\textsubscript{2}.

The biomass production over the entire duration of the experiment (number of cells/mL) was calculated to evaluate the cumulative effect of sequential pulse exposure. Since the experimental procedure required the daily dilution of algae, the daily biomass increases between the centrifugation steps were summed.

(B) Dose-response curves

The EC\textsubscript{50} values for growth rate inhibition, and 95% confidence interval were estimated using a 2 parameter logistic model (Eq. 2), with the aid of the statistics software Prism (1992-2003 GraphPad software, Inc).

\[
E = \frac{100}{1 + 10^{(\log EC50 - \log C) \times \text{Hillslope}}} \tag{2}
\]

E = Endpoint, inhibition of the growth rate; C = Nominal exposure concentration; Hillslope = slope of the dose-response curve at EC50.
(C) Difference in the sensitivity of the algae prior to and following sequential exposure

The significance of the difference between the EC50s from 2 experiments was tested with an F-test that compares the goodness-of-fit of two models by assessing the sum-of-squares (1992-2003 GraphPad software, Inc). The hypotheses were \( H_0: \) EC50\(_1\) = EC50\(_2\) and \( H_1: \) EC50\(_1\) \( \neq \) EC50\(_2\). Significant differences in EC50 values would indicate an increased or decreased sensitivity of the algae to isoproturon.

(D) Inhibition of the PSII effective quantum yield

The PSII effective quantum yield, \( Y \), is expressed as a percentage of the reduction in fluorescence, \( F \), with respect to the maximal fluorescence, \( Fm' \):

\[
Y = \frac{(Fm' - F)}{Fm'}
\]

and the inhibition, Inh(\%), is calculated as follows:

\[
Inh(\%) = \frac{Y_1 - Y_2}{Y_1},
\]

where \( Y_1 > Y_2 \), \( Y_1 \) being the average yield of the controls and \( Y_2 \) the yield of the exposed culture.
4.4 Results and discussion

4.4.1 Exposure scenario 1

Figure 4.1 presents the effects of sequential short pulse exposures at high isoproturon concentrations. During each of the six isoproturon pulses, the growth of the algae exposed to either 400 or 1000 μg/L was inhibited, as indicated by a cease in increase of cell density during the exposures (4.1 B and C). Inhibition of growth was expected since the chosen exposure concentrations were greater than the growth inhibition EC50 estimated following a 10 h pulse exposure [12]. Despite qualitative observation, growth inhibition during each exposure could not be quantitatively compared, because the duration of exposure was too short. During the exposures, the effective quantum yield (Figure 4.1 C and D) reached maximum inhibition within 1.5h of chemical addition. The effective quantum yield inhibition ranged from 48 to 66% at 400 μg/L isoproturon and from 60 to 74% at 1000 μg/L. These effect levels were similar to the effects observed during single pulse exposure experiments [12]. Inhibition of the effective quantum yield during each pulse was relatively constant, which indicated that previous pulse exposures did not influence the response during subsequent pulses. The inhibition of algal growth during pulses may be attributed to the rapid onset of inhibitory effects at the target site.

Similar to the fast onset of effects following chemical addition, a fast recovery of the effective quantum yield was repeatedly observed within 3 hours after chemical removal. This is in opposition to the effects observed on the seagrass Zostra capricorni following two sequential exposures to the triazine herbicide Irgarol [19]. In parallel to systematic recovery observed at the target site on the effective quantum yield, we observed a recovery of algal growth between the pulses, similar to that observed after single pulse exposures to PSII inhibitors in algae and duckweed [9-12]. In the experiments here, the average growth rate of the controls was not significantly different from that of the exposed cultures (p-value>0.05) (Table 4.1), except in recovery period no 4 (p-value=0.022), where the average growth rate of the exposed population was unexpectedly greater. However, neither an increasing nor a decreasing trend in average growth rates was observed during subsequent exposures, as the growth rates during the five recovery periods were not significantly different.
Figure 4.1: Short pulses at high isoproturon concentrations. A: Experimental design for the sequential exposure to 5h pulses (dotted line in graph B-D). The EC50 on algae growth was determined when indicated by the arrows. B: Effects on the cell density of a *Scenedesmus vacuolatus* population exposed to 1000µg/L pulses (○, solid line) compared to that of a control (▼, dashed line). After each chemical removal step, algal cultures were diluted to an optical density of 0.1, which explains the gap in the cell density. C: Effect of a sequential exposure to 1000 µg/l on the cell density of a culture (○, solid line), the inhibition of photosynthetic effective quantum yield of PSII (solid line), compared to the average inhibition of the control cultures (●) and its standard error (smaller than symbol, n=3). D: Effect of a 400 µg/l sequential exposure on the cell density (□), the inhibition of the effective quantum yield of PSII (dashed line), compared to the average inhibition of the controls (●) and its SE (smaller than symbol, n=3).
Table 4.1: Exposure scenario 1: Average and standard error (SE) of the growth rate during recovery periods of 3 replicate controls and 3 experimental cultures sequentially exposed to 5h pulses. The significance in the difference in the average growth rates of the two groups (p-value) was tested with a t-test.

<table>
<thead>
<tr>
<th></th>
<th>Recovery 1</th>
<th>Recovery 2</th>
<th>Recovery 3</th>
<th>Recovery 4</th>
<th>Recovery 5</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.049</td>
<td>0.048</td>
<td>0.049</td>
<td>0.049</td>
</tr>
<tr>
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<td>0.003</td>
<td>0.002</td>
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<td>0.050</td>
</tr>
<tr>
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<td>0.003</td>
<td>0.002</td>
<td>0.001</td>
<td>0.002</td>
</tr>
<tr>
<td>t-test with control, p-value</td>
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<td>0.56</td>
<td>0.02</td>
<td>0.97</td>
</tr>
<tr>
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<td>0.054</td>
<td>0.058</td>
<td>0.058</td>
<td>0.053</td>
</tr>
<tr>
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<td>0.003</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
</tr>
<tr>
<td>t-test with control, p-value</td>
<td>0.30</td>
<td>0.24</td>
<td>0.12</td>
<td>0.02</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Figure 4.2: A: Dose response curves of 24-h tests carried out with S. vacuolatus before (□, solid line) and after the six 5-hour sequential pulse experiment: non exposed algae (△, dashed line) and algae sequentially exposed at 1000µg/L (▽, dotted line). B: Dose response curves of 72-hour tests carried out at the end of exposure scenario 2, with cultures repeatedly exposed to 22.5h pulses at 120 µg/L (●, solid line) and with algae used as controls (○, dashed line).
The evolution in sensitivity of control and sequentially exposed algae was tested by determining the EC50 from 24h toxicity tests (Figure 4.2 A). The EC50 of the test performed at the beginning of the experiment was 225 μg/L with a 95% CI of (169 ; 300). At the end of sequential exposure, the EC50 obtained from a 24 h test performed with control algae was estimated at 177 μg/L (105; 296), while the EC50 of a second test run with algae that had undergone sequential 1000 μg/L isoproturon pulses was 164 μg/L (103; 261). The EC50 of these 3 tests (Figure 4.3 A) were not significantly different (p-value = 0.43) indicating that the population that underwent sequential exposure was neither more sensitive nor more resistant to isoproturon.

4.4.2 Exposure scenario 2

Figure 4.3 presents the effects of sequential long pulse exposures with short 1.5h recovery periods on the growth of *S. vacuolatus* and the inhibition of effective quantum yield (Figure 4.3 A). During each pulse exposure, isoproturon inhibited the growth rate of the exposed algal cultures. For example (Figure 4.3 B), the growth rate during the pulse exposures of a culture exposed to 120 μg/L ranged from 0.021 to 0.029 h\(^{-1}\), while that of the control varied between 0.042 and 0.047 h\(^{-1}\). Generally (Table 4.2), the average growth rates of the three controls ranged from 0.041 to 0.047 h\(^{-1}\) during the pulse exposures, while that of cultures exposed to 60 μg/L and 120 μg/L were reduced to 0.036-0.043 h\(^{-1}\) and to 0.021-0.029 h\(^{-1}\), respectively. Consequently, growth rates were inhibited by 2-20% at 60 μg/L and by 29-54% at 120 μg/L. Yet, the levels of inhibition during sequential pulses were not influenced by previous exposures, despite short recovery periods of 1.5 hours, since the average inhibition of the growth rate during the first pulse was not significantly different from the average inhibition during the last pulse (60 μg/L, p-value=0.63; 120 μg/L, p-value=0.22).

As in exposure scenario 1, the effective quantum yield of the exposed culture was inhibited directly following chemical addition and the response was similar among subsequent pulses (Figure 4.3 C). Within 0.5 h, the effective quantum yield was inhibited by 30% at 60 μg/L and by more than 40% at 120 μg/L. Inhibition of the effective quantum yield further increased during the first 7 hours of the pulse exposures, reaching a maximum inhibition of 42-46% at 60 μg/L and 51-58% at 120 μg/L. It then slightly decreased over the next 12 h of exposure. The decrease of effects at the target site during the exposures might be attributed to a decrease
in the dose of isoproturon per algae, since the cell density increased during the 22.5 h pulse. During the recovery period, the inhibition of the effective quantum yield dropped below 20%. The fast recovery between exposures may be attributed to the reversible binding at the target site and to repair mechanisms in PSII that replace the damaged D1 protein in PSII. In *Chlamydomonas reinhardtii*, following exposure to high intensity illumination, synthesis of D1 and its incorporation into functional PSII was estimated to take one hour [7, 20 ]. This repair interval is of the same order of magnitude as the recovery period observed for effective quantum yield inhibition.

Despite the very short recovery periods, the levels of inhibition observed during sequential pulses were not influenced by previous exposures. Furthermore, the pattern of inhibition was comparable among each pulse, supporting the hypothesis that longer pulse events (22.5 h) with short recovery periods could also be considered to be independent, as opposed to cumulative exposure events. However, the EC50 estimated from a 72 h test carried out at the end of sequential exposure with algae that had been sequentially exposed, significantly differed from the EC50 obtained with control cultures (p-value= 0.01; Figure 4.2 B). The EC50 of the test run with algae that had been repeatedly exposed (136 μg/L (110; 169)) was greater than the EC50 estimated for the control algae (99 μg/L (83; 119)), indicating a slight shift in sensitivity of the response of *S. vacuolatus* to isoproturon toxicity. The increase in EC50 suggested that sequential pulse exposures with short recovery periods may induce a physiological adaptation of the algal population, either by inducing a selection pressure on the population or by enhancing defence mechanisms against oxidative stress. This is in line with the increased tolerance observed in several *Scenedesmus subspicatus* clones, following a sixty-day exposure to atrazine (1-20 μg/L [21]). More generally, algal community tolerance increased following long-term exposure to 5-40 μg/L isoproturon [12, 22-24].
Figure 4.3: Long pulses at low isoproturon concentrations with short recovery time. A: Experimental design for the sequential exposure to 22.5h pulses (full lines in graph B-D). The EC50 on algae growth was determined when indicated by the arrows. B: Cell density and the growth rate regression of a *Scenedesmus vacuolatus* population exposed to 120 µg/L pulses (▲, solid line) compared to that of a control (■, dashed line). After each chemical removal step, algal cultures were diluted to an optical density of 0.1, which explains the gap in the cell density. C: Inhibition of the PSII effective quantum yield of algae exposed to 60 µg/L (◇, dotted line), 120 µg/L (▲, full line) and compared to the average of the controls (□, the SE is smaller than the symbol). D: The cumulative biomass production during the sequential pulses for the average controls (■) and cultures sequentially exposed to 120 µg/L isoproturon (▲). The error bars represent the standard error (n=3).
Table 4.2: Exposure scenario 2: Average and standard error (SE) of the growth rate during exposure of 3 replicate control and 3 experimental cultures, sequentially exposed to 22.5h pulses at 60 and 120μg/L. The inhibition of the growth rate is calculated for each pulse exposure.

<table>
<thead>
<tr>
<th></th>
<th>Pulse 1</th>
<th>Pulse 2</th>
<th>Pulse 3</th>
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<td>1</td>
<td>1</td>
<td>5</td>
</tr>
</tbody>
</table>

4.4.3 Implication of isoproturon sequential pulses

In both exposure scenarios, similar effects were observed. Algal growth was systematically inhibited during the exposures and the levels of effects were comparable during the course of the experiment. Furthermore, the response at the target site was highly consistent between exposure concentrations and expected concentration-response. Isoproturon inhibited the effective quantum yield within one hour after chemical addition, while recovery was observed within three hours following chemical removal. The response of the algae on both endpoints during a given pulse was not influenced by previous pulse exposures, even when the recovery periods were reduced to 1.5 hours. The highly reversible effects of isoproturon at the target site and the systematic recovery of the growth rate between pulse exposures, indicated that daily pulse exposures may be considered as independent exposure events, each followed by full recovery. However, two lasting effects on the algal population were observed. First when the population was exposed to a sequence of long pulses (scenario 2), which begins to approach a scenario of continuous exposure, there was an apparent increase in the tolerance of algae to isoproturon. Second in both scenarios, the growth of algae was inhibited during each exposure, resulting in cumulative losses in biomass production. In exposure scenario 2 (Figure 4.3 D), at 145 hours, the biomass was 7 times greater in the controls compared to that of sequentially exposed cultures. Such a loss in biomass production could influence the
competitiveness of species in surface water communities, e.g. periphyton [25]. Single pulse exposure (24 and 48h) to the triazine metribuzin was shown to induce a change in species composition and a loss of biomass by different algal groups [26].

For PSII herbicides, like isoproturon, that do not have delayed effects during the recovery phase and only a slight rise in tolerance, increase of biomass following sequential pulses may be predicted based upon the effects determined from single pulse exposure experiments [12]. Prediction of the total effect would be a function of the exposure concentration, the pulse duration, the recovery duration and the number of pulses. In the case of sequential exposure to a herbicide with a different mode of action, for example a chloroacetanilide, which inhibit the formation of long-chain fatty acids, the effect of a first pulse might influence the response to a second pulse. Cumulative effects over time might be induced, as a result of the delay in the recovery of the algae that was observed in single pulse exposure experiments [27].

4.5 Conclusion

Based upon the pulse and recovery intervals tested here, sequential pulse exposure to isoproturon could be considered as a sequence of independent events that impaired algal growth during exposure. The effects induced by isoproturon at the target site and on the growth of *S. vacuolatus* were highly reversible upon chemical removal, even after multiple pulses. Despite reversibility, the greatest effect was decreased net biomass production, which was proportional to the growth inhibition caused by each pulse. Furthermore, this study showed that sequential pulse exposure induced a slight increase in the tolerance of *S. vacuolatus* when the pulse exposures were on the order of one day and recovery durations were short. The effects measured in both scenarios showed that sequential pulses did effect *S. vacuolatus*, despite the fast recovery between exposures. Since each pulse exposure induced effects during exposure that lead to an accumulation of effects over time, short exceedances of chronic water quality criteria should be limited.

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4.6 References


Sequential isoproturon exposure


Chapter 4
Chapter 5

General discussion
General discussion

The aim of this thesis was to assess the effects of fluctuating exposures of herbicides on the algae *Scenedesmus vacuolatus*. These fluctuating exposures are typical of exposure in small waterways. They can be characterized by the concentration of the herbicide during exposure, the exposure duration, the number of exposures and the recovery periods between pulse exposures. The response of the algae to herbicides with different modes of action was investigated by varying these exposure characteristics, which can all influence the response of algae. Hence, the effects induced by the PSII inhibitors atrazine and isoproturon as well as the inhibitor of the formation of VLCFA S-metolachlor were all compared.

5.1 Effect of single pulse exposure to herbicides with different modes of action

An experimental method was developed to evaluate the effects of pulse exposure on *S. vacuolatus*, as well as the potential recovery following exposure. This method is adapted from the standard testing methods on algae [1, 2] and is quite easy to establish. The method could also be used to assess the effects of pulse exposure on different algae species with similar characteristics, such as an equivalent or greater growth rate. A requirement for the application of the method developed to any other algae strain is the verification that the growth of the chosen species is not affected by the method described for chemical removal, e.g. centrifugation.

During the exposure to the PSII inhibitors, the effects on the growth rate of algae were determined for exposure duration between 10 and 48 hours, yielding information on the time-dependency of effects. For both atrazine and isoproturon, the level of effects increased with increasing exposure concentration and duration, as was the case with most agrochemicals tested on macroinvertebrates and fish (Figure 5.1 [3-5]. Atrazine’s toxicity increased by a factor 3-5 between a 10 and a 48h exposure, while that of isoproturon increased to a smaller extent by a factor of 1.5.

The effect of a 10-hour isoproturon pulse was greater than that of atrazine, even if the toxicity of both herbicides was similar following 48 hours of exposure. This showed that atrazine and
isoproturon exerted dissimilar time-dependent toxicities in spite of their common primary site of action on photosystem II. This difference cannot be fully explained by measurements carried out in this study. On that account, further studies on the secondary effects and the defence mechanisms of this alga to both herbicides would be of interest.

At a subcellular level, the measurement inhibition of PSII effective quantum yield showed that both herbicides induced rapid effects at the target site after chemical addition while these effects decrease immediately after chemical removal. The rapid onset of effects and recovery corroborate observations of highly reversible binding displayed by both herbicides at their target sites [6].

The observation of effects on algae was continued during the recovery period since pulse exposure to some chemicals was shown to induce post-exposure effects on the tested organisms. Algae growth recovered following 10h and 24h pulses to both atrazine and isoproturon, as observed by Klaine following a 5-day exposure [7]. This recovery on growth may be attributed to the rapid recovery of effects observed at the target site, as already observed on corals [8]. The measurement of the photosynthetic yield was a valuable tool for the detection of effects at the target site and highlights the benefits of measuring effects both at the target site and on the population in order to refine the assessments of effects during and following exposure.

The effect assessment of pulse exposure was extended to the herbicide S-metolachlor, an inhibitor of lipid biosynthesis, to highlight similarities and dissimilarities in the response of the algae S. vacuolatus to herbicides with different modes of action. The EC50 for S-metolachlor on growth inhibition increased by a factor 7 between an 18 and a 48 hour exposure. This herbicide, which was less toxic to S. vacuolatus than the PSII inhibitors, induced a greater rise in toxicity with time (Figure 5.1). This suggests that the time-dependencies of effects may differ to a greater extent between herbicidal families like PSII inhibitors and the chloroacetanilides.
The time-dependence of the effects on the growth rate showed that S-metolachlor did inhibit the growth rate of algae, but only after 18h of exposure. Effects on the growth rate were not quantifiable for shorter exposure periods, even if the method allows their estimation at 10 hours. We deduce that there is an incipient time-to-effect, which was defined by Newman [9] as the minimum response time before the observed effect can be expressed. This concept was originally developed for lethality studies, but we assumed it to be applicable to other endpoints. The S-metolachlor time-to-effect on *S. vacuolatus* is estimated to 18 hours. In comparison, the time-to-effects of atrazine and isoproturon could not be precisely estimated with the current method, but is likely to be shorter than 10 hours.

The S-metolachlor time-to-effect is related to the exposure duration necessary to impair the growth of algae caused by the inhibition of the formation of VLFCA [10]. With synchronous algae, the algal development stage most sensitive to exposure to this herbicide was identified to be the last six hours of cell growth. Cells exposed during these six hours, or any exposure period including this stage, faced an inhibition of their reproduction. In continuous light, we observed an increase in the size of some cells, which led to an inhibition of the growth rate. The increase of the cell volume could be related to an accumulation of spores trapped within the algal mother cell wall, as observed by the increase in biomass of exposed cells and the increase of the total cell volume of the cultures. The formation of large cells was also
observed of the algae *Chlamydomonas reinhardtii* exposed to the chloroacetanilide alachlor [11]. For that reason, a similar time-to-effect might be expected for other chloroacetanilides herbicides that have the same primary mode of action [10]. The differences in the time-to-effects and the time-dependent toxicity of the three herbicides might be attributed to the physiological mode of action of the chemical. Based on this observation, the assessment of time-dependent effects for compounds with different modes should be carried out with at least one herbicide per herbicidal group. If a time-to-effect is observed, the assessment of the physiological mode of action could be refined by exposing synchronous grown algae.

We observed dissimilarities in the response of algae during the recovery period. In contrast to the reversible effects observed on the growth of algae during pulse exposure, we showed that the recovery following pulse exposure to S-metolachlor was delayed. The reproduction of synchronously cultured cells exposed for 24h exposure to S-metolachlor recovered 29 hours after chemical removal, while cells cultured in continuous light were able to release their spores within 24h following exposure. The difference in the capacity to recover between the herbicides studied may be related to the type of binding at the target site. While the binding of PSII inhibitors is reversible, S-metolachlor irreversibly binds to its target site, the VLCFA synthase [10]. We assume that the time-to-recovery will be similar for other chloroacetanilide herbicides as the recovery of *Chlamydomonas reinhardtii* following exposure to the chloroacetanilide alachlor was also delayed [11].

Our observations showed that a single pulse exposure does induce effects on growth during exposure, which are a function of the duration and the concentration of exposure. For chemicals like PSII inhibitors, these effects are limited to the time of exposure. For chloroacetanilides, the effects extend beyond the exposure period as the recovery of algae was delayed. For that reason, surpassing the CQC should be limited. The effect assessment of single pulse exposure was nevertheless a first step in the evaluation of effects during fluctuating exposures. To increase the environmental relevance, the effect assessment during sequential exposure is also of importance.
5.2 Effect of sequential exposure

The effects on both the growth of algae and at the target site of isoproturon were investigated with two different exposure scenarios: sequential short pulses and sequential long pulses. The effects of short isoproturon pulses were completely reversible following each pulse, which is in accordance with the observations made following a single pulse exposure. These experiments showed that sequential pulse exposure could be considered as a series of independent exposures. Nevertheless, sequential exposures to long pulses with short recovery periods, the characteristics of which resemble those of continuous exposure, induced a slight increase in the tolerance of the alga to this herbicide. However, the major effect of sequential exposure was the biomass reduction following each subsequent exposure, which resulted in a cumulative decrease overall. For PSII inhibitors, this loss in biomass production could be well predicted with the data obtained during observation of the effects during single pulse exposure.

Further experiments on the effects of sequential pulse exposure to S-metolachlor would be of interest to assess the effect of sequential exposure to herbicides that do not have a reversible binding at the target site. The delay in the recovery following S-metolachlor pulse exposure indicates that the response of a second exposure may be influenced by a first exposure, especially if the recovery period is shorter than 24 hours. In that case, the effects during subsequent pulses may be greater than those observed during a single pulse exposure of the same duration, as observed on macroinvertebrates during fluctuating exposure to the insecticides chlorpyrifos and carbaryl [12]. For a better estimation of the effects during S-metolachlor sequential exposure, we recommend including the measurements of effects at the sub-cellular level, in particular the content of certain fatty acids [13].
5.3 Conclusion and Outlook

The effect assessment of pulse exposure showed that peak exposures to the herbicides studied did impair the growth of algae, despite the short period of exposure. For that reason, short exceedances of the chronic water quality criteria (CQC), which are defined to protect the aquatic environment on a long term basis, are appreciable. The time-dependence of effects during exposure were shown to be compound specific, but differed to a greater extent for herbicides with different modes of action. The effects were either limited to the exposure period or induced a delayed effect following exposure, depending on the target site of the chemical and the reversibility of the binding at the target site. In the environment, algae are prone to be exposed sequentially. Multiple exposures to isoprotruron induce a cumulative reduction of biomass production over time. Based on these results, the level and the number of exceedances of the CQC should be limited. For chemicals that induce delayed effects, the number of exceedances should be more restrictive.

In order to estimate the risk of peak exposures on algae more successfully, the effects of pulse exposure should be measured on different algae strains. The methodology proposed for the effect assessment of single pulse exposure could be applied to other species. Exposing algae to repeated exposure might only be necessary in the case of delayed recovery following single pulse exposure. Furthermore, the assessment has so far focussed on the estimation of effects during pulse exposure to single chemicals. Aquatic organisms are nevertheless generally exposed not only to one, but several chemicals at a time. For that reason, the effects of pulse exposure should be estimated for other herbicides commonly detected in surface water, with the goal of predicting the effect of their mixture.

In surface waters, not only are PSII inhibitors and chloroacetanilides frequently detected. The assessment of pulse exposure to herbicide on algae should therefore be extended to herbicides with other modes of action susceptible of occurring at peak levels. This assessment would be of interest to confirm possible dissimilarities in the response of algae to herbicides with other primary modes of action.
5.4 References:


S-metolachlor pulse exposure on *Scenedesmus vacuolatus*: effects measured on the cell density, the cell volume and the biomass (dry weight) during exposure and the subsequent recovery
Appendix 1

6.1 Introduction

This appendix contains complementary results on the effects of S-metolachlor pulse exposure on the growth rate of *S. vacuolatus*. The determination of the growth rate of green algae are based either on biomass, defined as the dry weight per volume or cell density measurements [1;2]. A number of alternative techniques can be used to measure the cell density. Direct techniques are cell counts using a microscope and a cell counting chamber, or a particle counter. Indirect techniques comprise the measurement of the optical density or the fluorescence of a cell suspension. These measurements can be made if they correlate with the cell density in a defined measurement range.

In Chapter 3, we presented the effect of a 24h S-metolachlor pulse on the cell density measured by a particle counter during exposure and the subsequent recovery phase. Chloroacetanilide herbicides, like S-metolachlor, were shown to inhibit the cell division, a secondary effect of the inhibition of the formation of VLFCAs [3]. Another secondary effect observed was the loss of membrane stability in root cells that induced the swelling of those cells [4]. We therefore verified, if the increase in cell volume was due to increasing water content in the algae or, if the formation of large cells could be solely attributed to accumulation of autospores within the mother cell wall. Second, some authors showed that S-metolachlor also affect the photosynthesis [5], even if this herbicide does not primarily target the photosystems. We verified if an effect on the photosynthetic effective quantum yield on photosystem II could be detected at levels affecting growth during pulse exposure.

6.2 Material and Methods

*Chemicals*

S-metolachlor (S-metolachlor Pestanal®, 98.4%) stock solutions were prepared at 300 mg/l S-metolachlor in the algae culture medium (Chapter 3).
Effect of S-metolachlor on different endpoints

Alga

*Scenedesmus vacuolatus* (Chlorophyceae; strain 211-8b, Shihira and Krauss, Philadelphia, PA, USA) was cultured in batch cultures in a sterile inorganic medium prepared as described by Le Faucheur [6].

Pulse exposure

A) Effect on the growth rate and the photosynthetic efficiency

The effect of pulse exposure on the growth rate and the photosynthetic effective quantum yield were assessed according to the method described in Chapter 2, with the exception that the cell density and the cell volume were measured by particle counting using a coulter counter in the diameter range of 2.7 μm to 10 μm (Beckman Z2 Coulter® Particle Count and Size Analyzer, Germany). Algae were exposed to a 24h herbicidal pulse one day after the inoculation of the culture at concentrations ranging from 1.5 to 7.6 mg/l S-metolachlor. The experiments were run with 2 replicates per concentration and 2 controls. The growth rate was determined either based on an increase in cell density or on an increase in total cell volume per mL.

(B) Determination of the effects on the biomass (dry weight)

Prior the experiment, individual cellulose nitrate filters were placed in individual plastic petri dishes, dried at 60°C during 8 hours, and then weighed.

Since it was necessary to filter large volumes of cell suspension to reach an algal dry weight superior to 1 mg per filter, the volume of the algae cultures was increased to 150 mL. Batch cultures were grown in 150 ml of medium in sterile 300-ml Erlenmeyer flasks, inoculated with cells grown and maintained on an agar plate. The cultures were maintained in a shaker at 90 rpm at 25°C with continuous illumination at 105 μmol/m²/s by cool-white fluorescent lamps.

To determine the effects of S-metolachlor on the biomass production during the 24 hour exposure, S-metolachlor was diluted at concentrations ranging from 1-19mg/l in algal medium, and then algae were inoculated at a cell density of 1.3 *10⁶ cells/mL from cultures that had reached exponential growth. 50mL of algae cell suspension were filtered at the start of the exposure, whereas 30-50 mL was filtered 24h later depending on the degree of growth.
Appendix 1

inhibition. Filters were placed in an oven at 60°C during a minimum of 10 hours to dry, and weighed. In parallel to the filtration, the cell density of the algal cultures was measured by particle counting using a coulter counter in the diameter range of 2.7 μm to 10 μm (Beckman Z2 Coulter® Particle Count and Size Analyzer, Germany)

Data analysis

The growth rate was determined based on cell density or cell volume measurements. The natural logarithm of the measured parameter was plotted against time. The growth rate corresponds to the slope of the linear regression.

6.3 Results and discussion

6.3.1 Effect of S-metolachlor on the biomass production during a 24h exposure

The growth of algae based on cell density measurements was inhibited during a 24h exposure to S-metolachlor concentration ranging from 1-19mg/l. The cell density of control cultures increased by a factor 5 to in average $10^7$ cell/mL, while the density of cells exposed to S-metolachlor at most doubled (Figure 6.1). In opposition to the observed effect on cell density, the biomass of exposed cells augmented in average from 0.06 to 0.192 mg/mL, an increase just bellow that measured in the controls (0.21-0.28 mg/mL).

The increase in dry weight of all cultures during the 24h pulse (exposed and controls) showed that the increase in the volume of exposed cell is not due to an increase in water content of the cells, but to the increase in biomass production. This suggests that the enlargement of algae cells is solely due to the inhibition of cell division and not caused by any swelling of the cells that would be a consequence of an increase in permeability of the membranes [4].
6.3.2 Effects assessment of a S-metolachlor pulse on the cell density and the cell volume based growth rate

Figure 6.2 presents the response on the growth rate of *S. vacuolatus* based on the cell density or total cell volume measurements at different exposure concentrations. In the 2 experiments presented, the EC50 based on cell density measurements is 4.3 mg/L in the first experiment and 2.8 mg/L in the second (Figure 6.2A). Despite the significant difference in EC50 (p-value=0.016), the inhibition of the growth rate in both experiments clearly increased with increasing concentrations. During the recovery period subsequent to a 24h pulse, the growth rate was greater in exposed cultures than in controls (Figure 6.2 C), yielding negative inhibition of the growth rate. This might indicates either a compensation of growth during the 24h period following the pulse or a sudden release of spores trapped within the mother cell wall.

The same two experiments were also analyzed to evaluate the growth rate based on an increase in total cell volume. During pulse exposure (Figure 6.2 B), the inhibition of the growth rate was below 50% and did not increase with increasing exposure concentrations. This indicates that cells grew, but their division was inhibited. Furthermore, the volume based growth rate was constant in the recovery phase, suggesting that the increase in cell density was caused by a release of spores within the recovery period. The analysis of the cell size distribution (Figure 6.3) confirmed that the median diameter of cells shifted during recovery.
Appendix 1

The number of large cells present after exposure diminished, while the number of smaller cell augmented.

Figure 6.2: Growth rate based on the cell density \textit{S. vacuolatus} during exposure (A) and the subsequent 24h recovery phase (B) of replicate experiments (no. 1, □; no. 2, ○) and the growth rate based on the increase of total cell volume during a 24h pulse exposure (C) and the recovery (D). The controls are shown in full, while the exposed in empty symbols.

These findings are in accordance to results presented in the chapter 3 and to effects observed on the biomass. Cell density combined to cell volume measurements showed to be good substitutes to dry weigh measurements, which are difficult to carry out at a large scale [1]. S-metolachlor was shown to inhibit the cell division, but not the cell growth. Daughter cells were able to develop within the cell wall during exposure generating a greater number of large cells over time. During the 24h recovery period, the cells were able to release their daughter cells leading to a sudden increase in cell density.
6.3.3 Effect of S-metolachlor on the photosynthetic efficiency of PSII

S-metolachlor did not inhibit the photosynthetic effective quantum yield of *S. vacuolatus* even after 24h of exposure. The inhibition only reached 7.8% at 20 mg/L, a concentration strongly inhibiting growth. The inhibition of the PSII effective quantum yield is a sensitive measurement to detect the presence of herbicide targeting photosystem II [7], but not of herbicide like S-metolachlor that do not primarily target PSII [8].

6.4 Conclusion

These results confirm that the increase in cell volume during exposure to S-metolachlor is due to cell growth. The biomass increase is that of new spores formed within the mother cell wall. Their release is inhibited during exposure, but occurs within 24h following exposure. Finally, it was shown that the inhibition of the PSII effective quantum yield is an endpoint highly insensitive for the detection of effects induced by S-metolachlor, which does not primarily target the photosynthesis.


6.5 References


Effect of S-metolachlor on different endpoints
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Education

2004-2007 **PhD thesis** from the Swiss Federal Institute of Technology in Zürich (**ETHZ**) carried out at **Eawag**, the Swiss Federal Institutes of Aquatic Science and Technology in the department of Environmental toxicology.

**Subject:** Effect assessment of fluctuating exposure of herbicides with different modes of action on algae.

2004-2006 Continuing education in ecotoxicology.
- Attendance to all PEAK Ecotoxicology modules at Eawag and Cemagref in Lyon (evaluation, impacts, risk assessment)
- Short Courses organised by the Society of environmental toxicology and chemistry (SETAC); interspecies correlation and sediment toxicology

1997-2003 **Ingénieur en Génie rural**, Swiss Federal Institute of Technology in Lausanne (**EPFL**).
- Diploma thesis on the ecotoxicological risk assessment of contaminated sites in Ho Chi Minh city, Vietnam at EPFL and at the Centre for Environmental Technology (CEFINEA) of the National University of Ho Chi Minh City in Vietnam
- University exchange year at the ETHZ (1999-2000)

Work experience

2004-present Research assistant in the department of Environmental Toxicology at Eawag.
- Effect assessment carried out in the laboratory
- Presentations at international conference and to non specialist audience

2004-2006 Teaching assistant at Eawag, Ecole d'Ingénieur de Lullier and the University of Lausanne for microbiology and environmental toxicology courses.
- Lecturing, laboratory training, supervision of a master student and trainees

2003-2004 Research assistant at Eawag in the department «Water and agriculture ».
- Establishment of ecotoxicity tests to asses the effects of short herbicidal exposure, analysis of results and presentations

2003 Internship at B+C Ingénieurs.
- Hydraulic modelling of an alpine river, suggestion of flood mitigation measures

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- Development of a GIS database of coastal erosion hotspots in the state of Selangor using MapInfo

COWI Consulting Engineers and Planners AS, in the dept. of Environmental management in Aarhus, Denmark.
- Assisting in the preparation of a course on life cycle assessment and material flux analysis (software Simapro) for environmental managers in Asia

Internship at Ecoscan S.A in Lausanne.
- Data search and design of webpage on the environmental impacts of sports, i.e equipment, sport arenas, events

Trainee at the dept. of Environmental Microbiology at EAWAG.
- Laboratory training, characterisation of surfactant degrading microorganisms

Languages

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Teaching experience

2004-2006 Teaching assistant at Eawag, Ecole d’Ingénieur de Lullier and the University of Lausanne for microbiology and environmental toxicology courses.
- Lecturing, laboratory training, supervision of a master student and trainees.

2003 Teaching assistant at EPFL for the course Waste and Contaminated sites management.

Extra-curricula activities

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