Functional and compositional responses of stream microphytobenthic communities to multiple stressors increase and decrease

Inaugural
Dissertation for
the doctoral degree of
Dr. rer. nat.

from the Faculty of Biology
University of Duisburg-
Essen Germany

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Date of submission July 2024

Information on the Examination

This work was carried out within the framework of the SFB-RESIST 1439/1 founded by the Deutsche Forschungsgemeinschaft (DFG). All experiments in this work were conducted at the Faculty of Biology, Phycology Working Group at the University of Duisburg-Essen.

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Date of the oral examination: 30.10.2024

DOI: 10.17185/duepublico/82680
URN: urn:nbn:de:hbz:465-20241209-100131-8

List of abbreviations

ARC: Asymmetric Response Concept

WFD: Water Framework Directive

DNA: Deoxyribonucleic Acid

FPOM: fine particulate organic matter

OTU: Operational Taxonomic Unit

EPT: Ephemeroptera, Plecoptera and Trichopptera

AIC: Akaike's Information Criterion

ANOVA: Analysis of Variance

PERMANOVA: Permutational Multivariate Analysis of Variance

GLM: Generalised Linear Model

WWTP: Wastewater Treatment Plant

F0: Minimum fluorescence

Fm: Maximum fluorescence

PSII or Y(II) or Fv/Fm: Maximum quantum yield

ANCOVA: Analysis of covariance

NMDS: Non-metric multidimensional scaling

PCA : Principal correspondance analysis

RDA: Redundancy Analysis

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Summary

Climate change alongside existing chemical pollution and physical habitat disturbances affects inland rivers through a combination of multiple stressors. Microalgal communities of freshwater ecosystems have been found to respond to them, based on scarce field observations. However, natural rivers are not suitable for full-scale experimental trials, thus, the use of mesocosms can be used to understand how climate and anthropogenic stressors affect microalgae within the whole trophic network context. In this thesis I have straddled both scales, looking into four main subjects:

1) the effect of a salinity gradient and flow variation on the microphytobenthic community of an ExStream mesocosm within the urban Boye catchment,

2) the responses of benthic diatom communities (using digital microscopy and DNA metabarcoding) alongside photosynthesis-related traits in microphytobenthic communities in response to stressors increase and release (e.g. flow velocity, salinity, and temperature) in a large-scale ExStream mesocosm,

3) the responses of microalgae communities and a photosynthetic biomass proxy to multiple environmental stressors in the less impacted Kinzig River catchment and

4) how the restoration affects the ecosystems in general and the diatoms in particular in the urban Boye catchment.

In the mesocosm studies, results presented here illustrate how sensitive microalgae react to even minor changes in these environmental variables, particularly flow velocity and temperature. In the less degraded Kinzig catchment, results highlighted the complex relationship between various environmental variables and microalgae biomass and community composition. In contrast the freshly restored catchment of the Boye, characterized by natural to recently restored rivers, is urban and as such affected by anthropogenic pressures, showing increased degrees of degradation both in the water and the microphytobenthic community. Furthermore, this study showed no significant differences in composition according to time since restoration, which could relate to the quick generation time of the microphytobenthic community. In conclusion, the degree of degradation of the catchment will determine the responses of microalgae of the microphytobenthos to multiple stressors through all scales of study.

Zusammenfassung

Der Klimawandel, zusammen mit chemischer Verschmutzung und physikalischen Veränderungen, kann Flüsse durch den Effekt von multiplen Stressoren verändern. Gemäß der begrenzten Anzahl von Veröffentlichungen zu diesem Thema können sich die darin lebenden Mikroalgengemeinschaften darauf anpassen, wie aus Felduntersuchungen hervorgeht. Jedoch können Flüsse nicht für großangelegte experimentelle Versuche genutzt werden, wie Mesokosmen für die Effekte von klimatischen und anthropogenen Stressoren auf Mikroalgen in ihrem trophischen Netzwerk zum Verständnis genutzt werden können. In dieser Dissertation habe ich mich mit beiden Methoden, Feldproben und Mesokosmos-Experimenten befasst und habe dabei die folgenden Themen erschlossen:

1) Wie Salinitätsgradienten auf die mikrophytobenthische Gemeinschaft, im Kontext des Ökosystems in ExStream-Mesokosmos im stark anthropisch veränderten Boye Einzugsgebiet, einwirken.

2) Wie die in einem Mesokosmos-Experiment herausgefundenen multiplen Stressoren (Salzgehalt, Fließgeschwindigkeit und Temperatur) auf die Gemeinschaften und die photosynthetischen Traits der Mikroalgen des Mikrophytobenthos einwirken.

3) Die feldbasierten Erkenntnisse der multiplen Stressoren des weniger anthropisch veränderten Kinzig-Einzugsgebiets auf die Mikroalgen des Mikrophytobenthos und einen photosynthetischen Proxy

4) Wie die Renaturierung auf das Ökosystem und die Diatomeen im urbanen Boye-Einzugsgebiet wirkt.

In den Mesokosmosexperimenten wurde herausgefunden, dass die Mikroalgen im Mikrophytobenthos vor allem auf die Veränderungen in der Fließgeschwindigkeit und der Temperatur reagiert haben. Die Feldstudien der Kinzig ergaben, dass durch die geringe Degradierung des Einzugsgebietes die Mikroalgen und deren Biomasse im Mikrophytobenthos sehr stark von den Umweltfaktoren verändert wurden. Hingegen wurden im renaturierten Boye-Einzugsgebiet keine klaren gemeinschaftlichen Veränderungen festgestellt, weder durch die Zeit nach der Renaturierung noch durch Umweltfaktoren. Dadurch lässt sich schließen, dass

der Grad der Degradierung eines Einzugsgebiets die Mikroalgengemeinschaft im Mikrophytobenthos und ihre Reaktion auf Multiple Stressoren stark beeinflusst.

Introduction

1.1 Freshwater ecosystems in a changing environment

Freshwater ecosystems encompass lakes, rivers, streams and wetlands which contain 100,000 km^3 worldwide, making up less than 0.01% of all water on Earth (Jackson et al., 2001; Korenaga et al., 2017). They are vital to both human society and the natural world, supporting biodiversity and providing essential ecosystem services necessary for human well-being, such as provisioning (e.g. water supply for domestic, agricultural or industrial use, food processing, etc.), regulating (e.g. recharge of ground waters, flood control, etc.), supporting (e.g. trapping of sediments and pollutants, nutrient cycling, photosynthesis primary production, etc.) and cultural (e.g. aesthetic, recreational, educational, spiritual, etc.) services (B-Béres et al., 2023; Millennium Ecosystem Assessment, 2005).

However, since water is essential for domestic purposes, agriculture, energy production and industrial activities, the demand for water is expected to increase with the growth of the human population and the development of industry and agriculture, leading to a reduction in **quantity** (Vörösmarty et al., 2010; Zimmerman et al., 2008). Furthermore, water **quality** degradation has been documented because most freshwater ecosystems are exposed to multiple "anthropogenic stressors" (Alric et al., 2021; Castro-Català et al., 2020; Feld et al., 2016; Herrero et al., 2018; Nelson et al., 2009; Schürings et al., 2022) and is a predictable result given the current global change circumstances. The term "stressor" is often used interchangeably with "pollution", "pollutants" or "pressures" in ecological research because it is assumed that stressors are inherently harmful due to their association with "stress" (Folt et al., 1999; Piggott et al., 2015b). However, "stressor" has also sometimes been used interchangeably with "disturbance", although disturbance is a broader concept with a long history in ecology (Pickett and White, 1985; Sabater et al., 2018). While disturbances can be either natural or anthropogenic events that disrupt the ecosystem, community, or population structure (Resh et al., 1988; Sabater et al., 2018), stressors here refer specifically to anthropogenic disturbances that affect environmental variables, individuals, and cause populations, communities or ecosystem functions to exceed the range of normal variation compared to undisturbed reference conditions (Piggott et al., 2015b; Vos et al., 2023).

Anthropogenic stressors are broadly classified into chemical, physical, and biological types. Whereas chemical stressors include pollutants such as heavy metals, pesticides and nutrients (Schäfer et al., 2023; Schürings et al., 2022), physical stressors may include habitat modification, hydrological changes, fluctuations in temperature and precipitation patterns as a result of climate change, etc. (Fanelli et al., 2022). Biological stressors can include the introduction of invasive species, excessive use of aquatic resources (e.g. overfishing), spreading pathogens, etc. (Tockner et al., 2010).

At the local scale, land use changes such as deforestation and intensification of crop production have resulted to cropland and pasture becoming one of the world's predominant land use types, occupying nearly 50% of the planet's habitable land area (Schürings et al., 2022). According to Feld et al. (2016) changes in land use produce “cocktails of stressors”, like when agriculture affects aquatic biodiversity through diffuse pollution, siltation, and in-stream habitat degradation. Surface runoff from agricultural fields introduces nutrients into the rivers, which may lead to eutrophication, habitat loss, and severe ecological changes (Dahm et al., 2013). Agriculture is a major source of chemical stressors, e.g. excess nutrients and pesticides, which are actively transported into rivers and streams from adjacent agricultural lands by storm runoff after heavy rainfall events posing a significant threat to freshwater ecosystems and the services they provide.

On the other hand, increasing urbanization leads to the so-called wastewater production and pollution effect, which depends largely on the quality of treated and untreated industrial and municipal wastewater discharged into rivers and streams on a daily basis. Especially in densely populated areas, urban wastewater could be a significant point source of salt ions, nutrients and micropollutants such as pharmaceutical residues, microplastics and pesticides used in public or private facilities (Markert et al., 2024a). This leads to the degradation of water quality, habitat destruction and loss of biodiversity (Meyer et al., 2005). Urbanization also has significant impacts on water flows, it is also associated with discharge of wastewater at different points and erected barriers (e.g. dams) that impede the migration of plants and animals. Excessive use of key freshwater resources particularly in urban areas has altered the natural flow of many rivers and streams (Sabater et al., 2018). Habitat modification can make ecosystems more vulnerable to invasive species, which can outcompete or displace native species and alter food webs. Urbanization and climate change contribute to habitat fragmentation and altered hydrological regimes, further putting pressure on the ecosystem (Sabater et al., 2018; Szczepocka et al., 2021). In addition, industrial pollution, the introduction of invasive species, and climate change interact to result in significant ecological changes.

Besides local stressors, global stressors such as human induced climate change and its associated stressors, namely warming temperature and hydrological changes, also have a direct and significant impact on the quality and quantity of freshwater (Zimmerman et al., 2008). Climate change impacts habitat quality, species thermal tolerances, rates of ecosystem processes, and phenological cues, creating an urgent need for researchers to quantify these drivers and responses in ecologically relevant ways (Johnson and Penaluna, 2019).

It is challenging to decipher the effects of anthropogenic stressors in real-world scenarios. Due to the high frequencies of natural disturbances, it is difficult to identify and assess the impacts of anthropogenic stressors on riverine communities and ecosystems. A variety of anthropogenic and natural disturbances are nested at different temporal and spatial extents and interact to influence freshwater organisms (Sabater et al., 2018). For example, at the continental level, large-scale natural differences in climate and biogeography determine the distribution of fish in European lakes. Locally, however, fish distribution is driven by eutrophication, which is a consequence of both natural and human factors (Sabater et al., 2018). Therefore, it is difficult to distinguish between the effects of human-caused and natural disturbances on ecosystems (Feld et al., 2016). Thus, research should consider the effects of a specific set of stressors at different levels to accurately assess their impacts on freshwater ecosystems.

1.2 Multiple stressor effects in freshwater ecosystems

Anthropogenic activities contribute an important amount of nutrients loading into freshwater ecosystems, leading to eutrophication, which results in oxygen depletion. For example, eutrophication combined with increasing temperature favour algal blooms more than either stressor alone. These blooms reduce oxygen levels in water bodies, creating “dead zones” where aquatic life cannot survive. Algae use photosynthesis during the day to produce oxygen, which is good for the ecosystem. On the other hand, algae continue to breathe at night, using oxygen as fuel. Particularly in bloom areas with high population densities, this respiration can reduce oxygen levels. Dense blooms can also limit the amount of light that reaches the water, which will hinder algae and other aquatic plants from photosynthesizing (Somogyi et al., 2024). Because dissolved oxygen is essential to aquatic organisms' health, especially in slow-moving or stagnant waters with limited oxygen exchange, the combined effects of reduced light and respiration during the night can cause variations in dissolved oxygen levels.

Nutrient loading persists as a major stressor for many streams and rivers in Europe despite advancements in wastewater treatment technologies (Birk et al., 2020; Castro-Català et al., 2020; Feld et al., 2016; Lemm et al., 2021; Markert et al., 2024a, 2024b). Nõges et al., (2016) found that nutrient loading and hydrological alterations, either alone or in combination with additional stressors such as salt ions, were the most pervasive stressors in many rivers in Europe. Furthermore, numerous studies have provided thorough documentation of the effects of hydrological and morphological alterations on riverine communities (Hering et al., 2015; Markert et al., 2024b; Nõges et al., 2016). Several studies suggested that most rivers and streams in Europe suffer from the impact of multiple stressors, which pose serious threats to biota and constitute major challenges for freshwater ecosystem management (Alric et al., 2021; Castro-Català et al., 2020; Nõges et al., 2016; Schürings et al., 2024). Recent reports indicate that around 60% of European rivers do not meet ecological quality standards derived from biological criteria, mainly due to hydromorphological and water quality stressors (Markert et al., 2024b). Degradation in water quality remains a significant stressor for organisms (e.g. macroinvertebrates, diatoms and fish) in German rivers.

A decline in biodiversity can affect ecosystem functioning. Functional traits serve as proxies for adaptive strategies under specific environmental conditions and can be considered life strategies (Tapolczai et al., 2016). Organisms with similar life strategies that occupy analogous niches can be grouped into ecological or functional groups (Tapolczai et al., 2016). The functional roles that individual species play determine the effects of species loss on ecosystem functioning. For example, the phenotypic traits of an organism can influence how an ecosystem operates (Castro-Català et al., 2020). Therefore, ecosystem functions may decline due to the extinction of species that play important functional roles (Cardinale et al., 2012). However, the relationship between biodiversity and ecosystem functioning is complex and depends on various factors, including species interactions, community structure, rates of extinction, individual species characteristics, and environmental context. While more research and methodological development are needed, trait-based approaches in microalgae ecology are a useful tool for improving the analysis of environmental variability and advancing our understanding of multiple stressors impacts in aquatic ecosystems (B-Béres et al., 2024).

Predicting changes in biodiversity and ecosystem function is challenging yet crucial due to these interactions (Côté et al., 2016). Therefore, research focusing on multiple stressors integrating theory and application is a key area of interest in ecological studies. Additionally, there is a growing emphasis on understanding patterns of ecosystem recovery following stressor release (Vos et al., 2023). A key concept in ecosystem restoration is the return time, e.g. the rate at which recovery occurs following disruption from a stressor (Vos et al., 2023). Despite this, a comprehensive mechanistic understanding of degradation and recovery processes in rivers and streams remains elusive. Such mechanistic insights are essential for accurately forecasting the effects of stressors, their interactions, impacts on biodiversity and ecosystem functioning, and ultimately for facilitating the restoration of degraded freshwater ecosystems (Orr et al., 2020; Vos et al., 2023).

1.3 Microphytobenthic community responses to multiple stressors

Deciphering the responses organisms to the effects of multiple stressors, such as shifts in the community structure and functioning of benthic eukaryotic microalgae, is necessary to address the loss of freshwater biodiversity. There are five main ways in which two stressors can interact with species, communities, or ecosystems: stressor dominance, which is the situation in which one stressor has a greater impact on the response variable than the other; additive effects, in which the combined impact of the stressors is just the total of their individual effects; synergistic effects, in which the combined impact of the stressors is stronger than the sum of their impacts; antagonistic effects, in which the combined impact of the stressors is weaker than the combined effect; and reversal, in which the joint effect of the stressors is the opposite direction of their individual effects (Birk et al., 2020; Vos et al., 2023). These various types of responses highlight the complexity of comprehending the ways in which multiple stressors affect ecological systems.

According to Vos et al. (2023) introduced Asymmetric Response Concept (ARC), different ecological mechanisms predominate during periods of rising and falling stressor intensities, leading to different pathways for ecosystem functions and community structure. This can lead to asymmetrical responses to stressors. The ability of a species to disperse based on its characteristics and connectivity, species-specific stressor tolerance, and biotic interactions like competition, facilitation, predation, and parasitism are important variables. According to the ARC, these mechanisms affect how quickly or slowly an ecosystem returns to its pre-degradation state, recovers partially, stays degraded, or transforms into a new ecosystem with distinct communities (Vos et al., 2023).

River restoration aims to restore the functions, forms, and habitats of streams that have been degraded. This is often done to help recover specific riverine species that have become endangered due to changes in their environment. Stream recovery aims to restore degraded stream forms, functions, and habitats, often to facilitate the restoration of a particular endangered species that has been nearly extinct due to changes in the river environment (Edwards et al., 2020). The process of restoring an ecosystem involves several steps and mechanisms that determine whether it will transition to a new state or return to its pre-disturbance state (Brown and Manoylov, 2023; Edwards et al., 2020; Vos et al., 2023). Eliminating or reducing the stressors initially allows the ecosystem to respond, resulting in changes that occur immediately as species begin to recolonize and physical and chemical conditions return to normal. Tolerance to stressors varies by species; more tolerant species recover more quickly. Due to rapid recolonization of species with high dispersal ability, habitat connectivity and dispersal capacity are critical. During recovery, biotic interaction such as competition, facilitation, predation, and parasitism shape the composition of communities (Vos et al., 2023). Another factor is indirect effects, such as predators control of grazers, which promote algae recovery. Restoring ecosystem processes is critical, including primary production and nutrient cycling (Brown and Manoylov, 2023). Recovery outcomes vary, for example, ecosystems may fully recover, partially recover, remain in a degraded state, or transition to a new state with different species and functions (Brown and Manoylov, 2023; Vos et al., 2023). Understanding these dynamic processes is important for efficient management and restoration of ecosystems.

Following the Water Framework Directive (WFD), freshwater monitoring programmes have traditionally used structural changes within the biotic (e.g. macroinvertebrate, diatoms, fish and macrophytes) communities to evaluate the ecological status of freshwater bodies (European Commission, 2000). The WFD describes several ecological quality elements for assessing the ecological status of surface waters. These elements are classified into biological, hydromorphological and physico-chemical quality elements. Biological quality elements include benthic invertebrates, fish fauna, macrophytes, phytoplankton and phytobenthos.

Phytobenthos or periphyton biofilms are surface-attached communities of microscopic photosynthetic organisms. They host the majority of primary producers, mainly eukaryotic algae dominated by Bacillariophyta (diatoms), but also Chlorophyta and Euglenophyta, as well as cyanobacteria and other mixo- and heterotrophic protists and fungi (Underwood, 2010), which live on the sediment surface in almost all waters. River biofilms that grow on rock surfaces, including gravel and cobblestones, are called epilithic biofilms. These biofilms are more complex in structure and have higher algal biomass than other types of biofilms that grow on macroalgae and macrophytes (epiphytic biofilms), muddy or sandy sediments (epipelic or epipsammic biofilms), animals (epizoic biofilms), dead woods and all nontoxic submerged artificial objects (Round, 1991).

Diatoms (Bacillariophyta) frequently dominate all these biofilms. They occur worldwide in all aquatic, semi-aquatic, and even somewhat humid or semi-arid ecosystems (Round et al., 1990). As the base of trophic level in aquatic systems, algal communities control bottom-up processes and have an impact on the aquatic ecosystem as a whole. Diatoms are a class of algae that are found in instream environments and are distinguished by their hydrated silica cell wall. According to Smol and Stoermer (Smol and Stoermer, 2015), diatoms are regarded as sensitive and reliable bioindicators for a range of environmental stressors. The species composition of benthic diatom communities is a direct reflection of the particular combination of long-term environmental factors that prevail in their ecosystems. Additionally, their cell walls are well preserved; therefore, it is likely that they could be used effectively as proxies for evaluating past environmental conditions, and for monitoring the ongoing multidimensional changes caused by multiple stressors in our rivers and streams.

Multiple stressor effects on microphytobenthos communities are currently assessed using field surveys, mesocosms experiments and predictive modelling. Field observations form the basis of the majority of studies on microphytobenthic algal communities in freshwater ecosystems. They represent a useful approach to estimate photosynthetic biomass (Breuer et al., 2016; Kahlert and McKie, 2014; Kumar et al., 2014) and to study microalgae community composition through morphological observations (Bellinger et al., 2013; Blinn and Bailey, 2001; Breuer et al., 2017; Hering et al., 2006; Kelly and Wilson, 2004; Lavoie et al., 2018; Malinowska-Gniewosz et al., 2018; Markert et al., 2024b; Munn et al., 2018), and high-throughput amplicon sequencing (Kulaš et al., 2022; Minerovic et al., 2020). Recently, there has been growing interest in controlled mesocosm experiments testing the effects of stressors on microphytobenthic communities (Bondar-Kunze et al., 2016; Bondar-Kunze et al., 2021; Costello et al., 2018; Frost et al., 2023; Nuy et al., 2018; Piggott et al., 2015a; Salis et al., 2019), which provide valuable insights into our understanding of biofilm community responses to multiple stressors. However, the recovery process after alleviating the stressors is less studied. We were unable to find any previous mesocosm studies that examined the recovery of microphytobenthos communities after alleviating stressors application.

1.4 Aims and hypotheses of this thesis

This work aims to contribute to our understanding of stream microphytobenthic community composition and photosynthesis-related traits to multiple stressors increase and release using digital light microscopy, 18S V9 amplicon sequencing and chlorophyll fluorescence. It involves both controlled streamside mesocosm experiments and fieldwork in the Emscher/Boye and Kinzig catchments, two anthropogenically impacted river networks in Germany. Specifically, I attempted to answer the following questions:

1. How does salinization affect the ecological responses of an urban stream ecosystem under varying flow regimes?

The ExStream system (Piggott et al., 2015a) was used to look into this question. A streamside mesocosm was set up at the Boye River in the Western Germany Ruhr metropolitan area and applied a salinity gradient ranging from an ambient salinity of 0.76 mS/cm to 3 mS/cm under different regimes of flow velocity (normal: 20 cm/s, reduced: 10 cm/s). I evaluated changes in related ecosystem functions (microbial respiration, organic matter decomposition, and primary production) as well as effects on different organism groups (macroinvertebrates, algae, parasites, and fungi) reported in Chapter I.

2. How do stream microphytobenthic communities functionally and compositionally respond to the increase and release of multiple stressors in a mesocosm experiment?

This question was investigated using Exstream mesocosm experiment where I investigated how increases in temperature and salinity and decreases in flow velocity affect microphytobenthic communities (Chapter II). I hypothesized that stressors cause a shift in community composition towards lower diversity and greater dominance of disturbance-tolerant taxa, with a relatively limited impact on photosynthetic biomass. Additionally, I hypothesized that priority effects, where resident communities occupy physical and niche space, will initially hinder recolonization by immigrant species when the stressors load is alleviated.

3. How do stream microphytobenthos communities respond to anthropogenic stressors in the Kinzig catchment?

This question was investigated through field sampling in the Kinzig catchment located in Central Germany (Chapter III). I hypothesized that photosynthetic biomass and microphytobenthos community composition in the Kinzig catchment correlate with environmental factors. Higher biomass and reduced diversity are associated with increased nutrient availability and eutrophication in the system.

4. Are microphytobenthic community compositions in restored sewage channels primarily influenced by the time since restoration or by ongoing anthropogenic stressors?

This investigation was conducted through field sampling in the Emscher/Boye catchment (Chapter IV). I hypothesized that the degree of diatom diversity and community composition changes is correlated with the time elapsed since restoration, with older restoration sites showing more significant ecological recovery compared to newer restoration sites. However, despite restoration efforts in this urban streams, including both reference and restored sites, continue to experience degradation due to ongoing anthropogenic pressures.

1.5. Structure of this thesis

This thesis is a compilation of four different articles that collectively address the core objectives. Two articles have been published, one is currently under review and another is in the final stages of preparation for submission, as described below:

Chapter 1: Pimentel, I.M., Baikova, D., Buchner, D., Burfeid-Castillanos,A., David, G.M., Deep, A., Doliwa, A., Hadžiomerović, U., **Mayombo, N.A.S.**, Prati, S., Spyra, M.A., Vermiert, A.M., Beisser, D., Dunthorn, M., Piggott, J.J., Sures, B., Tiegs, S., Leese, F., Beermann, A. (2024). Assessing the response of an urban stream ecosystem to salinization under different flow regimes. Science of the Total Environment, 926, 171849. https://doi.org/10.1016/j.scitotenv.2024.171849

Chapter 2: **Mayombo, N.A.S.**, Burfeid-Castellanos, A.M., Vermiert, A.M., Pimentel, I.M., Rehsen, P.M., Dani, M., Jansinski, C., Spyra, M., Kloster, M., Vidakovic, D., Buchner, D. & Beszteri, B. 2024. Functional and compositional responses of stream microphytobenthic communities to multiple stressors increase and release in a mesocosm experiment. Science of the Total Environment, 943, 173670. https://doi.org/10.1016/j.scitotenv.2024.173670

Article 3: Mayombo, N.A.S., Mimoza, D., Burfeid-Castellanos, A.M., Kloster, M., Vidakovic, D., Buchner, D., Beszteri, B. Assessment microphytobenthos communities in the Kinzig Catchment using photosynthesis-related traits, digital light microscopy and 18S-V9 amplicon sequencing. Manuscript submitted for publication in *Frontiers in Ecology and Evolution* on April 24th, 2024 (currently under review). https://doi.org/10.1101/2024.06.17.599279

Article 4: Mayombo, N.A.S., Haverbeck, L., Dani, M., Kloster, M., Vidakovic, D., Beszteri, B., Burfeid-Castellanos, A.M. Characterization of diatom communities in restored sewage channels in the Boye catchment, Germany. (preprint). https://doi.org/10.1101/2024.07.03.601863

Together, these articles comprehensively examine the thesis objectives, each contributing unique perspectives and insights on the overarching research questions.

Each article is preceded by a declaration of the author's contribution.

Cumulative Thesis/Extent of Contribution

Cumulative thesis of Mr Ntambwe Albert Serge Mayombo

Author contributions

Paper 1. Science of the Total Environment

Assessing the response of an urban stream ecosystem to salinization under different flow regimes

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Contributions:

- Conception 0 %: Followed the hypotheses and study design drawn in the grant application.
- Conduction of experimental work 10 %: Not present during the experimental work due to COVID restrictions (in South Africa and the Democratic Republic of the Congo). Participated in the photosynthetic analysis in the lab from October on.
- Data analysis 100 %: of the microalgae data (~20% of the total).
- Species identification 50 %: Molecular identification shared with lead author.
- Statistical analysis 0 %: Done by the lead author.
- Writing the manuscript 100 %: of the microalgae sub-chapter (~20% of the total).
- Revision of the manuscript 6 %: Shared revision of all co-authors.

Signature of the Doctoral Candidate	Signature of the Doctoral Supervisor

Chapter I

Science of the Total Environment 926 (2024) 171849

Contents lists available at ScienceDirect

Science of the Total Environment

Assessing the response of an urban stream ecosystem to salinization under different flow regimes

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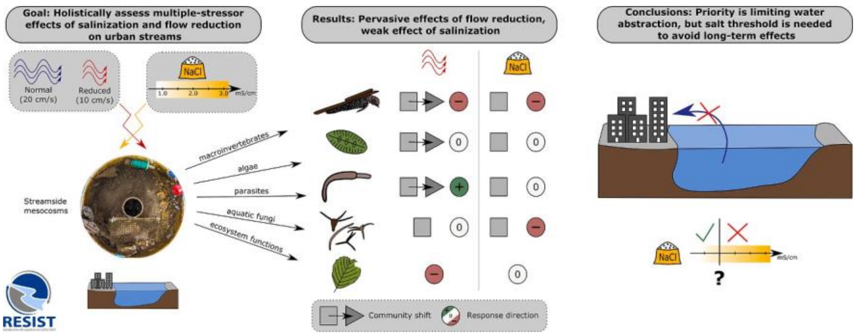
HIGHLIGHTS

- Urban streams increasingly exposed to salt and low flow due to climate change.
- Holistic assessment of multiple organism groups and ecosystem functions.
- Flow velocity reduction threatens stream ecosystem integrity.
- Minor impact of salt, probably due to stressor legacy from historic mining.
- Water abstraction in urban streams should be carefully limited to avoid low flow.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Macroinvertebrates
Algae

ABSTRACT

Urban streams are exposed to a variety of anthropogenic stressors. Freshwater salinization is a key stressor in these ecosystems that is predicted to be further exacerbated by climate change, which causes simultaneous

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https://doi.org/10.1016/j.scitotenv.2024.171849
Received 16 November 2023; Received in revised form 8 February 2024; Accepted 19 March 2024

Fungi
Parasites
Organic-matter decomposition
Primary production

changes in flow parameters, potentially resulting in non-additive effects on aquatic ecosystems. However, the effects of salinization and flow velocity on urban streams are still poorly understood as multiple-stressor experiments are often conducted at pristine rather than urban sites. Therefore, we conducted a mesocosm experiment at the Boye River, a recently restored stream located in a highly urbanized area in Western Germany, and applied recurrent pulses of salinity along a gradient (NaCl, 9 h daily of +0 to +2.5 mS/cm) in combination with normal and reduced current velocities (20 cm/s vs. 10 cm/s). Using a comprehensive assessment across multiple organism groups (macroinvertebrates, eukaryotic algae, fungi, parasites) and ecosystem functions (primary production, organic-matter decomposition), we show that flow velocity reduction has a pervasive impact, causing community shifts for almost all assessed organism groups (except fungi) and inhibiting organic-matter decomposition. Salinization affected only dynamic components of community assembly by enhancing invertebrate emigration via drift and reducing fungal reproduction. We caution that the comparatively small impact of salt in our study can be due to legacy effects from past salt pollution by coal mining activities >30 years ago. Nevertheless, our results suggest that urban stream management should prioritize the continuity of a minimum discharge to maintain ecosystem integrity. Our study exemplifies a holistic approach for the assessment of multiple-stressor impacts on streams, which is needed to inform the establishment of a salinity threshold above which mitigation actions must be taken.

1. Introduction

Ecosystems adjacent to, or located within, urban areas experience a wide range of anthropogenic impacts that result in environmental parameters exceeding their natural range of variation. Such changes are defined as ‘stressors’ to the ecosystem if they affect single taxa, impact entire communities, or alter ecosystem functions relative to reference conditions (Piggott et al., 2015b). As urbanization increases globally (Ritchie and Roser, 2018), ecosystems become more exposed to urban stressors. Given that human settlements depend upon access to potable water, they are often located close to freshwater sources. Therefore, streams are highly vulnerable because they act as vectors and collectors of various urbanization effects, such as pollution from domestic and industrial wastewater (Birk et al., 2020; Ormerod et al., 2010). Streams are also threatened by other stressors operating at larger spatial scales, such as global warming (Kaushal et al., 2010), heat waves (Tassone et al., 2022) and altered precipitation patterns (Schneider et al., 2013) caused by climate change. This vulnerability of streams is reflected in extraordinarily high species extinction rates and biodiversity loss (Collen et al., 2014; Reid et al., 2019).

Among the drivers threatening stream ecosystems, freshwater salinization is an emerging and serious concern for ecosystem health (Cañedo-Argüelles et al., 2019). Its sources are diverse and include agricultural irrigation practices, mining activities, and the application of de-icing salt (Cañedo-Argüelles et al., 2013). The latter can have a particularly strong impact in urban areas with cold winters (Moore et al., 2020). However, urbanization has been shown to foster salinization independently of winter temperature across a latitudinal gradient, potentially through wastewater effluents and weathering of infrastructure (Utz et al., 2022). Furthermore, the degree of freshwater salinization within urban watersheds is affected by climate change due to alterations in precipitation and temperature regimes (Stirpe et al., 2017). While milder winters may reduce the application of road salts for certain regions, drier and longer summers may exacerbate salinization (Cunillera-Montcusí et al., 2022; Jeppesen et al., 2020) and may outweigh the benefit of higher winter temperatures (Stirpe et al., 2017). Total or seasonal reductions in precipitation also result in flow reduction. This in turn reduces water depth and wetted perimeter and renders an increasing proportion of benthic habitats unavailable. In addition, low discharge alters the thermal regime of water bodies, influences water chemistry and nutrient availability, promotes sedimentation processes, and profoundly alters aquatic communities (Beermann et al., 2018a; Dewson et al., 2007). The detrimental effects of flow reduction can result in changes to ecosystem functioning (Arias Font et al., 2021). Therefore, the co-occurrence of salinization and flow reduction may have profound impacts on streams.

If stressors co-occur, they may interact and complicate impacts (Carrier-Belleau et al., 2023). An interaction is characterized by the effect of one stressor being modified by the presence or intensity of another, i.e., the effects of stressors do not merely add up when they co-occur. Stressor interactions are commonly observed in aquatic and terrestrial ecosystems (Birk et al., 2020; Darling and Côté, 2008; Dieleman et al., 2012; Jackson et al., 2016). Interactions can be classified as either ‘synergistic’ or ‘antagonistic’, denoting a more or a less extreme outcome than expected from the sum (additive) or product (multiplicative) of their individual effects (Folt et al., 1999; Piggott et al., 2015b). Their presence, intensity and direction strongly depend on the environmental context in which the ecosystem under study is embedded (Kefford et al., 2023). While many multiple-stressor experiments have explicitly chosen study sites in streams with diverse communities, good water quality, and low anthropogenic impact (e.g. Beermann et al., 2018a; Bondar-Kunze et al., 2021; Piggott et al., 2012), multiple-stressor interactions in urban streams are less studied.

Stressor impacts and stressor interactions in stream ecosystems are generally assessed via changes in community composition of target groups such as macroinvertebrates or diatoms (Birk et al., 2012; Cairns and Pratt, 1993; Carter et al., 2017; Martin et al., 2012). As many species within these groups are sensitive to environmental changes, they are also used as bioindicators for environmental monitoring and stressor management. Hence, experimental studies on multiple stressors often focus on these biological target groups (e.g. macroinvertebrates: Beermann et al., 2018a; Blöcher et al., 2020; periphyton: Bondar-Kunze et al., 2021; Costello et al., 2018; both: Piggott et al., 2012). However, by using a broader approach that considers other groups, such as non-diatom algae (Schneider et al., 2012), fish (Alric et al., 2021), parasites (Sures et al., 2023) or fungi (Solé et al., 2008), and by increasing taxonomic resolution for the analysis of largely disregarded organismal groups such as non-biting midges (Chironomidae; Beermann et al., 2018b), one can obtain a more nuanced view of ecological conditions. Indeed, sensitivities to stressors are differentially distributed among organism groups, thereby providing complementary, rather than redundant, information (Heino, 2010; Vilmi et al., 2016).

Ecosystem integrity relies on ecosystem functions that provide primary sources of carbon and energy in streams, such as primary production and organic-matter decomposition. Studying rates of change in these ecosystem functions can serve as an additional line of evidence for anthropogenically induced changes at the ecosystem level (He et al., 2023). For example, organic-matter decomposition is impacted by multiple stressors (Ferreira et al., 2020) and can be directly affected by alterations in physical processes, such as leaching and fragmentation, and indirectly affected through changes in biological activities mediated by the combined action of macroinvertebrates and microorganisms, such as aquatic fungi and bacteria (Marks, 2019; Salis et al., 2023).

Rather than focusing on any single response variable, the impacts of multiple stressors on streams is best addressed by evaluating changes across different biological communities and ecosystem functions to understand mechanistic relationships. We used the *ExStream* system (Piggott et al., 2015a), a streamside mesocosm setup, at the Boye River in the Ruhr metropolitan area in Western Germany and applied a salinity gradient ranging from an ambient salinity of 0.76 mS/cm to 3 mS/cm under different regimes of flow velocity (normal: 20 cm/s, reduced: 10 cm/s). By assessing effects across multiple organism groups (macroinvertebrates, algae, parasites, and fungi) along with changes in associated ecosystem functions (organic-matter decomposition, microbial respiration, and primary production), we aimed to holistically characterize the magnitude and diversity of single and multiple-stressor responses to salinization and flow velocity reduction.

2. Material and methods

2.1. Study site

The experiment was conducted in late spring between April 22 and May 28, 2021 at the Boye River (latitude, longitude: 51.5533°N, 6.9485°E). The Boye River is a carbonate-rich, sand-dominated lowland stream in North Rhine-Westphalia, Germany, and is part of the Emscher river system (www.elwasweb.nrw.de, last accessed 31.10.2023). It constitutes a stream in the process of ecological recovery; during the late 19th and early 20th century, the Emscher and its tributaries were transformed into a concrete channel system of open sewers for adjacent human settlements, because substantial coal mining activities rendered the construction of underground sewers unfeasible. In the late 20th century, coal mining activities stopped and, subsequently, the most economically ambitious river restoration project in Europe (Perini, 2017) was launched, relocating untreated wastewater to closed underground sewers and rebuilding near-natural river beds (Winking et al., 2014). The efficiency of restoration efforts in reducing historical salt pollution in the Boye catchment also became evident from time series data on salinity: while a chloride concentration of 15,500 mg/L and conductivity of 20.2 mS/cm was measured at the mouth of the Boye River in 1996, the latest measurements in 2021 was at 26 mg/L and 0.4 mS/cm (data downloaded from www.elwasweb.nrw.de, January 22,

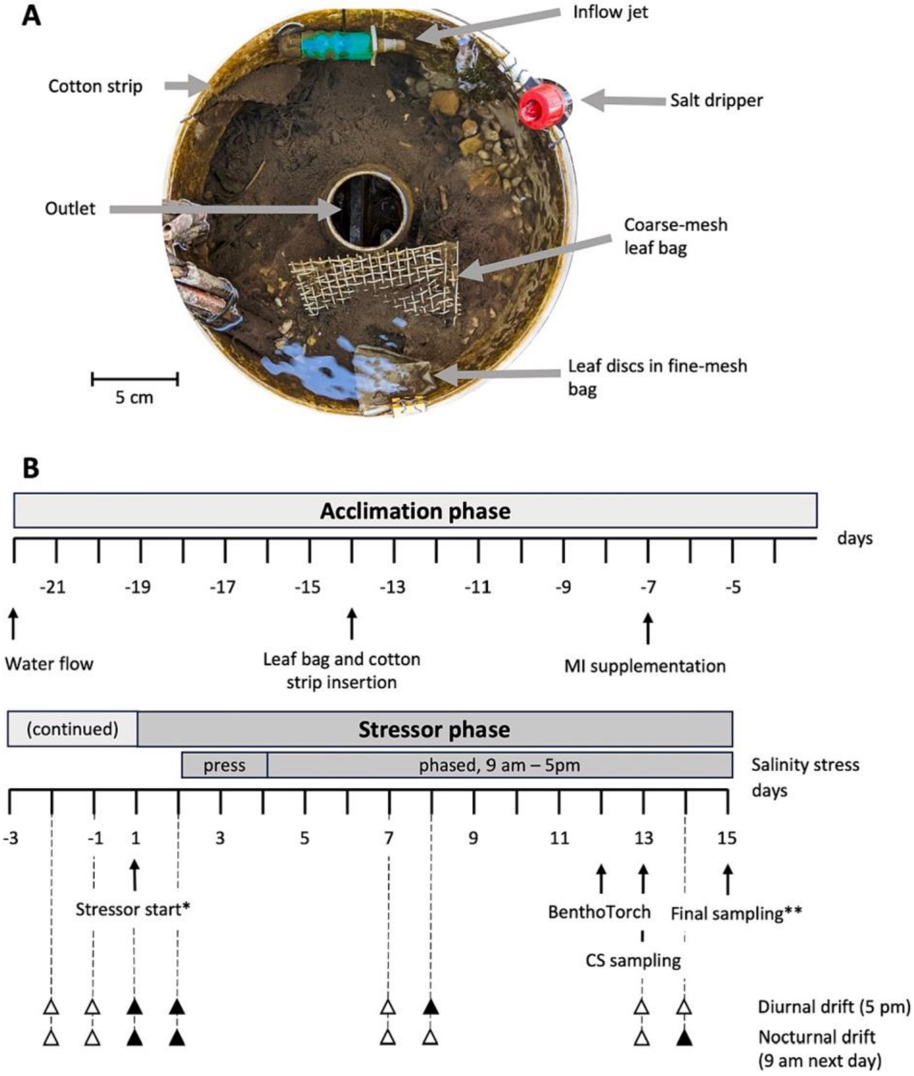

Fig. 1. ExStream overview. A: Circular mesocosms. The 64 mesocosms were constantly supplied with stream water through the inflow. A removable inflow jet allowed to alter flow velocity (with jet: 20 cm/s, without jet: 10 cm/s). Mesocosms were supplied with a coarse-mesh leaf bag, leaf discs protected by a fine-mesh tetrahedral bag and a standardized cotton strip. Water and drifting macroinvertebrates leave the mesocosms via a central outlet. B: Timeline of the experiment. * At the stressor start, salt was only applied for a few hours and subsequently turned off, as a salinity gradient developed within the dripper line. It was resumed after adaptation of the setup on the next day. ** Final sampling involved macroinvertebrates from benthic habitat and leaf bags, the biofilm from the mesocosm wall, and leaf discs. Abbreviations: MI - macroinvertebrates, CS - cotton strips. White triangles mark drift samples that have been collected, but not analyzed and black triangles mark the analyzed drift samples.

2024). The study site became sewage free in 2017; however, the river sections further upstream have been cleaned of sewage water since 2007. Since then, salinity has been moderate and fluctuated between 24.7 and 48.1 mg/L of chloride, without pronounced seasonal peaks that would indicate a strong influence of de-icing salt application on the water chemistry of the Boye River (for further details, see Supplement 1.1).

2.2. Experimental system

In the *ExStream* system, water is redirected from the stream into circular mesocosms. We used two pumps (NGAm 1A-Pro, max. capacity: 315 L/min) to move the water on top of a two-level scaffold. Perforated aluminum cages with a hole size of 5 mm surrounded the inflow, allowing migration of macroinvertebrates into the system while retaining coarse material. The water was first pumped into four 203-L sediment traps, where flow velocity was reduced to allow suspended material to settle at the bottom of the tank. The water then entered the header tanks, each of which gravity-fed 16 circular mesocosms (outer diameter: 25 cm, central outflow: 6 cm, volume 3.5 L, Fig. 1A) positioned on the lower level of the scaffold (total number: 64). Any excess water was directed to the system drain using an overflow at the top of each header tank. After passing through a retention filter basin, the system drain was returned to the Boye downstream of the system intake. The constant supply of fresh river water ensures that physico-chemical conditions within the mesocosms are comparable to those in the adjacent river and do not substantially differ among them. Temperature was recorded every 5 min using temperature loggers (HOBO Pendant Temp/Light and HOBO Pendant MX Temp/Light with ±0.5 °C accuracy) in the Boye River close to the pump intake, in all header tanks, and in thirteen mesocosms. Temperature minima and maxima in the mesocosms differed from those of the river due to short-term declines in the flow rate when tube blockages occurred (range in mesocosms: 8.7–24 °C, Boye River: 9.9–19.2 °C), however, the mean temperature was similar to the river water (mean ± SD in mesocosms: 13.1 ± 2.0 °C, Boyer River: 12.9 ± 1.8 °C, see also Supplement 1.2).

2.3. Experimental design

All mesocosms were supplied with 1 L of a mixture of store-bought, washed sand (0–1 mm) and a slurry of fine particulate organic matter (FPOM, ratio: 34:1). FPOM originated from Spechtsbach (latitude, longitude: 51.5627 °N, 6.9154 °E), a tributary to the Boye River, where the meandering course of the stream facilitated the accumulation of FPOM at pool sections. It was supplied as a carbon source and for inoculation with microbes and was thoroughly mixed with the sand to represent a typical substrate of the studied river. A patch of store-bought gravel (quartz stones, grain size: 6–8 mm, volume per mesocosm: 200 mL) was positioned directly behind the inflow along with three large stones (quartz, approx. diameter: 20–40 mm). As an additional habitat, two packages of dead wood from a nearby site (latitude, longitude: 51.5531 °N, 6.9491 °E) amounting to 30 ± 0.3 g were positioned at approx. 9 o'clock relative to the inflow of the mesocosm.

The experiment consisted of two phases (Fig. 1B). In the acclimation phase, environmental conditions were similar among all mesocosms and colonization by drifting invertebrates and microbes allowed for the assembly of natural communities. In the stressor phase, mesocosms were experimentally manipulated by increasing salinity and reducing flow velocity.

The acclimation phase started along with water flow, on April 22 (day —22) and lasted for 22 days. Discharge in the mesocosms was calibrated twice a day using shut-off valves. Initially, discharge was set to approx. 1.4 L/min, but was then increased to 2.1 L/min on April 30 (day —14) for the rest of the experiment to improve stability of the flow. On the same day, a coarse-mesh leaf-litter bag (leaf mass, air-dried: 4 ± 0.1 g, mesh size: 5 mm), a set of six leaf discs (diameter: 11 mm) within a tetrahedral fine-mesh gauze fabric (mesh size: 0.5 mm) and one standardized cotton strip (8 cm long, after Tiegs et al., 2013, 2019) were added. Leaf-litter bags and leaf discs were prepared from fallen alder leaves (*Alnus glutinosa*) that were air-dried for 2.5 weeks. The leaf discs serve as a substrate for growth of aquatic fungi and other microorganisms and for estimating microbial-driven leaf decomposition under exclusion of macroinvertebrates. The leaf-litter bags provide another microhabitat for macroinvertebrates and were used to estimate decomposition driven by micro- and macro-organisms in combination. Cotton strips were attached to the outer mesocosm wall in front of the inflow (Fig. 1A) and were used to estimate tensile-strength loss as a standardized and widely used measure for organic-matter decomposition (Tiegs et al., 2013, 2019).

Colonization by macroinvertebrates during the acclimation phase was augmented by manual seeding using multi-habitat kick-net samples from an upstream section of the Boye River (latitude, longitude: 51.5608 °N, 6.9330 °E) on May 7 (day —7). This allowed the inclusion of taxa that are typically underrepresented in the drift. The specific stream section was chosen for seeding because it has never received sewage water in the past and, thus, harbors near-natural communities. In total, 50 streambed patches of approx. 0.0625 m^2 were sampled using nets with a mesh size of 500 μm, the contents of which were sorted in the field, excluding large predators and fish that accidentally entered the kick-nets, and randomly distributed over the mesocosms. First, all samples were pooled and divided into eight buckets. The content of each bucket was randomly dispersed over eight truncated 1-L PET bottles using a rotator by steadily pouring the content over the rotating bottles. Each bottle was then emptied into a mesocosm. Consequently, each mesocosm was seeded with benthic invertebrates occurring in an equivalent of 0.05 m^2 benthic habitat. For the first three days after the seeding procedure, drifting invertebrates were captured with tea strainers (hole size: 1 mm) and reintroduced into the mesocosms.

The stressor phase began with the onset of the experimental manipulations on May 14 (day 1, Fig. 1B) and lasted for 14 days. In each spatial block, treatments were randomly arranged. We applied a full-factorial design with eight levels of increasing salinity (targeted addition of chloride: + 0, + 100, + 200, + 300, + 400, + 500, + 600 and +700 mg/L) and two levels of flow velocity (normal vs. reduced, 20 cm/s vs. 10 cm/s), with four replicates per treatment combination. We chose NaCl over other salts because chloride is an ion that is commonly associated with urban drivers of salinization, such as road salts and wastewater (Cormier et al., 2013; Kaushal et al., 2018; Utz et al., 2022). The selected levels of salinity cover predicted increases in electrical conductivities given future changes in land use and climate (Table 1): Olson (2018) predicted that, towards the end of the current century, >10 % of streams in America will potentially reach conductivities of 2

Table 1

Targeted and achieved chloride concentrations with the corresponding electrical conductivity (EC). Background chloride concentrations of the Boye water ranged between 28 and 36 mg/L and background conductivity ranged between 0.620 and 0.928 mS/cm. The conversion from chloride addition to EC increase and vice versa was calculated from a calibration curve based on three water samples taken from the Boye river: Chloride addition in mg/L = —6.4534 + 315.8 × EC increase in mS/cm.

Salt treatment	Target		Achieved	
	Chloride addition (mg/ L)	EC increase (mS/cm)	Chloride addition (mg/ L)	EC increase (mS/cm)
Level 1	+100	+0.337	+37	+0.138
Level 2	+200	+0.654	+141	+0.467
Level 3	+300	+0.970	+245	+0.797
Level 4	+400	+1.287	+251	+0.814
Level 5	+500	+1.604	+418	+1.345
Level 6	+600	+1.920	+537	+1.721
Level 7	+700	+2.237	+603	+1.931

mS/cm or more.

2.4. Details on experimental manipulations

2.4.1. Salinity

Mesocosms were supplied with NaCl solutions of varying concentrations at a constant rate of 4 L/h using a pressure-compensating dripper system. An oversaturated stock solution (>350 mg/L NaCl) was prepared by constantly mixing river water with boiling salt tablets (Claramat, >99.9 % NaCl) using a submersible pump (Gardena, 9000Dirt). Dosage pumps (GHL Doser 2.1) fed different volumes (pumping rates in mL/min: 4, 12, 20, 27, 35, 43, 51) of the stock solution into seven separate salt lines, which were supplied with filtered river water (mesh size: 0.1 mm). In the original setup, a strong salinity gradient developed within each line and the addition of salt on the first day of the stressor phase was stopped. The setup was optimized the following day by incorporating a mixing chamber behind the inflow of the dosage pumps, and the salt application was resumed at 8:30 pm on May 15 (day 2). Because fine sediment that did not settle in the sediment traps sometimes blocked the inflow tubes to the mesocosms, regular monitoring of the water supply was necessary. To avoid an excessive accumulation of salt during nighttime when this frequent monitoring was not feasible, the dosage pumps were turned off overnight from May 17 (day 4) onward, resulting in a phased stressor application between approx. 9:30 am and 6:30 pm.

Water samples were taken once during the acclimation phase (day 7) and thrice during the stressor phase (stressor days 5, 9, and 13) to measure the ambient chloride concentration and create a calibration curve. During the experiment, the Boye had an average chloride concentration of 31.8 ± 3.3 mg/L (range: 28–35 mg/L). More detailed information on basic water chemistry and physical measurements at the study site (e.g. ionic composition, concentration of metals, pH, dissolved oxygen) is to be found in Supplement 1.3. Electrical conductivity was measured twice a day in each mesocosm and converted into added chloride based on a calibration curve derived from the water samples of the Boye. Median conductivities in the mesocosms ranged from 0.761 to 3.074 mS/cm, and summary metrics for each mesocosm (mean, standard deviation, range and median) are publicly available on the Open Science Framework (doi.org/10.17605/OSF.IO/247JC).

Change in electrical conductivity was calculated daily by subtracting the mean conductivity of the control mesocosms ($N = 4$) from the measured conductivity of the mesocosms. The actual added chloride concentration (±SD) for the salinity levels was estimated to be 37.0 ± 15.1 (+100), 141.0 ± 29.3 (+200), 245.4 ± 57.9 (+300), 250.7 ± 60.3 (+400), 418.4 ± 24.3 (+500), 536.9 ± 62.7 (+600) and 603.4 ± 48.7 (+700) mg/L chloride added (Table 1). These deviations from the targeted chloride addition and the differences in spread among the levels was probably due to malfunctioning of the dosage pumps and the dripping system.

2.4.2. Flow velocity

To reduce flow velocity, the water inflow jet was removed for half of the mesocosms. Because discharge was kept constant, the same amount of water was forced through a larger diameter, resulting in lower flow velocity. The realized near-surface flow velocity was measured in empty mesocosms by measuring the time a stick took to encircle the circumference of a mesocosm for both treatments (mean ± SD for normal flow velocity: 19.0 ± 1 cm/s, $N = 8$; mean ± SD for reduced flow velocity: 9.7 ± 1.3 cm/s, N = 8).

2.5. Sampling procedures and sample processing

2.5.1. Macroinvertebrates

As a response to stress, macroinvertebrates commonly enter drift to migrate to sites offering better environmental conditions. Thus, drifting macroinvertebrates were captured with a tea strainer (hole size: 1 mm) for selected time frames (days −1, −2, 1, 2, 7, 8, 13 and 14) and sampled at 5 pm (diurnal drift, after 8 h) and the following morning at 9 am (nocturnal drift, after 16 h). For further sample processing, diurnal and nocturnal drift samples from day 1 and day 2 were selected to capture the immediate drift response to the stressors, along with the diurnal sample from day 7 and the nocturnal sample from day 14 (Fig. 1B). Because the frequent occurrence of early larval stages in drift samples prevented reliable identification at a low taxonomic level, invertebrates were identified to family level using order-specific keys (Supplement 1.4) and counted to derive drift abundances per hour.

Macroinvertebrate communities were sampled after day 14 from two habitats, leaf bags and benthic habitat. The coarse-mesh leaf bags were removed and transferred to a 1-L PET bottle filled with Boye water for transport to the laboratory. Leaves were thoroughly rinsed with distilled water over a white tray and frozen at −20 °C for preservation, whereas macroinvertebrates inhabiting the leaf bags were collected with a 500-μm sieve and preserved in 96 % ethanol. For the benthic community, water flow for a mesocosm was stopped and the substrate with its invertebrate inhabitants was immediately preserved in 96 % ethanol in the field. The preservative was fully exchanged within 24 h after sampling.

In the laboratory, the benthic community was elutriated from the substratum. Macroinvertebrates and organic material were retrieved with a 500-μm sieve and evenly distributed by carefully agitating in water. Each community was then subsampled by area into one quarter and three quarters of the original sample, and ethanol was exchanged 24 h later. In the smaller subsample, macroinvertebrates were separated from other organic material. The sorted benthic subsamples and the leaf-bag communities were prepared for metabarcoding by homogenizing for 30 s at approx. 20,000 rpm in the preservation liquid using the Ultra Turrax T25 (IKA, Stauffen, Germany) with the dispersing tool S25 N-18G (stator diameter: 18 mm). The dispersing tool was sterilized between two samples by consecutively processing three 4 % bleach solutions followed by three ddH_2O samples. Negative controls (11 for the benthic dataset, 9 for the leaf-litter dataset) used in the metabarcoding process described below have already been introduced at this step by processing technical ethanol (96 %).

2.5.2. Amphipod parasites

Amphipods were retrieved from each drift sample, and ten amphipods were collected from the benthic macroinvertebrate samples at the end of the stressor phase when possible. All amphipods were immediately preserved in 96 % ethanol. Amphipods were subsequently measured (fourth coxal plate length), morphologically identified to the lowest taxonomic level possible, dissected, and screened for parasites under the microscope. No amphipods were found infected with microscopically visible macro-parasites, such as acanthocephalan or nematode larvae. Thus, after gut removal, amphipod tissue was used for the molecular identification of both host and microsporidian parasites as described below.

2.5.3. Periphyton

At day 12 of the stressor phase, chlorophyll *a* concentration in the biofilm of each mesocosm was estimated with fluorometric measurements (BenthoTorch, BBE+ Moldaenke, Schwentinental, Germany). Along the mesocosm, five positions were selected and measured with an approximate angle of 45°. The five measurements were then averaged to give a final value per mesocosm.

At the end of the experiment, the biofilm was scraped from the mesocosm wall. Using plastic razor blades, a 100 cm^2 sample of biofilm (5 cm × 20 cm) was scraped into a 1.5-mL tube with 96 % ethanol to be used for metabarcoding of the biofilm.

2.5.4. Leaf discs

The fine-mesh bags were collected on the final day, transported to the laboratory, and opened immediately to retrieve the leaf discs. The leaf discs were cleaned with stream water and placed in petri dishes filled with 15 mL of filtered water from the stream with a salt addition equivalent to the treatment in the different mesocosms. The leaf discs were incubated for 24 h on a shaker table (50 rpm at 12.5 ± 1 °C, photoperiod 4 h light; 8 h dark; 12 h light). The resulting conidial suspensions (15 mL and 4 mL of washing) were fixed with 1 mL of formalin (37 %). Remaining leaf discs were stored at —20 °C until further processing. Subsequently, the leaf discs were freeze-dried and weighed to determine final dry mass (DM). The DM was used to determine mass loss (expressed in percentage) by subtracting it from the mean initial mass of the leaf discs (determined using control bags, 28.1 mg ± 1.3, $N = 8$). As ergosterol concentration is a proxy of fungal biomass, an ergosterol extraction was performed according to Gessner (2020). Briefly, lipid extraction and saponification were performed in 10 mL of methanol/KOH at 80 °C for 30 min. The resulting extract was purified using solid-phase extraction (Sep-Pak Vac-RC-500 mg, Waters, USA). Ergosterol was quantified using high-performance liquid chromatography at 282 nm and 33 °C (Infinity II 1260, Agilent Technologies, Santa Clara, CA, USA). Fungal biomass was expressed as μg ergosterol g^{-1} DM.

The fixed conidial suspensions were mixed in order to ensure an even distribution of the conidia. A 5-mL aliquot was filtered on 5-μm pore size filters (Cellulose nitrate, Whatmann, Little Chalfont, UK) and stained using 0.1 % Trypan blue in 60 % lactic acid. For each sample, a minimum of 65 randomly chosen microscope fields (200 x) was investigated in order to count and identify the conidia (Gulis et al., 2020). Sporulation rates were then expressed as the number of conidia mg^{-1} DM d^{-1}.

2.5.5. *Coarse-mesh leaf bags*

Leaf material retrieved from the coarse-mesh leaf bags was stored at —20 °C until further processing. The samples were freeze-dried for at least 48 h by a lyophilizer (Heto PowerDry LL3000, Thermo, USA) and weighed to the nearest 0.01 g (FX-1200i, A&D Co., Ltd.). Eight leaf bags were exposed to leaching with tap water for 24 h and were then immediately frozen. These control bags were processed and weighed together with the leaf material used in the experiment to estimate leaf mass loss due to freeze-drying and leaching. Leaf mass loss of the sample bags was expressed as % of the original air-dry mass of the leaf material minus the mass difference of the control bags.

2.5.6. *Cotton strips*

Cotton strips were sampled on day 13. For each mesocosm, two 50-mL Falcon tubes were wrapped in aluminum foil and filled with water from the mesocosm. The cotton strip was retrieved using sterile plastic forceps and then introduced into one of the Falcon tubes after carefully removing loose algae and sediment. The second falcon tube contained stream water only, serving as a control for respiration measurements. The lids were closed tightly and the tubes were incubated in a closed respiration tank that was continuously supplied with water from the Boye River for approx. 2.5 h. Subsequently, the cotton strips were removed from the tubes using sterile forceps. Dissolved oxygen concentrations were measured with a luminescent LDO101 electrode (Hach-Lange GmbH, Germany). The exact duration of incubation, the temperature within the respiration tank, and dissolved oxygen concentrations of the sample and control tubes were recorded to calculate respiration rates as oxygen consumed in mg g^{-1} DM h^{-1} (Tiegs et al., 2013).

The cotton strips were further processed for future genetic analyses and tensile-strength measurements. From one end of each strip, 2 cm were cut and immediately preserved in 99.8 % ethanol (Fisher Scientific, analytical grade, UK) while the other part was cleaned to remove biofilm and sediment using a paintbrush in an ethanol bath. The strips were wrapped in individual tin foil trays and dried at 40 °C for ≥24 h before shipping for tensile-strength measurements at the Aquatic Ecology Lab at Oakland University (Tiegs et al., 2013). Each strip was placed into the jaws of a Mark-10 tensiometer mounted to a motorized test stand and pulled at a rate of 20 mm/min. The dried part of the cotton strips was weighed to the closest mg, and the mass was extrapolated for the calculation of respiration rates described above.

2.6. *Processing of molecular samples*

2.6.1. *Metabarcoding*

Three different sample types were processed for metabarcoding: the benthic invertebrate subsample, the leaf-bag samples, and the biofilm samples. First, lysis of the dissolved tissue powder or biofilm was achieved by bead-beating the samples with a few zirconia beads (diameter: 2 mm) for 2 min at 2400 rpm in the Mini-Bead-Beater 96 (Biospec products, Bartlesville, USA). For the biofilm samples, 12 negative controls were introduced at this step by processing 96 % ethanol. The samples were then incubated for 20 min at 56 °C in lysis buffer (45 mM Tris Base, 360 mM NaCl, 18 mM EDTA, 0.5 % SDS, 1 mg/mL Proteinase K, 0.05 mg/mL RNase A). Lysates were split into extraction duplicates on two independent plates and subsequently processed on a Biomek FX^{P} Automated Workstation (Beckman Coulter, Indianapolis USA).

DNA extraction followed a silica bead-based extraction protocol (Buchner, 2022a). Target DNA fragments were amplified with a two-step PCR approach (Zizka et al., 2019): In the first step, common metabarcoding primers for the respective target taxa were used (biofilm: 1389F/1510R, Amaral-Zettler et al., 2009; macroinvertebrates: fwhF2/fwhR2n, Vamos et al., 2017), extended by a binding site for the second step primers on their 5′-end (Buchner et al., 2021). Amplification was done in 10-μL assays with 1 μL of the extracts using the Qiagen Multiplex Plus Kit (Qiagen, Hilden, Germany) with a final concentration of 1× Multiplex Mastermix and 200 nM of forward and reverse primer, respectively. Cycling conditions were 5 min of initial denaturation at 96 °C, 20 cycles of (i) 30 s denaturation at 95 °C, (ii) 90 s of annealing at 58 °C (fwh2/fwhR2n) or 59 °C (1389F/1510R), (iii) 30 s of extension at 72 °C, and a final elongation for 10 min at 68 °C. After a bead-based clean-up of the first-step PCR products (Buchner, 2022b), 2 μL were further amplified in a 10-μL assay. Forward and reverse primers for the 2nd step were complementary to the binding sites introduced in the 1st step and extended by a sample-specific index and the Illumina flow cell adapter on their 5′-end. Final concentrations of the ingredients were 1× Multiplex Mastermix and 100 nM of forward and reverse primer, respectively. The cycling conditions were identical to the first-step PCR with the exception of cycle number (25×) and annealing temperature (61 °C).

DNA concentrations were normalized to 2 ng/μL using a bead-based normalization protocol (Buchner, 2022c), and samples were pooled together to one final library per sample type (benthic invertebrate subsamples, leaf-bag samples, biofilm samples). The libraries were concentrated on silica spin columns (EconoSpin Mini Spin Columns, Epoch Life Sciences, Missouri City, USA) and eluted in a final volume of 100 μL. Library concentrations were measured on the Fragment Analyzer using the High Sensitivity NGS Fragment Analysis Kit (Advanced Analytical, Ankeny, USA) and then sent for paired-end sequencing (2 × 150 bp) on the HiSeqX platform to Macrogen Europe (Amsterdam, Netherlands).

2.6.2. *Barcoding*

Amphipod tissue was used for barcoding of hosts and microsporidian parasites. DNA was extracted using a modified salt precipitation protocol according to Grabner et al. (2015). Molecular identification of hosts was obtained with the universal metazoan primers LCO1490 and HCO2198 (Folmer et al., 1994), while that of microsporidians with the universal microsporidian-targeted primers V1 (Zhu et al., 1993) and mic-uni3R (Weigand et al., 2016). PCR reactions used for amphipod host amplification were prepared following Weigand et al. (2016). One reaction contained 10 μL of 2× AccuStart II PCR ToughMix (Quanta Bioscience), 0.5 μM of each primer, and 1 μL of DNA, and MilliQ water was added up to a total volume of 20 μL. Cycling conditions were 3 min initial denaturation at 94 °C, 35 cycles of (i) 35 s denaturation at 94 °C,

(ii) 40 s of combined elongation and annealing at 68 °C, and a final elongation for 5 min at 68 °C. PCR products were sent to Microsynth Seqlab (Göttingen, Germany) for Sanger sequencing using the forward primers LCO1490 and V1.

2.7. *Bioinformatic processing of sequencing data*

2.7.1. *Metabarcoding*

Raw sequences were demultiplexed by inline tags with the Python script demultiplexer (v1.1.0, https://github.com/DominikBuchner/demultiplexer). Bioinformatic processing from demultiplexed sequence data to an OTU (operational taxonomic unit) table was subsequently performed with apscale (v1.4, Buchner et al., 2022), applying default parameters. In this pipeline, paired-end read merging was done with VSEARCH (v2.22.1, Rognes et al., 2016), and removal of primer sequences was done with cutadapt (v4.1, Martin, 2011). The remaining sequences were filtered for a length of 205 ± 10 bp and a maximum expected error of 1. Reads were first dereplicated for individual samples and retained for global dereplication only if they had a minimum abundance of 4. After global dereplication, OTUs were clustered using VSEARCH with a minimum similarity threshold of 97 %. The resulting OTU table was taxonomically assigned using BOLDigger (version 2.1.1, Buchner and Leese, 2020), a python-based program that queries the CO1 identification engine of the BOLDsystems database (Ratnasingham and Hebert, 2007, queried on February 20, 2023). To improve the reliability of diversity estimates, the taxonomically assigned OTU table was further curated by removing potential sequencing errors with LULU (Frøslev et al., 2017). Default parameters were chosen with the exception of minimum similarity, which was set to 90 % in accordance with the authors' recommendations to increase the threshold for markers with lower variability than ITS (Frøslev et al., 2017). OTUs with species-level assignment which have been erroneously removed by the algorithm were recovered.

For metabarcoding of the biofilm, bioinformatic processing was done using the natrix2 workflow (Deep et al., 2023). Paired-end reads were assembled with the PANDAseq (v2.11, Masella et al., 2012) simple Bayesian algorithm, and primers were trimmed from the sequences with cutadapt (v3.2, Martin, 2011) before quality filtering with a pandaseq threshold score of 0.9, minimum length of 77, and maximum length of 196 nucleotides. Sequence dereplication was carried out at 100 % similarity using the cd-hit algorithm (v 4.8.1, Fu et al., 2012), and chimeric sequences were identified and removed. To reduce erroneous sequences without strict abundance cut-offs, the split-sample filtering approach in the AmpliconDuo (v1.1, Lange et al., 2015) R package was used. OTUs were generated by clustering sequences using swarm (v2.2.2, Mahé et al., 2014). Mothur (v1.40.5, Schloss et al., 2009) was used to align OTUs against the Protist Ribosomal Reference (PR2) database (v4.14.0, Guillou et al., 2013), and only taxonomic assignments with bootstrap values greater than or equal to 60 were retained. Post-clustering curation of amplicon sequencing data was performed using MUMU (https://github.com/frederic-mahe/mumu), a fast and robust C++ implementation of LULU (Frøslev et al., 2017).

In all three metabarcoding datasets, replicates of the same biological sample were merged by removing OTUs that occurred in only one of the replicates and summing up reads of respective replicates for all remaining OTUs. For each OTU, the maximum number of reads found in negative controls was subtracted from the read numbers in biological samples. OTU tables from benthic and leaf-bag samples were filtered for freshwater invertebrate taxa, retaining only OTUs with a family-level assignment (min. 90 % similarity to a reference sequence), and the biofilm dataset was filtered for and split into two different organism groups: One table with exclusively eukaryotic algal taxa and one table with relevant parasite taxa.

2.7.2. *Barcoding*

Raw sequences were quality-checked and edited using Geneious v2023.1.2 (Biomatters). Only sequences with a minimum length of 200 bp were used for the analyses. Host and parasite sequences were separately aligned using the MAFFT v7.490 algorithm with standard settings (Katoh et al., 2019). For microsporidians, a maximum likelihood phylogenetic tree with bootstrap support values (1000 replicates) was produced in IQ-Tree 2.2.0 (Minh et al., 2020), with the microsporidian *Metchnikovella dogieli* (MT969020) as an outgroup. The GTR + F + G4 substitution model was selected based on Bayesian information criterion scores. To identify hosts and parasites, obtained sequences were compared against records contained in GenBank using blastn. Due to a low number of infected amphipods, statistical analyses between different treatments, drift and stationary individuals, experimental phases, and amphipod taxa were not possible. Thus, the presented descriptive analyses are based on information inferred from the phylogenetic position of different parasites to highlight possible unaccounted bias in mesocosm experiments.

2.8. *Statistical analysis*

2.8.1. *Response variables*

Six distinct community datasets (benthic macroinvertebrate community, leaf-bag macroinvertebrate community, macroinvertebrate drift, eukaryotic algae in biofilm, parasites detected in biofilm, and conidia of aquatic fungi) and ecosystem functioning data were analyzed. For all metabarcoding datasets, OTU richness was calculated. The un- biased Simpson diversity index (Hurlbert, 1971) was used as an alpha- diversity metric if abundance data could be estimated. In the case of benthic and leaf-bag macroinvertebrate metabarcoding data, read counts and abundances cannot be assumed to be correlated due to a variety of species-specific biases (Lamb et al., 2019), such as different biomass between species (Elbrecht and Leese, 2015), primer biases (Stadhouders et al., 2010), and differential mitochondrial copy numbers. In addition, OTU richness among the sensitive insect orders Ephemeroptera, Plecoptera, and Trichoptera (EPT) was calculated. For drift, total abundance and taxon-specific drift for the five most abundant families was expressed as individuals per day. For aquatic fungi, ergosterol concentrations in μg/mg DM, total sporulation rate expressed as spores per mg DM, and taxon-specific sporulation rate for the four most abundant species was calculated.

Ecosystem functioning metrics for CPOM decomposition (leaf decomposition in leaf bags and leaf discs representing microbial-driven decomposition, tensile-strength loss in cotton strips) were expressed as % loss per day relative to the starting dry mass or tensile strength. Microbial respiration rate was expressed as mg O_2 per mg DM per hour and chlorophyll *a* concentration as mg per cm².

2.8.2. *Predictors*

As for salinity, the mesocosm-specific median conductivity across measurements was used as a proxy. The median was chosen over the mean to mitigate the influence of outliers on the predictor's value. Mesocosm-specific conductivity was chosen over the treatment-specific conductivity because mesocosm-specific medians performed significantly better as predictors of conductivity in a simple linear model according to Akaike's Information Criterion ($\Delta AIC > 2$). To assess whether high maximum conductivities in a given mesocosm (> 5 mS/cm) led to outliers in any response, a visual examination of scatter plots with the respective mesocosms being highlighted was done before analysis. However, across all response variables, none of the outliers were associated with high maximum conductivities (Supplement 2). Salinity was centered and deviation coding was used for flow velocity to use Type III sums of squares in ANOVA analyses.

2.8.3. *Univariate statistical analysis*

All statistical analyses were conducted in R (v4.1.2, R Core Team, 2021) and R-Studio (v2023.6.1.524, Posit team, 2023) using the pack- ages tidyverse (v2.0.0, Wickham et al., 2019) and readxl (v1.4.2, Wickham and Bryan, 2023) to load and handle data. A variety of regression techniques were employed as an analytical framework. In all regression models, the predictors were flow velocity specified as a factor, salinity as a continuous variable, and a product term of both predictors to analyze interactions:

$$Y_i = \beta_0 + \beta_1 \times Flow_i + \beta_2 \times Salinity_i + \beta_3 \times Flow_i \times Salinity_i + \varepsilon_i$$

Here, ε represents the model residuals, β_0 the intercept, while β_1, β_2 and β_3 represent the model coefficients of flow velocity reduction, salinization and their interaction, respectively. The application of a simple linear regression model was preferred over regression models using link-functions because it allowed testing of the predictor interactions on the additive scale. In this case, the statistical null hypothesis aligns with the simple addition model sensu Schäfer and Piggott (2018), and the effects of the predictors are expected to add up. When analyzing changes in biodiversity such as species richness or abundances, this reflects absolute changes and is often considered more informative, whereas log and logit-link functions or transformations can result in a non-detection or misinterpretation of interactions on the additive scale (Spake et al., 2023).

Therefore, ordinary least squares regression was used, and model assumptions were checked using residual plots generated by the packages car (v3.1.2, Fox and Weisberg, 2019) and performance (v 0.10.4, Lüdecke et al., 2021), the Shapiro-Wilk test for normality, and the Breusch-Pagan test for heteroscedasticity. Only if violations of model assumptions were detected, count data were modeled with a Poisson regression using the log-link function, whereas percentages and the Simpson diversity index were modeled with the package betareg (v3.1.4, Grün et al., 2012), applying beta regression with a logit-link function. If overdispersion was detected for Poisson regression models, the standard error was corrected using the estimated dispersion parameter in a quasi-GLM. If outlier values in the response variable caused violations of model assumptions, quantile regression from the quantreg package (v5.95, Koenker, 2023) was applied modeling the median of the response in dependence of the predictors.

Type-III ANOVA was conducted using F-tests for ordinary least squares regression and likelihood ratio $\chi 2$-test for beta regression and (quasi-)Poisson regression. In the case of quantile regression, t-statics for the model coefficients were calculated, with standard errors estimated by bootstrapping with the xy-pairs method (Koenker, 1994) with 200 replications. In addition to test statistics, effect sizes were presented as model coefficients along with their 95 % confidence intervals. For ordinary least square regression and quantile regression, model coefficients represent the absolute change of mean and median, respectively, in the response variable caused by reduced flow velocity or by an increase of 1 mS/cm in salinity. Model coefficients from (quasi-) Poisson regression models and beta regression were converted to represent the ratio or odds ratio (OR) between reduced and normal flow velocity and between a salinity increase of 1 mS/cm and ambient salinity. For statistically significant interactions, the significance of the salinity effect was assessed under normal and reduced flow velocity conditions, using pairwise comparisons in the emmeans package (v 1.8.8., Lenth, 2022), and t-statistics or z-scores are presented for linear or non-linear models, respectively.

Interpretation of the ordinary least squares regression model statis- tics has to be done with care for those response variables where viola- tions of model assumptions have been detected. Nevertheless, p-values for the interaction term from ordinary least square regression were evaluated in addition to those from nonlinear models to have an approximation for potential interactions on the additive scale. Signifi- cant main effects ($p \leq 0.05$) and tendencies ($p \leq 0.1$) were plotted as marginal means, and interactions as conditional means along with 95 % confidence intervals using the packages emmeans (v 1.8.8., Lenth, 2022) and ggplot2 (v 3.4.3 Wickham, 2016) to aid interpretation. All model specifications and residual plots are presented in Supplement 2.

2.8.4. Multivariate statistical analysis

A PERMANOVA approach was used to analyze changes in commu- nity composition caused by the predictors. Distance matrices were first constructed with the package vegan (v2.6.4, Oksanen et al., 2022) using Jaccard distance on presence-absence data in macroinvertebrate communities (benthic habitat and leaf bag) or Bray-Curtis distances on relative abundances in periphyton, periphyton parasite, and aquatic fungi communities. Multivariate homogeneity of variances was tested with the betadisper function, and the PERMANOVA was subsequently applied with 10,000 randomizations using the RRPP package (v1.4.0, Collyer and Adams, 2018) to enable an ANOVA type III-like analysis (Supplement 3). Standardized distances (Z-scores) were presented along with test statistics.

2.8.5. OTU level analysis

As an exploratory analysis, OTU responses were modeled for all metabarcoding datasets using presence-absence data and binomial regression models with a logit-link function. Only OTUs occurring in 25 to 75 % of the mesocosms were chosen. The purpose of this analysis was to characterize the distribution of OTU response directions (increase or reduction in the occurrence probabilities) in each organism group. Since the product term in the logit model has no straight-forward interpretation, it was explicitly not included and only the predictors flow velocity and salinity were used. Qualitative descriptors of response strength were derived from the estimated odds ratio (OR) according to Rosenthal (1996). For salinity, response strength categorization depends on the specified units of the continuous predictor. We chose to refer to a response caused by a salinity increase of 1 mS/cm, because this already resulted in brackish water conditions (> 1.5 mS/cm) at the Boye River.

For increasing occurrence probabilities, the minimum OR for a weak response is 1.5:1, for a moderate response is 2.5:1, for a strong response is 4:1, and for a very strong response is 10:1 (Rosenthal, 1996). If occurrence probabilities were decreasing, the maximum OR for a weak, moderate, strong, and very strong response were 1:0.667, 1:0.4, 1:0.25, and 1:0.1, respectively. All p-values were fdr-corrected (false discovery rate).

3. Results

Overall, flow velocity had stronger effects than salinity and altered macroinvertebrate, algal, and parasite community composition while also decreasing ecosystem functions (Table 2). Salinity primarily affected fungal sporulation rates and macroinvertebrate drift behavior. However, none of the assessed communities showed compositional re- sponses to the salinity gradient.

The raw data on taxonomic composition for each of the datasets (OTU tables for benthic and leaf-bag macroinvertebrates, eukaryotic algae and parasites, macroinvertebrate drift, fungal spores, and parasite infections in amphipods), and binomial regression results for all OTU responses are deposited in the Open Science Framework (doi.org/10,.17605/OSF.IO/247JC).

3.1. Macroinvertebrates

Metabarcoding of benthic and leaf-bag samples generated a total of 134,988,797 and 305,718,958 reads, respectively, with 0.639 % and 0.000 % of the reads found in negative controls. After bioinformatic processing and taxonomic filtering, 114,634,668 and 39,317,146 reads remained in the final datasets, with a total of 631 and 474 macroinvertebrate OTUs in the benthic and leaf-bag habitat. In both habitats, the order Diptera (flies and mosquitoes) comprised the highest proportion of OTUs (benthic: 61.7 %, leaf bag: 57.6 %), followed by Tubificida (freshwater worms, benthic: 10.8 %, leaf bag: 8.1 %), and Podocopida (ostracods, 5.2 %) in the benthic habitat or Coleoptera (beetles, 4.9 %) in the leaf bags.

No effect of applied stressors on OTU richness was detected

Table 2

Summary (p-values and effect sizes) of regression models for effects of salinity and flow velocity on biological communities and ecosystem functions. Summary statistics indicate the overall sample mean ± standard error and the sample size in brackets. Significant *p*-values ($p \leq 0.05$) are highlighted in bold font. *P*-values ≤0.1 are highlighted with italic font. Effect sizes are presented along with 95 % confidence intervals in square brackets. Depending on the applied regression model, the effect refers to the absolute difference in means (Δ) or medians (Δmed), the ratio (Ξ), the odds ratio (OR) or the *Z*-score (Z) associated with a predictor, and arrows indicate the direction of an effect. In the case of flow velocity, the effect sizes refer to the change produced by reduced flow velocities relative to normal flow velocities. For salinity, the effect sizes represent the change caused by an increase by 1 mS/cm during salt pulses. For interactions, the effect represents the change in the effect of salinity caused by a reduction in flow velocity. The second p-value in brackets for non-additive regression models refers to the result of ordinary least square regression to indicate if stressor interactions occur on the additive scale. Abbreviations: DM (dry mass), EPT (Ephemeroptera, Plecoptera, Trichoptera), MI (macroinvertebrates), OTU (operational taxonomic unit).

Flow velocity						Salinity			Flow velocity: Salinity	
Response variable	Summary	Effect	Effect size	p		Effect size	p		Effect size	p
Benthic MI OTU richness	186.5 ± 3.3 (63)	Δ	−2.6 [—16.0, 10.8]	0.699		4.7 [—5.1, 14.6]	0.340		−7.0 [—26.6, 12.7]	0.481
Benthic EPT OTU richness	7.8 ± 0.4 (63)	Ξ	*0.851* *[0.712, 1.017]*	*0.076*	↓	0.949 [0.832, 1.082]	0.435		0.907 [0.697, 1.179]	0.466 (0.524)
Benthic MI community	NA (63)	Z	**3.744**	**<0.001**		−1.071	0.860		−1.652	0.950
Leaf-bag MI OTU richness	128.9 ± 4.1 (63)	Ξ	0.928 [0.817, 1.054]	0.250		1.026 [0.935, 1.125]	0.592		1.032 [0.857, 1.241]	0.741 (0.757)
Leaf-bag EPT OTU richness	3.9 ± 0.3 (63)	Δ	−0.1 [—1.1, 0.9]	0.832		−0.4 [—1.1, 0.3]	0.287		0.3 [—1.2, 1.8]	0.696
Leaf-bag MI community	NA (63)	Z	**2.036**	**0.021**		−1.286	0.901		−0.769	0.778
Drift, MI abundance (d^{-1})	15.8 ± 0.5 (64)	Δ	1.2 [—0.7, 3.0]	0.212		**2.0** **[0.7, 3.4]**	**0.004**	↑	0.1 [—2.6, 2.8]	0.948
Baetidae (d^{-1})	2.0 ± 0.2 (64)	Ξ	**2.258** **[1.814, 2.829]**	**<0.001**	↑	**1.17** **[1.000, 1.368]**	**0.050**	↑	1.19 [0.871, 1.629]	0.275 *(0.097)*
Chironomidae (d^{-1})	7.5 ± 0.3 (64)	Ξ	**0.84** **[0.707, 0.997]**	**0.046**	↓	1.062 [0.937, 1.204]	0.345		0.927 [0.721, 1.191]	0.554 (0.467)
Gammaridae (d^{-1})	1.5 ± 0.2 (64)	Ξ	**2.304** **[1.471, 3.737]**	**<0.001**	↑	1.331 [0.973, 1.826]	*0.074*	↑	0.762 [0.404, 1.423]	0.393 (0.876)
Hydroptilidae (d^{-1})	1.9 ± 0.1 (64)	Δ	0.0 [—0.4, 0.5]	0.843		**0.4** **[0.0, 0.7]**	**0.034**	↑	−0.1 [—0.8, 0.6]	0.717
Naididae (d^{-1})	1.4 ± 0.1 (64)	Ξ	*0.798* *[0.627, 1.016]*	*0.067*	↓	1.143 [0.957, 1.362]	0.139		1.211 [0.851, 1.723]	0.287 (0.382)
Drift, MI Simpson index	0.708 ± 0.012 (64)	OR	**1.527** **[1.267, 1.842]**	**<0.001**	↑	**1.161** **[1.016, 1.327]**	**0.028**	↑	1.168 [0.895, 1.525]	0.253 (0.408)
Biofilm, algal OTU richness	498.9 ± 10.0 (64)	Δ	−6.1 [—47.1, 34.9]	0.767		1.9 [—28.0, 31.8]	0.899		14.9 [—45.0, 74.8]	0.621
Biofilm, algal Simpson index	0.925 ± 0.002 (64)	OR	0.971 [0.888, 1.062]	0.516		0.972 [0.911, 1.038]	0.400		1.014 [0.890, 1.155]	0.832 (0.785)
Biofilm, algal community	NA (64)	Z	**4.037**	**<0.001**		0.243	0.398		−1.002	0.837
Biofilm, parasite OTU richness	222.2 ± 7.1 (64)	Δ	−20.0 [—48.3, 8.3]	0.163		1.1 [—19.5, 21.8]	0.914		20.4 [—20.9, 61.7]	0.328
Biofilm, parasite Simpson index	0.934 ± 0.002 (64)	Δ_{med}	**0.012** **[0.006, 0.020]**	**0.001**	↓	0.003 [—0.005, 0.006]	0.347		0.002 [—0.005, 0.017]	0.701
Biofilm, parasite community	NA (64)	Z	**3.576**	**<0.001**		0.196	0.413		−0.725	0.759
Fungi, ergosterol concentration (μg g^{-1} DM)	48.3 ± 2.8 (62)	Δ_{med}	−3.3 [—14.3, 11.3]	0.603		−0.9 [—10.2, 7.1]	0.821		1.6 [—15.4, 19.9]	0.842
Fungi, sporulation rate (mg^{-1} DM d^{-1})	1798.4 ± 100.9 (61)	Δ	−100.4 [—486.2, 285.4]	0.604		**¡364.4** **[¡640.6, ¡88.2]**	**0.011**	↓	326.1 [—226.3, 878.5]	0.242
Amniculicola longissima (mg^{-1} DM d^{-1})	41.5 ± 3.6 (61)	Ξ	1.058 [0.743, 1.504]	0.752		**0.764** **[0.589, 0.980]**	**0.034**	↓	0.993 [0.595, 1.648]	0.978 (0.646)
Clavariopsis aquatica (mg^{-1} DM d^{-1})	102.2 ± 8.5 (61)	Ξ	1.108 [0.810, 1.519]	0.521		0.863 [0.686, 1.079]	0.199		**1.822** **[1.161, 2.875]**	**0.009** **(0.027)**
Sigmoïd 1 (mg^{-1} DM d^{-1})	1545.5 ± 88.5 (61)	Ξ	0.999 [0.807, 1.236]	0.995		**0.811** **[0.694, 0.944]**	**0.007**	↓	1.23 [0.904, 1.673]	0.188 (0.258)
Tetracladium marchalianum (mg^{-1} DM d^{-1})	63.2 ± 7.3 (61)	Ξ	0.789 [0.503, 1.220]	0.288		**0.612** **[0.439, 0.837]**	**0.002**	↓	1.043 [0.542, 1.972]	0.897 (0.827)
Fungi, Simpson index	0.258 ± 0.009 (62)	Δ_{med}	0.020 [—0.030, 0.076]	0.408		−0.016 [—0.050, 0.023]	0.397		0.007 [—0.074, 0.072]	0.848
Fungi, community composition	NA (62)	Z	−0.679	0.745		0.467	0.322		1.13	0.136
Microbial respiration rate (mg O$_2$ g^{-1} h^{-1})	0.299 ± 0.004 (64)	Δ	**¡0.027** **[¡0.043, ¡0.011]**	**0.001**	↓	−0.006 [—0.018, 0.005]	0.292		0.005 [—0.018, 0.029]	0.657
Tensile-strength loss rate (% d^{-1})	2.011 ± 0.043 (64)	Δ	**¡0.404** **[¡0.546, ¡0.262]**	**<0.001**	↓	−0.049 [—0.153, 0.054]	0.346		−0.017 [—0.224, 0.191]	0.873
Leaf pack, decomposition rate (% d^{-1})	0.743 ± 0.026 (63)	Δ	*−0.089* *[—0.189, 0.012]*	*0.082*	↓	0.054 [—0.018, 0.127]	0.140		−0.033 [—0.178, 0.112]	0.653
Leaf discs, decomposition rate (% d^{-1})	1.400 ± 0.060 (62)	OR	0.916 [0.784, 1.071]	0.272		1.094 [0.978, 1.224]	0.114		1.172 [0.937, 1.466]	0.166 (0.210)
Chlorophyll a concentration (mg cm^{-2})	2.717 ± 0.121 (64)	Ξ	0.894 [0.748, 1.068]	0.214		1.039 [0.912, 1.183]	0.560		1.077 [0.830, 1.397]	0.570 (0.628)

(Table 2), yet, richness among the sensitive orders Ephemeroptera, Plecoptera, and Trichoptera (EPT; mayflies, stoneflies, and caddisflies) tended to be lower under reduced flow velocity conditions ($\chi^2_1 = 3.144$, $p = 0.076$, Fig. 2A) in the benthic samples. Macroinvertebrate community composition based on presence-absence data strongly changed with flow velocity (benthic habitat: $F_{1,59} = 2.178$, $p < 0.001$; leaf bag: $F_{1,59} = 1.534$, $p = 0.021$) while being unaffected by salinity (Table 2).

In the drift samples, 2958 individuals were counted and allocated to 34 macroinvertebrate families. The five most abundant families, which made up 90 % of the drift (47.8 % Chironomidae, 12.6 % Baetidae, 12.3 % Hydroptilidae, 9.4 % Gammaridae, 9.1 % Naididae), were analyzed in addition to the aggregate metrics. Diversity in drift samples was higher for reduced flow velocity ($\chi^2_1 = 19.677$, $p < 0.001$, Fig. 2B) and increasing salinities ($\chi^2_1 = 4.833$, $p = 0.028$), while total drift abundances increased exclusively with higher salinities ($F_{1,60} = 9.062$, $p = 0.004$, Fig. 3A-D). In particular, the ephemeropteran family Baetidae ($\chi^2_1 = 3.858$, $p = 0.050$) and the trichopteran family Hydroptilidae ($F_{1,60} = 4.699$, $p = 0.034$) evaded higher salinities by drifting with the water current, and such a tendency was also observed for the amphipod family Gammaridae ($\chi^2_1 = 3.203$, $p = 0.074$). A potential stressor interaction on the additive scale was recorded for Baetidae ($F_{1,60} = 2.851$, $p = 0.097$); under normal flow velocity conditions, the number of drifting specimens remained constant ($z = 0.550$, $p = 0.583$), while drift abundances increased with higher salinities if flow velocity was reduced ($z = 2.557$, $p = 0.011$, Fig. 4A).

Contrarily, a reduction in flow velocity did not alter the number of drifting macroinvertebrates because the direction of flow velocity effects differed among taxa (Fig. 2D). While the families Baetidae ($\chi^2_1 = 56.354$, $p < 0.001$) and Gammaridae ($\chi^2_1 = 13.848$, $p < 0.001$) increased their propensity to drift, the dipteran family Chironomidae ($\chi^2_1 = 3.982$, $p = 0.046$) responded by reduced drift, and oligochaetes of the family Naididae showed a similar tendency ($\chi^2_1 = 3.359$, $p = 0.067$).

3.2. *Biofilm*

Metabarcoding of the biofilm generated a total of 78,757,274 reads with 1.98 % of the reads in negative controls. After bioinformatic processing and taxonomic filtration, 39,876,604 reads with 2097 OTUs remained in the eukaryotic algae dataset, and 2,692,710 reads with 1027 OTUs formed the parasite dataset. The most OTU rich groups among eukaryotic algae were Bacillariophyta (diatoms, 43.3 % of OTUs), Ulotrichales (green algae, 10.8 %), and Chrysophyceae (golden algae, 8.7 % of OTUs). In the parasite dataset, 1.9 % of the OTUs were metazoans with representatives from the classes Myxozoa, Platyhelminthes, Nematoda, and Annelida. The most diverse parasite groups were Oomycota (25.7 %), Amphifilida (18.8 %), and Chytridiomycotina (chytrid fungi, 14.2 %).

Algal OTU richness and diversity were unaffected by both stressors,

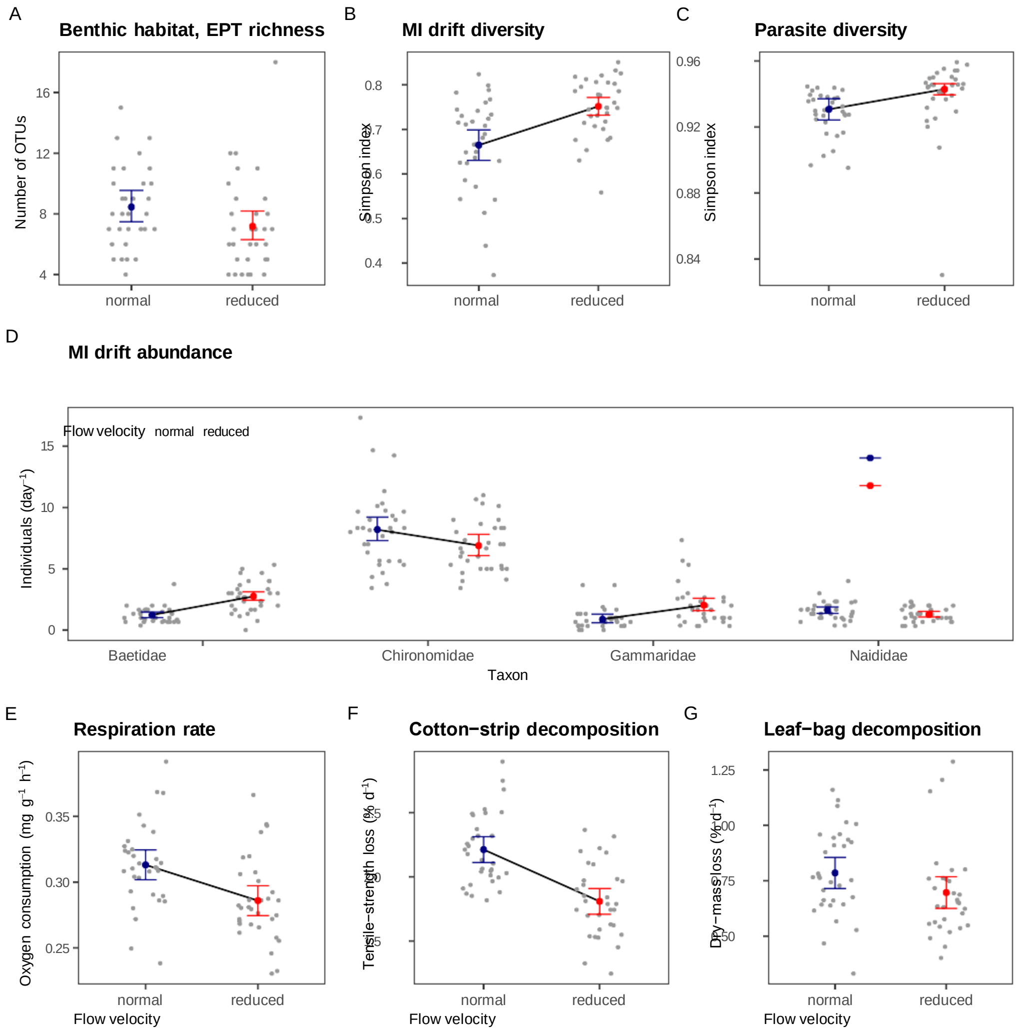

Fig. 2. Effects of flow velocity on community metrics, taxon-specific macroinvertebrate drift and ecosystem functions. Marginal means from (generalized) linear models are plotted along with their 95 % confidence interval. Significance ($p \leq 0.05$) is highlighted by a connecting line between the means, while such a line is missing for trends ($0.05 < p \leq 0.1$). Abbreviations: MI – macroinvertebrate.

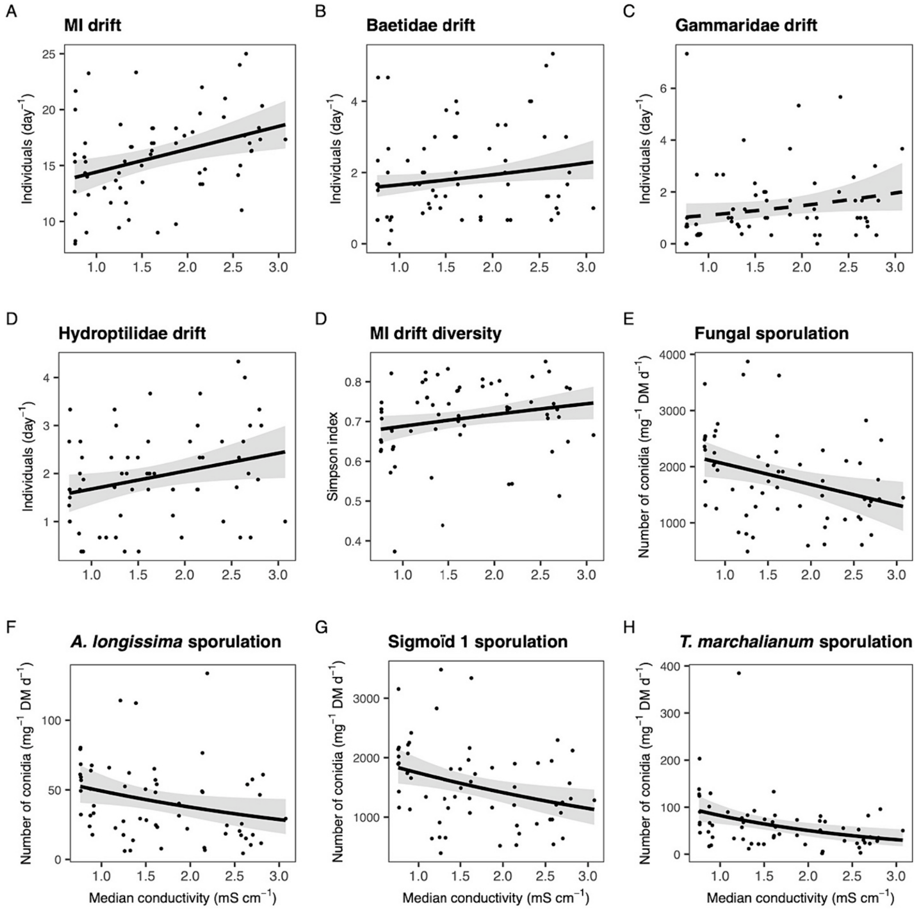

Fig. 3. Effects of salinity on community metrics, taxon-specific macroinvertebrate drift and taxon-specific fungal sporulation rates. Marginal effects from (generalized) linear models are plotted along with their 95 % confidence interval. Significant effects ($p \leq 0.05$) are plotted with a solid line and trends (0.05 $p \leq 0.1$) are plotted with a dashed line. Median conductivity refers to measurements during salt pulses. Abbreviations: MI - macroinvertebrate.

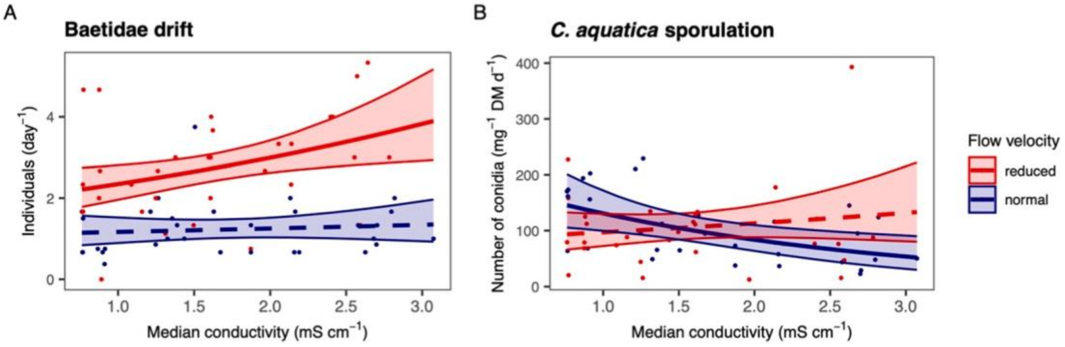

Fig. 4. Interaction effects between flow velocity and salinity on drift behavior of the ephemeropteran family Baetidae ($0.05 < p < 0.1$ on additive scale, $p > 0.1$ on logarithmic scale) and sporulation rates in the fungus ***Clavariopsis aquatica*** ($p < 0.05$ on both additive and logarithmic scale). Conditional salinity effects from (generalized) linear models are plotted along with their 95 % confidence interval. Dashed lines indicate a non-significant relationship in post-hoc tests, while solid lines indicate a significant relationship. Median conductivity refers to measurements during salt pulses.

while diversity of the parasite community increased with lower flow velocities ($t = 3.331$, $p = 0.001$, Fig. 2C). Both biofilm communities were affected in their composition only by a reduction in flow velocity (algae: $Z = 4.037$, $p < 0.001$, parasites: $Z = 3.576$, $p < 0.001$, Table 2).

3.3. Aquatic fungi

In total, 20 different fungal species were found by morphological determination among 88,130 counted conidia. The four most abundant species made up 97.4 % of all spores. These species were ***Amniculicola longissima*** (2.4 %), ***Clavariopsis aquatica*** (5.8 %), ***Tetracladium marchalianum*** (3.6 %), and an unidentified species hereafter referred to as Sigmoïd 1 (length: 60–70 μm, 85. 6 %). Contrary to the other organism groups, aquatic fungi did not respond to a reduction in flow velocity. Community composition, diversity, and total fungal biomass estimated by ergosterol concentrations remained unaltered by the stressors (Table 2). However, sporulation rates decreased with higher salinities ($F_{1,57} = 6.980$, $p = 0.011$, Fig. 3E-H). This negative response was observed consistently for the species *A. longissima* ($\chi^2_1 = 4.491$, $p = 0.034$), *T. marchalianum* ($\chi^2_1 = 9.732$, $p = 0.002$), and Sigmoid 1 ($\chi^2_1 = 7.322$, $p = 0.007$), while *C. aquatica* shows a context-dependent response ($\chi^2_1 = 6.800$, $p = 0.009$, Fig. 4B). Given normal flow velocities, its sporulation rate decreased with higher salinities ($z = 2.716$, $p = 0.007$), whereas this effect disappeared with reduced flow velocities ($z = 0.943$, $p = 0.346$).

3.4. Ecosystem functions

Organic-matter decomposition was measured in leaf bags and leaf discs as % loss of leaf mass and in a cotton-strip assay as % loss of tensile strength. The leaf discs and cotton strips largely represent microbial-driven decomposition, while the leaf bags were decomposed by both macro- and microorganisms. Reduced flow velocities negatively affected tensile-strength loss ($F_{1,60} = 32.314$, $p < 0.001$) and also tended to decrease leaf-bag decomposition ($F_{1,59} = 3.135$, $p = 0.082$, Fig. 2F, G), but decomposition of leaf discs did not show any evidence for stressor effects (Table 2). In addition, microbial respiration rates, measured from the cotton strips, exhibited a similar response pattern, showing lower respiration for reduced flow velocity ($F_{1,60} = 11.505$, $p = 0.001$, Fig. 2E). Primary production measured as chlorophyll *a* concentration was unaffected by all stressor conditions (Table 2).

3.5. Amphipod parasites

A total of 952 amphipods, identified as *Gammarus pulex* clade C, *Gammarus pulex* clade E, and *Gammarus fossarum* type B, were collected. Only a small proportion of amphipods (8.6 %, $n = 82$) was infected with species of the phylum Microsporidia. These belonged to 14 isolates ascribed to 3 different clades sensu Bojko et al. (2022) (see Supplement 4). Despite the large diversity of microsporidians found in the mesocosms, it was impossible to assess the influence of stressors due to low infections. Nevertheless, three microsporidian isolates new to science were detected among the infected amphipods. The undescribed microsporidian isolates, here named Microsporidium sp. EX01, Microsporidium sp. EX02, and Microsporidium sp. EX03, all belonged to the Enterocytozoonida clade. The closest isolates were, respectively, Microsporidium sp. BVIO (FJ756176; 98.1 % similarity), Microsporidium sp. BVOH10 (FJ756195; 98.6 % similarity), and Microsporidium sp. OTU51 (MG241431; 94.6 % similarity). Large amphipods (4th plate length above 2 mm, $n = 377$) had the bulk of infections (14.5

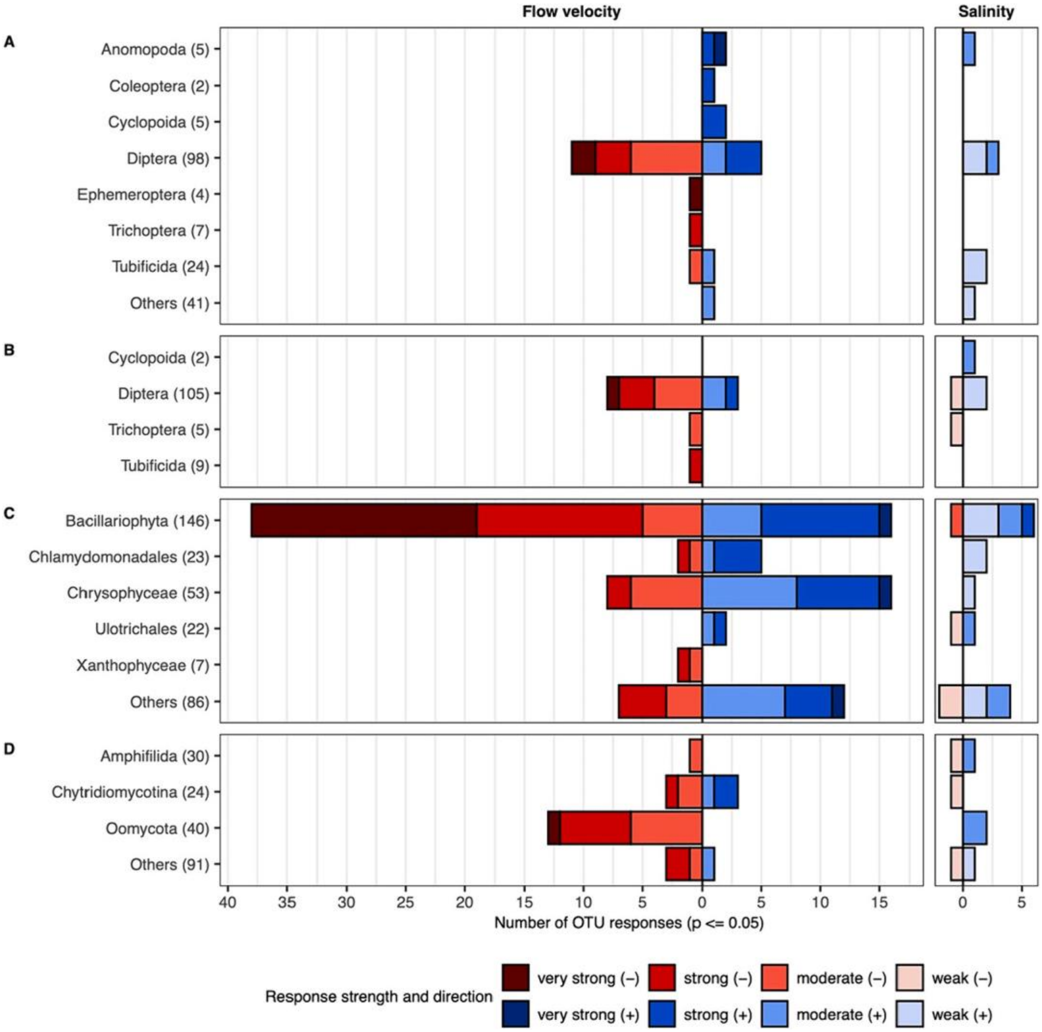

Fig. 5. Number of OTU responses ($p \leq 0.05$, false discovery rate adjusted) to flow velocity and salinity. Negative effects on the probability of presence of an OTU by the stressors flow velocity reduction (left panels) and salinization (right panels) are marked in red, and positive responses are marked in blue. The strength of a response is categorized based on odds ratios, according to Rosenthal (1996). For salinity, response strength refers to the relative change in odds caused by a salinity increase of 1 mS/cm during salt pulses, for flow velocity reduction it refers to the relative change in odds between normal and reduced flow velocity conditions. Counts of OTU responses were aggregated on order level or higher, if no order-level taxonomic assignment existed. The number of OTUs tested is given in brackets. A: Benthic macroinvertebrate community. B: Leaf-bag macroinvertebrate community. C: Algal community in biofilm. D: Parasite community in biofilm.

%) and hosted the most diverse parasite community with 12 different microsporidian isolates.

3.6. Exploratory analysis on OTU responses

The majority of OTUs were too rare (benthic macroinvertebrates: 59.3 %, leaf-bag macroinvertebrates: 61.2 %, algae: 71.2 %, parasites: 73.2 %) or too common (14.1 %, 10.5 %, 13.2 %, and 11.8 %, respectively) to allow for statistical analyses for their probability of presence. Only stressor responses for OTUs that occurred in 25 to 75 % of the mesocosms were assessed. This was the case for 168 macroinvertebrate OTUs from the benthic habitat, 134 from the leaf-bag habitat, 328 algal, and 154 parasite OTUs.

A reduction in flow velocity mostly caused decreases in the probability of presence for OTUs, while responses to salinity were exclusively positive, though rarely detected (Fig. 5). In contrast to the other organism groups, macroinvertebrates in the benthic habitat showed a similar number of positive and negative responses to reduced flow (Fig. 5A). OTUs belonging to flies and mosquitoes (Diptera) had varying response patterns, while only negative responses were reported for mayflies (Ephemeroptera) and only positive ones for microcrustaceans (Anomopoda and Cyclopoida). In the leaf-bag habitat, reduced flow velocity caused exclusively negative responses, affecting dipteran and freshwater worm (Tubificida) OTUs (Fig. 5B). Among eukaryotic algae, the majority of responses to reduced flow velocity was negative with a distinctly high number of very strong negative responses (OR < 0.1:1) being detected among diatom OTUs (Bacillariophyta, Fig. 5C). Positive responses to reduced flow velocity were also observed for diatom OTUs and all OTUs belonging to green algae (Chaetopeltidales and Chlorophyta) and golden algae (Chrysophyceae). Two diatom OTUs responded positively to salinity. Parasite responses to flow velocity were negative and detected for oomycete and fluke (Trematoda) OTUs (Fig. 5D). A positive response to salinity was also detected for an oomycete OTU.

4. Discussion

Climate and land use change are predicted to drive salinization of urban streams (Olson, 2018), while higher frequencies of drought pose an additional risk by altering flow parameters (Woodward et al., 2010). Here, we investigated the impacts of freshwater salinization (gradient from 0.8 to 2.8 mS/cm median conductivity during daily 9 h pulses) and flow velocity reduction (20 cm/s vs. 10 cm/s) across organism groups and functional metrics in an urban stream. Although different groups of organisms provide complementary information on the state of an ecosystem, mesocosm studies rarely consider more than one to two target groups. In our study, we employed a comprehensive approach by assessing multiple-stressor impacts across organism groups and on ecosystem functions. We found that flow velocity determined community composition of nearly all aquatic organism groups and affected ecosystem functions, while salinization exerted a lower impact on an urban stream. However, we caution that the weak effects of salinity in our 2-week stressor phase can cause more substantial effects in the long term.

4.1. Comparison of stressor importance

Although both applied stressors are known to have a strong impact on stream ecosystems (flow velocity: Bondar-Kunze et al., 2021; Hall et al., 2019; Juvigny-Khenafou et al., 2021; salinity: Cañedo-Argüelles et al., 2013; both: Beermann et al., 2018b; Sundermann et al., 2013), we only found evidence for impairment of ecosystem functions and structural changes in biological communities for reduced flow velocity. The underlying mechanisms by which reductions in flow velocity affect organisms are manifold (reviewed in Dewson et al., 2007), including direct effects through lowered shear stress, as well as indirect effects through limited supply of nutrients and oxygen and promotion of sedimentation, thereby changing microhabitat structure and filling interstitial spaces. This variety of mechanisms is reflected by the pervasiveness of the observed flow velocity effects on community structure across organism groups (macroinvertebrates, algae and parasites) as well as on some of the associated ecosystem functions (microbial respiration, cotton-strip decomposition and, as a trend, leaf decomposition in coarse-mesh bags). Ultimately, structural changes in community composition can impair crucial functions (Oliver et al., 2015), and even jeopardize ecosystem integrity. Decomposition of organic matter makes allochthonous resources from adjacent terrestrial ecosystems available for the aquatic food web (Ferreira et al., 2020) and its disruption severely limits the availability of basic resources. Respiration rate represents the activity of heterotrophic and autotrophic microbes (Tiegs et al., 2013), which in turn are involved in nutrient and carbon cycling. Thus, our findings highlight the critical role that flow velocity plays for stream ecosystem integrity.

In contrast to our observations, freshwater salinization frequently has a more severe impact than other abiotic stressors in experimental studies and generally exerts adverse effects on aquatic organisms (Velasco et al., 2019). We found negative responses to salt exclusively in abundance metrics representing dynamic aspects of community assembly, i.e. macroinvertebrate drift (migration) and fungal sporulation rates (reproduction). Spore production by aquatic hyphomycetes is particularly responsive to a variety of anthropogenic alterations (Lecerf and Chauvet, 2008) and can therefore be interpreted as an early stress indicator. Likewise, the number of drifting macroinvertebrates detects more nuanced changes in populations compared to presence-absence metrics. Previous experiments have detected a largely consistent inverse relationship between drift-related stressor responses and those derived from final macroinvertebrate abundance in mesocosm experiments (e.g. Beermann et al., 2018a; Blöcher et al., 2020; Magbanua et al., 2016; Piggott et al., 2015c). This implies that the number of drifting individuals can be used as a surrogate metric for final abundances in the mesocosm. While flow velocity effects were pronounced and altered macroinvertebrate community composition based on occurrences, salinization had a comparatively minor impact with increases in drift that imply population declines.

4.2. Stressor interactions

Overall, interactions between reduced flow velocity and salinization were rare and among the 32 response variables evaluated, we found only one interactive effect (antagonism for decreasing *C. aquatica* sporulation rate) and an additional interaction trend (synergism for increasing macroinvertebrate Baetidae drift abundance). Our observation is in agreement with a review by Velasco et al. (2019), who found that salinization more frequently shows additive than interactive effects with other stressors. In contrast, Kefford et al. (2023) have identified non-additive effects of conductivity with temperature, turbidity and terrain slope on EPT family richness. However, the occurrence and direction (synergism vs. antagonism) of these interactions were highly dependent on the environmental context, emphasizing that general statements concerning the potential of salinity for interactive effects cannot be made. The scarcity of deviations from additivity in our study indicates that the few detected stressor interactions likely did not occur on the abiotic level, i.e. one stressor modifying the intensity of the other or both affecting a third environmental variable (Boyd and Brown, 2015; Vos et al., 2023). Instead, we expect that the observed interactions occurred either on organism level and one stressor affected the organism's sensitivity to the other, or through interspecific interactions (Bray et al., 2018; Thompson et al., 2018). Therefore, we discuss potential mechanisms below within the context of organism groups.

4.3. Details on stressor responses in organism groups

4.3.1. Macroinvertebrates

For macroinvertebrates, negative effects on richness, diversity and abundance by flow velocity reduction are commonly reported (Beermann et al., 2018a; Blöcher et al., 2020; Elbrecht et al., 2016; Juvigny-Khenafou et al., 2021). EPT taxa (mayfly, stonefly and caddisfly larvae) have been shown to be particularly sensitive and prefer fast currents (Elbrecht et al., 2016), because slow currents cannot ensure a sufficient supply of oxygen for the larvae (Genkai-Kato et al., 2005) and higher sedimentation rates simultaneously interfere with respiration by clogging the organism's gills (Wood and Armitage, 1997). Accordingly, we observed community shifts when flow velocity was reduced, accompanied by greater drift abundances of baetid mayflies and, as a trend, a reduction in EPT richness in the benthic habitat. Modifications in habitat conditions due to reduced flow velocity also favor certain species (e.g. Elbrecht et al., 2016), if energy expenditure for locomotion and foraging is minimized, or if interspecific competition with more sensitive taxa is relaxed. For example, microcrustaceans are associated with slow flowing habitats in streams (Dole-Olivier et al., 2000), aligning with the positive microcrustacean OTU responses that we found in the benthic habitat. We observed that non-biting midges (Chironomidae) and freshwater oligochaetes (Naididae, not significantly) drifted less when flow velocity was reduced. However, this can also be explained by a reduction of random drift by dislocation, rather than an improvement in habitat suitability (Dewson et al., 2007).

Macroinvertebrates associated with the leaf-litter habitat might use it as a shelter and as a food source. Species that break down coarse particulate organic matter are categorized as ‘shredders’ and substantially contribute to leaf decomposition (Graça, 2001). We found exclusively negative OTU responses in the leaf bag to reduced flow velocity, and greater drift abundances of the freshwater amphipod family Gammaridae, which is a key shredder taxon (Englert et al., 2013). Shredders might be particularly sensitive to reduced flow velocity. For example, Cristiano and Di Sabatino (2023) demonstrated that shredder relative abundance was substantially lower in a slow flowing reach compared to a neighboring fast flowing one in a headwater section, and leaf decomposition rates were reduced. Likewise, in our experiment, macroinvertebrate-driven leaf decomposition tended to decrease. The parallel changes in macroinvertebrate communities and the lack of an effect of current alone on leaf fragmentation (5 cm/s vs. 20 cm/s in Ferreira et al., 2006) indicate that macroinvertebrate community shifts ultimately caused a deceleration of leaf decomposition rate.

In contrast, we found only limited evidence for salinity affecting macroinvertebrates in the present experiment. Field studies have reported community shifts starting at 1 mS/cm (Horrigan et al., 2005; Schröder et al., 2015), which was in the first quarter of the salinity gradient applied here. Even though we cannot exclude the possibility that abundance instead of presence-absence metrics would have detected community changes in the benthic and leaf-bag habitat, the lack of an identifiable response to salt for richness of sensitive EPT taxa is surprising. For example, other studies have reported losses of a quarter of genera belonging to such salt-sensitive orders when water conditions become brackish (Cormier et al., 2013).

Our drift metrics, however, captured salinity responses. Diversity of drifting invertebrates increased with salinity, suggesting that salt-sensitive individuals have emigrated in search for more favorable conditions. The latter was the case for baetid mayfly and hydroptilid caddisfly larvae, both being families that have previously been identified as salt-sensitive (Baetidae: Beermann et al., 2018a; Szőcs et al., 2012; both: Cañedo-Argüelles et al., 2012). Surprisingly, the freshwater crustacean Gammaridae tended to follow a similar pattern. Even though crustaceans are considered fairly tolerant to salinization due to their marine ancestry (Kefford et al., 2016; Le et al., 2021; Szőcs et al., 2014), highly differential gene regulation with increased salinities in *Gammarus fossarum* indicate physiological costs of acclimation to salinized environments (Brasseur et al., 2022) that can result in drift responses. Finally, we found a trend for a positive synergistic interaction (sensu Piggott et al., 2015b) between the two stressors affecting baetid drift. Greater baetid drift rates for salinization were augmented by reduced flow velocities, and the same response pattern was reported by Beermann et al. (2018a). As a response to reduced flow velocity, baetid mayfly larvae might have increased their respiratory gill movements to enable sufficient oxygen supply, which in turn increased exposure of their respiratory surfaces to salt ions, potentially causing local Na^+ poisoning (Kefford, 2019).

4.3.2. Eukaryotic algae

Changes in community composition by reduced flow velocities were particularly strong for eukaryotic algae. Filamentous green algae are susceptible to shear stress and dislodgement given high flow velocities (Dewson et al., 2007; Grimm and Fisher, 1989). Therefore, high streamflow favors prostrate diatoms in the periphyton, but filamentous green algae become dominant if flow recedes in nutrient rich streams (Suren et al., 2003). This pattern is also reflected in the periphyton communities exposed to reduced flow velocity in our experiment, since diatom OTUs mostly decreased in occurrences, while green algae and golden algae increased in occurrences.

Diatom species show pronounced differences in salinity preferences and sharp community shifts are commonly reported for 0.37 or 7.9 mS/cm and higher (reviewed in Stenger-Kovács et al., 2023). Thus, the lack of a response to elevated salinities in our eukaryotic algal community metrics can be partly explained by the fact that the salinity gradient explored here started above the low threshold, while ending far from the high threshold. If ambient salinities are elevated, periphyton communities show only weak responses to additional salt pollution (80 mg/L chloride in Costello et al., 2018; >0.725 mS/cm in Schröder et al., 2015), which potentially applies to many urban streams. The only responses to salinization which we detected in this organism group were increased occurrences of two diatom OTUs. In fact, moderate levels of salinization might favor salt-tolerant brackish-water species without extirpating more sensitive ones and result in higher diatom OTU richness (Nuy et al., 2018). An increased production of polymeric substances in the biofilm can protect sensitive species in the biofilm against the adverse effects of salinity (Decho, 2000; Steele et al., 2014).

4.3.3. Parasites

Parasites are rarely considered in multiple-stressor studies (He et al., 2023), even though they are useful biological indicators given their dependence on hosts (Lafferty and Kuris, 1999; Sures et al., 2017). Multiple stressors can directly impact free-living parasite stages or the hosts they rely upon, indirectly hindering life-cycle completion (Lafferty and Kuris, 1999) or facilitating infection (e.g. Buss and Hua, 2018). As a matter of fact, a variety of different traits including life-cycle complexity, *endo*- and ectoparasitism, host specialization and mode of transmission affects their ability to persist in degraded environments (Sures et al., 2023). However, some parasites may modulate the ability of their host to withstand multiple stressors by decreasing (Litchman and Thomas, 2023) or increasing (Piscart et al., 2007) their host's tolerances, or by altering its behavior (Sures et al., 2023). For instance, we detected microsporidian parasites, which are suspected to alter host drifting behavior (see Prati et al., 2023), in amphipods, suggesting that other hosts might potentially harbor similar parasites. Although not quantified, such alterations might partially account for some variability in the data as parasites are a known source of bias in ecological studies (Litchman and Thomas, 2023). It should be noted that the parasite metabarcoding dataset contains exclusively parasitic taxa, such as Apicomplexa, Trypanosomatida, Myxozoa or Trematoda, but also taxa that include not only parasites, but also organisms of other life strategies, like the Oomycota, Labyrinthulomycetes or Chytridiomycota. Nevertheless, we found the compositional changes in responses to anthropogenic stressors in the considered relevant parasite taxa to be similarly strong.

In addition, diversity increased with reduced flow velocity, which can be explained by higher infection rates due to a potential accumulation of infective stages in slow moving water (Marcogliese, 2016) and an enhanced susceptibility of organisms that languish in reduced flow conditions (Lenihan et al., 1999).

4.3.4. Fungi and microbial functions

In contrast to the other organism groups we assessed, fungi were only affected by salinity. This contradicts evidence from multiple studies reporting a negative effect of reduced flow on fungal spore production, richness, biomass and microbe-mediated litter decomposition (Bruder et al., 2016; Ferreira and Graça, 2006; Schlief and Mutz, 2009; but see Colas et al., 2016). Interestingly, our two metrics of microbial-driven organic-matter decomposition responded differently, as tensile- strength loss was affected by a reduction in flow velocity, while leaf disc decomposition was not. Multiple not mutually exclusive explana- tions can be given for this discrepancy. First, cotton strips have shorter incubation times than leaves (Ferreira et al., 2020), therefore, differential mass loss in leaf discs might simply not have become sufficiently pronounced to be detected. Second, the cotton strips were accessible for macroinvertebrates and, even though direct feeding on the cotton material is unlikely (Tiegs et al., 2013), developing biofilms might be grazed by them, resulting in top-down effects on the cotton strip. Third, the protective polyester mesh might have decreased the differentiation between flow velocity conditions and lowered abrasion by sediment (Tomczyk et al., 2022). The reduced respiration rate in cotton strips give further evidence for impairment of microbial functions by reduced flow velocities, indicating that the employment of fine-mesh bags might underestimate the effect of flow parameters on microbial communities and associated ecosystem functions, either by disrupting biological interactions or by protecting the leaf material from adverse effects of reduced flow.

Salinization inhibited spore production for three of the four most abundant species (*A. longissima*, *T. marchalianum* and Sigmoïd 1), while fungal biomass and microbial-driven decomposition remained un- changed. Berger et al. (2018) have found that leaf decomposition rates were consistently reduced by salinization across mesocosm studies and field surveys, however, this relationship was ambiguous given salinity ranges covering <3 mS/cm. Since the highest salinity explored in our study falls within this lower range, the results of our study are in no direct contrast with the general trend stated by Berger et al. (2018) and most probably relate to the stability of fungal biomass. The phenomenon of decreasing sporulation rates combined with constant fungal biomass accrual was also observed by Canhoto et al. (2017) across a wide salinity gradient (0 to 9.7 g/L Cl$^-$). In their study, *C. aquatica* sporulation rate was resistant to salinization and did not change up to an addition of 1.2 g/L Cl$^-$. Our results indicate context-dependency of this species' sensitivity to salt, since low flow velocity mitigated the negative salinization effect on its reproductive activity. We speculate that competitive and mutualistic interactions with other microbes (Khan, 1987; Romaní et al., 2006) are responsible for the observed stressor interaction.

4.4. Limitations of our study

We conducted our experiment at a restored stream reach in an urban area that has been impacted by coal mining in the past. The concept of 'ecological memory' (Padisak, 1992) or 'stressor legacy' (Jackson et al., 2021) suggests that past ecosystem states affect the response of biological components to current stressors. Therefore, historic salt pollution by coal mining, as it was observed for other German rivers (e.g. Petruck and Stöffler, 2011; Schulz and Cañedo-Argüelles, 2019), might have filtered for salt-tolerant taxa or caused evolutionary adaptation in local populations. Such local adaptations have been suggested by Schröder et al. (2015) in the formerly salt polluted Lippe catchment. Despite being typically referred to as salt sensitive, the caddisfly *Psychomyia pusilla* and the freshwater snail *Ancylus fluviatilis* showed increasing population sizes if salinity surpassed 1.6 mS/cm or 1.0 mS/cm, respectively (Schröder et al., 2015). However, evidence for populations adapting to salinity is mixed (Cañedo-Argüelles et al., 2019), and Kefford et al. (2012) found remarkably similar family-specific salt tolerances between macroinvertebrate populations of regions with different aridity. Therefore, the explanation that salt sensitive species have been removed from the system in the past appears to be more likely. Nevertheless, evidence for local evolutionary adaptation would be of considerable interest. Research in the Boye catchment could be directed towards comparing salt sensitivity in populations of typically sensitive taxa (e.g. mayfly or stonefly species) with conspecific populations from a catchment lacking any salt pollution history.

Furthermore, salt application in our study can be characterized as a daily recurring 9 h pulse exposure. This was not a design choice, but silt transported with the stream water involved the risk of blockages of the valve controlling the water supply for the mesocosms. Since salt application was not coupled to stream water supply, we decided to limit salt stress to those time frames during which we could closely monitor the system. As urban watersheds are characterized by highly variable conductivities (Zarnaghsh and Husic, 2023), the results of our experiment are still biologically relevant. Nevertheless, permanent salt exposure will likely cause different responses: Weaker effects of pulsed compared to continuous exposure may be expected if organisms are able to recover between salt pulses. On the other hand, pulse application could prevent acclimatization thereby causing elevated stress due to frequently changing environmental conditions. Experiments comparing pulse and continuous exposure suggest that recovery in between pulses facilitates resilience to salt-induced stress in macroinvertebrates, algae and fungi (3 h pulses, 2–3 days recovery: Cañedo-Argüelles et al., 2014; 2 days pulse, 5 days recovery: Canhoto et al., 2023), however, similar effects between the two stressor regimes have also been reported for biofilms (30 min pulse, 24 h recovery: Cochero et al., 2017). Ultimately, the difference between stressor regimes should depend on pulse duration and recovery time between pulses.

4.5. Conclusions and implications for urban stream management

By a comprehensive analysis across organism groups, we found that flow velocity reductions restructured eukaryotic algal and macro- invertebrate communities, increased the diversity of parasites and dis- rupted decomposing ecosystem functions. This aligns with a substantial body of research highlighting the importance that flow parameters have for structuring limnetic communities and ensuring ecosystem func- tioning (e.g. Beermann et al., 2018a; Bruder et al., 2016; Cristiano and Di Sabatino, 2023; Dewson et al., 2007; Elbrecht et al., 2016). Therefore, safeguarding a minimum flow of surface freshwaters by limiting water abstraction combined with restoration and conservation of stream hydro-morphology (Gillmann et al., 2023; Lin et al., 2020) should be the priority to prevent deterioration also in urban stream ecosystems. We measured only subtle effects of a salinity gradient covering future predictions of salinization by land use and climate change (Olson, 2018). Nevertheless, we urge caution in interpreting salinization as a minor stressor for urban streams. First, the focal stream of this study may show legacy effects of historic salt exposures and effects on urban streams in a different setting may be more profound. Second, lowered fungal reproduction and increased macroinvertebrate emigration as observed for high salinities will most probably be exacerbated by chronic long-term exposures. Comprehensive analyses across organism groups like the current study help to set salinity thresholds, which, if exceeded, require mitigation measures to be taken. These include a reduction of de-icing salt applications and improved management strategies for sewage water, potentially also its deionization if this process becomes affordable (Cañedo-Argüelles et al., 2016).

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.171849.

CRediT authorship contribution statement

Iris Madge Pimentel: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Daria Baikova:** Investigation, Writing – original draft. **Dominik Buchner:** Investigation, Methodology, Software. **Andrea Burfeid Castellanos:** Investigation, Writing – original draft. **Gwendoline M. David:** Investigation, Writing – original draft. **Aman Deep:** Investigation, Software, Writing – original draft. **Annemie Doliwa:** Investigation, Writing – original draft. **Una Hadžiomerović:** Investigation, Writing – original draft. **Ntambwe A. Serge Mayombo:** Investigation, Writing – original draft. **Sebastian Prati:** Investigation, Writing – original draft. **Marzena Agata Spyra:** Investigation, Writing – original draft. **Anna-Maria Vermiert:** Investigation. **Daniela Beisser:** Software, Supervision, Writing – review & editing. **Micah Dunthorn:** Supervision, Writing – review & editing. **Jeremy J. Piggott:** Methodology, Supervision, Writing – review & editing. **Bernd Sures:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Scott D. Tiegs:** Resources, Writing – review & editing. **Florian Leese:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Arne J. Beermann:** Conceptualization, Investigation, Supervision, Writing – review & editing.

Declaration of competing interest

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

Data availability

Sequencing data, processed data and scripts used for analyses are publicly available at osf.io: DOI 10.17605/OSF.IO/247JC

Acknowledgements

This work was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) - CRC 1439/1 (RESIST) [grant number 426547801]. We deeply appreciate the support for field and laboratory work given by many helpers and by the Emschergenossenschaft und Lippeverband (EGLV). A special thanks to Alina Kirchhof, Isabel Nadine Zydek and Ronja Hollenberg for their dedicated efforts during macroinvertebrate sample sorting and to two anonymous reviewers for their comments, which have substantially improved the article. We acknowledge support by the Open Access Publication Fund of the University of Duisburg-Essen.

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Paper 2. Science of the Total Environment

Functional and compositional responses of stream microphytobenthic communities to multiple stressors increase and release in a mesocosm experiment,

Ntambwe Albert Serge Mayombo, Andrea M. Burfeid-Castellanos, Anna-Maria Vermiert, Iris Madge Pimentel, Philipp M. Rehsen, Mimoza Dani, Christina Jasinski, Marzena Agata Spyra, Michael Kloster, Danijela Vidaković, Dominik Buchner, Bánk Beszteri

Contributions:

- Conception 25 %: Followed the hypotheses and study design drawn in the grant application.
- Conduction of experimental work 100 %: Field work and lab work lead by NASM.
- Data analysis 100 %: data curation and formatting.
- Species identification 25 %: Shared with student helpers, which NASM supervised.
- Statistical analysis 90 %: Statistical analysis designed and mostly executed by NASM.
- Writing the manuscript 90 %: Introduction, Results, Discussion and most of the Materials and Methods written by NASM.
- Revision of the manuscript 90 %: Shared revision of all co-authors.

Signature of the Doctoral Candidate

Signature of the Doctoral Supervisor

Chapter II

Science of the Total Environment 943 (2024) 173670

Contents lists available at ScienceDirect

Science of the Total Environment

Functional and compositional responses of stream microphytobenthic communities to multiple stressors increase and release in a mesocosm experiment

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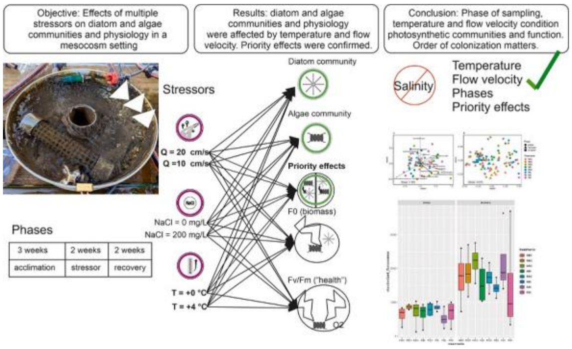
HIGHLIGHTS

- Multiple stressors (e.g. reduction in flow velocity, increase in salinity and temperature) threaten urban streams.
- Diatom and microalgae communities and their photosynthetic biomass were examined in a stream mesocosm experiment.
- Reduced flow velocity and increased temperature had a greater effect on microphytobenthic communities and biomass.
- Possible memory effect of salinity on eukaryotic microalgae assemblages.
- Following stressor treatment, recovery resulted in a convergence of community composition with priority effects.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Chlorophyll fluorescence
Diatoms
Microalgae ExStream
system Digital
microscopy
18S-V9 amplicon sequencing

ABSTRACT

Field observations form the basis of the majority of studies on microphytobenthic algal communities in freshwater ecosystems. Controlled mesocosm experiments data are comparatively uncommon. The few experimental mesocosm studies that have been conducted provide valuable insights into how multiple stressors affect the community structures and photosynthesis-related traits of benthic microalgae. The recovery process after the stressors have subsided, however, has received less attention in mesocosm studies. To close this gap, here we present the results of a riparian mesocosm experiment designed to investigate the effects of reduced flow velocity, increased salinity and increased temperature on microphytobenthic communities. We used a full factorial design with a semi-randomised distribution of treatments consisting of two levels of each stressor (2 × 2 × 2 treatments), with eight replicates making a total of 64 circular mesocosms, allowing a nuanced examination of their individual and combined influences. We aimed to elucidate the responses of microalgae communities seeded from stream water to the applied environmental stressors. Our results showed significant effects of

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https://doi.org/10.1016/j.scitotenv.2024.173670
Received 6 December 2023; Received in revised form 17 May 2024; Accepted 29 May 2024

reduced flow velocity and increased temperature on microphytobenthic communities. Recovery after stressor treatment led to a convergence in community composition, with priority effects (hypothesized to reflect competition for substrate between resident and newly arriving immigrant taxa) slowing down community shifts and biomass increase. Our study contributes to the growing body of literature on the ecological dynamics of microphytobenthos and emphasises the importance of rigorous experiments to validate hypotheses. These results encourage further investigation into the nuanced interactions between microphytobenthos and their environment and shed light on the complexity of ecological responses in benthic systems.

1. Introduction

Freshwater ecosystems are dynamic and rich in biodiversity. They are closely linked to all environmental ecosystems and are therefore vulnerable to anthropogenic activities in the catchment areas. These vital habitats face numerous challenges due to multiple anthropogenic stressors. In this context, any disturbance factor that causes environmental variables, individuals, populations, communities, or ecosystem functions to exceed the range of normal variation relative to undisturbed reference conditions is referred to as an anthropogenic stressor (Vos et al., 2023). Freshwater salinization is one of the most pressing stressors (Cañedo-Argüelles et al., 2013), and in addition to measuring and monitoring the ion contents of water bodies, it is important to know how salinization impacts ecosystems and their communities. Inland water salinization can occur naturally, as observed in permanent and ephemeral saline lakes where evaporation typically exceeds annual rainfall (Stenger-Kovács et al., 2023). This is referred to as primary salinization. Yet, secondary salinization of rivers and streams, resulting from human activities like agriculture, water withdrawal, road salt application, mining effluents, sea level rise, and climate change, can significantly alter freshwater communities (Cañedo-Argüelles et al., 2013; Cunillera-Montcusí et al., 2022). This human-induced salinization not only affects the structure and function of aquatic communities but also impacts the benefits, known as ecosystem services, that we derive from these ecosystems (Cañedo-Argüelles et al., 2013; Cunillera-Montcusí et al., 2022; Kelly et al., 2023).

In addition, global warming - a hallmark of anthropogenic climate change - adds thermal stress that has cascading effects on species distributions, metabolic rates, and interspecific interactions (Striebel et al., 2016; Lengyel et al., 2023b). There are several direct (e.g. changes in individual growth rates, photosynthetic efficiency, and cellular biochemistry) and indirect effects (e.g. via changes in nutrient availability, concentration of dissolved gases, pH, and water column stability) of water temperature on aquatic ecosystems (Bondar-Kunze et al., 2021; Siegel et al., 2023). Furthermore, changes in flow rates caused by factors such as erratic precipitation patterns leading to flooding or drought, dam constructions, diversions, and numerous other forms of infrastructure, resulting in the reduction or elimination of flooding and other ecologically important aspects of flow regimes, can affect habitat structures and nutrient availability (Tonkin et al., 2018; Bondar-Kunze et al., 2021). These anthropogenic stressors can have profound and often unpredictable impacts on freshwater ecosystems (Piggott et al., 2015). Microphytobenthic communities form an essential component of freshwater ecosystems. They are also known as periphyton, often dominated by diatoms (Bacillariophyta). Periphyton form the biofilm that inhabits the benthic interface of water bodies and contributes significantly to primary production through photosynthesis (Lengyel et al., 2023b). Their role extends beyond primary production to nutrient cycling and sediment stabilisation, and they form the basis of food webs (B-Béres et al., 2023; Lengyel et al., 2023a). Given their central role in ecosystem dynamics, it is crucial to decipher how they respond to the anthropogenic stressors mentioned above for both nature conservation and complying with the European Commission's Water Framework Directive (European Commission, 2000).

Understanding the responses of microphytobenthos (MPB) assemblages to multiple stressors is not only a scientific endeavour but an urgent ecological imperative. It is well established that the photosynthetic activity (Silva, 2000), and community structure of diatoms (Dixit et al., 1999; Virtanen and Soininen, 2012; Schröder et al., 2015; Heikkinen et al., 2022) are both affected by prolonged exposure to salt. Yet, characterising salinity's specific influence on species composition and freshwater ecosystem functioning remains challenging (Castillo et al., 2018; Stenger-Kovács et al., 2023). In addition, increasing water temperature and reduced water flow velocity are both important factors that influence periphyton, though water chemistry is perceived to play a more crucial role in driving their community composition (Hering et al., 2006; Marcel et al., 2017). The relatively low number of studies testing the effects of multiple stressors on freshwater microphytobenthos, compared to the diversity of stressors to be selected, makes it difficult to distinguish clear general patterns when it comes to their interactive effects. If a stressor is interactive it interacts positively (in the same direction, synergism) or negatively (in opposite direction, antagonism) with a second or third stressor. Very few studies have explored the interactions between stressors on one hand and the dynamics of recolonization on the other (Piggott et al., 2015).

Diatoms have a well-established global reputation in biomonitoring studies owing to their exceptional bioindication capabilities (Lobo et al., 2016). They are particularly valuable for evaluating ecosystem recovery following environmental disturbances, as evidenced in lotic ecosystem research (Steinman and McIntire, 1990; Brown and Manoylov, 2023). Notably, significant shifts in diatom communities have been well-documented in correlation with eutrophication in streams and rivers. Efforts to mitigate nutrient inputs, aimed at restoring these ecosystems to their original state have yielded ambiguous outcomes and often failed to achieve a full return to the pre-disturbance conditions (Brown and Manoylov, 2023). According to this study, ecosystem recovery is a complex process that involves more than just reversing the pathway of degradation. It may take several decades to complete or, in the case of some streams and rivers, may not occur at all (Brown and Manoylov, 2023). The recently introduced Asymmetric Response Concept (ARC, Vos et al., 2023) highlights the differences between degradation and recovery pathways of biological communities in terms of the relative contribution of processes that determine community assembly, such as abiotic tolerances, biotic interactions and dispersal. These processes can vary in speed and outcome, potentially leading to new ecosystem states or even failure. While some studies have suggested that diatom communities may require several decades to recover fully, others have reported signs of recolonization in a matter of weeks. However, these quick recolonization observations have typically relied on ecological indices rather than comprehensive community composition assessments (Peterson et al., 1990; Steinman and McIntire, 1990; Lacoursière et al., 2011; Duong et al., 2012).

Most freshwater microphytobenthic community studies are based on field observations of diatom assemblages, while data from controlled mesocosm experimental settings are relatively scarce. The few experimental studies that have been conducted have provided valuable insights into the impact of single stressors, such as light availability, flow velocity, sediment, nutrients, temperature, and salinity, as well as their combinations, on the community structures and functional traits of benthic diatoms (Piggott et al., 2015; Bondar-Kunze et al., 2016; Nuy et al., 2018; Costello et al., 2018; Salis et al., 2019; Bondar-Kunze et al., 2021; Frost et al., 2023). However, the recovery process after the alleviation of these stressors is less studied, we could not find any prior mesocosm studies that have examined the recovery of microphytobenthos communities following the alleviation of stressors.

In the present study, we attempt to address the question of how the microphytobenthic algal communities change, develop, and potentially restructure in the face of anthropogenic pressures and changing climatic conditions, using a highly replicated streamside mesocosm experiment. Our primary objective is to contribute valuable insights to the management and conservation of freshwater ecosystems by conducting a comprehensive investigation into the multifaceted impacts of various stressors on microphytobenthos communities. Our study is based on two specific hypotheses: First, we hypothesize that stressors lead to a shift in community composition toward lower diversity and increased dominance of disturbance-tolerant taxa, while having a relatively limited impact on photosynthetic biomass. Second, we expect that priority effects, i.e. occupancy of physical and niche space by a resident community, will initially hinder recolonization by immigrant species when stressor load is alleviated. This initial asymmetry in recovery is expected to be observable over weeks such that the microphytobenthos community structure in stressed mesocosms will remain different from that of the unstressed control.

2. Materials and methods

2.1. Study site and experimental design

The Emscher/Boye catchment, located in the highly urbanised Ruhr Metropolitan Region of Northern Germany, has a long history of severe anthropogenic impacts. For centuries, the densely populated Ruhr Metropolitan Area used this river system as an open sewer to help release untreated wastewater from domestic, industrial coal mining operations and agricultural surface runoff activities. Since 1990s, the Emschergenossenschaft, a local water management authority, initiated restoration projects at various parts of the catchment to stop and revert the negative impacts of anthropogenic disturbances in this river network (Winking et al., 2014, 2016). However, in the context of these habitat restoration efforts, the critical aspect of ecosystem dynamics of microphytobenthic communities after restoration of the Emscher/Boye catchment remains unexplored. The background physical and chemical measurements taken bi-weekly in the river during the experiment are listed in Table 1.

We used a streamside mesocosm experimental setup (ExStream System, Piggott et al., 2015) consisting of 64 circular mesocosms to decipher the functional and compositional responses of stream microphytobenthos communities to multiple stressors. The experiment was conducted in the Boye River (51.5533°N, 6.9485°E). In the experimental system, untreated water was redirected from the Boye River to flow through circular mesocosms (Fig. 1A/C). Water was pumped from the river using two pumps (Pedrollo NGAm 1A-pro) into two settling tanks placed on top of the scaffolding structure (Fig. 1B). The water entering the system was divided into four spatial blocks (B1-B4, each having 16 circular mesocosm channels) via a distributor and then passed through a 203 L sediment trap in which the flow velocity was reduced so that fine suspended material could settle to the bottom of the tank. The water was then passed into a second sediment trap, where further sedimentation could take place. The water then entered the header tank (Fig. 1B), which supplied the mesocosms (outer diameter: 25 cm, central outflow: 6 cm, volume 3.5 L, area: 450 cm^2; Microwave Ring Moulds, Interworld, Auckland, New Zealand, Fig. 1C). Using a shut-off valve, the inflow in the mesocosms was calibrated twice a day to roughly 2.1 L/min. Excess water was discharged via an overflow at the top of each collection tank into the system's outfall, where it was filtered through a retention basin and finally discharged back into the Boye River downstream of the system's intake.

The experiment ran for 48 days from 4th March to 21st April 2022, starting with 20 days (March 4 – March 24) of colonization and acclimation. After that, stressors were introduced into the mesocosms for 14 days (March 24 – April 7, the stressor phase), and then a 14-day recovery period (April 7 – April 21, the recovery phase) with no applied stressors. We employed a full factorial design (2 × 2 × 2) with a random distribution of flow velocity and salinity treatments, and a block-wise distribution of temperature treatments (Table 2), consisting of two levels of each stressor, with eight replicates. However, due to an error, a salinization treatment was incorrectly assigned to a different mesocosm, resulting in variations in the number of replicates for different treatments, such as nine replicates for the treatments reduced flow velocity combined with warming (C08, F + T, see Table 2) and salinization combined with warming (C07, S + T, see Table 2). During the 14-day stressor phase, we aimed to expose the stressed mesocosms to increased temperature + 4 °C (± 0.2 °C) vs ambient (control) (Fig. 2A, also see Fig. S1A), increased salinity (+ 200 mg/L chloride) vs background (Fig. 2B, also see Fig. S1B, C), and reduced flow velocity (~10 cm/s) vs normal (~20 cm/s) (Table 2).

To reduce flow velocity, the inflow jet was removed without affecting water volume. This resulted in two flow velocities: normal flow velocity (14.25 ± 7.59 cm/s, n = 4) with the inflow jet and reduced flow velocity (3.50 ± 3.32 cm/s, n = 4) without it. This significantly reduces flow velocity as would be caused by human activities such as water abstraction, morphological changes, etc. To induce salt stress, a concentrated NaCl solution (> 350 mg/L) was prepared by dissolving salt tablets (Claramat, >99.9 % NaCl) in stream water. This solution was fed into an irrigation pipe via dosage pumps (GHL Doser 2.1, at a rate of approx. 714 mL/min). The irrigation pipe was additionally supplied with filtered stream water (mesh size 100 μm) and pressure-compensated drippers released the water from the irrigation pipe to the salt-treated mesocosms at a rate of 4 L/h. Initially, a motor-driven agitator was used for salt dissolution, but it caused undissolved salt tablets to block the suction hoses of the dosing pumps. Therefore, the agitator was replaced with a submersible pump (Gardena, 9000Dirt) for mixing on the following day, delaying the start of salinization by one day. Heating was achieved by warming water in collection tanks, and a mixing module allowed quick temperature adjustments while minimizing high-temperature exposure to organisms. Temperature (Fig. 2A) was recorded every 5 min in two randomly selected mesocosms per block (HOBO Pendant MX Temp/Light) and electrical conductivity (EC,

Table 1
Background physical and chemical parameters measured bi-weekly during the experiment. Conductivity, pH, dissolved oxygen, water temperature and water level were measured in situ, while ortho-phosphate, total phosphate, ammonia, nitrite, nitrate, total nitrogen, chloride, sulfate and dissolved organic carbon (DOC) are lab measurements.

Date	25/02/ 2022	11/03/ 2022	25/03/ 2022	07/04/ 2022	21/04/ 2022
pH	8.18	8.13	NA	8.01	8.21
Conductivity (μS/ cm)	594	793	NA	600	796
Dissolved oxygen (mg/L)	7.3	10.7	NA	14.4	14.2
Water temperature (°C)	5.6	5.1	NA	7.8	10.6
Water level (cm)	56	36.7	NA	50	31.5
Ortho-phosphate (mg/L)	0.023	0	0.012	0.028	0.011
Total Phosphate (mg/L)	0.024	0.020	0.033	0.049	0.026
Ammonia (mg/L)	0.103	0.191	0.15	0.127	0.076
Nitrite (mg/L)	0.032	0.074	0.081	0.068	0.052
Nitrate (mg/L)	17.303	7.273	7.823	11.901	6.141
Total Nitrogen (mg/L)	3.998	1.813	1.908	2.808	1.462
Chloride (mg/L)	30.673	25	37	28	37
Sulfate (mg/L)	96	107	159	105	160
DOC (mg/L)	NA	17.24	16.69	21.21	16.4

Fig. 1. ExStream experimental setup show A) circular mesocosms; B) header tanks (green) and small heating bucket (black); C) Inside the circular mesocosm channel showing the dripping line for salt treatment, the area scraped for biofilm recovery in light green, area scraped for different biofilm samples analysed in this study red, and areas where MINI-PAM II measurement were done (dark green).

Table 2
Glossary and treatment distribution. In bold "problematic" channels explained in the experimental design.

Code	Short	Flow	Salinity	Temperature	Phase	Channels
C	Control	Normal	Background	Control	Stressor	C21, C23, C35, C43
					Recovery	C18, C19, C37, C42,
T	Increased temperature	Normal	Background	Increased	Stressor	C05, C62, C63
					Recovery	C04, C09, C52, C54
S	Increased salinity	Normal	Salt-treatment	Control	Stressor	C25, C26, C38, C47
					Recovery	C27, C31, C36, C44
F	Reduced flow	Reduced	Background	Control	Stressor	C28, C30, C33, C39
					Recovery	C22, C29, C34, C46
S + T	Salt + Temperature	Normal	Salt treatment	Increased	Stressor	**C07**, C13, C15, C49, C50
					Recovery	C14, C16, C53, C60
F + T	Flow + Temperature	Reduced	Background	Increased	Stressor	C06, **C08**, C10, C56, C59
					Recovery	C01, C12, C55, C57
F + S	Flow + Salinity	Reduced	Salt-treatment	Control	Stressor	C17, C24, C40, C48
					Recovery	C20, C32, C41, C46
F + S + T	Flow + Salinity + Temperature	Reduced	Salt-treatment	Increased	Stressor	C11, C61, C62
					Recovery	C02, C03, C58, C64

a proxy for salinity, Fig. 2B) was measured daily in all mesocosms using a WTW Cond 315i probe. If significant flow velocity reductions were observed during discharge calibration, an EC measurement was performed before making discharge adjustments.

The warming treatment was interrupted for three and a half days between stressor day 6 and the evening of stressor day 9 because a defective pump had to be replaced. Both warming and salt treatment were stopped for stressor day 13 and the night of stressor day 14 because heavy rainfall resulted in high sediment loads in stream water. This made maintaining the system challenging and the risk of blockages in the filter system and tubing leading to the mesocosms was too high to keep the stressors running. While the stressor treatments were effective (9 days of warming and 11 days of salinization), the average increase in water temperature between warmed and surrounding mesocosms was 3.45 °C (treatment means ± SD: ambient temperature = 8.71 ± 0.06 °C, warming = 12.16 ± 0.08 °C, n = 4). This is close to +3.26 °C of the projected temperature increase under SSP5–8.5 scenario in March (2080–2090). The average conductivity increase between background

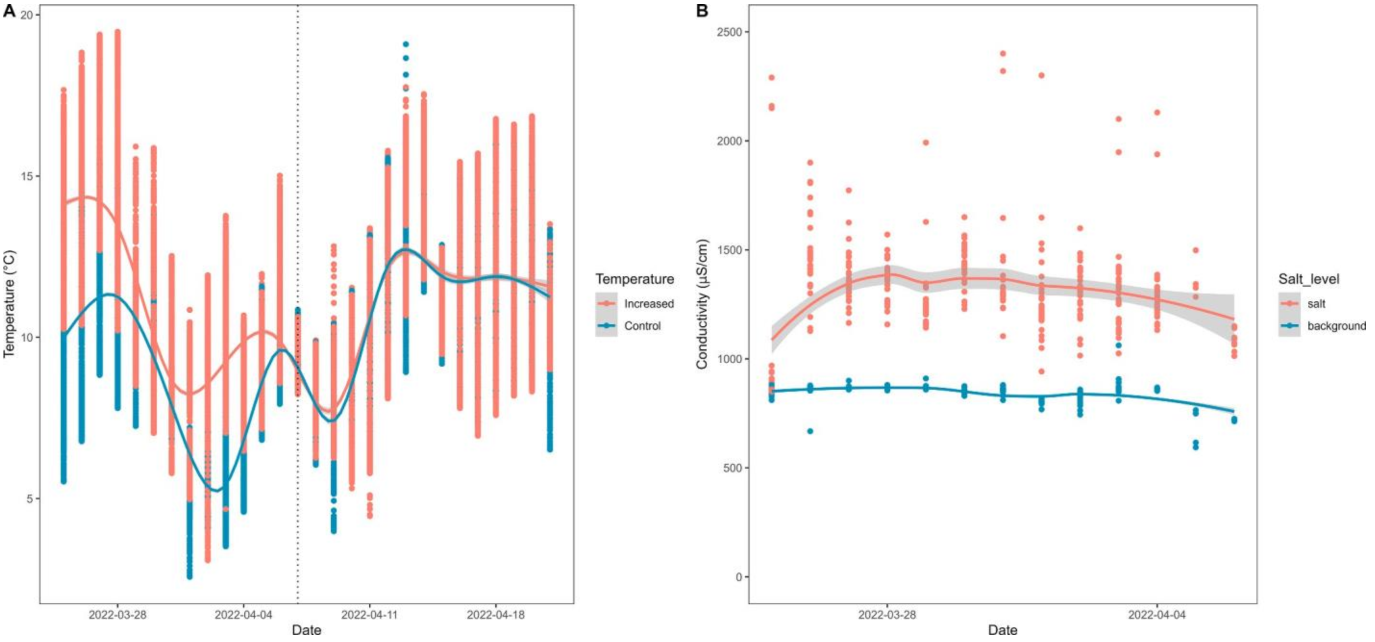

Fig. 2. Plot showing time-series dataset of A) temperature (stressor and recovery phases) and B) conductivity (only stressor phase) treatments applied in this study. The vertical dotted line separates the stressor application (left) from the stressor alleviation (right) phase.

salinity and elevated salinity was 0.529 mS/cm (treatment mean ± SD: $EC_{ambient} = 0.842 \pm 0.006$ mS/cm, $EC_{salt} = 1.343 \pm 0.151$ mS/cm), corresponding to 154.1 mg/L added chloride (see Fig. S1). For the above calculations, short-term salinity peaks due to flow reductions have been removed. Our goal was to slightly exceed the maximum observed conductivity in the Boye catchment, which averages 1413 µS/cm at Nattbach, our experiment site. At the end of the 14-day stressor phase, half of the mesocosms from each treatment and their combinations were sampled. The remaining half were allowed to recover for 14 days. During this time, the inflow jet was reattached in all mesocosms with a reduced flow velocity treatment and the heating and salinization treatments were discontinued. The experiment ended with sampling of the recovery mesocosms on April 21, 2022.

Chlorophyll fluorescence following dark adaptation of microphytobenthic biofilm was monitored every 3 to 4 days during the stressor and recovery phase using a MINI-PAM II fluorometer (Heinz Walz GmbH, Effeltrich, Germany) to accurately quantify photosynthetic health (Y(II) or Fv/Fm) and biomass (F_0 or Fm) in situ throughout the experiment (Schreiber, 1986; Honeywill et al., 2002). The maximum quantum yield of photosystem II, also known as maximum efficiency of light utilization (Y(II) or Fv/Fm) is an indicator of health, while the minimum (F_0) and the maximum (Fm) fluorescence yield are indicators of biomass (Honeywill et al., 2002). Four positions along the internal wall of the mesocosm channel were selected and measured for photosynthetic activity (Fig. 1C, dark green points). Saturation pulse measurements were made in the evenings, always at least 30 min after dusk, so that dark-adapted biofilms were measured. Biofilm samples were collected from half of the mesocosm channels (32) at the end of the stressor phase. The remaining half of the mesocosm channels (32) were allowed to recover after the release of the stressors and were sampled at the end of the recovery phase. In addition, the biofilm in the remaining channels was completely scraped over an area of approximately 2 cm × 2 cm at the end of the stressor application phase, to monitor de novo recolonization using MINI-PAM II.

During sampling (end of stressor and recovery phases), biofilm was scraped from the internal walls of the mesocosms using plastic razor blades. Two samples were collected; one was taken over an area of approximately 5 × 20 cm^2 of mesocosms into 30 mL falcons (Fig. 1C pink-red), and fixed with 20 mL 96 % ethanol for digital microscopy for detailed diatom community observations (Burfeid-Castellanos et al., 2022) and 18S−V9 amplicon sequencing to reveal the broader microphytobenthic community dynamics (Amaral-Zettler et al., 2009). Another sample was collected over an area of about 1 × 1 cm^2 and preserved in 1.5 mL Eppendorf tubes with 96 % ethanol for lab chlorophyll extraction and measurements using a Tecan microplate reader (Mandalakis et al., 2017). Separate samples of biofilms were collected at the scraped surface area (2 × 2 cm) for 18S−V9 amplicon sequencing.

2.2. *Lab sample preparation for chlorophyll extraction, digital microscopy and 18S−V9 amplicon sequencing*

Chlorophyll extraction was performed in absolute ethanol following standard protocols (Ritchie, 2008; Brito et al., 2009). First, samples were resuspended using a Pasteur pipette, approximately 1–1.5 mL of each sample was transferred to a Macherey & Nagel bead tube and then placed in a bead beater and homogenized for four 60-s cycles each at 40,000 rpm. After homogenization, samples were incubated in the dark for 1 h, then centrifuged at 2500 rpm for 3 min, and 250 µL of the supernatant was transferred into a 96-well plate. The remaining samples were put back at −20 °C for storage. The plate was loaded into a Tecan Infinite M Plex multimode microplate reader (Tecan Group Ltd., Männedorf, Switzerland). The main results from this part of the study were the estimation of photosynthetic biomass using Tecan microplate reader set up in fluorescence bottom reading mode with the following parameters: excitation wavelength at 460 nm, emission wavelength at 680 nm, gain manually set to 135 and 10 flashes per measurement. Chlorophyll fluorescence measurements were saved in an Excel spreadsheet for further statistical analyses.

The preparation and processing of biofilm samples for digital microscopy followed the procedures described by Burfeid-Castellanos et al. (2022). Hydrogen peroxide and hydrochloric acid were used to digest the samples after five prewash cycles (Taylor et al., 2007). The samples were rinsed seven times, centrifuged at 1200 rpm for 3 min (Eppendorf Centrifuge 5427 R; Hamburg, Germany) and decanted with distilled water before they were pipetted onto a coverslip (15 mm × 15 mm, # 1.5), allowed to dry, and then mounted on permanent glass slides with Naphrax resin (refractive index = 1.71, Biologie-Bedarf Thorns, Deggendorf, Germany). To create virtual slides, a 5 mm × 5 mm area on the permanent glass slide with the highest density of evenly distributed diatom valves was scanned at a magnification of 600× using a VS200 slide scanner (EVIDENT, Tokyo, Japan) with ASW 3.1 software (Olympus Soft Imaging Solutions GmbH, Münster, Germany). A web-browser based image annotation platform, BIIGLE 2.0, was used to count and identify diatoms on these virtual slides (Langenkämper et al., 2017; Burfeid-Castellanos et al., 2022). At least 400 diatom valves were counted and identified up to species level using available literature (Levkov et al., 2013; Trobajo et al., 2013; Cantonati et al., 2017). A list of diatom taxa and their relative abundances was created and utilized for additional analyses.

Sample preparation and DNA extraction for 18S−V9 amplicon sequencing were performed according to Buchner's silica bead-based extraction protocol (Buchner, 2022a, 2022b). Each sample was processed in two replicates, and the genomic DNA amplification of the V9 hypervariable region of the small subunit ribosomal RNA genes (approximately 130 base pairs) was carried out using the universal specific forward primer 1389F (5′-TTGTACACACCGCCC-3′) and the eukaryotic specific reverse primer 1510R (5′-CCTTCYGCAGGTTCACC-TAC-3′; Amaral-Zettler et al., 2009), following the protocol described by Stoeck et al. (2010). Sequencing of the resulting libraries was performed on Illumina MiSeq at CeGat GmbH (Tübingen, Germany).

2.3. Bioinformatic analysis of 18S−V9 amplicon sequencing data

The Natrix2 workflow (Welzel et al., 2020; Deep et al., 2023) was used to perform bioinformatic analyses of microphytobenthos Illumina amplicon sequencing data. The workflow's operational taxonomic units (OTUs) variant was implemented using the clustering algorithm Swarm v3.0.0 (Mahé et al., 2015). Paired-end reads were assembled using the simple Bayesian algorithm in PANDAseq v2.11 (Masella et al., 2012). Primers were trimmed using cutadapt v3.2 (Martin, 2011) and filtered with a PANDAseq threshold of 0.9, a minimum length of 77, and a maximum length of 196 nucleotides. Sequence dereplication was performed using the algorithm CD-HIT v4.8.1 (Fu et al., 2012) at 100 % similarity, and chimeric sequences were identified and removed using VSEARCH v2.15.2 (Rognes et al., 2016). The split-sample approach (Lange et al., 2015) was used to reduce erroneous sequences without strict abundance cut-offs. The AmpliconDuo v1.1 R package (Lange et al., 2015) was used for statistical analysis of the amplicon sequencing data resulting from the split-sample process. OTUs were generated by clustering sequences with Swarm v3.0.0 (Mahé et al., 2015) and were then aligned with mothur v1.40.5 (Schloss et al., 2009) against the protist ribosomal reference database (PR^2) v.4.14.0 (Guillou et al.,2013). MUMU (https://github.com/frederic-mahe/mumu), a C++ implementation of LULU (Frøslev et al., 2017), was used for post-clustering curation of the periphyton amplicon sequencing data. The replicates of sequenced samples were merged into one representative sample by calculating the sum of reads for each OTU. The negative controls with the maximum reads were subtracted from the sum of both samples to standardize the sequences' proportions. This step is important to reduce redundancy in the resulting dataset for subsequent statistical analyses. The resulting OTU table was filtered for only eukaryotic algal taxa.

2.4. Statistical analyses

Statistical analyses were performed in R v.4.3.0 (R Core Team, 2023) using the packages 'tidyverse' v2.0.0 (Wickham et al., 2019) and 'vegan' v2.6.4 (Oksanen et al., 2022). Diatom taxa with a relative abundance of at least 1 % were retained for further analysis. For amplicon data, filtering OTUs with read counts below a threshold of >10 vs. no filtering gave similar results in terms of significance; the results given below are from the comparisons of the non-filtered data set. The relative abundance of diatom taxa or OTU was calculated by dividing their count by the total count of the respective sample and then multiplying by 100 to convert it to a percentage. The relative abundances of the dominant diatom taxa and OTU reads were plotted in stacked bar plots and used for further multivariate analyses. Alpha diversity indices, e.g. species richness, Shannon and Simpson, were derived using the 'diversity()' function from 'vegan', based on diatom taxonomy from digital microscopy and microalgae amplicon sequencing. In addition, Gamma and Beta diversity indices, such as Nestedness, Turnover and Sorensen were calculated according to Baselga (2010). These indices and photosynthetic biomass data were compared across treatments using analysis of variance (ANOVA). Next, we conducted individual ANOVAs for each treatment-specific alpha, beta, gamma diversity indices, as well as F_0, Y(II) and standardized Tecan fluorescence measurements using flow velocity, salinity, and temperature as fixed factors. Using the lm() function, linear regression models were fitted with temperature, salinity, flow velocity as the predictor variables and F_0 and Y(II) as the response variables. Due to skewed distributions, Hellinger transformation of the abundance data and OTU reads was performed using the 'decostand()' function of the 'vegan' package to minimize the impact of very abundant taxa and OTUs (Anderson et al., 2006; Legendre and Gallagher, 2001). To determine stressor effects on MPB community composition, 'betadisper()' evaluated multivariate homogeneity of group dispersion and 'adonis2()' executed PERMANOVA. In addition, a pairwise comparison of each treatment against the control was conducted using the 'adonis2 ()' function to see if they were significantly different. We adjusted *p*-values for multiple comparisons using the Benjamini and Hochberg (1995) method. Associations between samples were visualized with non-metric multidimensional scaling (NMDS) using 'metaMDS()' based on the Bray-Curtis similarity index, with 999 permutations.

3. Results

3.1. Diatom community composition

A total of 257 different diatom taxa belonging to 61 genera were identified during the examination of biofilm samples using digital microscopy. The most dominant taxa in both stressor and recovery samples were *Achnanthidium minutissimum* (52.6 % and 56.5 %, respectively), *Achnanthidium jackii* (9.47 % and 10.65 %, respectively), and *Navicula gregaria* (4.6 % and 4.1 % respectively) of the total valve counts (Fig. 3A). The alpha and gamma diversity indices are listed in Table 3. During the stressor phase, linear regression revealed that both reductions in flow velocity ($t = -2.454$, $p = 0.021$) and increases in temperature ($t = -2.487$, $p = 0.019$) were associated with a significant decrease in the Shannon diversity index, indicating lower biodiversity under these stress conditions. After recovery, mesocosms previously treated with increases in temperature (T) were associated with a significant decrease in Simpson diversity index ($t = -3.329$, $p = 0.002$), suggesting that the lower biodiversity persisted after the increase temperature stress was released. When comparing both the stressor and recovery phases, significantly lower gamma diversity was observed during the recovery phase (ANOVA, $F = 7.4$, $p = 0.02$). Furthermore, we did not detect significant differences in species turnover, nestedness and Sorensen index between treatments during the stressor and recovery phases (all *p*-values >0.05, Table S1).

The analysis of multivariate homogeneity of group dispersions (variances) revealed significant heterogeneity of within-group variances only for the salt treatment (ANOVA, $F = 8.51$, $p = 0.005$). Significant differences in multivariate dispersion were also observed when comparing the two phases of the experiment (ANOVA, $F = 4.583$, $p = 0.036$). PERMANOVA partitioning testing of the effects of temperature, salinity and flow velocity on diatom communities showed a significant effect of temperature ($R^2 = 0.097$, $p = 0.005$) and flow velocity ($R^2 = 0.115$, $p = 0.002$, Table 4) during the stressor phase. A significant effect of temperature was also observed during the recovery phase ($R^2 = 0.094$, $p = 0.002$, Table 4). When testing the effects of experimental phases (stressor vs recovery) and treatments, the phase factor was highly significant (PERMANOVA, $R^2 = 0.092$, $p = 0.001$), indicating substantial differences in diatom communities between the phases, though a contribution by the heterogeneous dispersions (above) also cannot be excluded. Diatom communities were also significantly different between the treatments ($R^2 = 0.191$, $p = 0.001$, see Table 4), highlighting the influence of treatments on the assemblages. Furthermore, pairwise comparison of each treatment with the control (C) using PERMANOVA revealed a significant effect of increased temperature treatment (T), as well as the combined treatment of reduced flow velocity and increased temperature (F + T) and reduced flow velocity, increased salinity and increased temperature (F + S + T) during the stressor phase (Table S2), that can be summarized as a dominance effect of the temperature treatment. However, after applying corrections for multiple comparisons, all adjusted *p*-values were greater than the significance threshold of 0.05 (Table S2), suggesting that the observed effect may be due to random variation rather than a true effect. The non-metric multidimensional scaling (NMDS) plot showed scattered sample points without a clear treatment-grouping pattern (Fig. 4A).

3.2. Microalgae community composition

18S−V9 amplicon sequencing of microphytobenthos biofilm

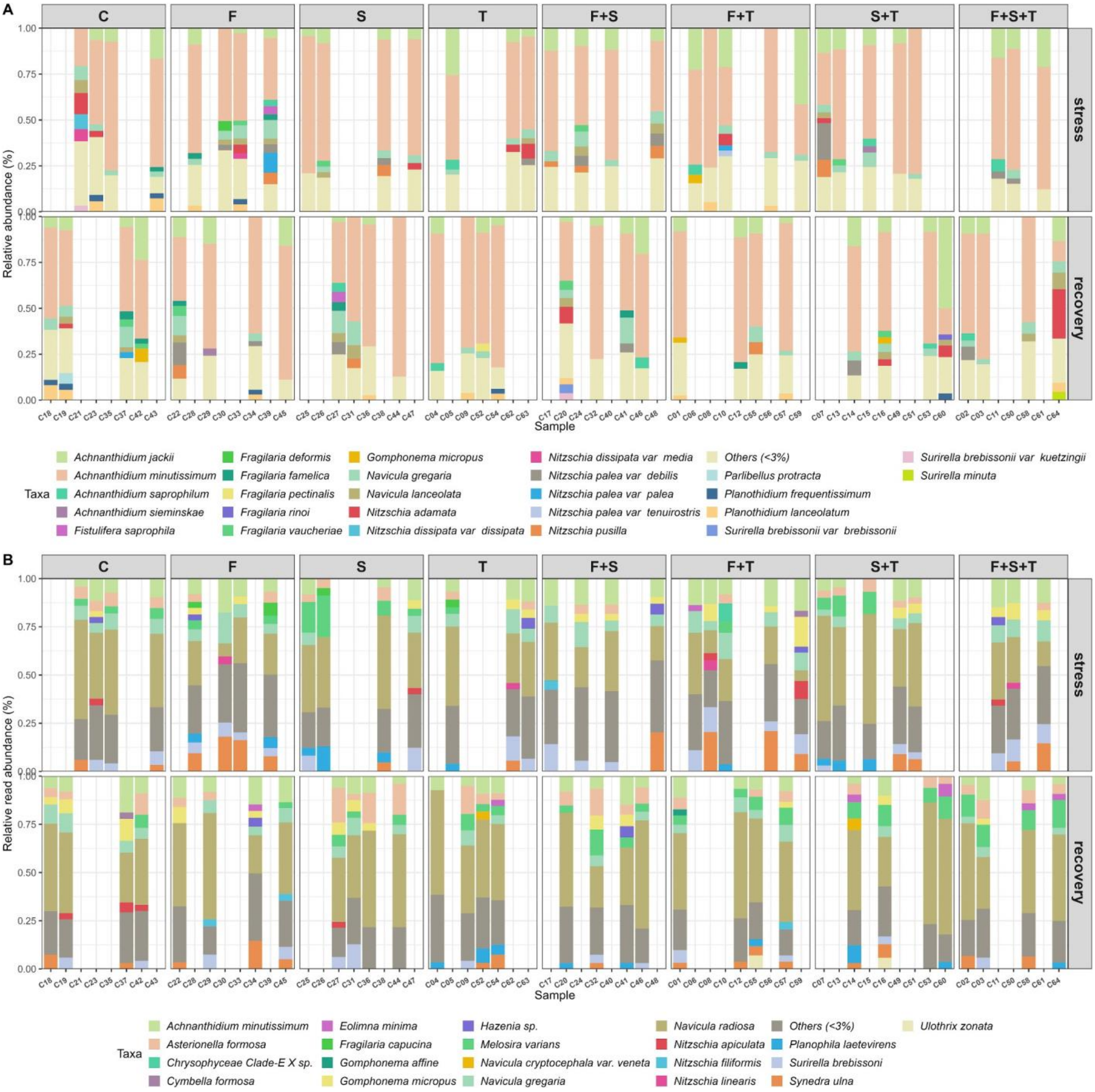

Fig. 3. Community composition of microalgae in different treatments. Relative abundance of the dominant microphytobenthos taxa during stressor and recovery phases. A) Diatom community as determined by digital microscopy. B) OTU read abundance from 18S−V9 amplicon sequencing. C: control; F: reduced flow velocity; S: increased salinity; T: increased temperature; F + S: reduced flow velocity + increased salinity; F + T: reduced flow velocity + increased temperature; S + T: increased salinity + increased temperature; F + S + T: reduced flow velocity + increased salinity + increased temperature. C + numbers (Sample): channel number.

samples, including the recovery samples from the completely scraped areas, generated a total of 34,063,298 reads. Among these reads 785,609 constituting 2.3 % of the total, matched negative controls and were discarded. Following taxonomic filtering, our analysis retained 19,603,405 reads representing 1854 Operational Taxonomic Units (OTUs) in the microalgae dataset. Of these, 13,034,618 reads were obtained from samples collected at the end of both the stress and recovery phases of the experiment, while approximately 6,568,787 reads originated from samples collected from areas that had been entirely stripped of biofilm at the beginning of the recovery phase (henceforth scraped area, to serve as a recolonization area free of priority effects). Diatoms (Bacillariophyta) accounted for 11,696,476 reads, which translates to approximately 89.7 %, of the total microalgae reads, associated with 751 OTUs (Fig. 3B).

The samples obtained from scraped areas exhibited even higher presence of diatoms, with 6,245,423 representing roughly 95 % of the total reads originating from these particular samples (Fig. S2). All samples were dominated by reads of *Achnanthidium minutissimum*, *Asterionella formosa*, *Navicula radiosa*, *Navicula gregaria*, *Gomphonema micropus*, *Ulnaria ulna* and *Surirella brebissonii* (Fig. 3B).

OTU alpha and gamma diversity indices are given in Table 3. During the stressor phase, linear regression revealed significant positive effect of reduced flow velocity on OTUs Shannon ($t = 4.265$, $p < 0.001$) and Simpson (4.757, $p < 0.001$) diversity index, suggesting that reduced flow velocity is associated with higher microalgae diversity. Simpson index was significantly different between the treatments as confirmed by ANOVA ($F = 4.73$, $p = 0.01$). It was lower in mesocosms treated with increased salinity and temperature (S + T) and higher in mesocosms treated with reduced flow velocity (F). Upon comparing the stressor and recovery phases, we observed statistically significant differences in Gamma diversity (ANOVA, $F = 108.7$, $p < 0.001$), OTU richness ($t_{welch} = 10.57$, $p < 0.001$), Shannon index ($t_{welch} = 4.53$, $p < 0.001$) and Simpson index ($t_{welch} = 3.75$, $p < 0.001$), with high mean values often recorded during the stressor phase (Table 3). However, we observed no significant differences in species turnover, nestedness and Sorensen index between the stressor and recovery phase (all $p > 0.05$) (Table S1).

Within-group variances were found to be consistent for all factors tested (ANOVA, all *p*-values above 0.05). Subsequently, PERMANOVA testing the effects of temperature, salt and flow velocity on microalgae communities showed only a significant effect of flow velocity ($R^2 = 0.144$, $p = 0.001$, Table 4) during the stressor phase. Temperature and salinity had a significant influence on the microalgae community composition during the recovery phase (PERMANOVA, $R^2 = 0.11$, $p = 0.001$ and $R^2 = 0.064$, $p = 0.023$, respectively, Table 4). We also observed significant effects of phase ($R^2 = 0.261$, $p = 0.001$) and treatments ($R^2 = 0.131$, $p = 0.010$). Furthermore, community composition between the scraped and non-scraped surfaces at the end of the recovery phase remained significantly different ($R^2 = 0.161$, $p = 0.001$, Table 4), suggesting priority effects. The comparisons of each treatment with the control confirmed the dominant effect of reduced flow velocity (F) on microalgae OTUs ($R^2 = 0.336$, $p = 0.033$), with a marginal effect of the combined treatment of reduced flow velocity and increased salinity (F + S, $R^2 = 0.295$, $p = 0.046$) and the three applied stressors (F + S + T, $R^2 = 0.255$, $p = 0.049$) during the stressor phase (Table S2). During the recovery phase, the reduced flow velocity and salinity (F + S, $R^2 = 0.297$, $p = 0.029$), reduced flow velocity and temperature (F + T, $R^2 = 0.246$, $p = 0.021$) and reduced flow velocity, salinity and temperature treatment (F + S + T, $R^2 = 0.323$, $p = 0.026$, see Table S2) showed significant effect on microalgae OTUs. However, correction for multiple comparisons resulted in adjusted *p*-values >0.05, which may indicate random variation rather than true effects of the treatments. The analysis of indicator species revealed that 18 microalgae OTUs were good indicators of increased temperature, nine of which were associated with diatoms. Six OTUs were good indicators of increased salinity, and finally 18 OTUs were good indicators of reduced velocity. The non-metric multidimensional scaling plot (NMDS) showed scattered sampling points with no discernible grouping pattern based on treatments. However, when comparing the two different experimental phases, there was a clear separation between the stressor (dots) and recovery phase (triangles and squares), and between scraped (square) and non-scraped (triangles) areas during the recovery phase (Fig. 4B).

3.3. Photosynthetic biomass and health

Both the minimum and maximum fluorescence intensity (F_0 and Fm) after dark adaptation of the biofilm responded in the same way in all mesocosms during the stressor and recovery phases. Therefore, we present here only the results related to F_0 and maximum quantum yield of Photosystem II (PSII, Y(II) or Fv/Fm), but, Fm data are also shown in Table 5. Analysis of covariance (ANCOVA) revealed that minimum fluorescence intensity (F_0) decreased during the stressor phase ($t = -20.23$, $p < 0.001$). Only temperature was significantly associated with a decrease in F_0 ($t = -3327$, $p < 0.001$) (Fig. 5A). During recovery phase, ANCOVA revealed that flow velocity ($t = 2.7$, $p = 0.007$), temperature ($t = -2.66$, $p = 0.008$), and the interaction term of time in days and flow velocity ($t = -2.451$, $p = 0.015$) were statistically significant (Fig. 5B).

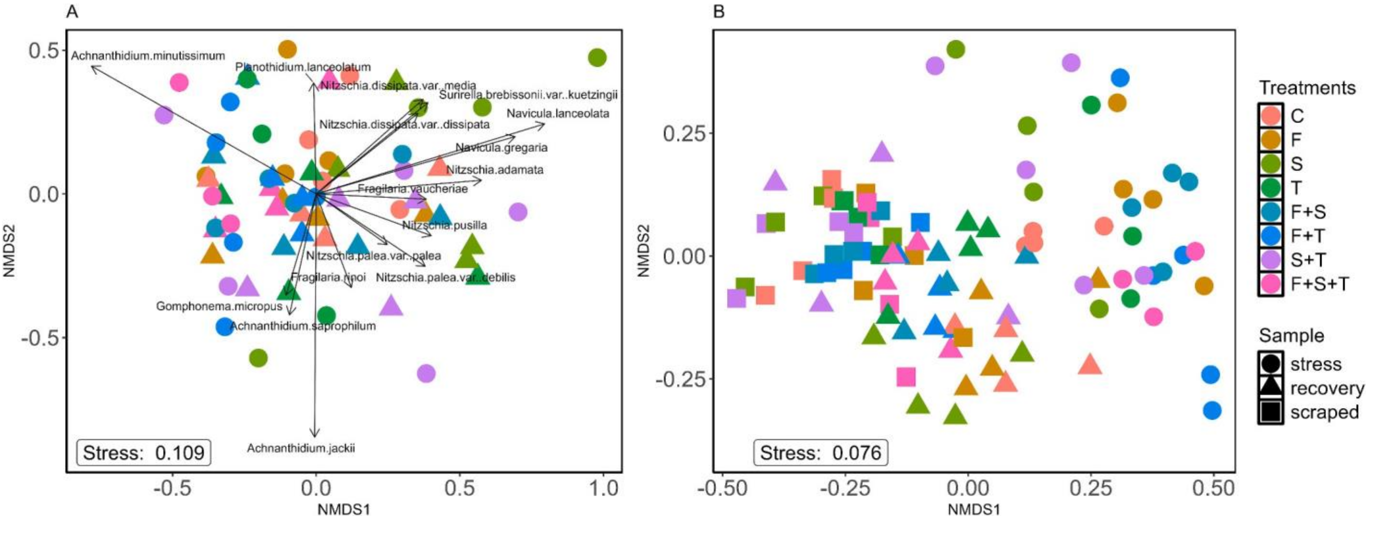

Fig. 4. Non-metric multidimensional scaling plots showing: A) diatom communities from digital microscopy and B) microphytobenthos assemblages as revealed by 18S–V9 amplicon sequencing.

Table 3
Mean Gamma diversity, Richness, Shannon and Simpson diversity indices in different treatments calculated based on digital microscopy and 18S−V9 amplicon sequencing data.

Treat.	Tot. gamma div.	Gamma div.		Richness		Shannon		Simpson	
		Stress	Recov.	Stress	Recov.	Stress	Recov.	stress	Recov.
Diatoms: digital	light microscopy								
C	115	92	75	44.50	35.75	2.16	1.79	0.70	0.62
F	106	85	71	42	38.75	1.91	2	0.61	0.68
S	103	85	67	46	35	2.60	2.07	0.82	0.73
T	133	85	92	39.50	41.50	1.86	1.92	0.60	0.66
F + S	111	88	76	40.25	40.75	1.78	2.21	0.61	0.73
F + T	124	92	86	36.50	40.50	1.58	1.73	0.53	0.59
S + T	130	93	91	38.75	44.75	1.84	2.29	0.64	0.76
F + S + T	134	110	76	40.25	39	1.45	1.71	0.50	0.57
Microalgae: 18S−V9 amplicon sequencing									
C	859	697	597	428.25	354.75	2.58	2.57	0.79	0.82
F	851	719	575	448.50	334.25	3.18	2.52	0.91	0.80
S	833	670	558	404.75	303	2.54	2.33	0.81	0.78
T	890	712	620	430	359.25	2.73	2.53	0.86	0.79
F + S	894	746	576	458.50	330	3.06	2.53	0.89	0.80
F + T	920	748	589	430	331.75	2.95	2.38	0.89	0.77
S + T	887	743	534	441.75	302.25	2.56	2.22	0.76	0.72
F + S + T	906	722	596	432.75	335.25	2.89	2.44	0.89	0.79

We observed a strong positive trend ($t = 4.98$, $p < 0.001$) in F_0 over the scraped surfaces during the recovery phase, although with no significant differences by treatment (Fig. 5C). When comparing the scraped with the unscraped areas, ANCOVA showed that the scraped area (t = —3.878, $p < 0.001$), flow velocity ($t = 2.832$, $p = 0.005$), temperature (t-value = —2.795, $p = 0.005$) and interactions scraped area and time in days ($t = 3.179$, $p = 0.002$) and flow velocity and time in days (t = —2.569, $p = 0.01$) had significant effect on F_0 (Fig. 5C).When comparing the scraped with the unscraped areas, the interaction between scraped area and time ($t = 3.179$, $p = 0.002$) may indicate that as the number of days increases, chlorophyll biomass tends to increase, specifically under the condition of recovery after scraping.

The maximum quantum yield of Photosystem II (Y(II) or Fv/Fm) showed a significant ($t = 4.3$, $p < 0.001$) but very small increase (adjusted $R^2 = 0.016$) during the stressor phase, as indicated by the linear regression (Fig. 6A). Y(II) increased significantly over time (t- value 2.118, $p = 0.034$) and with increasing temperature (t-value = 2.419, $p = 0.016$, Fig. 6A). During the recovery phase, Y(II) showed a small but positive trend over time ($t = 2.316$, $p = 0.021$), but no significant effects of all tested predictors (Fig. 6B). These results were also confirmed by ANOVA as all the p-values were also >0.05 (see Table 5). Over the scraped surface, linear regression showed an overall weak but not significant positive trend over time ($t = 1.79$, $p = 0.08$), with no significant effects of flow velocity, increased salinity and temperature, and their interactions (Fig. 6C). When comparing the scraped with the unscraped areas during the recovery phase, only time in days showed significant effects on Y(II) ($t = 2.843$, $p = 0.005$), all the other predictors namely flow velocity, salinity and temperature, and their interactions were not significant. Linear regression showed a positive trend of Y(II) on the final day of the recovery phase on non-scraped areas ($t = 3.33$, $p = 0.001$) and the scraped surfaces ($t = 2.7$, $p = 0.01$). However, the model's overall explanatory power was too small (p adjusted = 0.03). In addition, ANOVA followed by Tukey's multiple comparison test revealed that the mean value of Y(II) was significantly higher in the recovery (unscraped) than in the stressor phases (0.055 units, 95 % CI: 0.016 to 0.094, $p = 0.003$), as well as in the recovery (scraped) than in the stressor phases (0.077 units, 95 % CI: 0.010 to 0.143, $p = 0.02$). However, there was no significant difference between the recovery (scraped) and recovery (unscraped).

3.4. Chlorophyll fluorometry

In our study using Tecan microplate reader to measure chlorophyll fluorescence, we found that there were no significant differences in extracted chlorophyll fluorescence values among the different treatments on the last day of the stressor phase (ANOVA, all $p > 0.05$, Fig. 7). Similarly, when we examined the chlorophyll fluorescence values at the end of the recovery phase, we observed no significant distinction between treatments, as confirmed by our ANOVA analysis (all p values >0.05, Fig. 7). However, when we compared the two experimental phases, we noticed a noteworthy difference in chlorophyll fluorescence levels after the extraction process in the lab. Specifically, the end of the recovery phase exhibited higher chlorophyll fluorescence values ($F = 55.71$, $p < 0.001$), confirming what we observed with in situ chlorophyll fluorescence measurements.

4. Discussion

Our study aimed to unravel the impact of reduction in flow velocity, an increase in salinity and warming water on microphytobenthos communities, focusing on both taxonomic composition and photosynthesis-related functional traits in mesocosms. Our results showed that among 257 diatom taxa identified in this study, *Achnanthidium minutissimum*, *Achnanthidium jackii*, and *Navicula gregaria* were the most dominant in the majority of mesocosms, including the control (C), during the stressor and recovery phases. These taxa are known for their broad ecological range. For example, the prevalence of *A. minutissimum*, which accounted for 54.6 % of total number of counted valves, underlines this wide tolerance range. Taxa from the *A. minutissimum* complex have been reported to be early colonizers or pioneers, often occurring in sites with major anthropogenic impacts (Potapova and Hamilton, 2007; Jüttner et al., 2022). This may be the first indication of a lack of suitable conditions for more sensitive taxa in the system. These pioneer taxa are generally dominant in spring, as this season is characterised by various types of physical disturbances due to flooding and high river flow.

4.1. Stressor effects

4.1.1. Flow velocity

Our study showed that reduced flow velocity significantly influenced the composition, diversity, and biomass of microbial communities. Under reduced flow conditions, diatom-based microscopy assessments

Table 4

Results PERMANOVA testing the effects of temperature, salinity and flow velocity on diatom (digital microscopy) and microalgae (18S−V9 amplicon sequencing) communities df: degrees of freedom; SS: sum of squares; R^2: variance explained by the groups, F: F-statistic, p: p-values, * Significant ($p < 0.05$) main effects and interactions are highlighted in bold.

Factor	*df*	*SS*	*R^2*	*F* *	*Pr (>F)*
Diatoms: digital microscopy Stressor phase					
Temperature	1	0.266	0.097	3.605	**0.001**
Salinity	1	0.078	0.029	1.061	0.356
Velocity	1	0.314	0.115	4.258	**0.002**
Residual	28	2.068	0.753		
Total	31	2.744	1.000		
Recovery phase					
Temperature	1	0.187	0.094	3.187	**0.003**
Salinity	1	0.076	0.038	1.297	0.205
Velocity	1	0.087	0.044	1.492	0.127
Residual	28	1.640	0.824		
Total	31	1.980	1.000		
Phase + treatments					
Phase	1	0.481	0.092	7.075	**0.001**
Treatments	7	0.993	0.191	2.086	**0.001**
Residual	55	3.741	0.718		
Total	63	5.209	1.000		
Algae: 18S−V9 amplicon sequencing					
Stressor phase					
Temperature	1	0.053	0.041	1.465	0.153
Salinity	1	0.037	0.029	1.024	0.344
Velocity	1	0.187	0.144	5.185	**0.001**
Residual	28	1.068	0.753		
Total	31	2.744	1.000		
Recovery phase					
Temperature	1	0.131	0.110	3.851	**0.001**
Salinity	1	0.077	0.064	2.256	**0.023**
Velocity	1	0.033	0.028	0.965	0.455
Residual	28	0.953	0.798		
Total	31	1.194	1.000		
Interactions during recovery					
Temperature:Salinity	1	0.023	0.019	0.687	0.740
Temperature:Velocity	1	0.065	0.054	1.950	**0.039**
Salinity:Velocity	1	0.031	0.026	0.943	0.442
Residual	25	0.834	0.698		
Total	31	1.194	1.000		
Phase + treatments					
Phase	1	0.879	0.261	23.542	**0.001**
Treatments	7	0.440	0.131	1.683	**0.010**
Residual	55	2.054	0.609		
Total	63	3.371	1.000		
Scraped vs unscraped areas during recovery					
Area	1	0.408	0.161	12.695	**0.001**
Treatments	7	0.361	0.142	1.606	**0.007**
Residuals	55	1.767	0.697		
Total	63	2.537	1.000		

revealed a lower Shannon diversity index, indicating a decline in species diversity. In contrast, amplicon sequencing demonstrated a greater Shannon and Simpson diversity index, suggesting an improvement in the microalgae communities' richness. Microscopy and amplicon sequencing revealed that the community composition under reduced flow was significantly different from the other treatments at the end of the stressor and recovery phases, as confirmed by PERMANOVA analyses. Diatom morphology data notably indicate that the difference in community structure continued into the recovery phase. Moreover, under reduced flow conditions compared to other treatments, the minimum fluorescence F_0, a measure of photosynthetic biomass, recovered more quickly. This might indicate that reduced flow has less severe eco-physiological effects on periphyton than temperature or salinity, which is also in line with the observed lack of effect of salinity upon the maximum photosystem II yield (Y(II) or Fv/Fm) of the periphyton.

Table 5

Mean values of photosynthetic biomass (F_0, Fm) and physiological health (Fv/Fm) and ANOVA results comparing each treatment against the control. C: control; T: increased temperature; S: increased salinity; S + T: increased salinity + increased temperature; F: Reduced flow velocity; F + T: reduced flow velocity + increased temperature; F + S: reduced flow + increased salinity; F + S + T: reduced flow velocity + increased salinity +

Treat.	Phase	F_0					Fm					Y(II) or (Fv/Fm)				
		min	max	Estimate	t	p-value*	Min	max	Estimate	t	p-value*	min	Max	Estimate	t	p-value*
C	Stressor Recovery	35	1014	246.70	19.55	**<0.001**	120	2660	735.93	21.23	**<0.001**	0.13	0.85	0.65	52.79	**<0.001**
T	Stressor Recovery	50	563	194.69	16.86	**<0.001**	106	1874	640.25	14.33	**<0.001**	0.09	0.88	0.66	40.85	**<0.001**
S	Stressor Recovery	25	679	−39.09	−2.21	**0.03**	75	1452	−121.63	−2.50	**0.01**	0.19	0.88	0.002	0.16	0.87
S + T	Stressor Recovery	27	434	−47.46	−2.91	**0.004**	52	1603	−168.74	−2.67	**0.007**	0.11	0.91	−0.02	−0.88	0.38
F	Stressor Recovery	41	803	9.31	0.52	0.60	89	2552	−0.54	−0.01	0.99	0	0.88	0.01	0.50	0.62
F + T	Stressor Recovery	34	660	−13.07	−0.80	0.43	67	2461	28.11	0.44	0.66	0.44	0.90	0.03	1.442	0.15
F + S	Stressor Recovery	18	793	−36.07	−2.03	**0.04**	60	1578	−105.61	−2.17	**0.03**	0.06	0.90	−0.01	−0.44	0.66
F + S + T	Stressor Recovery	33	430	−47.43	−2.89	**0.004**	74	1981	−120.14	−1.89	0.05	0.04	0.84	−0.0008	−0.04	0.97
		[illegible]	802	[illegible]	[illegible]	0.28	[illegible]	1807	[illegible]	[illegible]	0.19	0.03	0.92	[illegible]	[illegible]	0.23

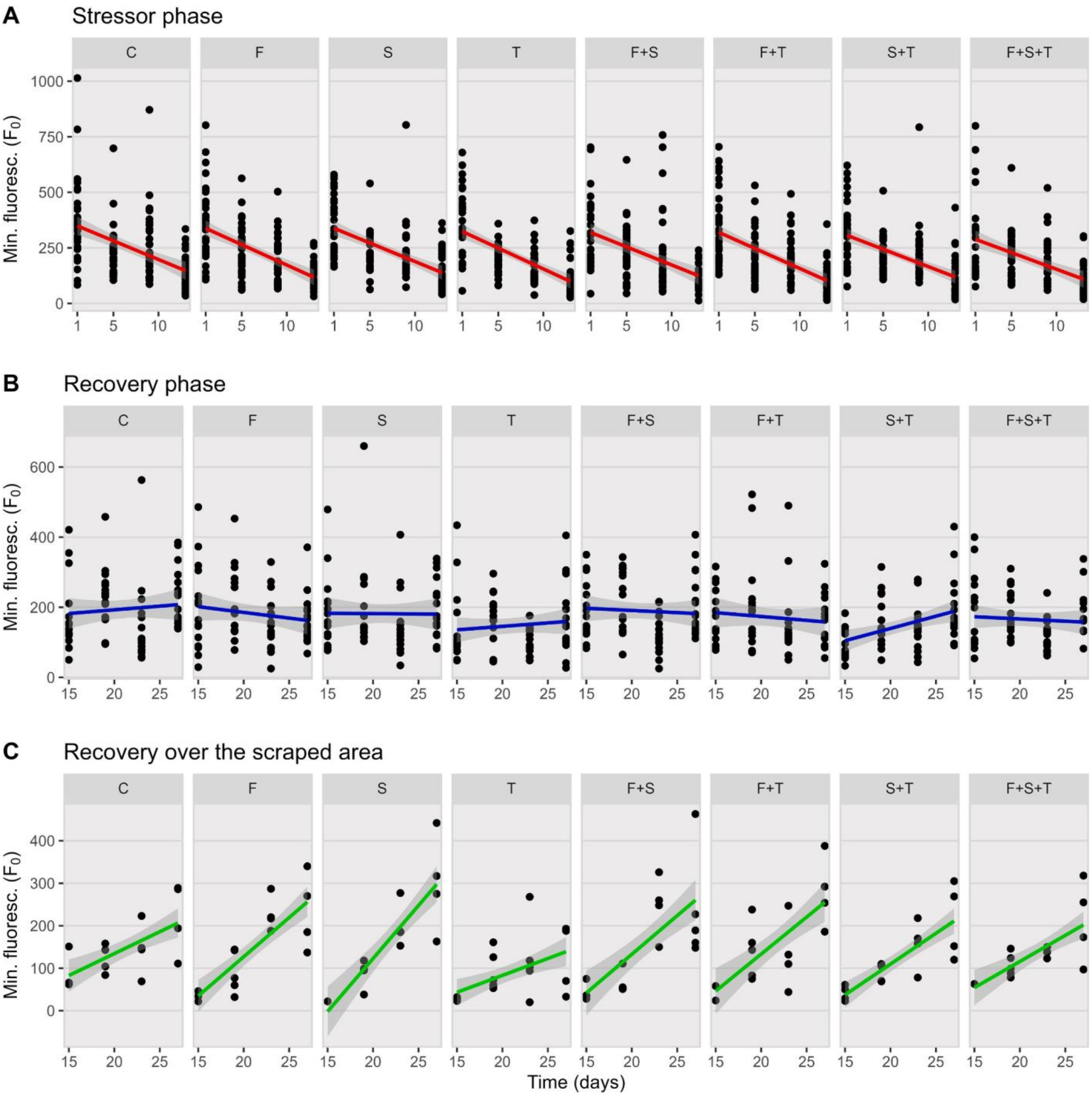

Fig. 5. Plot showing minimum fluorescence yield measurement in different treatments, with fitted linear regression lines. C: control; F: Reduced flow velocity; S: increased salinity; T: increased temperature; F + S: reduced flow + increased salinity; F + T: reduced flow velocity + increased temperature; S + T: increased salinity + increased temperature; F + S + T: reduced flow velocity + increased salinity + increased temperature.

Our amplicon, but not microscopy, results are consistent with previous studies, for example, Salis et al. (2019) found despite decreased flow, there was an increase in taxon richness and a decrease in algal biomass and cell density. Microalgae biofilms growing in slow flow velocity developed visible strands of diatoms and appeared to be thicker and more mature than biofilms in fast flow treatment (Battin et al., 2003a; Battin et al., 2003b; Bondar-Kunze et al., 2016). Rather than community structure, flow-related responses were associated with changes in biomass (Battin et al., 2003b). For example, slower river flow velocities had a major impact on the architecture and dynamics of natural biofilms (Battin et al., 2003b). Nuy et al. (2018) observed only weak community changes as responses to reduced flow velocity. They concluded that flow velocity, despite its known influence on nutrient and oxygen distribution in streams, may not necessarily lead to significant changes in community composition. Furthermore, a weak but significant correlation was found between photosynthetic biomass (F_0) and reduced flow velocity. One quantitative effect of flow velocity was a drop in photosynthetic biomass (Bondar-Kunze et al., 2016).

Summarizing, our analyses of reduced flow conditions revealed inconsistent results about increase / decrease in diversity indices compared to normal flow regimes according to our data, suggesting a decline in diatom biodiversity, alongside an increase in overall algal diversity, under low flow conditions. Furthermore, the composition of diatom and microalgal communities differed significantly under

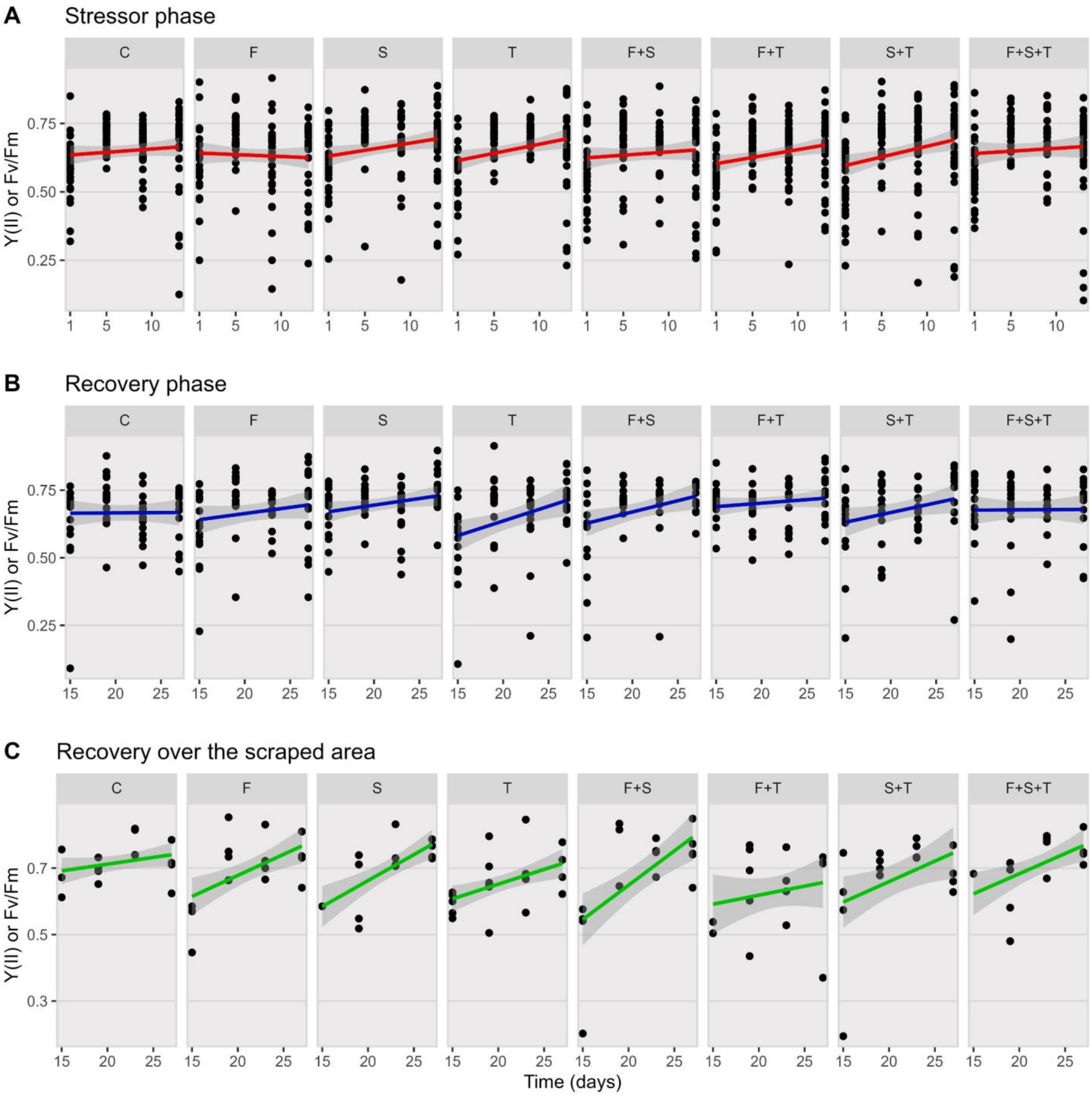

Fig. 6. Plot showing the quantum yield of Photosystem II (Y(II) or Fv/Fm) per treatment with fitted linear regression line. C: control; F: Reduced flow velocity; S: increased salinity; T: increased temperature; F + S: reduced flow + increased salinity; F + T: reduced flow velocity + increased temperature; S + T: increased salinity + increased temperature; F + S + T: reduced flow velocity + increased salinity + increased temperature.

reduced flow conditions compared to other treatments, indicating changes in community structure. Additionally, reduced flow conditions were associated with a slight decrease in photosynthetic biomass, suggesting a possible impact on primary productivity within the ecosystem. These results highlight the importance of considering flow velocity dynamics in understanding and managing aquatic ecosystems, as reductions in flow conditions resulting from low rainfall under climate change scenarios can have significant ecological impacts.

4.1.2. Salinity

We found no significant effect of salinity on microalgae biomass and community composition during stressor phase. This is in contrast to our hypothesis, which predicted a shift in community composition toward a dominance of salt tolerant taxa as a result of increased salinity in the system. However, one diatom taxon and few microalgae OTUs were significant indicators of increased salinity, highlighting their sensitivity as indicators. Microscopy analysis showed that only *Mayamaea permitis* was a significant indicator of increased salinity. This taxon is considered a good indicator of polluted waters (Cantonati et al., 2017). Although microalgae OTUs also showed no statistically significant response to increased salinity treatment during the stressor phase, six (2 Bacillariophyta, 2 Ochrophyta and 2 Chlorophyta) were significant indicators of salinity.

Notably, the average conductivity increase between background and

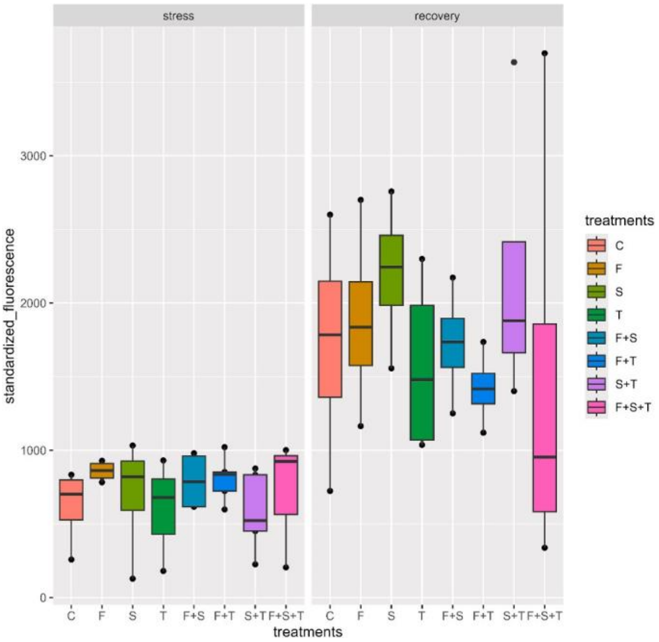

Fig. 7. Boxplot showing chlorophyll fluorescence in different treatments during stressor (Nstr) and recovery (Nrec) phases. C: control (Nstr = 4, Nrec = 4); F: Reduced flow velocity (Nstr = 4, Nrec = 4); S: increased salinity (Nstr = 4, Nrec = 4); T: increased temperature (Nstr = 3, Nrec = 4); F + S: reduced flow + increased salinity (Nstr = 4, Nrec = 4); F + T: reduced flow velocity + increased temperature (Nstr = 5, Nrec = 4); S + T: increased salinity + increased temperature (Nstr = 5, Nrec = 4); F + S + T: reduced flow velocity + increased salinity + increased temperature (Nstr = 3, Nrec = 4).

increased salinity treatment was 0.529 mS/cm, corresponding to an added chloride of 154 mg/L. Although in general, even an increase of chloride concentration by 0.5 mg/L can lead to a change in the structure of microalgae community (Stenger-Kovács et al., 2023), the stream investigated here regularly experiences salinity fluctuations that are substantially larger than we could simulate in our mesocosm experiment. Our results show that the salinity increase by ca. 154 mg/L did not constitute a significant stressor in the studied ecosystem. In spite of this, both microscopic and amplicon analyses revealed subtle but noticeable differences compared to control conditions. Altogether, salinity change of the magnitude applied here may be a subliminal stressor for the studied system, with only a few diatom and microalgal species responding, also highlighting the sensitivity of the few affected taxa as indicators of salinity change.

4.1.3. Temperature

We detected a significant effect of increased temperature treatment on diatom communities (Shannon diversity and taxonomic composition as determined by microscopy) but not on the whole microalgae assemblages (amplicon data). *Parlibellus protractoides* and *Fragilaria pectinalis* were good indicators of increase in temperature. Shannon diversity was lower in increased temperature mesocosms, in line with our hypothesis. Often, stressors cause a decline in diatom species richness as observed in this case with increasing temperature in mesocosms (Piggott et al., 2015; Da Silva et al., 2019; Lengyel et al., 2023b). In this study, we achieved stressor treatment by effectively increasing temperature for at least 9 days of warming. According to Lengyel et al. (2023b), a moderate temperature increase of 3 °C may be the main factor changing the composition of benthic algal community. It is important to note that this study was conducted in the summer with temperatures above 20 °C, making direct comparisons difficult as our study took place in the spring with the highest mean ambient temperature of 8.7 °C. Thus, our temperature increase treatment of +3.45 °C kept stressed mesocosms within the optimal range for the growth of diatoms. This probably favoured diatom growth since they usually prefer lower temperatures, having their optimum growth below 20 °C (Patrick et al., 1969). Although some diatoms can grow even at higher temperatures, they cannot compete with Cyanobacteria, due to their better resource utilization and, in some cases, production of allelochemicals (Leflaive and Ten-Hage, 2007). Many diatoms have an optimal growth temperature range between 5 and 20 °C, Chlorophyta between 15 and 30 °C and Cyanobacteria can also grow above 30 °C (Patrick et al., 1969). Therefore, to better simulate critical ecological effects of global warming, heated stream mesocosm experiments conducted in summer will be more suitable.

While photosynthetic biomass (F_0) decreased in all mesocosms during the stressor phase, the maximum quantum yield (Y(II) or Fv/Fm) showed a slight increase, with increased temperature showing a weak positive effect. The initial decrease in photosynthetic biomass could be due to an overall strong cooling during the stressor phase, which may have overridden treatment effects on overall periphyton growth (as seen in F_0). Contrary to Lengyel et al. (2023b), who found that higher temperature reduced the maximum quantum yield of photosystem II in microphytobenthos, we found a positive effect; the difference between both studies can again probably be explained by the different ambient temperatures.

4.1.4. Stressor interactions

Our results showed only one statistically significant interaction between temperature and flow velocity, which had a small but significant positive coefficient. This suggests a potentially synergistic effect between these two variables on microalgae OTUs. Thus, the combined influence of velocity and temperature may affect microalgae communities more than would be predicted from the effect of each variable acting alone (Vos et al., 2023). Synergistic interactions underscore the complexity of ecological interactions in stream ecosystems and highlight the importance of considering multiple environmental factors when assessing their impact on microalgae communities. There was no other significant two- or three-way interaction.

4.2. Recovery

Several observations (e.g. differences in multivariate dispersions, Gamma and Beta diversity indices, Table S1) indicate a homogenization of diatom communities during the recovery phase in comparison with the stressor phase. This is expected due to the more similar environmental conditions. Most treatment channels reached a recovery end time point community composition statistically indistinguishable from the control channels, indicating a close-to-complete recovery during the 2-week recovery phase (Table S2). Fluctuations in temperature likely played a significant role in shaping the composition and dynamics of periphyton communities and biomass during our experiment and the rather different ambient temperatures are suspected to play an important role in explaining this.

4.3. Priority effects

We observed faster microphytobentos growth on scraped surfaces compared to those on unscraped ones during the recovery phase, resulting in identical total in vivo chlorophyll fluorescence (as a proxy of photosynthetic biomass) at the end of the recovery phase. Community composition after the two weeks of the recovery phase was different in the scraped when compared with the non-scraped areas. This observation suggests that substrate occupancy by resident taxa slowed down recolonization after stressor effect, and influenced the taxonomic composition of the final communities. We interpret this to reflect transitional priority effects caused by competition for substrate between resident and immigrant taxa (Stroud et al., 2024). Further research into the mechanisms driving priority effects in microphytobenthos communities could provide valuable insights into asymmetric paths of recovery vs degradation.

5. Conclusion

This study examined the response of microalgae to reduction in flow velocity, and increase in salinity and temperature in a stream mesocosm experiment. We detected significant effects of flow velocity and temperature on microalgae communities, increase in salinity led to little change, probably because the applied stressor intensity was relatively mild for microphytobenthic organisms of the study system. Although we saw a rough convergence among stressor treatments during the recovery phase of the experiment, the different communities observed between pre-colonized vs. cleaned (scraped) areas at the recovery end time point indicate that a successional climax has probably not been reached by this time. The latter comparison also indicates that resident communities at least transiently influence recolonization dynamics in a post-stressor recovery, in line with the Asymmetric Response Concept (Vos et al., 2023).

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.173670.

CRediT authorship contribution statement

Ntambwe Albert Serge Mayombo: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Investigation, Formal analysis, Data curation, Conceptualization. **Andrea M. Burfeid-Castellanos:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Conceptualization. **Anna-Maria Vermiert:** Writing – review & editing, Project administration, Methodology, Investigation. **Iris Madge Pimentel:** Writing – review & editing, Visualization, Project administration, Methodology, Investigation, Data curation. **Philipp M. Rehsen:** Writing – review & editing, Project administration, Methodology, Investigation, Conceptualization. **Mimoza Dani:** Writing – review & editing, Investigation. **Christina Jasinski:** Writing – review & editing, Investigation. **Marzena Agata Spyra:** Writing – review & editing, Project administration, Investigation. **Michael Kloster:** Writing – review & editing, Software, Project administration, Methodology, Investigation. **Danijela Vidaković:** Writing – review & editing, Investigation. **Dominik Buchner:** Writing – review & editing, Project administration, Methodology, Investigation. **Bánk Beszteri:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

All the datasets and the R scripts used in this study are available in our GitHub repository: https://github.com/sergemayombo/exstream 2022.

Acknowledgements

This study was conducted within framework of the Collaborative Research Centre (CRC) 1439 RESIST (Multilevel Response to Stressor Increase and Decrease in Stream Ecosystems; www.sfb-resist.de) funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation; CRC 1439/1, project number: 426547801). We are very grateful for the support of many helpers during the experiment in the field and laboratory work. The editor and two anonymous reviewers are thanked for their comments that helped improve this manuscript. We acknowledge support by the Open Access Publication Fund of the University of Duisburg-Essen.

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Paper 3. Submitted to Frontiers in Ecology and Evolution

Assessment of microphytobenthos communities in the Kinzig catchment using photosynthesis-related traits, digital light microscopy and 18S-V9 amplicon sequencing

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Contributions:

- Conception 10 %: Followed the hypotheses and study design drawn in the grant application.
- Conduction of experimental work 0 %: Samples taken by CRC1439 project Z02
- Data analysis 100 %: Data curation completely done by NASM.

- Species identification 100 %: Species identification completely done by NASM.
- Statistical analysis 100 %: Statistical analysis designed and executed by NASM.
- Writing the manuscript 100 %: First draft of the manuscript written in its entirety by NASM.
- Revision of the manuscript 15 %: Shared revision of all co-authors.

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Chapter III

Assessment of microphytobenthos communities in the Kinzig catchment using photosynthesis-related traits, digital light microscopy and 18S-V9 amplicon sequencing

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Keywords: Microphytobenthos, biofilm, Bacillariophyta, environmental factors, digital microscopy, amplicon sequencing, photosynthetic biomass

Abstract

Microalgae form an essential group of benthic organisms that respond swiftly to environmental changes. They are widely used as bioindicators of anthropogenic stressors in freshwater ecosystems. We aimed to assess the responses of microalgae communities to multiple environmental stressors in the Kinzig River catchment, home to a long-term ecological monitoring site, in Germany. We used a photosynthetic biomass proxy alongside community composition of diatoms assessed by digital light microscopy, and of microalgae by 18S-V9 amplicon sequencing, to characterise microalgae at 19 sampling sites scattered across the catchment. Our results revealed significant effects of physical and chemical factors on microalgae biomass and community compositions. We found that conductivity, water temperature and pH were the most important factors affecting microalgae community composition, as observed in both microscopy and amplicon analysis. In addition to these three variables, the effect of total phosphate on all microalgae, together with water discharge on the diatom (Bacillariophyta) communities, as assessed by amplicon analysis, may reveal taxon-specific variations in the ecological responses of different microalgal groups. Our results highlighted the complex relationship between various environmental variables and microalgae biomass and community composition. Further investigations, involving the collection of time series data, are required to fully understand the underlying biotic and abiotic parameters that influence these microalgae communities.

1 Introduction

The complex assemblages of microorganisms colonizing all submerged substrates also referred to as benthic microalgae biofilms, periphyton, or microphytobenthos (MPB), play an essential role in the biogeochemical cycles, primary production, and sediment stabilisation (B-Béres et al., 2023). At first glance, these tiny organisms may seem insignificant, but their presence and importance in rivers should not be underestimated. Through photosynthesis, these microscopic organisms act as microforests, converting sunlight into energy, and producing oxygen and organic matter. They contribute substantially to the global carbon and nitrogen cycles and provide essential ecosystem services that sustain diverse forms of life (Castro-Català et al., 2020; B-Béres et al., 2023). The environment and other organisms with which MPB biofilms interact (such as grazers) affect their species richness, community composition, and productivity (Birk et al., 2020). The ability of MPB biofilms to grow in different habitats and conditions also explains the compositional variability and complexity observed in their assemblages.

MPB assemblages are mainly dominated by diatoms (Bacillariophyta), but also other eukaryotes, including other microalgae such as Chlorophyta, Cyanobacteria and Euglenophyta, as well as other mixo- and heterotrophic protists, and fungi, may be present in lesser proportions (Underwood, 2010; Lemley et al., 2016; Dalu et al., 2020). Diatoms are secondary endosymbiotic eukaryotic algae belonging to the phylum Stramenopiles. Their glassy, siliceous cell walls known as frustules have been the focus of MPB community studies, mainly by observing their morphology under a light or a scanning electron microscope (Morales et al., 2001; Smol and Stoermer, 2010). Due to their short life cycles and rapid response to abiotic and biotic changes, MPB, particularly diatoms, are valuable bioindicators that can provide important insights into the health of aquatic ecosystems (Smol and Stoermer, 2010). Therefore, since 2000, the European Commission Water Framework Directive (European Commission, 2000) recommends the use of Biological Quality Elements (BQE), including diatoms, for the ecological assessment of surface waters.

Generally, studies of MPB biofilms have focused on the study of diatoms using light microscopy to analyse their community compositions. As diatoms are often the main component of these benthic systems, this approach has provided valuable insights into their ecological dynamics because they swiftly respond to both present and past environmental effects (Soininen et al., 2016; Viso and Blanco, 2023). Several studies have examined the responses of diatom communities to various anthropogenic disturbances (Walsh and Wepener, 2009; Hlúbiková et al., 2014; Teittinen et al., 2015; Stenger-Kovács et al., 2020). Diatom responses to a range of stressors, including heavy metal pollution (Lavoie et al., 2018), habitat fragmentation (Szczepocka et al., 2021) and climate change (Tornés et al., 2022) are well documented. This wealth of knowledge has greatly improved our understanding of diatom ecology and its resilience or vulnerability under different stressful conditions.

However, these traditional methods present a somewhat biased perspective. They often overlook the diversity and functional potential of the wider range of other organism groups that make up MPB biofilms, which includes not only other microalgae but also mixotrophic and heterotrophic protists and even fungi. This narrow focus on diatoms may lead us to underestimate the true ecological complexity and functional diversity of MPB biofilms. Additionally, it is not only the community composition that necessitates investigation but also ecosystem functions (Stenger-Kovács et al., 2020). Among these functions, photosynthesis stands out as a primary process underpinning the productivity and biogeochemical cycling in freshwater ecosystems.

A crucial aspect that remains underexplored is the potential differential impact of anthropogenic activities on microphytobenthos communities. As we continue to reshape the planet in the Anthropocene, understanding the nuanced effects of multiple anthropogenic stressors has become increasingly important. Human activities introduce different types of anthropogenic pressures in river catchments. These human-induced stressors have various effects on microphytobenthos communities (Feld et al., 2016; Birk et al., 2020). For example, in urban areas, with high population densities, extensive infrastructure and industrial activities, cocktail of point source pollutants may enter in freshwater ecosystems, that include wastewater treatment plant effluents, channel erosion, nutrients load, and inflow of contaminants such as heavy metals and other chemicals (Trábert et al., 2020). This is in contrast with the rural landscapes dominated by agricultural land use, which is characterized typically by more diffuse pollution loads, such as the influx of nutrients, agrochemicals, pesticides and fine sediments, as well as hydromorphological alterations (Feld et al., 2016; Schürings et al., 2022). Yet, the effects of these multiple anthropogenic stressors (chemical and hydro-morphological) on microphytobenthos communities remain largely unknown.

In this study, we investigated how stream microphytobenthos communities respond to environmental variables in the Kinzig catchment, which is part of the Rhine-Main-Observatory (RMO), a long-term ecological research site (LTER) in central Germany. We focused on the primary environmental correlates of periphyton distribution in this river network. Our objectives were to: (1) assess the dynamics of photosynthetic biomass as a result of water physical and chemical conditions; (2) examine microalgae community composition and diversity metrics (abundances, richness, etc.) in relation to environmental variables and associated changes in ecological status; (3) identify the potential environmental factors that influence community composition in response to environmental conditions and ecological status, as compared to the macrozoobenthos studies of the LTER (Nguyen et al., 2023). We postulated that photosynthetic biomass and composition of MPB communities in the Kinzig catchment correlate with environmental factors. Higher biomass and reduced diversity will be associated with increased nutrient availability and eutrophication in the system. Similarly, hydrology (e.g. high flow velocity and depth, producing cloudy and low-light waters), will result also in a reduction in algal biomass and change in community composition. We seek to understand the resilience and adaptability of the MPB community by contrasting their responses to multiple anthropogenic stressors. In the face of numerous human pressures, this knowledge may help develop more potent conservation strategies for these essential freshwater ecosystem components. In addition to shedding light on the wider effects of multiple stressors on freshwater ecosystems, our study advances our understanding of MPB ecology.

2 Materials and methods

2.1 Study site

In spring 2021, nineteen samples were collected from various streams across the Kinzig catchment and examined using a range of methods, including a recently developed digital version of diatom light microscopy for improved resolution of community composition (Burfeid-Castellanos et al., 2022), 18S-V9 amplicon sequencing for more comprehensive taxonomic characterisation of MPB communities (Amaral-Zettler et al., 2009) and in situ chlorophyll fluorescence measurements as a proxy for overall and group-specific photosynthetic biomass estimation (Kahlert and McKie, 2014). The Kinzig catchment area (1058 km2) is a long-term ecosystem research site (LTER) in the Rhine-Main Observatory (RMO) (Nguyen et al., 2023). This low-mountain river system flows through different types of land use introducing various stressors. We are currently unaware of any ongoing restoration initiative in this river catchment, although land use in the study area has changed over time. Germany is actively working to reduce nutrient emissions in all catchment areas, in addition to ongoing efforts to address climate change (Nguyen et al., 2023). These factors could alter the structural composition of the microphytobenthos community over time. This study was part of the Collaborative Research Centre Multilevel Response to Stressor Increase and Release in Stream Ecosystems (CRC RESIST, https://sfb-resist.de/), which investigates the effects of global change in rivers and streams using field studies, mesocosm field and lab experiments and modelling approaches on several organism groups and scopes. We collected 19 samples in spring 2021 from ten tributaries selected within the Kinzig catchment (Figure 1). Sites were sampled based on adjacent land use.

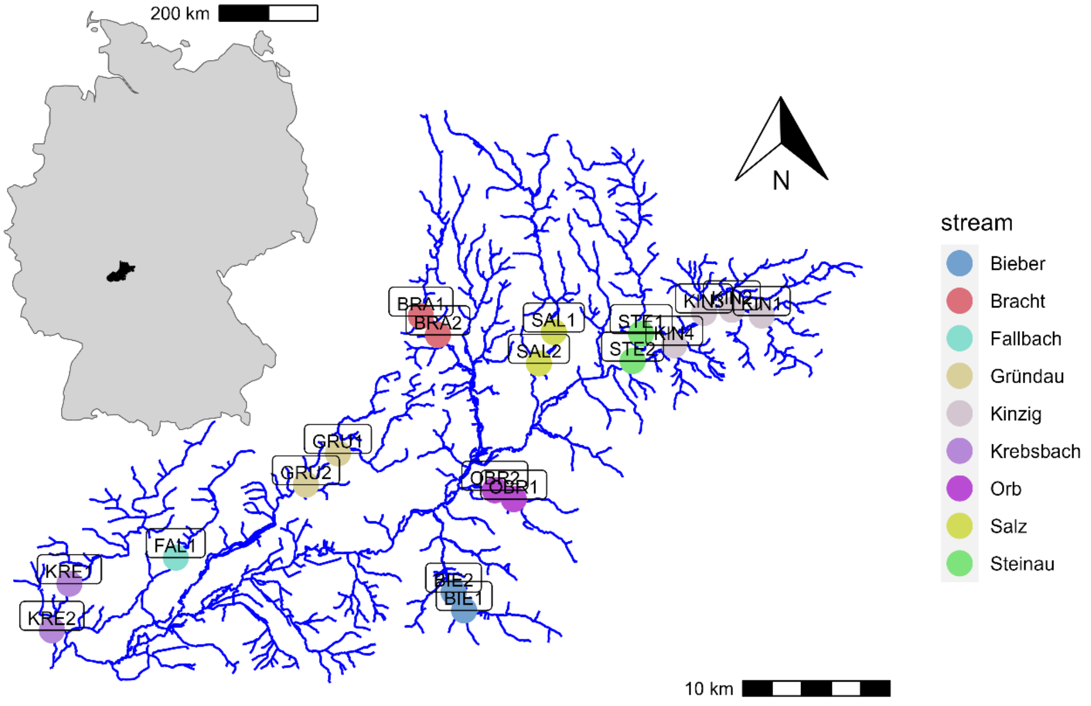

Figure 1 Map of the Kinzig catchment in Central Germany, showing sampling sites

2.2 Sample collection, preparation and processing

At least five separate chlorophyll (Chl) fluorescence measurements were made in situ on each sampled rock using a BenthoTorch (bbe Moldaenke GmbH, Schwentinental, Germany). The measurements were made using factory-programmed settings, and total Chl-a concentrations were given as the sum of biomass values for the three photosynthetic algal groups, namely cyanobacteria, green algae and diatoms. Biofilm samples were collected according to the recommendations and guidelines for sampling benthic diatoms from European rivers (Kelly et al., 1998; CEN. UNE-EN 13946:2014, 2014). A new, clean toothbrush was used to scrape approximately 20 cm2 from each of the five cobble stones sampled at each site. Approximately 20 mL of water from the river was used to collect the scrapings from the 5 cobble stones. As these samples were collected for both digital microscopy and DNA extraction, absolute ethanol (99%) was added, resulting in an approximate final concentration of approximately 75%, transported on ice and then frozen at -20˚C once in the laboratory until they were divided for preparation for further analysis by digital microscopy and molecular methods. Water temperature, electrical conductivity and pH were measured in situ using a PCE-PHD 1 pH meter (Meschede, Germany). Other physical and chemical properties of the water including dissolved oxygen (DO), oxygen saturation, average velocity, average depth, discharge and water level were also measured in situ during biofilm sampling. In the laboratory, the Deutsches Institut für Normung (DIN) ISO norms were followed to determine the content of ortho-phosphate by photometry using a test kit, total phosphorus (TP) via ICP-MS (PerkinElmer), ammonia by photometry according to DIN 38406-5 (E5 – 1), nitrite by photozometry according to DIN EN 26 777, nitrate chloride and sulfate via ion chromatography, total nitrogen (TN) was calculate from nitrite, nitrate and ammonium values.

2.3 Diatom analysis by digital microscopy

Sample preparation and processing for digital microscopy followed the procedures described by Burfeid-Castellanos and coworkers (Burfeid-Castellanos et al., 2022). After five prewash cycles, the biofilm samples were digested with hydrogen peroxide and hydrochloric acid on a hot plate (Taylor et al., 2007). After seven rinsing cycles, centrifugation at 1200 rpm for 3 minutes (Eppendorf Centrifuge 5427 R; Eppendorf, Hamburg, Germany) and decanting and replenishing with distilled water, a 400 µL aliquot of each cleaned sample was pipetted onto a coverslip (15 x 15 mm, #1,5), allowed to dry and then mounted onto permanent glass slides using Naphrax resin (refractive index = 1.71, Biologie-Bedarf Thorns, Deggendorf, Germany). To produce virtual slides, an area of approximately 5 x 5 mm2 on the part of the permanent glass slide with the highest density of evenly distributed diatom valves was scanned using a VS200 slide scanner (EVIDENT, Tokyo, Japan) at a magnification of 600x (UPlanXApo) with the ASW 3.1 software (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

These virtual slides were analysed for diatom counting and identification with the BIIGLE 2.0 web browser-based image annotation platform (Langenkämper et al., 2017; Zurowietz and Nattkemper, 2021) as described previously (Burfeid-Castellanos et al., 2022). Using a combination of general (Bey and Ector, 2013; Cantonati et al., 2017; Spaulding et al., 2022) and specific taxonomic literature (Cox, 1995; Levkov et al., 2013; Trobajo et al., 2013), at least 400 diatom valves were counted and identified at the highest level of possible taxonomic resolution, mostly at species level. For each sample, a list of the taxa and their relative abundances was generated to calculate the IPS (“Indice de Polluosensibilité Spécifique”) (Prygiel and Coste, 1993), using OMNIDIA software (Lecointe et al., 1993) and to run multivariate statistical analyses using R open source software environment for statistical analyses (R Core Team, 2023).

2.4 Molecular analyses

2.4.1 DNA extraction and sequencing

Sample preparation and DNA extraction were performed following the silica bead-based extraction protocol described by Buchner (Buchner, 2022a; 2022b). Each sample was processed in 2 replicates. Genomic DNA amplification of the V9 hypervariable region of the small subunit ribosomal RNA genes (ca. 130 bp) was performed using the universal specific forward primer 1389F (5′-TTGTACACACCGCCC-3′) and the eukaryotic specific reverse primer 1510R (5′-CCTTCYGCAGGTTCACCTAC-3′) (Amaral-Zettler et al., 2009), following the protocol of Stoeck et al. (Stoeck et al., 2010). The resulting library was sequenced on an Illumina MiSeq at CeGat GmbH (Tübingen, Germany).

2.4.2 Bioinformatics analysis

The Natrix2 workflow (Deep et al., 2023) was used to perform bioinformatics analyses on microphytobenthos Illumina amplicon sequencing data. The operational taxonomic units (OTUs) variant of the workflow was implemented using the SWARM v3.0.0 (Mahé et al., 2015) clustering algorithm. Paired-end reads were assembled using the simple Bayesian algorithm in PANDAseq v2.11 (Masella et al., 2012). Primers were trimmed with cutadapt v3.2 (Martin, 2011), and then filtered with a PANDAseq threshold score of 0.9, minimum length of 77, and maximum length 196 nucleotides. Sequence dereplication was performed using the CD-HIT v4.8.1 (Fu et al., 2012) algorithm at 100% similarity and chimeric sequences were identified and removed using VSEARCH v2.15.2 (Rognes et al., 2016). The split-sample approach (Lange et al., 2015) was applied to reduce erroneous sequences without strict abundance cut-offs. Statistical analysis of the amplicon sequencing data resulting from the split-sample process was conducted using the AmpliconDuo v1.1 R package (Lange et al., 2015). OTUs were generated by clustering sequences with Swarm v3.0.0 (Mahé et al., 2015). Then, mothur v1.40.5 (Schloss et al., 2009) was used to align OTUs against the protist ribosomal reference database (PR2) v.4.14.0 (Guillou et al., 2013). Finally, mumu (https://github.com/frederic-mahe/mumu), a C++ implementation of lulu (Frøslev et al., 2017), was used for post-clustering curation of the periphyton amplicon sequencing data. The replicates of sequenced samples were merged into one representative sample by calculating the sum of reads for each OTU and the negative controls with maximum reads were subtracted from the sum of both samples to standardize the proportion of sequences. This is important to reduce redundancy in the resulting dataset for subsequent statistical analyses. The resulting OTU table with their relative read abundances is used for subsequent multivariate statistical analyses.

2.5 Data analysis

We further examined the results from all above-described analyses using R v4.2.2 (R Core Team, 2023) and various packages including 'tidyverse' v2.0.0 (Wickham et al., 2019), 'vegan' v2.6-4 (Oksanen et al., 2022), 'factoextra' v1.0.7 (Kassambara A, 2020), 'ggstatsplot' v0.12.0 (Patil, 2021). Principal Components Analysis (PCA) was conducted after scaling the variables to have a mean of zero and a standard deviation of one, to show the correlation between environmental variables and sampling sites throughout the catchment. Correlation analysis was performed to determine the relationship between photosynthetic biomass and environmental variables, visualized using 'corrplot' package (v0.92, Wei and Simko, 2021). We used the Hellinger transformed abundance matrix for species composition and OTU reads (Legendre and Gallagher, 2001). PERMANOVA was conducted to determine which environmental variables influenced diatoms and microalgae OTU assemblages. Furthermore, to characterise the influence of environmental variables on diatom species composition and microalgae OTU assemblages, we conducted the Redundancy Analysis (RDA). To simplify the RDA model, we performed a stepwise selection of statistically important environmental variables using 'ordiR2step()' function. Only variables with significant correlations were visualized in the graphs.

3. Results

3.1. Environmental variables analysis

The physical and chemical parameters measured in this study are summarized in Table 1. The pH value of the water was neutral to relatively alkaline (7.03 to 9.55), with low to moderate electrical conductivity (108 to 862 µS/cm) (More details are given in Supplementary Table 1). Water temperature varied between 6 and 12.9˚C, mean 8.69˚C. The PCA performed on the matrix of physical and chemical variables showed that the first two axes explained 74.6% of the total variation in the data, so the first axis explained 58.2% of the variation and the second axis explained 16.4% of the variation (Figure 2). The most significant gradients in the environmental data were represented by chloride, conductivity, sulfate, nitrate and total nitrogen, which were the main contributors to the first PCA axis, while total phosphorus and average depth were the main contributor to the second PCA axis (Figure 2). The PCA ordination diagram showed two main groups of sampling locations. On the left side, the locations are distributed along a hydrological gradient (average depth vs. average velocity) that can be interpreted to roughly reflect altitude or upstream – downstream position in the catchment. The right side of the diagram shows three downstream sampling locations (FAL1, KRE1 and KRE2) offset from the others by increased nutrients and salinity (Figure 2, Table 1). This means that while our sampling locations seem to capture well the catchment-wide hydrological gradient, it does not resolve the nutrient and salinization gradient that appears close to orthogonal to the latter.

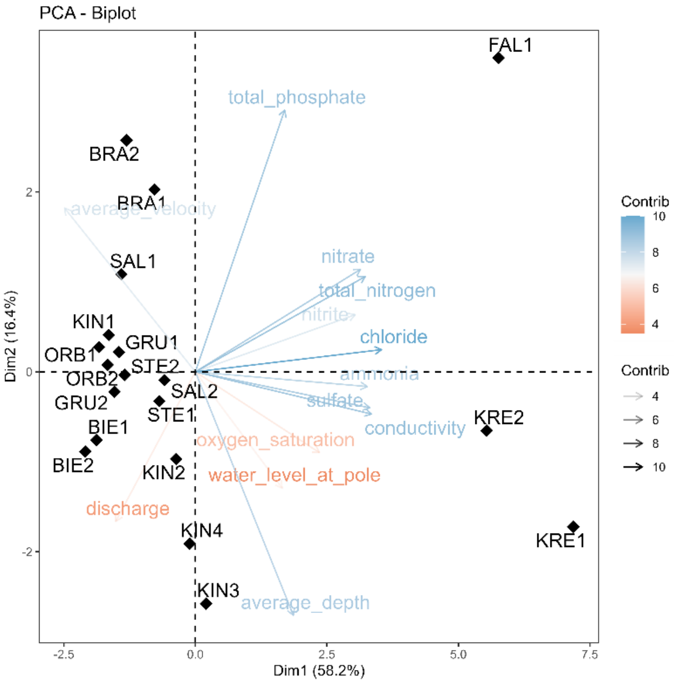

Figure 2 PCA biplot showing the relationship between the physical and chemical parameters and sampling sites The arrows and colour gradient show the contribution of different variables to the principal component.

Table 1 Summary statistics for physical and chemical parameters measured in the Kinzig catchment.

Parameter	**Unit**	**Min**	**Max**	**Mean**
pH		7.03	9.55	8.3
Water temperature	˚C	6	12.9	8.69
Dissolved oxygen	mg/L	11.1	15.3	12.69
Oxygen saturation	mg/L	97.03	145.4	109.4
Conductivity	µS/cm	108	862	334.26
Average velocity	m/s	0.07	0.70	0.39
Average depth	cm	11.4	50.9	26.4
Discharge	m^3/s	0.104	0.647	0.302
Water level at pole	cm	22	60	36.37
Total phosphate	mg/L	0.013	0.071	0.025
ammonia	mg/L	0.004	0.324	0.054
Nitrite	mg/L	0.0103	0.189	0.049
Nitrate	mg/L	3.3	16.0	7.55
Total nitrogen	mg/L	0.757	3.798	1.76
Chloride	mg/L	6.4	64.0	19.33
Sulfate	mg/L	6.5	47.0	16.5

3.2. Photosynthetic biomass

We found statistically significant correlations between photosynthetic pigment fluorescence attributed by the BenthoTorch algorithm to cyanobacteria, green algae, diatoms and total algae concentration between the streams (ANOVA, all p values < 0.05). The correlation matrix of the physical and chemical environmental variables and mean photosynthetic biomass of different microalgae groups showed that pH was positively correlated with total and cyanobacterial fluorescence; sulfate strongly negatively correlated with total, cyanobactreial, and diatom (but not with green algal) chlorophyll signal. Total and green algal pigment fluorescence was also negatively correlated with average depth, as well as cyanobacterial and green algal signal with average velocity. Besides, water temperature and ammonia both showed a negative correlation with the diatom signal (Figure 3).

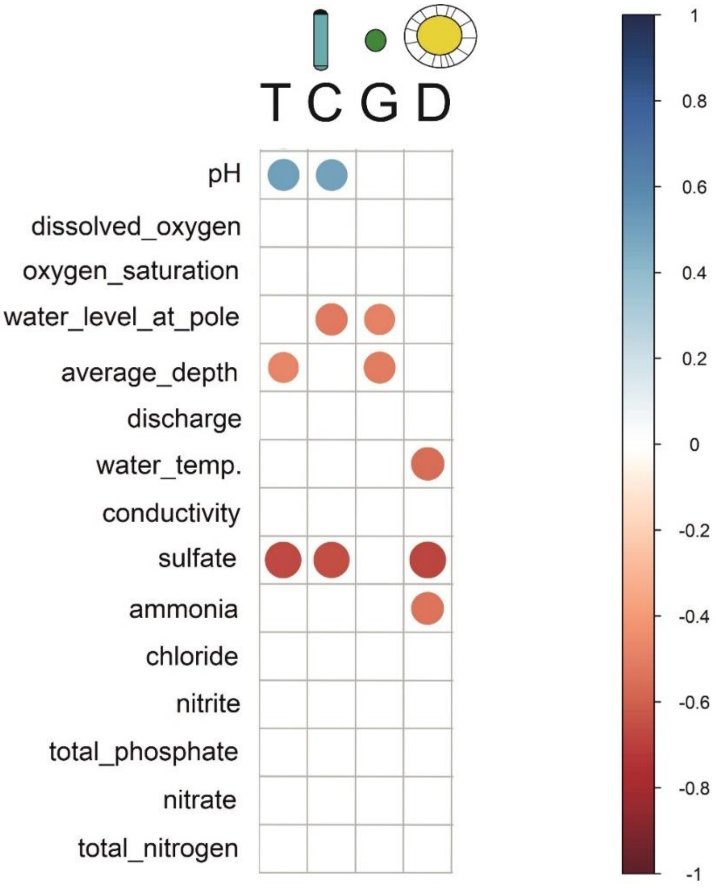

Figure 3 Correlation matrix showing the relationship between physical and chemical variables, as well as photosynthetic biomass of different microalgal groups. The variables in the matrix are ordered such that the

correlated ones are next to each other. Positive correlations are displayed in blue and negative correlations in red colour. Colour intensity and the size of the circle are proportional to the correlation coefficients. Correlation coefficients with a p-value > 0.05 are considered insignificant and their fields remain blank. T = Total microalgae concentration, C = Cyanobacteria, G = Green algae, D = Diatoms

3.3. Diatom community composition: morphological analysis

A total of 142 diatom taxa belonging to 46 genera were identified by digital microscopy. Diatom community composition was dominated by *Navicula lanceolata*, *Navicula gregaria*, *Nitzschia dissipata*, *Achnanthidium minutissimum* and *Fragilaria deformis* (Figure 4 and Supplementary Table 2). Species richness of individual samples ranged from 21 to 56 (mean 37.3) and Shannon diversity index from 1.26 to 3.25 (mean 2.56). Both species richness and Shannon were lower in KRE1 and higher in GRU2. PERMANOVA common model including all predictors showed that conductivity, pH, and water temperature had significant effects ($p < 0.05$) on diatom community composition. All other factors had no statistically significant effects on diatom community composition ($p > 0.05$, Table 2).

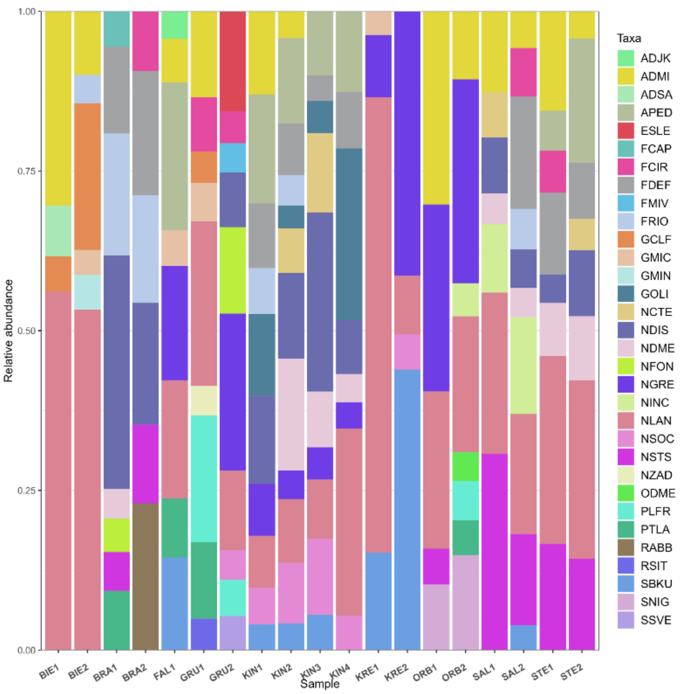

Figure 4 Relative abundance of dominant diatom taxa identified by digital light microscopy. Four letter code based on the OMNIDIA short code, explained in Supplementary Table 2.

Table 2 Results of PERMANOVA partitioning testing the effects of environmental variables on microalgae communities.

Factor	***df***	***SS***	**R^2**	***F***	***P*****
Diatoms (digital microscopy)					
pH	1	0.6901	0.1664	4.1280	**0.007**
Water temperature	1	0.6204	0.1496	3.7111	**0.017**
Dissolved oxygen	1	0.0741	0.0179	0.4431	0.860
Oxygen saturation	1	0.1741	0.0421	1.0445	0.416
Conductivity	1	0.5884	0.1419	3.5195	**0.021**
Average velocity	1	0.1455	0.0351	0.8700	0.535
Average depth	1	0.2064	0.0498	1.2345	0.311
Discharge	1	0.2811	0.0677	1.6816	0.195
Water level at pole	1	0.0822	0.0198	0.4920	0.808
Total phosphate	1	0.1957	0.0472	1.1706	0.355
Ammonia	1	0.1018	0.0245	0.6086	0.772
Nitrite	1	0.0713	0.0172	0.4263	0.863
Nitrate	1	0.1581	0.0381	0.9457	0.484
Chloride	1	0.0840	0.0202	0.5022	0.801
Sulfate	1	0.1728	0.0417	1.0334	0.437
Residual	3	0.5016	0.1209		
Total	18	4.1481	1.0000		
Diatom OTUs (18S-V9 amplicon sequencing)					
pH	1	0.2665	0.1329	5.0575	**0.001**
Water temperature	1	0.2264	0.1129	4.2969	**0.001**
Dissolved oxygen	1	0.0701	0.0350	1.3306	0.267
Oxygen saturation	1	0.0927	0.0462	1.7598	0.105
Conductivity	1	0.3972	0.1981	7.5385	**0.001**
Average velocity	1	0.0465	0.0232	0.8822	0.563
Average depth	1	0.1051	0.0524	1.9947	0.069
Discharge	1	0.1335	0.0666	2.5337	**0.018**
Water level at pole	1	0.0477	0.0238	0.9044	0.505
Total phosphate	1	0.1350	0.0674	2.5629	**0.021**
Ammonia	1	0.0788	0.0393	1.4956	0.190
Nitrite	1	0.0474	0.0236	0.8989	0.513
Nitrate	1	0.0894	0.0446	1.6974	0.112
Chloride	1	0.0651	0.0325	1.2359	0.290
Sulfate	1	0.0457	0.0228	0.8665	0.552
Residual	3	0.1581	0.0788		
Total	18	2.0050	1.0000		
All microalgae OTUs (18S-V9 amplicon sequencing)					
pH	1	0.4197	0.1389	4.6839	**0.001**
Water temperature	1	0.2914	0.0964	3.2521	**0.005**
Dissolved oxygen	1	0.1750	0.0579	19529	0.052
Oxygen saturation	1	0.1454	0.0481	1.6226	0.116
Conductivity	1	0.5216	0.1726	5.8201	**0.001**
Average velocity	1	0.0914	0.0303	1.0205	0.449
Average depth	1	0.1524	0.0504	1.7011	0.079
Discharge	1	0.1526	0.0505	1.7028	0.080

Water level at pole	1	0.0947	0.0313	1.0569	0.419
Total phosphate	1	0.1782	0.0590	1.9885	**0.044**
Ammonia	1	0.1373	0.0454	1.5326	0.116
Nitrite	1	0.0822	0.0272	0.9171	0.564
Nitrate	1	0.1209	0.0400	1.3489	0.191
Chloride	1	0.1103	0.0365	1.2310	0.283
Sulfate	1	0.0800	0.0265	0.8927	0.568
Residual	3	0.2688	0.0890		
Total	18	3.0217	1.0000		

df: degrees of freedom; *SS*: sum of squares; R^2: variance explained by the groups, *F*: *F*-statistic, *p*: p-values, *significant p-values are marked in bold

The RDA model explained a variation of 27% on the first axis and 17.8% on the second. The environmental factors pointed out by this analysis to show the most statistically significant association ($p < 0.05$) with the differentiation of diatom communities were pH, water temperature, oxygen saturation (oxygen_100%), conductivity, average velocity, average depth, ammonia, chloride and sulfate. Of these, only pH, water temperature and conductivity showed a strong effect, while the effects of all the other factors was weak. The RDA ordination diagram shows sites in the upper left quadrant that are largely influenced by pH and oxygen saturation, and were associated with *Nitzschia soratensis* and *Fragilaria rinoi*. Sites plotted in the lower right quadrant, were mainly influenced by ammonia, sulfate, chloride, conductivity and average depth. These sites were associated with taxa such as *Surirella brebissonii* var. *kuetzingii* and *Navicula gregaria* (Figure 5). The ecological status index of diatoms indicated by the IPS ranged from 10.5 to 15.9 (mean 14.3), indicating poor to good ecological status. Only one site, KRE2 had poor ecological status (Figure 5). The main component of the environmental gradient correlated with community composition was that of water temperature vs. pH and oxygen saturation, while the second axis was most strongly correlated with water depth, salinity components (chloride, sulfate and conductivity), and ammonia concentration. Interestingly, the magnitude of differentiation in diatom community composition appeared lower than the separation in environmental variables between the three high-nutrient, high-sulfate locations (FAL1, KRE1, KRE2) observed in the above PCA.

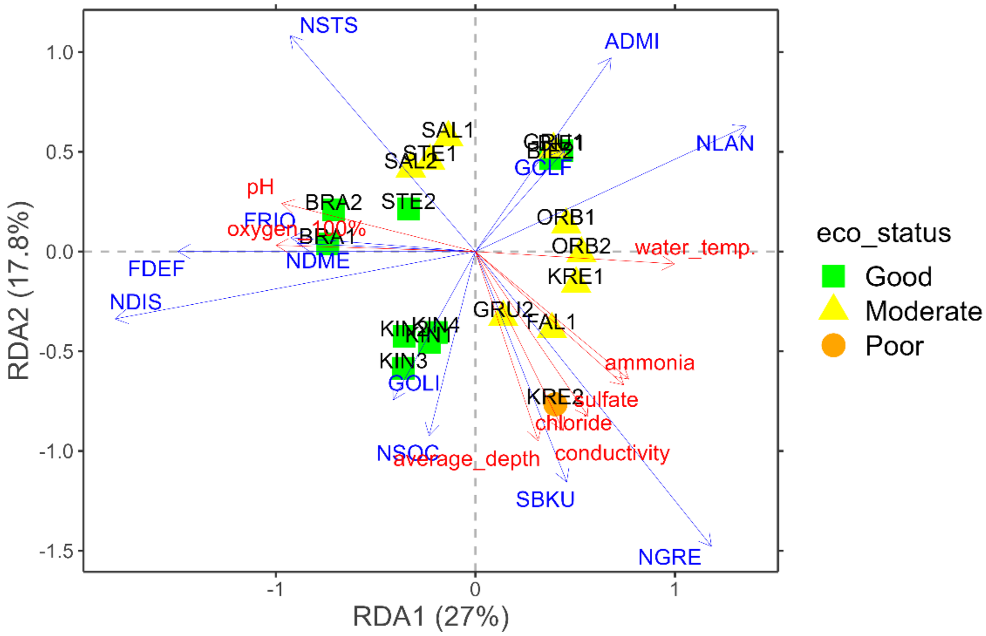

Figure 5 Redundancy analysis (RDA) plot presenting the relationships between the analysed diatom assemblages in the samples based on digital light microscopy and environmental variables. Four letter code based on the OMNIDIA short code, explained in Supplementary Table 2.

3.4. Microalgae community composition: 18S-V9 amplicon sequencing

A total of 21,293,230 reads were obtained after running the Natrix Bioinformatics pipeline on 18S-V9 amplicon sequencing data from the 19 sampling sites (average 1,120,696 reads per sample, minimum 533,819 reads and maximum 1,604,630 reads). These reads were clustered into 7294 OTUs from various protist groups and assigned taxonomy up to the highest possible level. Microalgae OTU richness ranged from 803 to 2295 (mean 1460), Shannon diversity from 6.69 to 7.74 (mean 7.24). The lowest alpha diversity values were recovered in STE1 and the highest in GRU2. Diatoms contributed most of the MPB OTUs, making up the majority of reads (13,322,277 or about 79.3% of total read counts; Figure 6A, Supplementary Table 2). We found 1400 different diatom OTUs, with OTU richness ranging from 140 to 392 (mean 247.59), Shannon diversity ranging from 1.52 to 2.66 (mean 2.16). The lowest value of diatom OTU richness was recorded in BIE2 and the highest in FAL1. The diatom OTUs with the highest read counts were assigned to *Navicula radiosa*, *Navicula gregaria*, *Nitzschia dissipata*, *Surirella brebissoni*, *Melosira varians* and *Asterionella formosa* (Figure 6B). Many of these taxa were also observed during the digital light microscopy analysis, except for *N. radiosa* and *A. formosa* (Supplementary Table 2).

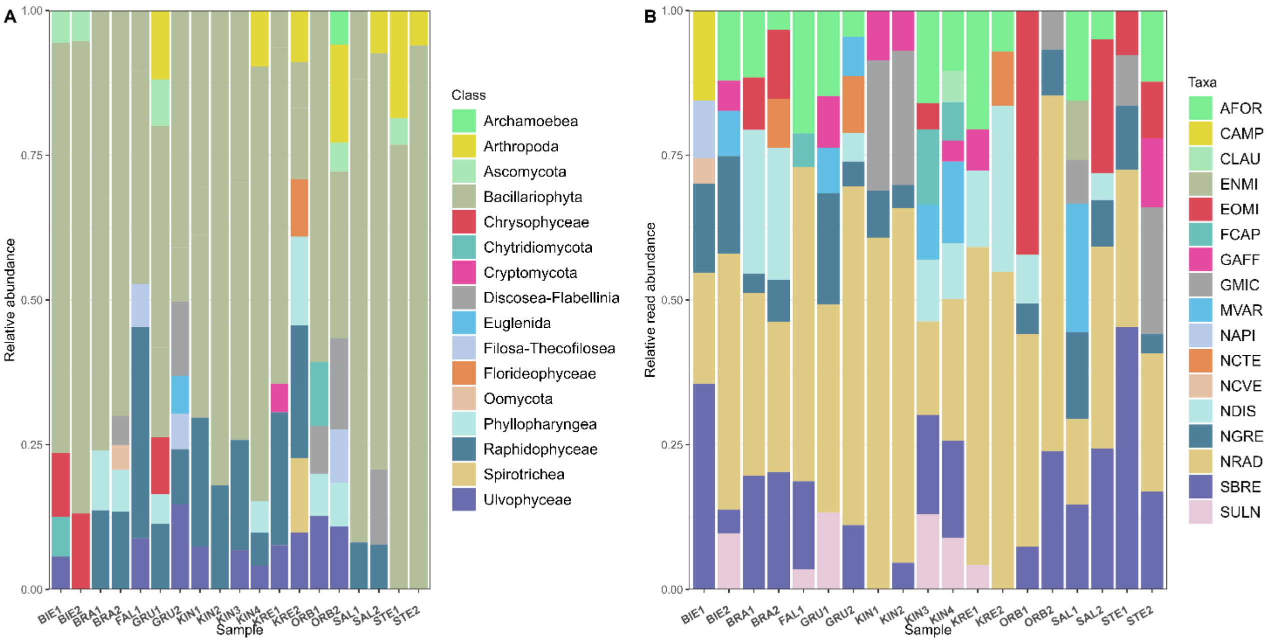

Figure 6 Relative reads abundance of **(A)** dominant OTU classes in the samples and **(B)** taxa associated with dominant diatom (Bacillariophyta) OTUs. Four letter code based on the OMNIDIA short code, explained in Supplementary Table 2.

PERMANOVA common model including all predictors run on the full microalgae OTU dataset revealed that water pH, temperature, conductivity had significant effect on community composition ($p < 0.05$, Table 2). Total phosphate had a weak effect on microalgae community. Similarly, the same model run on the diatom subset of OTU reads revealed significant effects of water pH, temperature, conductivity, discharge and total phosphate ($p < 0.05$, Table 2).

The RDA model examining the relationship between the diatom subset of OTU reads and environmental variables showed a lower explanatory power than the one for the diatom microscopy data set, with a fitted variation of 20.5% explained by the first axis and 17% by the second axis (Figure 7A). The major explanatory gradient here was a salinization – depth gradient that played a slightly lower role in explaining the diatom microscopy results, with the pH vs. water temperature gradient occurring here along the second axis (Figure 7A). In the upper left quadrant of the RDA ordination diagram, sites such as OBR1 and OBR2 were influenced by flow velocity, while pH was the most important predictor at sites such as KIN2 and KIN3 in the lower left quadrant. Sites BIE1, BIE2 and GRU1 in the upper right quadrant were mostly associated with higher water temperature. Finally, FAL1, KIN1 and KIN2 in the lower right quadrant were mainly influenced by chloride, conductivity and depth. The ecological status index (IPS) calculated from the diatom amplicon data set was between 11.4 and 18.1 (mean value 15.6), indicating poor to very good water quality (one has to note that this index was not calibrated for amplicon data sets). Only site KRE2 had a poor ecological status (Figure 7A). In this RDA, the high nutrient, salinized set of samples (FAL1, KRE1, KRE2) appeared with the highest RDA1 scores, but not so strongly offset from the rest of the samples as in the environmental PCA.

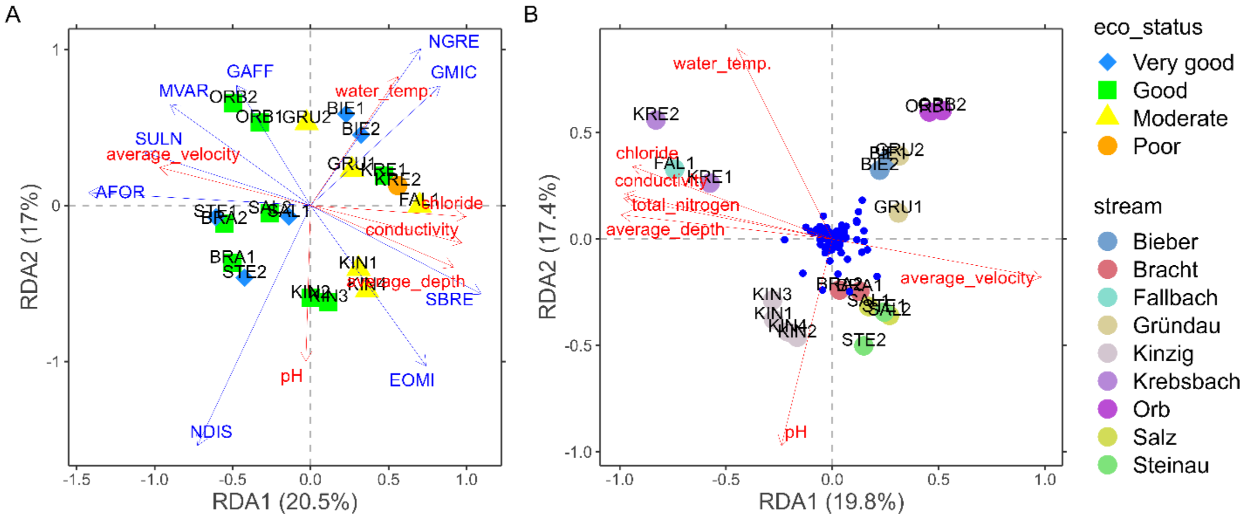

Figure 7 Redundancy analysis (RDA) plots presenting the relationships between the analysed OTUs assemblages and environmental variables: **(A)** diatom (Bacillariophyta) subset of OTUs (ecological status according to IPS index) and **(B)** the whole microalgae OTU dataset (small blue dots show significant OTUs). Four letter code based on the OMNIDIA short code, explained in Supplementary Table 2.

Similarly, the RDA model explained a fitted variation of 19.8% on the first axis and 17.4% on the second axis when analysing the relationship between the entire microalgae OTU read dataset and physical and chemical variables (Figure 7B). The main explanatory environmental gradients were highly similar to the diatom OTU data set (with opposite signs along the first axis). Major environmental factors contributing to these variations ($p < 0.05$) included pH, temperature, conductivity, average velocity, average depth, total nitrogen and chloride. The separation of FAL1, KRE1 and KRE2 from the rest of the samples was clearer in this case than for both above diatom community data sets. (Figure 7B). Sites located in the Kinzig Stream (KIN1 – KIN4), plotted in the lower left quadrant, were mainly influenced by pH, while sites plotted in the lower right quadrant (BRA1, BRA2, SAL1, SAL2, STE1 and STE2) were associated with flow velocity (Figure7B).

4. Discussion

4.1. Microalgae community composition in response to environmental variables

The effort to understand the effects of anthropogenic stressors on microalgal communities within a river catchment remains at the forefront of research in aquatic ecosystems. The compelling reason for this increasing interest is the widespread recognition that anthropogenic activities may have differential impacts on freshwater ecosystems. This study aimed to identify the main environmental drivers of variability in photosynthetic biomass and microalgal community compositions in the anthropogenically stressed Kinzig catchment. Alongside a set of candidate environmental predictors, we used for this purpose data sets capturing total and group-specific photosynthetic biomass and community composition of diatoms (characterized by microscopy vs. by amplicon sequencing) as well as of all microalgal groups (amplicon sequencing).

The PCA ordination of environmental predictors highlighted a hydrological gradient (depth and discharge vs. velocity) well resolved by our samples. Close to orthogonal to that, the PCA indicated a water chemistry gradient (nutrient and salts, the latter mainly sulphate, but also chloride) captured by two clusters of sites (the high nutrient, high salinity locations FAL1, KRE1 and KRE2 vs. all others). In addition, total phosphate concentration showed an aspect of variability somewhat decoupled from both of these gradients (Fig 2).

In terms of photosynthetic biomass (as estimated using a BenthoTorch), the depth gradient was slightly, but significantly negatively associated with total and green algal chlorophyll concentration (Fig. 3.), indicating a slightly decreasing trend in green algal biomass content with increasing depth which could be due to light limitations (Virtanen and Soininen, 2012). Total, cyanobacterial and diatom chlorophyll signals were strongly negatively correlated with sulfate, also being the main driver of the salinity gradient in the system. Thus, whereas biomass of green algae seems to respond more strongly along the hydrological gradient, complementary to this, the chlorophyll biomass of diatoms and cyanobacteria appears to be driven mainly by the negative effect of sulfate (presumably more due to its osmotic effect than to specific toxicity). Conductivity, widely acknowledged as a key factor influencing periphytic communities, particularly diatoms, is underscored by several studies (Soininen et al., 2004; Virtanen and Soininen, 2012; Stenger-Kovács et al., 2020). It mainly reflects the overall ionic concentrations and closely correlates with water pH, another important factor noted in this study, as besides these two main environmental gradients, pH was positively correlated with the total and cyanobacterial biomass estimates; and diatom biomass estimates negatively with water temperature (as well as ammonium that can be seen as a component of the above water chemistry gradient).

We used two statistical approaches (PERMANOVA and RDA) to identify the main environmental drivers of community variability in three data sets: diatoms as assessed by microscopy; diatoms assessed by amplicon sequencing; and all microalgal taxa by amplicon sequencing. Common to all PERMANOVA and RDA results for all three data sets was the identification of a strong role of conductivity in driving patterns of community variability in both diatoms and all microalgal taxa (Table 2, Figs 5 and 7), reflecting the main water chemistry gradient described. Both PERMANOVA and RDA highlighted a strong response of diatom and microalgae communities to pH and water temperature besides conductivity in the case of both the amplicon and the microscopy data sets.

In the case of the microalgal amplicon data, discharge, total phosphate concentrations showed significant explanatory power of community composition besides conductivity, water temperature and pH, indicating possible taxon-specific differences in ecological responses of different microalgal groups. Cyanobacteria, which were found to respond to some environmental gradient in terms of their pigment signatures, were not captured by our community data sets at all, leaving their specific responses open.

In addition, RDA showed that flow velocity was a significant factor for microalgae community composition. This is consistent with earlier research, several studies have reported that stream flow velocity is a major factor influencing the heterogeneity in the composition of microphytobenthic communities and biodiversity in streams (Boulêtreau et al., 2010; Townsend et al., 2012; Breuer et al., 2017). Benthic microalgae are directly exposed to increased flow velocity and material movement carried by water current. Furthermore, gas bubbles produced through metabolic processes like photosynthesis or respiration can further disrupt and upset the stability or integrity of the microphytobenthic communities under high flow velocity conditions (Boulêtreau et al., 2010). The microalgae communities tend to be highly specialized at high flow velocity (Soininen, 2005). For example, attached taxa (like *Achnantidium minutissimum*) can tolerate higher flow velocity better than biraphid taxa (like *Navicula lanceolata*), which live on the substrata. Flow velocity influences the availability of other resources, such as light, suspended particles and nutrients, as well as other abiotic factors (e.g. temperature) (Townsend et al., 2012; Breuer et al., 2017), thus having a significant impact on microalgae community composition.

Judged qualitatively by the spread of points in the RDA, as well as by the number of significant environmental correlations revealed, or also by the number of OTUs being almost an order of magnitude higher than the number of taxa observed microscopically, the amplicon data set provided a better resolution of the ecological signals in diatom communities than the microscopy data set (Zimmermann et al., 2015). Although neither taxa observed by microscopy nor OTUs can be expected to strictly depict “real” species, this might indicate that the higher richness captured by the amplicon method at least partially contains ecologically relevant information beyond that contained in the microscopy data. Since closely related species often have identical 18S rDNA sequences, it could be expected that even this signal could possibly be improved by the use of more variable markers (Zimmermann et al., 2014). By the same criteria, with slightly different main drivers revealed in the PERMANOVA, the ecological resolution of the microalgal amplicon data set seems to have been the highest. This might be expected simply due to the broader taxonomic coverage.

The main gradients explaining community variability in the RDA ordinations were highly similar across the three data sets (with most variation in depicted patterns corresponding to rotation and mirroring of highly similar configurations). Common to all three data sets, these ordinations differed from what might have been expected from the environmental PCA by showing a strong alignment between the effects of the above described main hydrological and water chemistry gradients, with water temperature and pH showing strong effects, close to orthogonal to this main axis of variability. This is in line with the PERMANOVA results, and could indicate that less prominent gradients (as identified by a PCA) can also have strong ecological effects. As to the alignment of the main hydrological and water chemistry axes of variability, it is possible that more extensive sampling could differentiate between their effects upon community composition more clearly, this aspect will need to be revisited in the future. These findings agree with other studies in which water physical and chemical variables were main drivers of changes in microalgae assemblages, often with shift from more sensitive species to more tolerant species composition (Soininen et al., 2004; Virtanen and Soininen, 2012; Teittinen et al., 2015; Breuer et al., 2017).

The community ordinations (RDAs) also delivered somewhat unexpected results with respect to the FAL1, KRE1, KRE2 group of samples. These appeared with a substantial offset from all other samples in the environmental PCA, indicating a large difference in terms of both nutrient and salt concentrations. Although they appeared in relatively close vicinity of each other also in the RDA plots, their outlier position compared to all other samples was less pronounced than in the environmental PCA. Also in this respect, one can observe a pattern of increasing resolution in the rank order of diatom microscopy; diatom amplicon; microalgal amplicon data set.

Our results are consistent with (Nguyen et al., 2023), who found that the macroinvertebrate communities in the Kinzig catchment followed an environmental gradient ranging from sites with good, moderate to poor water quality. For example, the majority of species in the macroinvertebrate communities found at good water quality sites were grazers that preferred clear water with coarser substrates, and oligotrophic nutrient content. On the other hand, species that were foragers or active filter feeders and had a preference for turbid water with finer substrates, brackish and eutrophic/mesotrophic conditions were more likely to be found in poor water quality sites (Nguyen et al., 2023).

Our IPS results from microscopy analysis showed poor to good ecological status, while amplicon analysis showed poor to high (very good) ecological status at the sampled sites throughout the catchment (Figures 5 and 7A). This could be explained by the discrepancies in community composition observed between the two methods. These discrepancies may be due to biases in both methods. For example, microscopy may ignore some barely visible taxa, while amplicon sequencing still miss many taxa in the PR2 reference library used in the present study (Zimmermann et al., 2014). Diatom ecological status indices such as the IPS are calculated on the notion that the ecological quality of a site is represented by the average ecological value of its taxa, weighted by their relative abundances (Stevenson et al., 2010). As a result, high abundance of a taxa strongly influences these indices, whereas low abundance of a taxa only slightly affects the final value (Tapolczai et al., 2024). For example, when our microscopic observations revealed that *Navicula lanceolata* was the most dominant taxa in most of the samples, amplicon analysis showed that the most abundant OTUs were associated with *Navicula radiosa*. And, the latter had a higher sensitivity to the IPS (S = 5.0) than the former (S = 3.8). The variations in water quality classes between the two methods may also be due to overestimation of the relative abundance of large species – for example, the *Achnanthidium minutissimum* case mentioned above. However, our results were consistent in the case of site KRE2, which had a poor ecological status as demonstrated by both microscopy and amplicon analysis. The substrate at this site was largely sediment and it is one of the three sites discriminated by the PCA, located at the lowest altitude, which followed a nutrients and salinity gradient.

4.2. Taxonomic inconsistencies between diatom microscopy and amplicon sequencing

Our results showed that microalgae communities characterized by different methods used in this study were influenced by various environmental factors, which agrees with previous studies (Virtanen and Soininen, 2012; Teittinen et al., 2015; Stenger-Kovács et al., 2020). Digital microscopy analysis revealed that *Navicula lanceolata* was the most dominant diatom taxon in the majority of our sampling sites, except KIN3 and KIN4. This taxon was reported to be the most dominant in an anthropogenically modified river in

Poland, reaching over 80% of the total number of counted valve in spring (Noga et al., 2013). The authors attributed this dominance to the sampling season rather than anthropogenic influences. It was also identified as one of the taxa that had a higher proportion in agricultural land, with a higher content of nutrients and ions, such as in the Carpathian Basin (Stenger-Kovács et al., 2020). It occurred massively in slightly eutrophic boreal streams (Virtanen and Soininen, 2012). Due to its broad trophic preference for electrolyte-rich water and cooler temperatures, *N. lanceolata* grows rapidly during the winter and spring months (Noga et al., 2013; Schröder et al., 2015; Cantonati et al., 2017). This may explain why this species was highly represented in our samples collected in the first weeks of spring 2021. Therefore, the dominance of this taxon in our samples could also be related to the sampling season.

In contrast, amplicon sequencing analysis showed that *Navicula radiosa* was the most dominant taxon in most of our samples. We did not identify this taxon in microscopic analysis, instead we found *N. lanceolata* to be the most abundant taxon. Given the common taxonomic grouping of these two diatom species in the genus *Navicula,* they certainly have some similar morphological features that could lead to confusion in identification. However, *N. lanceolata* has obtusely rounded ends and a roundish central area (Cantonati et al., 2017). The observed discrepancies in our results are likely due to misidentification in the reference database, suggesting that the name *N. radiosa* may have been incorrectly assigned to OTUs belonging to *N. lanceolata* (Zimmermann et al., 2014). Other taxa detected in high abundances through molecular analysis in this study, include *Navicula gregaria and Surirella brebisonii*. These two taxa were also identified in light microscopy analysis. They grow abundantly in various environmental conditions, from mesotrophic to eutrophic waters. For example, *N. gregaria* has been reported as one of the indicators of nutrient enrichment in boreal alkaline and eutrophic basins (Virtanen and Soininen, 2012). We suspect that the presence of this taxon in large quantities in our samples may be related to nutrients and salt concenration in the river catchment.

We found 1400 different diatom OTUs, about ten times the number of taxa we detected with morphological observations (142), demonstrating the broader taxonomic coverage of amplicon analysis. However, the reference sequences from the 18S-V9 region for many freshwater diatom taxa are still missing from the PR2 reference database used in the present study. This shows that a lot of work is still needed to complete the reference databases in this research area. Even the well-curated and updated diatom reference database as Diat.barcode (Rimet et al., 2019) is still incomplete, which can lead to both non-identification and misidentification of taxa (Zimmermann et al., 2014; Bailet et al., 2019). In addition, it is not clear whether the issues of cryptic and pseudocryptic species and infraspecific or intragenomic variation are covered by rDNA reference databases (Minerovic et al., 2020).

Our results showed that only a small number of diatom taxa could be identified through both morphological analysis and amplicon sequencing to the lowest taxonomic level. The reference database used for taxonomic assignment in our amplicon analysis is likely incomplete, which could explain the large differences in taxonomic composition between the two methods. Since diatoms are unicellular organisms, variables such as the number of genomes and the number of gene copies per genome influence the number of copies of a gene (Vasselon et al., 2018). This implies that gene copy number could be approximated by cell biovolume. From the smallest to the largest diatom species, there is variation in gene copy number that can have a major effect on metabarcoding quantification (Vasselon et al., 2018). This could explain why highly abundant small-size taxa observed in microscopy such as *Achnanthidium minutissimum* were not as dominant in amplicon analysis.

In conclusion, our study aimed to identify the key environmental variables that influence microalgal biomass and community composition in the Kinzig catchment. Our results showed that depth gradient slightly negatively correlated with total and green algal biomass. Sulfate showed a strong negative correlation with the total, cyanobacterial, and diatom biomass. Total and cyanobacterial biomass were positively associated with pH, while diatom biomass was negatively correlated with ammonium content and water temperature. PERMANOVA showed that conductivity, water temperature and pH were the most important factors influencing microalgae community composition, as observed in both microscopy and amplicon analysis. Together with these three variables, total phosphate in all microalgae OTU and water discharge in diatom (Bacillariophyta) OTU adatasets may suggest taxon-specific variations in the ecological responses of various microalgae groups. Our results highlighted the sensitivity of eukaryotic algae to physical and chemical variables, demonstrating their potential as good indicators of environmental gradients in the Kinzig catchment. These results suggest the complex relationship between multiple environmental factors and microalgal biomass and community composition. Further investigations, including collection of time series data, are required to fully understand the underlying biotic and abiotic factors driving these microalgal communities.

Data availability

The datasets presented in this study can be found in the online repository Zenodo:

https://doi.org/10.5281/zenodo.11061732

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

NASM: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualisation, Writing – original draft, Writing – review & editing. MD: Data curation, Formal analysis, Writing - review & editing. MK: Investigation, Methodology, Software, Writing: review & editing. DV: Data curation, Formal analysis, Writing: review and editing. DB: Investigation, Methodology, Software, Writing: review & editing. AMBC: Investigation, Methodology, Supervison, Formal analysis, Validation, Writing – review & editing. BB: Conceptualization, Funding acquisition, Supervision, Project administration, Writing – review & editing.

Funding

This study was performed within the framework of the Collaborative Research Centre (CRC) 1439 RESIST (Multilevel Response to Stressor Increase and Decrease in Stream Ecosystems; www.sfb-resist.de) funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation; CRC 1439/1, project number: 426547801).

Acknowledgements

We want to thank Marzena Spyra and all the students who helped with sample collection and in the laboratory with sample processing. The editor and two anonymous reviewers are thanked for their comments that helped improve this manuscript. We acknowledge support by the Open Access Publication Fund of the University of Duisburg-Essen.

Supplementary materials

Supplementary Table 1 Physical and chemical parameters analysed in this study

Supplementary Table 2 List of taxa idedntified by microscopy (_MIC) or amplicon sequencing (_DNA) with their 4 letters OMINIDIA codes

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Paper 4. Pre-Print in bioRXiv

Characterization of diatom communities in restored sewage channels in the Boye catchment, Germany

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Contributions:

- Conception 5 %: Followed the hypotheses and study design drawn in the grant application.
- Conduction of experimental work 0 %: Samples taken by CRC1439 project Z02 and members of the Phycology lab.
- Data analysis 100 %: Data curation and analysis completely executed by NASM.
- Species identification 20 %: Molecular species identification assigned by NASM.
- Statistical analysis 100 %: Statistical analysis designed and executed by NASM.
- Writing the manuscript 100 %: First draft of the manuscript written in its entirety by NASM.
- Revision of the manuscript 15 %: Shared revision of all co-authors.

Signature of the Doctoral Candidate

Signature of the Doctoral Supervisor

Chapter IV

Characterization of diatom communities in restored sewage channels in the Boye catchment, Germany

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Abstract

Restoration programs were initiated in different stream systems within the Boye catchment in the early 1990s to stop and reverse the negative impacts of anthropogenic disturbances. However, our knowledge of the effects of restoration works in this river network, specifically on benthic diatom communities, is still limited. Diatoms were used successfully to assess the impact of restoration works in a range of river networks around the world but received less attention in the Boye catchment. This study aimed to characterize benthic diatom communities in this restored river catchment, using digital microscopy methods. We collected samples in the spring and summer of 2020 at sites restored in different years (1995, 2005, 2010, 2012, and 2013) within the catchment. Our results showed no effect of season, restoration date (year), and location of the site along the streams on the most dominant diatom species. However, some rare taxa indicated significant variations between the seasons. Overall water quality in the streams ranged from moderate to very good, indicating the positive impacts of restoration works conducted in these former sewage channels.

Introduction

Anthropogenic activities have exacerbated the degradation of river catchments around the world, reducing their capacity to deliver the most basic goods and services [1,2]. These essential ecosystems are frequently exposed to a rising number of stressors [3,4] and remain among the most substantially altered, resulting in unprecedented biodiversity loss [5,6]. River restoration initiatives generally seek to halt and then reverse the historical trends of freshwater biodiversity depletion caused by river channel deterioration, restoring them to their natural condition by enhancing their ecological health status [7–9]. Despite the fact that restoration is an improvement method, it is undeniable that it also introduces some level of disturbances in unclean water bodies [7,10,11]. However, our understanding of the effects of river restoration is still limited due to the fact that just a few projects have included comprehensive monitoring. One of these projects include the rivers of the Boye catchment [12-14]. The Boye catchment is located in the densely populated and industrialized Ruhr Metropolitan Area, and they have been severely impacted by many anthropogenic stressors [12].

Previously, untreated wastewater from urban (e.g. open sewage channels), agricultural (e.g. surface runoff from farmlands), and industrial (e.g. coal mining) activities was discharged into these stream channels [12–14]. The morphology of the majority of these streams were altered since most of them were straightened, corseted in concrete and deepened. Additionally, previous intense coal mining activities in this region resulted in subsidence issues in large areas within the catchment [15]. Restoration programs were initiated in different stream systems within the Boye catchment in the early 1990s to stop and then reverse the negative impacts of anthropogenic disturbances [1], promote holistic management of these urban freshwater ecosystems, and comply with the European Framework Directive requirement of achieving good ecological health status [2,16], by the extended deadline of 2027. Therefore, an assessment of the success of recent restoration works within this watershed by evaluating the response of various biological quality components (e.g. fishes, macroinvertebrates, macrophytes, phytobenthos/phytoplankton; see [2] has become a necessity to determine whether all of the effort put in was worthwhile.

In stream ecosystems, diatoms are the most common, diverse, and best-studied group of biofilm-forming microalgae [17]. Their community composition reflects the state of their environment's ecology, with quick changes responding to water composition variations, making them ideal candidates for biomonitoring in a variety of aquatic habitats [18]. Diatoms revealed successfully the impact of restoration activities in a range of other river systems throughout the world [11,19–23].

Some studies have investigated the response of biota, particularly macroinvertebrates, to the recent restoration efforts within the Boye watershed [12,14,24,25]. However, microphytobenthic communities, such as diatoms received less attention in these river systems. The goal of this study was to close this gap by characterizing diatom communities in restored streams within the Boye catchment, using virtual slides prepared as described in details in [26,27], using Biigle v.2.0 [28,29]. This is the first study to present and discuss diatom community composition in these river systems as a function of the effects of time since restoration (year), sampling season (spring or summer), and site location (upstream, midstream, or downstream).

We hypothesize that we will be able to observe clear changes in diatom abundance and community compositions between sites within the Boye catchment based on sampling season, year of restoration, and location along the streams.

Materials and methods

Study site

For this investigation, we collected benthic diatom samples from various streams in the Boye catchment (Figure 1). These streams were once open sewage channels, but they have been restored over time, with some dating back to the early 1990s. Diatom biofilms were sampled in spring (April 24, 2020) and summer (July 2, 2020). Water temperature recorded *in situ* during sampling was 7.6 °C in spring (range from 6 °C to 10.7 °C) and 15.77 °C in summer (ranging from 15 °C to 17.8 °C) (Table 1). Conductivity ranged from 680 µS/cm to 1980 µS/cm, with a mean of 1021 µS/cm in spring, and 425 µS/cm to 1124 µS/cm, mean 805 µS/cm in summer (Table 1). Water pH ranged from 7.67 to 8.14 with a mean of 7.92, and from 7.26 to 7.98 with a mean of 7.65, in Spring and Summer respectively (Table 1). Only temperature and pH showed statistically significant differences (ANOVA $p < 0.05$) across seasons.

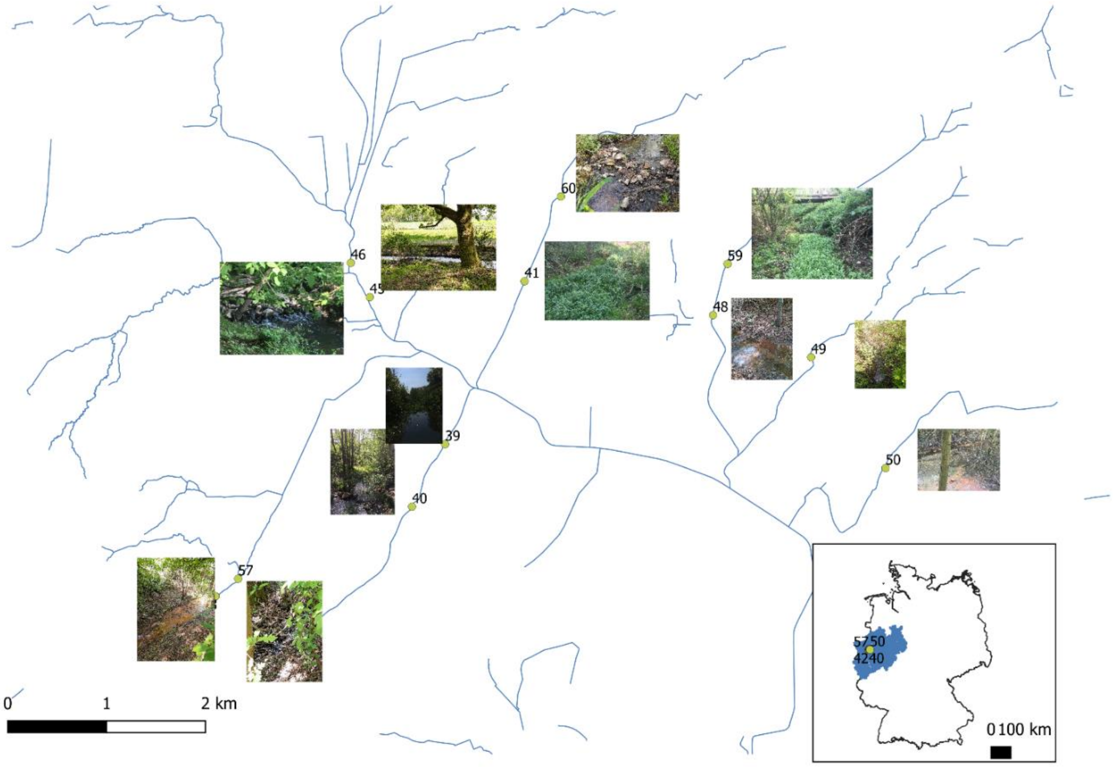

Figure 1: Figure 1: Map of the Boye catchment in Germany, the sampling sites are marked with green dots and numbered (more details in Table 1).

Table 1. Sampling site details

Site #	**Stream**	**Community**	**Season**	**Location**	**Restor. year**	**pH**	**Cond. (µS/cm)**	**Temp. (˚C)**
39	Kirchschemmsbach	Epilithon	spring	downstream	2010	8.08	970	8.0
40	Kirchschemmsbach	Epilithon	spring	upstream	2010	7.87	1072	7.2
41	Haarbach	Epiphython + Epidendron	spring	downstream	2013	7.86	1047	10.7
42	Vorthbach	Epilithon + Epidendron	spring	upstream	1995	8.14	843	6.9
45	Boye	Epilithon	spring	downstream	2005	7.81	680	8.1
46	Boye	Epilithon	spring	upstream	2005	7.84	710	9.3
48	Wittringer Mühlenbach	Epidendron	spring	downstream	2012	7.67	1171	6.0
50	Hahnenbach	Epidendron	spring	midstream	2013	7.90	1021	7.6
57	Vorthbach	Epidendron	spring	midstream	1995	7.99	950	6.7
59	Wittringer Mühlenbach	Epiphyton	spring	upstream	2012	8.08	1021	7.2
60	Haarbach	Epilithon	spring	upstream	2013	8.06	970	8.0
39	Kirchschemmsbach	Epilithon	summer	downstream	2010	7.74	974	15.0
41	Haarbach	Epidendron + Epiphyton	summer	downstream	2013	7.76	1124	16.4
42	Vorthbach	Epilithon + Epidendron	summer	upstream	1995	7.30	1005	15.0
43	Vorthbach	Epidendron	summer	downstream	1995	7.98	804	15.0
45	Boye	Epilithon	summer	downstream	2005	7.60	460	15.6
46	Boye	Epilithon	summer	upstream	2005	7.57	425	15.4
48	Wittringer Mühlenbach	Epidendron	summer	downstream	2012	7.80	621	16.7
57	Vorthbach	Epidendron + Epilithon	summer	midstream	1995	7.26	1007	15.0
60	Haarbach	Epilithon	summer	upstream	2013	7.87	825	17.8

Diatom biofilm sampling, processing and digital microscopy

Biofilm samples were collected by scrubbing a surface area of about 20 cm^2 of at least three cobbles with a tooth brush. In sites where we were unable to find stones (epilithon), other hard substrata such as woods (epidendron) or macrophytes (epiphyton) were sampled. More details about the sampling sites are given in table 1. Immediately after collection, the samples were fixed in a solution of ethanol and then taken to the laboratory for further processing and analyses. For qualitative and quantitative analyses of diatom communities, we treated 10 mL of each sample with 30 ml of concentrated hydrogen peroxide (H_2O_2) on a hot plate, before adding few drops of concentrated hydrochloric acid (HCl) as described in Taylor et al. [30]. Then, a drop of the sample was mounted on a microscope permanent slide using Norland Optical Adhesive 61 (NOA 61). Finally, we scanned permanent slides with an Olympus Vs200 slide scanner (Olympus, Tokyo, Japan) at 63x magnification apochromatic and 1.24 Numeric Aperture to get virtual slides following the protocols detailed in Kloster et al. [26,27]. Diatoms on digital slide were identified by morphometric observations on Biigle 2.0 [28,29], using standard literature [31–33]. Diatom abundance data were exported as excel sheet for further analysis. We used the OMNIDIA software [34] for the calculation of diatom indices, such as the Specific Pollution Sensitivity Index (IPS) [35] and the Biological Diatom Index (IBD) [36] for water ecological health status assessment. The four-letter code devised for this software is used on diatom taxa to clarify multidimensional scaling.

Statistical data analysis

We conducted all multivariate statistical analyses in the open-source statistical software R (v4.1.1) [37] using the 'vegan' package (v2.5-7) [38], and the 'ggstatsplot' package [39] . As proposed by Anderson et al. [40], diatom abundance data were log-transformed (log b (x) + 1 for x > 0, where b is the basis of the logarithm, here we used base 2) to minimize the weight of taxa with high abundance relative to those with low abundance to achieve multivariate normal distribution. All taxa with abundances less than 5% were put under a single label as "Others". We ran the vegan 'betadisper()' function to calculate differences in multivariate homogeneity of group dispersion (variances or average distance to centroids) based on the distance matrix, then used the vegan 'adonis()' function to run a Permutational

Multivatiate Analysis of Variance (PERMANOVA) to see how time since restoration, sampling season, and site location affected benthic diatom community composition. In order to visualize associations between samples based on distance matrices, non-metric multidimensional scaling (nMDS) was used with the vegan 'metaMDS()' function and the Bray–Curtis similarity index with 9999 permutations.

Results

Diatom community composition and diversity

In this study, we identified 232 distinct diatom taxa, from 52 genera. Only 31 taxa had abundance values greater than or equal to 5% (Figure 2, Table 2). The most dominant (abundance greater than or equal to 5 %) and frequent (occurring in at least 4 samples) diatom taxa in both sampling seasons were *Planothidium lanceolatum* (Brebisson ex Kützing) Lange-Bertalot (OMNIDIA code: PTLA), *Achnanthidium minutissimum* (Kützing) Czarnecki (ADMI), *Rhoicosphenia abbreviata* (C.Agardh) Lange-Bertalot (RABB), *Planothidium frequentissimum* (Lange-Bertalot) Lange-Bertalot (PLFR), *Cocconeis placentula* Ehrenberg (CPLA),*Gomphonema parvulum* var. *parvulum* (Kützing) Kützing (GPAR), and *Achnanthidium saprophilum* (Kobayasi et Mayama) Round & Bukhtiyarova (ADSA) (Figure 2). Each species is mostly found in a part of the biofilm.

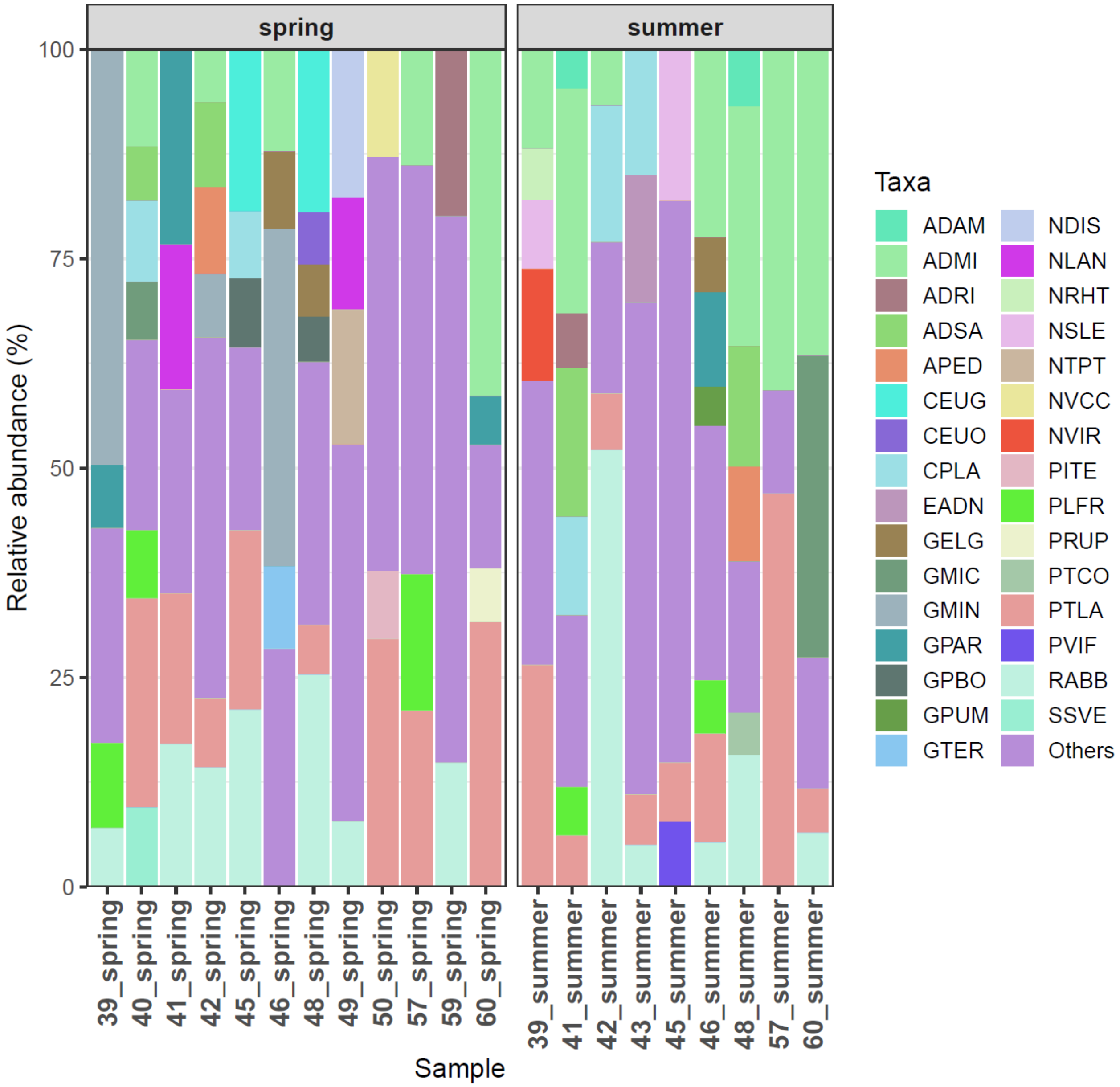

Figure 2: Figure 2: Relative abundance of dominant diatom species at different sites within the Boye catchment in spring and summer 2020

Table 2: Table 2: Most dominant diatom taxa identified in this study in decreasing order of abundances

Taxa	**OMNIDIA Code**
Planothidium lanceolatum (Brébisson ex Kützing) Lange-Bertalot var. *lanceolatum*	PTLA
Achnanthidium minutissimum (Kützing) Czarnecki var. *minutissimum*	ADMI
Rhoicosphenia abbreviata (C.Agardh) Lange-Bertalot	RABB
Gomphonema minutum (Agardh) Agardh f. *minutum*	GMIN
Cocconeis placentula Ehrenberg	CPLA
Achnanthidium saprophilum (Kobayasi et Mayama) Round & Bukhtiyarova	ADSA
Gomphonema parvulum var. *parvulum* f. *parvulum (Kützing) Kützing*	GPAR
Planothidium frequentissimum (Lange-Bertalot)Lange-Bertalot var. *frequentissimum*	PLFR
Gomphonema micropus Kützing var. *micropus*	GMIC
Cocconeis euglypta Ehrenberg	CEUG
Navicula lanceolata (Agardh) Ehrenberg var. *lanceolata*	NLAN
Achnanthidium rivulare Potapova &Ponader	ADRI
Navicula slesvicensis Grunow	NSLE
Gomphonema elegantissimum Reichardt & Lange-Bertalot in Hofmann & al.	GELG
Amphora pediculus (Kützing) Grunow var. *pediculus*	APED
Nitzschia dissipata subsp. dissipata (Kützing) Grunow var. *dissipata*	NDIS
Navicula tripunctata (O.F.Müller) Bory var. *tripunctata*	NTPT
Epithemia adnata (Kützing) Brébisson var. *adnata*	EADN
Gomphonema pseudobohemicum Lange-Bertalot & Reichardt	GPBO
Navicula viridula (Kützing) Ehrenberg var. *viridula*	NVIR
Navicula viridulacalcis Lange-Bertalot var. *viridulacalcis*	NVCC
Achnanthidium atomoides Monnier, Lange-Bertalot & Ector	ADAM
Gomphonema tergestinum (Grunow in Van Heurck) Schmidt in Schmidt et al. var. *tergestinum*	GTER
Staurosira venter (Ehrenberg) Cleve et Moeller var. *venter*	SSVE
Prestauroneis integra (W.Smith) Bruder	PITE
Pinnularia viridiformis Krammer	PVIF
Pinnularia rupestris Hantzsch in Rabenhorst var. *rupestris*	PRUP
Navicula rhynchotella Lange-Bertalot	NRHT
Cocconeis euglyptoides (Geitler) Lange-Bertalot	CEUO
Platessa conspicua (A.Mayer) Lange-Bertalot	PTCO
Gomphonema pumilum (Grunow) Reichardt et Lange-Bertalot var. *pumilum*	GPUM

Despite the fact that the low profile guild predominated in high proportions in both seasons, we observed a significant rise in motile guild relative abundances in the spring, indicating that season had a major impact on the motile guild (ANOVA $p < 0.001$, Figures 3&4C). All other diatom guilds, however, did not show a significant effect of season (ANOVA $p > 0.05$, Figures 4A, B, D, E&F). The high profile guild, on the other hand, showed a significant influence of time since restoration (ANOVA $p = 0.04$).

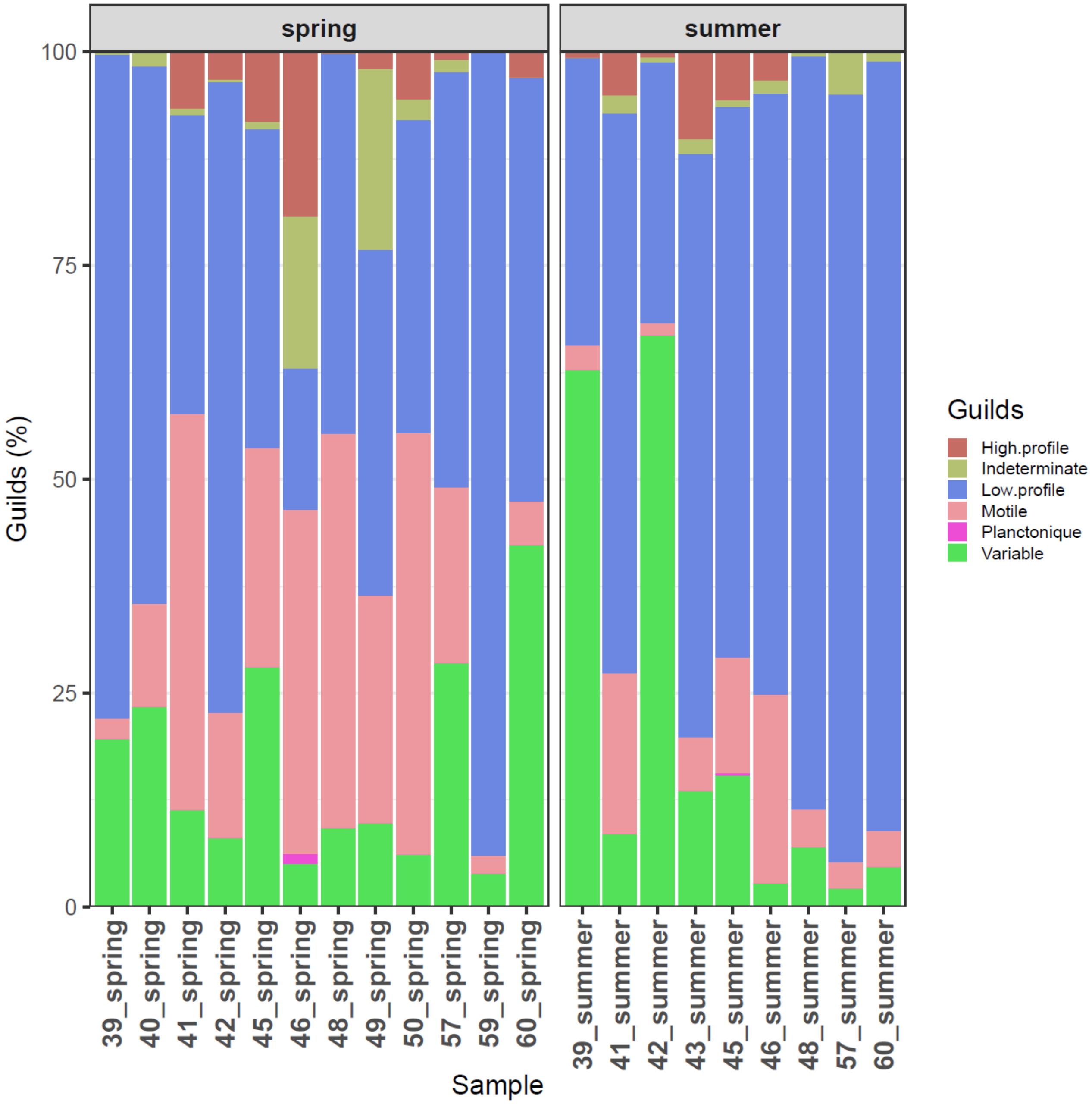

Figure 3: Figure 3: Relative abundance of different diatom guilds at different sites within the Bye catchment in spring and summer 2020

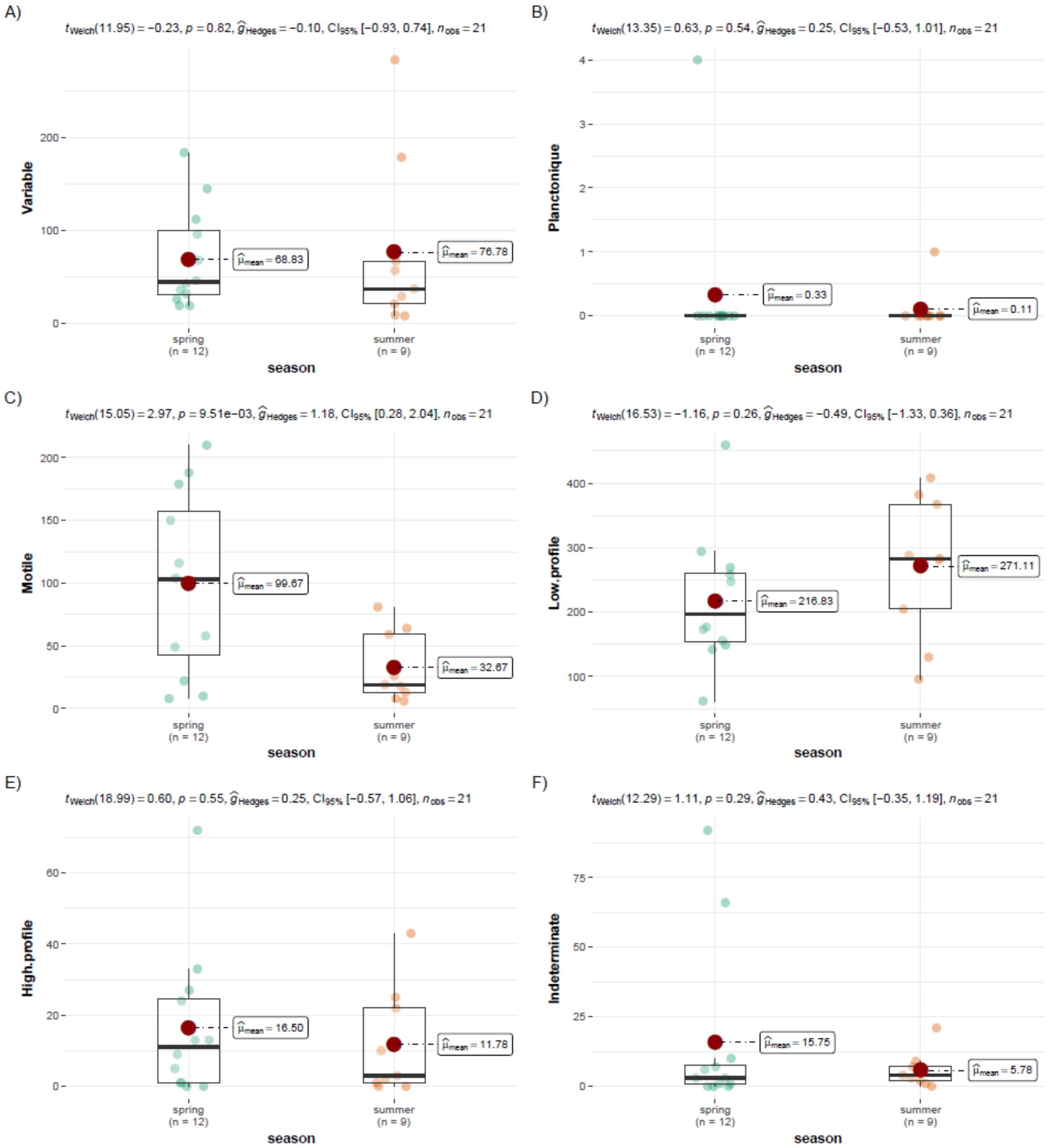

Figure 4: Figure 4: Variable (A), Planktonic (B), Motile guild (C), Low profile guild (D), High profile guild (E), and Intermediate (F) in spring and summer 2020. Results of ANOVA tests: $p < 0.001$ only for motile guild, $p > 0.05$ for all the other guilds.

During the spring season, species richness (S) ranged from 25 taxa in site 39 (Table 1), to 71 taxa in site 50, (Figure 5A). In the summer, we observed low species richness (22 taxa) downstream

Kirchschemmsbach and high species richness (61 taxa) downstream the Boye at sites 39 and 45, respectively (Figure 5A). Shannon-Wiener diversity index (H) ranged from 1.31 in site 59 to 3.55 in site 50 during the spring, with a mean of 2.54, and from 1.75 at site 48 to 3.26 at site 45 downstream the Boye in summer, with a mean of 2.49 (Figure 5B). Simpson diversity index peaked at 17.85 at site 50, and it was lowest 2.58 at site 59, upstream Wittringer Mühlenbach, in the spring (Figure 5C). In Summer, the greatest reading was 15.63 at site 45, and the lowest reading was 3.21 at site 48, downstream (Figure 5C). Pielou's eveness (J) ranged from 0.40 (site 59 upstream Wittringer Mühlenbach) to 0.83 (site 50) and from 0.55 (site 48) to 0.80 (site 46), in spring and summer respectively (Figure 4D). However, we failed to observe any substantial difference between both seasons for all these diversity indices (ANOVA $p > 0.05$, Figures 5A, B, C&D). Additionally, none of the studied factors had a significant effect on the Specific Pollution Sensitivity (IPS) Index. The index showed values ranging from 12.8 to 18.3 (mean 15.13) in spring, and 13.7 to 16.2 (mean 14.99) in summer. The Biological Diatom Index (IBD), on the other hand, showed significant changes between seasons (Anova $p = 0.04$). Overall the index ranged 12.4 to 16.4 (mean 14.8) and 15.4 to (mean 16) in spring and summer respectively.

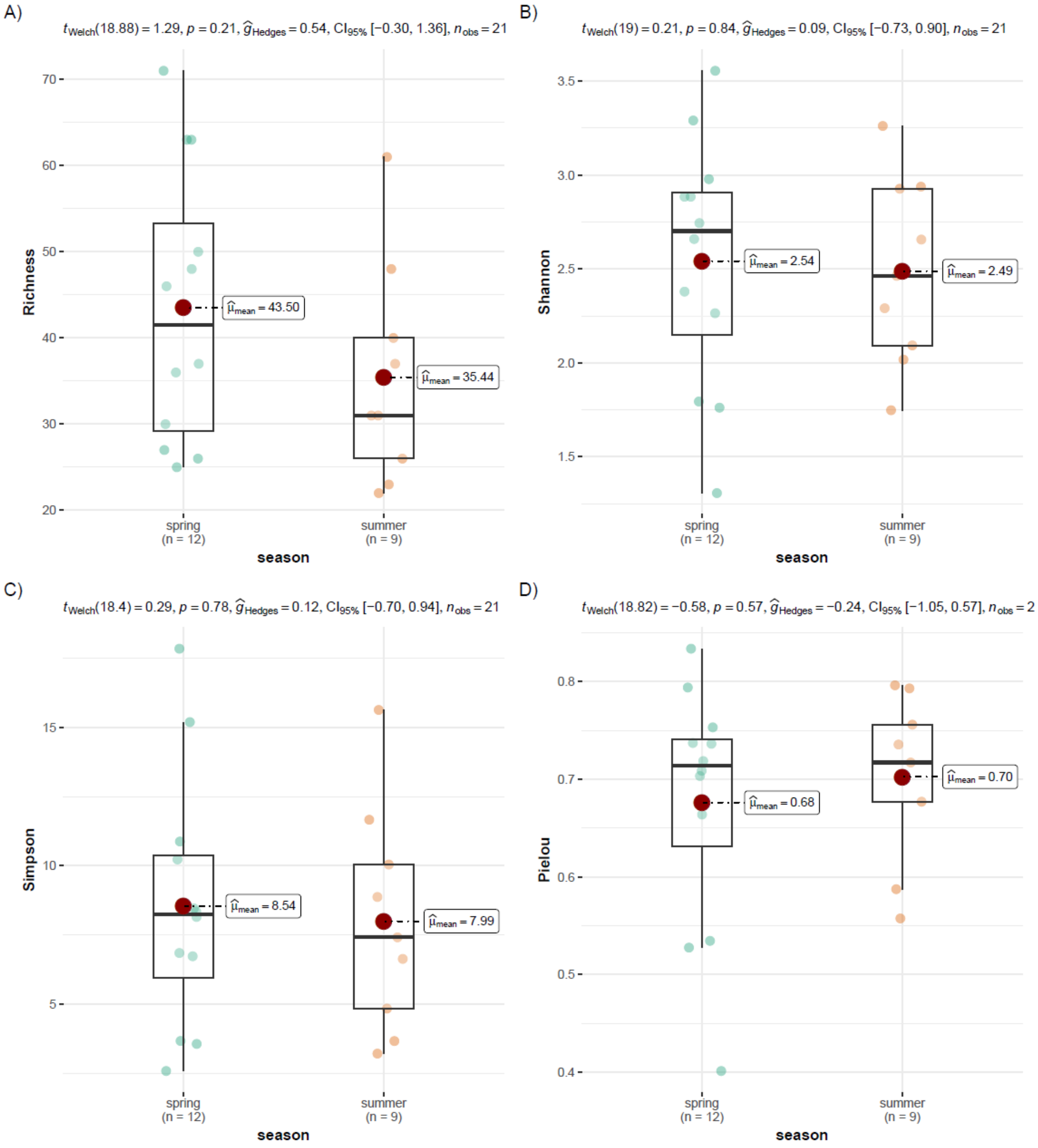

Figure 5: Species Richness (A), Shannon-Wiener diversity index (B), Simpson diversity index (C), and Pielou's evenness (D) in spring and summer 2020. Results of ANOVA tests: $p > 0.05$ in all the cases

Effects of sampling season, time since restoration and site location on diatom communities

The test for multivariate homogeneity of group dispersion (distance from centroids) on our diatom species abundance dataset revealed that all groups were homogenous ($p > 0.05$). PERMANOVA run on a dataset which included only species with abundance values greater than or equal to 5% in each sample, did not demonstrate any significant differences between neither sampling season, duration of time after restoration, site location along the streams, nor their interactions ($p > 0.05$). However, when we ran PERMANOVA on the entire dataset, which also included all rare taxa with abundances less than 5%, we found that only sampling season had a significant effect on diatom abundances (Sum of Squares (*SS*) = 1.0658, R^2 = 0.229, p = 0.0017; Table 3). However, all two- and three-way interactions, as well as the effects of site location and time after restoration, were still not significant ($p > 0.05$, Table 3).

Table 3: Table 3: Results of three-way crossed PERMANOVA of the effects of sampling season, site location, and time since restoration.

Factor	***df***	***SS***	***F***	R^2	***p****
Reduced dataset (only most abundant taxa)					
Season	1	0.2460	0.9282	0.0529	0.5147
Restoration_date	4	0.8603	0.8117	0.1849	0.7064
Site	2	0.4674	0.8820	0.1005	0.5767
Season x Restoration_date	4	1.1162	1.0531	0.2399	0.4050
Season x Site	2	0.4908	0.9261	0.1055	0.5367
Restoration_date x Site	5	1.0232	0.7723	0.2199	0.7788
Season x Restoration_date x Site	1	0.1840	0.6945	0.0396	0.7592
Residual	1	0.2650		0.0570	
Total	20	4.6528		1.0000	
Whole dataset (with rare taxa)					
Season	1	1.0658	6.0910	0.2291	**0.0017**
Restoration_date	4	1.1536	1.6482	0.2479	0.1545
Site	2	0.4357	1.2449	0.0936	0.2952
Season x Restoration_date	4	0.6925	0.9894	0.1488	0.4678
Season x Site	2	0.2956	0.8446	0.0635	0.6337
Restoration_date x Site	5	0.7342	0.8392	0.1578	0.6726
Season x Restoration_date x Site	1	0.1005	0.5743	0.0216	0.8827
Residuals	1	0.1750		0.0376	
Total	20	4.6528		1.0000	

* The only significant ($p < 0.05$) main effect of the season is highlighted in bold. *df*: degrees of freedom; *SS*: sum of squares; *F*: *F*-statistic, R^2: variance explained by the groups, *p*: p-values.

Indicator species analysis revealed that only *Achnanthidium minutissimum* (Kützing) Czarnecki var. *minutissimum* (ADMI) was the only most common taxa to have near significant ($p = 0.045$) differences in abundances between seasons, occurring in slightly higher numbers in summer. *Navicula cari* Ehrenberg ($p = 0.02$) and *Diploneis calcilacustris*.

Lange-Bertalot et A. Fuhrmann ($p = 0.02$) increased also significantly in abundances in summer, but they were classed as rare taxa together with all those that did not reach 5% in abundance. In the spring, *Hippodonta capitata* (Ehr.) Lange-Bertalot, Metzeltin and Witkowski (HCAP) and *Ulnaria ulna* (Nitzsch) Compere var. *ulna* showed nearly significant rise in abundance (p = 0,049 and 0.049, respectively). The nMDS plots revealed large seasonal overlapping and widely dispersed sample sets (Figures 6 A&B). Although the analysis yielded a high stress value (0.19), we observed a severe distortion in the arrangement of sample points scattered all over the two-dimensional (2D) space displayed on the graphs, despite the high correlation between the observed dissimilarity and the ordination distances (non-metric fit $R^2 = 0.99$, linear fit $R^2 = 0.98$, Figures 6 A&B).

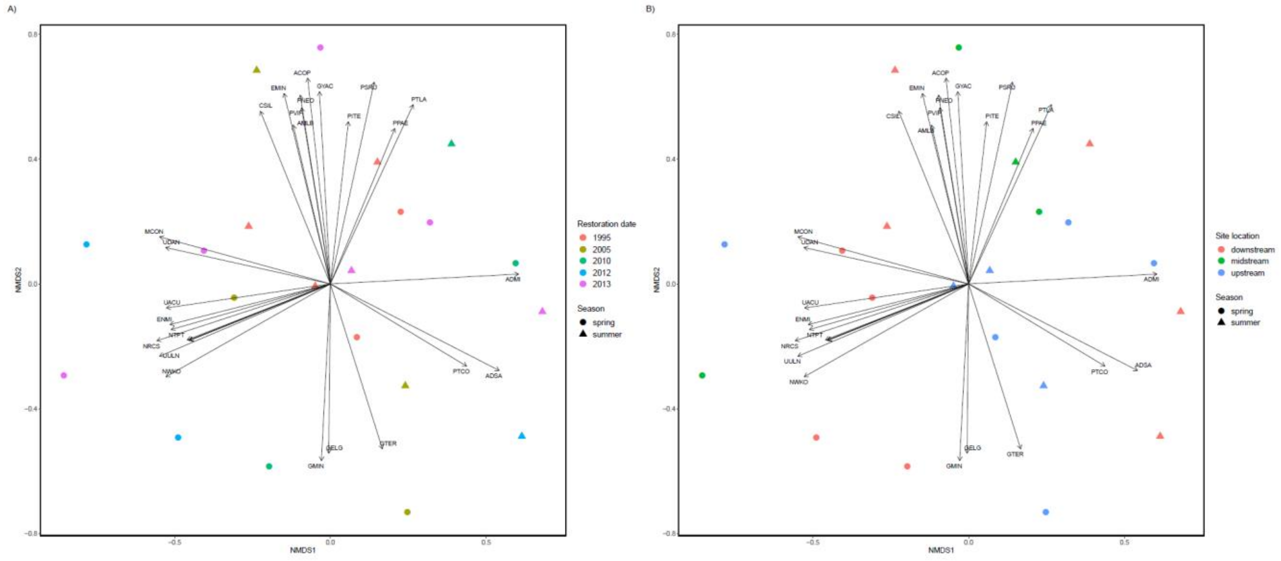

Figure 6: Figure 6: Non-metric multidimensional scaling (nMDS) graphs showing distances between diatom communities in samples based on (A) Restoration date and season and (B) site location and season.

Discussion

In the current study, we expected to observe clear differences in diatom abundance and community compositions between sites within the Boye catchment based on sampling season, date of restoration (year), and location along the streams. Our data demonstrated that the dominant benthic diatom taxa identified in this watershed, except *Achnanthidium minutissimum* were unaffected by any of the investigated factors. The sampling season, on the other hand, had a significant effect on the full dataset, which included rare taxa. *A. minutissimum* was the only most dominant and frequenttaxon to show nearly significant differences in abundances between the seasons. This is a pioneer diatom species [21,41], that occurs in large numbers at a variety of trophic levels [32]. It thrives abundantly in alkaline and acidic habitats, as well as in oligotrophic and hypertrophic waters [42]. Several studies reported *A. minutissimum* to be an indication of good water quality, for example low nutrients content [11,19,21,43– 45]. This diatom species was the most numerous and important, contributing to differences between sites based on restoration status in "boreal rivers" [21]. We speculate that the increase in abundance of *A. minutissimum* in Summer 2020 was an indication of better in water quality during this season. *Navicula cari* and *Diploneis calcilacustris*, the other two species that showed substantial seasonal differences, increased in relative abundance in summer, albeit they remained scarce.

All the other most prevalent diatom species were found in roughly the same amounts in both seasons, with no notable variations between them. Most of these taxa, such as *Planothidium lanceolatum, Rhoicosphenia abbreviata, Gomphonema minutum, Cocconeis placentula*, *Gomphonema parvulum* var. *parvulum, Planothidium frequentissimum, Gomphonema micropus* and *Achnanthidium saprophilum* have a wide range of tolerance to various ecological conditions. For example, *P. lanceolatum* and *P. frequentissimum* very often co-occur in electrolyte-poor to rich waters, as well as in oligotrophic to polytrophic, up to β-mesosaprobic waters [31,32]. *Rhoicospehenia abbreviata* and *Cocconeis placentula* were reported as characteristic indicator species of sites with intermediate level of pollution [46]. These dominant diatom species were also found in restored reaches with better water quality in the Bzura River, Poland [19]. The presence of these taxa in high abundance suggests some improvements in water quality within the Boye catchment even though there are still some levels of various anthropogenic stressors that could be entering the system as the waters flow through the densely populated urban areas.

Our dataset showed a clear dominance of low profile diatom ecological guild in both seasons. Low profile guild increase in relative abundance in nutrients-poor and high disturbances (e.g. high flow velocity) conditions [47,48]. Our results revealed no effect of neither season, restoration date, nor site location on low profile guilds. Trábert et al. [49] found no correlation between low profile guild abundance and any of the investigated factors, such as nutrients and environmental variables, in the Danube. The relative abundance of motile guild increased significantly throughout the spring season. Motile guild diatom species are more nutrients-dependent and susceptible to perturbations [47,48]. We speculate that water quality in the streams degraded slightly during the spring season, which favored motile guild diatoms to thrive. This degradation is also seen through the slight increase in conductivity and pH observed during this season.

The Biological Diatom Index (IBD) [36] revealed significant differences between the spring and summer seasons, indicating moderate to good ecological status in spring, and even higher ecological status in summer. Therefore, in the spring, water quality ranges from moderate to good, and in the summer, it rises to very good. The Specific Pollution Sensitivity Index (IPS) [35] on the other hand, was not responsive to any of the examined conditions but did indicate moderate to good ecological status in the spring and even higher ecological status in the summer. Similarly, according to IPS, water quality was satisfactory to good in the spring and improved to very good in the summer. These findings point to an increase in water quality within the Boye catchment, which has been repaired for at least seven years at some sites and even over a decade at others. According to PERMANOVA run on the most dominant taxa dataset, diatom community composition and abundances were not influenced by season, time since restoration and location of the site along the streams. However, season had a significant effect on the whole abundance dataset, including rare taxa. Our results imply that diatom community composition in these sites that we sampled were more or less similar, with no significant differences among them. We speculate that diatom communities in these sites evolved to much stable or climax communities over the years.

In conclusion, our results revealed no significant effects of season, time since restoration and location of the sites along the streams. The dominant diatom species in all the sites were very similar during spring and summer. Given the fact that all the sites we sampled in this study were restored for over at least seven years, it would be necessary? to also sample in sites that were restored more recently. This study is a baseline report of the ecological status of the rivers within the Boye watershed based on benthic diatom communities. Although we lack the knowledge of the conditions of these streams prior to the start of restoration works to assess their effectiveness at this point, these baseline data will be helpful for future monitoring. These results will be important for the ongoing research under the Collaborative Research Centre (CRC) 1439 RESIST, in which we are investigating the functional and compositional responses of stream microphytobenthic communities to multiple stressors increase and decrease within the Boye catchment using both field work and ExStream mesocosm experiment.

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General discussion

The research presented in this thesis provides insights into the functional and compositional responses of microphytobenthos biomass and community composition as a result of ecological degradation due to multiple stressors and the subsequent recovery process after stressor release using mesocosm experiments and field studies. In general, the results showed that microphytobenthos biomass and community compositions in the studied river systems was influenced by stressors such as changes in flow velocity and temperature. However, due to their rapid growth and short generation time, the biomass of microalgae recovered much faster than their community composition. Comparing the degradation and recovery processes showed that resident communities significantly influence recovery after a disturbance, supporting the Asymmetric Response Concept (Vos et al., 2023), which suggests that ecosystems respond unequally to degradation and recovery. Below I present and briefly discuss the main results of the individual article included in this thesis.

In the first chapter, we used the ExStream mesocosm experimental setup to study the salinization responses of urban river ecosystems under different flow regimes (normal vs. reduced flow velocity). Through a thorough evaluation of different groups of organisms, including microalgae, fungi, parasites and macroinvertebrates, and ecosystem functions such as primary production and decomposition of organic matter, this study showed that reductions in flow velocity influenced nearly all organism groups and several ecosystem functions, including microbial respiration and decomposition rates, but not photosynthetic biomass. This indicates that flow velocity is critical for maintaining stream ecosystem integrity. Flow velocity also appeared to be the main driver of community composition in the microphytobenthos, mainly in the distribution of algal groups. Thus we confirm previous findings that the ability rivers and their aquatic organisms to adapt to changes in water flow regimes is limited (Bondar-Kunze et al., 2021; Palmer et al., 2008).

Salinization primarily affected dynamic community aspects, such as macroinvertebrate drift and fungal sporulation, suggesting early stress responses but less immediate structural community impact. However, the microphytobenthos community did not reflect the same responsiveness, probably due to the algae selection being driven by the already high saline variation in the original catchment. In contrast, salinization increased invertebrate emigration and reduced fungal proliferation, with the effects of previous coal mining in the studied region possibly reducing the overall impact. These results highlight the limits of riverine ecosystems to changes in water flow and the critical need to maintain natural flow regimes to preserve ecological integrity and functionality, as these changes have observable effects on some ecosystem characteristics.

In the second chapter, I examined the effects of reduced flow velocity, increased salinity, in addition to warming water on microphytobenthos biomass and community composition in Exstream mesocosms experiment. Reduced flow velocity significantly affected the composition, diversity, and biomass of eukaryotic microalgal communities. Diatom-based microscopy data showed lower species diversity at reduced flow, while amplicon sequencing indicated higher OTU richness of all microalgae. Community composition was significantly different under reduced flow than other treatments. The reduced flow also slightly reduced photosynthetic biomass, suggesting minor effects on primary productivity. These results confirm once again the significant ecological impacts of streamflow reductions, which are likely to become more common with climate change(Bondar-Kunze et al., 2016; Bondar-Kunze et al., 2021; Palmer et al., 2008). Contrary to expectations, increased salinity did not have a significant impact onmicroalgae biomass or community composition. However, some taxa, such as *Mayamaea permitis*, and some microalgae OTUs were sensitive to increased salinity and acted as indicators of salinity changes. Even moderate levels of salinization can benefit salt-tolerant brackish water species without eliminating more sensitive species, potentially resulting in increased OTU richness (Nuy et al., 2018). The results showed that the applied salinity had less impact than natural fluctuations in the studied stream, suggesting that the applied salinity changes did not pose a significant threat to this ecosystem. Furthermore, the increased temperature treatment was not a real stressor due to an unexpected drop in ambient temperature during the stressor application period distancing the ecosystem from the algae temperature optimum, distance which decreased with the application if warming. Photosynthetic biomass (F_0) decreased in all mesocosms during the stressor phase, likely because of the drop in ambient temperature. I found one significant interaction between temperature and flow velocity on microalgae OTU community composition, suggesting a synergistic effect on microalgae communities and highlighting the complexity of the combined effects of environmental stressors.

During the recovery phase, diatom communities homogenized, with most treatment channels reaching a community composition similar to control channels, indicating near complete recovery. Scraped surfaces exhibited faster growth of microphytobenthos compared to unscraped surfaces with no significant differences after 2 weeks of recovery. In addition, microphytobenthos community composition between the scraped and unscraped surfaces remained significantly different, suggesting that competition for substrate between resident and immigrant taxa influenced community composition, which indicate priority effects. Fast growth of photosynthetic biomass was also reported in a previous study where no differences was observed after 4.5 days of recovery in controls and pesticide-treated flumes (Rosenkranz et al., 2023). Brown and Manoylov (Brown and Manoylov, 2023) observed different diatom communities 42 years after agricultural stream restoration compared to reference sites. The concluded that ecological degradation of the habitat was likely driven by sedimentation due to

run-off in the recovering agriculture stream. They highlighted the importance of protecting water quality, as the recovery of nutrients can be a decades-long process overlayed with many potentially new stressors influencing aquatic organisms (Brown et Manoylov, 2023). Thus, long-term restoration efforts can lead to persistent changes in community composition, possibly due to lasting alterations in habitat conditions or nutrient dynamics introduced during the restoration process.

In the third chapter, I examined the environmental factors that determine photosynthetic biomass and microalgae community composition in the Kinzig catchment. Using principal component analysis (PCA) of environmental predictors, I identified two main gradients within the catchment: a hydrological gradient (depth and discharge vs. flow velocity) and a chemical gradient (nutrients and salts, particularly sulfate and chloride). Furthermore, phosphate concentration showed independent variability. Photosynthetic biomass, measured using a BenthoTorch, was slightly negatively associated with depth, likely due to light limitations. Green algae biomass responded primarily to the hydrological gradient, while diatom and cyanobacterial biomass had a strong negative correlation with sulfate, the main drivers of the salinity gradient. Conductivity, reflecting overall ionic concentrations, correlated with water pH and was a significant factor for periphytic communities, including diatoms. Water temperature and ammonium also influenced diatom biomass. For microalgae, discharge and phosphate concentration also has a significant impact on community composition, suggesting taxon-specific ecological responses. Flow velocity was found to be a key factor in microalgal community composition and influenced resource availability and stability of microphytobenthic communities. These results are consistent with other studies showing that water variables drive changes in microalgae assemblages, often shifting from sensitive to tolerant species (Pillsbury et al., 2019; Soininen et al., 2004; Stenger-Kovács et al., 2020). I found that ecological status assessments using IPS on microscopy and the diatom subset of the amplicon sequencing data were different, possibly due to biases in both methods. Microscopy often misses barely visible taxa, while amplicon sequencing may not capture all taxa due to incomplete reference libraries (Kochoska et al., 2023). Discrepancies between these methods highlight the need for more comprehensive reference databases and the profit of using both methods for a clearer ecological picture.

In the fourth and last chapter of this thesis, I characterized diatom communities along restored sewers in the Boye catchment based on length of time since restoration. I found no significant differences in community composition in recovering streams based on time since restoration. The microscopy analysis data revealed that the taxa belonging to the *Achnanthidium minutissimum* group dominated in the majority of the samples including some of the rivers restored during the year 2021 (Boye oh B224 and Liesefeld oh Boye). This is a pioneer diatom species known for its resilience across various trophic levels and water conditions. The lack of community variability implies that the diatom community recovery process across the Boye catchment occurs much faster than the recovery of other organisms such as macroinvertebrates (Gillmann et al., 2023; Winking et al., 2014, 2016). I found only limited correlation between physical and chemical variables and microphytobenthos, with conductivity and average flow velocity being the most important factors affecting community composition. The lack of significant differences between our restored sites and reference sites suggests that although these reference sites were never used as open sewers, they were nevertheless compromised in some way, probably by some diffuse urban stressors. Further monitoring and comprehensive analyses are necessary to fully understand the long-term dynamics of microphytobenthic communities in response to ongoing restoration efforts and environmental changes in this urban river network.

In summary, the main drivers for microphytobenthos community composition in general and diatoms community in particular were hydrological in nature and to a lesser degree salinization based. The catchment of the sampling, be it field or mesocosms based, was influential on the relative importance of the driver (Kaijser et al., 2024). this further underlines the importance of the multiple stressors, as both studied catchments differed mostly on the degree anthropogenic degradation. On the other hand, while the total community can be responsing to variations in the rivers, the photosynthetic function of the microphytobenthos showed algal group responses.

Conclusion and outlook

This thesis comprehensively examined the functional and compositional responses of stream microphytobenthic communities to multiple stressors and provided compelling evidence on how microalgal communities respond to degradation caused by anthropogenic stressors as well as recovery following the release of stressors. By analyzing microalgal communities using morphology and amplicon sequencing methods, as well as examining photosynthesis-related traits in both mesocosm experiments and field studies, this research highlights the responses of these important biofilm-forming assemblages during the degradation and recovery of stream ecosystems.

Regardless of study setup, the mesocosm studies were driven by flow velocity. Microphytobenthic communities responded to hydrological parameters over other physicochemical stressors, in contrast to other organism groups. This may be due to the different generation scales of the organisms. As microalgae reproduce quickly their response times can supersede those of other organisms, such as invertebrates. A community thus adapted to a variable catchment like the Boye should be more resilient to changes in the common stressors, that in this case would be the salinization. This also is reflected by the photosynthetic function within the community.

Although the field studies had different aims, similar conclusions can be drawn. Comparing a little degraded catchment (Kinzig) to a highly degraded one (Boye), it becomes clear that the degradation drives the homogenization of the community. Thus, we could observe more variability between communities in the Kinzig catchment than in the Boye catchment. The physicochemical parameters had a bigger impact on the communities of the Kinzig because of the lack of urban driven degradation, while the reference sites of the Boye were still degraded by the urban influence, so that no significant differences occurred to newly restored rivers.

Future research should focus on long-term monitoring to capture the dynamics of community responses to environmental changes and restoration efforts. Understanding the interactions between multiple stressors, such as flow velocity, salinity and temperature, as well as nutrients is important for developing effective management strategies for urban streams. Further studies are needed to explore the complex relationships between environmental factors and microalgal communities to better predict and mitigate the impacts of climate change on aquatic ecosystems.

Additionally, future studies should include multiple sampling events throughout different seasons to capture the full spectrum of microalgae community dynamics. This will help to identify variations in community structure and function that may not be evident with spring-only sampling. Furthermore, to better understand the impact of nutrient levels, especially phosphorus, on microalgae communities, experiments should be conducted across a range of phosphorus concentrations. Comparing data from periods of both low and high phosphorus availability, particularly during the summer months, can provide insights into how nutrient stress influences microphytobenthic community composition and resilience. Integrating these insights will enhance our ability to maintain and restore healthy and resilient stream ecosystems in the face of ongoing environmental challenges.

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Please note that this section includes only the references cited in this work's introduction, general discussion, conclusion and outlook. References cited within the four chapters are provided at the end of each chapter. This approach was chosen to clearly distinguish the manuscripts that constitute this work from the other sections

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Acknowledgements

First of all, I would like to thank my supervisor Prof. Bánk Beszteri, who made it possible and gave me the opportunity to do my doctorate in his group. I am also very grateful to my second supervisor Prof. Daniel Hering and my mentor Dr. Julia Foerster from LANUV. Your expert ideas, generous support, guidance, advice and encouragement have been invaluable throughout my doctoral research and in my academic, professional and personal development.

I would like to express my sincere thanks to my collaborators and co-authors Andrea Burfeid-Castellanos, Michael Kloster, Marzena Spyra, Sara Beszteri, and Danijela Vidaković, as well as all the CRC RESIST doctoral students, for your support during my doctoral research. Your community spirit and insightful contributions have greatly increased the depth and breadth of this work.

My thanks also go to the entire Phycology group, particularly Kay Eibl, for fostering an environment beyond professional boundaries that makes every day a pleasant and enriching experience.

My deepest thanks go to my wife Dally Mayombo and sons Matthew Mayombo and Micah Mayombo. Thank you for your unconditional love and support. Your encouragement has been my driving force and I am fortunate to have a foundation of love and support.

I would like to thank the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG) for funding this research. Your financial support has helped me to continue my research based on personal interests and I am truly grateful for this opportunity.

The curriculum vitae is not included in the online version for data protection reasons.

Declarations required for the Application of Admission to the Doctoral Proceedings (*Promotionsbegehren*)

Declaration:

In accordance with § 6 (para. 2, clause g) of the Regulations Governing the Doctoral Proceedings of the Faculty of Biology for awarding the doctoral degree Dr. rer. nat., I hereby declare that I represent the field to which the topic "*Functional and compositional responses of stream microphytobenthic communities to multiple stressors increase and decrease"* is assigned in research and teaching and that I support the application of Ntambwe Albert Serge Mayombo.

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