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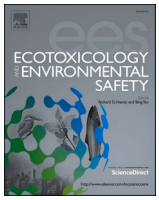

Effects of the herbicides metazachlor and flufenacet on phytoplankton communities – A microcosm assay

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ABSTRACT

Agrochemicals are the main pollutants in freshwater ecosystems. Metazachlor and flufenacet are two common herbicides applied in fall (i.e., August–October) to agricultural fields in Northern Germany. High concentrations of these herbicides are often found in adjacent aquatic ecosystems. Phytoplankton are one of the highly susceptible non-targeted aquatic organismal groups for herbicides and effects on phytoplankton may initiate a chain of consequences in meta communities through trophic interactions. Few studies have focused on responses of the phytoplankton community for metazachlor and, no studies have focused on flufenacet. We studied the effects of metazachlor and flufenacet on the phytoplankton community by conducting a microcosm experiment exposing natural fall phytoplankton communities to environmentally realistic concentrations as 0 (control), 0.5, 5 and 50 μg L^{-1} of metazachlor and flufenacet treatments over a 4-week period. We measured changes in density, composition (i.e., in phyla and species level), taxonomic diversity indices, and functional features of phytoplankton communities as a response to herbicides. A reduction in the density of Chlorophyta species (e.g., *Koliella longiseta*, *Selenastrum bibraianum*) and Cyanobacteria species (e.g., *Merismopedia tenuissima* and *Aphanocapsa elegans*) was observed in herbicide treatments compared to controls. The phytoplankton community shifted towards a high density of species from Bacillariophyta (e.g., *Nitzschia fonticola* and *Cyclotella meneghiniana*), Miozoa (i.e., *Peridinium willei*), and Euglenozoa (i.e., *Trachelomonas volvocina*) in herbicide treatments compared to controls. Metazachlor and flufenacet showed significant negative effects on taxonomic diversity indices (e.g., species richness, the Shannon-Wiener index) and functional features (e.g., functional dispersion and redundancy) of the phytoplankton communities, with increasing herbicide concentrations. Our study provides insights into direct, selective, and irrecoverable effects of metazachlor and flufenacet on phytoplankton communities in the short-term. The comprehensive understanding of these effects of environmentally realistic herbicide concentrations on aquatic biota is essential for a sustainable management of aquatic ecosystems in agricultural areas.

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1. Introduction

Agrochemicals are the main chemical stressors in freshwater ecosystems. Lentic and lotic freshwater ecosystems surrounded by agricultural areas frequently receive agricultural runoff contaminated by herbicides used for crop management. Metazachlor ($C_{14}H_{16}ClN_3O$) and flufenacet ($C_{14}H_{13}F_4N_3O_2S$) are two common herbicides used in European countries as pre- and early-postemergence control of a wide range of broad-leaved weeds and grasses in agricultural fields (Andreasen et al., 2020; Velisek et al., 2020). Metazachlor is mainly applied to rape, while flufenacet is applied to wheat, barley, rye, and other winter cereals (Dücker, 2020; Ulrich et al., 2018). Both herbicides are usually applied in fall (Dücker, 2020; Ulrich et al., 2018) in high quantities. For example, metazachlor application for rape was 750 g ha^{-1} and flufenacet application for winter wheat was 200–240 g ha^{-1}, according to the official recommendations. Therefore, there is a high probability of contaminating adjacent freshwater ecosystems with metazachlor and flufenacet in higher concentrations, which were frequently detected in aquatic ecosystems in Northern lowland German agricultural areas (Ulrich et al., 2021; Wijewardene et al., 2021). Concentrations as high as 35 μg L^{-1} and 1 μg L^{-1} of metazachlor and flufenacet were found in drainage waters, respectively (Ulrich et al., 2021). According to German environmental quality standards on surface waters, the maximum allowable concentrations for metazachlor and flufenacet are 0.40 μg L^{-1} and 0.20 μg L^{-1}, respectively (OGewV, 2010, 2016).

Herbicides can have dramatic consequences on species structure and function of the freshwater ecosystems (Lozano et al., 2018, 2019, 2021; Pérez et al., 2007; Sabio y García et al., 2022; Wijewardene et al., 2021). Metazachlor and flufenacet traces were already found in drinking water sources emphasizing risks for human health (Karier et al., 2017; Ulrich et al., 2021). Few studies have reported about metazachlor toxicity to non-target biotic communities in aquatic ecosystems, such as fish (Velisek et al., 2020), macrophytes, and plankton communities (Mohr et al., 2008). However, as far as we know, there are no studies about flufenacet with this scope. Phytoplankton are one of the main primary producers in freshwater ecosystems. They are susceptible to herbicides and changes in phytoplankton communities lead to many consequences on other aquatic biota as they are the basis of the food chains and food webs (Lozano et al., 2018, 2019; Pérez et al., 2007). Furthermore, phytoplankton are one of the best ecological indicators for aquatic stress responses (Wu et al., 2017). Despite the importance of phytoplankton in aquatic ecosystems, studies focusing on effects of herbicides on phytoplankton are lacking as only a few studies have focused on metazachlor and none on flufenacet.

Metazachlor belongs to the substance group chloroacetamide and flufenacet to the oxyacetamides (Mohr et al., 2008; Trenkamp et al., 2004). Metazachlor and flufenacet act as lipid biosynthesis inhibitors according to their mode of action (Faust et al., 1994; Trenkamp et al., 2004). According to Mohr et al. (2008), metazachlor inhibits the very long chain fatty acid (VLCFA) elongase enzyme and leads to the disruption of the VLCFA (> 18 C) production process. Then, the cell membrane loses the fatty acid incorporation to keep the cell rigidity and permeability functions resulting in leakage of cell membranes and cell division impairment. Therefore, it ultimately reduces growth and reproduction in autotrophs. Flufenacet is a herbicide, which inhibits all activities of the VLCFA elognase in higher plants (Trenkamp et al., 2004). The toxicity of metazachlor and flufenacet on algae is moderate and high, respectively, considering acute 72 h EC_{50} (the concentration cause 50% reduction in algae growth) as 0.0162 mg L^{-1} and 0.00204 mg L^{-1} (tested on *Raphidocelis subcapitata* (Kors.) Nyg., Kom., Kris. & Skul.) (Lewis et al., 2006). Both herbicides are degraded to oxalic acid (OA) and sulfonic acid (ESA). These transformation products are categorized as low toxic compounds compared to the original substance, considering acute 72 h EC_{50} for algae (Metazachlor OA: 25.7 mg L^{-1}; Metazachlor ESA: 93.8 mg L^{-1}; Flufenacet OA: > 100 mg L^{-1}; Flufenacet ESA: > 86.7 mg L^{-1}) (Lewis et al., 2006).

Metazachlor decreases phytoplankton density but it can recover 30–35 days after application (Noack et al., 2003). According to Mohr et al. (2008), metazachlor is highly toxic for chlorophytes and less toxic for diatoms and cryptophytes and therefore leads to changes in phytoplankton community. Species-specific responses of the phytoplankton community are often reported for herbicides due to their selective effects (Chang et al., 2011; Leboulanger et al., 2011; Lozano et al., 2018, 2019). For example, the abundance of *Cryptomonas erosa* Ehr. and *Rhodomonas minuta* Skuja (*Chroomonas minuta* (Skuja) Bour.) increases with increasing metazachlor concentration in lentic mesocosms (Mohr et al., 2008). Species composition may be further used to derive taxonomic diversity indices to gain insights on biodiversity, the key component of understanding ecosystem health, function, and integrity (Otero et al., 2020). Functional features, such as functional diversity indices and functional redundancy indices will further extend the understanding of relationships between biodiversity, ecosystem processes, functioning, and stability by incorporating both species and trait composition of the community (Mouchet et al., 2010). Biotic communities with higher taxonomic and functional diversity may provide more ecosystem services and may be more resilient to disturbances (Otero et al., 2020; Pakeman, 2014). To the best of our knowledge, there are no specific studies to tackle direct effects of metazachlor and flufenacet on phytoplankton taxonomic and functional diversity.

Understanding the overall community responses of phytoplankton to two commonly used herbicides (i.e., metazachlor and flufenacet) under realistic environmental concentrations is needed and helpful to disentangle the cause-and-effect of biotic communities in natural environments exposed to herbicides. The objectives of our study were (i) to explore the effects of metazachlor and flufenacet on phytoplankton community composition, (ii) to identify the effects of metazachlor and flufenacet on taxonomic diversity indices and functional features, and (iii) to study the dynamics of these effects over the short-term in a 4-week period. We hypothesized that (i) an increase in herbicide concentration shifts species composition towards herbicide tolerant phyla (e.g., diatoms) and species (H1), (ii) an increase in herbicide concentration reduces taxonomic diversity and functional diversity/redundancy of the phytoplankton community (H2), and (iii) effects of herbicide exposures on the phytoplankton community are irrecoverable in the shorter term, i.e., a 4-week period (H3).

2. Methods

2.1. Outdoor microcosms

A microcosm experiment with natural phytoplankton communities was carried out during the application period of the selected herbicides. Natural phytoplankton communities were sampled from a pond (54°44'20" N, 9°35'42" E) in the "Winderatter Lake" nature reserve area, in the Kielstau catchment, Northern Germany on August 17th, 2020. We collected phytoplankton communities from the ponds in the nature reserve area, which were less likely to have been exposed to herbicides. This was confirmed by the herbicide measurements in the pond water during trial experiments, which did not detect the herbicides we screened for in the pond water. We filtered pond water through 150 μm mesh to remove zooplankton in order to avoid grazing pressure on phytoplankton in microcosms (Mack et al., 2012). The microcosm experiment contained 87 glass vessels of 2.6 L volume filled with 2.4 L of the filtered pond water and placed outdoor under partially sheltered but natural light and temperature conditions (Fig. 1). Metazachlor and flufenacet concentrations were prepared by using the commercial products Butisan® and Cadou® SC respectively. Microcosms were treated once with the selected herbicides, metazachlor and flufenacet, at the beginning of the experiment. Exposure concentrations were selected as 0 (controls), 0.5 μg L^{-1} depicting a common concentration in surface water after application, 5 μg L^{-1} as a realistic concentration after a heavy rainfall event immediately after application, and 50 μg L^{-1} as

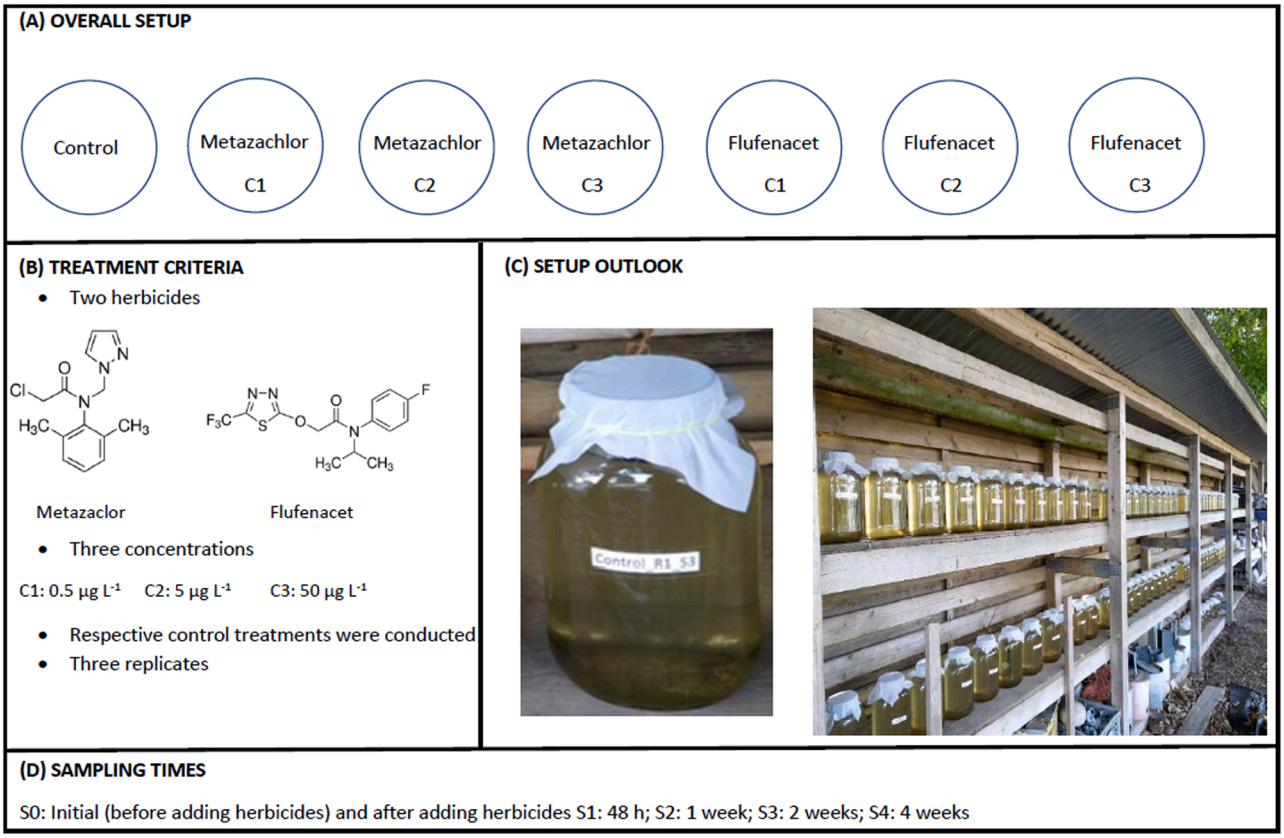

Fig. 1. Microcosm experiment design as overall setup with seven different treatments (A), description of treatment criteria (B), setup outlook (C) and description on sampling times (D). Treatments are represented as control and respective concentrations (C1: 0.5 µg L^{-1}; C2: 5 µg L^{-1}; C3: 50 µg L^{-1}) of exposed herbicides (M: Metazachlor and F: Flufenacet) here onwards.

concentration that can occur due to accidental spraying on the surface water during application (e.g., Ulrich et al., 2018; Ulrich et al., 2021; Wijewardene et al., 2021) (Fig. 1A–C). Control and treatments were conducted in triplicates. Microcosms were supplemented with nitrogen in the form of potassium nitrate and phosphorous as potassium phosphate with 10% of initial pond water concentration every other day to avoid nutrient limitation (Kasai and Hanazato, 1995; Spawn et al., 1997). Specifically, concentrations of 0.01 mg NO_3-N L^{-1} and 0.0025 mg PO_4-P L^{-1}, respectively, were applied. Water samples were taken to measure herbicide and nutrient concentrations (see Section 2.2 in Methods) and phytoplankton community attributes (see Section 2.3) at the beginning of the experiment before exposure to herbicides (S0), and 48 h (S1), 1 week (S2), 2 weeks (S3), and 4 weeks (S4) after exposure to the herbicides (Fig. 1D). At S0, all microcosms were similar. Therefore, we represented physicochemical and phytoplankton parameters at S0 as one sample named "control". To observe whether herbicide degradation occurs due to abiotic factors, such as UV light, we conducted one parallel microcosm for each herbicide composed of 5 µg L^{-1} of herbicide and distilled water under the same conditions as the other microcosms.

2.2. Physicochemical parameters

We collected daily measurements of pH, electrical conductivity (EC), and dissolved oxygen (DO) using two portable meters (WTM Multi 340i and WTW Cond 330i, Germany). Water temperature and light were measured at a 15-minute interval throughout the experiment by a HOBO Pendant data logger (Onset Computer Corporation, Pocasset, MA, USA), from which daily mean values were calculated. Samples for nutrient and herbicide measurements were collected at five sampling times recorded above (S0-S4). For analyzes of nutrients, filtered water samples (through GF/C Whatman glass microfiber and 0.45 µm cellulose acetate filter) were stored in pre-cleaned plastic bottles (50 mL) and kept frozen at − 18 °C until measurement. The concentrations of dissolved phosphate-phosphorus (PO_4-P), ammonium-nitrogen (NH_4-N), and nitrate-nitrogen (NO_3-N) were measured according to the standard methods of the DEV (Deutsche Einheitsverfahren, 1997). PO_4-P and NH_4-N were measured photometrically using a spectrophotometer (SHIMADZU UV-1800, Japan) according to DEV D11 and DEV E5 protocols, respectively. NO_3-N was determined ion chromatographically using an ion chromatography system (Metrohm ECO IC, Switzerland) according to DEV D19 protocol.

Water samples (30 mL) were collected for herbicide measurements from each microcosm and stored in glass bottles at 4 °C until the analysis. All samples were left for 24 h at 4 °C for sedimentation before the analysis and the supernatant water was analyzed without any further treatment according to DIN 38407-36:2014–09 by liquid chromatography-mass spectroscopy using an Agilent 1290 Multisampler and High Speed Pumps and an Agilent Triplequad 6495 with an injection volume of 100 µL. We used a Phenomenex column Synergi™, 4 µm, Hydro RP 80 Å, 50 × 3 mm and a security guard column Phenomenex AQ C18, 4 × 3 mm. Further quality parameters related to herbicide measurements are listed in Table S1 in Appendix.

2.3. Phytoplankton species and trait composition

Water samples (500 mL) were collected, preserved with Lugol's iodine solution, and sedimented for taxonomic identification based on standard methods (Wu et al., 2011). Soft microalgae (non-diatom) were observed using an optical microscope (Nikon Eclipse E200-LED, Germany) under × 400 magnification. We carried out taxonomic identification to species level based on current taxonomic criteria (Burchardt, 2014; Cantonati et al., 2017; Hu and Wei, 2006). Taxonomic

nomenclature follows the criteria set up by Guiry and Guiry (2020). Permanent slides were prepared to identify diatoms by using 5 mL of 30% hydrogen peroxide (H_2O_2) and 0.5 mL of 1 mol L^{-1} hydrochloric acid (HCl) for the oxidization processes of organic materials in the samples. When the oxidation process was complete, 0.1 mL of the diatom-ethanol mix was transferred to a 24 × 24 mm cover slip and a drop of naphrax was used to mount the slide. Diatoms were observed with the optical microscope (Nikon Eclipse E200-LED, Germany) under × 1000 magnification with oil immersion and were identified based on the key books by Bąk et al. (2012), Bey and Ector (2013), Cantonati et al. (2017), and Hofmann et al. (2011). Phytoplankton traits of the identified species were further investigated using literature. Phytoplankton species were assigned to three functional traits: biovolumes [nano: 5–100 μm^3, micro: 100–300 μm^3, meso: 300–600 μm^3, macro: 600–1500 μm^3 and large: > 1500 μm^3] (Abonyi et al., 2018; Kruk et al., 2017; Qu et al., 2018; Rimet and Bouchez, 2012), life form [unicellular, colonial and filamentous] (Abonyi et al., 2018; Kruk et al., 2017; Rimet and Bouchez, 2012) and ecological guild [low profile, high profile, motile and planktonic] (Guiry and Guiry, 2020; Rimet and Bouchez, 2012). More details on studied traits and traits composition of the phytoplankton community in our study can be found in Tables S2 and S3 in the Appendix.

2.4. Phytoplankton taxonomic diversity indices and functional features

Taxonomic diversity indices, such as species richness (Gleason, 1922), the Shannon-Wiener index and evenness (Shannon, 1963), and the Simpson index (Simpson, 1949) were calculated by function *div* in the R package *ecoloop* (Guo, 2019). Functional features are depicted with functional diversity indices and functional redundancy indices. Functional diversity indices, such as functional richness (FRic), functional evenness (FEve), functional dispersion (FDis), functional divergence (FDiv), and functional redundancy indices, such as FR01 and FR02 were computed using the function *dbFD* in R package *vegan* (Oksanen et al., 2019). The detailed descriptions of the calculations of the functional features are reported in Wu et al. (2019).

2.5. Statistical analyses

All data analyses were performed using R software version 4.0.2 (R Development Core Team, 2020). Phytoplankton species abundance data were used for all statistical analyses on species and trait data. Changes of phytoplankton density, diatom density, and diatom-to-phytoplankton ratio were investigated in different treatments throughout the experiment period. Further, composition of the phytoplankton community at the phyla level was analyzed as the mean relative abundance in each treatment at each sampling time.

Species abundance data were Hellinger-transformed using the function *decostand* in R package *vegan* to reduce the weight of the most abundant species and keep Euclidean distances between samples in the multidimensional space without interruptions. Differences or similarities of phytoplankton community composition in different treatments at each sampling time were studied by multivariate permutational ANOVA (PERMANOVA, Bray-Curtis method, permutations=999) using the *adonis* function in R package *vegan*. All the analyzes were statistically significant ($p < 0.05$). Multivariate homogeneity of group dispersions was assessed using function *betadisper* in R package *vegan* and the p values for all analyzes were > 0.05. Visual representations of the PERMANOVA analyses were illustrated in principal coordinate analysis (PCoA) plots. Species level responses of the phytoplankton community to different concentrations of the metazachlor and flufenacet compared to controls over the experiment period were further analyzed by principal response curves analysis (PRC) using function *prc* in R package *vegan*. The PRC model represents the responses of individual species to the treatments using treatment scores (effect) and species weight (Neif et al., 2017; Van den Brink and Ter Braak, 1998). Higher effect value represents the greater response of the community to the treatment. Species weights closer to zero indicate no influence or a different pattern of response compared to the overall PRC model. Positive weights indicate the species follow the pattern of the PRC model and species with higher positive weights follow the pattern strongly. Negative weights indicate the opposite pattern. The first axis of the PRC models was significant at $p < 0.1$ (model df = 1, residual df = 32, permutations = 999, function *anova* in R package *vegan*). Only the response of strongly affected species (species weight > 0.1 and species weight < − 0.1) was visualized for each herbicide treatment to maintain the clarity of representation.

The effects of metazachlor and flufenacet on phytoplankton community attributes (i.e., total phytoplankton density, diatom density, diatom-to-phytoplankton ratio, density of each phylum, taxonomic diversity indices, and functional features) were further explored using multiple linear regression models. The exposure time, light, and temperature were included in the models in addition to herbicide concentrations due to the high relevance observed in the two-by-two parameter explorations in initial data analyses. All variables were log [ln (x + 1)] and z-score transformed before linear regression analyses. It obtained standardized coefficients to compare magnitude within and between models.

3. Results

3.1. Herbicide concentrations and other physicochemical parameters

Metazachlor and flufenacet concentrations in the microcosms decreased with time (Fig. 2). On the other hand, we observed an increase in the metabolite concentrations: sulfonic acid (ESA) and oxalic acid (OA) of each herbicide. Chemical degradation of herbicides due to abiotic factors was not observed for metazachlor but was present for flufenacet in the microcosms only with herbicides and distilled water. Herbicide measurements of the lowest concentration (C1) showed lower concentrations than we added, probably due to matrix effects (herbicides attached to suspended matter). Changes in the other physicochemical parameters over the study period are recorded in Table 1. Detailed temporal changes in the other physicochemical parameters are recorded in Fig. S1.

3.2. Effects of metazachlor and flufenacet on phytoplankton density, diatom density, and the diatom-to-phytoplankton ratio

Phytoplankton density, diatom density, and the diatom-to-phytoplankton ratio changed during the study period and these changes were different among the treatments (Fig. S2). Phytoplankton densities of the controls decreased in the first week of the experiment and were stable thereafter. Compared to controls, phytoplankton densities in the herbicide treatments were considerably lower (Fig. S2A). Diatom density increased with exposure time and flufenacet treatments showed higher diatom density than metazachlor after 4 weeks of exposure (Fig. S2B). Diatom-to-phytoplankton ratios were higher in herbicide treatments compared to controls and the differences among treatments increased with exposure time. After 4 weeks of exposure, diatom-to-phytoplankton ratios were higher in flufenacet treatments compared to metazachlor treatments (Fig. S2C).

3.3. Effects of metazachlor and flufenacet on phytoplankton species composition

In total, 136 species were identified in the samples, which belonged to 9 phyla: Bacillariophyta (diatoms) (39), Charophyta (6), Chlorophyta (43), Cryptophyta (3), Cyanobacteria (16), Euglenozoa (class: Euglenophyceae) (23), Haptophyta (1), Miozoa (class: Dinophyceae) (2), and Ochrophyta (3). Changes in the relative abundance of phytoplankton phyla respective to different treatments are illustrated in Fig. 3. Relative

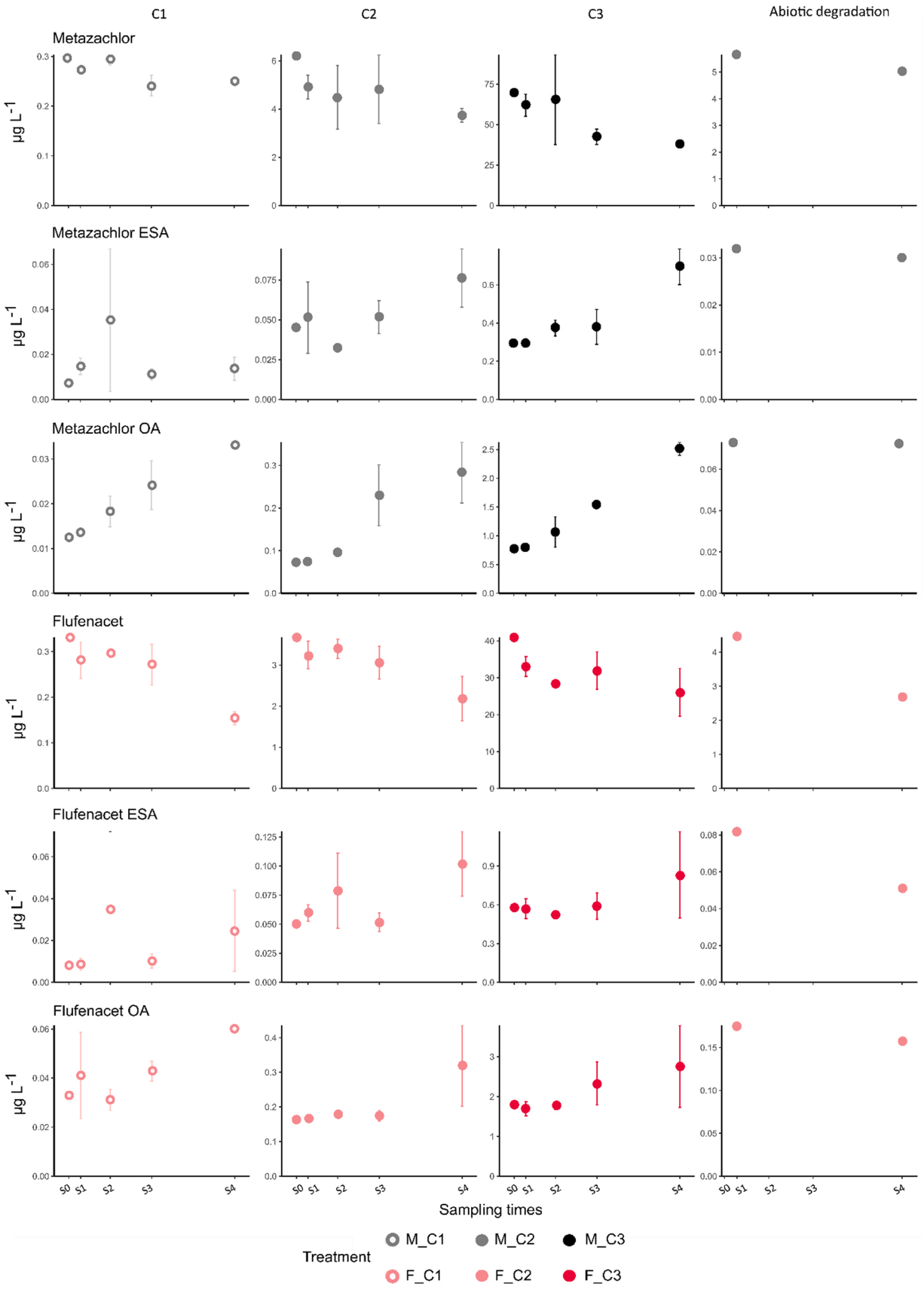

Fig. 2. Dynamics of metazachlor and flufenacet concentrations and their metabolite concentrations: sulfonic acid (ESA) and oxalic acid (OA), in microcosms during the experiment. Different herbicide treatments are represented according to respective concentrations as C1: 0.5 µg L^{-1}; C2: 5 µg L^{-1}; C3: 50 µg L^{-1} and specific samples were maintained to measure abiotic degradation of herbicides (5 µg L^{-1} herbicide + distilled water). Sampling times reported as S0: Before exposure; S1: 48 h after exposure; S2: 1 week after exposure; S3: 2 weeks after exposure and S4: 4 weeks after exposure. Dispersion bars denote standard deviation (SD).

Table 1
Changes of the physicochemical parameters as mean and range (min-max) in the microcosms during the experiment period.

Physicochemical parameter	Mean (Range)
Light intensity [Lux]	1604 (214–3910)
Temperature [°C]	17.5 (14.6–25.9)
pH	7.47 (6.90–8.67)
Conductivity [μS cm^{-1}]	239 (230–252)
Dissolved oxygen (DO) [mg L^{-1}]	6.54 (2.07–12.58)
NH_4-N [mg L^{-1}]	0.031 (0.001–0.162)
NO_3-N [mg L^{-1}]	0.080 (0.035–0.186)
PO_4-P [mg L^{-1}]	0.254 (0.167–0.347)

abundance of Chlorophyta was mostly lower in herbicide exposed treatments compared to respective control (except M_C1 and F_C1 at S2) (Fig. 3). An increase in metazachlor concentration led to a reduction of Chlorophyta throughout the experiment, while flufenacet followed the same pattern in S2 and S3 (Fig. 3).

The response of the whole phytoplankton composition to the different treatments along sampling times are illustrated in Fig. 4. Results of the permutational multivariate ANOVA at each sampling time (PERMANOVA: ADONIS, permutations = 999, Bray-Curtis method; Results: $p < 0.05$; model df = 6, residual df = 14; $F > 1.61$) confirmed that species composition significantly changed due to herbicide exposures and clusters in PCoA analysis, which emphasized the influence of herbicide toxicity. Species composition in the control was always different compared to herbicide exposed treatments and grouping of concentration dependent clusters was observed with the increased herbicide exposure time. The highest variation in species composition among treatments was observed after 4 weeks of exposure time. Species composition of M_C1 was the closest to respective controls and the distance of clusters to the controls increased as the herbicide concentration increased. At S3, clear grouping of the clusters according to concentration was noted regardless of the herbicide type, emphasizing a similar phytoplankton species composition for each herbicide concentration of both herbicides. At S4, the clusters were further apart and showed distinct grouping according to the toxicity of herbicides. The highest concentration of both herbicides (M_C3 and F_C3) overlapped, emphasizing the similar phytoplankton species composition after 2 weeks of exposure (S3 and S4 in Fig. 4).

Species-level responses to the herbicide concentrations compared to controls during the experiment duration is illustrated in Fig. 5 by the first axis of the principal response curves (PRC) analysis ($p < 0.1$), which represent the responses of the strongly affected species (species weight > 0.1 and species weight < -0.1) to the treatments. Overall, the PRC model for metazachlor explained 24% of phytoplankton community variation by treatments and 10% by time (p for all canonical axes = 0.57). The PRC model for flufenacet explained 25% of phytoplankton community variation by treatments and 12% by time (p for all canonical axes = 0.26). The responses were greater with the increase in herbicide concentrations and, even after 4 weeks of exposure, progressive response can still be observed without tending towards controls. In both herbicide exposed treatments, abundance of *Peridinium willei* Huit.-Kaas (Miozoa) and *Trachelomonas volvocina* (Ehr.) Ehr. (Euglenozoa) showed an increasing trend with increasing herbicide concentrations. Additionally, diatom species (Bacillariophyta), such as *Fragilaria capucina* Desm. showed an increasing trend with increasing metazachlor concentrations, while *Nitzschia fonticola* (Grun.) Grun. and *Cyclotella meneghiniana* Kütz. showed an increasing trend with increasing flufenacet concentrations. In comparison, abundance of green algae species

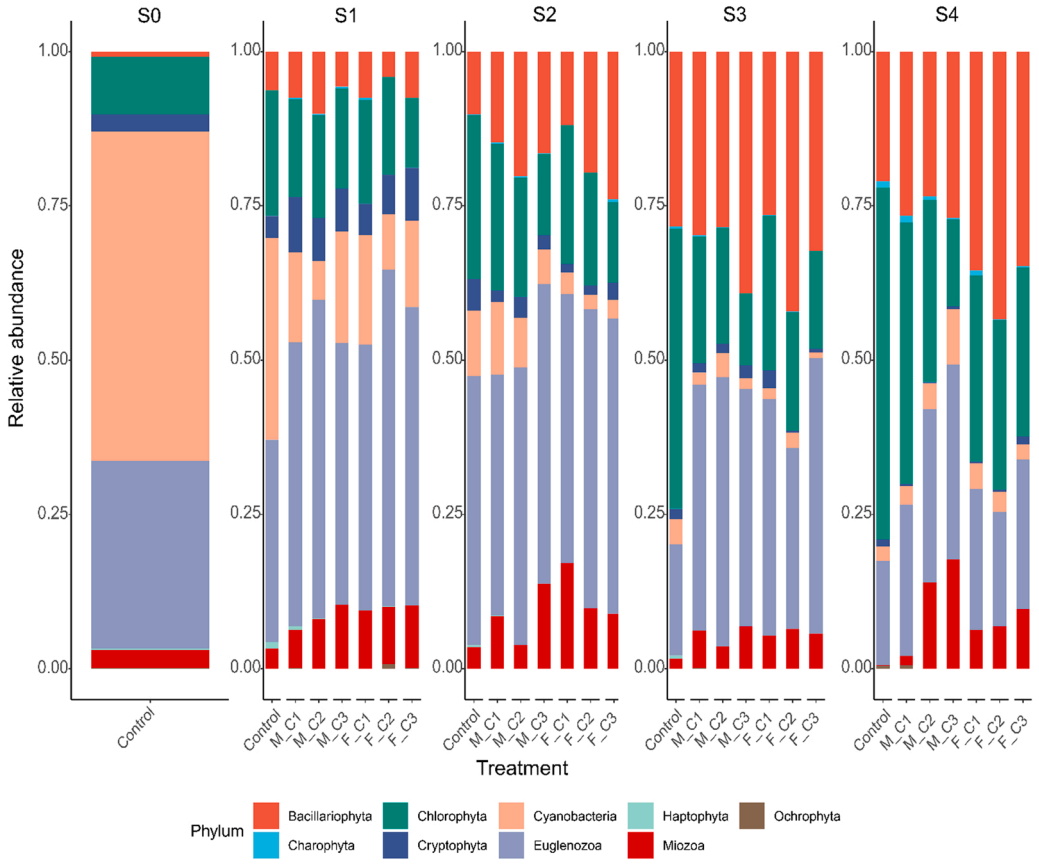

Fig. 3. Changes in the relative abundance of phytoplankton phyla across different treatments over the herbicide exposure time. Treatments are represented as control and respective concentrations (C1: 0.5 μg L^{-1}; C2: 5 μg L^{-1}; C3: 50 μg L^{-1}) of exposed herbicides (M: Metazachlor and F: Flufenacet). Sampling times reported as S0: before exposure; S1: 48 h after exposure; S2: 1 week after exposure; S3: 2 weeks after exposure and S4: 4 weeks after exposure.

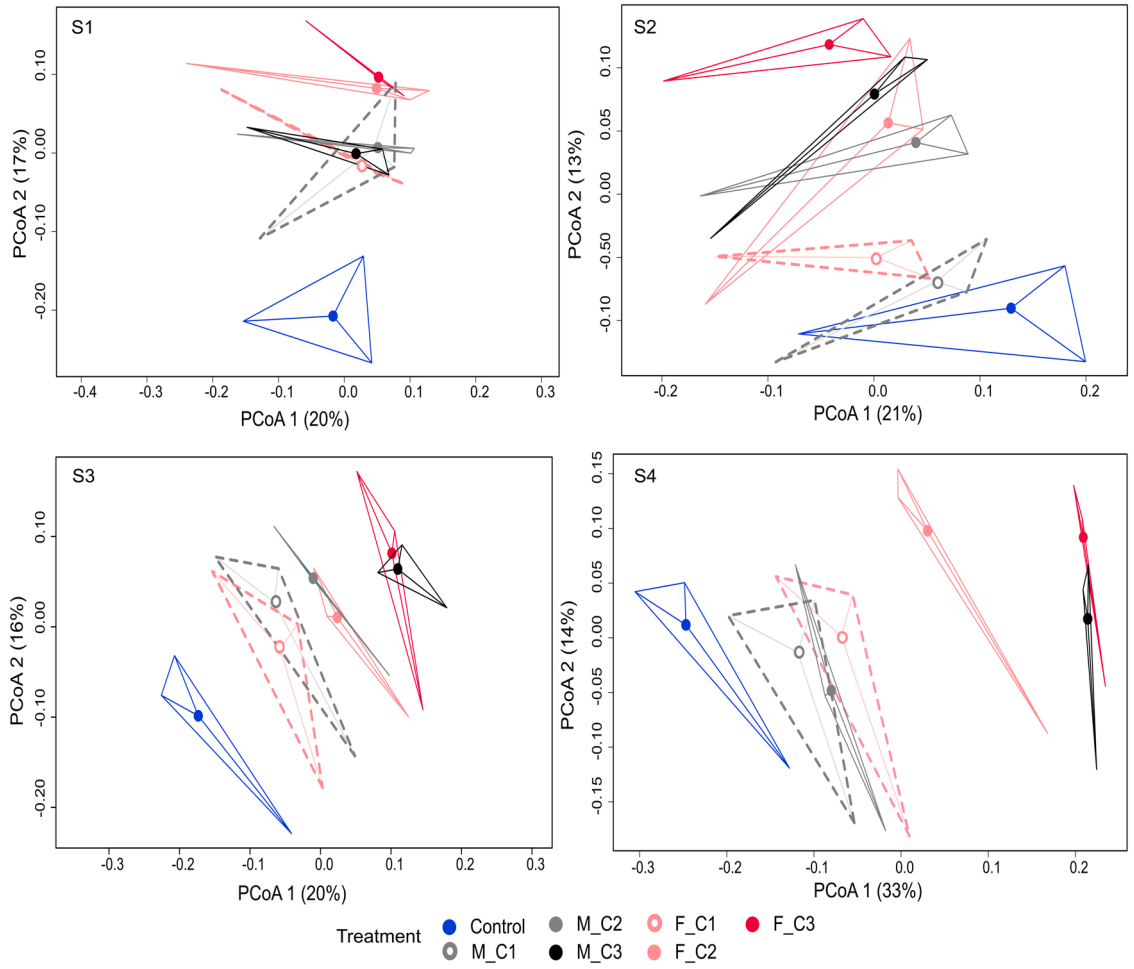

Fig. 4. Response of the whole phytoplankton composition to the different treatments. Treatments are represented as control and respective concentrations (C1: 0.5 μg L^{-1}; C2: 5 μg L^{-1}; C3: 50 μg L^{-1}) of exposed herbicides (M: Metazachlor and F: Flufenacet; see legend). Sampling times reported as S1: 48 h after exposure; S2: 1 week after exposure; S3: 2 weeks after exposure and S4: 4 weeks after exposure. Polygon edges represent replicates of the treatments and points illustrate the centroids of the polygons.

(Chlorophyta), such as *Koliella longiseta* (Vis.) Hin., *Chlorella minutissima* Fott & Nov., *Selenastrum bibraianum* Rein., *Chlamydomonas reinhardtii* Dang., *Tetraedron minimum* (A. Braun) Hans., *Eutetramorus planctonicus* Kors. and blue green algae species (Cyanobacteria) *Merismopedia tenuissima* Lemm. and *Aphanocapsa elegans* (Lemm.) Joo. were lower in herbicide-exposed treatments compared to controls. In contrast to this general pattern, one of the Chlorophyta species *Planctonema lauterbornii* Schm. showed an increasing trend with increasing herbicide concentrations over time compared to controls and this response was more prominent in flufenacet treatments. Density changes of the strongly affected phytoplankton species over the study period are illustrated in Fig. S3 in Appendix.

3.4. Effects of metazachlor and flufenacet on phytoplankton community attributes

Taxonomic diversity indices and functional features (functional diversity and functional redundancy) are other important attributes of the phytoplankton community. Variation of the taxonomic diversity indices and functional features among treatments are illustrated in Fig. S4 in the Appendix. Results of the multiple regression models emphasized significant effects of metazachlor and flufenacet on different phytoplankton community attributes (Table 2).

Phytoplankton density, Chlorophyta and Cyanobacteria densities in phytoplankton community, species richness, the Shannon-Wiener index, the Simpson index, FDis and FR02 were significantly reduced with an increase in metazachlor concentration. Some of these effects were further retrogressed with increased exposure time. For example, phytoplankton density and FR02 like attributes continued to significantly decrease with an increase in exposure time to metazachlor (Table 2). Contrasting trends were observed as the recovery of phytoplankton community attributes increased with an increase in exposure time, such as Chlorophyta density, the Shannon-Wiener index, and the Simpson index. In addition, the diatom-to-phytoplankton ratio, Miozoa density, FEve, and FDiv significantly increased with rising metazachlor concentrations. Among these attributes, the diatom-to-phytoplankton ratio and FEve continued to increase with an increase in exposure time to metazachlor. In contrast, Miozoa density and FDiv were significantly reduced with an increase in exposure time to the metazachlor (Table 2).

The increased flufenacet concentrations also resulted in negative effects on phytoplankton community attributes (Table 2). Phytoplankton density, Chlorophyta density, species richness, the Shannon-Wiener index, the Simpson index, evenness, FDis and FR02 were significantly reduced with an increase in flufenacet concentration. Among these attributes, phytoplankton density, species richness, and FR02 continued to decrease with increase of exposure time to flufenacet while the Shannon-Wiener index, the Simpson index, and evenness showed an increasing tendency with increase in exposure time to the flufenacet (Table 2). Similar to metazachlor, the diatom-to-

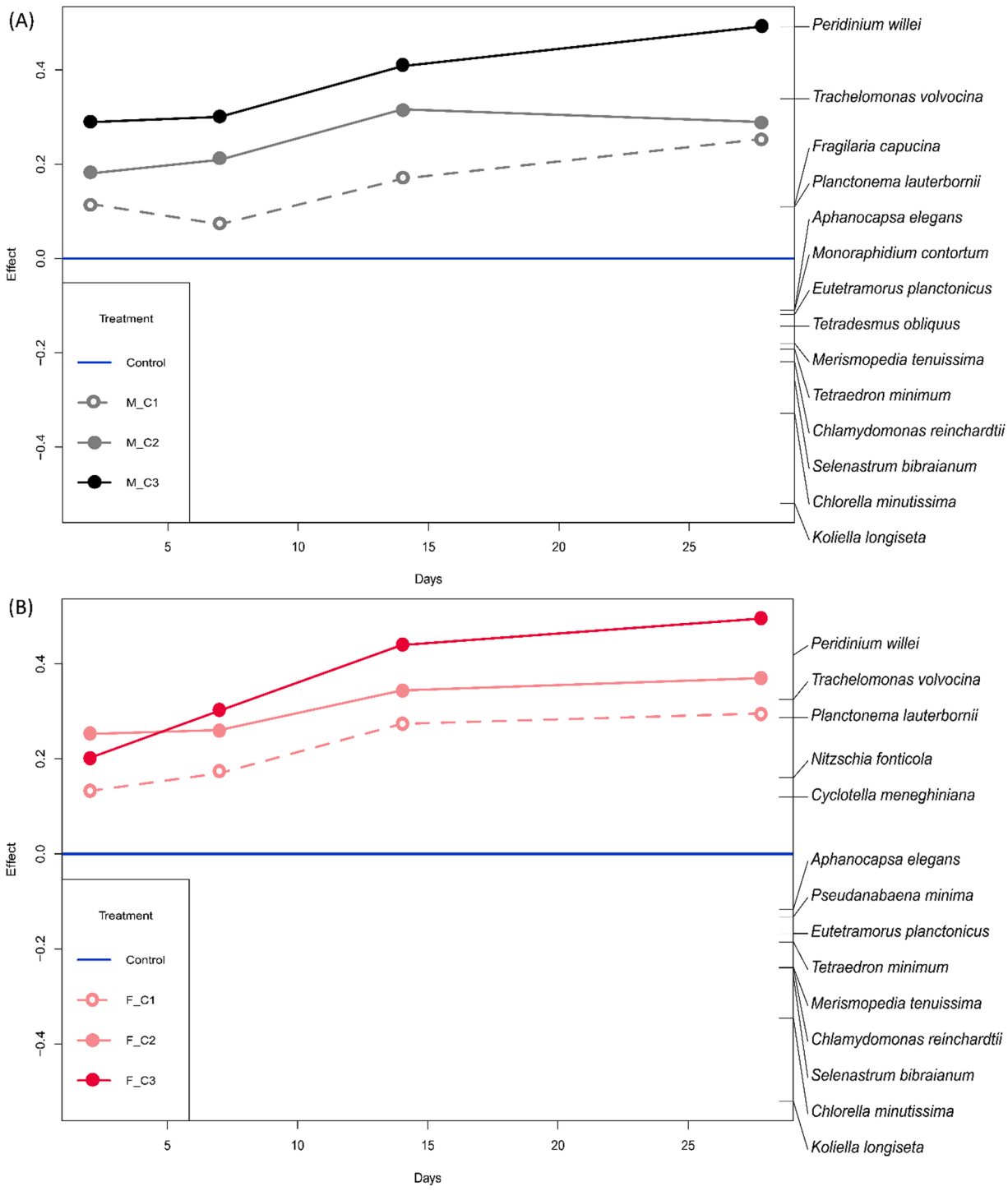

Fig. 5. Results of the PRC analysis. First axis of the PRC analysis is shown here. Response of the phytoplankton species to the metazachlor (A) and flufenacet (B) treatments compared to controls during the experiment period. Treatments are represented as control and respective concentrations (C1: 0.5 μg L^{-1}; C2: 5 μg L^{-1}; C3: 50 μg L^{-1}) of exposed herbicides (M: Metazachlor and F: Flufenacet; see legend). Symbols represent the mean effect (mean PRC score) for each treatment and sampling time (n = 3). Only strongly affected species (species weight > 0.1 and species weight < − 0.1) to the treatments are illustrated here. Species showing an increase in abundance in the herbicide-exposed treatments compared to controls can be found above the zero-effect line (control) and vice versa.

phytoplankton ratio and FDiv significantly increased with an increase in flufenacet concentration. The diatom-to-phytoplankton ratio continued to increase with an increase in exposure time to the flufenacet (Table 2). In contrast, FDiv was significantly decreased with an increase in exposure time to metazachlor. Furthermore, light and temperature significantly influenced the phytoplankton community attributes together with herbicide concentrations and exposure time (Table 2).

4. Discussion

4.1. Shift in phytoplankton species composition

Metazachlor and flufenacet significantly affected phytoplankton community composition resulting in the reduction of Chlorophyta species (e.g., *Koliella longiseta*, *Chlorella minutissima*, *Selenastrum*

Table 2
Multiple regression models on the effects of metazachlor and flufenacet on phytoplankton community attributes ($p < 0.05$). Explanatory variables with significant standard coefficients ($p < 0.05$) in the models are represented in bold. Exposure time, light, and temperature are included in all models as they emerged as significant variables on phytoplankton attributes in preliminary data analyses. All variables were log-transformed [ln (n + 1)] and standardized before the regression analyses. Significant multiple regression models ($p < 0.05$) were not obtained for Haptophyta density, Ochrophyta density, and FRic.

Phytoplankton community attributes	Metazachlor					Flufenacet				
	Standardized coefficients				Model R^2	Standardized coefficients				Model R^2
	Metazachlor	Time	Light	Temperature		Flufenacet	Time	Light	Temperature	
Phytoplankton density	**-0.379**	**-0.505**	**0.342**	**0.279**	0.48	**-0.408**	**-0.394**	**0.308**	**0.329**	0.46
Diatom density (Bacillariophyta)	-0.037	**0.736**	0.008	-0.161	0.67	-0.029	**0.793**	0.033	-0.041	0.69
Diatom: Phytoplankton	**0.164**	**0.398**	**0.390**	**-0.216**	0.73	**0.181**	**0.587**	**0.293**	-0.028	0.73
Phylum wise density										
Charophyta	-0.137	**0.636**	-0.061	0.271	0.31	-0.169	**0.566**	-0.054	0.149	0.26
Chlorophyta	**-0.654**	**0.297**	0.008	**0.264**	0.52	**-0.606**	**0.290**	0.150	0.293	0.52
Cryptophyta	-0.074	**-0.671**	-0.006	**-0.342**	0.39	-0.210	-0.283	-0.227	-0.017	0.26
Cyanobacteria	**-0.249**	-0.188	-0.241	0.224	0.35	-0.142	-0.278	-0.135	**0.315**	0.38
Miozoa	**0.317**	**-0.460**	0.046	-0.201	0.24	0.204	**-0.406**	0.007	0.184	0.17
Euglenozoa	-0.072	**-0.718**	0.027	-0.016	0.50	-0.108	**-0.633**	-0.034	-0.034	0.44
Taxonomic diversity indices										
Species richness	**-0.318**	-0.157	**-0.744**	**-0.430**	0.73	**-0.307**	**-0.369**	**-0.503**	**-0.482**	0.65
Shannon-Wiener index	**-0.372**	**0.683**	**-0.684**	-0.132	0.41	**-0.473**	**0.671**	**-0.473**	-0.204	0.48
Simpson index	**-0.309**	**0.669**	**-0.346**	0.189	0.29	**-0.425**	**0.741**	-0.183	0.060	0.50
Evenness	-0.093	**0.699**	0.087	**0.408**	0.49	**-0.147**	**0.846**	0.156	**0.364**	0.77
Functional features										
FEve	**0.242**	**0.839**	-0.215	-0.013	0.62	0.195	**0.707**	-0.051	-0.072	0.56
FDis	**-0.405**	0.292	0.150	**0.314**	0.33	**-0.275**	0.284	**0.487**	**0.384**	0.53
FDiv	**0.395**	**-0.464**	-0.070	0.098	0.44	**0.308**	**-0.435**	-0.046	0.187	0.39
FR01	0.191	-0.176	**-0.364**	**-0.342**	0.28	0.003	-0.217	**-0.599**	**-0.479**	0.54
FR02	**-0.288**	**-0.670**	0.204	0.108	0.49	**-0.243**	**-0.534**	0.037	0.157	0.44

bibraianum) and Cyanobacteria species (e.g., *Merismopedia tenuissima* and *Aphanocapsa elegans*). In addition, both herbicides changed the phytoplankton community towards a high abundance of species belonging to Bacillariophyta (e.g., *Fragilaria capucina, Nitzschia fonticola* and *Cyclotella meneghiniana*), Miozoa (i.e., *Peridinium willei*), and Euglenozoa (i.e., *Trachelomonas volvocina*) as we expected in hypothesis 1 (H1) (Fig. 5). Furthermore, a significant increase in the diatom-to-phytoplankton ratio and a decrease in Chlorophyta density with increasing herbicide concentrations further supported H1 (Table 2).

Selective effects of herbicides on phytoplankton species are frequently acknowledged in previous studies (Chang et al., 2011; Huertas et al., 2010; Lozano et al., 2018, 2019). These effects highly varied depending on initial species composition of the phytoplankton community (Debenest et al., 2010). Mohr et al. (2008) reported a high sensitivity of Chlorophytes to metazachlor emphasizing its significant effect at 5 μg L^{-1}, the smallest tested concentration in their study. We observed a similar trend in all our herbicide treatments starting from 0.5 μg L^{-1} compared to controls. Freshwater Chlorophyta and some Cyanobacteria species usually contain high amounts of VLCFA, specifically polyunsaturated 18 C acids of the Omega-3 type (Ahlgren et al., 1992). Therefore, they can be adversely affected by these herbicides as metazachlor and flufenacet strongly inhibit VLCFA synthesis. Moreover, Debenest et al. (2010) highlighted in their review that many previous studies on herbicides have shown that Chlorophyta and Cyanobacteria are 4–6 times more sensitive than diatoms. They also emphasized that eutrophic diatom species may be highly tolerant to the herbicides compared to other diatom species with respect to the observations in benthic algae community studies. In our study, *Fragilaria capucina* was tolerant to metazachlor while *Nitzschia fonticola* and *Cyclotella meneghiniana* seemed highly abundant in flufenacet exposed treatments among the diatom species and all three species are well known as eutrophic diatom species (Debenest et al., 2010; Yao et al., 2011). *Peridinium willei* and *Trachelomonas volvocina* were observed as the most tolerant species to both herbicides. We observed a higher abundance of these two species in small lentic water bodies, which are characterized by high pesticide and PO_4-P concentrations during our field study in agricultural landscape in Northern Germany (Wijewardene et al., 2021). Additionally, few studies have found a high abundance of *Peridinium* sp. in high herbicide concentrations (metazachlor: Noack et al., 2003; simetryn: Chang et al., 2011). In contrast to the general trend of high susceptibility of Chlorophytes to the studied herbicides, we observed that the filamentous Chlorophyte, *Planctonema lauterbornii* was abundant at S4 in herbicide-exposed treatments compared to controls. This species has shown a strong relationship with temperature (Nõges and Viirret, 2001). Furthermore, the potential appearance of filamentous green algae in periphytic algae community exposed to metazachlor during later stages was discussed in the study of Noack et al. (2003).

4.2. Effects on phytoplankton taxonomic diversity and functional features

Metazachlor and flufenacet showed mostly similar effects on taxonomic diversity indices, (e.g., species richness and the Shannon-Wiener index) and functional features (e.g., FDis and FR02) as we expected in hypothesis 2 (H2) (Table 2). Though there were no directly comparable studies about the effect of these two specific herbicides on phytoplankton taxonomic diversity indices, many herbicides have negative effects on phytoplankton taxonomic diversity (glyphosate: Fugère et al., 2020; paraquat: Leboulanger et al., 2011). Functional features of the biotic communities are helpful to understand how communities respond to stressors and give insights to potential impacts on ecosystem functioning (Pakeman, 2014). Functional diversity indices (e.g., FDis, FEve, and FDiv) provide insights on how the multidimensional functional space is filled (Schleuter et al., 2010). In our study, FDis significantly decreased with increasing herbicide concentrations indicating that dispersion or variation of functional space will be lower with herbicide exposures emphasizing potential under or over utilization of the resources in the ecosystem. Contrary to our expectations in H2, FDiv was positively affected by herbicide concentrations. An increase of FDiv could be the result of the dominance of extreme species in the functional space (Schleuter et al., 2010). FR02 represents functional redundancy and both herbicides have shown a negative impact on it implying that the ability to maintain stability or resistance of the ecosystem will decrease with increased herbicide concentrations.

4.3. Recovery potentials of phytoplankton communities

Exposure time plays an important role regarding effects on biotic communities exposed to herbicides and may allow communities to recover, adapt or extinct over time (Mohr et al., 2008; Noack et al., 2003). We expected no short-term recovery of the phytoplankton community due to herbicide exposures (H3) and this was partly verified. Changes in overall species composition of the phytoplankton community (Fig. 4) and the responses of the strongly affected species (Fig. 5) showed progressive trends without leaning towards phytoplankton community composition in controls throughout our 4-week study period. This emphasizes the irrecoverable short-term impacts of the herbicides on the phytoplankton community as we hypothesized in H3. Noack et al. (2003) observed a recovery of total phytoplankton density only after 30–35 days of metazachlor application. Furthermore, among taxonomic diversity indices, species richness decreased with exposure time, while the Shannon-Wiener index, evenness, and the Simpson index increased over exposure time showing the recovering potentials. Recovering densities of the strongly affected phytoplankton species, particularly 2 weeks after exposure, may lead to the recovery of the phytoplankton community's taxonomic diversity (Fig. S3 in Appendix). With an increasing exposure time, functional features, such as FR02 demonstrated continuous negative impacts and negative impacts on FDis revealed recovering potentials. Recovery of trait diversity over time complies with the recovery of taxonomic diversity and evenness and may lead to the recovery of FDis. These recovery potentials may be associated with the decrease in initial herbicide concentrations with increasing time (Fig. 2). Similarly, Noack et al. (2003) observed a remarkable decrease in initial metazachlor concentrations 2 weeks after application. A decrease in initial herbicide concentrations may occur mostly due to biotic degradation by microorganisms (DeLorenzo et al., 2001), and we identified candidates for bioremediation like *Pseudomonas alcaligenes* (Hölzel et al., unpublished data). In addition, degradation might be related to abiotic factors, such as UV light (Fig. 2). We detected a higher degradation in flufenacet compared to metazachlor. This complies with the stability of the herbicides in water reported as DT_{50} (degradation time for 50% of the initial concentration) of 216 and 54 days for metazachlor and flufenacet, respectively (Lewis et al., 2006).

4.4. Metazachlor and flufenacet

The mode of action of the pesticides on aquatic microbiota may be different from the target organisms (DeLorenzo et al., 2001). Both metazachlor and flufenacet have shown similar effects on species composition, taxonomic diversity indices, and functional features of the phytoplankton community in our study. Flufenacet has an eight-fold higher toxicity than metazachlor, but both are similar in mode of action (Lewis et al., 2006). Despite the difference in toxicity, we observed similar effects at each concentration of both herbicides. For example, species composition of both herbicides overlapped at the highest concentration and at each concentration in S3 (Fig. 4). Standardized coefficients of the multiple regression analyses for both herbicides were similar regarding effects on phytoplankton attributes, such as phytoplankton density, species richness, and functional redundancy (Table 2). This may be caused (i) by a higher degradation rate of flufenacet in water, compared to metazachlor, and/or (ii) by the fact that effects of herbicides with same mode of action may result in similar effects on phytoplankton communities after a certain threshold concentration. As we studied only two herbicides, further control studies with a higher number of herbicides with a similar mode of action are needed. We emphasize that the mode of action of pesticides would be a reasonable way to categorize data in field studies to disentangle the effects of multiple pesticide contaminations on non-target aquatic biota.

In addition, there was a significant influence of light and temperature on phytoplankton communities exposed to herbicides. For example, light and temperature had a greater effect on species richness than the herbicide concentrations when comparing the standardized coefficients in multiple regression models (Table 2). We kept all samples in the same outdoor environment to have similar light and temperature conditions, but temporal changes of light and temperature during the study period were high and highly influential to phytoplankton. Therefore, we emphasize the effect of temperature and light conditions on attributes of the phytoplankton community under herbicide exposures. The prominent influence of light and temperature on the structure of phytoplankton communities under multiple stressors are acknowledged in many studies (Arhonditsis et al., 2004; Wijewardene et al., 2021). We emphasize the importance of integrative studies to understand overall effects of herbicides on phytoplankton by expanding these experiments by combining multiple stressors and their interactions. This understanding would be useful to manage and conserve our aquatic ecosystems under continuous environmental threats, such as global warming and eutrophication.

In summary, metazachlor and flufenacet selectively affected phytoplankton community composition resulting in a reduction of species from Chlorophyta and Cyanobacteria and changed the community towards a high abundance of species from Bacillariophyta, Miozoa, and Euglenozoa. Furthermore, metazachlor and flufenacet showed negative effects on taxonomic diversity (e.g., species richness and the Shannon-Wiener index) and functional features (e.g., functional dispersion and functional redundancy) of the phytoplankton community. Light and temperature significantly influenced the observed changes in phytoplankton attributes under herbicide exposures. Most of the effects on the phytoplankton community were increasing throughout the exposure time without showing any recovery or reversing potentials during the 4-week period of our study.

5. Conclusion

In this study, we focus on effects of environmentally realistic concentrations of two common herbicides, metazachlor and flufenacet, on the phytoplankton community. According to our microcosm study, metazachlor and flufenacet cause structural changes in phytoplankton community composition, taxonomic diversity, and functional features. Even concentrations as low as 0.5 μg L^{-1} of herbicides in lentic aquatic ecosystems due to a single event may mostly remain for at least a 4-week period and may affect the phytoplankton community despite their chemical degradation due to biotic or abiotic factors. Both herbicides have similar impacts on phytoplankton communities particularly at 50 μg L^{-1} regardless of the differences in toxicity. Categorizing data according to the mode of action of the pesticides may be helpful to disentangle effects on non-target aquatic biota especially in field studies where we encounter contamination from multiple pesticides in high concentrations. Light and temperature play an important role in shaping the phytoplankton communities under herbicide exposures. This highlights the importance of multiple stressor studies to gain a comprehensive understanding of herbicide effects on phytoplankton communities in natural aquatic ecosystems. Furthermore, modeling these effects along the trophic interaction pathways will help evaluate the ecosystem level consequences of herbicides. This comprehensive understanding is needed for the management and conservation of aquatic ecosystems surrounded by agricultural land, which continue to expand worldwide to fulfill human demands.

CRediT authorship contribution statement

Lishani Wijewardene: Conceptualization, Methodology, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Naicheng Wu**: Conceptualization, Methodology, Supervision, Writing – review & editing. **Georg Hörmann**: Formal analysis, Writing – review & editing. **Beata Messyasz**: Data curation, Writing – review & editing. **Christina Hölzel**: Conceptualization, Writing – review & editing. **Tenna Riis**: Supervision, Writing – review & editing. **Uta Ulrich**:

Conceptualization, Methodology, Data curation, Supervision, Writing – review & editing. **Nicola Fohrer**: Conceptualization, Methodology, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.113036.

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