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Faculty of Natural Resources and
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Effects of the carotenoid inhibiting herbicide diflufenican on the photosynthesis of benthic algae

Jelena Rakovic

Department of Aquatic Sciences and Assessment
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Jelena Rakovic

Supervisor:	Maria Kahlert, SLU, Department of Aquatic Sciences and Assessment
Assistant Supervisor:	Alexander Feckler, SLU, Department of Aquatic Sciences and Assessment
Examiner:	Sarah Josefsson, SLU, Department of Aquatic Sciences and Assessment

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Abstract

Throughout the world, the large-scale use of pesticides in agriculture is an area of concern due to known harmful effects on human and environmental health. Mixtures of different pesticides are found in lakes and streams, where their presence endangers aquatic organisms, like benthic algae, and the ecosystem services they provide. Benthic algae are important primary producers and chemical modulators. Since they are photoautotrophs, they are especially threatened by photosynthesis inhibiting herbicides. Studies have shown that exposure to herbicides causes toxic effects on algae, although most chemical risk assessments are performed on planktonic algae. The aim of this study was to evaluate the effects of the herbicide diflufenican (2',4'-difluoro-2-(α,α,α-trifluoro-m-tolyloxy)nicotinanilide) on the photosynthetic efficiency of benthic algal communities. The choice of herbicide is based on the high prevalence of diflufenican above its water quality standard in Swedish surface waters as well as its known toxicity to aquatic organisms. Diflufenican inhibits the synthesis of carotenoids in weeds, which causes photo-bleaching of green tissues due to oxidative degradation of chlorophylls, the main light-harvesting pigments. The hypothesis of this study was that diflufenican would diminish the photosynthetic efficiency of the benthic algal community. The study was conducted using benthic algae grown for six weeks on tiles submerged in Lake Erken (59˚50'N, 18˚35'E). The algae were then exposed to diflufenican for twelve days. To evaluate the effects on photosynthesis throughout the experiment, chlorophyll fluorescence was measured with a Pulse Amplitude Modulated Fluorometer (PAM-FMS 1, Hansatech®). In contrast to my expectations, the photosynthetic efficiency of the algae exposed to the highest test concentration (10 µg/L) increased to a level above the control. Interestingly, the biomass did not seem to differ between the treatments and the control. These results indicate that diflufenican might have induced synthesis of more chlorophylls as a way to increase energy attainment to be able to cope with the toxic stress; a mechanism called the greening effect. However, diflufenican does not affect the existing pool of carotenoids, thus it only affects the growing parts of weeds. Since the algal biomass did not increase over the course of this study, the existing carotenoids might have been sufficient, at least for some of the algae. This could explain the lack of difference in photosynthetic efficiency between the lower treatments and the control. Additionally, it has been shown that diflufenican also inhibits synthesis of fatty acids in plants. This mechanism might have contributed to the induced greening effect despite the lack of growth. Further analysis will be performed to evaluate the effects on species composition and the pigment content, which will bring more insights regarding the effects of diflufenican on the community of benthic algae.

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Abbreviations

EC_{50} (growth)	The median test concentration that causes a 50 % reduction of growth (Norberg, 2004).
WQS	Water Quality Standard. Indicates the maximum concentration of a substance at which no negative effects on the environment can be expected (Swedish Chemical Agency, 2012).
Photoautotroph	Organisms that obtain energy from sun light and can fixate carbon from CO_2

1. Introduction

The large-scale use of pesticides in conventional agriculture is an area of concern due to known negative effects on human and environmental health (Horrigan *et al.*, 2002; Relyea & Hoverman, 2006; Corsini *et al.*, 2013). Pesticides are used to manage the pressures that monoculture farming systems are faced with, such as competitive weeds, animal pests and pathogens (Oerke & Dehne, 2004). One of the issues regarding pesticides is that the substances do not only affect target organisms (DeLorenzo *et al.*, 2001). After application to cultivated fields, pesticides can be transported via run-off, drainage and spray-drift eventually reaching freshwater ecosystems (Kreuger, 1998) exposing aquatic organisms to a mixture of these biologically active chemicals (DeLorenzo *et al.*, 2001). There are a vast variety of pesticides with different mechanisms of action and target organisms (Oerke & Dehne, 2004). Herbicides are used to combat weeds and many act by inhibiting photochemical reactions, thereby diminishing energy acquirement (Hess, 2000; Fischer *et al.*, 2010). The components of photosynthesis are the same in plants, algae and cyanobacteria (Tymoczko *et al.*, 2012), hence the presence of herbicides in surface waters poses a threat to aquatic photoautotrophic organisms (DeLorenzo *et al.*, 2001) and the ecosystem services they provide.

Algae are the main primary producers in aquatic ecosystems and are responsible for half of the oxygen production in the world (Chapman, 2013). If herbicide exposure alters the structure and functions of the algal community it can affect species of higher trophy levels and continually the aquatic ecosystem as a whole (Rohr & Crumrine, 2005; Rybicki *et al.*, 2012). In standard environmental risk assessment of the effects of chemicals on aquatic ecosystems, single-species test are performed on organisms from at least three different trophy levels. The model algal test organisms are planktonic green algae (Linders, 2010; PPDB, 2012); less is known of the herbicidal effects on algae with other lifestyles and different physiology, like benthic algae. Studies have shown that the sensitivity of aquatic microorganisms towards pesticides varies to a great extent (DeLorenzo *et al.*, 2001). Thus, to gain further knowledge of the effects of herbicides on aquatic photoautotrophs it is of relevance to evaluate the effects on benthic algae.

Benthic algae are important primary producers in lakes, streams and wetlands (Stevenson *et al.*, 1996, pp 10–11). The community composition of benthic algae varies with depth due to light quantity and quality as well as wave disturbance. However, the dominant classes are diatoms (Bacillariophyceae), green algae (Chlorophyceae) and blue-green algae (Cyanophyceae) (Stevenson *et al.*, 1996, pp 59–60). Benthic algae provide nutrients and oxygen as well as habitat for other aquatic organisms. Moreover, they take part in the biogeochemical cycles by transforming inorganic compounds to organic forms (Stevenson *et al.*, 1996, pp 10–11). Benthic algae form a community together with fungi and bacteria, which is called periphyton (Stevenson *et al.*, 1996, pp 59–60) or biofilm. The microorganisms live in close proximity in a matrix of extracellular polymeric substances (EPS) fixed on a solid surface (Sabater *et al.*, 2007; Tlili *et al.*, 2011). The EPS can adsorb heavy metals and organic pollutants as well as metabolize some synthetic compounds (DeLorenzo *et al.*, 2001; Lawrence *et al.*, 2001; Kang & Zhu, 2013). Furthermore, benthic algae are used as biomonitors in streams and lakes because they integrate the water chemistry over a long period of time and thus reflect nutrient concentrations, pH and pollution of a water course (Stevenson & Pan, 2010).

The aim of the present study was to evaluate the effects of the herbicide diflufenican (2',4'-difluoro-2-(α,α,α-trifluoro-m-tolyloxy)nicotinanilide) on the photosynthetic efficiency of natural communities of benthic algae. Diflufenican belongs to the group of herbicides that inhibit the synthesis of carotenoids, pigments with vital functions associated with photosynthesis (Boger & Sandmann, 1998; Dayan & Zaccaro, 2012). The choice of herbicide is based on the high prevalence of diflufenican above its WQS in surface waters of agriculturally intense regions in Sweden (Lindström *et al.*, 2013) and its known toxicity to aquatic organisms in in the low ug/L range (PPDB, 2012; Weyman *et al.*, 2012). In 2012 the concentration of diflufenican exceeded the WQS value on 59 test occasions out of 120 (Lindström *et al.*, 2013). Furthermore, the bioconcentration of diflufenican from water to aquatic organisms is at the threshold of concern according to European authorities (PPDB, 2012), however, biomagnification along the aquatic food chain is not expected to occur (Lazartigues *et al.*, 2013).

The inhibition of carotenoids is related to photosynthesis because carotenoids take part in non-photochemical quenching. In most photoautotrophs, chlorophylls are the main light-harvesting pigments. When chlorophyll molecules are struck by photons the energy is absorbed by excitation of electrons. The excitation energy has three possible pathways; it can be used in photochemical reactions or be dissipated as heat or chlorophyll fluorescence (Stevenson *et al.*, 1996, pp 122–123; Tymoczko *et al.*, 2012). The latter two are called non-photochemical quenching (Maxwell & Johnson, 2000; Frankart *et al.*, 2003). In situations of high light intensities, the harvested light energy can be higher than the capacity of the photosynthesis machinery. This generates excess excited chlorophylls, some of which turn into triplet chlorophyll, which can transfer the excitation energy to oxygen producing reactive oxygen species. Carotenoids can quench and re-emit the excess energy from triplet chlorophyll and reactive oxygen species, thereby preventing photo-oxidative damage of the photosynthetic apparatus (Haynes & Kirkwood, 1992; Salguero *et al.*, 2003; Fischer *et al.*, 2010). The inhibition of carotenoid synthesis in weeds causes photo-bleaching of green tissues due to oxidative degradation of chlorophylls (Boger & Sandmann, 1998; Dayan & Zaccaro, 2012). Consequently, the hypothesis of the present study was that diflufenican would diminish the photosynthetic efficiency of the benthic algal community due to degradation of the photosynthesis apparatus.

2. Materials and method

2.1 Chemicals

The herbicide diflufenican was applied in the form of Diflanil 500 SC (Schneiter AGRO AG; Seon, Switzerland), containing 500 g/L diflufenican. The final concentrations of 0.0, 0.04, 0.2, 1.0, 5.0 and 10.0 µg diflufenican/L were achieved by serial dilution of the formulation in water from Lake Erken, which was used as test medium during this study.

The test concentrations were chosen based on results from Weyman et al. (2012): The EC_{50} (growth) for the two green algae; *Scenedesmus subspicatus* and *Pseudokirchneriella subcapitata* are 0.25 µg/L and 0.27 µg/L respectively. While the diatom *Navicula pelliculosa* has an EC_{50}-value of 3.5 µg/L (Weyman *et al.*, 2012). However, these ecotoxicological studies were performed on cultures of single species of algae and not whole benthic communities. Bearing this in mind, a wide range of test concentrations was chosen.

2.2 Preparation of the benthic community

The benthic community was grown on tiles (16 cm^2) in Lake Erken (59°50'N, 18°35'E), a dimictic meso-eutrophic lake in Sweden (Kahlert *et al.*, 2002). The tiles were submerged in the lake at depths between 0.5 and 1.0 m in early March 2014. Six weeks later, tiles were retrieved and transported to the laboratory in petri dishes to avoid desiccation. Back in the laboratory, four aquaria were set up for each test concentration containing four litres of test medium and the respective concentration of diflufenican (0.0, 0.04, 0.2, 1.0, 5.0 and 10.0 µg/L). Per aquarium, 13 tiles were submerged at the beginning of the experiment. Subsequently, the aquaria were set up in a temperature-controlled chamber set to 20 ± 1 °C, aerated individually and covered with plastic film to minimize evaporation. The biofilms were illuminated (Osram FLUORA T8, 36 W/77) with an intensity of 75 ± 11 µmol/m^2s in a light:dark regime of 16:8 h. To ensure a constant level of exposure to diflufenican, a semi-static test design was applied: every third day (day 3, 6, and 9) the tiles were careful transferred into fresh test medium with the respective herbicide concentration.

In Lake Erken the benthic algae receive approximately 150 µmol/m^2s at a depth of 1.0 m (Kahlert *et al.*, 2002). In March 2014 the following parameters were obtained from the Erken Laboratory: pH was 7.72 (7.39 - 7.99); filtrated Abs (420 nm, 5cm) was 0.054 (0.027 - 0.058); P-PO_4 was 27.98 µg/L (5.33 - 36.03 µg/L); total-P was 41.8 µg/L (13.6 - 46.1 µg/L); total-N was 785.12 µg/L (721.2 - 1218.13 µg/L) (medians, minimum and maximum values). According to the Environmental Quality Criteria of the Swedish Environmental Protection Agency, Lake Erken has a good nutrient status. The filtrated absorbance is a good estimate of DOC and since the filtrated absorbance of Erken water is right in between the classifications "slightly coloured water" (0.02-0.05) and "moderately coloured water", the water is relatively clear (Swedish EPA, 1999).

2.3 Measuring Chlorophyll Fluorescence

Measuring of chlorophyll fluorescence has a wide range of applications, such as in studies of the effects of herbicides on algal species as reviewed by Juneau et al. (2007). The underlying principle of this method is that a quantum of harvested light energy can be used in one of the following processes; 1) photochemistry, 2) chlorophyll fluorescence or 3) heat dissipation. When the efficiency of the photochemical reactions is reduced, more energy is dissipated via non-photochemical quenching (Schreiber *et al.*, 1995; Maxwell & Johnson, 2000; Dayan & Zaccaro, 2012).

Chlorophyll fluorescence was measured with a Pulse Amplitude Modulated (PAM) Fluorometer (PAM-FMS 1, Hansatech®). This method is based on the theory that application of a short saturating pulse of light closes all reaction centres, thereby stopping photochemistry without causing any change in the ratio between heat dissipation and chlorophyll fluorescence. Most of the chlorophyll fluorescence comes from photosystem II (PSII) (Hansatech Instruments Ltd.), since the photochemical reactions are initiated there (Tymoczko *et al.*, 2012). When photochemical quenching is eliminated, the maximum fluorescence can be measured following dark-adaption (F_m) and light-adaption (F_m'). Prior to the saturating pulse, a reference point is established by measuring minimum level of fluorescence (F_o) or steady-state fluorescence (F_s) in dark- and light-adapted samples, respectively (Schreiber *et al.*, 1995). F_o is a good estimate of the algal biomass in the sample (Honeywill *et al.*, 2002). The maximum quantum yield of PSII photochemistry following dark-adaption (F_v/F_m) can then be calculated with the following equation:

$$F_v/F_m = (F_o - F_m)/F_m$$ (Schreiber *et al.*, 1995).

The effective quantum yield of PSII photochemistry following light-adaption (ϕPSII) can be calculated with the following equation:

$$\Phi PSII = (F_s - F'_m)/F'_m$$ (Schreiber *et al.*, 1995).

The software Modfluor was used together with PAM-FMS 1. Modfluor contains pre-programmed scripts for attaining F_v/ F_m and ϕPSII. The modulation beam was set to 4, whereas the gain and pulse were set to 50 and 60, respectively, ensuring a stable physiology of the samples, light saturation during measurements and maximum fluorescence yields being in the detection range (Hansatech Instruments Ltd.).

On days 4, 7, 10 and 12, one tile per aquaria (*n* = 4 per test concentration) was retrieved for analysis. The biofilm of each tile was scraped into a separate petri dish with a silicon headed scraper and some tap water was added. The algae were slightly separated and homogenized with the same scraper, then transferred into small beakers in which tap water was added up to 25 mL. From each of the four resulting beakers, 150 µL of the algae suspension was transferred to a 96 well black microwell plate and filled up to 300 µL with tap water. The algae suspensions were dark adapted for at least 10 min, prior to initiating the F_v/ F_m-script measuring F_0 and F_m and calculating F_v/ F_m. Afterwards, samples were light adapted under the conditions described above for at least 10 minutes. Subsequently, F_s and F_m was measured and ϕPSII calculated.

2.4 Statistics

Initially, relative maximum and effective PSII quantum yields of each treatment, compared to the diflufenican-free control at the respective measurement day (set to 100%), were calculated. Unpaired, two-sided 95% confidence intervals (CIs) were used for significance testing (Altman *et al.*, 2000). The basics behind this statistical test are; if the 95% CIs of the differences between the mean of the treatments and the corresponding control do not include zero, the result indicates that there is a statistically significant difference between the treatment and the control. For all comparisons at each measurement day, CIs were adjusted for multiple comparisons applying the Bonferroni adjustment to control the family wise error rate. This means that since the control treatment was used five times for comparison, once for each treatment, the alpha value (0.05) was divided by five. The relative minimum level of fluorescence (F_0) was also calculated following a significance test as above. For all statistical analyses and figures, R version 3.0.2 for Mac (R Development Core Team, 2013) was used.

3. Results

The results from the dark-adapted samples are indicating a rising trend in the relative F_v/F_m of the algae exposed to the highest test concentration (10 µg/L) with statistically significant higher values compared to the corresponding control after 12 days of exposure (difference of means: 20.0%; 95% CI 9.8 to 30.2; n = 4; Fig. 1e). The results from the algae exposed to the lowest test concentration (0.04 µg/L) also show a significantly higher F_v/F_m on day 12 compared to the corresponding control (difference of means: 17.2%; 95% CI 4.6 to 29.8; n = 4; Fig. 1a), although in this case there is no general pattern of increase throughout the period of exposure. Furthermore, the mean relative change in F_v/F_m from the second highest treatment (5 µg/L; Fig. 1d) is indicating a non-significant increase as it is higher than the control on most days. In contrast, in the lower treatments the mean relative F_v/F_m fluctuates around the level of the control (Fig. 1b & c).

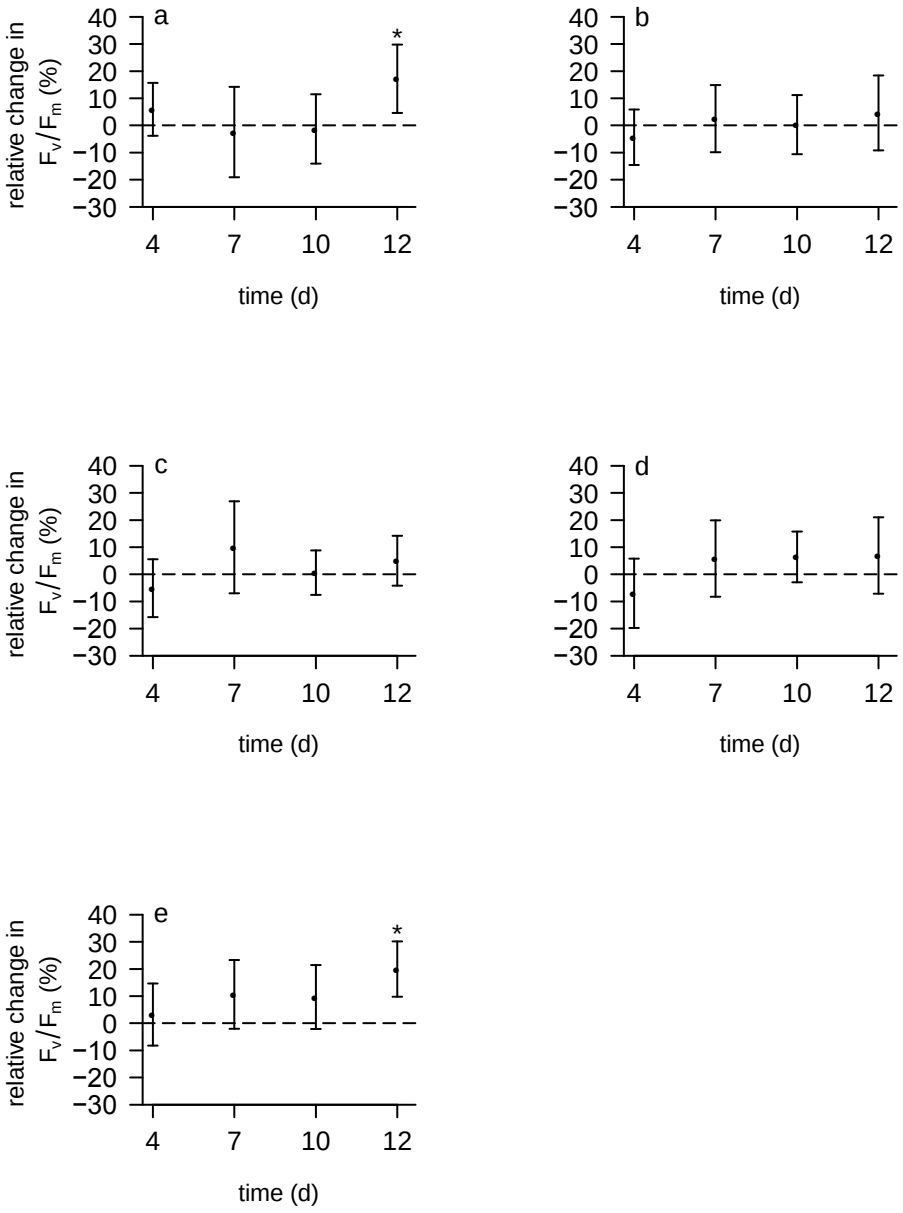

Figure 1. Mean (± 95% CI; n = 3-4) relative changes in the maximum PSII quantum yield (F_v/F_m) compared to the control at the respective measurement day. Each diagram shows the results from one test concentration, i.e. a) 0.04; b) 0.2; c) 1; d) 5 and e) 10 µg diflufenican/L. Asterisks denote a statistically significant difference between the respective treatment and control.

The results from the light-adapted samples (Fig. 2) show that the there is no significant difference between the treatments and control for all accept the highest treatment, where ϕPSII is statistically significantly higher at days 7 (difference of means: 12.5%; 95% CI 4.8 to 20.2; *n* = 4; Fig. 2e) and 12 (difference of means: 17.5%; 95% CI 3.5 to 31.6; *n* = 4; Fig. 2e).

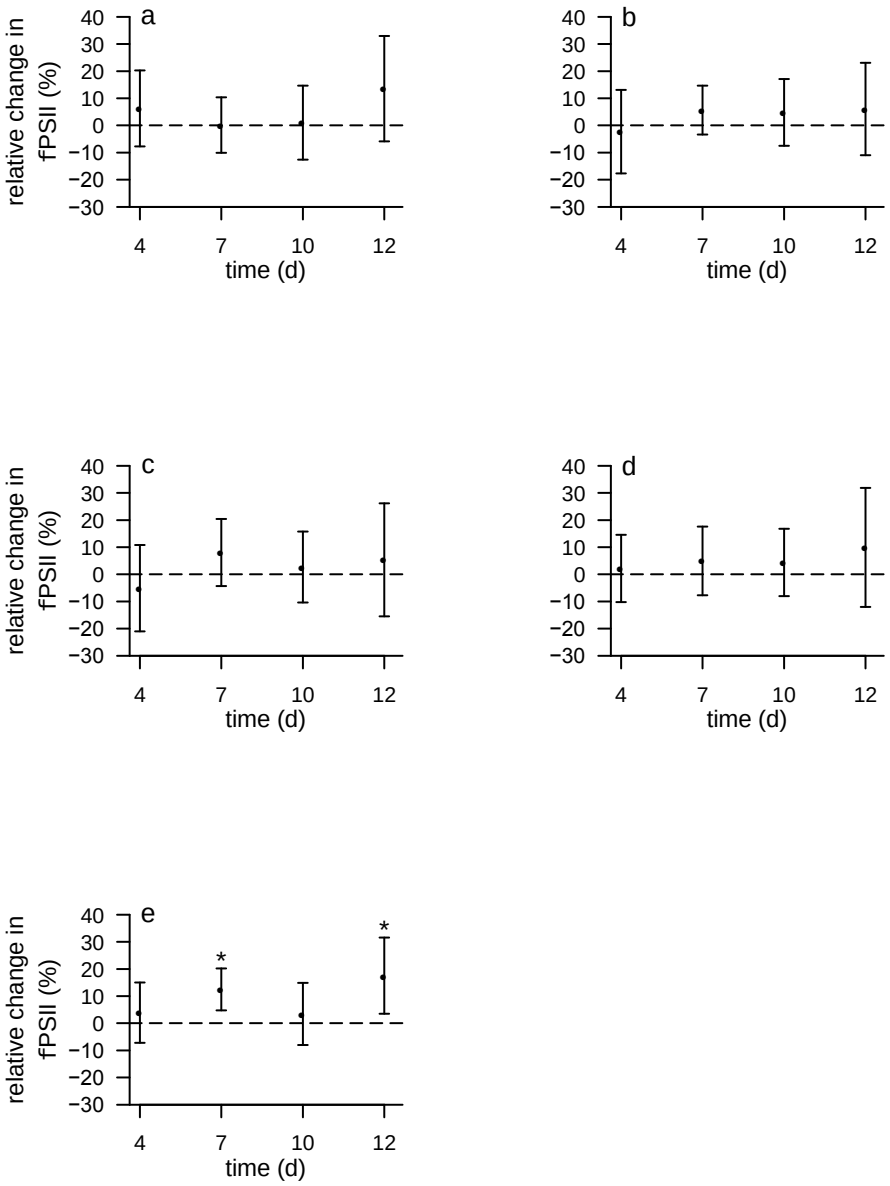

Figure 2. Mean (± 95% CI; n = 3-4) relative changes in the effective PSII quantum yield (ϕPSII) compared to the control at the respective measurement day. Each diagram shows results from one test concentration, i.e. a) 0.04; b) 0.2; c) 1; d) 5 and e) 10 µg diflufenican/L. Asterisks denote a significant difference between the respective treatment and control.

The minimum level of fluorescence did not statistically significantly differ between the treatments and the control on the respective measurement days (Fig. 3). In fact, absolute F_0 was decreasing in all treatments in the same order of magnitude (Fig. 4).

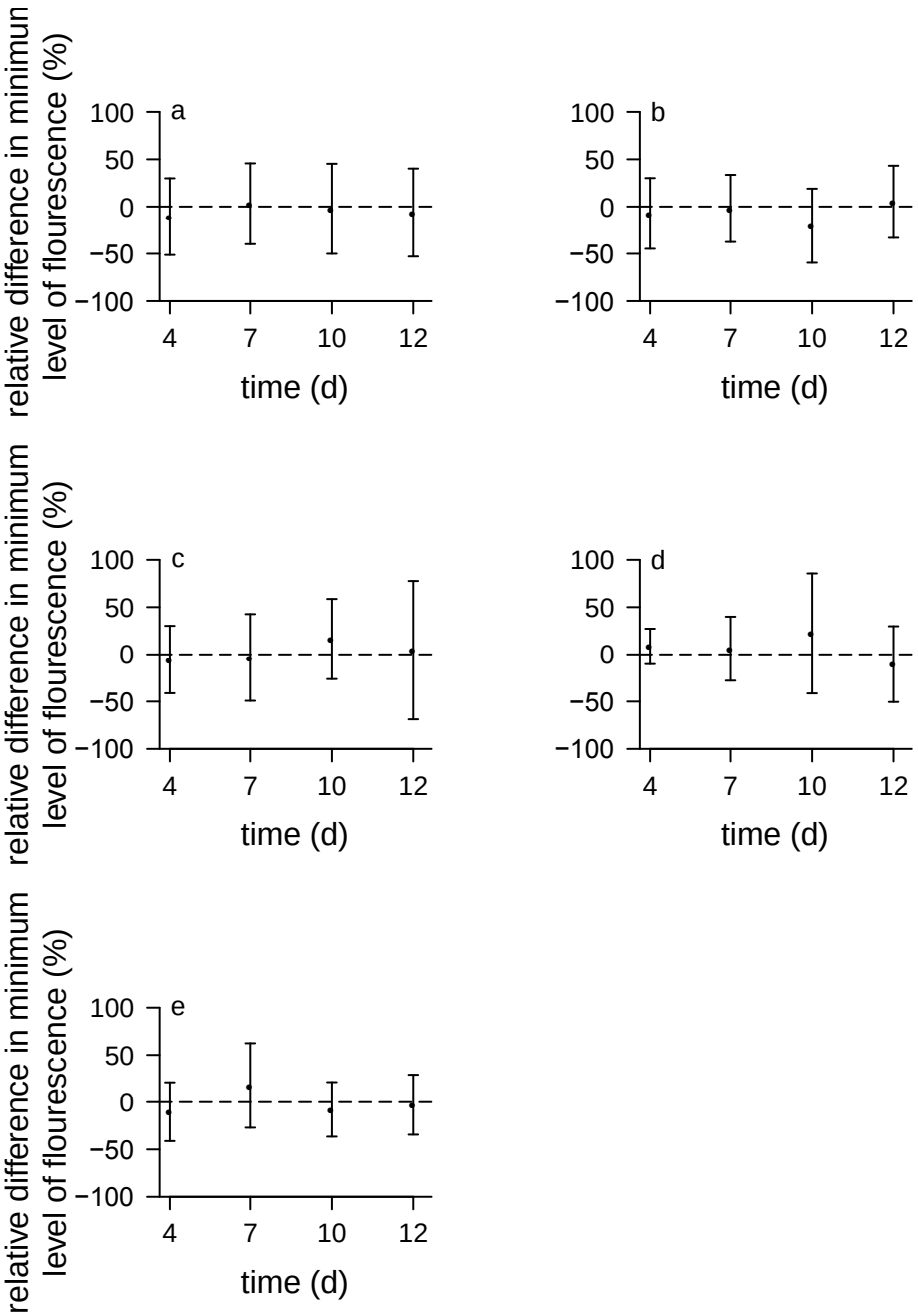

Figure 3. Mean (± 95% CI; n = 3-4) relative differences of the minimum level of fluorescence compared to the control at the respective measurement day. Each diagram shows results from one test concentration, i.e. a) 0.04; b) 0.2; c) 1; d) 5 and e) 10 µg diflufenican/L.

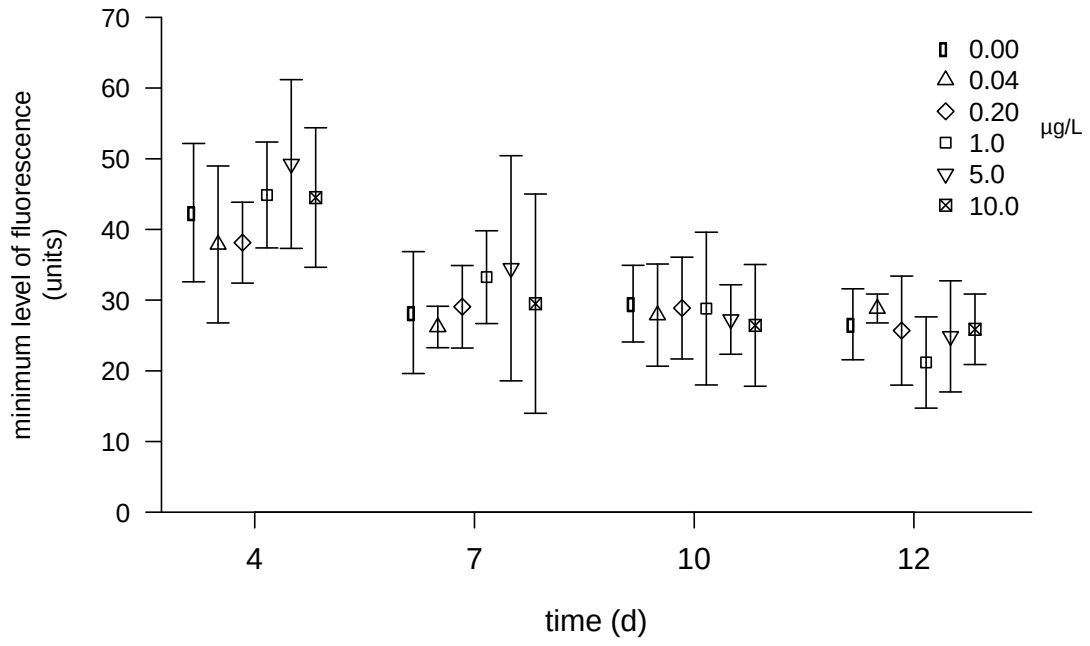

Figure 4. Mean (± 95% CI; n = 3-4) minimum level of fluorescence units (F_0) from the different treatments throughout the period of exposure.

4. Discussion

The expected outcome of the present study was that diflufenican would decrease both the maximum (F_v/F_m) and effective (ϕPSII) PSII quantum yields. Particularly, it was expected that the F_v/F_m and ϕPSII of the algae from the highest test concentrations (5 and 10 µg/L) would be lower compared to the control on the respective measurement day. However, the results were not in accordance with the hypothesis. The increase in PSII quantum yields of the algae exposed to the highest test concentrations indicates that they developed a higher capacity to convert light energy into chemical energy through photochemical reactions. The increase in F_v/F_m of the algae exposed to the lowest treatment on day 12 is not accompanied by a significant increase in ϕPSII, furthermore there is no general pattern of rising PSII quantum yields, therefore this might be a measurement error or a result of different test conditions. An important aspect to consider is the fact that the endpoint used in this study (PSII quantum yield) cannot alone explain the effects of diflufenican on the photosynthesis of benthic algae.

In the following subchapters I will discuss a possible explanation to the observed results. I will also discuss test conditions that might have influenced the results as well as provide some implications for further studies.

4.1 The Greening Effect

In many shaded benthic habitats light levels are extremely low. Benthic algae have evolved the ability to adapt to these conditions by synthesizing more light-harvesting pigments to maximize energy absorption (Stevenson *et al.*, 1996, p 130). The greening effect suggest that when algae are exposed to environmental stress they can utilize the mechanism of shade adaption to increase their light-harvesting capacity (cf. Hatfield *et al.*, 1989; Tlili *et al.*, 2011). In the present study, diflufenican might have induced an upsurge of chlorophyll synthesis, resulting in a higher photosynthetic efficiency and more energy available to cope with the toxic stress. In the study of Tlili et al. (2011), similar conclusions were made. They studied the chronic effects on biofilms exposed to a mixture of diuron (PSII inhibitor) and tebuconazole (triazole fungicide) for 63 days. The biofilms were pre-cultured for 28 days in contaminated or non-contaminated artificial channels before the analysis was initiated. Throughout the experiment, the levels of chlorophyll *a* in the algae from the contaminated channels were generally higher compared to those from the non-contaminated sites, while the biomass followed the reverse trend (Tlili *et al.*, 2011). Higher levels of chlorophyll *a* indicate a higher photosynthetic efficiency, which is in consistence with the results of this study (Fig. 1d & e, Fig. 2e). Tlili et al. (2011) concluded that exposure to diuron induced the greening effect. However, the energy gained from the additional light-harvesting pigments did not increase the biomass, thus it might have been used to deal with with the damage caused by the toxicant.

In contrast to the additional findings of Tlili et al. (2011), the relative biomass of the benthic algae exposed to diflufenican (estimated using F_0) did not differ substantially compared to the control (Fig. 3a-e, Fig. 4). This can be attributed to unfavourable test conditions which will be discussed below. Nevertheless, the results of this study – a higher photosynthetic efficiency at a similar biomass – still suggest that the greening effect occurred. However, this might not be a long-term solution to toxic stress. In fact Tlili et al. (2011) showed that the carbon incorporation of the chronically exposed algae was higher compared to the non-exposed reference in the beginning of the analysis (between days 28 and 35), after which the trend reversed. They concluded that the protective mechanism of an increase in light-harvesting pigments could not maintain functional stability after a long period of exposure (Tlili *et al.*, 2011). This suggests that the greening effect reached a maximum capacity after which the stress became too high for the algae to cope with.

4.2 The lack of growth and the other mechanism of action

An important aspect to consider is that diflufenican only induces photo-bleaching of the growing parts of weeds because it does not diminish the existing pool of carotenoids (Knight & Kirkwood, 1991). In the present study, F_0 indicates that the biomass did not increase throughout the experiment (Fig. 4), thus the algae might not have been kept in the growth phase. Considering this, the existing pool of carotenoids in the benthic algae might have been sufficient to quench the excess energy and protect the photosynthetic apparatus. This does however not explain the increased photosynthetic efficiency of the algae exposed to the

highest test concentration. Unless some of the benthic algae did grow or still needed more carotenoids. In that case the lack of growth might have merely reduced the toxic effects.

There is an addition possible explanation to the induced toxic response, in spite of a non-growing biomass. Diflufenican has an additional, less known, mechanism of action that is not related to carotenoid synthesis. According to the study of Ashton et al. (1994), diflufenican inhibits the synthesis of fatty acids in plants by inhibition of enoyl-ACP reductase enzymes. They also showed that diflufenican inhibits Type II fatty acid synthase in *Escherichia coli*, explaining that it shares many features with the plant fatty acid synthase. In view of this, diflufenican might have inhibited both the synthesis of carotenoids and fatty acids in the benthic algae of the present study. Both of these mechanisms might have induced the greening effect as a way to increase energy attainment needed to manage the toxic stress.

The lack of growth might have been a result of light limitation, since the light level in the experiment (75 ± 11 µmol/m^2s) was approximately half of that found in the field (approximately 150 µmol/m^2s). Furthermore, the communities might have already reached maturity, as evident by the thickness of the biofilms at the start of the experiment.

4.3 Possibility of Adsorption, Reduction and Metabolism

Considering that diflufenican is toxic to aquatic photoautotrophs in the low ug/L range (Weyman *et al.*, 2012) I expected toxic effects from exposure to the lower concentrations of diflufenican as well as the highest. The toxic effects might have been reduced due to certain test conditions. Indeed, several studies of the effects of herbicides on non-target species show that the test conditions can significantly affect the results gained from PAM fluorometry (Bengtson Nash & Quayle, 2007; Schreiber *et al.*, 2007; Sjollema *et al.*, 2014). A particular condition that might have affected the sensitivity of the benthic algae towards diflufenican in this study was the thickness of the biofilms at the start of the experiment (2 – 5 mm).

The matrix of EPS constitutes a major fraction of the biofilm and is a complex mixture of compounds, mainly polysaccharides and proteins although including humic substances, lipids and nucleic acids. EPS provide a large active area with both polar and hydrophobic elements, on which ions as well as organic molecules can adsorb. The EPS provides protection and facilitates accumulation of nutrients for the microorganisms (Lawrence *et al.*, 2001; Kang & Zhu, 2013). Kang & Zhu (2013) have shown that EPS secreted by different microorganisms can degrade a model nitro-aromatic compound (NAC). They propose that EPS might play an important role in the natural detoxification of organic contaminants. Furthermore, Lawrence et al. (2001) have shown that river biofilm communities can adsorb and metabolize the herbicides atrazine and diclofop. Besides this, herbicides may be adsorbed and subsequently desorbed by biofilms, as shown by Tlili et al. (2011) in the case of diuron (PSII inhibitor). In view of these studies, it is possible that a fraction of the diflufenican molecules were adsorbed by the EPS, some of which might have been degraded. This would diminish the diflufenican concentration in the water between the days of water exchange, which would explain the lack of significant toxic effects in the lower treatments.

4.4 Implications for further studies

I have some suggestions for further studies of the effects of herbicides on communities of benthic algae. Firstly, the algae should be kept in the growth phase to increase the perceptibility of effects on biomass. This can be achieved by preventing resource limitations. In the present study, the algae were most likely not limited by nutrients, since Lake Erken has a good nutrient status and the water was exchanged every third day. As mentioned above, the benthic algae might have been light limited although they can adapt to these conditions and still grow (Stevenson *et al.*, 1996, pp 129–131). I would however increase the light intensity if the herbicide used is a carotenoid synthesis inhibitor, since a vital function of carotenoids is protection from excess light energy.

Another important resource for benthic microorganisms is space (Stevenson *et al.*, 1996, p 64). At the start of the experiment most of the tiles were fully covered by thick biofilms since the benthic community had grown in nutrient and light-rich conditions for six weeks. Therefore, lack of growth might be a result of space-limitation. To reduce space-limitation one could retrieve pre-cultured biofilms when there is only a thin layer of growth. By making sure that the biofilms are thin, the amount of EPS and possible adsorption sites are also reduced. Thereby the possible accumulation of the herbicide in the matrix is reduced and the problem of decreasing water concentrations is diminished. It would be valuable to study if and to what extent the herbicide is adsorbed and if redox reactions occur or if there is any metabolism of the herbicide.

Conclusions

In this study the herbicide diflufenican increased the photosynthetic efficiency of benthic algae exposed to 10 µg/L during 12 days. These results can be explained by the ability of algae to adapt to environmental stress by increasing their capacity to acquire energy, which means synthesising more chlorophyll pigments. The thickness of the biofilms and the lack of growth might have reduced the toxic response, which could explain why there were no significant differences in photosynthetic efficiency between the lower treatments and the control. Therefore, I suggest using thin biofilms and higher light intensities in further studies of the effects of herbicides on benthic algal communities.

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