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Zur Erlangung des Doktorgrades

von der Mathematisch-Naturwissenschaftlich Fakultät

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Lishani Nisansala Wijewardene M.Sc.

geboren in Colombo, Sri Lanka

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Erste Gutachterin: Prof. Dr. Nicola Fohrer

Zweite Gutachterin: Prof. Dr. Eileen Eckmeier

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Abstract

Rural lowland areas are characterized by mixed land use (e.g., forest, grassland, residential), which is mostly dominated by agricultural lands. Scattered freshwater ecosystems in these rural areas are important for many provisions, such as drinking water supply, irrigation for agriculture, livelihoods, ground water recharge, flood control and recreation. Lotic systems, such as agricultural streams that flow through croplands and farms, and lentic small water bodies (LSWB) are prominent among them. Yet, these smaller lotic and lentic ecosystems receive less attention compared to large rivers and lakes, which are visibly appealing.

Agricultural streams and LSWB are globally abundant and ecologically significant habitats. These ecosystems are susceptible to numerous stressors originating from agricultural practices. Current agricultural trends are leading towards large, intensive systems with low crop diversity. Therefore, water abstraction and agrochemical use are rapidly increasing and causing significant impacts on freshwater ecosystems surrounding agricultural areas. This leads to the emergence of hydrological disturbances, nutrient enrichment, and pesticide contamination as multiple stressors on agricultural streams and LSWB - but the consequences of which are still ambiguous.

Algal communities are efficient indicators of ecological impacts of these stressors by revealing consequences to microbial biodiversity and ecosystem functioning. Periphytic algal communities, such as epiphyton and epilithon are dominant in agricultural streams while phytoplankton is the dominant algal community in LSWB. Therefore, studying the responses of these dominant algal communities in respective freshwater ecosystems may increase our understanding of the impacts of multiple stressors on ecosystem health and integrity.

This study aims to understand impacts of multiple stressors, particularly hydrological disturbances, nutrient enrichment, and pesticide contamination on algal communities in freshwater ecosystems in rural areas dominated by agricultural land use. We conducted (i) a thorough literature review, (ii) a field study in agricultural streams, (iii) a field study in LSWB and (iv) a microcosm experiment to gain a comprehensive understanding of the topic. The key findings of this study are:

(i) Epiphytic biofilms are understudied in freshwater ecosystems. Epiphytic biofilms and their interactions with macrophytes are essential to understand, maintain, and improve freshwater ecosystem health and integrity.

(ii) Epiphyton and epilithon communities show distinct structural differences during an annual cycle in agricultural streams in terms of biomass, algal composition, and diatom species composition. Structural properties of epiphyton are less affected by hydrological regimes and water nutrient concentration than epilithon, indicating that epilithon are more dependent on ambient nutrients while epiphyton can take advantage of macrophyte leachates. Light, temperature, and dissolved organic carbon are other key variables that drive structure of epiphyton and epilithon in agricultural streams.

(iii) Pesticides and nutrient concentrations are concurrent stressors in LSWB. High pesticide toxicity and PO_4-P concentrations can shift phytoplankton community composition to less-sensitive, fast-adapting generalists. Functional features can be altered by nutrient concentrations and pesticide toxicity leading to negative and positive feedback on the functionality of ecosystems from the former and latter stressors, respectively. However, these positive responses of pesticide toxicity on phytoplankton most likely occur due to the low level of pesticide concentrations and indirect positive effects of pesticides (i.e., suppression of grazing pressure due to insecticides) in the studied LSWB. Water level change, electrical conductivity, and dissolved oxygen also play important roles in shaping phytoplankton species composition and functional features.

(iv) As highlighted in the field study, pesticide toxicity on phytoplankton communities is primarily governed by herbicides. Environmentally realistic concentrations of two common herbicides, metazachlor and flufenacet, cause structural changes in phytoplankton community composition, taxonomic diversity, and functional features. 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 activities or abiotic factors. Light and temperature play an important role in shaping the phytoplankton communities under herbicide exposure. Categorizing data according to the mode of action of the pesticides will be helpful to disentangle effects on non-target aquatic biota, especially in field studies where we encounter contamination from multiple pesticides in high concentrations.

Overall, hydrological disturbances and agrochemicals significantly influence the structure of the algal communities [i.e., biomass, algal composition (green algae, cyanobacteria, and diatom concentrations), species composition, trait composition, taxonomical and functional diversity] in freshwater ecosystems in rural areas. Addressing the interactions within algal communities, interactions with their substrates, and interactions with other biota are necessary to gain a better understanding of the underlying controlling mechanisms of the algal community structure and to draw a holistic picture of the consequences of multiple stressors in these ecosystems.

Zusammenfassung

Ländliche Tieflandgebiete sind durch eine gemischte Landnutzung (z. B. Wald, Grünland, Wohngebiete) gekennzeichnet, die meist von landwirtschaftlichen Flächen dominiert wird. Die verstreuten Süßwasser-Ökosysteme in diesen ländlichen Gebieten sind für viele Zwecke, wie zum Beispiel die Trinkwasserversorgung, die Bewässerung in der Landwirtschaft, den Lebensunterhalt, die Grundwasseranreicherung, den Hochwasserschutz und die Freizeitgestaltung, wichtig. Lotische Systeme, wie landwirtschaftliche Bäche, die durch Ackerland und Bauernhöfe fließen, und lentische Kleingewässer (LSWB) spielen dabei eine wichtige Rolle. Dennoch wird diesen kleineren lotischen und lentischen Ökosystemen weniger Aufmerksamkeit geschenkt als den großen Flüssen und Seen, die eine große Anziehungskraft ausüben.

Landwirtschaftliche Fließgewässer und LSWB sind weltweit weit verbreitete und ökologisch bedeutende Lebensräume. Diese Ökosysteme sind anfällig für zahlreiche auf landwirtschaftliche Praktiken zurückzuführende Stressfaktoren. Die gegenwärtigen Trends in der Landwirtschaft führen zu großen, intensiven Systemen mit geringer Kulturpflanzenvielfalt. Die Wasserentnahme und der Einsatz von Agrochemikalien nehmen daher rapide zu und haben erhebliche Auswirkungen auf die Süßwasserökosysteme in der Umgebung landwirtschaftlicher Flächen. Dies führt dazu, dass hydrologische Störungen, Nährstoffanreicherung und Pestizidkontamination als Mehrfachstressoren für landwirtschaftlich genutzte Fließgewässer und LSWB auftreten, deren Folgen sind jedoch noch unklar.

Algengemeinschaften sind effiziente Indikatoren für die ökologischen Auswirkungen dieser Stressfaktoren, da sie die mikrobielle Artenvielfalt und die Ökosystemfunktion beeinflussen. Periphytische Algengemeinschaften wie Epiphyton und Epilithon sind in von Landwirtschaft umgebenen Fließgewässern vorherrschend, während Phytoplankton die vorherrschende Algengemeinschaft in LSWB ist. Die Untersuchung der Reaktionen dieser vorherrschenden Algengemeinschaften in den jeweiligen Süßwasserökosystemen kann daher zu einem besseren Verständnis der Auswirkungen verschiedener Stressfaktoren auf die Gesundheit und Unversehrtheit der Ökosysteme beitragen.

Diese Studie zielt darauf ab, die Auswirkungen verschiedener Stressoren, insbesondere hydrologischer Störungen, Nährstoffanreicherung und Pestizidkontamination, auf Algengemeinschaften in Süßwasserökosystemen in ländlichen, landwirtschaftlich geprägten

Gebieten zu verstehen. Wir haben (i) eine gründliche Literaturrecherche, (ii) eine Feldstudie in von Landwirtschaft umgebenen Bächen, (iii) eine Feldstudie in LSWB und (iv) ein Mikrokosmos-Experiment durchgeführt, um ein umfassendes Verständnis des Themas zu erlangen. Die wichtigsten Ergebnisse dieser Studie sind:

(i) Epiphytische Biofilme sind in Süßwasserökosystemen nicht ausreichend erforscht. Epiphytische Biofilme und ihre Interaktionen mit Makrophyten sind wichtig, um die Gesundheit und Integrität von Süßwasserökosystemen zu verstehen, zu erhalten und zu verbessern.

(ii) Epiphyton- und Epilithon-Gemeinschaften weisen während eines Jahreszyklus in landwirtschaftlich genutzten Fließgewässern deutliche strukturelle Unterschiede in Bezug auf Biomasse, Algenzusammensetzung und Zusammensetzung der Kieselalgenarten auf. Die strukturellen Eigenschaften von Epiphyton werden von den hydrologischen Bedingungen und der Nährstoffkonzentration im Wasser weniger stark beeinflusst als die von Epilithon, dies deutet darauf hin, dass Epilithon stärker von den Nährstoffen in der Umgebung abhängig ist, während Epiphyton die Auslaugung von Makrophyten nutzen kann. Licht, Temperatur und gelöster organischer Kohlenstoff sind weitere Schlüsselvariablen, die die Struktur von Epiphyton und Epilithon in landwirtschaftlich genutzten Bächen beeinflussen.

(iii) Pestizid- und Nährstoffkonzentrationen sind gleichzeitige Stressfaktoren in LSWB. Hohe Pestizidtoxizität und PO_4-P-Konzentrationen können die Zusammensetzung der Phytoplanktongemeinschaft in Richtung weniger empfindlicher, schnell anpassungsfähiger Generalisten verändern. Funktionelle Merkmale können durch Nährstoffkonzentrationen und Pestizidtoxizität verändert werden, was zu negativen und positiven Rückkopplungen auf die Funktionalität von Ökosystemen durch die erstgenannten Stressoren führt. Diese positiven Reaktionen der Pestizidtoxizität auf das Phytoplankton sind jedoch höchstwahrscheinlich auf die niedrigen Pestizidkonzentrationen und die indirekten positiven Auswirkungen von Pestiziden (d. h. die Unterdrückung des Weidedrucks durch Insektizide) in den untersuchten LSWB zurückzuführen. Wasserstandsänderungen, die elektrische Leitfähigkeit und der gelöste Sauerstoff spielen ebenfalls eine wichtige Rolle bei der Gestaltung der Artenzusammensetzung und der funktionellen Merkmale des Phytoplanktons.

(iv) Wie in der Feldstudie deutlich wurde, wird die Toxizität von Pestiziden auf Phytoplanktongemeinschaften in erster Linie durch Herbizide bestimmt. Zwei gängige

Herbizide, Metazachlor und Flufenacet, bewirken in umweltverträglichen Konzentrationen strukturelle Veränderungen in der Zusammensetzung der Phytoplanktongemeinschaft, der taxonomischen Vielfalt und den funktionellen Merkmalen. Herbizidkonzentrationen von bis zu 0,5 µg L^{-1} in lentischen aquatischen Ökosystemen, die durch ein einziges Ereignis verursacht werden, können in der Regel mindestens vier Wochen lang bestehen bleiben und die Phytoplanktongemeinschaft trotz ihres chemischen Abbaus durch biotische Aktivitäten oder abiotische Faktoren beeinflussen. Licht und Temperatur spielen eine wichtige Rolle bei der Gestaltung der Phytoplanktongemeinschaften unter Herbizidexposition. Eine Kategorisierung der Daten nach der Wirkungsweise der Pestizide wird insbesondere bei Feldstudien, bei denen wir eine Kontamination durch mehrere Pestizide in hohen Konzentrationen feststellen, hilfreich sein, um die Auswirkungen auf aquatische Nicht-Ziel-Biota zu entflechten.

Insgesamt haben hydrologische Störungen und Agrochemikalien einen erheblichen Einfluss auf die Struktur der Algengemeinschaften [d. h. Biomasse, Algenzusammensetzung (Grünalgen, Cyanobakterien und Kieselalgenkonzentrationen), Artenzusammensetzung, Merkmalszusammensetzung, taxonomische und funktionelle Vielfalt] in Süßwasserökosystemen in ländlichen Gebieten. Die Wechselwirkungen innerhalb der Algengemeinschaften, die Wechselwirkungen mit ihren Substraten und mit anderen Biota müssen untersucht werden, um ein besseres Verständnis der zugrundeliegenden Kontrollmechanismen für die Struktur der Algengemeinschaften zu erlangen und ein ganzheitliches Bild der Folgen der vielfältigen Stressfaktoren in diesen Ökosystemen zu schaffen.

Contents

Chapter 1 General Introduction

1.1 Freshwater ecosystems in rural areas

Agriculture is a predominant mode of land use in many temperate lowland rural areas (Kanianska, 2016; Winkler et al., 2021). In the agricultural landscape freshwater ecosystems such as ditches, streams, ponds, and lakes (Fig. 1.1) are essential to (i) irrigate crops, (ii) supply water for livestock, and (iii) drain water from agricultural fields (Oenema et al., 2005).

1.1.1 Lotic ecosystems

Lotic ecosystems surrounded by agricultural areas are known as "agricultural streams". They play an ecologically significant role by supporting a unique biota and biodiversity (Moore and Palmer, 2005; Williams et al., 2004), nutrient cycling and transport (Bouraoui and Grizzetti, 2011; Comer-Warner et al., 2020), sediment transport (Stenfert Kroese et al., 2020), carbon cycling and flux dynamics (Cornejo et al., 2020), and transporting pesticides and other contaminants (Weber et al., 2018). The ecological significance of agricultural streams has been more widely studied compared to lentic ecosystems in similar land use areas.

1.1.2 Lentic ecosystems

Lentic freshwater ecosystems are a main part of dense hydrological networks in rural areas. Among them, lentic small water bodies (LSWB) are abundant globally (Hill et al., 2018). For example, 8.6 % of ponds and lakes by area globally consist of very small lentic water bodies encompassing a surface area < 0.001 km^2 (Holgerson and Raymond, 2016). Particularly, the North Sea region has a large number of shallow and highly eutrophicated lentic water bodies in agricultural areas (Adrian et al., 2016). LSWB can be defined as stagnant water bodies with a surface area between 1 m^2 and 20000 m^2, natural or manmade, perennial or seasonal (Biggs et al., 2005b; Hill et al., 2018). They possess a high ecological significance due to (i) unique and high biodiversity (Davies et al., 2008; Gagné and Fahrig, 2007; Hill et al., 2017), (ii) providing of refugia for biota in human intervened landscapes (Chester and Robson, 2013), (iii) the storage/sink of pollutants coming from catchments (Biggs et al., 2005b), (iv) flood control (Takamura, 2012), and (v) their contribution to carbon flux in ecosystems (Gilbert et al., 2021; Holgerson and Raymond, 2016). Despite the importance of LSWB in agricultural areas, they are still largely neglected in environmental monitoring and conservation policy frameworks and legislation (Hill et al., 2018).

Fig. 1.1: Freshwater ecosystems in rural areas, which are focused on this study. A and C are lotic ecosystems or agricultural streams. B and D are lentic small water bodies (LSWB).

1.2 Multiple stressors in freshwater ecosystems

Many recent studies have focused on stressors in freshwater ecosystems in rural landscapes. In the last decade, researchers tended to study more than one simultaneous stressor at a time and the topic of 'multiple stressors' came into discussion, for example, from current collaborative research projects such as RESIST (https://sfb-resist.de). The understanding of combined effects of multiple stressors due to anthropogenic interventions on ecosystem health and integrity is a pressing need and an unprecedented challenge. Individual stressors may lead to linear negative responses while multiple stressors may result in enigmatic effects showing additive, synergistic or antagonistic responses (Piggott et al., 2012). Lotic and lentic freshwater ecosystems in rural landscapes are subjected to specific concurrent stressors, such as changes in hydrological regimes and exposure to agrochemicals, such as pesticides and nutrients. Soil erosion associated with agriculture is an additional stress on these ecosystems resulting increased sedimentation, turbidity, and alterations in hydrology (Stenfert Kroese et al., 2020; Sutherland et al., 2012). Agricultural runoff further degrades water quality in freshwater ecosystems. Having different agricultural practices (e.g., crops and livestock) in a catchment leads to complex and diverse stressors on freshwater ecosystems and produces point and non-point sources of pollutants (e.g., pesticides and fertilizer: Brenner et al., 2005; Ulrich et al., 2021). In addition, freshwater ecosystems are vulnerable to climate change impacts, such as increasing temperature and extreme events (Blackburn and Stanley, 2021; McDowell et al., 2017). Although increasing temperature, sedimentation, nutrient enrichment, and pesticide toxicity have been studied as multiple stressors in agricultural streams (Cornejo et al., 2019; Munn et al., 2018; Piggott et al., 2012), similar studies focussing on LSWB are still lacking.

1.2.1 Hydrological disturbances

Hydrological disturbances are one of the main stressors in both lentic and lotic freshwater ecosystems in rural areas (Riis and Biggs, 2003; Wu et al., 2019). Water level is the key hydrological component in lentic ecosystems, while in lotic ecosystems, hydrology becomes more complex as it varies in different aspects such as water level, discharge, frequency/duration of high/low flow events, and timing (Poff et al., 1997). For example, Ulrich et al. (2018) studied LSWB in a rural, agriculture-dominant lowland landscape in Northern Germany (i.e., the Kielstau catchment) and observed significant water level change in association with precipitation, which was responsible for pesticide dilution or concentration in LSWB. In lowland agricultural streams, the effects of hydrological regimes on biofilms have

been studied comprehensively (Guo et al., 2020; Wu et al., 2019). Among the hydrological components, median daily flow and short-term hydrological regimes seem to be strongly associated with structure of biotic communities under multiple stressor conditions (Guo et al., 2020; Qu et al., 2018a; Qu et al., 2018b; Wu et al., 2019).

1.2.2 Agrochemicals – pesticides and nutrients

Pesticides are often used for crop management in agriculture. Herbicides, fungicides, and insecticides are the main groups of pesticides depending on the target organisms to control. Intensive agriculture in rural areas is the main source of pesticide discharge to freshwater ecosystems. For example, the exceedance of pesticide risk thresholds decreased 3.7-fold in areas without agricultural land use (Szöcs et al., 2017). Neumann et al. (2003) detected high pesticide concentrations, (130 μg L^{-1} prosulfocarb, 92 μg L^{-1} metamitron, and 51 μg L^{-1} ethofumesate) in drainage channels and outlets from agricultural fields which flow into the surrounding freshwater ecosystems. These inflows contaminate both water and sediment. For example, Munn et al. (2018) detected 131 different pesticides in water and streambed sediment samples in agricultural streams and some of them were high in concentration (e.g., 2.15 μg L^{-1} acetanilide/amides and 0.9 μg L^{-1} triazines). Furthermore, pesticides have been reported in LSWB in higher concentrations than agricultural streams (e.g., 10.14 μg L^{-1} metazachlor) (Ulrich et al., 2018). Pesticide contamination, and their fate in freshwater habitats depends on many factors, such as precipitation, soil characteristics, topography, land-water interface characteristics, physicochemical properties of pesticides, and characteristics of the water body (Ulrich et al., 2018).

Dominant agricultural areas in rural landscapes are the main source of nutrients to freshwater ecosystems due to the high use of fertilizer to enhance crop growth and the high input of manure (Bouraoui and Grizzetti, 2011; Oenema et al., 2005; Weigelhofer, 2017). For example, agricultural areas in Europe are responsible for up to 80% of the nitrogen (N) and 40% of the phosphorous (P) runoff to river networks (Bouraoui and Grizzetti, 2011). High N and P runoff to freshwater ecosystems is not only occurring in Europe but is a global problem (Carpenter et al., 1998; Withers et al., 2014). For example, according to Lavoie et al. (2004) agricultural streams in Canada received 6-fold higher N and 9-fold higher P levels compared to reference sites. Nutrient enrichment is, thus, one of the main stressors in freshwater ecosystems.

1.3 Algal communities

Algae play a crucial role in freshwater ecosystems as they are the basal resource for higher trophic levels. Furthermore, microalgal groups are important as ecological indicators due to their high sensitivity to environmental gradients (Bellinger and Sigee, 2015; Wu et al., 2017). Easy sampling and well-known autecology further facilitate the use of micro algae as the ideal candidate for environmental monitoring and bio-assessments (Wu et al., 2017). Different algal communities have become dominant and important in different ecosystems to reveal stress-response relationships. For example, in lotic ecosystems "periphyton" algal communities are important (Larned, 2010), while in lentic ecosystems "phytoplankton" are vital (They et al., 2014) (Fig. 1.2).

1.3.1 Periphyton

Algae which are attached to submerged living or inert surfaces are called "periphyton" (Gubelit and Grossart, 2020; Larned, 2010). Periphyton are important ecological indicators in lotic freshwater ecosystems (Moresco and Rodrigues, 2014; Vis et al., 1998). Responses of periphytic communities in agricultural streams to multiple stressors have been studied. For example, Piggott et al. (2012) studied the impact of increasing temperature, sediment addition, and nutrient enrichment on periphytic algal communities in agricultural streams. Periphyton communities can be further specified according to their preferred substrate as macrophytes: epiphyton, sand: epipsammon, stone/rock: epilithon, and sediment: epipelon (Gubelit and Grossart, 2020).

1.3.1.1 Epiphyton

Epiphyton in agricultural streams are studied less than the other periphytic ecosystems. Structural changes in the epiphyton community are generally associated with the environmental variables such as water level, flow velocity, light intensity, temperature, pH, conductivity, dissolved oxygen, turbidity, nutrients, and chloride concentrations (Adam et al., 2017; Eriksson, 2001; Hempel et al., 2009; Lévesque et al., 2017; Morin and Kimball, 1983; Phiri et al., 2007). Biotic interactions with host macrophytes and grazing are also important in understanding of epiphyton responses (Wijewardene et al., 2022).

1.3.1.2 Epilithon

Epilithon community gained focus on multiple stressor studies in the agricultural streams (Piggott et al., 2012). Structural changes in the epilithon community are strongly linked with environmental variables, such as hydrology, temperature, nutrients, alkalinity, conductivity, suspended solids, and biological oxygen demand and biotic interactions such as grazing and competition (Casartelli and Ferragut, 2018; Guo et al., 2020; Winter and Duthie, 2000).

1.3.2 Phytoplankton

Phytoplankton are key phototrophic drifting organisms in freshwater ecosystems involved in primary production, trophic interactions, energy flow, and nutrient cycling (Brierley, 2017; Meng et al., 2020). They are the main primary producers in LSWB and an ideal model community to understand effects of multiple stressors in these ecosystems. Phytoplankton in LSWB are sensitive to environmental variables, such as water level, light availability, temperature, pH, conductivity, dissolved oxygen, nutrient concentrations, and various other biotic selection pressures, such as grazing and competition (Çelekli et al., 2014; Celewicz-Goldyn and Kuczynska-Kippen, 2017; Celewicz and Gołdyn, 2021; Chia et al., 2011; Zębek and Szymańska, 2017).

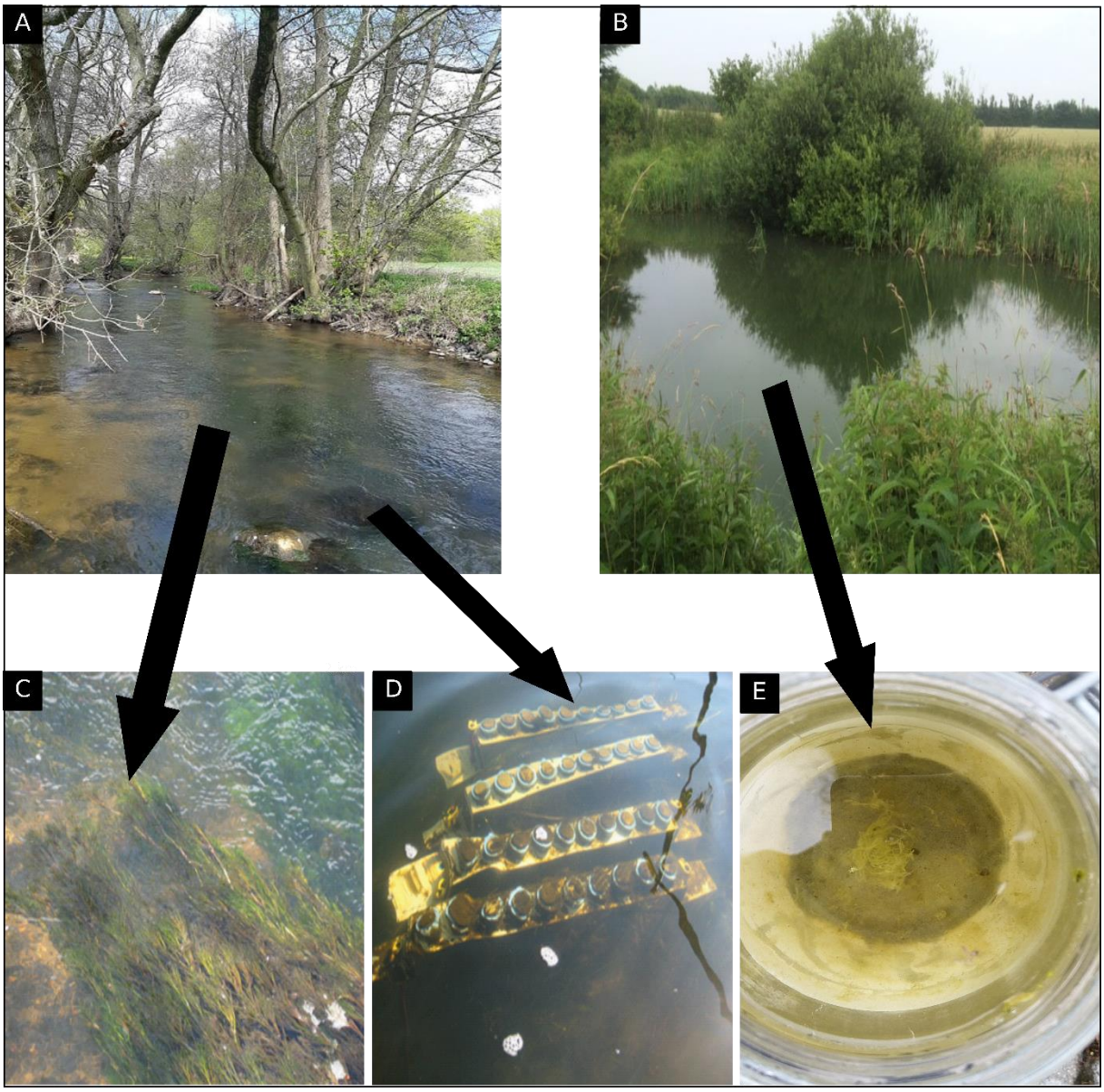

Fig. 1.2: Dominant algal communities in agricultural streams (A) and LSWB (B) in rural lowland areas of Aarhus, Denmark and the Kielstau catchment in Germany: epiphyton (C), epilithon (D), and phytoplankton (E).

1.4 Responses of algal communities to multiple stressors

Responses of algal communities to multiple stressors can be measured or studied using different macro to micro scale parameters, such as biomass (chlorophyll-*a* or ash-free dry mass), elemental stoichiometry (e.g., C: N: P ratio), concentration of different algal groups using pigment analyses (e.g., fucoxanthin concentration as a proxy for diatom concentration), species composition, and trait composition (Bellinger and Sigee, 2015; Steinman et al., 2017). Furthermore, species and trait composition can be integrated to indices, such as taxonomic diversity indices and functional features, to understand consequences of the multiple stressors in ecosystem health and integrity.

1.4.1 Species and trait composition

Changes in species composition is an initial response of the biotic communities to stressors (Butchart et al., 2010). Sensitive species will disappear from the community and only tolerant species can be persistent under particular stressors. Changes in the phytoplankton community due to multiple stressors in lentic freshwater ecosystems have been identified and consequences have been visible through the trophic levels, influencing ecosystem structure, function, and integrity (Taherzadeh et al., 2019). For example, Vinebrooke et al. (2003) found that acidification in lakes shifted the phytoplankton community towards larger, acid-tolerant dinoflagellates and filamentous green algae. They suggested that when global warming acts as a simultaneous stressor with acidification, the phytoplankton community further shifts towards a dinoflagellate-dominated system, which could have adverse impacts on energy transfer through food webs and ecosystem resilience. Therefore, changes in the species composition of algal communities are a key indicator for understanding the effects of multiple stressors on freshwater ecosystems.

Trait composition of algal communities also provides insights on the consequences of multiple stressors. Cottingham (1999) observed the effect of nutrient enrichment and zooplankton grazing as concurrent stressors on cell size of the phytoplankton communities in lakes. They witnessed that “increased phosphorus loading, and increased zooplankton size had positive effects on large phytoplankton, slope of size spectra and mean phytoplankton size, but negative effects on the relative abundance of small phytoplankton” (Cottingham, 1999, p. 810). Functional traits of algal communities are the key to maintain biodiversity and link to ecosystem functioning. Petchey and Gaston (2006) stated that “all traits are important for the function of

interest and no traits are functionally uninformative" (p. 743). Cell size, lifeform, ecological guild, motility, and spore formation are key functional traits of the algae used for the understanding of functional diversity and ecosystem functionality under multiple stressors (Table 1.1, adopted from Wu et al. (2017)).

Table 1.1: Algal traits and their expected responses to selected multiple stressors such as pesticides, nutrients, and hydrology. This table is adopted from Wu et al. (2017).

Traits	Categories	Expected responses under selected stressors (pesticides, nutrients, and hydrology)
Cell size (Abonyi et al., 2018; Kruk et al., 2017; Qu et al., 2018a; Rimet and Bouchez, 2012)	Nano (5-100 µm^3) Micro (100-300 µm^3) Meso (300-600 µm^3) Macro (600-1500 µm^3) Large (> 1500 µm^3)	Smaller cells have an advantage under nutrient-limiting and high flow conditions due to their higher nutrient uptake rates and growth rates that allow greater resilience to environmental stressors. Larger cells show a converse trend.
Life form (Abonyi et al., 2018; Kruk et al., 2017)	Colonial Filamentous Unicellular Flagellates	Filamentous algae have an advantage in resource gathering under nutrient limited environments, but they are susceptible to high flow disturbances. Unicellular algae have an advantage in depositional environments and high resource conditions.
Ecological guild (Rimet and Bouchez, 2012)	Low profile High profile Motile Planktonic	Low profile taxa have an advantage in low resource and high flow conditions. High profile taxa show the converse trend. Motile and planktonic taxa have an advantage in resource gathering, low flow, and depositional conditions, and can avoid pollutants.
Motility (Lange et al., 2016; Witteveen et al., 2020)		Motile taxa can actively move away from pollutants and have an advantage in low flow and depositional conditions.
Spore formation (Lange et al., 2016; Witteveen et al., 2020)		Spore forming taxa have an advantage in unfavourable conditions.

1.4.2 Taxonomic diversity indices

Traditional taxonomic diversity indices, including (i) alpha diversity indices (e.g., genus/species richness, the Shannon-Wiener index, the Simpson index, and evenness) and (ii) beta diversity indices (e.g., the Bray-Curtis index), are helpful to understand implications of the multiple stressors on biodiversity of the ecosystems (Wang et al., 2020). For example, Filiz et

al. (2020) found that under high nutrient concentrations, the genus richness of phytoplankton communities reduced regardless of the other simultaneous stressors, such as increasing temperature and the heatwave effect.

1.4.3 Functional features: functional diversity/redundancy indices

Functional features of algal communities can be expressed in both functional diversity (FD) and redundancy (FR) indices. FD and FR are components of biodiversity which give insights on ecosystem functionality. FD implies the range of things that organisms do in communities and ecosystems (Petchey and Gaston, 2006). Several studies provide different definitions of functional diversity, i.e., (i) 'the functional multiplicity within a community' (Tesfaye et al., 2003), (ii) 'the number, type and distribution of functions performed by organisms within an ecosystem' (Díaz and Cabido, 2001), and (iii) 'the value and range of those species and organismal traits that influence ecosystem functioning' (Tilman, 2001). FR indicates the ability of compensating species loss by other species who perform a similar function in the ecosystem (Fetzer et al., 2015). Therefore, FD and FR provide insights on ecosystem stability and persistency.

FD mainly represents four main indices, i.e., functional richness (FRic), functional evenness (FEve), functional divergence (FDiv), and functional dispersion (FDis) (Table 4.2). FRic implies how much of the functional niche space is filled by the existing species (Mouchet et al., 2010). FEve indicates how regularly functional niche space is filled by the existing species (Mouchet et al., 2010). FDiv shows how far species abundances of existing species distribute from the centre of functional niche space (Mouchet et al., 2010). In other words, FDiv implies whether abundance distribution in trait space maximizes towards the extremes or the centre of the trait space (Karadimou et al., 2016). FDis measures the degree of functional dissimilarity in the trait space among the species of the community (Karadimou et al., 2016). Among these four indices only FRic does not consider species abundances in calculations and all indices are statistically independent from each other and taxonomic diversity indices (Mason et al., 2005).

Under multiple stressors, it is essential to have species that can compensate for the functions of the losing species due to one stressor to keep ecosystem functionality intact. FR indices are the indicators of the above-mentioned mechanism which links diversity with ecosystem stability and provides insights on future ecosystem resistance or resilience potentials

(Bruno et al., 2016). There are many methods to measure FR like FR indices, such as (i) FR01: difference between taxonomic diversity and FD (i.e., the difference between the Simpson diversity index and Rao's quadratic entropy), (ii) FR02: the mean number of species per functional group (FG), and (iii) FR03: the difference between taxonomic species evenness and functional evenness (i.e., FEve) (Table 1.2, adopted from Wu et al. (2019)). FGs can be determined by the classification of the species by means of Ward's clustering (k-means) method and the optimum number of FGs can be established by the Calinski-Harabasz criterion (Pomerleau et al., 2015). Further details on FD and FR indices can be found in Bruno et al. (2016) and Cadotte et al. (2011).

Table 1.2: Functional features: functional diversity and redundancy indices. This table is adopted from Wu et al. (2019).

Functional features	Codes	Description
Functional diversity (FD)	FRic	Functional Richness
	FEve	Functional Evenness: weights the pairwise distances by the summed relative abundance of species i and j.
	FDis	Functional Dispersion: sums the abundance-weighted functional dispersion of the response traits.
	FDiv	Functional Divergence: represents how abundance is distributed in multivariate trait space.
Functional redundancy (FR)	FR01	Simpson taxonomic diversity index-Rao's quadratic entropy (Rao's quadratic entropy: standardized by the maximum value to constrain the values within the range of 0 - 1).
	FR02	Species richness/number of Functional Groups (i.e., FG).
	FR03	Species evenness-Functional evenness (i.e., FEve).

1.5 Motivation

1.5.1 Research gaps

Freshwater ecosystems in lowland rural areas dominated by agricultural land use are typically subjected to multiple stressors. Lotic ecosystems, particularly agricultural streams, have gained little attention on evaluating the impacts of multiple stressors (Cornejo et al., 2019; Piggott et al., 2012) and LSWB have been totally neglected on this topic. Hydrology (e.g., water level and discharge) and agrochemicals (e.g., pesticides and nutrients) are important concurrent stressors in these ecosystems (Cornejo et al., 2019; Munn et al., 2018) and their impacts on aquatic biota are still not fully investigated. Also, available studies on these multiple stressors mostly focused on responses of macroinvertebrate communities (e.g., Cornejo et al., 2019; Davis et al., 2018; Juvigny-Khenafou et al., 2021). Algal communities are key indicators to assess effects of hydrology and agrochemicals on ecosystem structure, function, and integrity (Andrus et al., 2013; Munn et al., 2018). Studies focusing on the responses of dominant algal communities in specific freshwater ecosystems (e.g., lentic ecosystems: phytoplankton, lotic

ecosystems: epiphyton and epilithon) in rural areas under hydrology and agrochemicals as multiple stressors are rare (Fig. 1.3).

Periphyton communities are dominant in lowland agricultural streams and studies on multiple stressors in these ecosystems have mostly focused on the epilithon community. However, macrophytes are often abundant in agricultural streams and, therefore, epiphyton communities are as important as the epilithon community. But what do we know about epiphyton communities in freshwater ecosystems? Studies of epiphyton communities in freshwater ecosystems are scattered and no comprehensive review exists bridging available literature. Therefore, reviewing current literature is necessary to identify knowledge gaps in the subject matter and to design future studies.

Epiphyton studies in agricultural streams are rare and restricted to only a few months in the summer or one sampling in each season. Furthermore, hydrology itself has been recognized as a key driver for benthic algal communities in agricultural streams (Guo et al., 2021; Guo et al., 2020; Wu et al., 2019). Structural responses of the epilithic diatom community were more strongly associated with short-term hydrological indices than with physicochemical variables (Guo et al., 2020). A similar understanding is lacking on epiphyton. Additionally, concurrent evaluations of both epiphyton and epilithon in same natural stream ecosystems are rare. Lastly, although nutrient enrichment is a typical simultaneous stressor in agricultural streams, there are no attempts to combine high frequency (daily) hydrology and nutrient data to understand their contribution on controlling structural properties of epiphyton and epilithon in agricultural streams continuously over one year.

In addition to these knowledge gaps, there is little known about LSWB (Biggs et al., 2005b). Available ecological studies in LSWB focused mostly on amphibians (e.g., Gagné and Fahrig, 2007) and macroinvertebrate communities (e.g., Onandia et al., 2021). Pesticide contaminations and high nutrient concentrations are often reported in these ecosystems (Indermuehle et al., 2008; Ulrich et al., 2018). Though they are globally abundant and ecologically significant habitats, to the best of our knowledge, there are no studies to tackle impacts of multiple stressors (e.g., pesticides, nutrients, and water level change) particularly focused on the phytoplankton community, which is the dominant algal community in LSWB. Phytoplankton community composition and functional features can be used to understand the effects of multiple stressors on biodiversity and ecosystem functioning in LSWB and will be essential to manage LSWB sustainably.

Field studies on LSWB in German lowland rural areas revealed that metazachlor and flufenacet are the most frequently contaminating herbicides in these ecosystems (Ulrich et al., 2018; Ulrich et al., 2021). A few studies have reported about metazachlor toxicity on non-target biotic communities in aquatic ecosystems, such as fish (Velisek et al., 2020), macrophytes, and plankton communities (Mohr et al., 2008). However, to the best of our understanding, there are no studies about flufenacet with this scope. Changes in species composition of the phytoplankton community is evident due to metazachlor exposures under the lowest tested concentrations in previous studies (5 µg L^{-1}: Mohr et al. (2008) and 32 µg L^{-1}: Noack et al. (2003)). There are no specific studies to tackle direct effects of metazachlor and flufenacet on phytoplankton taxonomic and functional diversity. Understanding the overall community response 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.

1.5.2 Research questions

Overall, lotic and lentic freshwater ecosystems in rural areas dominated by with agricultural land use are subjected to concurrent stressors, such as hydrological disturbances, nutrient enrichment and pesticide contamination. Addressing these "multiple stressors" is needed to understand impacts on aquatic biota instead of focusing on individual stressors separately. Dominant algal groups in these ecosystems are ideal candidates to study multiple stressors. Knowing the above-mentioned knowledge gaps associated with the multiple stressors on algal communities in freshwater ecosystems in rural areas, it is understood that studies are needed to address the knowledge gaps swiftly. A comprehensive understanding of impacts on algal communities, which are the main primary producers in lotic and lentic freshwater ecosystems in rural areas, will help us to foresee consequences at the ecosystem level, which is under constant threat of anthropogenic pressures, holistically.

The main aim of the study is, therefore, to determine impacts of multiple stressors on algal communities in freshwater ecosystems in rural areas dominated by agricultural land use. In this dissertation, the main research questions addressed are:

(i) What do we know about epiphyton in freshwater ecosystems and what are their interactions with macrophytes?

(ii) What are the effects of multiple stressors on epiphyton and epilithon in agricultural streams?

(iii) What are the effects of multiple stressors on phytoplankton in lentic small water bodies in agricultural areas?

(iv) What are the effects of the herbicides, metazachlor and flufenacet, on phytoplankton?

Fig. 1.3: Schematic diagram of the focus of the PhD project, "Impacts of multiple stressors on algal communities in freshwater ecosystems in rural areas". Selected multiple stressors were hydrological disturbances (A) and agrochemicals (B). Responses were studied on periphyton (C) and phytoplankton (D).

1.6 Introduction to methods of characterization of algal communities

1.6.1 Sampling of algal communities

Different methods were followed to collect epiphyton, epilithon, and phytoplankton communities in this study (Fig. 1.4). Epiphyton and epilithon were sampled every three weeks (21 days) in the two agricultural streams for a one-year period (February 2019 to January 2020). For epiphyton, 10-15 apical shoots (5 cm long) of *Ranunculus aquaticus* (predominant macrophyte in lowland agricultural streams in Denmark) were harvested to have a composite sample of epiphyton. For epilithon, inorganic fritted glass disks (3.8 cm^2; catalogue no. 528-042; LECO Corporation, St Joseph, Michigan) deployed six weeks prior to sampling were collected (Steinman et al., 2017). In the field, all samples were immediately placed in a dark container with stream water and transported to the laboratory. Epiphyton attached to the macrophytes were extracted by gently brushing stems and leaves. For epilithon, the biofilms from the disks were removed through careful brushing. Phytoplankton samples were collected from the LSWB following the standard protocols mentioned in Wu et al. (2011). 20 L of water was filtered using phytoplankton nets (mesh size: 20 μm) to collect phytoplankton samples from the LSWB.

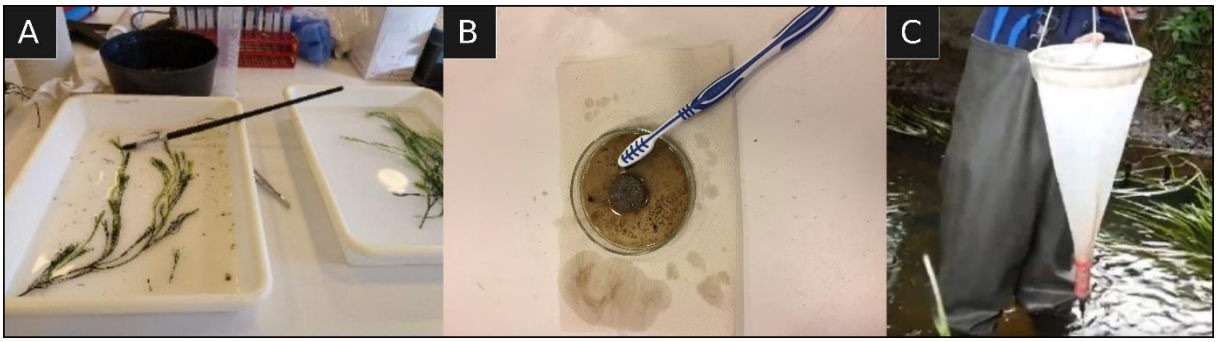

Fig. 1.4: Sampling methods of different algal communities: epiphyton (A), epilithon (B), and phytoplankton (C).

1.6.2 Determination of biomass

Ash-free dry mass (AFDM) and chlorophyll-*a* (Chl-a) are the most common biomass measurements which provide insights on total and autotrophic biomass, respectively. Chl-a was extracted to 95% ethanol from triplicate aliquot samples and estimated according to the method described in Steinman et al. (2017). The AFDM of biofilms was also measured according to the

method recommended in Steinman et al. (2017). The Autotrophic Index (AI) is the proportion between AFDM and Chl-a (Steinman et al., 2017) and describes the trophic nature (heterotrophic: autotrophic composition) of the biofilm such that values ≥ 200 indicate heterotrophic associations, whereas values below this point indicate an autotrophic nature (Lakatos, 1989).

1.6.3 Determination of different algal groups

The methods described in Li et al. (2002) were used to determine algal composition in terms of diatoms, green algae, and cyanobacteria (blue green algae) through pigment analysis. Pigment analysis is an ideal way to determine the algal composition of samples in slurry form (i.e., epiphyton and phytoplankton) (Fig. 1.5A). Algal samples were filtered through glass fibre membrane filters (Whatman^{TM} GF/F, 0.7 µm) which were frozen at -18 ^{o}C until the pigment extraction. For pigment extraction, defrosted membrane filters were placed in 6 mL acetone and shaken at 4 °C under dark conditions for 8 h. The supernatant of the samples was used for pigment analysis by high performance liquid chromatography (HPLC) following high speed centrifugation. The HPLC system included a Thermo SCIENTIFIC Dionex UltiMate 3000 pump (flow rate: 1 mL min^{-1}), a Diode array detector, an autosampler (20 µL sampling loop, at 4 °C), and a column compartment (Column Luna, 3 µm C8). Fucoxanthin, chlorophyll-*b*, and zeaxanthin were selected as marker pigments for diatoms, green algae, and cyanobacteria, respectively (Li et al., 2002). Algal composition of epilithon in corresponding algal groups were obtained by an *in-situ* fluorometer (BenthoTorch, bbe Moldaenke, Schwentinental, Germany) (Fig. 1.5B). The BenthoTorch has been shown to compare well with lab-derived conventional spectrophotometric/HPLC-based methods (Kahlert and McKie, 2014; Steinman et al., 2017).

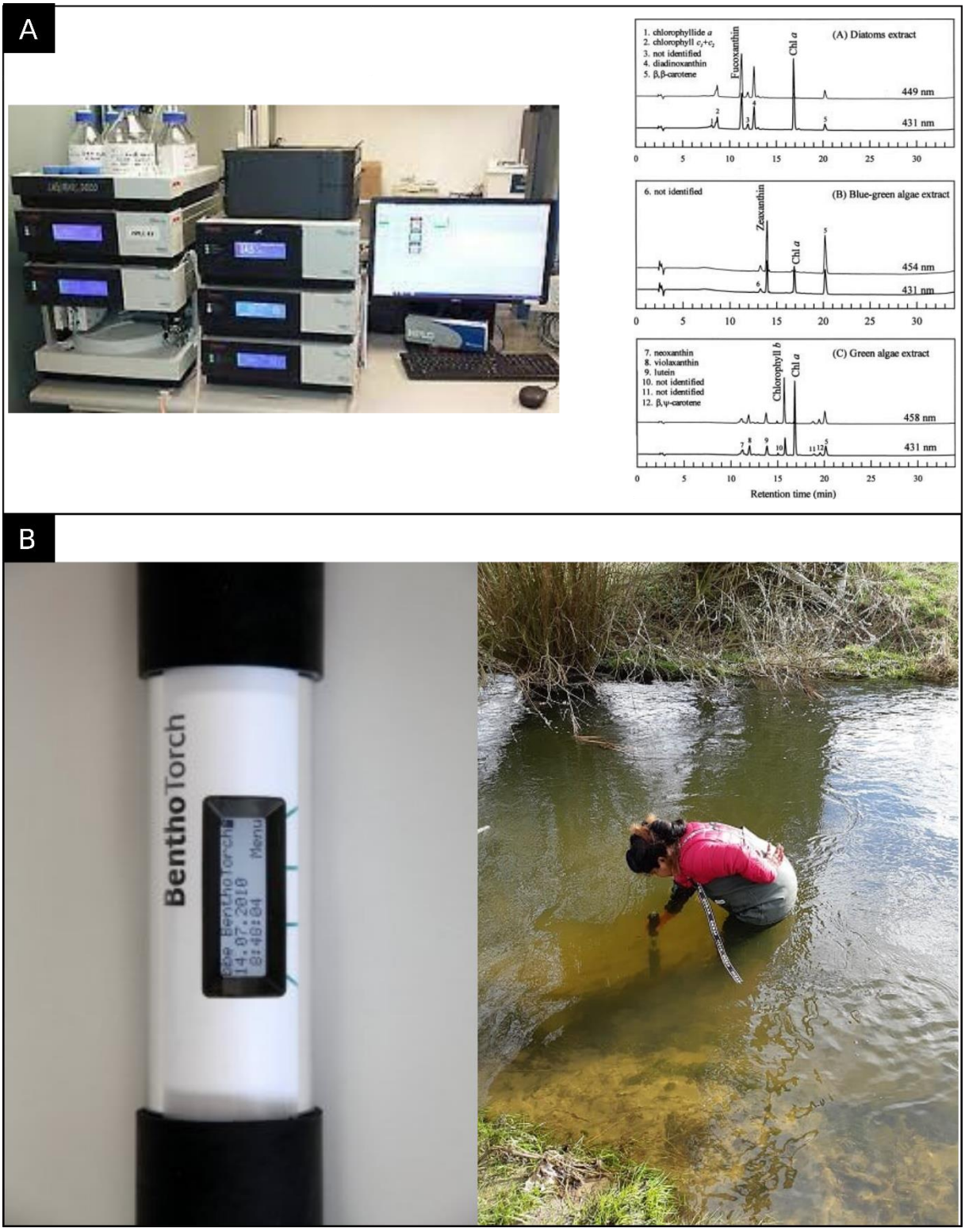

Fig. 1.5: Determination of different algal groups (i.e., green algae, blue green algae, and diatoms) in algal communities. Two different methods can be used e.g., epiphyton/phytoplankton: pigment analysis (A) (adopted from Li et al. (2002)) and epilithon: fluorescence measurements by Benthotorch (B).

1.6.4 Species composition

Algal samples preserved in Lugol's iodine were used to identify algae species (Fig. 1.6). Non-diatom soft microalgae were observed using an optical microscope (Nikon Eclipse E200-LED, Tokyo, Japan) under a magnification of 400×. Identification of algae were performed down to the species level based on current taxonomical criteria (Burchardt, 2014; Cantonati et al., 2017; Hu and Wei, 2006). The nomenclature follows criteria described in Guiry and Guiry (2020).

To identify diatoms, permanent slides were prepared (Fig. 1.6). For the oxidization processes of organic materials in the samples, 5 mL of 30% hydrogen peroxide [H_2O_2] and 0.5 mL of 1 mol/L hydrochloric acid [HCl] were used. After oxidation, 0.1 mL of the diatom-ethanol mix was transferred on a 24 × 24 mm cover slip and a drop of Naphrax was used to mount the slide. Diatoms were identified with an optical microscope (Nikon Eclipse E200-LED, Tokyo, Japan) under 1000× magnification with oil immersion based on the identification guides by Bey and Ector (2013), Hofmann et al. (2011), Cantonati et al. (2017), and Bąk et al. (2012).

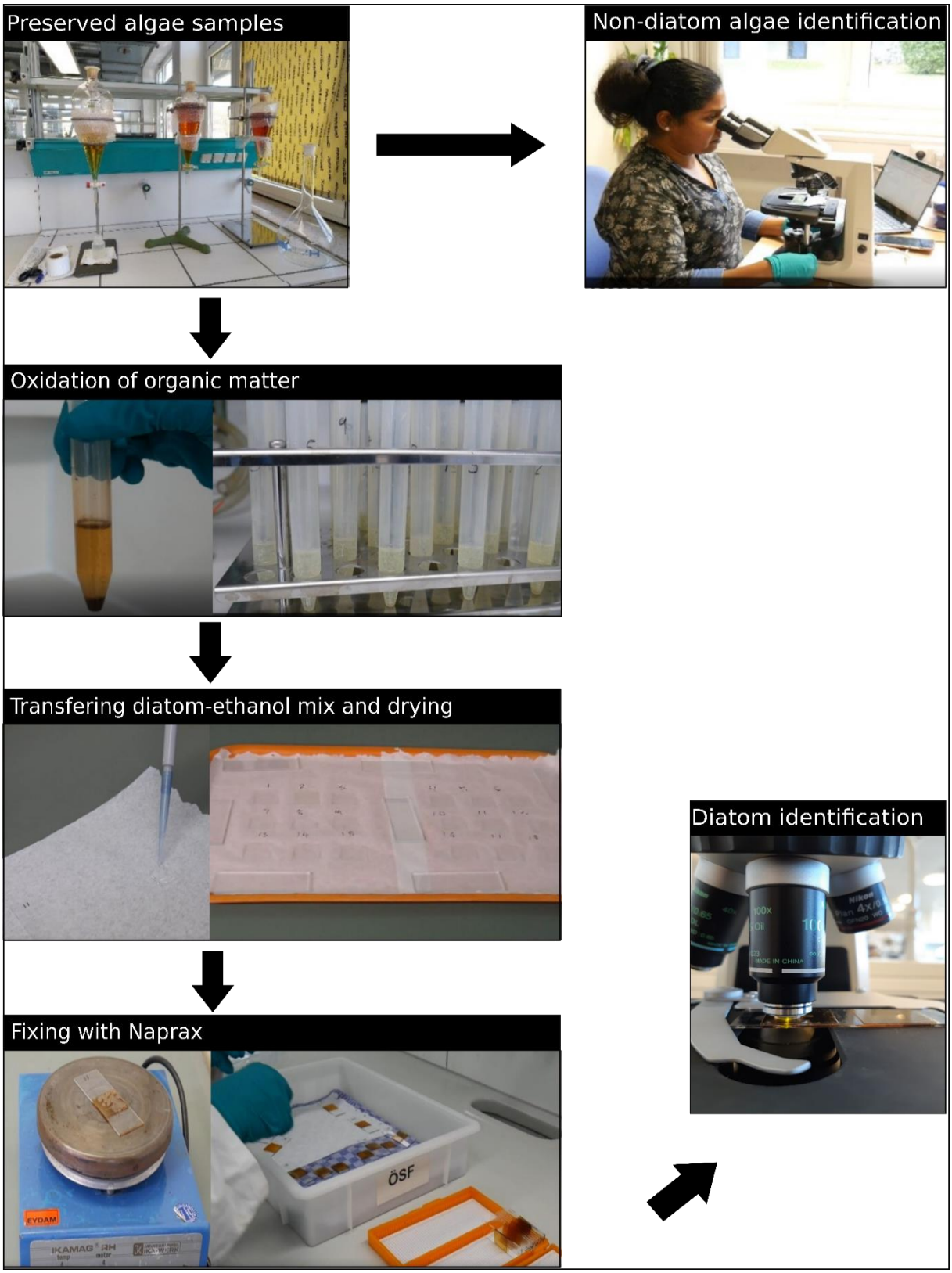

Fig. 1.6: Preparation of algal samples for algae species identification through a microscope. Organic matter in the samples was oxidized before using the samples for diatom species identification.

1.6.5 Functional analyses

1.6.5.1 Metabolism

The changes in dissolved oxygen concentrations (DO) were measured to estimate metabolism using the light-dark bottle technique (Bott, 2006). For epiphyton replicates, 10 mL of slurry in 25 mL glass vessels were incubated and filled them headspace-free with standardized growth media. For each epilithon replicate, one disk was placed in a 72 mL glass container and was filled headspace-free with standardized growth media. For each incubation, three replicate controls (i.e., only with standardized growth media) were included. Each vial was incubated under light conditions (photosynthetically active radiation, PAR: 300 µmol photons m^{-2} s^{-1}) for 4 h and under dark conditions for 20 h in a water bath at 15 ºC. Vials containing epiphyton samples were incubated using a rotating wheel whereas epilithon samples were directly placed in the water bath (Fig. 1.7). The new fresh standardized growth media was used for epilithon dark incubations.

The oxygen saturation was measured at the beginning and end of the light incubation and dark incubation using an oxygen microsensor (Unisense PA2000, Aarhus, Denmark) to measure epiphyton oxygen measurements and oxygen probe (YSI, ProODO Optical Dissolved Oxygen Instrument) for epilithon measurements. The difference in DO over time in light and dark incubation were regarded as net biofilm production (NPP) and biofilm community respiration (ER), respectively. The gross primary production (GPP) was calculated by summing up ER and NPP.

1.6.5.2 Nutrient uptake

Nutrient uptake rates were estimated by incubating epiphyton slurries and epilithon disks for 3 hours (15 ºC; PAR: 300 µmol photons m^{-2} s^{-1} light intensity). For epiphyton, 10 mL of slurry was added to 25 mL glass vessels and filled with standardized growth media. For epilithon, each replicate disk was placed in 72 mL glass containers filled with standardized growth media. For each incubation, three controls were also included. Vials were incubated in a water bath using a rotating wheel for epiphyton and directly placed within the water tank for epilithon. At the beginning of the experiment, subsamples from the control treatments were obtained and used at T0 concentration for all samples. During the incubation, water subsamples were obtained every hour for each vial (T1, T2 and T3) for 3 hours. Sampled water was filtered through GFF filters and preserved in 15 mL tubes (at least 5 mL samples) by storing it at -18

^{o}C. Ammonium (NH_4^+), nitrate (NO_3^-), and phosphate (PO_4^{3-}) concentrations in the samples were measured by flow injection analysis (Lachat QC-8000 Flow Injection Autoanalyzer, Lachat Instruments, USA). The slope of the regression between the nutrient concentrations and time (T0, T1, T2 and T3) for each of the nutrient components was calculated as nutrient uptake rates for each replicate. The average uptake rate of three replicates from each site and each sampling date were used for further analysis.

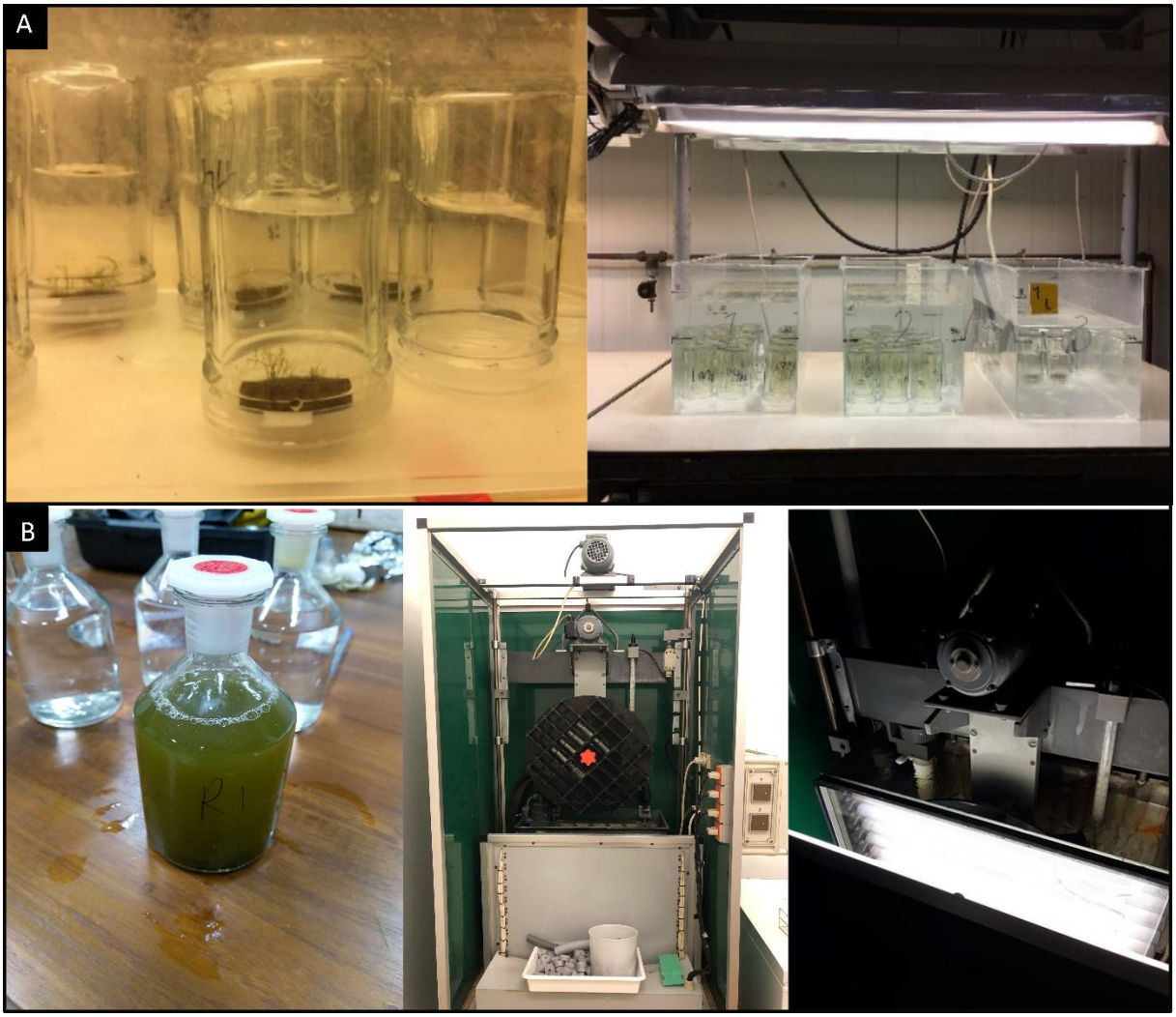

Fig. 1.7: Setups for functional analyses (metabolism and nutrient uptake) of epilithon (A) and epiphyton (B).

Chapter 2 Epiphytic biofilms in freshwater and interactions with macrophytes: current understanding and future directions

Lishani Wijewardene[1,2,3], Naicheng Wu[1,4*], Nicola Fohrer[1], Tenna Riis[2,5*]

[1] Department of Hydrology and Water Resources Management, Institute for Natural Resource Conservation, Kiel University, 24118 Kiel, Germany

[2] Department of Biology, Aarhus University, Ole Worms Allé 1, 8000 Aarhus C, Denmark

[3] Department of Limnology and Water Technology, Faculty of Fisheries and Marine Sciences & Technology, University of Ruhuna, 81000 Matara, Sri Lanka

[4] Department of Geography and Spatial Information Techniques, Ningbo University, 315211 Ningbo, China

[5] WATEC, Aarhus University Centre for Water Technology, Department of Biology, 8000 Aarhus C, Denmark

[*]Corresponding authors: T. Riis (tenna.riis@bio.au.dk) and N. Wu (nwu@hydrology.uni-kiel.de)

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Abstract

Epiphytic biofilm is an important component in freshwater ecosystems and is one of the main primary producers in shallow freshwater ecosystems. The epiphytic biofilm is comprised of an autotrophic community made up of diatoms, green algae, and cyanobacteria, and a heterotrophic community consisting of bacteria, protozoa, fungi, and other microorganisms. Macrophytes are the host domain for epiphytic biofilm, providing substrate and influencing epiphytic biofilm via structural characteristics. Strong competitive, mutualistic, and commensalistic relationships between epiphytic biofilm and macrophytes have resulted from interactions for resources (e.g., light and nutrients) and trophic and allelopathic dynamics. Even though these interactions have wider implications on ecosystem structure, function, and integrity, the current understanding of epiphytic biofilm-macrophyte interactions is limited. In this review, we highlight the current understanding of epiphytic biofilms in freshwater ecosystems and synthesize their different interactions with macrophytes by providing illustrative examples. Furthermore, we identify key areas where research is currently lacking and provide directions for future research in this field, which will allow for better integrated aquatic ecosystem management and conservation strategies.

Keywords: Competition, Nutrients, Light, Trophic interactions, Allelopathy, Freshwater

Highlights

- Epiphytic biofilm plays an important role in freshwater aquatic ecosystems
- Macrophyte morphology and characteristics influence epiphytic biofilm
- Epiphytic biofilm and macrophytes form a highly interactive and complex platform
- Competitive, mutualistic, and commensalistic relationships exist
- Knowledge gaps are highlighted for future research

2.1 Introduction

Biofilms are complex microbial assemblages with a pronounced three-dimensional architecture that attach to solid surfaces and are surrounded by a self-produced matrix composed of extracellular polymeric substance (EPS) (Castiblanco and Sundin, 2016). Periphyton are biofilms attached to any submerged surfaces (Gubelit and Grossart, 2020), whereas 'epiphytic biofilm' occurs on aboveground surfaces of macrophytes. Macrophytes are macroscopic autotrophs growing as submerged, emergent, and floating forms in aquatic ecosystems (Chambers et al., 2007).

Epiphytic biofilm plays multiple roles in aquatic ecosystems (Fig. 2.1) and is important for maintaining ecosystem structure, specifically community composition and diversity (Jones and Thornber, 2010) and functions, such as primary production and respiration (Allen, 1971; Alnoee et al., 2016; Cattaneo and Kalff, 1979; Sand-Jensen et al., 1989; Shamsudin and Sleigh, 1995; Squires et al., 2009; Vadeboncoeur and Steinman, 2002), trophic interactions (Brönmark, 1985; Jones and Sayer, 2003; Vadeboncoeur and Steinman, 2002), nutrient uptake and cycling (Levi et al., 2015; Levi et al., 2017; Sudo et al., 1978; Vadeboncoeur and Steinman, 2002), decomposition (Rybakova, 2010; Sudo et al., 1978), pollutant removal (Lindell et al., 2016; Phillips et al., 2010), and microbial gene pool preservation (Levi et al., 2017; Rusznyák et al., 2008). Macrophytes are 'ecosystem engineers' as they shape the physical properties of aquatic ecosystems; they alter hydraulics by resisting water flow, aid in sediment particle settlement, and influence light availability by shading and maintaining clear water status (Polvi and Sarneel, 2018). Furthermore, macrophytes regulate water chemistry (e.g., dissolved oxygen, carbon, and nutrients) and support other aquatic biota and biological processes such as primary production and grazing (Lacoul and Freedman, 2006; O'Hare et al., 2018; Thomaz and Cunha, 2010). In addition, macrophytes are ideal substrates for microbial growth forming macrophyte-biofilm platforms which display unique, complex, and interdependent biological interactions (Eriksson, 2001). The broader periphyton structure and function has been previously reviewed (Gubelit and Grossart, 2020; Larned, 2010; Sand-Jensen and Borum, 1991; Vadeboncoeur and Steinman, 2002), but here we focus on the epiphytic biofilm on freshwater macrophytes. Despite the importance of macrophytes and their biofilms in freshwater systems, there are no comprehensive reviews on their interactions.

Epiphytic biofilms on live macrophytes are different and unique in both structure and function compared to the other periphytic biofilms in inert freshwater habitats (e.g., sand:

epipsammon, stone/rock: epilithon, and sediment: epipelon) (Levi et al., 2017). Autotrophic communities in epiphytic biofilm are usually dominated by diatoms, green algae, cyanobacteria, and euglenoids (Costică et al., 2018; Shamsudin and Sleigh, 1995; Xia et al., 2020), and dominant algal groups may differ with season and grazing pressure (Jones and Sayer, 2003; Roberts et al., 2003). The heterotrophic community comprises bacteria, protozoa, fungi, and other microorganisms, whereas the bacterial community of epiphytic biofilm is typically dominated by Bacteroidetes, Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, and a low abundance of Actinobacteria and Planctomycetes (Hempel et al., 2008; Hempel et al., 2009; Levi et al., 2017; Xia et al., 2020). Some studies have shown that the epiphytic biofilm has a higher species diversity and presence of unique species than epilithon and epipsammon (Bojorge-García et al., 2014; Levi et al., 2017). In addition, some studies have emphasized that the epiphytic biofilm has lower algal biomass and carbon to nitrogen to phosphorous (C:N:P) ratios compared to epilithon in both lentic (Kahlert and Pettersson, 2002; Wolters et al., 2019) and lotic ecosystems (Belyaeva, 2017). However, the significance of these differences of epiphytic biofilms compared to biofilms on inert substrates in eutrophic freshwater ecosystems is still being debated (Eminson and Moss, 1980; Kahlert and Pettersson, 2002). With regard to reach-scale metabolism in streams, macrophyte habitats (i.e., consisting of both macrophyte and epiphytic biofilm) have shown considerably higher metabolic rates than inert habitats, such as epipsammon and epilithon (Alnoee et al., 2016). Furthermore, a comparative assessment of biomass-specific summertime nutrient uptake rates in streams has shown that epiphytic biofilm is more efficient in NH_4-N and PO_4-P uptake than benthic biofilms (Levi et al., 2015, Wijewardene et al., unpublished data). Epiphytic biofilm also plays an active role in the nitrification/denitrification processes (Eriksson, 2001; Eriksson and Weisner, 1999) where nitrate assimilation is lower in epiphytic biofilms compared to epipelon in summer stream biofilms (Kreiling et al., 2011).

Although epiphyton on terrestrial plants have been studied for several centuries, surveys on aquatic epiphyton only came into prominence at the beginning of the 20th century. Fritsch (1907a) and Fritsch (1907b) studied epiphytic communities on aquatic plants in the former British colonial island of Ceylon. Since then, investigations on aquatic epiphytes have expanded rapidly (Fig. S2.1). Epiphytic structural-functional characteristics are correlated with environmental variables, such as water level, flow velocity, light intensity, temperature, pH, conductivity, dissolved oxygen, turbidity, nutrients, and chloride concentrations (Adam et al., 2017; Eriksson, 2001; Hempel et al., 2009; Lévesque et al., 2017; Morin and Kimball, 1983;

Phiri et al., 2007). Environmental variables can affect epiphytic biofilm directly and indirectly via changes to the macrophyte vegetation (O'Hare et al., 2018; Sultana et al., 2010). Some studies suggest that epiphytic biofilm is less sensitive to ambient environmental variables and more dependent on the interactions between macrophyte and biofilm (Lv et al., 2019; Morin and Kimball, 1983). There is still little understanding on epiphytic biofilm-macrophyte specific relationships.

Epiphytic biofilms are understudied compared to other periphytic biofilms in freshwater ecosystems. This is surprising as epiphytic biofilm-macrophyte specific interactions interfere with important ecosystem processes and these interactions are highly complex. To understand the dynamics of macrophyte-dominated ecosystems under continuous anthropogenic influences, we need to gain a better understanding of biofilm-macrophyte interactions, their link with environmental variables, and ecosystem scale implications. Lack of understanding of these interactions may underestimate the importance of macrophyte habitats in freshwater ecosystems due to ignorance of the role macrophytes play as a substrate for microbial biofilm. Therefore, our objectives in this review are (i) to describe the present understanding of the epiphytic biofilm and their interactions with macrophytes. This includes how freshwater macrophytes influence their epiphytic biofilms, how the biofilms are influenced by environmental variables, and how biofilm-macrophyte interactions are impacted by different types of resources. Objective (ii) is to highlight knowledge gaps on this subject and provide directions on future research.

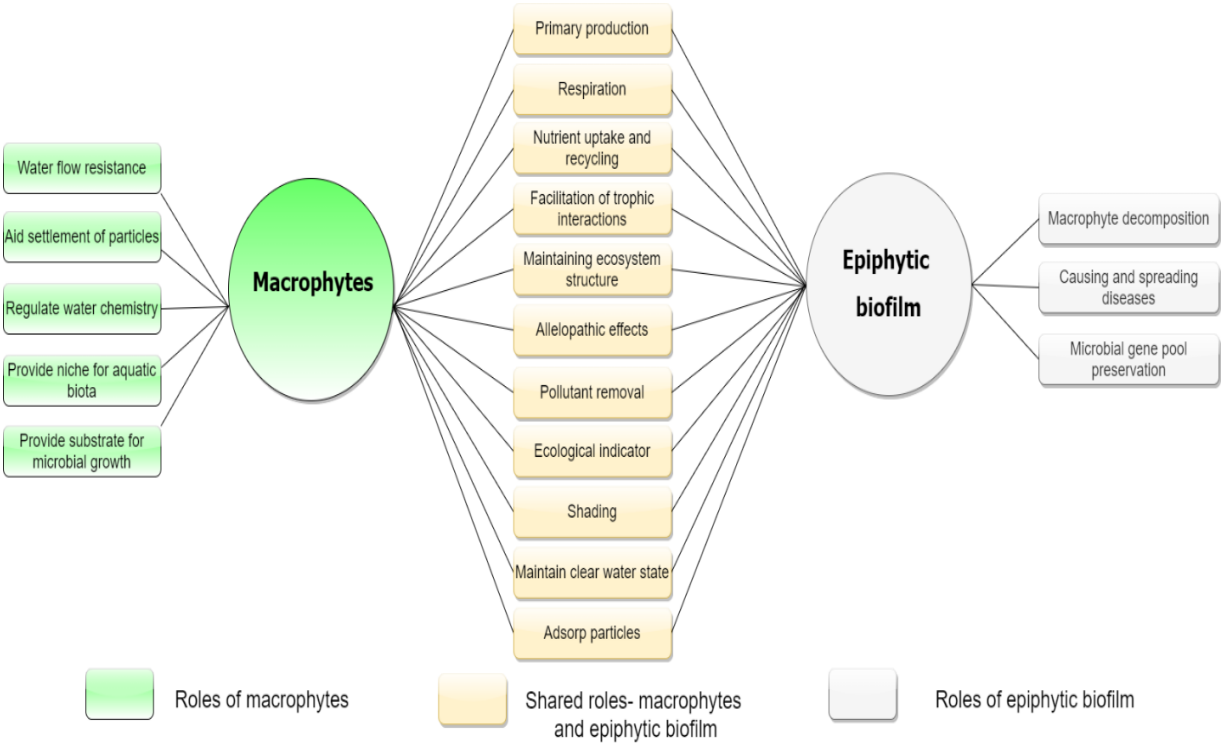

Fig. 2.1: Separate and combined roles of epiphytic biofilm and macrophytes in aquatic ecosystems.

2.2 Methods

Literature was searched on Web of Science (Thomson Reuters) using keywords "macrophyte" and "biofilm" and other relevant keywords in the titles of articles published from 1955 to 2020. The search query was built as below: TI = (macrophyte OR aquatic plant OR hydrophyte OR plant OR macrophytic OR macroalgae OR higher aquatic plant OR aquatic macrophyte OR emergent plant OR submerged plant OR submersed plant OR submerged vegetation OR floating-leaved plant OR free-floating plant OR aquatic autotrophs OR aquatic vascular plants) AND TI = (biofilm OR microbial community OR autotrophic biofilm community OR heterotrophic biofilm community OR epiphytic algae OR epiphytic diatom OR algae OR algal OR diatom OR cyanobacteria OR epiphyte OR epiphyton OR periphyton OR attached alga OR microalgae OR epiphytic flora OR ephiphytism OR bacterial community OR bacterioplankton OR biofilm metacommunity). 3425 references were initially extracted based on keywords defined. We supplied 28 older references (1900-1954) by using the same keywords in the advanced search query on Google Scholar™. By carefully reading titles, abstracts, and the papers (main text), we identified 810 relevant marine and freshwater ecosystem references. We focused on only the 251 freshwater references in this overview,

which included studies in artificial freshwater systems such as mesocosms and theoretical modelling studies. We emphasize that this is not an exhaustive review, but rather an overview of the subject matter.

Temporal trends of the publications are illustrated in Fig. S2.1. Both the annual number of publications and their proportion to the total scientific articles of the databases showed a linear increase over time. Geographical distribution of selected articles for this study are represented in Fig. S2.2. Most of the studies were performed in lakes, whereas the structural diversity and functional capabilities of the epiphytic biofilm in stream ecosystems has received less attention, even though they are the main sites for solute cycling in the landscape. In addition, when describing the epiphytic community structure, most studies have solely paid attention to either autotrophic or heterotrophic communities. Of these two, the autotrophic community of the epiphytic biofilm has been more comprehensively studied compared to the heterotrophic community, and community-wide investigations covering both autotrophs and heterotrophs are rare (but see: Gubelit and Grossart (2020); Levi et al. (2017)). The main interactions were identified as the provision of substrate, interactions with resources (light and nutrients), trophic interactions, allelopathy and other interactions on flow, diseases, and pollutants.

2.3 Interactions between epiphytic biofilm and macrophytes

Epiphytic biofilm and macrophytes form a highly interactive unit with the provision of substrate, competition for resources (e.g., light and nutrients), trophic interactions (e.g., herbivory and carnivory), allelopathic interactions, interactions related to flow, diseases, and pollutants – all of which can be categorized into competitive (-/-), mutualistic (+/+), and commensalistic (+/0) interactions under different scenarios (Fig. 2.2 and Fig. 2.3).

2.3.1 Provision of substrate

Direct interaction between macrophyte and epiphytic biofilm are the result of the provision of substrate for attachment (Fig. 2.2). Most of the reviewed studies indicate host-plant species specificity on structure and function of the epiphytic biofilm (Adam et al., 2017; Calheiros et al., 2010; Ferreiro et al., 2013; Hempel et al., 2008; Lalonde and Downing, 1991; Prowse, 1959; Toporowska et al., 2008; Tóth, 2013; Tunca et al., 2014). Toporowska et al. (2008) found that species composition of epiphytic algae was different according to the host macrophyte (i.e., *Stratiotes aloides*, *Potamogeton lucens*, *Ceratophyllum demersum*, and *Chara* spp). In contrast, some other studies claimed macrophytes to be a neutral substrate for epiphytes (Cattaneo and Kalff, 1979; Frankova et al., 2017; Millie and Lowe, 1983; Shamsudin and Sleigh, 1995). Cattaneo and Kalff (1979) studied epiphyton biomass and primary production on natural *Potamogeton richardsonii* plants and morphologically equivalent plastic plants and found no difference in studied parameters between natural and artificial plants. Host-species specificity is high in oligotrophic waters compared to eutrophic waters (Eminson and Moss, 1980; Lalonde and Downing, 1991), which emphasizes that the environmental variables are a potential cause for the lack of host-species specificity.

Structural characteristics of macrophytes (e.g., complexity, growth form, life stage, vertical distribution of biomass, leaf architecture, and leaf age) directly influence the epiphytic biofilm structure (Ferreiro et al., 2013; Lalonde and Downing, 1991; Laugaste and Reunanen, 2005; Pettit et al., 2016; Tóth, 2013) (Fig. 2.2). Higher morphological complexity of macrophytes (e.g., high perimeter to surface area ratio, high fractal dimension, high species complexity index) supports high epiphytic biofilm biomass and diversity due to enhanced niche diversity (Ferreiro et al., 2013; Hinojosa-Garro et al., 2010; Levi et al., 2017; Pettit et al., 2016). Levi et al. (2017) found that the least complex macrophyte, *Sparganium emersum*, had lower richness and evenness compared to the more morphologically complex macrophyte, *Callitriche*

spp. However, Casartelli and Ferragut (2018) have highlighted that these differences in epiphyte density or diversity related to macrophyte complexity may highly depend on the colonization time (e.g., early colonization vs. mature biofilm). Depending on the growth form of macrophytes, submerged plants tend to possess the highest epiphyte abundance, chlorophyll-*a*, biomass, and diversity compared to other growth forms of the aquatic macrophytes since they grow just below the water surface, which allows higher light penetration and provides a complex and large surface area for epiphyte development (Laugaste and Reunanen, 2005; Pettit et al., 2016). Leaf architecture (e.g., size, shape, flexibility) may affect epiphytic biofilm biomass, abundance, and diversity. For example, ribbon like flexible leaves (e.g., *Vallisneria americana*) had lower epiphytic algal biomass compared to broad-leaved (e.g., *Elodea canadensis*) or whorled leaved macrophytes (e.g., *Myriophyllum spicatum)* (Lalonde and Downing, 1991). The number of bacterial cells per plant area was higher in *Myriophyllum spicatum* than in *Potamogeton perfoliatus* due to a higher surface to volume ratio and whorl-like structure (Hempel et al., 2009).

In addition to plant and leaf complexity, the abundance and diversity of epiphytic algae and bacteria also increases with age of the macrophyte leaves and some of the primary and secondary colonizers even stay present after the death of the host leaves (Rogers and Breen, 1981). These senescing macrophytes are important nutrient reserves for the epiphytic biofilm (Borrego-Ramos et al., 2019; Brönmark, 1989; Carpenter and Lodge, 1986; Xia et al., 2020). Borrego-Ramos et al. (2019) observed higher diatom richness on dead macrophyte stems compared to live macrophytes. Vertical biomass distribution of aquatic macrophytes also tends to influence the mean epiphyton abundance, biomass, cell size, and rate of species succession (Romo and Galanti, 1998), in particular, due to changes in light availability from the edge to the bottom of a macrophyte bed. Apart from macrophyte structural characteristics, the chemical composition of macrophytes, such as the content of carbon, calcium carbonate ($CaCO_3$) encrustations, and total phenolic compounds, affect biofilm community composition (Hempel et al., 2008; Hempel et al., 2009; Wolters et al., 2019). In the study of Wolters et al. (2019), the density of epiphytic bacteria negatively correlates with biofilm $CaCO_3$ content from macrophytes. They reasoned that $CaCO_3$ encrustations may adsorb free dissolved organic carbon (DOC), amino and fatty acids, and then limit them for use by the bacterial community. The links between the characteristics of macrophyte species and the epiphytic biofilm structure means that macrophyte richness and coverage will affect the epiphytic algal abundance and

taxonomic composition on the ecosystem scale (Casartelli and Ferragut, 2015; de Souza et al., 2015).

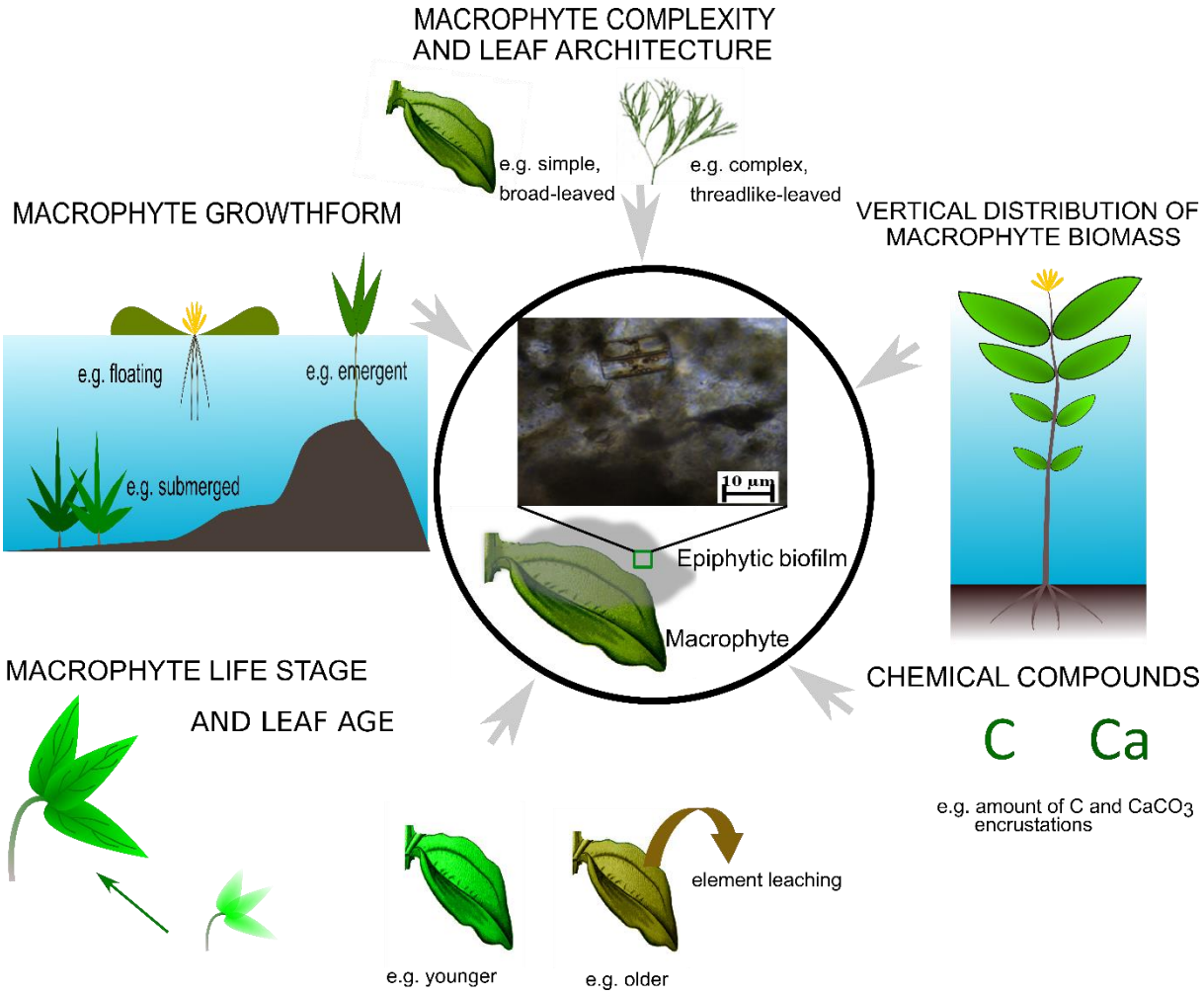

Fig. 2.2: Macrophyte characteristics affecting the epiphytic biofilm.

2.3.2 Interactions on resources: Light

The autotrophic community in the epiphytic biofilm shows a rapid response to light. Epiphytic algal density and biomass show a positive relationship with light intensity up to a saturation level or until another limiting factor for photosynthesis emerges (Lévesque et al., 2017; Sultana et al., 2004). Sultana et al. (2004) investigated colonization and growth of epiphytic algae under two light regimes, i.e., low: 80 µmol m^{-2} s^{-2} and high: 200 µmol m^{-2} s^{-2}. While the species composition of the most abundant epiphytic algae did not change under the two light regimes, a subset of unique rare species had developed under each light regime. Furthermore, vertical distribution of the epiphytic algae under both light levels showed that the

basal part of macrophytes were inhabited by a homogenous community of the epiphytic algae while the apical plant parts were occupied with a mosaic community.

Asaeda et al. (2004) and Tóth (2013) showed a 60-80% reduction in macrophyte production due to direct competition for light between epiphytic biofilm and macrophytes (Fig. 2.3A). A large part of the light reaching the leaf surface of macrophytes is attenuated by the epiphytic biofilm, which can be > 80% under high nutrient conditions (Raeder et al., 2010; Sand-Jensen and Søndergaard, 1981). In eutrophic freshwater ecosystems, shading by the epiphytic biofilm has been identified as a main cause for drastic reduction of submerged vegetation in late summer (Min et al., 2017; O'Hare et al., 2018; Phillips et al., 1978; Song et al., 2017a). Additionally, the shading effect of epiphytic biofilm becomes crucial for macrophytes in shaded or light-limited environments (Köhler et al., 2010; Sand-Jensen and Revsbech, 1987). Contrary to the well-known negative effect of epiphytic biofilm on macrophyte light availability, epiphyton has been identified as a protective cover for macrophytes from desiccation and harmful short-wave radiation such as UV (Gaiser et al., 2011; Klančnik et al., 2015). Klančnik et al. (2015) studied the effects of epiphyton on the quality and quantity of radiation transmitted through the leaf tissue of submerged macrophytes. They have found that removal of epiphyton significantly increased the transmittance of short-wave radiation and have emphasized the role of the epiphyton dominated by diatoms for the prevention of potential harmful effects of short-wave radiation.

Compared to studies on the shading effects of epiphytic biofilm on macrophytes, investigations into the macrophyte shading effect on epiphytes are limited. However, where macrophyte biomass is dense, epiphytic production is strongly constrained by macrophyte shading (Morin and Kimball, 1983; Squires et al., 2009). Alteration of light penetration from macrophytes is identified as major determinant of the epiphytic biomass, while macrophyte biomass and their epiphyton were inversely correlated (Cattaneo et al., 1998; Gosselain et al., 2005; Pettit et al., 2016). This tends to also affect the functional role of epiphytes, for example, strong vertical gradients in metabolism (Vis et al., 2006) and nutrient uptake (T. Riis, unpublished data). Generally, macrophytes show morphological (e.g., average number of leaves, total shoot length, number of newly recruited shoots, and stem diameter) and physiological (e.g., expansion or contraction of leaf area) adaptations to optimize light availability (Asaeda et al., 2004; Riis et al., 2012; Sultana et al., 2010). Although macrophytes shaded by epiphytic algae do not show significant adaptations to overcome the light limitation (Asaeda et al., 2004), long-term colonization of epiphyton and its shading effect can induce

morphological changes in aquatic macrophytes (Sultana et al., 2010). Recent studies on light competition between epiphytic biofilm and macrophytes, focus on modelling approaches to tackle this complex relationship (Zhang et al., 2015; Zhang et al., 2018).

2.3.3 Interactions on resources: Nutrients

Elevated chlorophyll-*a* content, biomass, primary production, and a shift in species composition or decreased diversity are the initial responses of the epiphytic biofilm to nutrient enrichment in the surrounding water (Becares et al., 2008; Mei and Zhang, 2015; Min et al., 2017; Romo et al., 2007; Song et al., 2017a). In mesocosm experiments, the increment of epiphytic biomass and chlorophyll-*a* were higher in the combined N and P nutrient treatments compared to individual N or P nutrient treatments (Ray et al., 2014). Nevertheless, P is considered a key nutrient in most studies, as it plays a major role in freshwater systems. Romo et al. (2007) stated that levels above 0.1-0.2 mg L^{-1} P prevent the coexistence of macrophyte, epiphytic biofilm, and phytoplankton resulting in a reduction of submerged macrophyte biomass. According to Lalonde and Downing (1991), a weak and non-linear relationship was found between total P (TP) and epiphytic biomass, where epiphytic biomass increased until 0.039 mg L^{-1} TP but decreased at higher levels in their study conducted in 11 lakes in Canada based on macrophytes such as *Elodea canadensis*, *Myriophyllum spicatum*, *Vallisneria americana*, and *Potamogeton* spp.. However, in Romo et al. (2007), epiphytic biomass increased linearly with increased TP up to 0.35 mg L^{-1} TP in their mesocosms study conducted in a Mediterranean lake dominated with *Chara* spp. and *Phragmites australis*. In addition, different epiphytic algae show differences in P source dependency. For example, filamentous *Mougeotia* and long-stalked *Gomphonema* depend on external water for P while small adnate forms like *Acanthes* depend on macrophytes for 20-60% of their P requirement (Moeller et al., 1988).

The direct interaction between epiphytic biofilm and macrophytes for nutrients is difficult to isolate since the consequences are shared with different compartments of the environment, such as the epiphytic biofilm, macrophytes, phytoplankton in surrounding water, and sediments. Rooted macrophytes may exclusively depend on sediment nutrients, while epiphytic biofilms have limited access to sediment nutrients and mostly depend on nutrients in the water column, nutrient release from macrophytes or internal nutrient sources (Allen, 1971; Moeller et al., 1988; Périllon and Hilt, 2019). There is overwhelming evidence to support the hypothesis that epiphytic biofilm and macrophytes are competitors for nutrients (e.g., O'Hare

et al., 2018; Périllon and Hilt, 2019; Romo et al., 2007; Xie et al., 2013) (Fig. 2.3A). Mechanisms underlying the suppression of aquatic vegetation through a rapid increase of epiphyte biomass and its shading effect under mesotrophic and eutrophic scenarios are well-studied (e.g., Becares et al., 2008; Phillips et al., 1978) and were revised recently by adding the roles of competitive and non-competitive macrophytes (O'Hare et al., 2018). Initially, abundance, density, and biomass of both epiphytic algae and macrophytes increase with nutrient enrichment, but with increasing eutrophication macrophytes lose the competition due to light limitation by epiphytic shading. Moreover, recent studies suggest that physiological changes occur in macrophytes (e.g., increased antioxidant enzyme activities, reduced chlorophyll content, and promoted peroxidation of membrane lipids) due to nutrient enrichments, which further enhances these deleterious effects (Min et al., 2017; Song et al., 2017a). In addition to N and P, the epiphytic biofilm and macrophytes are also competing for dissolved inorganic carbon (DIC) (Jones et al., 2002; Wolters et al., 2019; Xie et al., 2013). Epiphytic algae and macrophyte density were negatively correlated and the steepness of the slope decreased with increasing DIC concentrations, emphasizing competition for DIC between them (Jones et al., 2002).

However, studies have suggested mutualistic interactions also for nutrients between epiphytic biofilm and macrophytes (Fig. 2.3B). The epiphytic biofilm benefits from living macrophyte nutrient exudations (Kahlert and Pettersson, 2002; Wolters et al., 2019) resulting in effects on epiphytic biofilm biomass and nutritional value (e.g., lower C:N:P molar ratio). According to Burkholder and Wetzel (1990), the main source of P for epiphytes is the host macrophyte during the growing season, but Carignan and Kalff (1982) and Moeller et al. (1988) found that living macrophytes release very little P for epiphytes (e.g., ca. 3.4-9%: Carignan and Kalff (1982) and ca. 2%: Moeller et al. (1988)). Wolters et al. (2019) stated that nutrient release (e.g., N, P, and DOC) of both living and senescing macrophytes may affect associated epiphytic biofilm. DOC exudations of both living and senescing macrophytes may support the heterotrophic community of the epiphytic biofilm (Demarty and Prairie, 2009; Xia et al., 2020). In this mutualistic interaction, macrophytes can benefit from N fixation (Srivastava et al., 2017) occurring in epiphytic biofilm community. Hempel et al. (2008) listed the positive effects of epiphytic biofilm on macrophytes linked with nutrient interactions as (i) providing organic compounds and carbon dioxide and (ii) enhancing nutrient recycling.

2.3.4 Trophic interactions

Epiphytic biofilm is important for primary production in freshwater systems and as a site of trophic interactions benefitting both macrophytes and the epiphytic biofilm. Fast growth and high nutrition value of epiphytic algae make it an important food source for secondary producers in shallow aquatic ecosystems (Jaschinski et al., 2011; Jones et al., 1999). The epiphytic algae initiate a crucial food web that include lower trophic level invertebrates (e.g., micrograzers, meiofauna, herbivore macroinvertebrates) and higher trophic level organisms, such as fish (Brönmark and Vermaat, 1998; Jones and Sayer, 2003). Invertebrates show differential preference toward various epiphytic algae types: stalked and tubular diatoms are usually preferred by nematodes, rotifers induce grazing pressure on prostrate diatoms, while both rotifers and ciliates show a preference for *Cocconeis*-type diatoms (Albay and Aykulu, 2002).

Macrophytes are susceptible for direct herbivory by grazers. However, macrophytes and grazers have a mutualistic relationship driven by epiphyte-dependent trophic interactions (Jones et al., 1999; Underwood et al., 1992) (Fig. 2.3C). Macrophytes benefit from grazers (e.g., by increased survival, growth, and biomass) since they can release the macrophytes from epiphytes that compete for resources and provide nutrients to the macrophytes from their excretory by-products (Brönmark, 1985; Brönmark, 1989; Jones et al., 1999; Underwood et al., 1992). In return, macrophytes support macroinvertebrate grazers "by providing a large surface area for colonization by epiphytic algae and bacteria, by improving biofilm stoichiometry and by stimulating bacterial growth" (quote: Wolters et al., 2019). The epiphytic biofilm acts as a protective cover that shields macrophytes from grazer-induced damages (Dudley, 1992). Further, carnivorous macrophytes (e.g., *Utricularia* spp.) use the epiphytic biofilm to facilitate prey utilization resulting in a commensalistic trophic relationship (Caravieri et al., 2014; Diaz-Olarte and Duque, 2009; Diaz-Olarte et al., 2007; Pitsch et al., 2017; Simek et al., 2017) (Fig. 2.3D). Epiphytes also benefit from grazers as the physical disturbance created by grazers allows fast regeneration of epiphytic biofilm while shedding off the thick, old, and dead biofilm (Rodrigues and Bicudo, 2001). The aforementioned trophic interactions are usually considered as an adaptive evolutionary advantage for macrophytes, given that dissolved organic matter released by macrophytes can attract grazers, which can feed on epiphytes (Brönmark, 1985). However, some studies rejected this hypothesis, while stating that chemical signals (e.g., organic compounds) released by certain species of algae in the epiphytic biofilm can attract

invertebrates. For example, the epiphytic algae of *Egeria najas* attract the snail, *Hebetancylus moricandi* (Mormul et al., 2010).

2.3.5 Allelopathic interactions

Allelopathy, the secretion of chemical compounds to inhibit growth of other organisms, is another direct interaction between specific macrophytes and epiphytic biofilm for the benefit of their competitive interactions (Fig. 2.3E) (See Gross, 2003; Mohamed, 2017 for detailed reviews). Macrophytes, such as *Myriophyllum*, *Ceratophyllum*, *Elodea*, *Najas*, *Stratiotes*, and *Chara* genera, are identified as allelopathic active macrophytes which are able to secrete toxic compounds (e.g., polyphenolic compounds, sulfur compounds) to inhibit the formation, growth, and establishment of epiphytic biofilms (Gross et al., 2003; Hilt, 2006; Hilt and Gross, 2008; Mulderij et al., 2009) and reduce species richness and diversity of epiphytic biofilms (Hai-ting et al., 2013). Among different epiphytic algal groups, diatoms and cyanobacteria show a higher sensitivity to macrophyte allelopathic substances compared to green algae (Erhard and Gross, 2006; Hilt, 2006; Hilt and Gross, 2008). Epiphytic bacterial community composition may differ due to the macrophyte allelopathic compounds and some of these bacterial communities are capable of degrading allelopathic substances (Hempel et al., 2009). Conversely, the epiphytic biofilm might release compounds that are toxic to macrophytes and mainly cyanobacterial species of epiphytic biofilm express this allelopathic interactions (Mohamed, 2017).

Hilt (2006) tested the hypothesis that "epiphyton has higher vulnerability to macrophyte allelopathy than phytoplankton", but the results indicated low vulnerability of epiphyton to macrophyte (*Myriophyllum spicatum*) allelopathy, showing no impact on epiphytic algal species (i.e., the green algae *Stigeoclonium tenue* and diatom *Gomphonema parvulum*) and even showed increased growth of an epiphytic cyanobacterium (*Oscillatoria limosa*). Similarly, Mohamed and Al Shehri (2010) highlighted that allelopathic compounds of *Stratiotes aloides* supported growth and toxic production of epiphytic cyanobacteria such as *Merismopedia tennuissima* and *Leptolyngbya boryana.* Epiphyton may develop resistance to the allelopathic substances of macrophytes and the mechanism behind this is still being debated as co-evolution and local adaptation (Gross, 2003; Reigosa et al., 1999) or only an algal strain-specific response (Eigemann et al., 2013). Despite numerous studies on allelopathic interaction of macrophytes on epiphytic biofilm and vice versa, the results are often contradictory and differ among macrophyte species and their epiphytes. These differences may be due to differences in (i) scale of experiments (e.g., laboratory studies, mesocosm studies, and studies conducted in natural

ecosystems), (ii) extraction methods of allopathic compounds from the macrophytes, and (iii) source of epiphytic species (e.g., single species or mixed communities).

2.3.6 Interactions with flow velocity

The water flow is crucial to freshwater macrophytes and epiphytic biofilm and derives direct interactions. Macrophytes cause resistance to the water flow and low water velocity aids the colonization and growth of epiphytic biofilm (Fig. 2.3F). On the other hand, high velocities are beneficial to macrophyte growth as the result of reduced resource competition by sloughing of the epiphytic biofilm (Špoljar et al., 2017). Furthermore, epiphytic biofilm-macrophyte interactions with regards to metabolism of dissolved oxygen/inorganic carbon, nitrification, and denitrification processes may be altered by flow velocities. This is manifested by the flow-induced variations of diffusion rates to and from epiphytic biofilms and by alterations of metabolic rates within the epiphytic community (Eriksson, 2001). Eriksson (2001) studied the macrophyte-epiphytic biofilm complex of *Potamogeton pectinatus* at flow velocities of 0, 0.03, and 9 cm s^{-1} and noted a progressive increase of photosynthesis and respiration rates with the flow velocity. Further, flow velocity significantly affected denitrification in epiphytic biofilms. High flow velocities facilitate efficient transport of organic matter to and from epiphytic biofilm-macrophyte interfaces and stagnant water conditions support the bacterial community within the epiphytic biofilm aiding to internal metabolic processes.

2.3.7 Interactions regarding diseases

Diseases can be another direct interaction between epiphytic biofilm and macrophytes (Fig. 2.3G). Some evidence suggests that heterotrophic communities in the epiphytic biofilm may cause disease or malformations in macrophytes. Extensive inward swelling, disorganization of the epidermal walls, and degradation of epidermis and mesophyll cell walls were observed following an increase in density and diversity of epiphytic bacteria with macrophyte leaf age (Rogers and Breen, 1981). Contrary to the general negative impact of epiphytic biofilm causing a disease in macrophytes, some bacterial genera (e.g., *Pseudomonas*) in epiphytic biofilm may prevent disease in macrophytes by suppressing pathogenic microorganisms in the biofilm and promote macrophyte growth (Zhao et al., 2017). Xia et al. (2020) observed the presence of bacterial genera such as *Exiguobacterium*, *Pseudomonas*, and *Chryseobacterium* in epiphytic biofilms, which have the potential to inhibit phytopathogenic fungi.

2.3.8 Interactions with water pollutants

Trace metal elements, pesticides, and other pollutants in aquatic ecosystems may adversely affect the structure and function of the epiphytic community in aquatic ecosystems (Mingchao et al., 2013; Wendt-Rasch et al., 2004). This has led to the use of epiphytic communities as an indicator to assess aquatic pollution and biomonitoring of aquatic ecosystems (Kiss et al., 2003; Mingchao et al., 2013; Phiri et al., 2007). There is a direct interaction between a macrophyte and its epiphytic biofilm not only for macroelements like C, N, and P, but also for trace metal elements (Fig. 2.3H). The trace metal transformation pathway from sediment to macrophyte to epiphytes was traced by Jackson et al. (1994) experimenting on *Myriophyllum spicatum*; they found that ^{60}Co and ^{54}Mn of the epiphytes were mostly derived from their host macrophytes.

Moreover, interactions between epiphytic biofilms and macrophytes can cause an increase or decrease in the toxicity of trace metal elements to macrophytes, particularly by the heterotrophic community in the epiphytic biofilm, which contributes to trace metal accumulation and biomagnification through food chains. Epiphytic bacteria tend to accumulate mercury (Hg) and produce methylmercury (MeHg), the latter being much more toxic than the former (Coelho-Souza et al., 2011; Dranguet et al., 2017; Gentes et al., 2017). Beauvais-Flück et al. (2018) found negative impacts of MeHg on the macrophyte, *Elodea nuttallii*, in which antioxidant responses were induced. In contrast, epiphytic bacteria have shown the ability to oxidize trace metal elements and these oxidized compounds are less toxic to the macrophytes than their original state. The production of biogenic Mn oxides in epiphytic biofilms composed of bacterial strains, such as *Acidovorax, Comamonas, Pseudomonas,* and *Rhizobium*, was reported on the leaf surfaces of *Egeria densa* (Tsuji et al., 2017). Furthermore, to make practical use of their accumulation ability, the use of epiphytic biofilms have been tested and recommended as part of remedial treatment of water polluted with trace metal elements and organic compounds (e.g., *Salvinia minima* recommended to treat coal pile runoff: Lindell et al. (2016); *Typha latifolia* recommended to treat naphthenic acids: Phillips et al. (2010)). According to Zhang et al. (2014), macrophyte species may play a more significant role in the removal of pollutants than the epiphytic bacterial community. The specific roles of epiphytic biofilms on the removal of pollutants have been highlighted in recent studies. Further, macrophyte-epiphytic biofilm interactions towards breaking down complex compounds to simple nutrients, metal ion mobilization and inducing uptake of pollutants by macrophytes are also recognized (Srivastava et al., 2017).

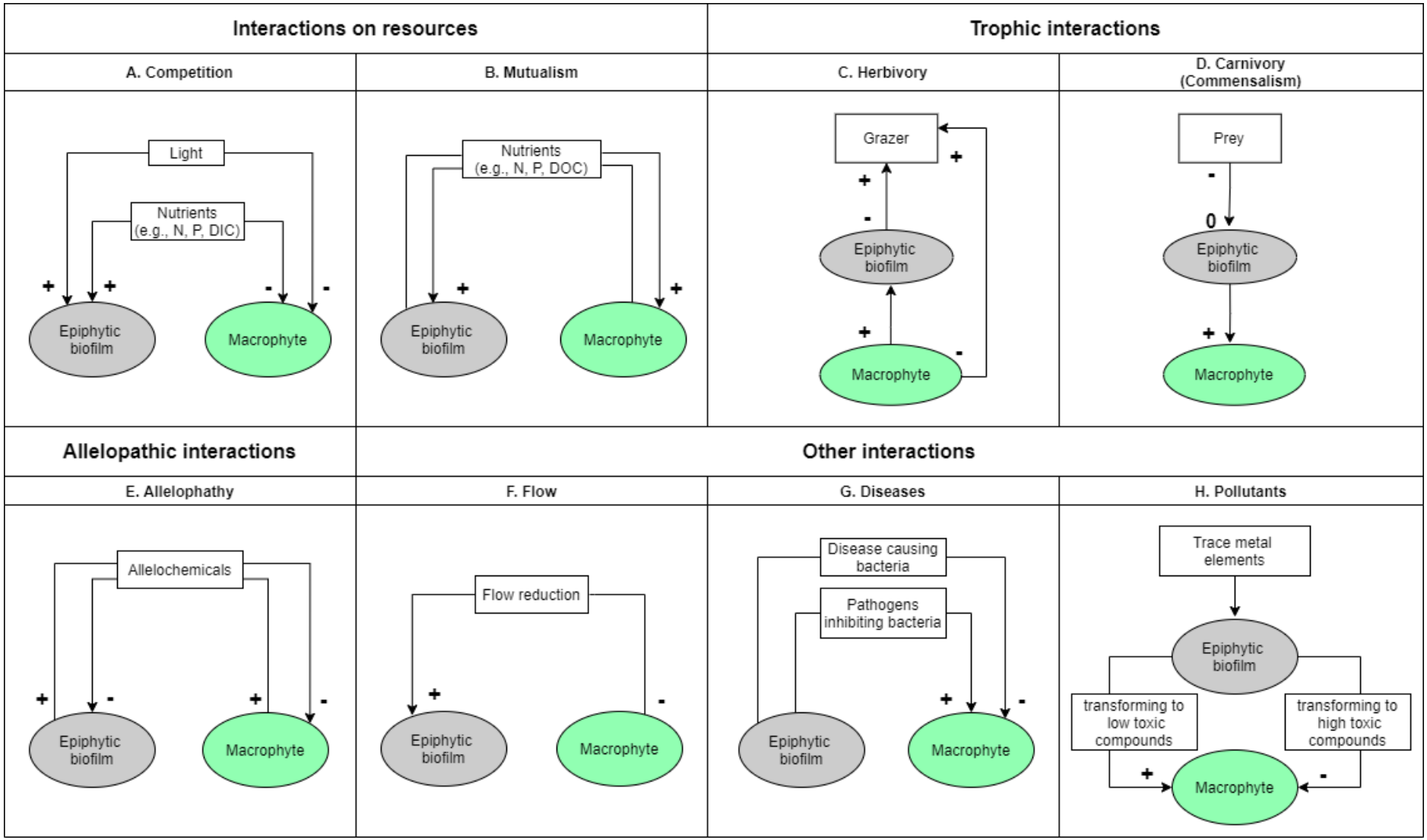

Fig. 2.3: Interactions between epiphytic biofilm and macrophytes. N, P, DIC and DOC indicate the nitrogen, phosphorous, dissolved inorganic carbon and dissolved organic carbon, respectively. In interactions: +, - and 0 signs imply the positive, negative, and neutral effects, respectively.

2.4 Knowledge gaps and future research directions

Although environmental variables are important drivers of structural and functional properties of epiphytic biofilm, interrelationships among these environmental variables and the effects of combined environmental variables on epiphytic biofilm need more attention in future studies (Flynn et al., 2002). Rather than considering environmental variables individually, experiments should be designed to observe their combined influence on the epiphytic biofilm, such as shown for epilithon by Guo et al. (2020). In addition, seasonal dynamics of the epiphytic biofilm have only been addressed in a few studies (e.g., Toporowska et al., 2008; Tunca et al., 2014). Most seasonal studies focused on summer months or were largely restricted to four samplings representing the four seasons of the year. Therefore, correlations of environmental variables with structural and functional properties of the epiphytic biofilm should be studied at a high temporal resolution (e.g., Wijewardene et al., 2021a). Studies on biofilm community trait composition and functional features, such as functional diversity and redundancy, have been performed in epilithic biofilm in order to understand environmental drivers and processes structuring the community (Guo et al., 2020; Wu et al., 2019), but studies related to trait composition of epiphytic biofilm are still rare (e.g., Ács et al., 2019). Including functional traits in future research would greatly expand our current understanding of environmental drivers and processes governing the functionality of epiphytic communities.

Host plant-species specificity and influences of macrophyte characteristics on the epiphytic biofilm are still to be determined. Current studies are restricted to few main genera of macrophytes, such as *Myriophyllum*, *Potamogeton*, *Ceratophyllum*, *Vallisneria*, *Phragmites,* and *Nymphaea,* and investigations are needed to focus on other important macrophyte species to formulate a comprehensive view on the epiphytic biofilm. Furthermore, studies on the reverse scenario, the effect of the epiphytic biofilm structure on macrophyte morphology and other characteristics is greatly lacking (but see, Sultana et al. (2010)).

Interactions within the epiphytic biofilm-macrophyte complex for nutrients have received little attention. For example, the use and dependency of macrophytes on nutrients derived from epiphytic biofilm and the use and dependency of epiphytic biofilm on leaching nutrients from macrophytes still need more research. More investigations should be designed to isolate nutrient relationships within this unique platform under various ambient environmental settings, e.g., using advanced tracer experiments of stable isotopes (^{15}N, ^{32}P, and ^{13}C) coupling with nutrient uptake kinetic models (e.g., differentiate abiotic uptake by

adsorbing and biotic uptake) (e.g., Scinto and Reddy, 2003; Song et al., 2017b). The importance of the epiphytic biofilm in trophic interactions and its involvement in trophic cascades are emphasized in many studies, but the hypotheses regarding underlying mechanisms of these trophic interactions are usually contradictory, e.g., whether grazers are attracted by macrophytes or epiphytic biofilm (Brönmark, 1985; Mormul et al., 2010). Therefore, more research is needed to clarify the triggering factor of these trophic interactions. The availability of increased imaging technology, molecular markers, and using stable isotopes as tracers to track C, N, and P in the complex food chains will aid in the exploration of trophic interactions (Bakker et al., 2016).

Allelopathic effects of macrophytes on phytoplankton and bacterioplankton are well-studied, but studies on epiphytic biofilm are limited. The responses of epiphytic heterotrophs to macrophyte allopathic compounds are unknown. In terms of the epiphytic biofilm, only cyanobacteria are usually highlighted as candidates capable of inducing allelopathic reactions in the plant (Mohamed, 2017). More studies are needed to test the allelopathic potential of other groups of the epiphytic biofilm such as green algae, diatoms, and bacteria. Moreover, since the results of allelopathy experiments are often depending on the specific epiphyte-macrophyte combination under investigation, there is a need to investigate the allelopathic activity through a wide range of epiphyte-macrophyte combinations. Most of the allelopathic studies have been conducted in the laboratory using extracts from macrophytes and cultures of certain species of the epiphytic biofilm (Erhard and Gross, 2006; Mohamed and Al Shehri, 2010), but whether these observations are consistent with those in their natural habitats (e.g., Mulderij et al., 2009) is not yet fully known. Therefore, more field studies based on metacommunity ecology (i.e., not on individual species but the community) should be conducted to understand allelopathic relationships under natural conditions and scale-up effects on ecosystem level. Rather than growth, effects of allelopathy at the molecular and genetic level also should be studied. Other interactions discussed in this review, i.e., flow, diseases, and pollutants, have great potential for improving applications of epiphytic biofilm-macrophyte interactions in wastewater treatment and managing freshwater aquatic ecosystems under anthropogenic pressures such as pollution.

2.5 Concluding remarks

Epiphytic biofilms play a key role in shallow aquatic ecosystems by contributing to ecosystem structure, function, and integrity. The structure and function of the epiphytic biofilm is largely related to its host (e.g., macrophyte species, morphology, and characteristics).

Consequently, a myriad of interactions between the epiphytic biofilm and host macrophytes have been documented, such as interactions on resources, trophic interactions, and allelopathic interactions. These interactions can often be complex in natural habitats, manifested through competitive, mutualistic, and commensalistic relationships. Despite these findings, there are several key areas where research is currently lacking. This overview not only attempts to identify such knowledge gaps, but also acts as a basis for designing future studies – with a particular emphasis on including epiphytic biofilm to understand, maintain, and improve freshwater ecosystem health and integrity (Adam et al., 2017; Costică et al., 2018; Lorch and Ottow, 1986; Phiri et al., 2007). Improved knowledge of the biofilm-macrophyte relationship can be used to enhance our understanding of the costs and benefits of current management practices, such as removing of natural vegetation and re-oligotrophication (Baattrup-Pedersen et al., 2002; Geist and Hawkins, 2016). This can also lead to incorporation of epiphytic biofilm-macrophyte interactions in modelling approaches to predict future dynamics in aquatic ecosystems and guiding conservation strategies (Wade et al., 2002; Ward et al., 2016; Zhang et al., 2018).

Chapter 3 Epiphyton in agricultural streams: structural control and comparison to epilithon

Lishani Wijewardene[1,2,3*], Naicheng Wu[1,4*], Pau Giménez-Grau[2], Cecilie Holmboe[2], Nicola Fohrer[1], Annette Baattrup-Pedersen[5], Tenna Riis[2,6]

[1] Department of Hydrology and Water Resources Management, Institute for Natural Resource Conservation, Kiel University, 24118 Kiel, Germany

[2] Department of Biology, Aarhus University, Ole Worms Allé 1, 8000 Aarhus C, Denmark

[3] Department of Limnology and Water Technology, Faculty of Fisheries and Marine Sciences & Technology, University of Ruhuna, 81000 Matara, Sri Lanka

[4] Department of Geography and Spatial Information Techniques, Ningbo University, Ningbo 315211, China

[5] Department of Bioscience, Aarhus University, Vejlsøvej 25, 8800 Silkeborg, Denmark

[6] WATEC, Aarhus University Centre for Water Technology, Department of Biology, 8000 Aarhus C, Denmark

[*]Corresponding authors: L. Wijewardene (lishani@hydrology.uni-kiel.de) and N. Wu (nwu@hydrology.uni-kiel.de)

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Abstract

Stream biofilms play an important role in the structure, functioning, and integrity of agricultural streams. In many lowland streams macrophyte vegetation is abundant and functions as an important substrate for biofilm (epiphyton) in addition to the gravel and stone substrate for epilithon on the stream bed. We expect that reach-scale habitat conditions in streams (e.g., nutrient availability, hydraulic conditions) affect the epiphyton and epilithon biomass and composition, and that this effect will be substrate-specific (macrophytes and stones). The objectives of our study were (i) to describe concurrent changes in epiphyton and epilithon biomass and composition over a year in agricultural streams, and (ii) to determine the substrate specific reach-scale habitat drivers for the epiphyton and epilithon structure. We monitored epiphyton and epilithon biofilm biomass and composition at three-week intervals and reach-scale environmental conditions daily during a year for two agricultural streams. The results showed that epiphyton and epilithon communities differed in biomass, having high substrate specific biomass in epilithon compared to epiphyton. Epiphyton was mainly composed of diatom and green algae, while cyanobacteria were more important in epilithon, and the diatom species composition varied between the two biofilm types. Epiphyton structural properties were less influenced by reach-scale hydrology and nutrient availability compared to epilithon. The overall explanatory power of the measured environmental variables was low, probably due to micro-scale habitat effects and interactive processes within stream biofilms. Knowledge of biofilm control in agricultural streams is important in order to improve management strategies, and future studies should improve the understanding of micro-scale habitat conditions, interactive relationships within biofilms and between the biofilm and the substrates.

Key words: Stream biofilms, Biomass, Algal composition, Hydrology, Nutrients, Macrophytes

3.1 Introduction

Stream biofilms play a unique and key role in aquatic ecosystems due to their involvement in biogeochemical cycles through primary production, ecosystem metabolism, nutrient uptake and trophic interactions (Battin et al., 2003; Besemer et al., 2012). Biofilms are complex in their structure and function, and are composed of autotrophic microalgae dominated by diatoms, green algae and cyanobacteria, as well as heterotrophic organisms such as bacteria, protozoa, and fungi (Romaní, 2009). Biofilm grows on various substrates in the stream including macrophytes (epiphyton) and stone and gravel (epilithon), and their development is controlled by a complex array of factors and interactions (Biggs and Thomsen, 1995) with irradiance, nutrient availability, physical disturbance and grazing being the most important (Biggs, 1996).

Most studies on biofilm structure in lowland agricultural streams have been on epilithic biofilm (e.g., Guo et al., 2020; Wu et al., 2019) and much less is known about the epiphytic biofilm, although we know it might be the primary site for microalgae growth in macrophyte rich streams and may be the main contributor to reach-scale metabolism (Alnoee et al., 2016) and nutrient uptake (Levi et al., 2015). Furthermore, macrophytes as biofilm substrate offer very different conditions for auto- and heterotrophic biofilm compared to gravel, and stones and the communities may therefore differ. First, macrophytes are organic substrates and thus may leach organic carbon (Demarty and Prairie, 2009; Zhai et al., 2013) and nutrients (Bojorge-García et al., 2014; Burkholder and Wetzel, 1990; Wijewardene et al., 2022). Macrophytes may also exchange CO_2 and O_2 with epiphytic biofilm, as a product of photosynthesis and respiration (Brodersen et al., 2020). In addition, internal biofilm recycling of nutrient and gas may occur (Allen, 1971) and affect both the biofilm and the host. Second, macrophytes grow as dense beds, creating a strong gradient in hydraulic conditions from the more exposed outside to the inside with reduced hydraulic disturbance (i.e., reduced alternation in water velocity and turbulence) (Cantonati and Spitale, 2009). In contrast, nutrient and gas exchange in epilithic biofilm occur via the open stream water or internal recycling in the biofilm. Therefore, hydrological disturbance and resource availability can be very different for epiphytic and epilithic biofilm at a given time and the differences may vary with temporal changes in hydrology and resource availability in the streams on a daily and seasonal scale (Roberts et al., 2007). Ultimately, it may lead to structural differences in epiphyton and epilithon communities in terms of biomass and composition, driven by substrate-specific drivers.

Epilithon is typically dominated by firmly attached diatoms or green filamentous algae (Lowe and LaLiberte, 2017; Tang et al., 2002), but higher abundances of cyanobacteria may occur depending on the environmental conditions (Zlatanović et al., 2018). Diatoms are also considered to be the predominant group of algae in the epiphyton community (Costică et al., 2018; Shamsudin and Sleigh, 1995), but the composition changes over seasons depending on both environmental conditions and the growth pattern of the host plant (Shamsudin and Sleigh, 1995; Xia et al., 2020). Some studies argue that epiphyton and epilithon diatom species composition is remarkably different (e.g., Cantonati and Spitale, 2009) while others state the opposite (e.g., Winter and Duthie, 2000). Overall, Biggs (1996); Biggs et al. (2005a) and Biggs et al. (1998) link reach-scale hydrological and hydraulic factors to periphyton abundance and composition. Nutrients such as phosphate (PO_4^{3-}) and organic contaminants are the main drivers of diatom assemblages in streams (e.g., Guo et al., 2020; Munn et al., 2018; Soininen, 2007; Yang et al., 2015) and their composition can therefore be closely related to land use in the stream catchment and reach-scale habitat factors. More specifically, Cantonati and Spitale (2009) found that diatom species composition was predominantly driven by reach-scale temperature, water velocity, nitrate (NO_3^-) and PO_4^{3-} in both the epiphyton and epilithon in mountainous streams surrounded by a pristine environment, while Winter and Duthie (2000) identified alkalinity, conductivity, suspended solids, and biological oxygen demand as the main drivers in streams surrounded by the mixed land-use of urban, agriculture and woodlands. One study with concurrent measurements of epiphyton and epilithon (Shamsudin and Sleigh, 1995), found that the species composition of epiphytic algae on *Ranunculus* sp. were overall similar to the epilithic algae species on stream bed gravel, and also that the dominant species differed between the two substrata.

The objectives of our study were to further explore the epiphyton and epilithon community in streams (i) to describe concurrent changes of the epiphyton and epilithon biomass and composition during a year in agricultural streams, and (ii) to determine the substrate-specific reach-scale habitat drivers for the epiphyton and epilithon structure. We described epiphyton and epilithon composition in terms of biomass, auto-heterotrophic composition, microalgae groups, and diatom species composition, whereas reach-scale habitat conditions were described for short-term (3 weeks) environmental regimes based on daily measurements. We hypothesized that (i) structural components of epiphyton and epilithon are significantly different due to the main habitat differences between macrophyte and gravel/stone, e.g., organic versus inorganic substrates and ease of hydraulic disturbances (H1); and therefore that (ii)

epiphyton biomass and composition are less affected by the short-term hydrological regime due to a hydraulic gradient within the macrophyte bed, and are thus less directly disturbed by reach-scale water velocity at high discharge compared to epilithon (H2), and (iii) epiphyton biomass and composition are less dependent on reach-scale water nutrients compared to epilithon due to organic exudates from macrophyte substrates (H3).

3.2 Methods

3.2.1 Study area

We selected two lowland agricultural streams, namely, Aarhus (56º13'N, 10º04'E) and Lyngbygård (56º15'N, 10º03'E) located in Jutland, Denmark (Fig. S3.1), with a watershed area of 118.6 km^2 and 131.5 km^2, respectively, dominated by agricultural land cover (72.7 % and 71.6 %, respectively) (https://oda.ft.dk/, 12/11/2019). Stream substrate was a mix of sand, gravel, and stones. *Ranunculus aquaticus* was the predominant macrophyte in both streams (Riis, 2008). Stream water pH typically ranged from 7.0 to 7.9 with an alkalinity from 2.81 to 2.85 mEq L^{-1}. We conducted field measurements and sampling from February 2019 to January 2020. Although the two streams had similar catchment size and land use, there was significant differences in light availability, dissolved organic carbon and PO_4^{3-} (see Section 3.3.1). Therefore, we treat the two streams separately in the data analyses.

3.2.2 Environmental variables

Incident light above the water surface was recorded every 5 minutes by a HOBO Pendant data logger (Onset Computer Corporation, Pocasset, MA, USA). Recorded light in Lux units was converted to photosynthetically active radiation (PAR, mols photons m^{-2} day^{-1}) by applying a conversion factor of 0.019 (Thimijan and Heins, 1983). Water temperature was recorded every 15 minutes with a YSI EXO3 multiparameter sonde (Yellow Springs, OH, USA). At each stream, discharge data were obtained from a nearby gauging station (Danish Environmental Protection Agency) that records in 15-minute intervals.

Water samples for nutrient and dissolved organic carbon (DOC) concentration analyses were collected eight times a day using an automated ISCO 3700 Portable Sampler (Teledyne ISCO), pooled together to obtain a daily composite sample, and filtered through pre-combusted GFF filters (Whatman, UK). Samples for phosphate (PO_4^{3-}), ammonium (NH_4^+) and nitrate (NO_3^-) analyses were frozen, whereas DOC samples were acidified using 10% HCl to pH = 2 -

3. Inorganic nutrient concentrations were analysed using a Lachat QC-8000 Flow Injection Autoanalyzer (Lachat Instruments, USA). Concentrations of DOC were analysed through combustion catalytic oxidation on a Shimadzu TOC Analyzer TOC-VCSH. Dissolved inorganic nitrogen (DIN) was calculated as the sum of NH_4^+ and NO_3^-.

Using the daily averaged data of the above measurements, nine environmental variables were calculated to describe short-term environmental regimes covering the period of 21 days before the biofilm sampling date following the descriptions of Guo et al. (2020) (Table S3.1). These environmental variables included other environmental parameters (other env.): cumulative light (photons m^{-2}), mean temperature (ºC), mean DOC (mg L^{-1}); hydrological regime parameters: median discharge (Q_{med}, L s^{-1}), coefficient of variation of discharge (CV of Q, %), frequency of low flow (Fre_{Low}, days) and frequency of high flow (Fre_{High}, days); and nutrients parameters: mean water PO_4^{3-} concentration (mg L^{-1}) and mean water DIN concentration (mg L^{-1}).

3.2.3 Epiphyton and epilithon sampling

We sampled epiphyton and epilithon every three weeks (21 days) in the two study streams. For epiphyton, we harvested 10-15 apical shoots (5 cm long) of *R. aquaticus* across the macrophyte bed including both the edges and middle areas to obtain a composite sample. For epilithon, we collected 20 inorganic fritted glass disks (3.8 cm^2; catalogue no. 528-042; LECO Corporation, St Joseph, Michigan) deployed six weeks prior to sampling for each time (Fig. S3.1, Steinman et al. (2017)). Inorganic fritted glass disks were deployed in an open reach-section close (<5 m) to the macrophyte beds without overlap. In the field, all samples were immediately placed in a dark container with very little stream water and transported to the laboratory. We extracted the epiphyton attached to the macrophytes by gently brushing the stems and leaves. For epilithon characterization, we removed the biofilm from the disks through careful brushing.

3.2.4 Epiphyton and epilithon structure characterization

Chlorophyll-*a* (Chl-a) was extracted through 95% ethanol from triplicate aliquot samples and estimated according to the method described by Steinman et al. (2017). Biofilm ash-free dry mass (AFDM) was measured according to the method recommended in Steinman et al. (2017). The dry weight of the harvested macrophytes was measured after drying it at 70 ^oC for 48 h. Initially epiphyton measurements were calculated per dry weight of macrophyte,

and the epilithon measurements were calculated per disc area (cm^{-2}) and converted to per substrate area (m^{-2}) for further analyses. The conversion of epiphyton measurements from per dry weight of macrophyte to per area of macrophyte was performed using the known relationship between dry weight to area 22.4 g m^{-2} for *R. aquaticus* (T. Riis, unpublished data). The Autotrophic Index (AI) is the proportion between AFDM and Chl-a (Steinman et al., 2017), and describes the trophic nature (heterotrophic: autotrophic composition) of the biofilm such that values ≥200 indicate heterotrophic associations, whereas values below this point indicate an autotrophic nature (Lakatos, 1989).

The epiphyton algal group composition (i.e., diatoms, green algae and cyanobacteria) was obtained using pigment analysis (adopted from Li et al. (2002)). Epiphyton slurries were filtered through glass fiber membranes (Whatman GF/F, 0.7 µm), and membrane filters were immediately frozen at -18 ^{o}C until pigment extraction. Membrane filters were extracted in acetone (grade: HPLC Plus, purity: ≥ 99.9%) for 8 h. The supernatant of the samples was used for pigment analysis by high performance liquid chromatography (HPLC) following high speed centrifugation. The HPLC system included a Thermo SCIENTIFIC Dionex UltiMate 3000 pump (flow rate: 1 mL min^{-1}), Diode array detector, autosampler (20 µL sampling loop, at 4°C) and column compartment (Column Luna, 3 µm C8). Fucoxanthin, chlorophyll-*b* and zeaxanthin were selected as marker pigments for diatoms, green algae and cyanobacteria, respectively (Li et al., 2002). The algal composition of epilithon were obtained by an *in-situ* fluorometer (BenthoTorch, bbe Moldaenke, Schwentinental, Germany). The BenthoTorch compares reasonably well with lab derived conventional spectrophotometric/HPLC based methods (Garrido et al., 2019; Kahlert and McKie, 2014; Rosero-López et al., 2021; Steinman et al., 2017). These collected pigment/fluorometric measurements for each algal group were converted to organic carbon and then to an organic biomass by using the known relationships among Chl-a to organic carbon (1:30) and organic carbon to organic biomass (1:2) (T. Riis, unpublished data). To calculate the heterotrophic biomass, first the AFDM was subtracted from each of the algae groups' organic biomass. Then, 20% of it was considered as living heterotrophic biomass and the remaining 80% of the biomass was considered as dead organic matter (Sanzone et al., 2001). Finally, the ratios of diatoms, cyanobacteria, green algae, and heterotrophs to AFDM were calculated and visualized.

To identify diatoms, permanent slides were prepared after oxidization using 5 mL of 30% hydrogen peroxide [H_2O_2] and 0.5 mL of 1 mol L^{-1} hydrochloric acid [HCl], and then 0.1 mL of the diatom-ethanol mix was transferred on a 24 × 24 mm cover slip. A drop of Naphrax

was used to mount the slides. Diatoms were identified with the optical microscope (Nikon Eclipse E200-LED, Tokyo, Japan) under 1000× magnification with oil immersion, based on recommendations in Bey (Bey and Ector, 2013), Hofmann (Hofmann et al., 2011), Cantonati (Cantonati et al., 2017) and Bak (Bąk et al., 2012).

3.2.5 Statistical analyses

All statistical analyses were performed using R software version 4.0.2 (R Core Team, 2020) and figures were made using R package *ggplot2* (Wickham, 2016). Relationships between environmental variables were identified by a Kendall correlations coefficient with a significance level of $p < 0.05$ (Fig. S.2; using function *cor* from the R package *corrplot* (Wei and Simko, 2017)). Highly correlated environmental variables ($r > 0.70$) were excluded from the further analyses. Significant differences of environmental variables between two streams were identified by t test/Wilcoxon rank sum test, depending on the fulfilment of the associated hypotheses of the statistical tests. As we found significant differences in the environmental variables of the two streams, all the below-mentioned statistical analyses were repeated for different data subsets, including Aarhus epiphyton, Lyngbygård epiphyton, Aarhus epilithon, Lyngbygård epilithon in addition to our main two datasets of epiphyton and epilithon.

The diatom species composition (relative abundance of species) was Hellinger-transformed using the function *decostand* in R package *vegan* (Oksanen et al., 2020). This maintained the Euclidean distances between samples in the multidimensional space, avoiding interruptions by reducing the weight of abundant species. To identify differences of diatom species composition between epiphyton and epilithon, we conducted a non-metric multidimensional scaling (NMDS) analysis based on the Bray – Curtis similarity using the *metaMDS* function from the R package *vegan*. These differences between studied community assemblages were further statistically tested by a permutational multivariate analysis of variance using distance matrices (ADONIS, permutations = 999, using the function *adonis* from the R package *vegan*). A community trajectory analysis was conducted on the NMDS distance matrix to understand how each community changes over time throughout our one-year study period (using function *trajectoryPlot* from the R package *vegclust*; (De Caceres, 2010)).

We followed the below-mentioned steps to identify the main drivers of diatom species composition. First, a preliminary detrended correspondence analysis (DCA, using function *decorana* from the R package *vegan*) on the Hellinger-transformed species data was conducted.

The longest DCA gradient lengths along the axes were below 2, suggesting that a redundancy analysis (RDA) was suitable for describing species composition (Lepš and Šmilauer, 2003). We conducted a partial redundancy analysis (pRDA) to quantify the amount of variability in diatom assemblages explained by the environmental variables collectively as three categories, i.e., other env: (light, temperature and DOC), hydrology (Q_{med}, CV of Q) and nutrients (PO_4^{3-} and DIN) and their shared contributions (Cornejo et al., 2019). Hellinger-transformed species data was used in this analysis. The adjusted R^2 (adj. R^2) values of the pRDA analysis were used to explain the variability associated with each environmental category and their shared contributions. Variation partitioning was conducted by using the *varpart* function from the R package *vegan*. The statistical significance of the pRDA models were tested using the *anova* function from the R package *vegan* (permutations = 999). Results were represented in Venn diagrams that drawn using Inkscape software (Inkscape Project, 2020).

To assess the relationship between environmental variables and other structural responses such as biomass (i.e., Chl-a, AFDM), AI, and algal composition (i.e., diatom, green algae, and cyanobacteria), we first conducted simple linear regressions between the selected responses and environmental variables. Variables with significant linear regressions were used to conduct multiple regressions. For each biofilm response variable, the best models out of the multiple regressions were selected through a stepwise model selections by AICc (function *stepAIC* in R package *MASS* (Ripley et al., 2013)) for model simplification i.e., the model with minimum AICc value was considered as the best fitted. All structural responses and environmental variables were transformed to ln (x + 1) and scaled before the regression analyses.

3.3 Results

3.3.1 Changes in environmental variables

Environmental variables varied significantly over the study period (Fig. 3.1; Table S3.1). High median discharge (Q_{med}), frequency of high flow (Fre_{High}), DOC and DIN concentrations were observed in the winter months while high light, temperature, and a frequency of low flow (Fre_{Low}) characterized summer conditions. PO_4^{3-} concentration was lowest during spring, and after May it increased in both streams. Furthermore, DOC and Q_{med} revealed a positive correlation ($r = 0.5$, $p < 0.05$), emphasizing co-occurring high discharge and turbid water conditions (Fig. S3.2). The light availability was significantly higher in Aarhus

due to less shading from riparian vegetation than the Lyngbygård (Aarhus: 369.93 (mean) ± 271.34 (SD) photons m^{-2}, Lyngbygård: 153.89 ± 112.62 photons m^{-2}) and higher PO_4^{3-} concentrations (Aarhus: 0.04 ± 0.01 mg L^{-1}, Lyngbygård: 0.01 ± 0.01 mg L^{-1}) (t test/Wilcoxon rank sum test, $p < 0.05$). In contrast, DOC concentrations were significantly higher in Lyngbygård (Aarhus: 5.28 ± 0.95 mg L^{-1}, Lyngbygård: 6.22 ± 1.45 mg L^{-1}) compared to Aarhus (t test/Wilcoxon rank sum test, $p < 0.05$). Hydrological variables were not significantly different between the two streams (t test, $p > 0.05$).

3.3.2 Biomass, AI and main drivers

The epiphyton and epilithon biomass (per substrate area) changed over the year in both streams (Fig. 3.2). The concentrations of Chl-a (mean: 0.78 mg m^{-2}, range: 0.02-5.22 mg m^{-2}) and AFDM (mean: 0.39 g m^{-2}, range: 0.04-3.03 g m^{-2}) in the epiphyton were much lower than in the epilithon (mean Chl-a: 55.55 mg m^{-2}, range: 10.60-148.05 mg m^{-2} and mean AFDM: 26.27 g m^{-2}, range: 8.60-93.33 g m^{-2}). The AI of both biofilm types was generally higher than 200, indicating a high heterotrophic dominance (Fig. 3.2), and AI was generally higher in the epiphyton (mean: 902, range: 134 - 2703) than in the epilithon (mean: 677, range: 177-1965). In both communities, AFDM peaked during the summer months (i.e., July), whereas the Chl-a of the epiphyton peaked twice, in spring (i.e., April) and in autumn (i.e., September).

When comparing epiphyton in the two streams, we found that Chl-a concentrations in Aarhus (1.15 mg m^{-2}, range: 0.10 - 6.23 mg m^{-2}) generally doubled the concentrations in Lyngbygård (0.52 mg m^{-2}, range: 0.01 - 2.53 mg m^{-2}). AFDM was also mostly higher in Aarhus (mean: 0.64 g m^{-2}, range: 0.07 - 4.49 g m^{-2}) than in Lyngbygård (mean: 0.21 g m^{-2}, range: 0.03 - 0.47 g m^{-2}), and AIs were 778 (range: 239 - 1787) and 993 (range: 103 - 3475), respectively. A similar pattern was observed for epilithon. The mean Chl-a concentrations in Aarhus doubled (71.87 mg m^{-2}, range: 16.60 – 148.05 mg m^{-2}) the concentrations in Lyngbygård (38.14 mg m^{-2}, range: 10.60 - 113.66 mg m^{-2}); AFDM was higher in Aarhus (mean: 30.28 g m^{-2}; range: 8.95 – 93.33 g m^{-2}) than in Lyngbygård (mean: 22.00 g m^{-2}; range: 8.59 – 63.77 g m^{-2}), and AIs in Aarhus and Lyngbygård streams were 677 (range: 171 - 1965) and 579 (range: 171 - 1649), respectively.

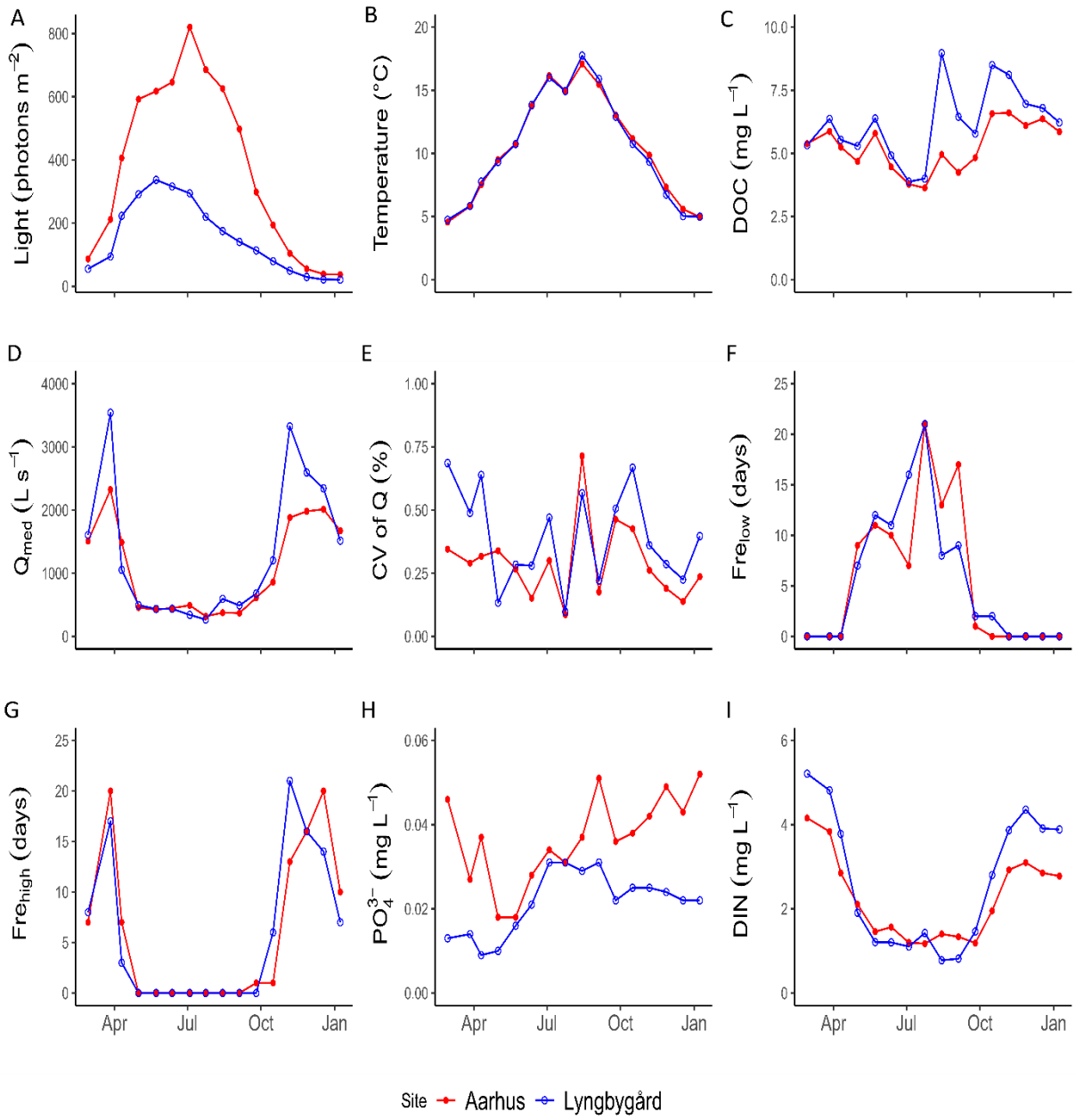

Fig. 3.1: Changes in environmental variables over the study period. Description of calculations of these variables can be found in Table S3.1. All environmental data derived from measurements obtained in the period of 21 days before the sampling date: A: cumulative light (Photons m^{-2} day^{-1}); B: mean temperature (ºC); C: mean DOC (mg L^{-1}); D: median discharge (Q_{med}, L s^{-1}); E: coefficient of variation of discharge (CV of Q, %); F: frequency of low flow (Fre_{Low}, days); G: frequency of high flow (Fre_{High}, days); H: mean PO_4^{3-} concentration (mg L^{-1}) in water and I: mean DIN concentration (mg L^{-1}) in water.

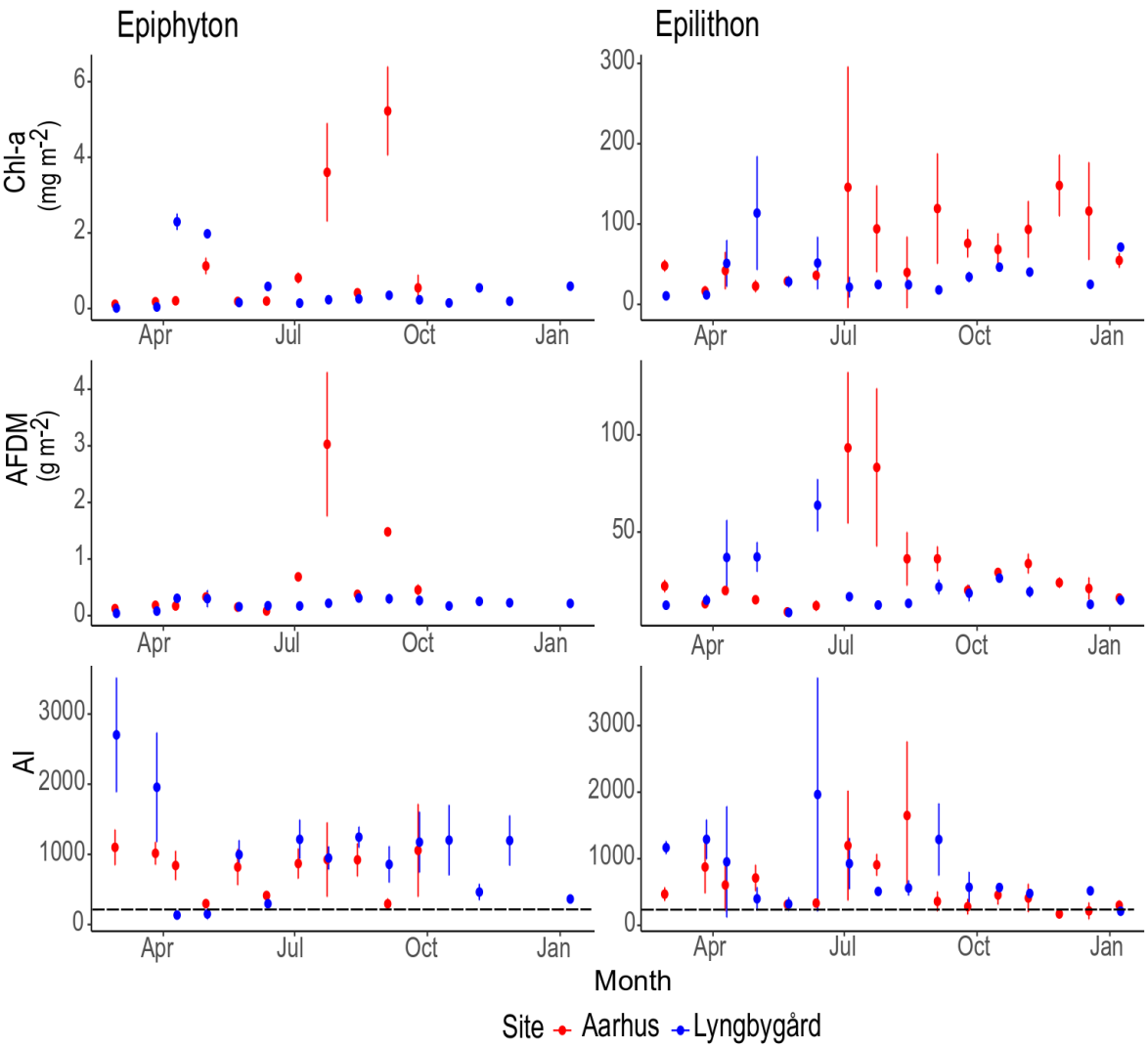

Fig. 3.2: Changes in epiphytic and epilithic biomass and AI (per substrate area) throughout the annual study of the two streams. Dots and error bars denote mean and standard deviation values, respectively (n = 3). The horizontal dashed lines in AI graphs show the index value of 200, which mark the limit between heterotrophic (above) or autotrophic (below) predominance in the biofilms.

We found weak correlations between the environmental variables and the epiphytic and epilithic biomass and AI when analysing data from both streams together (Table S3.2). Only the epilithon Chl-a showed a significant and positive relationship with water PO_4^{3-} concentration (Table 3.1). No significant models were obtained for epiphyton and epilithon biomass and AI when streams were analysed separately.

Table 3.1: Multiple regression models on biomass and AI of epiphyton and epilithon ($p < 0.05$ and lowest AIC) for all data from the two study streams. Significant variables in the models are shown in bold. All environmental data derived from measurements obtained in the period of 21 days before the sampling date (Fig. 3.1).

Model	Response variables	Environmental variables	Estimate	p value	Model adj.R^2	Model significance	AIC
Epiphyton	Chl-a	No significant model					
	AFDM	Temperature	0.306	0.112	0.218	0.023	72.215
		DOC	-0.352	0.069			
	AI	No significant model					
Epilithon	Chl-a	CV of Q	-0.259	0.128	0.224	0.01	87.531
		PO_4^{3-}	**0.386**	**0.027**			
	AFDM	No significant model					
	AI	CV of Q	0.298	0.093	0.164	0.029	89.946
		PO_4^{3-}	-0.282	0.111			

3.3.3 Algal composition and main drivers

Over 50% of the epiphyton and epilithon consisted of dead organic matter and algal composition changed throughout the year (Fig. 3.3). As a general trend, the autotrophic community of epiphyton consisted of diatoms > green algae > cyanobacteria (very low), while the epilithon community consisted of diatoms > cyanobacteria > green algae. Peaks of diatoms in epiphyton were found in April at Lyngbygård and in September at Aarhus. Green algae were present in high percentage in epiphyton community throughout the year around except for two

peak times of the diatoms. In the epilithon community, the highest autotrophic community (diatoms and cyanobacteria) was observed in late May and the presence of green algae was only observed in summer in Aarhus stream.

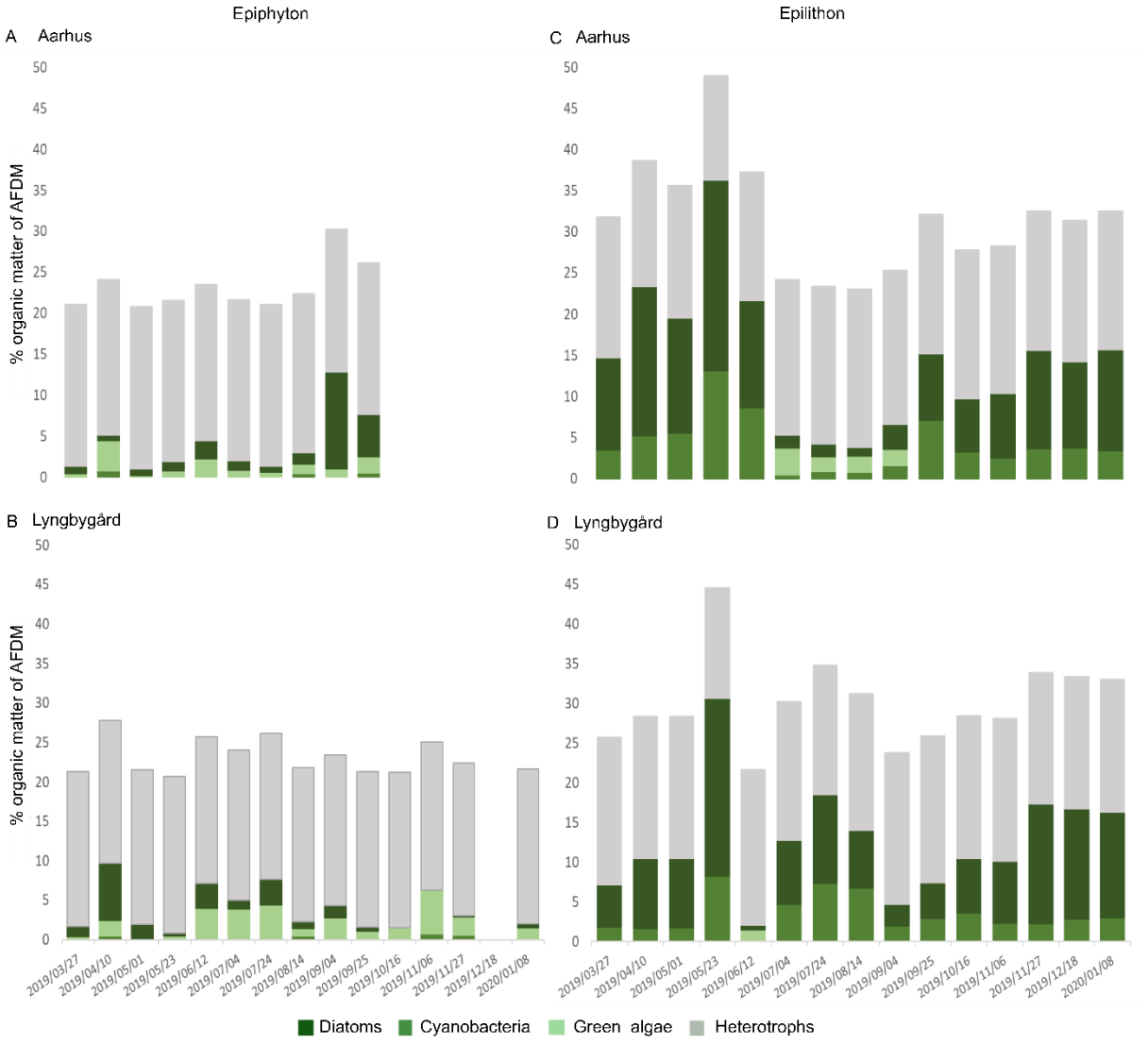

Fig. 3.3: Changes of biofilm composition over the year. A: epiphyton at Aarhus stream; B: epiphyton at Lyngbygård stream; C: epilithon at Aarhus stream and D: epilithon at Lyngbygård stream. Epiphyton only present at samplings date where macrophytes are present.

Overall, DOC negatively correlated with epiphytic diatom biomass but positively correlated with epiphytic cyanobacteria biomass in Lyngbygård (Table 3.2). In epilithon, temperature negatively correlated with diatom biomass. Furthermore, epilithic green algae biomass positively linked with temperature and negatively correlated to DOC (Table 3.2). No significant models were obtained for epiphyton in Aarhus and epilithon in Lyngbygård.

Table 3.2: Multiple regression models on algal composition in epiphyton and epilithon ($p < 0.05$ and lowest AIC). Significant variables in the models are shown in bold. All environmental data derived from measurements obtained in the period of 21 days before the sampling date (Fig. 3.1).

Model	Response variables	Environmental variables	Estimate	p value	Model adj.R^2	Model significance	AIC
Epiphyton	Diatom	**DOC**	**-0.502**	**0.012**	0.218	0.012	66.106
	Cyanobacteria	Q_{med}	0.349	0.087	0.217	0.03	67.048
		CV of Q	0.309	0.127			
	Green algae	No significant model					
Epilithon	Diatom	**Temperature**	**-0.562**	**0.001**	0.291	0.001	78.752
	Cyanobacteria	No significant model					
	Green algae	**Temperature**	**0.624**	**0.028**	0.365	0.002	77.231
		DOC	**-0.596**	**0.006**			
		Q_{med}	0.460	0.161			
Epiphyton - Lyngbygård	Diatom	Light	0.390	0.147	0.388	0.027	37.480
		DOC	-0.413	0.127			
	Cyanobacteria	**DOC**	**0.570**	**0.033**	0.269	0.033	39.183
	Green algae	No significant model					
Epilithon - Aarhus	Diatom	**Temperature**	**-0.679**	**0.005**	0.419	0.005	38.270
	Cyanobacteria	No significant model					
	Green algae	Temperature	0.353	0.179	0.543	0.004	35.466
		DOC	-0.495	0.069			

3.3.4 Diatom species composition and main drivers

In total, 193 diatom species were found in our study belonging to seven different families (i.e., Monoraphidees, Naviculacees, Araphidees, Centrophycidees, Surirellacees,

Nitzschiacees and Brachyraphydees). We identified 135 species in epiphyton and 164 species in epilithon. The epiphyton diatom community was dominated by *Cocconeis placentula* var. *euglypta* Ehr. (25% in relative abundance), followed by *Navicula lanceolate* Ehr. (9%), *Achnanthidium minutissimum* Kütz. (7%), *Navicula tripunctata* (Müller) Bory (6%) and *Gomphonema parvulum* Kütz. (5%) (Table S3.3). The epilithon diatom community was dominated by *Achnanthidium minutissimum* (37% in relative abundance), followed by, *Navicula lanceolate* (10%), *Cocconeis placentula* var. *euglypta* (8%), *Planothidium lanceolatum* (Brébisson ex Kütz.) Lange-Bertalot (4%) and *Planothidium frequentissimum* Lange-Bertalot (3%) (Table S3.3).

A non-metric multidimensional scaling (NMDS) ordination of the biofilm diatom community composition revealed two distinct clusters (stress: 0.01, Fig. 3.4A) suggesting that the diatom species composition of epiphyton and epilithon communities were significantly different from each other (Adonis, $F = 7.08$, $p = 0.001$). Additionally, the epiphyton and epilithon diatom community showed distinct separation according to stream (Fig. 3.4B; stress: 0.16 and Adonis, $F = 4.56$, $p = 0.001$ and Fig 4D; stress: 0.14 and Adonis, $F = 2.87$, $p = 0.008$). Furthermore, the temporal dynamics of the epiphyton and epilithon diatom community showed greater variation in Lyngbygård compared to in Aarhus over the sampling year (Fig. 3.4C and 3.4E). Epilithic diatom communities tended to come back to first sample completing a cyclic path while the epiphytic diatom communities had more distinctive start and end points.

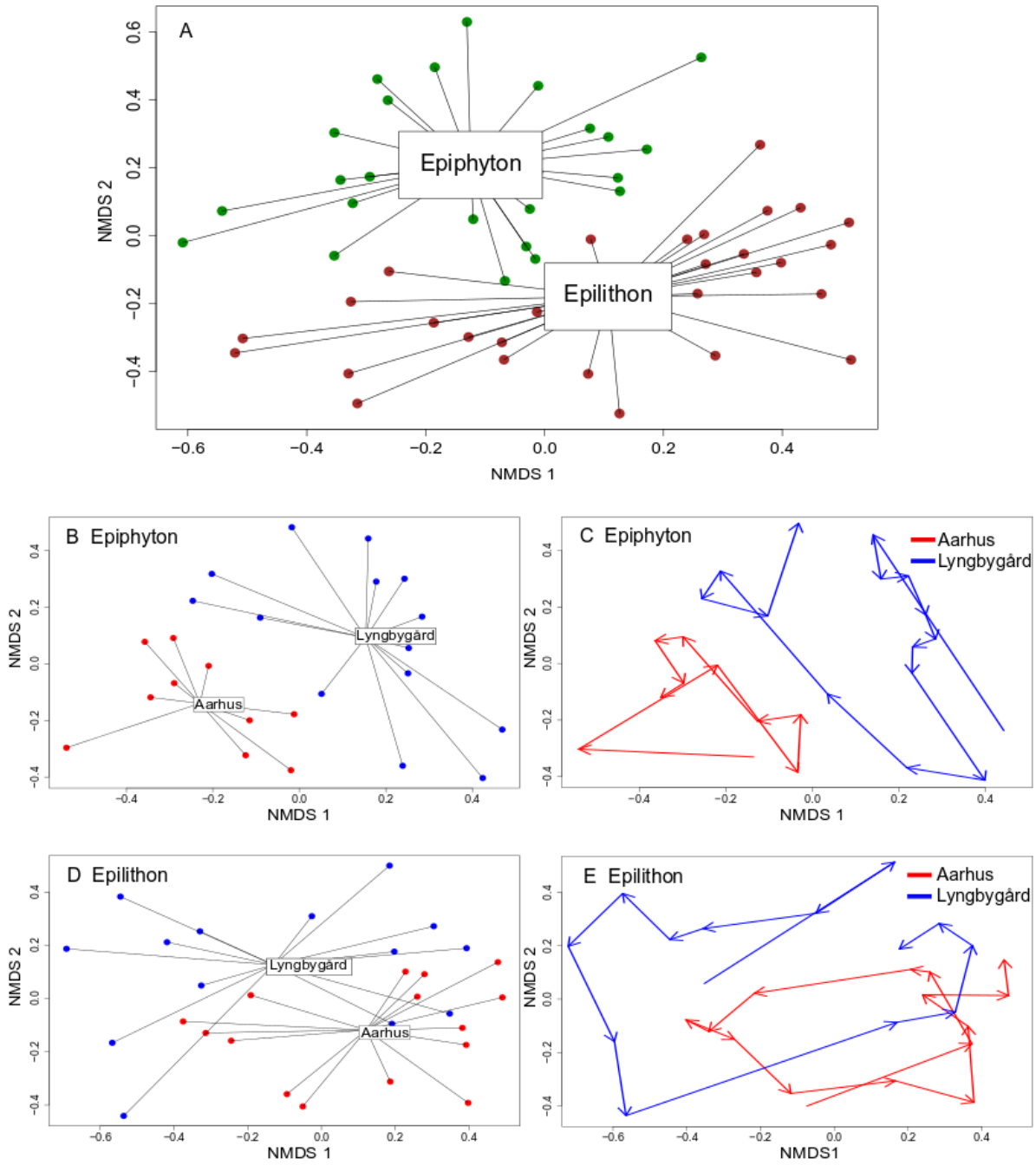

Fig. 3.4: Differences in diatom species composition and trajectories of community change over the study year. A: epiphyton and epilithon diatom species composition; B: epiphytic diatom communities in two streams; C: trajectories of epiphytic community change over the study year; D: epilithic diatom communities in two streams and E: trajectories of epilithic community change over the year.

According to the partial redundancy analysis (pRDA) the variation in the epiphytic diatom community can be mainly explained by nutrients and other environmental variables (adj. R^2 = 0.20 and 0.28, respectively, Fig. 3.5A), with hydrology less important in both streams (Fig. 3.5C and 3.5E). For epilithon, all three groups of variables were responsible for an equal amount of variation (adj. R^2 = 0.30; 0.25, and 0.21), indicating that hydrology was more important for epilithon than for epiphyton (Fig. 3.5B). The effect of hydrology on epilithon was especially pronounced in Aarhus (Fig. 3.5D). The combination of any two and all three environmental variable categories better explained diatom species composition in both communities than any individual categories, and the highest variability of the diatom community composition was explained by the shared contribution of all three categories in both epiphyton and epilithon, as 0.37 and 0.36 (adj. R^2), respectively (Fig. 3.5A and 3.5B).

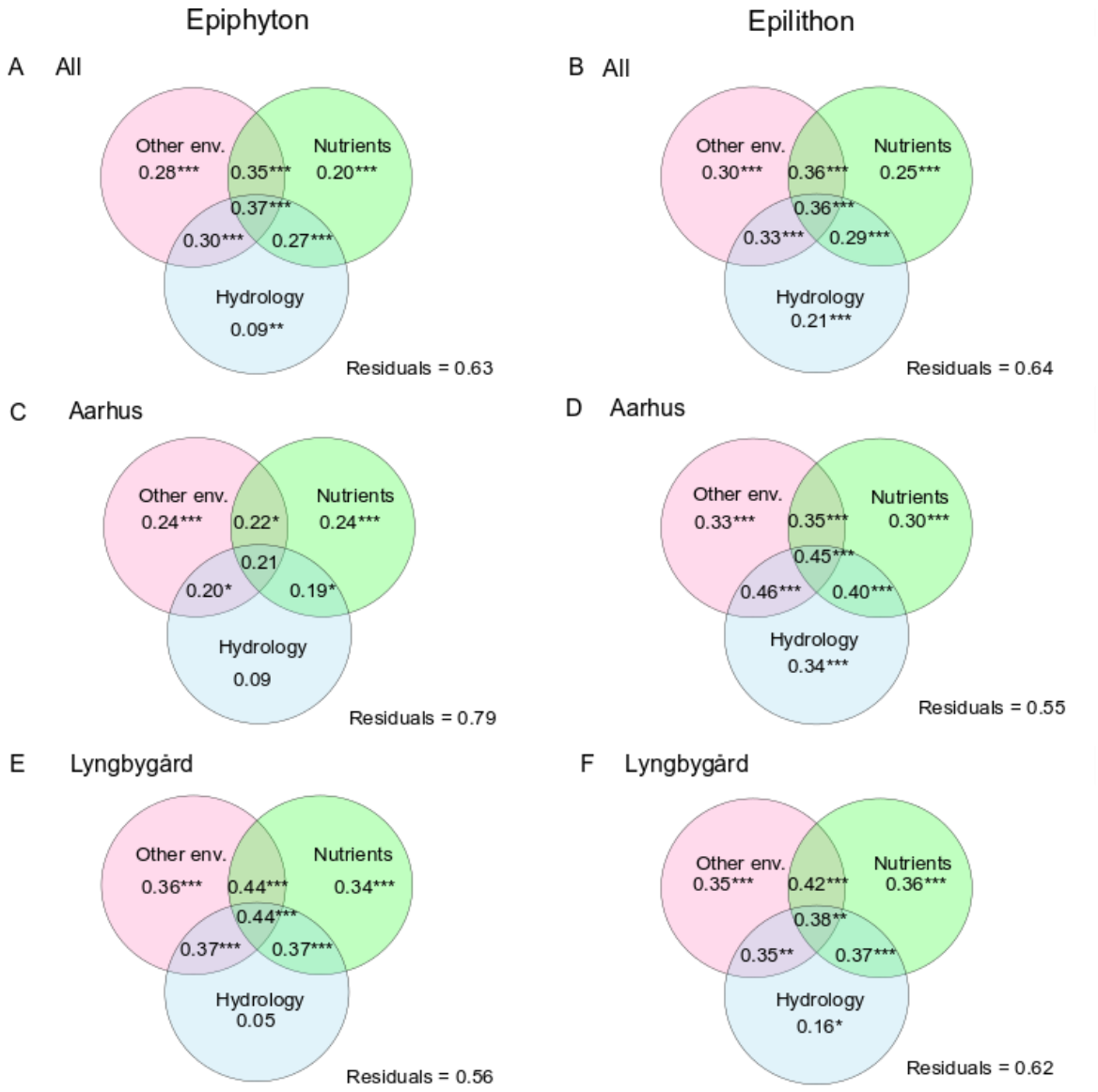

Fig. 3.5: Partial redundancy analysis (pRDA) for quantifying the variation of diatom community composition explained by hydrology (Q_{med}, CV of Q), nutrients (PO_4^{3-}, DIN) and other environmental variables (other env: light, temperature, DOC), their shared contribution and unexplained variance (i.e., Residual). Each subplot represents the studied diatom assemblages as A: epiphyton; B: epilithon; C: epiphyton in Aarhus stream; D: epilithon in Aarhus stream; E: epiphyton in Lyngbygård stream and F: epilithon in Lyngbygård stream. Significance codes: *p < 0.05, **p < 0.01, ***p < 0.001. All environmental data derived from measurements obtained in the period of 21 days before the sampling date (Fig. 3.1).

3.4 Discussion

The results of the study revealed that the biofilm structural components, such as biomass, algal composition and diatom species composition were highly different for epiphyton and epilithon supporting H1. Further, some of our observations supported that epiphyton was less influenced by hydrological and nutrient regimes compared to epilithon (H2 and H3). The explanatory power using daily environmental measurements in a 3-week regime was lower than expected, but it was still within the range of previous studies.

The main structural differences between epiphyton and epilithon found in our study were that in epiphyton, unlike in epilithon, (1) the area-based biomass was about 50-fold lower, (2) the heterotrophic composition was higher, (3) the abundance of cyanobacteria was lower, and (4) the dominant diatom species were associated with low disturbance and high nutrient concentrations. In H1, we expected the biomass to be different between the two biofilms, which is shown in the results, but the expected difference was higher biomass in epiphyton than epilithon focusing on hydrological disturbances and nutrients solely. However, the results revealed an opposite trend. A higher biomass per substrate area for epilithon, compared with epiphyton have previously been found in river ecosystem (Belyaeva, 2017) and lakes (Kahlert and Pettersson, 2002). They emphasize that a higher epilithon biomass is most likely due higher stability (Soininen and Eloranta, 2004; Zelnik and Sušin, 2020) and the durability of gravel and stone substrates, whereas plant-decaying process and macrophyte allelopathic substances may lead to less biomass accrual on epiphyton (Kahlert and Pettersson, 2002). Both biofilm communities showed a dominant heterotrophy similar to previous studies (e.g., Fernandes and Esteves, 2003; Lock et al., 1984). However, we also found that epiphyton showed higher heterotrophy compared to epilithon, which suggests that macrophytes to be a more favourable

substrate for the heterotrophic community compared to the gravel/stone substrate (Wolters et al., 2019).

Diatoms and green algae were the main algal communities in epiphyton, which is consistent with previous studies (Costică et al., 2018; Piirsoo et al., 2007), and the presence of cyanobacteria was negligible. In contrast, cyanobacteria were more abundant than green algae in epilithon, particularly during summer months in Aarhus, where discharge and DIN concentrations were low. This is supported by Zlatanović et al. (2018) that noted a change in the epilithon community from diatoms to green algae and cyanobacteria under low flow periods. Diatom species composition was significantly different between epiphyton and epilithon in our study, which agrees with previous studies by Cantonati and Spitale (2009) and Soininen and Eloranta (2004). In contrast, Winter and Duthie (2000) concluded that the diatom community structure of the two communities is not consistently different in streams surrounded by mixed-land use such as urban, agriculture and woodlands. Furthermore, the dominant epiphyton diatom species found was *Cocconeis placentula* var. *euglypta* whereas *Achnanthidium minutissimum* dominated in epilithon diatom community, which follows the findings in (Shamsudin and Sleigh, 1995; Soininen and Eloranta, 2004; Zelnik and Sušin, 2020).

Differences in the dominant diatom species in epiphyton and epilithon supported hypotheses H2 and H3 stating that epilithon is more strongly related to hydrology and nutrient concentrations in streams than epiphyton. The dominating species in epilithon, *Achnanthidium minutissimum*, is well-known as a species adapted to a high velocity environment (Shen et al., 2018), whereas the species that were found in high abundances in the epiphyton community are often found in a high nutrient environment under agricultural influence (e.g., *Gomphonema* spp. and *Encyonema* sp., Table S3.3) (Lu et al., 2020). Therefore, differences in species composition of the epiphyton and epilithon support that the epilithon is more dependent on the water nutrient concentration and hydraulic disturbances validating the H2 and H3 hypotheses. This was further supported by the fact that the temporal trajectories of diatom community compositions showed the epilithon community completing a cyclic path, moving back to its original state at the end of year while the epiphyton start and end points were different. Closer associations of the epiphytic community with the macrophyte characteristics and the life cycle were found than with environmental factors, which may cause the high turnover rate of the species and the lower persistence of communities compared to epilithon as reported by (Soininen and Eloranta, 2004) and the discontinuity of the cyclic path found in our study, despite the reoccurrence of environmental conditions (Ferreiro et al., 2013; Pettit et al., 2016).

We also found direct support for H3 in the multiple regression analyses. We found that the epilithic biomass (Chl-a) was better related to nutrients than epiphyton (Table 3.1). Epilithic Chl-a demonstrated positive association with PO_4^{3-} and a similar relationship was found in many previous studies (Bowden et al., 1992; Hill et al., 2009). Further, H2 and H3 were directly confirmed by the results of the pRDA analyses, showing variation of the epilithic diatom species composition, more associated with hydrology and nutrients than epiphyton (Fig. 3.5). The influence of hydrology (i.e., discharge, current velocity, low flow, high flow) on the epilithon structure was highly recognized in many previous studies in lotic systems (Ács and Kiss, 1993; Guo et al., 2020; Matthaei et al., 2010; Moulton et al., 2009) and nutrients were identified as an important factor in driving epilithic diatom composition (Munn et al., 2018; Soininen and Eloranta, 2004). Furthermore, the low association of epiphyton with water nutrient concentrations supports the suggestion that the epiphyton community may depend on nutrients released from the macrophyte (Bojorge-García et al., 2014; Wolters et al., 2019). Gosselain et al. (2005) also highlighted that epiphyton was related to physical variables, such as light, macrophyte architecture and hydrology (i.e., seasonal water level variations) in the order of decreasing importance.

Overall, we observed considerable differences in the biofilm structural components between the two study streams, and these differences were predominantly driven by differences in light availability, DOC and PO_4^{3-} (Fig. 3.1). For example, the biofilm biomass was twice as high in Aarhus compared to Lyngbygård, due to higher light availability caused by lower riparian vegetation cover and higher PO_4^{3-} concentrations in Aarhus. High biofilm Chl-a in Lyngbygård was observed only in spring under low riparian shading prior to leaf out, following the Tank et al. (2018) where it had maximum light availability due to low shading by spring riparian vegetation. In Aarhus, we observed a higher abundance of *Cladophora* sp. (filamentous green algae) during summer months, and the eutrophic indicator species *Achnanthidium minutissimum* showed high abundance (relative abundance doubled in epilithon and four times higher in epiphyton) at high PO_4^{3-} concentration compared to Lyngbygård (Lu et al., 2020). Thus, although the two study streams showed similar land use and catchment size, local differences driven by light, DOC and PO_4^{3-} were important to the site-specific biofilm structure.

The explanatory power of the environmental factors on the epiphytic and epilithic structural responses was overall low across the two streams (36-37%), and lower in Aarhus (21-45%) than in Lyngbygård (38-44%). Usually, nutrient concentrations are only measured once or a few times during biofilm accrual, and by using daily measurements we expected a higher

explanatory power. However, the explanatory power was not significantly different from earlier studies such as (Biggs, 2000), who found that 44-49% of variation in mean monthly biofilm Chl-a was explained by hydrology and nutrient concentration in New Zealand streams. Another study by Lévesque et al. (2017) found that, environmental variables were only able to explain 15.4% of the variation of epiphyton biomass in a fluvial system in Canada. Part of the general high unexplained variation in models on biofilm biomass, could result from the fact that (i) reach-scale variables do not reflect micro-scale variables as strongly as expected from previous studies (Biggs, 1996; Biggs et al., 2005a; Biggs et al., 1998), and that biofilms thus may be more closely linked to the micro-habitat mediated environmental variables than the reach-scale measurements (Morin and Kimball, 1983). Furthermore, (ii) biofilms constitute complex communities where individual components respond differently to environmental variables, e.g., DOC acts as a nutrient for the heterotrophic community but for autotrophs high DOC may lead to limited light availability for autotrophs and their photosynthesis (Lévesque et al., 2017; Sobczak and Findlay, 2002). In addition, (iii) biofilm community compartments are highly interactive; for example, autotrophic communities in the biofilm may use CO_2 for their primary production, derived from the respiration of the heterotrophic community internally (Allen, 1971). Moreover, (iv) interactions between biofilms and their substrate such as, epiphytic biofilm and host macrophytes may also show competitive and mutualistic relationships for nutrients on a smaller spatial scale, which may be more important than the nutrient concentrations in the surrounding flowing water (Wijewardene et al., 2022). In order to improve the predictive strength of models on epiphytic and epilithic biofilm, measurements of environmental variables at the micro-habitat scale may be required, or more sensitive data analyses, such as a time series analysis (e.g., Lange, 2006).

3.5 Conclusion

Epiphyton and epilithon showed distinct structural differences during a year in two agricultural streams in terms of biomass, algal composition, and diatom species composition. Epiphyton structural properties were less affected by hydrological regimes and water nutrient concentrations than epilithon, indicating that epilithon is more dependent on external water nutrients to fulfil nutrient requirements, while epiphyton can take advantage of macrophyte leachates. Other environmental variables such as light, temperature and DOC played an important role in driving epiphyton and epilithon structural differences between the two streams. We observed a generally low explanatory power of the included environmental

variables on the biofilm structure even though we used daily measurements. Future studies should address the interactions within biofilm communities, interactions with their substrates and interactions with other biota to better understand the underlying controlling mechanisms of the epiphyton and epilithon structure in agricultural streams. Using micro-scale measurements instead of reach-scale measurements may further enhance the understanding of environmental drivers of the epiphytic and epilithic community structure.

Chapter 4 Influences of pesticides, nutrients, and local environmental variables on phytoplankton communities in lentic small water bodies in a German lowland agricultural area

LishaniWijewardene[1,2], Naicheng Wu[1,3*], Yueming Qu[1], Kun Guo[2], Beata Messyasz[4], Stefan Lorenz[5], Tenna Riis[2,6], Uta Ulrich[1], Nicola Fohrer[1]

[1] Department of Hydrology and Water Resources Management, Institute for Natural Resource Conservation, Kiel University, 24118 Kiel, Germany

[2] Department of Biology, Aarhus University, Ole Worms Allé 1, 8000 Aarhus C, Denmark

[3] Department of Geography and Spatial Information Techniques, Ningbo University, 315211 Ningbo, China

[4] Department of Hydrobiology, Institute of Environmental Biology, Faculty of Biology, Adam Mickiewicz University in Poznan, Uniwersytetu Poznanskiego 6, 61-614 Poznan, Poland

[5] Julius Kuehn-Institute, Institute for Ecological Chemistry, Plant Analysis and Stored Product Protection, Koenigin-Luise-Str. 19, 14195 Berlin, Germany

[6] WATEC, Aarhus University Centre for Water Technology, Department of Biology, 8000 Aarhus C, Denmark

[*]Corresponding authors: N. Wu (nwu@hydrology.uni-kiel.de)

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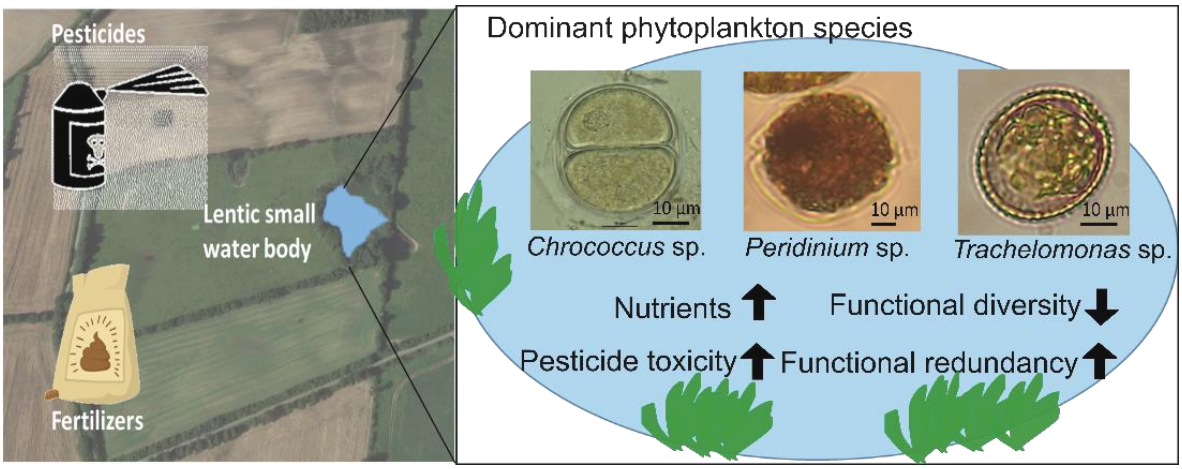
Abstract

Agrochemicals such as pesticides and nutrients are concurrent chemical stressors in freshwater aquatic ecosystems surrounded by agricultural areas. Lentic small water bodies (LSWB) are ecologically significant habitats especially for maintaining biodiversity but highly understudied. Phytoplankton are ideal indicator species for stress responses. Functional features of the phytoplankton are important in revealing the processes that determine the structure of the communities. In this study, we investigated the effects of pesticides, nutrients, and local environmental variables on the species composition and functional features of phytoplankton communities in LSWB. We studied pesticide toxicity of ninety-four pesticides, three nutrients (NH_4-N, NO_3-N and PO_4-P) and local environment variables (precipitation, water level change, temperature, dissolved oxygen concentration, electrical conductivity, pH) in five LSWB over twelve weeks during the spring pesticide application period. We explored respective changes in species composition of phytoplankton community and functional features. Redundancy analysis and variance partitioning analysis were applied to correlate phytoplankton community compositions with the pesticide toxicity (as maximum toxicity in toxic units), nutrients and local environment variables. We used multiple linear regression models to identify the main environmental variables driving the functional features of phytoplankton communities. Pesticide toxicity, nutrients, and local environmental variables significantly ($p < 0.001$) contributed to shaping phytoplankton community composition individually. Local environment variables showed the highest pure contribution for driving phytoplankton composition (12%), followed by nutrients (8%) and pesticide toxicity (2%). Functional features (represent by functional diversity and functional redundancy) of the phytoplankton community were significantly affected by pesticide toxicity and nutrients concentrations. The functional richness and functional evenness were negatively affected by PO_4-P concentrations. Pesticide toxicity was positively correlated with functional redundancy indices. Our findings emphasized the relative importance of concurrent multiple stressors (e.g., pesticides and nutrients) on phytoplankton community structure, directing potential effects on metacommunity structures in aquatic ecosystems subjected to agricultural runoff.

Keywords: Multiple stressors, Agrochemicals, Nutrient and pesticide enrichment, Species composition, Functional diversity, Functional redundancy

Graphical abstract



Highlights

- Pesticides and nutrients are concurrent stressors in lentic small water bodies.
- Pesticide toxicity and nutrients affect phytoplankton community composition.
- Functional diversity reduced with increasing nutrient concentrations.
- Functional redundancy increased with increasing pesticide toxicity.

4.1 Introduction

Freshwater aquatic ecosystems are vulnerable to simultaneous multiple stressors (Aljerf, 2017). Pesticides and nutrients are the main co-occurring chemical stressors in aquatic environments (Aktar et al., 2009; Cooper, 1993; Stehle and Schulz, 2015). Aquatic ecosystems in agricultural areas are frequently exposed to these chemical stressors with agricultural runoffs that contain agrochemicals such as fertilizers and pesticides, which are used for crop growth and plant management (Aktar et al., 2009). Agricultural runoffs can alter the structure and functions of biota in both lotic and lentic aquatic ecosystems. The impacts of pesticides and nutrients on aquatic biota in lotic ecosystems (e.g., Andrus et al., 2013; Cornejo et al., 2019) are better understood compared to lentic systems in agricultural areas (Lorenz et al., 2017). Our study ecosystems, lentic small water bodies (LSWB) can be defined as pond ecosystems which are between 1 m^2 and 2 ha in area, permanent or seasonal, manmade or natural (Biggs et al., 2005b) and also comply with the EU Water Framework Directive stating as lentic water bodies with a surface area <50 ha (WFD, 2009). LSWB are abundant at a global scale and important as habitats for maintaining biodiversity not only for aquatic species but also amphibians and migratory birds (Hill et al., 2017; Hornbach et al., 2020; Ulrich et al., 2018). In addition, LSWB are natural sinks for substances receiving from their catchments and sensitive to local conditions and variations in geology, hydrology, climate and vegetation due to their small catchment size (Biggs et al., 2005b). These LSWB are still neglected by environmental monitoring programs despite their high ecological significance and a high potential for being contaminated with pesticides and nutrients in intensive agricultural areas (Indermuehle et al., 2008). Therefore, monitoring the dynamics of pesticides and nutrients and understanding their potential impacts on associated biota are essential to preserving ecosystem integrity, functions and services of LSWB (Schreiner et al., 2016).

Phytoplankton are important primary producers in freshwater ecosystems and play a vital role in nutrient cycling and energy flow (Brierley, 2017). Further, phytoplankton are ideal for stress-response studies due to their high sensitivity to environmental gradients, short generation time and well-known autecology (Qu et al., 2018b; Wu et al., 2017). Pesticides are the synthetic products using for crop management in agriculture and mainly consist of herbicides, insecticides and fungicides (Schäfer et al., 2011). The effects of pesticides can influence the phytoplankton community both positively and negatively. Direct effects of pesticides often result in adverse negative impacts on phytoplankton through inhibition of

growth, photosynthesis, and reproduction (Rico-Martínez et al., 2012). Indirect effects can often mask the direct negative effects of pesticides (e.g., insecticides) through altering top-down selection pressures, such as predation and competition (Fleeger et al., 2003). Both direct and indirect effects of pesticides on phytoplankton affect densities, growth and traits which ultimately change the composition, structure and function of the phytoplankton community (Cedergreen and Rasmussen, 2017; Noack et al., 2003; Schäfer et al., 2011; Staley et al., 2015). Nitrogen (NH_4-N and NO_3-N) and phosphorous (PO_4-P) are the main nutrients regulating the phytoplankton communities. Fertilizers used in agriculture for enhancing crop growth lead to enrichment of these nutrients in aquatic ecosystems surrounded by agricultural areas (Guignard et al., 2017; Serediak et al., 2014). In contrast to pesticides, nutrient enrichment (e.g., NO_3-N and PO_4-P) often causes phytoplankton abundance and biomass increment which, trigger algal blooms and alter species composition (Heisler et al., 2008; Serediak et al., 2014). On the other hand, low levels of nutrients have also been regarded as a stressor on phytoplankton in previous studies (Bestion et al., 2018; Cunha and Calijuri, 2011). Therefore, when pesticides and nutrients act as simultaneous multiple stressors, this may result in complex responses on the phytoplankton community compositions under natural ecosystem conditions and we are still lacking a comprehensive understanding of these interactions (Vinebrooke et al., 2004). Other than pesticides and nutrients, local environmental factors such as light (Cunha and Calijuri, 2011), water temperature (Rasconi et al., 2015; Schabhüttl et al., 2013), pH (Holopainen, 1991), electric conductivity (EC, Sgarzi et al., 2019), and dissolved oxygen (DO, Kunlasak et al., 2013) also play important roles in shaping phytoplankton communities.

The taxonomic and trait composition of phytoplankton can be altered by pesticides and nutrients (Borics et al., 2020). Traditional biodiversity measurements (e.g., species richness or diversity indices, such as Shannon and Simpson) are solely based on the taxonomic composition and may misinterpret the status of ecosystems (Mouchet et al., 2010). Trait-based (morphological, physiological and behavioural features) approaches are widely used now in the context of describing phytoplankton communities due to their closer relationships with environmental conditions than taxonomic composition (Weithoff and Beisner, 2019). Further, trait-based approaches are robust in predicting ecosystem structure and functions (e.g., Guo et al., 2019; Weithoff, 2003; Zwart et al., 2015). Functional features such as indices of functional diversity (FD) and functional redundancy (FR) have been developed incorporating taxonomic and trait composition and enhancing the potential to reveal the processes that structure biological communities (Mouchet et al., 2010). FD indices reflect the connectivity between

diversity and ecological functions while FR indices are positively associated with stability, resistance and resilience of ecosystems (Schleuter et al., 2010; Wu et al., 2019). The use of phytoplankton community composition and functional features to understand the effects of multiple stressors on biodiversity and ecosystem functioning are still scarce (but see Wu et al., 2019).

Despite the growing need of understanding the effect of pesticides and nutrients as concurrent multiple stressors on phytoplankton communities, there are only a few attempts to answer the question (e.g., Andrus et al., 2013; Baker et al., 2016; Leboulanger et al., 2011). Andrus et al. (2013) focused on atrazine concentrations, nutrients and sediments in agricultural streams and found no effects on algal abundance, diversity, or assemblage structure. Phytoplankton affected by different ways in the study of two herbicides (e.g., Diuron: increase biomass, biovolume and no effect on diversity; Paraquat: decrease biomass, biovolume and diversity) and the insecticide (Fenitrothion: no effect on biomass and biovolume, but reduced diversity) in nutrient-enriched microcosms (Leboulanger et al., 2011). Baker et al. (2016) studied co-application of glyphosate and nutrients on phytoplankton and observed declining nutritional value (e.g., edible carbon concentration) of phytoplankton. To our best knowledge, this study is the first attempt to understand the impacts of pesticides and nutrients on phytoplankton communities in LSWB. LSWB are typically neglected in macro-scale ecological surveys regardless of the ecological importance of these unique ecosystems that are abundant in rural agricultural areas (e.g., in the lowlands of northeast Germany) (Ulrich et al., 2018). Further, the list of pesticides measured in our study is also neglected from almost every monitoring program even though they are commonly used in agricultural practices. We investigated (i) the contribution of pesticide toxicity, nutrients, and local environmental variables on driving species composition of phytoplankton communities and (ii) how these affect the functional features of phytoplankton communities. We hypothesised that pesticide toxicity and nutrient concentrations significantly affect the species composition of phytoplankton communities (e.g., dominance of pollutant tolerant eutrophic indicator species with the increase of pesticide toxicity and nutrient concentrations) (H1) and functional features of phytoplankton communities (e.g., functional richness, evenness and redundancy reduce with increase of pesticide toxicity and nutrient concentrations) (H2).

4.2 Methods

4.2.1 Study area

The Kielstau catchment is a lowland watershed with an area of 50 km^2 and is located in the Northern part of Germany (Schleswig–Holstein). In 2010, the Kielstau catchment was appointed as UNESCO demonstration site (Fohrer and Schmalz, 2012). The land use in the Kielstau catchment is dominated by agriculture (63.7%) and grassland (20.3%) (Wagner et al., 2018). Agricultural fields (~38%) are drained by drainage systems, such as ditches and tile drainage pipes to improve crop growth and management practices (Fohrer et al., 2007). The average annual temperature and precipitation are approximately 8.9 ^oC and 885 mm respectively (DWD, 2017). The soil type in the Kielstau catchment is dominated with Luvisols and relatively homogenous (Ulrich et al., 2018). Further, the slopes range from 0.2 to 3.7% representing flattened lowland topography (Ulrich et al., 2018). Five LSWB (A1, A2, A3, A4, A5) were selected to explore the impacts of pesticides and nutrients on phytoplankton communities due to (i) their similarities in classification as "shallow-storage type" (Kalettka and Rudat, 2006) and (ii) located in same agriculture dominated catchment but surrounded by different crop types (Table 4.1 and Fig. 4.1).

Table 4.1: Description of studied LSWB

LSWB	Area (m^2)	Depth (cm)	Surrounded crop cultivations
A1	205	108	Broad beans
A2	109	160	Winter wheat and oilseed rape
A3	90	100	Strawberries and oilseed rape
A4	236	110	Winter barley
A5	41	110	Corn

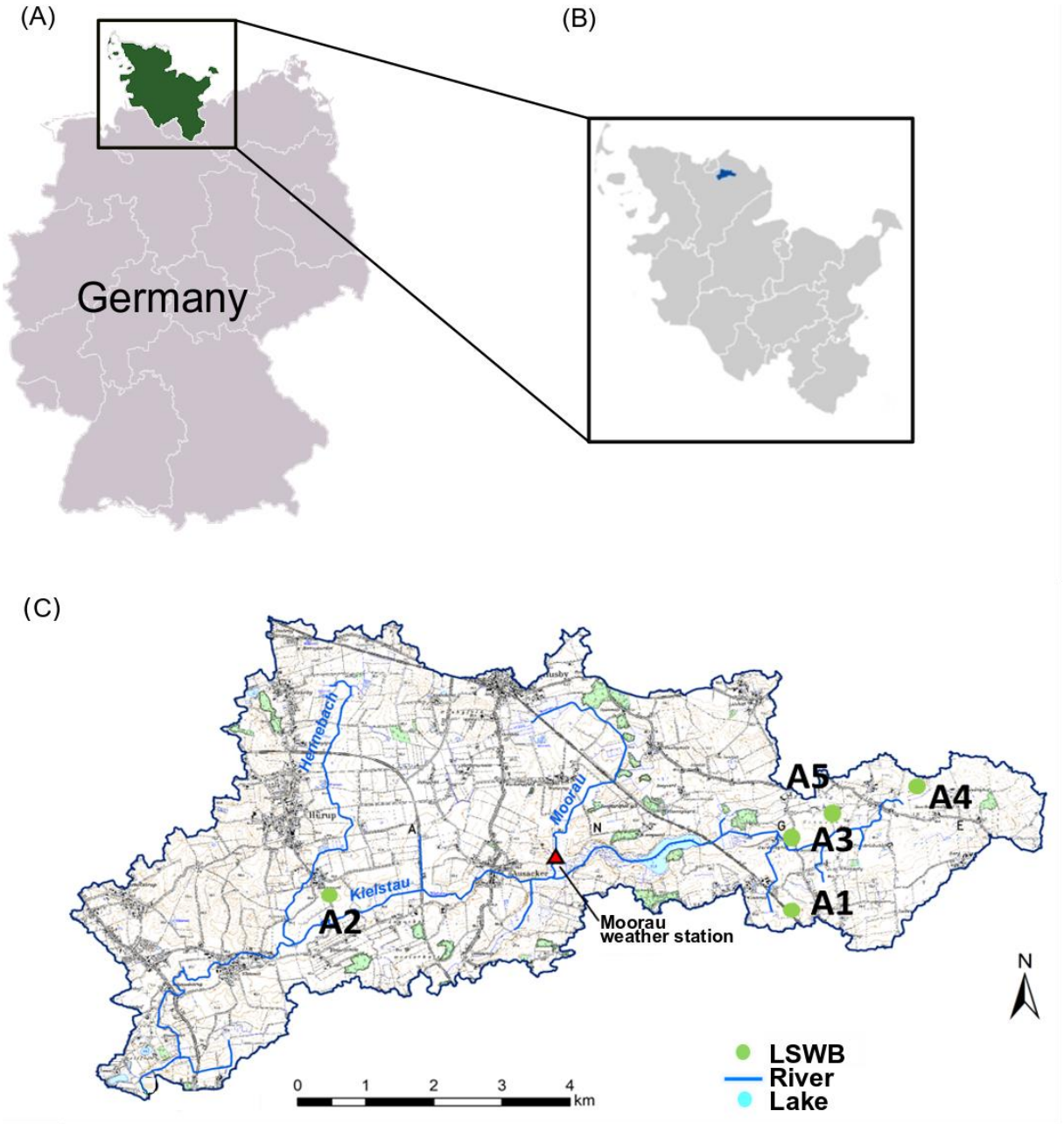


Fig. 4.1: Federal state of Schleswig–Holstein in Germany (highlighted in green colour) (A), Kielstau catchment in Schleswig–Holstein (highlighted in blue colour) (B) and Location of the monitored lentic small water bodies (LSWB) in Kielstau catchment (C). (Source: LVermGeo, 2005; Wagner et al., 2018)

4.2.2 Sample collection and measurements of pesticides, nutrients, and local environmental variables

LSWB were monitored throughout 12 weeks (except A5 which was only monitored the last 6 weeks of the study period due to the late start of cultivation in the adjacent agricultural area) during the spring pesticide application period (April to July 2018) to obtain an exposure gradient of the selected stressors: pesticides and nutrients (Ulrich et al., 2021). Water samples were collected simultaneously three prescribed places of the LSWB once per week. Collected water samples were mixed before storing for different analyses. Water samples for pesticides analysis were stored in glass bottles at 4 °C. Ninety-four pesticides were analysed in the water samples, which are commonly used in agricultural practices (Table S4.1). The solid-phase extraction method was followed to concentrate pesticides in water and measure pesticide concentrations using LC-MS/MS (UltiMate 3000 RS, Dionex). Detector: QTRAP 5500, AB SCIEX and column: Phenomenex Kinetex C18 (particle size: 2.6 µm; length: 100 mm; diameter: 3 mm) were used to detect pesticide concentrations. The recovery of pesticides was in the range of 60 – 120 %. We converted pesticide concentrations detected above the limit of quantification (LOQ): 0.001 µg/L (Fig. S4.1; Ulrich et al., 2021) to toxic unit (TU; Fig. S4.2), a widely used approach for standardization concentration-related pesticide toxicity (Peterson, 1994). TUs were given as maximum TU (TU_max) for each sample. TU_max was calculated for each sample by considering all pesticides found in the samples. We calculated TU_max based on data available for algae toxicity (acute 72 h EC50 for algae, Table S4.2) from Pesticide Properties Data Base (Lewis et al., 2006) and using the below equation adopted from Cornejo et al. (2019):

$$\text{TU\_max} = max_{i=1}^{n}\left[log\left(\frac{C_i}{EC50_i}\right)\right]$$

Here, TU_max is the maximum toxicity of n pesticides detected in the sample, C_i is the concentration of the pesticide i (µg/L), and $EC50_i$ is the acute 72 h median effective concentration for algae (µg/L) reported for pesticide i.

For analyses 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 -20 °C until measurement. The concentrations of phosphate-phosphorus (PO_4-P), ammonium-nitrogen (NH_4-N) 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 ion chromatography system (Metrohm ECO IC, Switzerland) according to DEV D19 protocol.

Data of local environmental variables were collected from on-site measurements. Precipitation data during the study period were obtained from the Moorau weather station using an unheated tipping bucket rain gauge (Campbell BWS-200, UK) in the Kielstau catchment and total precipitation during 7 days before sampling was calculated (T_Pre_7d). To measure water level changes of LSWB, we installed a wooden bar in each LSWB noting initial water level on the starting day of the field campaign. Change of water level was measured using measuring tape with reference to the initially recorded water level. Water temperature, pH, electric conductivity (EC), and dissolved oxygen (DO) were measured *in situ* using a portable meter (WTM Multi 340i and WTW Cond 330i, Germany).

4.2.3 Phytoplankton sample collection, morphological identification, species traits, functional diversity (FD) and redundancy (FR) indices

At each sampling site and date, algae samples were collected based on standard methods (Wu et al., 2011) and used for taxonomical identification. Non-diatom soft microalgae were observed using an optical microscope (Nikon Eclipse E200-LED, Tokyo, Japan) under 400× magnifications. Taxonomic identification to species level was carried out based on current taxonomical criteria (Burchardt, 2014; Cantonati et al., 2017; Hu and Wei, 2006). Nomenclature follows criteria set up by Guiry and Guiry (2020). Permanent slides were prepared to identify diatoms. We used 5 mL of 30% hydrogen peroxide [H_2O_2] and 0.5 mL of 1 mol/L hydrochloric acid [HCl] for the oxidization processes of organic materials in the samples. After oxidation, 0.1 mL of the diatom-ethanol mix was transferred on a 24 × 24 mm cover slip and a drop of Naphrax was used to mount the slide. Diatoms were identified with the optical microscope (Nikon Eclipse E200-LED, Tokyo, Japan) under 1000× magnification with oil immersion, based on the key books by Bey (Bey and Ector, 2013) , Hofmann (Hofmann et al., 2011), Cantonati (Cantonati et al., 2017) and Bak (Bąk et al., 2012).

Further, traits information was obtained from the literature for all the 160 species observed in our study. Phytoplankton species were assigned to four functional traits: biovolumes (nano, micro, meso, macro and large) (Abonyi et al., 2018; Kruk et al., 2017; Qu

et al., 2018a; Rimet and Bouchez, 2012), life form (unicellular, colonial, filamentous and flagellates) (Abonyi et al., 2018; Kruk et al., 2017; Rimet and Bouchez, 2012), ecological guild (low profile, high profile, motile and planktonic) (Guiry and Guiry, 2020; Rimet and Bouchez, 2012), motility (Lange et al., 2016; Witteveen et al., 2020) and spore formation (Lange et al., 2016; Witteveen et al., 2020) (see Table S4.3 for details).

Using species composition and traits data, functional features (functional diversity (FD) and redundancy (FR) indices) were calculated for each sample. Four FD indices were calculated such as functional richness (FRic), functional evenness (FEve), functional dispersion (FDis) and functional divergence (FDiv) using R package FD (Laliberté et al., 2014). Further, two FR indices were calculated as FR01 and FR02. FR01 was the difference between taxonomic diversity and functional diversity (i.e., the difference between the Simpson diversity index and Rao's quadratic entropy). FR02 was calculated as the mean number of species per functional group (FG). FGs were determined by the classification of the species by means of Ward's clustering (*k*-means) method. The optimum number of FGs were governed by the Calinski-Harabasz criterion (Pomerleau et al., 2015). Further details about descriptions and calculations of functional features can be found in previous studies (Bruno et al., 2016; Cadotte et al., 2011; Wu et al., 2019).

4.2.4 Statistical analyses

We performed all data analyses with the R software version 4.0.2 (R Core Team, 2020). The Kruskal–Wallis non-parametric ANOVA by ranks test was used to compare the environmental variables and functional features of phytoplankton communities among LSWB. The following data analyses were carried out to explore the possible impacts of pesticide toxicity (TU_max), nutrients (NH_4-N, NO_3-N and PO_4-P) and local environment variables (Local: T_Pre_7d, temperature, pH, EC, DO, Water level change) on species compositions (H1). Species composition (abundance) were Hellinger-transformed using function *decostand* in R package *vegan* (Oksanen et al., 2019). It was used to keep Euclidean distances between samples in the multidimensional space avoiding interruptions by reducing the weight of abundant species. The co-linear variables (VIF >= 5) in environmental variables (Local and Nutrients) were excluded using a stepwise procedure until all predictors of VIF's were < 5 (R package *usdm*, function: *vifstep*, Naimi (2015)). A preliminary detrended correspondence analysis (DCA; R package *vegan*, function: *decorana*) on the Hellinger-transformed species data was conducted. The longest DCA gradient length was 3.31 along the second axis,

suggesting that both redundancy analysis (RDA) and canonical correspondence analysis (CCA) were suitable for describing species composition (Lepš and Šmilauer, 2003). We performed RDA and tested the significance using ANOVA. Only when it was significant, a forward selection was conducted to choose well-fitted variables in local and nutrients categories to describe the species composition with two stopping criteria: significance level and the adjusted coefficient of determination (Adj R^2) of the global model (Blanchet et al., 2008). Forward selection was performed using function *forward.sel* in the R package *adespatial* (Dray et al., 2020). The selected environmental variables in each category (Local: water level change, EC and DO; Nutrients: NH_4-N, NO_3-N and PO_4-P; Pesticide toxicity: TU_max) were then used as explanatory variables for further analyses. Variance partitioning analysis (VPA) was performed by using function *varpart* in the R package *vegan* to quantify the variability explained by each set of the categories which were Local, Nutrients and Pesticide toxicity.

We explored the relationships between the functional features (FD and FR indices) and selected environmental variables (H2), using multiple linear regression models between biotic indices and selected environmental variables after forward selection as mentioned above. Simplified and well-fitted multiple linear regression models were obtained through stepwise model selections by AICc (R package MASS, function: stepAIC, Ripley et al. (2013)). The model with the minimum AICc value was considered as the best-fitted one. All environmental variables and functional features were transformed to ln (x+1) (except TU_max which is already in log scale) and then z-score transformed before linear regression analyses. It allowed acquiring standardized coefficients that can compare the magnitude within and between multiple linear regression models (Schielzeth, 2010; Zhou et al., 2020).

4.3 Results

4.3.1 Variations of pesticide toxicity, nutrient concentrations, and local environmental variables

The environmental variables were highly variable across samples and among LSWB (Table 4.2). Altogether, 41 pesticides and pesticide metabolite products were detected above 0.001 µg/L in studied LSWB (Fig. S4.1). We found 15 pesticides per sample on average (range: 10 – 22). It included 25 herbicides such as Dimethachlor in all samples and mostly Bentazone, Quinmerac, and Diflufenican which were present at 83.33%, 75.93% and 70.37% of studied samples respectively. Ten fungicides were found such as Bixafen in all samples and mostly Tebuconazole and Epoxiconazole found at 81.48% and 61.11% of samples respectively.

Clothianidin, Pirimicarb and Thiacloprid were the three insecticides found at 24.07%, 16.67% and 12.96% of samples respectively. Two metabolites of Metazachlor (herbicide) were frequently found in studied samples which were Metazachlor-oxalic acid (Metazachlor-OA) 81.48% and Metazachlor-sulfonic acid (Metazachlor-ESA) 74.07%. Desamino-metamitron was another metabolite found at 46.29% of studied samples (Ulrich et al., 2021). TU_max of the samples predominantly governed by herbicides such as Diflufenican and Dimethachlor which are highly toxic to algae and also by Terbuthylazine which is moderately toxic to algae but found in high concentrations in studied LSWB (Fig. S4.2). T_Pre_7d was on average 10.59 mm (range: 0 - 52.10 mm) during the study period.

Considering the variation of the environmental variables among the LSWB (Table 4.2), the average number of pesticides discovered in each LSWB were 16, 15, 16, 13, 18 in A1, A2, A3, A4 and A5 respectively. Among the detected 25 herbicides, 10 herbicides such as 2,4-D, Bentazone, Bromoxynil, Dimethachlor, Florasulam, MCPA, Nicosulfuron, Quinmerac, S-metolachlor and Terbuthylazine were present in all studied LSWB. From the detected 10 fungicides, 6 fungicides such as Azoxystrobin, Bixafen, Boscalid, Epoxiconazole, Fludioxonil and Tebuconazole were found at all LSWB. Considering insecticides, Pirimicarb was detected at 4 studied LSWB except for A4. From the detected metabolites, Desamino-metamitron and Metazachlor-OA were found in all LSWB and Metazachlor-ESA was found in 4 LSWB except A5. Considering nutrients concentrations, A2 and A3 showed the lowest NO_3-N levels. In contrast, the highest NH_4-N concentrations were recorded in A3. The highest NO_3-N concentration and second highest NH_4-N concentration were observed at A4. A5 was significantly different from the other LSWB with remarkably highest PO_4-P concentration and pesticide toxicity (TU_max). Among the local environmental variables of five LSWB, water level reduced significantly in A2 and A3 and remained relatively constant in other LSWB during the study period. The pH was similar among LSWB showing neutral average pH levels. EC was significantly different among LSWB indicating highest in A4 and lowest in A2.

Table 4.2: Variation of environmental factors in all studied samples and among LSWB (Mean ± SD)

Parameter	All samples	LSWB				
		A1	A2	A3	A4	A5
Pesticide toxicity (TU_max)	1.00 ± 0.53	0.46 ± 0.43^{c}	0.68 ± 0.20^{c}	1.07 ± 0.11^{b}	1.33 ± 0.13^{b}	1.90 ± 0.46^{a}
Ammonium-nitrogen (NH_4-N) (mg/L)	0.139 ± 0.302	0.035 ± 0.027^{ab}	0.037 ± 0.032^{ab}	0.339 ± 0.528^{a}	0.209 ± 0.279^{a}	0.016 ± 0.018^{b}
Nitrate-nitrogen (NO_3-N) (mg/L)	1.428 ± 2.809	0.610 ± 0.950^{b}	0.010 ± 0.036^{c}	0.000 ± 0.000^{c}	5.780 ± 3.200^{a}	0.048 ± 0.096^{bc}
Phosphate-phosphorus (PO_4-P) (mg/L)	0.055 ± 0.068	0.054 ± 0.048^{b}	0.031 ± 0.019^{bc}	0.034 ± 0.037^{bc}	0.028 ± 0.056^{c}	0.203 ± 0.062^{a}
Water level change (cm)	-13.83 ± 20.41	-1.88 ± 2.89^{a}	-36.04 ± 25.22^{b}	-24.87 ± 14.04^{b}	0.083 ± 1.02^{a}	0.92 ± 4.34^{a}
Temperature (^{o}C)	16.01 ± 4.04	16.63 ± 3.80^{ab}	17.58 ± 4.05^{a}	14.25 ± 3.59^{bc}	13.90 ± 3.54^{c}	19.33 ± 3.31^{a}
pH	7.31 ± 0.59	7.36 ± 0.70	7.24 ± 0.70	7.36 ± 0.63	7.15 ± 0.36	7.62 ± 0.47
Conductivity (EC) (µS/cm)	384.63 ± 104.54	374.25 ± 28.92^{c}	246.75 ± 18.24^{e}	466.83 ± 71.97^{b}	494.25 ± 21.22^{a}	297.5 ± 28.54^{d}
Dissolved Oxygen (DO) (mg/L)	7.57 ± 2.96	8.98 ± 2.73^{a}	6.59 ± 2.58^{ab}	6.32 ± 3.20^{b}	7.71 ± 3.11^{ab}	8.94 ± 2.22^{ab}

Different subscript letters show significant different among LSWB in monitored parameters (Kruskal-Wallis test; $p < 0.05$). (-) sign in the water level change indicates reduction of the water level and vice versa.

4.3.2 Characteristics of phytoplankton communities

We identified 160 phytoplankton taxa in the studied LSWB. Phytoplankton species were distributed across 10 phyla, i.e., Bacillariophyta (46), Charophyta (3), Chlorophyta (34), Chrysophyta (1), Cryptophyta (7), Cyanophyta (28), Euglenophyta (32), Haptophyta (2), Dinophyta (3), Ochrophyta (4). The dominant species was *Chroococcus turgidus* (Kütz.) Nägeli (Cyanophyta) contributing to 63.19% of the total abundance integrated over the study period for all LSWB. *Achnanthidium minutissimum* (Kütz.) Czarnecki, *Rhodomonas lacustris* var. *nannoplanctica* (Skuja) Javornicky and *Navicula lanceolata* (Ag.) Ehr. also occurred frequently (e.g., found in > 40 samples out of 54). Dominant species integrated over the study period for each LSWB also was *Chroococcus turgidus*. But species composition at A5 showed co-dominance of other species such as *Peridinium willei* Huitfeldt-Kaas (Dinophyta), *Lepocinclis ovum* (Ehr.) Lemm. and *Trachelomonas* spp. (Euglenophyta). Functional features of the phytoplankton community varied highly between samples and LSWB (Table 4.3 and Fig. 4.2). Functional diversity indices of phytoplankton community in A5 significantly differed from other LSWB showing the lowest FRic, FEve, FDis and highest FDiv. Considering functional redundancy indices, A5 varied from others indicating the highest values for both FR01 and FR02 indices.

Table 4.3: Functional features (functional diversity and redundancy indices) in phytoplankton communities: variations among samples (n = 54) as mean (range) in this study. The detailed descriptions of the indices can be found in Bruno et al. (2016), Cadotte et al. (2011) and Wu et al. (2019).

Categories	Functional feature	Mean (Range)
Functional diversity (FD)	Functional Richness (FRic)	0.25 (0.03 - 0.36)
	Functional Evenness (FEve)	0.44 (0.08 - 0.61)
	Functional Dispersion (FDis)	0.44 (0.08 - 0.61)
	Functional Divergence (FDiv)	0.79 (0.24 - 0.99)
Functional redundancy (FR)	FR01	0.22 (-0.22 - 0.63)
	FR02	4.01 (1.83 - 6.5)

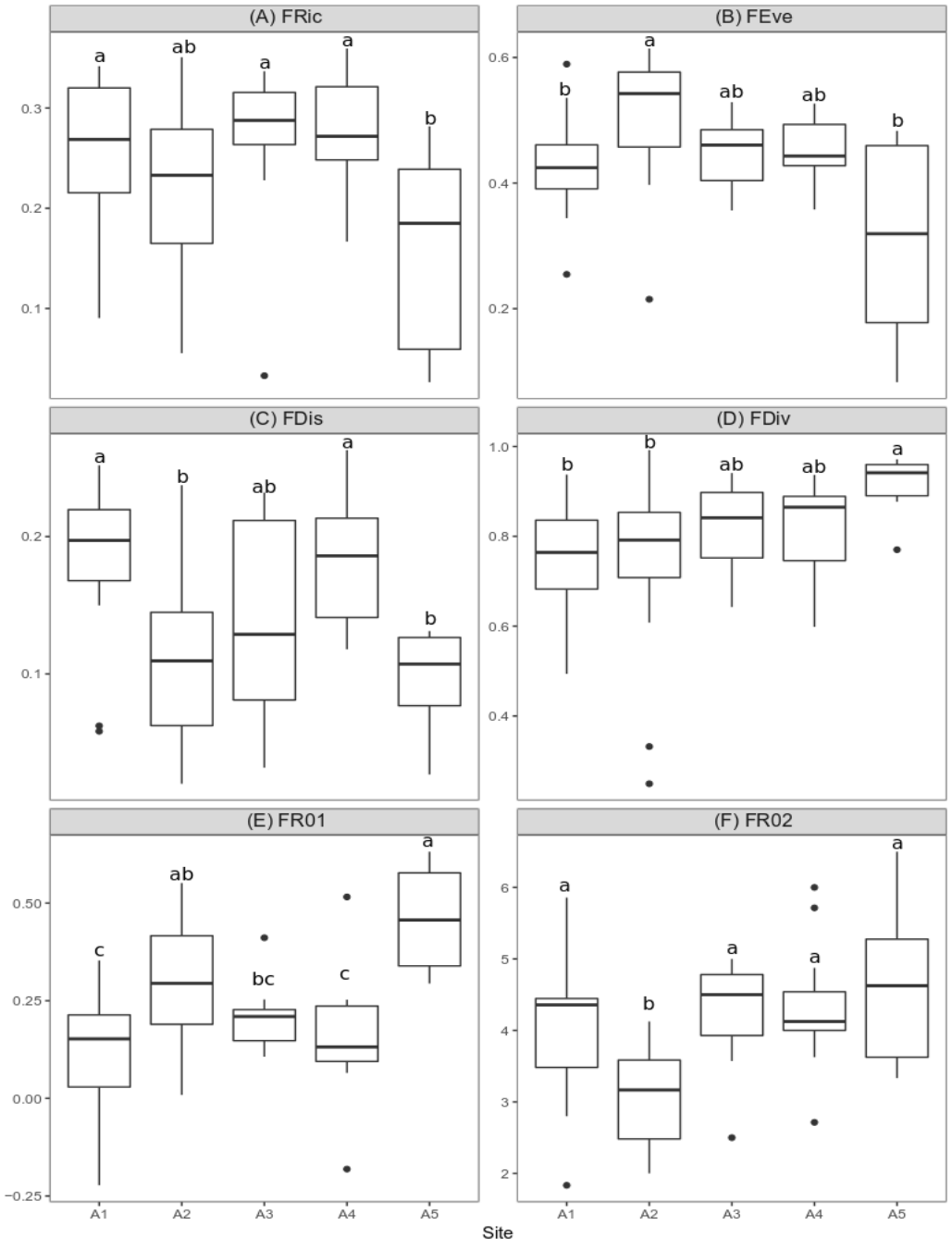


Fig 4.2: Variation of functional diversity indices (functional diversity ((A) FRic, (B) FEve, (C) FDis and (D) FDiv) and functional redundancy ((E) FR01 and (F) FR02)) among studied LSWB. Different subscript letters show significant differences among LSWB in monitored parameters (Kruskal-Wallis test; $p < 0.05$).

4.3.3 Main drivers of species composition

Redundancy analysis (RDA) explained 34.54% (adj R^2) of the total variation of the phytoplankton community by the selected environmental variables. All the canonical axes were significant in the test of significance (F = 4.99, p = 0.001, permutations = 999). The first two canonical axes explained together 24.61% of the total variance of phytoplankton composition, the first axis (RDA1) alone explaining 18.97% and 5.64% from the second axis (RDA2). Furthermore, any dominant residual structure of phytoplankton composition was not observed in the model as the first unconstrained eigenvalue was comparatively low (0.072) than the first constrained eigenvalue (0.146). As shown in Fig. 4.3, pesticide toxicity (TU_max), PO_4-P, NH_4-N, DO and water level change played important roles in the dispersion of samples along the RDA1. NO_3-N and EC were associated with RDA2. Samples with high TU_max placed together with high nutrients such as PO_4-P and NH_4-N opposite to DO and water level changed along RDA1. Samples from A5, A1 and A2 were mostly associated with high PO4-P and pesticide toxicity (TU_max) compared with samples from A3 and A4 (Fig. 4.3A). Phytoplankton species (10% most frequent and 80% best axis fit in the RDA model) were represented in Fig. 4.3B. Phytoplankton species such as *Chroccoccus turgidus* (Cyanophyta), *Peridinium willei* (Dinophyta) and *Trachelomonas* spp. (Euglenophyta) were correlated with high PO_4-P and pesticide toxicity (TU_max). Species belong to Chlorophyta such as *Chloromonas angustissima* (Ettl) Gerlaff & Ettl and Bacillariophyta (Diatoms) like *Navicula lanceolata* and *Gomphonema parvulum* (Kütz.) Kütz. were placed on the opposite side and correlated with high DO and water level change.

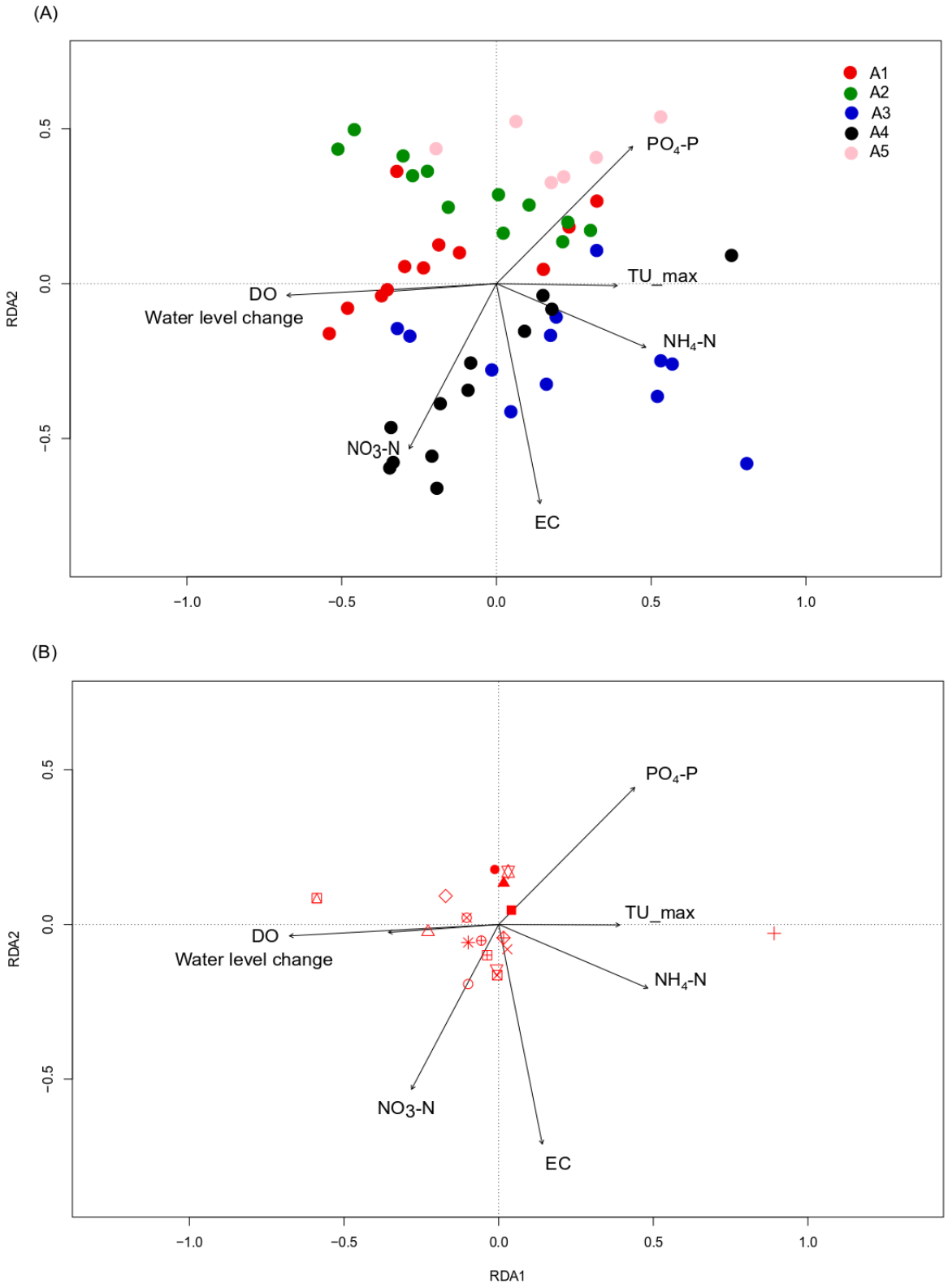

(A)
A1
A2
A3
A4
A5
PO4-P
TU_max
DO
Water level change
NH4-N
NO3-N
EC
RDA2
0.5
0.0
−0.5
−1.0
−0.5
0.0
0.5
1.0
(B)
PO4-P
TU_max
DO
Water level change
NH4-N
NO3-N
EC
RDA2
RDA1

Fig. 4.3: Redundancy analysis (RDA) for the phytoplankton species composition. (A) Biplot of the sampling sites and environmental variables. (B) Biplot of the phytoplankton species (10% most frequent and 80% best axis fit) and environmental variables. Phytoplankton species represent in different symbols as +: *Chroococcus turgidus* (Kütz.) Nägeli; ✡: *Peridinium willei* Huitfeldt-Kaas; ▲: *Trachelomonas volvocinopsis* Svirenko; ■: *Trachelomonas planctonica* Svirenko; ⌖: *Lemnicola hungarica* (Grunow) Round & Basson; ×: *Cocconeis placentula* Ehr.; ▽: *Eunotia minor* (Kütz.) Grunow in Van Heurck; ⊠: *Fragilaria mesolepta* Rab.; ○: *Achnanthidium minutissimum* (Kütz.) Czarnecki; ⊞: *Planothidium frequentissimum* (Lange-Bertalot) Lange-Bertalot; ⊕: *Navicula lanceolate* (Ag.) Ehr.; ✳: *Gomphonema parvulum* (Kütz.) Kütz.; △: *Chloromonas angustissima* (Ettl) Gerlaff & Ettl; ⊠: *Pseudanabaena minima* (G.S. An) Anag.; ◇: *Cryptomonas curvata* Ehr. and ⧈: *Rhodomonas lacustris* var. *nannoplanctica* (Skuja) Javornicky. The abbreviated environmental variables stand for, EC: electric conductivity; DO: dissolved oxygen; NH_4-N: ammonium-nitrogen; NO_3-N: nitrate-nitrogen; PO_4-P: phosphate-phosphorus and TU_max: pesticide toxicity.

Variance partitioning analysis (VPA) indicated all three individual fractions of local environmental (Local), nutrients and pesticide toxicity were statistically significant ($p < 0.001$) (Fig. 4.4). Local environment variables showed the highest pure contribution for driving phytoplankton composition (12%), followed by nutrients (8%) and pesticide toxicity (2%). The combination of all three categories well explained the phytoplankton communities (34%) as it was higher than the sum of individual categories (22%). The highest variability of the phytoplankton community was explained by the interaction between local environmental variables and nutrients as 8% compared to other interactions, such as pesticide toxicity × nutrients 3%; local × pesticide toxicity 1% and local × nutrients × pesticide toxicity 1%. The proportion of variance associated with the interaction of pesticide toxicity and nutrients (3%) is higher than the individual effect of pesticide toxicity (Fig. 4.4). As hypothesised in H1, it appeared that nutrients and pesticide toxicity significantly affected the phytoplankton species composition.

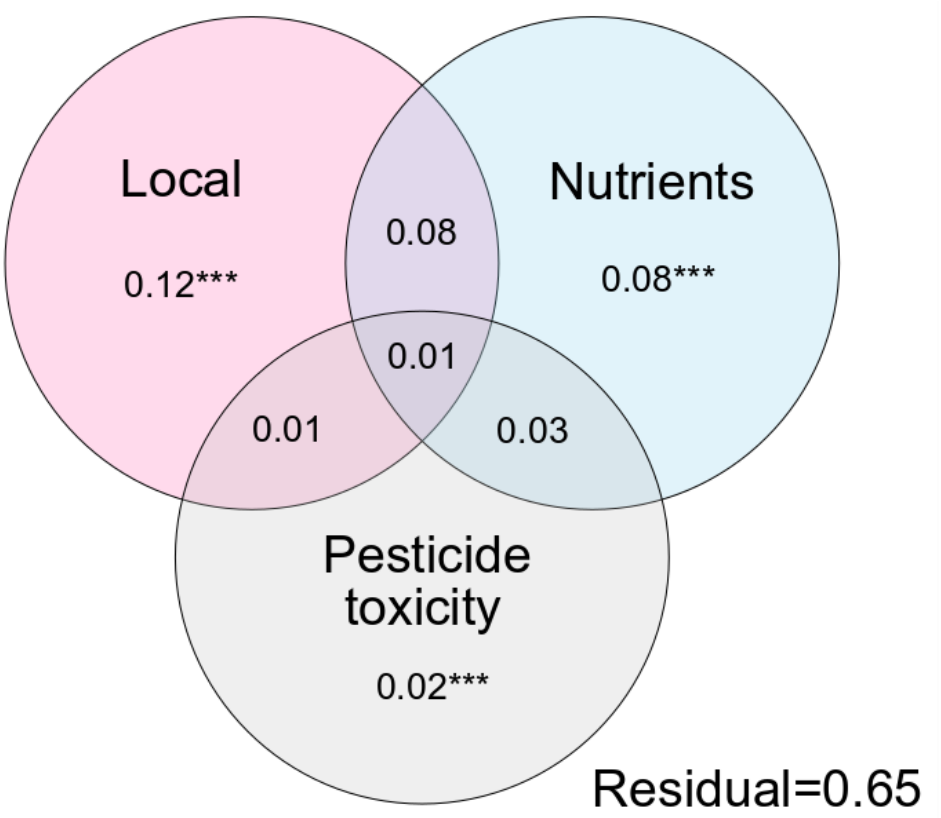


Fig. 4.4: The proportion of variation of the phytoplankton community composition explained purely by pesticide toxicity (TU_max), nutrients (NH_4-N, NO_3-N and PO_4-P) and local environmental variables (EC, DO and water level change), interaction between two categories (i.e. Local × Nutrients, Local × Pesticide toxicity and Nutrients × Pesticide toxicity), interaction of all three categories, and unexplained variance (i.e. Residual). The abbreviated environmental variables stand for, EC: electric conductivity; DO: dissolved oxygen; NH_4-N: ammonium-nitrogen; NO_3-N: nitrate-nitrogen and PO_4-P: phosphate-phosphorus. Total variance = 100 and significance codes represented as *p < 0.05, **p < 0.01, ***p < 0.001.

4.3.4 Relationships between functional features and environmental variables

Overall, functional features of the phytoplankton community were significantly affected by PO_4-P, NO_3-N, pesticide toxicity (TU_max) and EC (Fig. 4.5). Functional

richness (FRic) was positively correlated with EC (regression coefficient: 0.211; $p = 0.12$) and negatively correlated with PO_4-P concentration (regression coefficient: -0.269; $p < 0.05$). Functional evenness (FEve) was negatively correlated with both water level change (regression coefficient: -0.224, $p < 0.05$) and PO_4-P (regression coefficient: -0.533, $p < 0.001$). Functional dispersion (FDis) showed positive relationship with NO_3-N (regression coefficient: 0.423, $p < 0.001$) while functional divergence (FDiv) indicated positive association with PO_4-P (regression coefficient: 0.228, $p = 0.09$) and negative association with DO (regression coefficient: -0.262, $p < 0.05$). In general, FD indices were mainly governed by nutrients (e.g., PO_4-P and NO_3-N) showing richness and evenness of the phytoplankton community were reduced with increasing PO_4-P and dispersion increased with increasing NO_3-N. In functional redundancy indices, FR01 showed positive association with TU_max (regression coefficient: 0.416, $p < 0.001$) and negative association with EC (regression coefficient: -0.293, $p < 0.05$) and NO_3-N (regression coefficient: -0.287, $p < 0.05$). FR02 index indicated positive association with TU_max (regression coefficient: 0.327, $p < 0.05$) and EC (regression coefficient: 0.267, $p < 0.05$). To summarize, as expected by H2, nutrient concentrations and pesticide toxicity had a significant effect on functional features of the phytoplankton community. Functional diversity reduced with increasing nutrient concentrations, especially with PO_4-P. But in contrast to our expectations, functional diversity was increased with pesticide toxicity as it positively linked to FR indices.

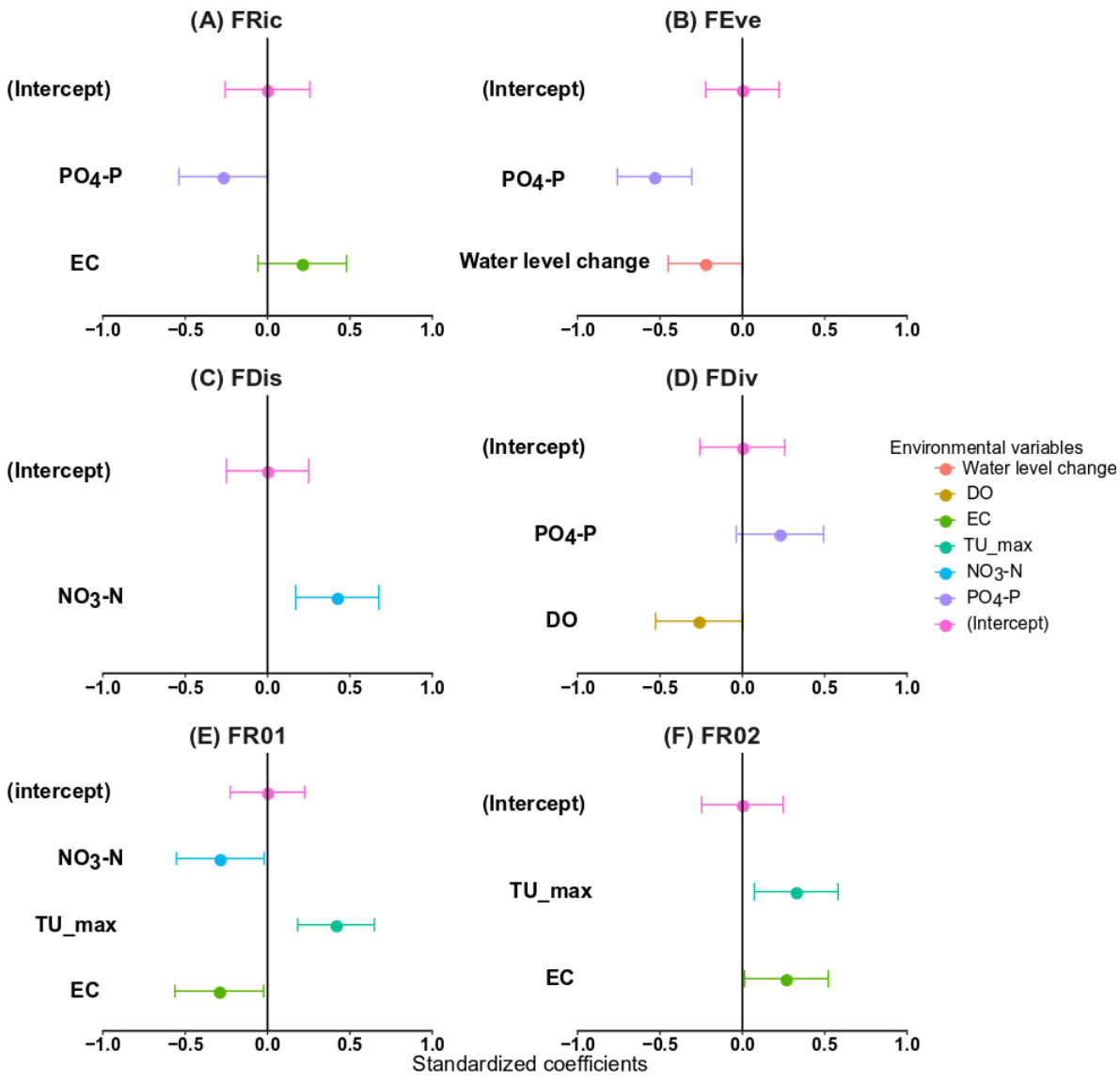


Fig. 4.5: Linear regression models of the functional features (functional diversity indices ((A) FRic, (B) FEve, (C) FDis and (D) FDiv) and functional redundancy indices ((E) FR01 and (F) FR02)) of the phytoplankton community. The standardized regression coefficients and 95% confidence intervals of the environmental variables that associated with functional features are shown here. The confidence intervals of environmental variables which are not crossing the zero line represent the significant associations ($p < 0.05$) with functional features. The abbreviated environmental variables stand for, EC: electric conductivity; DO: dissolved

oxygen; NH_4-N: ammonium-nitrogen; NO_3-N: nitrate-nitrogen; PO_4-P: phosphate-phosphorus and TU_max: pesticide toxicity.

4.4 Discussion

4.4.1 Pesticides, nutrients, and local environmental variables

Pesticide toxicity (TU_max) of the samples was predominantly governed by herbicides such as Diflufenican and Dimethachlor, which are highly toxic to algae, and Terbuthylazine, which is moderately toxic to algae but found in high concentrations in the studied LSWB (Fig. S4.2). The highest TU_max was recorded in A5 where we found high concentrations of Terbuthylazine (e.g., 2.34 µg/L). In other LSWB, Diflufenican concentration was reduced respectively across A4, A3 and A2. The lowest pesticide toxicity was recorded in A1 samples and it contained less toxic Dimethachlor compared to Diflufenican (Fig. S4.2 and Table S4.2). In 2008, the highest concentration of Terbuthylazine was observed as 0.15 µg/L in aquatic ecosystems in Kielstau catchment (Ulrich, 2011). In 2018, we observed Terbuthylazine concentration in 15-fold higher concentration compared to 2008. The Kielstau catchment is an area dominated by agriculture and subjected to agrochemical applications such as pesticides and fertilizers over decades and shows a further increasing trend of exposing aquatic ecosystems to higher pesticide concentrations compared to previous studies (Ulrich, 2011; Ulrich et al., 2018) and varying nutrient concentrations (Wagner et al., 2018). Further, samples with high TU_max associated with high PO_4-P concentrations (Table 4.1: e.g., A5 samples and Fig. 4.3A). This emphasizes the high probability of occurrence of these two stressors as simultaneous multiple stressors in lentic small water bodies. In addition, the high variability of EC among LSWB may occur due to contamination of the other inorganic fertilizers applied to the crops such as Potassium, Boron, Magnesium, Magnesium sulfate and Manganese (Lueck, 2015; Mascianica, 1983).

The high TU_max and nutrient concentrations such as PO_4-P and NH_4-N were associated with low DO and water level (Fig. 4.3A). Similarly, Noack et al. (2003) observed

lower oxygen levels in higher pesticide applied mesocosms in their study which may be probably associated with inhibition of phytoplankton photosynthesis at high pesticide concentrations. Moreover, our samples indicated eutrophic conditions in the studied LSWB as its PO_4-P > 0.005 mg/L according to OECD (1992) classification. A low level of dissolved oxygen is one particular characteristic of the eutrophicated lentic ecosystems (Fareed and Abid Ali, 2005). Reducing water levels in lentic ecosystems may concentrate pesticides and nutrients resulting in negative relationships between water level and TU_max, PO_4-P and NH_4-N. This result in our study foresees the impact of future climate change associated with predicted reduction of water levels in freshwater ecosystems. Therefore, our work provides insights on potential changes in phytoplankton community composition in future decades under climate change. Future studies which combine these baseline observations with modelling attempts will lead to predicting the fate of aquatic biota under climate change scenarios.

4.4.2 Species composition of the phytoplankton community

Alteration of the species composition is one of the key impacts ensued by multiple stressors. Species that can perform well under one stressor may fail to survive when the second stressor present at the same time (Litchman et al., 2012). Therefore, the species-specific response for both single and combined stressors play an important role in composition variations of the phytoplankton communities. Due to the tendency for eutrophic conditions in the studied LSWB, we expected to see indicator species for eutrophication throughout our whole samples. In our study, among the most frequent phytoplankton species, abundance of *Chroccoccus turgidus* (Cyanophyta), *Peridinium willei* (Dinophyta) and *Trachelomonas* spp. (Euglenophyta) were correlated positively with PO_4-P and pesticide toxicity (TU_max) while in the opposite we were still able to detect strong indicator species for eutrophication such as *Chloromonas angustissima*, *Navicula lanceolata* and *Gomphonema parvulum* (Fig. 4.3B). *Chroccoccus turgidus* and *Peridinium willei* are often identified as generalists found in deep and shallow, oligo to eutrophic, medium to large lentic ecosystems, whereas the habitat of *Trachelomonas* spp. described as meso-eutrophic, shallow ponds (Padisák et al., 2009).

Therefore, as far as the nutrient enrichment with PO_4-P is considered, the species associated with high PO_4-P levels are rather generalists for nutrient conditions compared to species placed in the opposite. Therefore, it seems that the combined effect of pesticides and nutrients suppresses eutrophic indicator species, and consequently the species that can tolerate both eutrophic conditions and pesticide toxicity became dominant. Our results provide evidence that when the pesticide effect was low, eutrophic conditions govern the species composition (Fig. 4.3B). Moreover, pesticide toxicity of the studied samples was mainly governed by herbicides (e.g., Diflufenican, Dimethachlor and Terbuthylazine) (see Fig. S4.2). Further, these herbicides were low in concentrations (Fig. S4.1, maximum pesticide concentration: Terbuthylazine – 2.34 µg/L) and these herbicides mixtures may act as dissimilarity mixtures due to their different modes of action (e.g., photosynthesis inhibitors and cell division inhibitors; Table S4.2). Similar to our results, in the mesocosm study of Noack et al. (2003), they observed *Peridinium* spp. dominating phytoplankton community at the highest herbicide concentration. Further, Pesce et al. (2011) highlighted many studies on herbicides contaminated aquatic systems directed towards the initial inhibition of the photosynthesis of primary producers (including macrophytes) following a significant release of nutrients into the water. It ultimately caused an increase in the abundance of less-sensitive or fast-adapting phytoplankton species, particularly flagellates. Therefore, we emphasize the importance of studying the ecological significance of combined environmental variables rather than individual variables to understand the ongoing underlying processes of the biotic community structuring.

As the effects of categories of combined environmental variables (VPA results), we identified the significant effect of local environmental variables (EC, DO and water level change), pesticide toxicity (TU_max) and nutrients (PO_4-P, NO_3-N and NH_4-N) on driving species composition of the phytoplankton communities individually (Fig. 4.4). It confirms our H1, that nutrients and pesticides significantly affect the phytoplankton species composition. Nutrients contributed to shaping the phytoplankton community higher than pesticide toxicity. Similarly, many studies in lentic ecosystems highlighted the strong association of nutrients

with the phytoplankton composition (e.g., Liu et al., 2010; Wang et al., 2012). Additionally, we observed that the highest variability of individual effects was associated with local environmental variables such as DO, water level change and EC. Correspondingly, Çelekli et al. (2014), Grabowska et al. (2014) and Chia et al. (2011) observed the closed association of phytoplankton with the same physicochemical factors in lentic ecosystems. Andrus et al. (2013) observed algal composition alterations associated with water flow (local habitat variable) in agricultural streams and but not with pesticides, which may be potentially due to limited exposure (concentration or duration limitations). We re-emphasized, the importance of the interaction of pesticide toxicity and nutrients as simultaneous stressors (multiple stressors) rather than the individual effect of pesticide toxicity on structuring phytoplankton community (Fig. 4.4). A similar study on aquatic macroinvertebrates in agricultural streams identified synergistic interaction between nutrient enrichment and pesticide toxicity on structuring species composition (Cornejo et al., 2019). According to Pesce et al. (2011), benthic algal communities in artificial streams showed the additive effect between pesticide toxicity and nutrients when the communities were exposed to dissimilarity mode of herbicides similar to our study. With the lack of studies on pesticides and nutrients as multiple stressors in lentic ecosystems, we could not compare our findings with similar ecosystems to gain a better understanding. With this, we highlight pesticide toxicity and nutrients as important multiple stressors on shaping species composition of the phytoplankton. Further, the change of phytoplankton community structure under environmental disturbances depends on their flexibility in eco-physiological traits (Litchman et al., 2012; Taherzadeh et al., 2019). Therefore, studies to identify adaptive and irreversible thresholds of the impacts of pesticides and nutrients as multiple stressors are needed (e.g., Taherzadeh et al., 2019). This understanding of community dynamics is highly necessary towards policy making with regards to sustainable environmental management (Aljerf and Choukaife, 2016). In addition, VPA explained a relatively low fraction (35%) of the phytoplankton community by the studied parameters in our study. Including other important factors that control phytoplankton community, such as light availability, grazing pressure and macrophyte density may help to improve future studies (Arhonditsis et al., 2004; Iacarella et al., 2018).

4.4.3 Functional features of phytoplankton community

Functional features such as functional diversity and functional redundancy indices are considered better predictors of ecosystem productivity and vulnerability than species diversity (Schleuter et al., 2010; Wu et al., 2019). These functional features usually provide insights into a broad understanding of ecosystem functionality: (i) how much of the functional niche space is filled by the existing species (functional richness) and (ii) how functional niche space is filled by the existing species (functional evenness, functional divergence, functional variance) and (iii) ability of stability, resistance and resilience (functional redundancy) (Bruno et al., 2016; Schleuter et al., 2010). According to our findings, functional features are negatively affected by nutrient concentrations (e.g., PO_4-P and NO_3-N). Functional richness and evenness were significantly reduced with the increase of PO_4-P while divergence significantly increased with NO_3-N. It re-emphasizes the most common phenomenon of eutrophication (Heisler et al., 2008; Xu et al., 2010). Schleuter et al. (2010) emphasized the high functional divergence or dispersion can be occurred due to the clustering of abundant species at the edges of the traits space, which is the reflection of the predominance of extreme/tolerant species.

In contrast to our expectation, pesticide toxicity (TU_max) linked positively with the functional redundancy showing that increased pesticide toxicity can increase stability, resistance, and resilience of the ecosystem. This positive feedback of pesticide toxicity on phytoplankton functionality can be explained as (i) indirect positive effect of pesticides through suppressing top-down selection pressures on phytoplankton communities, such as predation and competition (e.g., van Donk et al., 1995), (ii) pesticides concentrations may not be high enough to decline phytoplankton abundance, diversity or alter assemblage structure (e.g., Andrus et al., 2013; Relyea, 2009; Waiser and Robarts, 1997) and/or (iii) functional diversity and redundancy are maximized when disturbances are not too low or too high as described by the ‘‘intermediate disturbance hypothesis’’ (Connell, 1978).

Indirect positive effects of pesticides on phytoplankton can be expected in our study due to the presence of insecticides (i.e., Clothianidin, Pirimicarb and Thiacloprid, see Fig. S4.1) which may be toxic to zooplankton and hence reduce grazing pressure on phytoplankton. Among them, Pirimicarb is highly toxic to zooplankton while Clothianidin and Thiacloprid are moderately toxic (Acute 48 h EC_{50} (mg/L) of *Daphnia magna*; Lewis et al. (2006)). Direct toxicity of these insecticides on algae can order as Clothianidin > Thiacloprid > Pirimicarb (Table S4.2). Clothianidin was present only at A1 (range: 0 – 0.0094 µg/L) and A4 (range: 0 – 0.0047 µg/L). Thiacloprid was present at A1, A3 and A5 in very low concentrations (range: 0 – 0.001 µg/L). Pirimicarb was present at four LSWB except in A4 in high concentrations as A1 (range: 0 – 0.016 µg/L), A2 (range: 0 – 0.001 µg/L), A3 (range: 0 – 0.005 µg/L), A5 (range: 0 – 0.018 µg/L). Overall, we can expect that A1 and A5 may have benefited from the presence of insecticides compared to the other LSWB. Phytoplankton communities subjected to direct high pesticide toxicity due to high concentrations of herbicides, at the same time benefited with the presence of insecticides in high concentrations (e.g., A5) that can reduce zooplankton grazing pressure. On the other hand, phytoplankton communities exposed to lowest direct pesticide toxicity further benefited with high concentrations of insecticides (e.g., A1). This phenomenon may be the most likely cause for the resulting positive feedback of pesticide toxicity on the phytoplankton community in our study.

Furthermore, we assume that the low pesticide concentrations observed in our study likely not to lead the expected negative effect of pesticide toxicity on functional features of phytoplankton communities as hypothesized in H2. The highest pesticide toxicity in our study was caused by Terbuthylazine (Fig. S4.3) and corresponded to 2.34 µg/L in concentration. The five percent hazard concentration (HC_5: which indicate pesticide concentration protecting 95% of species) for Terbuthylazine stated as high as 39 µg/L in the study on epiphytic algae community (Cedergreen et al., 2004). Species composition alterations of phytoplankton due to pesticides have been observed only in higher concentrations in previous studies (e.g.,

Metazachlor > 5 µg/L (Mohr et al., 2008); Nicosulfuron: 10 µg/L (Leboulanger et al., 2001); Isoproturon > 30 µg/L and S-metolachlor: 5 µg/L (Debenest et al., 2009).

In addition, considering the involvement of local environmental variables on functional features, only conductivity played a significant but inconsistent role with functional redundancy. As mentioned in 4.1, we assume that other mineral fertilizers may cause changes in EC in our studied LSWB. These mineral fertilizers may act positively under low amount supplying essential nutrients but may act toxic at high concentrations on the phytoplankton community (Hutchinson, 1961; Talling, 2010). Future studies on phytoplankton community need to include measurements of ions such as K^+, Mg^{2+}, Br^+, Mn^{2+} to disentangle the effect of EC on functional features and to understand the overall effect of fertilizers applied in agriculture. Overall, as we expected from our hypothesis 2 (H2), functional features of phytoplankton communities were significantly affected by pesticides and nutrients, but the direction of the effects was only partially verified as functional features such as functional diversity indices decreased with increased nutrient concentrations and functional redundancy increased with increased pesticide toxicity. Therefore, negative impacts of nutrients are prominent on phytoplankton functional features in our study than negative impacts of pesticides.

Furthermore, historical data on agrochemical exposures and respective responses of the phytoplankton communities of the LSWB are needed to understand the current status of the phytoplankton communities comprehensively. Our previous work (e.g., Qu et al., 2018a; Qu et al., 2018b; Wu et al., 2018) shown that the riverine phytoplankton are shaped by both historical factors and local environmental variables. However, we are lacking the historical information on studied LSWB due to unavailability of long-term continuous monitoring. Therefore, we emphasize the importance of long-term studies and mesocosm experiments (to tackle before and after agrochemical exposure conditions clearly) to a better understanding of the current context.

4.5 Conclusion

Overall, our findings emphasize the role of pesticide toxicity and nutrient enrichment as multiple stressors on the phytoplankton community structure of lentic small water bodies in agricultural areas. Rather than individual effects of pesticides, the interactive effect of the pesticide toxicity and nutrient concentrations contributed to shaping the species composition of the phytoplankton. Under concurrent stressors of PO_4-P and pesticide toxicity, phytoplankton species composition can reverse from dominance of indicator species of eutrophic status (i.e., *Chloromonas angustissima*, *Navicula lanceolata* and *Gomphonema parvulum*) to co-dominance of less-sensitive, fast adapting generalist species (i.e., *Chroccoccus turgidus*, *Peridinium willei* and *Trachelomonas* spp.). Further, functional features can be altered by nutrient concentrations and pesticide toxicity leading to negative and positive feedback on the functionality of ecosystems respectively from former and later stressors. However, these positive responses of pesticide toxicity on phytoplankton most likely occurred due to the low level of pesticide concentrations and indirect positive effects of pesticides in the studied LSWB. Local environmental variables also play an important role in shaping phytoplankton species composition and functional features. Studies addressing adaptive and irreversible thresholds of the consequences of pesticides and nutrients as multiple stressors are needed to gain a deeper understanding of phytoplankton community dynamics (e.g., Taherzadeh et al., 2019). In addition, these studies should be extended towards multiple trophic levels to generate a holistic picture of the effects of ecosystem structure and functioning (e.g., Zhou et al., 2020).

Chapter 5 Effects of the herbicides metazachlor and flufenacet on phytoplankton communities - a microcosm assay

Lishani Wijewardene[1,2*], Naicheng Wu[1,3*], Georg Hörmann[1], Beata Messyasz[4], Tenna Riis[5,6], Christina Hölzel[7], Uta Ulrich[1], Nicola Fohrer[1]

[1] Department of Hydrology and Water Resources Management, Institute for Natural Resource Conservation, Kiel University, 24118 Kiel, Germany

[2] Department of Limnology and Water Technology, Faculty of Fisheries and Marine Sciences & Technology, University of Ruhuna, 81000 Matara, Sri Lanka

[3] Department of Geography and Spatial Information Techniques, Ningbo University, 315211 Ningbo, China

[4] Department of Hydrobiology, Institute of Environmental Biology, Faculty of Biology, Adam Mickiewicz University in Poznan, Uniwersytetu Poznanskiego 6, 61-614 Poznan, Poland

[5] Department of Biology, Aarhus University, Ole Worms Allé 1, 8000 Aarhus C, Denmark

[6] WATEC, Aarhus University Centre for Water Technology, Department of Biology, 8000 Aarhus C, Denmark

[7] Institute of Animal Breeding and Husbandry, Kiel University, Hermann-Rodewald-Str. 6, 24118 Kiel, Germany

[*]Corresponding authors: L. Wijewardene (lishani@hydrology.uni-kiel.de) and N. Wu (nwu@hydrology.uni-kiel.de)

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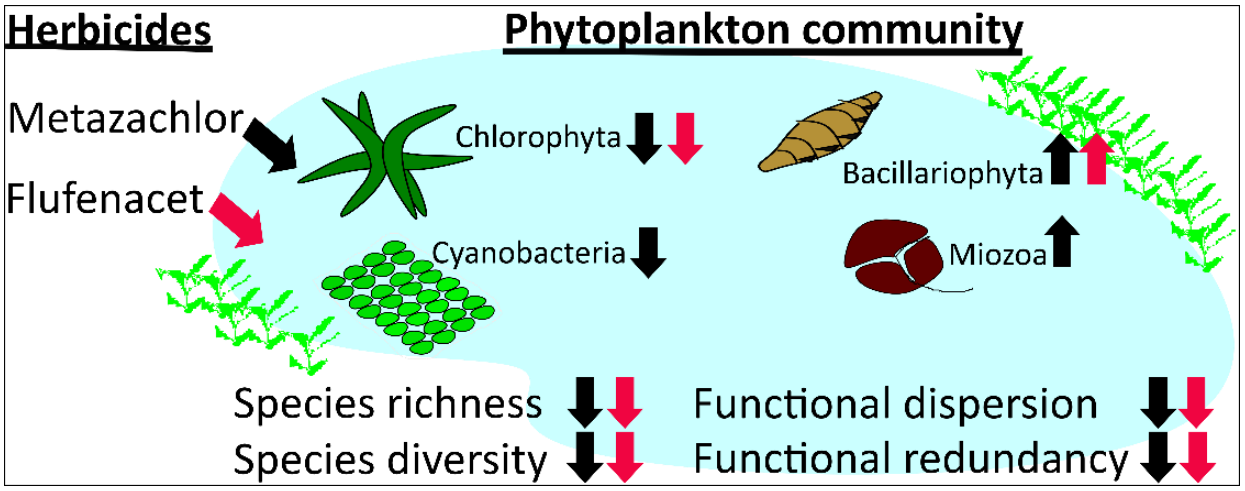
Abstract

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.

Keywords: Herbicides, Algae, Species composition, Biodiversity, Functional features

Graphical abstract



Highlights

- Even traces of metazachlor and flufenacet affect phytoplankton communities
- Increase of herbicide concentrations reduced Chlorophyta density
- Species diversity and functional features were affected by herbicides
- Effects on phytoplankton communities did not recover in the short-term

5.1 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., 2021b). 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; OGewV, 2016).

Herbicides can have dramatic consequences on species structure and function of the freshwater ecosystems (Lozano et al., 2019; Lozano et al., 2021; Lozano et al., 2018; Pérez et al., 2007; Sabio y García et al., 2022; Wijewardene et al., 2021b). 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., 2019; Lozano et al., 2018; 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 causes 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 (e.g., Chang et al., 2011; Leboulanger et al., 2011; Lozano et al., 2019; Lozano et al., 2018). 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).

5.2 Methods

5.2.1 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. 5.1). 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 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., 2021b) (Fig. 5.1A, B and 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 5.2.2 in Methods) and phytoplankton community attributes (see Section 5.2.3 in Methods) 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. 5.1D). 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.

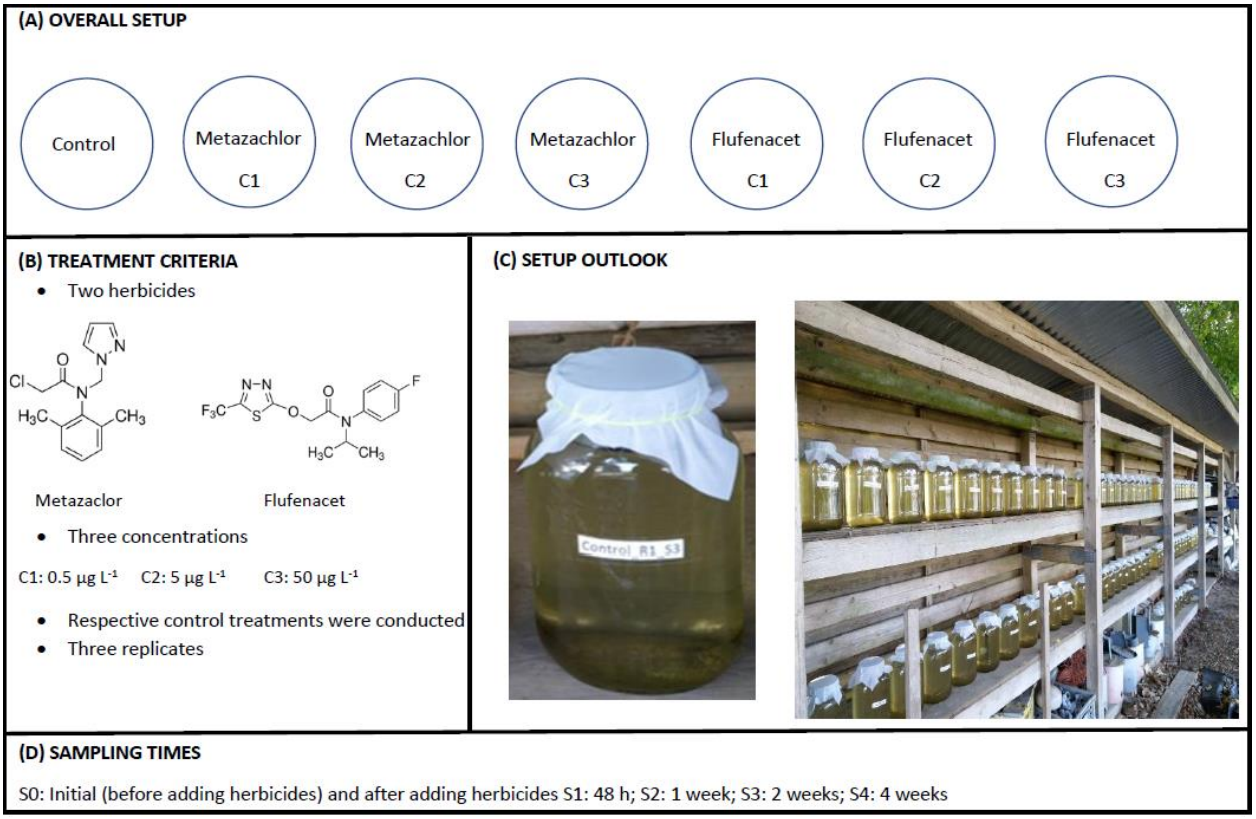


Fig. 5.1: Microcosm experiment design as overall setup with seven different treatments (A), description of treatment criteria (B), setup outlook (C) and description on sampling times (D). Treatments are represented as control and respective concentrations (C1: 0.5 µg L^{-1}; C2: 5 µg L^{-1}; C3: 50 µg L^{-1}) of exposed herbicides (M: Metazachlor and F: Flufenacet) here onwards.

5.2.2 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 analyses 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 ℃ 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 ^{o}C until the analysis. All samples were left for 24 h at 4 ^{o}C for sedimentation before the analysis and the supernatant water was analysed 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^{TM}, 4 µm, Hydro RP 80 Å, 50x3 mm and a security guard column Phenomenex AQ C18, 4x3 mm. Further quality parameters related to herbicide measurements are listed in Table S5.1 in Appendix.

5.2.3 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, Tokyo, Japan) 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, Tokyo, Japan) 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., 2018a; 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 S5.2 and S5.3 in the Appendix.

5.2.4 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).

5.2.5 Statistical analyses

All data analyses were performed using R software version 4.0.2 (R 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 analysed 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 analyses 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 analyses 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 analysed 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 ln (x+1) and z-score transformed

before linear regression analyses. It obtained standardised coefficients to compare magnitude within and between models.

5.3 Results

5.3.1 Herbicide concentrations and other physicochemical parameters

Metazachlor and flufenacet concentrations in the microcosms decreased with time (Fig. 5.2). 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 5.1. Detailed temporal changes in the other physicochemical parameters are recorded in Fig. S5.1.

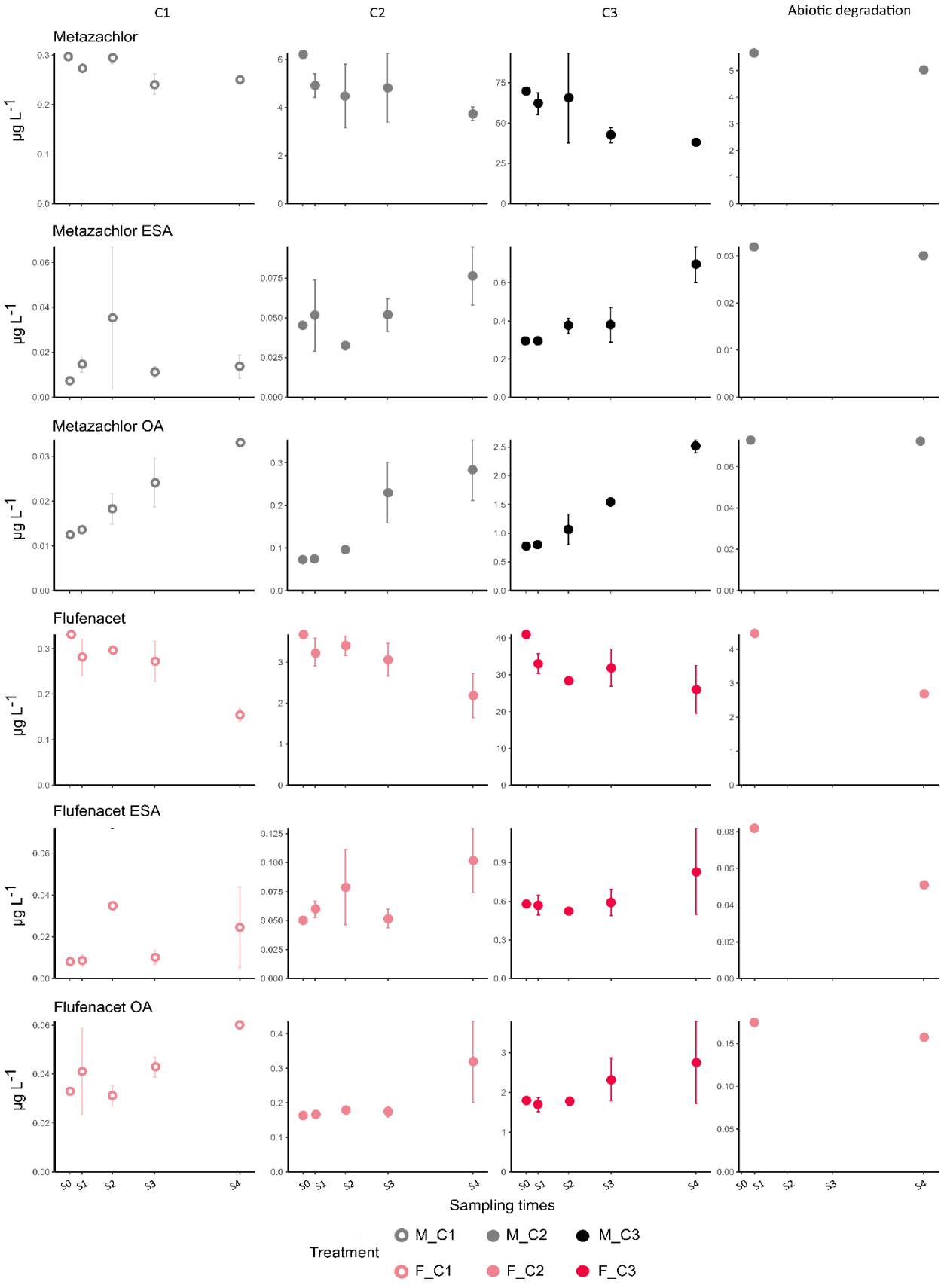

C1
C2
C3
Abiotic degradation
Metazachlor
Metazachlor ESA
Metazachlor OA
Flufenacet
Flufenacet ESA
Flufenacet OA
µg L-1
S0 S1 S2 S3 S4
Sampling times
Treatment
M_C1
M_C2
M_C3
F_C1
F_C2
F_C3

Fig. 5.2: 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: 48h after exposure; S2: 1 week after exposure; S3: 2 weeks after exposure; S4: 4 weeks after exposure. Dispersion bars denote standard deviation (SD).

Table 5.1: 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 [ºC]	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)

5.3.2 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. S5.2). 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. S5.2A). Diatom density increased with exposure time and flufenacet treatments showed higher diatom density than metazachlor after 4 weeks of exposure (Fig. S5.2B). 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. S5.2C).

5.3.3 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. 5.3. Relative abundance of Chlorophyta was mostly lower in herbicide exposed treatments compared to respective control (except M_C1 and F_C1 at S2) (Fig. 5.3). 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. 5.3).

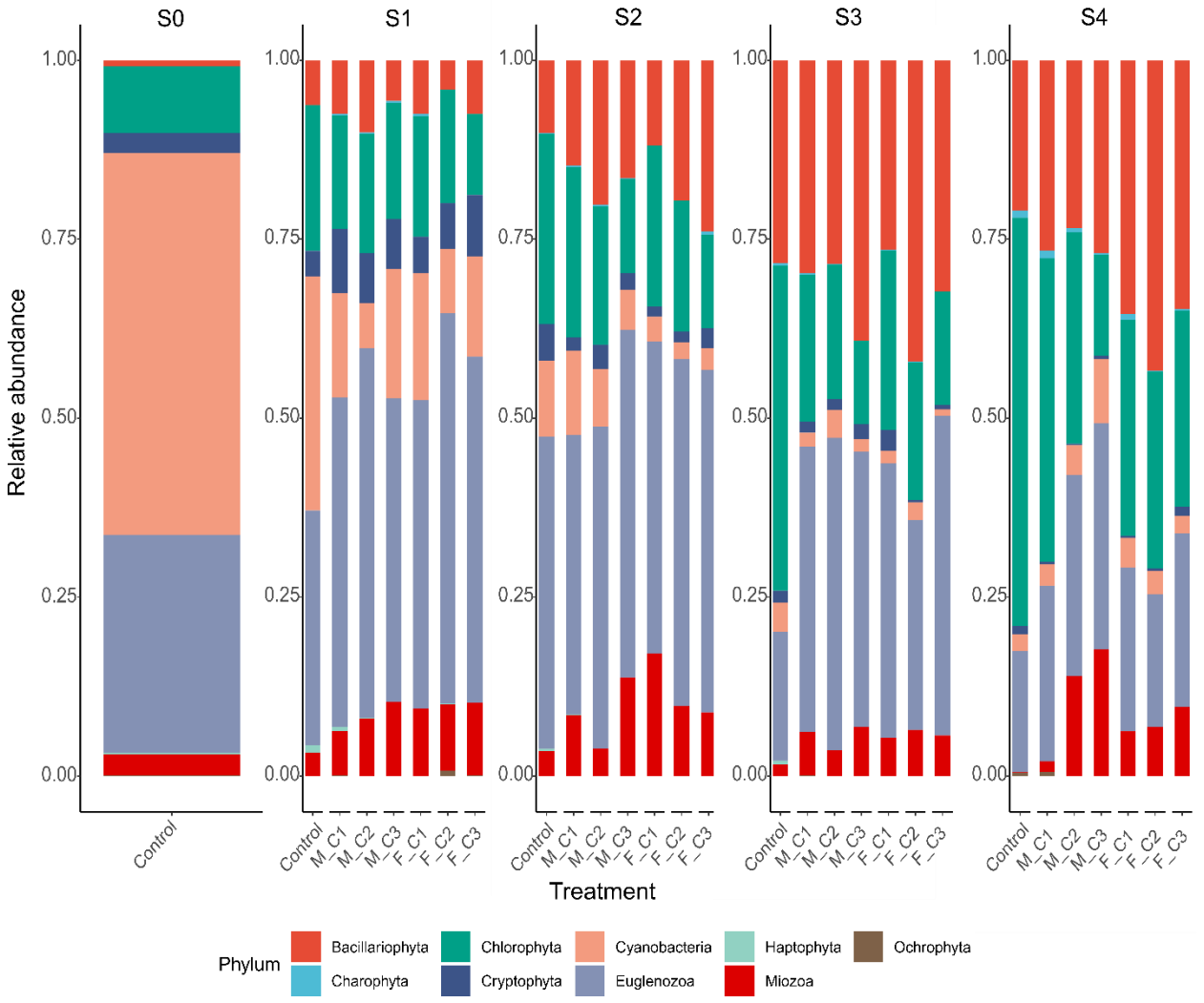


Fig. 5.3: Changes in the relative abundance of phytoplankton phyla across different treatments over the herbicide exposure time. Treatments are represented as control and respective concentrations (C1: 0.5 µg L^{-1}; C2: 5 µg L^{-1}; C3: 50 µg L^{-1}) of exposed herbicides (M: Metazachlor and F: Flufenacet). Sampling times reported as S0: before exposure; S1: 48h after exposure; S2: 1 week after exposure; S3: 2 weeks after exposure; S4: 4 weeks after exposure.

The response of the whole phytoplankton composition to the different treatments along sampling times are illustrated in Fig. 5.4. 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. 5.4).

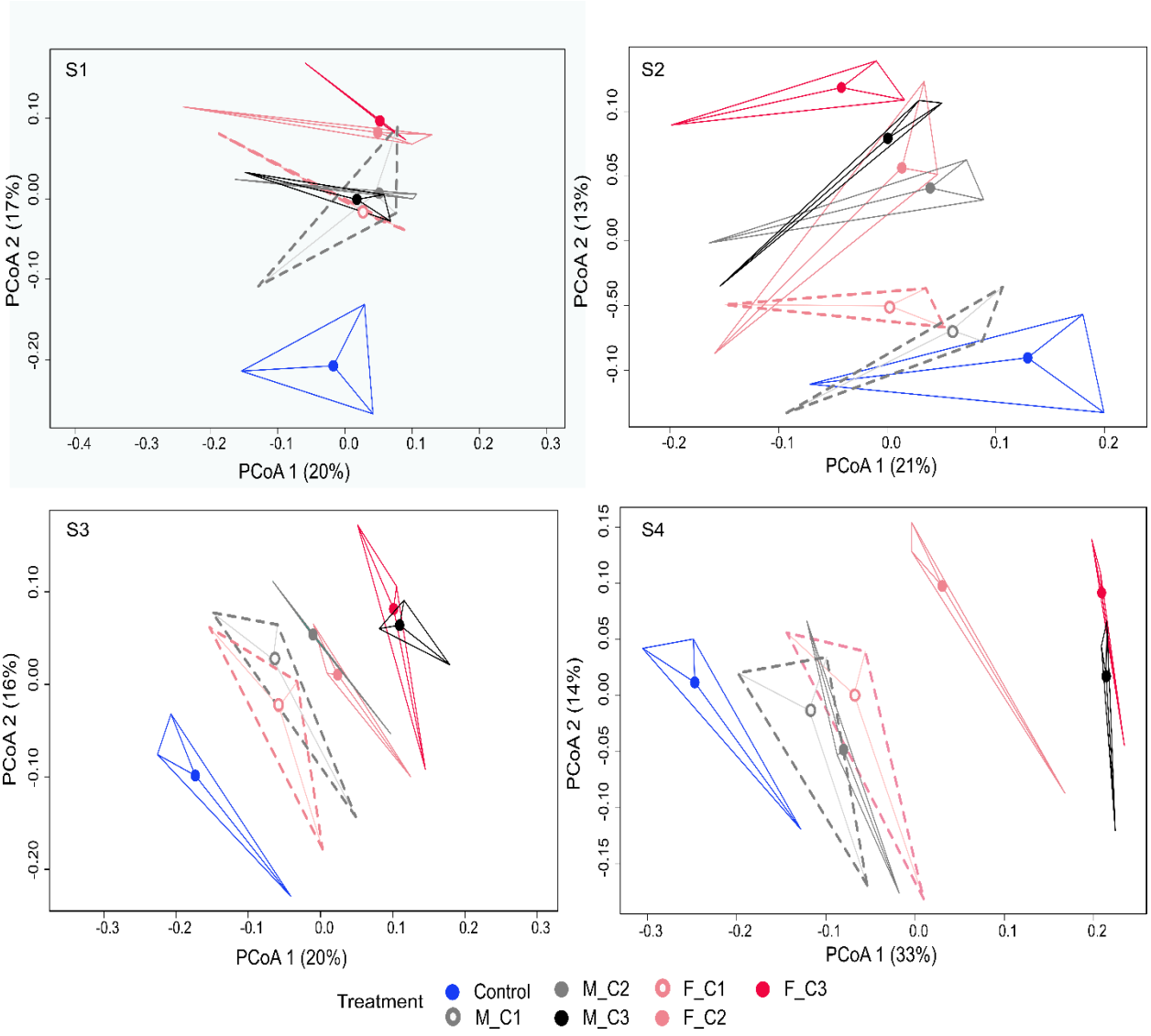


Fig. 5.4: Response of the whole phytoplankton composition to the different treatments. Treatments are represented as control and respective concentrations (C1: 0.5 µg L^{-1}; C2: 5 µg L^{-1}; C3: 50 µg L^{-1}) of exposed herbicides (M: Metazachlor and F: Flufenacet; see legend). Sampling times reported 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.

Species-level responses to the herbicide concentrations compared to controls during the experiment duration is illustrated in Fig. 5.5 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 (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. S5.3 in Appendix.

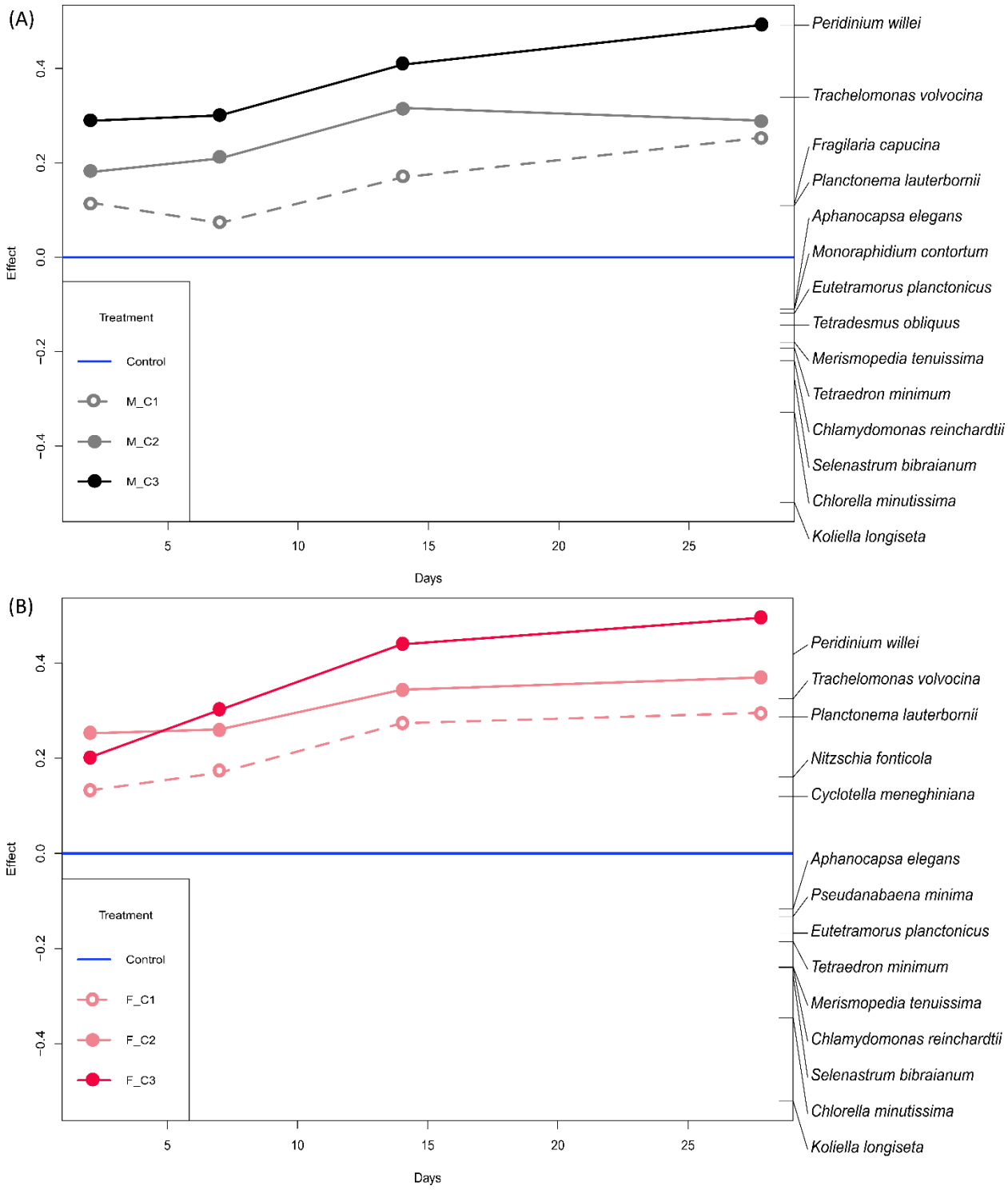


Fig. 5.5: Results of the PRC analysis. First axis of the PRC analysis is shown here. Response of the phytoplankton species to the metazachlor (A) and flufenacet (B) treatments compared to

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.

5.3.4 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. S5.4 in the Appendix. Results of the multiple regression models emphasized significant effects of metazachlor and flufenacet on different phytoplankton community attributes (Table 5.2).

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 5.2). 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 5.2).

The increased flufenacet concentrations also resulted in negative effects on phytoplankton community attributes (Table 5.2). 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 5.2). Similar to metazachlor, the diatom-to-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 5.2). In contrast, FDiv was significantly decreased with an increase in exposure time to flufenacet. Furthermore, light and temperature significantly influenced the phytoplankton community attributes together with herbicide concentrations and exposure time (Table 5.2).

Table 5.2: 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 standardised 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

5.4 Discussion

5.4.1 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 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. 5.5). Furthermore, a significant increase in the diatom-to-phytoplankton ratio and a decrease in Chlorophyta density with increasing herbicide concentrations further supported H1 (Table 5.2).

Selective effects of herbicides on phytoplankton species are frequently acknowledged in previous studies (e.g., Chang et al., 2011; Huertas et al., 2010; Lozano et al., 2019; Lozano et al., 2018). 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 $\mu g\ L^{-1}$, the smallest tested concentration in their study. We observed a similar trend in all our herbicide treatments starting from 0.5 $\mu g\ L^{-1}$ 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 to 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 lentic small 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., 2021b). 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).

5.4.2 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 5.2). 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.

5.4.3 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. 5.4) and the responses of the strongly affected species (Fig. 5.5) 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 hypothesised 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 some strongly affected phytoplankton species, particularly 2 weeks after exposure, may lead to the recovery of the phytoplankton community's taxonomic diversity (Fig. S5.3 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. 5.2). 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. 5.2). 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).

5.4.4 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. 5.4). 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 5.2). 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 categorise 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 5.2). 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., 2021b). 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.

5.5 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, modelling 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 fulfil human demands.

Chapter 6 General discussion

Globally, many lowland rural areas are used as agricultural lands and, thus, both lotic and lentic freshwater ecosystems in these areas are subjected to multiple stressors, particularly, hydrological disturbances and agrochemicals. Impacts of these multiple stressors can be identified with assessments based on algal communities. Given ample effort, we answered the specific questions formulated under 1.5.2 Section. This thesis reveals the effects of multiple stressors on structure, function, and integrity of freshwater ecosystems. The findings in this study shed light on the use of algal communities as ecological indicators in order to understand and manage freshwater ecosystems under threat of multiple stressors in rural lotic and lentic freshwater habitats.

6.1 Answers to the research questions

(i) What do we know about epiphyton in freshwater ecosystems and what are their interactions with macrophytes?

Epiphyton play a key role in shallow aquatic ecosystems while contributing to ecosystem structure, function, and integrity mainly through primary production, ecosystem respiration, nutrient uptake and recycling, pollutant removal as well as causing and aiding diseases (Chapter 2, Fig. 2.1). The structure and function of epiphyton are largely related to the host (e.g., macrophyte species, morphology, and characteristics) (Chapter 2, Fig. 2.2). Consequently, a myriad of interactions between epiphyton and host macrophytes have been documented (Chapter 2, Fig. 2.3). Interactions on resources, trophic interactions, and allelopathic interactions are highlighted among them. The interactive platform between epiphyton and aquatic macrophytes signifies the complexity of interactions in natural habitats through competitive, mutualistic, and commensalistic relationships. Yet, there are some key areas where research is currently lacking. Section 2.4 of Chapter 2 of this thesis provides directions to conduct future research toward developing a better understanding of the subject matter. This improved understanding on epiphyton in freshwater ecosystems and their interactions with macrophytes will aid in developing new management and conservation strategies for shallow freshwater ecosystems, which are under constant threat of human interference.

(ii) What are the effects of multiple stressors on epiphyton and epilithon in agricultural streams?

Stream biofilm communities play an important role on structure, function, and integrity of agricultural streams (Battin et al., 2003). In many lowland streams in rural areas, macrophyte vegetation is abundant and functions as an important substrate for biofilm (epiphyton) in addition to the gravel and stone substrate for epilithon on the stream bed (Levi et al., 2017; Riis et al., 2000). Hydrological disturbances and nutrient enrichment are concurrent stressors in these agricultural streams (Munn et al., 2018; Piggott et al., 2012).

The use of periphytic algal communities to assess the ecological state of freshwater ecosystems has a long history. However, we still lack evidence on (i) whether we can solely depend on sampling of one type of periphytic community for assessment and (ii) whether different periphytic communities in same environment respond similarly to multiple stressors. For example, some studies suggest that epiphyton and epilithon diatom species composition is remarkably different (e.g., Cantonati and Spitale, 2009) while others state the opposite (e.g., Winter and Duthie, 2000). Winter and Duthie (2000) studied epiphytic, epilithic and epiphelic diatom communities in streams surrounded by mixed land use, such as urban, agriculture and woodlands and concluded that there is no apparent benefit of sampling diatom communities separately on substrates for water quality assessments. However, in our study, epiphyton and epilithon showed distinct structural differences during a year in two agricultural streams in terms of biomass, algal composition, and diatom species composition. For example, high substrate specific biomass was observed in epilithon compared to epiphyton (Chapter 3, Fig. 3.2). Epiphyton was mainly composed of diatoms and green algae, while cyanobacteria were more important in epilithon (Chapter 3, Fig. 3.3). Both overall diatom species composition and dominant diatom species in each algal community were different (Chapter 3, Fig. 3.4). Therefore, substrate specific differences were emphasized in epiphyton and epilithon in agricultural streams.

On the other hand, epiphyton and epilithon communities responded differently to multiple stressors in agricultural streams. Epiphyton structural properties were less affected by hydrological regimes and water nutrient concentrations than epilithon. For example, epilithic Chl-a concentrations showed a positive association with PO_4^{3-} concentration, while none of the biomass measurements of epiphyton were associated with the hydrological regime or nutrient concentrations (Chapter 3, Table 3.1). Furthermore, the variation in the epiphytic diatom

community was mainly explained by the nutrients and other environmental factors (Chapter 3, Fig. 3.5A), but hydrology was less important in both streams (Chapter 3, Fig. 3.5C and 3.5E). For epilithon, all three groups of variables explained equal amounts of variation, indicating that hydrology was more important for epilithon than for epiphyton (Chapter 3, Fig. 3.5B). These findings indicate that epilithon is more dependent on external water nutrients to fulfil its nutrient requirements while epiphyton can take advantage of macrophyte leachates. The influence of hydrology and hydraulics (i.e., discharge, current velocity, low flow, high flow) on epilithon structure was highly recognized in many previous studies in lotic systems (Ács and Kiss, 1993; Guo et al., 2020; Matthaei et al., 2010; Moulton et al., 2009) and nutrients were identified as an important factor in driving epilithic diatom composition (Munn et al., 2018; Soininen and Eloranta, 2004). Additionally, according to Bojorge-García et al. (2014) and Wolters et al. (2019), the epiphyton community may depend on nutrients released from macrophytes. Other environmental variables such as light, temperature, and DOC played an important role in driving epiphyton and epilithon structural differences between the two streams. Despite the above findings, we acknowledge a generally low explanatory power of the included environmental variables on the biofilm structure although we used daily measurements. Our findings support that (i) sampling of different periphytic communities are needed in ecological assessments of agricultural streams, and (ii) the sensitivity of epiphyton and epilithon to multiple stressors are different.

Furthermore, we provided the following insights on future management practices of the agricultural streams; (i) Species composition of biofilms is more sensitive to the environmental variables than macro-scale parameters such as Chl-a and AFDM. Therefore, including measurements on species composition is needed to identify and monitor the status of agricultural streams; (ii) Epilithon closely link with environmental variables, while epiphyton closely link with the conditions altered by macrophytes. Besemer (2015) stated that biofilm communities exposed to the same environmental conditions can still be different among habitats, while emphasizing the importance of the nature of the substratum (e.g., chemical composition, surface area, and stability). Therefore, maintaining macrophytes are important in agricultural streams to continue the benefits of epiphyton on stream functioning such as metabolism and nutrient uptake (Wijewardene et al., unpublished data). Current management practices in agricultural streams, such as weed cutting (Baattrup-Pedersen et al., 2002; Baattrup-Pedersen and Riis, 2004) may need re-evaluation considering the importance of macrophytes as substrate for epiphyton; (iii) We observed considerable differences between the two studied

agricultural streams even though they are representing a similar catchment area and land use. Therefore, small-scale management practices of local watersheds are important in managing agricultural streams.

(iii) What are the effects of multiple stressors on phytoplankton in lentic small water bodies (LSWB) in rural areas?

LSWB are abundant and ecologically significant habitats in rural areas (Bolpagni et al., 2019; Hill et al., 2018). In addition, LSWB are natural sinks for substance runoff from their catchments and are sensitive to local conditions and variations in geology, hydrology, climate, and vegetation due to their small catchment size (Biggs et al., 2005b). In our field study (Chapter 4), we identified pesticide toxicity and nutrient enrichment as multiple stressors on the phytoplankton community structure in LSWB in rural areas (Chapter 4, Table 4.2, and Fig. 4.3A). Furthermore, the high pesticide toxicity (TU_max) and nutrient concentrations such as PO_4-P and NH_4-N were associated with decreasing water level (Chapter 4, Fig. 4.3A). Decreasing water levels in lentic ecosystems may concentrate pesticides and nutrients resulting in negative relationships between water level and TU_max, PO_4-P, and NH_4-N. Hence, our field study foresees the impact of potential future climate changes associated with predicted reduction of water levels in freshwater ecosystems. Therefore, the observed effects on phytoplankton community in our study provides insights on potential changes in phytoplankton community composition expected in the future.

TU_max, nutrients (NH_4-N, NO_3-N and PO_4-P) and local environmental variables (EC, DO, and water level change) significantly contributed to shaping the phytoplankton community composition individually in LSWB (Chapter 4, Fig. 4.4). Local environmental variables showed the highest pure contribution for driving phytoplankton composition followed by nutrients and pesticide toxicity. The interactive effect of pesticide toxicity and nutrient concentrations contributed to shaping the species composition of the phytoplankton (Chapter 4, Fig. 4.4) rather than individual effects of pesticides. Eutrophic small diatom species are recognized as tolerant or indicator species for pesticide contamination (Debenest et al., 2010). Under concurrent stressors of PO_4-P and pesticide toxicity, phytoplankton species composition can reverse from dominance of indicator species of eutrophic status (i.e., *Chloromonas angustissima*, *Navicula lanceolata* and *Gomphonema parvulum*) to co-dominance of less-sensitive, fast adapting generalist species (i.e., *Chroccoccus turgidus* (Cyanophyta), *Peridinium willei* (Dinophyta) and *Trachelomonas* spp. (Euglenophyta)) (Chapter 4, Fig. 4.3B). Previous studies reported

dinophytes, such as *Peridinium* spp., dominating phytoplankton communities under high herbicide concentrations (Noack et al., 2003) and euglenophytes as pesticide-resistant species (DeLorenzo et al., 2001). According to Pesce et al. (2011), herbicides contaminating aquatic systems showed the initial inhibition of the photosynthesis of primary producers (including macrophytes) following a significant release of nutrients into the water causing an increase in the abundance of less-sensitive or fast-adapting phytoplankton species, particularly flagellates.

Functional features (FD and FR) of the phytoplankton community were significantly affected by pesticide toxicity and nutrient concentrations (Chapter 4, Fig. 4.5). The functional richness and functional evenness were negatively affected by PO_4-P concentrations. Pesticide toxicity was positively correlated with functional redundancy indices. Therefore, functional features can be altered by nutrient concentrations and pesticide toxicity leading to negative and positive feedbacks on the functionality of ecosystems from former and latter stressors, respectively. However, these positive feedbacks of pesticide toxicity on phytoplankton most likely occurred due to the low level of pesticide concentrations (Cedergreen et al., 2004) and indirect positive effects of pesticides (i.e., reduced grazing pressure due to insecticides) (van Donk et al., 1995) in the studied LSWB. Local environmental variables also play an important role in shaping phytoplankton species composition and functional features. Based on our phytoplankton-based study, we foresee nutrient enrichment due to extensive use of fertilizers as the dominant negative cause for ecosystem functionality in LSWB under the current situation in the Kielstau catchment.

(iv) What are the effects of the herbicides, metazachlor and flufenacet, on phytoplankton?

As highlighted in the field study (Chapter 4), pesticide toxicity on phytoplankton communities is primarily governed by herbicides. Environmentally realistic concentrations of two common herbicides, metazachlor and flufenacet, caused structural changes in phytoplankton community composition, taxonomic diversity, and functional features. Metazachlor and flufenacet selectively affected phytoplankton community composition resulting in a reduction of species from Chlorophyta (e.g., *Koliella longiseta*, *Selenastrum bibraianum*) and Cyanobacteria (e.g., *Merismopedia tenuissima* and *Aphanocapsa elegans*) and changed the community towards a high abundance of species from Bacillariophyta (e.g., *Nitzschia fonticola* and *Cyclotella meneghiniana*), Miozoa (i.e., *Peridinium willei*), and Euglenozoa (i.e., *Trachelomonas volvocina*) (Chapter 5, Fig. 5.3). Selective effects of herbicides on phytoplankton species are frequently acknowledged in previous studies (e.g.,

Chang et al., 2011; Huertas et al., 2010). These effects highly varied depending on initial species composition of the phytoplankton community (Debenest et al., 2010). Similar to our results, Mohr et al. (2008) also reported a high sensitivity of chlorophytes to metazachlor emphasizing its significant effect at 5 μg L^{-1}, the smallest tested concentration in their study. 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 (Chapter 5, Table 5.1). Although there were no directly comparable studies about the effect of these two specific herbicides on phytoplankton taxonomic or functional diversity indices, many herbicides negatively affect phytoplankton taxonomic diversity (glyphosate: Fugère et al. (2020); paraquat: Leboulanger et al. (2011)).

Most of the effects on the phytoplankton community were increased throughout the exposure time without showing any recovery or reversing potentials during the 4-week period of our study. 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. Noack et al. (2003) observed a recovery of total phytoplankton density only after 30-35 days of metazachlor application. Flufenacet has an eight-fold higher toxicity than metazachlor, but both are similar in their mode of action (Lewis et al., 2006). Both herbicides showed similar impacts on phytoplankton communities particularly at 50 μg L^{-1} regardless of the differences in toxicity. Therefore, 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 significantly influenced the observed changes in phytoplankton attributes 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. This comprehensive understanding is needed for the management and conservation of aquatic ecosystems surrounded by agricultural land, which continues to expand worldwide to fulfil human demands.

6.2 Outlook

Impacts of multiple stressors on algal communities in freshwater ecosystems in rural areas dominated by agricultural land use have been investigated in this study. We focused on abiotic stressors, but biological stressors were not included. A multiple stressor study in a large lake found that the amount of variance associated with phytoplankton community can be explained by biological stressors, water quality, temperature, and climate in reducing order (Kelly et al., 2017). Therefore, including biological stressors into these studies may greatly enhance the explanation of the algae community responses further. Grazing pressure, competition within algal communities, interactions between autotrophic-heterotrophic communities within biofilms and interactions with macrophytes would be the important biological counterparts to address to understand responses of the algal communities even more comprehensively. For example, future studies should address the interactions within autotrophic and heterotrophic communities in epiphyton/epilithon, interactions with their substrates and interactions with other biota to better understand the underlying controlling mechanisms of the epiphyton and epilithon structure in agricultural streams.

Even though we tackled most of the important environmental variables in our study that can influence the studied algal communities, dynamics of a few more environmental variables may be important. For example, Si concentrations are important for diatoms. Also, EC varied highly in the LSWB and influenced the phytoplankton community. Minerals included in fertilizers may cause changes in EC. Further, these minerals may act positively under a low amount supplying essential nutrients but may have toxic effects at high concentrations on the phytoplankton (Hutchinson, 1961; Talling, 2010). Future studies on phytoplankton community need to include measurements of ions such as K^+, Mg^{2+}, Br^+, Mn^{2+} to disentangle the effect of EC on phytoplankton and to understand the overall effect of fertilizers applied in agriculture.

Our study has a great potential to expand further with modelling approaches. For example, our field study in LSWB foresees the impact of future climate change associated with predicted reduction of water levels in freshwater ecosystems. Therefore, our work provides insights on potential changes in phytoplankton community composition in future decades under climate change. Future studies which combine these baseline observations with modelling attempts will lead to predicting the fate of aquatic biota under climate change scenarios. Studies addressing adaptive and irreversible thresholds of the consequences of pesticides and nutrients as multiple stressors are needed to gain a deeper understanding of phytoplankton community

dynamics. In addition, these studies should be extended towards multiple trophic levels to generate a holistic picture of the effects of ecosystem structure and functioning.

6.3 Conclusion

The tasks of this thesis were dedicated to understanding of the impacts of multiple stressors on algal communities in freshwater ecosystems in rural areas dominated with agricultural land use. Lentic and lotic freshwater ecosystems are subject to hydrological disturbances and agrochemicals as simultaneous multiple stressors. Dominant algal communities in the ecosystem serve as the best indicators to reveal the impacts of these stressors and consequences on ecosystem biodiversity and functioning. The key findings of the study are listed below.

(i) Hydrological disturbances and agrochemicals are significantly contributing to shaping algal communities in freshwater ecosystems in lowland rural areas dominated by agricultural land use.

(ii) Effects of these multiple stressors are more evident in micro scale parameters, such as species and trait composition, and are reflected in related indices rather than in macro scale measurements, such as biomass. Therefore, including micro scale parameters to monitoring and assessments based on algal communities in these ecosystems are essential to understand, manage, and conserve ecosystem health, function, and integrity.

(iii) Addressing the interactions within algal communities, interactions with their substrates and interactions with other biota are necessary to increase our understanding of the underlying controlling mechanisms of the structure of algal communities in agricultural streams and LSWB in rural areas.

This thesis gives insights on potential alterations in periphytic and phytoplankton composition and diversity under the concurrent stressors, e.g., hydrological disturbances and agrochemicals in freshwater ecosystems in rural areas dominated by agricultural land use. Combining biological stressors and extending this study towards multiple trophic levels may further improve our understanding and will be helpful to manage and conserve aquatic ecosystems surrounded by agricultural land, which continue to expand worldwide to fulfil human demands.

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Appendix

Supplementary materials: Chapter 2

Fig. S2.1: The trend of number of publications (solid line) and its proportion to the total number of scientific articles published in databases (dash line) on epiphytes on macrophytes from 1955 to 2020. Data source: Web of science (Thomson Reuters) [Accessed on 02/01/2021].

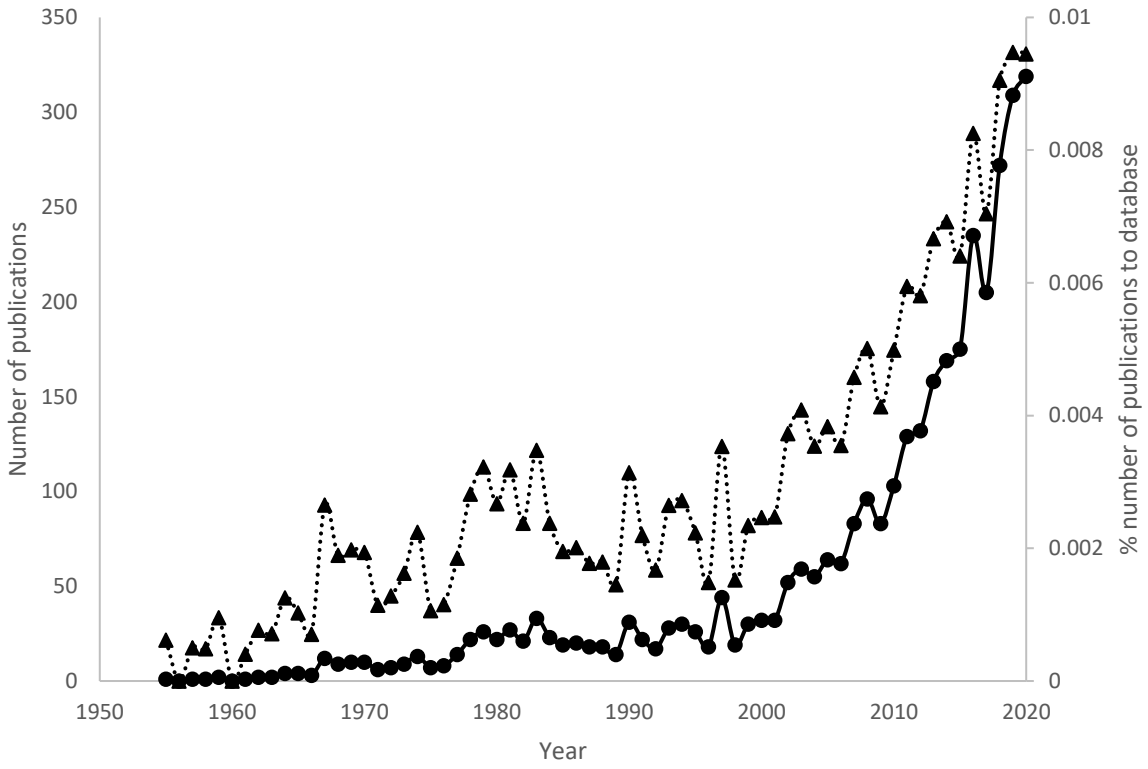
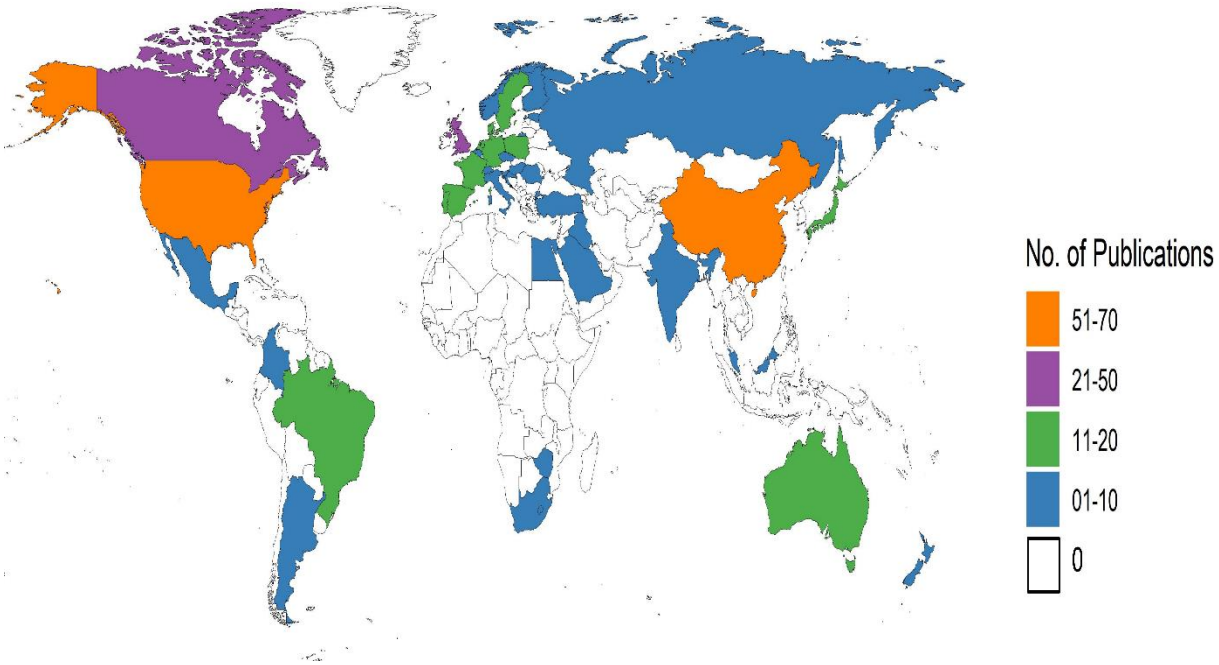


Fig. S2.2: The global distribution of the selected articles in our study



Supplementary materials: Chapter 3

Table S3.1: Description of nine environmental variables used in our study

Environmental variable code	Unit	Description
Other environmental variables (Other env.)		
Light	Photons m^{-2} day^{-1}	Cumulative light in 21 days
Temperature	^{o}C	Mean temperature in 21 days
DOC	mg L^{-1}	Mean of daily average dissolved organic carbon (DOC) concentrations in 21 days period before sampling date
Hydrology		
Q_{med}	L s^{-1}	Median of the discharge in 21 days period before sampling date
CV of Q	%	Coefficient of variation of discharge in 21 days period before sampling date
Fre_{Low}	days	Frequency of low discharge: number of days during 21 days period, where the magnitude of discharge remains below a lower threshold. Low discharge is defined as daily mean flow below the 25th percentile of all daily values for the time period 2019 - 2020
Fre_{High}	days	Frequency of high discharge: number of days during 21 days period, where the magnitude of discharge remains above a higher threshold. High discharge is defined as flow above the 75th percentile of all daily values for the time period 2019 – 2020
Nutrients		
PO_4^{3-}	mg L^{-1}	Mean of daily average phosphate (PO_4^{3-}) concentrations in 21 days period before sampling date
DIN	mg L^{-1}	Mean of daily average dissolved inorganic nitrogen (DIN) concentrations in 21 days period before sampling date

Table S3.2: Epiphyton and epilithon biomass and AI related to environmental variables (Kendall correlation coefficients; displayed only significant co-relations $p < 0.05$). Correlations are significant but weak (<0.40).

	Epiphyton			Epilithon		
Variables	Chl-a	AFDM	AI	Chl-a	AFDM	AI
Light	0.27	0.22	-0.19		0.16	0.18
Temperature		0.36			0.19	
DOC	-0.20	-0.16			-0.07	
Q_{med}	-0.22	-0.24	0.13		-0.10	
CV of Q	-0.23	-0.09	0.25	-0.20		0.2
PO_4^{3-}	0.07	0.25		0.30		-0.19
DIN		-0.35	0.02		-0.14	

Table S3.3: Relative abundance (%) of 20 most abundant diatom species in the biofilms

Diatom species name	Epiphyton		Epilithon	
	Aarhus	Lyngbygård	Aarhus	Lyngbygård
Achnanthidium minutissimum (Kützing) Czarnecki 1994	12.01	3.43	46.91	27.58
Amphora pediculus (Kützing) Grunow 1875	2.08	1.03	1.67	3.50
Aulacoseira ambigua (Grunow) Simonsen 1979		3.15		1.38
Cocconeis pediculus Ehrenberg 1838	1.77	4.05	1.52	
Cocconeis placentula Ehrenberg 1838	0.97			
Cocconeis placentula var. *euglypta* (Ehrenberg) Grunow 1884	26.98	24.44	7.32	7.97
Cyclostephanos dubius (Fricke) Round 1987	1.53	3.98	0.43	3.70
Diatoma vulgaris Bory 1824		1.30		
Encyonema silesiacum (Bleisch) Mann 1990	1.27			
Fragilaria capucina var. *Gracilis* (Østrup) Hustedt 1950		1.02		0.96
Fragilaria vaucheriae (Kützing) Petersen 1938	4.68		3.25	1.47
Gomphonema olivaceum (Hornemann) Ehrenberg 1838	1.97	6.83	2.93	1.79
Gomphonema parvulum (Kützing) Kützing 1849	2.45	7.15	1.42	2.75
Gomphonema pumilum (Grunow) Reichardt & Lange-Bertalot 1991		1.60		
Gomphonema pumilum var. *rigidum* Reichardt & Lange-Bertalot 1997		1.69		
Gomphonema sp. Ehrenberg, 1832	0.78	3.24		1.05

Melosira varians Agardh 1827	3.83		0.57	
Navicula capitatoradiata Germain 1981	0.73			
Navicula cryptotenella Lange-Bertalot 1985	3.71	2.15	2.33	0.92
Navicula gregaria Donkin 1861	2.72	1.45	3.25	2.59
Navicula lanceolata Ehrenberg 1838	7.49	9.87	8.51	10.91
Navicula minima Grunow 1880				1.51
Navicula reichardtiana Lange-Bertalot 1989	4.27		1.58	
Navicula tripunctata (Müller) Bory 1822	3.65	7.19	1.43	3.11
Nitzschia frustulum (Kützing) Grunow 1880			0.43	
Nitzschia palea (Kützing) Smith 1856	1.54	1.30	1.22	1.11
Planothidium frequentissimum (Lange-Bertalot) Lange-Bertalot 1999			1.11	5.36
Planothidium lanceolatum (Brébisson ex Kützing) Lange-Bertalot 1999	1.39	1.01	3.46	4.07
Rhoicosphenia abbreviata (Agardh) Lange-Bertalot 1980		1.77		
Staurosira construens f. *venter* (Ehrenberg) Bukhtiyarova 1995			0.68	1.33
Staurosira construens var. *construens* Ehrenberg 1843				0.98
Staurosirella pinnata (Ehrenberg) Williams & Round 1988			0.53	

Fig. S3.1: Location of monitored two agricultural streams at Aarhus, Denmark (A), study sites (B: Aarhus stream and Lyngbygård stream) and studied stream algal communities (C: epiphyton and epilithon).

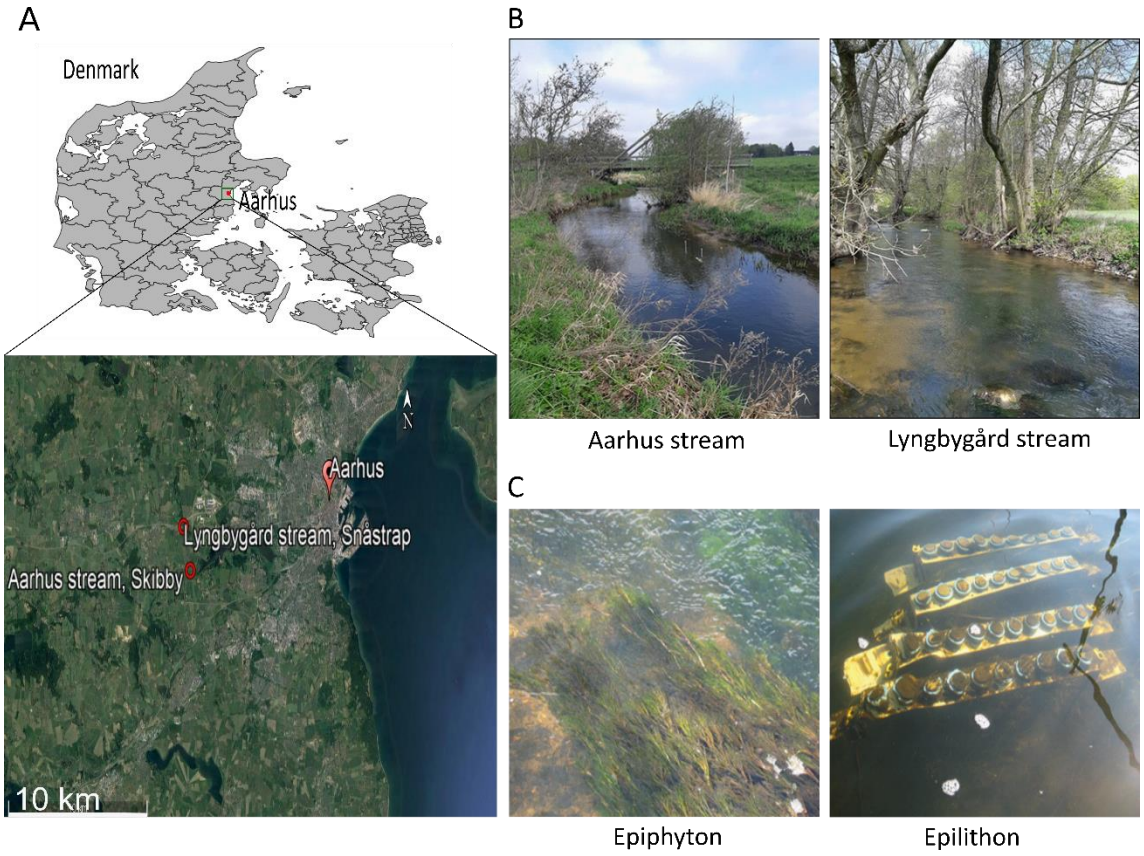
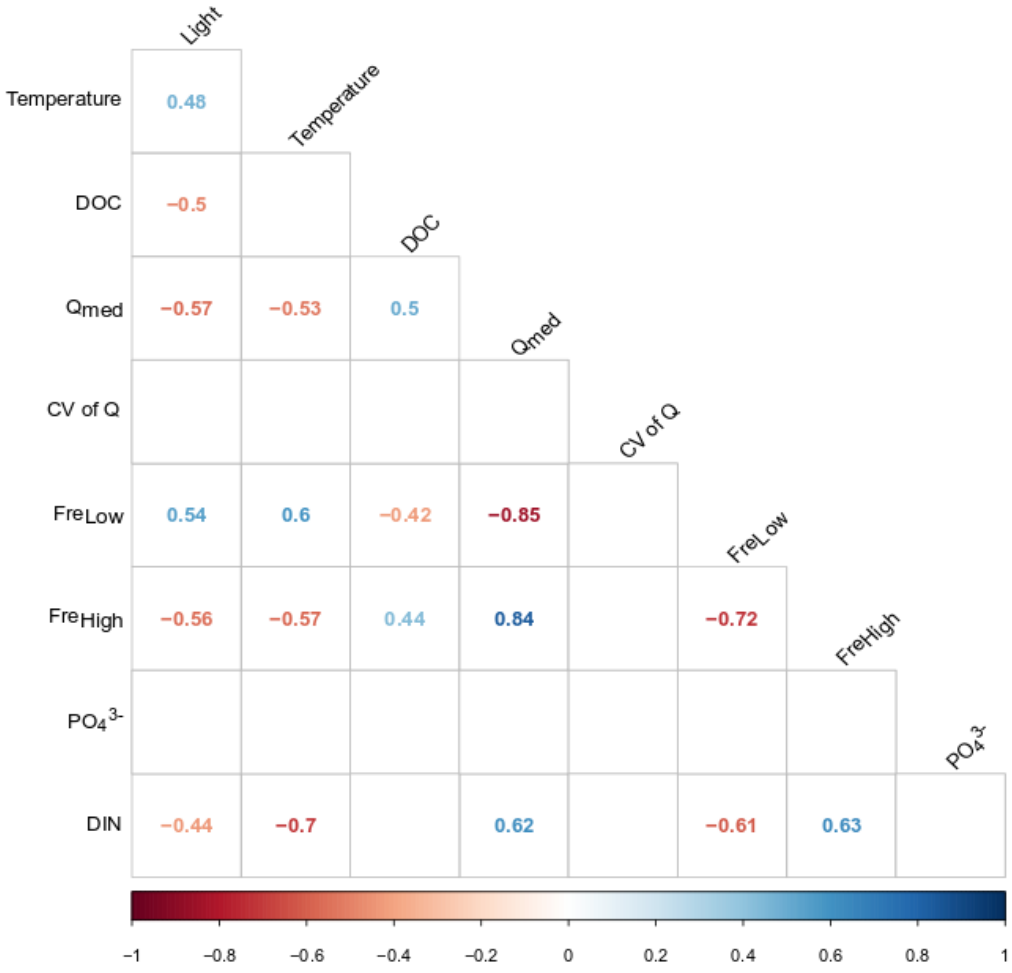


Fig. S3.2: Correlations between environmental variables

Only significant correlations ($p < 0.05$, Kendall correlation coefficients) are shown in here.



Supplementary materials: Chapter 4

Table S4.1: List of the measured pesticides in this study
List of the 94 measured pesticides and metabolites in monitored LSWB during weekly sampling from 11.04.2018 to 03.07.2018.

Herbicides	**Fungicides**	**Insecticides**	**Metabolites**
2,4-D	Amisulbrom	Acetamiprid	Desamino-metamitron
Aclonifen	Azoxystrobin	Chlorantraniliprole	Metazachlor-ESA
Bentazone	Bixafen	Chlorpyrifos	Metazachlor-OA
Bifenox	Boscalid	Clothianidin	
Bromoxynil	Cyazofamid	Dimethoate	
Chloridazon	Cyprodinil	Imidacloprid	
Chlortoluron	Difenoconazole	Methiocarb	
Dichlorprop	Dimethomorph	Pirimicarb	
Diflufenican	Dimoxystrobin	Pymetrozine	
Dimethachlor	Epoxiconazole	Spinosyn A	
Dimethenamid-P	Famoxadone	Spinosyn D	
Florasulam	Fenpropidin	Thiacloprid	
Flufenacet	Fenpropimorph	Thiamethoxam	
Flumioxazin	Fludioxonil		
Flupyrsulfuron-methyl	Fluopicolide		
Fluroxypyr	Fluquinconazole		
Flurtamone	Fluxapyroxad		
Foramsulfuron	Isopyrazam		
Imazosulfuron	Metrafenone		
Isoproturon	Picoxystrobin		
Lenacil	Prochloraz		

MCPA	Propamocarb		
Mecoprop	Propiconazole		
Mesosulfuron-methyl	Proquinazid		
Mesotrione	Prothioconazole		
Metamitron	Pyraclostrobin		
Metazachlor	Quinoxyfen		
Metosulam	Spiroxamine		
Metribuzin	Tebuconazole		
Metsulfuron-methyl	Trifloxystrobin		
Napropamide	Zoxamide		
Nicosulfuron			
Pendimethalin			
Pethoxamid			
Phenmedipham			
Picolinafen			
Propyzamide			
Prosulfocarb			
Prosulfuron			
Pyraflufen-ethyl			
Pyroxsulam			
Quinmerac			
S-metolachlor			
Terbuthylazine			
Thifensulfuron-methyl			
Triasulfuron			
Tritosulfuron			

Table S4.2: Properties of pesticides and metabolites

Properties of detected pesticides and metabolites (>0.001 µg/L) in LSWB were summarized here as type, chemical composition, mode of action, acute 72 h EC50 for algae and toxicity.

Type of pesticide	Pesticides	Chemical composition	Mode of action	Acute 72 h EC50 for Algae (mg/L)	Toxicity*
Herbicides	2,4-D	$C_8H_6Cl_2O_3$	Increases biosynthesis and production of ethylene causing uncontrolled cell division leading to damages in vascular tissue	24.2	Low
	Aclonifen	$C_{12}H_9ClN_2O_3$	Inhibition of carotenoid biosynthesis	0.47	Moderate
	Bentazone	$C_{10}H_{12}N_2O_3S$	Inhibits photosynthesis (photosystem II)	10.1	Low
	Bromoxynil	$C_7H_3Br_2NO$	Inhibits photosynthesis (photosystem II)	0.12	Moderate
	Chloridazon	$C_{10}H_8ClN_3O$	Inhibits photosynthesis (photosystem II)	3.0	Moderate
	Diflufenican	$C_{19}H_{11}F_5N_2O_2$	Bleaching: inhibition of carotenoid biosynthesis	0.00025	High
	Dimethachlor	$C_{13}H_{18}ClNO_2$	Inhibition of cell division	0.0065	High
	Dimethenamid-P	$C_{12}H_{18}ClNO_2S$	Fatty acid inhibitor	0.019	Moderate
	Florasulam	$C_{12}H_8F_3N_5O_3S$	Inhibits plant amino acid synthesis	0.00894	High
	Flufenacet	$C_{14}H_{13}F_4N_3O_2S$	Inhibition of cell division	0.00204	High

Flumioxazin	$C_{19}H_{15}FN_2O_4$	Inhibition of protoporphyrinogen oxidase	0.00085	High
Foramsulfuron	$C_{17}H_{20}N_6O_7S$	Acetolactate synthase inhibitor, stunting growth and causing death	8.1	Moderate
Isoproturon	$C_{12}H_{18}N_2O$	Inhibits photosynthesis (photosystem II)	0.013	Moderate
MCPA	$C_9H_9ClO_3$	Synthetic auxin	79.8	Low
Mecoprop	$C_10H_{11}ClO_3$	Synthetic auxin	237	Low
Mesosulfuron-methyl	$C_{17}H_{21}N_5O_9S_2$	Inhibits plant amino acid synthesis	0.2	Moderate
Metazachlor	$C_{14}H_{16}ClN_3O$	Ergosterol inhibitor and inhibition of cell division	0.0162	Moderate
Napropamide	$C_{17}H_{21}NO_2$	Inhibition of cell division	3.4	Moderate
Nicosulfuron	$C_{15}H_{18}N_6O_6S$	Inhibits plant amino acid synthesis	7.8	Moderate
Propyzamide	$C_{12}H_{11}Cl_2NO$	Microtubule assembly inhibition	2.8	Moderate
Prosulfocarb	$C_{14}H_{21}NOS$	Lipid synthesis inhibitor	0.049	Moderate
Prosulfuron	$C_{15}H_{16}F_3N_5O_4S$	Inhibits plant amino acid synthesis	0.0089	High
Quinmerac	$C_{11}H_8ClNO_2$	Phytotoxic effects	48.5	Low
S-metolachlor	$C_{15}H_{22}ClNO_2$	Inhibition of cell division	0.017	Moderate
Terbuthylazine	$C_9H_{16}ClN_5$	Inhibits photosynthesis (photosystem II)	0.012	Moderate

Fungicides	Azoxystrobin	$C_{22}H_{17}N_3O_5$	Respiration inhibitor	0.36	Moderate
	Bixafen	$C_{18}H_{12}Cl_2F_3N_3O$	Succinate DeHydrogenase Inhibitor	0.097	Moderate
	Boscalid	$C_{18}H_{12}Cl_2N_2O$	Inhibits spore germination and germ tube elongation. Succinate DeHydrogenase Inhibitor	3.75	Moderate
	Dimethomorph	$C_{21}H_{22}ClNO_4$	Cellulose synthesis inhibitor	29.2	Low
	Dimoxystrobin	$C_{19}H_{22}N_2O_3$	Respiration inhibitor	0.017	Moderate
	Epoxiconazole	$C_{17}H_{13}ClFN_3O$	Sterol biosynthesis inhibitor	> 10.69	Low
	Fenpropimorph	$C_20H_{33}NO$	Disrupts membrane function	0.327	Moderate
	Fludioxonil	$C_{12}H_6F_2N_2O_2$	Inhibits transport-associated phosphorylation of glucose, reducing mycelial growth	0.024	Moderate
	Spiroxamine	$C_{18}H_{35}NO_2$	Disrupts membrane function and inhibits sterol biosynthesis in membranes	0.003	High
	Tebuconazole	$C_{16}H_{22}ClN_3O$	Disrupts membrane function and sterol biosynthesis inhibitor	1.96	Moderate
Insecticides	Clothianidin	$C_6H_8ClN_5O_2S$	Acetylcholine receptor agonist	55	Low
	Pirimicarb	$C_{11}H_{18}N_4O_2$	Acetylcholinesterase inhibitor	140	Low
	Thiacloprid	$C_10H_9ClN_4S$	Acetylcholine receptor agonist	60.6	Low
Metabolites	Desamino-metamitron	$C_10H_9N_3O$	Not applicable	73.5	Low

	Metazachlor-ESA	$C_{14}H_{17}N_3SO_4$	Not applicable	93.8	Low
	Metazachlor-OA	$C_{14}H_{15}N_3O_3$	Not applicable	25.7	Low

Source: PPDB: Pesticide Properties DataBase (FOOTPRINT PPDB, Lewis et al. (2006), https://sitem.herts.ac.uk/aeru/ppdb, accessed date - 19/06/2020. *Toxicity: Classification of toxicity according to PPDB guidelines considering acute 72 h EC50 for algae (> 10 = Low; 0.01 - 10 = Moderate; < 0.01 = High)

Table S4.3: Phytoplankton traitsPhytoplankton traits, their categories and their expected responses to pesticides and nutrients stressors in this study.

Traits	Categories	Codes	Expected responses under selected stressors (pesticides and nutrients)
1. Cell size ((Abonyi et al., 2018; Kruk et al., 2017; Qu et al., 2018a; Rimet and Bouchez, 2012)	Nano (5-100 µm3)	BioVol_C1	Smaller cells have higher nutrient uptake rates and growth rates that allow greater resilience to environmental stressors making them advantage under nutrient-limiting and high disturbance conditions; Larger cells show converse trend
	Micro (100-300 µm3)	BioVol_C2	
	Meso (300-600 µm3)	BioVol_C3	
	Macro (600-1500 µm3)	BioVol_C4	
	Large (> 1500 µm3)	BioVol_C5	
2. Life form (Abonyi et al., 2018; Kruk et al., 2017; Rimet and Bouchez, 2012)	Colonial	LifFor_col	LifFor_fil has advantage in resource gathering under nutrient limited environment. LifFor_uni has advantage in high resource conditions.
	Filamentous	LifFor_fil	
	Unicellular	LifFor_uni	
	Flagellates	LifFor_fil	
3. Ecological guild (Guiry and Guiry, 2020; Rimet and Bouchez, 2012)	Low profile	LowPro	Motile and planktonic taxa have advantage in resource gathering and avoid pollutants.
	High profile	HigPro	
	Motile	MotTax	
	Planktonic	PlaTax	

3. Motility (Lange et al., 2016; Witteveen et al., 2020)	Yes (1) or No (0)	Motility	Motile taxa have ability to actively move away from pollutants
4. Spore formation (Lange et al., 2016; Witteveen et al., 2020)	Yes (1) or No (0)	SprFor	Spore forming taxa have advantage in unfavourable conditions

Fig. S4.1: Pesticide and metabolite concentrations

Pesticide and metabolite concentrations (above the LOQ: 0.001 µg/L) in monitored five lentic small water bodies (LSWB: A1 to A5) during weekly sampling from 11.04.2018 to 03.07.2018 (12 weeks: S1 to S12).

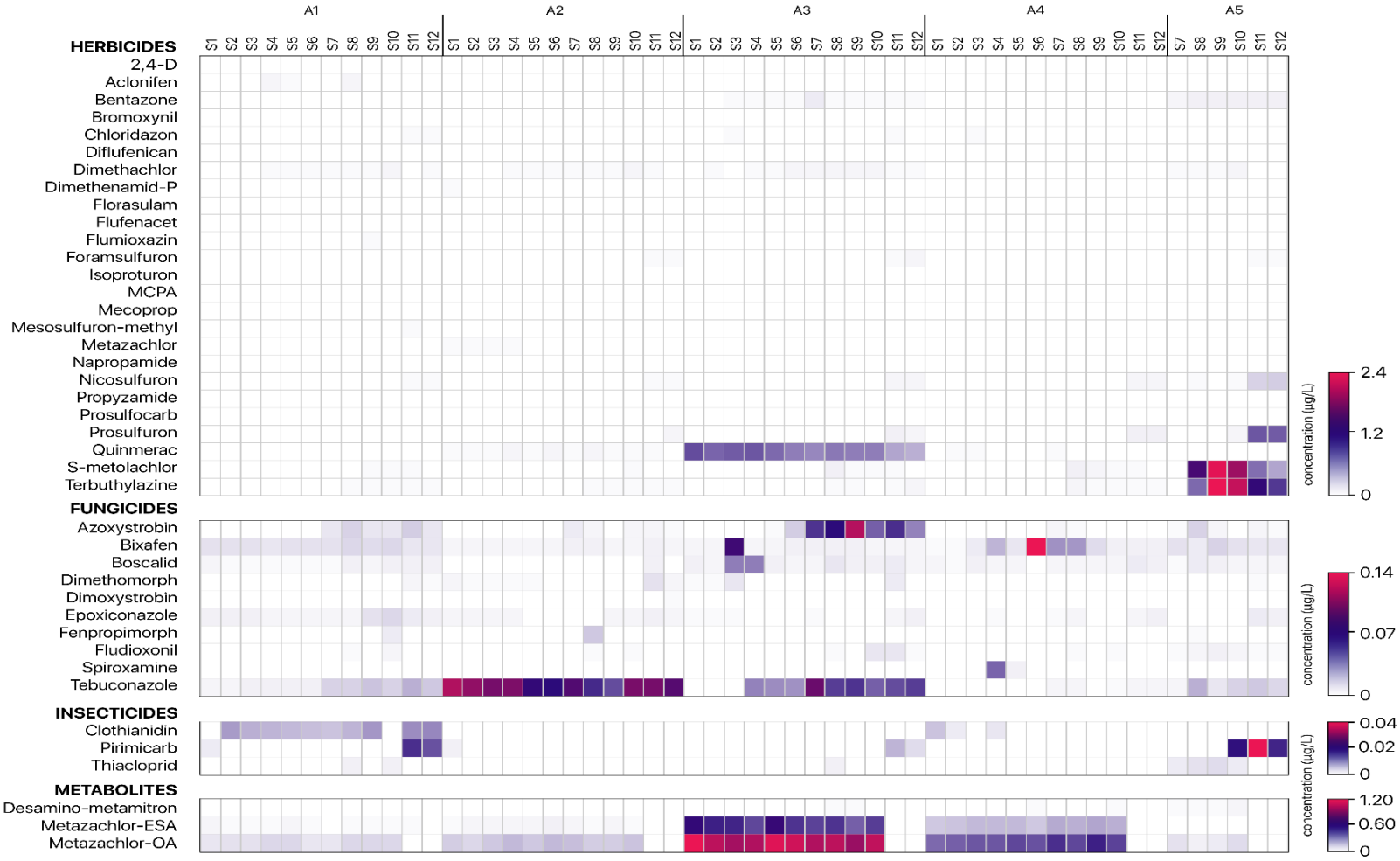
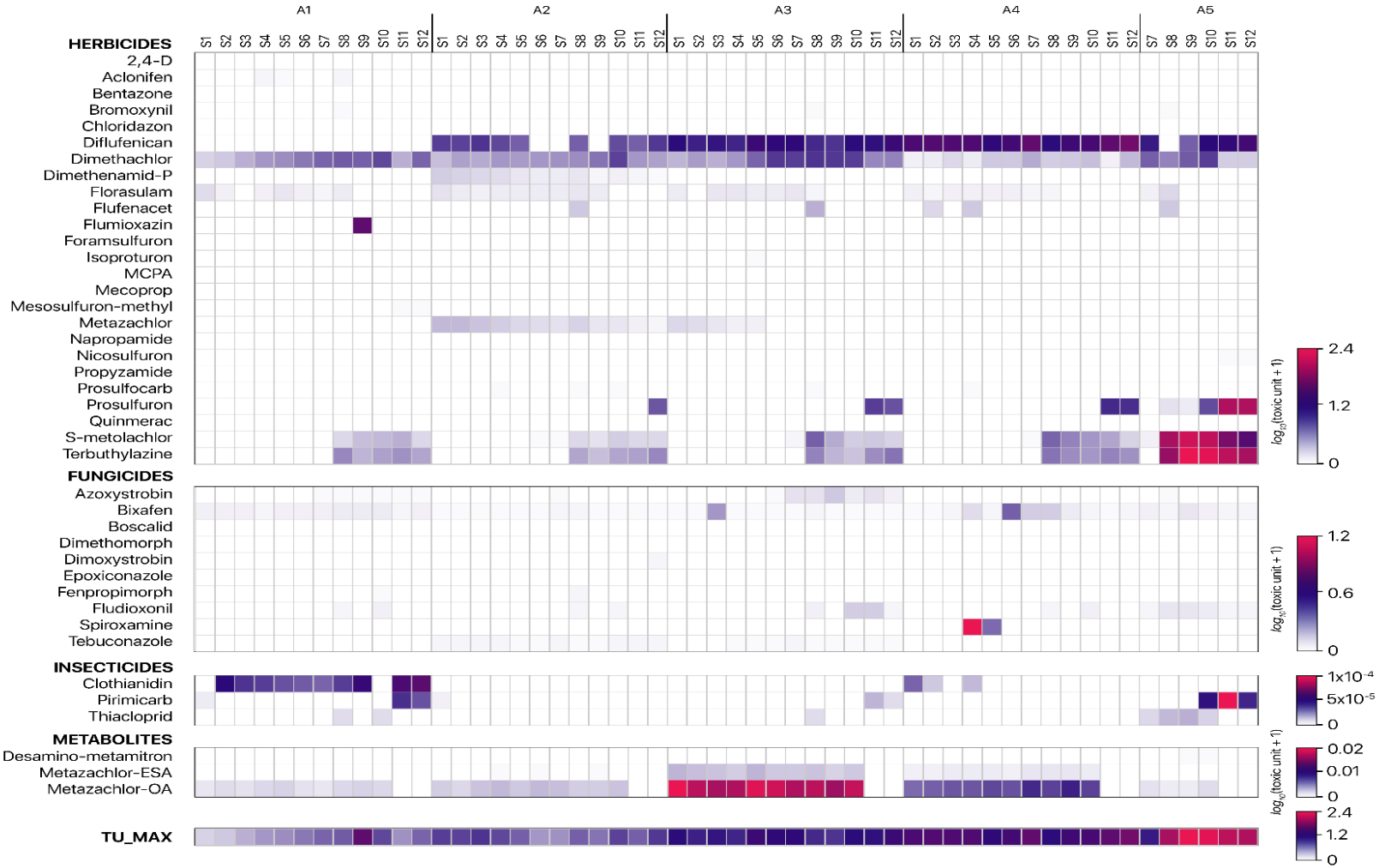


Fig. S4.2: Toxicity of pesticides and metabolites

Toxicity of pesticides and metabolites expressed as toxic units (transformed to log (x+1)) in monitored five lentic small water bodies (LSWB: A1 to A5) during weekly sampling from 11.04.2018 to 03.07.2018 (12 weeks: S1 to S12). Variation of maximum toxicity (TU_max) among samples also illustrated here.



Supplementary materials: Chapter 5

Table S5.1: Quality parameters associated with herbicides measurements

Herbicides and transformation products	Limit of Quantification [µg L^{-1}]	Repeatability (Relative standard deviation) [%]	Reproducibility [%]	Linearity [µg L^{-1}]	Recovery [%]
Metazachlor	0.005	2	8	0.005-1	91
Metazachlor-ESA	0.025	4	9	0.005-1	89
Metazachlor-OA	0.025	6	5	0.005-1	86
Flufenacet	0.005	5	5	0.005-1	123
Flufenacet-ESA	0.025	6	10	0.005-1	94
Flufenacet-OA	0.025	7	8	0.005-1	96

Table S5.2: Phytoplankton traits

Phytoplankton traits and categories in this study.

Traits	Categories	Codes
1. Biovolume	Nano (5-100 µm^3)	BioVol_C1
(Abonyi et al., 2018; Kruk et al., 2017; Qu et al., 2018a; Rimet and Bouchez, 2012)	Micro (100-300 µm^3)	BioVol_C2
	Meso (300-600 µm^3)	BioVol_C3
	Macro (600-1500 µm^3)	BioVol_C4
	Large (> 1500 µm^3)	BioVol_C5
2. Life form	Colonial	LifFor_col
(Abonyi et al., 2018; Kruk et al., 2017; Rimet and Bouchez, 2012)	Filamentous	LifFor_fil
	Unicellular	LifFor_uni
3. Ecological guild	Low profile	LowPro
(Guiry and Guiry, 2020; Rimet and Bouchez, 2012)	High profile	HigPro
	Motile	MotTax
	Planktonic	PlaTax

Table S5.3: Identified phytoplankton species in the study and their traits. The presence of the traits is represented as "1" and the absence of the traits is represented as "0". Descriptions of the codes use for traits can be found in Table S5.2.

Sp_no	Species name	BioVol_C1	BioVol_C2	BioVol_C3	BioVol_C4	BioVol_C5	LifFor_col	LifFor_fil	LifFor_uni	LowPro	HigPro	TotTax	PlaTax
Sp01	*Achnanthidium exiguum*	0	1	0	0	0	0	0	1	1	0	0	0
Sp02	*Achnanthidium minutissimum*	1	0	0	0	0	0	0	1	1	0	0	0
Sp03	*Achroonema articulatum*	0	1	0	0	0	0	1	0	0	0	0	1
Sp04	*Amphora ovalis*	0	0	0	0	1	0	0	1	1	0	0	0
Sp05	*Ankyra ancora*	0	1	0	0	0	0	0	1	0	0	0	1
Sp06	*Anomoeoneis sphaerophora*	0	0	0	0	1	0	0	1	0	0	1	0
Sp07	*Aphanocapsa elegans*	0	1	0	0	0	1	0	0	0	0	0	1
Sp08	*Aphanocapsa grevillei*	0	1	0	0	0	1	0	0	0	0	0	1
Sp09	*Brachysira serians*	0	1	0	0	0	0	0	1	1	0	0	0
Sp10	*Caloneis bacillum*	0	0	1	0	0	0	0	1	0	0	1	0
Sp11	*Chlamydomonas reinchardtii*	0	1	0	0	0	0	0	1	0	0	1	0
Sp12	*Chlorella minutissima*	1	0	0	0	0	0	0	1	0	0	0	1
Sp13	*Chloromonas angustissima*	1	0	0	0	0	0	0	1	0	0	1	0
Sp14	*Chroococcus limneticus*	0	0	0	1	0	1	0	0	0	0	0	1
Sp15	*Chroococcus vacuolatus*	0	0	1	0	0	1	0	0	0	0	0	1
Sp16	*Chrysochromulina parva*	1	0	0	0	0	0	0	1	0	0	1	0
Sp17	*Closterium ehrenbergii*	0	0	0	0	1	0	0	1	0	0	0	1
Sp18	*Cocconeis placentula*	0	0	0	0	1	0	0	1	1	0	0	0
Sp19	*Coelastrum astroideum*	0	0	0	1	0	1	0	0	0	0	0	1
Sp20	*Coenochloris helvetica*	0	1	0	0	0	1	0	0	0	0	0	1
Sp21	*Coenocystis planctonica*	0	1	0	0	0	1	0	0	0	0	0	1
Sp22	*Cosmarium carinthiacum*	0	0	0	0	1	0	0	1	0	0	0	1
Sp23	*Cosmarium trilobulatum*	0	0	0	0	1	0	0	1	0	0	0	1
Sp24	*Cryptomonas ovata*	0	0	0	0	1	0	0	1	0	0	1	0
Sp25	*Cyclotella meneghiniana*	0	0	0	1	0	0	0	1	0	0	0	1

Sp26	*Cymatopleura solea*	0	0	0	0	1	0	0	1	0	0	1	0
Sp27	*Desmodesmus opoliensis*	0	0	1	0	0	1	0	0	0	0	0	1
Sp28	*Diatoma vulgaris*	0	0	0	0	1	1	0	0	0	1	0	0
Sp29	*Dictosphaerium ehrenbergainum*	0	0	1	0	0	1	0	0	0	0	0	1
Sp30	*Diplostauron elegans*	0	1	0	0	0	0	0	1	0	0	1	0
Sp31	*Euglenaria clavata*	0	0	0	0	1	0	0	1	0	0	1	0
Sp32	*Euglenaformis proxima*	0	0	0	0	1	0	0	1	0	0	1	0
Sp33	*Euglena viridis*	0	0	0	0	1	0	0	1	0	0	1	0
Sp34	*Eunotia bilunaris*	0	0	0	1	0	0	0	1	0	1	0	0
Sp35	*Eunotia minor*	0	0	0	1	0	0	0	1	0	1	0	0
Sp36	*Eutetramorus planctonicus*	0	0	0	0	1	1	0	0	0	0	0	1
Sp37	*Eutetramorus polycoccus*	0	1	0	0	0	1	0	0	0	0	0	1
Sp38	*Fragilaria capucina*	0	1	0	0	0	1	0	0	0	1	0	0
Sp39	*Fragilaria gracilis*	1	0	0	0	0	1	0	0	0	0	0	1
Sp40	*Fragilaria mesolepta*	0	1	0	0	0	1	0	0	0	1	0	0
Sp41	*Fragilaria pararumpens*	0	0	1	0	0	1	0	0	0	1	0	0
Sp42	*Geminella terricola*	0	1	0	0	0	0	1	0	0	0	0	1
Sp43	*Gomphonema affine*	0	0	0	1	0	0	0	1	0	1	0	0
Sp44	*Gomphonema acidoclinatum*	0	0	1	0	0	0	0	1	0	1	0	0
Sp45	*Gomphonema acuminatum*	0	0	0	0	1	1	0	0	0	1	0	0
Sp46	*Gomphonema angusticephalum*	0	0	1	0	0	0	0	1	0	1	0	0
Sp47	*Gomphonema parvulum*	0	0	1	0	0	0	0	1	0	1	0	0
Sp48	*Gomphonema truncatum*	0	0	0	1	0	1	0	0	0	1	0	0
Sp49	*Hippodonta capitata*	0	0	1	0	0	0	0	1	0	0	1	0
Sp50	*Jaaginema geminata*	1	0	0	0	0	0	1	0	0	0	0	1
Sp51	*Kirchneriella obesa*	0	0	1	0	0	1	0	0	0	0	0	1
Sp52	*Koliella longiseta*	0	1	0	0	0	0	0	1	0	0	0	1
Sp53	*Koliella sempervirens*	0	1	0	0	0	0	0	1	0	0	0	1
Sp54	*Lemnicola hungarica*	0	0	1	0	0	0	0	1	1	0	0	0
Sp55	*Lepocinclis acus*	0	0	0	0	1	0	0	1	0	0	1	0

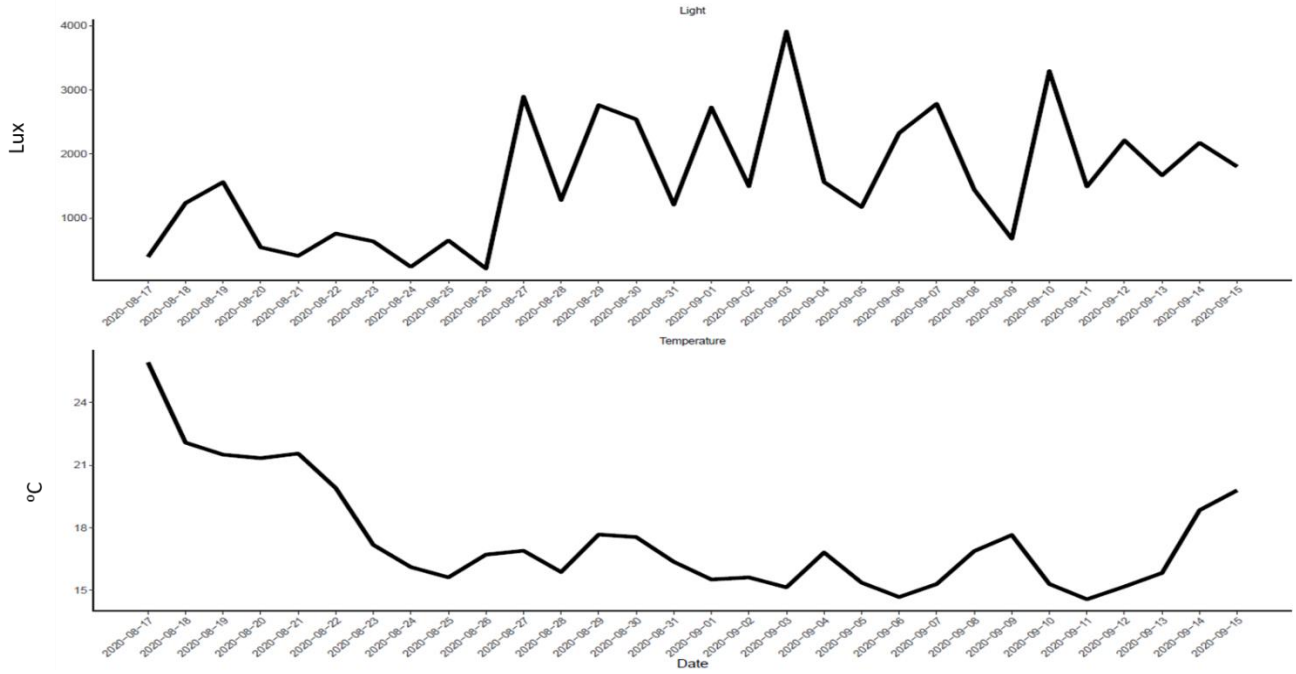
Sp56	*Lepocinclis marssonii*	0	0	0	0	1	0	0	1	0	0	1	0
Sp57	*Lepocinclis ovum*	0	1	0	0	1	0	0	1	0	0	1	0
Sp58	*Lepocinclis texta*	0	0	0	0	1	0	0	1	0	0	1	0
Sp59	*Melosira varians*	0	0	0	0	1	0	1	0	0	1	0	0
Sp60	*Meridion circulare*	0	0	0	1	0	1	0	0	1	0	0	0
Sp61	*Merismopedia elegans*	0	1	0	0	0	1	0	0	0	0	0	1
Sp62	*Merismopedia tenuissima*	1	0	0	0	0	1	0	0	0	0	0	1
Sp63	*Microcystis flos-aquae*	0	0	0	0	1	1	0	0	0	0	0	1
Sp64	*Microspora floccosa*	0	0	0	0	1	0	1	0	0	0	0	1
Sp65	*Monomorphina pyrum*	0	0	0	0	1	0	0	1	0	0	1	0
Sp66	*Monoraphidium contortum*	1	0	0	0	0	0	0	1	0	0	0	1
Sp67	*Monoraphidium griffithii*	0	0	1	0	0	0	0	1	0	0	0	1
Sp68	*Navicula cincta*	0	0	1	0	0	0	0	1	0	0	1	0
Sp69	*Navicula lanceolata*	0	0	0	1	0	0	0	1	0	0	1	0
Sp70	*Neglectella solitaria*	0	1	0	0	0	1	0	0	0	0	0	1
Sp71	*Nitzschia fonticola*	0	0	1	0	0	0	0	1	0	0	1	0
Sp72	*Oocystidium polymammilatum*	0	0	0	0	1	0	0	1	0	0	0	1
Sp73	*Oocystis borgei*	0	0	0	0	1	1	0	0	0	0	0	1
Sp74	*Oocystis lacustris*	0	0	1	0	0	1	0	0	0	0	0	1
Sp75	*Parapediastrum biradiatum*	0	0	0	1	0	1	0	0	0	0	0	1
Sp76	*Pediastrum boryanum*	0	0	0	1	0	1	0	0	0	0	0	1
Sp77	*Pediastrum duplex*	0	0	0	1	0	1	0	0	0	0	0	1
Sp78	*Pediastrum tetras*	0	0	0	1	0	1	0	0	0	0	0	1
Sp79	*Peridiniopsis cunningtonii*	0	0	0	0	1	0	0	1	0	0	1	0
Sp80	*Peridinium willei*	0	0	0	0	1	0	0	1	0	0	1	0
Sp81	*Phacus monilatus*	0	0	0	0	1	0	0	1	0	0	1	0
Sp82	*Phacus helicoides*	0	0	0	0	1	0	0	1	0	0	1	0
Sp83	*Phacus longicauda*	0	0	0	0	1	0	0	1	0	0	1	0
Sp84	*Phacus orbicularis*	0	0	0	0	1	0	0	1	0	0	1	0
Sp85	*Phacus parvulus*	0	0	0	0	1	0	0	1	0	0	1	0
Sp86	*Phormidium tenue*	1	0	0	0	0	0	1	0	0	0	0	1

Sp87	*Pinnularia subcapitata*	0	0	1	0	0	0	0	1	0	0	1	0
Sp88	*Pinnularia viridis*	0	0	0	0	1	0	0	1	0	0	1	0
Sp89	*Planctonema lauterbornii*	0	0	0	1	0	0	1	0	0	0	0	1
Sp90	*Planothidium frequentissimum*	0	1	0	0	0	0	0	1	1	0	0	0
Sp91	*Planothidium lanceolatum*	0	0	1	0	0	0	0	1	1	0	0	0
Sp92	*Polyedriopsis spinulosa*	0	0	1	0	0	0	0	1	0	0	0	1
Sp93	*Pseudanabaena minima*	0	1	0	0	0	0	1	0	0	0	0	1
Sp94	*Psudodidymocystis planctonica*	0	1	0	0	0	1	0	0	0	0	0	1
Sp95	*Pteromonas cordiformis*	0	0	0	0	1	0	0	1	0	0	1	0
Sp96	*Rhodomonas lacustris* var. *nannoplanctica*	0	0	1	0	0	0	0	1	0	0	1	0
Sp97	*Comasiella arcuata*	1	0	0	0	0	1	0	0	0	0	0	1
Sp98	*Tetradesmus obliquus*	1	0	0	0	0	1	0	0	0	0	0	1
Sp99	*Selenastrum bibraianum*	0	0	0	0	1	1	0	0	0	0	0	1
Sp100	*Sellaphora pupula*	0	0	0	1	0	0	0	1	0	0	1	0
Sp101	*Senedesmus acuminatus*	0	0	0	1	0	1	0	0	0	0	0	1
Sp102	*Spirulina major*	1	0	0	0	0	0	1	0	0	0	0	1
Sp103	*Staurastrum gracile*	0	0	0	0	1	0	0	1	0	0	0	1
Sp104	*Staurastrum tetracerum*	0	1	0	0	0	0	0	1	0	0	0	1
Sp105	*Stauroneis gracilis*	0	0	0	1	0	0	0	1	0	0	1	0
Sp106	*Stichococcus minor*	0	0	0	0	0	0	1	0	0	0	0	1
Sp107	*Strombomonas acuminata*	0	0	0	1	0	0	0	1	0	0	1	0
Sp108	*Strombomonas borysteniensis*	0	0	0	0	1	0	0	1	0	0	1	0
Sp109	*Surirella angusta*	0	0	0	1	0	0	0	1	0	0	1	0
Sp110	*Synechococcus nidulans*	1	0	0	0	0	0	0	1	0	0	0	1
Sp111	*Synura globosa*	0	0	0	1	0	1	0	0	0	0	1	0
Sp112	*Tabularia fasciculata*	0	0	1	0	0	0	0	1	0	1	0	0
Sp113	*Tetraedron minimum*	0	1	0	0	0	0	0	1	0	0	0	1
Sp114	*Tetrastrum komarekii*	1	0	0	0	0	1	0	0	0	0	0	1
Sp115	*Trachelomonas armata*	0	0	0	0	1	0	0	1	0	0	1	0
Sp116	*Trachelomonas hispida*	0	0	0	0	1	0	0	1	0	0	1	0

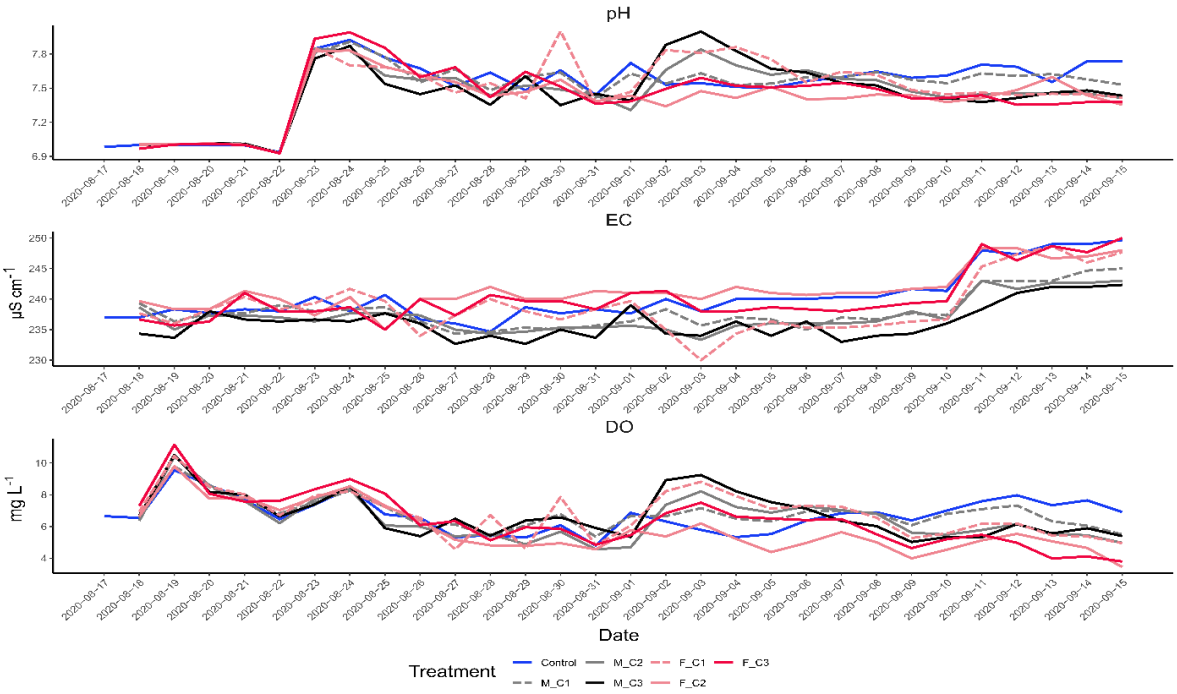
Sp117	*Trachelomonas intermedia*	0	0	0	0	1	0	0	1	0	0	1	0
Sp118	*Trachelomonas planctonica*	0	0	0	0	1	0	0	1	0	0	1	0
Sp119	*Trachelomonas planctonica* var. *hyalina*	0	0	0	0	1	0	0	1	0	0	1	0
Sp120	*Trachelomonas volvocina*	0	0	0	0	1	0	0	1	0	0	1	0
Sp121	*Trachelomonas volvocinopsis*	0	0	0	0	1	0	0	1	0	0	1	0
Sp122	*Treubaria plantonica*	0	0	0	1	0	0	0	1	0	0	0	1
Sp123	*Treubaria schmidlei*	0	1	0	0	0	0	0	1	0	0	0	1
Sp124	*Ulnaria acus*	0	0	0	0	1	0	0	1	0	1	0	0
Sp125	*Ulnaria ulna*	0	0	0	0	1	1	0	0	0	1	0	0
Sp126	*Uronema confervicolum*	0	0	0	0	0	0	0	0	0	0	0	0
Sp127	*Woronichinia obtusa*	0	0	0	1	0	1	0	0	0	0	0	1
Sp128	*Chrooceoccus turgidus*	0	0	0	0	1	1	0	0	0	0	0	1
Sp129	*Phacus caudatus*	0	0	0	0	1	0	0	1	0	0	1	0
Sp130	*Cryptomonas curvata*	0	0	1	0	0	0	0	1	0	0	1	0
Sp131	*Chromulina ovalis*	1	0	0	0	0	0	0	1	0	0	1	0
Sp132	*Tribonema vulgare*	0	0	0	1	0	0	1	0	0	0	0	1
Sp133	*Sorastrum spinulosum*	0	0	1	0	0	1	0	0	0	0	0	1
Sp134	*Lagerheimia ciliata*	0	1	0	0	0	0	0	1	0	0	1	0
Sp135	*Mougeotia* sp.	0	0	0	0	1	0	1	0	0	0	0	1
Sp136	*Dolichospermum flos-aquae*	0	1	0	0	0	0	1	0	0	0	0	1

Fig. S5.1: Temporal changes of physicochemical parameters during the study 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).

Light and Temperature



pH, Conductivity (EC) and Dissolved oxygen (DO) – Mean values



Nutrients

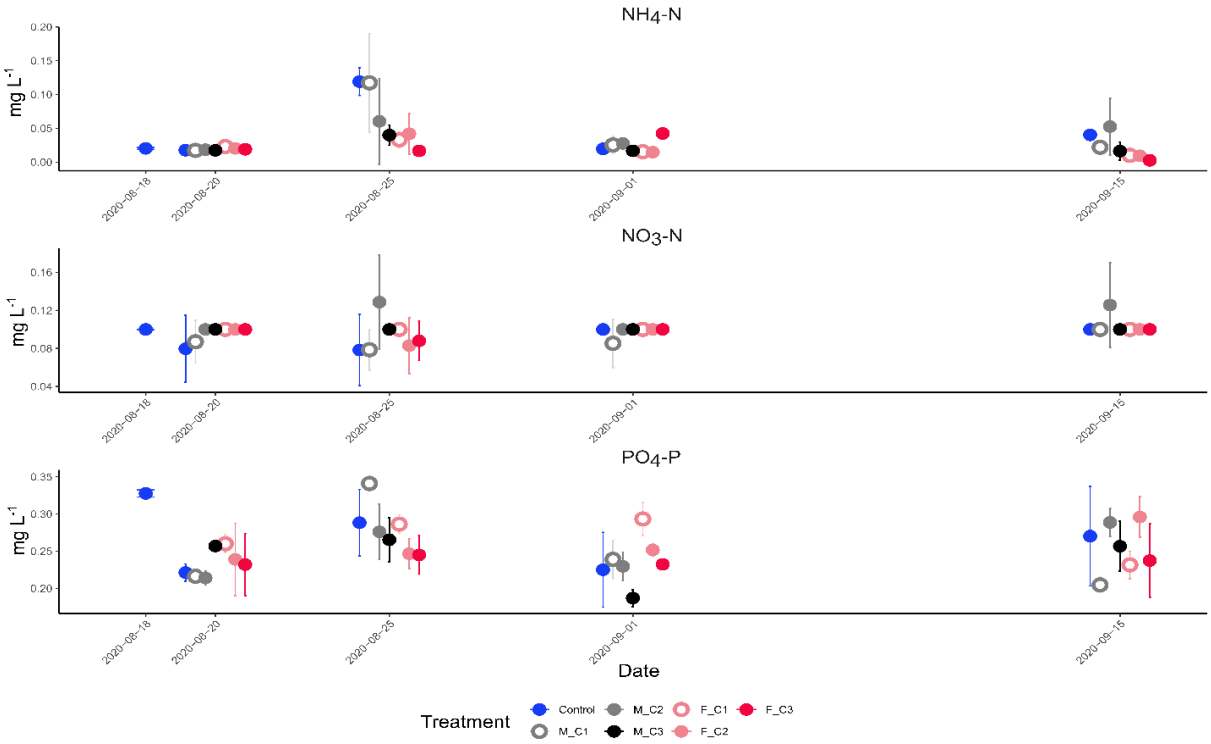


Fig. S5.2: Changes of the phytoplankton density (A), diatom density (B), and diatom-to-phytoplankton ratio (C) across different treatments over the herbicide exposure time. Treatments are represented as control and respective concentrations (C1: 0.5 µg L^{-1}; C2: 5 µg L^{-1}; C3: 50 µg L^{-1}) of exposed herbicides (M: Metazachlor and F: Flufenacet; see legend). Sampling times reported as S0: Before exposure; S1: 48h after exposure; S2: 1 week after exposure; S3: 2 weeks after exposure; S4: 4 weeks after exposure. Dispersion bars denote standard deviation.

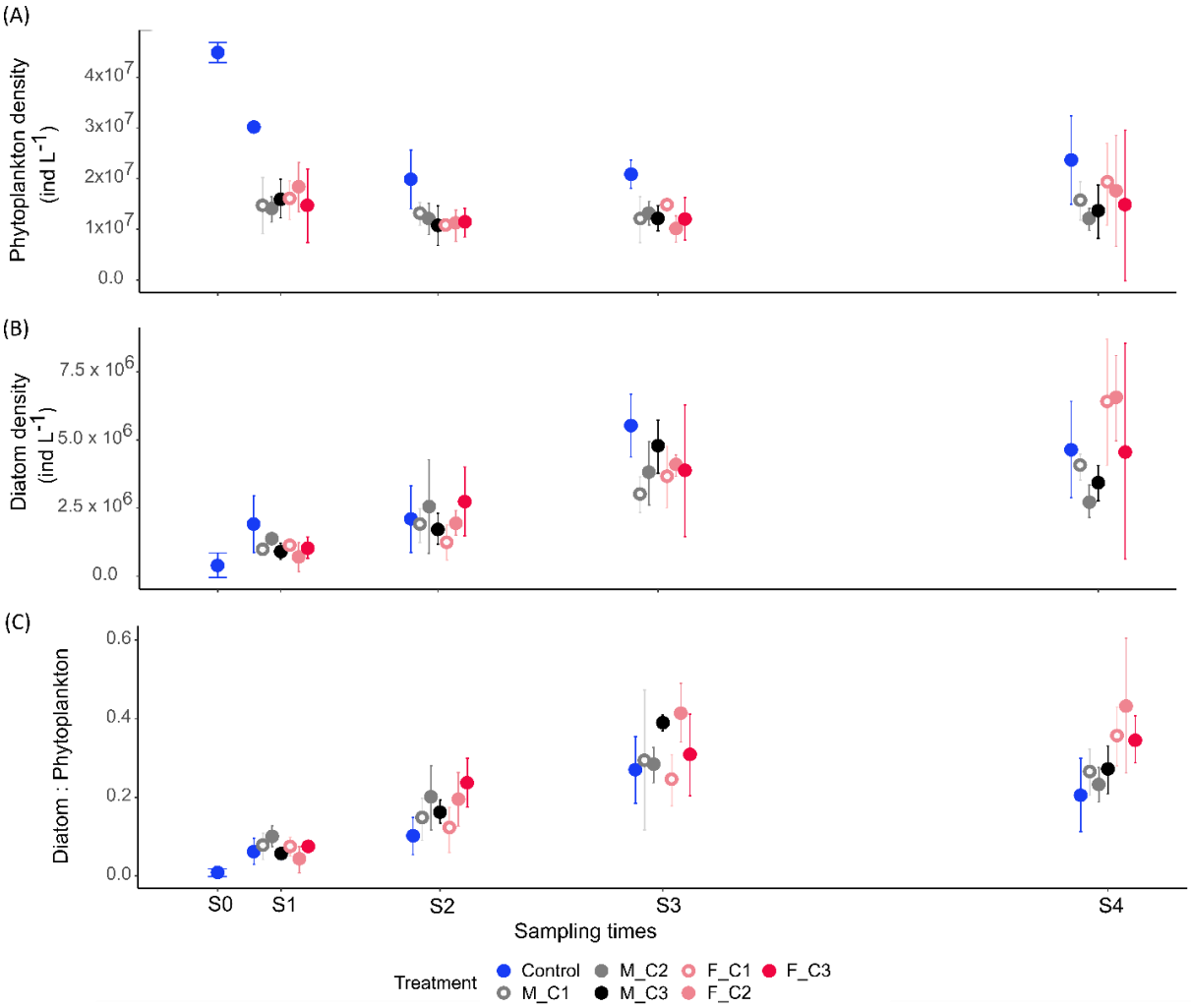


Fig. S5.3: Density changes of the strongly affected phytoplankton species identified in PRC analysis (species weight > 0.1 and species weight < -0.1) during the study period. Treatments are represented as control and respective concentrations (C1: 0.5 µg L^{-1}; C2: 5 µg L^{-1}; C3: 50 µg L^{-1}) of exposed herbicides (M: Metazachlor and F: Flufenacet; see legend). Dispersion bars denote standard deviation. Sampling times reported as S0: Before exposure; S1: 48h after exposure; S2: 1 week after exposure; S3: 2 weeks after exposure; S4: 4 weeks after exposure. Dispersion bars denote standard deviation.

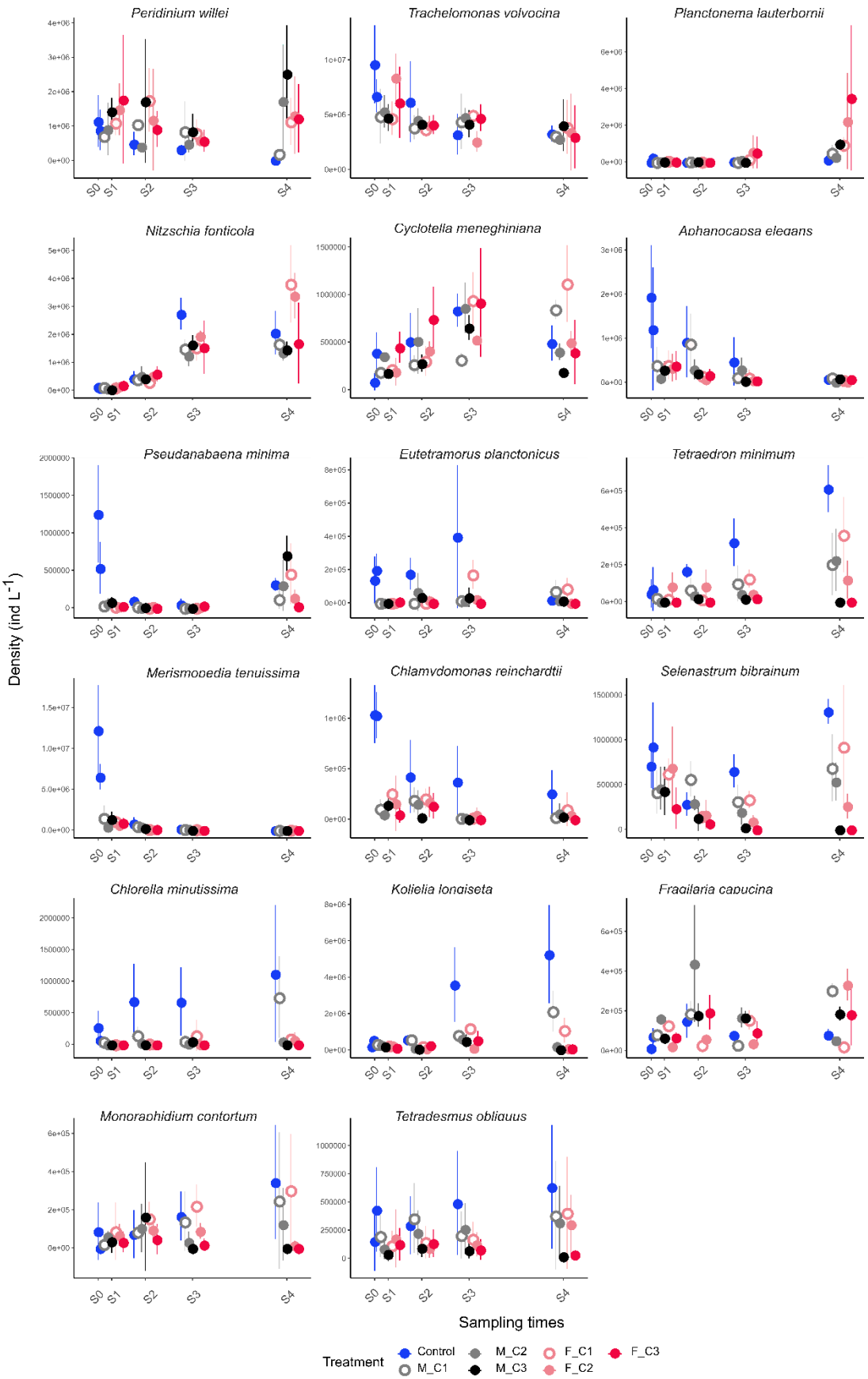
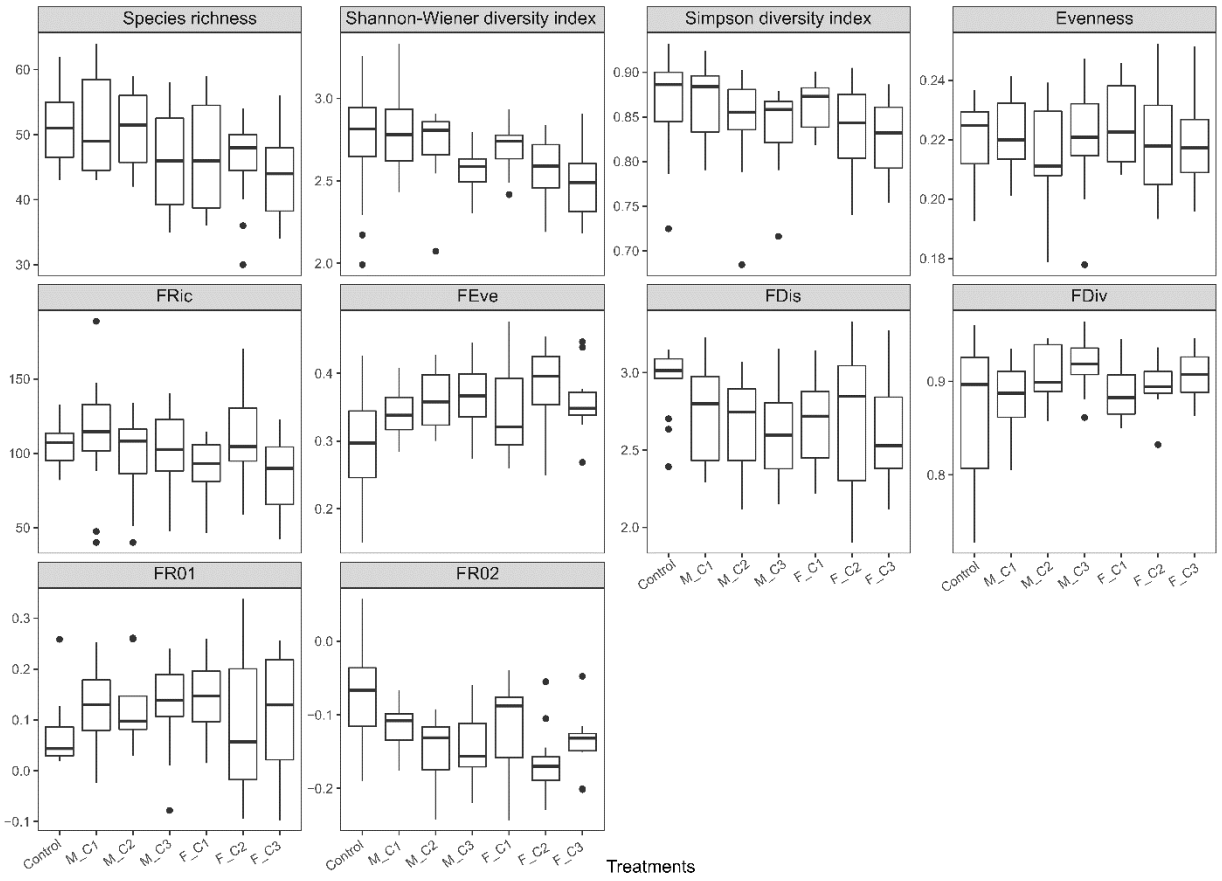

Peridinium willei
Trachelomonas volvocina
Planctonema lauterbornii
Nitzschia fonticola
Cyclotella meneghiniana
Aphanocapsa elegans
Pseudanabaena minima
Eutetramorus planctonicus
Tetraedron minimum
Merismopedia tenuissima
Chlamydomonas reinchardtii
Selenastrum bibrainum
Chlorella minutissima
Kolielia longiseta
Fragilaria capucina
Monoraphidium contortum
Tetradesmus obliquus
Density (ind L-1)
S0
S1
S2
S3
S4
Sampling times
Treatment
Control
M_C1
M_C2
M_C3
F_C1
F_C2
F_C3

Fig. S5.4: Taxonomic diversity indices and functional features of the phytoplankton community at 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). Dispersion bars denote standard deviation.



Declaration

I, Lishani Nisansala Wijewardene, hereby declare that the dissertation submitted, entitled "Impacts of multiple stressors on algal communities in freshwater ecosystems in rural areas" was written independently by me. The content and design of this thesis, apart from the supervisor's guidance, is my own work. The thesis has not been submitted either partially or wholly as a part of a doctoral degree to another examining body and is my first and only doctoral procedure. Chapter 2, 3, 4 and 5 of the thesis have been published in peer-reviewed journals. This work has been prepared respecting the Rules of Good Scientific Practice of the German Research Foundation. I have not been deprived of an academic degree.

Kiel, January-2022

Lishani Nisansala Wijewardene