Lancaster Environment Centre

Lancaster University

Responses of freshwater phytoplankton exposed to chemical contaminants: tolerance acquisition, physiological trade-offs and environmental controls

Simone Rizzuto, MSc

Submitted for the degree of

Doctor of Philosophy in Environmental Science

November 2021

Acknowledgements

Working on this Doctoral Thesis represented an incredible journey, very challenging but at the same time extremely rewarding.

I will start by thanking my wonderful supervisors from LEC and NIVA. Thank you to Kevin C. Jones, Hao Zhang, Luca Nizzetto, and Eva Leu. I can't find the words to thank you enough for the invaluable time spent together into constructive scientific discussions, for your illuminated guidance, and for the friendly chats when they were needed the most. Thank you for making me feel at home in both Lancaster and Oslo. You truly helped me growing professionally into a better scientist.

Thank you to all my co-authors, which contributed to the making of these PhD Chapters by adding their insightful comments: Didier Baho, Jan-Erik Thrane, Francesco Pomati, Jon Norberg, Dag O. Hessen, Birger Skjelbred. I am particularly grateful to Didier Baho, who showed me the ropes of "R", taught me how to handle complex statistical analysis, and spent days and nights (literally) with me in the laboratory in Oslo. A big thank you also to Jan-Erik Thrane for the experimental support in maintain algal cultures in long-term controlled environments. I also wish to thank Runmei Wang and Yanying Li for their guidance into setting up the "tricky" LC-MS/MS in LEC. A huge thanks to all my colleagues, office and lab mates at LEC and NIVA for their friendship, laughter and moral support along this journey.

Last but not least, I thank my wife Marina for sharing this experience with me. Thank you for being there, supporting me, encouraging me, and making my life easier every day. Thanks to Babbo e Mamma, among my biggest supporters. A voi che ci siete stati fin dall'inizio. Senza di voi questo non sarebbe stato possibile. Grazie.

Statement of Contribution

This is to certify that, to the best of my knowledge, the intellectual content of this PhD thesis represents the product of my own work and that all the assistance in preparing this thesis and sources have been acknowledged. This thesis includes collaborations with my supervisors Kevin C. Jones, Hao Zhang, Luca Nizzetto and Eva Leu, and other co-authors such as Didier Baho, Jan-Erik Thrane, Francesco Pomati, Dag O. Hessen, Birger Skjelbred and Jon Norberg. I was responsible for the project's conceptual and experimental design, data collection, analysis, interpretation and synthesis into final form for publication. My supervisors provided intellectual guidance, equipment, funding support, and editorial assistance. This thesis has not been submitted by this candidate for the award of Doctor of Philosophy elsewhere.

Funding for this PhD Project was provided the Research Council of Norway, project grant no. 244460, Pollution and Ecosystem Adaptation to Changes in the Environment (PEACE). The research was co-funded by a discretional internal grant from the Norwegian Institute of Water Research (NIVA), under the Institute Strategic Priorities (ISS) on ecosystem remediation (TRADE-OFF). The PhD candidate was also supported by the Lancaster University Faculty of Science and Technology and a Lancaster Environment Centre PhD Scholarship.

Simone Rizzuto

Abstract

Chemical pollution of freshwaters represents a large-scale environmental problem which needs to be reduced to avoid global or regional change. Integrating ecological theory in ecotoxicological approaches is instrumental to understand how the widespread presence of contaminants influence biota in complex natural systems. In this thesis, the concept of ecosystems as complex adaptive systems (CAS) is integrated as a guiding framework into ecotoxicological approaches, to unravel the implications arising from adaptation of freshwater phytoplankton to waterborne chemical contamination under realistic environmental conditions.

The first experimental study examined the influence of two key environmental factors (dissolved organic matter – DOM and pH) on the responses, tolerance acquisition and associated trade-offs in a population of phytoplankton exposed to sub-lethal concentrations of a mix of twelve organic micropollutants (pharmaceuticals and personal care products - PPCPs) over multiple generations. DOM reduced the toxic effect of the mix of PPCPs at environmentally relevant concentrations and modulated tolerance acquisition and associated trade-offs in the microalgal population, possibly by complexing micropollutants.

The concept of ecological memory (EM) recognizes the importance of previous stress encounters in promoting community tolerance and thereby enhances ecosystem stability, provided that gained tolerances are preserved during non-stress periods. It was hypothesized that the recruitment of tolerant species can be facilitated by imposing an initial sorting process (conditioning) during the early stages of community assembly, which should result in higher production (biomass development and photosynthetic efficiency) and stable community composition. To test this, phytoplankton resting stages were germinated from lake sediments originating from two catchments that differed in contamination history: one impacted by long-term herbicide and pesticide exposures (historically contaminated lake) from an agricultural

catchment, compared to a low-impacted one (near-pristine lake) from a forested catchment. Conditioning was achieved by adding an herbicide (Isoproturon, which was commonly used in the catchment of the historically contaminated lake) during germination. Afterwards, the communities obtained from germination were exposed to an increasing gradient of Isoproturon. As hypothesized, upon conditioning, the phytoplankton assemblages from the historically contaminated lake were able to rapidly restore photosynthetic efficiency ($p > 0.01$) and became structurally (community composition) more resistant to Isoproturon. The communities of the near-pristine lake did not yield these positive effects regardless of conditioning. Moreover, assemblages that displayed higher structural resistance concurrently yielded lower biomass, indicating that benefits of EM in increasing structural stability may trade-off with production. The results indicate that EM can foster ecosystem stability to a recurring stressor.

The third study investigated how EM influences the functions (growth) and structure (diversity) of early-stage assemblages of phytoplankton by using a trait-based approach. Phytoplankton assemblages were germinated from the sediments of two lakes which differed in contamination history: a historically contaminated lake and a forested, near-pristine one, in presence/absence of Isoproturon. Results showed that the functions and responses of the two communities were dependent on the previous history of contamination, confirming the study's expectations. The EM of previous contamination allowed the community originating from the sediments of the historically contaminated lake to maintain growth and diversity when germinated in presence of the herbicide. In contrast, sub-lethal concentrations of the herbicide caused negative effects on the growth and diversity of the community from the near-pristine lake.

The last two studies arose from the need to explain the results observed in the first study, where the DOM appeared to decrease the toxicity/bioavailability of some PPCPs on microalgae. The main hypothesis was complexation mediated by the DOM lowering toxic effect or bioavailability, however the experimental design used in the firsts study did not allow the

verification of this hypothesis. Hence, the binding of DOM with contaminants was examined in detail in two different studies, using an improved dialysis equilibrium-method. The method's performance was critically evaluated through a series of rigorous QA/QC criteria, across a range of DOM concentrations and pH conditions, using the herbicide Isoproturon as model compound. Good measurement reproducibility, mass balance closure, and successful trans-membrane equilibrium of ISU were obtained. The improved equilibrium-method was therefore applied to examine the interactions between DOM and a selection of compounds from the mix of PPCPs used in the first study, under the same conditions of DOM and pH. Association with DOM was confirmed for the more hydrophobic PPCPs at high pH. The results suggest the binding was driven by i) the presence of carboxylic groups of PPCPs, ii) high pH shifting the structural configuration of DOM, making it more suited to bind some of the PPCPs. A non-linear change of binding capacity with increasing DOM concentration was also observed among the tested PPCPs.

The thesis demonstrates that the concept of ecosystems as complex adaptive system can be an important addition in moving ecotoxicological approaches towards "chemical stress ecology". Recommendations are made in support of future research to further support this transition.

Table of Contents

Aims of the thesis and conceptual guide

This thesis presents a multidisciplinary approach to address complex questions arising from the global problem of chemical pollution in freshwater ecosystems by using innovative experimental methods. Due to the intricacy of the different ecological and chemical processes examined, a conceptual guide is developed to help the reader and is schematized in Figure i. The thesis comprises two main parts, each identifying a different research area:

- **Part I – Integration of ecological concepts in ecotoxicology**.
- **Part II – Environmental Chemistry**.

In Part I, the main aim is to explore different fundamental ecological processes to help increase the understanding of how populations, communities and ecosystems deal with chemical pollution in freshwaters. The concept of ecosystems as complex adaptive systems (CAS) is integrated as a guiding framework into ecotoxicological approaches, to unravel the ecological implications arising from adaptation of freshwater phytoplankton to waterborne chemical contamination under realistic environmental conditions. After the introductory review chapter (Chapter I), the concepts of tolerance acquisition, physiological trade-offs, ecological memory and trait diversity are examined in three chapters (Chapters II, III and IV). The overarching questions posed in Part I are:

- *What are the ecological and physiological trade-offs occurring from adaptation to chemical stress?*
- *What are the roles of environmental factors in tolerance acquisition and associated trade-offs?*
- *Are tolerance and trade-offs retained over time and can they be recalled - even if they are not always expressed?*

Studying such ecological processes in freshwater ecosystems, where the potential co-existence of multiple chemical pollutants under heterogeneous environmental factors is the norm, is important for many reasons, especially in the context of global environmental change (Niinemets et al., 2017; Woodward et al., 2010), where numerous pressures (e.g. eutrophication, increased sediment load, altered nutrient balance, water browning, differences in toxicity/bioavailability of pollutants) create suboptimal conditions for primary producers, consumers and decomposers, ultimately leading to complex feedbacks between climate change and freshwater ecosystem performance (Niinemets et al., 2017). The introductory chapter (**Chapter I**) presents the ecological concepts examined in the thesis. This is done after summarising the current impact of chemical pollution on freshwaters, the state of the art of European legislation on environmental protection, the current state of integration of ecology in ecotoxicology with knowledge gaps, and the potential benefits of integrating ecological theories in ecotoxicological approaches. In **Chapter II**, the process of tolerance acquisition to chemical pollutants is tested experimentally, in controlled experiments with freshwater phytoplankton populations. The occurrence of physiological trade-offs that arise from acquiring tolerance to the stressors, and the influence of environmental factors on this process, are investigated. The environmental factors selected are varying concentrations of natural dissolved organic matter (DOM) and water pH, due to their crucial role in the fate, behaviour, and bioavailability of contaminants. In **Chapter III**, the theory of ecological memory of long-term contamination was experimentally tested through a germination study on freshwater phytoplankton communities originating from catchments with different histories of contamination. In **Chapter IV**, the influence of ecological memory of historical contamination on the process of ecological succession of freshwater phytoplankton communities was assessed, through a trait-based approach. The ecological theories tested in these Chapters were assessed using phytoplankton as model organism(s), because they are relatively easy to handle

in laboratory conditions (Merchant et al., 2007), and represent a good example where adaptation processes are likely to co-occur over human timescales (Collins and De Meaux, 2009; Fogg, 2001; Thibodeau et al., 2015), making them widely used model organisms for toxicological and evolutionary studies at population and community levels. The choice was also dictated by their immense importance from an ecological perspective, being the dominant primary producers on the planet and responsible for half of the global primary production - despite their microscopic size (Field et al., 1998).

Part II of the thesis (Chapters V, VI) was developed to examine in detail the chemical processes (i.e. complexation) arising between DOM and the investigated contaminants, as well as identifying the influence of water pH in this interaction. In particular, Part II was designed to address specific questions originating from the toxicity outcome induced by the contaminant – environmental factors interactions on phytoplankton (Chapter II), where the influence of DOM and water pH decreased the toxic effect of the investigated contaminants. It was hypothesized that the bioavailability of these chemicals was lowered by their complexation with DOM, which required a specifically designed experimental method to test the hypothesis. Hence, in **Chapter V**, an improved equilibrium-based method to assess the binding of DOM with chemical contaminants was developed and critically evaluated. The effectiveness of the method was tested on a model compound, under a wide range of environmentally realistic DOM concentrations and pH levels, against defined quality control/quality assurance (QA/QC) conditions. After proving its efficacy, the method was applied in **Chapter VI**, where the binding of a selection of the contaminants used in Chapter II was tested under the same environmental conditions of DOM and pH as used in Chapter II.

Chapter VII concludes the thesis, gathering all the main results arising from Chapters II-VI, summarising the key messages, recommendations and future work.

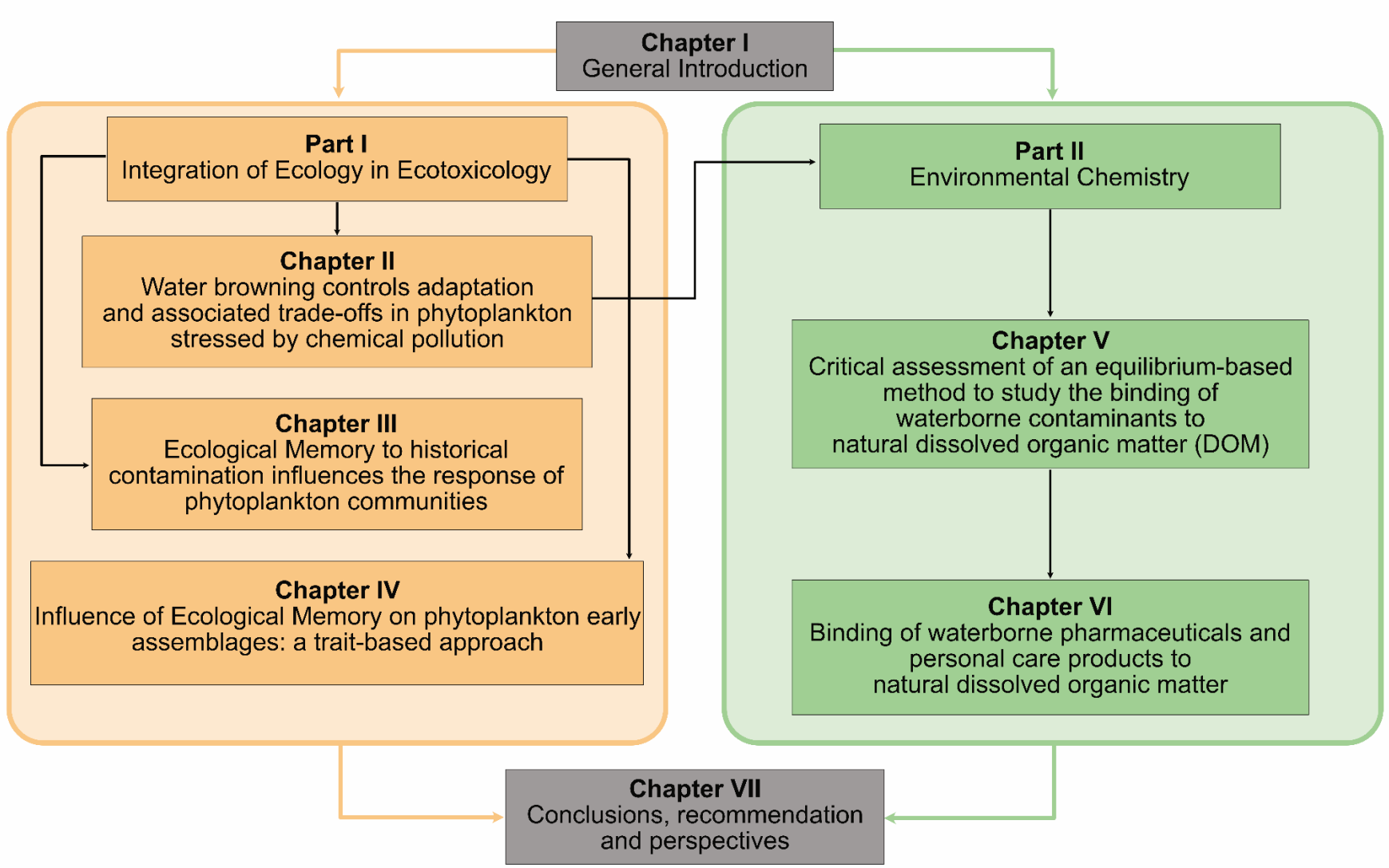


Figure i. **Conceptual guide of the thesis.**

List of Papers

This thesis contains 4 papers published in international peer-reviewed journals and 1 in preparation for submission to an appropriate journal. These correspond to Chapters II – VI. They are listed below, with a brief description of the contribution made by the candidate and co-authors (*italic*). Links to the published articles are provided at the beginning of each Chapter. The published version of the articles can also be found in the separate Appendix section "Appendices and Separate files".

1. **S. Rizzuto**, J. E. Thrane, D. L. Baho, K. C. Jones, H. Zhang, D. O. Hessen, L. Nizzetto, E. Leu, 2020. Water Browning Controls Adaptation and Associated Trade-Offs in Phytoplankton Stressed by Chemical Pollution. Environ. Sci. Technol. 54, 5569–5579 (**Chapter II**). ***S. Rizzuto*** *designed and performed the experiment with suggestions from J. E. Thrane, carried out the data analysis and wrote the manuscript. K.C. Jones, H. Zhang, L. Nizzetto, E. Leu, and D. L. Baho supervised the process. D. O. Hessen was involved in the preliminary design of the research questions.*

2. D. L. Baho, **S. Rizzuto**, L. Nizzetto, D. O. Hessen, J. Norberg, B. Skjelbred, K. C. Jones, H. Zhang, E. Leu, 2021. Ecological memory to historical contamination influences the response of phytoplankton communities. Ecosystems. https://doi.org/10.1007/s10021-021-00604-0 (**Chapter III**). ***S. Rizzuto*** *and D.L. Baho designed and performed the experiment, carried out the data analysis and wrote the manuscript. L. Nizzetto, E. Leu, K.C. Jones and H. Zhang supervised the process. D. O. Hessen and J. Norberg were involved in the preliminary design of the research questions. B. Skjelbred performed the taxonomic analysis.*

3. **S. Rizzuto**, D.L. Baho, K. C. Jones, H. Zhang, F. Pomati, E. Leu, L. Nizzetto, 2021. Influence of ecological memory on phytoplankton early assemblages: a trait-based

approach. Sci. Tot. Environ. In prep. (**Chapter IV**). ***S. Rizzuto*** *and D.L. Baho designed and performed the experiment.* ***S. Rizzuto*** *carried out the data analysis and wrote the manuscript, with the supervision of L. Nizzetto, E. Leu, F. Pomati, K.C. Jones and H. Zhang.*

4. **S. Rizzuto**, D. L. Baho, K. C. Jones, E. Leu, L. Nizzetto, H. Zhang. Critical assessment of an equilibrium-based method to study the binding of waterborne contaminants to natural dissolved organic matter (DOM). 2021. Chemosphere. 285, 131524. (**Chapter V**). ***S. Rizzuto*** *designed and performed the experiment, carried out the data analysis and wrote the manuscript, with the supervision of K.C. Jones, H. Zhang, L. Nizzetto and E. Leu.*

5. **S. Rizzuto**, D. L. Baho, K. C. Jones, H. Zhang, E. Leu, L. Nizzetto, 2021. Binding of Waterborne Pharmaceutical and Personal Care Products to Natural Dissolved Organic Matter under different pH. Sci. Tot. Environ.784, 147208. (**Chapter VI**). ***S. Rizzuto*** *designed and performed the experiment, carried out the data analysis and wrote the manuscript, with the supervision of K.C. Jones, H. Zhang, L. Nizzetto and E. Leu*

List of Tables

List of Figures

List of Appendices

All the Appendices and Supplementary Materials have been included in the separate file "Appendices and Supplementary Materials" to facilitate the reading of this thesis. For convenience, links to the published Supplementary Material has been added at the beginning of every Chapter.

Chapter I – General Introduction

1.1 Chemical pollution in freshwaters

Water is an invaluable resource. Approximatively 93,000 km^3 of water is in rivers and lakes and even more can be found stored in ice or groundwater (Allan, 2004). It can be argued that such richness of freshwaters may easily guarantee the survival and prosperity of humankind and the demands for the homeostasis of natural ecosystems (Figure 1,1), but, unfortunately, this is not the case.

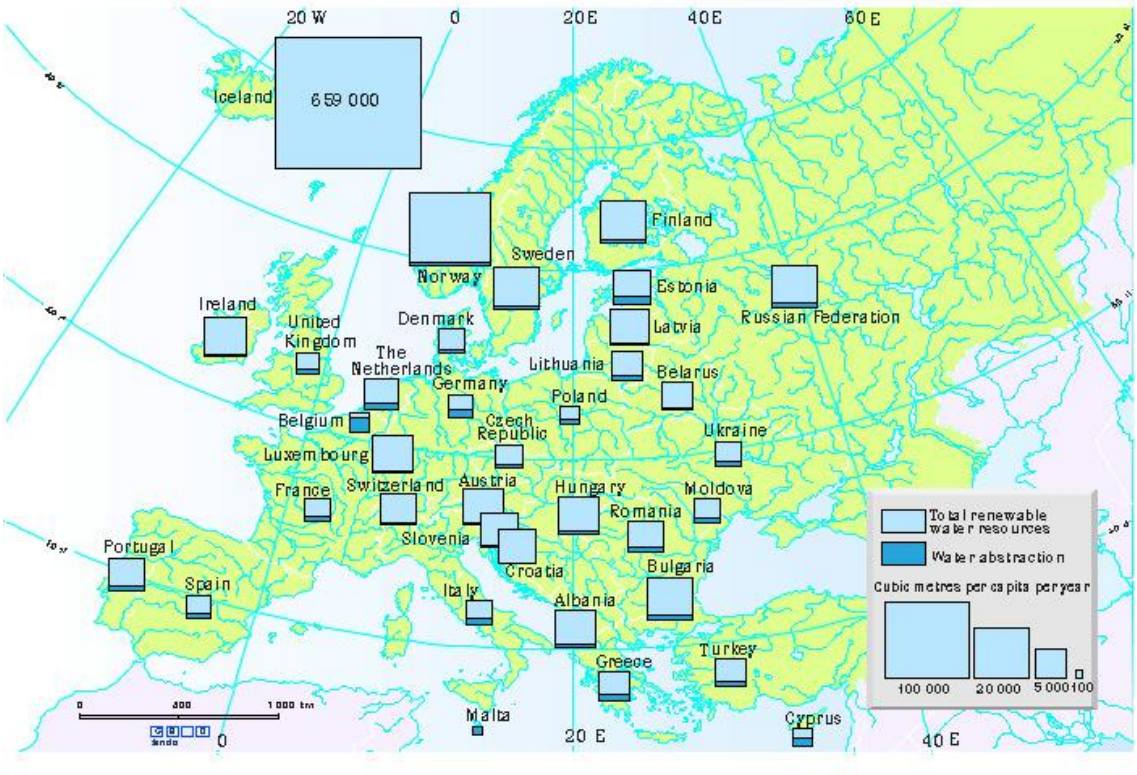


Figure 1.1. Water resource distribution in Europe. Copyright holder: European Environment Agency (EEA).

The distribution of water is not homogenous around the globe, and to compound this problem, the impacts of human activities are rapidly transforming inland water bodies through overuse and pollution, which has been estimated to be threatening the water security of more than 80% of human populations (Vörösmarty et al., 2010). Chemical pollution is one of the major

challenges faced in the last decades (Vörösmarty et al., 2015). The global chemical production from anthropogenic activities such as agricultural practice, industry and urban settlements amounted to 400 million tonnes in the year 2000, which corresponded to 400,000 times more than 70 years earlier in 1930 (Gessner and Tlili, 2016). Figure 1.2 shows the total EU consumption and production of chemical substances in the last decade.

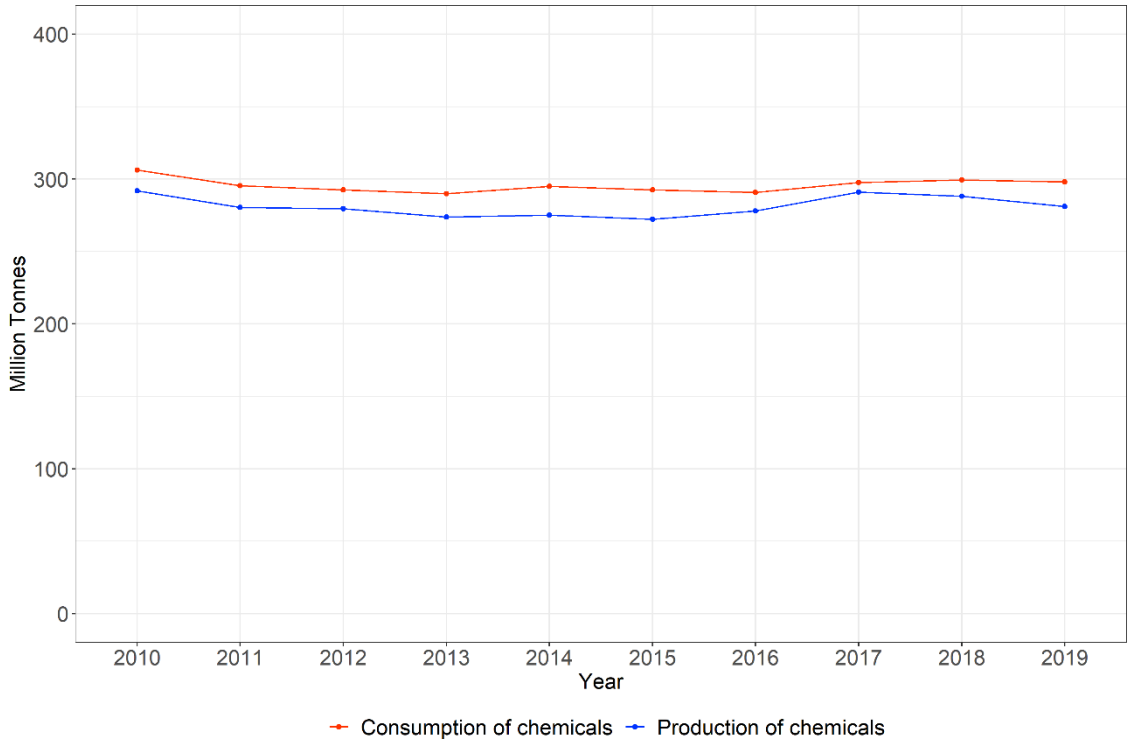


Figure 1.2 EU consumption and production of chemical compounds from 2010 to 2019. Data from European Commission statistics. https://ec.europa.eu/eurostat/web/products-eurostat-news/-/ddn-20200121-1

Such extensive use inevitably directs chemicals towards freshwaters via direct application or run-off of agrochemicals (Elias et al., 2018), permitted or accidental industrial releases (Batty and Hallberg, 2010), through treated or untreated wastewater infrastructures (Gardner et al., 2012), or in leachates from landfills (Kümmerer et al., 2019), causing severe threats towards human health, the integrity of freshwater ecosystems and associated services (Rockström et al., 2009; Vörösmarty et al., 2005). For instance, pollution-related diseases caused over 9 million premature deaths in 2015, which is 16% of global deaths, three times more than those caused

by AIDS, tuberculosis and malaria combined and 15 times more than the deaths related to wars and other forms of violence. In the most polluted countries, diseases associated with pollution are responsible for more than one death in four (Landrigan et al., 2018).

The negative effects on wildlife and ecological communities also place real costs on ecosystem functions and services in financial terms (Wang et al., 2019). An example was reported by Pretty and others (2000), who estimated around £140 million of external cost for clean-up, purification and recovery of drinking water caused by fertilizers and pesticides application in UK agricultural activities. Among the widespread occurrence of anthropogenic contaminants in freshwaters, the group of pharmaceutical and personal care products (PPCPs - Boxall *et al.* 2012) are of particular concern and scientific interest. PPCPs are specifically designed to address human and veterinary care, and include human and animal therapeutic drugs and their subsequent metabolites and conjugates, as well as sunscreen, soaps, moisturizers, lipsticks ingredients, fragrances, insect repellents, antibacterial agents, etc., (Daughton and Ternes, 1999). Their global production went from 1 million tons in 1993 (Daughton and Ternes, 1999) to 20 million tonnes in 2016 (Wang and Wang, 2016), and is still increasing due to the high demands of PPCPs in preventing or curing diseases and sustaining the development of economic activities such as aquaculture and livestock farming. PPCPs are continuously discharged in surface water mainly as by-products of modern life from treated and un-treated wastewaters (Daughton and Ternes, 1999). Due to their partial resistance to biological and physical degradation and the biological activity that several of these compounds exert, even at low concentration (Boxall et al., 2012), they can interfere with non-target aquatic organisms (Grzesiuk et al., 2018). Phytoplankton is particularly affected as PPCPs can interfere with fundamental metabolic pathways related to chlorophyll a and fatty acid synthesis (Zhang et al., 2012, 2019). Modern agriculture is another anthropogenic activity which particularly impacts freshwater systems; it accounts for 70% of the water abstractions worldwide (FAO, 2017) and

is recognized as exerting substantial pressure on aquatic ecosystems (Foley et al., 2005). Almost 16 million km^2 of available croplands are cultivated worldwide, making agriculture the world's largest terrestrial biome (Foley et al., 2011). The expansion and intensification of agriculture resulted in an increase of more than 750% in the global production of pesticides from the 1950's to 2000 (Tilman et al., 2002), representing a 50 billion US dollars global market (Stehle and Schulz, 2015). The extensive use and runoff of fertilizers and pesticides (Tilman et al., 2002) has threatened the integrity of many freshwater ecosystems (Vörösmarty et al., 2005; Weatherhead and Howden, 2009). Pesticides detected in receiving waters can alter enzymatic activity and cellular metabolism (Sturm et al., 2007) in non-target freshwater organisms, thereby decreasing their fitness (Beketov and Liess, 2008). Herbicides are intentionally used to produce ecological effects by filtering-out undesired species (Schütte et al., 2017). Triazine and phenylurea compounds are among the most extensively used herbicides, adopted for the annual control of grasses and broadleaf weeds (Alvarsson, 2012; Böttcher and Schroll, 2007; Fernandez and Gardinali, 2016). Their mode of action inhibits the electron transport chain of photosystem-II by competing with plastoquinone for binding to the D1 protein in the thylakoid membrane (Arnaud et al., 1994). Typically, herbicides are used in pre-emergence phase, to avoid negatively impacting the crop itself. In receiving waters, however, most photosynthetic organisms (including both vascular plants and microalgae) can be affected. At sub-lethal concentrations, hindrance of the photosynthetic process results in growth inhibition and increased mortality rates (Schroer et al., 2004) in more susceptible organisms. Different responses in different species alters the natural assemblage of species in the community (Rohr and Crumrine, 2005), and this can ultimately impair ecosystem functioning (Schäfer et al., 2007). During a recent risk assessment conducted on 4000 monitoring sites in European lakes and rivers, it was estimated that organic chemicals are likely to exert acute lethal or chronic long-term effects on biota in 14% and 42% of the sites,

respectively (Malaj et al., 2014). These results clearly demonstrate that chemical pollution represents a large-scale environmental problem that needs solutions (Rockström et al., 2009). The planet's capacity to deal with chemical pollution has been listed as one of the nine planetary or regional critical anthropogenic stressors which need to be reduced to avoid global or regional change (Rockström et al., 2009; Steffen et al., 2015). However, the planetary boundary of chemical pollution has not been yet quantified (Rockström et al., 2009), and along with other global challenges, still has poorly understood significance and implications (Campbell et al., 2017; Stehle and Schulz, 2015).

1.2 Environmental protection from water pollutants: approaches in ecological risk assessment

Driven by safety concerns for human and ecosystem health caused by chemical pollution, governments and stakeholders are striving to protect the environment by reducing their dependence on chemicals, implementing the efficiency of wastewater treatment plants, as well as assessing and managing chemical contaminants throughout their lifecycles (WHO, 2011). Monitoring the water quality of freshwater systems is now a worldwide legal requirement (WHO, 2011). The need to investigate the effects induced by anthropogenic contaminants on natural systems led towards the establishment of a branch of science called ecotoxicology (Moriarty, 1999). Ecotoxicology was firstly established in the 1970s as the environmental branch of toxicology, studying the potential negative effects of chemicals through the systematic study of their fate, exposure and effects (Truhaut, 1977). Due to the close connections with medical toxicology and environmental chemistry, the core principles of ecotoxicology are experimental testing, analysis of dose-effect relationships, estimation of effects concentrations and extrapolation of risk for ecosystems. It is closely coupled to knowledge and understanding of environmental chemistry and the effects of environmental conditions/variables on chemical form, fate, behaviour and degradation. This approach paved

the way to understand the effects of chemical contaminants, and to assess their environmental consequences (Moriarty, 1999). In regulatory risk assessment, science-based chemical management faces the need to provide robust, representative, cost-effective data to predict the impact of chemical pollutants on ecosystems. Regulatory ecotoxicology generally defines “no unacceptable" contaminant concentrations for ecosystems relying on the implementation of arbitrary toxicity endpoints (OECD, 2006a), which establish threshold concentrations below which No Observable Effect Concentration (NOEC), LCxx (lethal concentration on xx% of population), or ECxx (effect concentration on xx% of population) impact on selected organisms occurs (OECD, 2006a). The threshold values are usually obtained through laboratory based ecotoxicity tests, typically involving representative single species (in aquatic risk assessment usually *Daphnia*, fish, phytoplankton), and single chemicals, under controlled environmental conditions (OECD, 2006a). Such an approach allows for comparative studies and quantitative ranking of toxicity among compounds, and currently underpins the development and implementation of chemical management and environmental protection acts regulating the use of chemicals with potential ecological risk. Two key examples are the chemical control regulation in the European Union for the Registration, Evaluation, Authorisation and Restriction of Chemicals manufactured in or imported into the European Union (EU REACH), and the major body of legislation and sustainable use of European freshwater resources, the Water Framework Directive (WFD, 2000/60/EC). The EU REACH (Figure 1.3) was adopted to improve the protection of human health and the environment from the risks that can be posed by chemicals, while enhancing the competitiveness of the EU chemicals industry. REACH established a framework for EU action in the field of water policy by introducing rules for the production, commercialisation, usage, shipment and release for more than 23,000 chemical substances (Article 16 2000/60/EC).

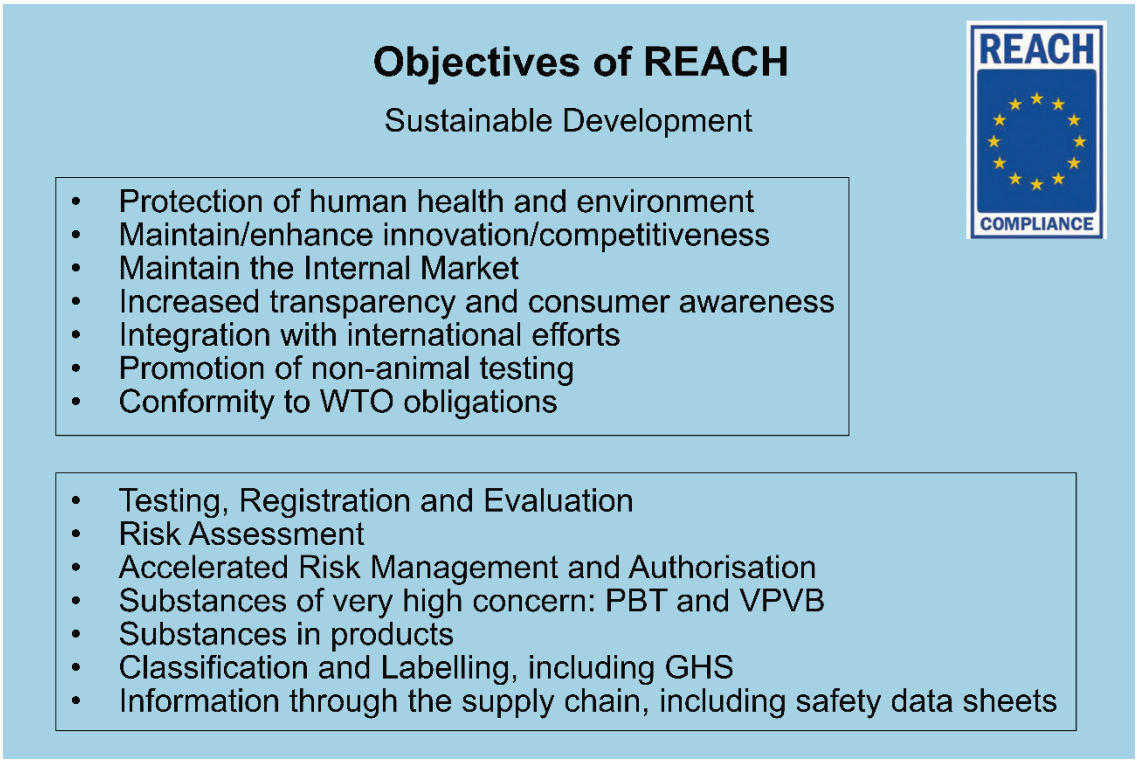


Figure 1.3. REACH objectives**.**

The WFD (Figure 1.4) was introduced in 2000 to protect, defend, and restore our water heritage against anthropogenic pressures, with the aim to reach "good ecological and chemical status" of European freshwaters by 2015 (European Union, 2000).

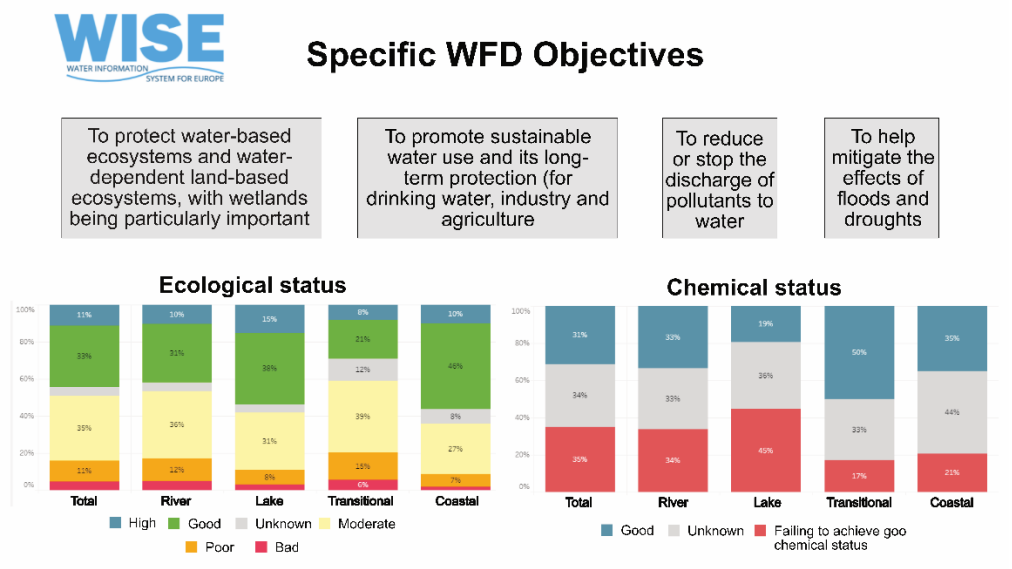


Figure 1.4. Water Framework Directive objectives and status.

The ecological quality assessment introduced a radical shift from other methods, focusing on a holistic ecosystem approach rather than on a few chemical quality parameters, which was recognized as a more effective (although more complex), integrative method compared to those proposed by other regulations (Hering et al., 2010; Moss, 2007). In contrast, while the regulation generally acknowledges the importance of an ecosystem approach to protect waterbodies, in the area of the assessment of chemical pollution such an approach has not been sufficiently assimilated yet. For instance, the approach used to assess the chemical status of European freshwaters in the WFD reflected the more classic "standard-conditions" ecotoxicology (Beyer et al., 2014). To this end, the WFD introduces EQS which must not be exceeded if good chemical status is to be met (2008/105/EC) on a watch list of 33 priority substances and 8 other pollutants believed to represent a significant risk to or via the aquatic environment (Article 16, WFD 2000/60/EC). Similarly to the EU REACH, these EQS are set based on the results of standard laboratory procedures using single species, and single chemicals, under controlled laboratory conditions (OECD, 2006a).

To date, the WFD is widely acknowledged as the most substantial and ambitious piece of European environmental legislation. However, over 20 years since its implementation, the WFD has not delivered its main objectives of non-deterioration of water status and did not achieve good status for all European freshwaters (Figure 1.5). Apart from some good results observed in groundwaters, where the majority was judged to be of good overall status in 2015 (European Commission, 2019a), 60% of European surface water bodies were still falling below the required standard in 2015, which translates to about 130,000 waterbodies being far from reaching "good ecological and chemical status" in 2019. Concerning chemical pollution aspects, about 67% of water bodies failed to achieve good chemical status, based on the current WFD method for chemical status classification (European Commission, 2015).

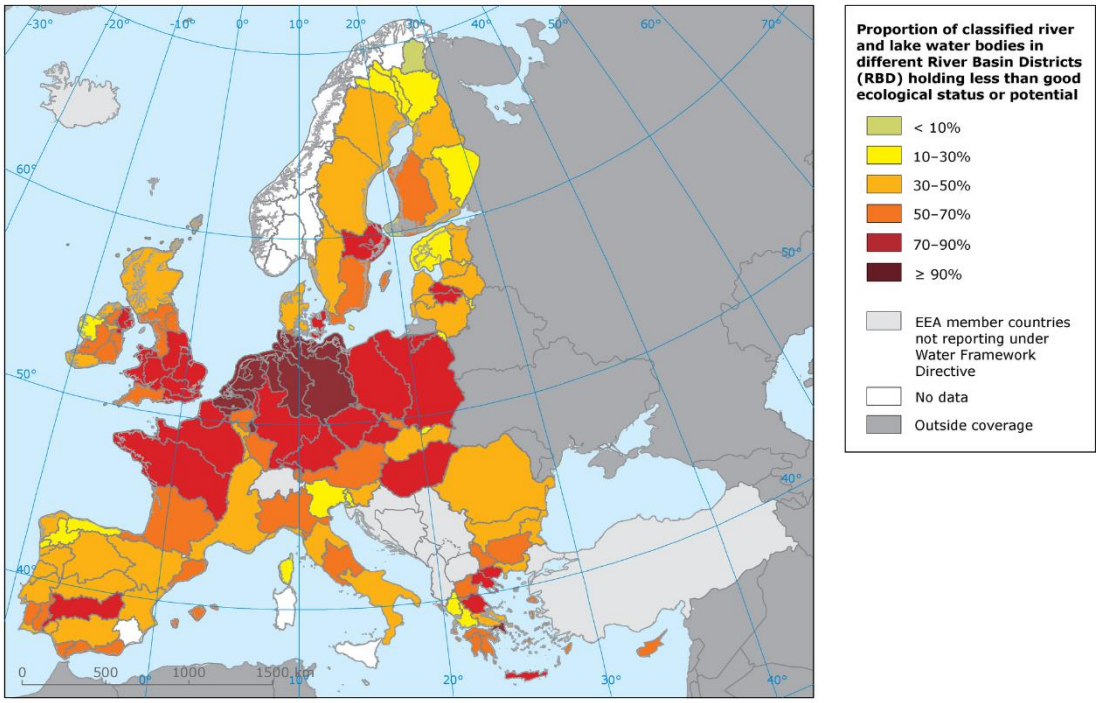


Figure 1.5. Proportion of classified river and lake water bodies in different River Basin Districts (RBD) holding less than good ecological status or potential. The figure shows percentage of the total number of classified water bodies. Copyright holder: European Commission.

Although EU funds will continue to support implementation efforts, the path towards the full compliance of the WFD's plans by 2027 represents a huge challenge. In the last progress report on the WFD from the EU parliament in 2015, further measures were proposed to reach the primary objective of the WFD by its deadline (European Commission, 2015). Among others, these measures recommended to improve and expand monitoring and assessment tools to ensure a statistically robust and comprehensive picture of the status of the aquatic environment, and to continue the consolidation of integrated multidisciplinary water management (European Commission, 2015). The EU concluded its review on the WFD with "…the key area where there is room to improve and to achieve better results is on chemicals" (European Commission, 2019b), pointing towards a deficiency of the regulatory assessment on effect exposure of chemical pollution (Voulvoulis et al., 2017). The substantial environmental impacts of chemical stressors on freshwaters reported by Malaj and others (2014), and the exceedance of

regulatory thresholds of pesticides in 50% of European waters and sediments (Stehle and Schulz, 2015) are undeniable evidence of the shortcomings of the current legislations and their take-up. Also, sub-optimal regulatory methods failing to tackle chemical pollution have been demonstrated, imposing limitations to the ecological status of European surface waters (Posthuma et al., 2020). A main criticism is that while the robustness and representativeness of the "standard-conditions" in regulatory ecotoxicology is sound and continues to be a mainstay today (i.e. screening large numbers of substances and environmental media to identify those that may be hazardous www.epa.gov/ecotox), this approach is too rigid and views ecosystems as static entities - ignoring the complex network of interactions occurring between chemical contaminants, biota and environmental conditions (Chapman, 2002; van der Brink, 2008; van Straalen, 2003).

1.3 From ecotoxicology to "chemical stress ecology": state of the art and knowledge gaps

A major challenge for ecotoxicology is to understand how the widespread presence of pesticides translates into affecting ecosystem integrity from populations, to community, to ecosystem scale (Köhler and Triebskorn, 2013; Stehle and Schulz, 2015). Analysing patterns, processes and relationships at high levels of biological organisation - such as populations, communities, and whole ecosystems in the field - has always been the domain of ecology. The integration of ecological theory is assuming increasing importance in ecotoxicology and is driving this discipline towards a crucial paradigm shift (Chapman, 2002). Chapman (2002), van Straalen (2003), and van der Brink (2008) described this integration process as the transition from the paradigm of "standard-conditions toxicology" towards the more advanced "chemical stress ecology", defining the latter as "the study of the consequences of chemically induced changes in a biological system and the resultant effects on organisms, such as their abundance, distribution and interactions with other organisms and the environment" (van der Brink, 2008; van Straalen, 2003). To this end, ecotoxicology is progressing and broadening

concepts to develop further far-reaching, holistic measures to fill these gaps (Fischer et al., 2013; van der Brink, 2008; van Straalen, 2003). For example, the need to investigate the mechanisms underlying the influence of chemical pollution from species to communities led to the development of community ecotoxicology (Clements and Rohr, 2009). One main advantage of this branch of ecotoxicology is that since communities are composed by many species showing different sensitivity to stressors, they can fairly reflect the variable and complex response at ecosystems level (Clements and Rohr, 2009). This approach has been initially addressed by focusing on microbial communities as they provided a manageable ecological model enabling observations for thousands of individuals distributed in tens of interacting species in a small space (Clements and Rohr, 2009). However, the theoretical framework behind this concept is in principle applicable to all communities of organisms. Following exposure to a variety of chemical stressors, community ecotoxicology has been used to show evidence of demographic alterations in populations, structural changes in communities, and functional responses of ecosystems (Newmann, 2001). The capacity of a community to host different species with different sensitivity towards stressors stablished the basis of a fundamental concept in community ecotoxicology; the pollution-induced community tolerance (PICT) (Blanck and Wangberg, 1988). For instance, the PICT (Box 1) introduced the theory that exposure to chemical stress may act as a selection process, whereby toxic chemicals will eliminate or hinder the success of the most sensitive species to the advantage of the most tolerant ones, or may induce shifts in species composition due to altered competitive interactions under contaminant exposure (Blanck, 2002; Blanck and Wangberg, 1988). To this end, the acquisition of tolerance to contaminants is quantified by measuring responses of physiological endpoints in acute short-term bioassays by comparing the responses of the reference community and the chronically pre-exposed one (Blanck 2002, Box 1, Figure 1.6). The PICT theoretical framework successfully established a causal relationship between

exposure and community effects, integrating both structural and functional complexity within ecosystems, which also had the potential to link assessments of the ecological and chemical status of ecosystems in environmental risk assessment (Tlili et al., 2016). To this end, a critical review of PICT in biotic communities was presented by Blanck (2002).

The PICT approach not only represents a useful tool in ecotoxicology, but is also instrumental for the integration of ecological theory in ecotoxicology as it contributed to understanding of the underlying adaptation mechanisms within community structural changes due to recurrent chemical stress exposure through replacement of more sensitive species with more tolerant ones (Blanck, 2002; Tlili et al., 2016). This framework successfully demonstrated that communities with a history of exposure to the same stressor were strongly buffered against negative impacts of exposure to chemical pollutants because of previous replacement of sensitive species with more tolerant ones (Feckler et al., 2018).

BOX 1

Pollution-Induced Community Tolerance: The PICT concept (Blanck, 2002) determines whether pollutants have exerted a selection pressure on natural communities by eliminating the most sensitive species and thereby increasing their tolerance. In the figure below (modified from Tlili et al., 2016) the two phases of the PICT approach: a) selection and b) detection. During the selection phase, inter- and intraspecific selections occur under exposure to pollutants, leading to the restructuring of the community by disappearance of sensitive species and dominance of tolerant ones when exposure reaches critical levels for a sufficient period of time. In the detection phase, community tolerance is quantified in the laboratory. Responses of functional endpoints in short-term bioassays with increasing concentrations of the pollutant of interest are measured, allowing the establishment of concentration–response curves for the reference and the pollutant-selected communities.

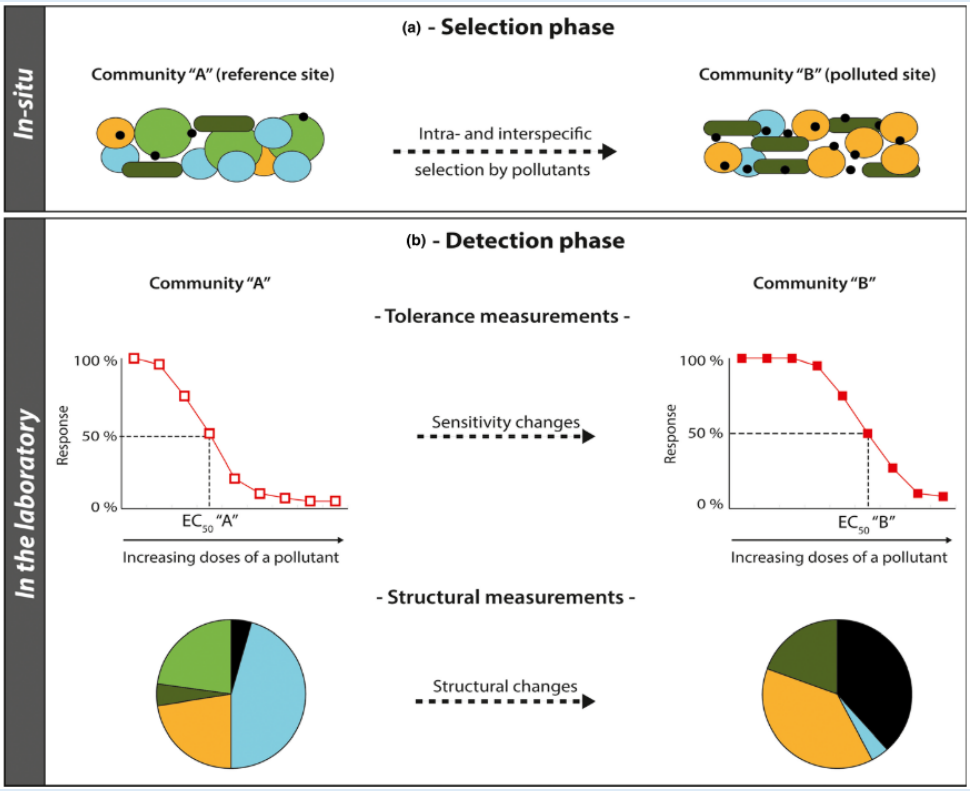


Figure 1.6. The two phases of the PICT approach.

At the same time, the PICT approach alone cannot explain other adaptation processes such as physiological (Bradshaw & K 1989) and evolutionary adaptation (Bell, 2017). A clear definition for these processes is necessary here and is recalled in BOX 2. Physiological adaptation (also known as acclimation) involves a rapid physiological adjustment resulting in improved tolerance towards the stress, which occurs within the generation of the organism, and it is generally reversible after removal of the stressor (Bradshaw & K 1989). Evolutionary adaptation generally occurs over extended time scales and involves the selection of strains of organisms carrying genetic variants or modifications that are more tolerant to the contaminant (Bell 2017). More recently, the classic concept of evolutionary adaptation was updated by evidence reporting that this process can also be rapid, occurring a few generations after the initiation of the stress (Hairston et al. 2005; Ellner et al. 2011).

BOX 2

Adaptation: the capacity of organisms/biological organisation to acquire tolerance to a stressor through physiological (Bradshaw & K 1989), ecological (Blanck and Wangberg, 1988) or evolutionary processes (Bell, 2017). At lower ranks of biological organisations (e.g. populations, species), evidence of adaptation to chemical stressors stems from studies reporting physiological (Bradshaw and K, 1989) and evolutionary processes (Gomulkiewicz and Holt, 1995). Ecological adaptation instead emphasizes the replacement of stress sensitive species with tolerant ones (PICT - Blanck & Wangberg 1988).

One potential limitation of the PICT approach is that it typically focuses on communities that are incidentally available at a given time point, giving a snapshot of the phenotypic changes induced by the tolerance acquisition to the contaminant (Blanck, 2002). Hence, such approaches cannot account for the presence of latent adaptations that might not be fully expressed or detectable in stress-free periods, but might be still present in an inactive form, such as a dormant stage (Orsini et al., 2013), and be recalled when the stressful conditions

reappear. Other studies are crucial to understand the ecological consequences for ecosystem responses to toxic stress induced by the full spectrum of adaptation processes. Very good examples are the studies from Feckler et al. (2018) and Jacquet & Altermatt (2020). Both studies report empirical evidence that previous histories of exposure to contaminants could trigger different adaptation processes thereby screening the communities from the negative effect of contamination. Another example dates back to 2007, when Medina et al. produced an extensive review article, where they reported evidence that exposure to chemical pollution drove rapid, evolutionary adaptation in several species, enabling them to persist in polluted environments (Medina et al., 2007). At the same time, the authors acknowledged that such a process might also represent an ecological cost with possible long-term disrupting effects, which required further investigation (Medina et al., 2007). This hypothesis arises from a well-rooted concept in ecology, which is known as trade-off (BOX 3). According to this theory, more tolerant species might not be the more productive (Bazzaz et al., 1987; Lerdau and Gershenzon, 1997).

BOX 3

Trade-off: this process is a fundamental postulate of the resource-based allocation theory (Bazzaz et al., 1987; Lerdau and Gershenzon, 1997), which acknowledges that any use of energy for some purposes different from reproduction and growth will decrease the intrinsic growth rate of the species.

Due to the potential ecological consequences caused by the occurrence of trade-offs, Medina et al. (2007) suggested to test this process further experimentally in ecotoxicological approaches, and to include them in ERA. However, apart from some other studies which experimentally tested the occurrence of trade-offs (Horne et al., 2014; Jin and Agustí, 2018a), the degree to which adaptation processes lead to trade-offs is not well documented at the

moment in ecotoxicology, despite this being a crucial aspect for the inclusion of rapid adaptation to pollution in future environmental risk assessment (Arnaud et al., 2005).

To further increase the degree of complexity, the impact of chemical contaminants on biota may not depend only on their concentrations (Fischer et al., 2013). Key environmental factors such as temperature and underlying water chemistry (e.g. water pH, DOM) may dramatically influence the chemicals form, the way organisms are exposed to chemical pollutants in water, and the way they respond to it. For instance, some environmental factors may act antagonistically against the toxic effect of the chemical stressors (Holmstrup et al., 2010; Laskowski et al., 2010). Environmental factors can also exert a physiological background stress, acting synergistically with contaminants in a multiple stressor effect (Crain et al., 2008). Hence, correlating the combined effect of environmental factors and chemical contaminants and linking them across different biological levels has become a crucial challenge for modern ecotoxicology. For example, Schäfer and others (2012) studied how the effects arising from both anthropogenic and environmental stressors can propagate from freshwater communities to ecosystems. Similarly, Beyer and others (2014) overviewed the combined effects of environmental factors and contaminants in aquatic ecotoxicology, suggesting that the inclusion of the interaction of environmental factors with chemical contaminants would greatly benefit regulatory risk assessment. Gessner and Tlili (2016) discussed fundamental steps for the integration of freshwater ecological theory with ecotoxicology. Central themes of their paper were the relevance of indirect effects induced by chemical contaminants on interspecies interactions and food webs, along with the influence of multiple stressors, in particular interactions between contaminants and environmental factors (Gessner and Tlili, 2016). Thanks to such studies, the dynamics of biotic and abiotic environmental factors influencing the effects of anthropogenic chemical pollutants in natural systems are relatively well-known

at different levels of biological complexity (from cells to organisms, populations, communities and ecosystems).

Fischer and others (2013) went one step further towards the integration of ecological theory in ecotoxicology, and took up the challenge of integrating environmental factors, biological complexity and adaptation processes to address the toxicity of chemical pollutants in dynamic natural systems. The authors proposed the integration of three key concepts from ecotoxicology and ecology, namely the explicit consideration of adaptation processes, an energy-based approach accounting for the costs (trade-offs) and benefits of such processes, and the use of traits as descriptors. Definition of traits and their importance in ecology are outlined below in Box 4.

BOX 4

Traits are measurable individual attributes present in organisms or species encompassing genetic, morphological or life-history features such as size, metabolic activity or development stage. The traits of organisms are the target of natural selection, and their analysis can reveal important information on selection process induced by chemical pollutants (Fischer et al., 2013). Traits can therefore be used to categorize the biological characteristics of organisms allowing to describe them on a continuous scale. In addition, since many traits respond independently to specific factors, they can be used to investigate and scale the effects of chemical pollutants, abiotic and biotic factors on individual fitness, population dynamics and community characters (Violle et al., 2007). For this purpose, traits have already been extensively used in ecology to assess several processes such as i) mechanistically understand community organisation and the response of specific organisms under given environmental conditions (McGill et al., 2006); ii) individually assess the influence of overlapping factors responsible for changes in community structure by identifying specific traits responding to selection (Suding et al., 2008); and iii) relate the phenotypic characteristics from individual or population-level to higher level functions (Reiss et al., 2009).

In summary, traits represent the common currency in ecology to study responses of organisms to the influence of environmental drivers (Reiss et al., 2009; Violle et al., 2007), and have the potential to become crucially important to investigate the effects of chemical pollutants in ecotoxicology. The framework proposed by Fischer et al. (2013) is an example of trait-based approach applied to the field of ecotoxicological risk assessment, to improve the description of ecological responses to pollutants and extrapolate between different species or populations using traits related to vulnerability to stressors. However, despite the efforts made in recent years by many other researchers, a framework that correlates the combined effects of environmental factors with chemical contaminants, incorporates adaptation processes at the higher levels of biological organisations, and uses trait-based approach is still not sufficiently developed to be translated into regulatory risk assessment procedures. Hence, a main objective of this thesis is to contribute to the development of a framework that is based on these concepts, to better elucidate the impact of chemical pollution in freshwater ecosystems and help increase the integration of ecotoxicology into chemical stress ecology. This thesis builds on the ecological conceptual framework of ecosystems as complex adaptive systems (Norberg, 2004), which is described in the next section.

1.4 The complex adaptive system (CAS) framework: integration in ecotoxicology

In complex natural systems, responses to environmental stressors can be influenced by the different sensitivity of species and interspecific interactions (Clements and Rohr, 2009), which can lead to unexpected effects following chemical exposure, resulting in various indirect and cascading effects, with positive or negative consequences on both individual species and communities (Hooper et al., 2005; Forbes and Calow, 2012). The buffering capacity of ecological processes, together with other aspects of ecosystem dynamics and stability can therefore shield the effects of stressors on ecosystems (Sheenan, 1984), often resulting in blurred direct effects compared to the ones detected on species or individuals (Vinebrooke et

al., 2004). This process is particularly relevant if multiple species benefit for the same resources, that is, share similar, if not identical, roles in ecosystem functions (e.g. primary production, nitrogen fixing, algae scraping, scavenging, etc.), therefore revealing some functional equivalence, or redundancy in ecological functions (Hubbell, 2005; Salmaso et al., 2015). In ecological theory, this pool of different species can also be defined as a functional group (Solbrig, 1993). Tackling this concept in risk assessment is challenging. A fundamental problem is how to extrapolate data produced for a small number of "standard" species to predict the impacts of contaminants on a larger number of species differing in their sensitivity. Generally, regulatory ecotoxicology applies a pragmatic solution to address the uncertainties related to the lack of knowledge on the basis and range of species sensitivity. For instance, threshold contaminant concentrations are defined on the basis of "safety factors", placed on data available from standard ecotoxicity tests established on single species, or on species sensitivity distributions (Posthuma et al., 2020). However, these approaches show consistent technical flaws. For example, the limitations of the species sensitivity distribution approach are thoroughly discussed by Spurgeon and others (2020). In traditional regulatory approaches, limitations are mostly based on the fact that the derivation of any EQS value for regulatory application (i.e. NOEC, LCxx, etc.) could be driven by the most sensitive species. As a consequence, this may underestimate the ecosystem's ability to withstand the stressor, with potential impacts on restoration cost-effectiveness. Alternatively, if the choice of the representative species is particularly unlucky, (e.g. by selecting only the most resistant species), regulatory assessment can dangerously under-protect the most vulnerable species, and therefore overestimate the ecosystem's ability to withstand the stress (Lau et al., 2014). Such failures are usually driven by chemicals impacting on specific physiological traits that are not usually measured in standard regulatory ecotoxicity tests, such as behaviour, physiology, genotoxicity and whose unforeseen effects are reported causing concerning impacts on

different organisms, such as marine molluscs (Matthiessen and Law, 2002), bees (Woodcock et al., 2017), birds of prey (Newton and Wyllie, 1992), and vultures (Oaks et al., 2004).

Hence, it is clear that in order to capture the subtle nuances of the effects of chemical contaminants, it is necessary to overcome the flaws induced by this species-centred view. One possible way could be to start integrating more ecological theory into ecotoxicological approaches. Examples of this approach have been illustrated by the work of Fischer and others (2013), who suggested that assessing the impact of chemical stressors on the community composition, by selecting sets of species from the same functional groups, allowed for a better understanding of the complex biota-contaminants interactions at community and ecosystem levels. Other authors framed their ecotoxicological approaches around fundamental ecological concepts such as resilience (Bundschuh et al., 2017), resistance and recovery (Box 5). The ecological concept of resilience is a property that emerges from considering high-level biological aggregations and is therefore difficult to observe and demonstrate experimentally (Bundschuh et al., 2017). For this reason, despite the efforts of researchers, the approach of integrating state of the art ecology has not been fully assimilated into regulatory ecotoxicology yet. To this end, it is still possible to address more accessible and easily measurable characteristics of the ecosystem that are proxies of the ecosystem resilience response. One possible way would be to simultaneously measure the change in biological diversity and the rate of a function. While this approach is applicable to any group of organisms (producers, consumers, prey-predator, etc.), an ecological approach to risk assessment could focus on addressing the changes induced by chemical stressors on the relative abundances of species, as well as the functioning of the community (e.g. primary production rate). An example might be ‘*how does the species diversity in phytoplankton change in relation to the rate of primary production when the community is exposed to chemical stressors*?”.

The main aim of this thesis is actually to build on ecological concepts, such as the CAS to integrate ecological theory in ecotoxicological approach, and investigate on the complex responses of biota arising from the exposure to chemical contaminants.

BOX 5

Resilience: in 1973, C. S. Holling introduced the word resilience into the ecological literature as a way of helping to understand the non-linear dynamics observed in ecosystems. Ecological resilience was defined as the amount of disturbance that an ecosystem could withstand without changing self-organized processes and structures (defined as alternative stable states) (Holling, 1973). In ecotoxicology, the prevailing view is that resilience is the ability of organisms to resist or rebound from chemical stress (Clements and Rohr, 2009).

Resistance & Recovery: resistance has been used within ecological stability research to characterize the property of communities or populations to withstand stress, by remaining unchanged when subject to disturbance. The inverse of resistance is sensitivity (Grimm and Wissel, 1997). Hillebrand and others (2018) broadened the use of this concept to consider resistance to be a component of ecological resilience, together with recovery, which in turn is the ability of a population or community to regain normal functions and structures after being impacted. Both these concepts can be measured in terms of functional (e.g. biomass production and resource use) and structural (e.g. community composition) characteristics (Hillebrand et al., 2018).

The concept of complex adaptive system (CAS, Norberg *et al.* 2001), provides a theoretical framework to elucidate how different responses of organisms in a community to a stressor or an environmental gradient result in changing the community composition. For instance, this theory acknowledges that the assemblage of species within a community changes towards a dominance of those whose functional characteristics (or more appropriately “traits”) are best suited to cope with the selective forces of the environment (Norberg et al., 2001). Traits (Box 4) can be individual properties, such as optimal growth temperature, sensitivity curve to a

stressor, etc., and ultimately depend on the physiological and/or morphological characteristics of the individual (e.g. shape and dimensions of the individuals, amount and typology of pigments, composition of cell membranes, membrane receptor characteristics, etc.). Hence, traits influence the behaviour and the response of individuals in their environment, and determine the demographic rates of populations and the development of the community structure in response to the selective force of the environment (McGill et al., 2006). For this purpose, trait diversity is receiving increasing attention, since several studies indicate that it better relates ecological structure with ecosystem functioning compared to taxonomic diversity (Hillebrand and Matthiessen, 2009). Ultimately, trait diversity provides functionality and stability of ecosystem processes, overall determining the resilience and response capacity (Enquist et al., 2015).

In highly dynamic systems, such as microbial communities for example, the CAS concept can be exploited to analyse resilience of the ecosystem. Within this context, the trait variance, or the measure of the width of the distribution of traits in the community, is proportional to the rate at which species within functional groups can replace each other in response to environmental change (Norberg, 2004). This capacity (also known as response capacity), can be crucial for the community's (or ecosystem's) ability to maintain certain processes (for example a rate of a function such as primary productivity in phytoplankton communities), under a broad gradient of a stressor. Addressing response capacity could therefore be extremely helpful for an ecotoxicological approach that aims to investigate the effects of chemical contaminants at ecosystem level. For example, integrating response capacity could help improve the understanding of how far a chemical stress can affect a given community function (i.e. phytoplankton productivity) before the function rate drops. Answering such questions is certainly challenging, but ecological theory can help by creating expectations and generating the driving hypothesis.

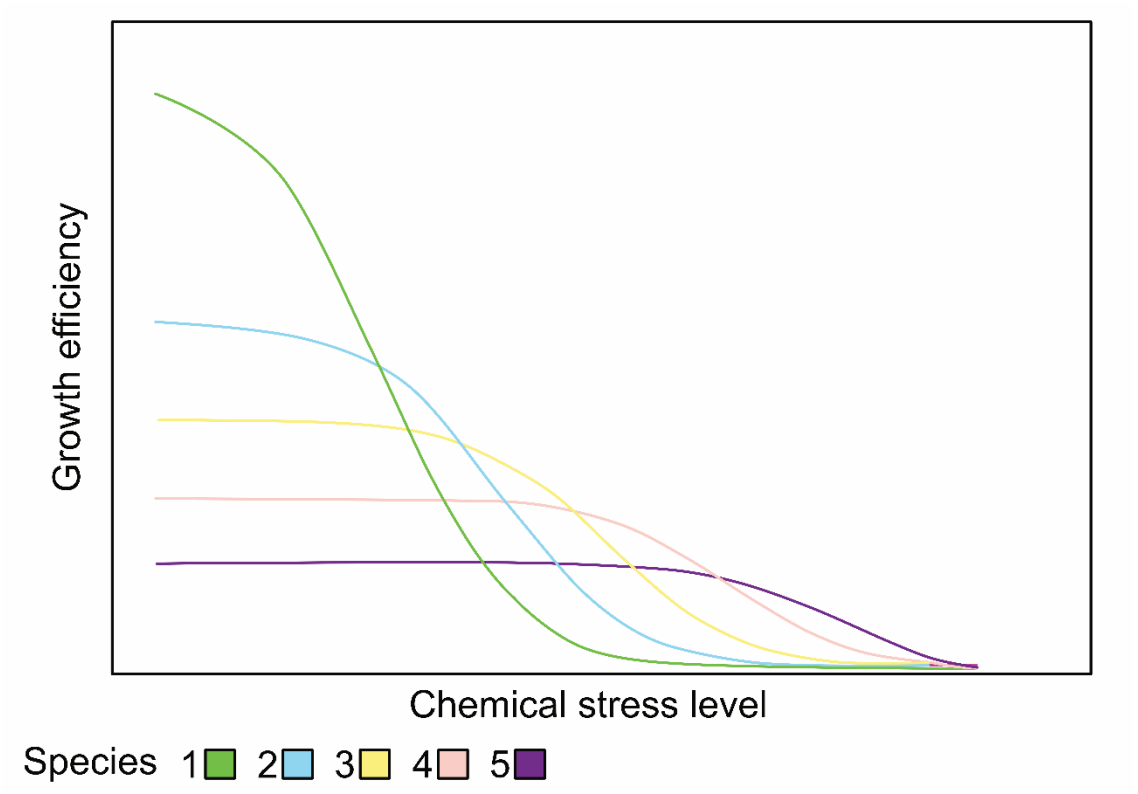


Figure 1.7. Expectations of a community response in a chemical stress context.

Norberg (2001) described the response capacity theory and provided an example for community responses to a temperature gradient. The same concept can be adopted in a chemical stress context. To this end, the expectations of a community response to chemical stress are illustrated in Figure 1.7. The five different colours represent different species in a functional group, for example phytoplankton species in the functional group of primary producers. The shape of each curve describes the different species sensitivity under an increasing gradient of a chemical stressor. The growth efficiency is high when there is no stress, whilst their growth is impaired when the chemical stress increases. The most sensitive species (represented by the green colour, Species 1, Figure 1.7) should be the most abundant in the community in absence of the stressor and should dominate in executing their function in this environment (e.g. community production). In contrast, the other species show higher growth efficiency at higher stress levels. For instance, the species indicated by the purple colour (Species 5, Figure 1.7) is the most tolerant one, which would be dominating under higher stress

conditions. At the same time, the conceptual scheme shows that no species is expected to be tolerant to a broad range of environmental levels of a toxicant and at the same time the main performer in the absence of the stress (Norberg, 2004). The existence of these trade-offs can therefore be seen as the results of investments a species undertook to fit the main environmental conditions it was exposed to during its history. Allocating resources to maximize fitness in a given range of conditions, trade-off with the possibility of allocating resources for fitness in other environmental conditions (Norberg, 2004; Norberg et al., 2001). This process is a fundamental postulate of the resource-based allocation theory (Bazzaz et al., 1987; Lerdau and Gershenzon, 1997), which acknowledges that any use of energy for some purposes different from reproduction and growth will decrease the intrinsic growth rate of the species.

Hence, an ecological approach to chemical risk would certainly benefit by considering the diversity of responses of species to chemical stressors underpinning important ecological functions. For example, Pomati and others (2017) successfully implemented this framework in their study, where they demonstrated the effect of waterborne PPCPs in reducing the phenotypic diversity of a phytoplankton community, thereby altering its response capacity, and potentially affecting aquatic ecosystem processes. Nevertheless, the evolution of this framework in ecotoxicology is still in its early stages and there is certainly space for further development. The response capacity theory can certainly be useful to scale up from physiological responses to large-scale integrated ecosystem responses to contaminants, and to examine complex processes induced by the presence of chemical stressors in a heterogeneous environment. For example, the different elements of an ecosystem respond to contaminants' exposure through physiological (Bradshaw & K 1989), ecological (Blanck and Wangberg, 1988) or evolutionary (Bell, 2017) processes. In addition, environmental conditions may affect the interactions between chemicals and biota (Holmstrup et al., 2010; Laskowski et al., 2010), which can theoretically influence both the response and emergence of adaptations to

contaminants. Hence, these processes introduce additional levels of complexity that can theoretically influence the response capacity of a community, and need to be carefully investigated to facilitate the integration of the CAS framework in ecotoxicology. The main objective of this thesis is to study these processes, and their implications, under the CAS framework, with the ultimate aim of pushing ecotoxicology one step further towards its transformation into "chemical stress ecology". All the processes studied in this thesis are briefly introduced in the next sections, before being discussed in the relative chapters.

1.4.1 Rapid adaptation

The concept of rapid adaptation is tested and discussed in Chapter II: "Water browning controls adaptation and associated trade-offs in phytoplankton stressed by chemical pollution".

At lower ranks of biological organisations (e.g. populations, species), evidence of adaptation to chemical stressors stems from studies reporting physiological (Bradshaw and K, 1989) and evolutionary processes (Gomulkiewicz and Holt, 1995). For instance, the first process (also known as acclimation) involves a rapid physiological adjustment resulting in improved tolerance towards the stress, which occurs within the generation of the organism, and it is generally reversible after removal of the stressor (Bradshaw and K, 1989). The second process generally occurs over extended time scales and involves the selection of strains of organisms carrying genetic variants or modifications that are more tolerant to the contaminant (Bell, 2017). More recently, the classic concept of evolutionary adaptation was updated by evidence reporting that this process can also be rapid, occurring a few generations after the initiation of the stress (Ellner et al., 2011; Hairston et al., 2005). Bell et al. (2017) reported a very detailed review on the direct and indirect effect of medical therapy, agricultural practice and industrial activities on rapid adaptation. For instance, Costelloe et al. (2010) and Bell et al. (2014) produced two extensive meta-analyses on the evolution of antibiotic resistance in pathogenic

bacteria, concluding that resistance was stronger among bacteria isolated from patients who had recently (within 1 month) received antibiotic therapy than from those who had not done so recently (within the previous year). Busi & Powles (2009) observed that sub-lethal exposure to glyphosate of susceptible strains of *Lolium* (a grass) induced tolerance after three or four cycles of selection and crossing. Manalil et al. (2011) also observed the rapid adaptation of *Lolium* populations to dichlofos, a widely used postemergence herbicide. Other authors reported that the large populations and prolific spore production of pathogenic fungi led to the rapid development of resistant types after strong selection was imposed by highly toxic chemicals (Hocart et al., 1990; Hollomon et al., 1997; Reijo et al., 1994). Experimental evolution of pesticide resistance has been reported in model organisms such as *Caenorhabditis* (Lopes et al., 2008) and *Daphnia* (Jansen et al., 2011). The concept of rapid adaptation is crucially important for ecological theory and is consequently adopted in ecotoxicology to describe rapid evolutionary adaptation induced by exposure to anthropogenic contaminants and defined "Micro-evolution due to pollution" by Medina and others (2007).

1.4.2 Long-term consequences of adaptation - Ecological Memory

The theory of ecological memory is experimentally tested in Chapter III: "Ecological Memory of historical contamination influences the response of phytoplankton communities".

Over extended time scales, adaptations involving ecological and evolutionary processes prevail. The result of sorting of species (ecological adaptation – PICT, Blanck 2002) and/or mutated strains of organisms (evolutionary adaptation, Bell 2017) co-occurring during long-term exposure to stressors, influences community structure and ultimately the ability of ecosystems to withstand or recover from recurring stressors, thus preventing ecosystems from collapsing (Johnstone et al., 2016; Padisák, 1992; Scheffer et al., 2001). This concept is acknowledged by the Ecological Memory theory (EM, Padisák 1992), which recognizes that

past experiences influence present day ecosystem responses, thereby enabling communities to cope better with recurrent stress (Johnstone et al., 2016; Turner, 2010). EM is central to understand how ecosystems respond to disturbance. The suite of species life-history traits that represent the adaptive response to a disturbance acquired over long-time scales are defined as information legacies of the EM. For example, species with tolerance traits that are well aligned with a given disturbance regime may have an immediate and powerful recruitment advantage after a typical disturbance even (Johnstone et al., 2016; Turner, 2010). Disturbances with characteristics that fall within the range of past variation will tend to perpetuate the same set of species traits that performed well in the past, creating a reinforcing eco-evolutionary feed-back that supports ecological resilience (Johnstone et al., 2016; Turner, 2010). Hence, if the disturbance characteristics allow adaptations to be partly or fully maintained during periods of non-stress, EM is established, which allow the community to cope efficiently when the recurring stressor reappears (Scheffer and Carpenter, 2003). EM can contribute to enhance ecosystem stability by promoting resistance and recovery (Donohue et al., 2016; Hillebrand et al., 2018), two fundamental aspects of ecosystem resilience. In contrast, a change in disturbance regimes and/or environmental conditions can induce a loss in information legacies, which may generate a resilience debt that manifests itself only after the system is disturbed. Hence, forecasting the future response of freshwater ecosystems to disturbances is increasingly important, especially considering the influence of global environmental change (Niinemets et al., 2017; Woodward et al., 2010). To some extent, the EM and the PICT approaches could be considered as similar, as they both observe how previous exposure to stressors influence the response of organisms. However, the two concepts are different and have their own advantages and disadvantages. The PICT approach typically focuses on communities that are incidentally available at a given time point, giving a snapshot of the phenotypic changes induced by tolerance acquisition to the contaminant (Blanck, 2002). This approach is revealed to be

extremely useful in the context of ecotoxicological approaches, in particular in understanding how communities can cope with the recurrent pressure of contaminants by restructuring and selecting tolerant species. Instead, EM could be particularly useful in including the effects of latent adaptations, which might not be fully expressed or detectable in stress-free periods, but might be still present in an inactive form, such as a dormant stage (Orsini et al., 2013), and they can be recalled when the stressful conditions reappear. Hughes and others (2019) reported evidence of how EM of previous heat-waves in coral populations protected the same populations against recurrent events of the same kind. In particular, the authors showed that the mortality of coral reefs decreased after the second heat episode (in 2017) compared to the first one (in 2016), where the first event increased the proportion of resistant species (Hughes et al., 2019). Due to these properties, the theory of EM is considered as extremely important to observe the resilience of communities induced by adaptation processes (Johnstone et al., 2016). The application of EM in ecotoxicological approaches could be instrumental to understand the response of communities to long-term exposure to chemical contaminants. Nonetheless, it is not entirely understood to what extent latent adaptations could be preserved, which could hinder the capacity of the community to recall adaptation to the stressors. For example, ecosystems environmental conditions can be extremely plastic (Johnstone et al., 2016). Changes in environmental conditions could not allow the preservation of latent adaptation present in dormient stages, which could represent a limitation for the influence of EM, and deserve to be investigated further.

At the same time, evidence of EM is relatively rare, and stems mostly from theoretical studies or observational studies, while experimental approaches are rare. For example, Hughes and others (2019) described beneficial effects of EM in coral reefs exposed to two successive heat wave events causing bleaching. One potential limitation of these studies is that they focus on communities available at that time, where the causal relationship between previous exposure

and response might be blurred by other drivers (Cochran and Chambers, 1965). A useful experimental model to overcome this issue is represented by organisms that have the ability to produce long lasting resting stages that can act like " seed banks" containing previous species assemblages spanning over extended period of time (Orsini et al., 2013). Phytoplankton germination experiments offer a good toolkit to study EM (Ellegaard et al., 2013; Ellegaard and Ribeiro, 2018).

1.4.3 The cost of adaptation

The occurrence of trade-offs is investigated in all Chapters of Part I: Ecological integration in Ecotoxicology.

Trade-offs are results of adaptation, and can theoretically arise from physiological, ecological or evolutionary processes. At population level, intraspecific trade-offs are relatively easy to understand (Figure 1.7). For example, investing energy in acquiring tolerance towards a stressor may indeed favour species under stressful conditions, but it could represent an ecological disadvantage when the selective force changes, such as lower growth in the absence of the stressor (Bazzaz et al., 1987; Lerdau and Gershenzon, 1997). As an example, Ghalambor et al. (2004) reported the occurrence of a trade-off between reproduction and fast-swimming performance to avoid predation in Trinidadian Guppies. Vila-Aiub et al. (2009) demonstrated the ecological cost arising from an enhanced herbicide tolerance in *Lolium rigidum*, indicating a trade-off with its capacity to exploit resources in the absence of the stressor. Agra et al (2011) observed trade-offs originating from adaptation to historically contaminated acid mine drainage (Copper) in clones of *Daphnia longispina*. Resistant clones showed lower reproduction and somatic growth rates in unpolluted sites relative to the sensitive ones. However, in general, the degree to which adaptation processes lead to trade-offs is not well documented at the moment, despite this being a crucial aspect for the inclusion of rapid

adaptation to pollution in future environmental risk assessment (Arnaud et al., 2005). At community level, interspecific trade-offs are indeed more complex to predict, as they include intraspecific processes as well as interactions among species. For instance, a trade-off in one species could change the way other species exploit their ecological niches and participate in overall ecosystem functioning, potentially escalating effects on higher biological associations. Norberg (2004) provided a mathematical interpretation of interspecific functional trade-offs, concluding that such functions may provide additional information about the processes of species sorting occurring due to environmental change, and therefore on the dynamics of fundamental community attributes such as trait distribution and total productivity. At the same time, trait distribution in communities can provide information on past environmental regimes and also give an indication of the ability to cope with future response to environmental changes (Enquist et al., 2015). Hence, traits not only shape the community structure in response to environmental changes, but through the intrinsic trade-offs, they also alter the way it will respond to future change, and ultimately define the system resilience (Edwards et al., 2013). Some recent studies showed the impact of diffuse micro-pollutants on the trait diversity of microbial communities, indicating their ability to alter trait diversity, potentially affecting aquatic ecosystem processes (Baho et al., 2019; Baho et al., 2019; Pomati et al., 2017). In the context of this thesis, the term “diffuse” refers to a non-point source contamination, in contrast with point source, which refers specifically to a precise location of contamination emission. However, few studies have focused on functional trade-offs directly. Investigating trade-offs between functions is notoriously challenging, due to their intrinsic nature. However it is vital to make predictions on the way populations and communities will respond to the selective pressure of many different chemicals, in highly heterogeneous environmental conditions (Woodward et al., 2010).

1.4.4 Influence of environmental conditions on chemical pollutants

The binding of DOM with contaminants is investigated in both Chapters V and VI of Part II of this thesis: Environmental Chemistry.

Understanding and predicting community response(s) to chemical stressor(s) is further complicated by the variability of the environment. For instance, the influence of contaminants on aquatic biota may not depend only on their concentrations and inherent toxicological properties, but also on the surrounding environmental conditions (Fischer et al., 2013). Key environmental factors such as temperature and underlying water chemistry (e.g. water pH, Dissolved Organic Matter - DOM) may dramatically influence the chemicals form, the way organisms are exposed to chemical pollutants in water, and the way they respond to it. For instance, some environmental factors may act antagonistically against the toxic effect of the chemical stressors (Holmstrup et al., 2010; Laskowski et al., 2010), or can exert a physiological background stress, acting synergistically with contaminants in a multiple stressor effect (Crain et al., 2008). Temperature, for example, was found to have mainly synergistic interactions with chemical pollutants (Holmstrup et al., 2010; Laskowski et al., 2010). The DOM plays a major role in this context, due to its ability to alter the levels of toxicity and bioavailability of several groups of contaminants in freshwaters (Akkanen et al., 2001; Akkanen and Kukkonen, 2003). Hence, understanding the interactions of key environmental factors (including the role of constituents of natural freshwaters and the water chemistry) with chemical contaminants is therefore crucial for a correct prediction of ecological risk, and is one of the main objectives of this thesis. Given the crucial role of DOM in the biogeochemical processes of freshwater ecosystems (Monteith et al., 2007), this thesis investigates the ability of this environmental factor to interact with chemicals contaminants and eventually influence their toxicity/bioavailability.

1.4.4.1 Dissolved Organic Matter

DOM represents a ubiquitous and compositionally diverse key environmental factor in freshwater ecosystems. Generally analysed as concentration of dissolved organic carbon (DOC mg L^{-1}), DOM consists of soluble organic materials (up to 90% humic acids) derived from the partial decomposition of organic materials, including bacteria, algae and plants (Thurman, 1985). Due to its multifaceted nature, DOM serves as a powerful connection within aquatic ecosystems, associating them with the surrounding landscapes through the flux of materials and energy (Raymond and Spencer, 2015). During the last decades, climate and land-use change and recovery from past acidification have caused water browning (Monteith et al., 2007), leading towards a diffuse increase of DOM and changed pH in many northern ecosystems (Monteith et al., 2007; Williamson et al., 2016). DOM can bind and transport anthropogenic contaminants by forming complexes that are too large or too polar to cross biological membranes, thereby reducing bioavailability and toxic outcomes in contaminants (Böhm et al., 2016; Liu et al., 2016; Lu et al., 2018; Pan et al., 2008; Rowett et al., 2016).

Early evidence that DOM interacted with organic contaminants dates back to the end of 60s, when Wershaw et al. (1969), showed a relationship between NOM, DDT and 2, 4, 5-T. They demonstrated that sodium humate (a potassium salt of humic acid) can solubilize an otherwise insoluble insecticide, DDT, and humic acid strongly sorbs 2, 4, 5-T (2, 4, 5-trichlorophenoxyacetic acid) from a water solution. Other authors reported that DOM can increase aqueous solubility of phthalates esters (Matsuda and Schnitzer, 1971) and alkanes (Boehm and Quinn, 1973). Boehm & Quinn (1976) reported that DOM in sea-water reduces biological uptake of petroleum hydrocarbons. A few years later Hassett and Anderson (1982) highlighted the effects of river and sewage-borne DOM on the adsorption of hydrophobic organic compounds such as cholesterol and 2,2',5,5'-tetrachlorobiphenyl. This behaviour could be explained either with the formation of soluble complexes between hydrophobic compounds

and DOM or the competition between these species for surface adsorption sites. Several other authors demonstrated that particles with high organic matter content have greater affinity for hydrophobic organic compounds than do particles with low organic content (Dong et al., 2014; Schulten, 1999; Wei-Hass et al., 2014). There is some evidence that the chemical - adsorbing capacity of DOM is due to the presence of small organic particles (≈110 nm largest dimension; Österberg *et al.* 1994) called humic substances. This has been reported by Herbert et al. (1993), who used fluorescence-quenching spectroscopy to quantify the interactions between pyrene and ultrafiltration fractions of soil water-soluble organic carbon, humic and fulvic acids. Except from the largest water-soluble organic carbon fraction (100000 Nominal Molecular Weight Cut Off - MWCO), water-soluble organic carbon adsorbed less pyrene than humic and fulvic acids. These humic substances, components of the acid-hydrophobic fraction of DOM, such as humic and fulvic acids, carbohydrates, lipids, proteins, lignin, and a variety of low molecular weight compounds, carried several reactive functional groups that can create ligands with metals ions (such as Cu, Mn, Al...) to form metal complexes (van Dijk, 1971; Zunino et al., 1979), to bind to non-ionic pesticides (Herbert et al., 1993; Lee and Farmer, 1989) or influence the bioavailability of herbicides in waters and soils (Cox et al., 2000; Herbert et al., 1993; Seol and Lee, 2000).

The DOM binding affinity (generally expressed as distribution coefficient K_{DOC} or K_d) can be controlled by several factors such as the water chemistry (e.g. pH), the physicochemical properties of the compounds (e.g. hydrophobicity, presence of functional groups) (Ashauer and Escher, 2010; Behera et al., 2010; Sun et al., 2020a), and of the DOM (e.g. molecular size, aromaticity, presence of functional groups, concentration) (C Gu et al., 2007; Lin et al., 2018b; Tanaka et al., 2005). For instance, higher molecular size constituents (e.g. humic acids) generally show higher affinity for binding with chemical compounds (Bai et al., 2019; Xu et al., 2019), while the opposite can occur for lower molecular size constituents (Ding et al., 2011;

Pokrovsky et al., 2016). This process is usually related to the molecular size influencing the physicochemical properties of DOM, where larger fractions of DOM are usually connected with the more condensed structure, stronger hydrophobicity, abundant aromaticity and therefore higher affinity with organic contaminants (Bai et al., 2019; Ma and Yates, 2018). The binding of DOM may not be simply dependent on its concentration. For instance, increasing concentrations of DOM could generate tighter molecular rearrangements, preventing the chemical compounds to access more hydrophobic areas of the DOM where the binding generally takes place (Akkanen et al., 2001; Akkanen and Kukkonen, 2003). In addition, the binding of DOM with contaminants can be influenced by different molecular size of DOM constituents. The binding process induced by the DOM can be pH dependent (Pan et al., 2009), for two main reasons. The first is that ionizable organic contaminants exist as ionic and/or neutral forms in the aqueous phase (Ashauer and Escher, 2010; Rozman and Doull, 2000a). Furthermore, the neutral species are more hydrophobic (measured as octanol-water partition coefficient log K_{ow}) than the ionized (Karlsson et al., 2017; Nakamura et al., 2008; Valenti et al., 2009), which increases the likelihood of complexation by DOM (Rowett et al., 2016). The second reason is that a change in pH in the water column can cause a re-organization in the macromolecular structure of dissolved humic substances (Myeni et al., 1999b), which can be responsible for the alteration of their binding capacities with hydrophobic organic chemicals (Schlautman and Morgan, 1993).

Increasing levels of DOM may also cause a stressor-like behaviour on biota. For instance, the productivity of freshwater phytoplankton may be altered by increasing concentrations of DOM in various ways (Creed et al., 2018). It can reduce algal growth by: i) hindering light availability (Hagman et al., 2018; Thrane et al., 2014); ii) adding organically bound nutrients in nutrient-limited environments (e.g. P) (Creed et al., 2018); iii) complex or adsorb key elements (e.g. Fe) (Creed et al., 2018); iv) promote the growth of heterotrophic bacteria with higher affinity

for limiting nutrients (e.g. P) (Creed et al., 2018); v) produce harmful free radicals and reactive species of oxygen from photoactivation (Wolf et al., 2017); vi) cause negative effects on photosynthesis (Pflugmacher et al., 2006).

The analytical techniques used to investigate the binding effects of DOM on contaminants have included solubility enhancement (Wei-Hass et al., 2014; Wershaw et al., 1969), fluorescence-quenching (Herbert et al., 1993; Hong et al., 2021; Pan et al., 2007), purging or sparging (Hassett and Milicic, 1985), solid-phase micro-extraction (Poerschmann et al., 1997; Ramos et al., 1998; Ripszam and Haglund, 2015), reverse-phase HPLC separation (Laundrum et al., 1984), size exclusion chromatography (Hassett and Milicic, 1985), liquid-liquid extraction (Hassett and Milicic, 1985; Johnsen, 1987) and equilibrium dialysis (Carter and Suffet, 1982; Seol and Lee, 2000).

1.4.5 Influence of environmental conditions on acquiring tolerance

The influence of environmental conditions on the process of tolerance acquisition to chemical stress is investigated in Chapter II: "Water browning controls adaptation and associated trade-offs in phytoplankton stressed by chemical pollution".

Following the response capacity framework, organisms co-evolve simultaneously, responding to all driving forces of the environment (i.e. chemical pollutants, environmental factors). According to the resource based allocation theory (Bazzaz et al., 1987; Lerdau and Gershenzon, 1997), the development of traits that define sensitivity to chemical stress is also mediated by allocation of energy to other types of adaptations (e.g. to low pH, low temperature, etc.). Any allocation of resources for a given adaptation (yielding a given trait) has implications for all other traits of an organism (Norberg, 2004). Hence, by interacting with the selective pressure of the contaminants, environmental factors can ultimately influence the development of tolerance towards the stressors and subsequent trade-offs. For example, in the case of

antagonistic interactions between chemical stress and environmental factors, mitigation of the toxicity of the chemical stress can result in a species which is less adapted towards the contaminant. Proportional response of tolerance acquisition in relation to stress intensity is reported elsewhere by other authors (Gassmann and Futuyma, 2005; Medina et al., 2007). An ecological approach to ecotoxicology must therefore necessarily take into consideration these confounding factors arising from the environment to elucidate or 'scale' present and future responses of ecosystems to chemical pollution (Fischer et al., 2013).

1.4.6 Trait diversity: trait richness, evenness and divergence

The trait-based approach is applied in Chapter III: "Influence of ecological memory on phytoplankton early assemblages: a trait-based approach".

Community dynamics in response to the effect of chemical pollutants are traditionally described through the taxonomic approach. However, a species-centred view can hinder the importance of inter- and intra-specific phenotypic variance between populations and its significance in ecological and evolutionary response of communities to stressor(s) (Enquist et al., 2015; McGill et al., 2006). Trait-based approaches represent a promising tool to overcome these hindrances, as they have the potential to incorporate variance within and between populations, to capture time-dependent responses, and to link their effects on community-level and ecosystem processes (Hillebrand and Matthiessen, 2009; Norberg, 2004; Norberg et al., 2001). Traits have already been recognised as the common currency to explain ecosystem functioning, even at the global scale (Reichstein et al., 2014), and individual-resolved models can also explain ecosystem processes (Lasky et al., 2014). Hence, the concept of functional diversity, or trait diversity (TD), is receiving increasing attention as it can better reflect the ecological structure with ecosystem functioning compared to the taxonomic diversity (Enquist et al., 2015; Hillebrand and Matthiessen, 2009; Reiss et al., 2009).

Multiple traits measured community-wide at the individual level are the most promising and straightforward way to combine inter- and intra-specific variation in one single approach (Fontana et al., 2014). At the same time, monitoring large numbers of traits with potential functional properties in ecological communities (i.e. phytoplankton) presents a number of challenges. For example, given the fast generation time of phytoplankton and the relatively small spatial scale at which the event occurs, this task requires high-frequency sampling of fluctuating small-scale environments (Pomati et al., 2011). The recent developments in the application of scanning-flow cytometry offers promising tools for the automated counting, characterisation and identification of phytoplankton traits (Baho et al., 2019). In addition, while the general concept of TD is generally accepted, agreement on a single TD measure is still far from being reached, such that existing measures range from metrics involving the calculation of the volume of the convex hull containing all taxa of a community, to the number of functional groups (Fontana et al., 2016; Mason et al., 2013; Petchey and Gaston, 2006). In this thesis, the set of measures validated by Fontana and others (2016) were used to study trait richness and evenness. For instance, TOP indices (trait onion peeling) was used for trait richness, TED (trait even distribution) for trait evenness. In the study by Fontana et al. (2016), both TOP and TED were selected as they showed better performances compared to other trait diversity indices such as Functional Richness (FRic) and Functional Evenness (FEve) (Villéger et al., 2008), whose bigger limitations appeared to be computationally heavy when in the presence of a high number of samples (such as in phytoplankton communities), and do not respond according to *a priori* expectations (Fontana et al., 2016). Trait divergence was calculated by using the FDis indices, originally developed by Laliberte and Legendre (2010) and later modified by Fontana and others (2016). For more information on the calculation of the indices, please refer to Appendix Chapter IV (*Trait diversity calculations*).

1.5 Selection of chemicals

The choice of chemical contaminants for this thesis was carried out in order to represent two well-known sources of contamination in freshwaters: namely urban settlements (PPCPs) and agricultural practice (herbicides). The impact of these anthropogenic activities on ecosystems is treated in detail in the previous paragraph "Chemical pollution in freshwaters" of this Chapter. Both PPCPs and herbicides present relatively well-known toxic effects on phytoplankton. PPCPs are not specifically designed to affect phytoplankton, nonetheless they can interfere with their fundamental metabolic pathways related to chlorophyll a and fatty acid synthesis even at low concentrations, hindering the algal growth (Zhang et al., 2012, 2019). For example, some PPCPs such as triclosan, carbamazepine and diclofenac have demonstrated effects in inhibiting lipid synthesis, which can potentially induce destabilizing effects on the cell membrane of algae (Franz et al., 2008; Zhang et al., 2019). This process can transcend to higher organizational levels, causing alterations in phytoplankton community structure (Pomati and Nizzetto, 2013). Hence, the repercussions of the impact of chemical pollution on phytoplankton populations will undoubtedly affect the rest of the components of the trophic web, and therefore their toxic effects need to be carefully investigated (Schäfer et al., 2007). In contrast, herbicides are intentionally used to produce ecological effects by filtering-out undesired species (Schütte et al., 2017). For instance, triazine and phenylurea compounds such as Isoproturon affect phytoplankton growth by inhibiting the electron transport chain of photosystem-II, by competing with plastoquinone for binding to the D1 protein in the thylakoid membrane (Arnaud et al., 1994). At sub-lethal concentrations, hindrance of the photosynthetic process results in growth inhibition and increased mortality rates (Schroer et al., 2004) in more susceptible organisms. Different responses in different species alters the natural assemblage of species in the community (Rohr and Crumrine, 2005), and this can ultimately impair ecosystem functioning (Schäfer et al., 2007). The effects of these two groups of contaminants on

phytoplankton are relatively well-known, however information on the influence of their long-term exposure on the response of biota are still scarce, and need to be investigated further.

A mixture of twelve PPCPs reflected the most commonly detected substances in European wastewater and surface water (Pomati et al., 2017). The exposure levels of the PPCPs in Chapters 2 and 6 were chosen as the concentrations that yielded 30% decrease in growth rate (sub-lethal) in a pilot toxicity test, conducted following the OECD Guidelines (OECD, 2006b). The concentrations of individuals PPCPs were in the range of concentrations commonly detected in freshwaters (Pomati et al., 2017) and can be found in Table 1.1. Isoproturon (ISU), widely used for its inhibitor properties disrupting photosystem II until its ban in European Union in 2016, was selected as a model stressor for Chapters 3, 4 and 5. As for the PPCPs, the concentrations of ISU applied in this thesis were determined using an eco-toxicological test for growth inhibition (OECD, 2006b) and were selected to cause sub-lethal effects. The major physicochemical properties of the contaminants and the experimental concentrations used in this thesis can be found in Table 1.1. For more information on their chemical structure and more detailed physicochemical properties, please refer to material and methods sections and supplementary materials of the relevant Chapters of this thesis.

Table 1.1. List of chemical substances applied in this thesis reporting their CAS ID, formula, mode of action, functional groups, molecular weight (g-mol), acid dissociation constant (pKa), octanol-water partition coefficient (log K_{ow}) and concentrations applied (µg/L).

Chemical	CAS ID	Formula	Mode of action	Functional group	mm (g/mol)	pKa	Log K_{ow}	Spiked conc. (µg/L)
Atenolol	29122-68-7	$C_{14}H_{22}N_2O_3$	beta-blocker	Amine	266.34	9	0.16	22
Bezafibrate	42859-67-0	$C_{19}H_{20}ClNO_4$	lipid-lowering agent	Carboxylic	361.822	3.83	4.25	2.2
Carbamazepine	298-46-4	$C_{15}H_{12}N_2O$	anticonvulsant	Carbamoyl	236.274	13.9	2.45	22
Clarithromycin	81103-11-9	$C_{38}H_{69}NO_{13}$	antibiotic	Amine	747.964	8.99	3.16	22
Diclofenac	15307-86-5	$C_{14}H_{11}Cl_2NO_2$	non-steroidal anti-inflammatory	Carboxylic	296.147	4.15	4.51	22
Furosemide	54-31-9	$C_{12}H_{11}ClN_2O_5S$	anti-hypertension	Chlorobenzoic	330.739	4.25	2.03	2.22
Hydrochlorothiazole	58-93-5	$C_7H_8ClN_3O_4S_2$	diuretic	Chloro	297.728	7.9	-0.07	22
Ibuprofen	15867-27-1	$C_{13}H_{18}O_2$	non-steroidal anti-inflammatory	Carboxylic	206.285	4.91	3.97	22
Isoproturon	34123-59-6	$C_{12}H_{18}N_{20}$	herbicide	p-cumenyl	206.82	na	2.87	9.5
Ranitidine	66357-35-5	$C_{13}H_{23}ClN_4O_3S$	anti-acid	Amine	314.404	7.8	0.08	2.2
Sulfamethoxazole	723-46-6	$C_{10}H_{11}N_3O_3S$	antibiotic	Amine	253.276	1.6/5.7	0.89	2.2
Benzophenone-4	4065-45-6	$C_{14}H_{12}O_6S$	sunscreen	Carboxylic	308.304	7.6	0.37	22
Triclosan	3380-34-5	$C_{12}H_7Cl_3O_2$	anti-bacterial	Phenols/ethers	289.536	7.9	4.76	2.2

Part I: Ecological Integration in Ecotoxicology

Chapter II: Water Browning Controls Adaptation and Associated Trade-Offs in Phytoplankton Stressed by Chemical Pollution

Simone Rizzuto[1], Jan-Erik Thrane[2], Didier L. Baho[2,3], Kevin C. Jones[1], Hao Zhang[1], Dag O. Hessen[4,5], Luca Nizzetto[2, 6*], Eva Leu[7]

[1] Lancaster Environment Centre, Lancaster University, Lancaster, LA1 4YQ, UK.

[2] Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, 0349, Oslo, Norway.

[3] Department of Aquatic Sciences and Assessment, Swedish University of Agricultural Sciences, P.O. Box 7050, SE-750-07 Uppsala, Sweden.

[4] Section for Aquatic Biology and Ecotoxicology, University of Oslo, Post box 1066, Blindern, 0316 Oslo, Norway.

[5] Center of Biogeochemistry in the Anthropocene (CAB), University of Oslo, Post box 1066, Blindern, 0316 Oslo, Norway.

[6] RECETOX, Masaryk University, Kamenice 753/5, 625 00 Brno, Czech Republic.

[7] Akvaplan-niva, CIENS, Science Park, Gaustadalléen 21, 0349, Oslo, Norway.

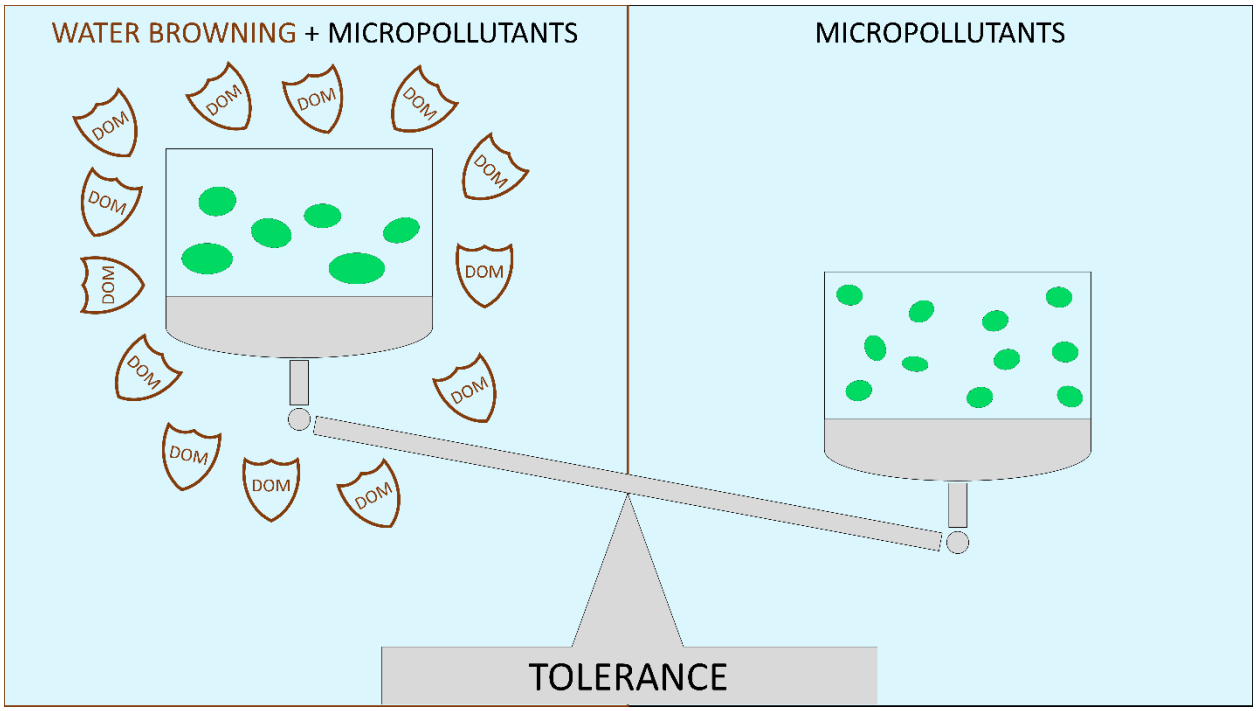
* corresponding author: luca.nizzetto@niva.no

Published in: **S. Rizzuto**, J. E. Thrane, D. L. Baho, K. C. Jones, H. Zhang, D. O. Hessen, L. Nizzetto, E. Leu, 2020. Water Browning Controls Adaptation and Associated Trade-Offs in Phytoplankton Stressed by Chemical Pollution. Environ. Sci. Technol. 54, 5569–5579. https://doi.org/10.1021/acs.est.0c00548**.** To access the Supplementary Material please consult page S2 of the "Appendices and Supplementary Material" file or visit https://doi.org/10.1021/acs.est.0c00548. Appendix Chapter II on page S15 shows the poster presented at SETAC SciCon 2020.

Abstract

Acquisition of tolerance to an environmental stressor can result in organisms displaying slower growth after stress release. While well-grounded in the theory, empirical evidence of trade-off between stress tolerance and organism fitness is scarce and blurred by the interaction with different environmental factors. Here, we report the effects of water browning on the responses, tolerance acquisition and associated trade-offs in a population of microalgae exposed to sub-lethal concentrations of organic micropollutants over multiple generations. Our results show that dissolved organic matter (DOM) reduces toxic responses and modulates tolerance acquisition by the algae, possibly by complexing micropollutants. Microalgae that acquire tolerance allocate resources to fitness at the cost of reduced cell size. They yield higher productivity than non-adapted ones when grown in presence of micropollutants, but lower in their absence. The net trade-off was positive, indicating that adaptation can result in higher productivity and fitness in tolerant species in recurrently stressed environments.

Table of Contents (TOC) / Abstract Art



2.1 Introduction

Populations that have been exposed over multiple generations to the selective pressure of a recurrent stressor may acquire tolerance through physiological and evolutionary adaptations (Bell, 2017). Although these processes usually occur at different time scales (Collins and Gardner, 2009), evolutionary adaptation can also be rapid, arising a few generations after the stress onset (Gonzalez and Bell, 2013). Populations that acquire tolerance towards a specific stressor often show lower growth in another context, such as in the absence of the stressor (Bazzaz et al., 1987; Gassmann and Futuyma, 2005; Lerdau and Gershenzon, 1997; Vila-Aiub et al., 2009a) (maladaptation) (Jin and Agustí, 2018b; Kottuparambil et al., 2019). Existence of these trade-offs is a fundamental postulate of resource-based allocation theory (Bazzaz et al., 1987; Lerdau and Gershenzon, 1997). Drawing predictions of the net positive effect of tolerance acquisition on the functioning of a population requires accounting for these antagonistic processes and is therefore complex. In addition, the magnitude of the stress can be modulated by other environmental factors. This is the case for example, for water pollutants, the availability and/or toxic action of which can be affected by interaction with natural dissolved organic matter (DOM) or water pH (Holmstrup et al., 2010; Laskowski et al., 2010). How the interaction of chemical stressors with environmental factors influences tolerance acquisition and associated costs is mostly uncharted.

Chemical pollution acts as an important selective pressure on aquatic biota (Rockström et al., 2009). Among the range of widespread freshwater chemical pollutants, pharmaceutical and personal care products (PPCPs) are concerning as they are continuously discharged from wastewater effluents, and are biologically active at low concentrations (Schwarzenbach et al., 2006). PPCPs can interfere with fundamental metabolic pathways related to chlorophyll-a and lipids synthesis (Zhang et al., 2012, 2019), which increases their likelihood to adversely impact phytoplankton (Escher et al., 2005; Grzesiuk et al., 2018; Neuwoehner et al., 2006; Wilson et

al., 2003). Evidence that microalgae can adapt to diffuse anthropogenic contaminants is available (Bell, 2017; Huertas et al., 2010; Marvá et al., 2014), but documentation on the environmental controls on tolerance acquisition and on the occurrence and nature of trade-offs is scant (Medina et al., 2007). As also specified in an earlier paragraph, in the context of this thesis, the term "diffuse" refers to a non-point source of contamination, in contrast with point source, which refers specifically to a precise location of contamination emission.

During the last decades, climate and land-use change and recovery from past acidification have caused water browning which haven a diffuse increase of natural DOM and changed pH in many ecosystems (Monteith et al., 2007; Roulet and Moore, 2006; Williamson et al., 2016). DOM (commonly analysed as the concentration of dissolved organic carbon – DOC) can adsorb, bind and/or transform PPCPs by forming less bioavailable and toxic complexes (Pan et al., 2009; Rowett et al., 2016). This process can be pH dependent since many fresh water contaminants, including many PPCPs, exist simultaneously as ionic and neutral forms in the aqueous phase at environmental conditions (Ashauer and Escher, 2010; Rozman and Doull, 2000b). Neutral species dominate at water pH lower than the compound's acid dissociation constant (pKa) and tend to be more toxic, possibly because the organisms' lipid membranes are often more permeable to non-polar molecules (Lipnick, 1995). Neutrality in the molecular charge can in turn increase the likelihood of hydrophobic interactions with DOM (Rowett et al., 2016), possibly resulting in lower bioavailability and toxicity. The influence of these environmental factors on the form, availability and toxicity of PPCPs has been the subject of research (Albanese et al., 2017; Pan et al., 2009; Rowett et al., 2016). Several of these studies report that ecological risk assessment for these micropollutants is based on toxicity data from standardized tests, which rarely account for the effects of natural water chemistry, hence, not taking into account the bioavailable fraction of micropollutants. This concern has been widely recognized for metals in water bodies (Erickson, 2013; Niyogi and Wood, 2004), resulting in

the introduction of the Biotic Ligand Model (Environment Agency, 2009). Similar considerations apply also to certain organic contaminants, calling for the need of a better understanding of their interaction with constituents of aquatic systems such as dissolved organic matter". In addition, the potential implications for driving adaptation and related trade-off are currently unexplored. Given the current widespread browning and the wide range of DOM concentrations in natural surface waters, a better understanding of this factor's role as a modulator of toxic responses and the development of tolerant strains is needed.

In order to address these gaps, we designed a two-phase experiment assessing the role of DOM on the toxic outcomes and adaptation (in terms of tolerance acquisition) and associated fitness trade-offs in a microalgae population exposed to a mixture of PPCPs. First, we postulated that:

i) DOM reduces the negative effects of PPCPs on algal growth (Pan et al., 2009; Rowett et al., 2016);

ii) prolonged exposure to sub-lethal concentrations of PPCPs induces tolerance in microalgae.

Then, after testing these preconditions, we hypothesized that:

i) acquisition of tolerance to PPCPs trades-off with growth efficiency in the absence of the pollutants;

ii) DOM during the adaptation period controls both acquisition of tolerance and emergence of fitness trade-offs.

The experiment was designed as follows (see Figure 2.1):

- In phase I we assessed microalgal growth and cell size response to PPCPs under different conditions of DOM and pH;

- Then we subjected the microalgae to a two-month adaptation period, where they were exposed to sub-lethal PPCP levels and different levels of DOM, under the pH conditions that in phase I yielded highest growth inhibition;

- Finally, in phase II the growth and cell size of non-adapted and adapted populations to PPCPs under different levels of DOM were compared in the presence and absence of PPCPs.

Addressing the implications of the two-way interaction between environment and environmental stressors on biota growth and fitness represents a challenge of considerable complexity. This multiple stressor – multiple interaction situation prevails in nature and it is important to understand and quantitatively balance synergistic/antagonistic effects, inform realistic extrapolations of results to real environmental conditions, and ultimately address the broader ecological implications of these interactions.

2.2 Materials and Methods

2.2.1 Experimental Design

The experiment consisted of two-phases (Figure 2.1), interposed by an adaptation period. An acclimation phase preceded the first phase of the experiment, where the cultures were acclimated for five days to combinations of DOM (0, 5 and 15 mg L^{-1} DOC) and pH (6.5 and 8). During phase I, the growth response of the microalgae population to the mix of PPCPs was tested for combinations of three DOM levels (0, 5, 15 mg L^{-1} DOC) and pH (6.5, 8) in a factorial design (Figure 2.1). Then, the algae were allowed to adapt for 2 months under the same experimental conditions of PPCPs and DOM (Figure 2.1) at pH 8 only (following results from phase I). In phase II, the adapted populations were sub-sampled and grown in the presence and absence of the mix of PPCPs (at the same concentrations used in phase I and during the adaptation period, but in the absence of DOM, Figure 2.1), to assess acquisition of tolerance, growth performance and ultimately trade-offs in growth efficiency.

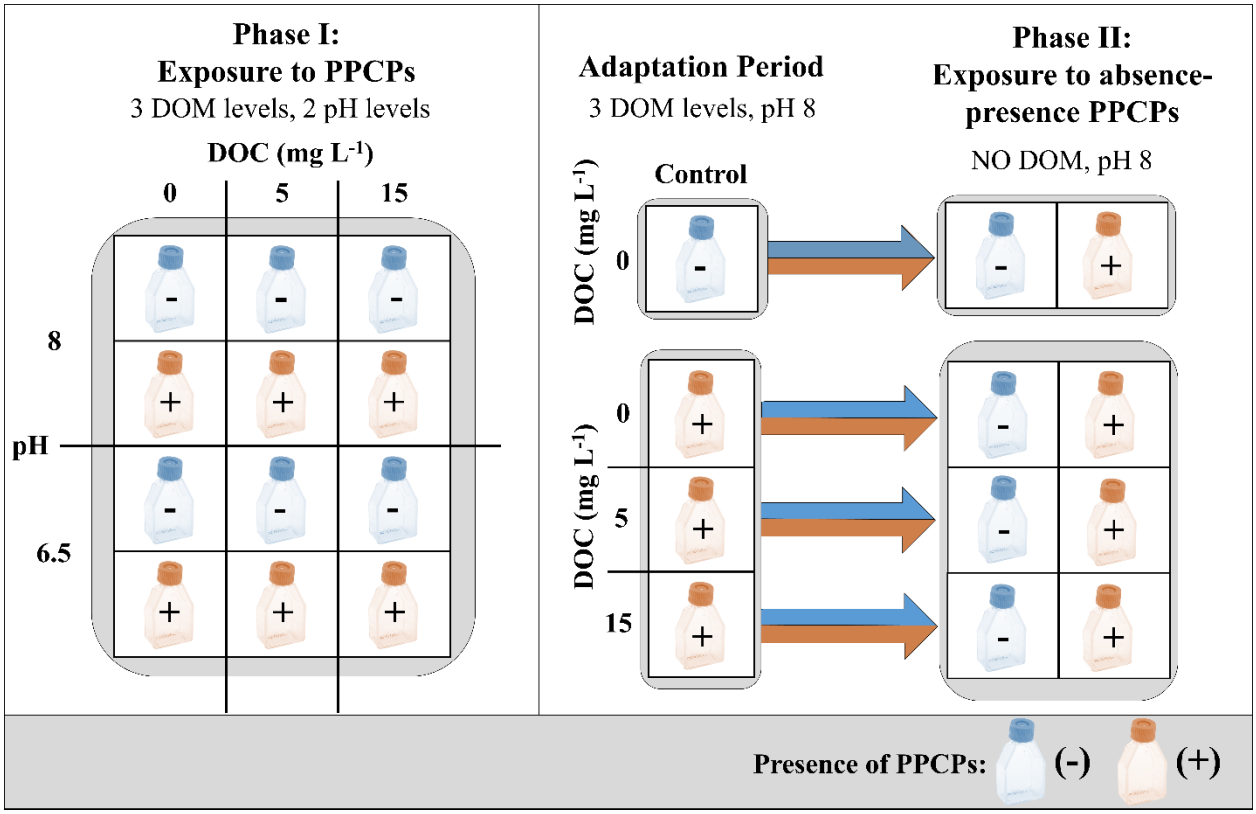


Figure 2.1. Factorial experimental design. Phase I; exposure of the algal population to the absence (-) and the presence (+) of a mix of 12 PPCPs under different DOM and pH levels. Adaptation period; multi-generational exposure of the algal population to the presence (+) of PPCPs under different levels of DOM (0, 5, 15 mg L^{-1} DOC) at pH 8. Phase II; exposure of the algal population previously adapted to the presence of PPCPs under different levels of DOM, and of the control population which never experienced the contaminants and/or the DOM during the adaptation period, to the absence (-) and the presence (+) of PPCPs.

2.2.1.1. Selection of algal culture

The chlorophyte *Chlamydomonas reinhardtii* (strain CC-1690 21 gr mt+) was used in laboratory growth experiments. This is a widely used model organism for toxicological and evolution studies (Gonzalez and Bell, 2013).

2.2.1.2. Selection of DOM and pH

DOM originated from the Hellerudmyra tarn (Norway) and was previously isolated through reverse osmosis (Gonzalez and Bell, 2013). All the physical-chemical properties of this DOM are reported by Gjessing et al. (Gjessing et al., 1999a). The levels of DOM and pH applied represent the range typically found in Northern European lakes (Henriksen et al., 1998; Thrane et al., 2014). The effect of the algal photosynthesis on the sequestration of carbon dioxide could

cause an increase of the pH of the cultures due to increasing levels of hydroxide. The choice of nutrient concentrations (mesotrophic lakes, P= 30 µg L^{-1}) minimized this effect to a very modest increase (average increase in both set of cultures at the end of bot exposure phases was of 0.5±0.08).

2.2.1.3 Selection of chemical contaminants

A mixture of 12 PPCPs was taken as chemical stressor model (Table S2.2), according to a number of previous studies (Pomati et al., 2017, 2006) and reflecting most commonly detected substances in European wastewater and surface water (Table S2.1). PPCPs analytical standards were purchased from Sigma-Aldrich (USA), mixed and diluted in dimethyl sulfoxide (DMSO, Sigma-Aldrich) to create a stock solution. The exposure level used in this experiment to induce toxic effects from PPCPs in phase I and II and during the adaptation period (Table S2.2) was chosen as the concentration that yielded a 30% decrease in growth rate in a pilot toxicity test (Table S2.3), following the OCED guidelines (OECD, 2006a). The concentrations of individual PPCPs ranged from 2.2 to 22 µg L^{-1} (Table S2.2), within the range of concentrations detected in European freshwaters (Table S2.1). The concentration of individual PPCPs was determined at the end of both experimental phases (Table S2.4) as described in Text S2.1.

2.2.1.4 Algal culturing and biomass measurements

The algae were grown as batch cultures in 60 mL non-treated polystyrene cell culture flasks (Nunc, Thermo-scientific, US), using WC medium (Guillard and Lorenzen, 1972) with P concentration of 30 µg L^{-1}. Cultures were incubated at 16 °C in a temperature controlled room under constant white light (100 µmoles of photons m^{-2} s^{-1}; this resulted in no light limitation, based on earlier experiments with *C. reinhardtii* (Thrane et al., 2016)). Each treatment was replicated four times (total number of experimental units was 48 in both phases).

The relative biomass development was monitored in both phases as the chlorophyll a *in vivo* fluorescence (excitation at 460 nm and emission at 680 nm, Figure S2.1, S2.2), using a plate reader equipped with a spectrophotometer (BioTek Synergy MX; Winosky, VT, USA). Triplicates from each experimental unit were loaded on clear flat bottom 96 well black microplates (300 µL in each well) (Corning, USA). Biomass assessments were further constrained through cell number and size distribution determination, measured by a coulter counter (Multisizer 3, Beckman Coulter Life Sciences, USA). For phase I, samples of 1 mL were collected from each experimental unit at the end of the exponential growth phase (on day 5, as judged from the chlorophyll *in vivo* fluorescence, Figure S2.1). For phase II, samples for cell counting were taken daily.

2.2.1.5 Phase I

DOM enriched medium was prepared by spiking a solution of DOM diluted in MQ, in two bulk solutions of modified WC medium (mesotrophic lakes, P= 30 µg L^{-1}) to reach concentrations of 5 and 15 mg DOC/L, respectively. A third batch (control) with no added DOM was also prepared (only MQ water was added with no DOM). The volume of the three bulk solutions was split into two separate sets, the pH of which was adjusted by titration with HCl or NaOH to 6.5 and 8, respectively. Finally, 20 µL of PPCPs stock solution was added to half of the units, to reach the concentrations shown in Table S2.3. 40 mL of each of the 12 different media (3 DOM-levels x 2 pH levels x 2 PPCPs levels) were added to four replicate culture flasks and inoculated with 100 µL of algal stock culture. This resulted in a starting concentration of ca. 1000 cells per mL (measured in a coulter counter). Phase I was run for 7 days under the light and temperature conditions described earlier.

2.2.1.6 Experimental adaptation period

Following phase I, the algal cultures from the pH=8 set were grown for 2 months in the presence of PPCPs, under the same experimental conditions as in phase I. Only the higher pH

conditions was chosen because these conditions only induced growth inhibition by PPCPs in phase I. Such a prolonged sub-lethal exposure was aimed at inducing selection of resistant traits and promote adaptations that could affect population dynamics and result in the postulated fitness trade-offs. Exposure was conducted under three DOM levels (0, 5 and 15 mg L^{-1} DOC), to account on the influence of DOC on the emergence of tolerance and growth trade-offs. A control culture was grown at the same level of pH, in the absence of PPCPs and DOM. The cultures were transferred to new growth medium every week (0.5 mL culture to 40 mL of fresh medium) during the adaptation period.

2.2.1.7 Phase II

Following the adaptation phase, subsamples (100 µL) from each culture were inoculated in two separate sets of four replicate culture flasks and diluted with 40 mL of DOM-free growth medium. One set was spiked with 20 µL of the PPCP solution (at the same concentrations used in phase I), while the other one was spiked with 20 µL of the carrier solvent (DMSO) only (excluding the contaminants). Phase II was run for 7 days during which cultures were grown exponentially under the same light, nutrient and temperature conditions used in phase I.

2.2.2 Data treatment, response parameters and statistical analysis

All the analyses were conducted using R (version 3.5.1) statistical software (R Core Development Team 2015). Growth rate was calculated using the total algal biovolume as determined from the cell counter. Total algal biovolume (BV_t) was calculated based on the number of cells (N) and their radius (r), assuming a spherical shape of the cells:

$$BV_t = \sum_{i=1}^{n} \frac{4}{3} \pi {r_i}^3 \, N_i$$

Specific growth rate μ (d^{-1}) of each experimental unit was calculated as the slope of a linear regression of log-transformed biovolume against time, using data from the exponential growth phase (Figure S2.3). For the comparison of cell size between treatments, we calculated peak cell diameter (μm) as the mode of cell size distribution. The peak cell diameter values were then used to calculate the cellular biovolume (μm^3, "hereon called "cell size") as the volume of the sphere ($4/3\ \pi r^3$). Growth rate based on the cell count (here called "recruitment rate"), was also calculated to disentangle the growth of the microalgae from the variation of the cell size caused by the treatments. Recruitment rates were taken here as a proxy of fitness, whereby fitness is fundamentally defined as the probability of producing off-spring and is measured through the increase in the population cell number over time (Hagman et al., 2018). As we used culture batches in microcosms, recruitment depended only on the generation of off-spring and dispersal was absent.

A rigorous definition of trade-off was also formulated. First, we defined the benefit of the adaptation (B_{adp}, t^{-1}) as the gain in growth rate the adapted population displayed when growing in the presence of PPCPs. This was calculated as:

$$B_{adp} = gr_{A,+} + gr_{nonA,+}$$

Where $gr_{A,+}$ and $gr_{nonA,+}$ represented the growth rates of the adapted population and non-adapted population in the presence of PPCPs in phase II. Similarly, we defined the cost of adaptation (D_{adp}, t^{-1}) as the reduction in growth rate the adapted population displayed when growing in the absence of PPCPs in phase II, calculated as:

$$D_{adp} = gr_{A,-} + gr_{nonA,-}$$

Their net trade-off was therefore calculated as their sum, after bootstrapping estimated growth rate values from gaussian distributions fitted to the experimental growth rate data. Data

variability and uncertainties were tracked down to the final values of gap and trade-off using a Monte Carlo frame ($N=10^5$).

$$Net\ trade-off = B_{adp} + D_{adp}$$

Power analysis was performed to support that the number of replicates planned for this experiment was sufficient to detect the potential differences in endpoints in the multiple groups. With a total amount of 144 samples and 12 groups, the power value for the three-way ANOVA interaction was 0.86, 0.84 for the two-way and 0.86 for the one-way. In phase I, the toxic responses of the algal population to PPCPs under different combinations of DOM and pH was evaluated by a two-step procedure. As response variables we used total algal biovolume and cell size. First, we used a three-way ANOVA to test the significance of the treatment factors (PPCPs, DOM and pH) and their interactions. Secondly, we used linear modelling (with all predictor variables coded as factors) to test for significant differences in toxic responses between groups of interest (e.g. whether the response of total algal biovolume or cell size to contaminants differed significantly between different DOC levels at a given pH).

In phase II, we first tested whether the adaptation period had caused algae to develop tolerance to PPCPs, and whether an eventual adaptation led the trade-off (i.e. reduced growth rate and/or cell size when grown without contaminants). We did this by modelling specific growth rate, cell size and recruitment rate as a function of contaminants exposure (factor variable with two levels; yes/no) and whether they were allowed to adapt to PPCPs in the adaptation period (factor variable with two levels; yes/no). We tested for main effects and interactions between the two treatment factors. For the populations that underwent the adaptation phase under different levels of DOM, we tested how specific growth rate and cell size responded to contaminant exposure in phase II in the absence of DOM. This was done by modelling specific growth rate and cell size as a function of two factors: the presence/absence of PPCPs and DOM-

level during the adaptation period (factor variable with three levels; 0, 5 and 15 mg L^{-1} DOC). We tested for main effects and interactions between two treatment factors.

2.3 Results

2.3.1 Phase I

2.3.1.1 Effects of DOM and PPCPs on biomass

The three-way interaction between PPCPs, DOM and pH did not have a significant effect on the total algal biovolume yield (Table 2.1). The mix of PPCPs had a highly significant effect on the total algal biovolume yield ($F = 97.025$, $p < 0.001$; Table 2.1 and Figure 2.2A-B). This effect was strongly dependent on pH and DOM, as shown by the significant interactions between PPCPs and pH ($F = 20.807$, $p < 0.001$) and PPCPs and DOM ($F = 5.684$, $p < 0.05$). While exposure to PPCPs generally reduced the total biovolume yield ($t = -6.07$, $p < 0.05$), the toxic effect was significantly stronger at pH 8 than at pH 6.5 ($t = -4.55$, $p < 0.001$). Low concentrations of DOM (5 mg L^{-1} DOC) at pH 8 decreased the negative effect of contaminants exposure on the total biovolume yield, relative to the control without DOM ($t = 2.272$, $p < 0.05$). A similar positive effect was not observed at the higher level of DOM (15 mg L^{-1} DOC), where the total biovolume did not differ from the control with no DOM ($t = 0.56$, $p = 0.586$). At pH 6.5, the detrimental effect of PPCPs was not influenced by the DOM ($F = 0.1865$, $df = 2.9$, $p = 0.83$).

In the absence of PPCPs, the total biovolume yield was significantly lower at the higher level of DOM (15 mg L^{-1} DOC) compared to the control without DOM ($t = -3.45$, $p = 0.0027$) at both pH levels. Total biovolume tended to be more sensitive to high DOM levels at pH 6.5 than at pH 8 (Figure 2.2A), but the difference was border-line significant ($p = 0.08$).

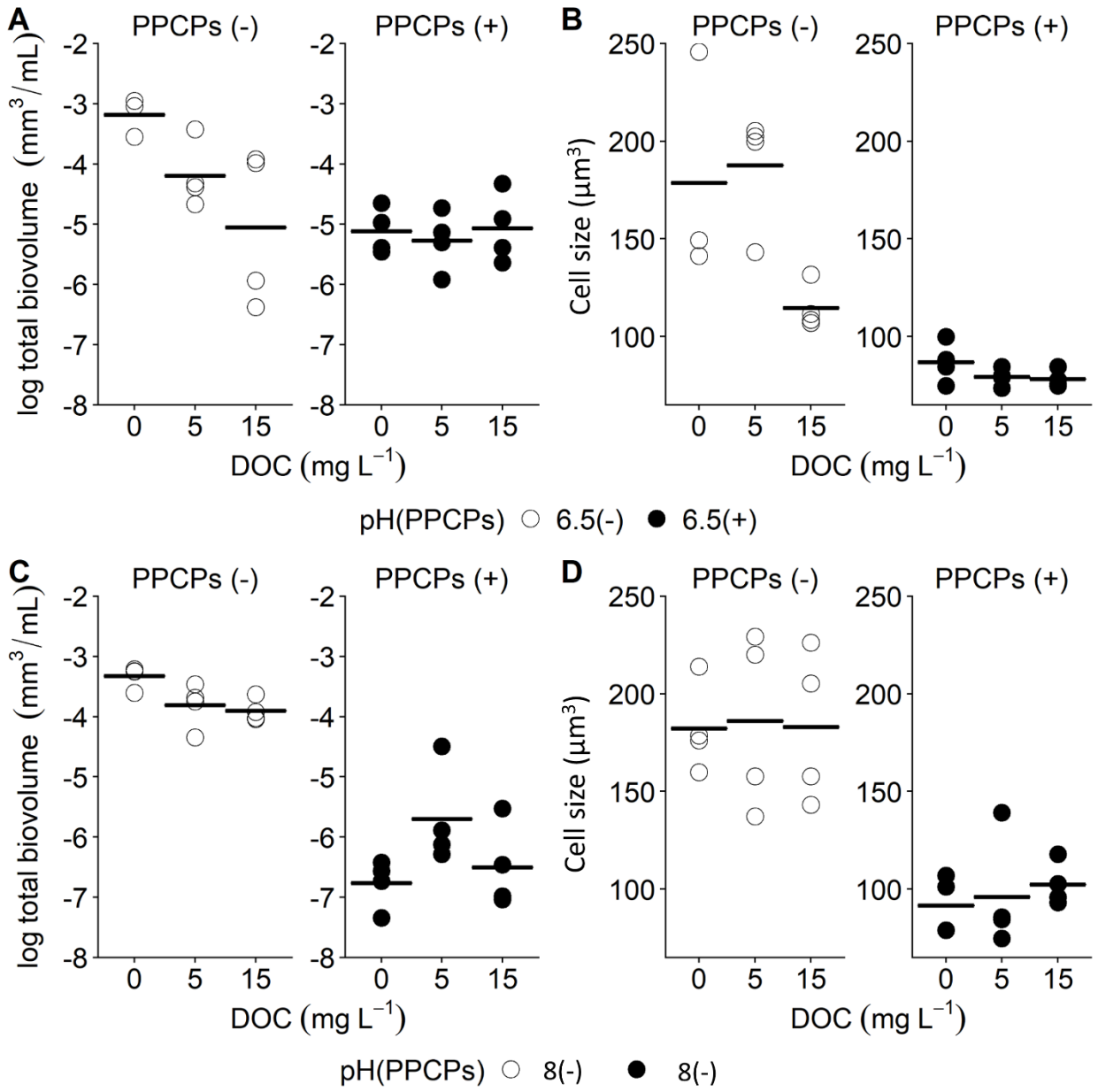


Figure 2.2. Phase I results. Log total biovolume yield (mm^3/mL) and cell size (μm^3) of *C. reinhardtii* as a function of DOM (0, 5, 15 mg L^{-1} DOC) in the absence (-) and the presence (+) of the mix of PPCPs, at pH 6.5 (A, B) and 8 (C, D). Short horizontal bars represent the mean for each group.

Table 2.1. ANOVA table of phase I results. Main outcome from a three-way ANOVA which tested the effects of PPCPs (the absence/presence), DOM (0, 5, 15, mg L^{-1} DOC) and pH (6.5, 8) on log total algal biovolume yield (mm^3 mL^{-1}) and cell size (μm^3). R2; proportion of variation explained by the interactions and the main effects. df; degree of freedom. SS; Sum of square means. F; F value. Significant values are reported in bold.

Variables	Factors and interactions	R^2	df	SS	F	*p*
log total biovolume (mm^3 mL^{-1})	PPCPs:DOM:pH	0.81	2	1.58	2.16	0.13
	PPCPs:DOM	0.63	**2**	**4.43**	**5.68**	**<0.01**
	PPCPs:pH	0.68	**1**	**8.1**	**20.21**	**<0.001**
	DOM:pH	0.06	2	1.7	2.18	0.13
	PPCPs	0.54	**1**	**37.77**	**97.02**	**<0.001**
	DOM	0.03	2	2.15	2.76	0.08
	pH	0.01	1	1.33	3.42	0.07
cell size (μm^3)	PPCPs:DOM:pH	0.82	2	0.37	1.25	0.3
	PPCPs:DOM	0.74	**2**	**0.81**	**2.75**	**<0.05**
	PPCPs:pH	0.74	1	0.01	0.07	0.79
	DOM:pH	0.1	**2**	**1.05**	**3.56**	**0.04**
	PPCPs	0.69	**1**	**20.77**	**141.2**	**<0.001**
	DOM	0.02	2	0.55	1.92	0.16
	pH	0.05	1	1.29	8.8	0.005

2.3.1.2 DOM and PPCPs effects on cell size

The interaction between PPCPs, DOM and pH did not significantly affect the cell size of the microalgae (Table 2.1). The mix of PPCPs consistently decreased the mean cell size of the population ($F = 141.20$, $p < 0.001$, Table 2.1 and Figure 2.2B). This effect was also modified by the presence of DOM, as shown by the significant interaction term ($F = 2.75$, $p < 0.05$), while the interaction with pH was not significant. The negative effect of PPCPs on cell size ($t = -7.323$, $p < 0.001$) was lower ($t = 2.579$, $p < 0.05$) at pH 8 than at pH 6.5. In the absence of contaminants, the higher level of DOM (15mg L^{-1} DOC) negatively affected the cell size only in the treatment with pH 6.5 ($t = -3.20$, $p < 0.05$).

2.3.2 Phase II

2.3.2.1 Trade-offs of tolerance acquisition in the absence of DOM

Exposure to PPCPs in phase II in absence of DOM decreased algal growth rates (defined as the increase in total algal biovolume over time) in all cultures, regardless of previous adaptation ($F = 43.68$, $p < 0.001$; Figure 2.3A and Table 2.2). The growth inhibition effect was, however, significantly lower for the adapted cultures (df = 12, estimated mean difference = 0.51 μ (d^{-1}), $p < 0.05$). At the same time, when grown in the absence of PPCPs in phase II, adapted cultures had a significant slower growth than not-adapted ones (df =12, estimated mean difference = -0.27 μ (d^{-1}), $p < 0.05$, Figure 2.3A and B, Table S2.7).

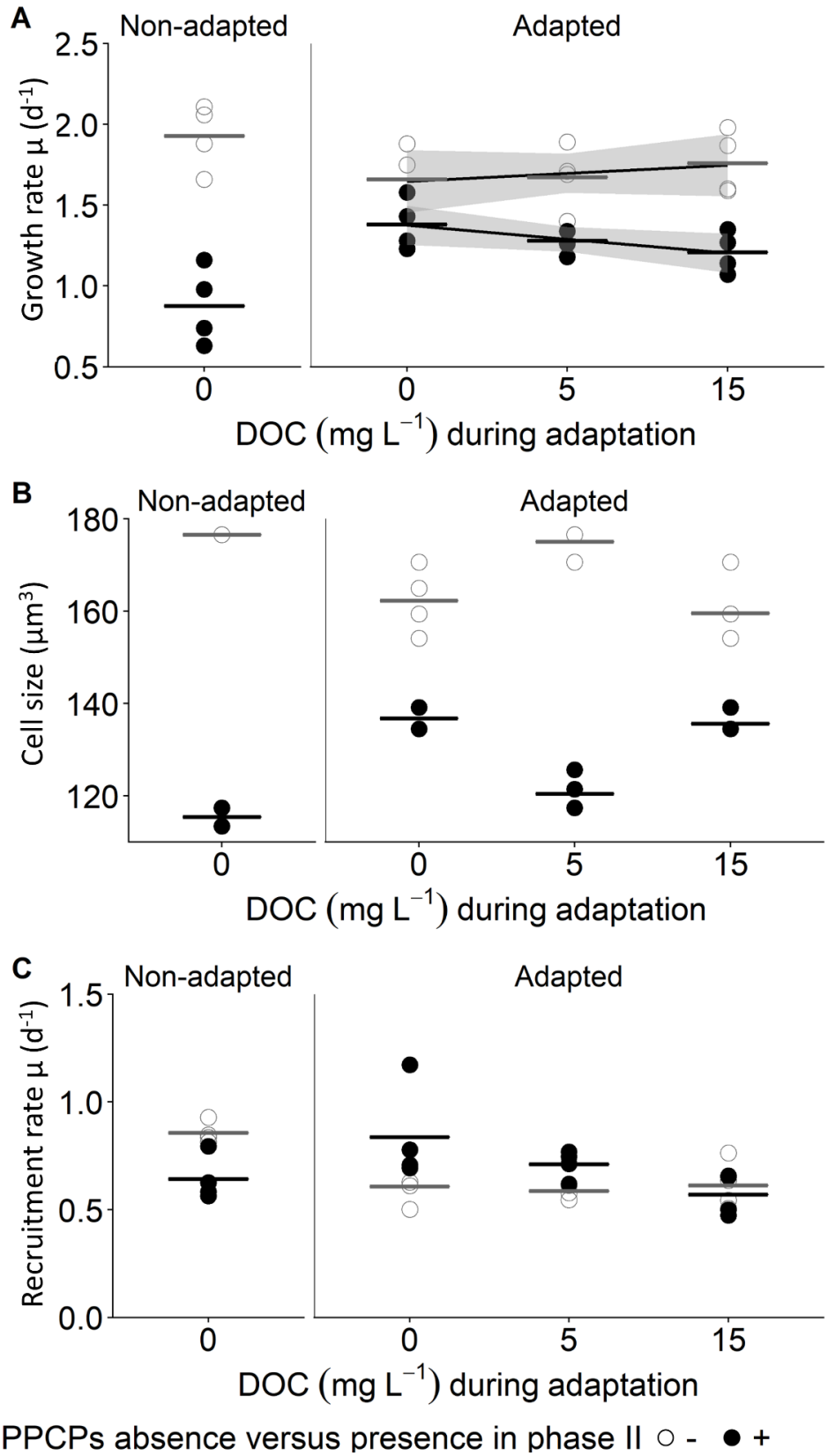


Figure 2.3. Phase II results, growth rate and cell size. Growth rate μ (d^{-1}) (A), cell size (μm^3) (B) and recruitment rate μ (d^{-1}) (C) of the population which did not experiment PPCPs and DOM during the adaptation period (non-adapted), and the population cultivated with PPCPs and DOM levels during the adaptation period (adapted), in response to the absence (-) and the presence (+) of the mix of PPCPs in phase II. Short bar report the mean values.

Table 2.2. Effect of the presence of PPCPs and DOM during the adaptation period. ANOVA-table showing the main outcome from the two-way ANOVA which tested the effects of the presence of PPCPs during the adaptation period on the growth rate μ (d^{-1}), cell size (μm^3) and recruitment rate μ (d^{-1}) of the algal populations exposed to the absence/presence of PPCPs in phase II (non-adapted vs. adapted), and the effects induced by the presence of DOM during the adaptation period with PPCPs on the growth rate and cell size of the algal population exposed to the absence/presence of PPCPs in phase II (adapted with no DOM vs. adapted with DOM). R2; proportion of variation explained by the interactions and the main effects; df; degree of freedom. SS; Sum of square means. F; F value. Significant values are reported in bold.

Variables	Factors and interactions	R^2	df	SS	F	*p*
growth rate μ (d^{-1})	PPCPs during adaptation	0.01	1	0.05	1.36	0.26
	PPCPs in phase II	**0.45**	**1**	**1.77**	**43.68**	**<0.001**
	PPCPs during adaptation : PPCPs in phase II	**0.83**	**1**	**0.59**	**14.64**	**<0.01**
	residuals		*12*	*0.49*		
cell size (μm^3)	**PPCPs during adaptation**	**0.01**	**1**	**0.02**	**7.64**	**<0.05**
	PPCPs in phase II	**0.45**	**1**	**1.67**	**509.56**	**<0.001**
	PPCPs during adaptation : PPCPs in phase II	0.84	**1**	**0.3**	**90.97**	**<0.001**
	residuals		*12*	*0.04*		
recruitment rate μ (d^{-1})	PPCPs during adaptation	0.06	1	0.002	0.15	0.7
	PPCPs in phase II	0.38	1	0.001	0.01	0.9
	PPCPs during adaptation : PPCPs in phase II	0.48	**1**	**0.2**	**11.9**	**<0.05**
	residuals		12	0.21		
growth rate μ (d^{-1})	**PPCs in phase II**	0.63	**1**	**1**	**36.43**	**<0.001**
	DOM during adaptation with PPCPs	0.005	2	0.009	0.16	0.85
	PPCPs in phase II : DOM during adaptation with PPCPs	0.69	2	0.07	1.37	0.28
	residuals		*18*	*0.49*		
cell size (μm^3)	**PPCPs in phase II**	0.81	**1**	**1.58**	**309.84**	**<0.001**
	DOM during adaptation with PPCPs	0.004	2	0.008	0.84	0.45
	PPCPs in phase II : DOM during adaptation with PPCPs	**0.95**	**2**	**0.27**	**26.52**	**<0.001**
	residuals		*18*	*0.092*		

recruitment rate μ (d^{-1})	**PPCPs in phase II**	**0.14**	**1**	**0.06**	**4.58**	**<0.05**
	DOM during adaptation with PPCPs	0.15	1	0.07	2.46	0.11
	PPCPs in phase II : DOM during adaptation with PPCPs	0.45	1	0.07	2.61	0.11
	residuals		*18*	0.25		

Cell size response was similar to that of growth rate. Exposure to PPCPs in phase II yielded smaller cells in all treatments (F = 509.56, $p < 0.001$; Figure 2.3B and Table 2.2). The magnitude of the effect, however, was strongly dependent on the adaptation (F = 90.97, $p < 0.001$). In phase II experiments, the mean cell size of cultures exposed to PPCPs during the adaptation period was significantly larger in the presence of the contaminants than that of non-adapted cultures (df = 12, estimated mean difference = 0.352 µm, $p < 0.001$, Table S2.7). Concurrently, their cell size was smaller than the non-adapted ones when grown in phase II in absence of PPCPs (df = 12, estimated mean difference = -0.194 µm, $p < 0.001$, Table S2.7).

PPCP exposure during phase II decreased recruitment rates (taken as a proxy of fitness and measured here simply as the increase of cell number over time) of the non-adapted population (F = 4.58, $p < 0.05$). The adapted population, on the contrary, yielded higher recruitment rate when algae were exposed in phase II to the PPCPs (df = 12, estimated mean difference= -0.229 μ (d^{-1}), $p < 0.05$). The effect of adaption on the recruitment rate mirrored observed growth rate and cell size patterns (Figure 2.3C, Table S2.7). For instance, the adapted population yielded a higher recruitment rate when exposed to the PPCPs in phase II (df = 12, estimated mean difference = 0.194 μ (d^{-1}), $p < 0.001$, Table S2.7), but lower in the absence of the contaminants (df= 12, estimated mean difference = -0.25 μ (d^{-1}), $p < 0.05$, Table S2.7), relative to the non-adapted population.

2.3.2.2 Effects of DOM on tolerance acquisition and trade-offs

Similar to the response of adapted algae in absence of DOM, the exposure to PPCPs in phase II significantly affected growth rate, cell size and recruitment rate of algae adapted in presence of DOM (Figure 2.3, Table 2.2). Growth rates and recruitment rates of adapted algae exposed to PPCPs declined along the DOM gradient applied during the adaptation period. The recruitment rate of algae that acquired adaptation in presence of the highest level of DOM was significantly lower relative to that of algae adapted in its absence ($t = -2.27$, $p < 0.05$). The DOM gradient during the adaptation period did not significantly affect growth rates, cell size and recruitment rates of the adapted algae in absence of contaminants (Table 2.2, Figure 2.3), despite those were, altogether, lower than that of non-adapted algae (Table S2.7).

2.3.2.3 Benefits, deficits and net trade-off in the growth rate of the adapted population

The interaction between the presence of DOM and PPCPs during the adaptation affected the gain in growth rate of the adapted population either when exposed to the presence (B_{adp}) or in absence (D_{adp}) of PPCPs in phase II (Figure 2.4A). In presence of PPCPs during phase II, the B_{adp} followed an increasing pattern along the DOM gradient (Figure 2.4A). In contrast, the D_{adp} followed a decreasing pattern when in absence of PPCPs in phase II (Figure 2.4A). This effect was however not significant ($F = 0.45$, $p = 0.65$). The exposure to PPCPs in phase II affected the gain in growth rate of the adapted population compared to the non-adapted one ($F = 29.52$, $p < 0.001$), while no significant effect was yielded by the presence of DOM during the adaptation period ($F = 0.05$, $p = 0.95$). The microalgae adapted to the presence of PPCPs during the adaptation period yielded a positive gain in growth rate (B_{adp}) when exposed to the presence of PPCPs in phase II, and a negative gain (D_{adp}) in their absence, compared to the non-adapted population (Figure 2.4A). The difference between B_{adp} and D_{adp} is also significant ($t = 3.76$, $p < 0.05$). The net trade-off in growth rate of the adapted population showed to be positive, but not significantly affected by the presence of DOM during the adaptation period (Figure 2.4B).

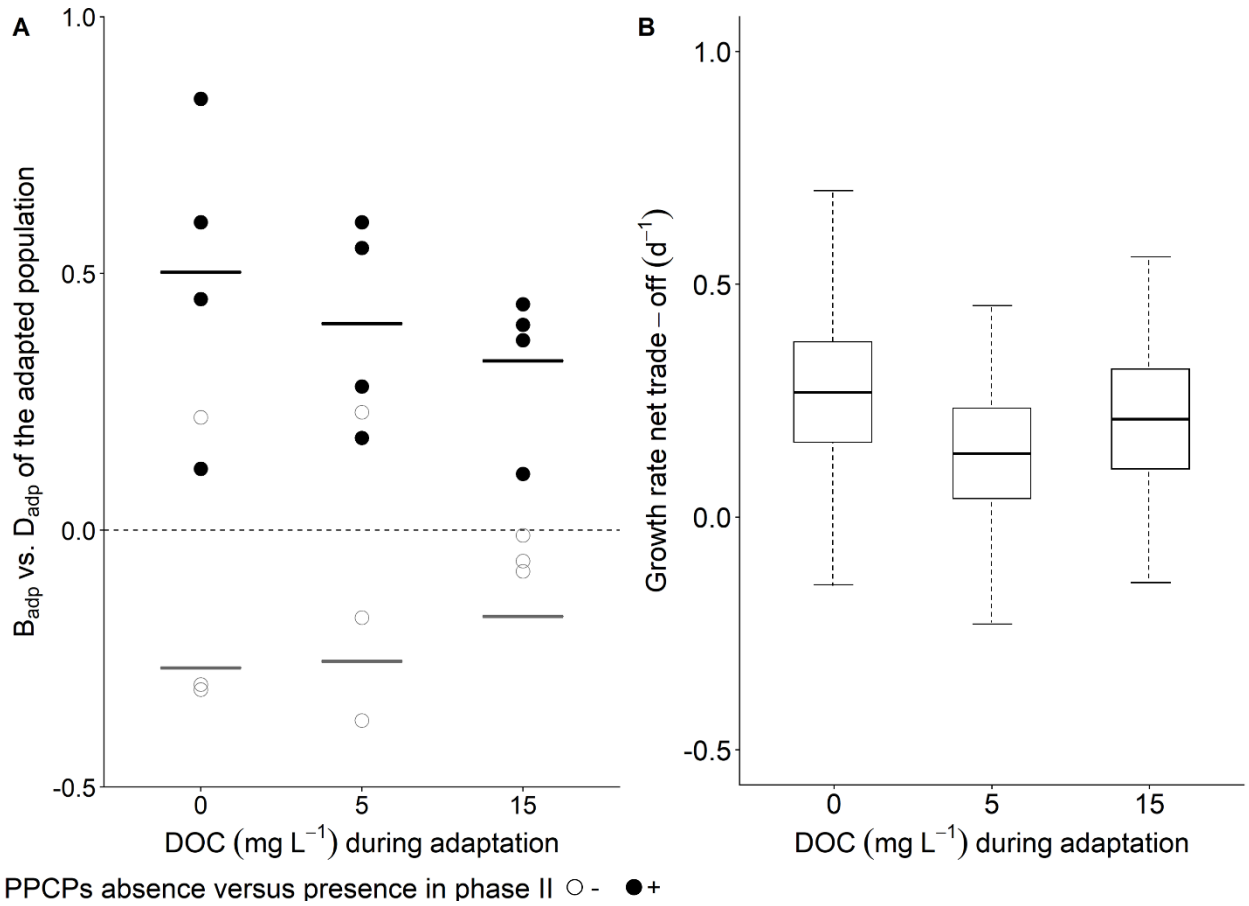


Figure 2.4. (A) Benefits (B_{adp}) and Deficits (D_{adp}) from tolerance acquisition in the growth rate μ (d^{-1}) of the adapted population exposed to the presence and the absence of PPCPs in phase II. (B) The net trade-off from tolerance acquisition. These variables were calculated after bootstrapping estimated growth rate μ (d^{-1}) values from gaussian distributions fitted to the experimental growth rate data. Data variability and uncertainties were tracked down to the final values of gap and trade-off using a Montecarlo frame ($N=10^5$).

2.4 Discussion

We assessed the effects of the interaction of micropollutants and DOM on growth, cell size and fitness (through the use of recruitment rate as a proxy) of a freshwater microalgae population. We focused in particular on the emergence of trade-offs associated to adaptation acquisition (i.e. whether tolerance acquisition to chemical stress (Bell, 2017; Gonzalez and Bell, 2017) influences these variables when algae grow in the absence of the stressor) as well as the role of

an important environmental factor (namely, DOM) on the development of tolerance acquisition and related costs. Our results showed that algae responses depended on PPCPs, DOM and their interaction during the adaptation period. In particular we observed:

i) a mitigating effect induced by the combination of DOM and pH on the toxic effect of the PPCPs (Figure 2.2, Table 2.1);

ii) Emergence of tolerant populations upon the adaptation period;

iii) tolerance acquisition and emergence of related trade-off were influenced by DOM levels during the adaptation period (Figure 2.3, Tables 2.2, S2.5-S2.6);

whereby, points i) and ii) verified the study's postulates, and point iii) supported our main hypothesis. The following sections discuss these findings and their implications in detail.

2.4.1 Phase I - Effects of DOM and pH on algal population responses to PPCPs

PPCPs negatively affected total biovolume and cell size of the tested population during phase I. Previous studies showed that PPCPs can affect growth of microalgae (Zhang et al., 2012, 2019). Our findings showed that the interaction between DOM, pH and PPCPs had a significant effect on the algal biovolume. This translated into a positive effect of the interaction of DOM, pH and PPCPs on algal growth that was observed in particular at the lower DOM concentration (5 mg L^{-1} DOC) and pH 8. Under these conditions observed growth hindrance effects by PPCPs were minimal. This verified the first of our postulates. pH could vary the speciation/form of both contaminants and DOM, and modify contaminants' ionic configuration. These, in turn, could affect both their toxicological properties and/or their complexation with DOM, and thereby their availability. The majority of the compounds (7 out of 12) within the mix of PPCPs used in the present study (Table S2.2), were in their associated form, moderately to highly hydrophobic (log_{kow} ranging from 2.03 to 4.76), while the remaining were highly hydrophilic (log_{kow} ranging from -0.07 to 0.89, Table S2.2). Hydrophobic compounds usually have a

significant interaction with DOM (Pan et al., 2009) that likely influenced our results. In addition, higher pH (8) forms neutral species also for some of the more hydrophilic compounds, promoting their toxicity and their complexation. Among the PPCPs in the mixture, carbamazepine, clarithromycin and triclosan had pKa between 7.9 and 13.9 (Table S2.2). This explained the dependency of the toxicity results on pH. Our findings were in line with previous studies (Pan et al., 2009; Rowett et al., 2016).

At higher DOM concentration (15 mg L^{-1} DOC), such a toxicity inhibition effect vanished. We argue that this is caused by direct, negative impacts of DOM on the algae. For instance, DOM could actually directly stress algae (Creed et al., 2018; Wolf et al., 2017; Pflugmacher et al, 2006) in various ways (an effect that was found in our experiment to be more pronounced where algae were grown in absence of PPCPs at lower pH). In particular, DOM could i) reduce growth by reducing light availability (Creed et al., 2018); ii) in nutrient-limited environments, affect algal growth by adding organically bound nutrients (e.g. P (Creed et al., 2018)), hinder it by complexing or adsorbing key elements (e.g. Fe (Creed et al., 2018)), or promote the growth of heterotrophic bacteria with higher affinity for limiting nutrients (e.g. P (Creed et al., 2018)); iii) produce of harmful free radicals and reactive oxygen species from photoactivation stressing the algae (Wolf et al., 2017); and iv) affect directly the photosynthetic machinery (Pflugmacher et al., 2006). In the experimental conditions, lack of short-wave irradiation and nutrient saturated conditions excluded negative impacts due to formation of reactive species and nutrient limitations. Direct negative effects of high DOM levels on algae were more plausible mechanisms. This explanation was consistent with the observed interactive effect between pH and DOM on growth inhibition in absence of PPCPs.

While the focus of phase I was on the impacts on growth, negative effects on cell size were consistently observed. Although, the mode of action of PPCPs on algae is not fully understood, some PPCPs (i.e. triclosan, carbamazepine, diclofenac) have the ability to impair fundamental

metabolic pathways related to chlorophyll-a and lipid synthesis (Franz et al., 2008; Zhang et al., 2012, 2019), which may induce destabilizing effects on the cell membrane of algae.

2.4.2 Phase II – Tolerance acquisition and trade-offs

During the adaptation period the algae were exposed over multiple generations to the mix of PCPPs. This resulted in acquisition of tolerance as demonstrated by the higher growth rate of the adapted population in phase II compared to non-adapted ones under PPCPs exposure. Considering the time frame of the adaptation period (> 2 months) (Bell, 2017), PPCPs might have favoured the emergence of tolerant strains through selective filtering. While this could be the result of rapid evolution, a physiological component of this response could not be excluded, in principle. To disentangle the nature of the adaptation process is notoriously difficult and was outside the scope of this study. However, the rapid changes in cell size observed in experimental phase II as response to PPCPs especially in the non-adapted population pointed at fast physiological responses that could affect resource allocation. Similar findings indicating tolerance acquisition triggered by rapid adaptation to chemical stress were also reported by others (Vila-Aiub et al., 2009b), including attempts to isolate physiological, ecological and evolutionary processes (Medina et al., 2007).

Our results showed that acquiring tolerance introduced a cost. This was evident when the adapted population was grown in absence of PPCPs, yielding a lower growth rate (relative to the non-adapted one). Physiological and evolutionary trade-offs are broadly treated and described in biological literature, and different theoretical bodies provide explanation or acknowledge their existence as a postulate (Bazzaz et al., 1987; Lerdau and Gershenzon, 1997). Trade-offs between growth and cell size could reflect the need to balance investment in tolerance at expense of energy expenditure on other fundamental processes. Trade-offs could theoretically originate both from physiological acclimation, or ecological and evolutionary adaptations. Their effects on population demographic rates emerge when individuals capable

of expressing metabolic paths or molecular arrangements conferring stress tolerance (at the expense of other fundamental functions) increase their frequency in the population. Here we showed that a two-month continuous sub-lethal exposure to PPCPs set a new environmental optimum selecting tolerant organisms with a significantly different morphology (i.e. cell size) and higher fitness (i.e. higher recruitment rate). This resulted in a stress tolerant population with growth dynamics that were different from the wild type both in the presence and in the absence of the stressors. Similar findings indicating emergence of trade-offs in rapidly adapted phytoplankton were also reported elsewhere (Jin and Agustí, 2018b). Our study complemented and expanded these results showing that the selectivity of the environment was significantly controlled by ambient DOM levels.

The co-variance between growth rates, recruitment rates and cell size indicated a tight interconnection between stress response of these variables and the acquisition of tolerance. Cell size results basically mirrored the patterns observed for growth rates. Similar to growth rate, tolerance acquisition reduced negative effects of PPCPs on cell size. At the same time, the trade-off led to a smaller cell size of the adapted population in the absence of the contaminants, relative to the non-adapted population. Recruitment rates responded similarly but in this case the benefits of tolerance acquisition appeared more clearly. Adapted microalgae growing in the presence of the contaminants yielded recruitment rates comparable to those of the wild type growing in absence of stress.

Recruitment rates results demonstrated that tolerance acquisition fundamentally concerned allocation of resources toward maximizing fitness in the selective environment (i.e. in the presence of PPCPs) at the cost of a smaller cell size. Cell size changes accounted for a considerable fraction of the biovolume-derived growth rate response. Reduced cell volume explained almost 100% of the observed growth rate inhibition in phase II of the population adapted in the absence of DOM (not shown). In contrast, the relative contribution of cell size

change in the growth rate loss in the presence of PPCPs ranged 20-50% (not shown). Disentangling the influence of recruitment rate and cell size on the growth rate allowed to reveal another interesting effect related to tolerance acquisition. When grown in presence of PPCPs, the adapted population yielded a higher recruitment of larger-sized cells, compared with the non-adapted population. This was especially visible for the treatment with no DOM addition during the adaptation period.

Whether the acquisition of tolerance implied a net advantage when balanced against its costs was a question deserving attention. Based on the experimental results, B_{adp} and D_{adp} were positive and negative, respectively. The net trade-off of the adapted population tended to be positive, suggesting that the acquisition of tolerance generally resulted in a net benefit for the population. This had implications on how adapted populations would behave in variable environments in which phases of stress periodically followed phases of non-stress (i.e. a lake receiving contaminated waters intermittently). In such an environment (assuming stress periods were equivalent to periods of non-stress) the adapted population could theoretically have a two-fold competitive advantage: first, by yielding higher biomass over time that the non-adapted one. Second, by having a net fitness advantage. This indicated that PPCPs potentially represented an important selective force in impacted ecosystems, and that chemical pollution should be included more frequently in the study of multi-stressor ecosystem responses.

2.4.2.1 Phase II - Effects of DOM on tolerance acquisition and trade-off

The presence of DOM during the adaptation period reduced tolerance acquisition (both in terms of growth and recruitment rates) and appeared to lower both B_{adp} and D_{adp}, in line with our hypotheses. Based on the results of phase I, DOM and high pH mitigated the selective pressure hindering tolerance acquisition by the stressed algae. Similar findings suggesting a proportional response of tolerance acquisition in relation to stress intensity were reported elsewhere

(Kirkpatrick and Peischl, 2012; Uecker and Hermisson, 2011). In our case, stress mitigation depended upon an environmental factor (DOM) of great relevance for freshwater ecosystems and under fundamental biogeochemical control (Monteith et al., 2007; Roulet and Moore, 2006). While growth rate and recruitment rate were dependent on DOM levels during the adaptation period, the net trade-off was not. This is obviously because both B_{adp} and D_{adp} grown in their absolute value at increasing level of DOM during the adaptation period, compensating for their off-set. As discussed above, despite a positive net trade-off of adaptation that was apparently independent from DOM, the increasingly large gap in the growth rate response of adapted algae in the presence and the absence of PPCPs had interesting implications. It suggested that the population that gained tolerance in the absence of DOM, developed faster response dynamics to changes in stress levels. As a result of a similar net trade-off, this population was expected to experience more rapid biomass losses at the onset of the stressor, and to recover faster at the stress release, compared to the populations that partially acquired tolerance in the presence of DOM. In contrast, this population was expected to respond to changes in stress levels smoothing biomass loss and gains. These different behaviours, embodied in the different growth dynamics and trade-offs, represented two alternative strategies to stress-response. In the broader ecological context, the co-existence of adapted and non-adapted populations in a community could have implication on community structuring, functioning and ultimately ecosystem resilience (Enquist et al., 2015).

2.4.3 Environmental significance

Through the use of sub-lethal concentrations of a mixture of PPCPs as stressor model and DOM as model of environmental control, we showed that the interaction of stressors and the environment modulated adaptation processes and the unfolding of associated functional trade-offs. We added here more empirical evidence for the key role of DOM and pH in mediating toxic responses to PPCPs (Rowett et al., 2016), showing that both the direct effect of DOM, as

well as its interaction with chemical pollutants on algae growth were highly dependent on pH. These findings suggested that laboratory toxicity tests conducted through standardized methodologies could lead to a wrong estimation of the toxicity of PPCPs and other trace organics by ignoring effects of speciation / complexation on the contaminants' bioavailability induced by water chemistry (DOM and pH). As the effects of DOM and pH have been widely recognized in the BLM set for ecological risk assessment in metals (Environment Agency, 2009), we support the recommendations raised by other relevant studies (Rowett et al., 2016), namely that a similar approach should be used for micropollutants to provide the most relevant standards.

Furthermore, our results complemented the findings of other recent studies showing acquisition of tolerance to chemical stress triggered by multi-generational exposure to the same stressor (Bell, 2017; Medina et al., 2007). In addition, we reported new empirical evidence of the costs and net trade-offs associated to tolerance acquisition. DOM could counteract the process of tolerance acquisition when algae were exposed to sub-lethal levels of chemical stressors for multiple generations. This, in turn, had implications also for costs associated to tolerance acquisition. Adapted algae had relatively higher growth rates when growing in the presence of the stressor compared to non-adapted ones, and, on the contrary, had lower growth rate in pristine conditions. While DOM affected these rates, their net trade-off was positive and DOM-independent, suggesting that acquiring tolerance was generally advantageous for the algae, and could represent a significant selective pressure in impacted ecosystems.

Tolerant microalgae displayed higher recruitment rates and smaller cell size when grown in the presence of PPCPs, indicating tolerance acquisition coincided with allocation in fitness at the cost of a smaller cell size. This strategy allowed tolerant microalgae to compensate a considerable part of the growth rate loss due to PPCPs.

At the same time, our study illustrates the response of only one single species under standardised laboratories conditions. While extremely useful to understand the process of rapid adaptation and trade-offs, such a study cannot entirely capture the complexity of the real-world conditions. For instance, freshwater ecosystems are constantly exposed to a multitude of different chemicals contaminants and environmental factors, which may have a number of antagonistic and synergistic effects on biota (Fischer et al., 2013), not to mention a series of intra- and inter-species interactions occurring at community level (Enquist et al., 2015). In addition, under real-world conditions, adaptation to a certain group of contaminants does not guarantee the same level of tolerance towards other groups of stressors. For instance, according to energy allocation theory (Bazzaz et al., 1987; Lerdau and Gershenzon, 1997), adaptation to a certain stress may decrease the tolerance of biota when in the presence of other novel stressors (Carlson et al., 2014; Samani and Bell, 2016). Hence, more research should be oriented into this direction.

Our results also added new insights to the impacts of water browning. Since browning is caused by increasing levels of DOM (Monteith et al., 2007), our findings suggest that while this process might mitigate the detrimental effects caused by ubiquitous organic contaminants, at the same time antagonistic effects on the tolerance acquisition of stressed populations should be considered as one of its potential implications. In addition, since the presence of micropollutants might be associated with excess nutrient loading, a potential interaction between these two stressors would be worthwhile to study.

In conclusion, results presented here could be useful to guide future assessments on the ecological and evolutionary consequences induced by the process of browning in freshwater ecosystems that are also recipient of wastewater discharges, and might be beneficial to inform environmental management in a multi-stressor context.

2.5 Acknowledgments

2.5.1 General

The authors thank Rolf Vogt from the Department of Chemistry, University of Oslo for providing the DOM used in this study. The authors wish to thank Per Færøvig of the Section of Aquatic Biology and Ecotoxicology, University of Oslo for helping with the technical aspect of the experimental setup.

2.5.2 Funding

This study was financed by the Research Council of Norway, project grant no. 244460, Pollution and Ecosystem Adaptation to Changes in the Environment (PEACE). The research was co-funded by a discretional internal grant from the Norwegian Institute for Water Research (NIVA), under the Institute Strategic Priorities (SIS) on ecosystem remediation (TRADE-OFF). SR was also supported by the Lancaster University Faculty of Science and Technology and a Lancaster Environment Centre PhD Scholarship.

Chapter III: Ecological memory to historical contamination influences the response of phytoplankton communities.

Didier L. Baho[1,2*], Simone Rizzuto[3], Luca Nizzetto[1,4*], Dag O. Hessen[5], Jon Norberg[6], Birger Skjelbred[1], Kevin C. Jones[3], Hao Zhang[3] and Eva Leu[7]

[1] Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, 0349, Oslo, Norway

[2] Department of Aquatic Sciences and Assessment, Swedish University of Agricultural Sciences, Uppsala, Sweden

[3] Lancaster University, Lancaster Environment Centre, Lancaster, United Kingdom

[4] RECETOX, Masarik University, Kamenice 753/5, 625 00 Brno, Czech Republic

[5] University of Oslo, Dept. Biosciences, Box 1066 Blindern, 0316, Oslo, Norway

[6]Stockholm Resilience Centre, Stockholm University, Kräftriket 2B, SE-10691, Stockholm, Sweden

[7] Akvaplan-niva, CIENS, Science Park, Gaustadalléen 21, 0349, Oslo, Norway

* Corresponding authors email: didier.baho@slu.se, luca.nizzetto@niva.no

Published in: D. L. Baho, S. Rizzuto, L. Nizzetto, D. O. Hessen, J. Norberg, B. Skjelbred, K. C. Jones, H. Zhang, E. Leu, 2021. Ecological memory to historical contamination influences the response of phytoplankton communities. Ecosystems. https://doi.org/10.1007/s10021-021-00604-0**.** To access the Supplementary Material please consult page S16 of the "Appendices and Supplementary Material" file or visit https://doi.org/10.1007/s10021-021-00604-0. Part of this paper was presented at SETAC 2019 and won best presentation award (Appendix Chapter III on page S31).

Abstract

Ecological memory (EM) recognizes the importance of previous stress encounters in promoting community tolerance and thereby enhances ecosystem stability, provided that gained tolerances are preserved during non-stress periods. Drawing from this concept we hypothesized that the recruitment of tolerant species can be facilitated by imposing an initial sorting process (conditioning) during the early stages of community assembly, which should result in higher production (biomass development and photosynthetic efficiency) and stable community composition. To test this, phytoplankton resting stages were germinated from lake sediments originating from two catchments that differed in contamination history: one impacted by long-term herbicides and pesticides exposures (historically contaminated lake) from an agricultural catchment compared to a low-impacted one (near-pristine lake) from a forested catchment. Conditioning was achieved by adding an herbicide (Isoproturon, which was commonly used in the catchment of the historically contaminated lake) during germination. Afterwards, the communities obtained from germination were exposed to an increasing gradient of Isoproturon. As hypothesized, upon conditioning, the phytoplankton assemblages from the historically contaminated lake were able to rapidly restore photosynthetic efficiency ($p > 0.01$) and became structurally (community composition) more resistant to Isoproturon. The communities of the near-pristine lake did not yield these positive effects regardless of conditioning, supporting that EM was a unique attribute of the historically stressed ecosystem. Moreover, assemblages that displayed higher structural resistance concurrently yielded lower biomass, indicating that benefits of EM in increasing structural stability may trade-off with production. Our results clearly indicate that EM can foster ecosystem stability to a recurring stressor.

Keywords: Ecological memory, phytoplankton communities, stability, recurrent stressor, community tolerance, trade-off

Highlights

Contamination history determines community responses when a stressor recur

Communities that had previous encounters with stressor(s) show higher resistance

Higher community resistance can result in a trade-off with biomass production

3.1 Introduction

The concept of ecological memory (EM – Padisak, 1992) proposes that past experience influences present day responses of ecosystems, thereby enabling communities to cope better with recurrent stress (Turner, 2010, Johnstone et al., 2016). Ecosystems that have been exposed to recurrent stressor(s) can acclimatize and eventually adapt (Ogle et al., 2015; Samani and Bell, 2016) through physiological, ecological and evolutionary processes (Collins and Gardner, 2009). Over longer time scales, adaptations involving ecological (Blanck, 2002) and evolutionary (Samani and Bell, 2016; Bell, 2017) processes are more likely to underpin EM. Ecological adaptation emphasizes the replacement of stress sensitive species with tolerant ones (a phenomenon described, amongst others, in the concept of pollution-induced community tolerance [PICT]; Blanck 2002). Evolutionary adaptation involves the selection of strains or organisms carrying genetic variants or modifications that confer resistance (Bell, 2017). If these adaptations are partly or fully maintained during periods of non-stress, EM is established and the community can cope efficiently when the recurring stressor reappears (Padisak, 1992; Scheffer and Carpenter, 2003; Johnstone et al., 2016). EM can contribute to enhance the stability of the ecosystem by promoting resistance and recovery (Donohue et al., 2016; Hillebrand et al., 2018). Resistance can be expressed by the ability to withstand stress whereas recovery addresses the ability to regain normal functions and structures after being impacted (Hillebrand et al., 2018). Resistance and recovery can be measured in terms of functional (e.g.

biomass production and resource use) and structural (community composition) characteristics (Hillebrand et al., 2018).

Evidence of the causal relationship between earlier encounters to a stressor and present-day tolerance stems mostly from observational studies or theoretical models (Peterson, 2002; Ogle et al., 2015; Hughes e al., 2019), while experimental approaches targeting natural communities are rare (Feckler et al., 2018). An inherent limitation of observational approaches is that they typically focus on communities that are incidentally available at a given time point (snapshot), which might be blurred by other drivers (Cochran and Chambers, 1965). Under such circumstances, the acquisition of adaptation may not be fully expressed or detectable during stress-free periods, despite still being present in an inactive form, i.e., as dormant stage (Orsini et al., 2013). Organisms that have the ability to produce long lasting resting stages represent a useful experimental model since these can act like "seed banks" containing previous species assemblages that span over an extended period of time stage (Orsini et al., 2013). In phytoplankton, the formation of resting spores or cysts is a common strategy stage (Orsini et al., 2013) and can be considered as natural biological archives that offer a good opportunity to study whether or how EM helps to recruit species that gained tolerance through past adaptations (Ellegaard et al., 2018). Hence, phytoplankton germination experiments offer a good model to study EM (Padisak, 1992). Moreover, some anthropogenic stressors, such as pesticides, are relatively well monitored and offer the prospect of investigating how communities that have been repeatedly exposed to the same stressor can develop tolerance (Blanck, 2002). The contamination of freshwaters by pesticides from agricultural fields (Fölster et al., 2014) is one of the few stressors that have been monitored over time scales (decades) relevant for ecological and evolutionary adaptation (Thompson 1998).

Pesticide (including insecticides, fungicides and herbicides) runoff from agricultural fields can adversely affect diversity (Tilman et al, 2002), functioning and ecosystem services in

freshwaters (Vörösmarty et al., 2005, 2010; Weatherhead and Howden 2009). Pesticides can decrease the fitness of non-target aquatic organisms (Beketov and Liess, 2008) by altering their enzyme activity and metabolism (Sturm et al., 2007). They can also alter community structures (Rohr and Crumrine, 2005) by increasing mortality of sensitive species (Schroer et al., 2004). Herbicides specifically target groups of organisms that carry out photosynthesis such as phytoplankton (Brock et al., 2000). Nevertheless, the long-term effects of herbicides exposure on algae still remain unclear (Schäfer et al., 2011). Empirical evidence showed that certain herbicides (e.g. Atrazine) can shift the distribution of sensitive species towards more tolerant species and thereby increase community tolerance (Bérard and Benninghoff, 2001; Seguin et al., 2002). However, the net cost of acquiring tolerance may involve a trade-off, for instance, with production. The most tolerant species may not be the most productive ones (Moe et al., 2013; Rizzuto et al., 2020). Such trade-offs are generally overlooked (Medina et al., 2007).

Here, we used a two-phase experiment to evaluate the significance of EM in influencing the responses of natural phytoplankton from a lake that has been historically exposed to various herbicides that leached from the surrounding agricultural catchment. During the first phase of the experiment phytoplankton assemblages were germinated (from sediments) and simultaneously conditioned to an herbicide (presence vs. absence of Isoproturon: 12 µg/L), for 17 days. In the second phase of the experiment, communities that were obtained from the previous germination stage were exposed to a broader concentration gradient (0 µg/L, 7 µg/L, 12 µg/L, 61 µg/L and 92 µg/L) of the same herbicide for 7 days. During the second phase of the experiment functional endpoints related to production (total biomass and photosynthetic efficiency), and structural characterization of the phytoplankton assemblages (community composition) were monitored. We hypothesized (H1) that the presence of the herbicide (hereafter named conditioning) during germination of phytoplankton originating from the historically contaminated lake yields communities that are more structurally resistant and able

to maintain a higher production under stress. The underlying assumption is that conditioning facilitates the recruitment of tolerant species (Kraft et al., 2015), which were selectively favored by previous stress episodes. The selection process of tolerant species is captured by the PICT concept (Blanck, 2002), whereas EM (Padisak, 1992) adds a temporal dimension to the process and emphasizes the persistence of tolerant species over time. During non-stress periods tolerant species might lose their advantages to more competitive non-tolerant species (Tilman, 1982), but can still be present in seed banks and brought back during unfavorable conditions. In order to contrast with H1, the same conditioning and exposure procedures were applied to phytoplankton assemblages that were germinated from lake sediments of a near-pristine, forested catchment, that had no historical exposure to the herbicide and therefore potentially lacked tolerant species and were potentially more vulnerable. In this case, we hypothesized (H2) that conditioning is ineffective in yielding a more productive and structurally resistant community, due of the lack of EM and this can increase their sensitivity to the second herbicide exposure.

3.2 Materials and Methods

3.2.1 Sediment collection

During August 2017, sediment was collected with a corer from two Swedish lakes that mainly differed in their catchments. The lakes have similar ambient climate, physical and chemical characteristics (trophic status, water depth and submerged aquatic macrophytes consisting mostly of *Myriophyllum* genus [watermilfoil]; Text S3.1, Figure S3.1). Finnsjön (60°21'45.1"N, 17°52'56.1"E,) is a near-pristine lake with a forest dominated catchment and Tåkern (58°21'07.0"N, 14°49'42.7"E) is a historically contaminated lake that drains from an area associated with intensive large-scale agricultural use (Text S3.2, Table S3.1). At least 15 different cores were collected from each lake. The upper oxic layer (ca. 5cm) from the sediment cores was carefully sectioned and temporarily stored in a cooler. Once in the laboratory,

sediment samples from the same catchment were mixed to obtain an aggregated seed bank, then sieved (mesh size of 5 mm) to remove large materials (stone, roots and debris) and stored in the dark at 4 °C until the start of the experiment.

3.2.2 Model stressor and pilot study

The phenylurea herbicide Isoproturon was selected as a model stressor as it was commonly used and previously analyzed in the catchment of the historically impacted lake (Table S3.1). Isoproturon was widely used, for its inhibitory properties that disrupt the electron transport in photosystem II by binding to the protein D1 in the thylakoid membrane (Arnaud et al., 1994), until its ban from use in the European Union in 2016. The concentrations of Isoproturon used during the experiment were determined using an eco-toxicological test for growth inhibition (OECD guideline, Test No. 201) using lab-cultured algae (*Pseudokirchneriella subcapitata*, recently revised and renamed to *Raphidocelis subcapitata* (Suzuki et al., 2018)) and phytoplankton community assemblages from the two selected lakes. Based on the results of the growth inhibition test (Text S3.2, Figure S3.2), four concentration levels were selected: 7, 12, 62 and 92 µg/L causing approximately 5, 10, 70 and 90% growth inhibition, respectively. During the first phase of the experiment a single Isoproturon concentration (12 µg/L) was used for conditioning while four increasing levels (L1: 7, L2: 12, L3: 61 and L4: 92 µg/L) of the same herbicide were used during the second phase of the experiment. The stability of Isoproturon was assessed during the experiment using liquid chromatography mass spectrometry (Text S3.3). The measured Isoproturon concentrations varied moderately between the replicates of the different tested concentrations (Table 3.1). The difference between the nominal and measured concentrations on average varied by 26 % (Table 3.1). This difference was mainly caused by the behaviour of the solvent carrier (DMSO), which at the low temperature conditions of the climate room (4°C) tended to change its liquid consistence to a semi-solid one, inducing some difficulties into the spiking procedure. At the same time,

this difficulty did not influence the estimated magnitude of the inhibitory effect of the herbicide that was originally planned with the selection of the nominal concentrations.

Table 3.1. Comparison of the Nominal and Measured concentrations, expressed as time-weighted mean, of Isoproturon during the two different phases of the experiment.

Phase	Lake	Nominal concentrations (µg/L)	Time-weighted mean concentrations (µg/L)
I	Near-pristine	12	17.25
	Historically contaminated	12	17.81
II	Near-pristine	7	9.19
	Historically contaminated	7	11.22
	Near-pristine	61	46.94
	Historically contaminated	61	54.67

3.2.3 Experimental details

3.2.3.1 Phase I: Germination and conditioning phase

Phytoplankton communities were germinated from sediments in bioreactors (Figure 3.1). Bioreactors were divided into two equal sets. In one set germination and conditioning to the presence (+) of sub-lethal concentrations of Isoproturon (12 µg/L) occurred whereas in the other set Isoproturon was absent (-). Each unit (bioreactor) had 5 replicates, resulting in a total of 20 germination microcosms. Well-homogenized subsamples of 3 mL sediment were transferred into 250 mL glass jars (total 20). The glass jars (250 mL) containing the sediment were covered with steel woven nets (mesh size 60 µm) and were carefully placed into larger glass flasks (2.2L, Ikea, Sweden) before slowly adding 1.4L of Z8 medium (phosphate concentration of ca. 60 µg/L) to the bioreactors. The steel net was used as a barrier for zooplankton grazers to prevent them from reaching the outer flask, if they emerged from their

resting stages. Once the bioreactors were filled with culture media, they were sealed with acrylic clip-lock caps fitted with two air inlets (glass tubes) and an air outlet to facilitate resuspension of algae by gently bubbling filtered (0.2µm, Whatman, UK) air in the flasks for 3 minutes at regular intervals of 15 minutes. Isoproturon (Sigma-Aldrich, US) spiking solutions were prepared using dimethyl sulfoxide (DMSO) as a carrier solvent. Conditioning to Isoproturon during germination was achieved by pipetting 20 µL of a dimethyl sulfoxide (DMSO) solution containing 16.8 µg Isoproturon to half of the bioreactors, to reach a final concentration of 12 µg/L. Germination occurring in the absence of the Isoproturon (non-conditioned) was achieved by adding an equivalent volume of solvent only (DMSO) to the other half of the bioreactors, so as to rule out possible solvent related effects. The bioreactors were kept overnight at 4°C. The following day the temperature was programmed to increase at an approximate rate of 0.5 °C/hour to reach 13°C. Once the ambient temperature was reached, a diel light cycle was applied (light/dark: 16/8 hours, 30 µmol photons $m^{-2}s^{-1}$ light irradiance). The germination phase lasted for 17 days and at the end, the replicates were bulked according to the four experimental units (Figure 3.1). Bulking of replicates according to the four different germination scenarios was deemed necessary as it gives the opportunity to standardize the starting conditions for phase II.

3.2.3.2 Phase II: Isoproturon exposure

In the second part of the experiment, subsamples from the bulk of each individual treatment were inoculated in Erlenmeyer flasks and exposed to an increasing gradient of Isoproturon levels (L1-L4) and a control (0 µg/L), each in triplicate (total of 60 experimental units; Figure 3.1). The inoculum was standardized using chlorophyll-a concentrations measured as in vivo fluorescence. The inocula were diluted with freshly prepared Z8 medium to reach a final volume of 300 ml using the following dilution scheme: near-pristine (-) 1:5, near-pristine (+) 1:7, historically contaminated (-) 1:8, historically contaminated (+) 1:8. The standardized

inocula were exposed to the Isoproturon gradient (L1-L4). The exposure phase lasted for 7 days.

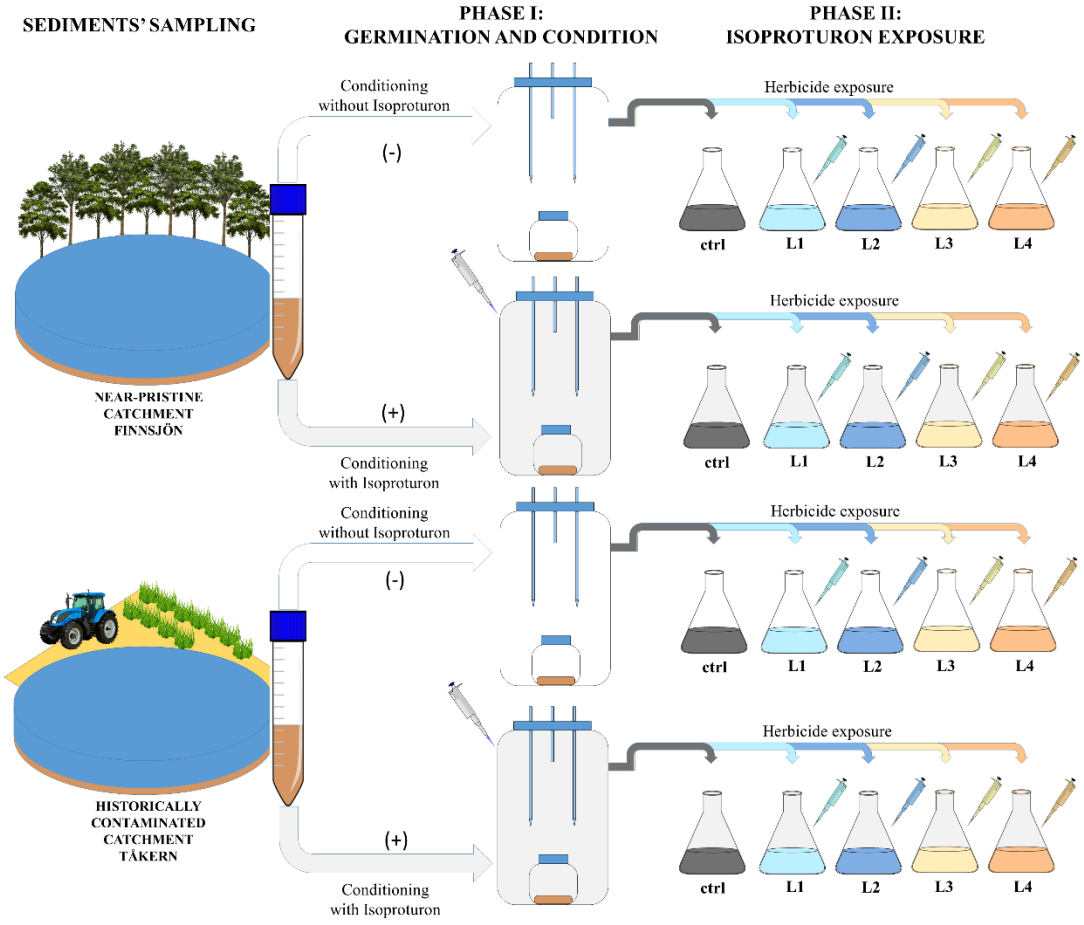


Figure 3.1. Conceptual figure illustrating the experiment design and the workflow divided into two phases: germination and exposure. During the first phase of the experiment, phytoplankton were germinated from seed banks while a subset (half) of the culture units was simultaneously conditioned with a sub-lethal concentration of Isoproturon (12 µg/L). The communities obtained at the end pf the germination phase were exposed to an increasing exposure concentration of Isoproturon (Ctrl: 0, L1: 7, L2:12, L3: 61, L4: 92 µg/L).

3.2.3.3 Phytoplankton responses: growth rate, total biomass, species composition and photosynthetic efficiency

The growth of phytoplankton was monitored daily during both phases using in vivo fluorescence, which was measured using a spectrophotometer (BioTek Synergy MX; Winosky, VT, USA). Triplicates from each sample (300 µL in each well) were loaded on 96 well clear flat-bottomed microplates (Corning, USA). Fluorescence was analyzed using the integrated

software Gen 5 (BioTek, US) with excitation and emission wavelengths of 440 nm and 685 nm, respectively. The growth rate µg (d^{-1}) was calculated as the slope of a linear regression for log-transformed in vivo fluorescence data against time (Hagman et al., 2019.

Samples were collected for species taxonomic identification at the end of both phases. They were analyzed from the bulked samples of phase I and from each replicate from phase II. The bulked samples of phase I were analyzed to downscale the effort used to identify species while providing adequate information that can be used: a) to evaluate differences across the two lakes and the effects of conditioning; b) and help to characterize the starting community composition of the second phase of the experiment. Phytoplankton were identified using the Utermöhl technique (protocol: CEN – EN 15204) and a light microscope, generally to the lowest taxonomic level (species). Total biomass (mg/m^3) was calculated from geometric conversions based on a standard protocol (CEN - EN 16695). Three species diversity matrices were derived from the taxonomy data: species richness, Shannon diversity index and evenness.

The effects of Isoproturon on photosynthetic efficiency (maximum quantum yield (F_v/F_m) of photosystem II), were quantified by using a modified procedure developed by Hanelt (1998) that involved measuring in vivo chlorophyll-a fluorescence by means of a portable pulse-amplitude modulated fluorometer (Water-PAM, Walz, Germany) and the software (WinControl, Walz) provided by the manufacturer. Prior to each measurement, the samples (aliquots of 10 ml) were incubated in the dark for 3 minutes. Thereafter, the minimum fluorescence F_o was determined by applying weak red light pulses, followed by short pulses (0.6 s) of strong saturating light to record maximum florescence F_m. F_v was calculated as the difference between the maximum and minimum fluorescence ($F_v = F_m-F_o$), where the yield (F_v/F_m) was indicative of the physiological status of the communities. The photosynthetic efficiency was measured at three different time points (day 1, 3 and 7) during phase II of the experiment.

3.2.4 Statistical analysis

All analyses were conducted using R (version 3.5.1) statistical software (R Core Development Team, 2015).

3.2.4.1 Phytoplankton growth rate, total biomass, species diversity matrices

The growth rate, total biomass, species richness, Shannon diversity index and evenness of phytoplankton were analyzed using linear mixed-effect regression models. During phase I, the effects of two main factors: contamination history (2 groups: near-pristine lake vs. historically contaminated lake) and conditioning (2 groups: presence and absence of Isoproturon during germination) were tested. During phase II, the effects of the following three factors: contamination history (2 groups: near-pristine lake vs. historically contaminated lake), conditioning (2 groups: presence and absence of Isoproturon during germination) and Isoproturon exposure (5 groups: control, L1-L4) were tested. Log transformation was used in some cases to fulfil the assumptions of normality. The interaction terms between the fixed factors were considered important for inference. When significant main effects were detected, pairwise comparisons based on estimated marginal means were used. Pairwise comparisons were complemented with effect sizes in some cases to assess the magnitude of treatment effects. Effect sizes based on Cohen's *d* values were calculated by taking the mean difference between two groups that was then divided by the pooled standard deviation (Cohen, 2013).

3.2.4.2 Photosynthetic efficiency

Time series data for the photosynthetic efficiency measurements were analyzed using a repeated measurement analysis of variance (ANOVA). Huynh-Feldt correction was applied when the assumptions of sphericity were breached. The photosynthetic efficiency data recorded during the last sampling (day 7) point were analyzed using a mixed model to assess if the effects of the Isoproturon exposures of phase II were significant.

3.2.4.3 Phytoplankton community composition and structural resistance

Multivariate analyses were used to evaluate the effects of the Isoproturon exposure (phase II) on the phytoplankton community composition and measure structural resistance. Non-metric multidimensional scaling (NMDS) based on Bray-Curtis similarity and square-root-transformed species matrix data obtained from the taxonomic analysis, was used to assess the effect of Isoproturon exposure. NMDS analyses were complemented with permutational multivariate ANOVA using Bray-Curtis similarity matrix, with 9,999 unrestricted permutations and applying Monte Carlo p-values corrections. Structural resistance was calculated using a similar method as previously described by Hillebrand and others (2018) that is based on geometric distance. Structural resistance was calculated as Euclidean distance between the centroid coordinates of the control, relative to those of Isoproturon exposures levels (L1- L4). The centroid coordinates were extracted from the NMDS plots. The closer the distances between phytoplankton communities of the control and the respective Isoproturon exposure levels (L1-L4) the higher the resistance.

3.3 Results

3.3.1 Comparing phytoplankton communities from the two lakes and assessing the influence of conditioning (phase I)

Germination started a few days earlier for the historically contaminated lake compared to the near-pristine lake (Figure S3.3), however both lakes achieved similar growth rate (Figure 3.2A). The earlier initiation of phytoplankton growth from the historically contaminated lake subsequently led to a higher total biomass compared to assemblages that originated from the near-pristine lake ($F_{1,8}$=1164.94, p <0.001, Figure 3.2B). Species richness (Figure S3.4A), Shannon diversity index (Figure 3.2C) and evenness (Figure S3.4B) of phytoplankton assemblages from the historically contaminated lake were substantially higher than the near-pristine lake (Table S3.2 and Figure S3.5). The species composition also differed between lakes

(Figure 3.2D), the proportion of Chrysophyceae was considerably higher in the near-pristine lake whereas Chlorophyceae were the most abundant algae group in both lakes.

The effects of conditioning (i.e., the presence of Isoproturon during germination) led to a decrease in Shannon diversity index ($F_{1,8}$= 65.80, $p < 0.001$) and evenness ($F_{1,8}$= 30.70, $p < 0.001$) in both lakes. In contrast, the effects of conditioning on the growth rate and species richness were negligible. Conditioning had a significant effect on the total phytoplankton biomass ($F_{1,8}$= 8.60, $p < 0.05$), which also appeared to significantly interact with the contamination history ($F_{1,8}$= 48.99, $p < 0.001$, Table S3.2). For instance, conditioning had a significant (Cohen' d = 3.89), negative effect on the total phytoplankton biomass of the historically contaminated lake whereas the opposite trend was observed for the near-pristine lake (Cohen' d = 2.1 and Figure 3.2B). Besides total biomass, significant interaction between conditioning and contamination history were not observed for; growth rate, species richness, Shannon diversity and evenness (Table S3.2). The species composition (Figure 3.2D) was marginally affected by conditioning.

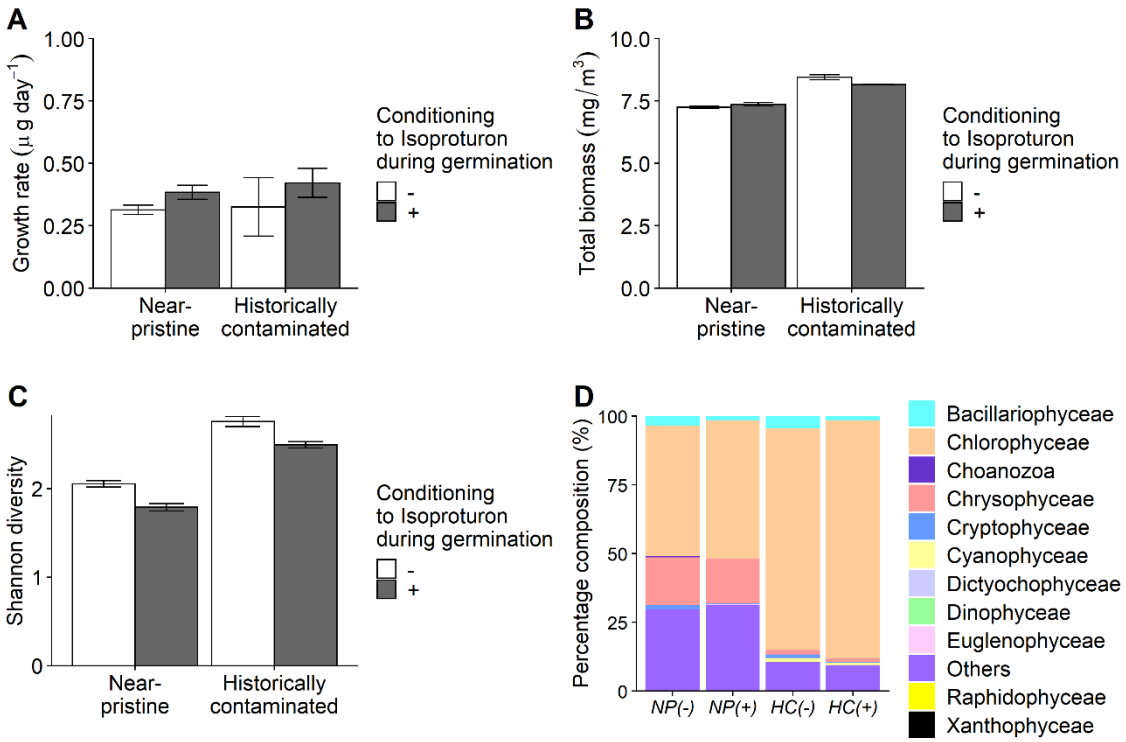


Figure 3.2. Effects of conditioning, during the first phase of the experiment, on growth rate (A), total biomass (B), Shannon diversity (C) and relative proportion of phytoplankton groups (D) in the communities originated from the two lakes (NP: near-pristine, HC: historically contaminated). Error bars when present indicate standard error.

3.3.2 Effects of Isoproturon exposure gradient on growth rate, total biomass and species diversity (phase II)

Exposure to the Isoproturon gradient (L1-L4) during the second phase of the experiment significantly affected total phytoplankton biomass, growth rate, species richness, Shannon diversity and evenness (Table 3.2, Figure 3.3). The interaction between Isoproturon exposure and conditioning was significant for species richness and Shannon diversity. The interaction between Isoproturon exposure and contamination history was significant for total biomass, species richness, Shannon diversity and evenness, whereas interaction between conditioning and contamination history was significant for growth rate, species richness, Shannon diversity and evenness. Interactions involving all three factors (Isoproturon exposure, conditioning and contamination history) were not significant for any of measured variables (Table 3.2).

Table 3.2. Summary of the effects of the contamination history, conditioning, Isoproturon exposure and the interaction term between the three factors on different endpoints; growth rate, total biomass, species richness, Shannon diversity and evenness of phytoplankton recorded during the second phase of the experiment. Significant values are reported in bold.

Variables	Effects	df	SS	F	p
Growth rate	**Isoproturon exposure**	**4, 60**	**2.09**	**89.25**	**< 0.001**
	Conditioning	1, 60	0.01	2.27	0.14
	Contamination history (Con his)	**1, 60**	**0.09**	**14.92**	**< 0.001**
	Isoproturon exposure: Conditioning	4, 60	0.01	0.61	0.66
	Isoproturon exposure: Con his	4, 60	0.01	0.34	0.85
	Conditioning: Con his	**1, 60**	**0.03**	**4.88**	**<0.05**
	Isoproturon exposure: Conditioning: Con his	4, 60	0.02	0.76	0.55
Total biomass	**Isoproturon exposure**	**4, 60**	**19.89**	**149.98**	**< 0.001**
	Conditioning	**1, 60**	**0.43**	**13.00**	**< 0.001**
	Contamination history (Con his)	**1, 60**	**1.81**	**54.46**	**< 0.001**
	Isoproturon exposure: Conditioning	4, 60	0.01	0.10	0.98
	Isoproturon exposure: Con his	**4, 60**	**0.57**	**4.31**	**< 0.01**
	Conditioning: Con his	1, 60	0.001	0.03	0.87
	Isoproturon exposure: Conditioning: Con his	4, 60	0.03	0.22	0.93
Species richness	**Isoproturon exposure**	**4, 60**	**146.83**	3.82	**< 0.05**
	Conditioning	**1, 60**	**93.75**	9.75	**< 0.01**
	Contamination history (Con his)	1, 60	0.42	0.04	0.84
	Isoproturon exposure: Conditioning	4, 60	79.17	2.06	0.10
	Isoproturon exposure: Con his	**4, 60**	**117.83**	3.06	**< 0.05**
	Conditioning: Con his	**1, 60**	**150.42**	15.64	**< 0.001**
	Isoproturon exposure: Conditioning: Con his	4, 60	46.17	1.20	0.33
Shannon diversity	Isoproturon exposure	4, 60	0.10	1.92	0.13
	Conditioning	**1, 60**	**0.23**	**17.10**	**< 0.001**
	Contamination history (Con his)	**1, 60**	**0.69**	**51.80**	**< 0.001**
	Isoproturon exposure: Conditioning	4, 60	0.13	2.36	0.07
	Isoproturon exposure: Con his	**4, 60**	**1.44**	**26.77**	**< 0.001**
	Conditioning: Con his	**1, 60**	**0.20**	**14.78**	**< 0.001**

	Isoproturon exposure: Conditioning: Con his	4, 60	0.07	1.36	0.26
Evenness	**Isoproturon exposure**	**4, 60**	**0.02**	**4.03**	**< 0.01**
	Conditioning	**1, 60**	**0.01**	**5.24**	**< 0.05**
	Contamination history (Con his)	**1, 60**	**0.06**	**44.10**	**< 0.001**
	Isoproturon exposure: Conditioning	4, 60	0.01	1.18	0.33
	Isoproturon exposure: Con his	**4, 60**	**0.09**	**17.75**	**< 0.001**
	Conditioning: Con his	1, 60	0.004	3.23	0.08
	Isoproturon exposure: Conditioning: Con his	4, 60	0.01	1.46	0.23

In addition, pairwise comparisons revealed some general patterns. For instance, exposure to the two highest Isoproturon levels (L3 and L4) led to a significant decrease in growth rate (Figure 3.4A) and total biomass (Figure 3.4B) of phytoplankton compared to the control and the low exposure levels (L1 and L2). The decrease in total biomass was more pronounced for the historically contaminated lake (Cohen's d values; Table S3.3) when exposed to the highest level of Isoproturon (L4; Figure 3.3B and Figure 3.4B). The effects of conditioning on growth rate varied across the different Isoproturon exposure levels for the historically contaminated lake, whereas conditioning generally led to a slight increase in growth rate of phytoplankton for the near-pristine lake (Figure 3.3A and Figure 3.4A). Nevertheless, growth rates of phytoplankton from the historically contaminated lake were consistently higher for the two highest exposure levels (L3 and L4) compared to the near-pristine lake (Figure 3.4A). The magnitude of change in the growth rate was also consistently higher (larger Cohen's d values between the control and the Isoproturon exposure levels L1-L4) for the near-pristine lake compared to the historically contaminated lake (Table S3.3). The highest exposure levels L3 and L4 did not reduce species richness (Figure S3.6), except for communities originating from the near-pristine lake that had been conditioned to Isoproturon during germination. Shannon diversity and evenness of the historically contaminated lake increased with increasing herbicide

levels, while the opposite was observed for the near-pristine lake (Figure 3.3C-D and Figure 3.4C-D).

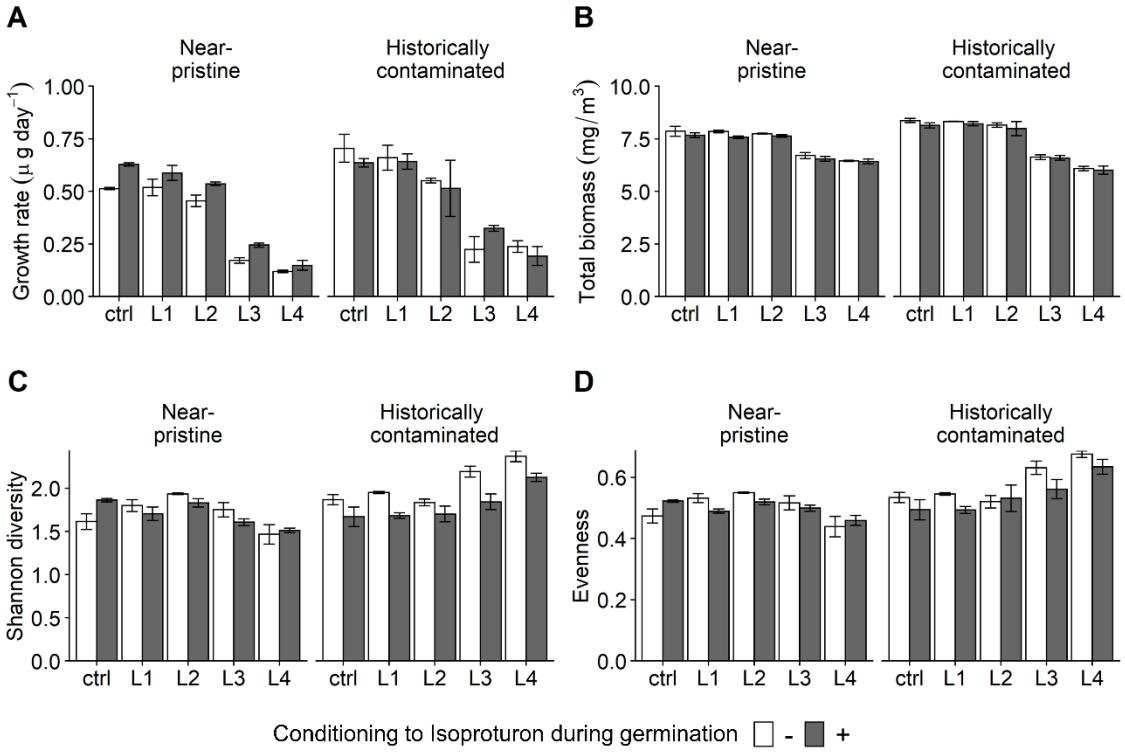


Figure 3.3. Comparison of growth rate (A), total biomass (B), Shannon diversity index (C) and evenness (D) of phytoplankton originating from the near-pristine and the historically contaminated lake, along the Isoproturon exposure gradient (L1-L4) used during the second phase of the experiment. Error bars indicate standard error.

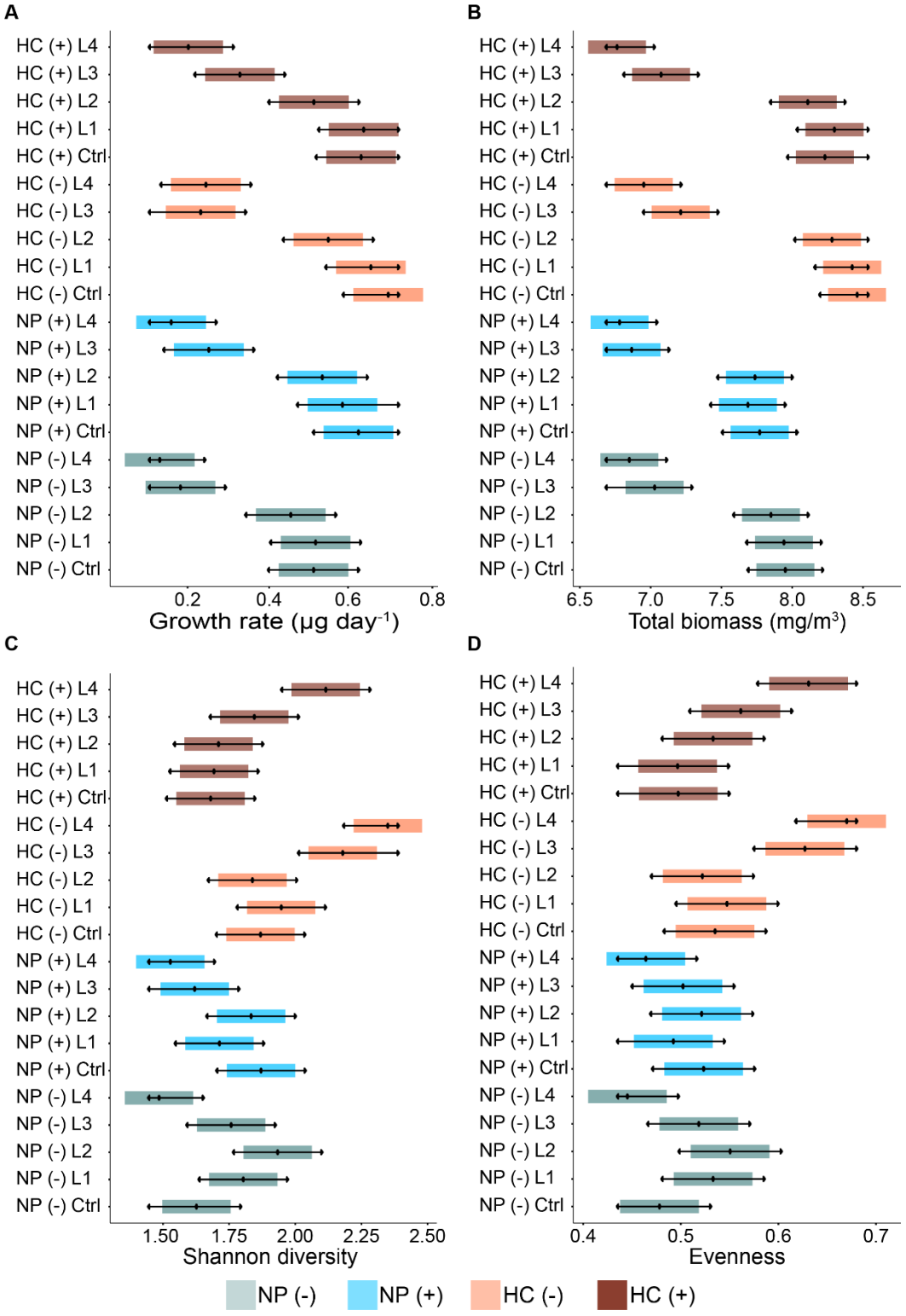


Figure 3.4. Pairwise comparison based on the estimated marginal means for the growth rate (A), total biomass (B), Shannon diversity index (C) and evenness (D) of phytoplankton observed during the second phase of the experiment. The central points in the figure indicate the mean response with 95% confidence interval for the combined main effects (contamination history, conditioning, Isoproturon exposure) for the near-pristine (NP) lake and the historically contaminated (HC) lake that were conditioned with (+) and without (-) Isoproturon during germination.

3.3.3 Effects of Isoproturon exposure gradient on photosynthetic efficiency (phase II)

Exposure to the Isoproturon concentration gradient significantly decreased the photosynthetic yield of the phytoplankton of both lakes (near-pristine lake; $F_{4, 20}$=51.4, $p < 0.01$, historically contaminated lake; $F_{4, 20}$ = 15.2, $p < 0.01$ and Figure 3.5). Furthermore, significant time effects and the interaction between time and Isoproturon exposure were observed in both lakes (Table S3.4). The relative decrease of the photosynthetic yield was generally stronger at higher Isoproturon exposure levels (L3-L4). The changes in photosynthetic yield over time were remarkably distinct between the two lakes and the conditioning scenarios (Figure 3.5), in particular for the last time point (day 7). The effects of the herbicide were still significant in the near-pristine lake on the last sampling event (day 7; Table S3.5). Furthermore, the differences between the control and the two highest levels (L3 and L4) of the near-pristine phytoplankton assemblages were larger when they were conditioned to the herbicide (L3: t = -11.8, $p < 0.01$, L4: t = -14.4, $p < 0.01$) compared to the non-conditioned scheme (L3: t = -0.34, $p = 0.73$, L4: t = -5.0, $p < 0.01$). In the historically contaminated lake, the effects of the Isoproturon exposure on day 7 (Table S3.5) were still significant ($F_{4,15}$=12.3, $p < 0.01$) when conditioning was omitted during germination.

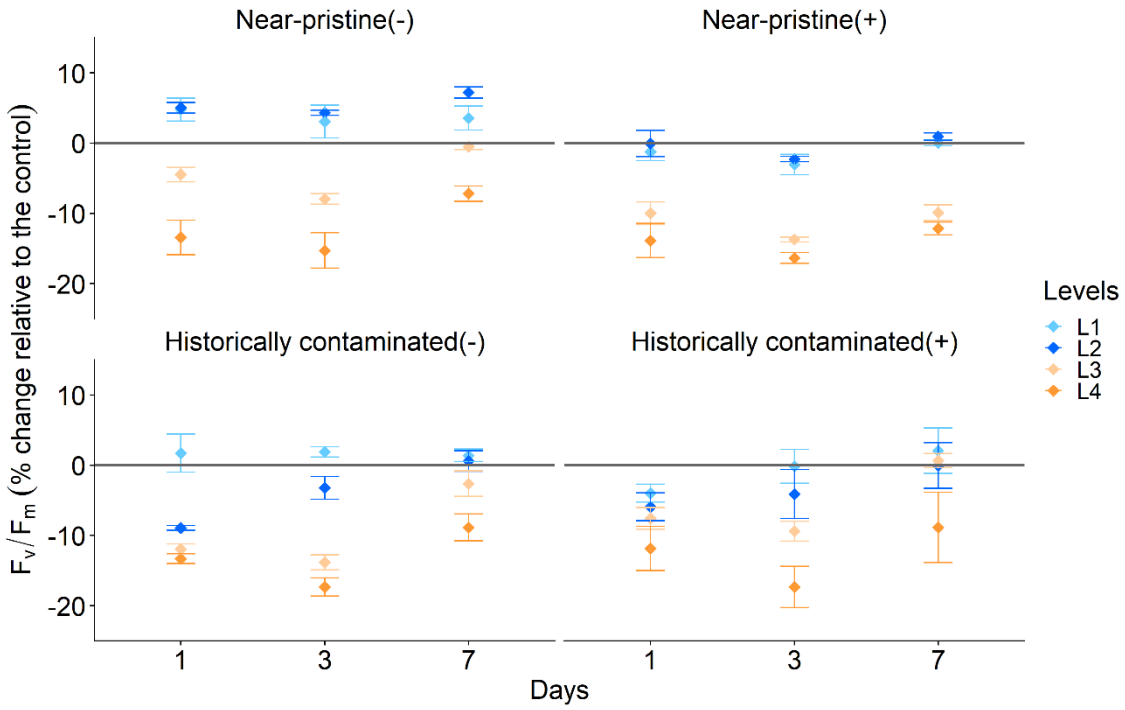


Figure 3.5. Photosynthetic efficiency (Fv/Fm): percentage of change relative to control in the near-pristine and historically contaminated phytoplankton communities that were conditioned with (+) and without (-) Isoproturon during germination. Error bars indicate standard error.

3.3.4 Effects of Isoproturon exposure gradient on community composition (phase II)

The composition of communities changed significantly (PERMANOVA: Table S3.6) in response to Isoproturon exposure. The relative proportions of most taxa decreased with increasing exposure levels (Figure 3.6A), apart from two groups (Chrysophyceae and unidentified group (others)) that increased in relative proportion at the two highest herbicide treatment levels.

Changes in community composition, depicted by the non-metric multidimensional scaling (NMDS) analyses (Figure 3.6B), were most pronounced at the highest exposure levels and reflected both the contamination history and conditioning during germination. Phytoplankton communities from the historically contaminated lake that underwent conditioning displayed higher structural resistance to the Isoproturon exposure. The distance between the control and the highest Isoproturon exposure level (L4) was shortened (Table 3.3, Figure 3.6B) when

conditioning was applied during germination compared to the non-conditioned analogues. The opposite was observed for the communities originating from the near-pristine lake system with regard to conditioning (Table 3.3, Figure 3.6B).

Table 3.3. Comparing community composition of the near-pristine and the historically contaminated lakes that were exposed to the Isoproturon concentration gradient.

Contamination history	Conditioning	Isoproturon exposure	t-value	P-value	Euclidean distance
Near-pristine	(-)	L1	0.93	0.46	0.16
		L2	1.37	0.19	0.29
		L3	**2.46**	**0.02**	0.44
		L4	**3.21**	**0.01**	0.38
	(+)	L1	1.32	0.19	0.15
		L2	1.61	0.09	0.26
		L3	**3.51**	**0.004**	0.53
		L4	**4.43**	**0.002**	0.53
Historically contaminated	(-)	L1	1.23	0.25	0.14
		L2	1.66	0.08	0.08
		L3	**4.01**	**0.002**	0.29
		L4	**4.23**	**0.002**	0.33
	(+)	L1	1.10	0.34	0.18
		L2	0.88	0.48	0.23
		L3	**3.91**	**0.002**	0.32
		L4	**3.98**	**0.004**	0.20

The Euclidean distance between the control and the different exposure levels (L1, L2, L3 and L4) centroids based on the NMDS plots, across the two lakes and the two different conditioning scenarios. Significant results are highlighted in bold.

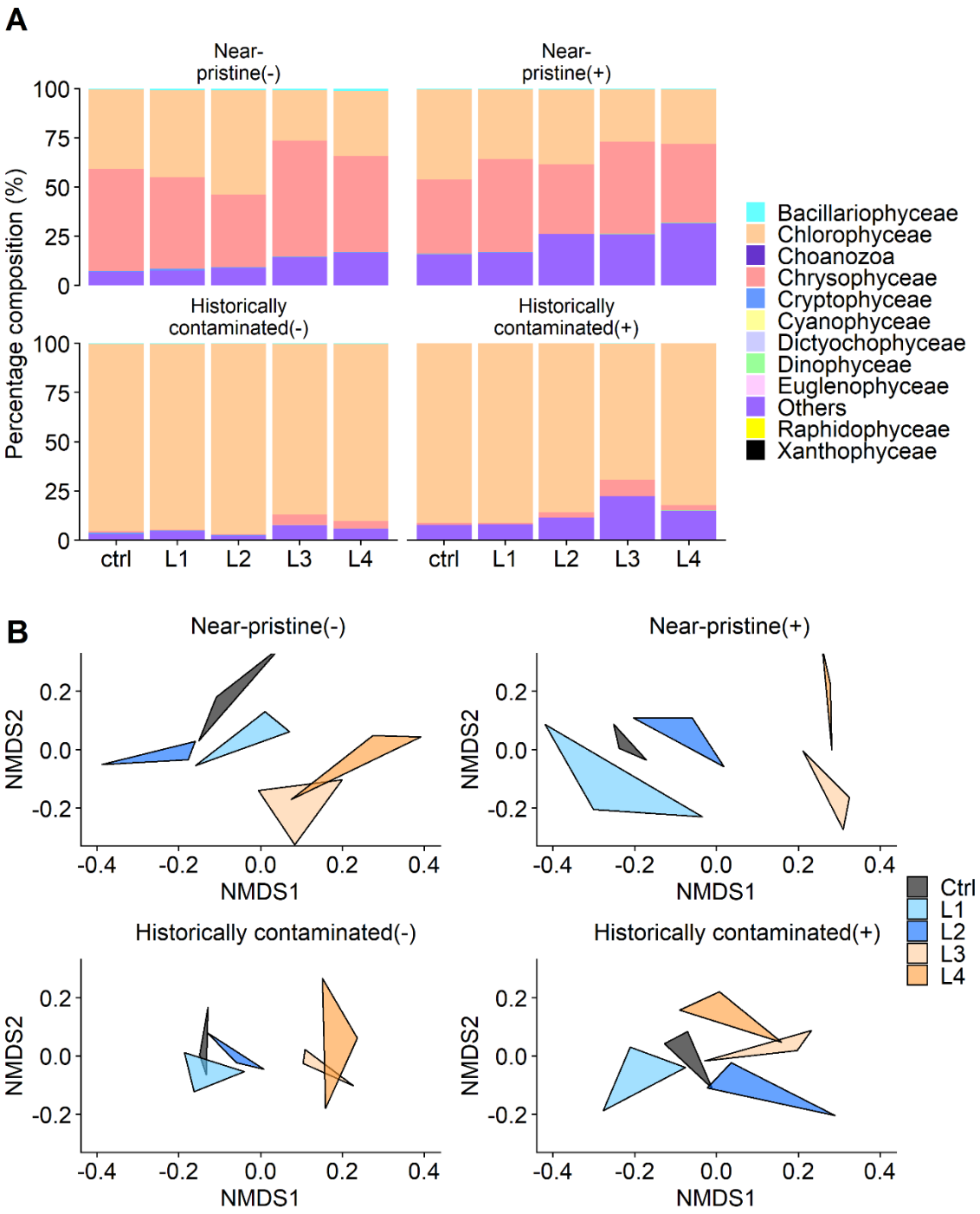


Figure 3.6. Changes in community structure observed during the exposure phase. (A) Percentage composition (relative abundances) of the major phytoplankton groups identified in the two lakes (near-pristine and historically contaminate) conditioned without (-) and with (+) Isoproturon; (B) non-metric multidimensional scaling plot (NMDS) from the four experimental units. The corners of the polygons represent the replicates. The stress values of the experimental units are 0.09, 0.07, 0.07 and 0.08 for NP(-), NP(+), HC(-) and HC(+), respectively.

3.4 Discussion

This study provides a proof of concept of the relevance of EM in influencing ecosystem stability during the resurgence of a recurrent stressor. In particular, we focused here on conditioning as a trigger for retrieving adaptations nested in EM that may not be expressed in the community assemblages during periods of non-stress. We took advantage of lakes with different herbicide exposure histories to obtain communities with and without "memory" of Isoproturon stress to test our hypotheses. However, it is important to acknowledge that beyond historical exposure to herbicides, the lakes also differ in other respects (e.g., differences in catchment characteristics, level of connectedness with other systems, etc.). To rule out confounding effects posed by these factors we focused on studying EM to a specific stressor by "retrieving" EM for Isoproturon through conditioning (i.e., by applying the same stressor during germination). Conditioning was used as a means to facilitate the recruitment of tolerant strains/species that were already present in dormant stages in the sediment of the historically contaminated lake. The experiment with phytoplankton communities from the near-pristine lake should therefore not be considered as a direct term of comparison but as a means to refute H1, and therefore test its robustness. Phase I of the experimental design was conceived to assemble natural communities harbouring different genotypes from two lakes that differ in contamination histories. During phase II, EM is evaluated by assessing the ability of phytoplankton assemblages from the historically contaminated lake to maintain high production and stable composition under the Isoproturon gradient.

The results from phase II of the experiment support our expectations that conditioning of the community from the historically contaminated lake facilitates the recruitment of tolerant species that had acquired adaptation in the past. Phytoplankton assemblages from the historically contaminated lake, upon conditioning, were in fact able to restore photosynthetic efficiency (Figure 3.5) and proved to be structurally (Figure 3.6 and Table 3.3) more resistant

when exposed to an increasing gradient of Isoproturon. At the same time, conditioning did not lead to the same effect in the near pristine lake (Figures 3.5-6 and Table 3.3).

3.4.1 Implications of EM for production: total biomass and photosynthetic efficiency

Total biomass and photosynthetic efficiency were monitored during the second phase of the experiment. The behaviour of the two parameters differed markedly and were dependent on the lake contamination background and conditioning. Conditioning did not increase the total phytoplankton biomass (Figure 3.3B) irrespective of the lakes' contamination history. In contrast, photosynthetic efficiency was restored within a few days, despite being impacted in the earlier stage of the second phase of the experiment (Figure 3.5), while Isoproturon was still present (Table 3.1). However, such an improvement in restoring photosynthetic efficiency was only observed in phytoplankton assemblages that originated from the historically contaminated lake following conditioning during gemination (Figure 3.5). The contrasting responses between photosynthetic efficiency and biomass development can reflect differences in the temporal scales of these processes (Kriegman et al., 2018). Physiological responses, such as photosynthetic efficiency, occur more rapidly, and most likely reflect changes in the regulation of photosystem reaction centers (Antonacci et al., 2018). This type of response is expected for organisms exposed to Isoproturon, as this herbicide is a photosystem II inhibitor (Antonacci et al., 2018). Herbicide-resistance has been shown to occur following a substitution mutation that altered structure of the targeted intracellular site, e.g., the D1 protein of Photosystem II (Antonacci et al., 2018). The improvement in photosynthetic efficiency might be linked to an increase in the prevalence of species/strains that express a mutation of the D1 protein of the photosystem reaction center to counteract the inhibitory effects of Isoproturon (Antonacci et al., 2018). In contrast to photosynthetic efficiency, biomass development may reflect changes in energy and resource allocation within organisms that might occur at a slower pace(van Straalen and Hoffmann, 2000). This time lag might explain the lack of higher total biomass

mediated by EM (Tilmon, 2008; Hertz et al., 2013), perhaps also due to the relatively short duration (7 days) of the second phase the experiment.

3.4.2 Implications of ecological memory for structural resistance

Following conditioning, the community composition of phytoplankton from the historically contaminated lake displayed a higher structural resistance (Figure 3.6 and Table 3.3), compared to the non-conditioned ones. Here, we observed that the distance between the centroids of the control and Isoproturon exposure levels (especially L4) for the phytoplankton communities decreased when conditioning was applied to phytoplankton from the historically contaminated lake. These results support our initial hypothesis (H1) concerning the structural stability aspects, and suggest that phytoplankton communities that have been previously exposed to a stressor can boost their capacity to cope with subsequent encounters to the same stressor (Johnstone et al., 2016). Such an increase in structural resistance was not observed in either the conditioned or the non-conditioned communities from the near-pristine lake, thus supporting H2. Regardless of conditioning, phytoplankton from the near-pristine lake showed weaker structural resistance (Figure 3.6B, Table 3.3). In this case the distance between the centroids of controls and the two highest treatment levels (L3 and L4; Figure 3.6B, Table 3.3) actually increased as a consequence of conditioning, suggesting an increase in community sensitivity.

Moreover, the benefits of ecological memory in promoting structural resistance might involve a fundamental trade-off with the ability to maintain high biomass production (Vinebrooke et al., 2004). Our results only provide some evidence of such a trade-off under a short-term designed experiment. For instance, the communities from the historically contaminated lake had a higher growth rate than the near-pristine lake (Figure 3.3) for the two highest treatment levels, but this did not result in larger biomass; this may relate to the occurrence of a trade-off. Similar trade-offs (i.e. the negative relationship between acquiring tolerance and building biomass) were observed by others (Coley et al., 1985; Strauss et al., 2002; Boivin et al., 2003;

Vila-Aiub and others 2009) as the most tolerant species might not necessarily be the most productive ones (Moe et al., 2013; Rizzuto et al., 2020). Further research is needed to fully elucidate trade-offs in the context of EM and over longer time scales.

Long-term herbicide exposure can favour the selection of tolerant strains (Schäfer et al., 2011) or species that could be stored and eventually retrieved from seed banks. The selection of tolerant species can occur through ecological adaptation. Ecological adaptation acknowledges the replacement of sensitive species with tolerant ones (i.e., pollution-induced community tolerance [PICT]; Blanck 2002), thereby helping to maintain key processes and structures. Previous empirical evidence showed that certain herbicides can shift the distribution of sensitive species towards more tolerant species and thereby increase the community tolerance (Bérard and Benninghoff, 2001; Seguin et al., 2002). In the present study, the hypothesis of an increase in the prevalence of tolerant species after conditioning was supported by the observed increase in Shannon diversity and evenness along the herbicide exposure gradient, which only occurred in phytoplankton assemblages of the historically contaminated lake (Figure 3.3C-D and Figure 3.4C-D). Changes in evenness have previously been shown to be a more robust indicator of ecological change than species richness (Hillebrand et al., 2008), which is in line with our findings.

Altogether, these results indicate that: a) acquired structural resistance by the community is a consequence of retrieving EM through conditioning; and b) EM is an attribute only of the systems that had historically been exposed to Isoproturon or other herbicides with a similar mode of toxic action. Since EM was retained in dormant stages, the role of conditioning was crucial in facilitating the recruitment of tolerant species, which were selectively favoured by previous stress episodes.

These results complement previous findings and provide new insights on the concept of EM. Hughes and others (2019) described beneficial effects of EM in coral reefs exposed to two successive heat wave events causing bleaching. The authors showed that the mortality of coral reefs decreased after the second heat episode (in 2017) compared to the first one (in 2016), where the first event increased the proportion of resistant species (Hughes et al., 2019). In another study, Feckler and others (2018), showed that the performance of microbial communities in decomposing leaf-litter from an agricultural stream was enhanced under exposure to pesticides compared to a community from a near-pristine stream. In addition to earlier assessments, our results strengthen the perception that previous encounters with a stressor matter (Samani and Bell, 2016) and can increase structural resistance, even when these adaptations are not present or prevalent in the standing crop community.

3.5 Conclusion

We used an experimental approach to test an ecological concept (EM) that has previously remained elusive to empirical testing. We showed that adaptations from past experience that are present in dormant phytoplankton stages in lake sediments, can be readily expressed when stressors reappear. Our results show the beneficial effects of EM in restoring certain processes (e.g., photosynthetic efficiency) and increasing structural resistance of the phytoplankton communities. However, they also indicate a trade-off between resistance and other processes related to biomass production. The lack of EM to herbicide in the phytoplankton from the near-pristine lake system did not measurably yield stress tolerant species and structurally resistant communities. In addition, other processes related to spatial distribution and dispersal of species (e.g., dispersal of meta-communities: Leibold et al., 2004) can also influence ecosystems and contribute to stability toward the stressors. Better knowledge on these temporal (EM) and spatial (dispersal of meta-communities) processes are essential to gain a deeper understanding of the abilities of ecosystems to cope with recurrent stress.

3.6 Acknowledgments

The authors wish to thank Per Færøvig of the Section of Aquatic Biology and Ecotoxicology, University of Oslo for helping with the experimental setup. The authors thank: Vladyslava Hostyeva and Petra Mutinova from Culture Collection of Algae (NIVA-CCA) of the Norwegian Institute for Water Research (NIVA) for preparing the nutrient medium used during the experiment; and the personnel from the visitor centre of Tåkern Naturum for lending us their boat during sampling. This study was co-funded by the Research Council of Norway (contract 244460) for the PEACE (Pollution and Ecosystem Adaptation to Changes in the Environment) project and an internal grant from NIVA under the Institute Strategic Priorities (SIS) on ecosystem remediation (TRADE-OFF). SR was supported by the Lancaster University Faculty of Science and Technology and a Lancaster Environment Centre PhD scholarship.

Data accessibility statement: Should the manuscript be accepted, all data supporting the results will be archived in an appropriate public repository (Figshare).

Author contributions: DLB, LN, DOH, JN and EL conceived the study and designed the experiment. DLB, SR, LN and EL carried out the experiment. The taxonomic classification and enumeration of phytoplankton was performed by BS. DLB and SR analysed the data and took the lead in writing the manuscript. All authors provided critical analysis of results, feedbacks, helped structuring and editing the manuscript.

Chapter IV: Influence of Ecological Memory on phytoplankton early assemblages: a trait-based approach

Simone Rizzuto[1*], Didier Baho[2], Kevin C. Jones[1], Hao Zhang[1], Birger Skjelbred[2], Francesco Pomati[3], Eva Leu[4], Luca Nizzetto[2,5*]

[1] Lancaster Environment Centre, Lancaster University, Lancaster, LA1 4YQ, UK.

[2] Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, 0349, Oslo, Norway.

[3] Department of Aquatic Ecology, Eawag: Swiss Federal Institute of Aquatic Science and Technology, Überlandstrasse 133, 8600 Dübendorf, Switzerland.

[4] Akvaplan-niva, CIENS, Science Park, Gaustadalléen 21, 0349, Oslo, Norway.

[5] RECETOX, Masaryk University, Kamenice 753/5, 625 00 Brno, Czech Republic.

*Corresponding authors email: s.rizzuto@lancaster.ac.uk.; luca.nizzetto@niva.com.

In submission: S. Rizzuto, D.L. Baho, K. C. Jones, H. Zhang, F. Pomati, E. Leu, L. Nizzetto, 2021. Influence of ecological memory on phytoplankton early assemblages: a trait-based approach. Sci. Tot. Environ. To access the Supplementary Material please consult page S32 of the "Appendices and Supplementary Material" file. Appendix Chapter IV on page S37 illustrates the mathematical concepts behind the calculation of the trait diversity indices used in this paper.

Abstract

Understanding the mechanisms influencing the process of ecological succession (ES) in ecosystems is crucial to predict the responses of ecosystems to environmental change. Chemical contaminants may significantly affect ES by hindering the emergence of more sensitive species. This process is particularly relevant in freshwaters, where phytoplankton is target of waterborne micropollutants and effects on community assembling during ES may produce repercussions on food web structure and trophic energy transfer. Communities historically exposed to chemical stressors can develop tolerance. The concept of Ecological Memory (EM) describes the process of tolerance development by communities (including, the tolerance induced by chemical stress). In this study, it was investigated, for the first time, the influence of EM of historical exposure to an herbicide in native environment on the early assembling of a phytoplankton community. Phytoplankton resting stages were germinated in presence and absence of an herbicide from lake sediments originating from two catchments with different contamination histories: one impacted by long-term herbicides and pesticides exposures (historically contaminated - HC) and one that did not previously experience such a stressor (near pristine - NP). The development of the community assemblages was evaluated and compared structurally and functionally through the analysis of trait distribution and trait diversity (TD) indexes (i.e. trait richness, evenness and divergence). The focus on traits enables using trait ecology to draw semiquantitative expectations on the responses, including comparing responses of communities hosting different sets of species. Results showed that EM of historical contamination allowed the HC community to maintain functions (growth rate) and structure (unaffected trait richness, evenness and divergence) during the early stage of ES under herbicide exposure. In contrast, assembling of the NP community was sensitive to the herbicides yielding lower production and diversity. Results support that EM of herbicide was

a unique attribute of the HC community, indicating historical exposure to agricultural runoff resulted in persistent adaptations likely through sorting of organisms with certain traits.

4.1 Introduction

Ecological succession (ES) is a series of changes in species composition and abundance unfolding over time in an ecological community in unsteady condition (Margalef, 1978; Reynolds et al., 1983). Colonization of habitats and algal blooms embodies ES. ES is driven by both deterministic and stochastic processes (Zhou et al., 2014), the comprehension of which can provide insights into how communities develop over time (i.e. primary succession) or recover from a disturbance (i.e. secondary succession) (Chang et al., 2018), and predict the responses of ecosystems to environmental change (Zhou et al., 2014). Multiple factors may influence ES, including site history, dispersal limitation, abiotic stressors, and biotic interactions that operates at a range of spatial scales (Blois et al., 2013; Chang et al., 2018). Anthropogenic stressors may have an impact on ES, too (David et al., 2020; Jiang et al., 2017; Jiao et al., 2016; Nikolova et al., 2021). There is evidence that chemical contaminants can directly affect community assembly mechanisms in different groups of organisms, with potential consequences for their community development (David et al., 2020; Jiang et al., 2017; Jiao et al., 2016; Nikolova et al., 2021). However, while the influence of natural factors on ES is relatively well-known, the impact of chemical contaminants on this process is still scarcely explored. In chronically polluted ecosystems, the chemical stressor may represent a dominant driver of community assembling, by sorting for more tolerant species or strains. In this study it is hypothesized that the history of chemical stress exposure can affect the way community structures and functioning unfold during an ES.

Contamination is a concern for the functioning and development of freshwater ecosystems (Vörösmarty et al., 2015, 2010). For example, many water bodies in Europe have received

loads of pesticides and herbicides over several decades through off-site drift or run-off from fields. Herbicides in particular can alter enzymatic activities and/or cellular metabolism in phytoplankton, thereby decreasing fitness of intolerant species (Beketov et al., 2013). Triazine and phenyl-urea herbicides are the most extensively used herbicides (Fernandez and Gardinali, 2016), specifically designed to inhibit the electron transport chain of photosystem-II through binding with a protein that is conserved across photosynthetic organisms (Arnaud et al., 1994). While the influence of chemical contaminants on phytoplankton is extensively studied (e.g. Beketov et al., 2013), their influence on ES in phytoplankton is scarcely studied. Effects of chemical pollution on ES in phytoplankton assembling processes can potentially indirectly induce effects on trophic transfer and eventually on food web structure and dynamics (Vellend, 2016, 2010).

Chronic exposure to sublethal concentrations of stressors can however induce adaptive processes that can influence the entire community composition and characteristics over long terms (Baho et al., 2021; Feckler et al., 2018). The theory of ecological memory (EM - Padisák, 1992) proposes that past experience influences present day response of ecosystems enabling communities to cope better with recurrent stress (Johnstone et al., 2016; Turner, 2010). EM to a given stressor operates over long time scales and it is retained in stress-free periods, embedding the influence of both ecological (Blanck, 2002) (e.g. through sorting of stress-tolerant individuals/populations) or evolutionary (Bell, 2017) processes. Such a theory fully assimilates the concept of Pollution-Induced Community Tolerance (PICT - Blanck, 2002).

Resting stages of phytoplankton are a useful experimental model to assess both EM and its influence on ES. They can in fact act as “seed banks” containing assemblages of species reflecting previous ecosystem states (Orsini et al., 2013). They can also be easily germinated obtaining a self-assembling community reflecting ecological and physiological adaptations from past conditions. Such a process embodies the early stage of an ES. Germinating resting

stages from sediments of historically contaminated lakes, both in presence/absence of the stressor, yields a valid experimental model to study the effect of EM on ES under controlled exposure conditions (Baho et al., 2021; Ellegaard et al., 2018). Previous studies indicated that EM can foster tolerance in functions (i.e. photosynthetic efficiency) and structure (i.e. community composition) in phytoplankton communities historically exposed to a phenyl-urea herbicide (Baho et al., 2021). The extent to which EM of previous stress can influence the process of ES is currently unknown. Investigating EM phenomenon on ES of natural phytoplankton is useful to elucidate whether adaptation to historical contamination contributes to dynamically shape the development of ecosystems.

Community dynamics are traditionally described based on the changing relative abundances of species. While this approach is informative, it prevents a direct comparison between different ecosystems (e.g. a historically contaminated lake (HC), vs. a near-pristine (NP) lake control). Furthermore, a species-centred view may blindfold the importance of inter- and intra-specific phenotypic variance between populations and its influence on ecological and evolutionary responses of communities to stressors. Trait-based approaches will instead bring these elements at the core of the analysis focus (Enquist et al., 2015; Hillebrand and Matthiessen, 2009; Reiss et al., 2009). Multiple traits measured community-wide at the individual level are the most promising and straightforward way to combine inter- and intra-specific variation in one single approach (Fontana et al., 2014). Organisms' traits determine their responses to the environment, and changes in trait diversity (TD), unlike species composition, can be mechanistically linked to the selective forces of the environments. Because of this, trait-based analyses can be used to compare responses of ecosystems carrying different species assemblages to a given stressor.

In the present study, the effect of the EM of an herbicide (Isoproturon (ISU)) occurring in the native environment of a historically contaminated lake, was investigated on the early

community assembly of phytoplankton from that same environment, via trait-based approach. The ecological theories of EM (Padisák, 1992) and ES (Margalef, 1978; Reynolds et al., 1983; Zhou et al., 2014) are instrumental to build a series of expectations from the experiment, which can be found in the next section (Experimental Expectations).

4.2 Material and Methods

This study is part of a larger two-phase experiment (Chapter 3, Baho et al., 2021). More details on the set up of the experiment and the germination method can be found in Baho et al. (2021).

4.2.1 Lake selection

Sediments were collected from lakes located in Sweden during August 2017. Lakes have similar ambient characteristics such as trophic status, water depth and submerged aquatic macrophytes (mostly *Myriophyllum* genus [watermilfoil]), but mainly differed in their catchments. Finnsjön (60° 21’ 45.1’’ N, 17° 52’ 56.1’’ E) is taken here as a eutrophic near-pristine (NP) system, being characterized by a forested dominated catchment that never received treatments with pesticides. Tåkern (58° 21’ 07.0’’ N, 14° 49’ 42.7’’ E) is a historically contaminated (HC) lake located in an area associated with intensive large scale agricultural use. At least 15 different sediment cores were collected from each lake. The upper oxic layer (ca. 5 cm) from the sediment cores was carefully sectioned and temporarily stored in a cooler. Once in the laboratory, sediment samples from the same catchment were mixed to obtain an aggregated seed bank, then sieved (mesh size of 5 mm) to remove large materials (stone, roots and debris) and stored in the dark at 4 °C until the start of the experiment.

4.2.2 Stressor

Isoproturon (ISU, CAS 34123-59-6) is a phenyl-urea herbicide that was commonly used and previously analysed in the HC lake (Baho et al., 2021), and was selected as a stressor. ISU had extensive use for its photosynthetic inhibitory properties (Arnaud et al., 1994) until its ban from

European Union in 2016. The sub-lethal concentrations applied during the germination phase (12 µg/L) were determined through a standard ecotoxicological test for algal growth inhibition (OECD, 2009), as reported by Baho and others (2021). The concentrations of the herbicide varied moderately between replicates but did not show evidence of degradation (Baho et al., 2021).

4.2.3 Experimental design and measured endpoints

Phytoplankton communities were germinated from the sediments of the NP and HC lake (Figure 4.1), in bioreactors, following the method reported by Baho and others (2021). The experiment was carried out for 17 days, where five replicates of both communities were germinated in presence/absence of 12 µg/L ISU. During the experiment, samples were collected from each replicate on day 0, 7, 14, 17, fixed with a solution of paraformaldehyde and glutaraldehyde (respective concentrations of 0.01 and 0.1%, pH 7 and total volume of 100 µL) and stored in the dark at 4°C prior to analysis using scanning flow-cytometry.

Community response measured at each time point included functional aspects (e.g. growth, through the total count of living phytoplankton cells) and trait diversity. The set of trait diversity (TD) indexes validated by Fontana and others (2014) and calculated from observations performed at individual level through a scanning flow cytometer (see below) were used. These included measurements of trait richness (trait onion peeling - TOP index) and evenness (trait even distribution - TED index) (Fontana et al., 2014). Trait divergence was calculated using the FDis index, originally developed by Laliberte and Legendre (2010) and modified by Fontana and others (2014). Trait-based approach was selected here to allow formulating theoretical expectations even when comparing responses from different ecosystems (HC vs. NP). More information on the calculation of trait indices can be found in Appendix Chapter IV (*Trait diversity calculation*).

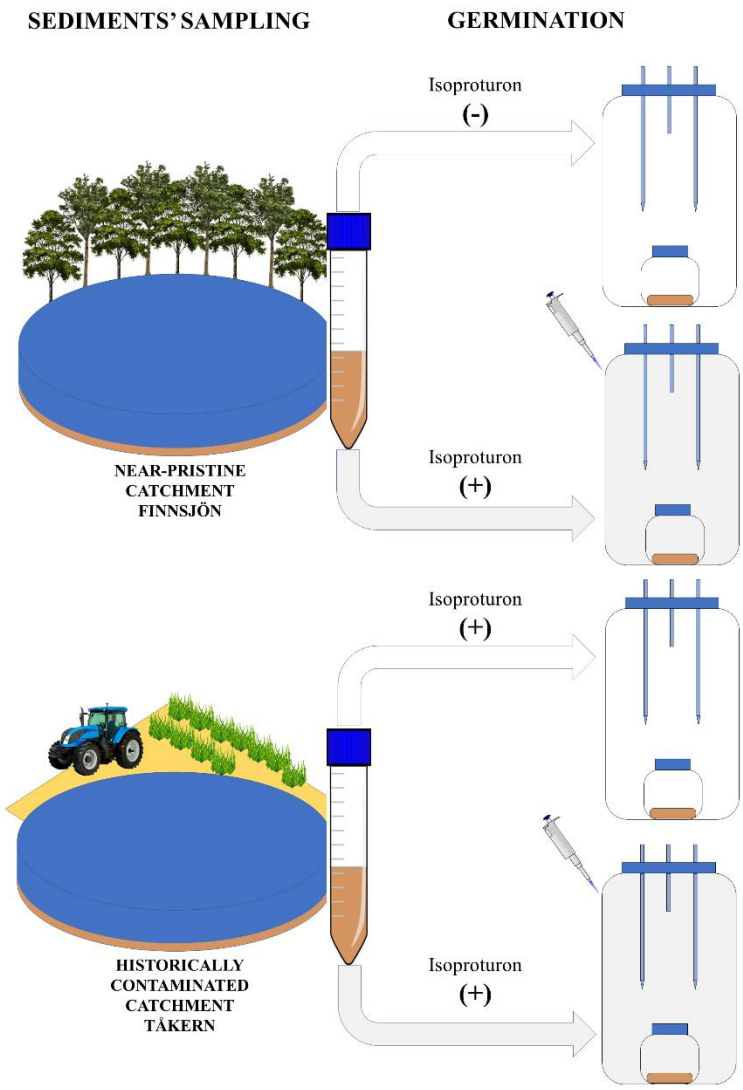


Figure 4.1. Experimental design. Modified from Baho et al. (2021).

4.2.4 Experimental expectations

Concerning functional responses, the null hypothesis that the growth (measured by change in the total number of individuals over time) will be significantly hindered by ISU similarly in NP and HC, was tested. The EM theory helps formulating the following expectation:

- The null hypothesis is rejected. The growth inhibition induced by the herbicide during germination would be lower in the HC community compared to the NP community due to the presence of more tolerant species recruited from the HC sediments.

Concerning TD measurements, ecological theory helps formulating a series of expectations from the experiment (Margalef, 1978; Reynolds et al., 1983; Zhou et al., 2014). For instance, in the early stages of the community assembly (embodied here by the germination phase) an increase of trait richness is expected with time as more species are recruited. A decrease in trait evenness can also be postulated as in the initial state the trait space is small and rapidly filled by pioneer organisms likely carrying similar characteristics (Fontana et al., 2017). With time, random processes will enable insurgence of organisms with different set of traits expanding the trait space of the community and resulting in lower evenness in the trait value distribution. Also trait divergence is expected to decrease as organisms with value of the traits distant from the dominant pioneering organisms increases through random or niche events with time (Gerisch et al., 2012; Gusmao et al., 2016). The experiment of the present work introduces, for the first time, EM from historical exposure to herbicide (deemed to be present in the HC community) as a factor. Based on these general expectations the following null hypothesis (e.g. assuming no effects of EM on ES of phytoplankton) was assessed: HC and NP communities shows similar responses in the time change of TOP, TED and FDIs when germinated in presence of ISU with both lower level of trait richness and higher levels of trait evenness and divergence compared to their germination in absence of ISU. The expectation is that the null hypothesis is not verified demonstrating a significant effect of EM on trait assemblages reflected by higher sensitivity in the variation of TD indexes in the NP community when germinated in presence of ISU. More in detail, it is expected that:

- In NP, TOP will be lower when germination occurs in presence of ISU, because of the ecological filter imposed. In HC such an effect will expectedly not result in significant changes of TOP values, regardless germination conditions. It is however expected that in absence of ISU, TOP will be (at the end of the germination experiments) higher in

NP than HC. This stems from the theoretical assumption that more pristine environments harbour higher biodiversity than strongly stressed ones.

- TED will be higher at the end of the germination experiment in HC than NP because of the expected smaller size of the trait space, resulting in a higher likelihood that organisms in the community will be spread evenly. When ISU is applied however, it is expected that TED will decline in both communities because of ISU filter being more effective on organisms with an optimal growth rate in absence of the herbicide. However, because NP is expected to have a larger trait space than HC, ISU is more likely to have a stronger negative effect on NP than HC.

- In NP FDis will be higher when germination occurs in presence of ISU. FDis is in fact higher when organisms with "extreme values" of the measured traits (e.g. distant from the centroid of the trait space) become more abundant. It can be in fact expected that ISU will primarily filter out the organisms that have an optimal growth in absence of stressor (which are assumed to be prevalent in this community), resulting in increased relative abundance of underperforming "peripheral" organisms. In contrast, it is expected that in HC, because of the prevalence of tolerant organisms, such an effect will be less pronounced or completely absent.

4.2.5 Individual-level trait measurements

A scanning flow cytometer (SCF) from Cytobuoy (Woerden, the Netherlands) was used to count and characterise the trait distribution of phytoplankton at individual level. The instrument is equipped with two solid-state lasers (488 nm and 635 nm) and designed to analyse phytoplankton ranging from picoplankton to larger phytoplankton (0.5 to 700 µm in diameter and about 1 mm in length). Size of individual plankton cells was determined by scattering, where the light scattered from particles (≥ 1µm length) while passing the laser beams was

measured at two different angles: forward (FWS) and sideward scatter (SWS). The fluorescence emitted by the photosynthetic pigments from algal cells were measured at three different wavelengths: red (chlorophyll a), orange (phycocyanin), and yellow (phycoerythrin). The raw Cytobuoy data were processed with R statistical computer software using distribution of fluorescence as a filter to retain fluorescence particle relevant for phytoplankton with a size ≥ 1 µm in length. Additional information on Cytobuoy analysis can be found elsewhere (Fontana et al., 2017; Thomas et al., 2018).

The total organismal abundance was based on the number of individual phytoplankton cells with size ≥ 1 µm detected through SCF. Specific growth rate µ ($time^{-1}$) of each replicate was calculated as the slope of a linear regression of log-transformed total organismal abundance against time. For the comparison of cell size between lakes and germination scenarios, we calculated peak cell diameter (µm) as the mode of cell size distribution, modelled trough generalized additive modelling (GAM) to control the random effect caused by the slight bimodal distribution showed in the HC community (Figure S4.1).

Four individual-level trait data were used to calculate the trait diversity indices: 3 pigments (chlorophyll a, phycocyanin and phycoerythrin) and size. Additional information on the calculation of the indices, can be found in the Appendix Chapter IV (*Trait diversity calculations*).

4.2.6 Statistical analyses

Time series data for the total abundance and trait diversity measurements were analysed using a repeated measurement analysis of variance (ANOVA), to test the significance of the treatment factors (germination, lake and time) and their interactions. Huynh–Feldt correction was applied when the assumptions of sphericity were breached. Second, we used linear modelling (with all predictor variables coded as factors) to test for significant differences in

toxic responses between groups of interest (e.g., whether the response of the total abundance, trait diversity or cell size to contaminants differed significantly between different lakes and germination levels). Total abundance was modelled differently from the other variables as present count data. For this purpose, a glm model with Poisson distribution was used to compare the results from the last day of sampling. Multivariate analyses were used to evaluate the differences in traits' composition between the NP and HC community, as well as the effects of the ISU exposure during the germination phase on the phytoplankton community traits composition. Non-metric multidimensional scaling (NMDS) based on Bray–Curtis similarity and square-root-transformed species matrix data obtained from the taxonomic analysis was used to assess the effect of Isoproturon expo- sure. NMDS analyses were complemented with permutational multivariate ANOVA using Bray-Curtis similarity matrix, with 9,999 unrestricted permutations and applying Monte Carlo p-values corrections.

4.3 Results

4.3.1 Total abundance and growth rate

The change in total organisms' abundance during early ES of the phytoplankton was compared between the communities originated from the two lakes with and without ISU during germination. Expectedly, total organisms' abundance followed an increasing trend with time in both communities starting from day 7 from the beginning of the incubation ($F_{1,16}$ = 188.93; $p < 0.05$; Table 4.1), as shown in Figure 4.2. Overall, the total abundance of the NP and HC communities was not different ($F_{1,\ 16}$ = 1.64; p = 0.22; Table 4.1), however significant differences were observed in the response of the two communities to different germination scenarios ($F_{1,\ 16}$ = 7.84; $p < 0.05$; Table 4.1) (Figure 4.2A). For instance, in the NP community the presence of ISU during germination yielded a significantly lower total abundance compared to when the community was germinated in absence of the stressor (t = -4.31; $p < 0.01$). In contrast, the HC community yielded comparable total abundance results in both germination

scenarios (t = -0.41; p = 0.69). This is in accordance with the expectation on the functional response.

The results of growth rate reflected those of the total abundance (Table 4.2, Figure 4.2B) showing a significant effect induced by the two-way interaction between lake and germination scenario ($F_{1,16}$ = 4.15, p < 0.05, Table 4.2). Figure 4.2B shows that the two communities followed opposite patterns in relationship to the presence of ISU during germination. A significant negative effect of ISU was observed only in the NP community, compared to germinated in absence of the herbicide (t = - 2.543, p < 0.05). In contrast, the growth rate of the HC community did not show significant differences when the community was germinated in absence/presence of ISU (t = 1.4, p = 0.19).

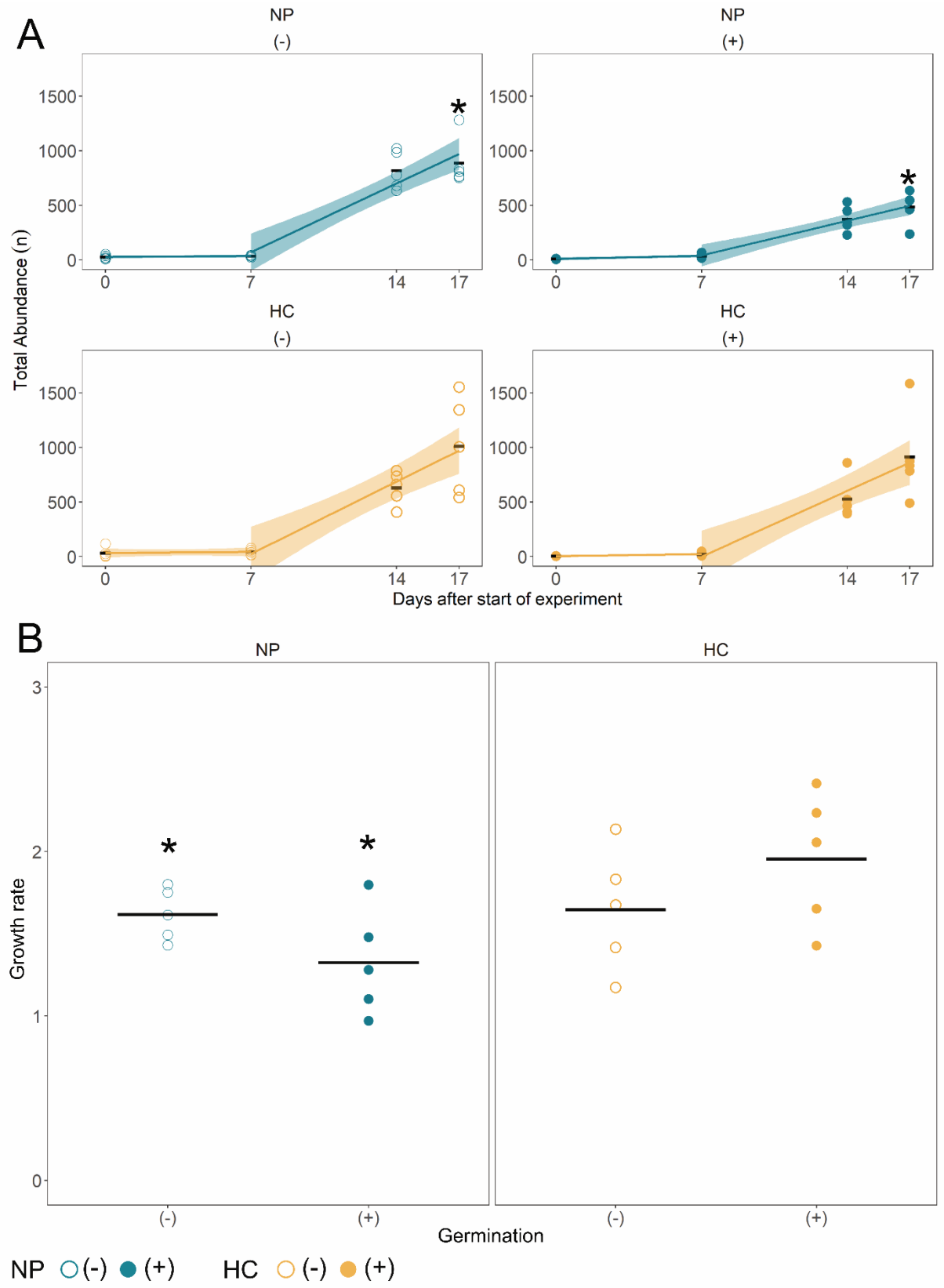


Figure 4.2. A) Temporal development of total abundance (n) of phytoplankton cells and B) growth rate at the end of the experiment in the communities arising from the near-pristine (NP) and historically-contaminated (HC) lakes, germinated in absence (-) and presence (+) of the herbicide. The presence of (*) indicates statistically significant differences.

Table 4.1. Repeated measurement ANOVA testing the effects of lake (historically-contaminated / near-pristine), germination scenario (ger: with or without herbicide), and time of exposure (day) and their interaction terms on the total abundance, trait richness (TOP), evenness (TED) and divergence (FDis) of the two phytoplankton communities.

variables	Factors and interactions	df	SSn	SSd	F	p
total abundance	lake:germ:day	16	116258.30	702783.90	2.65	0.12
	germ:day	**16**	**239108.00**	**702783.90**	**5.44**	**< 0.05**
	lake:day	16	100860.20	702783.90	2.30	0.15
	lake:germ	16	118734.00	791342.40	2.40	0.14
	day	**16**	**8298565.30**	**702783.90**	**188.93**	**< 0.05**
	germ	**16**	**387532.80**	**791342.40**	**7.84**	**< 0.05**
	lake	16	80899.20	791342.40	1.64	0.22
TOP	lake:germ:day	16	14.25	64.70	3.53	0.08
	germ:day	**16**	**42.54**	**64.70**	**10.52**	**< 0.05**
	lake:day	**16**	**37.81**	**64.70**	**9.35**	**< 0.05**
	lake:germ	16	17.26	78.16	3.53	0.08
	day	**16**	**135.21**	**64.70**	**33.44**	**< 0.05**
	germ	**16**	**51.44**	**78.16**	**10.53**	**< 0.05**
	lake	**16**	**48.48**	**78.16**	**9.92**	**< 0.05**
TED	lake:germ:day	16	0.12	0.41	4.57	**< 0.05**
	germ:day	16	0.01	0.41	0.28	0.60
	lake:day	16	0.00	0.41	0.08	0.78
	lake:germ	16	0.00	0.45	0.13	0.72
	day	**16**	**3.61**	**0.41**	**141.12**	**< 0.05**
	germ	16	0.06	0.45	2.25	0.15
	lake	16	0.11	0.45	3.87	0.07
Fdis	lake:germ:day	16	0.02	0.38	0.98	0.34
	germ:day	16	0.06	0.38	2.44	0.14
	lake:day	**16**	**0.29**	**0.38**	**12.05**	**< 0.05**
	lake:germ	16	0.03	0.49	1.02	0.33
	day	**16**	**0.36**	**0.38**	**15.10**	**< 0.05**
	germ	16	0.00	0.49	0.08	0.78
	lake	16	0.02	0.49	0.63	0.44

Table 4.2. ANOVA analyses testing the effects of lake (historically-contaminated / near-pristine), germination scenario (ger: with or without herbicide), and their interaction terms on the growth rate and peak cell size of the two phytoplankton communities.

variables	Factors and interactions	df	SS	MS	F	p
Growth rate	**lake:germ**	**1**	**0.45**	**0.45**	**4.15**	**<0.05**
	germ	1	0.00	0.00	0.00	0.96
	lake	**1**	**0.54**	**0.54**	**4.97**	**<0.05**
	Residuals	16	1.74	0.11		
Peak cell size	lake:germ	1	0.00	0.00	0.03	0.86
	germ	1	0.00	0.00	0.04	0.84
	lake	**1**	**2199.60**	**2199.60**	**3426.19**	**<0.001**
	Residuals	16	10.30	0.60		

4.3.2 Trait distribution in the communities

The distribution of organismal traits measured at individual-level in the community was compared during the germination phase across the two treatments (presence/absence of ISU). Multi-variate permutational analyses (Permanova) indicate that the trait assemblages of the NP and HC community on the last day of the germination period were significantly different (df: 1, 3998, SS: 11.21, MS: 11.21, R^2: 0.2, $p < 0.001$), while the presence of ISU during the germination period did not have significant effects within a given community (df: 1, 2998, SS: 3.2, MS: 3.2, R^2: 0.15, $p = 0.74$). The non-metric multidimensional scaling (NMDS) analyses depicted in Figure 4.3A, suggest that the different trait assemblages between the communities occurring between the NP and HC was mainly caused by differences in size distribution, while the influence from the other pigments measured in the experiment (chlorophyll a, phycocyanin and phycoerythrin) appeared to be negligible (Figures S4.2-4). Results from the peak cell size analyses (Figure 4.3B) confirm significant differences in organisms' size distribution between the two communities (df1:17, SS: 2199.6, F: 3632.51, $p < 0.001$, Table 4.2), and show that the NP community has a significantly smaller cell size compared to the HC community ($t = -46.13$, $p < 0.001$). Cell size and pigments development results are reported in the supplementary

information (Figures S4.1-4). Within the communities, the germination with the herbicide did not show significant effects on the cell size ($df_{1:16}$; F = 0.04, p = 0.84, Table 4.2).

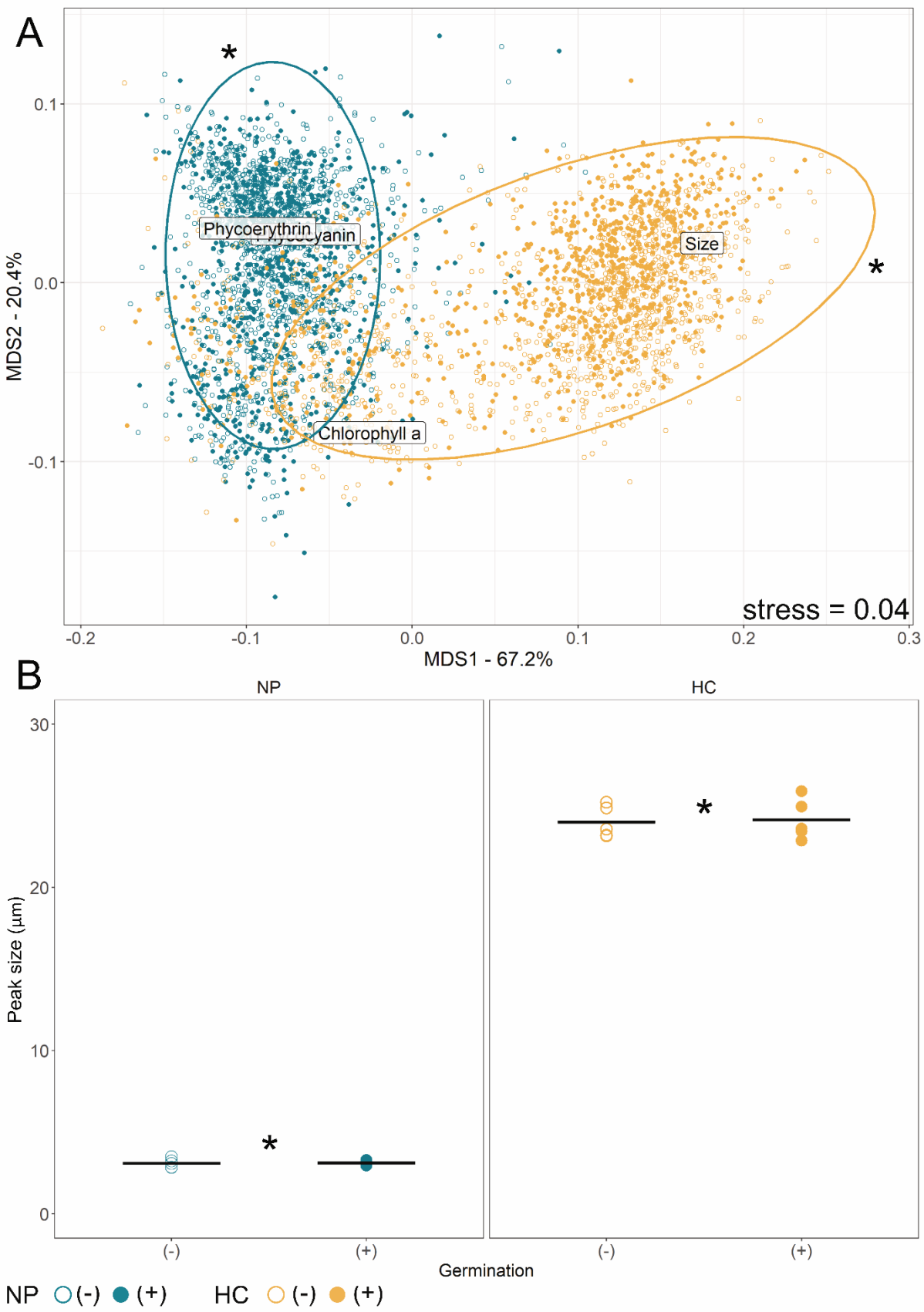


Figure 4.3. A) Non-Metrical Multidimensional scaling plot of individual-level trait data of phytoplankton communities germinated from the near-pristine (NP) and historically contaminated (HC)

catchments both in absence (-) and presence (+) of herbicide. Sub-selection of 4000 data points sampled on day 23 of germination. Ellipses represent 95% confidence interval. Labels indicate the continuous variables tested. Stress value is reported in the plot. B) Peak cell size (µm) of the phytoplankton communities arising from the near-pristine (NP) and historically-contaminated (HC) catchments, germinated in absence (-) and in presence (+) of the herbicide. The presence of (*) indicates statistically significant differences.

4.3.3 Trait Diversity Indices

4.3.3.1 Trait richness - TOP

As expected, TOP increased with time in both NP and HC communities ($F_{1,16}$ = 33.44, $p < 0.05$; Table 4.1; Figure 4.4A). different trends were however observed between the two communities ($F_{1,16}$ = 9.92; $p < 0.05$, Table 4.1), indicating a significant role of the interaction between germination and time ($F_{1,16}$ = 10.52, $p < 0.05$, Table 4.1) and between lake and time ($F_{1,16}$ = 9.35, $p < 0.05$, Table 4.1). As in the total abundance results, a significant effect of the presence of ISU during germination was observed for TOP in the NP community ($F_{1,8}$ = 8.44, $p < 0.05$). Here, the TOP value on the final day of the germination period was significantly lower in presence than in absence of the herbicide ($t = -3.99$, $p < 0.05$). In contrast, no significant differences between the germination scenarios were observed in the HC community.

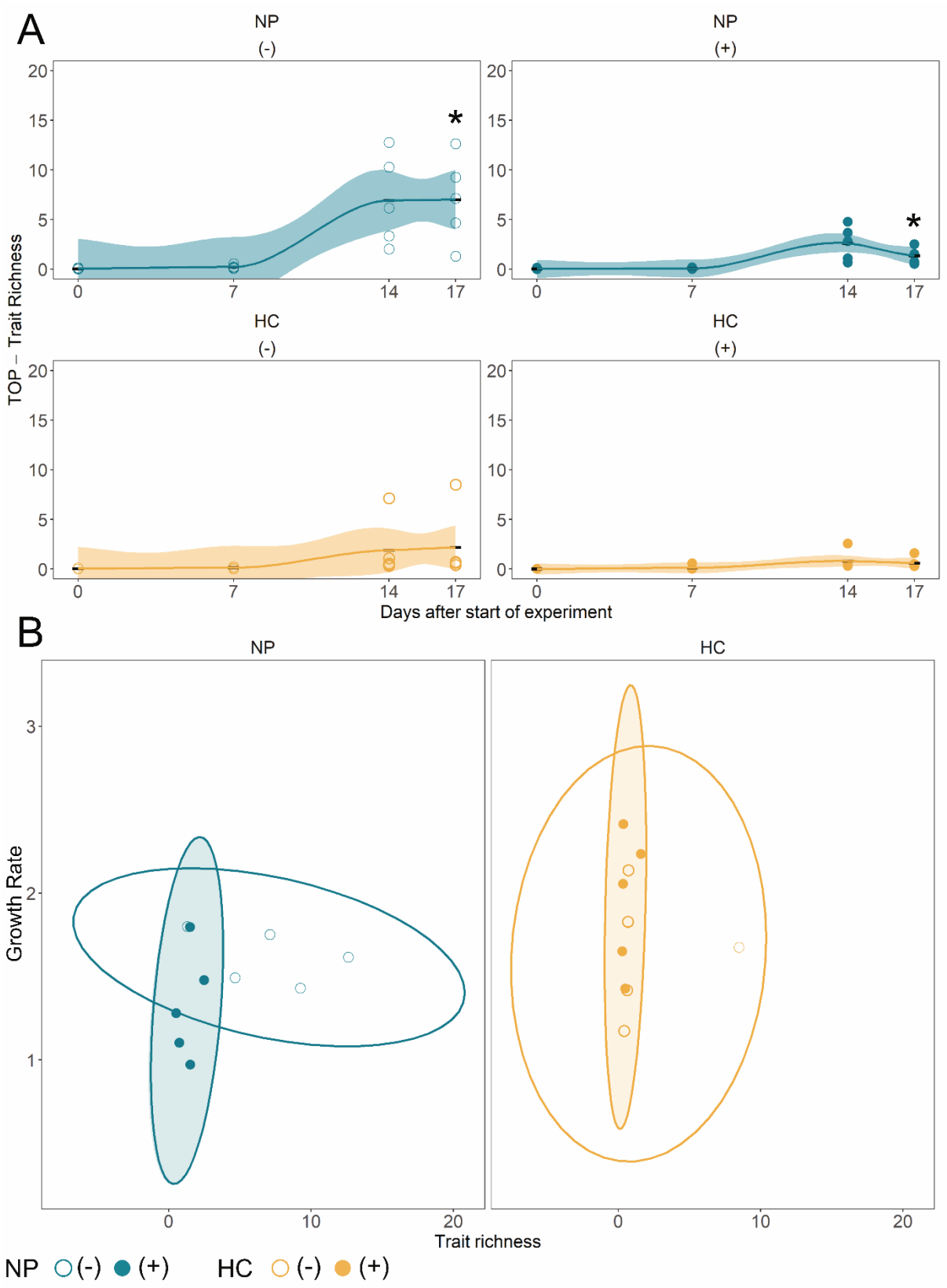


Figure 4.4. A) TOP development during the germination period and B) bivariate plot of Growth rate and TOP in the phytoplankton communities arising from the near-pristine (NP) and historically contaminated (HC) lakes, germinated in absence (-) and presence (+) of the herbicide. The presence of (*) indicates statistically significant differences.

4.3.3.2 Trait evenness - TED

TED of both the NP and HC community was significantly influenced by the effect of time during the ecological succession ($F_{1,16}$ = 141.12, $p < 0.05$, Table 4.1), which induced a significantly decreasing pattern in both the NP community ($F_{1,8}$ = 80.50, $p < 0.05$), and the HC one ($F_{1,8}$ = 66.11, $p < 0.05$), as depicted in Figure 4.5. TED values did however show significant differences between the two communities ($F_{1,16}$ = 3.87, $p = 0.07$, Table 4.1). No differences induced by the different germination scenarios were observed on the last day of the germination period in either the NP community ($F_{1,5}$ = 4.11, $p = 0.1$) or the HC one ($F_{1,5}$ = 0.09, $p = 0.78$).

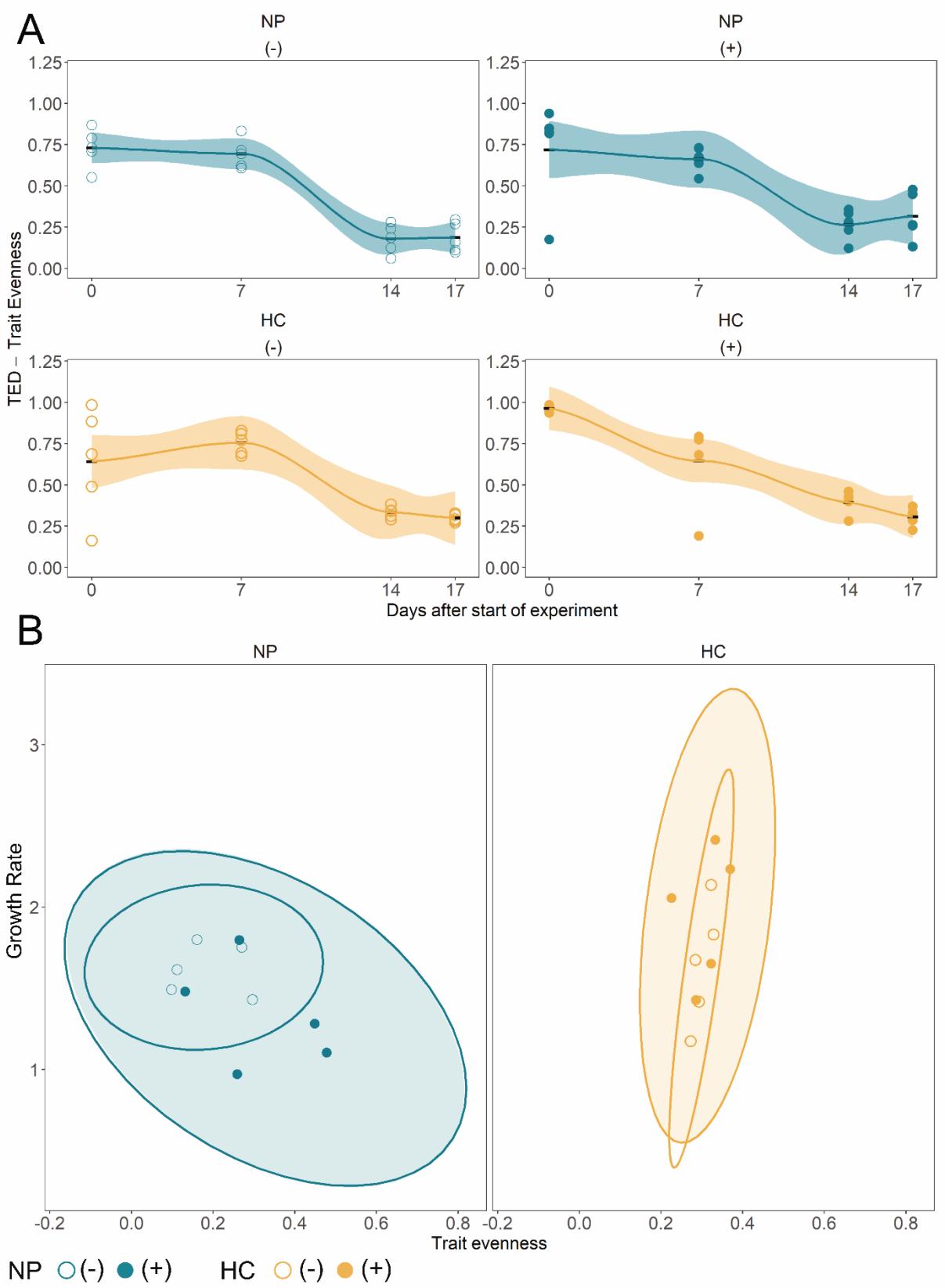


Figure 4.5. TED Development the germination period in the phytoplankton communities arising from the near-pristine (NP) and historically contaminated (HC) lakes, germinated in absence (-) and presence (+) of the herbicide.

4.3.3.3 Trait divergence - FDis

FDis showed a different development over time for the two communities, as indicated by the significant effect induced by time ($F_{1,16}$ = 13.41, $p < 0.05$), and by the two-way interaction between lake and time ($F_{1,16}$ = 15.82, $p < 0.05$, Table 4.1). For instance, while the FDis of the NP community significantly increased over time ($F_{1,8}$ = 35.28, $p < 0.05$, Figure 4.6A), the one of the HC community was stable, yielding comparable values between the beginning and the end of the experiment ($F_{1,8}$ = 0.84, $p = 0.9$, Figure 4.6A). The responses of the two communities to the presence of the herbicide during germination were remarkably different (Figure 4.6A). In the NP community, the FDis on the last day of sampling was significantly affected by the presence of ISU ($F_{1,10}$ = 80.07, $p < 0.001$), with FDis being higher in the germination scenario in presence of the herbicide compared than in its absence ($t = 8.95$, $p < 0.001$). In contrast, such an effect was not observed in the HC community ($F_{1,10}$ = 0.02, $p = 0.90$).

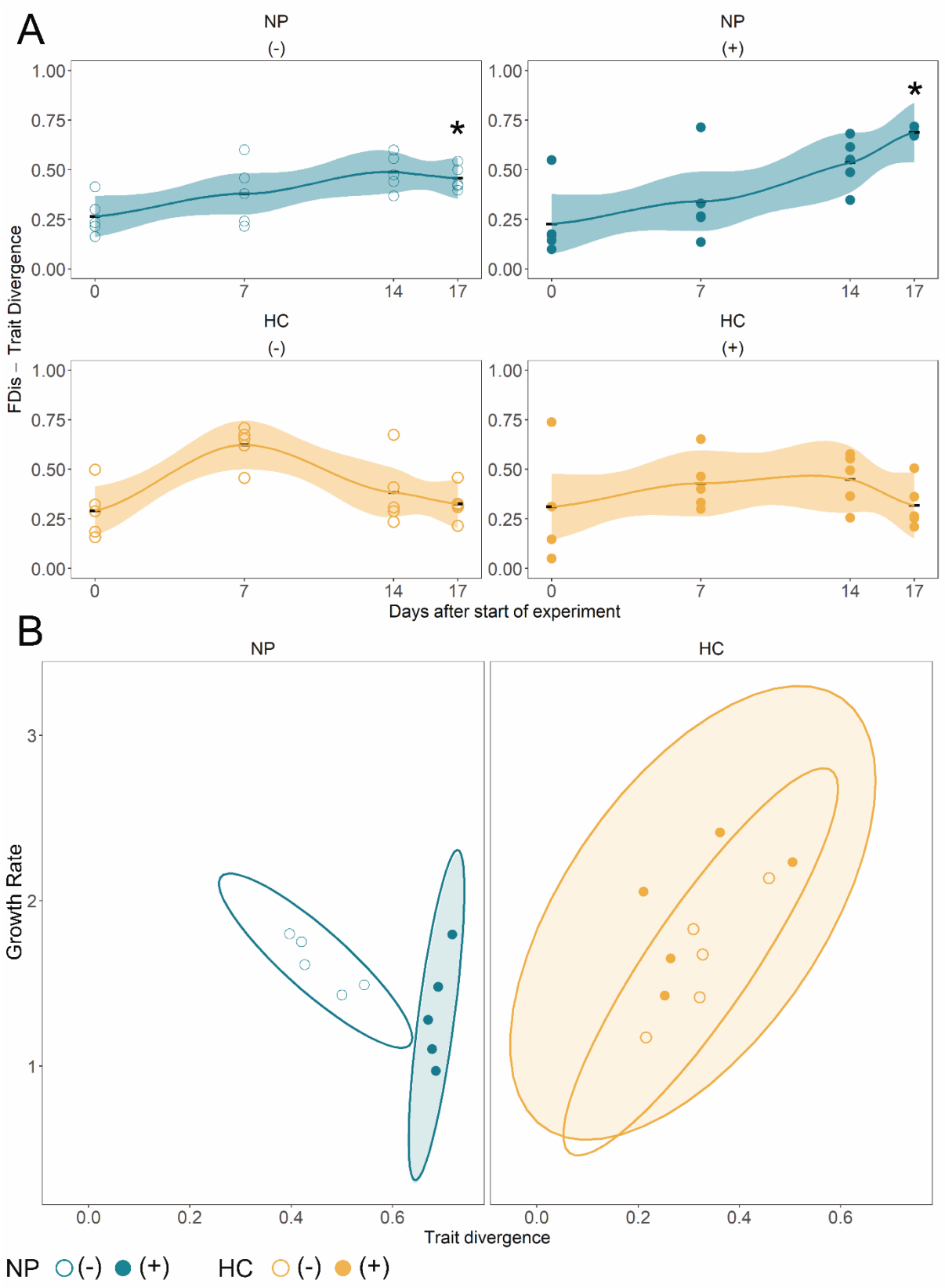


Figure 4.6. A) FDis development during the germination period and B) bivariate plot of Growth rate and FDis in the phytoplankton communities arising from the near-pristine (NP) and historically contaminated (HC) lakes, germinated in absence (-) and presence (+) of the herbicide. The presence of (*) indicates statistically significant differences. (*) significant difference between treatments.

4.4 Discussions

This study empirically assessed the influence of EM of historical contamination on the functioning (growth) and structure (trait diversity) of natural phytoplankton communities during the early stages of an ES. A germination experiment from lake sediments offered the opportunity to examine natural phytoplankton communities obtained from lakes with different historical herbicide exposure, carrying a comprehensive load of genetic material accumulated over the years (i.e. in comparison to using standing crop communities). Hence the community assemblages used here harboured different strains/species (and their physiological adaptations) present in the native lakes. The germination experiments enabled also to run observation of the community self-assembling process under highly controlled and reproducible conditions (e.g. dramatically reducing the influence of neutral random processes). Finally, individual level trait-based observations allowed drawing mechanistic expectations on the evolution of the community structure and a comparison of responses across different ecosystems (HC vs. NP).

Results obtained here mostly fulfilled (or were broadly consistent) with the expectations detailed above for the responses of growth and TD indices. For instance, NP community showed higher level of sensitivity towards the herbicide added during the germination phase compared to the HC community, this being in line with the expectations of EM controlling structural and functional responses in HC during early ES in presence of the historical stressor. For instance, the HC community displayed higher functional and structural resistance while assembling under the influence of ISU, during the germination period. While the observed pattern may in principle be driven by an unknown driver (other than EM), the strong consistency between observed dynamics of trait distribution and the mechanistically derived expectations introduced above, strongly indicated that ISU applied during germination acted as a selective filter, whereby recruitment of tolerant strains or species unfolded only in the HC

community as a result of EM. In the following sections, these findings will be discussed in detail.

4.4.1 Influence of EM on community functioning

The patterns of total organisms' abundance and growth rate were sensitive to the historical contamination background. In fact, the presence of ISU during germination did not affect the biomass and growth of the HC community compared to germination without it. In contrast, the presence of the herbicide induced a marked negative effect on both endpoints in the NP community. These findings are consistent with our first expectation. In the HC community, EM of ISU (and possibly other phenylurea herbicides used in this lake catchment) historically selected for tolerant strains or species assemblages. These were preserved in dormant stages in the lake sediments. When during the germination ISU was applied as filter, the HC community ability of recruiting individuals (i.e. growth rate) and its total standing crop (total abundance) at the end of the experiment were unaffected. In contrast, both growth rate and total abundance of the NP community were significantly hindered by sub-lethal concentrations of ISU during the germination. This is the expected effect of ISU, whose mode of action directly inhibits photosynthesis of microalgae (Arnaud et al., 1994), resulting in lower growth.

It has been shown that acquisition of tolerance towards phenyl urea pesticides is associated to a mutation of the D1 protein of the photosystem reaction centre counteracting the inhibitory effects of Isoproturon (Antonacci et al., 2018). Herbicide resistance has been shown to occur following a substitution mutation that altered the structure of the intracellular site generally targeted by the herbicide, for example, the D1 protein of Photosystem II (Antonacci et al., 2018). However, species composition was very different between the two communities, with the NP community showing a considerably higher portion of Chrysophyceae, whereas Chlorophyceae were the most abundant algae group in both lakes (Baho et al., 2021). This suggest that EM in the HC community is not necessarily carried by the presence of mutants,

but certainly also by the presence of more tolerant species selected through the ecological filter imposed by the historical use of phenyl urea herbicides in the HC catchment. This hypothesis is corroborated by the distinct species composition of the two assemblages obtained during germination, and the fact that prevailing organisms in the HC community were characterized by a substantially larger body size. A larger body size in microorganisms has been previously associated to lower sensitivity to chemical stressors, following both theoretical and empirical assessments (Del Vento and Dachs, 2009; Tambi et al., 2009).

4.4.2 Influence of EM on community trait diversity

The analysis of trait distribution was used to assess structural resistance of the communities during early ES in presence of ISU. Results confirms observation on the effect of EM on growth and organisms' abundance. The two communities exhibited a neat difference in the way they responded to the herbicide during germination. For instance, in the HC community all the investigated trait indices (richness, evenness and divergence) were remarkably unaffected regardless the presence or absence of the herbicide. This denotes a structural resistance of HC and reinforce confidence on the role of EM. Acquisition of EM appear to positively increase resistance of historically stressed ecosystem (at least concerning a specific stress). EM conferred HC resistance in both functional and structural terms.

In contrast, in the NP community, the TD diversity indexes were significantly affected by ISU during germination. For instance, TOP decreased and FDis increased (compared to the germination without ISU), while TED appeared to remain unaffected regardless the germination conditions. While TOP was higher in NP than HC in the treatment without ISU, in NP it decreased significantly when the herbicide was added during germination. Such an effect is consistent with expectations from the effect of a strong ecological filter in a non-tolerant community. The expectation that TED would decline more markedly in NP than HC

under the effect of ISU was instead not verified, suggesting, perhaps, that the trait space at the early ES phase was still too limited to observe significant responses.

All together, these results clearly point at the rejection of the null hypothesis that EM of ISU does not influence present days responses of the HC to this stressor, indicating that past uses of phenyl urea herbicides in the HC lake significantly affected structures and functioning of this ecosystem by sorting for tolerant strains or species. A decrease of trait diversity in phytoplankton induced by chemical contaminants was previously described on another study (Pomati et al., 2017). The increase in FDis observed during the germination with the herbicide indicate that filtering operated by ISU caused tendency of sorting organisms carrying values of the measured traits departing from the central value of the distributions. In a multidimensional space of traits (e.g. when simultaneously considering measures of several traits), this means that organisms that depart from the space of the centroids tended to become prevalent over time as the community assembling progressed (Gerisch et al., 2012; Gusmao et al., 2016). This reinforce the notion that the selection posed by the herbicide on the NP community sorted for a set of more tolerant organisms that are not prevailing in this community. Evidence of trait combinations differing from the centre of gravity (the 'average' trait composition) in relationship to disturbed habitats have been reported by other authors (De Castro-Català et al., 2020; Gusmao et al., 2016).

The lack of structural resistance observed in the NP community under germination with ISU probably contributed to the observed lack of functional resistance (e.g. negative effects on the growth of the community). This may suggest that ISU modified the fundamental relationship between phytoplankton community structure (trait diversity) and biomass production. A similar observation was made earlier in another study for atrazine (Baert et al., 2016), another phenyl urea herbicide. Drawing from complexity theory (Enquist et al., 2015), it can be said that because of the lack of resistance to the stressor, and unlike the HC community, the NP

community was forced to structurally respond (through the trait sorting posed by the ISU) to the unfavourable contaminated environment. Such a passive adaptive process can be seen to have a cost (measurable for example through the growth deficit observed in NP during germination with ISU). In this case the NP community, that did not harboured adaptations from historical encounter with the stressor, was forced to respond to the sorting imposed by the ecological filter (ISU). This favoured more tolerant populations to the detriment of dominant top performers, which instead prevailed in absence of the ecological filter.

As expected, TED decreased in both communities along time. However, unlike expected, TED was apparently unaffected by the presence of the herbicide during germination in both communities. Trait indices represent tools to lower the dimensionality of complex multidimensional trait data arrays. While they are clearly useful to condense complex information in a single numerical datum, the dimensional scaling can easily result in lower signal resolution (Fontana et al., 2016). In this case TED appeared not to yield any observable different behaviour across the two communities in the treatment with and without ISU. This can be attributed to lack of resolution and therefore was not discuss further. Notably, the trait indices analysed here are independent and orthogonal (Fontana et al., 2016), and lack of response in one of them does not invalidate the results obtained for the others.

4.4.3 Differences between communities and effect of herbicide during germination: individual-level traits vs. trait indices vs. taxonomic analysis results

The trait-based approach used in this study enabled drawing prediction on community responses and compare them between different ecosystems (i.e. HC vs. NP). This would not have been conceptually possible through a traditional taxonomic approach. While species compositions of different ecosystems are the results of several complex processes where temporal and geographical boundary conditions are determinant, it can be argued that organismal responses to the environment are always mediated by traits and traits have been

seen earlier as more sensible features to track ecosystem changes than changes in species composition (Enquist et al., 2015; Hillebrand and Matthiessen, 2009; Reiss et al., 2009). Organisms belonging to different species will therefore have similar responses to the environment if they carry similar traits. This axiom enables cross-comparison of trait patterns results in the present study, by using both individual-level trait data and TD indices.

Results from the NMDS and Permanova analysis indicate that the communities originated from the near-pristine and historically contaminated lake had a significantly different trait distribution. This is not surprising and suggests that, while care was taken to select lakes with similar ambient climate, physical and chemical characteristics (i.e. water depth, trophic status, etc.), the lake clearly differed in several respects (and not only in contamination histories). Catchment characteristics, level of connectedness with other systems are, among others, potentially driving elements of different species composition. Detailed analyses of the individual-level trait data (pigments and size) suggest that the differences between the communities may be caused by their significant difference in size, while no difference in pigments was detected between the two communities (Figure S4.2-4). Size is arguably a very important characteristic of an organism as it relates to other facets of life-history and ecological processes including metabolism, development, resource requirements and trophic interactions (Acevedo-Trejos et al., 2015; White et al., 2007).

In addition, the results arising from this approach can be compared to the findings from the first phase of the study from Baho and others (2021), which was performed by using the more traditional taxonomic analyses. For instance, Baho et al. (2021) showed that the two communities had a different species composition (data not shown here, please refer to Figure 3.2 in Chapter 3), and suggested a weak effect of the herbicide on the species richness and evenness of both communities. However, results also showed that the influence of the herbicide on the species composition did not differ between the two and resulted independent on the

previous history of contamination (Baho et al., 2021). These results indicate that the trait indices represent a powerful dimensional scaling tool to unfold mathematical terms significant patterns lying in the changes in the distributions of multiple traits taking place simultaneously as a response to the selective forces of the environment. Multiple dimensional trait analysis had the advantage to capture the subtle changes and effects operated by the herbicide that remained subtle or unseen by using the individual-level traits only or species composition data, while assessing more general functional structural aspects of the communities which make them inter-comparable across systems to a greater extent rather than individual-level trait data. These results also contribute to other findings reporting multiple traits measured community-wide at the individual level as the most promising and straightforward way to combine inter- and intraspecific results into one single approach giving meaningful results (Cianciaruso et al., 2009).

The effect of the herbicide on the NP community ES may also be more concerning as are observed on early-stage assemblages of phytoplankton. A small effect at an early stage of a dynamic process can cascade in amplified effects later in time, contributing to affect the shape of the community structure of phytoplankton, which could have repercussions on both food web and trophic energy transfer in freshwater ecosystems (Vellend, 2016, 2010). Hence, analysis of trait distribution responses in ecosystems in unsteady conditions can represent a useful sensible parameter to monitor and assess ecological quality of ecosystems.

4.5 Conclusions

The experimental approach used in this study successfully illustrated the influence of EM on complex community level responses and adaptation to chemical stress. While Pollution Induced Community Tolerance has been empirically studied in several previous study, the underlying concept of EM is nested in ecological theory and acknowledges the fundamental

mechanisms of adaptation through not only physiological or evolutionary process but also through ecological sorting of strains, population or species replacing stress intolerant functional groups.

This study addressed, for the first time, the role of EM on the dynamic assembling of communities during ES. It was showed that adaptations from past experience are readily available in phytoplankton at a new recurrence of a stressor, even when harboured in dormant stages in sediments. These adaptations can enable structural and functional resistance in the presence of stress. On the other hand, the historically stressed community expressed a lower level of trait richness compared to NP community when growth in absence of the stressor. The absence of EM in the community originated from the sediments of the NP lake yielded communities lacking resistance to this stressor. Remarkably, even at low levels (sub-lethal concentrations) of the herbicide there was a negative influence on both structure and function of this community. Such a response can be considered as evidence of an effect of a chemical stressor to modify the relationship between structure and functioning. The present study showed that through the analysis of trait distribution (in contrast to taxonomic analysis) such a complex response could be observed already at a very early stage of an ES. This is relevant as effects on early community development (especially on primary producers) may translate in large cascading effects on the broader ecosystem in later stages of the succession.

Part II: Environmental Chemistry

Chapter V: Critical assessment of an equilibrium-based method to study the binding of waterborne contaminants to natural dissolved organic matter (DOM).

Simone Rizzuto [1], Kevin C. Jones [1*], Hao Zhang [1*], Didier L. Baho [2], Eva Leu [3], Luca Nizzetto [2, 4]

[1] Lancaster Environment Centre, Lancaster University, Lancaster, LA1 4YQ, UK.

[2] Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, 0349, Oslo, Norway.

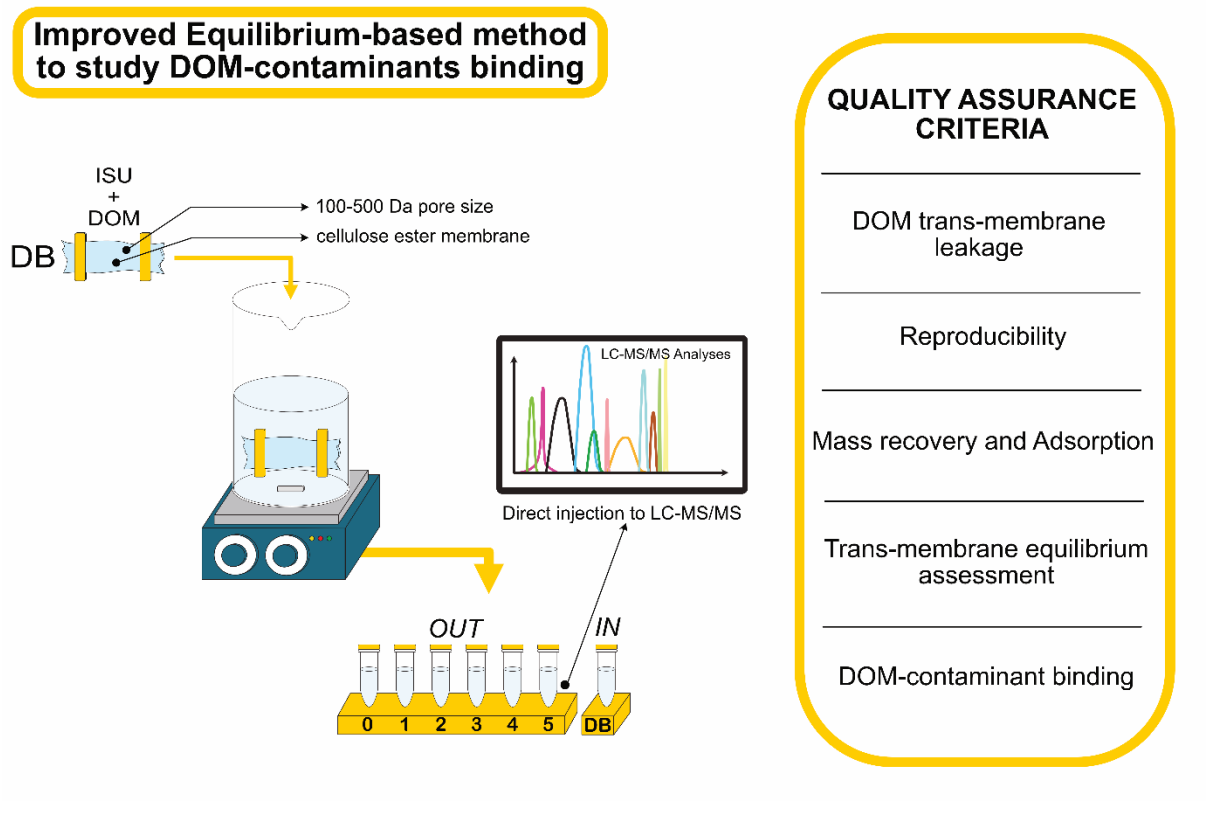
[3] Akvaplan-niva, CIENS, Science Park, Gaustadalléen 21, 0349, Oslo, Norway.

[4] RECETOX, Masaryk University, Kamenice 753/5, 625 00 Brno, Czech Republic.

*corresponding authors: k.c.jones@lancaster.ac.uk; h.zhang@lancaster.ac.uk.

Published in: S. Rizzuto, D. L. Baho, K. C. Jones, E. Leu, L. Nizzetto, H. Zhang. Critical assessment of an equilibrium-based method to study the binding of waterborne contaminants to natural dissolved organic matter (DOM). 2021. Chemosphere. 285, 131524. **https://doi.org/10.1016/j.chemosphere.2021.131524.** To access the Supplementary Material please consult page S43 of the "Appendices and Supplementary Material" file or visit **https://doi.org/10.1016/j.chemosphere.2021.131524.**

Graphical Abstract (TOC)



Abstract

Dissolved organic matter (DOM) can play a major role in determining availability of pollutants to aquatic biota. Equilibrium dialysis is the most commonly used method to assess the interaction between DOM and organic contaminants. However, results obtained through this method can be affected by confounding factors linked to the diffusion of DOM through the membrane or the interaction of DOM and/or the compounds with the membrane itself. In this study, we propose an improved experimental approach, where highly hydrophilic cellulose-ester membranes with small molecular cut-off (100-500 Da) were used to overcome some of these hindrances. The performance of the method to determine the binding of a commonly used moderately hydrophobic herbicide (Isoproturon - ISU) with natural DOM was critically evaluated through a set of quality assurance criteria, across a range of DOM concentrations and pH conditions. DOM trans-membrane diffusion was prevented by the smaller pore size of the dialysis membrane. Good measurement reproducibility, mass balance closure, and successful trans-membrane equilibrium of ISU were obtained. ISU showed relatively low affinity with DOM (log K_{DOC} 1-2 L g^{-1}), which was significantly influenced by varying pH and DOM concentration. An alternative membrane may be needed for higher pH conditions as the greater adsorption effect blurred the observation of trans-membrane equilibrium and confounding mass balance closure. The paper makes recommendations on how to avoid measurement artefacts, while considering criteria for the expected mass distribution of compounds at equilibrium and for sorption onto the membrane and surfaces of the experimental units.

5.1 Introduction

Large amounts of chemical contaminants are continuously discharged to freshwaters from anthropogenic activities (FAO, 2017), threatening the integrity of ecosystems (Pal et al., 2010; Schulz et al., 2021). The effects of chemical contaminants on aquatic biota do not only depend on their concentrations and inherent toxicological properties, but can also be influenced by environmental factors that can alter their bioavailability (Fischer et al., 2013). Assessments of the effects of waterborne contaminants on aquatic biota typically overlook environmental determinants, considering simplified standard exposure scenarios (OECD, 2009). This leads to assessments of chemical risk that can be poorly representative of real environmental conditions (Rowett et al., 2016). Understanding the interactions of key environmental factors (including the role of constituents of natural freshwaters and the water chemistry) with chemical contaminants is crucial for a correct prediction of ecological risk.

Dissolved organic matter (DOM) can play a major role in affecting the form and effects of waterborne contaminants. DOM is an heterogeneous mixture of a wide range of natural organic substances that can be found in all aquatic environments (Leenheer and Croue', 2003). It has the ability to bind, adsorb and/or transform contaminants by forming complexes that are too large or too polar to cross biological membranes (Lipnick, 1995), thereby reducing the bioavailability and toxic outcome of contaminants (Lin et al., 2018a; Rowett et al., 2016). The affinity of DOM with contaminants (generally expressed as a distribution coefficient K_{DOC}) can be affected by the water chemistry (e.g. pH), temperature, physicochemical properties of both the contaminants (i.e. hydrophobicity – presence of functional groups), and DOM (e.g. molecular size, aromaticity, type of functional groups in the DOM molecules and concentration) (Lin et al., 2018b; Pokrovsky et al., 2016; Xu and Guo, 2017). For instance, higher molecular size DOM constituents (i.e. humic acids) generally show higher affinity for binding with chemical compounds (Bai et al., 2019; Xu et al., 2019), while the opposite can

occur for lower molecular size constituents (Ding et al., 2011; Pokrovsky et al., 2016). This is usually related to the molecular size altering the physicochemical properties of DOM, where larger size fractions of DOM are usually connected with more condensed structure, stronger hydrophobicity, abundant aromaticity and therefore higher affinity with organic chemicals (Bai et al., 2019; Ma and Yates, 2018). A change in water pH may affect the complexation of DOM and contaminant, since many chemicals, including pesticides, exists simultaneously as ionic and neutral forms n the aquatic environment (Ashauer and Escher, 2010; Rozman and Doull, 2000a). Neutral species dominate at water pH lower than the compound's acid dissociation constant (pKa) and tend to be more toxic, possibly because the organisms' lipid membranes are often more permeable to non-polar molecules (Lipnick, 1995). Neutrality in the molecular charge can in turn increase the likelihood of hydrophobic interactions with DOM (Rowett et al., 2016), possibly resulting in lower bioavailability and toxicity. Water pH can also alter the physicochemical properties of the DOM, by altering its chemical configuration and therefore its binding affinity with the contaminants (Longstaffe et al., 2013; Myeni et al., 1999a). The binding capacity of the DOM is generally dependent on its concentration (Burkhard, 2000; Krop et al., 2001), however higher concentrations of DOM could also influence the supramolecular configuration of the DOM itself (Vialykh et al., 2020), preventing contaminants from accessing binding domains in the DOM (Akkanen and Kukkonen, 2003; Cao et al., 2018). These complex behaviours cannot be easily predicted. Addressing the role of DOM on chemical risk assessment therefore requires dedicated studies and reliable experimental approaches.

Several techniques have been used to investigate DOM and contaminants interactions. These include fluorescence quenching (Hong et al., 2021), solubility enhancement (Wei-Hass et al., 2014), purging or sparging (Hassett and Milicic, 1985), solid-phase micro-extraction (Ren et al., 2020; Ripszam and Haglund, 2015), reverse-phase HPLC separation (Hsieh et al., 2015;

Laundrum et al., 1984), size-exclusion chromatography (Jota and Hassett, 1991; Lee and Hur, 2017), liquid-liquid extraction (Johnsen, 1987) and equilibrium dialysis (Akkanen et al., 2001; Yamada and Katoh, 2020; Zhao et al., 2014). Equilibrium dialysis has been the most common approach due to its apparent simplicity and the possibility of easily providing confirmation of measurement validity through mass balance closure. This method exploits the osmosis process where a solute moves from an area at higher concentration to one at lower concentration until it reaches equilibrium between two sides of a semipermeable membrane. Contaminants and DOM are usually spiked inside a dialysis membrane ideally made of an inert material, and with pores of a defined molecular cut-off (e.g. 1000 Da). In principle, this setup allows the contaminant molecules to freely diffuse across the membrane, whereas the larger-sized DOM complexes are confined inside the dialysis bag (Akkanen and Kukkonen, 2003; Yamada and Katoh, 2020). If binding occurs, the concentration of contaminant inside the bag is higher than that outside, as it represents the sum of the freely dissolved fraction (at trans-membrane equilibrium) and the amount bound to DOM. In this case calculation of distribution coefficients is, in principle, possible.

While conceptually simple, several confounding factors can blur experimental results and potentially produce artefacts and errors. One technical hindrance is that the pore size typically used in these experiments (1000 Da) does not completely prevent the crossing of smaller DOM molecules from inside the dialysis bag, leading to an under-estimation of the binding effect of DOM (Akkanen et al., 2004; Akkanen and Kukkonen, 2003, 2001). The use of membrane with a smaller molecular cut-off could solve this issue, but it may also present other challenges, such as an amplified interaction of the membrane with both DOM and contaminants (Thevenot et al., 2009). Membranes made of polymers with different compositions and properties can be used (Dias and Duarte, 2013). The choice of material is key for preventing interactions of DOM and/or contaminants with the membrane. Polymers used in dialysis include polycarbonate,

polysulfones, polyacrylonitrile and - more commonly - a range of cellulose-based membranes (Dias and Duarte, 2013; Tolkoff-Rubin, 2011). Electrochemical properties or hydrophobicity of the membranes can therefore vary markedly with their composition. This can affect the interaction of both contaminants and DOM with the membrane, which may alter permeability and mass recovery, and therefore affecting mass balance results, invalidating the measurements.

Dialysis equilibrium experiments usually overlook adsorption of the investigated chemicals on the experimental units (e.g. glassware, membrane and any material in contact with the solution), under the assumption that such an interaction would not interfere with the trans-membrane equilibrium (Akkanen and Kukkonen, 2003). Nevertheless, significant adsorption could negatively affect the quality of the experiment (Thevenot et al., 2009).

To stress the performance and viability of the equilibrium dialysis method to measure binding between contaminants and DOM, we introduce an approach based on the following improvements:

- Reduced membrane pore size (100-500 Da) to prevent DOM crossing the membrane.
- Use of cellulose ester membrane to reduce hydrophobic interactions between membrane and DOM/contaminants.
- Measurements of test compound concentrations by direct injection to LC-MS/MS;
- Adoption of a rigorous set of quality assurance criteria, aimed at preventing leakage of DOM through dialysis membrane, verifying equilibrium conditions, ensuring system mass-balance closure, assessing the sorption of the contaminants on the membrane and experimental unit components.

The herbicide Isoproturon (ISU) was used as a model compound. It was selected for two main reasons. Firstly, ISU was one the most extensively used herbicide applied in agricultural practice, before its ban from the European Union in 2016. ISU was widely used for its inhibitory properties that disrupt the electron transport in photosystem II by binding to the protein D1 in the thylakoid membrane (Arnaud et al., 1994). Due to the focus of this thesis on phytoplankton, the choice of ISU as a model for this study was obviously due to its documented negative effects on these organisms (Brock et al., 2000). Understanding the capacity of DOM to bind ISU and reduce its toxicity is therefore important. Secondly, ISU represents an optimal model compound to test the capacity of this improved equilibrium-based method for its chemical structure, with hydrophobic and partly charged molecular domains, providing possibilities for both hydrophobic and weak electrostatic interactions between the chemical and the functional groups of the DOM. The binding was tested with DOM isolated from a natural lake (Gjessing et al., 1999a) to ensure a natural complex spectrum of DOM constituents from boreal ecosystems (Leenheer and Croue', 2003). The experiments were carried out for a range of DOM concentrations and water pH, to challenge the method's ability to provide consistent and reliable results and elucidate its possible limitations.

5.2 Materials and Methods

5.2.1 Selection and settings of experimental conditions

Chemicals: ISU (CAS 34123-59-6) is a 1,1-dimethyl-3-(4-isopropylphenyl)-urea substituted by a p-cumenyl group at position 3, and is an herbicide acting as a photosynthesis inhibitor for weed control (Tomlin, 2000). Its molecular formula is $C_{12}H_{18}N_{20}$, and its molecular weight of 206.82 g mol^{-1}. It is non-ionic and relatively hydrophilic (octanol-water partition coefficient log K_{ow} 2.87 - Hansch et al., 1995). Other important physicochemical properties are: water solubility of 65 mg L^{-1} at 22°C (MacBean, 2008); Henry's Law constant of $1.9x10^{-9}$ atm-cu m/mole (MacBean, 2008); melting point of 158.0 °C (O'Neil, 2013); flash point of 100 °C (212

°F) (MacBean, 2008); density of 1.2 at 20 °C (MacBean, 2008); vapour pressure of $9.1x10^{-3}$ mPa /$2.47x10^{-8}$ mm Hg/ at 25 °C (MacBean, 2008); atmospheric OH rate constant: 1.20e-^{11} cm3/molecule*sec (Palm et al., 1998). ISU's chemical structure is shown in Figure 5.1. The interaction of ISU and DOM has been studied previously, but focusing on soil organic matter (Beck and Jones, 1996; Ertli et al., 2004). Beyond hydrophobic interaction driven by the moderate K_{ow} value, the ability of ISU to weakly bind with O, N and H atoms of the DOM (Ertli et al., 2004), and possibly the membrane, makes this compound a useful and challenging molecular model to test the experimental method. ISU standard reference material (99.21% HPLC purity) was purchased from Sigma-Aldrich (US). The compound was prepared with methanol to create a 1 mg mL^{-1} stock solution. Two serial dilution procedures were carried out to create a 10 µg mL^{-1} ISU working solution.

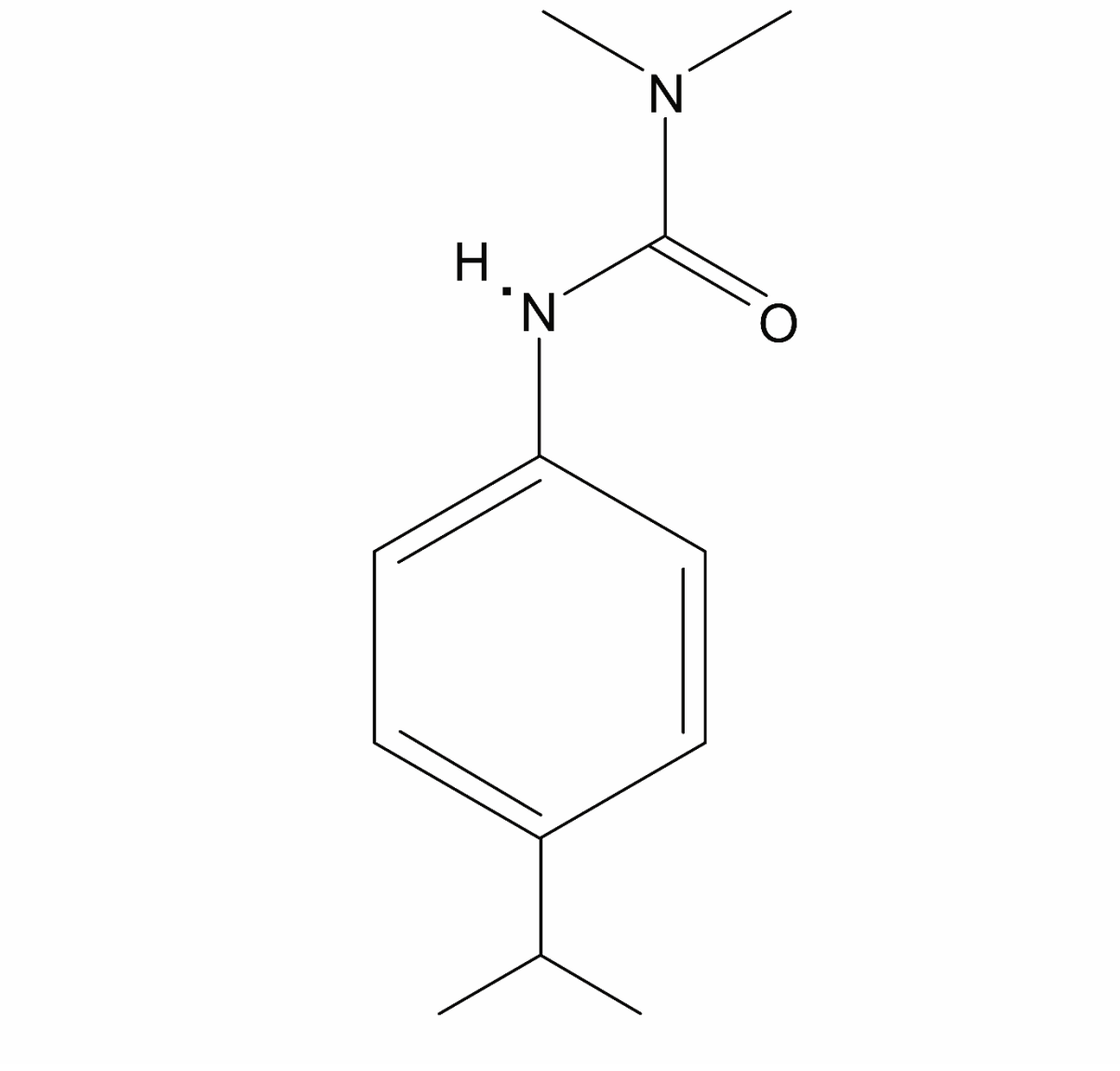


Figure 5.1. Chemical structure of Isoproturon.

Natural DOM and water pH: The DOM used in this study was previously isolated with reversed osmosis from the water of Hellerudmyra (Norway), a small catchment (0.08 km^2) used for most natural DOM research at the Norwegian Institute for Water Research (NIVA, Norway), providing samples for the (IHSS) Nordic Fulvic and Humic Reference Material (Gjessing et al., 1999a). The most relevant physicochemical properties of this natural DOM are reported in Table 5.1. The levels of DOM used in this experiment were 0, 5, 10 and 20 mg L^{-1}

DOC, which represent low to mid-high concentrations typically found in boreal lakes (Henriksen et al., 1998). The DOM working solution was prepared by weighing the dry DOM and dissolving in equivalent MQ water to reach 1 mg mL^{-1} concentration. The solution pH used (4, 5, 6, 7 and 8) represent the range expected within boreal freshwater systems (Bååth and Kritzberg, 2015). The pH was monitored daily during the experiment. No significant changes were observed (data not shown).

Table 5.1. Physicochemical properties of the natural DOM used in this study when prepared at typical concentration found in the source lake (see Gjessing et al., 1999 for details).

pH	Conductivity (mS m^{-1})	Colour (mg Pt L^{-1})	UV absorbance 254 nm	($SUVA_{254}$)* (mgC x 10^2)	%Corg**	molecular weight (Da)	C_{ar}/C_{al}***
5.17	2.49	166	0.813	4.59	50.3	3900	0.22

Lake DOC concentration typically of 17.7 mg L^{-1}.

(*) Specific UV – Absorbance at 254 nm. (**) Percent of Carbon content. (***) Ratio of aromatic to aliphatic carbon.

Dialysis experiment: Cellulose ester dialysis bags, with a flat-width of 31 mm and molecular cut-off 100-500 Da (Spectra/Por, Spectrum Europe, Breda, The Netherlands) were used; 10 cm-long sections were cut and prepared by thoroughly washing with Milli-Q water to remove any excess of the preserving agents and were maintained soaked in Milli-Q water overnight before the experiment. Ten litres of soft artificial freshwater (SAF) were prepared by adding 1.17 g NaCl to Milli-Q water to reach 0.01 M (0.58 g L^{-1}), to give an ionic strength commonly detected in boreal freshwaters. SAF was then split into five bottles (2 litres each) where the pH was adjusted by titration with HCl or NaOH to reach pH 4, 5, 6, 7 and 8 respectively. At the start of the experiment, each bag was spiked with 200 µL of ISU 10 µg mL^{-1} working solution. The control bag with no DOM was topped up with SAF to reach 10 mL total volume. The other bags were spiked with 170 µL, 340 µL and 640 µL of DOM working solutions to reach 5, 10

and 20 mg L^{-1} DOC respectively, and then SAF was added to each bag to reach 10 mL total volume. Each dialysis bag was then sealed with standard closures (Spectra/Por, Spectrum Europe, Breda, The Netherlands), before being placed in a 250 mL glass beaker, containing 200 mL of SAF (Figure 5.2). This procedure was repeated for each pH level, in triplicates, for a total of 60 units. After adding glass-coated metal stirrer bars (20 mm), the experimental units were closed at the top with Teflon linen plugs, and placed on a magnetic stirrer (Multistirrer15, Progen Scientific, UK) in the dark at 18 ± 1.6 °C for 48 hours. Samples (2.0 mL) were collected from the beakers (external to the dialysis bag) at 6-time intervals (t0 = 0 hrs, t1 = 4hrs, t2 = 8 hrs, t3 = 12 hrs, t4 = 24 hrs, t5 = 48 hrs) and split into two 1 mL aliquots for the determination of ISU and to check for potential leakage of DOM through the dialysis membrane, respectively. At 48 hours, 1 mL sample was also collected from inside the bag of each unit (Figure 5.2). Samples for chemical analyses were filtered through a 0.2 µm filter (Whatman, UK) placed on a syringe, transferred into a 2.5 mL amber glass vial, and stored at -20° C until analyses. Expected concentration of the herbicide at the end of the experiment at equilibrium conditions was 9.5 µg L^{-1}. This concentration was selected *a priori* through a standard ecotoxicological test based on OECD guidelines of 2009 causing sub-lethal effects on phytoplankton (Text S5.1). Mass recovery was determined by following the method reported in Text S5.2. Mass loss due to adsorption of ISU was quantified in each experimental unit inside and outside the dialysis bag. Each experimental unit was rinsed with a total amount of 10 mL of methanol. 5 mL were used to rinse the internal part of the unit (inside the bag). The other 5 mL were used to rinse the external part (outside wall of the dialysis bag, internal wall of the beaker, clips and stirrer bars). The respective solvents were collected in amber vials, to be later gently dried with nitrogen stream, and re-suspended in 1 mL methanol, than filtered and stored at -20° C in 2.5 mL amber glass vials. The results were further checked by mass balance.

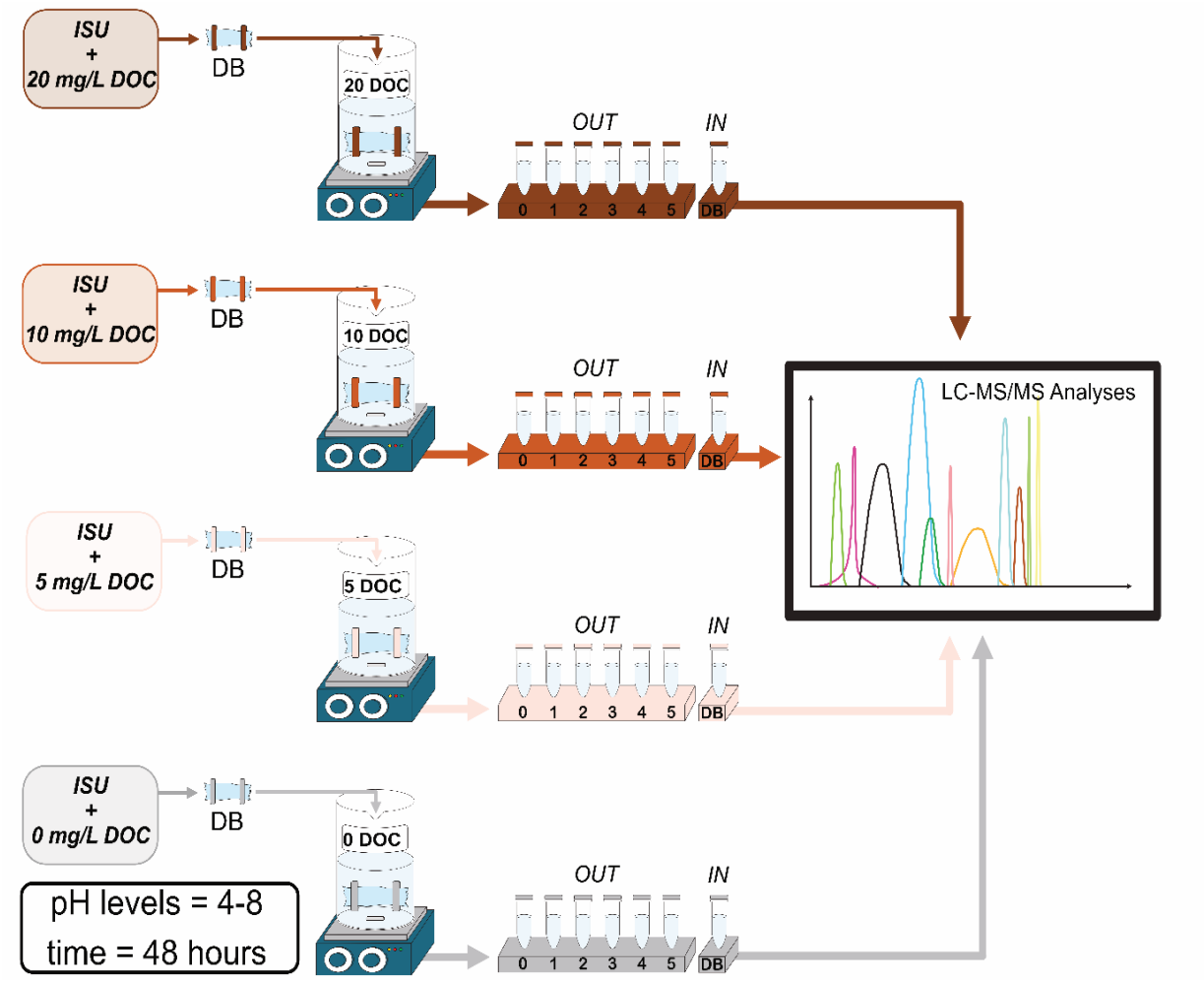


Figure 5.2. Experimental design. The association of ISU to DOM (0, 5, 10 and 20 mg L^{-1} DOC) was tested through equilibrium dialysis technique at 5 pH levels (4, 5, 6, 7 and 8). DB; dialysis bags. OUT; sampled collected at six time intervals (0 = 0hrs, 1 = 4hrs, 2 = 8hrs, 3 = 12hrs, 4 = 24hrs, 5 = 48hrs) outside the dialysis bag. IN; sampled collected inside the dialysis bag 48 hours after the start of the experiment.

5.2.2 Chemical analyses

ISU was quantified by direct injection liquid chromatography mass spectrometry (Shimadzu, 8040), using an XBridge BEH C18 column (2.1 mm X 100 mm, 3.5 µm). The mobile media were A, 0.2% ammonium formate in Milli-Q water, and B, acetonitrile. The gradient procedure was optimized at: 0-1 min 20% B, then increased to 100% within 8 min, held at 100% for 5 min, after that decreased to the initial conditions (20% B) within 1 min. Finally, 6 minutes of post-run ensured re-equilibration of the column before the next injection. The injection volume was 10 µL, while the column and the tray temperature were set to 25°C. The quantification of

ISU was based on internal standard method (Isoproturon-d6, Sigma Aldrich), and the instrument detection limit of 0.96 ng mL^{-1}. To check on possible ion suppression, standard ISU samples were analysed after every sample injection. Samples with quantification accuracy below 98% were discarded.

5.2.3 Assessment of method performance and quality of the measurements

A set of criteria concerned with system functionality, equilibrium condition and mass recovery were used to critically assess the method performance under varying pH and DOM concentration. These were:

DOM trans-membrane leakage: The absence of traces of DOM outside the dialysis bag was a quality assurance criterion to exclude artefacts linked to DOM trans-membrane leakage. DOM concentration outside the dialysis bag was analysed immediately after each experiment using a plate reader coupled with a spectrophotometer (BioTek Synergy MX; Winosky, VT, US). Triplicates of solution samples from outside the bag of each experimental unit were directly loaded on clear flat-bottom 96-well black microplates (300 µL in each well) (Corning, US). Samples from standard DOM solutions of 5 and 20 mg L^{-1} DOC were also loaded for comparison. Absorbance wavelengths between 250-280 nm were measured, in accordance with other studies (Hagman et al., 2018; Rizzuto et al., 2020; Thrane et al., 2014).

Reproducibility: The quality of replicate measurement of ISU concentration in and outside the bags was checked for the presence of outliers. The total variability among each set of triplicates was considered acceptable when it was below 25% (of which 10% attributable to analytical variability of the direct injection analysis method and 15% of acceptable system results variability).

Mass recovery and adsorption: Total mass recovery of the herbicide (i.e. mass balance closure) was tested by comparing ISU masses recovered inside and outside the bag. In addition,

the mass of ISU adsorbed to components of the experimental units were analysed (both on the internal wall of the membrane, from here referred as A_{in} (µg)) and on the outside surfaces (i.e. sum of external wall of the bag, glassware, clips and stirring bars, A_{out} (µg)). These recovered masses were summed together and compared with the spiked mass to calculate recovery and mass balance closure.

Assessment of trans-membrane equilibrium of ISU: The fundamental criterion for checking trans-membrane equilibrium was that in the control units (e.g. the experimental units with no added DOM), the concentration of ISU inside (C_{in}, µg L^{-1}) and outside (C_{out}, µg L^{-1}) the dialysis bag was the same. However, because the solution volumes inside and outside the dialysis bags were very different (nominally 10 ml inside vs. 200 mL outside), an additional criterion based on comparing experimental observation with theoretical expectation of mass distribution inside and outside the bag was introduced. For instance, the mass (µg) of the herbicide was calculated inside (mass detected inside - $M_{D/in}$) and outside (mass detected outside - $M_{D/out}$) the bag by multiplying measured C_{in} and C_{out} (respectively) with the volumes of the solution measured in the control units in the respective compartments at the end of the experiments. $M_{D}/_{in}$ and $M_{D/out}$ were then compared with the masses inside and outside the dialysis bag of the control units theoretically expected at equilibrium ($M_{E/in}$ and $M_{E/out}$, respectively), considering the experimentally measured adsorption on the membrane and glassware. Only when all equilibrium criteria were met, the DOM-ISU binding was assessed.

5.2.4 DOM-ISU binding

When the equilibrium conditions and all other quality criteria were verified, the DOM-ISU binding was assessed in the experimental units containing DOM. Theoretically, if DOM bound the herbicide, a significantly higher concentration will be detected inside the dialysis bags than outside, because complexation by the DOM prevents a certain amount of compound from crossing the membrane due the larger molecular size of the DOM. Hence, significantly higher

C_{in} than C_{out} was deemed as required evidence for partial or complete binding of ISU to DOM. A second criteria was also considered, whereby C_{in} in the experimental units with DOM should be significantly higher than C_{in} in the control units. Furthermore, to rule out the confounding factor of different dilutions inside and outside the bag, another condition to verify ISU binding to DOM was that the mass $M_{D/in}$ in the experimental units containing DOM should be higher than the mass $M_{D/in}$ of the control units.

In summary, three scenarios were considered to determine complexation in the units containing DOM:

- No binding: $C_{in} \sim C_{out}$; $M_{D/in} \sim M_{E/in,}$ and $M_{D/out} \sim M_{E/out}$.
- Complete binding: $M_{D/in}$ represented 100% of the ISU mass spiked in the experimental unit, $C_{in} >> C_{out}$ and $C_{out} \sim 0$, $M_{D/in}$ DOM units > $M_{D/in}$ control units, while $M_{D/out} = 0$.
- Partial binding: $C_{in} > C_{out}$, $M_{D/in}$ DOM units > $M_{D/in}$ control units.

In the units where partial or complete binding was observed, the magnitude of the complexation of ISU with DOM was also expressed by calculating the conditional distribution coefficient (K_{DOC}, L g^{-1}). K_{DOC} was calculated as follows (from Buschmann et al., 2006) assuming at equilibrium:

$$K_{DOC} = \frac{[C_{in}\,] - [C_{out}]}{[C_{out}]\; x\; [DOC]} * \; 1000 \qquad \text{(Equation 1)}$$

where the term [C_{in}]-[C_{out}] is the concentration of compound complexed with DOM and [C_{out}] is the concentration of free (unbound) compound. DOC and DOM values can be inter-converted in the equation by using the DOM's % of C content value of 50.3 (Table 5.1).

The percentage of bound compound (B_{DOM}), defined by mass of bound compound divided by the total mass of the compound inside the bag, was calculated as follows:

$$B_{DOM} = \frac{M_{in} - C_{out} * V_{in}}{M_{in}} * 100 \qquad \text{(Equation 2)}$$

where V_{in} is the solution volume inside the dialysis bag and $C_{out}*V_{in}$ is the mass of unbound inside the bag. M_{in} is the total mass of compound inside the bag.

5.2.5 Statistical analyses

Data analyses and statistics were conducted using R (version 3.5.1) statistical software (R Core Development Team, 2015). The single comparison analyses between C_{in} and C_{out}, K_{DOC} and B_{DOM} between different DOM levels were tested by one-way ANOVA. The graphs were prepared by using the R package "ggplot2" (Wickham, 2006).

5.3 Results and Discussions

5.3.1 Quality of measurements: trans-membrane diffusion of DOM

The absorbance measurements (A_{280}) made after 24 and 48 hours showed no differences between the samples collected outside the dialysis bag from the DOM units with 5 and 20 mg L^{-1} DOC and the control unit in the absence of DOM, at all pH levels (Figure S5.1B, $p = 0.76$). Samples from standard DOM solutions at 5 and 20 mg L^{-1} DOC are also reported for comparison in Figure S5.1A. These results proved that the low molecular cut-off membrane used in this experiment (100-500 Da) confined all the DOM inside the bag throughout the duration of the experiment. The efficacy of smaller pore size membrane (also previously reported by others - Buschmann et al., 2006), represents a substantial improvement. For instance, Thevenot et al. (2009) reported DOM losses during their experiments with dialysis membrane with 1000 Da molecular weight cut-off. Akkanen et al. (2003) reported a 15% decrease in the DOM content (20 mg L^{-1} DOC) inside the dialysis bag after 4 days of dialysis using a membrane with 1000 Da pore size. Williams and others had similar results (1999). Carter and Suffet (1982) also observed 5% losses with Aldrich humic acids. Those inappropriate pore sizes have led to the misestimation of the interaction between DOM and

contaminants due to the leakage of DOM-contaminants complexes from the dialysis bags (Thevenot et al., 2009; Williams et al., 1999; Yamada and Katoh, 2020). While trans-membrane transfer of DOM molecules smaller than 100 Da cannot be excluded in principle, no measurable amounts were detected outside the membrane, suggesting that - if present - these artefacts had negligible influence on the measurement quality. It must be also noted that there is preferential binding of organic chemicals to the high molecular weight fraction of DOM (Vialykh et al., 2020). In addition, while the reduced pore size of the membrane used in this study prevented the diffusion of DOM through the dialysis membrane, the results may also vary with other types of DOM with different molecular size.

5.3.2 Quality of measurements: reproducibility, mass balance and adsorption

The variability of the replicates was ≤25%, which was the threshold set *a priori* for measurement acceptability. The total mass recovery of the herbicide, which combined the mass measured in the experimental solutions and the one adsorbed to the different compartments of the experimental units (dialysis membranes, glassware and bag closures), yielded an overall 100 ± 0.3% at all DOM concentrations and pH levels (Table 5.2, S5.1). Such optimal mass balance closure of the herbicide indicated negligible ISU degradation/volatilisation during the experiment, in agreement with expectation from data on ISU degradation half-life (Bi et al., 2012; Böttcher and Schroll, 2007).

The mass of the herbicide recovered from adsorption to the different experimental compartments ranged from 5% to 42% and differed across pH and DOM levels (Table 5.2, S5.1). Two distinct patterns were observed at pH 4-6 and 7-8, respectively. At lower pH levels (4-6), the A_{in} was below 20% in the control units, ranging between 10-17% (Table 5.2, S5.1). Notably, a significant increasing pattern of A_{in} was observed across the DOM levels, reaching values of 30-38% at the highest levels of DOM (20 mg L^{-1} DOC, Table 5.2). The A_{out} remained consistently below 20% in all units, ranging between 5% and 17%, with no significant

differences between controls and units with DOM (Table 5.2). At the higher pH levels (7-8) in contrast, the A_{in} values of the control units were significantly higher ($p < 0.01$) compared to those observed at pH 4-6, ranging between 37% and 40% (Table 5.2, S5.1). No differences in A_{in} were observed between the control and the DOM units. In addition, A_{out} at the higher pH levels was significantly higher compared to those at pH 4-6 ($p < 0.01$), yielding values between 30% and 42%, with no significant differences between the control and DOM units (Table 5.2, S5.1).

The different adsorption observed across the pH levels could be attributed to the influence of water pH on the speciation/form of the herbicide, and/or the dialysis membrane, which could vary their ionic configuration, modify their physical-chemical properties, and in turn affect their interactions (Ashauer and Escher, 2010; Rizzuto et al., 2021, 2020). A modification in the properties of ISU is the least likely, due to its non-ionic characteristics at the pH values considered here. Instead, several cellulose esters of the dialysis membranes are carriers of carboxylic or phthalates groups (such as trimellitate and phthalates-functionalized cellulose) with pKa values in the range of conditions studied here (Dias and Duarte, 2013). Changes in pH levels can confer wettability and hydrophilic character to the membrane (which limit hydrophobic interactions) and likely promote pH-dependent weak interactions with the herbicide (Dias and Duarte, 2013). Hence, higher pH values (7-8) could have modified the membrane properties, leading to higher adsorption of the herbicide to both the inner and outer walls of the dialysis membrane in all experimental units compared to the lowest pH levels (Table 5.2, S5.1). High degrees of adsorption may affect the quality of the experiments and the accuracy of the binding results. As the binding of ISU with DOM is not significant at high pH conditions (C_{in} and C_{out} are similar), the adsorption issue is irrelevant for this work at high pH. However, an alternative membrane with lower adsorption may be needed for assessing binding

accurately at higher pH conditions as the greater adsorption effect blurred the observation of trans-membrane equilibrium and confounding mass balance closure.

The increasing pattern of A_{in} across the DOM levels observed only at the lower pH levels can be explained with the interaction between the herbicide, the dialysis membrane, and the DOM itself. For instance, DOM includes humic and fulvic acids rich in carboxylic groups and electronegative domains such as oxygen atoms in alcohols and ketones functional groups, whose dipolar moment and interaction with partly charged domains of the ISU or the dialysis bag can be strongly influenced by water pH (Dias and Duarte, 2013; Pace et al., 2012; Tanaka et al., 2005). Hence, the lower pH may have changed the chemical configuration of the DOM, increased its binding affinity with both the herbicide and the dialysis membrane. This hypothesis is corroborated by the fact that the A_{in} in the control units and the A_{out} in all experimental units is consistently below 20%. Hence, while the influence of A_{out} on the quality of measurements can be considered negligible, the increasing pattern of A_{in} in the DOM units at pH 4-6 should be taken in consideration during the evaluation of the method performance to avoid blurring the true effect of the DOM and cause artefacts in the analysis of DOM-ISU binding. Some previous studies of DOM-contaminant binding have overlooked adsorption to glassware and the membrane, leading to uncertainties over mass balance closure and measurement validity (Lee and Farmer, 1989; Thevenot et al., 2009). These results showed that assessing mass recovery and adsorption to the experimental unit surfaces is crucial for ensuring high confidence in the measurement quality, as also suggested in other studies (Rizzuto et al., 2021).

Table 5.2. Percentages of ISU mass recovered (mean ± standard deviation) a) inside, b) outside the bag, adsorbed c) inside (Ain) and d) outside the bag (Aout), and e) total recovery.

pH	DOM	% recovery in	% recovery out	% A_{in}	%A_{out}	%total

		(a)		(b)		(c)		(d)		recovery (e)	
		mean	**sd**	**mean**	**sd**	**mean**	**sd**	**mean**	**sd**	**mean**	**sd**
4	0	89.0	0.6	89.7	2.8	10.5	2.7	10.8	1.0	100.2	0.1
	5	102.4	0.6	74.4	1.4	25.6	1.4	10.8	0.5	99.9	0.0
	10	107.7	2.0	72.2	1.0	27.8	1.0	7.7	1.8	99.9	0.01
	20	103.9	2.6	69.9	1.5	30.0	1.5	11.9	2.8	99.9	0.1
5	0	84.8	1.3	80.5	4.7	17.3	1.5	15.7	0.9	100.3	0.5
	5	133.3	2.8	73.9	2.0	16.0	2.0	5.9	0.5	101.8	0.1
	10	108.7	0.6	66.4	1.5	13.5	1.6	5.6	0.5	100.6	0.1
	20	88.6	0.7	78.4	3.6	22.0	3.9	15.1	0.4	100.4	0.5
6	0	86.3	0.7	73.3	1.6	15.7	1.1	12.9	0.5	99.9	0.01
	5	96.9	0.9	73.9	2.0	26.0	2.0	11.5	0.9	99.9	0.0
	10	94.2	0.3	64.6	0.8	35.3	0.8	13.6	0.1	99.9	0.0
	20	77.7	1.2	61.9	0.4	38.0	0.4	23.9	1.0	99.9	0.0
7	0	74.9	5.8	61.5	4.7	38.5	4.7	24.5	5.5	99.9	0.0
	5	68.7	1.9	59.9	2.4	40.2	2.4	30.8	1.8	100.1	0.2
	10	67.6	1.4	62.9	1.2	37.3	1.2	32.2	1.3	100.1	0.2
	20	63.9	1.4	61.9	1.9	38.0	1.9	35.3	1.3	99.9	0.0
8	0	62.0	0.8	62.8	1.3	37.2	1.3	37.4	1.0	99.9	0.0
	5	63.6	1.1	62.6	0.8	37.3	0.8	36.0	1.3	99.9	0.0
	10	62.2	0.4	62.6	0.8	37.5	1.0	37.4	0.5	100.1	0.1
	20	57.1	3.6	59.8	0.9	40.1	0.8	42.3	3.5	99.9	0.3

5.3.3 Method performance: trans-membrane equilibrium

Equilibration across the membrane was checked in the control units after 48 hours, based on the criteria enunciated above (for C_{out} during the 48 hours experiment see Figure 5.3). C_{out} and C_{in} measured after 48 hours are shown in Figure 5.4 for the control units, the different DOM levels, and pH conditions. The control units displayed no significant differences between ISU C_{in} and C_{out} after 48 hours at all pH levels (Table S5.2) indicating equilibrium had been attained by this time. Furthermore, in compliance with the evaluation parameters, the herbicide $M_{D/in}$ values in the control units were not significantly higher than $M_{E/in}$, confirming that the equilibrium conditions were met at all pH levels. These results therefore confirm that the smaller pore size membrane allowed ISU trans-membrane diffusion while preventing DOM diffusion through the membrane pores. In addition, these results indicate that the adsorption

losses reported in the previous section had no observable effect on the trans-membrane equilibrium of the herbicide in the control units.

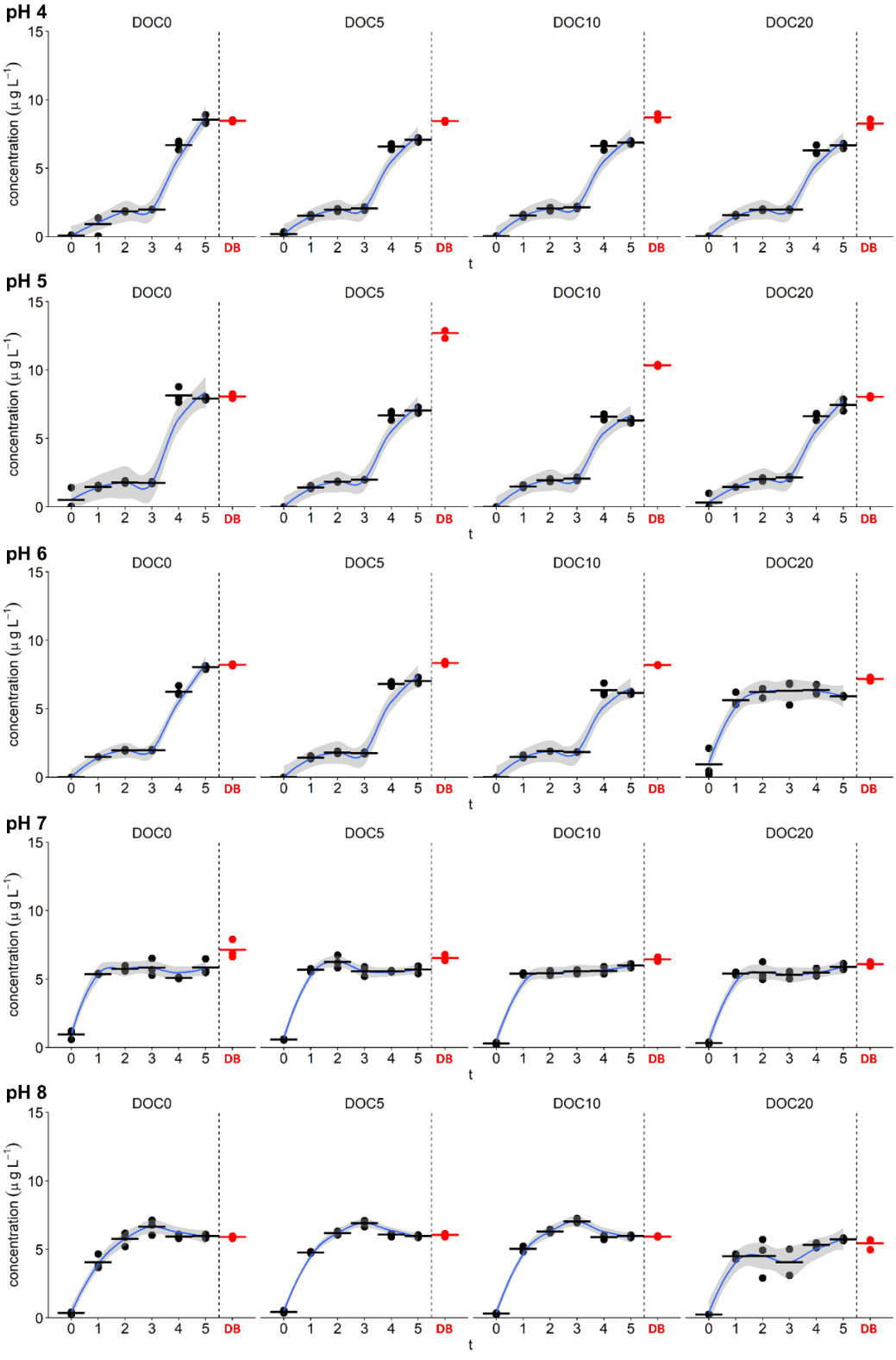


Figure 5.3. ISU concentrations detected outside the dialysis bag at six time intervals (0 = 0hrs, 1 = 4hrs, 2 = 8hrs, 3 = 12hrs, 4 = 24hrs, 5 = 48hrs) and inside the bag at 48 h (DB), at 4 levels of DOM (0, 5, 10, 20 mg L^{-1} DOC) and 5 levels pH (4-8). Horizontal bars represent the mean values. Locally estimated scatterplot smoothing (LOESS) was fitted to the data points to generate line plots. Grey areas indicate 95% confidence interval.

5.3.4 Method performance: DOM-ISU binding

The final step of the critical evaluation was to test the degree of ISU binding with DOM. The first criterion of the framework was that $C_{in} > C_{out}$ in the experimental units containing DOM, which was fulfilled under most of the pH and DOM conditions. For instance, results showed that C_{in} was significantly higher than C_{out} in all the experimental units containing DOM at pH 4, 5, 6 ($p < 0.001$, Tables S5.3-S5.5) and 7 ($p < 0.05$, Tables S5.3-S5.5), while no significant differences were observed at pH 8 (Tables S5.3-S5.5). However, while the results observed at pH 4-7 may be interpreted as evidence of DOM-ISU binding, the high A_{in} observed in the control units and the high A_{out} reported in all experimental units at pH 7 (ca. 38%) could not allow a high level of confidence in the evaluation of the DOM-ISU binding in the DOM units. It is plausible that such high levels of A_{out} measured at this pH level could have driven the equilibrium between the two sides of the membranes, causing the higher C_{in} compared to C_{out} (Table S5.1). Hence, to avoid the occurrence of false positive DOM-ISU binding, the data at pH 7 were not analysed further. In contrast, for the other three levels of pH where C_{in} was found higher than C_{out} (pH 4-6), the low A_{out} in all experimental units (<17%) and the minor A_{in} in the controls (<17%) ensured high confidence in the quality of measurements and therefore in the evaluation of the binding of DOM with the herbicide. Hence, the experimental units at pH 4-6 were admitted for evaluation to the second criterion of the framework, where C_{in} in the DOM units must be significantly higher than the control ones. Since the A_{in} was higher in the DOM units compared to the controls at pH 4-6 (Table 5.2, S5.1), the extra ISU adsorbed inside the dialysis bag was assumed to be complexed with the DOM (for more information on this process please refer to paragraph 'Quality of measurements: reproducibility, mass balance and adsorption'). By applying this assumption, C_{in} in the units treated with DOM yielded significantly higher values compared to the C_{in} of the control units at pH 4, 5 and 6 ($p < 0.001$) (Figure 5.4). Thus, all the units at pH 4-6 satisfied the second

criterion and were admitted to the last part of the framework for the evaluation of the DOM-ISU binding, where the $M_{D/in}$ of the experimental units must be higher than the $M_{D/in}$ in the control units. Results showed that by accounting for the extra ISU adsorbed inside the bag as complexed with DOM, the ISU $M_{D/in}$ of the DOM units yielded significantly higher values than $M_{D/in}$ of the controls at all concentrations of DOM at pH 4-6 (Table 5.2, S5.1). In addition, to further corroborate the choice to exclude the data at pH 7 from further analyses, results showed that ISU $M_{D/in}$ of the DOM units yielded significantly lower values than $M_{D/in}$ of the controls, which infringes the assumption set in the quality criteria. In contrast, results observed at pH 4, 5 and 6 fulfilled all the quality assurance criteria, indicating partial DOM-ISU binding. The complete binding scenario was not observed for any of the pH levels investigated.

The results presented here highlight that the dialysis method can yield potential false positives, and that quality assurance criteria based on mass distribution, rather than only on concentration comparison across the membrane is key to avoid significant errors in assessing binding of DOM with contaminants.

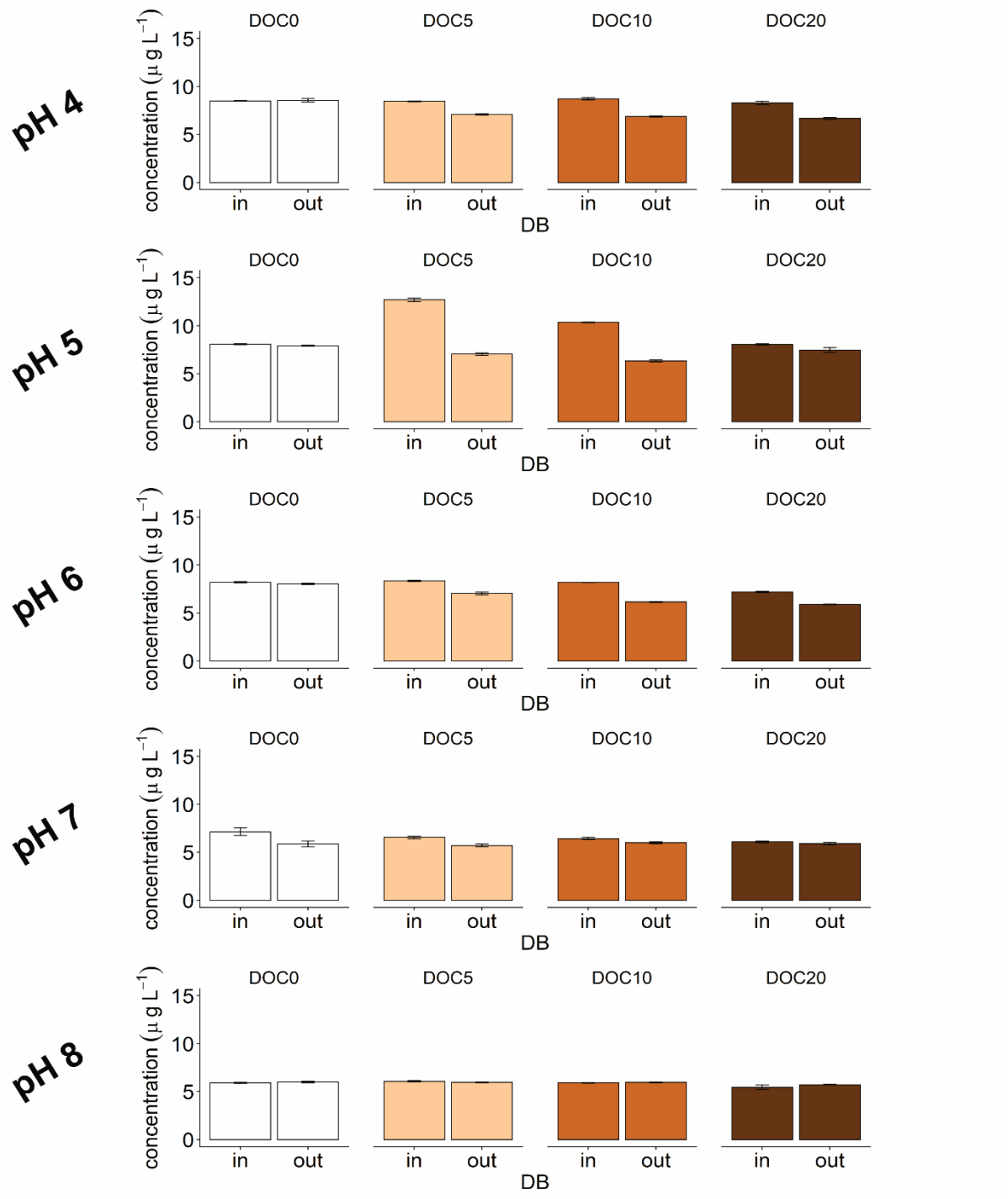


Figure 5.4. ISU concentration inside (Cin) and outside (Cout) the dialysis bag (DB) after 48 hours of the experiment, at 4 levels of DOM (0, 5, 10 and 20 mg L^{-1} DOC) and 5 levels of pH (4, 5, 6, 7 and 8). Black error bars represent standard deviation.

5.3.5 Method performance: K_{DOC} and B_{DOM}

In general, the hydrophobicity of a compound is considered as a determinant of its affinity for DOM (Pan et al., 2008). However, our experiments showed that an herbicide with relatively low hydrophobicity such as ISU (log K_{ow} 2.87) can partially bind with natural DOM possibly due to pH-dependent weak electrostatic interactions. Equation 1 and 2 were used to calculate K_{DOC} and B_{DOM} respectively, using the data obtained for the treatment conducted at pH 4, 5 and 6. The results are shown in Table 5.3. For instance, log K_{DOC} and B_{DOM} were 1.6 L g^{-1} and 18.9%, 1.6 L g^{-1} and 30.2%, 1.5 L g^{-1} and 19.5% respectively at pH 4, 5 and 6 (average among DOM levels, Table 5.3). The association of ISU with DOM was already reported for soil-water systems (Beck and Jones, 1996; Ertli et al., 2004), but – to the best of our knowledge – there are no other studies reporting the direct DOM-ISU binding in natural water environments. In another study, Akkanen and others (2001) tested the binding of natural DOM with atrazine, a phenylurea herbicide with very similar physical-chemical properties to ISU, using the equilibrium-dialysis method. The authors reported that the binding between the herbicide and DOM was too low or too weak to be detected (Akkanen and Kukkonen, 2003, 2001). The larger cut-off of the membranes used in that study (e.g. > 1000 Da) could result in DOM breakthrough, leading to an underestimation of DOM binding (Thevenot et al., 2009). In our study, the smaller pore size used (100-500 Da) prevented DOM molecules from crossing the dialysis membrane (Figure S5.1) and ensured a better assessment of natural DOM interaction with a phenylurea herbicide.

The K_{DOC} values of ISU previously measured for the interaction with organic matter in soil leachates varied greatly, and this variation may be caused by the different properties of organic matter, soil chemistry, as well as the different methods used (Burkhard, 2000; Krop et al., 2001). Cook et al. (2004) reported log K_{DOC} values of ISU ranging 1.96-5.75 in UK agricultural soil using standard batch adsorption procedures, suggesting that the interaction of ISU with

DOM was directly related to soil organic carbon content. Other authors observed ISU log K_{OC} values ranging between 1-3 in experiments of sorption/desorption of the herbicide to DOM originated from compost (Barriuso et al., 2011). These assessments are generally consistent with the present study results.

5.3.6 Influence of pH and DOM concentration on DOM-ISU binding

The partial binding between ISU and DOM was observed only at the lower levels of pH (4, 5 and 6) across all DOM levels (Table S5.3-S5.5), while no binding occurred at the higher pH levels (7-8) according to the quality criteria. The effect of water pH on the speciation/form of both the herbicide and the DOM could have played a key role in this process, as also reported in the previous paragraph ('3.2 Quality of measurements: reproducibility, mass balance and adsorption'). Most likely, the binding of ISU with DOM could have been favoured at the lower pH levels, where the alteration of the configuration of ionizable groups operated by the lower water pH can in turn form hydrogen bonds with electronegative domains of the ISU molecules or other types of weak electrostatic interactions (e.g. with the induced dipole of the phenylurea group in ISU). This hypothesis is supported by other studies, which report evidence of the effect of lower water pH on the ISU association with DOM. For instance, Beck & Jones (1996) showed that the affinity between ISU and DOM increased in more acidic soils compared to more alkaline ones. In another study on the effect of pH on association of ISU with natural DOM, Ertli and others (2004) showed a strong pH dependence, where adsorption of ISU was higher at lower pH in acidic soils.

The binding of ISU with DOM did not show significant differences between pH levels 4, 5 and 6. For instance, the average log K_{DOC} and B_{DOM} calculated among the different levels of DOM were of 1.7 ± 0.2, 1.6 ± 0.6 and 1.5 ± 0.3 L g^{-1}, and of 18.9 ± 2.1%, 30.2 ± 16.5% and 19.5 ± 3.9 % at pH 4, 5 and 6, respectively. At the same time, the binding of ISU across the DOM concentrations differed between the pH levels. While no significant differences were observed

between pH 4 and 6 (Table 5.3), at pH 5 the association of ISU with the lower levels of DOM (5 and 10 mg L^{-1}) was significantly higher ($p < 0.01$) than that measured at pH 4 and 6, as reported by the K_{DOC} and B_{DOM} values (Table 5.3). The influence of pH in altering the configuration of ISU, DOM and the dialysis membrane at different degrees for different pH could be the explanation for the increased binding between DOM and ISU at pH 5. Most likely, pH 5 represented an ideal condition for both the herbicide and DOM to interact, which resulted in the higher binding (Table 5.3). Preferred interaction of the herbicide ISU with natural DOM at pH 5 was also reported elsewhere (Ertli et al., 2004). Also, pH 5 gave the best experimental conditions, with the lowest adsorption of the herbicide compared to the other pH levels (Table 5.2). At the same time, the exceptionally low binding data at pH 5 for the DOM concentration of 20 mg L^{-1} cannot be explained logically. It is possible that the concentration of ISU in the sample taken from outside of the dialysis bag, C_{out}, was contaminated during sample treatment or analysis, which will result in incorrect binding data when using equations 1 and 2.

Notably, at all the pH levels investigated in this study, the association of ISU with DOM decreased ($p < 0.001$) with increasing concentration of DOM. For instance, the log K_{DOC} values were significantly higher at the lower levels of DOM (5 mg L^{-1} DOC), and lower at the higher levels of DOM (20 mg L^{-1} DOC) at pH 4, 5 and 6 (Table 5.3). The effect of DOM concentration on the percentage of bound compound (B_{DOM}) is less clear (Table 5.3). However, there is a general trend of lower B_{DOM} at 5 mg L^{-1} DOC. The lack of linear relationship between the DOM concentration and both the K_{DOC} and B_{DOM} results may stem from cross interaction between DOM constituents at higher DOM concentrations (Carter and Suffet, 1982; Deng et al., 2021), affecting the density of available binding sites for ISU on the DOM. Previous studies have discussed that higher activity of DOM in a solution may alter the DOM macromolecular configuration (Engebretson and von Wandruszka, 1994; Jiang et al., 2020), hindering access of ISU to the more reactive binding molecular domains. Behaviour similar to what was

observed here was described in previous studies using humic acids (Akkanen et al., 2004; Akkanen and Kukkonen, 2001), and natural DOM extracted from soils (Ling et al., 2005; Wang et al., 2018).

Table 5.3. Log conditional distribution coefficient (log KDOC), and percentage of bound compound (BDOM) for DOM-ISU binding at three different levels of DOM (5, 10 and 20 mg L^{-1} DOC), and water pH 4, 5 and 6.

pH	DOM	K_{DOC}	Log K_{DOC}	B_{DOM}
4	5	74.6	1.9	16.2
	10	48.8	1.7	21.1
	20	24.1	1.4	19.4
5	5	161	2.2	44.6
	10	63.8	1.8	38.9
	20	6.4	0.8	11.2
6	5	61.6	1.8	15.7
	10	45.5	1.7	24.8
	20	12.6	1.1	18.0

5.4 Conclusions

In this study, the performance of an optimized dialysis equilibrium-based method for measuring the binding of organic contaminants to natural dissolved organic matter (DOM) was improved using:

- reduced membrane pore size (100-500 Da) to prevent DOM crossing the membrane;
- highly hydrophilic cellulose-ester membrane to reduce hydrophobic interactions between DOM and the contaminants;
- direct injection to LC-MS/MS to quantify DOM-contaminant binding;

It was assessed against a set of quality assurance criteria based on: the evaluation of the mass distribution of the compounds in the system; the assessment of adsorption of the compounds on components of the experimental unit; and evaluation of DOM trans-membrane leakage. This assessment was carried out while measuring the binding between natural DOM and the

herbicide Isoproturon. The diffusion of DOM through the dialysis membrane was successfully prevented by the reduced pore size of the dialysis membrane. The method demonstrated good reproducibility, optimal mass balance closure, and successful fulfilment of trans-membrane equilibrium at all pH levels. Our results also suggest that water pH influenced the physical-chemical properties of the dialysis membrane, driving to substantially different levels of adsorption of the herbicide between the lower pH (4-6) compared to the higher (7-8). Therefore, the conditional distribution coefficient of the herbicide was successfully measured at the lower pH levels (4-6), while the high levels of adsorption observed inside and outside the walls of the dialysis membrane at the higher pH (7-8) did not allow high confidence in the method performance, as could potentially lead towards false positive of the DOM-ISU binding. This study demonstrated that without the implementation of strict quality assurance criteria (e.g. reproducibility, mass balance and adsorption values), rather than only through comparing concentrations at the two sides of the membrane, artefacts and false results could be produced. In addition, by providing an optimization of the experimental set-up, the present results demonstrate that the resolution and sensitivity of the equilibrium dialysis method could be substantially improved, even for challenging compounds such as phenylurea herbicides, characterized by low hydrophobicity but capable of engaging into weak-electrostatic interactions with the DOM. The approach used in this study may not be suitable for all classes of chemical compounds. This study tested a compound with moderate aqueous solubility, relatively low volatility and slow biodegradation such as ISU. More volatile/semi-volatile compounds would need controls to avoid compound volatilisation, while compounds with lower aqueous solubility could have stronger interactions with cellulose-ester dialysis membranes or the glassware, requiring further/different QA/QC procedures and checks. However, the current procedure is currently suitable for other compounds in the intermediate range of solubility and low volatility. One technical improvement in this study was to use

natural DOM extracted from the relevant waters to ensure a natural complex spectrum of DOM constituents. However, as the physicochemical properties of DOM are known to vary greatly among different ecosystems (i.e. molecular size, functional groups), it would be useful to compare the binding capacity over a selection of DOM, isolated from different locations to understand the binding variation in relationship with different physicochemical properties of DOM.

The present study confirms the binding of ISU to natural DOM, highlighting a controlling effect operated both by water pH and DOM concentrations. Our results also corroborate other studies indicating preferential binding of ISU with DOM at lower pH, and a relationship between the amount of ISU bound to the DOM which was not dependent on the DOM concentration. These results add information to earlier assessments of DOM-organic chemicals interactions and highlight the importance of considering more realistic environmental conditions when estimating availability of organic substances to biota during risk assessment, through the use of reliable methods. In particular, it is shown that despite being hydrophilic, phenylurea pesticides such as ISU, can establish complex interactions with naturally occurring DOM. Hence, the equilibrium-based approach used in this study can be used not only as a useful research tool for the investigation of the DOM-contaminants binding, but also as a systematic assessment of new/existing contaminants for risk assessment purposes.

5.5 Acknowledgements

The authors thank Rolf Vogt from the Department of Chemistry, University of Oslo for providing the DOM used in this study. We also thank David Rochester from the Department of Chemistry, Yanying Li and Runmei Wang from Lancaster Environment Centre, Lancaster University (UK), for helping with the setup of the LC/MS-MS. The authors also wish to thank the anonymous reviewers whose comments helped improving the manuscript.

5.6 Funding

This study was financed by the Research Council of Norway, project grant no. 244460, Pollution and Ecosystem Adaptation to Changes in the Environment (PEACE). The research was co-funded by a discretional internal grant from the Norwegian Institute for Water Research (NIVA), under the Institute Strategic Priorities (SIS) on ecosystem remediation (TRADE-OFF). SR was also supported by the Lancaster University Faculty of Science and Technology and a Lancaster Environment Centre PhD Scholarship.

5.7 Credit author statement

SR, LN, EL, DLB, KCJ and HZ conceived the idea. SR, LN, KCJ and HZ designed the experiment. SR collected and analyzed the data. SR took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analyses and manuscript.

5.8 Competing interest statement

The authors declare no conflicts of interest.

Chapter VI: Binding of Waterborne Pharmaceutical and Personal Care Products to Natural Dissolved Organic Matter under different pH

Simone Rizzuto [1], Didier L. Baho [2, 3], Kevin C. Jones [1*], Hao Zhang [1*], Eva Leu [4], Luca Nizzetto [3, 5]

[1] Lancaster Environment Centre, Lancaster University, Lancaster, LA1 4YQ, UK.

[2] Department of Aquatic Sciences and Assessment, Swedish University of Agricultural Sciences, Uppsala, Sweden.

[3] Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, 0349, Oslo, Norway.

[4] Akvaplan-niva, CIENS, Science Park, Gaustadalléen 21, 0349, Oslo, Norway.

[5] RECETOX, Masaryk University, Kamenice 753/5, 625 00 Brno, Czech Republic.

* Corresponding authors; k.c.jones@lancaster.ac.uk; h.zhang@lancaster.ac.uk

Published in: S. Rizzuto, D. L. Baho, K. C. Jones, H. Zhang, E. Leu, L. Nizzetto, 2021. Binding of Waterborne Pharmaceutical and Personal Care Products to Natural Dissolved Organic Matter under different pH. Sci. Tot. Environ.784, 147208. **https://doi.org/10.1016/j.scitotenv.2021.147208.** To access the Supplementary Material please consult page S51 of the "Appendices and Supplementary Material" file or visit **https://doi.org/10.1016/j.scitotenv.2021.147208.**

Abstract

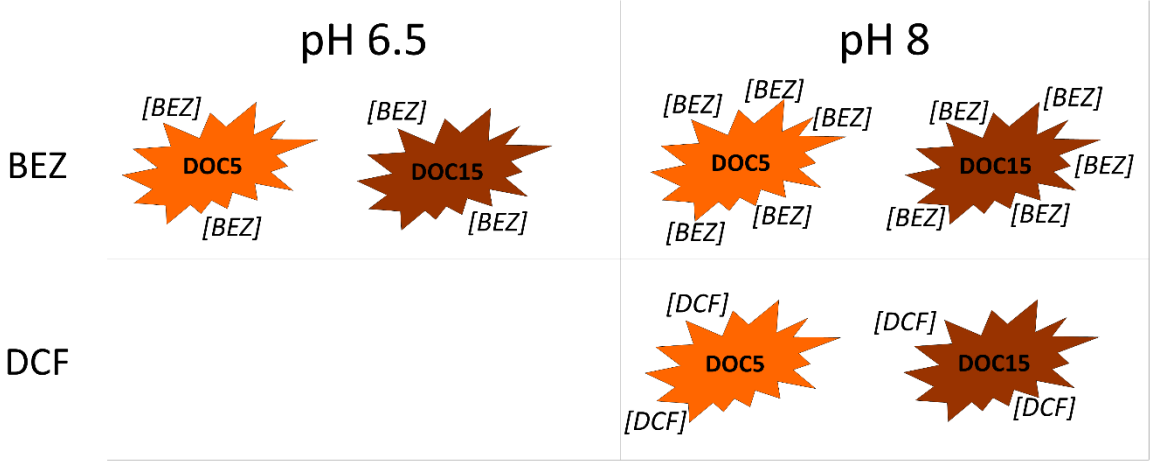
Information on how key environmental conditions such as natural dissolved organic matter (DOM) and water pH alter the possible risks posed by pharmaceuticals (PPCPs) is still scarce. In our previous study, the presence of natural DOM at high pH reduced the toxicity of a mix of waterborne PPCPs to algae. DOM-complexation and pH effect on speciation of the more hydrophobic and neutral compounds of the mix was suggested to be driving this behaviour. However, the study design did not allow the verification of this hypothesis. Here, the DOM-PPCPs interaction at different pH was investigated for 6 PPCPs through equilibrium dialysis, under the same conditions of DOM and pH as our previous study. Association with DOM was confirmed for the more hydrophobic PPCPs at high pH. The results suggest the binding was driven by i) the presence of carboxylic groups of PPCPs, ii) high pH shifting the structural configuration of DOM, making it more suited to bind some of the PPCPs. A non-linear change of binding capacity with increasing DOM concentration was also observed among the tested PPCPs.

Abstract Art / TOC

PPCP	Log K_{ow}	pKa
ATE	0.16	9.00
SMX	0.89	1.60/5.70
FUR	2.03	4.25
CLA	3.16	8.99
BEZ	4.25	3.83
DCF	4.51	4.15

NO BINDING WITH DOM

PARTIAL BINDING WITH DOM



6.1 Introduction

The widespread occurrence of anthropogenic contaminants in freshwater ecosystems is a major concern (Pal et al., 2010). Emerging contaminants such as the group of pharmaceutical and personal care products (PPCPs (Boxall et al., 2012)), widely used and continuously discharged in treated and untreated wastewater (Schwarzenbach et al., 2006), are particularly worrisome as they are biologically active at low concentration. In the environment, PPCPs can interfere with fundamental metabolic pathways (e.g. chlorophyll-a and lipid synthesis) in non-target organisms such as freshwater phytoplankton (Zhang et al., 2012, 2019); this could possibly translate into severe repercussions for the functioning of freshwater ecosystems (Arnold et al., 2013) considering the key ecological functions underpinned by microalgae. Knowledge of freshwater biota sensitivity to these stressors is mostly derived from experiments conducted under controlled laboratory conditions (OECD, 2009). There is a paucity of information on how the risk posed by these contaminants is modulated by the prevailing environmental conditions (Holmstrup et al., 2010; Laskowski et al., 2010). Dissolved organic matter (DOM) is present in all surface freshwaters (Leenheer and Croue', 2003) and has the ability to bind, adsorb and/or transform contaminants by forming complexes that are too large or too polar to cross biological membranes (Lipnick, 1995). Through this process, DOM (generally analysed as concentration of dissolved organic carbon – DOC) may reduce the bioavailable fraction and the toxicity of contaminants (Chen et al., 2017; Pan et al., 2009; Rowett et al., 2016).

The DOM binding affinity (usually expressed as distribution coefficient K_{DOC} or K_d) can be controlled by several factors, such as the water chemistry (i.e. pH), the physicochemical properties of the compounds (i.e. hydrophobicity, presence of functional groups) (Ashauer and Escher, 2010; Behera et al., 2010; Sun et al., 2020b) and of the DOM (i.e. molecular size, aromaticity, presence of functional groups and concentration; Gu et al., 2007; Tanaka et al., 2005). For example, a change in water pH can affect the compound-DOM complexation by

altering the compound and/or the DOM molecular configuration (Engebretson and von Wandruszka, 1994; Ghosh and Schnitzer, 1980; Myeni et al., 1999b). The relationship between DOM binding capacity and its concentration is also unclear. For instance, while linear correlations are generally reported (Burkhard, 2000; Krop et al., 2001), other studies observed that increasing concentrations of DOM could generate tighter molecular rearrangements, preventing the chemical compounds accessing the more hydrophobic areas of the DOM where binding generally takes place (Akkanen et al., 2001; Akkanen and Kukkonen, 2003). Hence, the effect of these complex interactions on toxicity of contaminants cannot be predicted easily. This is particularly important in boreal freshwater ecosystems. Here, climate and land-use changes - together with recovery from past acidification - have caused an increase in the levels of DOM and altered the water pH over recent decades, a process also known as water browning (Monteith et al., 2007; Williamson et al., 2016).

The influence of the interaction between DOM and water pH on the toxicity of PPCPs has been a subject of research (Pan et al., 2009). In a recent study (Rizzuto et al., 2020), we found that the toxicity of an environmentally realistic mix of 12 PPCPs to algae was reduced in water with low levels of natural DOM and high pH. We suggested that high pH conditions increased the DOM-PPCPs binding affinity by controlling the physicochemical properties of both PPCPs and DOM. In addition, a direct effect of DOM in hindering algal growth was observed with a non-linear dependence on DOM concentration (Rizzuto et al., 2020). However, that study design did not allow evaluation of the hypothesis on the mechanisms of interaction between PPCPs and DOM.

In this study we therefore explicitly addressed the interaction between PPCPs and the natural DOM at two different pH. Six PPCPs with demonstrated toxic effects on phytoplankton, with a range of different physicochemical properties were selected from the original mix; the same pH values, natural DOM (extracted from the same lake, Gjessing et al., 1999) and

concentrations were used as in the previous study (Rizzuto et al., 2020). An equilibrium dialysis technique was used to investigate the chemical-DOM binding (Akkanen et al., 2001; Akkanen and Kukkonen, 2001). With this method, the contaminant molecules can freely diffuse across the dialysis membrane, whereas the larger-sized DOM complexes are restricted to one side of the membrane. This method has been commonly used, but usually with a commercially available DOM only (i.e. Suwannee River, isolated humic or fulvic acids, Böhm et al., 2016). However, natural DOM is polydisperse, and may contain different constituents with varying levels of hydrophilicity, as well as several functional groups with different chemistry (Leenheer and Croue', 2003). Hence, while it is important to have a standardized DOM and/or to investigate the influence of different functional groups, it is also crucial to test the effects arising from DOM naturally occurring in local freshwater ecosystems that is relevant to the investigation (Akkanen et al., 2001). In addition, one technical hindrance recognized by several authors concerns the pore size of the membrane generally used in these experiments (>1000 Da); this could cause leakage of smaller molecular size DOM across the dialysis membrane, leading to an over-underestimation of the binding effect of DOM (Akkanen and Kukkonen, 2003). In the present study we overcame these limitations by using: i) natural DOM isolated from a boreal catchment (Gjessing et al., 1999b), containing virtually the full spectrum of constituents native of boreal systems; ii) hydrophilic semipermeable membranes with a smaller pore size (100-500 Da), enabling only relatively small and free chemicals (e.g. in the range of the PPCPs used here) to permeate.

Results from the equilibrium dialysis studies are used to test the hypotheses arising from our previous study, considering the implications for the potential effects on freshwater ecosystems.

6.2 Materials and Methods

The association of a mix of 6 PPCPs to three levels of natural DOM (0, 5 and 15 mg L^{-1} DOC), at two water pH values (6.5, 8) was tested using the equilibrium dialysis technique (Akkanen and Kukkonen, 2003) (Figure 6.1).

6.2.1 Selection of experimental conditions

Chemical compounds: Six PPCPs were selected from the mix used in our previous study (Rizzuto et al., 2020), namely: Atenolol (ATE), Sulfamethoxazole (SMX), Furosemide (FUR), Clarithromycin (CLA), Bezafibrate (BEZ), and Diclofenac (DCF) (chemical structures in Figure S6.1). They are among the most commonly detected PPCPs in European wastewaters and freshwaters (concentrations in natural freshwaters ranging from 0.1 to 380 µg L^{-1}, Table S6.1). The selection of the six PPCPs out of the mix of twelve from Chapter II (Rizzuto et al., 2020) was caried out according to specific parameters. The choice of the selection parameters was deemed necessary to guarantee the wider range possible of chemical-physical properties from the mix of the 12 PPCPs, with documented toxic effect on phytoplankton:

i) a broad range of hydrophobicity (measured as the octanol-water partition coefficient - log K_{ow}); the six PPCPs ranged from relatively high values of lipophilicity (log k_{ow} 4.51) to high levels of hydrophilicity (log k_{ow} 0.16) from the mix (Table 6.1).

ii) a range of acid dissociation constant (pKa) values; the selection ranged from pKa values of 1.6 to 9 (Table 6.1).

iii) presence of different functional groups on the analytes; the selection included compounds carrying amine, isoxazole, chlorobenzoic, and carboxylic groups (Table 6.1)

iv) Negative effects on primary producers; the six PPCPs were selected according to documented toxic effects on phytoplankton.

Expected concentrations of each chemical at the end of the experiment at equilibrium conditions (C_E, Table 6.1) are within the range of those detected in European freshwaters (Table S6.1).

Table 6.1. Physicochemical properties of the 6 investigated compounds.

PPCPs	Use	Log K_{ow}	Functional groups	Molecular weight (g mol^{-1})	water solubility (mg L^{-1})	pKa	C_E (µg L^{-1})
Atenolol (ATE)	Beta-blocker	0.16 (a)	Amine	266.34	13300	9 (f)	22
Sulfamethoxazole (SMX)	Antibiotic	0.89 (a)	Isoxazole	253.28	610	1.6/5.7 (g)	2.2
Furosemide (FUR)	Diuretic	2.03 (b)	Chlorobenzoic	330.74	73.1	3.9 (h)	2.2
Clarithromycin (CLA)	Antibiotic	3.16 (c)	Amine	747.96	1.63	8.99 (c)	22
Bezafibrate (BEZ)	Lipid-lowering	4.25 (d)	Carboxylic	361.82	1.55	3.83 (i)	22
Diclofenac (DCF)	Non-steroidal anti-inflammatory	4.51 (e)	Carboxylic	296.14	2.37	4.15 (b)	44

For reference on Log K_{ow} and pKa values:
a. (Hansch et al., 1995),
b. (Sangster, 1997),
c. (McFarland et al., 1997),
d. (Tang et al., 2014),
e. (Avdeef, 2005),
f. (O'Neil, 2013),
g. (Boreen et al., 2004),
h. (Khan and Ongerth, 2004),
i. (ChemAxon, 2021).

DOM and pH: The DOM used in this experiment was previously isolated through reverse osmosis from the water of the Hellerudmyra tarn (Norway) (Gjessing et al., 1999b), and

donated by the University of Oslo (Norway). Hellerudmyra tarn is a small catchment (0.08 km^2) that provided DOM samples for the IHSS Nordic Fulvic and Humic Reference Material (Gjessing et al., 1999b). The most relevant physical-chemical properties of this natural DOM are reported in Table 6.2. Other properties of the DOM were detailed in Gjessing et al. (1999). The levels of DOM applied here represent low to medium-high concentrations typically found in Northern European lakes (Henriksen et al., 1998). The pH levels were used to represent the range typically found in Northern European Lakes (Henriksen et al., 1998). They were monitored in all experimental units. No significant change in pH occurred in any of the units during the experiments (data not shown).

Table 6.2. Physicochemical properties of the natural DOM used in this study when prepared at typical concentration found in the source lake (see Gjessing et al., 1999 for details).

pH	Conductivity ($mS\ m^{-1}$)	Colour (mg Pt L^{-1})	UV absorbance 254 nm	($SUVA_{254}$)* (mgC x 10^2)	%Corg**	molecular weight (Da)	C_{ar}/C_{al}***
5.17	2.49	166	0.813	4.59	50.3	3900	0.22

Lake DOC concentration typically of 17.7 mg L^{-1}. (*) Specific UV – Absorbance at 254 nm. (**) Percentage of Carbon content. (***) Ratio of aromatic to aliphatic carbon.

6.2.2 Experimental setting

Preparation of PPCPs and DOM working solutions: Analytical standards of the 6 compounds were purchased from Sigma-Aldrich (USA) and individually diluted in methanol (Sigma-Aldrich) to create 4 stock solutions of 1 mg mL^{-1} for ATE, CLA, BEZ and DCF, and 2 stock solutions of 100 µg mL^{-1} for SMX and FUR. To prepare the working solution of the mix, 100 µL from the ATE, SMX, FUR, CLA and BEZ stock solutions and 200 µL from DCF stock solution was spiked into an amber glass vial, to reach the following concentrations in 10 mL total volume: ATE, CLA and BEZ 10 µg mL^{-1}, DCF 20 µg mL^{-1} and SMX and FUR 1 µg

mL^{-1}. The DOM working solution was prepared by weighing 10 mg of dry DOM and adding 10 mL of MQ water to reach a concentration of 1 mg DOM mL^{-1}.

Preparation of artificial water solutions at two different pH: Four litres of soft artificial water (SAF) was prepared by adding MQ water with 1.17 g NaCl to reach 0.01 M (0.58 g L^{-1}) ionic strength, commonly detected in boreal freshwaters. The solution was then split in two 2L bottles, and then adjusted by titration with HCl or NaOH to reach 6.5 and 8 respectively. The SAF solution was used inside and outside the dialysis bags.

The experimental units: Cellulose ester dialysis bags, with a flat-width of 31 mm and molecular cut-off of 100-500 Da (Spectra/por, Spectrum Europe, Breda, The Netherlands), were cut to 10 cm lengths and thoroughly washed with Milli-Q water. 460 µL aliquots of the PPCPs mix working solution were spiked in each dialysis bag. For the control units with no DOM, SAF was then added to each bag, to reach the final volume of 10 mL. For the 5 mg L^{-1} and 15 mg L^{-1} DOC units, 170 µL and 510 µL of DOM working solution were spiked into the respective bags, before adding SAF to reach the final volume of 10 mL. Dialysis bags were sealed with standard closures (Spectra/por, Spectrum Europe, Breda, The Netherlands) and placed into a 200 mL of SAF solution (Figure 6.1). After adding glass-coated metal stirrer bars (20 mm) the experimental units were closed on top with Teflon linen plugs, and placed on a magnetic stirrer (Multistirrer15, Progen Scientific, UK) in the dark at 19 ± 1.2 °C for 1 week. The experimental procedure was repeated at both pH levels (6.5, 8), in triplicates (Figure 6.1). Every day for 7 days, 2 mL samples were collected from the units (externally from the dialysis bag) and split into two 1 mL aliquots for the determination of PPCPs and to check for a potential break-through of DOM through the dialysis membrane, respectively. On the last day of the experiment, 1 mL sample was also collected from each unit, from inside the bags. Samples for chemical analyses were filtered through a 0.2 µm syringe, placed into a 2.5 mL amber glass vial, and stored at -20° C until analyses. Chemical analyses were carried out through direct

injection in LC-MS/MS (Shimadzu, 8081) following the method reported in Text S6.1. MS/MS acquisition parameters are reported in Table S6.2. The samples for DOM loss were processed immediately following the method reported in Text S6.2. No detectable release of DOM from the dialysis bags was observed at either pH level (Figure S6.2). Mass recovery (Text S6.3) and mass loss due to adsorption of the PPCPs to all the components of the experimental units (dialysis bag, glassware, clips and stirrer bars; Text S6.4) was also tested.

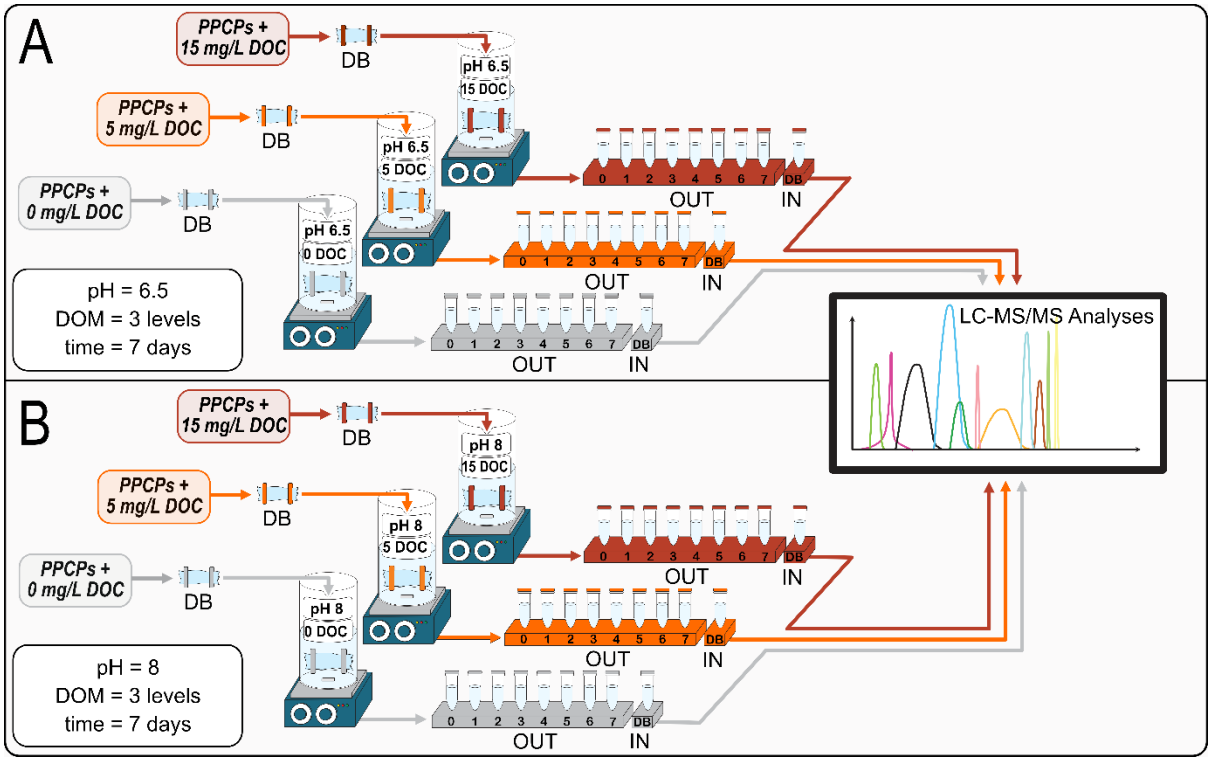


Figure 6.1. Experimental design. The association of the mix of 6 PPCPs to DOM (0, 5 and 15 mg L^{-1} DOC) was tested through equilibrium dialysis technique at pH 6.5(A) and 8(B). DB; dialysis bags, OUT; samples collected each day over 7 days outside the dialysis bag, IN; samples collected inside the dialysis bag on the seventh day of the experiment.

6.2.3 Data management scheme and quality assurance criteria

Data were treated according to the scheme presented in Figure 6.2. In summary, quality thresholds for reproducibility, mass balance closure in the experimental units, adsorption of PPCPs on the glassware and membranes and quality criteria to verify transmembrane equilibrium were considered as described in the following sections:

Reproducibility, mass balance and adsorption to glassware and membrane: The quality of replicates, mass recovery of the compounds (i.e. mass balance closure), and their mass loss due to adsorption to components of the experiment unit were checked both inside (internal wall of the bag, hereon refereed as A_{in} (µg)) and outside the bag (sum of external wall of the bag, glassware, clips and stirring bars, A_{out} (µg)). Acceptable mass recovery was set to be at 100 ± 10% of the mass of the compounds. Equilibrium dialysis studies do not generally report adsorption parameters, because they are based on the assumption that any chemical adsorbed to the bag, the glassware or other components should not interfere with the equilibrium between the compound inside and outside the bag (Akkanen et al., 2001; Akkanen and Kukkonen, 2003). However, excessive adsorption could impose a gradient preventing the compound from reaching equilibrium, such that the true effect of DOM may not be observable. In addition, our experimental setup differs from the others since we used a smaller pore size of the dialysis membrane (100-500 Da) to prevent DOM leakage, which can potentially influence the adsorption of the compounds by slowing their diffusion. Hence, mass loss due to adsorption (A_{in}, A_{out}) was included in the QA/QC parameters and set as acceptable in the range of 0-35% of the total added mass of individual PPCPs.

Equilibrium: A condition for the data to be considered for the next steps of the assessment of DOM complexation was that the concentration of PPCP in the control units reached equilibrium across the dialysis membrane by the end of the experiment. The criteria used to establish equilibrium conditions were:

- No significant differences were found between the concentration of the compound inside the control dialysis bag, C_{in} (µg L^{-1}), and concentration outside the control bag, C_{out} (µg L^{-1}). C_{in} could include both complexed and free compound, while C_{out} only represents free compound. Because the environments inside and outside the dialysis bags had very different volumes (10 mL inside vs. 200 mL outside), the mass (µg) of

each chemical was calculated inside ($M_{D/in}$) and outside ($M_{D/out}$) the bag to avoid any potential under-overestimation induced by dilution. The mass was calculated from the concentrations detected inside and outside the bag, not including any associated with the glassware and dialysis bag.

- The value of $M_{D}/_{in}$ and $M_{D/out}$ should not be significantly different from the mass expected inside and outside the dialysis bag of the control units at the end of the experiment ($M_{E/in}$ and $M_{E/out}$, respectively). The values of $M_{E/in}$ and $M_{E/out}$ were calculated from the values of the final concentration expected inside and outside the dialysis bag of the control units at the end of the experiment (C_E), assuming that C_{in} equilibrated with C_{out}, and allowing A_{in} and A_{out} values ranging 0-35%.

Failure to meet these conditions for the control units prompted the exclusion of the compound at that pH level from the study.

DOM-PPCPs binding: When the PPCPs in the control units reached equilibrium and all the quality criteria were fulfilled, the DOM-PPCPs binding was also assessed for the units containing DOM. Theoretically, if DOM bound the compounds, a significantly higher concentration will be detected inside the dialysis bags than outside, because complexation by the DOM prevents the compound crossing the membrane of the dialysis bag due the larger size. Analogously to the comparison of $M_{D/in}$ and $M_{D/out}$ performed on the control units to assess equilibrium conditions, a difference of $M_{D/in}$ and $M_{D/out}$ whereby $M_{D/in}$ being in significant excess of $M_{D/out}$ was deemed as one required piece of evidence for partial or complete binding of PPCPs to DOM. Furthermore, to rule out the confounding factor of different dilution between the bag inner and outer environment (and following the conceptual scheme of Figure 6.2), another condition for PPCP binding to DOM was that $M_{D/in}$ should be higher than $M_{E/in}$ for the experimental units with DOM and, concurrently, $M_{D/out}$ should be lower than $M_{E/out}$.

$M_{E/out}$ was calculated on the same assumptions used for $M_{E/in}$ (see paragraph *2.3.2 Equilibrium*). In summary, after checking for equilibration in the control units, three scenarios were considered to determine complexation in the units containing DOM:

- No binding: $C_{in} \sim C_{out}$; $M_{D/in} \sim M_{E/in,}$ and $M_{D/out} \leq M_{E/out}$.
- Complete binding: $M_{D/in}$ represented 100% of the PPCP mass spiked in the experimental unit, $M_{D/in} > M_{E/in}$, while $M_{D/out} = 0$.
- Partial binding: $M_{D/in} > M_{E/in}$ and $M_{D/out} < M_{E/out}$.

The magnitude of the effect of DOM was also tested by calculating the conditional distribution coefficient (K_{DOC}, L g^{-1}) at both levels of DOM. K_{DOC} was calculated as follows (from (Buschmann et al., 2006)):

$$K_{DOC} = \frac{[C_{in}] - [C_{out}]}{[C_{out}]\ x\ [DOC]} * \ 1000$$

Where the term [C_{in}]-[C_{out}] is the concentration of compound complexed with DOM and [C_{out}] is the concentration of free (unbound) compound. DOC and DOM values can be inter-converted in the equation by using the DOM's % of C content value of 50.3 (see Table 6.2).

The percentage of bound compound (B_{DOM}), defined by mass of bound compound divided by the total mass of the compound inside the bag, was calculated as follows:

$$B_{DOM} = \frac{M_{in} - C_{out} * V_{in}}{M_{in}} * 100$$

Where V_{in} is the solution volume inside the dialysis bag and C_{out}*V_{in} is the mass of unbound inside the bag. M_{in} is the total mass of compound inside the bag.

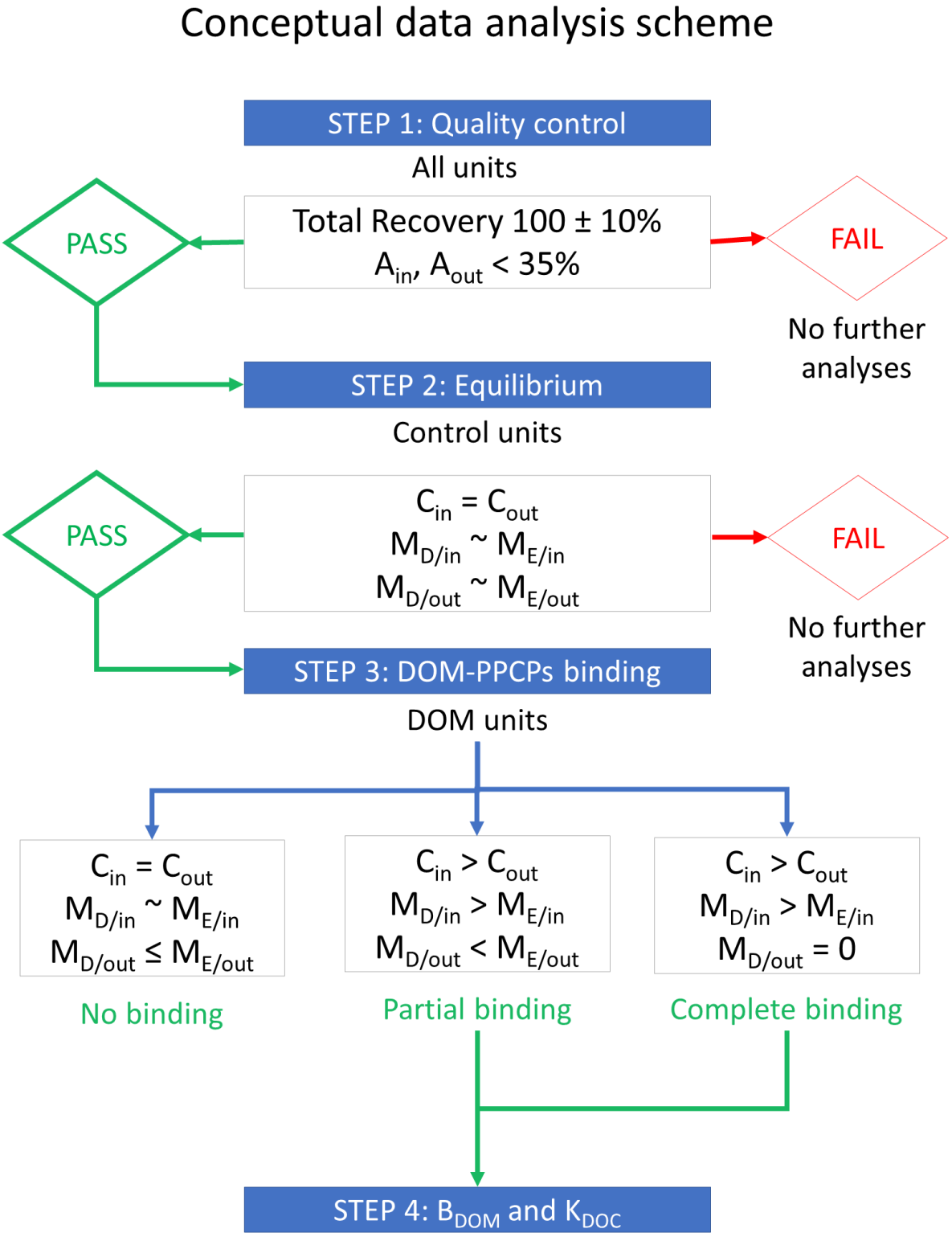


Figure 6.2. Conceptual data analysis scheme. Cin and Cout are the concentrations of each PPCP inside/outside the dialysis bags. MD/in is the mass of each PPCP detected inside the dialysis bag. MD/out is the mass of each PPCP detected outside the dialysis bag. ME/in is the mass of each PPCP expected inside the bag of the control units at equilibrium.

6.2.4 Statistical analyses

Data analyses and statistics were conducted using R (version 3.5.1) statistical software (R Core Development Team, 2015). The single comparison analyses between C_{in} and C_{out}, K_{DOC}, and B_{DOM} between different DOM levels were tested by one-way ANOVA test. The graphs were prepared using the R package "ggplot2" (Wickham, 2006).

6.3 Results and Discussions

6.3.1 Step 1: Quality control

All the compounds had 100 ± 10% total mass recovery at all pH and DOM levels in all units, apart from 3 outliers (Tables S6.3-S6.9). The optimal recovery rates shown by each targeted PPCP (Table S6.3-S6.8) indicated that ion suppression, a technical hindrance that can affect the accuracy of LC-MS/MS analyses using direct injection (Antignac et al., 2005; George et al., 2018), was negligible. Results from ATE, SMX, FUR, BEZ and DCF showed that A_{in} and A_{out} were minor or acceptable (2-35% mass loss (Tables S6.3-S6.8). In contrast, CLA showed higher A_{in} and A_{out} than the other compounds (Table S6.6), yielding values of ca. 32% at pH 6.5, and ca. 70% at pH 8 (Table S6.6). Higher adsorption of this compound at pH 8 probably links to its relative hydrophobicity (log K_{ow} 3.16 in the associated form with a pKa of 8.99). At pH 8, 91% of CLA was expected to be present in the solution in the associated form, compared to 99.7% at pH 6.5. The presence of more neutral (and consequently more hydrophobic) forms of the compound available at pH 8 may have enhanced its adsorption to the glassware, plastic clips or stirrer bars. The chemical configuration of CLA is very complex (see Figure S6.1) and offers the presence of different domains that can create H-bonds. At the same time, H-bonds are also sensitive to pH, as a consequence of the pH-induced charge density and the conformation change of CLA or of the dialysis membrane. Variable adsorption of CLA to glassware has been reported previously (Wibawa et al., 2003). Daily monitoring of water pH

reported no significant changes (data not shown), which excluded possible confounding effects induced by water pH drifting during the experiment.

6.3.2 Step 2: Equilibrium

Secondly, we checked transmembrane equilibration in the control units, based on the criteria mentioned above (Figure 6.2). C_{out} and C_{in} measured on the seventh day are depicted in Figure 6.3 for the different DOM and pH conditions (for C_{out} during the seven-day experiment see also Figure S6.3). The control units displayed no significant differences between C_{in} and C_{out} on day 7 at pH 6.5 or pH 8 for ATE, SMX, CLA (at pH 6.5 only), BEZ and DCF (Figure 6.3, Table S6.10). Furthermore, in compliance with the set quality assurance criteria, the $M_{D/in}$ values of these compounds observed in the control units were not higher than $M_{E/in}$ (Table S6.3-S6.4, S6.6-S6.8). This confirms that the equilibrium conditions were met for free chemical compounds (not complexed) at both pH levels. In contrast, significant differences were observed for FUR at pH 8, where C_{in} in the control unit was significantly higher than C_{out} (Figure 6.3, Table S6.10), while no differences were observed at pH 6.5. In addition, $M_{D/in}$ of this compound was approximatively 20% higher than $M_{E/in}$ (Table S6.5). These results indicate that FUR did not reach equilibrium before the end of the experiment at pH 8. Water pH was found to significantly influence the behaviour of FUR ($F = 5.66$, $p < 0.05$). This is not surprising because FUR is a relatively large molecule, with MW close to the lower boundary of the molecular cut off of the membrane and includes a carboxylic group with an estimated pKa=4.25. Beyond this ionizable functional group, FUR contains two domains capable of forming N-H···O hydrogen bonds. These domains can obviously form hydrogen bonds with groups of the cellulose ester, making FUR-membrane interactions sensitive to pH. Furthermore, the sulphamoil group at position 2 of the chlorobenzoic acid domain of FUR and the amine group in position 5 interacting with the oxygen of the furan group will both affect steric configuration and intramolecular interaction of this compound, depending on solution

pH. Because of these characteristics FUR is an example of a conformational polymorph (Thakuria et al., 2017). Examples of the effect of pH on steric arrangements of compounds used in drugs formulations are illustrated elsewhere (Frenkel et al., 2005). FUR at pH 8 failed to meet the equilibrium conditions and was therefore excluded from further analysis and discussion. The compounds reaching equilibrium (ATE, SMX, CLA at pH 6.5, FUR at pH 6.5, BEZ and DCF) were admitted to the next procedural steps, where the DOM-PPCP binding was assessed.

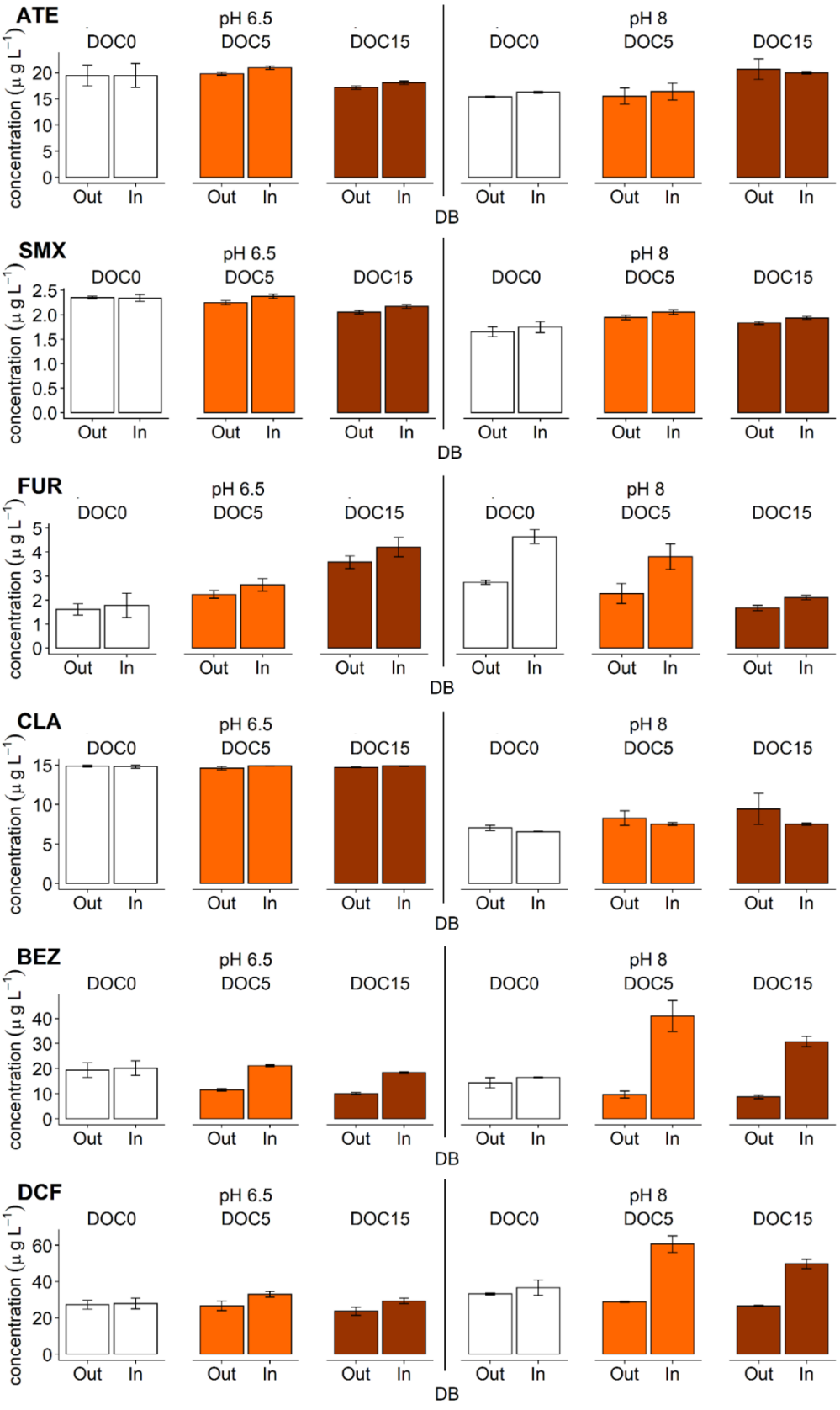


Figure 6.3. Concentration outside (Cout) and inside (Cin) the dialysis bag (DB) on the last day of the experiment for the six investigated PPCPs, at three levels of DOM (0, 5 and 15 mg L^{-1} DOC) and two levels of pH (6.5, 8). Error bars represent standard deviation.

6.3.3 Step 3 DOM-PPCPs binding

The third step of the data processing scheme was to assess the degree of binding with DOM for those chemicals that passed steps 1 and 2. ATE, SMX, CLA (pH 6.5) and FUR (pH 6.5) did not show significant differences between C_{in} and C_{out} for different DOM levels (Table S6.11-12), which indicates no binding according to the experimental design criteria and conceptual analysis scheme in Figure 6.2. In contrast, BEZ and DCF showed significantly higher C_{in} than C_{out} in the water containing DOM (Figure 6.3, Table S6.11-12). $M_{D/in}$ of the DOM units was significantly higher than $M_{E/in}$ for both the compounds (Tables S6.7-8). In addition, the $M_{D/out}$ was > 0 for both compounds at both DOM levels (Table S6.7-8), indicating partial binding with DOM, in line with the conceptual analysis scheme (Figure 6.2). Complete binding was not observed for any of the investigated compounds, according to the conceptual analysis scheme.

6.3.4 Factors influencing DOM-PPCPs binding affinity

Physicochemical properties of PPCPs and the role of DOM composition: Generally, the interaction between organic contaminants and DOM can be driven by different processes which may act both individually or synergistically. These may be hydrophobic interactions, weak H-bond interactions and covalent bonds.

The linear free energy concept, whereby the DOM-contaminant affinity – K_{DOC} or K_d - is positively correlated with the degree of hydrophobicity of contaminants – K_{ow} - (Pan et al., 2009) describes hydrophobic interactions. The results broadly indicate an influence of this interaction, with the higher hydrophobicity compounds (DCF and BEZ, log K_{ow} of 4.5 and 4.25, respectively – Table 6.1) showing association/partial binding with the DOM. In contrast, no DOM-binding was observed for the 4 compounds with lower hydrophobicity (ATE, SMX, FUR (pH 6.5) and CLA (pH 6.5), log K_{ow} of 0.16, 0.89, 2.03 and 3.16, respectively – Table 6.1). Nevertheless, interpretation only based on hydrophobic interaction is over simplistic.

Firstly, because these compounds all have weak acid groups which can interact with electron donors in the DOM and functional groups (such as amines) that can form N-H···O hydrogen bonds with oxidized groups in the DOM. Furthermore, all these PPCPs have aromatic rings which may engage in ion-dipole induced or dipole-dipole induced interactions with a range of DOM domains (especially aromatic and carboxylic groups as well as ketones, abundant in DOM) (Holbrook et al., 2004). In principal, cation exchange, cation bridging, surface complexation, and hydrogen binding can represent different forms of interaction between these PPCPs and DOM (Hernandez-Ruiz et al., 2012; Kwon and Armbrust, 2008; Pan et al., 2009). This is indicated by the B_{DOM} and K_{DOC} results (Table S6.13), that show BEZ having a higher degree of association with DOM than DCF, despite the latter having greater hydrophobicity. BEZ showed average B_{DOM} and log K_{DOC} ranging 45-74% and 1.98-2.52 respectively, while DCF indicated average values of 49% for B_{DOM} and 2.05 for log K_{DOC} (Table S6.13). The association of DCF and BEZ with DOM has been reported previously (Lu et al., 2018). Yamamoto et al. (2003) reported that other moderately lipophilic pharmaceuticals - 17β-estradiol, estriol and 17β ethynylestradiol (log K_{ow} of 3.94, 2.45 and 3.67, respectively) - with carboxyl groups similar to DCF - bound to DOM with sorption energy comparable to that of hydrogen bonding. Other studies report evidence of BEZ interacting with DOM, under experimental conditions designed to study degradation or removal of PPCPs from drinking water (Maeng et al., 2012; Vieno et al., 2010). To the best of our knowledge, the results presented here are the first on the interactions of naturally occurring DOM with BEZ.

Our study differs from others in reporting evidence of weak interactions for the other 4 compounds with DOM. Yamamoto et al. (2005) showed very low sorption of ATE to DOM (0.2% bound fraction, through fluorescence quenching observations) probably due to specific sorption forces other than hydrophobic interactions, or π- π interactions with Gibbs free energy contributions as an alternative (Delgado et al., 2015; Keiluweit and Kleber, 2009; Zeng et al.,

2012). Some other evidence suggested that binding mechanisms of sulphonamide antibiotics such as SMX tended to be hydrophobic partitioning, despite their low K_{ow} values (Chen et al., 2017). FUR was found to form complexes with fulvic acids, even though the mechanism behind the complexation remains unclear (Prakash Agarwal et al., 2008). Interactions of CLA with terrestrial humic acids (Elliot soil humic acid) through cationic species – low proton affinity sites of humic acids have also been reported (Christl et al., 2016). Despite their relevance for the topic treated in the present studies, none of these cited works used the equilibrium dialysis membrane method or were directly focused on investigating DOM-PPCPs binding. Rather, they were aimed at investigating the photodegradation of the compounds (Zeng et al., 2012), or chemical removal from drinking waters (Delgado et al., 2015; Zeng et al., 2012). Hence the present study introduces novel elements compared to previous assessments. There is no claim that the present approach and results can shed full mechanistic light on the physical-chemical processes controlling the interaction between PPCPs and DOM. The focus here was primarily on assessing the occurrence of binding and the potential relevance for exposure and risk assessment, while providing some fundamental evidence of the role of environmental conditions (e.g. by means of varying pH and DOM levels) and of the complexity of such interactions.

Previous assessments of the PPCPs-DOM interactions utilized commercial DOM standards such as Suwannee River Fulvic Acid, Suwannee River Humic Acid, Nordic Lake Fulvic Acid and Nordic Lake Humic Acid (Chen et al., 2004; Delgado et al., 2015; Keiluweit and Kleber, 2009; Zeng et al., 2012; Zhu and Pignatello, 2005). In the present study natural DOM extracted from a boreal lake was utilized. Chemical composition and the molecular configuration of DOM are key to determine modalities and level of interaction with other chemical species in the solution (Chin et al., 1997; Tanaka et al., 2005). The binding affinity of DOM was previously shown to positively correlate with its aromaticity and/or molecular weight

(Akkanen et al., 2004; Ripszam et al., 2015; Tanaka et al., 2005). The natural DOM used in this experiment has a medium-high degree of aromaticity ($SUVA_{254}$ 4.59) and molecular weight (3900 Da) compared to that used in previous studies (Akkanen et al., 2004; Ripszam et al., 2015). Relatively high molecular weight DOM in humic-rich lakes was also reported in other studies (Tulonen et al., 1992; Wang et al., 2020).

Effects of pH on DOM-PPCPs binding affinity: The results indicated that the association of DOM with BEZ and DCF was influenced by the water pH. BEZ had higher C_{in} than C_{out} at both levels of DOM, at pH 6.5 (ANOVA, F = 227.9, $p < 0.001$, Table S6.11-12) and 8 (ANOVA, F = 101.8, $p < 0.001$, Table S6.11-12). However, the results showed that the water pH affected the binding of the DOM and BEZ differently. While at pH 6.5 the $M_{D/out}$ was lower than $M_{E/out}$ (which is in line with our experimental criteria), the $M_{D/in}$ value was lower than the $M_{E/in}$ (Table S6.9, S6.7). This could be caused by the higher adsorption to the dialysis bag observed at lower pH, as indicated by the higher A_{in} at pH 6.5 compared to pH 8 (Table S6.9, S6.7). In contrast, at pH 8 the $M_{D/in}$ yielded significantly higher values than $M_{E/in}$ at both DOM levels, and the $M_{D/out}$ was lower than $M_{E/out}$ (Table S6.9, S6.7). BEZ yielded significantly lower B_{DOM} and log K_{DOC} values at pH 6.5 (45% and 1.98, respectively; average values between the two DOM levels) compared to pH 8 (74% and 2.52 respectively; average values between the two DOM levels) (Table S6.13). Hence, according to the BEZ results at pH 6.5 and 8, and criteria for binding (Figure 6.2), BEZ associated with DOM at both pH levels, but the interaction was stronger at pH 8.

DCF showed significantly higher C_{in} compared to C_{out} at pH 8 ($F=82.53$, $p < 0.001$), while the difference between C_{in} and C_{out} was too small to be significant at pH 6.5 (Table S6.11). The primary criterion for DOM-association was not met at pH 6.5, which means that no binding occurred. Significant binding with DOM at pH 8 was instead confirmed by $M_{D/in}$ yielding higher values than $M_{E/in}$ at both DOM levels, and by the $M_{D/out}$ lower than $M_{E/out}$ (Table S6.9,

S6.8). Losses (ca. 35%) were observed from the solution outside the bag due to adsorption of the compound onto to the beaker for both BEZ and DCF (Tables S6.7-8).

The effect of high pH increasing the binding with DOM observed for both compounds could be driven by two different processes. The first is a direct effect of water pH altering the ionic configuration and speciation of chemicals (Ashauer and Escher, 2010), changing their chemical properties and/or association with DOM. At higher pH weak acid groups in both PPCPs and DOM will be more in the dissociated form. While this may reduce the contribution of hydrophobic interaction on the binding process, it may simultaneously promote ionic or dipole-dipole interactions. This can explain the results for BEZ. However, BEZ and DCF are weak carboxylic acids (pKa = 3.83 and 4.15, respectively) and the difference in their speciation between pH 6.5 and 8 is negligible. For instance, both BEZ and DCF show 99% of ionized form at both pH 6.5 and 8 (data not shown). Hence, a more likely driving process will be the effect of pH on DOM physicochemical properties and molecular configuration (Engebretson and von Wandruszka, 1994; Ghosh and Schnitzer, 1980; Myeni et al., 1999b). For example, a change in pH may modulate the speciation of DOM functional groups (Tanaka et al., 2005), altering the fraction of protonated carboxylic groups, modulating the intra- and intermolecular H-bonding and leading to a different binding affinity (Cheng Gu et al., 2007; Pace et al., 2012). More acidic environments generally induce a more tightly condensed structure of DOM polymers and colloids, while a more alkaline environment usually causes an expansion of these structures (Pace et al., 2012). It was suggested that the primary mechanism responsible of this shift may not be the change in pH as in H^+ concentration, but a modulation in base cation concentration promoting the expansion and stabilisation of DOM structures (Engebretson and von Wandruszka, 1994; Ghosh and Schnitzer, 1980; Myeni et al., 1999b).

6.3.5 Effects of DOM concentrations on B_{DOM} and K_{DOC}

The binding of BEZ and DCF with the DOM was not substantially different between the two different concentrations of DOM. The results of the percentage of bound compound, B_{DOM}, showed that the effect of increasing DOM concentration on them was negligible. For instance, B_{DOM} values yielded 76% at the lower level of DOM, and 72% at the higher level for BEZ. For DCF, B_{DOM} was 46% at 5 mg L^{-1} DOC, and 52% at 15 mg L^{-1} DOC (Table S6.13). These results indicate that the maximum capacity to bind PPCPs by the natural DOM was already reached at 5 mg L^{-1} DOC. The results of the conditional distribution coefficient, $K_{DOC,}$ showed higher values at lower DOM concentrations. For instance, BEZ log K_{DOC} was 2.22 at 5 mg L^{-1} DOC and 1.74 at 15 mg L^{-1} DOC at pH 6.5, and 2.81 at 5 mg L^{-1} DOC and 2.22 at 15 mg L^{-1} DOC at pH 8, while for DCF log K_{DOC} was 2.34 at the lower level of DOM, and 1.76 at the higher one, at pH 8 (Table S6.13). Generally, log K_{DOC} results should not be affected by the DOM concentration, unless the binding capacity has reached its limit with excessive amount of DOM. The fact that higher amounts of DOM in the solution did not bind more PPCP might be because higher cross interaction of DOM constituents at higher DOM concentrations (Carter and Suffet, 1982) can cause changes in the DOM macromolecular structure (Engebretson and von Wandruszka, 1994; Ghosh and Schnitzer, 1980), hindering contaminant access to binding domains of the DOM. A non-linear binding pattern of DOM has been reported previously, showing decreasing K_{DOC} with increasing concentrations of humic acids (Carter and Suffet, 1982). These results were in line with earlier results reporting lower distribution coefficients with increasing DOM concentrations (Akkanen and Kukkonen, 2003).

The K_{DOC} values for PPCPs vary greatly in literature, depending on the different composition and source of OM and the different methodologies used. Our results were in the range of previously reported studies (Carballa et al., 2008; Lobo et al., 2014; Maeng et al., 2012). Nevertheless, it was not possible to directly compare our results with others because, to the

best of our knowledge, no other paper reported K_{DOC} values for BEZ and DCF in water. Many K_{DOC} values have been obtained for the association of BEZ and DCF to DOM in soil, using different methodologies. For example, Lyman et al. (1990) reported a K_{DOC} for BEZ of 2.62 through estimation from K_{OW} values. Barron et al. (2009) reported DCF K_{OC} values of 2.39 in agricultural soil by using combined pressurised liquid extraction and solid phase extraction methods prior to LC-MS/MS. These values are similar to those reported in this paper and also indicate that BEZ may have higher affinity for DOM compared to DCF. Rewitt et al. (2015) reported values of K_{DOC} for BEZ and DCF from batch adsorption experiments with DOM extracted from two different soils, where both compounds had higher K_{DOC} values in the soil with higher pH (4.22 ± 0.01; 7.22 ± 0.05) and higher carbon content (83 ± 0.3; 36.5 ± 2.7 g kg-1). DCF also indicated higher K_{OC} values than BEZ (BEZ; K_{OC} 1.6 – 2.80, DCF; K_{OC} 2.11 – 3.33). This is further evidence that organic matter originating from different sources can have different level of interaction with chemical pollutants. The use of different methodologies presented in the above-mentioned studies such as fluorescence quenching, equilibrium dialysis, or batch adsorption can also yield different results.

6.3.6 Implications for PPCP toxicity assessments.

Toxicity tests for informing risk assessments of pollutants are conducted under standardized conditions, which typically do not include an analysis of key environmental variables such as the level of DOM in the solution. Hence, more detailed ecological risk assessments for waterborne PPCPs will benefit from a better understanding of how interaction with DOM under a range of environmentally relevant pH levels influences availability of these compounds. In a previous study (Rizzuto et al., 2020), we found that the inhibition effect of a mix of PPCPs on the growth of a micro-algae population was reduced by the presence of natural DOM at pH 8. We hypothesized that decreased toxicity could be attributed to the formation of less bioavailable/toxic DOM-complexes. As several of the PPCPs in the tested mix were weak acids

with pKa ranging from 7.99 to 13, we postulated that pH could have a significant influence in determining the interaction and lead to the reduced toxic effects. The study also showed that the effect of DOM in hindering PPCP toxicity was not linearly dependent on its concentration.

Here, by using the same DOM and pH conditions, we empirically demonstrated that the combined effect of low concentrations of natural DOM (5 mg L^{-1} DOC) and high water pH (8) can control availability of some compounds, especially those that are more hydrophobic and more likely to diffuse through cell walls and biological membranes of algae - such as DCF and BEZ (Del Vento and Dachs, 2009). The present results also help explain the observed toxicological outcomes, in that they show a complexing ability of DOM that is not dependent on DOM concentration and is dependent on pH. Since the binding of DOM with PPCPs results in the formation of complexes which are too large or too polar to cross cell membranes (Chalew and Halden, 2009; Rowett et al., 2016), such interactions can reduce the bioavailability and toxic effect of PPCP mixtures (Alsop and Wilson, 2019; Maeng et al., 2012; Rizzuto et al., 2020), especially for compounds such as DCF, which has a demonstrated toxic effect on algae (Doležalová Weissmannová et al., 2018).

6.4 Conclusions

In summary, the present study empirically confirms the hypothesis emerging from our previous study on the complexing role of DOM for PPCPs, even at low DOM concentrations and at pH typical of freshwater environments during the development of algal blooms (Doležalová Weissmannová et al., 2018; Isidori et al., 2007). These results highlight the importance of considering more realistic environmental conditions when addressing the toxicological effects of PPCPs micro-pollutants, by showing that despite their relatively hydrophilic character, they can establish complex interactions with DOM that occurs in all-natural waterbodies. We

believe there is a need to expand knowledge on micro-pollutants effects on biota in waters, through a better understanding of the influence of the key environmental variables.

6.5 Acknowledgements

The authors thank Rolf Vogt from the Department of Chemistry, University of Oslo for providing the DOM used in this study. The authors wish to thank David Rochester from the Department of Chemistry and Runmei Wang from Lancaster Environment Centre, Lancaster University (UK), for helping with the setup of the LC/MSMS. The authors also want to thank the anonymous reviewers, whose comments and suggestions helped to improve the manuscript.

6.6 Funding

This study was financed by the Research Council of Norway, project grant no. 244460, Pollution and Ecosystem Adaptation to Changes in the Environment (PEACE). The research was co-funded by a discretional internal grant from the Norwegian Institute for Water Research (NIVA), under the Institute Strategic Priorities (SIS) on ecosystem remediation (TRADE-OFF). SR was also supported by the Lancaster University Faculty of Science and Technology and a Lancaster Environment Centre PhD Scholarship.

6.7 Credit author statement

SR, LN, EL, DLB, KCJ and HZ conceived the idea. SR, LN, KCJ and HZ designed the experiment. SR collected and analyzed the data. SR took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analyses and manuscript.

6.8 Competing interest statement

The authors declare no conflicts of interest.

Chapter VII: Conclusions, final remarks and recommendations

7.1 General conclusions

Despite the global effort made by regulatory agencies and researchers, chemical pollution still represents a worldwide issue which needs to be reduced to avoid global or regional scale change. This PhD thesis applied a multidisciplinary method to integrate ecological theories in ecotoxicological approaches, to help improve understanding of how populations, communities and ecosystems deal with chemical pollution in freshwaters. Five pilot studies were developed to track the implications of tolerance acquisition of biota to chemical pollutants in freshwaters, investigating the influence of environmental factors in this process.

In Part I, the integration of the concept of ecosystems as complex adaptive systems in ecotoxicological approaches addressed the main research questions of this thesis:

- *Question 1: What are the ecological and physiological trade-offs occurring from adaptation to chemical stress?*

Findings from **Chapter II and III** showed that repeated exposure to chemical stressors, either for a few generations or over longer time periods, can trigger adaptation processes inducing tolerance to the contaminants. In **Chapter II**, a two-months exposure to sublethal concentrations of a mix of PPCPs selected more tolerant organisms with a significantly different morphology (i.e. cell size), and higher fitness (i.e. higher recruitment rate) in presence of the chemicals. In **Chapter III**, the ecological memory of previous contamination promoted resistance in the community when exposed to similar stressors. At the same time, results from the first two chapters show that the process of tolerance acquisition carried a cost. This was evident in **Chapter II**, where the population adapted to the presence of PPCPs had a lower growth rate when grown in their absence, reflecting a trade-off between tolerance and productivity. In addition, findings from **Chapter II** suggested the occurrence of physiological

trade-offs between growth and cell size, which could reflect the need for the species to balance the investment in tolerance acquisition with the expense of energy expenditure on other fundamental processes. In **Chapter III**, the beneficial effects induced by the ecological memory of historical contamination in promoting structural resistance appeared to involve a fundamental trade-off with the ability to maintain high biomass production, as shown by the communities from the historically contaminated lake yielding higher growth rate compared to the near-pristine lake for the two highest treatments levels, which did not translate in larger biomass.

- *Question 2: What are the roles of environmental factors in tolerance acquisition and associated trade-offs?*

The main hypothesis behind this question is that since environmental factors can influence the toxicity/bioavailability of contaminants towards biota, they also may alter their selective pressure, and with it both the process of tolerance acquisition and associated trade-offs. Results arising from this thesis experimentally confirm this hypothesis. In **Chapter II**, the interaction between chemical contaminants and key components of water browning (DOM) altered the toxic outcome of the chemicals, modulated the adaptation process and the occurrence of associated trade-offs in freshwater phytoplankton. The combination of DOM and water pH probably mitigated the selective pressure operated by the PPCPs, thereby hindering the process of tolerance acquisition by the stressed algae. The importance of these results is amplified by the fact that stress mitigation depended on an environmental factor (DOM) of great relevance for freshwater ecosystems and under fundamental biogeochemical control. Results arising from this chapter are also crucial to help make predictions about the way populations and communities will respond to the selective pressure of many different chemicals, in highly heterogeneous environmental conditions.

- *Question 3: Are tolerance and trade-offs retained over time and can they be recalled - even if they are not always expressed?*

According to the results presented in this thesis, the answer to this question is yes. Results from **Chapter III** provide evidence that adaptations from past experience that are present in the dormant stage of phytoplankton in lake sediments can be readily expressed when stressors reappear. For instance, the ecological memory of historical exposure to contaminants shows beneficial effects in restoring fundamental processes such as photosynthetic efficiency, and increasing structural resistance of the phytoplankton communities. At the same time, trade-offs between resistance and other processes related to biomass production were also observed, as also reported in the response to the first research question ("*What are the ecological and physiological trade-offs occurring from adaptation to chemical stress?*"). Due to the implicit differences with the PICT concept, which is extremely useful to understand how communities can cope with the recurrent pressure of contaminants by restructuring and selecting tolerant species, EM could also be used as a complementary approach to study community responses to chemical pollutants, in particular to observe the ecosystem resilience induced by adaptation processes. **Chapter IV** addressed the challenge to experimentally assess ecological memory using a trait ecology approach and focussing on the fundamental ecological process of ealy ecological succession. Results from **Chapter IV** showed adaptation from past experience that are present in lake sediments as dormant phytoplankton stages can be readily expressed when the stressor reappears, showing beneficial effects in terms of maintaining functions and structure in the presence of recurring stress. **Chapter IV** also highlights the importance of integrating a trait-based approach in ecotoxicology, showing how trait diversity is the common currency to describe inter- and intra-specific variation in one single approach, and most crucially confirms and expands the conclusions from the previous **Chapter III**.

Part II of the thesis was dedicated to an in-depth examination of the binding process between the DOM and chemical contaminants. For this purpose, an improved equilibrium-based method was firstly developed and critically assessed, so that it could then be applied to test the binding of DOM with contaminants, investigating the influence of water pH and DOM concentration in this process. Findings from **Chapter V** show that the technical improvements in terms of dialysis membrane material and pore size, and the application of a strict QA/QC protocol ensured good reproducibility, optimal mass balance closure and successful fulfilment of trans-membrane equilibrium at all the tested pH levels. The use of a dialysis membrane with reduced pore size ensured the restriction of DOM to one side of the membrane, virtually eliminating DOM breakthrough, a technical hindrance which could cause uncertainties in the measurements. Results report the binding of a commonly used herbicide (Isoproturon) with natural DOM, highlighting a controlling effect of water pH in this process, and an effect of DOM not dependent on its concentration. In **Chapter VI**, the improved equilibrium-based method was applied to test the hypotheses arising in **Chapter II**, where the complexation operated by the DOM was proposed to have lowered toxicity/bioavailability of a mix of PPCPs on microalgae. The hypotheses were confirmed; results from **Chapter VI** suggest that the binding was driven by the presence of carboxylic groups in the PPCPs (which also occurred to be present in the most hydrophobic compounds of the mix), and that high pH may have shifted the structural configuration of DOM, potentially increasing its binding with some PPCPs. In addition, results confirm what was previously observed in **Chapter V**, where the binding capacity of the DOM was not dependent on its concentration across the narrow range tested.

7.2 Final remarks, future needs and recommendations

The main objective of this thesis was to experimentally investigate if fundamental ecological concepts linked to the process of tolerance acquisition to contaminants (i.e. rapid adaptation, ecological memory, trade-offs), and the hypothesis of a control operated by environmental

factors in this process, could occur in the real environment. The studies presented in this thesis successfully confirmed all the tested ecological concepts and hypotheses. It should be cautioned that such results cannot be used to define the common response of all freshwaters, as every ecosystem is inherently unique. The experiments carried out in this thesis attempt to reproduce conditions commonly found in boreal temperate freshwaters (e.g. in Scandinavia), such as relatively low temperature, low nutrients (oligotrophic), together with specific levels of pH and DOM sources and concentrations. The responses observed here may be very different in ecosystems with different characteristics, such as climatic areas (i.e. temperate, tropical, etc.), physicochemical properties (i.e. water chemistry, nutrients, different DOM), anthropogenic exposure conditions (i.e. history of contaminations, exposure profiles, exposure sources), which profoundly define the inherent properties of each ecosystem, and may alter the way it responds to chemical pollution. Stochastic components may play a fundamental role too. Hence, further research is required to test the ecological concepts investigated in this thesis in different environmental scenarios, to evaluate how intrinsic differences of freshwaters may influence these processes. For example, freshwaters are generally not exposed to a single group of contaminants that they can adapt to, but to multiple groups, each one with a different mode of action. Adaptation to a certain group of contaminants does not guarantee the same level of tolerance towards other groups of stressors. For instance, according to energy allocation theory (Bazzaz et al., 1987; Lerdau and Gershenzon, 1997), adaptation to a certain stress may decrease the tolerance of biota when in the presence of other novel stressors (Carlson et al., 2014; Samani and Bell, 2016). In addition, the mixture of different chemicals can interact with different heterogeneous environmental conditions which can change the bioavailability/toxicity of contaminants (either increasing or decreasing it), altering the tolerance acquisition process. Hence, this thesis uncovers other important research questions, such as to study how adaptation processes to certain stressors influence the response of biota to different environmental

stressors (and vice versa), in order to understand how populations and communities may persist in the context of global environmental change (Carlson et al., 2014). Temperature has a great influence on contaminants, generally showing synergistic toxic effects on biota. With climate change increasing global temperature, understanding the influence of this key environmental factor on the way organisms adapt to chemical stress would help to forecast the response of freshwaters to global change.

Another reason to further investigate the implications of adaptation processes is that exposure profiles of chemicals can vary in relationship of their release in the environment, so that the concentrations to which the target organisms will be exposed can vary too. For example, pollution in waters can be 'diffuse' (non-point source such as in WWTP effluent), 'pulsed' (e.g. in storm waters/runoff), 'seasonal' (e.g. pesticides) or 'very high/short-term' (e.g. accidental releases/spillages). The exposure profiles used in Chapter II simulated diffuse wastewater contamination of sub-lethal concentrations of PPCPs, which allowed the phytoplankton population to adapt and better tolerate the stress when re-exposed to the same concentrations of PPCPs. In Chapter III, the diffuse herbicide contamination during the germination phase 'awoke' the ecological memory of previous contamination in the community from the historically contaminated lake, allowing better resistance when re-exposed to sub-lethal concentrations of the herbicide in the second phase. An interesting research question would be to understand if and how organisms can develop tolerance in an environment characterised by pulsed exposure, which is generally identified by quick, highly concentrated release of contaminants alternated to short-time periods of lower concentrations/absence of stress.

This thesis provide evidence on the effect of DOM on reducing toxicity/bioavailability of chemicals and thereby screening biota against their negative effects. However, while this process can be considered as positive for exposed biota, it should not be generalized for all

freshwaters. For example, the interactions observed between DOM and chemicals in this thesis may be different in freshwaters with different type of DOM and/or other contaminants at play. As was previously reported in Chapters II, V-VI, DOM originating from different sources can have different physicochemical properties and therefore different binding capacity with the chemicals. In addition, DOM can also have negative effects on the toxicity of contaminants and/or on the growth of phytoplankton (as shown in Chapter II). This double negative effect can influence not only the toxic effect of chemicals but also the process of tolerance acquisition, and therefore it should be investigated further. These results corroborate the suggestions proposed by many researchers that laboratory toxicity tests on chemical substances should include studies assessing the effects of speciation/complexation on the contaminants' toxicity/bioavailability induced by the water chemistry (i.e. DOM and water pH). Conducting standardized methodologies ignoring the influence of environmental factors could lead to a wrong estimation of the toxicity of chemical contaminants.

A crucial message arising from this thesis is that the presence of underlying adaptation processes may represent an inherent protective response against contaminants. From a regulatory perspective, this is further evidence that the concentrations of contaminants detected in the environment should not be necessarily translated into a negative effect for the exposed organisms. For instance, according to the evidence presented here, contaminants levels that may be significantly impairing the growth of a community unaccustomed to that stress, can be tolerated by a community that has already encountered that stress and has developed adaptation allowing better resistance. However, does that mean that the protection of freshwaters with a history of contamination can be overlooked because they are more tolerant? Of course not. This thesis provides evidence that acquiring tolerance may offer some level of protection for the ecosystem against sub-lethal concentrations of chemical, however it may not be as protective against higher levels of stress. Hence, it would be crucial to study how much additional stress

an adapted ecosystem can tolerate before negative effects will manifest, compared to a non-adapted one. One implication for regulatory risk assessment would be increasing threshold values for those contaminants of which there is evidence that ecosystems have developed adaptation. At the same time, as also stated before, acquiring tolerance to a certain group of contaminants does not guarantee the same level of tolerance towards other groups of stressors, such as the occurrence of novel contaminants, or changing environmental factors. Hence, the results arising from this thesis reinforce the importance of conducting individual studies, carefully weighting the intrinsic characteristics (i.e. historical exposure, environmental conditions influencing chemicals toxicity) of each freshwater ecosystem before taking decisions regarding its protection/management. Performing *ad hoc* studies for different freshwater systems could help ensure no under or over-estimation of the risks for biota exposed to chemical pollution ecosystems, and should always be considered in ecotoxicological regulatory approaches.

Another important implication for regulatory risk assessment arising from this thesis could be the occurrence of trade-offs between tolerance and productivity. For instance, results from Chapters II and III provide evidence that a population/community that is more tolerant to the stress may not be as productive in the absence of the stress. What would be the consequences of these results in remediation projects? Would it be always the right solution to remove the stressor in a system that has developed tolerance and perhaps found its balance? Further research is therefore required in this direction.

Trait-based approaches already represent a huge addition for ecotoxicology for its fundamental role of describing inter- and intra-specific variation in one single approach. The findings presented in this thesis stress the necessity for trait-based approaches to be integrated in the description of community dynamics, combining ecological and evolutionary responses.

Further consideration should also be given to the different setup used in the experiments of this thesis. The innovative experimental designs proposed here, all enabled laboratory studies to be performed with the aim of inducing adaptation, awakening dormient adaptation stages in phytoplankton, and testing the binding between DOM and contaminants. However, while great effort was applied in order to replicate real environmental conditions found in lakes, the experiments cannot reproduce them entirely. Hence, there is still space for improvements. One example could be to conduct germination experiment directly in lakes rather than in the laboratory, perhaps using a similar technique as in Baho et al. (2017). For instance, these researchers used dialysis bags to grow algal communities directly in lakes, with the advantage to work in a real environment, while controlling the experimental conditions of temperature, light, and contaminants exposure. Further research could be done in this direction in order to improve the methods.

In conclusion, the concept of ecosystems as complex adaptive system can be crucial to shed further light on the role of adaptation processes to contaminants and their consequences, under the influence of environmental factors. Ultimately, results arising from this thesis increased knowledge on how populations and communities deal with chemical pollution in freshwaters, and allows ecotoxicology to take another step towards its transformation into "chemical stress ecology", a process that was started a couple of decades ago and it is still being actively pursued by many researchers. This work has implications for legislation and regulatory risk assessments. For instance, a framework that correlates the combined effects of environmental factors with chemical contaminants, incorporates adaptation processes at the higher levels of biological organisations, and uses trait-based approaches could help improvements to be carried out in the Water Framework Directive, a regulation has received critics for still using basic ecotoxicological approaches (Voulvoulis et al., 2017). The same EU Commission pointed towards a deficiency in the regulatory assessment on effect exposure of chemical pollution,

reporting that "…the key area where there is room to improve and to achieve better results is on chemicals" (European Commission, 2019b). There is still a great research space for joining state-of-the-art ecotoxicology with new elements from ecological theory, in order to develop a framework sufficiently evolved to translate into regulatory risk assessment.

8 References

Acevedo-Trejos, E., Brandt, G., Bruggeman, J., Merico, A., 2015. Mechanisms shaping size structure and functional diversity of phytoplankton communities in the ocean. https://doi.org/10.1038/srep08918

Agra, A.R., Soares, A.M.V.M., Barata, C., 2011. Life-history consequences of adaptation to pollution. "'Daphnia longispina clones historically exposed to copper.'" Ecotoxicology 20, 552–562. https://doi.org/10.1007/s10646-011-0621-5

Akkanen, J., Kukkonen, J.V.K., 2003. Measuring the bioavailability of two hydrophobic organic compounds in the presence of dissolved organic matter. Environ. Toxicol. Chem. 22, 518–524. https://doi.org/10.1897/1551-5028(2003)022<0518:MTBOTH>2.0.CO;2

Akkanen, J., Kukkonen, J.V.K., 2001. Effects of water hardness and dissolved organic material on bioavailability of selected organic chemicals. Environ. Toxicol. Chem. 20, 2303–2308.

Akkanen, J., Penttinen, S., Haitzer, M., Kukkonen, J.V.K., 2001. Bioavailability of atrazine, pyrene and benzo[a]pyrene in European river waters. Chemosphere 45, 453–462. https://doi.org/10.1016/S0045-6535(01)00038-8

Akkanen, J., Vogt, R.D., Kukkonen, J.V.K., 2004. Essential characteristics of natural dissolved organic matter affecting the sorption of hydrophobic organic contaminants. Aquat. Sci. 66, 171–177. https://doi.org/10.1007/s00027-004-0705-x

Albanese, K.A., Lanno, R.P., Hadad, C.M., Chin, Y.-P., 2017. Photolysis-and Dissolved Organic Matter-Induced Toxicity of Triclocarban to Daphnia magna. https://doi.org/10.1021/acs.estlett.7b00429

Allan, J.D., 2004. Landscapes and riverscapes: The influence of land use on stream ecosystems.

Annu. Rev. Ecol. Evol. Syst. 35, 257–284. https://doi.org/10.1146/annurev.ecolsys.35.120202.110122

Alsop, D., Wilson, J.Y., 2019. Waterborne pharmaceutical uptake and toxicity is modified by pH and dissolved organic carbon in zebrafish. Aquat. Toxicol. 210, 11–18. https://doi.org/10.1016/j.aquatox.2019.02.008

Alvarsson, A., 2012. Assessing the environmental impact of pesticides ; Effects of photosystem II inhibiting herbicides on primary production and ecosystems. Degree Proj. Master Sci. Ecotoxicol. 30 ECTS, Dep. Biol. Environ. Sci. Univ. Gothenburg, Sweden, 1–58.

Antignac, J.P., De Wasch, K., Monteau, F., De Brabander, H., Andre, F., Le Bizec, B., 2005. The ion suppression phenomenon in liquid chromatography-mass spectrometry and its consequences in the field of residue analysis, in: Analytica Chimica Acta. Elsevier, pp. 129–136. https://doi.org/10.1016/j.aca.2004.08.055

Antonacci, A., Lambreva, M.D., Margonelli, A., Sobolev, V., Pastorelli, S., Bertalan, I., Johanningmeier, U., Sobolev, A.P., Samish, I., Edelman, M., Havurinne, V., Tyystjärvi, E., Giardi, M.T., Mattoo, A.K., Rea, G., 2018. Photosystem-II D1 protein mutants of Chlamydomonas reinhardtii in relation to metabolic rewiring and remodelling of H-bond network at QB site. Sci. Rep. 8, 1–14. https://doi.org/10.1038/s41598-018-33146-y

Arnaud, L., Haubruge, E., Gage, M.J.G., 2005. The malathion-specific resistance gene confers a sperm competition advantage in Tribolium castaneum. Funct. Ecol. 19, 1032–1039. https://doi.org/10.1111/j.1365-2435.2005.01055.x

Arnaud, L., Taillander, G., Kaouadji, M., Ravanel, P., Tissut, M., 1994. Photosynthesis Inhibition by Phenylureas: a QSAR approach. Ecotoxicol. Environ. Saf. 28, 121–133.

Arnold, K.E., Boxall, A., Brown, A.R., Cuthbert, R., Gaw, S., Hutchinson, T.H., Jobling, S., Madden, J.C., Metcalfe, C.D., Naidoo, V., Shore, R.F., Smits, J.E., Taggart, M.A., Thompson, H.M., 2013. Assessing the exposure risk and impacts of pharmaceuticals in the environment on individuals and ecosystems. Biol. Lett. 9, 20130492–20130492. https://doi.org/10.1098/rsbl.2013.0492

Ashauer, R., Escher, B.I., 2010. Advantages of toxicokinetic and toxicodynamic modelling in aquatic ecotoxicology and risk assessment. J. Environ. Monit. 12, 2056–2061. https://doi.org/10.1039/c0em00234h

Avdeef, A., 2005. Physicochemical Profiling (Solubility, Permeability and Charge State). Curr. Top. Med. Chem. 1, 277–351. https://doi.org/10.2174/1568026013395100

Bååth, E., Kritzberg, E., 2015. pH Tolerance in Freshwater Bacterioplankton: Trait Variation of the Community as Measured by Leucine Incorporation. Appl. Environ. Microbiol. 81, 7411–7419.

Baert, J.M., Janssen, C.R., Sabbe, K., De Laender, F., 2016. Per capita interactions and stress tolerance drive stress-induced changes in biodiversity effects on ecosystem functions. Nat. Commun. 7, 12486.

Baho, Didier L, Leu, E., Pomati, F., Hessen, D.O., Norberg, J., Moe, S.J., Skjelbred, B., Nizzetto, L., Baho, D., 2019. Resilience of natural lake phytoplankton communities to pulse disturbances from micropollutant exposure and vertical mixing. Environ. Toxicol. Chem. 38, 2197–2208. https://doi.org/10.1002/etc.4536

Baho, Didier L., Pomati, F., Leu, E., Hessen, D.O., Moe, J.S., Norberg, J., Nizzetto, L., 2019. A single pulse of diffuse contaminants alters the size distribution of natural phytoplankton communities. Sci. Total Environ. 683, 578–588.

https://doi.org/https://doi.org/10.1016/j.scitotenv.2019.05.229

Baho, D.L., Rizzuto, S., Nizzetto, L., Hessen, D.O., Norberg, J., Skjelbred, B., Jones, K.C., Zhang, H., Leu, E., 2021. Ecological Memory of Historical Contamination Influences the Response of Phytoplankton Communities. Ecosystems 1–17. https://doi.org/10.1007/s10021-021-00604-0

Bai, L., Zhang, Q., Wang, C., Yao, X., Zhang, H., Jiang, H., 2019. Effects of natural dissolved organic matter on the complexation and biodegradation of 17A-ethinylestradiol in freshwater lakes. Environ. Pollut. 246, 782–789. https://doi.org/10.1016/j.envpol.2018.12.098

Barriuso, E., Andrades, M.S., Benoit, P., Houot, S., 2011. Pesticide desorption from soils facilitated by dissolved organic matter coming from composts: experimental data and modelling approach. Biogeochemistry 106, 117–133.

Barron, L., Havel, J., Purcell, M., Szpak, M., Keller, B., Paull, B., 2009. Predicting sorption of pharmaceuticals and personal care products onto soil and digested sludge using artificial neural networks. Analyst 134, 663–670. https://doi.org/10.1039/b817822d

Batty, L.C., Hallberg, K.B., 2010. Ecology of Industrial Pollution. Cambridge University Press, The Edinburgh Building, Cambridge CB2 8RU, UK Published.

Bazzaz, F.A., Chiariello, N.R., Coley, P.D., Pitelka, L.F., 1987. Allocating Resources to Reproduction and Defense. Bioscience 37, 58–67.

Beck, A.J., Jones, K.C., 1996. The effects of particle size, organic matter content, crop residues and dissolved organic matter on the sorption kinetics of atrazine and isoproturon by clay soil. Chemosphere 32, 2345–2358. https://doi.org/10.1016/0045-6535(96)00147-6

Behera, S.K., Oh, S.Y., Park, H.S., 2010. Sorption of triclosan onto activated carbon, kaolinite and montmorillonite: Effects of pH, ionic strength, and humic acid. J. Hazard. Mater. 179, 684–691. https://doi.org/10.1016/j.jhazmat.2010.03.056

Beketov, M.A., Kefford, B.J., Schafer, R.B., Liess, M., 2013. Pesticides reduce regional biodiversity of stream invertebrates. Proc. Natl. Acad. Sci. 110, 11039–11043. https://doi.org/10.1073/pnas.1305618110

Beketov, M.A., Liess, M., 2008. Potential of 11 Pesticides to Initiate Downstream Drift of Stream Macroinvertebrates. Arch Env. Contam Toxicol 55, 247–253. https://doi.org/10.1007/s00244-007-9104-3

Bell, B.G., Schellevis, F., Stobberingh, E., Goossens, H., Pringle, M., 2014. A systematic review and meta-analysis of the effects of antibiotic consumption on antibiotic resistance. BMC Infect. Dis. 14. https://doi.org/10.1186/1471-2334-14-13

Bell, G., 2017. Evolutionary Rescue. Annu. Rev. Ecol. Evol. Syst. 48, 605–627. https://doi.org/10.1146/annurev-ecolsys-110316-023011

Berard, A., Benninghoff, C., 2001. Pollution-Induced Community Tolerance (PICT) and seasonal variations in the sensitivity of phytoplanton to atrazine in nanocosms. Chemoshere. 45:427-437.

Beyer, J., Petersen, K., Song, Y., Ruus, A., Grung, M., Bakke, T., Tollefsen, K.E., 2014. Environmental risk assessment of combined effects in aquatic ecotoxicology: A discussion paper. Mar. Environ. Res. 96, 81–91. https://doi.org/10.1016/j.marenvres.2013.10.008

Bi, Y.F., Miao, S.S., Lu, Y.C., Qiu, C. Bin, Zhou, Y., Yang, H., 2012. Phytotoxicity,

bioaccumulation and degradation of isoproturon in green algae. J. Hazard. Mater. 243, 242–249. https://doi.org/10.1016/j.jhazmat.2012.10.021

Blanck, H., 2002. A critical review of procedures and approaches used for assessing pollution-induced community tolerance (PICT) in biotic communities. Hum. Ecol. Risk Assess. 8, 1003–1034. https://doi.org/10.1080/1080-700291905792

Blanck, H., Wangberg, S.A., 1988. Induced community tol- erance in marine periphyton established under arsenate stress. Can. J. Fish. Aquat. Sci. 45, 1816–1819.

Blois, J.L., Zarnetske, P.L., Fitzpatrick, M.C., Finnegan, S., 2013. Climate change and the past, present, and future of biotic interactions. Science (80-.). 341, 499–504. https://doi.org/10.1126/SCIENCE.1237184

Boehm, P.D., Quinn, J.G., 1976. The effect of dissolved organic matter in sea water on the uptake of mixed individual hydrocarbons and number 2 fuel oil by a marine filter-feeding bivalve (Mercenaria mercenaria). Estuar. Coast. Mar. Sci. 4, 93–105. https://doi.org/10.1016/0302-3524(76)90010-4

Boehm, P.D., Quinn, J.G., 1973. Solubilization of hydrocarbons by the dissolved organic matter in sea water. Geochim. Cosmochim. Acta 37, 2459–2477. https://doi.org/10.1016/0016-7037(73)90292-5

Böhm, L., Schlechtriem, C., Düring, R.A., 2016. Sorption of Highly Hydrophobic Organic Chemicals to Organic Matter Relevant for Fish Bioconcentration Studies. Environ. Sci. Technol. 50, 8316–8323. https://doi.org/10.1021/acs.est.6b01778

Boivin T, Bouvier JC, Chadœuf J, Beslay D, Sauphanor B. 2003. Constraints on adaptive mutations in the codling moth Cydia pomonella (L.): measuring fitness trade-offs and

natural selection. Heredity 90:107–113.

Boreen, A.L., Arnold, W.A., McNeill, K., 2004. Photochemical fate of sulfa drugs in then aquatic environment: Sulfa drugs containing five-membered heterocyclic groups. Environ. Sci. Technol. 38, 3933–3940. https://doi.org/10.1021/es0353053

Böttcher, T., Schroll, R., 2007. The fate of isoproturon in a freshwater microcosm with Lemna minor as a model organism. Chemosphere 66, 684–689. https://doi.org/10.1016/j.chemosphere.2006.07.087

Boxall, A., Rudd, M., Brooks, B., Hickmann, S., Snape, J., Parrott, J., Tetreault, G., Innes, E., McLaughlin, A., Choi, K., Ostapyk, K., Berninger, J., Szabo, N., Trudeau, V., Moore, R., Hallstrom, L., Ericson, J., Dyer, S., Verslycke, T., Coors, A., Belanger, S., Servos, M., Larsson, D., McMaster, M., Karlsson, M., Straub, J., Sibley, P., Staveley, J., Meyerhoff, R., Carriquiriborde, P., DeLeo, P., Giesy, J., Beazley, K., Gagné, F., Ankley, G., Caldwell, D., Van Der Kraak, G., Murray-Smith, R., Mastrocco, F., Topp, E., Gouin, T., Lazorchak, J., 2012. Pharmaceuticals and Personal Care Products in the Environment: What Are the Big Questions? Environ. Health Perspect. 120, 1221–1229. https://doi.org/10.1289/ehp.1104477

Bradshaw, A.D., K, H., 1989. Evolution and stress – genotype and phenotype components. Biol. J. Linn. Soc. 37, 137–155.

Brock TCM, Lahr J, Van den Brink PJ. 2000. Ecological risks of pesticides in freshwater ecosystems; Part 1: herbicides. Alterra.

Bundschuh, M., Schulz, R., Schäfer, R.B., Allen, C.R., Angeler, D.G., 2017. Resilience in ecotoxicology: Toward a multiple equilibrium concept. Environ. Toxicol. Chem. 36, 2574–2580. https://doi.org/10.1002/etc.3845

Burkhard, L.P., 2000. Estimating dissolved organic carbon partition coefficients for nonionic organic chemicals. Environ. Sci. Technol. 34, 4663–4668. https://doi.org/10.1021/es001269l

Buschmann, J., Kappeler, A., Lindauer, U., Kistler, D., Berg, M., Sigg, L., 2006. Arsenite and arsenate binding to dissolved humic acids: influence of pH, type of humicacid, and aluminum. Environ. Sci. Technol 40, 6015–6020.

Busi, R., Powles, S., 2009. Evolution of glyphosate resistance in a Lolium rigidum population by glyphosate selection at sublethal doses. Heredity (Edinb). 103, 318–325.

Campbell, B.M., Bear, D.J., Bennet, E.M., Hall-Spencer, J.M., Ingram, J.S.L., Jaramillo, F., Ortiz, R., Ramankutty, N., Sayer, J.A., Shindell, D., 2017. Agriculture production as a major driver of the Earth system exceeding planetary boundaries. Ecol. Soc. 22, 8.

Cao, B., Jiang, Z., Li, J., Zhang, X., Hu, Y., Chen, J., Zhang, Y., 2018. Different dissolved organic matter (DOM) characteristics lead to diverse atrazine adsorption traits on the non-rhizosphere and rhizosphere soil of Pennisetum americanum (L.) K. Schum. Chemosphere 209, 608–616. https://doi.org/10.1016/j.chemosphere.2018.06.069

Carballa, M., Fink, G., Omil, F., Lema, J.M., Ternes, T., 2008. Determination of the solid–water distribution coefficient(Kd) for pharmaceuticals, estrogens and musk fragrances indigested sludge. Water Res. 42, 287–295.

Carlson, S.M., Cunningham, C.J., Westley, P.A.H., 2014. Evolutionary rescue in a changing world. Trends Ecol. Evol. 29, 521–530. https://doi.org/10.1016/j.tree.2014.06.005

Carter, C.W., Suffet, I.H., 1982. Binding of DDT to Dissolved Humic Materials. Am. Chem. Soc. Environ. Sel. Technol 16, 735.

Chalew, T.E.A., Halden, R.U., 2009. Environmental exposure of aquatic and terrestrial biota to triclosan and triclocarban. J. Am. Water Resour. Assoc. 45, 4–13. https://doi.org/10.1111/j.1752-1688.2008.00284.x

Chang, C.C., Halpern, C.B., Antos, J.A., Avolio, M.L., Biswas, A., Cook, J.E., del Moral, R., Fischer, D.G., Holz, A., Pabst, R.J., Swanson, M.E., Zobel, D.B., 2018. Testing conceptual models of early plant succession across a disturbance gradient. J. Ecol. 107, 517–530.

Chapman, P.M., 2002. Integrating toxicology and ecology: Putting the "eco" into ecotoxicology. Mar. Pollut. Bull. 44, 7–15. https://doi.org/10.1016/S0025-326X(01)00253-3

ChemAxon, 2021. Calculators and Predictors | ChemAxon [WWW Document]. URL https://chemaxon.com/products/calculators-and-predictors#pka (accessed 1.27.21).

Chen, H., Zhou, W., Zhu, K., Zhan, H., Jiang, M., 2004. Sorption of ionizable organic compounds on HDTMA-modified loess soil. Sci. Total Environ. 326, 217–223. https://doi.org/10.1016/j.scitotenv.2003.12.011

Chen, K.L., Liu, L.C., Chen, W.R., 2017. Adsorption of sulfamethoxazole and sulfapyridine antibiotics in highorganic content soils. Environ. Pollut. 1163–1171. https://doi.org/https://dx.doi.org/10.1016/j.envpol.2017.08.011

Chin, Y.-P., Aiken, G.R., Danielsen, K.M., 1997. Binding of Pyrene to Aquatic and Commercial Humic Substances: The Role of Molecular Weight and Aromaticity. Environ. Sci. Technol 31, 1630–1635.

Christl, I., Ruiz, M., Schmidt, J.R., Pedersen, J.A., 2016. Clarithromycin and Tetracycline

Binding to Soil Humic Acid in the Absence and Presence of Calcium. Environ. Sci. Technol. 50, 9933–9942. https://doi.org/10.1021/acs.est.5b04693

Cianciaruso, M. V., Batalha, M.A., Gaston, K.J., Petchey, O.L., 2009. Including intraspecific variability in functional diversity. Ecology 90, 81–89. https://doi.org/10.1890/07-1864.1

Clements, W.H., Rohr, J.R., 2009. Community responses to contaminants: using basic ecological principles to predict ecotoxicological effects. Environ. Toxicol. Chem. 28, 1789–1800. https://doi.org/10.1897/09-140.1

Cochran, W.G., Chambers, S.P., 1965. The planning of Observational Studies of Human Populations. J. R. Stat. Soc. Ser. Gen. 128, 234–266.

Cohen J. 2013. Statistical power analysis for the behavioral sciences, Revised. New York: Academic Press.

Coley PD, Bryant JP, Chapin FS. 1985. Resource availability and plant antiherbivore defense. Science 230:895.

Collins, S., De Meaux, J., 2009. Adaptation to different rates of environmental change in Chlamydomonas. Evolution (N. Y). 63, 2952–2965. https://doi.org/10.1111/j.1558-5646.2009.00770.x

Collins, S., Gardner, A., 2009. Integrating physiological, ecological and evolutionary change: A Price equation approach. Ecol. Lett. 12, 744–757. https://doi.org/10.1111/j.1461-0248.2009.01340.x

Cooke, C.M., Shaw, G., Collins, C.D., 2004. Determination of solid–liquid partition coefficients (Kd)for the herbicides isoproturon and trifluralin infive UK agricultural soils. Environ. Pollut. 132, 541–552.

Costelloe, C., Metcalfe, C., Lovering, A., Mant, D., Hay, A., 2010. Effect of antibiotic prescribing in primary care on antimicrobial resistance in individual patients: systematic review and meta-analysis. Br. Med. J. 1–11. https://doi.org/10.1136/bmj.c2096

Cox, L., Celis, R., Hermosin, M.C., Cornejo, J., Zsolnay, A., Zeller, K., 2000. Effect of organic amendments on herbicide sorption as related to the nature of the dissolved organic matter. Environ. Sci. Technol. 34, 4600–4605. https://doi.org/10.1021/es0000293

Crain, C.M., Kroeker, K., Halpern, B.S., 2008. Interactive and cumulative effects of multiple human stressors in marine systems. Ecol. Lett. 11, 1304–1315. https://doi.org/10.1111/j.1461-0248.2008.01253.x

Creed, I.F., Bergström, A.K., Trick, C.G., Grimm, N.B., Hessen, D.O., Karlsson, J., Kidd, K.A., Kritzberg, E., McKnight, D.M., Freeman, E.C., Senar, O.E., Andersson, A., Ask, J., Berggren, M., Cherif, M., Giesler, R., Hotchkiss, E.R., Kortelainen, P., Palta, M.M., Vrede, T., Weyhenmeyer, G.A., 2018. Global change-driven effects on dissolved organic matter composition: Implications for food webs of northern lakes. Glob. Chang. Biol. https://doi.org/10.1111/gcb.14129

Daughton, C.G., Ternes, T.A., 1999. Pharmaceuticals and personal care products in the environment: Agents of subtle change? Environ. Health Perspect. 107, 907–938. https://doi.org/10.1289/ehp.99107s6907

David, V., Tortajada, S., Philippine, O., Bréret, M., Barnett, A., Agogué, H., Robin, F.X., Dupuy, C., 2020. Ecological succession and resilience of plankton recovering from an acute disturbance in freshwater marshes. Sci. Total Environ. 709, 135997. https://doi.org/10.1016/J.SCITOTENV.2019.135997

De Castro-Català, N., Dolédec, S., Kalogianni, E., Skoulikidis, N.T., Paunovic, M., Vasiljević,

B., Sabater, S., Tornés, E., Muñoz, I., 2020. Unravelling the effects of multiple stressors on diatom and macroinvertebrate communities in European river basins using structural and functional approaches. Sci. Total Environ. 742, 140543. https://doi.org/10.1016/J.SCITOTENV.2020.140543

Del Vento, S., Dachs, J., 2009. Prediction of uptake dynamics of persistent organic pollutants by bacteria and phytoplankton. Environ. Toxicol. Chem. 21, 2099–2107. https://doi.org/10.1002/etc.5620211013

Delgado, L.F., Charles, P., Glucina, K., Morlay, C., 2015. Adsorption of Ibuprofen and Atenolol at Trace Concentration on Activated Carbon. Sep. Sci. Technol. 50, 14887–1496. https://doi.org/10.1080/01496395.2014.975360

Deng, Y., Yan, C., Nie, M., Ding, M., 2021. Bisphenol A adsorption behavior on soil and biochar: impact of dissolved organic matter. Environ. Sci. Pollut. Res. 1–12. https://doi.org/10.1007/s11356-021-12723-1

Dias, F., Duarte, C., 2013. Cellulose and Its Derivatives Use in the Pharmaceutical Compounding Practice, in: Cellulose - Medical, Pharmaceutical and Electronic Applications. InTech. https://doi.org/10.5772/56637

Ding, Q., Wu, H.L., Xu, Y., Guo, L.J., Liu, K., Gao, H.M., Yang, H., 2011. Impact of low molecular weight organic acids and dissolved organic matter on sorption and mobility of isoproturon in two soils. J. Hazard. Mater. 190, 823–832. https://doi.org/10.1016/j.jhazmat.2011.04.003

Doležalová Weissmannová, H., Pavlovský, J., Fišerová, L., Kosárová, H., 2018. Toxicity of Diclofenac: Cadmium Binary Mixtures to Algae Desmodesmus subspicatus Using Normalization Method. Bull. Environ. Contam. Toxicol. 101, 205–213.

https://doi.org/10.1007/s00128-018-2384-7

Dong, Q., Li, P., Huang, Q., Abdelhafez, A.A., Chen, L., 2014. Occurrence, polarity and bioavailability of dissolved organic matter in the Huangpu River, China. J. Environ. Sci. (China) 26, 1843–1850.

Donohue, I., Hillebrand, H., Montoya, J.M., Petchey, O.L., Pimm, S.L., Fowler, M.S., Healy, K., Jackson, A.L., Lurgi, M., McClean, D., O'Connor, N.E., O'Gorman, E.J., Yang, Q., 2016. Navigating the complexity of ecological stability. Ecol. Lett. 19, 1172–1185. https://doi.org/10.1111/ele.12648

Edwards, K.F., Litchman, E., Klausmeier, C.A., Edwards, K.F., Litchmak, E., Klausmeier, C.A., 2013. Functional traits explain phytoplankton responses to environmental gradients across lakes of the United States. Ecology 94, 1626–1635.

Eggen, R.I.L., Hollender, J., Joss, A., Scha, M., STamm, C. 2014. Reducing the dishcarge of micropollutants in the aquatic environment: the benefits of upgrading wastewater treatment plants. Environ. Sci. Technol. 48, 7683-7689.

Elias, D., Wang, L., Jacinthe, P.A., 2018. A meta-analysis of pesticide loss in runoff under conventional tillage and no-till management. Environ. Monit. Assess. 190, 79. https://doi.org/10.1007/s10661-017-6441-1

Ellegaard, M., Godhe, A., Ribeiro, S., 2018. Time capsules in natural sediment archives—Tracking phytoplankton population genetic diversity and adaptation over multidecadal timescales in the face of environmental change. Evol. Appl. 11, 11–16. https://doi.org/10.1111/eva.12513

Ellegaard, M., Ribeiro, S., 2018. The long-term persistence of phytoplankton resting stages in

aquatic ‘seed banks.’ Biol. Rev. 93, 166–183. https://doi.org/10.1111/brv.12338

Ellegaard, M., Ribeiro, S., Lundholm, N., Andersen, T., Berge, T., Ekelund, F., Harnstrom, K., Godhe, A., 2013. Using the sediment archive of living dinoflagellate cysts and other protist resting stages to study dynamics through time, in: Lewis, J., Marret, F., Bradley, L. (Eds.), Biological and Geological Perspectives of Dinoflagellate. The Micropalaeontological Society, Special Publications. Geological Society, London, pp. 149–153.

Ellner, S.P., Geber, M.A., Hairston, N.G., 2011. Does rapid evolution matter? Measuring the rate of contemporary evolution and its impacts on ecological dynamics. Ecol. Lett. 14, 603–614. https://doi.org/10.1111/j.1461-0248.2011.01616.x

Engebretson, R.R., von Wandruszka, R., 1994. Microorganization in dissolved humic acids. Environ. Sci. Technol 28, 1934–1941.

Enquist, B.J., Norberg, J., Bonser, S.P., Violle, C., Webb, C.T., Henderson, A., Sloat, L.L., Savage, V.M., 2015. Scaling from Traits to Ecosystems: Developing a General Trait Driver Theory via Integrating Trait-Based and Metabolic Scaling Theories. Adv. Ecol. Res. 52, 249–318. https://doi.org/10.1016/bs.aecr.2015.02.001

Environment Agency, 2009. Using biotic ligand models to implement environmental quality standards for metals under the Water Framework Directive.

Erickson, R.J., 2013. The biotic ligand model approach for addressing effects of exposure water chemistry on aquatic toxicity of metals: genesis and challenges. Environ. Toxicol. Chem. 32, 1212–1214.

Ertli, T., Marton, A., Földényi, R., 2004. Effect of pH and the role of organic matter in the

adsorption of isoproturon on soils. Chemosphere 57, 771–779. https://doi.org/10.1016/j.chemosphere.2004.07.009

Escher, B.I., Bramaz, N., Eggen, R.I.L., Richter, M., 2005. In Vitro Assessment of Modes of Toxic Action of Pharmaceuticals in Aquatic Life. Environ. Sci. Technol. 39, 3090–3100. https://doi.org/10.1021/es048590e

European Commission, 2019a. Implementation report Water Framework Directive and Floods Directive - Questions and Answers [WWW Document]. URL https://ec.europa.eu/info/news/implementation-report-water-framework-directive-and-floods-directive-questions-and-answers-2019-feb-26_en#:~:text=The maximum deadline possible is,and reduce those flood risks. (accessed 1.7.21).

European Commission, 2019b. Fitness Check of the Water Framework Directive and the Floods Directive. Https://Ec.Europa.Eu/ 1–184.

European Commission, 2015. COMMUNICATION FROM THE COMMISSION TO THE EUROPEAN PARLIAMENT AND THE COUNCIL The Water Framework Directive and the Floods Directive: Actions towards the “good status” of EU water and to reduce flood risks.

European Union, 2000. Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 Establishing a Framework for Community Action in the Field of Water Policy.

FAO, 2017. Water pollution from agriculture: a global review. The Food and Agriculture Organization of the United Nations (FAO) and the International Water Management Institute (IWMI) on behalf of the Water Land and Ecosystems research program, Rome & Colombo.

Feckler, A., Goedkoop, W., Konschak, M., Bundschuh, R., Kenngott, K.G.J., Schulz, R., Zubrod, J.P., Bundschuh, M., 2018. History matters: Heterotrophic microbial community structure and function adapt to multiple stressors. Glob. Chang. Biol. 24, e402–e415. https://doi.org/10.1111/gcb.13859

Fernandez, M.V., Gardinali, P.R., 2016. Risk assessment of triazine herbicides in surface waters and bioaccumulation of Irgarol and M1 by submerged aquatic vegetation in Southeast Florida. Sci. Total Environ. 541, 1556–1571. https://doi.org/10.1016/j.scitotenv.2015.09.035

Field, C.B., Behrenfeld, M.J., Randerson, J.T., Fallowski, P., 1998. Primary production of the biosphere: integrating terrestrial and oceanic components. Science (80-.). 281, 237–240.

Fischer, B.B., Pomati, F., Eggen, R.I.L., 2013. The toxicity of chemical pollutants in dynamic natural systems: The challenge of integrating environmental factors and biological complexity. Sci. Total Environ. 449, 253–259. https://doi.org/10.1016/j.scitotenv.2013.01.066

Fogg, G.E., 2001. Algal adaptation to stress – some general remarks, in: Rai, L.C., Gaur, J.P. (Eds.), Algal Adaptation to Environmental Stresses. Physiological, Biochemical and Molecular Mechanisms. Springer, Berlin, Germany, pp. 1–20.

Foley, J.A., DeFries, R., Asner, G.P., Barford, C., Bonan, G., Carpenter, S.R., Stuart Chapin, F., Coe, M.T., Daily, G.C., Gibbs, H.K., Helkowski, J.H., Holloway, T., Howard, E.A., Kucharik, C.J., Monfreda, C., Patz, J.A., Colin Prentice, I., Ramankutty, N., Snyder, P.K., 2005. Global Consequences of Land Use. Science (80-.). 309, 570–574.

Foley, J.A., Ramankutty, N., Brauman, K.A., Cassidy, E.S., Gerber, J.S., Johnston, M., Mueller, N.D., O'Connell, C., Ray, D.K., West, P.C., Balzer, C., Bennett, E.M.,

Carpenter, S.R., Hill, J., Monfreda, C., Polasky, S., Rockström, J., Sheehan, J., Siebert, S., Tilman, D., Zaks, D.P.M., 2011. Solutions for a cultivated planet. Nature 478, 337–342. https://doi.org/10.1038/nature10452

Fo¨ lster J, Johnson RK, Futter MN, Wilander A. 2014. The Swedish monitoring of surface waters: 50 years of adaptive monitoring. AMBIO 43:3–18.

Fontana, S., Jokela, J., Pomati, F., 2014. Opportunities and challenges in deriving phytoplankton diversity measures from individual trait-based data obtained by scanning flow-cytometry. Front. Microbiol. 5, 12. https://doi.org/10.3389/fmicb.2014.00324

Fontana, S., Petchey, O.L., Pomati, F., 2016. Individual-level trait diversity concepts and indices to comprehensively describe community change in multidimensional trait space. Funct. Ecol. 30, 808–818. https://doi.org/10.1111/1365-2435.12551

Fontana, S., Thomas, M.K., Moldoveanu, M., Spaak, P., Pomati, F., 2017. Individual-level trait diversity predicts phytoplankton community properties better than species richness or evenness. ISME J. 2018 122 12, 356–366. https://doi.org/10.1038/ismej.2017.160

Forbes, V.E., Calow, P., 2012. Promises and problems for the new paradigm for risk assessment and an alternative approach involving predictive systems models. Environ. Toxicol. Chem. 31, 2663–2671. https://doi.org/10.1002/etc.2009

Franz, S., Altenburger, R., Heilmeier, H., Schimitt-Jansen, M., 2008. What contributes to the sensitivity of microalgae to triclosan? Aquat. Toxicol. 90, 102–108. https://doi.org/10.1016/j.aquatox.2008.08.003

Frenkel, Y.V., Clark, A.D., Das, K., Wang, Y.-H., Lewi, P.J., Janssen, P.A.J., Arnold, E., 2005. Concentration and pH Dependent Aggregation of Hydrophobic Drug Molecules and

Relevance to Oral Bioavailability. https://doi.org/10.1021/jm049439i

Gardner, M., Comber, S., Scrimshaw, M.D., Cartmell, E., Lester, J., Ellor, B., 2012. The significance of hazardous chemicals in wastewater treatment works effluents. Sci. Total Environ. 437, 363–372. https://doi.org/10.1016/j.scitotenv.2012.07.086

Gassmann, A.J., Futuyma, D.J., 2005. Consequence of herbivory for the fitness cost of herbicide resistance: Photosynthetic variation in the context of plant-herbivore interactions. J. Evol. Biol. 18, 447–454. https://doi.org/10.1111/j.1420-9101.2004.00819.x

George, R., Haywood, A., Khan, S., Radovanovic, M., Simmonds, J., Norris, R., 2018. Enhancement and Suppression of Ionization in Drug Analysis Using HPLC-MS/MS in Support of Therapeutic Drug Monitoring: A Review of Current Knowledge of Its Minimization and Assessment. Ther. Drug Monit. 40, 1–8. https://doi.org/10.1097/FTD.0000000000000471

Gerisch, M., Agostinelli, V., Henle, K., Dziock, F., 2012. More species, but all do the same: contrasting effects of flood disturbance on ground beetle functional and species diversity. Oikos 121, 508–515. https://doi.org/10.1111/J.1600-0706.2011.19749.X

Gessner, M.O., Tlili, A., 2016. Fostering integration of freshwater ecology with ecotoxicology. Freshw. Biol. 61, 1991–2001. https://doi.org/10.1111/fwb.12852

Ghalambor, C.K., Reznick, D.N., Walker, J.A., 2004. Constraints on Adaptive Evolution: The Functional Trade-Off between Reproduction and Fast-Start Swimming Performance in the Trinidadian Guppy (Poecilia reticulata). Source Am. Nat. 164, 38–50. https://doi.org/10.1086/421412

Ghosh, K., Schnitzer, M., 1980. Macromolecular structures of humic substances. Soil Sci. 129, 266–276.

Gjessing, E.T., Egeberg, P.K., Håkedal, J., 1999a. Natural organic matter in drinking water - The “NOM-typing project”, background and basic characteristics of original water samples and NOM isolates. Environ. Int. https://doi.org/10.1016/S0160-4120(98)00119-6

Gjessing, E.T., Egeberg, P.K., Håkedal, J., 1999b. Natural organic matter in drinking water - The “NOM-typing project”, background and basic characteristics of original water samples and NOM isolates. Environ. Int. 25, 145–159. https://doi.org/10.1016/S0160-4120(98)00119-6

Gomulkiewicz, R., Holt, R.D., 1995. When does Evolution by Natural Selection Prevent Extinction ? Evolution (N. Y). 49, 201–207.

Gonzalez, A., Bell, G., 2013. Evolutionary rescue and adaptation to abrupt environmental change depends upon the history of stress. Philos. Trans. R. Soc. B Biol. Sci. 368. https://doi.org/10.1098/rstb.2012.0079

Grimm, V., Wissel, C., 1997. Babel, or the ecological stability discussions: An inventory and analysis of terminology and a guide for avoiding confusion. Oecologia 109, 323–334. https://doi.org/10.1007/s004420050090

Grzesiuk, M., Spijkerman, E., Lachmann, S.C., Wacker, A., 2018. Environmental concentrations of pharmaceuticals directly affect phytoplankton and effects propagate through trophic interactions. Ecotoxicol. Environ. Saf. 156, 271–278. https://doi.org/10.1016/j.ecoenv.2018.03.019

Gu, C, Karthikeyan, K.G., Sibley, S.D., Pedersen, J.A., 2007. Complexation of the antibiotic tetracycline with humic acid. Chemosphere2 66, 1494–1501.

Gu, Cheng, Karthikeyan, K.G., Sibley, S.D., Pedersen, J.A., 2007. Complexation of the antibiotic tetracycline with humic acid. Chemosphere 66, 1494–1501. https://doi.org/10.1016/j.chemosphere.2006.08.028

Guillard, R.R.L., Lorenzen, C.J., 1972. Yellow-green algase with chlorophyllide C12. J. Phycol. 8, 10–14.

Gusmao, J.B., Brauko, K.M., Eriksson, B.K., Lana, P.C., 2016. Functional diversity of macrobenthic assemblages decreases in response to sewage discharges. Ecol. Indic. 66, 65–75. https://doi.org/10.1016/J.ECOLIND.2016.01.003

Hagman, C., Skjelbred, B., Thrane, J., Andersen, T., de Wit, H., 2018. Growth responses of the nuisance algae Gonyostomum semen (Raphidophyceae) to DOC and associated alterations of light quality and quantity. Aquat. Microb. Ecol. 82, 241–251. https://doi.org/10.3354/ame01894

Hairston, N.G., Ellner, S.P., Geber, M.A., Yoshida, T., Fox, J.A., 2005. Rapid evolution and the convergence of ecological and evolutionary time. Ecol. Lett. 8, 1114–1127. https://doi.org/10.1111/j.1461-0248.2005.00812.x

Hanelt D. 1998. Capability of dynamic photoinhibition in Arctic macroalgae is related to their depth distribution. Mar Biol 131:361–369.

Hansch, C., Leo, A., Hoekman, D., 1995. Exploring QSAR: Hydrophobic, electronic, and steric constants. American Chemical Society, Washington, DC.

Hasset, J.P., Anderson, M.A., 1982. Effect of dissolved organic matter on adsorption of

hydrophobic organic compounds by river- and sewageborne particles. Water Res. 16, 681–686.

Hassett, J.P., Milicic, E., 1985. Determination of equilibrium and rate constant for binding of a polychlorinated biphenyl congener by dissolved humic substances. Environ. Sci. Technol 19, 638–648.

Henriksen, A., Skjelvåle, B.L., Mannio, J., Wilander, A., Harriman, R., Curtis, C., Jensen, J.P., Fjeld, E., Moiseenkon, T., 1998. Northern European lake survey, 1995. Ambio 27, 80–91.

Herbert, B.E., Bertsch, P.M., Novak, J.M., 1993. Pyrene Sorption by Water-Soluble Organic Carbon. Environ. Sci. Technol. 27, 398–403. https://doi.org/10.1021/es00039a021

Hering, D., Borja, A., Carstensen, J., Carvalho, L., Elliott, M., Feld, C.K., Heiskanen, A.S., Johnson, R.K., Moe, J., Pont, D., Solheim, A.L., de Bund, W. van, 2010. The European Water Framework Directive at the age of 10: A critical review of the achievements with recommendations for the future. Sci. Total Environ. 408, 4007–4019. https://doi.org/10.1016/j.scitotenv.2010.05.031

Hernandez-Ruiz, S., Abrell, L., Wickramasekara, S., Chefetz, B., Chorover, J., 2012. Quantifying PPCP interaction with dissolved organic matter in aqueous solution: Combined use of fluorescence quenching and tandem mass spectrometry. Water Res. 46, 943–954. https://doi.org/10.1016/j.watres.2011.11.061

Hertz, P.E., Arima, Y., Harrison, A., Huey, R.B., Losos, J.B., Glor RE. 2013. Asynchronous evolution of physiology and morphology in anolis lizards. Evolution 67:2101–2113.

Hillebrand, H., Langenheder, S., Lebret, K., Lindström, E., Östman, Ö., Striebel, M., 2018. Decomposing multiple dimensions of stability in global change experiments. Ecol. Lett.

21, 21–30. https://doi.org/10.1111/ele.12867

Hillebrand, H., Matthiessen, B., 2009. Biodiversity in a complex world: consolidation and progress in functional biodiversity research. Ecol. Lett. 12, 1405–1419. https://doi.org/10.1111/j.1461-0248.2009.01388.x

Hillebrand, H., Bennett, D.M., Cadotte, M.W. 2008. Consequences of dominance: a review of evenness effects on local and regional ecosystem processes. Ecology 89:1510–1520.

Hocart, M.J., Lucas, J.A., Peberdy, J.F., 1990. Resistance to fungicides in field isolates and laboratory-induced mutants of Pseudocercosporella herpotrichoides. Mycol. Res. 94, 9–17.

Holbrook, R.D., Love, N.G., Novak, J.T., 2004. Sorption of 17β-estradiol and 17α-ethinylestradiol by colloidal organic carbon derived from biological wastewater treatment systems. Environ. Sci. Technol. 38, 3322–3329. https://doi.org/10.1021/es035122g

Holling, C.S., 1973. Resilience and stability of ecological systems. Annu. Reveiew Ecol. Syst. 4, 1–23.

Hollomon, D.W., Wheeler, I., Dixon, K., Longhurts, C., Skylakakis, G., 1997. Defining the resistance risk of the new powdery mildew fungicide quinoxyfen. Pestic. Sci. 51, 347–351.

Holmstrup, M., Bindesbøl, A.M., Oostingh, G.J., Duschl, A., Scheil, V., Köhler, H.R., Loureiro, S., Soares, A.M.V.M., Ferreira, A.L.G., Kienle, C., Gerhardt, A., Laskowski, R., Kramarz, P.E., Bayley, M., Svendsen, C., Spurgeon, D.J., 2010. Interactions between effects of environmental chemicals and natural stressors: A review. Sci. Total Environ. 408, 3746–3762. https://doi.org/10.1016/j.scitotenv.2009.10.067

Hong, H., Wu, S., Wang, Q., Dai, M., Qian, L., Zhu, H., Li, Junwei, Zhang, J., Liu, J., Li, Jian, Lu, H., Yan, C., 2021. Fluorescent dissolved organic matter facilitates the phytoavailability of copper in the coastal wetlands influenced by artificial topography. Sci. Total Environ. 790, 147855. https://doi.org/10.1016/j.scitotenv.2021.147855

Hooper, D.U., Chapin, F.S., Ewel, J.J., Hector, A., Inchausti, P., Lavorel, S., Lawton, J.H., Lodge, D.M., Loreau, M., Naeem, S., Schmid, B., Setälä, H., Symstad, A.J., Vandermeer, J., Wardle, D.A., 2005. Effects of biodiversity on ecosystem functioning: A consensus of current knowledge. Ecol. Monogr. 75, 3–35. https://doi.org/10.1890/04-0922

Horne, S.D., Chowdhury, S.K., Heng, H.H.Q., 2014. Stress, genomic adaptation, and the evolutionary trade-off. Front. Genet. 5, 1–6. https://doi.org/10.3389/fgene.2014.00092

Hsieh, P.C., Lee, C.L., Jen, J.F., Chang, K.C., 2015. Complexation-flocculation combined with microwave-assisted headspace solid-phase microextraction in determining the binding constants of hydrophobic organic pollutants to dissolved humic substances. Analyst 140, 1275–1280. https://doi.org/10.1039/c4an01923g

Hubbell, S.P., 2005. Neutral theory in community ecology and the hypothesis of functional equivalence. Funct. Ecol. 19, 166–172. https://doi.org/10.1111/j.0269-8463.2005.00965.x

Huertas, I.E., Rouco, M., López-Rodas, V., Costas, E., 2010. Estimating the capability of different phytoplankton groups to adapt to contamination: Herbicides will affect phytoplankton species differently. New Phytol. 188, 478–487. https://doi.org/10.1111/j.1469-8137.2010.03370.x

Hughes, T.P., Kerry, J.T., Connolly, S.R., Baird, A.H., Eakin, C.M., Heron, S.F., Hoey, A.S., Hoogenboom, M.O., Jacobson, M., Liu, G., Pratchett, M.S., Skirving, W., Torda, G.,

2019. Ecological memory modifies the cumulative impact of recurrent climate extremes. Nat. Clim. Chang. 9, 40–43. https://doi.org/10.1038/s41558-018-0351-2

Isidori, M., Nardelli, A., Pascarella, L., Rubino, M., Parrella, A., 2007. Toxic and genotoxic impact of fibrates and their photoproducts on non-target organisms. Environ. Int. 33, 635–641. https://doi.org/10.1016/j.envint.2007.01.006

Jacquet, C., Altermatt, F., 2020. The ghost of disturbance past: long-term effects of pulse disturbances on community biomass and composition. Proc. R. Soc. B Biol. Sci. 287, 20200678. https://doi.org/10.1098/rspb.2020.0678

Jansen, M., Coors, A., Stoks, R., De Meester, L., 2011. Evolutionary ecotoxicology of pesticide resistance: a case study in Daphnia. Ecotoxicology 20, 543–551.

Jiang, C., Geng, J., Hu, H., Ma, H., Gao, X., Ren, H., 2017. Impact of selected non-steroidal anti-inflammatory pharmaceuticals on microbial community assembly and activity in sequencing batch reactors. PLoS One 12, e0179236. https://doi.org/10.1371/JOURNAL.PONE.0179236

Jiang, T., Wang, D., Meng, B., Chi, J., Laudon, H., Liu, J., 2020. The concentrations and characteristics of dissolved organic matter in high-latitude lakes determine its ambient reducing capacity. Water Res. 169, 115217. https://doi.org/10.1016/j.watres.2019.115217

Jiao, S., Chen, W., Wang, E., Wang, J., Liu, Z., Li, Y., Wei, G., 2016. Microbial succession in response to pollutants in batch-enrichment culture. Sci. Reports 2016 61 6, 1–11. https://doi.org/10.1038/srep21791

Jin, P., Agustí, S., 2018a. Fast adaptation of tropical diatoms to increased warming with trade-offs. Sci. Rep. 8, 17771. https://doi.org/10.1038/s41598-018-36091-y

Jin, P., Agustí, S., 2018b. Fast adaptation of tropical diatoms to increased warming with trade-offs. Sci. Rep. 8, 17771. https://doi.org/10.1038/s41598-018-36091-y

Johnsen, S., 1987. Interactions between polycyclic aromatic hydrocarbons and natural aquatic humic substances. Contact time relationship. Sci. Total Environ. 67, 269–278.

Johnstone, J.F., Allen, C.D., Franklin, J.F., Frelich, L.E., Harvey, B.J., Higuera, P.E., Mack, M.C., Meentemeyer, R.K., Metz, M.R., Perry, G.L.W., Schoennagel, T., Turner, M.G., 2016. Changing disturbance regimes, ecological memory, and forest resilience. Front. Ecol. Environ. 14, 369–378. https://doi.org/10.1002/fee.1311

Jota, M.A.T., Hassett, J.P., 1991. Effects of environmental variables on binding of a PCB congener by dissolved humic substances. Environ. Toxicol. Chem. 10.

Karlsson, M. V, Carter, L.J., Agatz, A., Boxall, A.B.A., 2017. Novel Approach for Characterizing pH-Dependent Uptake of Ionizable Chemicals in Aquatic Organisms. Environ. Sci. Technol 51, 6971. https://doi.org/10.1021/acs.est.7b01265

Keiluweit, M., Kleber, M., 2009. Molecular-level interactions in soils and sediments: the role of aromatic π-systems. Environ. Sci. Technol 43.

Khan, S.J., Ongerth, J.E., 2004. Modelling of pharmaceutical residues in Australian sewage by quantities of use and fugacity calculations. Chemosphere 54, 355–367. https://doi.org/10.1016/j.chemosphere.2003.07.001

Kirkpatrick, M., Peischl, S., 2012. Evolutionary rescue by beneficial mutations in environments that change in space and time. Philos. Trans. R. Soc. https://doi.org/10.1098/rstb.2012.0082

Koelmans, A.A, van der Hejide, A., Knijff, L.M., Aalderink, R.H. 2001. Integrated modelling

of eurotrophication and organic contaminant fate & effects in aquatic ecosystems. A review. Water Res. 35, 3517-3536.

Köhler, H.R., Triebskorn, R., 2013. Wildlife ecotoxicology of pesticides: Can we track effects to the population level and beyond? Science (80-.). 341, 759–765. https://doi.org/10.1126/science.1237591

Kottuparambil, S., Jin, P., Agusti, S., 2019. Adaptation of Red Sea phytoplankton to experimental warming increases their tolerance to toxic metal exposure. Front. Environ. Sci. 7. https://doi.org/doi: 10.3389/fenvs.2019.00125

Kraft, N.J.B., Adler, P.B., Godoy, O., James, E.C., Fuller, S., Levine, J.M. 2015. Community assembly, coexistence and the environ- mental filtering metaphor. Funct Ecol 29:592–599.

Kriegman, S., Cheney, N., Bongard, J. 2018. How morphological development can guide evolution. Sci Rep 8:13934.

Krop, H.B., van Noort, P.C., Govers, H.A., 2001. Determination and theoretical aspects of the equilibrium between dissolved organic matter and hydrophobic organic micropollutants in water (Kdoc). Rev. Environ. Contam. Toxicol. https://doi.org/10.1007/978-1-4613-0107-3_1

Kümmerer, K., Dionysiou, D.D., Olsson, O., Fatta-Kassinos, D., 2019. Reducing aquatic micropollutants – Increasing the focus on input prevention and integrated emission management. Sci. Total Environ. 652, 836–850. https://doi.org/10.1016/j.scitotenv.2018.10.219

Kwon, J.W., Armbrust, K.L., 2008. Aqueous solubility, n-octanol-water partition coefficient,

and sorption of five selective serotonin reuptake inhibitors to sediments and soils. Bull. Environ. Contam. Toxicol. 81, 128–135.

Laliberte, E., Legendre, P., 2010. A distance-based framework for measuring functional diversity from multiple traits. Ecology 91, 299–305. https://doi.org/10.1890/08-2244.1

Landrigan, P.J., Fuller, R., Acosta, N.J.R., Adeyi, O., Arnold, R., Basu, N. (Nil), Baldé, A.B., Bertollini, R., Bose-O'Reilly, S., Boufford, J.I., Breysse, P.N., Chiles, T., Mahidol, C., Coll-Seck, A.M., Cropper, M.L., Fobil, J., Fuster, V., Greenstone, M., Haines, A., Hanrahan, D., Hunter, D., Khare, M., Krupnick, A., Lanphear, B., Lohani, B., Martin, K., Mathiasen, K. V., McTeer, M.A., Murray, C.J.L., Ndahimananjara, J.D., Perera, F., Potočnik, J., Preker, A.S., Ramesh, J., Rockström, J., Salinas, C., Samson, L.D., Sandilya, K., Sly, P.D., Smith, K.R., Steiner, A., Stewart, R.B., Suk, W.A., van Schayck, O.C.P., Yadama, G.N., Yumkella, K., Zhong, M., 2018. The Lancet Commission on pollution and health. Lancet 391, 462–512. https://doi.org/10.1016/S0140-6736(17)32345-0

Laskowski, R., Bednarska, A.J., Kramarz, P.E., Loureiro, S., Scheil, V., Kudłek, J., Holmstrup, M., 2010. Interactions between toxic chemicals and natural environmental factors - A meta-analysis and case studies. Sci. Total Environ. 408, 3763–3774. https://doi.org/10.1016/j.scitotenv.2010.01.043

Lasky, J.R., Uriarte, M., Boukili, V.K., Chazdon, R.L., 2014. Trait-mediated assembly processes predict successional changes in community diversity of tropical forests. Proc. Natl. Acad. Sci. 111, 5616–5621. https://doi.org/10.1073/pnas.1319342111

Lau, E.T.C., Yung, M.M.N., Karraker, N.E., Leung, K.M.Y., 2014. Is an assessment factor of 10 appropriate to account for the variation in chemical toxicity to freshwater ectotherms under different thermal conditions? Environ. Sci. Pollut. Res. 21, 95–104.

https://doi.org/10.1007/s11356-013-1708-8

Laundrum, P.F., Nihart, S.R., Eadie, B.J., Gardner, W.S., 1984. Reverse phase separation method for determining pollutant binding to Aldrich humic acid and dissolved organic carbon of natural waters. Environ. Sci. Technol 19, 187–192.

Lee, D.Y., Farmer, W.J., 1989. Dissolved organic matter interaction with napropamide and four other nonionic pesticides. J. Environ. Qual. 18, 468.

Lee, Y.K., Hur, J., 2017. Using two-dimensional correlation size exclusion chromatography (2D-CoSEC) to explore the size-dependent heterogeneity of humic substances for copper binding. Environ. Pollut. 227, 490–497. https://doi.org/10.1016/j.envpol.2017.04.099

Leenheer, J., Croue', J., 2003. Characterizing dissolved aquatic organic matter. Environ. Sci. Technol.

Leibold, M.A., Holyoak, M., Mouquet, N., Amarasekare, P., Chase, J.M., Hoopes, M.F., Holt, R.D., Shurin, J.B., Law, R., Tilman, D., Loreau, M., Gonzalez, A. 2004. The metacommunity concept: a framework for multi-scale community ecology. Ecol Lett 7:601–613.

Lerdau, M., Gershenzon, J., 1997. Allocation theory and chemical defence, in: Bazzaz, F.A., Grace, J. (Eds.), Plant Resource Allocation. pp. 256–277.

Lin, H., Xia, X., Bi, S., Jiang, X., Wang, H., Zhai, Y., Wen, W., 2018a. Quantifying Bioavailability of Pyrene Associated with Dissolved Organic Matter of Various Molecular Weights to Daphnia magna. Environ. Sci. Technol. 52, 644–653. https://doi.org/10.1021/acs.est.7b05520

Lin, H., Xia, X., Jiang, X., Bi, S., Wang, H., Zhai, Y., Wen, W., Guo, X., 2018b. Bioavailability

of Pyrene Associated with Different Types of Protein Compounds: Direct Evidence for Its Uptake by Daphnia magna. Environ. Sci. Technol. 52, 9851–9860. https://doi.org/10.1021/acs.est.8b03349

Ling, W., Xu, J., Gao, Y., 2005. Effects of dissolved organic matter from sewage sludge on the atrazine sorption by soils. Sci. China, Ser. C Life Sci. 48, 57–66. https://doi.org/10.1360/04yc0129

Lipnick, R.L., 1995. Structure-activity relationships, in: Rand, G.M. (Ed.), Fundamentals of Aquatic Toxicology - Effects, Environmental Fate, and Risk Assessment. Taylor & Francis, Washington, pp. 609–655.

Liu, Y., Yang, C., Cheng, P., He, X., Zhu, Y., Zhang, Y., 2016. Influences of humic acid on the bioavailability of phenanthrene and alkyl phenanthrenes to early life stages of marine medaka (Oryzias melastigma). Environ. Pollut. 210, 211–216. https://doi.org/10.1016/j.envpol.2015.12.011

Lobo, S., Li, H., Farhan, N., Yan, G., 2014. Evaluation of Diclofenac Prodrugs for Enhancing Transdermal Delivery. Drug Dev. Ind. Pharm. 40, 425–432. https://doi.org/10.3109/03639045.2013.767828

Longstaffe, J.G., Courtier-Murias, D., Simpson, A.J., 2013. The pH-dependence of organofluorine binding domain preference in dissolved humic acid. Chemosphere 90, 270–275. https://doi.org/10.1016/j.chemosphere.2012.07.004

Lopes, P.C., Sucena, E., Santos, M.E., Magalhaes, S., 2008. Rapid experimental evolution of pesticide resistance in C. elegans entails no costs and affects the mating system. PLoS One 3, e3741.

Lu, G., Xie, Z., Zhang, Z., 2018. Effects of dissolved organic matter, feeding, and water flow on the bioconcentration of diclofenac in crucian carp (Carassius auratus). Environ. Sci. Pollut. Res. 25, 7776–7784. https://doi.org/10.1007/s11356-017-1081-0

Lyman, W.J., Reehl, W.F., Rosenblatt, D.H., 1990. Handbook of Chemical Property Estimation Methods: Environmental Behaviour of Organic Compounds. American Chemical Society. American Chemical Society.

Ma, L., Yates, S.R., 2018. Dissolved organic matter and estrogen interactions regulate estrogen removal in the aqueous environment: A review. Sci. Total Environ. 640–641, 529–542. https://doi.org/10.1016/j.scitotenv.2018.05.301

MacBean, C., 2008. Isoproturon (34123-59-6), in: E-Pesticide Manual. British Crop Protection Council, Alton, UK.

Maeng, S.K., Sharma, S.K., Abel, C.D.T., Magic-Knezev, A., Song, K.G., Amy, G.L., 2012. Effects of effluent organic matter characteristics on the removal of bulk organic matter and selected pharmaceutically active compounds during managed aquifer recharge: Column study. J. Contam. Hydrol. https://doi.org/10.1016/j.jconhyd.2012.08.005

Malaj, E., von der Ohe, P.C., Grote, M., Kühne, R., Mondy, C.P., Usseglio-Polatera, P., Brack, W., Schäfer, R.B., 2014. Organic chemicals jeopardize the health of freshwater ecosystems on the continental scale. Proc. Natl. Acad. Sci. 111, 9549–9554. https://doi.org/10.1073/pnas.1321082111

Manalil, S., Busi, R., Renton, M., Powles, S.B., 2011. Rapid Evolution of Herbicide Resistance by Low Herbicide Dosages. Weed Sci. 59, 210–217. https://doi.org/10.1614/ws-d-10-00111.1

Margalef, R., 1978. Life-forms of phytoplankton as survival alternatives in an unstable environment. Oceanol. Acta 1, 493–509.

Marvá, F., García-Balboa, C., Baselga-Cervera, B., Costas, E., 2014. Rapid adaptation of some phytoplankton species to osmium as a result of spontaneous mutations. Ecotoxicology 23, 213–220. https://doi.org/10.1007/s10646-013-1164-8

Mason, N.W.H., De Bello, F., Mouillot, D., Pavoine, S., Dray, S., 2013. A guide for using functional diversity indices to reveal changes in assembly processes along ecological gradients. J. Veg. Sci. 24, 794–806. https://doi.org/10.1111/jvs.12013

Matsuda, K., Schnitzer, M., 1971. Reactions between fulvic acid, a soil humic material, and dialkyl phthalates. Bull. enrir. contam. toxic. 6, 200–204.

Matthiessen, P., Law, R.J., 2002. Contaminants and their effects on estuarine and coastal organisms in the United Kingdom in the late twentieth century. Environ. Pollut. 120, 739–757. https://doi.org/10.1016/S0269-7491(02)00175-6

McFarland, J.W., Berger, C.M., Froshauer, S.A., Hayashi, S.F., Hecker, S.J., Jaynes, B.H., Jefson, M.R., Kamicker, B.J., Lipinski, C.A., Lundy, K.M., Reese, C.P., Vu, C.B., 1997. Quantitative structure-activity relationships among macrolide antibacterial agents: In vitro and in vivo potency against Pasteurella multocida. J. Med. Chem. 40, 1340–1346. https://doi.org/10.1021/jm960436i

McGill, B., Enquist, B., Weiher, E., Westoby, M., 2006. Rebuilding community ecology from functional traits. Trends Ecol. Evol. 21, 178–185. https://doi.org/10.1016/j.tree.2006.02.002

Medina, M.H., Correa, J.A., Barata, C., 2007. Micro-evolution due to pollution: Possible

consequences for ecosystem responses to toxic stress. Chemosphere 67, 2105–2114. https://doi.org/10.1016/j.chemosphere.2006.12.024

Merchant, S.S., Prochnik, S.E., Vallon, O., Harris, E.H., Karpowicz, S.J., Witman, G.B., Terry, A., Salamov, A., Fritz-Laylin, L.K., Maréchal-Drouard, L., Marshall, W.F., Qu, L.-H., Nelson, D.R., Sanderfoot, A.A., Spalding, M.H., Kapitonov, V. V, Ren, Q., Ferris, P., Lindquist, E., Shapiro, H., Lucas, S.M., Grimwood, J., Schmutz, J., Cardol, P., Cerutti, H., Chanfreau, G., Jorgensen, R., Mayfield, S., Mueller-Roeber, B., Rajamani, S., Sayre, R.T., Brokstein, P., Dubchak, I., Goodstein, D., Hornick, L., Wayne Huang, Y., Jhaveri, J., Luo, Y., Martínez, D., Chi Abby Ngau, W., Otillar, B., Poliakov, A., Porter, A., Szajkowski, L., Werner, G., Zhou, K., Grigoriev, I. V, Rokhsar, D.S., Grossman, A.R., 2007. The Chlamydomonas Genome Reveals the Evolution of Key Animal and Plant Functions. Science (80-.). 318, 245–250. https://doi.org/10.1126/science.1143609

Moe, S.J., De Schamphelaere, K., Clements, W.H., Sorensen, M.T., Van den Brink, P.J., Liess, M. 2013. Combined and interactive effects of global climate change and toxicants on populations and communities. Environ Toxicol Chem Setac 32:49–61.

Monteith, D.T., Stoddard, J.L., Evans, C.D., De Wit, H.A., Forsius, M., Høgåsen, T., Wilander, A., Skjelkvåle, B.L., Jeffries, D.S., Vuorenmaa, J., Keller, B., Kopácek, J., Vesely, J., 2007. Dissolved organic carbon trends resulting from changes in atmospheric deposition chemistry. Nature 450, 537–541. https://doi.org/10.1038/nature06316

Moriarty, F., 1999. Ecotoxicology, 3rd ed. Academic Press.

Moss, B., 2007. Shallow lakes, the water framework directive and life. What should it all be about?, in: Shallow Lakes in a Changing World. Springer Netherlands, pp. 381–394. https://doi.org/10.1007/978-1-4020-6399-2_34

Myeni, S.C.B., Brown, J.T., Martinez, G.A., Meyer-Ilse, W., 1999a. Imaging of humic substance macromolecular structures in water and soils. Science (80-.). 286, 1335–1337.

Myeni, S.C.B., Brown, J.T., Matinez, G.A., Meyer-Ilse, W., 1999b. Imaging of humic substance macromolecular structures in water and soils. Science (80-.). 286, 1335–1337.

Nakamura, Y., Yamamoto, H., Sekizawa, J., Kondo, T., Hirai, N., Tatarazako, N., 2008. The effects of pH on fluoxetine in Japanese medaka (Oryzias latipes): Acute toxicity in fish larvae and bioaccumulation in juvenile fish. Chemosphere 70, 865–873. https://doi.org/10.1016/j.chemosphere.2007.06.089

Neuwoehner, J., Fenner, K., Escher, B.I., 2006. Physiological Modes of Action of Fluoxetine and its Human Metabolites in Algae. Environ. Sci. Technol. 43, 6830–6837. https://doi.org/10.1021/es9005493

Newmann, M.C., 2001. Fundamentals of Ecotoxicology. Lewis Publisher, Boca Raton, FL.

Newton, I., Wyllie, I., 1992. Recovery of a Sparrowhawk Population in Relation to Declining Pesticide Contamination. J. Appl. Ecol. 29, 476. https://doi.org/10.2307/2404515

Niinemets, Ü., Kahru, A., Mander, Ü., Nõges, P., Nõges, T., Tuvikene, A., Vasemägi, A., 2017. Interacting environmental and chemical stresses under global change in temperate aquatic ecosystems: stress responses, adaptation, and scaling. Reg. Environ. Chang. 17, 2061–2077. https://doi.org/10.1007/s10113-017-1196-3

Nikolova, C., Ijaz, U.Z., Gutierrez, T., 2021. Exploration of marine bacterioplankton community assembly mechanisms during chemical dispersant and surfactant-assisted oil biodegradation. Ecol. Evol. 00, ece3.8091. https://doi.org/10.1002/ECE3.8091

Niyogi, S., Wood, C.M., 2004. Biotic ligand model, a flexible tool for developing site-specific

water quality guidelines for metals. Environ. Sci. Technol 38, 6177–6192.

Norberg, J., 2004. Biodiversity and ecosystem functioning: A complex adaptive systems approach. Limnol. Oceanogr. 49, 1269–1277. https://doi.org/10.4319/lo.2004.49.4_part_2.1269

Norberg, J., Swaney, D.P., Dushoff, J., Lin, J., Casagrandi, R., Levin, S.A., 2001. Phenotypic diversity and ecosystem functioning in changing environments: A theoretical framework. Proc. Natl. Acad. Sci. 98, 11376–11381. https://doi.org/10.1073/pnas.171315998

O'Neil, M.J., 2013. The Merck index : an encyclopedia of chemicals, drugs, and biologicals. CamRoyal Society of Chemistry, Cambridge, UK.

Oaks, J.L., Gilbert, M., Virani, M.Z., Watson, R.T., Meteyer, C.U., Rideout, B.A., Shivaprasad, H.L., Ahmed, S., Chaudhry, M.J.I., Arshad, M., Mahmood, S., Ali, A., Khan, A.A., 2004. Diclofenac residues as the cause of vulture population decline in Pakistan. Nature 427, 630–633. https://doi.org/10.1038/nature02317

OECD, 2009. OECD Guidelines for the testing of chemicals. Proposal for updating guideline 201, Freshwater alga and cyanobacteria, Growth Inhibition Test 1–21.

OECD, 2006a. Test No. 201: Alga, Growth Inhibition Test. OECD Guidelines for the Testing of Chemicals, Section 2: Effects on Biotic Systems. https://doi.org/10.1787/9789264069923-en

OECD, 2006b. Test No. 201: Alga, Growth Inhibition Test, OECD Guidelines for the Testing of Chemicals, Section 2: Effects on Biotic Systems. OECD Publishing. https://doi.org/10.1787/9789264069923-en

Ogle, K., Barber, J.J., Barron-Gafford, G.A., Bentley, L.P., Young, J.M., Huxman, T.E., Loik,

M.E., Tissue, D.T. 2015. Quantifying ecological memory in plant and ecosystem processes. Ecol Lett 18:221– 235.

Orsini, L., Schwenk, K., De Meester, L., Coulborne, J.K., Pfrender, M.E., Weider, L.J., 2013. The evolutionary time machine: using dormant propagules to forecast how populations can adapt to changing environments. Trends Ecol. Evol. 28, 274–282.

Österberg, R., Szajdak, L., Mortensen, K., 1994. Temperature-dependent restructuring of fractal humic acids: a proton dependent process. Environ. Int. 20, 77–80.

Pace, M.L., Reche, I., Cole, J.J., Fernández-Barbero, A., Mazuecos, I.P., Prairie, Y.T., 2012. pH change induces shifts in the size and light absorption of dissolved organic matter. Biogeochemistry 108, 109–118. https://doi.org/10.1007/S10533-01

Padisák, J., 1992. Seasonal Succession of Phytoplankton in a Large Shallow Lake (Balaton , Hungary) --A Dynamic Approach to Ecological Memory , Its Possible Role and Mechanisms 80, 217–230.

Pal, A., Gin, K.Y.H., Lin, A.Y.C., Reinhard, M., 2010. Impacts of emerging organic contaminants on freshwater resources: Review of recent occurrences, sources, fate and effects. Sci. Total Environ. https://doi.org/10.1016/j.scitotenv.2010.09.026

Palm, W.U., Millet, M., Zetzsch, C., 1998. OH radical reactivity of pesticides adsorbed on aerosol materials: First results of experiments with filter samples, in: Ecotoxicology and Environmental Safety. Academic Press Inc., pp. 36–43. https://doi.org/10.1006/eesa.1998.1664

Pan, B., Ning, P., Xing, B., 2009. Part V - Sorption of pharmaceuticals and personal care products. Environ. Sci. Pollut. Res. 16, 106–116. https://doi.org/10.1007/s11356-008-

0052-x

Pan, B., Ning, P., Xing, B., 2008. Part IV - Sorption of hydrophobic organic contaminants. Environ. Sci. Pollut. Res. 15, 554–564. https://doi.org/10.1007/s11356-008-0051-y

Pan, B., Xing, B., Liu, W., Xing, G., Tao, S., 2007. Investigating interactions of phenanthrene with dissolved organic matter: Limitations of Stern-Volmer plot. Chemosphere 69, 1555–1562. https://doi.org/10.1016/j.chemosphere.2007.05.059

Peacock, K.A. 2011. The three faces of Ecological fitness. Stud. Hist. Philos. Biol. Biomed. Sci. 42, 99-105.

Petchey, O.L., Gaston, K.J., 2006. Functional diversity: Back to basics and looking forward. Ecol. Lett. 9, 741–758. https://doi.org/10.1111/j.1461-0248.2006.00924.x

Peterson, G.D. 2002. Contagious disturbance, ecological memory, and the emergence of landscape pattern. Ecosystems 5:329– 338.

Pflugmacher, S., Pietsch, C., Rieger, W., Steinberg, C.E.W., 2006. Dissolved natural organic matter (NOM) impacts photosynthetic oxygen production and electron transport in coontail Ceratophyllum demersum. Sci. Total Environ. 357, 169–175. https://doi.org/10.1016/j.scitotenv.2005.03.021

Poerschmann, J., Zhang, Z., Kopinke, F.D., Pawliszyn, J., 1997. Solid phase microextraction for determining the distribution of chemicals in aqueous matrices. Anal. Chem. 69, 597–600.

Pokrovsky, O.S., Manasypov, R.M., Loiko, S. V., Shirokova, L.S., 2016. Organic and organo-mineral colloids in discontinuous permafrost zone. Geochim. Cosmochim. Acta 188, 1–20. https://doi.org/10.1016/j.gca.2016.05.035

Pomati, F., Castiglioni, S., Zuccato, E., Fanelli, R., Vigetti, D., Rossetti, C., Calamari, D., 2006. Effects of a complex mixture of therapeutic drugs at environmental levels on human embryonic cells. Environ. Sci. Technol. 40, 2442–2447. https://doi.org/10.1021/es051715a

Pomati, F., Jokela, J., Castiglioni, S., Thomas, M.K., Nizzetto, L., 2017. Water-borne pharmaceuticals reduce phenotypic diversity and response capacity of natural phytoplankton communities. PLoS One 12, 1–18. https://doi.org/10.1371/journal.pone.0174207

Pomati, F., Jokela, J., Simona, M., Veronesi, M., Ibelings, B.W., 2011. An Automated Platform for Phytoplankton Ecology and Aquatic Ecosystem Monitoring. Environ. Sci. Technol 45, 9658–9665. https://doi.org/10.1021/es201934n

Pomati, F., Nizzetto, L., 2013. Assessing triclosan-induced ecological and trans-generational effects in natural phytoplankton communities: A trait-based field method. Ecotoxicology 22, 779–794. https://doi.org/10.1007/s10646-013-1068-7

Posthuma, L., Zijp, M.C., De Zwart, D., Van de Meent, D., Globevnik, L., Koprivsek, M., Focks, A., Van Gils, J., Birk, S., 2020. Chemical pollution imposes limitations to the ecological status of European surface waters. Sci. Rep. 10, 1–12. https://doi.org/10.1038/s41598-020-71537-2

Prakash Agarwal, S., Khalid Anwer, M., Aqil, M., 2008. Complexation of Furosemide with Fulvic Acid Extracted from Shilajit: A Novel Approach. Drug Dev. Ind. Pharm. 34, 506–511. https://doi.org/10.1080/03639040701744053

Pretty, J.N., Brett, C., Gee, D., Hine, R.E., Mason, C.F., Morison, J.I.L., Raven, H., Rayment, M.D., Van Der Bijl, G., 2000. An assessment of the total external costs of UK agriculture.

Agric. Syst. 65, 113–136. https://doi.org/10.1016/S0308-521X(00)00031-7

R Core Development Team, 2015. R: A Language and Environment for Statistical Computing. https://doi.org/10.1007/978-3-540-74686-7

Ramos, E.U., Meijer, S.N., Vaes, W.H.J., Verhaar, H.J.M., Hermens, J.L.M., 1998. Using solid-phase microextraction to determine partition coefficients to humic acids and bioavailable concentrations of hydrophobic chemicals. Environ. Sci. Technol 32, 3430–3435.

Raymond, P.A., Spencer, R.G.M., 2015. Riverine DOM, in: Hansell, D.A., Carlson, C.A. (Eds.), Biogeochemistry of Marine Dissolved Organic Matter. Academic press, San Diego, USA, pp. 509–533.

Reichstein, M., Bahn, M., Mahecha, M.D., Kattge, J., Baldocchi, D.D., 2014. Linking plant and ecosystem functional biogeography. Proc. Natl. Acad. Sci. 111, 13697–13702. https://doi.org/10.1073/PNAS.1216065111

Reijo, R.A., Cooper, E.M., Beagle, G.J., Huffaker, T.C., 1994. Systematic mutational analysis of the yeast beta-tubulin gene. Mol. Biol. Cell 5, 29–43.

Reiss, J., Bridle, J.R., Montoya, J.M., Woodward, G., 2009. Emerging horizons in biodiversity and ecosystem functioning research. Trends Ecol. Evol. 24, 505–514. https://doi.org/10.1016/j.tree.2009.03.018

Ren, H., Ma, F., Yao, X., Shao, K., Yang, L., 2020. Multi-spectroscopic investigation on the spatial distribution and copper binding ability of sediment dissolved organic matter in Nansi Lake, China. J. Hydrol. 591, 125289. https://doi.org/10.1016/j.jhydrol.2020.125289

Rewitt, D.M., Balogh, T., Jones, H., 2015. Sorption behaviours and transport potentials for selected pharmaceuticals and triclosan in two sterilised soils. Remediat. Manag. Contam. or Degrad. lands 15, 594–606.

Reynolds, C.S., Wiseman, S.W., Godfrey, B.M., Butterwick, C., 1983. Some effects of artificial mixing on the dynamics of phytoplankton populations in large limnetic enclosures. J. Plankton Res. 5, 203–234. https://doi.org/10.1093/PLANKT/5.2.203

Ripszam, M., Haglund, P., 2015. Automated method for determination of dissolved organic carbon-water distribution constants of structurally diverse pollutants using pre-equilibrium solid-phase microextraction. Environ. Toxicol. Chem. 34, 266–274. https://doi.org/10.1002/etc.2805

Ripszam, M., Paczkowska, J., Figueira, J., Veenaas, C., Haglund, P., 2015. Dissolved organic carbon quality and sorption of organic pollutants in the baltic sea in light of future climate change. Environ. Sci. Technol. 49, 1445–1452. https://doi.org/10.1021/es504437s

Rizzuto, S., Baho, D.L., Jones, K.C., Zhang, H., Leu, E., Nizzetto, L., 2021. Binding of waterborne pharmaceutical and personal care products to natural dissolved organic matter. Sci. Total Environ. 784, 147208. https://doi.org/10.1016/j.scitotenv.2021.147208

Rizzuto, S., Thrane, J.-E., Baho, D.L., Jones, K.C., Zhang, H., Hessen, D.O., Nizzetto, L., Leu, E., 2020. Water Browning Controls Adaptation and Associated Trade-Offs in Phytoplankton Stressed by Chemical Pollution. Environ. Sci. Technol. 54, 5569–5579. https://doi.org/10.1021/ACS.EST.0C00548

Rockström, J., Steffen, W., Noone, K., Persson, A., Chapin, F.., Lambin, E.., Lenton, T.., M, S., Folke, C., Schellnhuber, H.., Nykvist, B., de Wit, C.A., Hughes, T., van der Leeuw, S., H, R., Sorlin, S., Snyder, P, K., Costanza, R., Svedin, U., Falkenmark, M., Karlberg,

L., Corell, R.., Fabry, V.., Hansen, J., Walker, B., Liverman, D., Richardson, K., Crutzen, P., Foley, J.., 2009. A safe operating space for humanity. Nature 461, 472–475. https://doi.org/10.1016/j.jen.2014.01.005

Rohr, J.R., Crumrine, P.W., 2005. Effects of an herbicide and an insecticide on pond community structure and processes. Ecol. Appl. 15, 1135–1147.

Roulet, N., Moore, T.R., 2006. Browning the waters. Nature 444.

Rowett, C.J., Hutchinson, T.H., Comber, S.D.W., 2016. The impact of natural and anthropogenic Dissolved Organic Carbon (DOC), and pH on the toxicity of triclosan to the crustacean Gammarus pulex (L.). Sci. Total Environ. 565, 222–231. https://doi.org/10.1016/j.scitotenv.2016.04.170

Rozman, K.K., Doull, J., 2000a. Dose and time as variables of toxicity. Toxicology 144, 169–178. https://doi.org/10.1016/S0300-483X(99)00204-8

Rozman, K.K., Doull, J., 2000b. Dose and time as variables of toxicity. Toxicology 144, 169–178. https://doi.org/10.1016/S0300-483X(99)00204-8

Salmaso, N., Naselli-Flores, L., Padisák, J., 2015. Functional classifications and their application in phytoplankton ecology. Freshw. Biol. 60, 603–619. https://doi.org/10.1111/fwb.12520

Samani, P., Bell, G., 2016. The ghosts of selection past reduces the probability of plastic rescue but increases the likelihood of evolutionary rescue to novel stressors in experimental populations of wild yeast. Ecol. Lett. 19, 289–298. https://doi.org/10.1111/ele.12566

Sangster, J., 1997. LOGKOW A Databank of Evaluated Octanol-Water Partition Coefficients.

Schäfer, R.B., Bundschuh, M., Rouch, D.A., Szöcs, E., von der Ohe, P.C., Pettigrove, V.,

Schulz, R., Nugegoda, D., Kefford, B.J., 2012. Effects of pesticide toxicity, salinity and other environmental variables on selected ecosystem functions in streams and the relevance for ecosystem services. Sci. Total Environ. 415, 69–78. https://doi.org/10.1016/j.scitotenv.2011.05.063

Schäfer, R.B., van den Brink, P.J., Liess, M. 2011. Impacts of pesti- cides on freshwater ecosystems. Ecol Impacts Toxic Chem:111–37.

Schäfer, R.B., Caquet, T., Siimes, K., Mueller, R., Lagadic, L., Liess, M., 2007. Effects of pesticides on community structure and ecosystem functions in agricultural streams of three biogeographical regions in Europe. Sci. Total Environ. 382, 272–285. https://doi.org/10.1016/j.scitotenv.2007.04.040

Scheffer, M., Carpenter, S., Foley, J.A., Folke, C., Walker, B., 2001. Catastrophic shifts in ecosystems. Nature 413, 591–596. https://doi.org/10.1038/35098000

Scheffer, M., Carpenter, S.R., 2003. Catastrophic regime shifts in ecosystems: linking theory to observation. Trends Ecol. Evol. 18, 648–656. https://doi.org/10.1016/j.tree.2003.09.002

Schlautman, M.A., Morgan, J.J., 1993. Effects of aqueous chemistry on the binding of polycyclic aromatic hydrocarbons by dissolved humic materials. Environ. Sci. Technol 27, 961–969.

Schroer, A.F.W., Belgers, J.D.M., Brock, C.M., Matser, A.M., Maund, S.J., Van den Brink, P.J., 2004. Comparison of Laboratory Single Species and Field Population-Level Effects of the Pyrethroid Insecticide λ-Cyhalothrin on Freshwater Invertebrates. Arch. Environ. Contam. Toxicol. 46, 324–335. https://doi.org/10.1007/s00244-003-2315-3

Schulten, H.R., 1999. Interactions of dissolved organic matter with xenobiotic compounds: Molecular modeling in water. Environ. Toxicol. Chem. 18, 1643–1655. https://doi.org/10.1007/s11269-017-1757-8

Schulz, R., Bub, S., Petschick, L.L., Stehle, S., Wolfram, J., 2021. Applied pesticide toxicity shifts toward plants and invertebrates, even in GM crops. Science (80-.). 372, 81–84. https://doi.org/10.1126/science.abe1148

Schütte, G., Eckerstorfer, M., Rastelli, V., Reichenbecher, W., Restrepo-Vassalli, S., Ruohonen-Lehto, M., Saucy, A.-G.W., Mertens, M., 2017. Herbicide resistance and biodiversity: agronomic and environmental aspects of genetically modified herbicide-resistant plants. Environ. Sci. Eur. 29, 5. https://doi.org/10.1186/s12302-016-0100-y

Schwarzenbach, R., Escher, B.I., Fenner, K., Hofstetter, R., Johnson, C., Von Gunten, U., Wehrli, B., 2006. The Challenge of Micropollutants in Aquatic System. Science (80-.). 313, 1072–1077. https://doi.org/10.1126/science.1127291

Seguin, F., Le Bihan, F., Leboulanger, C., Berard, A. 2002. A risk assessment of pollution: induction of atrazine tolerance in phytoplankton communities in freshwater outdoor meso-cosms, using chlorophyll fluorescence as an endpoint. Water Res 36:3227–3236.

Seol, Y., Lee, L.S., 2000. Effect of Dissolved Organic Matter in Treated Effluents on Sorption of Atrazine and Prometryn by Soils. Soil Sci Soc Am J 64, 1976–1983. https://doi.org/10.2136/sssaj2000.6461976x

Sheenan, P.J., 1984. Effects on community and ecosystem structure and dynamics, in: Miller, P.J.. (Ed.), Effects of Pollutants at the Ecosystem Level. John Wiley & Sons Ltd, New York, USA, pp. 51–100.

Solbrig, O.T., 1993. Plant traits and adaptive strategies: their role in ecosystem function, in: Schulze, E.D., Mooney, H.A. (Eds.), Biodiversity and Ecosystem Function. Springer, Berlin, Germany, pp. 97–116.

Spurgeon, D., Lahive, E., Robinson, A., Short, S., Kille, P., 2020. Species Sensitivity to Toxic Substances: Evolution, Ecology and Applications. Front. Environ. Sci. 8, 237. https://doi.org/10.3389/fenvs.2020.588380

Steffen, W., Richardson, K., Rockstrom, J., Cornell, S.E., Fetzer, I., Bennett, E.M., Biggs, R., Carpenter, S.R., de Vries, W., de Wit, C.A., Folke, C., Gerten, D., Heinke, J., Mace, G.M., Persson, L.M., Ramanathan, V., Reyers, B., Sorlin, S., 2015. Planetary boundaries: Guiding human development on a changing planet. Science (80-.). 347, 1259855–1259855. https://doi.org/10.1126/science.1259855

Stehle, S., Schulz, R., 2015. Agricultural insecticides threaten surface waters at the global scale. Proc. Natl. Acad. Sci. U. S. A. 112, 5750–5755. https://doi.org/10.1073/pnas.1500232112

Strauss, S.Y., Rudgers, J.A., Lau, J.A., Irwin, R.E. 2002. Direct and ecological costs of resistance to herbivory. Trends Ecol Evol 17:278–285.

Sturm, A., Radau, T.S., Hahn, T., Schulz, R., 2007. Inhibition of rainbow trout acetylcholinesterase by aqueous and suspended particle-associated organophosphorous insecticides. Chemosphere 68, 605–612. https://doi.org/10.1016/j.chemosphere.2007.02.056

Suding, K.N., Lavorel, S., Chapin III, F.S., Cornelissen, C., Diaz, S., Garnier, E., Goldberg, D., Hooper, D.U., Jackson, S.T., Navas, M., 2008. Scaling environmental change through the community-level: a trait-based response-and-effect framework for plants. Glob. Chang. Biol. 14, 1125–1140.

Sun, M., Duker, R.Q., Gillissen, F., Van den Brink, P.J., Focks, A., Rico, A., 2020a. Influence of pH on the toxicity of ionisable pharmaceuticals and personal care products to freshwater invertebrates. Ecotoxicol. Environ. Saf. 191. https://doi.org/10.1016/j.ecoenv.2020.110172

Sun, M., Duker, R.Q., Gillissen, F., Van den Brink, P.J., Focks, A., Rico, A., 2020b. Influence of pH on the toxicity of ionisable pharmaceuticals and personal care products to freshwater invertebrates. Ecotoxicol. Environ. Saf. 191, 110172. https://doi.org/10.1016/j.ecoenv.2020.110172

Suzuki, S., Yamaguchi, H., Nakajima, N., Kawachi, M. 2018. Raphidocelis subcapitata (=*Pseudokirchneriella subcapitata*) provides an insight into genome evolution and environmental adaptations in the Sphaeropleales. Sci Rep 8:8058.

Tambi, H., Flaten, G.A.F., Egge, J.K., Bødtker, G., Jacobsen, A., Thingstad, T.F., 2009. Relationship between phosphate affinities and cell size and shape in various bacteria and phytoplankton 311–320. https://doi.org/10.3354/ame01369

Tanaka, F., Fukushima, M., Kikuchi, A., Yabuta, H., Ichikawa, H., Tatsumi, K., 2005. Influence of chemical characteristics of humic substances on the partition coefficient of a chlorinated dioxin. Chemosphere 58, 1319–1326. https://doi.org/10.1016/j.chemosphere.2004.10.008

Tang, Y., Li, X.-M., Xu, Z.-C., Guo, Q.-W., Hong, C.-Y., Bing, Y.-X., 2014. Removal of naproxen and bezafibrate by activated sludge under aerobic conditions: Kinetics and effect of substrates. Biotechnol. Appl. Biochem. 61, n/a-n/a. https://doi.org/10.1002/bab.1168

Thakuria, R., Sarma, B., Nangia, A., 2017. Hydrogen Bonding in Molecular Crystals, in: Comprehensive Supramolecular Chemistry II. Elsevier Inc., pp. 25–48.

https://doi.org/10.1016/B978-0-12-409547-2.12598-3

Thevenot, M., Dousset, S., Hertkorn, N., Schmitt-Kopplin, P., Andreux, F., 2009. Interactions of diuron with dissolved organic matter from organic amendments. Sci. Total Environ. 407, 4297–4302. https://doi.org/10.1016/j.scitotenv.2009.04.021

Thibodeau, G., Walsh, D.A., Beisner, B.E., 2015. Rapid eco-evolutionary responses in perturbed phytoplankton communities. Proc. R. Soc. B Biol. Sci. 282, 20151215. https://doi.org/10.1098/rspb.2015.1215

Thomas, M.K., Fontana, S., Reyes, M., Pomati, F., 2018. Quantifying cell densities and biovolumes of phytoplankton communities and functional groups using scanning flow citometry, machine learning and unsupervised clustering. PLoS One 22.

Thompson, J.N. 1998. Rapid evolution as an ecological process. Trends Ecol Evol 13:329–332.

Thrane, J.-E., Hessen, D.O., Andersen, T., 2016. The impact of irradiance on optimal and cellular nitrogen to phosphorus ratios in phytoplankton. Ecol. Lett. 19, 880–888. https://doi.org/10.1111/ele.12623

Thrane, J.E., Hessen, D.O., Andersen, T., 2014. The Absorption of Light in Lakes: Negative Impact of Dissolved Organic Carbon on Primary Productivity. Ecosystems 17, 1040–1052. https://doi.org/10.1007/s10021-014-9776-2

Thurman, E.M., 1985. Organic Geochemistry of Natural Waters. Dordrecht, The Netherlands.

Tilman, D., Cassman, K.G., Matson, P.A., Naylor, R., Polasky, S., 2002. Agricultural sustainability and intensive production practices. Nature 418, 671–677.

Tilman, D. 1982. Resource competition and community struc- ture. Princeton University Press.

Tilmon, K.J. 2008. Specialization, Speciation, and Radiation. 1st ed. University of California Press. https://doi.org/10.1525/j.c tt1pnq3k.

Tlili, A., Berard, A., Blanck, H., Bouchez, A., Cássio, F., Eriksson, K.M., Morin, S., Montuelle, B., Navarro, E., Pascoal, C., Pesce, S., Schmitt-Jansen, M., Behra, R., 2016. Pollution-induced community tolerance (PICT): towards an ecologically relevant risk assessment of chemicals in aquatic systems. Freshw. Biol. 61, 2141–2151. https://doi.org/10.1111/fwb.12558

Tolkoff-Rubin, N., 2011. Treatment of Irreversible Renal Failure, in: Goldman's Cecil Medicine: Twenty Fourth Edition. Elsevier Inc., pp. 818–826. https://doi.org/10.1016/B978-1-4377-1604-7.00133-0

Tomlin, C.S.D., 2000. Isoproturon (464), in: The E-Pesticide Manual (12th Ed.) Version 2.0. British Crop Protection Council, Farnham, UK3.

Truhaut, R., 1977. Ecotoxicology: Objectives, principles and perspectives. Ecotoxicol. Environ. Saf. 1, 151–173. https://doi.org/10.1016/0147-6513(77)90033-1

Tulonen, T., Salonen, K., Arvola, L., 1992. Effects of different molecular weight fractions of dissolved organic matter on the growth of bacteria, algae and protozoa from a highly humic lake. Hydrobiologia 229, 239–252. https://doi.org/10.1007/BF00007003

Turner, M.G., 2010. Disturbance and landscape dynamics in a changing world. Ecology 91, 2833–2849. https://doi.org/10.1890/10-0097.1

Uecker, H., Hermisson, J., 2011. On the Fixation Process of a Beneficial Mutation in a Variable Environment. Genetics 188, 915–930. https://doi.org/10.1534/genetics.110.124297

Valenti, T.W., Pilar Perez-hurtado, I., Kevin Chambliss, C., Brooksi, B.W., 2009.

Pharmaceuticals and Personal Care Products in the Environment, Aquatic toxicity of sertraline to Pimephales promelas at environmentally relevant surface water pH. Environ. Toxicol. Chem. 28, 2685–2694.

van der Brink, P.J., 2008. Ecological Risk Assessment: From Book-Keeping to Chemical Stress Ecology. Environ. Sci. Technol 42, 8999–9004. https://doi.org/10.1021/es801991c

van Dijk, H., 1971. Cation binding of humic acids. Geoderma 5, 53–67.

van Straalen, N., 2003. Ecotoxicology becomes stress ecology. Environ. Sci. Technol. 30, 324–330.

van Straalen, N.M., Hoffmann, A.A. 2000. Review of experimental evidence for physiological costs of tolerance to toxicants. In: Kammenga JE, Laskowski R, editors. Demography in Eco- toxicology. John Wiley and Sons. pp 147–61. https://research. vu.nl/en/publications/ccdfbf36-ee1c-4050-a0ef-e6ed63169b91.

Vellend, M., 2016. The Theory of Ecological Communities (MPB-57). Princeton University Press.

Vellend, M., 2010. Conceptual synthesis in community ecology. Q. Rev. Biol. 85, 183–206. https://doi.org/10.1086/652373

Vialykh, E.A., Mckay, G., Rosario-Ortiz, F.L., 2020. Computational Assessment of the Three-Dimensional Configuration of Dissolved Organic Matter Chromophores and Influence on Absorption Spectra. Environ. Sci. Technol. 54, 15904–15913. https://doi.org/10.1021/acs.est.0c05860

Vieno, N., Tuhkanen, T., Kronberg, L., 2010. Removal of Pharmaceuticals in Drinking Water Treatment: Effect of Chemical Coagulation. Environ. Technol. 27, 183–192.

Vila-Aiub, M.M., Neve, P., Powles, S.B., 2009b. Evidence for an ecological cost of enhanced herbicide metabolism in *Lolium rigidum*. J. Ecol. 97, 772–780. https://doi.org/10.1111/j.1365-2745.2009.01511.x

Villéger, S., Mason, N.W.H., Mouillot, D., 2008. New multidimensional functional diversity indices for a multifaceted framework in functional ecology. Ecology 89, 2290–2301. https://doi.org/10.1890/07-1206.1

Vinebrooke, R.D., Cottingham, K.L., Norberg, J., Scheffer, M., Dodson, S.I., Maberly, S.C., Sommer, U., 2004. Impacts of multiple stressors on biodiversity and ecosystem functioning: the role of species co-tolerance. Oikos 3, 451–457.

Violle, C., Navas, M.-L., Vile, D., Kazakou, E., Fortunel, C., Hummel, I., Garnier, E., 2007. Let the concept of trait be functional! Oikos 116, 882–892. https://doi.org/10.1111/j.0030-1299.2007.15559.x

Vörösmarty, C.J., Hoekstra, A.Y., Bunn, S.E., Conway, D., Gupta, J., 2015. Fresh water goes global. Science (80-.). 349, 478–479. https://doi.org/10.1126/science.aac6009

Vörösmarty, C.J., Léveque, C., Revenga, C., Bos, R., Caudill, C., Chilton, J., 2005. Fresh water. Millenn. Ecosyst. Assess. 1, 165–207.

Vörösmarty, C.J., McIntyre, P.B., Gessner, M.O., Dudgeon, D., Prusevich, A., Green, P., Glidden, S., Bunn, S.E., Sullivan, C.A., Liermann, C.R., Davies, P.M., 2010. Global threats to human water security and river biodiversity. Nature 467, 555–561. https://doi.org/10.1038/nature09440

Voulvoulis, N., Arpon, K.D., Giakoumis, T., 2017. The EU Water Framework Directive: From great expectations to problems with implementation. Sci. Total Environ. 575, 358–366.

https://doi.org/10.1016/j.scitotenv.2016.09.228

Wang, J., Wang, S., 2016. Removal of pharmaceuticals and personal care products (PPCPs) from wastewater: A review. J. Environ. Manage. 182, 620–640. https://doi.org/10.1016/j.jenvman.2016.07.049

Wang, M.X., Liang, L.N., Siu, W.S., Fan, D., Sun, H.R., Zhao, H.H., Zhou, G.J., Wu, W.J., 2019. Loss accounting of environmental pollution within Pearl River Delta region, South China. Environ. Pollut. 249, 676–685. https://doi.org/10.1016/j.envpol.2019.03.081

Wang, W., Zheng, B., Jiang, X., Chen, J., Wang, S., 2020. Characteristics and Source of Dissolved Organic Matter in Lake Hulun, A Large Shallow Eutrophic Steppe Lake in Northern China. Water 12. https://doi.org/doi:10.3390/w12040953

Wang, Z., Jiang, Q., Wang, R., Yuan, X., Yang, S., Wang, W., Zhao, Y., 2018. Effects of Dissolved Organic Matter on Sorption of Oxytetracycline to Sediments. Geofluids 2018. https://doi.org/10.1155/2018/1254529

Weatherhead, E.K., Howden, N.J.K., 2009. The relationship between land use and surface water resources in the UK. Land use policy 26, S243–S250. https://doi.org/10.1016/j.landusepol.2009.08.007

Wei-Hass, M.L., Hageman, K.J., Chin, Y.P., 2014. Partitioning of polybrominated diphenyl ethers to dissolved organic matter isolated from arctic surface waters. Environ. Sci. Technol 48, 4825–4859.

Wershaw, R.L., Burear, P.J., Goldberg, M.C., 1969. Interaction of Pesticides with Natural Organic Material. Environ. Sci. Technol. 3, 271–273. https://doi.org/10.1021/es60026a010

White, E.P., Ernest, S.K.M., Kerkhoff, A.J., Enquist, B.J., 2007. Relationships between body size and abundance in ecology. Trends Ecol. Evol. 22, 323–330. https://doi.org/10.1016/j.tree.2007.03.007

WHO, 2011. Guidelines for Drinking-water Quality, Fourth Edition, Vol. 1. WHO Libr. Cat. 1. https://doi.org/10.1016/S1462-0758(00)00006-6

Wibawa, J.I.D., Shaw, P.N., Barrett, D.A., 2003. Quantification of clarithromycin, its 14-hydroxy and decladinose metabolites in rat plasma, gastric juice and gastric tissue using high-performance liquid chromatography with electrochemical detection. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 783, 359–366. https://doi.org/10.1016/S1570-0232(02)00765-1

Wickham, H., 2006. ggplot2: Elegant Graphics for Data Analysis 35, 2006.

Williams, C.F., Farmer, W.J., Letey, J., Nelson, S.D., 1999. Design and Characterization of a New Dialysis Chamber for Investigating Dissolved Organic Matter-Organic Complexes. J. Environ. Qual. 28, 1757–1760. https://doi.org/10.2134/jeq1999.00472425002800060010x

Williamson, C.E., Overholt, E.P., Pilla, R.M., Leach, T.H., Brentrup, J.A., Knoll, L.B., Mette, E.M., Moeller, R.E., 2016. Ecological consequences of long-term browning in lakes. Sci. Rep. 5, 18666. https://doi.org/10.1038/srep18666

Wilson, B.A., Smith, V.H., Denoyelles, F., Larive, C.K., 2003. Effects of three pharmaceutical and personal care products on natural freshwater algal assemblages. Environ. Sci. Technol. 37, 1713–1719. https://doi.org/10.1021/es0259741

Wolf, R., Andersen, T., Hessen, D.O., Hylland, K., 2017. The influence of dissolved organic

carbon and ultraviolet radiation on the genomic integrity of Daphnia magna. Funct. Ecol. 31, 848–855. https://doi.org/10.1111/1365-2435.12730

Woodcock, B.A., Bullock, J.M., Shore, R.F., Heard, M.S., Pereira, M.G., Redhead, J., Ridding, L., Dean, H., Sleep, D., Henrys, P., Peyton, J., Hulmes, S., Hulmes, L., Sárospataki, M., Saure, C., Edwards, M., Genersch, E., Knäbe, S., Pywell, R.F., 2017. Country-specific effects of neonicotinoid pesticides on honey bees and wild bees. Science (80-.). 356, 1393–1395. https://doi.org/10.1126/science.aaa1190

Woodward, G., Perkins, D.M., Brown, L.E., 2010. Climate change and freshwater ecosystems: Impacts across multiple levels of organization. Philos. Trans. R. Soc. B Biol. Sci. 365, 2093–2106. https://doi.org/10.1098/rstb.2010.0055

Xu, H., Guo, L., 2017. Molecular size-dependent abundance and composition of dissolved organic matter in river, lake and sea waters. Water Res. 117, 115–126. https://doi.org/10.1016/j.watres.2017.04.006

Xu, H., Zou, L., Guan, D., Li, W., Jiang, H., 2019. Molecular weight-dependent spectral and metal binding properties of sediment dissolved organic matter from different origins. Sci. Total Environ. 665, 828–835. https://doi.org/10.1016/j.scitotenv.2019.02.186

Yamada, N., Katoh, M., 2020. Feature of lead complexed with dissolved organic matter on lead immobilization by hydroxyapatite in aqueous solutions and soils. Chemosphere 249, 126122. https://doi.org/10.1016/j.chemosphere.2020.126122

Yamamoto, H., Liljestrand, H.M., Shimizu, Y., Morita, M., 2003. Effects of physical−chemical characteristics on the sorption of selected endocrine disruptors by dissolved organic matter surrogates. Environ. Sci. Technol 37, 2646–2657.

Yamamoto, H, Yamamoto, Hiroshi, Hayashi, A., Nakamura, Y., Sekizawa, J., 2005. Fate and Partitioning of Selected Pharmaceuticals in Aquatic Environment, Environmental Sciences.

Zeng, C., Ji, Y., Zhou, L., Zhang, Y., Yang, X., 2012. The role of dissolved organic matters in the aquatic photodegradation of atenolol. J. Hazard. Mater. 239–240, 340–347. https://doi.org/10.1016/j.jhazmat.2012.09.005

Zhang, W., Zhang, M., Lin, K., Sun, W., Xiong, B., Guo, M., Cui, X., Fu, R., 2012. Eco-toxicological effect of Carbamazepine on Scenedesmus obliquus and Chlorella pyrenoidosa. Environ. Toxicol. Pharmacol. 33, 344–352. https://doi.org/10.1016/j.etap.2011.12.024

Zhang, Yonggang, Guo, J., Yao, T., Zhang, Yalei, Zhou, X., Chu, H., 2019. The influence of four pharmaceuticals on Chlorellapyrenoidosa culture. Sci. Rep. 9:1624, 1–10. https://doi.org/10.1038/s41598-018-36609-4

Zhao, J., Wang, Z., Ghosh, S., Xing, B., 2014. Phenanthrene binding by humic acid-protein complexes as studied by passive dosing technique. Environ. Pollut. 184, 145–153. https://doi.org/10.1016/j.envpol.2013.08.028

Zhou, J., Deng, Y., Zhang, P., Xue, K., Liang, Y., Van Nostrand, J.D., Yang, Y., He, Z., Wu, L., Stahl, D.A., Hazen, T, C., Tiedje, J.M., Arkin, A.P., 2014. Stochasticity, succession, and environmental perturbations in a fluidic ecosystem. PNAS 836–845. https://doi.org/10.1073/pnas.1324044111

Zhu, D., Pignatello, J., 2005. Characterizatiion of aromatic compound sorptive interactions with black carbon (charcoal) assisted by graphite as a model. Environ. Sci. Technol 35.

Zunino, H., Aguilera, M., Caiozzi, M., Peirano, P., Borde, F., Martin, J.P., 1979. Metalbinding organic macromolecules in soil, 3. Competition of Mg (II) and Zn(II) for binding sites in humic and fulvic-type model polymers. Soil Sci. 128, 257–266.

"…L'amor che move il sole e l'altre stelle.".

Lancaster Environment Centre

Lancaster University

Responses of freshwater phytoplankton exposed to chemical contaminants: tolerance acquisition, physiological trade-offs and environmental controls

(Appendices and Supplementary Material)

Simone Rizzuto, MSc

Submitted for the degree of

Doctor of Philosophy in Environmental Science

November 2021

Table of Contents – Appendices and Supplementary Material

Supplementary Information Chapter II: Water browning controls adaptation an associated trade-offs in phytoplankton stressed by chemical pollution.

Simone Rizzuto[1], Jan-Erik Thrane[2], Didier L. Baho[2,3], Kevin C. Jones[2], Hao Zhang[2], Dag Hessen[4,5], Luca Nizzetto[*2,6], Eva Leu[7]

[1] Lancaster Environment Centre, Lancaster University, Lancaster, LA1 4YQ, UK.

[2] Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, 0349, Oslo, Norway.

[3] Department of Aquatic Sciences and Assessment, Swedish University of Agricultural Sciences, P.O. Box 7050, SE-750-07 Uppsala, Sweden.

[4] Section for Aquatic Biology and Ecotoxicology, University of Oslo, Post box 1066, Blindern, 0316 Oslo, Norway.

[5] Center of Biogeochemistry in the Anthropocene (CAB), University of Oslo, Post box 1066, Blindern, 0316 Oslo, Norway.

[6] RECETOX, Masaryk University, Kamenice 753/5, 625 00 Brno, Czech Republic.

[7] Akvaplan-niva, CIENS, Science Park, Gaustadalléen 21, 0349, Oslo, Norway.

* Corresponding author. Email: luca.nizzetto@niva.no

List of Tables

List of Figures

List of Text:

Table S2.1. Summary data on the occurrence and concentration (ng/L) of PPCPs used in this study found in European freshwaters (lakes and rivers). The data was obtained from the Norman database. Norman is the Network of reference laboratories, research centres and related organisations for monitoring of emerging environmental substances (www.Norman-network.net). This table was modified from the paper published by Pomati et al. (2017) and Baho et al. (2019).

Chemical	Time analyzed	Times detected	Percentage detection (%)	Min conc. (ng/L)	Max conc. (ng/L)	Mean conc. (ng/L)	standard deviation (ng/L)	Q1 conc. (ng/L)	Median conc. (ng/L)	Q3 conc. (ng/L)
Atenolol	977	723	74	0.1	900	26.3	70.7	6	11	19
Bezafibrate	1384	764	55.2	0.3	21200	108.5	1162.7	8	13	28
Carbamazepine	22270	19361	86.9	0.8	7600	158.3	295.8	33	70	160
Clarithromycin	945	730	77.2	0.9	1100	21	44.7	10	13	21
Diclofenac	6320	4439	70.2	0.2	110000	785	5977.4	23	57	130
Furosemide	507	84	16.6	0.5	283000	9253.7	44732.1	12.25	35	76
Hydrochlorothiazide	484	235	48.6	4	389000	4425	36594.8	22	41	85.5
Ibuprofen	5154	3668	71.2	1.2	303000	214.5	5167.9	15	32	70
Ranitidine	50	29	58	1.3	200	33.4	55.1	2.3	5.4	40
Sulfamethoxazole	2616	2133	81.5	0.7	700	33.3	46	12	20	40
Benzophenone-4	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Triclosan	11565	9053	78.3	1	3060	20.4	56.9	8	12	20

Table S2.2. Chemical properties (acid dissociation constant – pKa, and octanol/water partition coefficient – log Kow), spiked concentrations (µg/L), and reported effective concentrations (µg/L) inhibiting 50% of growth (EC50) in phytoplankton species for the 12 studied chemical compounds. Toxicity values were obtained from the U.S. Environmental Protection Agency ECOTOXicology Database System (2015, Version 4.0, www.epa.gov/ecotox/). This table was modified from the papers published by Baho et al. (2019) and Pomati et al. (2017).

chemical	CAS ID	mm (g mol^{-1})	pKa	Log K_{ow}	water solubility (mg L^{-1})	Spiked conc. (µg L^{-1})	Mean EC50 (µg L^{-1})	SD (µg L^{-1})	Num. studies
Atenolol	29122-68-7	266.34	9	0.16	13300	22	3.18E+05	2.63E+05	3
Bezafibrate	42859-67-0	361.82	3.83	4.25	1.55	2.2	3.50E+04	2.63E+03	3
Carbamazepine	298-46-4	236.27	13.9	2.45	18	22	1.37E+05	2.83E+05	24
Clarithromycin	81103-11-9	747.96	8.99	3.16	1.63	22	1.97E+01	2.33E+01	3
Diclofenac	15307-86-5	296.14	4.15	4.51	2.37	22	6.27E+04	6.73E+04	6
Furosemide	54-31-5	330.74	4.25	2.03	73.1	2.2	> 7.000E+04	NA	1
Hydrochlorothiazide	58-93-5	297.73	7.9	-0.07	722	22	NA	NA	NA
Ibuprofen	15867-27-1	206.28	4.91	3.97	21	22	3.29E+05	1.92E+04	2
Ranitidine	66357-35-5	314.4	7.8	0.08	660000	2.2	2.70E+04	4.87E+04	2
Sulfamethoxazole	723-46-6	253.28	1.6/5.7	0.89	610	2.2	2.15E+03	3.10E+03	7
Benzophenone-4	4065-45-6	308.3	7.6	0.37	249.7	22	1.00E+04	NA	1
Triclosan	3380-34-5	289.53	7.9	4.76	10	2.2	5.86E+02	7.82E+02	24

Table S2.3. Growth inhibition test of *Chlamydomonas reinhardtii* exposed to the mix of PPCPs. The exposure levels used in our study were based on a preliminary test conducted on *C. reinhardtii* following the OECD guidelines (OECD, 2007). Eight levels of exposure were applied following a factorial increase (0, 1, 3, 10, 30, 100, 300, 1000). The concentrations used in this study were the one from level 5 (in bold), causing 28.6% growth inhibition.

	Concentrations (µg/L)							
Chemical	**Ctrl**	**L1**	**L2**	**L3**	**L4**	**L5**	**L6**	**L7**
Atenolol	0	0.22	0.66	2.2	6.6	**22**	66	220
Bezafibrate	0	0.022	0.066	0.22	0.66	**2.2**	6.6	22
Carbamazepine	0	0.22	0.66	2.2	6.6	**22**	66	220
Clarithromycin	0	0.22	0.66	2.2	6.6	**22**	66	220
Diclofenac	0	0.22	0.66	2.2	6.6	**22**	66	220
Furosemide	0	0.022	0.066	0.22	0.66	**2.2**	6.6	22
Hydrochlorothiazide	0	0.22	0.66	2.2	6.6	**22**	66	220
Ibuprofen	0	0.22	0.66	2.2	6.6	**22**	66	220
Ranitidine	0	0.022	0.066	0.22	0.66	**2.2**	6.6	22
Sulfamethoxazole	0	0.022	0.066	0.22	0.66	**2.2**	6.6	22
Benzophenone-3	0	0.22	0.66	2.2	6.6	**22**	66	220
Triclosan	0	0.022	0.066	0.22	0.66	**2.2**	6.6	22
n	6	3	3	3	3	**3**	3	3
mean growth rate µ (d^{-1})	1.62	1.79	1.61	1.64	1.66	**1.16**	0.90	0.55
SD	0.05	0.05	0.05	0.09	0.02	**0.01**	0.06	0.01
% growth inhibition		-10.7	0.8	-1.6	-2.8	**28.6**	44.4	65.7

Text S2.2. PPCPs stability test

In order to check for degradation of the mix of PPCPs, the experimental units exposed to the contaminants were sampled during both phases of the experiment as follows. 1 mL samples were collected in triplicates, stored in 2 mL GC amber glass vials at -20°C in the dark. The compounds were extracted through SPE extraction using HLB cartridges (Oasis) in 5 mL of MeOH. The extract was blown down to dryness with a gentle N^2 flow, reconstituted in 1 mL MeOH, and filtered through 0.2 µm PP syringes filters (Pall, UK) into a 2 mL GC vial. The samples were analysed by HPLC-MS (Shimadzu, 8040), using an XBridge BEH C18 column (2.1 mm x 100 mm, 3.5 µm) to separate the compounds. The mobile phases were A, 0.2% Ammonium hydroxide in MQ water, and B, 50% Methanol and Acetonitrile. The gradient procedure was optimized at: 0-1 min 20% B, then increased to 100% within 8 min, held at 100% for 5 min, after that decreased to the initial conditions (20% B) within 1 min. Finally, 6 minutes of post-run ensured re-equilibration of the column before the next injection. The injection volume was 15 µL and the column and the tray temperature were set to 35°C. The quantification of the compounds was based on internal standard method (Atenolol d7 and Ibuprofen d3, Sigma Aldrich), and the instrument detection limit was 3.87 ng/mL.

Table S2.4. Percentage of recovery (± standard deviation) of the mix of PPCPs at different levels of DOM and pH at the end of phase I and phase II.

	Chemical	Spiked conc. (ng/L)	Recovery DOC 0 (% ± sd)	Recovery DOC 5 (% ± sd)	Recovery DOC 15 (% ± sd)	
phase I	Atenolol	22	100 ± 0.3	94.3 ± 6.3	99.4 ± 0.1	pH 6.5
	Bezafibrate	2.2	99.3 ± 2.2	100.3 ± 1.0	97.6 ± 1.0	
	Carbamazepine	22	102.9 ± 1.2	101.2 ± 2.3	104.3 ± 3.3	
	Clarithromycin	22	98.2 ± 1.9	104.3 ± 2.3	105.2 ± 4.2	
	Diclofenac	22	99.4 ± 2.1	102.2 ± 2.0	100.3 ± 1.1	
	Furosemide	2.22	96.4 ± 4.1	99.3 ± 2.4	98.2 ± 2.4	
	Hydrochlorothiazide	22	103.7 ± 1.3	98.4 ± 2.5	98.8± 6.7	
	Ibuprofen	22	100.3 ± 0.3	99.4 ± 4.1	96.8 ± 7.4	
	Ranitidine	2.2	99.4 ± 2.8	98.7 ± 4.4	102.3 ± 2.3	
	Sulfamethoxazole	2.2	97.3 ± 2.1	95.6 ± 6.3	104.2 ± 4.0	
	Benzophenone-4	22	104.4 ± 3.4	99.2 ± 4.4	105.3 ± 4.0	
	Triclosan	2.2	99.2 ± 2.1	104.3 ± 5.4	99.7 ± 1.0	
	Atenolol	22	99.3 ± 0.9	100.4 ± 0.8	100.9 ± 1.0	pH 8
	Bezafibrate	2.2	102.4 ± 1.0	102.9 ± 3.2	101.7 ± 0.4	
	Carbamazepine	22	100.2 ± 2.0	98.2 ± 2.2	100.3 ± 0.8	
	Clarithromycin	22	103.3 ± 0.4	99.2 ± 4.2	105.3 ± 5.7	
	Diclofenac	22	98.4 ± 2.4	101.0 ± 1.2	104.5 ± 0.4	
	Furosemide	2.22	99.7 ± 2.4	104.2 ± 5.0	101.5 ± 6.3	
	Hydrochlorothiazide	22	95.4 ± 5.2	100.4 ± 1.0	98.5 ± 3.7	
	Ibuprofen	22	100.9 ± 1.3	99.8 ± 1.4	100.0 ± 1.2	
	Ranitidine	2.2	102.5 ± 3.3	98.9 ± 0.2	100.2 ± 3.2	
	Sulfamethoxazole	2.2	101.0 ± 3.0	96.2 ± 5.0	104.7 ± 7.0	
	Benzophenone-4	22	97.6 ± 2.2	102.8 ± 2.0	98.5 ± 0.3	
	Triclosan	2.2	96.6 ± 4.4	101.2 ± 0.2	99.3 ± 3.3	
phase II	Atenolol	22	99.7 ± 2.8	104.3 ± 6.4	100.0 ± 1.0	pH 8
	Bezafibrate	2.2	104.2 ± 1.5	102.3 ± 2.6	100.2 ± 1.0	
	Carbamazepine	22	100.3 ± 2.2	103.2 ± 3.7	102.0 ± 2.4	
	Clarithromycin	22	97.8 ± 2.5	99.8 ± 1.1	102.4 ± 1.0	
	Diclofenac	22	98.3 ± 1.1	98.8 ± 2.0	101.3 ± 0.2	
	Furosemide	2.22	99.1 ± 1.4	97.3 ± 4.0	99.2 ± 0.2	
	Hydrochlorothiazide	22	102.2 ± 2.7	98.6 ± 2.1	100.8 ± 1.0	
	Ibuprofen	22	101.3 ± 3.5	100.2 ± 2.4	101.2 ± 1.0	
	Ranitidine	2.2	98.8 ± 4.4	101.2 ± 2.3	99.6 ± 2.0	
	Sulfamethoxazole	2.2	102.4 ± 0.3	101.0 ± 2.1	99.3 ± 3.2	
	Benzophenone-4	22	101.0 ± 1.1	95.6 ± 4.2	96.6 ± 3.1	
	Triclosan	2.2	97.1 ± 3.0	99.0 ± 1.5	100.2 ± 2.0	

Table S2.5. Pairwise comparison post-hoc Tukey test on the gap between the growth rate μ (d^{-1}) in the absence/presence of the PPCPs in phase II in the non-adapted population, and in the population adapted to PPCPs at different levels of DOM. Significant values are reported in bold.

Population	DOC (mg L^{-1})	contrast PPCPs	estimate	df	t ratio	*p*
non-adapted	0	(-) vs (+)	**1.05**	**12**	**7.38**	**< 0.001**
adapted	0		**0.28**	**18**	**2.39**	**0.03**
	5		**0.39**	**18**	**3.35**	**0.04**
	15		**0.55**	**18**	**4.71**	**< 0.001**

Table S2.6. Pairwise comparison post-hoc Tukey test between the populations adapted in presence of PPCPs at different levels of DOC, in the absence/presence of PPCPs in phase II. In the table are reported the growth rate μ (d^{-1}), cell size (μm^3) and recruitment rate μ (d^{-1}). Significant values are reported in bold.

Variable	PPCPs	contrast (DOC levels)	estimate	df	t ratio	*p*
growth rate μ (d^{-1})	**(-)**	0-5	-0.0125	18	-0.107	0.994
		0-15	-0.1	18	-0.853	0.675
	(+)	0-5	0.1	18	0.853	0.675
		0-15	0.172	18	1.472	0.327
cell size (μm^3)	**(-)**	**0-5**	**-0.174**	**18**	**-3.451**	**< 0.05**
		0-15	0.038	18	0.755	0.735
	(+)	**0-5**	**0.266**	**18**	**5.264**	**< 0.001**
		0-15	0.018	18	0.359	0.932
recruitment rate μ (d^{-1})	**(-)**	0-5	0.022	18	0.257	0.964
		0-15	-0.003	18	-0.04	0.999
	(+)	0-5	0.126	18	1.496	0.316
		0-15	**0.267**	**18**	**3.168**	**0.014**

Table S2.7. Pairwise comparison post-hoc Tukey test between the populations adapted in presence of PPCPs at different levels of DOM (mg L^{-1} DOC) and the non-adapted population, in the absence/presence of PPCPs in phase II. In the table are reported growth rate, cell size and recruitment rate. Significant values are reported in bold.

Variable	Contrast	PPCPS	df	estimated mean difference	t ratio	*p*
growth rate μ (d^{-1})	adapted with 0 mg L^{-1} DOC vs. Non-adapted	(+)	**12**	**0.51**	**3.53**	**<0.05**
		(-)	**12**	**-0.27**	**-1.88**	**<0.05**
cell size (μm^3)		(+)	**12**	**0.35**	**8.7**	**<0.001**
		(-)	**12**	**-0.19**	**-4.79**	**<0.001**
recruitment rate μ (d^{-1})		(+)	**12**	**0.19**	**2.08**	**<0.05**
		(-)	**12**	**-0.25**	**-2.63**	**<0.05**
Variable	**Contrast**	**PPCPS**	**df**	**estimated mean difference**	**t ratio**	***p***
growth rate μ (d^{-1})	adapted with 5 mg L^{-1} DOC vs. Non-adapted	(+)	**18**	**0.4**	**2.99**	**<0.05**
		(-)	18	-0.25	-1.89	0.08
cell size (μm^3)		(+)	**18**	**0.09**	**0.69**	**<0.01**
		(-)	18	-0.02	-2.81	0.5
recruitment rate μ (d^{-1})		(+)	18	0.06	1.43	0.18
		(-)	**18**	**-0.27**	**-5.52**	**<0.001**
Variable	**Contrast**	**PPCPS**	**df**	**estimated mean difference**	**t ratio**	***p***
growth rate μ (d^{-1})	adapted with 15 mg L^{-1} DOC vs. Non-adapted	(+)	**18**	**0.33**	**2.39**	**<0.05**
		(-)	18	-0.17	-1.21	0.24
cell size (μm^3)		(+)	**18**	**0.33**	**7.81**	**<0.001**
		(-)	**18**	**0.23**	**-5.42**	**<0.001**
recruitment rate μ (d^{-1})		(+)	18	-0.07	1.06	0.3
		(-)	**18**	**-0.24**	**-3.62**	**<0.05**

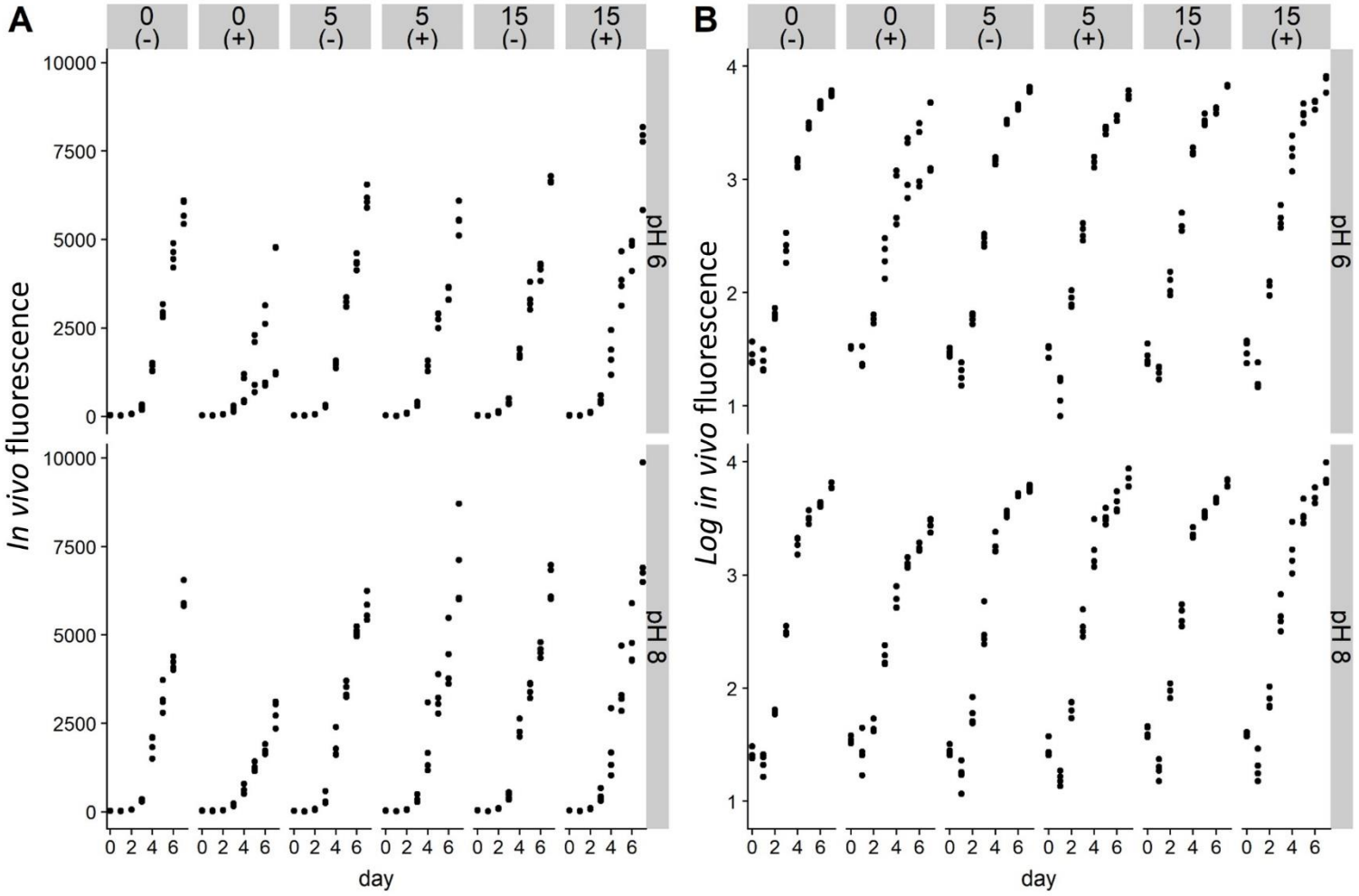


Figure S2.1. (A) Daily biomass development measured as the in vivo fluorescence and (B) log in vivo fluorescence data of the phytoplankton population under different DOM (DOC 0, 5, 15 mg L^{-1}) and pH levels (6.5, 8), in the absence (-) and the presence (+) of PPCPs, during phase I.

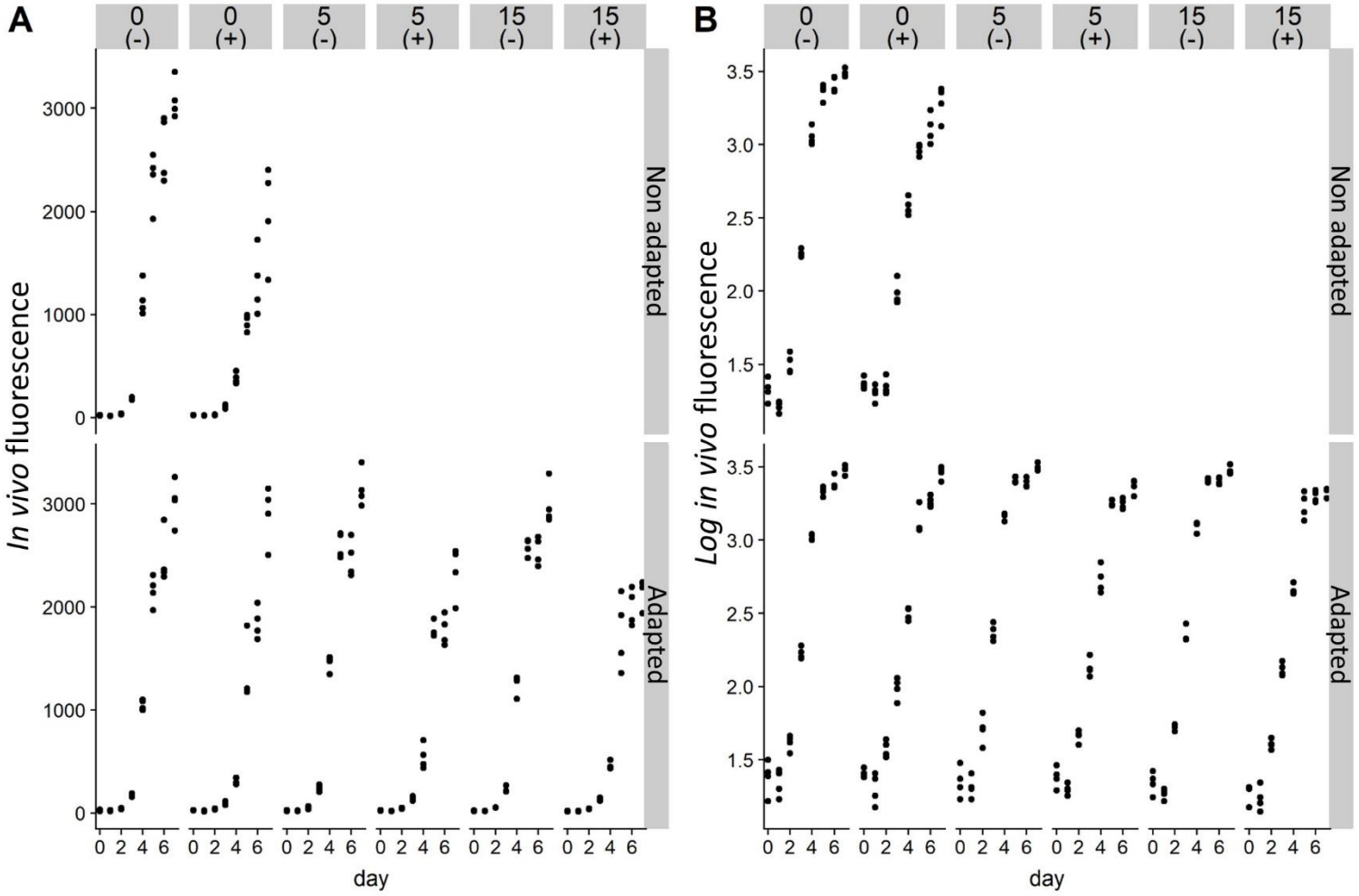


Figure S2.2. (A) Daily biomass development measured as the in vivo fluorescence and (B) log in vivo fluorescence data of the phytoplankton populations under different DOM levels (DOC 0, 5, 15 mg L^{-1}), in the absence (-) and the presence (+) of PPCPs, in the non-adapted and adapted populations during phase II.

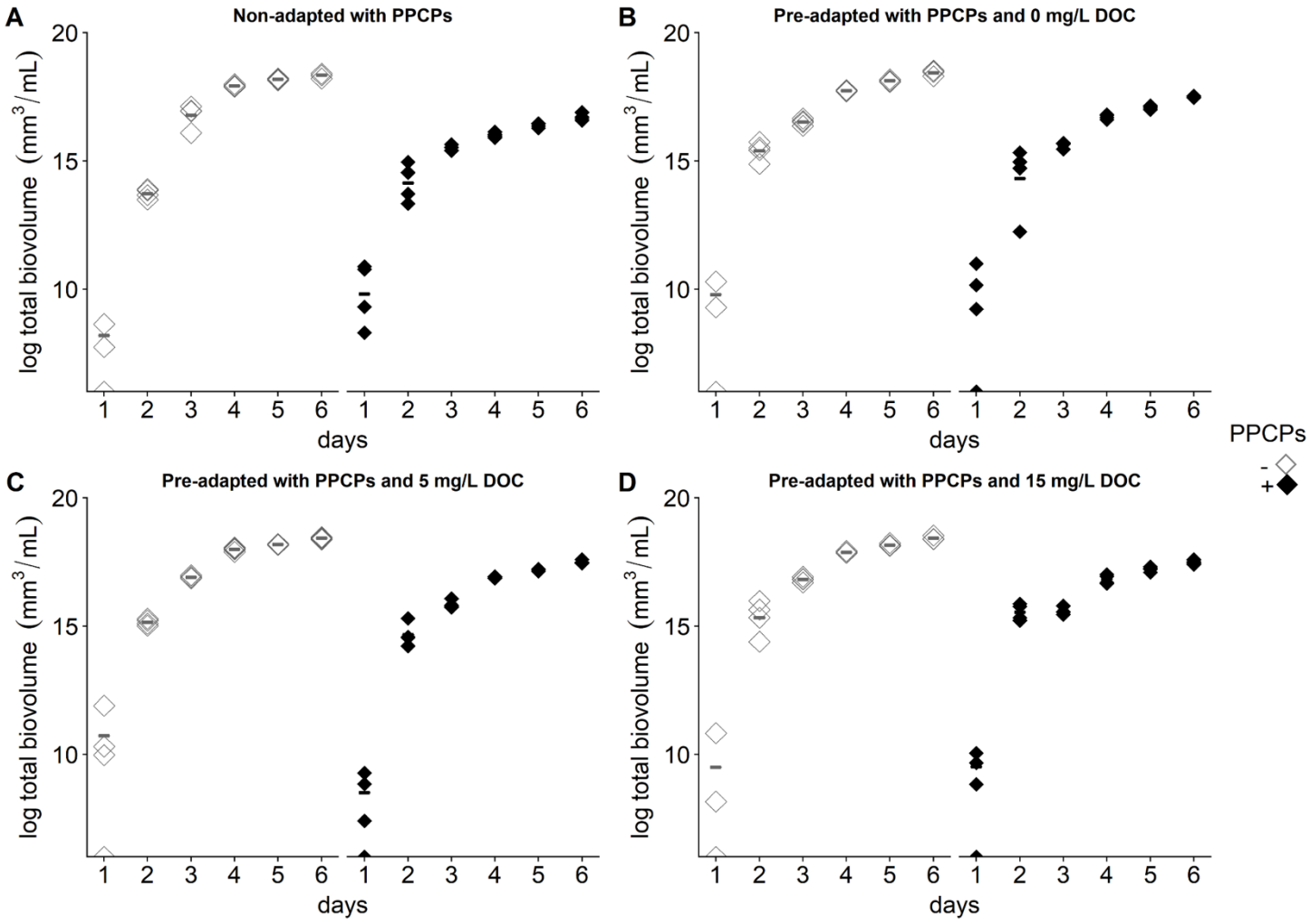


Figure S2.3. Log daily biovolume development (mm3/mL) of C. reinhardtii in the non-adapted (A), and adapted to PPCPs at 0 mg L^{-1} DOC (B), 5 mg L^{-1} DOC (C) and 15 mg L^{-1} DOC (D), in the absence (-) and the presence (+) of PPCPs during phase II. Short horizontal bars represent the mean of each group.

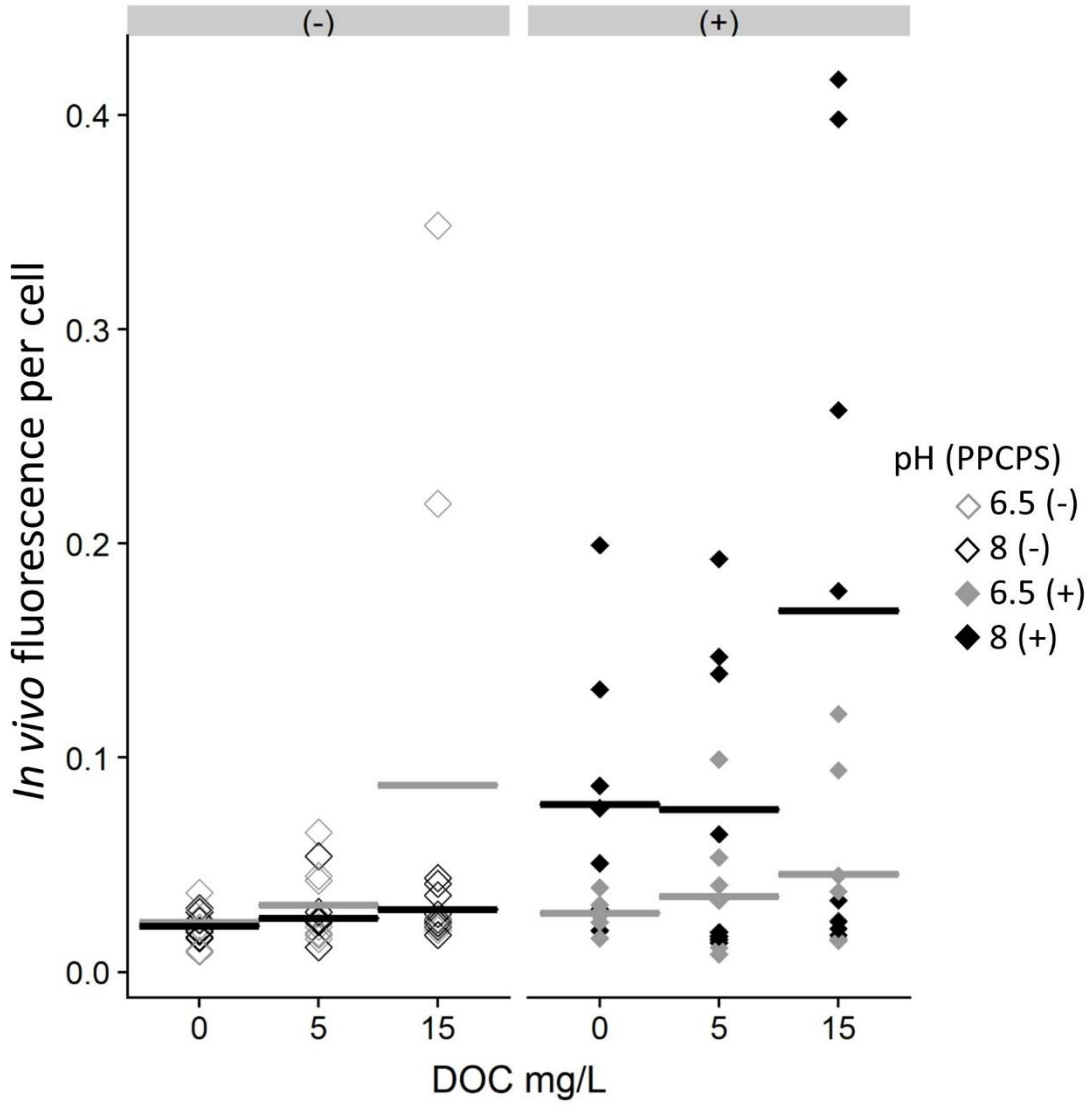
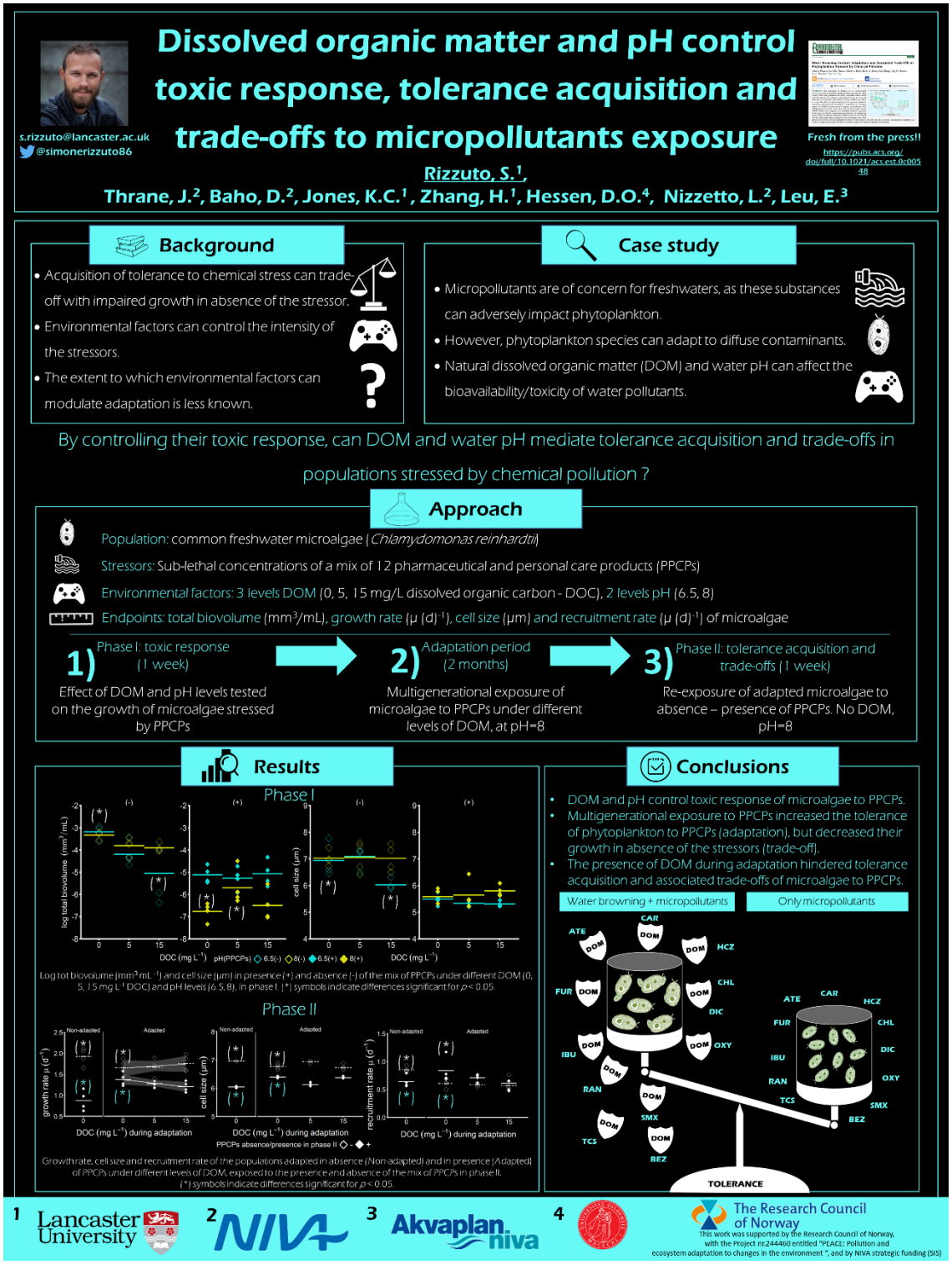


Figure S2.4. In vivo fluorescence per unit of biomass data of C. reinhardtii under different DOM (0, 5, 15 mg L^{-1} DOC) and pH (6.5, 8) levels, in the absence (-) and the presence (+) of PPCPs, during phase I.

Appendix Chapter II: Dissolved Organic Matter and pH control toxic response, tolerance acquisition and fitness trade-offs to micropollutants exposure.

Poster presentation at SETAC SciCon 2020



Supplementary Information Chapter III: Ecological Memory of historical contamination influences the response of phytoplankton communities.

Didier L. Baho[a,b*], Simone Rizzuto[c], Luca Nizzetto[a,d*], Dag O. Hessen[e], Jon Norberg[f], Birger Skjelbred[a], Kevin C. Jones[c], Hao Zhang[c] and Eva Leu[g]

[a] Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, 0349, Oslo, Norway

[b] Department of Aquatic Sciences and Assessment, Swedish University of Agricultural Sciences, Uppsala, Sweden

[c] Lancaster University, Lancaster Environment Centre, Lancaster, United Kingdom

[d] RECETOX, Masarik University, Kamenice 753/5, 625 00 Brno, Czech Republic

[e] University of Oslo, Dept. Biosciences, Box 1066 Blindern, 0316, Oslo, Norway

[f] Stockholm Resilience Centre, Stockholm University, Kräftriket 2B, SE-10691, Stockholm, Sweden

[g] Akvaplan-niva, CIENS, Science Park, Gaustadalléen 21, 0349, Oslo, Norway

* Corresponding authors. Email: didier.baho@slu.se, luca.nizzetto@niva.no

List of Tables:

List of Figures:

List of Text:

Text S3.1. Selection of study sites and their characterization

The lakes were selected using information from the Swedish monitoring programs on inland freshwater ecosystems. These data are freely accessible from the host webpage: https://www.slu.se/institutioner/vatten-miljo/datavardskap/. Both lakes shared similar physical-chemical characteristics including water depth (ca. 1m depth), pH (ca. 7.0), similar dominance of submerged aquatic macrophytes (*Myriophyllum* genus), and total phosphorus above 25 µg/L (eutrophic condition). The lakes differed mainly in their contamination history. Lake Finnsjön is a near-pristine lake located in a forested catchment in Uppland (Fig. S1). Lake Tårkern (Fig. S1) is located in the County of Östergötland and has an been exposed to many pesticides (Boström and others 2016) including Isoproturon (Table S1) for a relatively long time (decades). The herbicide (Isoproturon) was not detected in the water column of the two selected lakes when the sediments were sampled.

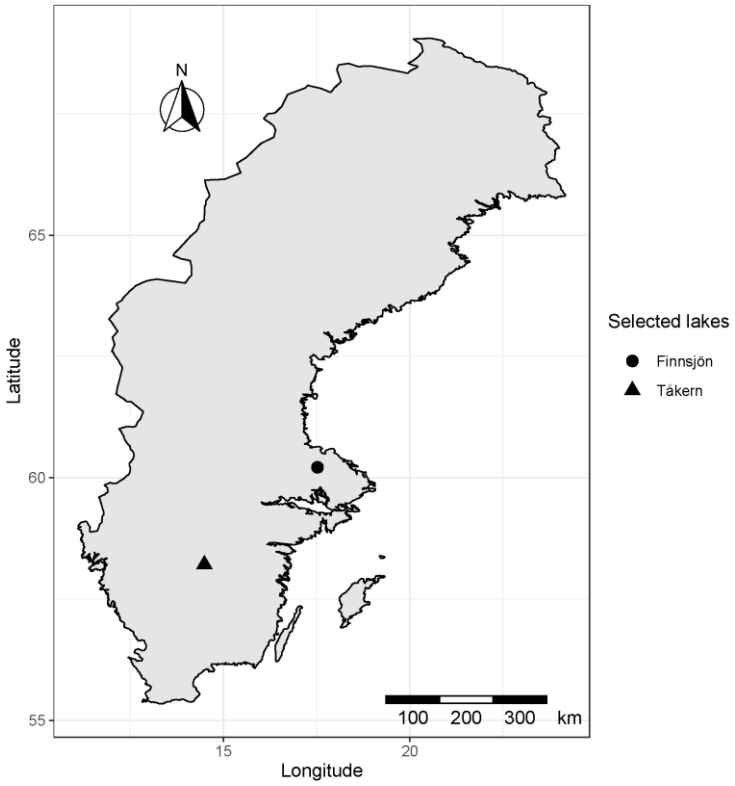


Figure S3.1. Location of the selected sampling sites: Lake Finnsjön (60°21'45.1"N, 17°52'56.1"E) is a near-pristine lake and Lake Tåkern is impacted by herbicides runoff (58°21'07.0"N, 14°49'42.7"E).

Table S3.1. List of selected herbicides (including some secondary metabolites; AMPA and Terbuthylazine) found in the catchment of Lake Tårkern, located in the County of Östergötland, between 2002–2015. The data was downloaded from the database (http://jordbruksvatten.slu.se/pesticider_start.cfm) on 25th July 2017.

Substances	Times detected	Min conc. (µg/L)	Median conc. (µg/L)	90th Percent. conc. (µg/L)	Max conc. (µg/L)
Aminomethylphosphonic acid (AMPA)	92	0.05	0.12	0.78	3
2, 6-Dichlorobenzamide (BAM)	34	0.01	0.01	0.03	0.04
Bentazon	295	0.02	0.17	0.56	21
Cyprodinil	6	0.01	0.04	0.07	0.07
Glyphosate	169	0.03	0.08	0.24	2.27
Isoproturon	119	0.002	0.01	0.35	5
Clopyralid	246	0.01	0.10	0.44	2.2
2-Methyl-4-chlorophenoxyacetic acid (MCPA)	141	0.01	0.09	2.6	28
Metribuzin	119	0.01	0.06	0.3	2.6
Terbutylazindesetyl	10	0.002	0.003	0.004	0.01

Text S3.2. **Isoproturon growth inhibition.**

The choice of the Isoproturon concentrations applied during the germination and exposure phase of the experiment was conceived to encompass the wide range of concentrations usually recorded in the field. Isoproturon runoff concentrations has been found to reach up to 60 mg/L in freshwater systems (Lecomte and others 2001). Moreover, Nitsche and Schlüsser (1998) showed that concentrations of the herbicide up to 42 µg/L were observed in rural-effluent wastewater in the spring months from April to May, which are the periods when the herbicide is applied, and runoff events are more likely to occur. For this purpose, different Isoproturon levels (L1=0.06, L2=0.12, L3=0.24, L4=0.48, L5=0.96, L6=1.92, L7=3.84, L8=7.68, L9=15.36, L10=30.72, L11=61.44 µg/L) were tested for 96 hours on the growth of a model phytoplankton species (*Pseudokirchneriella subcapitata* that has recently revised and renamed to *Raphidocelis subcapitata*), and of phytoplankton communities from both lakes. The results

from the three test cultures (*Pseudokirchneriella subcapitata*, phytoplankton communities from the two lakes obtained after germination from sediments) showed no effects from the first 7 exposure levels, whereas L8 (7.68 µg/L) caused 5-10%, L9 (15.36 µg/L) 20-25%, L10 (30.72 µg/L) 45-50% and L11 (61.44 µg/L) 70-75% growth inhibition on the model species and both the communities (Fig. S2).

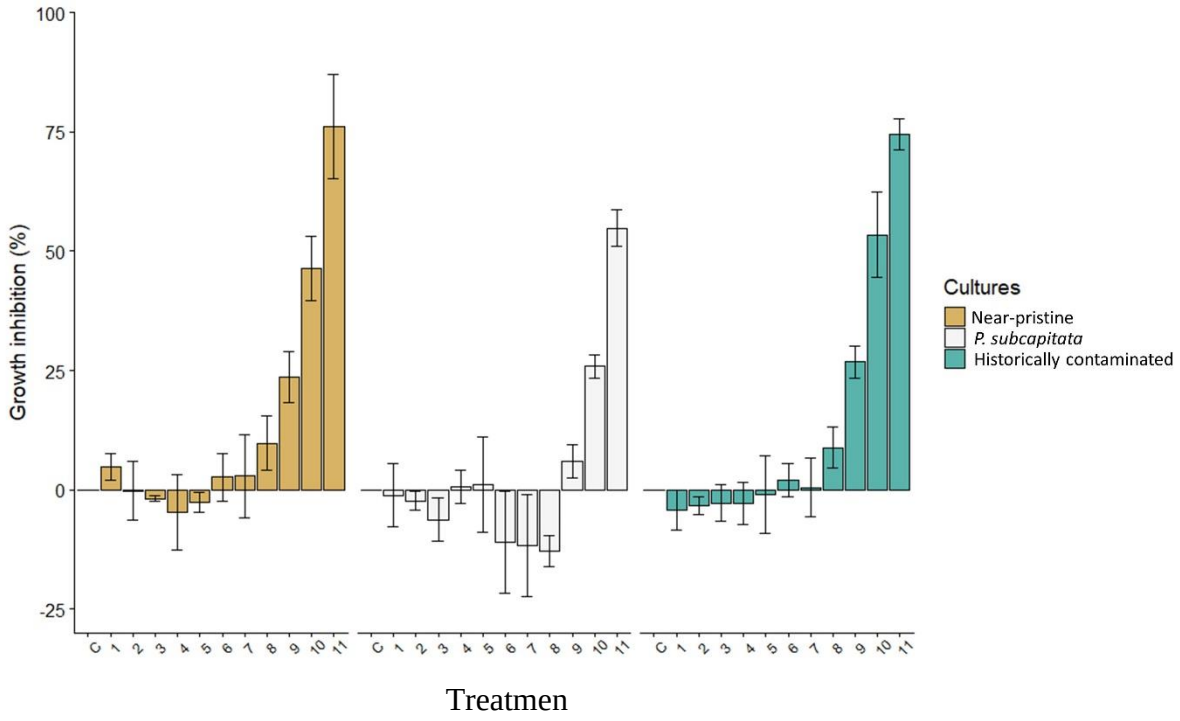


Figure S3.2. Growth inhibition (%) of the near-pristine, the historically contaminated community and the model species Pseudokirchneriella subcapitata along an increasing Isoproturon concentration that summed up to 11 exposure levels (0.06, 0.12, 0.24, 0.48, 0.96, 1.92, 3.84, 7.68, 15.36, 30.72, 61.44 µg/L) and the control.

Text S3.3. Measuring Isoproturon concentration during the experiment

In order to check for degradation of the herbicide, samples were taken during both phases of the experiment. Water samples of 20 mL were collected from the bioreactors with 12 µg/L Isoproturon from the germination phase, and from the experimental two experiment units; L1 (7 µg/L) and L3 (61 µg/L), from the exposure phase and stored in amber glass bottles at -20°C in the dark. The herbicide was extracted through SPE extraction using HLB cartridges (Oasis) in 5 mL of MeOH. The extract was dried with a gentle N_2 flow, reconstituted in 1 mL MeOH, and filtered through 0.2 µm PP syringes filters (Pall, UK) into a 2 mL GC vial. The samples were analyzed using liquid chromatography mass spectrometry (Shimadzu, 8040), using an XBridge BEH C18 column (2.1 mm x 100 mm, 3.5 µm) to separate the compounds. The mobile media used were A, 0.2% Ammonium formate in MQ water, and B, acetonitrile. The gradient procedure was optimized at: 0-1 min 20% B, then increased to 100% within 8 min, held at 100% for 5 min, after that decreased to the initial conditions (20% B) within 1 min. Finally, 6 minutes of post-run ensured re-equilibration of the column before the next injection. The injection volume was 10 µL, while the column and the tray temperature were set to 25°C. The quantification of ISU was based on internal standard method (Isoproturon-d6, Sigma Aldrich), and the instrument detection limit of 1.82 ng/mL.

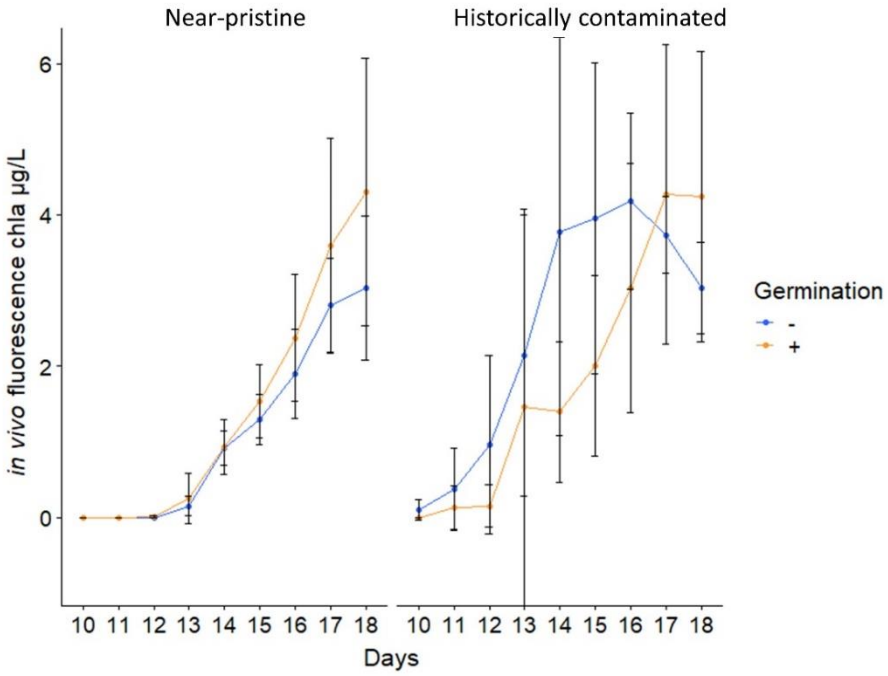


Figure S3.3. Growth of phytoplankton monitored on a daily basis during phase I (gemination and conditioning phase) measured as the in vivo fluorescence emission in the near-pristine and historically contaminated communities germinated without (-) and with (+) Isoproturon in the germination phase. Bars represent standard deviation.

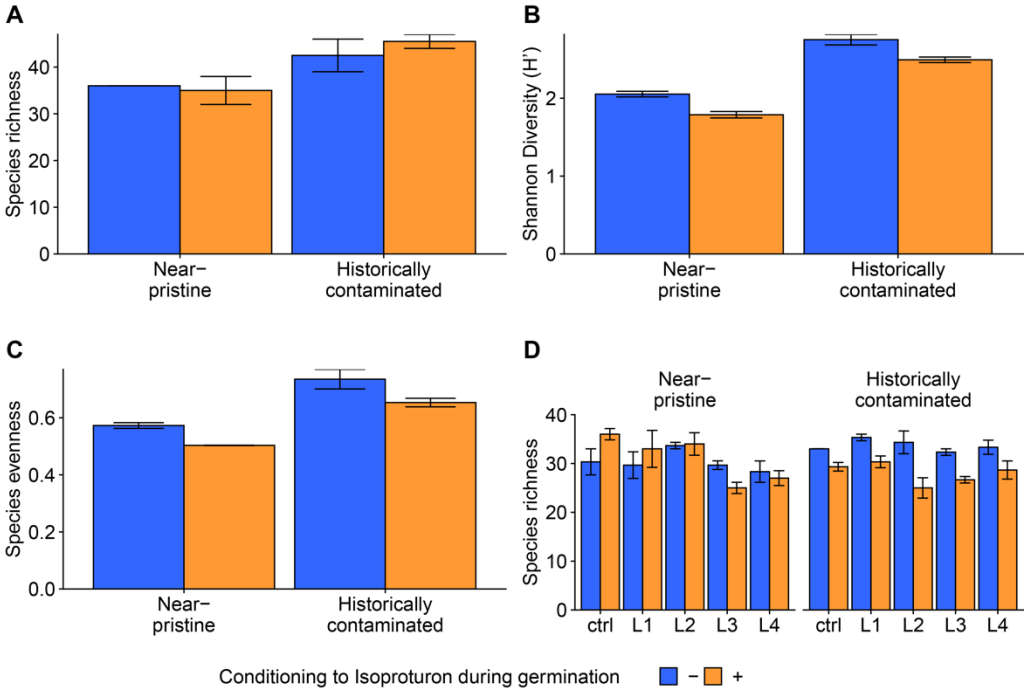


Figure S3.4. Comparison of (a) Species richness, (b) Shannon diversity and (c) evenness of the near-pristine and historically contaminated phytoplankton germinated without (-) and with (+) herbicide in the phase I. Panel (d) shows the species richness that was observed during phase II. The error bars represent standard error. Shannon diversity (b) is also presented in the main text.

Table S3.2. Summary of the effects of the contamination history, conditioning and their interaction on; growth rate, total biomass, species richness, Shannon diversity and evenness of phytoplankton recorded during phase I. Significant values are reported in bold.

Endpoints	Effects	df	SS	F	p
Growth rate	Contamination history	1, 20	0.003	0.13	0.73
	Conditioning	1, 20	0.03	1.52	0.24
	Contamination history: Conditioning	1, 20	0.0009	0.04	0.85
Total biomass	**Contamination history**	**1, 8**	**1.99**	**230.08**	**< 0.001**
	Conditioning	1, 8	0.01	1.70	0.26
	Contamination history: Conditioning	**1, 8**	**0.08**	**9.68**	**< 0.05**
Species richness	**Contamination history**	**1, 8**	**144.5**	**12.30**	**<0.05**
	Conditioning	1, 8	2.0	0.17	0.70
	Contamination history: Conditioning	1, 8	8.0	0.68	0.46
Shannon diversity	**Contamination history**	**1, 8**	**0.11**	**234.10**	**< 0.001**
	Conditioning	**1, 8**	**0.02**	**32.90**	**< 0.01**
	Contamination history: Conditioning	1, 8	0.0001	0.27	0.63
Evenness	**Contamination history**	**1, 8**	**0.05**	**65.31**	**< 0.01**
	Conditioning	**1, 8**	**0.01**	**15.35**	**< 0.05**
	Contamination history: Conditioning	1, 8	0.00008	0.11	0.76

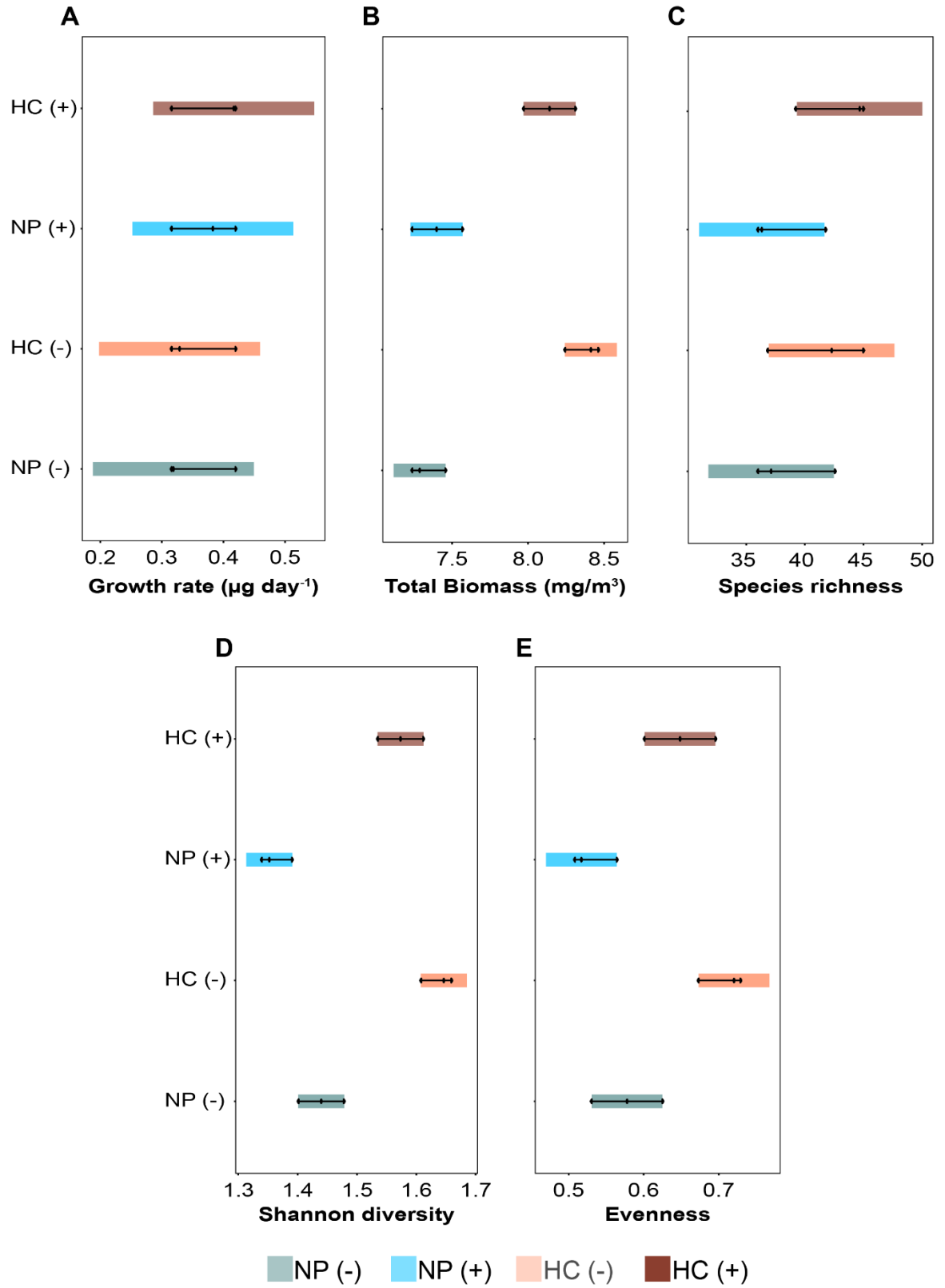


Figure S3.5. Estimated marginal means for (a) growth rate, (b) total biomass, (c) species richness, (d) Shannon diversity and (e) evenness of phytoplankton observed at the end phase I. The central points in the figure indicate the mean response with 95 % confidence interval for the combined main effects (contamination history, germination treatment) for the historically contaminated (HC) lake and near-pristine (NP) conditioned with (+) and without (-) Isoproturon during.

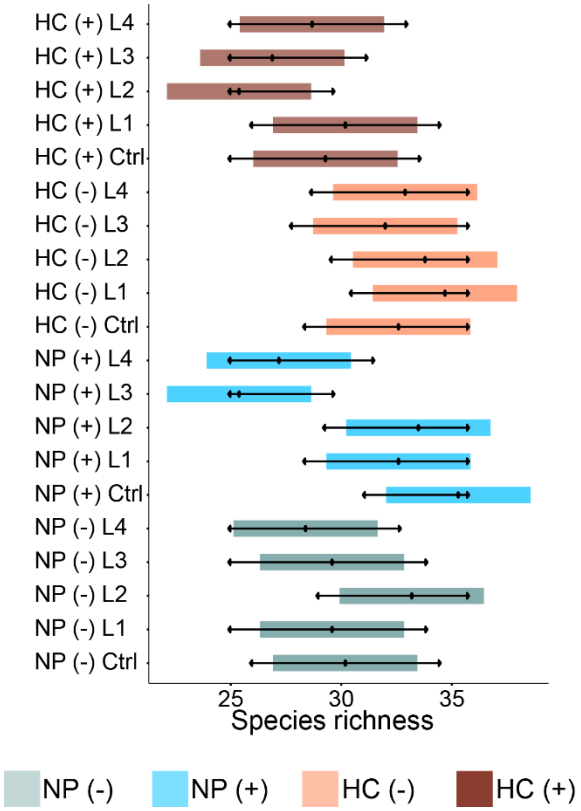


Figure S3.6. Estimated marginal means for species richness phytoplankton observed at the end phase II. The central points in the figure indicate the mean response with 95 % confidence interval for the combined main effects (contamination history, conditioning, Isoproturon exposure) for the historically contaminated (HC) lake and near-pristine (NP) conditioned with (+) and without (-) Isoproturon during germination and the five different Isoproturon exposure levels used during phase II.

Table S3.3. Summarizing the results of size Cohen's d effect size, comparing the control and four different exposure levels (L1-L4), for growth rate and total biomass of phytoplankton from phase II.

			Cohen's d	
Contamination History	**Conditioning**	**Comparisons**	**Growth rate**	**Total biomass**
Near-pristine	(-)	Ctrl vs L1	0.13	0.05
		Ctrl vs L2	2.11	0.51
		Ctrl vs L3	24.32	4.13
		Ctrl vs L4	46.73	5.50
Historically contaminated	(-)	Ctrl vs L1	0.5	0.41
		Ctrl vs L2	2.31	1.51
		Ctrl vs L3	5.37	11.52
		Ctrl vs L4	6.55	17.22
Near-pristine	(+)	Ctrl vs L1	1.12	0.81
		Ctrl vs L2	8.42	0.32
		Ctrl vs L3	30.89	7.67
		Ctrl vs L4	19.8	8.59
Historically contaminated	(+)	Ctrl vs L1	0.16	0.50
		Ctrl vs L2	0.89	0.41
		Ctrl vs L3	12.96	9.20
		Ctrl vs L4	8.97	10.57

Table S3.4. Summarizing the results of the repeated measures analysis of variance during phase II. The main effects included: herbicide exposure, germination treatment, time and the interaction terms on the photosynthetic efficiency of the phytoplankton communities across the two lakes that differed in contamination histories. Huynd-Feldt correction was applied when assumption of sphericity was breached, significant values are reported in bold.

Contamination History	Effects	df	F	p
Near-pristine	Isoproturon exposure	**4, 20**	**51.44**	**< 0.01**
	Conditioning	1, 20	1.43	0.24
	Day	**2, 40**	**30.18**	**< 0.01**
	Isoproturon exposure: Conditioning	**4, 20**	**3.23**	**0.03**
	Isoproturon exposure: Day	**8, 40**	**17.91**	**< 0.01**
	Germination treatment: Day	**2, 40**	**35.32**	**< 0.01**
	Isoproturon exposure: Conditioning: Day	8, 40	1.92	0.08
Historically contaminated	Isoproturon exposure	**4, 20**	**15.19**	**< 0.01**
	Conditioning	1, 20	0.14	0.71
	Day	**2, 40**	**119.4**	**< 0.01**
	Isoproturon exposure: Conditioning	4, 20	0.71	0.59
	Isoproturon exposure: Day	**8, 40**	**21.35**	**< 0.01**
	Germination treatment: Day	**2, 40**	**14.67**	**< 0.01**
	Isoproturon exposure: Conditioning: Day	8, 40	1.73	0.12

Table S3.5. Summary of the effects of Isoproturon exposure on the day 7 of the phase II on the photosynthetic efficiency of the phytoplankton assemblages germinated without (-) and with (+) herbicide from the sediments of the near-pristine and historically contaminated catchments. Significant values are reported in bold.

Contamination History	Conditioning	Effect	df	SS	F	p
Near-pristine	(-)	Treatment	**4, 15**	**0.01**	**15.32**	**< 0.001**
	(+)		**4, 15**	**0.02**	**74.15**	**< 0.001**
Historically contaminated	(-)		**4, 15**	**0.01**	**7.91**	**< 0.01**
	(+)		4, 15	0.01	1.96	0.17

Table S3.6. PERMANOVA test showing significant effect of the Isoproturon exposure gradient during the phase II. Shown are: df: degrees of freedom, SS: sum of squares, MS: mean of squares, F-statistic. Significant results ($p < 0.01$) are reported in bold.

Contamination History	Conditioning	df	SS	MS	F	P
Near-pristine	(-)	**4, 14**	**3537.36**	**884.34**	**8.25**	**<0.001**
	(+)	**4, 14**	**3968.25**	**992.06**	**8.75**	**<0.001**
Historically contaminated	(-)	**4, 14**	**3784.00**	**946.00**	**12.81**	**<0.001**
	(+)	**4, 14**	**3842.44**	**960.61**	**6.73**	**<0.001**

Appendix Chapter III: Freshwater phytoplankton community response across different historical contamination backgrounds.

Oral presentation at SETAC Helsinki 2019 – Winner Best Presentation Award. https://awards.setac.org/past-europe-award-winners/

Supplementary Information Chapter IV: Influence of ecological memory on phytoplankton early assemblages: a trait-based approach

Simone Rizzuto[1], Didier Baho[2], Kevin C. Jones[1], Hao Zhang[1], Francesco Pomati[3], Eva Leu[4], Luca Nizzetto[2,5]

[1] Lancaster Environment Centre, Lancaster University, Lancaster, LA1 4YQ, UK.

[2] Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, 0349, Oslo, Norway.

[3] Department of Aquatic Ecology, Eawag: Swiss Federal Institute of Aquatic Science and Technology, Überlandstrasse 133, 8600 Dübendorf, Switzerland.

[4] Akvaplan-niva, CIENS, Science Park, Gaustadalléen 21, 0349, Oslo, Norway.

[5] RECETOX, Masaryk University, Kamenice 753/5, 625 00 Brno, Czech Republic.

List of Figure:

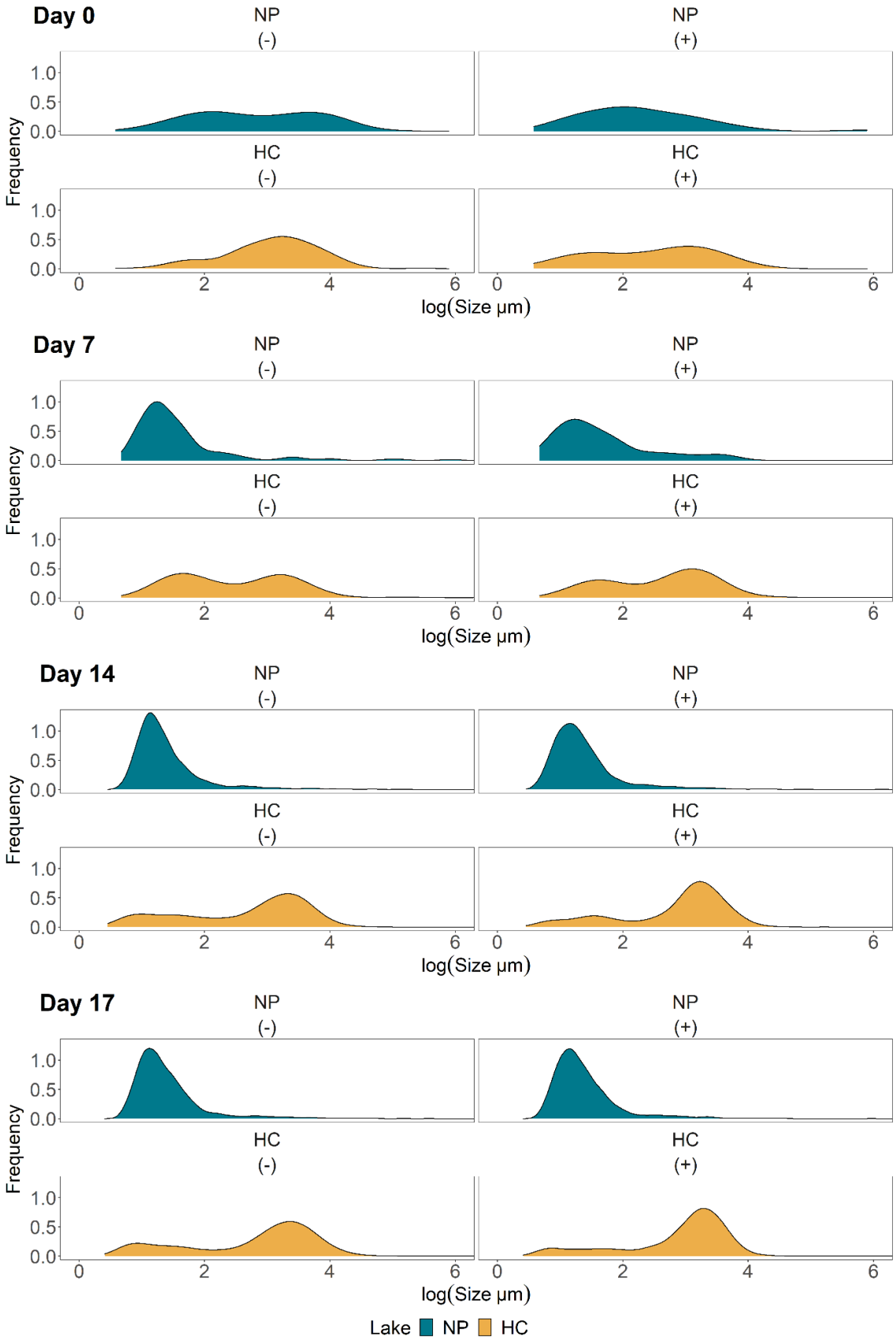


Figure S4.1. Size distribution of the phytoplankton communities from the NP and HC catchments germinates with and without herbicide.

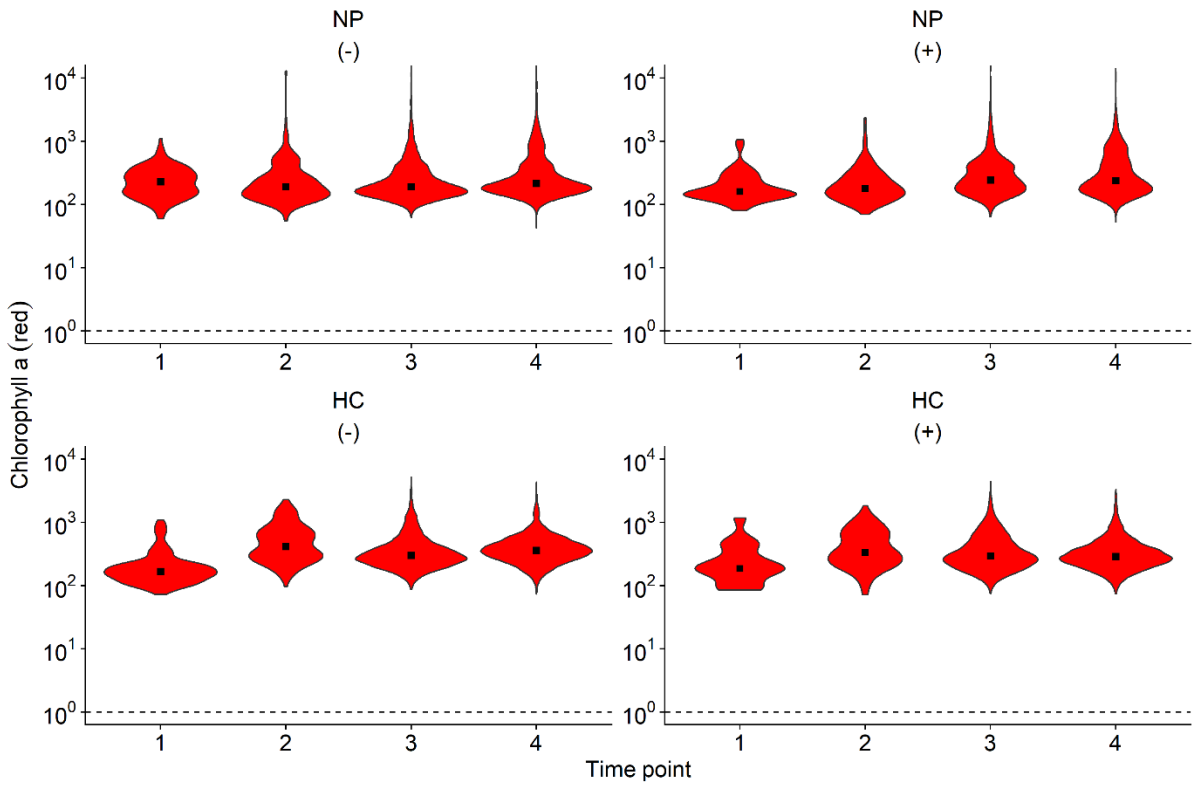


Figure S4.2. Violin plot of chlorophyll a of the phytoplankton communities from the NP and HC catchments germinates with and without herbicide.

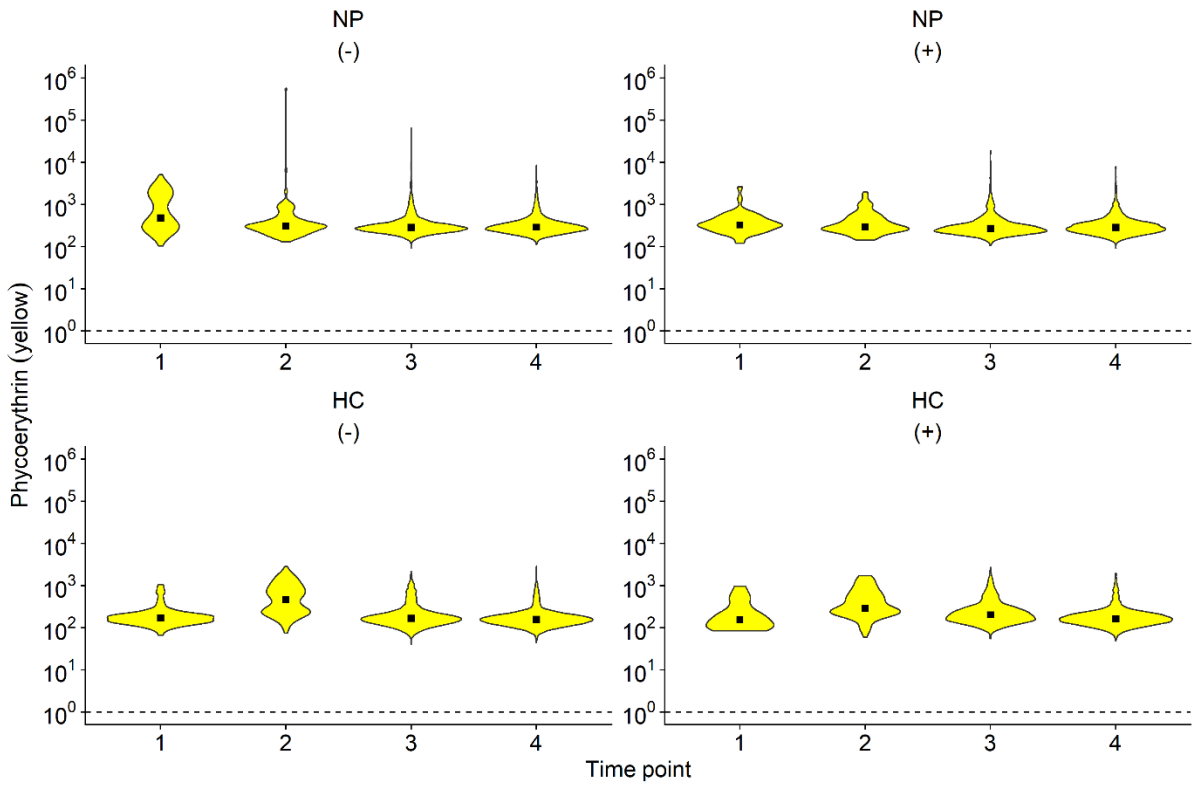


Figure S4.3. Violin plot of phycoerythrin of the phytoplankton communities from the NP and HC catchments germinates with and without herbicide.

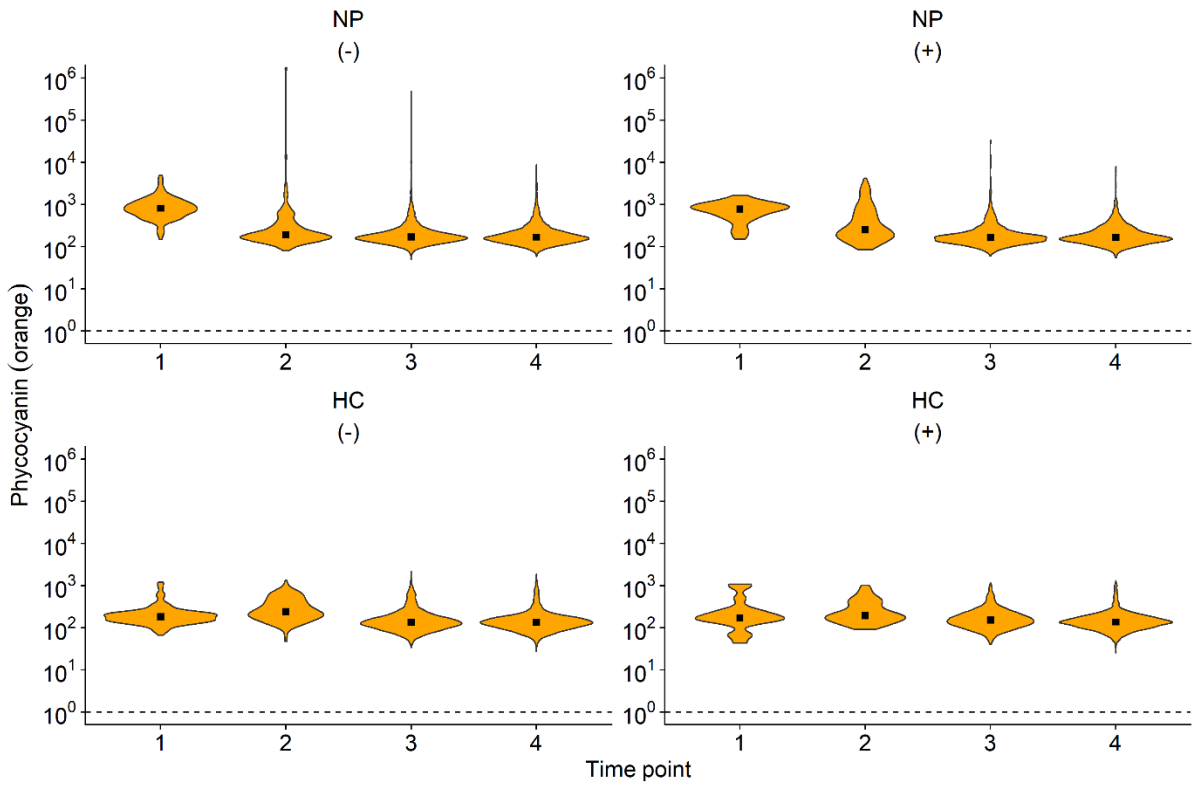


Figure S4.4. Violin plot of phycocyanin of the phytoplankton communities from the NP and HC catchments germinates with and without herbicide.

Appendix Chapter IV: Trait diversity calculation.

TOP Richness Index

The richness index TOP (Figure) was calculated as the sum of all successive areas touching all the points in the trait distribution, as reported by Fontana et al. (2016). The calculation is performed as follows: after the first minimum convex hull containing the outermost points has been built and its area has been measured, these points are deleted from the trait distribution and a second convex hull is calculated with the new outermost points (Figure A1).

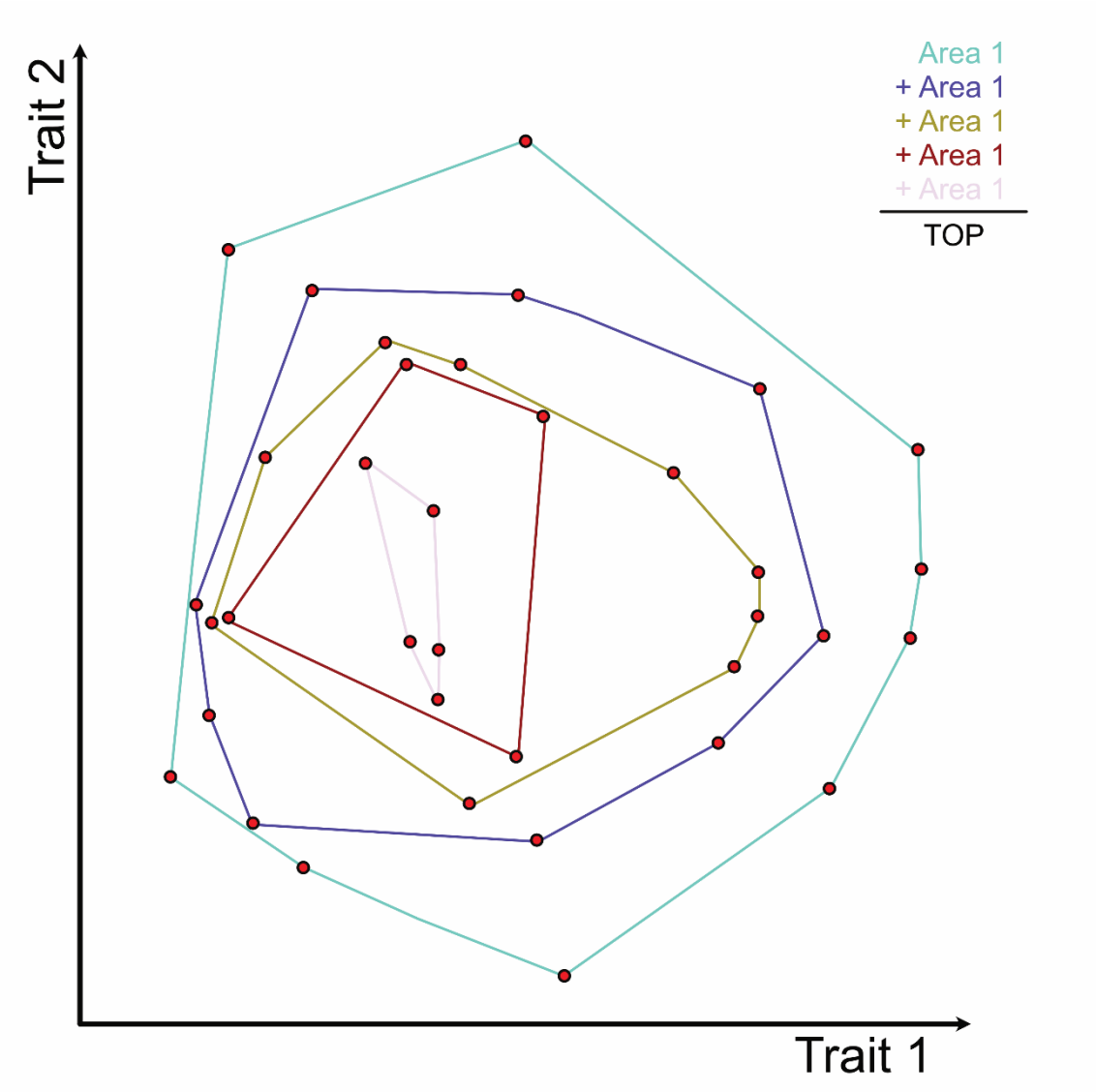


Figure A1. Simplified exemplification (2D) of the calculation of the TOP richness index, modified from Fontana et al. (2016). Red points represent an individual organism and its position is defined by

different traits (axes). The perimeters of the five convex polygons (indicated by the different colours) would represent areas of convex hulls in a multidimensional space. The TOP index of trait richness is then calculated as the sum of all areas.

This process carries on, similarly to peeling off layers of an onion, until the number of remaining points is insufficient for a convex hull (at least $n + 1$ points are needed to build a convex hull in an n-dimensional trait space). Since the number of individuals is generally much bigger than the number of traits considered, the influence of the remaining points (besides the ones accounting for the smallest area) can be considered negligible. The sum of all areas obtained this way represents the TOP index, which is more sensitive to the loss of individuals at the edges of the distribution, but also consider changes in the middle of the cloud of data points. Although TOP is conceptually similar to the convex onion-peeling approach proposed by Chazelle (1985) and Abellanas and others (1996), it has been developed independently and for a different application in a multidimensional trait space.

TED Evenness Index

The TED evenness index is a measure of how evenly distributed individuals are within the trait space (Fontana et al., 2016). It uses a reference distribution obtained starting from equidistant (evenly distributed) points in a n-dimensional space, where n is the number of traits considered. Here, a n-dimensional sphere with evenly distributed points is used as model reference (*geozoo* R-package), but it is also possible to use any n-dimensional geometric shape, provided that the same reference distribution is used for all communities for comparison. Since the number of points forming an n-dimensional sphere cannot be varied at will, the sphere with the lowest number of excess points relative to the test sample is automatically selected. Then, the most distant points from the centroid of the distribution (outermost points) are deleted, in order to obtain a cloud of evenly distributed points that is as similar as possible to a sphere and has exactly the same number of data points as in the test sample. Distance matrices among all

individual data points in the test sample and in the (even) reference distribution are calculated. The Kullback-Leibler divergence (KLdiv) (Kullback and Leibler, 1951) between the two probability distributions of distances (default settings of density functions are used in R) is inversely proportional to the evenness of the test sample. Hence, TED is calculated as:

$$1 - log_{10}(KLdiv + 1)$$

TED maximum value is therefore 1 (minimum *KLdiv* being 0). Graphical representation can be found below in Figure A2.

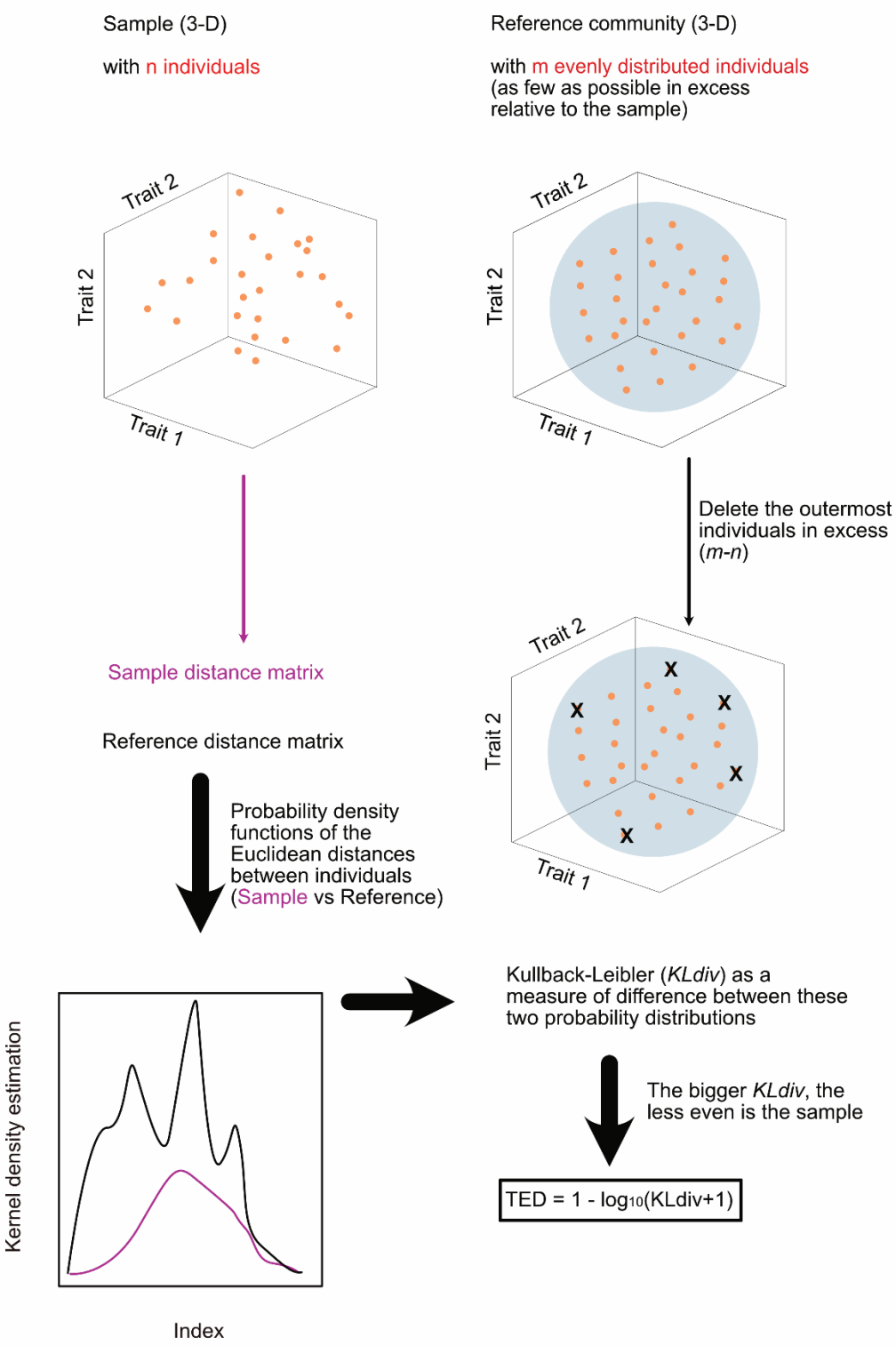


Figure A2. Representation of the steps required to calculate the TED index of trait evenness, modified from Fontana et al. (2016). The proposed example shows a community of 27 individuals distributed in a 3D trait space. Each orange point represent an individual organism, and its position is defined by different traits (axes).

FDis divergence index

According to its developers (Laliberte and Legendre, 2010), FDis represents the mean distance in a multidimensional trait space of individuals species to the centroid of all species; it accounts for species abundances by shifting the position of the centroid toward the more abundant species and weighting distances of individual species by their relative abundances. FDis is the multivariate analogue of the weighted mean absolute deviation, which makes the index unaffected by species richness.

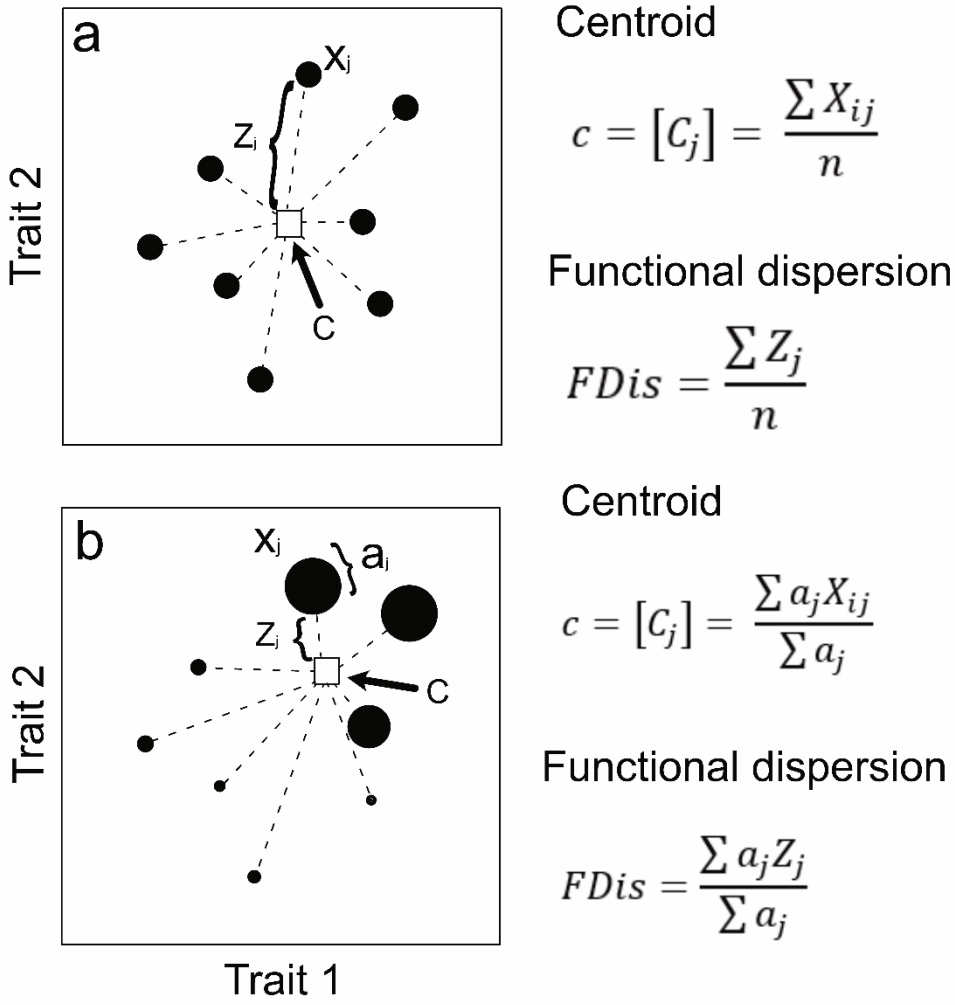


Figure A3. Example showing the computation of FDis (modified from Laliberte & Legendre, 2010).

An example of how FDis is computed is reported in Figure A3. The *n* individual species in a 2D trait space are represented by black circles whose sizes are proportional to their abundances.

Vector $\boldsymbol{X}_j$ represents the position of species j, vector c is the centroid of the n species (white square), Z_j is the distance of species *j* to the centroid **c**, and a_j is the abundance of species *j*. In panel (a), all species have equal abundances (i.e. presence – absence data). In that case, $\mathbf{c} = [c_i]$, where c_i is the mean value of trait *i*, and FDis is the mean of distances *Z* of individual species to **c**. In panel (b), species have different abundances. In that case, the position of **c** is weighted by the species relative abundances, such as it shifts towards the more abundant species. Individual distances *Z* of species to **c** are weighted by their relative abundances to compute FDis.

Supplementary Information Chapter V: Critical assessment of an equilibrium-based method to study the binding of waterborne contaminants to natural dissolved organic matter (DOM).

Simone Rizzuto [1], Kevin C. Jones [1*], Hao Zhang [1*], Didier L. Baho [2], Eva Leu [3], Luca Nizzetto [2, 4]

[1] Lancaster Environment Centre, Lancaster University, Lancaster, LA1 4YQ, UK.

[2] Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, 0349, Oslo, Norway.

[3] Akvaplan-niva, CIENS, Science Park, Gaustadalléen 21, 0349, Oslo, Norway.

[4] RECETOX, Masaryk University, Kamenice 753/5, 625 00 Brno, Czech Republic.

List of Tables:

List of Figures:

List of Text:

Text S5.1. Ecotoxicological test

Different Isoproturon levels (L1=0.06, L2=0.12, L3=0.24, L4=0.48, L5=0.96, L6=1.92, L7=3.84, L8=7.68, L9=15.36, L10=30.72, L11=61.44 µg/L) were tested for 96 hours on the growth of a model phytoplankton species (*Pseudokirchneriella subcapitata*), following a standard eco-toxicological test based on the OECD guidelines of 2009. The results showed no effects from the first 7 exposure levels, whereas L8 (7.68 µg/L) caused 5-10%, L9 (15.36 µg/L) 20-25%, L10 (30.72 µg/L) 45-50% and L11 (61.44 µg/L) 70-75% growth inhibition on the model species and both the communities (Figure S5.2). Therefore, a sub-lethal concentration of 9.5 µg L^{-1} was selected as final concentration.

Text S5.2. Mass recovery

The mass recovery was calculated to account for adsorption, volatilization or degradation of the compounds, as the difference in percentage between the mass expected and the mass detected in each experimental compartment.

$$Mass\ recovery\ (\%) = \left[\frac{Mass_{measured} * 100}{Mass_{expected}}\right] - 100$$

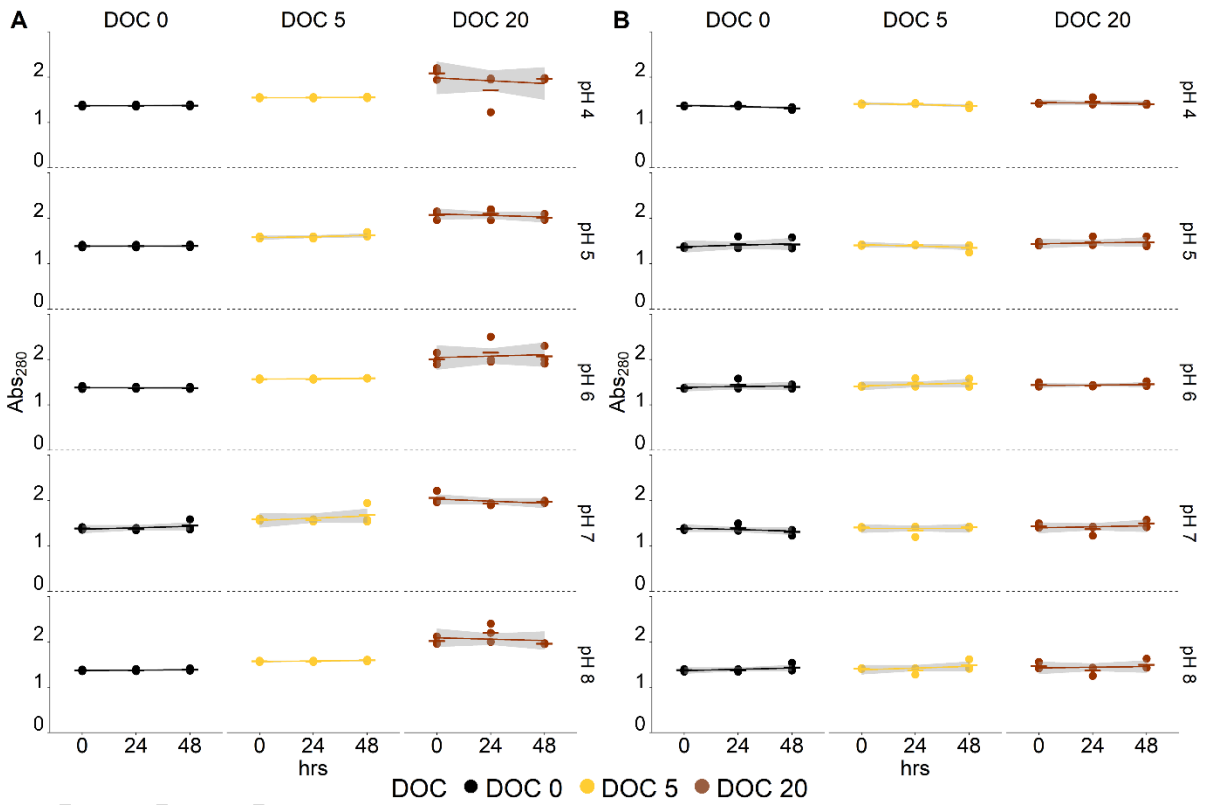


Figure S5.1. Absorbance (280 nm) of the lower (DOC5 = 5 mg L-1 DOC) and higher (DOC20 = 20 mg L-1 DOC) DOM levels measured on (A) the DOM standards and (B) the samples outside the bag at 0, 24 and 48 hours of the experiment at all pH levels. Locally estimated scatterplot smoothing (LOESS) was fitted to the data points to generate line plots. The grey area represents the 95% confidence interval.

Table S5.1. Summary table reporting mean and standard values of; Total recovery (%), Cin, Cout, MD/in and MD/out of ISU at 4 levels of DOM (0, 5, 10, 20 mg L-1 DOC) and 5 levels of pH (4, 5, 6, 7, 8). CE was 9.5 µg L-1. ME/in and ME/out were 0.95 and 0.05 µg.

pH	DOC (mg L^{-1})	Tot recovery (%)		C_{in} (µg L^{-1})		C_{out} (µg L^{-1})		$M_{det/in}$ (µg)		$M_{det/out}$ (µg)	
		mean	sd	mean	sd	mean	sd	mean	sd	mean	sd
4	0	100.2	0.1	8.5	0.1	8.6	0.3	0.1	0.0	1.7	0.1
	5	99.9	0.0	9.7	0.1	7.1	0.1	0.1	0.0	1.4	0.0
	10	99.9	0.0	10.2	0.2	6.9	0.1	0.1	0.0	1.4	0.0
	20	99.9	0.1	9.9	0.2	6.7	0.2	0.1	0.0	1.3	0.0
5	0	100.5	0.6	8.0	0.1	7.9	0.1	0.1	0.0	1.6	0.0
	5	101.8	0.1	12.7	0.3	7.0	0.2	0.1	0.0	1.4	0.0
	10	100.6	0.1	10.4	0.1	6.3	0.1	0.1	0.0	1.3	0.0
	20	100.4	0.5	8.4	0.1	7.5	0.3	0.1	0.0	1.5	0.1
6	0	100.2	0.2	8.2	0.1	8.1	0.1	0.1	0.0	1.6	0.0
	5	99.9	0.0	9.2	0.1	7.0	0.2	0.1	0.0	1.4	0.0
	10	99.9	0.0	9.0	0.0	6.2	0.1	0.1	0.0	1.2	0.0
	20	99.9	0.0	7.4	0.1	5.9	0.0	0.1	0.0	1.2	0.0
7	0	99.9	0.0	7.1	0.6	5.9	0.4	0.1	0.0	1.2	0.1
	5	100.1	0.2	6.5	0.2	5.7	0.2	0.1	0.0	1.1	0.1
	10	100.2	0.2	6.4	0.1	6.0	0.1	0.1	0.0	1.2	0.0
	20	99.9	0.0	6.1	0.1	5.9	0.2	0.1	0.0	1.2	0.0
8	0	99.9	0.0	5.9	0.1	6.0	0.1	0.1	0.0	1.2	0.0
	5	100.0	0.0	6.1	0.1	6.0	0.1	0.1	0.0	1.2	0.0
	10	100.1	0.1	5.9	0.0	6.0	0.1	0.1	0.0	1.2	0.0
	20	99.9	0.3	5.4	0.3	5.7	0.1	0.1	0.0	1.1	0.0

Table S5.2. Equilibrium conditions. One-way ANOVA testing of the difference between the concentration of ISU inside (Cin) and outside (Cout) the bag of the control units at the end of the experiment.

pH	variable	df	SS	F	p
4	**C_{in}-C_{out}**	1	0.007	0.133	0.734
	Residuals	4	0.22		
5	**C_{in}-C_{out}**	1	0.043	2.551	0.171
	Residuals	4	0.086		
6	**C_{in}-C_{out}**	1	0.034	2.251	0.231
	Residuals	4	0.045		
7	**C_{in}-C_{out}**	1	2.458	6.557	0.062
	Residuals	4	1.499		
8	**C_{in}-C_{out}**	1	0.008	0.522	0.51
	Residuals	4	0.06187		

Table S5.3. ANOVA table reporting the differences between the concentration of the compounds detected inside (Cin) and outside the dialysis bag (Cout), at the lower level of DOM (5 mg L-1 DOC), at pH 4-8.

pH	variable	df	SS	F	p
4	**C_{in}-C_{out}**	1	2.8	187.8	**<0.001**
	Residuals	4	0.06		
5	**C_{in}-C_{out}**	1	48	597.2	**<0.001**
	Residuals	4	0.32		
6	**C_{in}-C_{out}**	1	2.57	78.72	**<0.001**
	Residuals	4	0.13		
7	**C_{in}-C_{out}**	1	1.05	16.18	**<0.05**
	Residuals	4	0.26		
8	**C_{in}-C_{out}**	1	0.01	1.01	0.37
	Residuals	4	0.05		

Table S5.4. ANOVA table reporting the differences between the concentration of the compounds detected inside (Cin) and outside the dialysis bag (Cout), at the 10 mg L-1 DOC, at pH 4-8.

pH	variable	df	SS	F	p
4	**C_{in}-C_{out}**	1	5.1	148.5	**<0.001**
	Residuals	4	0.14		
5	**C_{in}-C_{out}**	1	24.36	1384	**<0.001**
	Residuals	4	0.07		
6	**C_{in}-C_{out}**	1	6.2	1244	**<0.001**
	Residuals	4	0.02		
7	**C_{in}-C_{out}**	1	0.3	13.36	**<0.05**
	Residuals	4	0.09		
8	**Cin-Cout**	1	0.01	0.39	0.57
	Residuals	4	0.02		

Table S5.5. ANOVA table reporting the differences between the concentration of the compounds detected inside (Cin) and outside the dialysis bag (Cout), at the higher level of DOM (20 mg L-1 DOC), at pH 4- 8.

pH	variable	df	SS	F	p
4	**C_{in}-C_{out}**	1	3.86	63.4	**<0.01**
	Residuals	4	0.24		
5	**C_{in}-C_{out}**	1	0.5	5.39	0.08
	Residuals	4	0.37		
6	**C_{in}-C_{out}**	1	2.51	214.1	**<0.001**
	Residuals	4	0.05		
7	**C_{in}-C_{out}**	1	0.05	1.48	0.29
	Residuals	4	0.15		
8	**C_{in}-C_{out}**	1	0.14	1.82	0.24
	Residuals	4	0.38		

Supplementary Information Chapter VI: Binding of Waterborne Pharmaceutical and Personal Care Products to Natural Dissolved Organic Matter

Simone Rizzuto [1], Didier L. Baho [2, 3], Kevin C. Jones [1*], Hao Zhang [1*], Eva Leu [4], Luca Nizzetto [3, 5]

[1] Lancaster Environment Centre, Lancaster University, Lancaster, LA1 4YQ, UK.

[2] Department of Aquatic Sciences and Assessment, Swedish University of Agricultural Sciences, Uppsala, Sweden.

[3] Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, 0349, Oslo, Norway.

[4] Akvaplan-niva, CIENS, Science Park, Gaustadalléen 21, 0349, Oslo, Norway.

[5] RECETOX, Masaryk University, Kamenice 753/5, 625 00 Brno, Czech Republic.

*corresponding authors: k.c.jones@lancaster.ac.uk; h.zhang@lancaster.ac.uk

List of Tables:

List of Figures:

List of Text:

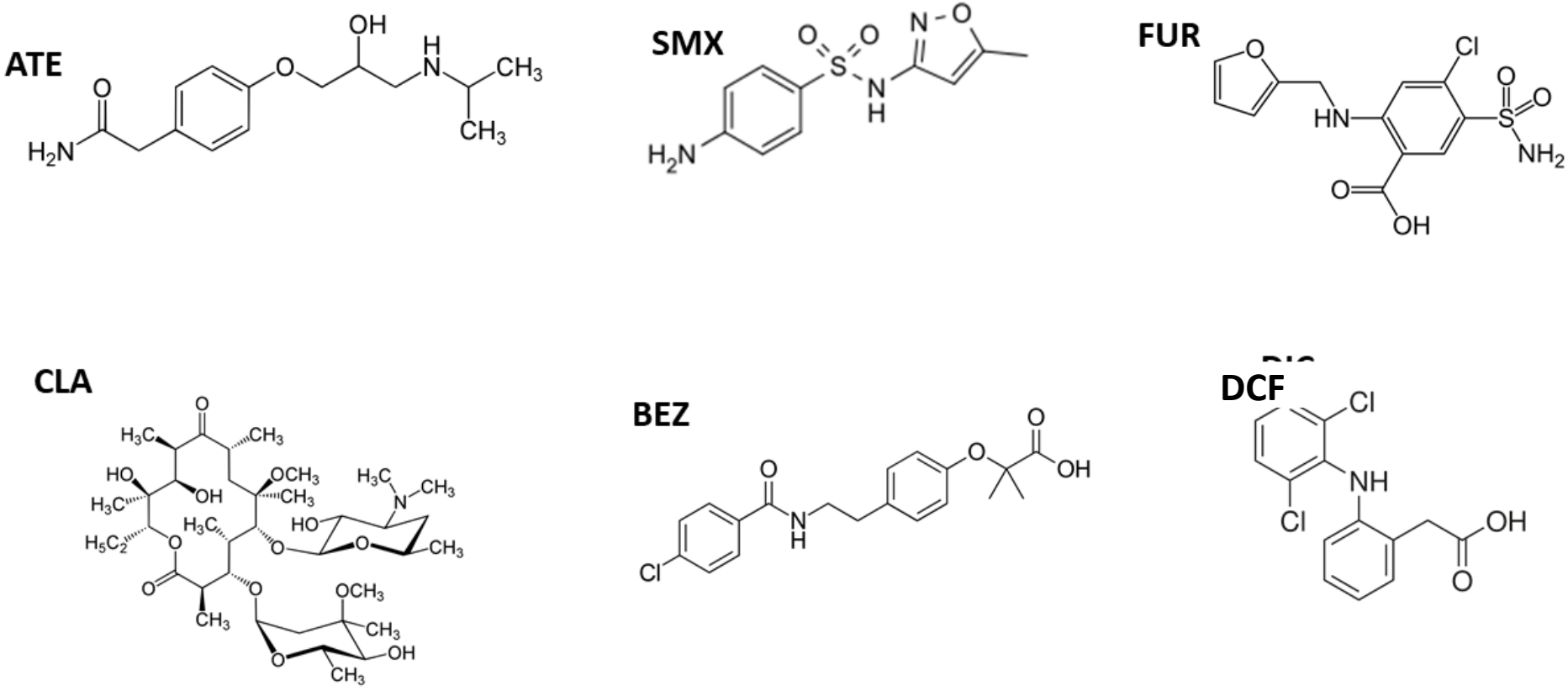


Figure S6.1. Chemical structures of the six investigated compounds.

Table S6.1. Summary data on the occurrence and concentration (ng/L) of PPCPs used in this study found in European freshwaters (lakes and rivers). The data was obtained from the Norman database. Norman is the Network of reference laboratories, research centres and related organisations for monitoring of emerging environmental substances (www.Norman-network.net). This table was modified from the paper published by Baho et al. (2019). Modified from Rizzuto et al. (2021).

Chemical	Time analyzed	Times detected	Percentage detection (%)	Min conc. (ng/L)	Max conc. (ng/L)	Mean conc. (ng/L)	standard deviation (ng/L)	Q1 conc. (ng/L)	Median conc. (ng/L)	Q3 conc. (ng/L)
Atenolol	977	723	74	0.1	900	26.3	70.7	6	11	19
Sulfamethoxazole	2616	2133	81.5	0.7	700	33.3	46	12	20	40
Furosemide	507	84	16.6	0.5	283000	9253.7	44732.1	12.25	35	76
Clarithromycin	945	730	77.2	0.9	1100	21	44.7	10	13	21
Bezafibrate	1384	764	55.2	0.3	21200	108.5	1162.7	8	13	28
Diclofenac	6320	4439	70.2	0.2	110000	785	5977.4	23	57	130

Text S6.1. Chemical analyses

PPCPs were analysed by LC-MS/MS (Shimadzu, 8040), using an XBridge BEH C18 column (2.1 mm x 100 mm, 3.5 μm, Waters) to separate the compounds. Samples were directly injected into the LC system. The mobile phases were A; 0.1% ammonium hydroxide in MQ water, and B; 50% methanol and 50% acetonitrile. The gradient procedure was optimized at: 0-1 min 15% B, increased to 90% within 5 min, then increased to 100% within 3 min, held at 100% for 3 min, after that decreased to the initial conditions (15% B) within 1 min. Finally, 6 minutes of post-run ensured re-equilibration of the column before the next injection. Acquisition parameters were both positive and negative ionization, dynamic MRM (Table S6.2). Gas temperature was 250°C, heat block temperature 400°C, drying gas flow 15 L/min, nebulizing gas flow 2 L/min. The injection volume was 15 μL and the column and the tray temperature were set to 35°C. The quantification of the compounds was based on internal standard method (Atenolol d7 for the positive ionization and Ibuprofen d3 for the negative, Sigma Aldrich); the instrument detection limit ranged between 0.56 - 0.87 ng/mL.

Table S6.2. MS/MS acquisition parameters of the investigated compounds.

Compound	ESI mode	precursor ion	product ion	retention time
ATE	(+)	267.1	145.10, 190.10	4.89
SMX	(+)	254.3	156.00, 92.15, 65.10	1.05
CLA	(+)	748.95	158.05, 591.10, 159.25	8.67
FUR	(-)	329.00	285.00, 205.00	3.57
BEZ	(-)	360.10	274.05, 154.10, 42.20	4.17
DCF	(-)	294.00	250.55, 214.00, 178.10	4.48

Text S6.2. Testing DOM's loss from dialysis bags

Other studies reported consistent loss of DOM at high concentration of DOM in the dialysis bags (Akkanen and Kukkonen, 2003, 2001). This issue recurred by using dialysis bags with 1000 Da pore size. In order to prevent this issue, we used smaller pore size (100-500 Da) and monitored on day 0, 1, 5 and 7 the loss of DOM from the dialysis bags, using a plate reader coupled with a spectrophotometer (BioTek Synergy MX; Winosky, VT, US). Triplicates of solution samples from outside the bag of each experimental unit were loaded on clear flat bottom 96 well black microplates (300 µL in each well) (Corning, US). Samples from standard DOM solutions of 5 and 15 mg L^{-1} DOC were also loaded for comparison. Absorbance wave lengths between 250-280 nm were measured, accordingly to other studies (Hagman et al., 2018). No loss was observed from either of the DOM levels over the 1-week period of the experiment (Figure S6.2).

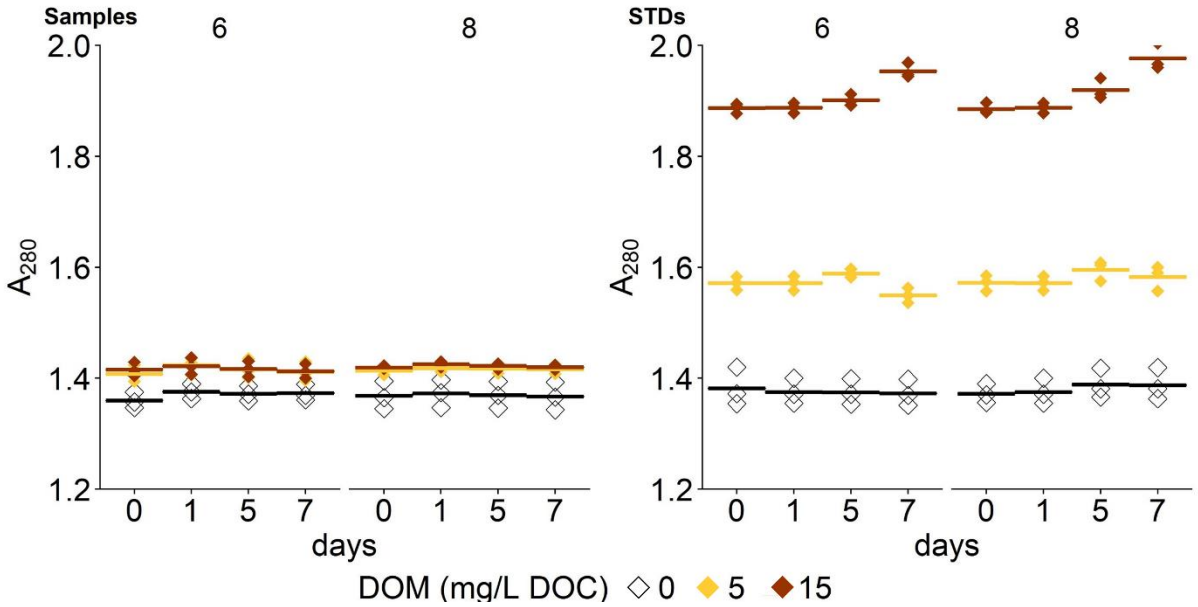


Figure S6.2. Absorbance (280 nm) of DOM levels measured on the samples and on standards on day 0, 1, 5 and 7 of the experiment, at pH 6.5 and 8.

Text S6.3. Mass recovery

The mass recovery was calculated to account for adsorption, volatilization or degradation of the compounds, as the difference in percentage between the mass expected (Table 6.1) and the mass detected in each experimental compartment.

$$Mass\ recovery\ (\%) = \left[\frac{Mass_{measured} * 100}{Mass_{expected}}\right] - 100$$

Text S6.4. Testing adsorption of PPCPs to experimental unit's components

During the experiment, more hydrophobic chemicals may adsorb to the glassware or to the bag. Hence, adsorption of the compounds to either the bag, the glassware, or other component of each experimental unit (clips, stirrer bars) was also tested. This was carried out by rinsing each experimental unit with a total amount of 10 mL of methanol. 5 mL were used to rinse the internal part of the unit (inside the bag). The other 5 mL to rinse the external part (outside the bag, internal wall of the beaker, stirrer bar and clips). The respective solvents were collected in amber vials, to be later gently dried with nitrogen stream, and re-suspended in 1 mL methanol, before being filtered and stored at -20° C in 2.5 mL amber glass vials. Chemical analyses using LC-MS/MS reported a recovery ranging from 98 – 110%.

Table S6.3. Atenolol. Table reporting the mean and standard deviation values of: percentage of mass loss compared to expected mass inside (a) and outside (b) the dialysis bag; mass recovered through adsorption inside (c) and outside (d) the bag; recovery percentages of the compound inside (e) and outside (f) the bag; total recovery percentage of the compound (g).

pH	DOM	(a) %Mass lost in		(b) %Mass lost out		(c) % A_{in}		(d) % A_{out}		(e) %Tot mass rec in		(f) %Tot mass rec out		(g) %Tot mass recovered	
		mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	Mean	sd	mean	sd
6.5	0	11.54	14.86	11.48	12.79	4.26	3.72	11.48	12.79	92.72	14.91	99.87	1.25	99.65	0.71
	5	4.83	2.03	9.95	1.93	4.67	1.80	9.95	1.93	99.83	0.54	101.25	0.85	99.99	0.03
	15	17.50	2.04	22.05	1.94	17.27	2.23	22.05	1.94	99.77	0.19	99.84	0.98	99.99	0.01
8	0	26.06	1.19	30.04	1.13	26.52	1.07	30.04	1.13	100.45	2.05	101.12	1.25	100.02	0.10
	5	25.44	10.49	29.45	9.93	18.03	13.54	29.45	9.93	92.59	19.94	99.74	0.97	99.65	0.95
	15	9.12	1.47	6.00	12.57	10.91	3.86	6.00	12.57	101.79	2.68	100.81	1.12	100.09	0.13

Table S6.4. Sulfamethoxazole. Table reporting the mean and standard deviation values of: percentage of mass loss compared to expected mass inside (a) and outside (b) the dialysis bag; mass recovered through adsorption inside (c) and outside (d) the bag; recovery percentages of the compound inside (e) and outside (f) the bag; total recovery percentage of the compound (g).

pH	DOM	(a) %Mass lost in		(b) %Mass lost out		(c) % A_{in}		(d) % A_{out}		(e) %Tot mass rec in		(f) %Tot mass rec out		(g) %Tot mass recovered	
		mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd
6.5	0	6.52	4.79	6.86	1.72	6.45	3.12	6.02	1.84	106.97	4.79	102.88	1.72	101.89	1.87
	5	7.85	2.60	2.19	2.54	8.45	2.25	7.32	2.42	108.31	2.60	102.51	2.19	102.78	2.21
	15	1.06	2.41	6.73	2.32	1.06	0.57	6.73	2.32	100.00	2.32	101.33	3.12	100.00	0.11
8	0	20.61	6.54	24.86	6.60	12.73	7.71	24.86	6.60	92.12	1.19	99.45	1.23	99.62	0.06
	5	6.76	2.93	11.72	2.83	13.64	12.86	11.72	2.83	106.87	15.46	100.64	0.98	100.33	0.74
	15	11.97	1.87	16.82	1.69	8.98	5.73	16.82	1.69	97.02	7.39	101.16	1.12	99.86	0.35

Table S6.5. Furosemide. Table reporting the mean and standard deviation values of: percentage of mass loss compared to expected mass inside (a) and outside (b) the dialysis bag; mass recovered through adsorption inside (c) and outside (d) the bag; recovery percentages of the compound inside (e) and outside (f) the bag; total recovery percentage of the compound (g).

pH	DOM	(a) %Mass lost in		(b) %Mass lost out		(c) % A_{in}		(d) % A_{out}		(e) %Tot mass rec in		(f) %Tot mass rec out		(g) %Tot mass recovered	
		mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd
6.5	0	19.24	2.30	26.86	4.86	18.21	2.22	26.86	4.86	101.97	1.12	100.00	1.01	100.57	1.48
	5	19.45	6.51	26.47	0.50	18.45	3.32	23.93	3.13	99.90	3.51	102.40	2.63	101.09	3.04
	15	19.12	6.41	26.35	6.80	20.45	6.00	24.02	6.30	98.57	3.41	102.37	1.80	101.76	2.26
8	0	-20.77	8.69	24.53	5.13	21.45	5.99	24.02	5.89	125.22	3.69	95.55	4.13	101.68	3.22
	5	22.96	6.78	23.31	7.03	20.45	3.88	22.60	4.96	102.42	3.78	103.91	4.57	103.75	3.34
	15	24.65	5.80	24.05	7.39	24.09	3.66	24.05	7.39	99.44	2.14	100.00	3.28	99.97	0.10

Table S6.6. Clarithromycin. Table reporting the mean and standard deviation values of: percentage of mass loss compared to expected mass inside (a) and outside (b) the dialysis bag; mass recovered through adsorption inside (c) and outside (d) the bag; recovery percentages of the compound inside (e) and outside (f) the bag; total recovery percentage of the compound (g).

pH	DOM	(a) %Mass lost in		(b) %Mass lost out		(c) % A_{in}		(d) % A_{out}		(e) %Tot mass rec in		(f) %Tot mass rec out		(g) %Tot mass recovered	
		mean	**sd**	**mean**	**sd**	**mean**	**sd**	**mean**	**sd**	**mean**	**sd**	**mean**	**sd**	**mean**	**sd**
7	**0**	32.66	0.42	32.41	0.83	33.12	0.75	32.18	0.79	100.46	0.52	101.20	0.80	100.02	0.02
	5	32.35	0.20	32.94	0.04	27.27	0.00	32.64	0.06	94.92	0.20	99.88	0.05	99.76	0.01
	15	32.35	0.26	33.05	0.28	30.92	1.00	34.05	0.34	98.57	0.77	98.12	0.38	99.93	0.04
8	**0**	70.42	0.44	67.99	2.17	64.18	4.39	66.16	2.16	93.75	4.76	99.14	2.15	99.70	0.23
	5	66.57	0.08	62.37	6.01	61.60	3.55	62.34	5.98	95.04	3.51	99.97	4.89	99.76	0.17
	15	66.76	0.14	57.12	12.65	66.67	2.14	59.16	11.65	99.90	2.11	102.16	12.16	100.00	0.10

Table S6.7. Bezafibrate. Table reporting the mean and standard deviation values of: percentage of mass loss compared to expected mass inside (a) and outside (b) the dialysis bag; mass recovered through adsorption inside (c) and outside (d) the bag; recovery percentages of the compound inside (e) and outside (f) the bag; total recovery percentage of the compound (g).

pH	DOM	(a) %Mass lost in		(b) %Mass lost out		(c) % A_{in}		(d) % A_{out}		(e) %Tot mass rec in		(f) %Tot mass rec out		(g) %Tot mass recovered	
		mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd
6.5	0	8.32	18.84	11.82	18.90	12.23	15.34	11.64	15.39	103.92	5.54	102.82	4.51	102.87	4.56
	5	3.78	2.65	37.45	3.12	8.86	6.56	37.12	3.64	105.09	7.66	99.67	0.52	99.92	0.85
	15	16.29	2.31	34.29	2.71	14.09	2.95	34.17	1.21	97.80	4.33	99.88	1.51	99.78	1.29
8	0	24.89	0.63	34.79	13.29	23.60	1.29	35.08	13.46	99.71	1.82	100.29	0.19	100.21	0.25
	5	-85.95	39.95	56.00	8.76	28.05	0.02	34.24	23.96	186.00	39.95	98.24	5.22	98.85	3.67
	15	-39.66	13.20	60.17	4.65	25.05	0.03	33.95	4.82	139.70	13.20	97.79	0.17	99.78	0.56

Table S6.8. Diclofenac. Table reporting the mean and standard deviation values of: percentage of mass loss compared to expected mass inside (a) and outside (b) the dialysis bag; mass recovered through adsorption inside (c) and outside (d) the bag; recovery percentages of the compound inside (e) and outside (f) the bag; total recovery percentage of the compound (g).

pH	DOM	(a) %Mass lost in		(b) %Mass lost out		(c) % A_{in}		(d) % A_{out}		(e) %Tot mass rec in		(f) %Tot mass rec out		(g) %Tot mass recovered	
		mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd
6.5	0	30.23	10.49	31.72	8.55	27.50	3.48	33.25	9.25	97.27	10.49	101.53	1.22	100.85	1.01
	5	17.46	5.77	33.31	9.21	19.17	5.89	34.58	9.26	101.70	0.49	101.27	0.17	101.29	0.16
	15	26.54	5.14	30.65	8.19	26.67	5.89	31.17	9.47	100.13	0.88	100.52	1.34	100.50	1.31
8	0	8.26	14.83	16.96	1.57	18.17	0.94	16.79	1.59	109.91	14.25	99.83	0.03	100.31	0.71
	5	-51.60	16.10	27.88	1.67	17.52	0.02	17.96	1.66	169.10	16.10	100.08	0.07	103.37	0.70
	15	-24.58	8.96	33.49	1.20	18.40	0.05	13.58	1.25	142.08	8.96	100.09	0.05	102.09	0.47

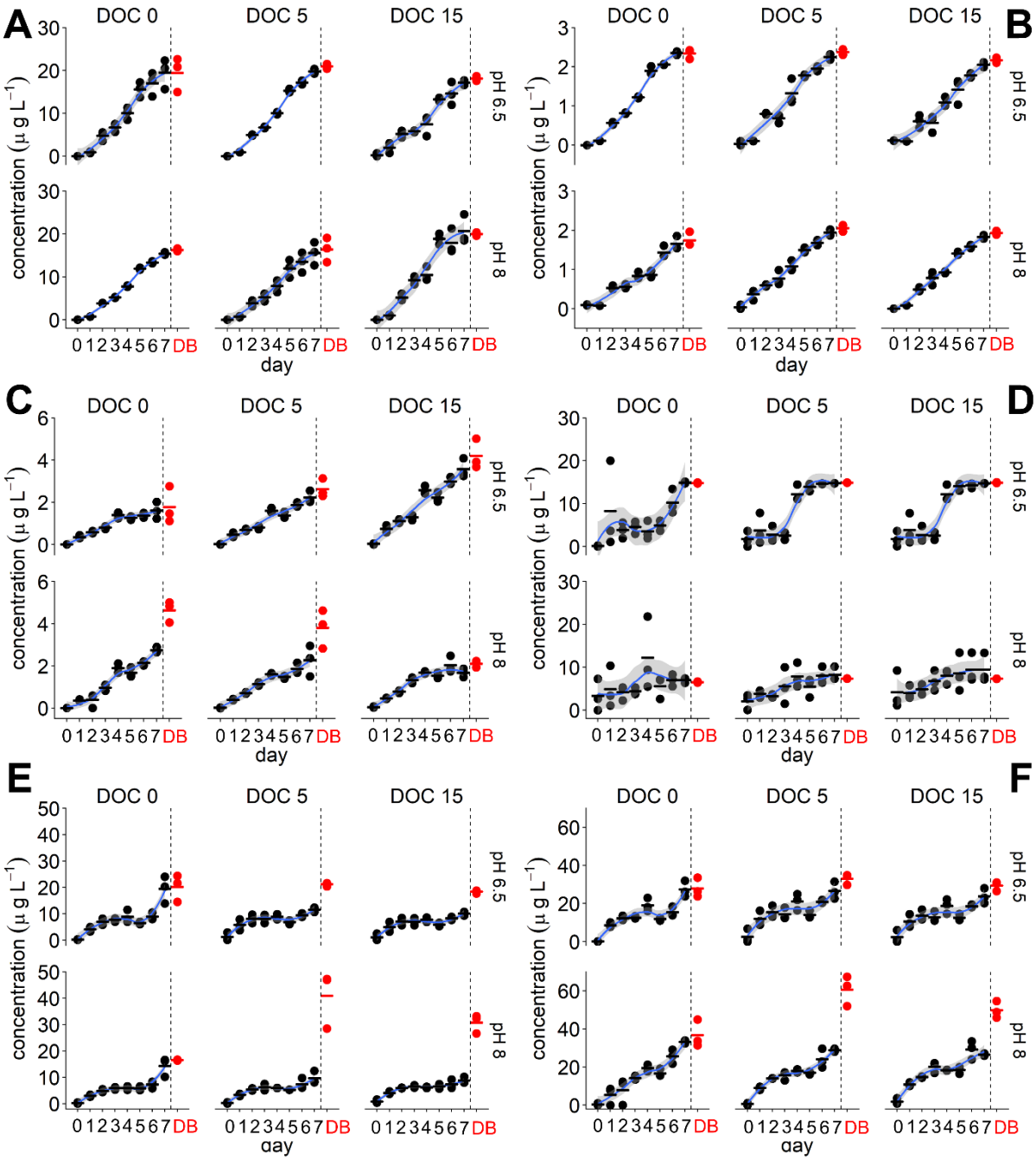


Figure S6.3. Daily concentrations (µg L-1) of (A) Atenolol, (B) Sulfamethoxazole, (C) Furosemide, (D) Clarithromycin, (E) Bezafibrate, and (F) Diclofenac outside the bag during the 7 days experiment, and inside the bag on the 7th day (DB) at 3 levels of DOM (0, 5, 15 mg L-1 DOC) and 2 levels of water pH (6.5, 8).

Table S6.9. Summary table of the 6 investigated compounds reporting mean and standard deviation values of; Total Recovery (%), concentration detected inside the bag (Cin), concentration detected outside the bag (Cout), the mass detected inside the bag (Mdet/in), and the mass detected outside the bag (Mdet/out) on the last day of the experiment, at the three levels of DOM (0, 5 and 15 mg L-1 DOC) and two levels of pH (6.5, 8). Mexp/in (µg) was 0.022 for SMX and FUR, 0.22 for ATE, CLA and BEZ, and 0.44 for DCF. Mexp/out (µg) was 0.44 for SMX and FUR, 4.4 for ATE, CH and BEZ, and 8.8 for DCF

Chem	pH	DOM (mg L^{-1} DOC)	Tot Recovery %		C_{in} (µg L^{-1})		C_{out} (µg L^{-1})		$M_{D/in}$ (µg)		$M_{D/out}$ (µg)	
			mean	sd	mean	sd	mean	sd	mean	sd	mean	sd
ATE	6.5	0	99.65	0.71	19.46	3.27	19.47	2.81	0.19	0.03	3.89	0.56
		5	99.99	0.03	20.94	0.45	19.81	0.42	0.21	0.00	3.96	0.08
		15	99.99	0.01	18.15	0.45	17.15	0.43	0.18	0.00	3.43	0.09
	8	0	100.02	0.10	16.27	0.26	15.39	0.25	0.16	0.00	3.08	0.05
		5	99.65	0.95	16.40	2.31	15.52	2.19	0.16	0.02	3.10	0.44
		15	100.09	0.13	19.99	0.32	20.68	2.77	0.20	0.00	4.14	0.55
SMX	6.5	0	106.89	1.87	2.34	0.11	2.35	0.04	0.02	0.00	0.47	0.01
		5	102.78	2.21	2.37	0.06	2.25	0.06	0.02	0.00	0.45	0.01
		15	100.00	0.11	2.18	0.05	2.05	0.05	0.02	0.00	0.41	0.01
	8	0	99.62	0.06	1.75	0.14	1.65	0.15	0.02	0.00	0.33	0.03
		5	100.33	0.74	2.05	0.06	1.94	0.06	0.02	0.00	0.39	0.01
		15	99.86	0.35	1.94	0.04	1.83	0.04	0.02	0.00	0.37	0.01
FUR	6.5	0	100.57	1.48	1.78	0.71	1.61	0.33	0.02	0.01	0.32	0.07
		5	106.09	8.04	2.63	0.36	2.23	0.23	0.03	0.00	0.45	0.05
		15	163.76	17.26	4.20	0.58	3.57	0.37	0.04	0.01	0.71	0.07
	8	0	128.68	5.22	4.64	0.41	2.74	0.11	0.05	0.00	0.55	0.02
		5	116.75	15.34	3.81	0.74	2.27	0.59	0.04	0.01	0.45	0.12
		15	99.97	0.10	2.10	0.13	1.67	0.16	0.02	0.00	0.33	0.03
CLA	6.5	0	100.02	0.02	14.82	0.09	14.87	0.18	0.15	0.00	2.97	0.04
		5	99.76	0.01	14.88	0.04	14.75	0.01	0.15	0.00	2.95	0.00
		15	99.93	0.04	14.88	0.06	14.73	0.06	0.15	0.00	2.95	0.01
	8	0	99.70	0.23	6.51	0.10	7.04	0.48	0.07	0.00	1.41	0.10
		5	99.76	0.17	7.36	0.02	8.28	1.32	0.07	0.00	1.66	0.26
		15	100.00	0.10	7.31	0.03	9.43	2.78	0.07	0.00	1.89	0.56
BEZ	6.5	0	102.87	4.56	20.17	4.14	19.40	4.16	0.20	0.04	3.88	0.83
		5	99.92	0.85	21.17	0.58	11.56	0.69	0.21	0.01	2.31	0.14
		15	99.78	1.29	18.42	0.51	10.06	0.60	0.18	0.01	2.01	0.12
	8	0	100.21	0.25	16.52	0.14	14.35	2.92	0.17	0.00	2.87	0.58
		5	93.85	13.67	40.91	8.79	9.68	1.93	0.41	0.09	1.94	0.39
		15	99.78	0.56	30.72	2.90	8.76	1.02	0.31	0.03	1.75	0.20
DCF	6.5	0	100.85	1.01	27.91	4.19	27.31	3.42	0.28	0.04	5.46	0.68
		5	101.29	0.16	33.02	2.31	26.67	3.68	0.33	0.02	5.33	0.74
		15	100.50	1.31	29.38	2.06	23.74	3.28	0.29	0.02	4.75	0.66
	8	0	100.31	0.71	36.70	5.93	33.22	0.63	0.37	0.06	6.64	0.13
		5	103.37	0.70	60.64	6.44	28.85	0.67	0.61	0.06	5.77	0.13

		15	102.09	0.47	49.83	3.58	26.60	0.48	0.50	0.04	5.32	0.10

Table S6.10. Equilibrium conditions. One-way ANOVA testing of the difference between the concentration of PPCPs inside (Cin) and outside (Cout) the bag of the control units at the end of the experiment. df; degree of freedom, F; F-statistic, SS; sum of squares, p; p-value.

PPCPs	variable	pH	df	SS	F	p
ATE	**C_{in} - C_{out}**	**6.5**	1	0.0002	0.00014	0.99
	Residuals		4	55.81		
	C_{in} - C_{out}	**8**	1	1.14	11.71	0.45
	Residuals		4	0.34		
SMX	**C_{in} - C_{out}**	**6.5**	1	0.0001	0.016	0.91
	Residuals		4	0.03	0.01	
	C_{in} - C_{out}	**8**	1	0.01	0.39	0.56
	Residuals		4	0.13		
FUR	**C_{in} - C_{out}**	**6.5**	1	0.04	0.09	0.77
	Residuals		4	1.83		
	C_{in} - C_{out}	**8**	**1**	**5.4**	**39.62**	**<0.05**
	Residuals		**4**	**0.54**		
CLA	**C_{in} - C_{out}**	**6.5**	1	0.004	0.15	0.72
	Residuals		4	0.13		
	C_{in} - C_{out}	**8**	1	0.43	2.4	0.2
	Residuals		4	0.71		
BEZ	**C_{in} - C_{out}**	**6.5**	1	0.89	0.03	0.86
	Residuals		4	103.4		
	C_{in} - C_{out}	**8**	1	7.12	1.11	0.35
	Residuals		4	25.69		
DCF	**C_{in} - C_{out}**	**6.5**	1	0.53	0.02	0.88
	Residuals		4	87.9		
	C_{in} - C_{out}	**8**	1	18.19	0.68	0.46
	Residuals		4	106.8	26.7	

Table S6.11. ANOVA table reporting the differences between the concentration of the compounds detected inside (Cin) and outside the dialysis bag (Cout), at the lower level of DOM (5 mg L-1 DOC), at pH 6.5 and 8.

PPCPs	variable	pH	df	SS	F	p
ATE	**C_{in} - C_{out}**	**6.5**	1	1.89	6.59	0.06
	Residuals		4	1.15		
	C_{in} - C_{out}	**8**	1	1.16	0.15	0.72
	Residuals		4	30.32		
SMX	**C_{in} - C_{out}**	**6.5**	1	0.02	4.9	0.09
	Residuals		4	0.02		
	C_{in} - C_{out}	**8**	1	0.02	2.96	0.16
	Residuals		4	0.02		
FUR	**C_{in} - C_{out}**	**6.5**	1	0.23	1.69	0.26
	Residuals		4	0.56		
CLA	**C_{in} - C_{out}**	**6.5**	1	0.11	1.88	0.24
	Residuals		4	0.24		
BEZ	**C_{in} - C_{out}**	**6.5**	**1**	**138.5**	**227.9**	**<0.001**
	Residuals		**4**	**2.43**		
	C_{in} - C_{out}	**8**	**1**	**1426.9**	**24.09**	**<0.01**
	Residuals		**4**	**242.9**		
DCF	**C_{in} - C_{out}**	**6.5**	1	60.31	4.26	0.11
	Residuals		4	56.69		
	C_{in} - C_{out}	**8**	**1**	**1516**	**48.24**	**<0.01**
	Residuals		**4**	**125.7**		

Table S6.12. ANOVA table reporting the differences between the concentration of the compounds detected inside (Cin) and outside the dialysis bag (Cout), at the higher level of DOM (15 mg L-1 DOC), at pH 6.5 and 8.

PPCPs	variable	pH	df	SS	F	p
ATE	**C_{in} - C_{out}**	**6.5**	1	1.42	4.9	0.09
	Residuals		4	1.16		
	C_{in} - C_{out}	**8**	1	0.72	0.12	0.74
	Residuals		4	23.27		
SMX	**C_{in} - C_{out}**	**6.5**	1	0.02	4.91	0.09
	Residuals		4	0.02		
	C_{in} - C_{out}	**8**	1	0.02	7.34	0.06
	Residuals		4	0.01		
FUR	**C_{in} - C_{out}**	**6.5**	1	0.6	1.69	0.26
	Residuals		4	1.42		
CLA	**C_{in} - C_{out}**	**6.5**	1	0.04	6.79	0.06
	Residuals		4	0.02		
BEZ	**C_{in} - C_{out}**	**6.5**	**1**	**104.83**	**227.9**	**<0.001**
	Residuals		**4**	**1.84**		
	C_{in} - C_{out}	**8**	**1**	**723.4**	**101.8**	**<0.001**
	Residuals		**4**	**28.4**		
DCF	**C_{in} - C_{out}**	**6.5**	1	47.77	4.26	0.11
	Residuals		4	44.9		
	C_{in} - C_{out}	**8**	**1**	**809.2**	**82.53**	**<0.001**
	Residuals		**4**	**39.2**		

Table S6.13. Log conditional distribution coefficient (log KDOC), and percentage of bound compound to DOM (BDOM) for the two PPCPs with binding to DOM, at two different levels of DOM (5, 15 mg L-1 DOC), and two water pH (6.5, 8).

PPCP	pH	DOM (mg L^{-1} DOC)	$logK_{DOC}$	B_{DOM}
BEZ	6.5	5	2.22	45
		15	1.74	45
	8	5	2.81	76
		15	2.22	71
DCF	8	5	2.34	52
		15	1.76	46

Appendix – Published Papers

Environmental Science & Technology

pubs.acs.org/est

Article

Water Browning Controls Adaptation and Associated Trade-Offs in Phytoplankton Stressed by Chemical Pollution

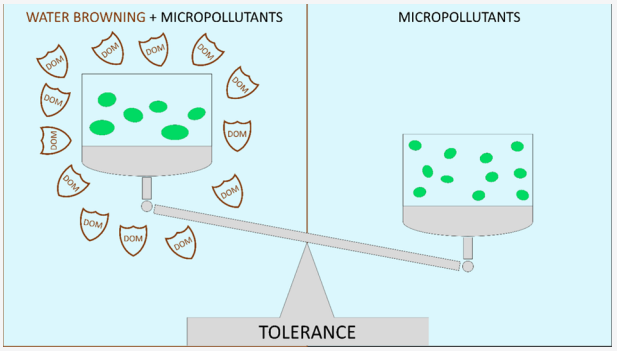
Simone Rizzuto, Jan-Erik Thrane, Didier L. Baho, Kevin C. Jones, Hao Zhang, Dag O. Hessen, Luca Nizzetto,* and Eva Leu

Cite This: *Environ. Sci. Technol.* 2020, 54, 5569−5579

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: The acquisition of tolerance to an environmental stressor can result in organisms displaying slower growth after stress release. While well-grounded in the theory, empirical evidence of the trade-off between stress tolerance and organism fitness is scarce and blurred by the interaction with different environmental factors. Here, we report the effects of water browning on the responses, tolerance acquisition, and associated trade-offs in a population of microalgae exposed to sublethal concentrations of organic micropollutants over multiple generations. Our results show that dissolved organic matter (DOM) reduces toxic responses and modulates tolerance acquisition by the algae, possibly by complexing micropollutants. Microalgae that acquire tolerance allocate resources to fitness at the cost of reduced cell size. They yield higher productivity than nonadapted ones when grown in the presence of micropollutants but lower in their absence. The net trade-off was positive, indicating that adaptation can result in a higher productivity and fitness in tolerant species in recurrently stressed environments.



1. INTRODUCTION

Populations that have been exposed over multiple generations to the selective pressure of a recurrent stressor may acquire tolerance through physiological and evolutionary adaptations.[1] Although these processes usually occur at different time scales,[2] evolutionary adaptation can also be rapid, arising a few generations after the stress onset.[3] Populations that acquire tolerance toward a specific stressor often show lower growth in another context, such as in the absence of the stressor[4−7] (maladaptation).[8,9] The existence of these trade-offs is a fundamental postulate of resource-based allocation theory.[6,7] Drawing predictions of the net positive effect of tolerance acquisition on the functioning of a population requires accounting for these antagonistic processes and is therefore complex. In addition, the magnitude of the stress can be modulated by other environmental factors. This is the case, for example, for water pollutants, the availability and/or toxic action of which can be affected by interaction with natural dissolved organic matter (DOM) or water pH.[10,11] How the interaction of chemical stressors with environmental factors influences tolerance acquisition and associated costs is mostly uncharted.

Chemical pollution acts as an important selective pressure on aquatic biota.[12] Among the range of widespread freshwater chemical pollutants, pharmaceutical and personal care products (PPCPs) are concerning as they are continuously discharged from wastewater effluents and are biologically active at low concentrations.[13] PPCPs can interfere with fundamental metabolic pathways related to chlorophyll *a* and lipid synthesis,[14,15] which increases their likelihood to adversely impact phytoplankton.[16−19] Evidence that microalgae can adapt to diffuse anthropogenic contaminants is available,[1,20,21] but documentation on the environmental controls on tolerance acquisition and on the occurrence and nature of trade-offs is scant.[22]

Over the last decades, climate and land-use change and recovery from past acidification have caused water browning,[23−25] which lead to a diffuse increase of natural DOM and changed pH in many ecosystems.[23−25] DOM (commonly analyzed as the concentration of dissolved organic carbon, DOC) can adsorb, bind, and/or transform PPCPs by forming less bioavailable and toxic complexes.[26,27] This process can be pH dependent since many freshwater contaminants, including many PPCPs, exist simultaneously as ionic and neutral forms in the aqueous phase at environmental conditions.[28,29] Neutral species dominate at water pH lower than the compound's acid dissociation constant (pK_a) and tend to be more toxic, possibly

Received: January 28, 2020
Revised: March 19, 2020
Accepted: April 15, 2020
Published: April 15, 2020

https://dx.doi.org/10.1021/acs.est.0c00548
Environ. Sci. Technol. 2020, 54, 5569−5579

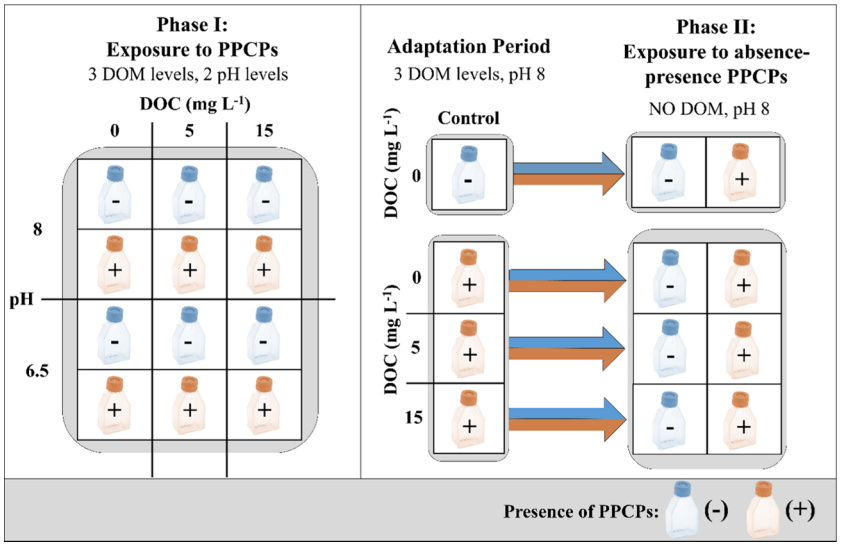


Figure 1. Factorial experimental design. Phase I: exposure of the algal population to the absence (−) and the presence (+) of a mix of 12 PPCPs under different DOM and pH levels. Adaptation period: multigenerational exposure of the algal population to the presence (+) of PPCPs under different levels of DOM (0, 5, 15 mg L^{-1} DOC) at pH 8. Phase II: exposure of the algal population previously adapted to the presence of PPCPs under different levels of DOM and of the control population, which never experienced the contaminants and/or the DOM during the adaptation period, to the absence (−) and the presence (+) of PPCPs.

because the organisms' lipid membranes are often more permeable to nonpolar molecules.[30] Neutrality in the molecular charge can in turn increase the likelihood of hydrophobic interactions with DOM,[27] possibly resulting in lower bioavailability and toxicity. The influence of these environmental factors on the form, availability, and toxicity of PPCPs has been the subject of research.[26,27,31,32] Several of these studies report that ecological risk assessment for these micropollutants is based on toxicity data from standardized tests, which rarely account for the effects of natural water chemistry, hence not taking into account the bioavailable fraction of micropollutants. This concern has been widely recognized for metals in water bodies,[33,34] resulting in the introduction of the biotic ligand model.[35] Similar considerations also apply to certain organic contaminants, calling for the need for a better understanding of their interaction with constituents of aquatic systems such as dissolved organic matter. In addition, the potential implications for driving adaptation and related trade-off are currently unexplored. Given the current widespread browning and the wide range of DOM concentrations in natural surface water, a better understanding of this factor's role as a modulator of toxic responses and the development of tolerant strains is needed.

To address these gaps, we designed a two-phase experiment, assessing the role of DOM in the toxic outcomes and adaptation (in terms of tolerance acquisition) and associated fitness trade-offs in a microalgae population exposed to a mixture of PPCPs. First, we postulated that

(i) DOM reduces the negative effects of PPCPs on algal growth;[26-27]

(ii) prolonged exposure to sublethal concentrations of PPCPs induces tolerance in microalgae.

Then, after testing these preconditions, we hypothesized that

(i) the acquisition of tolerance to PPCPs trades off with growth efficiency in the absence of the pollutants;

(ii) DOM during the adaptation period controls both the tolerance and the emergence of fitness trade-offs.

The experiment was designed as follows (see Figure 1):

- In phase I, we assessed microalgal growth and cell size response to PPCPs under different conditions of DOM and pH.
- Then, we subjected the microalgae to a two-month adaptation period, where they were exposed to sublethal PPCP levels and different levels of DOM, under the pH conditions that in phase I yielded the highest growth inhibition.
- Finally, in phase II, the growth and cell size of nonadapted and adapted populations to PPCPs under different levels of DOM were compared in the presence and absence of PPCPs.

Addressing the implications of the two-way interaction between environment and environmental stressors in biota growth and fitness represents a challenge of considerable complexity. This multiple stressors−multiple interaction situation prevails in nature, and it is important to understand and quantitatively balance synergistic/antagonistic effects, inform realistic extrapolations of results to real environmental conditions, and ultimately address the broader ecological implications of these interactions.

2. MATERIALS AND METHODS

2.1. Experimental Design. The experiment consisted of two phases (Figure 1), interposed by an adaptation period. An acclimation phase preceded the first phase of the experiment, where the cultures were acclimated for five days to combinations of DOM (0, 5, and 15 mg L^{-1} DOC) and pH (6.5 and 8). During phase I, the growth response of the microalgal population to the mix of PPCPs was tested for combinations of three DOM levels (0, 5, 15 mg L^{-1} DOC) and pH (6.5 and 8) in a factorial design (Figure 1). Then, the algae were allowed to adapt for 2 months under the same experimental conditions of PPCPs and DOM (Figure 1) at pH 8 only (following results from phase I). In phase II, the adapted populations were subsampled and grown in the

https://dx.doi.org/10.1021/acs.est.0c00548
Environ. Sci. Technol. 2020, 54, 5569−5579

presence and absence of the mix of PPCPs (at the same concentrations used in phase I and during the adaptation period but in the absence of DOM, Figure 1), to assess the acquisition of tolerance, growth performance, and ultimately trade-offs in growth efficiency.

2.2. Selection of Algal Culture. The chlorophyte *Chlamydomonas reinhardtii* (strain CC-1690 21 gr mt+) was used in laboratory growth experiments. This is a widely used model organism for toxicological and evolution studies.[37]

2.3. Selection of DOM and pH. DOM originated from the Hellerudmyra tarn (Norway) and was previously isolated through reverse osmosis.[38] All of the physical−chemical properties of this DOM are reported by Gjessing et al.[38] The levels of DOM and pH applied represent the range typically found in Northern European lakes.[39−40] The nutrient concentrations (mesotrophic lakes, P = 30 μg L^{-1}) minimized the effect induced by the algal photosynthesis on the sequestration of carbon dioxide, increasing the level of hydroxide and therefore the pH of the cultures. The increase in pH for the algal cultures exposed to the effect of PCPPs was very modest (not shown).

2.4. Selection of Chemical Contaminants. A mixture of 12 PPCPs was taken as a chemical stressor model (Table S2), according to a number of previous studies[41−44] and reflecting the most commonly detected substances in European wastewater and surface water (Table S1). PPCP analytical standards were purchased from Sigma-Aldrich, mixed, and diluted in dimethyl sulfoxide (DMSO, Sigma-Aldrich) to create a stock solution. The exposure level used in this experiment to induce toxic effects from PPCPs in phases I and II and during the adaptation period (Table S2) was chosen as the concentration that yielded a 30% decrease in growth rate in a pilot toxicity test (Table S3), following the OCED guidelines.[45] The concentrations of individual PPCPs ranged from 2.2 to 22 μg L^{-1} (Table S2), within the range of concentrations detected in European freshwater (Table S1). The concentration of individual PPCPs was determined at the end of both experimental phases (Table S4) as described in Text S2.

2.5. Algal Culturing and Biomass Measurements. The algae were grown as batch cultures in 60 mL nontreated polystyrene cell culture flasks (Nunc, Thermo Scientific), using WC medium[46] with a P concentration of 30 μg L^{-1}. Cultures were incubated at 16 °C in a temperature-controlled room under constant white light (100 μmoles of photons m^{-2} s^{-1}; this resulted in no light limitation, based on earlier experiments with *C. reinhardtii*[47−51]). Each treatment was replicated four times (the total number of experimental units was 48 in both phases).

The relative biomass development was monitored in both phases as the chlorophyll *a* in vivo fluorescence (excitation at 460 nm and emission at 680 nm, Figures S1 and S2), using a plate reader equipped with a spectrophotometer (BioTek Synergy MX; Winosky, VT). Triplicates from each experimental unit were loaded on clear flat-bottom 96-well black microplates (300 μL in each well, Corning). Biomass assessments were further constrained through cell number and size distribution determination, measured by a Coulter counter (Multisizer 3, Beckman Coulter Life Sciences). For phase I, samples of 1 mL were collected from each experimental unit at the end of the exponential growth phase (on day 5, as judged from the chlorophyll in vivo fluorescence, Figure S1). For phase II, samples for cell counting were taken daily.

2.6. Phase I. DOM-enriched medium was prepared by spiking MQ-diluted DOM in two bulk solutions of modified WC medium (see Section 2.1) to reach concentrations of 5 and 15 mg L^{-1} DOC. A third batch (control) with no added DOM was also prepared. The volume of the three bulk solutions was split into two separate sets, the pH of which was adjusted by titration with HCl or NaOH to 6.5 and 8, respectively. Finally, 20 μL of PPCP stock solution was added to half of the units, to reach the concentrations shown in Table S3. A total of 40 mL of each of the 12 different media (3 DOM levels × 2 pH levels × 2 PPCP levels) was added to four replicate culture flasks and inoculated with 100 μL of algal stock culture. This resulted in a starting concentration of ca. 1000 cells per mL (measured in a Coulter counter). Phase I was run for 7 days under the light and temperature conditions described earlier.

2.7. Experimental Adaptation Period. Following phase I, the algal cultures from the pH = 8 set were grown for 2 months in the presence of PPCPs, under the same experimental conditions as in phase I. Only the higher-pH conditions were chosen because these conditions only induced growth inhibition by PPCPs in phase I. Such a prolonged sublethal exposure was aimed at inducing selection of resistant traits and promote adaptations that could affect population dynamics and result in the postulated fitness trade-offs. Exposure was conducted under three DOM levels (0, 5, and 15 mg L^{-1} DOC), to account for the influence of DOC on the emergence of tolerance and growth trade-offs. A control culture was grown at the same level of pH in the absence of PPCPs and DOM. The cultures were transferred to new growth medium every week (0.5 mL of culture to 40 mL of fresh medium) during the adaptation period.

2.8. Phase II. Following the adaptation phase, subsamples (100 μL) from each culture were inoculated in two separate sets of four replicate culture flasks and diluted with 40 mL of DOM-free growth medium. One set was spiked with 20 μL of the PPCP solution (at the same concentrations used in phase I), while the other one was spiked with 20 μL of the carrier solvent (DMSO) only (excluding the contaminants). Phase II was run for 7 days during which cultures were grown exponentially under the same light, nutrient, and temperature conditions used in phase I.

2.9. Data Treatment, Response Parameters, and Statistical Analysis. All of the analyses were conducted using R (version 3.5.1) statistical software (R Core Development Team 2015). The growth rate was calculated using the total algal biovolume as determined from the cell counter. Total algal biovolume (BV_t) was calculated based on the number of cells (N) and their radius (r), assuming a spherical shape of the cells

$$BV_t = \sum_{i=1}^{n} \frac{4}{3}\pi r_i^3 N_i$$

Specific growth rate μ (day^{-1}) of each experimental unit was calculated as the slope of a linear regression of log-transformed biovolume against time, using data from the exponential growth phase (Figure S3). For the comparison of cell size between treatments, we calculated peak cell diameter (μm) as the mode of cell size distribution. The peak cell diameter values were then used to calculate the cellular biovolume (μm^3, hereon called "cell size") as the volume of the sphere ($4/3\ \pi r^3$). The growth rate based on the cell count (here called

https://dx.doi.org/10.1021/acs.est.0c00548
Environ. Sci. Technol. 2020, 54, 5569−5579

Table 1. ANOVA Table of Phase I Results[a]

variables	factors and interactions	R^2	df	SS	F	p
log total biovolume (mm^3 mL^{-1})	PPCPs:DOM:pH	0.81	2	1.58	2.16	0.13
	PPCPs:DOM	0.63	**2**	**4.43**	**5.68**	**<0.01**
	PPCPs:pH	0.68	**1**	**8.1**	**20.21**	**<0.001**
	DOM:pH	0.06	2	1.7	2.18	0.13
	PPCPs	0.54	**1**	**37.77**	**97.02**	**<0.001**
	DOM	0.03	2	2.15	2.76	0.08
	pH	0.01	1	1.33	3.42	0.07
cell size (μm^3)	PPCPs:DOM:pH	0.82	2	0.37	1.25	0.3
	PPCPs:DOM	0.74	**2**	**0.81**	**2.75**	**<0.05**
	PPCPs:pH	0.74	1	0.01	0.07	0.79
	DOM:pH	0.1	**2**	**1.05**	**3.56**	**0.04**
	PPCPs	0.69	**1**	**20.77**	**141.2**	**<0.001**
	DOM	0.02	2	0.55	1.92	0.16
	pH	0.05	1	1.29	8.8	0.005

[a]The main outcome from a three-way ANOVA, which tested the effects of PPCPs (the absence/presence), DOM (0, 5, 15 mg L^{-1} DOC), and pH (6.5 and 8) on log total algal biovolume yield (mm^3 mL^{-1}) and cell size (μm^3). R^2: proportion of variation explained by the interactions and the main effects. df: degree of freedom. SS: sum of square means. *F*: *F* value. Significant values are reported in bold.

"recruitment rate") was also calculated to disentangle the growth of the microalgae from the variation of the cell size caused by the treatments. Recruitment rates were taken here as a proxy of fitness, whereby fitness is fundamentally defined as the probability of producing offspring and measured through the increase in the population cell number over time.[5] As we used culture batches in microcosms, recruitment depended only on the generation of offspring and dispersal was absent.

A rigorous definition of trade-off was also formulated. First, we defined the benefit of the adaptation (B_{adp}, t^{-1}) as the gain in growth rate the adapted population displayed when growing in the presence of PPCPs. This was calculated as

$$B_{adp} = gr_{A,+} + gr_{nonA,+}$$

where $gr_{A,+}$ and $gr_{nonA,+}$ represented the growth rates of the adapted population and nonadapted population in the presence of PPCPs in phase II, respectively. Similarly, we defined the cost of adaptation (D_{adp}, t^{-1}) as the reduction in growth rate the adapted population displayed when growing in the absence of PPCPs in phase II, calculated as

$$D_{adp} = gr_{A,-} + gr_{nonA,-}$$

Their net trade-off was therefore calculated as their sum, after bootstrapping estimated growth rate values from Gaussian distributions fitted to the experimental growth rate data. Data variability and uncertainties were tracked down to the final values of gap and trade-off using a Monte Carlo frame ($N = 10^5$).

$$\text{net trade-off} = B_{adp} + D_{adp}$$

Power analysis was performed to support that the number of replicates planned for this experiment was sufficient to detect the potential differences in end points in the multiple groups. With a total amount of 144 samples and 12 groups, the power value for the three-way analysis of variance (ANOVA) interaction was 0.86, 0.84 for the two-way and 0.86 for the one-way. In phase I, the toxic responses of the algal population to PPCPs under different combinations of DOM and pH were evaluated by a two-step procedure. We used total algal biovolume and cell size as response variables. First, we used a three-way ANOVA to test the significance of the treatment factors (PPCPs, DOM, and pH) and their interactions. Second, we used linear modeling (with all predictor variables coded as factors) to test for significant differences in toxic responses between groups of interest (e.g., whether the response of the total algal biovolume or cell size to contaminants differed significantly between different DOC levels at a given pH).

In phase II, we first tested whether the adaptation period had caused algae to develop tolerance to PPCPs and whether an eventual adaptation led to the trade-off (i.e., reduced growth rate and/or cell size when grown without contaminants). We did this by modeling specific growth rate, cell size, and recruitment rate as a function of contaminant exposure (factor variable with two levels; yes/no) and whether they were allowed to adapt to PPCPs in the adaptation period (factor variable with two levels; yes/no). We tested for main effects and interactions between the two treatment factors. For the populations that underwent the adaptation phase under different levels of DOM, we tested how specific growth rate and cell size responded to contaminant exposure in phase II in the absence of DOM. This was done by modeling specific growth rate and cell size as a function of two factors: the presence/absence of PPCPs and DOM level during the adaptation period (factor variable with three levels; 0, 5, and 15 mg L^{-1} DOC). We tested for main effects and interactions between two treatment factors.

3. RESULTS

3.1. Phase I. *3.1.1. Effects of DOM and PPCPs on Biomass.* The three-way interaction between PPCPs, DOM, and pH did not have a significant effect on the total algal biovolume yield (Table 1). The mix of PPCPs had a highly significant effect on the total algal biovolume yield ($F = 97.025$, $p < 0.001$; Table 1 and Figure 2A–C). This effect was strongly dependent on pH and DOM, as shown by the significant interactions between PPCPs and pH ($F = 20.807$, $p < 0.001$) and PPCPs and DOM ($F = 5.684$, $p < 0.05$). While exposure to PPCPs generally reduced the total biovolume yield ($t = -6.07$, $p < 0.05$), the toxic effect was significantly stronger at pH 8 than at pH 6.5 ($t = -4.55$, $p < 0.001$). Low concentrations of DOM (5 mg L^{-1} DOC) at pH 8 decreased the negative effect of contaminant exposure on the total biovolume yield, relative to the control without DOM ($t =$

https://dx.doi.org/10.1021/acs.est.0c00548
Environ. Sci. Technol. 2020, 54, 5569–5579

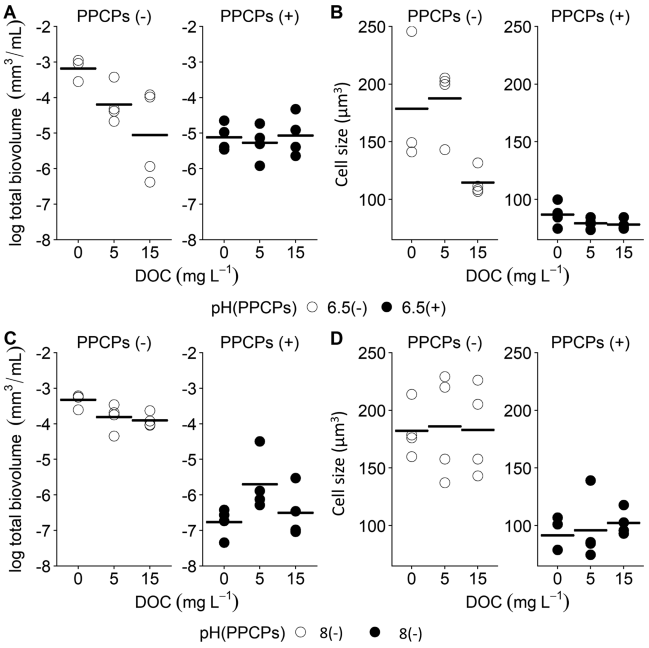


Figure 2. Phase I results. Log total biovolume yield (mm^3 mL^{-1}) and cell size (μm^3) of *C. reinhardtii* as a function of DOM (0, 5, 15 mg L^{-1} DOC) in the absence (−) and the presence (+) of the mix of PPCPs, at pH 6.5 (A, B) and 8 (C, D). Short horizontal bars represent the mean for each group.

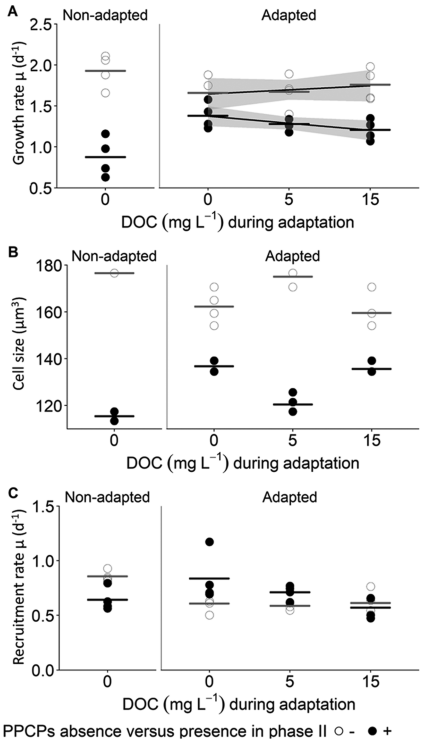


Figure 3. Phase II results. (A) Growth rate μ (day^{-1}), (B) cell size (μm^3), and (C) recruitment rate μ (day^{-1}) of the population that did not experience PPCPs and DOM during the adaptation period (nonadapted) and the population cultivated with PPCPs and DOM levels during the adaptation period (adapted), in response to the absence (−) and the presence (+) of the mix of PPCPs in phase II. Short bars report the mean values.

2.272, $p < 0.05$). A similar positive effect was not observed at the higher level of DOM (15 mg L^{-1} DOC), where the total biovolume did not differ from the control with no DOM (t = 0.56, p = 0.586). At pH 6.5, the detrimental effect of PPCPs was not influenced by the DOM (F = 0.1865, df = 2.9, p = 0.83).

In the absence of PPCPs, the total biovolume yield was significantly lower at the higher level of DOM (15 mg L^{-1} DOC) compared to the control without DOM (t = −3.45, p = 0.0027) at both pH levels. Total biovolume tended to be more sensitive to high DOM levels at pH 6.5 than at pH 8 (Figure 2A−C), but the difference was borderline significant (p = 0.08).

3.1.2. DOM and PPCP Effects on Cell Size. The interaction between PPCPs, DOM, and pH did not significantly affect the cell size of the microalgae (Table 1). The mix of PPCPs consistently decreased the mean cell size of the population (F = 141.20, $p < 0.001$, Table 1 and Figure 2B−D). This effect was also modified by the presence of DOM, as shown by the significant interaction term (F = 2.75, $p < 0.05$), while the interaction with pH was not significant. The negative effect of PPCPs on cell size (t = −7.323, $p < 0.001$) was lower (t = 2.579, $p < 0.05$) at pH 8 than at pH 6.5. In the absence of contaminants, the higher level of DOM (15 mg L^{-1} DOC) negatively affected the cell size only in the treatment with pH 6.5 (t = −3.20, $p < 0.05$).

3.2. Phase II. *3.2.1. Trade-Offs of Tolerance Acquisition in the Absence of DOM.* Exposure to PPCPs in phase II in the absence of DOM decreased algal growth rates (defined as the increase in total algal biovolume over time) in all cultures, regardless of previous adaptation (F = 43.68, $p < 0.001$; Figure 3A and Table 2). The growth inhibition effect was, however, significantly lower for the adapted cultures (df = 12, estimated mean difference = 0.51μ (day^{-1}), $p < 0.05$). At the same time, when grown in the absence of PPCPs in phase II, adapted cultures had a significantly slower growth than not-adapted ones (df = 12, estimated mean difference = −0.27μ (day^{-1}), $p < 0.05$, Figure 3A,B, Table S7).

The cell size response was similar to that of the growth rate. Exposure to PPCPs in phase II yielded smaller cells in all treatments (F = 509.56, $p < 0.001$; Figure 3B and Table 2). The magnitude of the effect, however, was strongly dependent on the adaptation (F = 90.97, $p < 0.001$). In phase II experiments, the mean cell size of cultures exposed to PPCPs during the adaptation period was significantly larger in the presence of the contaminants than that of nonadapted cultures (df = 12, estimated mean difference = 0.352 μm, $p < 0.001$, Table S7). Concurrently, their cell size was smaller than that of the nonadapted ones when grown in phase II in the absence of PPCPs (df = 12, estimated mean difference = −0.194 μm, $p < 0.001$, Table S7).

PPCP exposure during phase II decreased recruitment rates (taken as a proxy of fitness and measured here simply as the increase in cell number over time) of the nonadapted population (F = 4.58, $p < 0.05$). The adapted population, on the contrary, yielded a higher recruitment rate when algae were exposed in phase II to the PPCPs (df = 12, estimated mean difference = −0.229μ (day^{-1}), $p < 0.05$). The effect of adaption on the recruitment rate mirrored observed growth rate and cell size patterns (Figure 3C and Table S7). For instance, the adapted population yielded a higher recruitment rate when exposed to the PPCPs in phase II (df = 12, estimated mean difference = 0.194μ (day^{-1}), $p < 0.001$, Table S7) but lower in the absence of the contaminants (df = 12, estimated mean

https://dx.doi.org/10.1021/acs.est.0c00548
Environ. Sci. Technol. 2020, 54, 5569−5579

Table 2. Phase II: Effect of the Presence of PPCPs and DOM during the Adaptation Period[a]

variables	factors and interactions	R^2	df	SS	F	p
growth rate μ (day^{-1})	PPCPs during adaptation	0.01	1	0.05	1.36	0.26
	PPCPs in phase II	**0.45**	**1**	**1.77**	**43.68**	**<0.001**
	PPCPs during adaptation:PPCPs in phase II	**0.83**	**1**	**0.59**	**14.64**	**<0.01**
	residuals		12	0.49		
cell size (μm^3)	**PPCPs during adaptation**	**0.01**	**1**	**0.02**	**7.64**	**<0.05**
	PPCPs in phase II	**0.45**	**1**	**1.67**	**509.56**	**<0.001**
	PPCPs during adaptation:PPCPs in phase II	0.84	**1**	**0.3**	**90.97**	**<0.001**
	residuals		12	0.04		
recruitment rate μ (day^{-1})	PPCPs during adaptation	0.06	1	0.002	0.15	0.7
	PPCPs in phase II	0.38	1	0.001	0.01	0.9
	PPCPs during adaptation: PPCPs in phase II	0.48	**1**	**0.2**	**11.9**	**<0.05**
	residuals		12	0.21		
growth rate μ (day^{-1})	**PPCs in phase II**	0.63	**1**	**1**	**36.43**	**<0.001**
	DOM during adaptation with PPCPs	0.005	2	0.009	0.16	0.85
	PPCPs in phase II:DOM during adaptation with PPCPs	0.69	2	0.07	1.37	0.28
	residuals		18	0.49		
cell size (μm^3)	**PPCPs in phase II**	0.81	**1**	**1.58**	**309.84**	**<0.001**
	DOM during adaptation with PPCPs	0.004	2	0.008	0.84	0.45
	PPCPs in phase II:DOM during adaptation with PPCPs	**0.95**	**2**	**0.27**	**26.52**	**<0.001**
	residuals		18	0.092		
recruitment rate μ (day^{-1})	**PPCPs in phase II**	**0.14**	**1**	**0.06**	**4.58**	**<0.05**
	DOM during adaptation with PPCPs	0.15	1	0.07	2.46	0.11
	PPCPs in phase II:DOM during adaptation with PPCPs	0.45	1	0.07	2.61	0.11
	residuals		18	0.25		

[a]The ANOVA table showing the main outcome from the two-way ANOVA, which tested the effects of the presence of PPCPs during the adaptation period on the growth rate μ (day^{-1}), cell size (μm^3), and recruitment rate μ (day^{-1}) of the algal populations exposed to the absence/presence of PPCPs in phase II (nonadapted vs adapted) and the effects induced by the presence of DOM during the adaptation period with PPCPs on the growth rate and cell size of the algal population exposed to the absence/presence of PPCPs in phase II (adapted with no DOM vs adapted with DOM). R^2: proportion of variation explained by the interactions and the main effects; df: degree of freedom. SS: sum of square means. F: F value. Significant values are reported in bold.

difference = -0.25μ (day^{-1}), $p < 0.05$, Table S7), relative to the nonadapted population.

3.2.2. Effects of DOM on Tolerance Acquisition and Trade-Offs. Similar to the response of adapted algae in the absence of DOM, the exposure to PPCPs in phase II significantly affected growth rate, cell size, and recruitment rate of algae adapted in the presence of DOM (Figure 3 and Table 2). Growth rates and recruitment rates of adapted algae exposed to PPCPs declined along the DOM gradient applied during the adaptation period. The recruitment rate of algae that acquired adaptation in the presence of the highest level of DOM was significantly lower relative to that of algae adapted in its absence ($t = -2.27$, $p < 0.05$). The DOM gradient during the adaptation period did not significantly affect growth rates, cell size, and recruitment rates of the adapted algae in the absence of contaminants (Table 2 and Figure 3), despite being, altogether, lower than those of nonadapted algae (Table S7).

3.2.3. Benefits, Deficits, and Net Trade-Off in the Growth Rate of the Adapted Population. The interaction between the presence of DOM and PPCPs during the adaptation affected the gain in the growth rate of the adapted population either when exposed to the presence (B_{adp}) or in the absence (D_{adp}) of PPCPs in phase II (Figure 4A). In the presence of PPCPs during phase II, the B_{adp} followed an increasing pattern along the DOM gradient (Figure 4A). In contrast, the D_{adp} followed a decreasing pattern when in the absence of PPCPs in phase II (Figure 4A). This effect was, however, not significant (F = 0.45, p = 0.65). The exposure to PPCPs in phase II affected the gain in the growth rate of the adapted population compared to

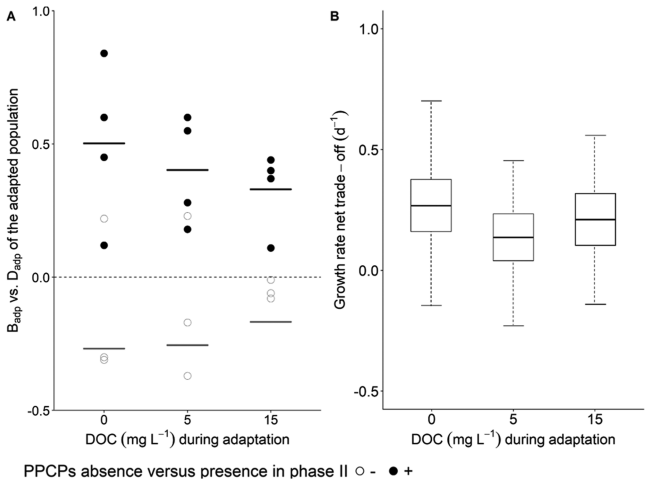


Figure 4. (A) Benefits (B_{adp}) and deficits (D_{adp}) from tolerance acquisition in the growth rate μ (day^{-1}) of the adapted population exposed to the presence and the absence of PPCPs in phase II. (B) Net trade-off from tolerance acquisition. These variables were calculated after bootstrapping estimated growth rate μ (day^{-1}) values from Gaussian distributions fitted to the experimental growth rate data. Data variability and uncertainties were tracked down to the final values of gap and trade-off using a Monte Carlo frame ($N = 10^5$).

the nonadapted one (F = 29.52, $p < 0.001$), while no significant effect was yielded by the presence of DOM during the adaptation period (F = 0.05, p = 0.95). The microalgae adapted to the presence of PPCPs during the adaptation

https://dx.doi.org/10.1021/acs.est.0c00548
Environ. Sci. Technol. 2020, 54, 5569−5579

period yielded a positive gain in growth rate (B_{adp}) when exposed to the presence of PPCPs in phase II and a negative gain (D_{adp}) in their absence, compared to the nonadapted population (Figure 4A). The difference between B_{adp} and D_{adp} is also significant ($t = 3.76$, $p < 0.05$). The net trade-off in the growth rate of the adapted population showed to be positive but not significantly affected by the presence of DOM during the adaptation period (Figure 4B).

4. DISCUSSION

We assessed the effects of the interaction of micropollutants and DOM on the growth, cell size, and fitness (through the use of recruitment rate as a proxy) of a freshwater microalgal population. We focused in particular on the emergence of trade-offs associated with adaptation acquisition (i.e., whether tolerance acquisition to chemical stress[2] influences these variables when algae grow in the absence of the stressor) as well as the role of an important environmental factor (namely, DOM) in the development of tolerance acquisition and related costs. Our results showed that algae responses depended on PPCPs, DOM, and their interaction during the adaptation period. In particular, we observed:

(i) a mitigating effect induced by the combination of DOM and pH on the toxic effect of the PPCPs (Figure 2 and Table 1);
(ii) the emergence of tolerant populations upon the adaptation period;
(iii) that tolerance acquisition and the emergence of related trade-off were influenced by DOM levels during the adaptation period (Figure 3, Table 2, and S5−S6)

Points (i) and (ii) verified the study's postulates, and point (iii) supported our main hypothesis. The following sections discuss these findings and their implications in detail.

4.1. Phase I: Effects of DOM and pH on Algal Population Responses to PPCPs. PPCPs negatively affected the total biovolume and cell size of the tested population during phase I. Previous studies showed that PPCPs could affect the growth of microalgae.[1] Our findings showed that the interaction between DOM, pH, and PPCPs had a significant effect on the algal biovolume. This translated into a positive effect of the interaction of DOM, pH, and PPCPs on algal growth that was observed in particular at the lower DOM concentration (5 mg L^{-1} DOC) and pH 8. Under these conditions, observed growth hindrance effects by PPCPs were minimal. This verified the first of our postulates. pH could vary the speciation/form of both contaminants and DOM and modify contaminants' ionic configuration. These, in turn, could affect both their toxicological properties and/or their complexation with DOM and thereby their availability. The majority of the compounds (7 out of 12) within the mix of PPCPs used in this study (Table S2) were in their associated form, moderately to highly hydrophobic (log_{kow} ranging from 2.03 to 4.76), while the remaining were highly hydrophilic (log_{kow} ranging from −0.07 to 0.89, Table S2). Hydrophobic compounds usually have a significant interaction with DOM,[26] which likely influenced our results. In addition, higher pH (8) forms neutral species also for some of the more hydrophilic compounds, promoting their toxicity and complexation. Among the PPCPs in the mixture, carbamazepine, clarithromycin, and triclosan had pK_a between 7.9 and 13.9 (Table S2). This explained the dependency of the toxicity results on pH. Our findings were in line with those of previous studies.[26,27]

At a higher DOM concentration (15 mg L^{-1} DOC), such a toxicity inhibition effect vanished. We argue that this is caused by direct, negative impacts of DOM on the algae. For instance, DOM could actually directly stress algae[36] in various ways (an effect that was found in our experiment to be more pronounced where algae were grown in the absence of PPCPs at a lower pH). In particular, DOM could (i) reduce growth by reducing light availability;[36] (ii) in nutrient-limited environments affect algal growth by adding organically bound nutrients (e.g., P[36]), hinder growth by complexing or adsorbing key elements (e.g., Fe[36]), or promote the growth of heterotrophic bacteria with higher affinity for limiting nutrients (e.g., P[36]); (iii) produce harmful free radicals and reactive oxygen species from photoactivation, stressing the algae;[52] and (iv) affect directly the photosynthetic machinery.[53] In the experimental conditions, the lack of short-wave irradiation and nutrient-saturated conditions excluded negative impacts due to the formation of reactive species and nutrient limitations. Direct negative effects of high DOM levels on algae were more plausible mechanisms. This explanation was consistent with the observed interactive effect between pH and DOM on growth inhibition in the absence of PPCPs.

While the focus of phase I was on the impacts on growth, negative effects on cell size were consistently observed. Although the mode of action of PPCPs on algae is not fully understood, some PPCPs (i.e., triclosan, carbamazepine, diclofenac) have the ability to impair fundamental metabolic pathways related to chlorophyll *a* and lipid synthesis,[14,15,54,55] which may induce destabilizing effects on the cell membrane of algae.

4.2. Phase II: Tolerance Acquisition and Trade-Offs. During the adaptation period, the algae were exposed over multiple generations to the mix of PCPPs. This resulted in the acquisition of tolerance as demonstrated by the higher growth rate of the adapted population in phase II compared to nonadapted ones under PPCP exposure. Considering the time frame of the adaptation period (>2 months),[1] PPCPs might have favored the emergence of tolerant strains through selective filtering. While this could be the result of rapid evolution, a physiological component of this response could not be excluded, in principle. To disentangle the nature of the adaptation process is notoriously difficult and was outside the scope of this study. However, the rapid changes in cell size observed in experimental phase II as a response to PPCPs, especially in the nonadapted population, pointed at fast physiological responses that could affect resource allocation. Similar findings indicating tolerance acquisition triggered by rapid adaptation to chemical stress were also reported by others,[2] including attempts to isolate physiological, ecological, and evolutionary processes.[5]

Our results showed that acquiring tolerance introduced a cost. This was evident when the adapted population was grown in the absence of PPCPs, yielding a lower growth rate (relative to the nonadapted one). Physiological and evolutionary trade-offs are broadly treated and described in the biological literature, and different theoretical bodies provide an explanation or acknowledge their existence as a postulate.[5,7] Trade-offs between growth and cell size could reflect the need to balance the investment in tolerance at the expense of energy expenditure on other fundamental processes. Trade-offs could theoretically originate both from physiological acclimation or ecological and evolutionary adaptations. Their effects on population demographic rates emerge when individuals

https://dx.doi.org/10.1021/acs.est.0c00548
Environ. Sci. Technol. 2020, 54, 5569−5579

capable of expressing metabolic paths or molecular arrangements conferring stress tolerance (at the expense of other fundamental functions) increase their frequency in the population. Here, we showed that a two-month continuous sublethal exposure to PPCPs set a new environmental optimum, selecting tolerant organisms with a significantly different morphology (i.e., cell size) and higher fitness (i.e., a higher recruitment rate). This resulted in a stress-tolerant population with growth dynamics that were different from the wild-type, both in the presence and in the absence of the stressors. Similar findings indicating the emergence of trade-offs in rapidly adapted phytoplankton were also reported elsewhere.[8] Our study complemented and expanded these results, showing that the selectivity of the environment was significantly controlled by ambient DOM levels.

The co-variance between growth rates, recruitment rates, and cell size indicated a tight interconnection between the stress response of these variables and the acquisition of tolerance. Cell size results basically mirrored the patterns observed for growth rates. Similar to growth rate, tolerance acquisition reduced the negative effects of PPCPs on cell size. At the same time, the trade-off led to a smaller cell size of the adapted population in the absence of the contaminants, relative to the nonadapted population. Recruitment rates responded similarly, but in this case, the benefits of tolerance acquisition appeared more clearly. Adapted microalgae growing in the presence of the contaminants yielded recruitment rates comparable to those of the wild-type growing in the absence of stress.

Recruitment rate results demonstrated that tolerance acquisition fundamentally concerned the allocation of resources toward maximizing fitness in the selective environment (i.e., in the presence of PPCPs) at the cost of a smaller cell size. Cell size changes accounted for a considerable fraction of the biovolume-derived growth rate response. Reduced cell volume explained almost 100% of the observed growth rate inhibition in phase II of the population adapted in the absence of DOM (not shown). In contrast, the relative contribution of cell size change in the growth rate loss in the presence of PPCPs ranged from 20 to 50% (not shown). Disentangling the influence of recruitment rate and cell size on the growth rate allowed us to reveal another interesting effect related to tolerance acquisition. When grown in the presence of PPCPs, the adapted population yielded a higher recruitment of larger-sized cells, compared with the nonadapted population. This was especially visible for the treatment with no DOM addition during the adaptation period.

Whether the acquisition of tolerance implied a net advantage when balanced against its costs was a question deserving attention. Based on the experimental results, B_{adp} and D_{adp} were positive and negative, respectively. The net trade-off of the adapted population tended to be positive, suggesting that the acquisition of tolerance generally resulted in a net benefit for the population. This had implications on how adapted populations would behave in variable environments in which phases of stress periodically followed phases of nonstress (i.e., a lake receiving contaminated waters intermittently). In such an environment (assuming that stress periods were equivalent to periods of nonstress), the adapted population could theoretically have a 2-fold competitive advantage: first, by yielding a higher biomass over time than the nonadapted one; second, by having a net fitness advantage. This indicated that PPCPs potentially represented an important selective force in impacted ecosystems and that chemical pollution should be included more frequently in the study of multistressor ecosystem responses.

4.3. Phase II: Effects of DOM on Tolerance Acquisition and Trade-Offs. The presence of DOM during the adaptation period reduced tolerance acquisition (both in terms of growth and recruitment rates) and appeared to lower both B_{adp} and D_{adp}, in line with our hypotheses. Based on the results of phase I, DOM and high pH mitigated the selective pressure, hindering tolerance acquisition by the stressed algae. Similar findings suggesting a proportional response of tolerance acquisition in relation to stress intensity were reported elsewhere.[5,22] In our case, stress mitigation depended on an environmental factor (DOM) of great relevance for freshwater ecosystems and under fundamental biogeochemical control.[23,25] While growth rate and recruitment rate were dependent on DOM levels during the adaptation period, the net trade-off was not. This is obviously because both B_{adp} and D_{adp} grow in their absolute value at the increasing level of DOM during the adaptation period, compensating for their offset. As discussed above, despite a positive net trade-off of adaptation that was apparently independent of DOM, the increasingly large gap in the growth rate response of adapted algae in the presence and the absence of PPCPs had interesting implications. This suggested that the population that gained tolerance in the absence of DOM developed faster response dynamics to changes in stress levels. As a result of a similar net trade-off, this population was expected to experience more rapid biomass losses at the onset of the stressor, and to recover faster at the stress release, compared to the populations that partially acquired tolerance in the presence of DOM. In contrast, this population was expected to respond to changes in stress levels, smoothing biomass loss and gains. These different behaviors, embodied in the different growth dynamics and trade-offs, represented two alternative strategies to stress response. In the broader ecological context, the co-existence of adapted and nonadapted populations in a community could have implications in community structuring and functioning and ultimately ecosystem resilience.[56−58]

4.4. Environmental Significance. Through the use of sublethal concentrations of a mixture of PPCPs as a stressor model and DOM as a model of environmental control, we showed that the interaction of stressors and the environment-modulated adaptation processes and the unfolding of associated functional trade-offs. We added here more empirical evidence for the key role of DOM and pH in mediating toxic responses to PPCPs,[2,26] showing that both the direct effect of DOM and the effect of its interaction with chemical pollutants on algae growth were highly dependent on pH. These findings suggested that laboratory toxicity tests conducted through standardized methodologies could lead to a wrong estimation of the toxicity of PPCPs and other trace organics by ignoring effects of speciation/complexation on the contaminants' bioavailability induced by water chemistry (DOM and pH). As the effects of DOM and pH have been widely recognized in the BLM set for ecological risk assessment in metals,[35] we support the recommendations raised by other relevant studies,[27] namely that a similar approach should be used for micropollutants to provide the most relevant standards.

Furthermore, our results complemented the findings of other recent studies showing the acquisition of tolerance to chemical stress triggered by multigenerational exposure to the same stressor.[1,22] At the same time, we reported new empirical

https://dx.doi.org/10.1021/acs.est.0c00548
Environ. Sci. Technol. 2020, 54, 5569−5579

evidence of the costs and net trade-offs associated with tolerance acquisition. DOM could counteract the process of tolerance acquisition when algae were exposed to sublethal levels of chemical stressors for multiple generations. This, in turn, had implications also for costs associated with tolerance acquisition. Adapted algae had relatively higher growth rates when growing in the presence of the stressor compared to nonadapted ones and, on the contrary, had a lower growth rate in pristine conditions. While DOM affected these rates, their net trade-off was positive and DOM-independent, suggesting that acquiring tolerance was generally advantageous for the algae and could represent a significant selective pressure in impacted ecosystems.

Tolerant microalgae displayed higher recruitment rates and smaller cell size when grown in the presence of PPCPs, indicating that tolerance acquisition coincided with allocation in fitness at the cost of a smaller cell size. This strategy allowed tolerant microalgae to compensate for a considerable part of the growth rate loss due to PPCPs.

Our results also added new insights to the impacts of water browning. Since browning is caused by increasing levels of DOM,[23-25] our findings suggest that while this process might mitigate the detrimental effects caused by ubiquitous organic contaminants, at the same time, antagonistic effects on the tolerance acquisition of stressed populations should be considered as one of its potential implications. In addition, since the presence of micropollutants might be associated with excess nutrient loading,[59,60] a potential interaction between these two stressors would be worthwhile to study.

In conclusion, results presented here could be useful to guide future assessments on the ecological and evolutionary consequences induced by the process of browning in freshwater ecosystems that are also the recipient of wastewater discharges and might be beneficial to inform environmental management in a multistressor context.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.0c00548.

Environmental concentrations; chemical properties and concentrations; toxicological test for the selection of the concentrations of the PPCPs; stability test; percentage of the recovery of the mix of PPCPs at different levels of DOM and pH at the end of phase I and phase II; pairwise comparison post-hoc Tukey test; in vivo fluorescence biomass development during phase I and phase II; log daily biovolume development during phase II; and in vivo fluorescence per unit of biomass data of the microalgae population during phase I (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Luca Nizzetto − *Norwegian Institute for Water Research (NIVA), 0349 Oslo, Norway; RECETOX, Masaryk University, 625 00 Brno, Czech Republic*; Email: luca.nizzetto@niva.no

Authors

Simone Rizzuto − *Lancaster Environment Centre, Lancaster University, Lancaster LA1 4YQ, U.K.;* orcid.org/0000-0002-5443-1274

Jan-Erik Thrane − *Norwegian Institute for Water Research (NIVA), 0349 Oslo, Norway*

Didier L. Baho − *Norwegian Institute for Water Research (NIVA), 0349 Oslo, Norway; Department of Aquatic Sciences and Assessment, Swedish University of Agricultural Sciences, SE-750-07 Uppsala, Sweden*

Kevin C. Jones − *Lancaster Environment Centre, Lancaster University, Lancaster LA1 4YQ, U.K.;* orcid.org/0000-0001-7108-9776

Hao Zhang − *Lancaster Environment Centre, Lancaster University, Lancaster LA1 4YQ, U.K.;* orcid.org/0000-0003-3641-1816

Dag O. Hessen − *Section for Aquatic Biology and Ecotoxicology and Center of Biogeochemistry in the Anthropocene (CAB), University of Oslo, 0316 Oslo, Norway*

Eva Leu − *Akvaplan-niva, CIENS, 0349 Oslo, Norway*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.est.0c00548

Funding

This study was financed by the Research Council of Norway, project grant no. 244460, Pollution and Ecosystem Adaptation to Changes in the Environment (PEACE). The research was co-funded by a discretional internal grant from the Norwegian Institute for Water Research (NIVA), under the Institute Strategic Priorities (SIS) on ecosystem remediation (TRADE-OFF). SR was also supported by the Lancaster University Faculty of Science and Technology and a Lancaster Environment Centre PhD Scholarship.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank Rolf Vogt from the Department of Chemistry, University of Oslo, for providing the DOM used in this study. The authors wish to thank Per Færøvig of the Section of Aquatic Biology and Ecotoxicology, University of Oslo, for helping with the technical aspect of the experimental setup.

■ REFERENCES

(1) Bell, G. Evolutionary Rescue. *Annu. Rev. Ecol., Evol., Syst.* **2017**, *48*, 605−627.

(2) Collins, S.; Gardner, A. Integrating Physiological, Ecological and Evolutionary Change: A Price Equation Approach. *Ecol. Lett.* **2009**, *12*, 744−757.

(3) Gonzalez, A.; Bell, G. Evolutionary Rescue and Adaptation to Abrupt Environmental Change Depends upon the History of Stress. *Philos. Trans. R. Soc., B* **2013**, *368*, No. 20120079.

(4) Vila-Aiub, M. M.; Neve, P.; Powles, S. B. Evidence for an Ecological Cost of Enhanced Herbicide Metabolism in Lolium Rigidum. *J. Ecol.* **2009**, *97*, 772−780.

(5) Gassmann, A. J.; Futuyma, D. J. Consequence of Herbivory for the Fitness Cost of Herbicide Resistance: Photosynthetic Variation in the Context of Plant-Herbivore Interactions. *J. Evol. Biol.* **2005**, *18*, 447−454.

(6) Lerdau, M.; Gershenzon, J. Allocation Theory and Chemical Defence. In *Plant Resource Allocation*; Bazzaz, F. A.; Grace, J., Eds.; Academic Press, 1997; pp 256−277.

(7) Bazzaz, F. A.; Chiariello, N. R.; Coley, P. D.; Pitelka, L. F. Allocating Resources to Reproduction and Defense. *Bioscience* **1987**, *37*, 58−67.

(8) Jin, P.; Agustí, S. Fast Adaptation of Tropical Diatoms to Increased Warming with Trade-Offs. *Sci. Rep.* **2018**, *8*, No. 17771.

(9) Kottuparambil, S.; Jin, P.; Agusti, S. Adaptation of Red Sea Phytoplankton to Experimental Warming Increases Their Tolerance to Toxic Metal Exposure. *Front. Environ. Sci.* **2019**, *7*, No. 125.
(10) Holmstrup, M.; Bindesbøl, A. M.; Oostingh, G. J.; Duschl, A.; Scheil, V.; Köhler, H. R.; Loureiro, S.; Soares, A. M. V. M.; Ferreira, A. L. G.; Kienle, C.; et al. Interactions between Effects of Environmental Chemicals and Natural Stressors: A Review. *Sci. Total Environ.* **2010**, *408*, 3746−3762.
(11) Laskowski, R.; Bednarska, A. J.; Kramarz, P. E.; Loureiro, S.; Scheil, V.; Kudłek, J.; Holmstrup, M. Interactions between Toxic Chemicals and Natural Environmental Factors - A Meta-Analysis and Case Studies. *Sci. Total Environ.* **2010**, *408*, 3763−3774.
(12) Rockström, J.; Steffen, W.; Noone, K.; Persson, A.; Chapin, F.; Lambin, E.; Lenton, T.; M, S.; Folke, C.; Schellnhuber, H.; et al. A Safe Operating Space for Humanity. *Nature* **2009**, *461*, 472−475.
(13) Schwarzenbach, R.; Escher, B. I.; Fenner, K.; Hofstetter, R.; Johnson, C.; Von Gunten, U.; Wehrli, B. The Challenge of Micropollutants in Aquatic System. *Science* **2006**, *313*, 1072−1077.
(14) Zhang, W.; Zhang, M.; Lin, K.; Sun, W.; Xiong, B.; Guo, M.; Cui, X.; Fu, R. Eco-Toxicological Effect of Carbamazepine on Scenedesmus Obliquus and Chlorella Pyrenoidosa. *Environ. Toxicol. Pharmacol.* **2012**, *33*, 344−352.
(15) Zhang, Y.; Guo, J.; Yao, T.; Zhang, Y.; Zhou, X.; Chu, H. The Influence of Four Pharmaceuticals on Chlorellapyrenoidosa Culture. *Sci. Rep.* **2019**, *9*, No. 1624.
(16) Grzesiuk, M.; Spijkerman, E.; Lachmann, S. C.; Wacker, A. Environmental Concentrations of Pharmaceuticals Directly Affect Phytoplankton and Effects Propagate through Trophic Interactions. *Ecotoxicol. Environ. Saf.* **2018**, *156*, 271−278.
(17) Wilson, B. A.; Smith, V. H.; Denoyelles, F.; Larive, C. K. Effects of Three Pharmaceutical and Personal Care Products on Natural Freshwater Algal Assemblages. *Environ. Sci. Technol.* **2003**, *37*, 1713−1719.
(18) Neuwoehner, J.; Fenner, K.; Escher, B. I. Physiological Modes of Action of Fluoxetine and Its Human Metabolites in Algae. *Environ. Sci. Technol.* **2009**, *43*, 6830−6837.
(19) Escher, B. I.; Bramaz, N.; Eggen, R. I. L.; Richter, M. In Vitro Assessment of Modes of Toxic Action of Pharmaceuticals in Aquatic Life. *Environ. Sci. Technol.* **2005**, *39*, 3090−3100.
(20) Huertas, I. E.; Rouco, M.; López-Rodas, V.; Costas, E. Estimating the Capability of Different Phytoplankton Groups to Adapt to Contamination: Herbicides Will Affect Phytoplankton Species Differently. *New Phytol.* **2010**, *188*, 478−487.
(21) Marvá, F.; García-Balboa, C.; Baselga-Cervera, B.; Costas, E. Rapid Adaptation of Some Phytoplankton Species to Osmium as a Result of Spontaneous Mutations. *Ecotoxicology* **2014**, *23*, 213−220.
(22) Medina, M. H.; Correa, J. A.; Barata, C. Micro-Evolution Due to Pollution: Possible Consequences for Ecosystem Responses to Toxic Stress. *Chemosphere* **2007**, *67*, 2105−2114.
(23) Monteith, D. T.; Stoddard, J. L.; Evans, C. D.; De Wit, H. A.; Forsius, M.; Høgåsen, T.; Wilander, A.; Skjelkvåle, B. L.; Jeffries, D. S.; Vuorenmaa, J.; et al. Dissolved Organic Carbon Trends Resulting from Changes in Atmospheric Deposition Chemistry. *Nature* **2007**, *450*, 537−541.
(24) Williamson, C. E.; Overholt, E. P.; Pilla, R. M.; Leach, T. H.; Brentrup, J. A.; Knoll, L. B.; Mette, E. M.; Moeller, R. E. Ecological Consequences of Long-Term Browning in Lakes. *Sci. Rep.* **2016**, *5*, No. 18666.
(25) Roulet, N.; Moore, T. R. Browning the Waters. *Nature* **2006**, *444*, 283−284.
(26) Pan, B.; Ning, P.; Xing, B. Part V - Sorption of Pharmaceuticals and Personal Care Products. *Environ. Sci. Pollut. Res.* **2009**, *16*, 106−116.
(27) Rowett, C. J.; Hutchinson, T. H.; Comber, S. D. W. The Impact of Natural and Anthropogenic Dissolved Organic Carbon (DOC), and PH on the Toxicity of Triclosan to the Crustacean Gammarus Pulex (L.). *Sci. Total Environ.* **2016**, *565*, 222−231.
(28) Ashauer, R.; Escher, B. I. Advantages of Toxicokinetic and Toxicodynamic Modelling in Aquatic Ecotoxicology and Risk Assessment. *J. Environ. Monit.* **2010**, *12*, 2056−2061.
(29) Rozman, K. K.; Doull, J. Dose and Time as Variables of Toxicity. *Toxicology* **2000**, *144*, 169−178.
(30) Lipnick, R. L. Structure-Activity Relationships. In *Fundamentals of Aquatic Toxicology - Effects, Environmental Fate, and Risk Assessment*;Rand, G. M., Ed.; Taylor & Francis: Washington, 1995; pp 609−655.
(31) Albanese, K. A.; Lanno, R. P.; Hadad, C. M.; Chin, Y.-P. Photolysis- and Dissolved Organic Matter-Induced Toxicity of Triclocarban to Daphnia Magna. **2017**, *4*, 457 462. DOI: 10.1021/acs.estlett.7b00429.
(32) Environment Agency. The Water Framework Directive (Standards and Classification) Directions (England and Wales), Water Framework Directive. 2015, https://doi.org/10.1136/bmjopen-2017-019199.
(33) Niyogi, S.; Wood, C. M. Biotic Ligand Model, a Flexible Tool for Developing Site-Specific Water Quality Guidelines for Metals. *Environ. Sci. Technol* **2004**, *38*, 6177−6192.
(34) Erickson, R. J. The Biotic Ligand Model Approach for Addressing Effects of Exposure Water Chemistry on Aquatic Toxicity of Metals: Genesis and Challenges. *Environ. Toxicol. Chem.* **2013**, *32*, 1212−1214.
(35) Environment Agency. *Using Biotic Ligand Models to Implement Environmental Quality Standards for Metals under the Water Framework Directive*, 2009.
(36) Creed, I. F.; Bergström, A. K.; Trick, C. G.; Grimm, N. B.; Hessen, D. O.; Karlsson, J.; Kidd, K. A.; Kritzberg, E.; McKnight, D. M.; Freeman, E. C. et al. Global Change-Driven Effects on Dissolved Organic Matter Composition: Implications for Food Webs of Northern Lakes. In *Global Change Biology*; Blackwell Publishing Ltd, 2018; pp 3692−3714.
(37) Merchant, S. S.; Prochnik, S. E.; Vallon, O.; Harris, E. H.; Karpowicz, S. J.; Witman, G. B.; Terry, A.; Salamov, A.; Fritz-Laylin, L. K.; Maréchal-Drouard, L.; et al. The Chlamydomonas Genome Reveals the Evolution of Key Animal and Plant Functions. *Science* **2007**, *318*, 245−250.
(38) Gjessing, E. T.; Egeberg, P. K.; Håkedal, J. Natural Organic Matter in Drinking Water - The "NOM-Typing Project", Background and Basic Characteristics of Original Water Samples and NOM Isolates. *Environ. Int.* **1999**, *25*, 145−159.
(39) Henriksen, A.; Skjelvåle, B. L.; Mannio, J.; Wilander, A.; Harriman, R.; Curtis, C.; Jensen, J. P.; Fjeld, E.; Moiseenkon, T. Northern European Lake Survey, 1995. *Ambio* **1998**, *27*, 80−91.
(40) Thrane, J. E.; Hessen, D. O.; Andersen, T. The Absorption of Light in Lakes: Negative Impact of Dissolved Organic Carbon on Primary Productivity. *Ecosystems* **2014**, *17*, 1040−1052.
(41) Baho, D. L.; Pomati, F.; Leu, E.; Hessen, D. O.; Moe, J. S.; Norberg, J.; Nizzetto, L. A Single Pulse of Diffuse Contaminants Alters the Size Distribution of Natural Phytoplankton Communities. *Sci. Total Environ.* **2019**, *683*, 578−588.
(42) Pomati, F.; Jokela, J.; Castiglioni, S.; Thomas, M. K.; Nizzetto, L. Water-Borne Pharmaceuticals Reduce Phenotypic Diversity and Response Capacity of Natural Phytoplankton Communities. *PLoS One* **2017**, *12*, No. e0174207.
(43) Pomati, F.; Castiglioni, S.; Zuccato, E.; Fanelli, R.; Vigetti, D.; Rossetti, C.; Calamari, D. Effects of a Complex Mixture of Therapeutic Drugs at Environmental Levels on Human Embryonic Cells. *Environ. Sci. Technol.* **2006**, *40*, 2442−2447.
(44) Baho, D. L.; Leu, E.; Pomati, F.; Hessen, D. O.; Norberg, J.; Moe, S. J.; Skjelbred, B.; Nizzetto, L.; Baho, D. Resilience of Natural Lake Phytoplankton Communities to Pulse Disturbances from Micropollutant Exposure and Vertical Mixing. *Environ. Toxicol. Chem.* **2019**, *38*, 2197−2208.
(45) OECD; Test No. 201: Alga, Growth Inhibition Test. In *OECD Guidelines for the Testing of Chemicals, Section 2: Effects on Biotic Systems*; OECD Publishing, 2006.

(46) Guillard, R. R. L.; Lorenzen, C. J. Yellow-Green Algase with Chlorophyllide C12. *J. Phycol.* **1972**, *8*, 10−14.
(47) Thrane, J.-E.; Hessen, D. O.; Andersen, T. The Impact of Irradiance on Optimal and Cellular Nitrogen to Phosphorus Ratios in Phytoplankton. *Ecol. Lett.* **2016**, *19*, 880−888.
(48) Peacock, K. A. The Three Faces of Ecological Fitness. *Stud. Hist. Philos. Sci. Part C, Stud. Hist. Philos. Biol. Biomed. Sci.* **2011**, *42*, 99−105.
(49) Nakamura, Y.; Yamamoto, H.; Sekizawa, J.; Kondo, T.; Hirai, N.; Tatarazako, N. The Effects of PH on Fluoxetine in Japanese Medaka (*Oryzias latipes*): Acute Toxicity in Fish Larvae and Bioaccumulation in Juvenile Fish. *Chemosphere* **2008**, *70*, 865−873.
(50) Karlsson, M. V.; Carter, L. J.; Agatz, A.; Boxall, A. B. A. Novel Approach for Characterizing PH-Dependent Uptake of Ionizable Chemicals in Aquatic Organisms. *Environ. Sci. Technol.* **2017**, *51*, 6965−6971.
(51) Valenti, T. W.; Pilar Perez-hurtado, I.; Kevin Chambliss, C.; Brooksi, B. W. Pharmaceuticals and Personal Care Products in the Environment, Aquatic Toxicity of Sertraline to Pimephales Promelas at Environmentally Relevant Surface Water PH. *Environ. Toxicol. Chem.* **2009**, *28*, 2685−2694.
(52) Wolf, R.; Andersen, T.; Hessen, D. O.; Hylland, K. The Influence of Dissolved Organic Carbon and Ultraviolet Radiation on the Genomic Integrity of Daphnia Magna. *Funct. Ecol.* **2017**, *31*, 848−855.
(53) Pflugmacher, S.; Pietsch, C.; Rieger, W.; Steinberg, C. E. W. Dissolved Natural Organic Matter (NOM) Impacts Photosynthetic Oxygen Production and Electron Transport in Coontail Ceratophyllum Demersum. *Sci. Total Environ.* **2006**, *357*, 169−175.
(54) Franz, S.; Altenburger, R.; Heilmeier, H.; Schmitt-Jansen, M. What Contributes to the Sensitivity of Microalgae to Triclosan? *Aquat. Toxicol.* **2008**, *90*, 102−108.
(55) Pomati, F.; Nizzetto, L. Assessing Triclosan-Induced Ecological and Trans-Generational Effects in Natural Phytoplankton Communities: A Trait-Based Field Method. *Ecotoxicology* **2013**, *22*, 779−794.
(56) Enquist, B. J.; Norberg, J.; Bonser, S. P.; Violle, C.; Webb, C. T.; Henderson, A.; Sloat, L. L.; Savage, V. M. *Scaling from Traits to Ecosystems: Developing a General Trait Driver Theory via Integrating Trait-Based and Metabolic Scaling Theories*; 1st ed.; Elsevier Ltd., 2015; Vol *52*.
(57) Uecker, H.; Hermisson, J. On the Fixation Process of a Beneficial Mutation in a Variable Environment. *Genetics* **2011**, *188*, 915−930.
(58) Kirkpatrick, M.; Peischl, S. Evolutionary Rescue by Beneficial Mutations in Environments That Change in Space and Time. *Philos. Trans. R. Soc., B* **2013**, *368*, No. 20120082.
(59) Koelmans, A. A.; Van der Heijde, A.; Knijff, L. M.; Aalderink, R. H. Integrated Modelling of Eutrophication and Organic Contaminant Fate & Effects in Aquatic Ecosystems. A Review. *Water Res.* **2001**, *35*, 3517−3536.
(60) L Eggen, R. I. L.; Hollender, J.; Joss, A.; Scha, M.; Stamm, C. Reducing the Discharge of Micropollutants in the Aquatic Environment: The Benefits of Upgrading Wastewater Treatment Plants. *Environ. Sci. Technol.* **2014**, *48*, 7683−7689.

https://dx.doi.org/10.1021/acs.est.0c00548
Environ. Sci. Technol. 2020, 54, 5569−5579

Ecosystems
https://doi.org/10.1007/s10021-021-00604-0

Ecological Memory of Historical Contamination Influences the Response of Phytoplankton Communities

Didier L. Baho,[1,2]* Simone Rizzuto,[3] Luca Nizzetto,[1,4]* Dag O. Hessen,[5] Jon Norberg,[6] Birger Skjelbred,[1] Kevin C. Jones,[3] Hao Zhang,[3] and Eva Leu[7]

[1]*Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, 0349 Oslo, Norway;* [2]*Department of Aquatic Sciences and Assessment, Swedish University of Agricultural Sciences, Uppsala, Sweden;* [3]*Lancaster Environment Centre, Lancaster University, Lancaster, UK;* [4]*RECETOX, Masarik University, Kamenice 753/5, 625 00 Brno, Czech Republic;* [5]*Department of Biosciences, University of Oslo, Blindern, Box 1066, 0316 Oslo, Norway;* [6]*Stockholm Resilience Centre, Stockholm University, Kräftriket 2B, 10691 Stockholm, Sweden;* [7]*Akvaplan-Niva, CIENS, Science Park, Gaustadalléen 21, 0349 Oslo, Norway*

Abstract

Ecological memory (EM) recognizes the importance of previous stress encounters in promoting community tolerance and thereby enhances ecosystem stability, provided that gained tolerances are preserved during non-stress periods. Drawing from this concept, we hypothesized that the recruitment of tolerant species can be facilitated by imposing an initial sorting process (conditioning) during the early stages of community assembly, which should result in higher production (biomass development and photosynthetic efficiency) and stable community composition. To test this, phytoplankton resting stages were germinated from lake sediments originating from two catchments that differed in contamination history: one impacted by long-term herbicides and pesticides exposures (historically contaminated lake) from an agricultural catchment compared to a low-impacted one (near-pristine lake) from a forested catchment. Conditioning was achieved by adding an herbicide (Isoproturon, which was commonly used in the catchment of the historically contaminated lake) during germination. Afterward, the communities obtained from germination were exposed to an increasing gradient of Isoproturon. As hypothesized, upon conditioning, the phytoplankton assemblages from the historically contaminated lake were able to rapidly restore photosynthetic efficiency ($p > 0.01$) and became structurally (community composition) more resistant to Isoproturon. The communities of the near-pristine lake did not yield these positive effects regardless of conditioning, supporting that EM was a unique attribute of the historically stressed ecosystem. Moreover, assemblages that displayed higher structural resistance concurrently yielded lower biomass, indicating that benefits of EM in increasing structural stability may trade-off with production. Our results clearly indicate that EM can foster ecosystem stability to a recurring stressor.

Received 8 July 2020; accepted 10 January 2021

Supplementary Information: The online version contains supplementary material available at https://doi.org/10.1007/s10021-021-00604-0.

Author contributions DLB, LN, DOH, JN and EL conceived the study and designed the experiment. DLB, SR, LN and EL carried out the experiment. The taxonomic classification and enumeration of phytoplankton was performed by BS. DLB and SR analyzed the data and took the lead in writing the manuscript. All authors provided critical analysis of results, feedbacks, helped structuring and editing the manuscript.

**Corresponding author; e-mail:* didier.baho@slu.se; luca.nizzetto@niva.no

Published online: 22 March 2021

Key words: Ecological memory; Phytoplankton communities; Stability; Recurrent stressor; Community tolerance; Trade-off.

Highlights

- Contamination history determines community responses when a stressor recur.
- Communities that had previous encounters with stressor(s) show higher resistance.
- Higher community resistance can result in a trade-off with biomass production.

Introduction

The concept of ecological memory (EM; Padisak 1992) proposes that past experience influences present day responses of ecosystems, thereby enabling communities to cope better with recurrent stress (Turner 2010; Johnstone and others 2016). Ecosystems that have been exposed to recurrent stressor(s) can acclimatize and eventually adapt (Ogle and others 2015; Samani and Bell 2016) through physiological, ecological and evolutionary processes (Collins and Gardner 2009). Over longer time scales, adaptations involving ecological (Blanck 2002) and evolutionary (Samani and Bell 2016; Bell 2017) processes are more likely to underpin EM. Ecological adaptation emphasizes the replacement of stress sensitive species with tolerant ones (a phenomenon described, amongst others, in the concept of pollution-induced community tolerance [PICT]; Blanck 2002). Evolutionary adaptation involves the selection of strains or organisms carrying genetic variants or modifications that confer resistance (Bell 2017). If these adaptations are partly or fully maintained during periods of non-stress, EM is established and the community can cope efficiently when the recurring stressor reappears (Padisak 1992; Scheffer and Carpenter 2003; Johnstone and others 2016). EM can contribute to enhance the stability of the ecosystem by promoting resistance and recovery (Donohue and others 2016; Hillebrand and others 2018). Resistance can be expressed by the ability to withstand stress, whereas recovery addresses the ability to regain normal functions and structures after being impacted (Hillebrand and others 2018). Resistance and recovery can be measured in terms of functional (for example, biomass production and resource use) and structural (community composition) characteristics (Hillebrand and others 2018).

Evidence of the causal relationship between earlier encounters to a stressor and present-day tolerance stems mostly from observational studies or theoretical models (Peterson 2002; Ogle and others 2015; Hughes and others 2019), while experimental approaches targeting natural communities are rare (Feckler and others 2018). An inherent limitation of observational approaches is that they typically focus on communities that are incidentally available at a given time point (snapshot), which might be blurred by other drivers (Cochran and Chambers 1965). Under such circumstances, the acquisition of adaptation may not be fully expressed or detectable during stress-free periods, despite still being present in an inactive form, that is, as dormant stage (Orsini and others 2013). Organisms that have the ability to produce long lasting resting stages represent a useful experimental model since these can act like ''seed banks'' containing previous species assemblages that span over an extended period of time (Orsini and others 2013). In phytoplankton, the formation of resting spores or cysts is a common strategy (Orsini and others 2013) and can be considered as natural biological archives that offer a good opportunity to study whether or how EM helps to recruit species that gained tolerance through past adaptations (Ellegaard and others 2018). Hence, phytoplankton germination experiments offer a good model to study EM (Padisak 1992). Moreover, some anthropogenic stressors, such as pesticides, are relatively well monitored and offer the prospect of investigating how communities that have been repeatedly exposed to the same stressor can develop tolerance (Blanck 2002). The contamination of freshwaters by pesticides from agricultural fields is one of the few stressors that have been monitored (Fölster and others 2014) over time scales (decades) relevant for ecological and evolutionary adaptation (Thompson 1998).

Pesticide (including insecticides, fungicides and herbicides) runoff from agricultural fields can adversely affect diversity (Tilman and others 2002), functioning and ecosystem services in freshwaters (Vörösmarty and others 2005, 2010; Weatherhead and Howden 2009). Pesticides can decrease the fitness of non-target aquatic organisms (Beketov and Liess 2008) by altering their enzyme activity and metabolism (Sturm and others 2007). They can also alter community structures (Rohr and Crumrine 2005) by increasing mortality of sensitive species (Schroer and others 2004). Herbicides specifically target groups of organisms that carry

out photosynthesis such as phytoplankton (Brock and others 2000). Nevertheless, the long-term effects of herbicides exposure on algae still remain unclear (Schäfer and others 2011). Empirical evidence showed that certain herbicides (for example, Atrazine) can shift the distribution of sensitive species toward more tolerant species and thereby increase community tolerance (Bérard and Benninghoff 2001; Seguin and others 2002). However, the net cost of acquiring tolerance may involve a trade-off, for instance, with production. The most tolerant species may not be the most productive ones (Moe and others 2013; Rizzuto and others 2020). Such trade-offs are generally overlooked (Medina and others 2007).

Here, we used a two-phase experiment to evaluate the significance of EM in influencing the responses of natural phytoplankton from a lake that has been historically exposed to various herbicides that leached from the surrounding agricultural catchment. During the first phase of the experiment, phytoplankton assemblages were germinated (from sediments) and simultaneously conditioned to an herbicide (presence vs. absence of Isoproturon: 12 µg/L), for 17 days. In the second phase of the experiment, communities that were obtained from the previous germination stage were exposed to a broader concentration gradient (0 µg/L, 7 µg/L, 12 µg/L, 61 µg/L, and 92 µg/L) of the same herbicide for 7 days. During the second phase of the experiment, functional endpoints related to production (total biomass and photosynthetic efficiency), and structural characterization of the phytoplankton assemblages (community composition) were monitored. We hypothesized (H1) that the presence of the herbicide (hereafter named conditioning) during germination of phytoplankton originating from the historically contaminated lake yields communities that are more structurally resistant and able to maintain a higher production under stress. The underlying assumption is that conditioning facilitates the recruitment of tolerant species (Kraft and others 2015), which were selectively favored by previous stress episodes. The selection process of tolerant species is captured by the PICT concept (Blanck 2002), whereas EM (Padisak 1992) adds a temporal dimension to the process and emphasizes the persistence of tolerant species over time. During non-stress periods, tolerant species might lose their advantages to more competitive non-tolerant species (Tilman 1982), but can still be present in seed banks and brought back during unfavorable conditions. To contrast with H1, the same conditioning and exposure procedures were applied to phytoplankton assemblages that were germinated from lake sediments of a near-pristine, forested catchment, that had no historical exposure to the herbicide and therefore potentially lacked tolerant species and were potentially more vulnerable. In this case, we hypothesized (H2) that conditioning is ineffective in yielding a more productive and structurally resistant community, due of the lack of EM and this can increase their sensitivity to the second herbicide exposure.

Materials and Methods

Sediment Collection

During August 2017, sediment was collected with a corer from two Swedish lakes that mainly differed in their catchments. The lakes have similar ambient climate, physical and chemical characteristics (trophic status, water depth and submerged aquatic macrophytes consisting mostly of *Myriophyllum* genus [watermilfoil]; Text S1, Figure S1). Finnsjön (60° 21′ 45.1″ N, 17° 52′ 56.1″ E,) is a near-pristine lake with a forest dominated catchment, and Tåkern (58° 21′ 07.0″ N, 14° 49′ 42.7″ E) is a historically contaminated lake that drains from an area associated with intensive large-scale agricultural use (Text S2, Table S1). At least 15 different cores were collected from each lake. The upper oxic layer (ca. 5 cm) from the sediment cores was carefully sectioned and temporarily stored in a cooler. Once in the laboratory, sediment samples from the same catchment were mixed to obtain an aggregated seed bank, then sieved (mesh size of 5 mm) to remove large materials (stone, roots and debris) and stored in the dark at 4 °C until the start of the experiment.

Model Stressor and Pilot Study

The phenylurea herbicide Isoproturon was selected as a model stressor as it was commonly used and previously analyzed in the catchment of the historically impacted lake (Table S1). Isoproturon was widely used, for its inhibitory properties that disrupt the electron transport in photosystem II by binding to the protein D1 in the thylakoid membrane (Arnaud and others 1994), until its ban from use in the European Union in 2016. The concentrations of Isoproturon applied during the experiment were determined using an eco-toxicological test for growth inhibition (OECD guideline, Test No. 201) using laboratory-cultured algae (*Pseudokirchneriella subcapitata,* recently revised and renamed to *Raphidocelis subcapitata* (Suzuki and others 2018)) and phytoplankton community

assemblages from the two selected lakes. Based on the results of the growth inhibition test (Text S2, Figure S2), four concentration levels were selected: 7, 12, 62 and 92 μg/L causing approximately 5, 10, 70 and 90% growth inhibition, respectively. During the first phase of the experiment, a single Isoproturon concentration (12 μg/L) was used for conditioning, while four increasing levels (L1: 7, L2: 12, L3: 61 and L4: 92 μg/L) of the same herbicide were used during the second phase of the experiment. The stability of Isoproturon was assessed during the experiment using liquid chromatography mass spectrometry (Text S3). The measured Isoproturon concentrations varied moderately between the replicates of the different tested concentrations (Table 1). The difference between the nominal and measured concentrations on average varied by 26% (Table 1).

Experimental Details

Phase I: Germination and conditioning phase

Phytoplankton communities were germinated from sediments in bioreactors (Figure 1). Bioreactors were divided into two equal sets. In one set, germination and conditioning to the presence (+) of sub-lethal concentrations of Isoproturon (12 μg/L) occurred, whereas in the other set, Isoproturon was absent (–). Each unit (bioreactor) was replicated 5 times, resulting in a total of 20 germination microcosms. Well-homogenized subsamples of 3 mL sediment were transferred into 250 mL glass jars (total 20). The glass jars (250 mL) containing the sediment were covered with steel woven nets (mesh size 60 μm) and were carefully placed into larger glass flasks (2.2L, Ikea, Sweden) before slowly adding 1.4L of Z8 medium (phosphate concentration of ca. 60 μg/L) to the bioreactors. The steel net was used as a barrier for zooplankton grazers to prevent them from reaching the outer flask, if they emerged from resting stages. Once the bioreactors were filled with culture media, they were sealed with acrylic clip-lock caps fitted with two air inlets (glass tubes) and an air outlet to facilitate resuspension of algae by gently bubbling filtered (0.2 μm, Whatman, UK) air in the flasks for 3 min at regular intervals of 15 min. Isoproturon (Sigma-Aldrich, US) spiking solutions were prepared using dimethyl sulfoxide (DMSO) as a carrier solvent. Conditioning to Isoproturon during germination was achieved by pipetting 20 μL of a DMSO solution containing 16.8 μg Isoproturon to half of the bioreactors, to reach a final concentration of 12 μg/L. Germination occurring in the absence of the Isoproturon (non-conditioned) was achieved by adding an equivalent volume of solvent only (DMSO) to the other half of the bioreactors, so as to rule out possible solvent-related effects. The bioreactors were kept overnight at 4 °C. The following day the temperature was programmed to increase at an approximate rate of 0.5 °C/hour to reach 13 °C. Once the ambient temperature was reached, a diel light cycle was applied (light/dark: 16/8 h, 30 μmol photons m^{-2} s^{-1} light irradiance). The germination phase lasted for 17 days, and at the end, the replicates were bulked according to the four experimental units (Figure 1). Bulking of replicates according to the four different germination scenarios was deemed necessary as it gives the opportunity to standardize the starting conditions for phase II.

Phase II: Isoproturon exposure

In the second part of the experiment, subsamples from the bulk of each individual treatment were inoculated in Erlenmeyer flasks and exposed to an increasing gradient of Isoproturon levels (L1–L4) and a control (0 μg/L), each in triplicate (total of 60 experimental units; Figure 1). The inoculum was standardized using chlorophyll-a concentrations measured as in vivo fluorescence. The inocula were diluted with freshly prepared Z8 medium to reach a

Table 1. Comparison of the Nominal and Measured Concentrations, Expressed as Time Weighted Mean, of Isoproturon During the Two Different Phases of the Experiment.

Phase	Lake	Nominal concentrations (μg/L)	Time-weighted mean concentrations (μg/L)
I	Near-pristine	12	17.25
	Historically contaminated	12	17.81
II	Near-pristine	7	9.19
	Historically contaminated	7	11.22
	Near-pristine	61	46.94
	Historically contaminated	61	54.67

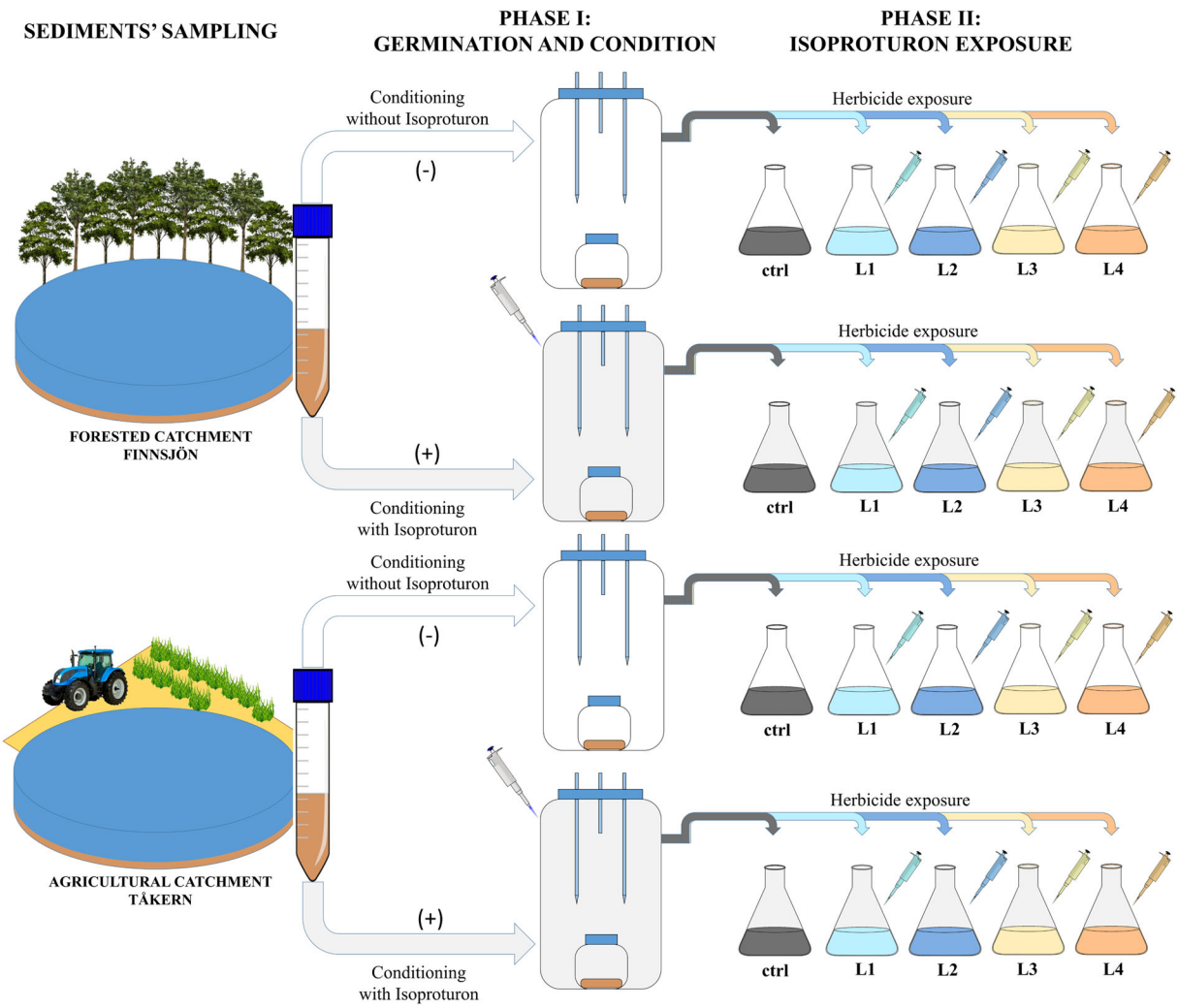


Figure 1. Conceptual figure illustrating the experiment design and the workflow divided into two phases: germination and exposure. During the first phase of the experiment, phytoplankton were germinated from seed banks while a subset (half) of the culture units was simultaneously conditioned with a sub-lethal concentration of Isoproturon (12 µg/L). The communities obtained at the end of the germination phase were exposed to an increasing exposure concentration of Isoproturon (Ctrl: 0, L1: 7, L2: 12, L3: 61 and L4: 92 µg/L).

final volume of 300 mL using the following dilution scheme: near-pristine (–) 1:5, near-pristine (+) 1:7, historically contaminated (−) 1:8, historically contaminated (+) 1:8. The standardized inocula were exposed to the Isoproturon gradient (L1–L4). The exposure phase lasted for 7 days.

Phytoplankton Responses: Growth Rate, Total Biomass, Species Composition and Photosynthetic Efficiency

The growth of phytoplankton was monitored daily during both phases using in vivo fluorescence, which was measured using a spectrophotometer (BioTek Synergy MX; Winosky, VT, USA). Triplicates from each sample (300 µL in each well) were loaded on 96 well clear flat-bottomed microplates (Corning, USA). Fluorescence was analyzed using the integrated software Gen 5 (BioTek, US) with excitation and emission wavelengths of 440 nm and 685 nm, respectively. The growth rate µg (d^{-1}) was calculated as the slope of a linear regression for log-transformed in vivo fluorescence data against time (Hagman and others 2019).

Samples were collected for species taxonomic identification at the end of both phases. They were analyzed from the bulked samples of phase I and from each replicate from phase II. The bulked samples of phase I were analyzed to downscale the effort used to identify species while providing adequate information to: (a) to evaluate differences

across the two lakes and the effects of conditioning; and (b) help to characterize the starting community composition of the second phase of the experiment. Phytoplankton were identified using the Utermöhl technique (protocol: CEN–EN 15,204) and a light microscope, generally to the lowest taxonomic level (species). Total biomass (mg/m^3) was calculated from geometric conversions based on a standard protocol (CEN—EN 16,695). Three diversity matrices were derived from the taxonomy data: species richness, Shannon diversity index and evenness.

The effects of Isoproturon on photosynthetic efficiency (maximum quantum yield (F_v/F_m) of photosystem II) were quantified by using a modified procedure developed by Hanelt (1998), that involved measuring in vivo chlorophyll-a fluorescence by means of a portable pulse-amplitude modulated fluorometer (Water-PAM, Walz, Germany) and the software (WinControl, Walz) provided by the manufacturer. Prior to each measurement, the samples (aliquots of less than 10 mL) were incubated in the dark for 3 min. Thereafter, the minimum fluorescence F_o was determined by applying weak red light pulses, followed by short pulses (0.6 s) of strong saturating light to record maximum florescence F_m. F_v was calculated as the difference between the maximum and minimum fluorescence ($F_v = F_m - F_o$), where the yield (F_v/F_m) was indicative of the physiological status of the communities. The photosynthetic efficiency was measured at three different time points (day 1, 3 and 7) during phase II of the experiment.

Statistical Analysis

All analyses were conducted using R (version 3.5.1) statistical software (R Core Development Team 2015).

Phytoplankton Growth Rate, Total Biomass, Species Diversity Matrices

The growth rate, total biomass, species richness, Shannon diversity index and evenness of phytoplankton were analyzed using linear regression models. During phase I, the effects of two main factors: contamination history (2 groups: near-pristine lake vs. historically contaminated lake) and conditioning (2 groups: presence and absence of Isoproturon during germination) were tested. During phase II, the effects of the following three factors: contamination history (2 groups: near-pristine lake vs. historically contaminated lake), conditioning (2 groups: presence and absence of Isoproturon during germination) and Isoproturon exposure (5 groups: control, L1–L4) were tested. Log transformation was used in some cases to fulfill the assumptions of normality. The interaction terms between the main factors were considered important for inference. When significant main effects were detected, pairwise comparisons based on estimated marginal means were used. Pairwise comparisons were complemented with effect sizes in some cases to assess the magnitude of treatment effects. Effect sizes based on Cohen's *d* values were calculated by taking the mean difference between two groups that was then divided by the pooled standard deviation (Cohen 2013).

Photosynthetic Efficiency

Time series data for the photosynthetic efficiency measurements were analyzed using a repeated measurement analysis of variance (ANOVA). Huynh–Feldt correction was applied when the assumptions of sphericity were breached. The photosynthetic efficiency data recorded during the last sampling (day 7) point were analyzed using a linear model to assess if the effects of the Isoproturon exposures of phase II were significant.

Phytoplankton Community Composition and Structural Resistance

Multivariate analyses were used to evaluate the effects of the Isoproturon exposure (phase II) on the phytoplankton community composition and measure structural resistance. Non-metric multidimensional scaling (NMDS) based on Bray–Curtis similarity and square-root-transformed species matrix data obtained from the taxonomic analysis was used to assess the effect of Isoproturon exposure. NMDS analyses were complemented with permutational multivariate ANOVA using Bray–Curtis similarity matrix, with 9,999 unrestricted permutations and applying Monte Carlo p-values corrections. Structural resistance was calculated using a similar method as previously described by Hillebrand and others (2018) that is based on geometric distance. Structural resistance was derived as Euclidean distance between the centroid coordinates of the control, relative to those of Isoproturon exposures levels (L1–L4). The centroid coordinates were extracted from the NMDS plots. The closer the distances between phytoplankton communities of the control and the respective Isoproturon exposure levels (L1–L4), the higher the resistance.

Results

Comparing Phytoplankton Communities from the Two Lakes and Assessing the Influence of Conditioning (Phase I)

Germination started a few days earlier for the historically contaminated lake compared to the near-pristine lake (Figure S3); however, both lakes achieved similar growth rates (Figure 2A). The earlier initiation of phytoplankton growth from the historically contaminated lake subsequently led to a higher total biomass compared to assemblages that originated from the near-pristine lake ($F_{1,8} = 230.08$, $p < 0.001$, Figure 2B). Species richness (Figure S4A), Shannon diversity index (Figure 2C) and evenness (Figure S4B) of phytoplankton assemblages from the historically contaminated lake were substantially higher than the near-pristine lake (Table S2 and Figure S5). The species composition also differed between lakes (Figure 2D), and the proportion of Chrysophyceae was considerably higher in the near-pristine lake, whereas Chlorophyceae were the most abundant algae group in both lakes.

The effects of conditioning (that is, the presence of Isoproturon during germination) led to a decrease in Shannon diversity index ($F_{1,8} = 32.90$, $p < 0.01$) and evenness ($F_{1,8} = 15.35$, $p < 0.05$) in both lakes. In contrast, the effects of conditioning on the growth rate and species richness were negligible. Conditioning had a marginal effect on the total phytoplankton biomass ($F_{1,8} = 1.70$, $p > 0.05$), but appeared to significantly interact with the contamination history ($F_{1,8} = 9.68$, $p < 0.05$, Table S2). For instance, conditioning had a pronounced negative effect (Cohen' $d = 3.89$) on the total phytoplankton biomass of the historically contaminated lake, whereas the opposite trend was observed for the near-pristine lake (Cohen' $d = 2.1$ and Figure 2B). Besides total biomass, significant interaction between conditioning and contamination history was not observed for growth rate, species richness, Shannon diversity and evenness (Table S2). The species composition (Figure 2D) was marginally affected by conditioning.

Effects of Isoproturon Exposure Gradient on Growth Rate, Total Biomass and Species Diversity (Phase II)

Exposure to the Isoproturon gradient (L1–L4) during the second phase of the experiment signif-

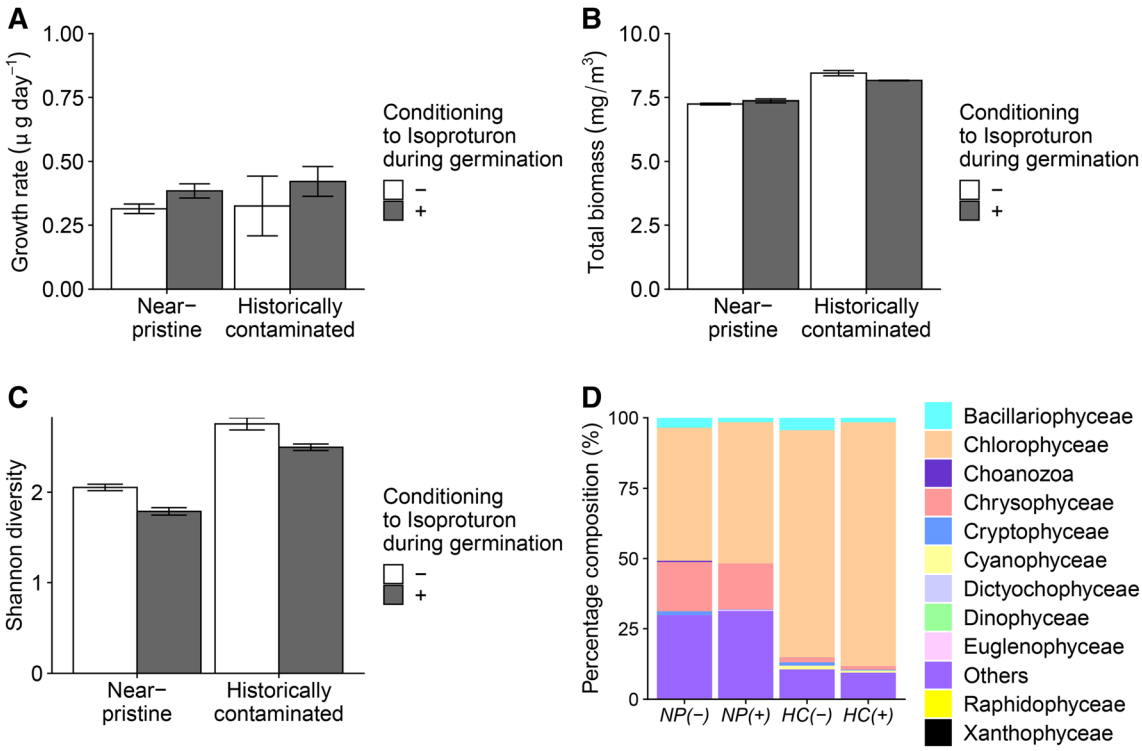


Figure 2. Effects of conditioning, during the first phase of the experiment, on growth rate (**A**), total biomass (**B**), Shannon diversity (**C**) and the relative proportion of phytoplankton groups (**D**) in the communities originated from the two lakes (NP: near-pristine, HC: historically contaminated). Error bars when present indicate standard error.

Table 2. Summary of the Effects of the Contamination History, Conditioning, Isoproturon Exposure and the Interaction Term Between the Three Factors on Different Endpoints; Growth Rate, Total Biomass, Species Richness, Shannon Diversity and Evenness of Phytoplankton Recorded During the Second Phase of the Experiment.

Variables	Effects	*df*	SS	*F*	*p*
Growth rate	**Isoproturon exposure**	**4, 60**	**2.09**	**89.25**	**< 0.001**
	Conditioning	1, 60	0.01	2.27	0.14
	Contamination history (Con his.)	**1, 60**	**0.09**	**14.92**	**< 0.001**
	Isoproturon exposure: Conditioning	4, 60	0.01	0.61	0.66
	Isoproturon exposure: Con his	4, 60	0.01	0.34	0.85
	Conditioning: Con his	**1, 60**	**0.03**	**4.88**	**< 0.05**
	Isoproturon exposure: Conditioning: Con his	4, 60	0.02	0.76	0.55
Total biomass	**Isoproturon exposure**	**4, 60**	**19.89**	**149.98**	**< 0.001**
	Conditioning	**1, 60**	**0.43**	**13.00**	**< 0.001**
	Contamination history (Con his.)	**1, 60**	**1.81**	**54.46**	**< 0.001**
	Isoproturon exposure: Conditioning	4, 60	0.01	0.10	0.98
	Isoproturon exposure: Con his	**4, 60**	**0.57**	**4.31**	**< 0.01**
	Conditioning: Con his	1, 60	0.001	0.03	0.87
	Isoproturon exposure: Conditioning: Con his	4, 60	0.03	0.22	0.93
Species richness	**Isoproturon exposure**	**4, 60**	**146.83**	3.82	**< 0.05**
	Conditioning	**1, 60**	**93.75**	9.75	**< 0.01**
	Contamination history (Con his.)	1, 60	0.42	0.04	0.84
	Isoproturon exposure: Conditioning	4, 60	79.17	2.06	0.10
	Isoproturon exposure: Con his	**4, 60**	**117.83**	3.06	**< 0.05**
	Conditioning: Con his	**1, 60**	**150.42**	15.64	**< 0.001**
	Isoproturon exposure: Conditioning: Con his	4, 60	46.17	1.20	0.33
Shannon diversity	Isoproturon exposure	4, 60	0.10	1.92	0.13
	Conditioning	**1, 60**	**0.23**	**17.10**	**< 0.001**
	Contamination history (Con his.)	**1, 60**	**0.69**	**51.80**	**< 0.001**
	Isoproturon exposure: Conditioning	4, 60	0.13	2.36	0.07
	Isoproturon exposure: Con his	**4, 60**	**1.44**	**26.77**	**< 0.001**
	Conditioning: Con his	**1, 60**	**0.20**	**14.78**	**< 0.001**
	Isoproturon exposure: Conditioning: Con his	4, 60	0.07	1.36	0.26
Evenness	**Isoproturon exposure**	**4, 60**	**0.02**	**4.03**	**< 0.01**
	Conditioning	**1, 60**	**0.01**	**5.24**	**< 0.05**
	Contamination history (Con his.)	**1, 60**	**0.06**	**44.10**	**< 0.001**
	Isoproturon exposure: Conditioning	4, 60	0.01	1.18	0.33
	Isoproturon exposure: Con his	**4, 60**	**0.09**	**17.75**	**< 0.001**
	Conditioning: Con his	1, 60	0.004	3.23	0.08
	Isoproturon exposure: Conditioning: Con his	4, 60	0.01	1.46	0.23

Significant values are reported in bold.

icantly affected total phytoplankton biomass, growth rate, species richness, and evenness (Table 2, Figure 3). The interaction between Isoproturon exposure and contamination history was significant for total biomass, species richness, Shannon diversity and evenness, whereas interaction between conditioning and contamination history was significant for growth rate, species richness and Shannon diversity. Interactions involving all three factors (Isoproturon exposure, conditioning and contamination history) were not significant for any of measured variables (Table 2).

In addition, pairwise comparisons revealed some general patterns. For instance, exposure to the two highest Isoproturon levels (L3 and L4) led to a significant decrease in growth rate (Figure 4A) and total biomass (Figure 4B) of phytoplankton compared to the control and the low exposure levels (L1 and L2). The decrease in total biomass was more pronounced for the historically contaminated

Table 3. Comparing Community Composition of the Near-pristine and the Historically Contaminated Lakes that Were Exposed to the Isoproturon Concentration Gradient.

Contamination history	Conditioning	Isoproturon exposure	t-value	P-value	Euclidean distance
Near-pristine	**(−)**	L1	0.93	0.46	0.16
		L2	1.37	0.19	0.29
		L3	**2.46**	**0.02**	0.44
		L4	**3.21**	**0.01**	0.38
	(+)	L1	1.32	0.19	0.15
		L2	1.61	0.09	0.26
		L3	**3.51**	**0.004**	0.53
		L4	**4.43**	**0.002**	0.53
Historically Contaminated	**(−)**	L1	1.23	0.25	0.14
		L2	1.66	0.08	0.08
		L3	**4.01**	**0.002**	0.29
		L4	**4.23**	**0.002**	0.33
	(+)	L1	1.10	0.34	0.18
		L2	0.88	0.48	0.23
		L3	**3.91**	**0.002**	0.32
		L4	**3.98**	**0.004**	0.20

The Euclidean distance between the control and the different exposure levels (L1, L2, L3 and L4) centroids based on the NMDS plots, across the two lakes and the two different conditioning scenarios. Significant results are highlighted in bold.

lake (Cohen's d values; Table S3) when exposed to the highest levels of Isoproturon (L3-L4; Figures 3B, 4B). The effects of conditioning on growth rate varied across the different Isoproturon exposure levels for the historically contaminated lake, whereas conditioning generally led to a slight increase in growth rate of phytoplankton for the near-pristine lake (Figures 3A, 4A). Nevertheless, growth rates of phytoplankton from the historically contaminated lake were consistently higher for the two highest exposure levels (L3 and L4) compared to the near-pristine lake (Figure 4A). The magnitude of change in the growth rate was also consistently higher (larger Cohen's d values between the control and the Isoproturon exposure levels L1–L4) for the near-pristine lake compared to the historically contaminated lake (Table S3). The highest exposure levels (L3 and L4) reduced species richness only for communities of the near-pristine lake (Figure S6), that were conditioned to Isoproturon during germination. Shannon diversity and evenness of the historically contaminated lake increased with increasing herbicide levels, while the opposite was observed for the near-pristine lake (Figures 3C,D, 4C, D).

Effects of Isoproturon Exposure Gradient on Photosynthetic Efficiency (Phase II)

Exposure to the Isoproturon concentration gradient significantly decreased the photosynthetic yield of the phytoplankton of both lakes (near-pristine lake; $F_{4,\ 20} = 51.4$, $p < 0.01$, historically contaminated lake; $F_{4,\ 20} = 15.2$, $p < 0.01$ and Figure 5). Furthermore, significant time effects and the interaction between time and Isoproturon exposure were observed in both lakes (Table S4). The relative decrease in the photosynthetic yield was generally stronger at higher Isoproturon exposure levels (L3–L4). The changes in photosynthetic yield over time were remarkably distinct between the two lakes and the conditioning scenarios (Figure 5), in particular for the last time point (day 7). The effects of the herbicide were still significant in the near-pristine lake on the last sampling event (day 7; Table S5). Furthermore, the differences between the control and the two highest levels (L3 and L4) of the near-pristine phytoplankton assemblages were larger when they were conditioned to the herbicide (L3: $t = -9.6$, $p < 0.01$, L4: $t = -11.8$, $p < 0.01$) compared to the non-conditioned scheme (L3: $t = -0.25$, $p = 0.8$, L4: $t = -3.7$, $p < 0.01$). In the historically contaminated lake, the effects of the Isoproturon exposure on day 7 (Table S5) were still significant

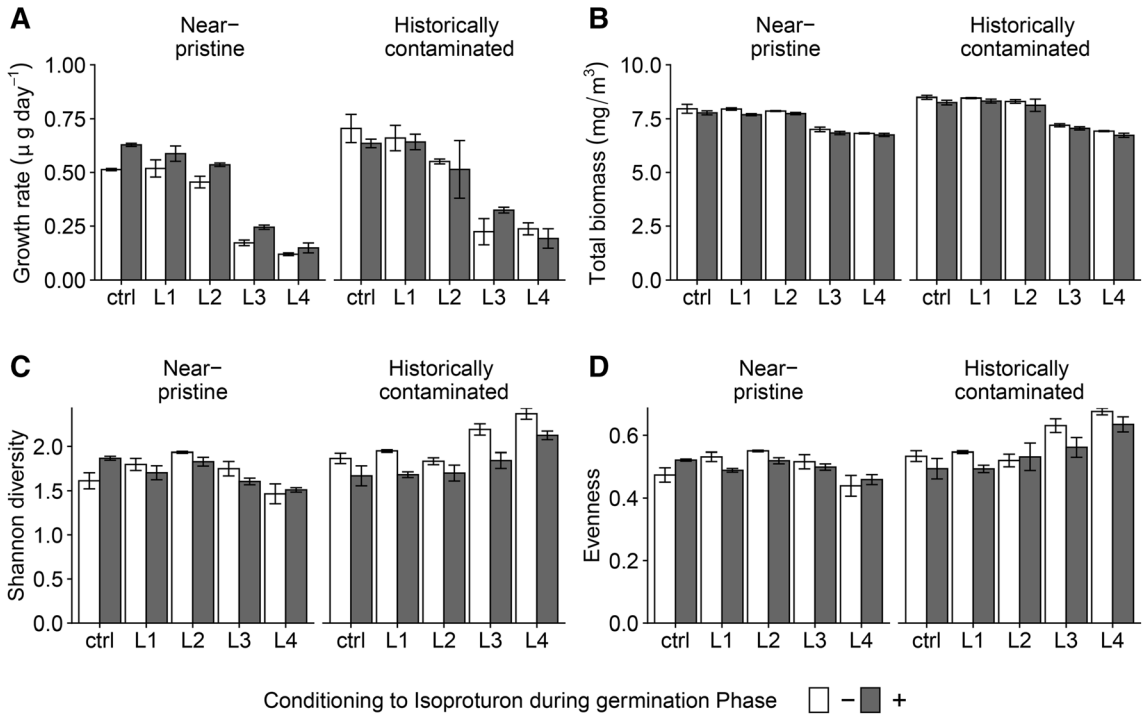


Figure 3. Comparison of growth rate (**A**), total biomass (**B**), Shannon diversity index (**C**) and evenness (**D**) of phytoplankton originating from the near-pristine and the historically contaminated lake, along the Isoproturon exposure gradient (L1–L4) used during the second phase of the experiment. Error bars indicate standard error.

($F_{4,15} = 7.91$, $p < 0.01$) when conditioning was omitted during germination.

Effects of Isoproturon Exposure Gradient on Community Composition (Phase II)

The composition of communities changed significantly (PERMANOVA: Table S6) in response to Isoproturon exposure. The relative proportions of most taxa decreased with increasing exposure levels (Figure 6A), apart from two groups (Chrysophyceae and unidentified group (others)) that increased in relative proportion at the two highest herbicide treatment levels.

Changes in community composition, depicted by the non-metric multidimensional scaling (NMDS) analyses (Figure 6B), were most pronounced at the highest exposure levels and reflected both the contamination history and conditioning during germination. Phytoplankton communities from the historically contaminated lake that underwent conditioning displayed higher structural resistance to the Isoproturon exposure. The distance between the control and the highest Isoproturon exposure level (L4) was shortened (Table 3, Figure 6B) when conditioning was applied during germination compared to the non-conditioned analogs. The opposite was observed for the communities originating from the near-pristine lake system with regard to conditioning (Table 3, Figure 6B).

Discussion

This study provides a proof of concept of the relevance of EM in influencing ecosystem stability during the resurgence of a recurrent stressor. In particular, we focused here on conditioning as a trigger for retrieving adaptations nested in EM that may not be expressed in the community assemblages during periods of non-stress. We took advantage of lakes with different herbicide exposure histories to obtain communities with and without ''memory'' of Isoproturon stress to test our hypotheses. However, it is important to acknowledge that beyond historical exposure to herbicides, the lakes also differ in other respects (for example, differences in catchment characteristics, level of connectedness with other systems and so on). To rule out confounding effects posed by these factors, we focused on studying EM to a specific stressor by ''retrieving'' EM for Isoproturon through conditioning (that is, by applying the same stressor during germination). Conditioning was used as a means to facilitate the recruitment of tolerant strains/species that were already present in dor-

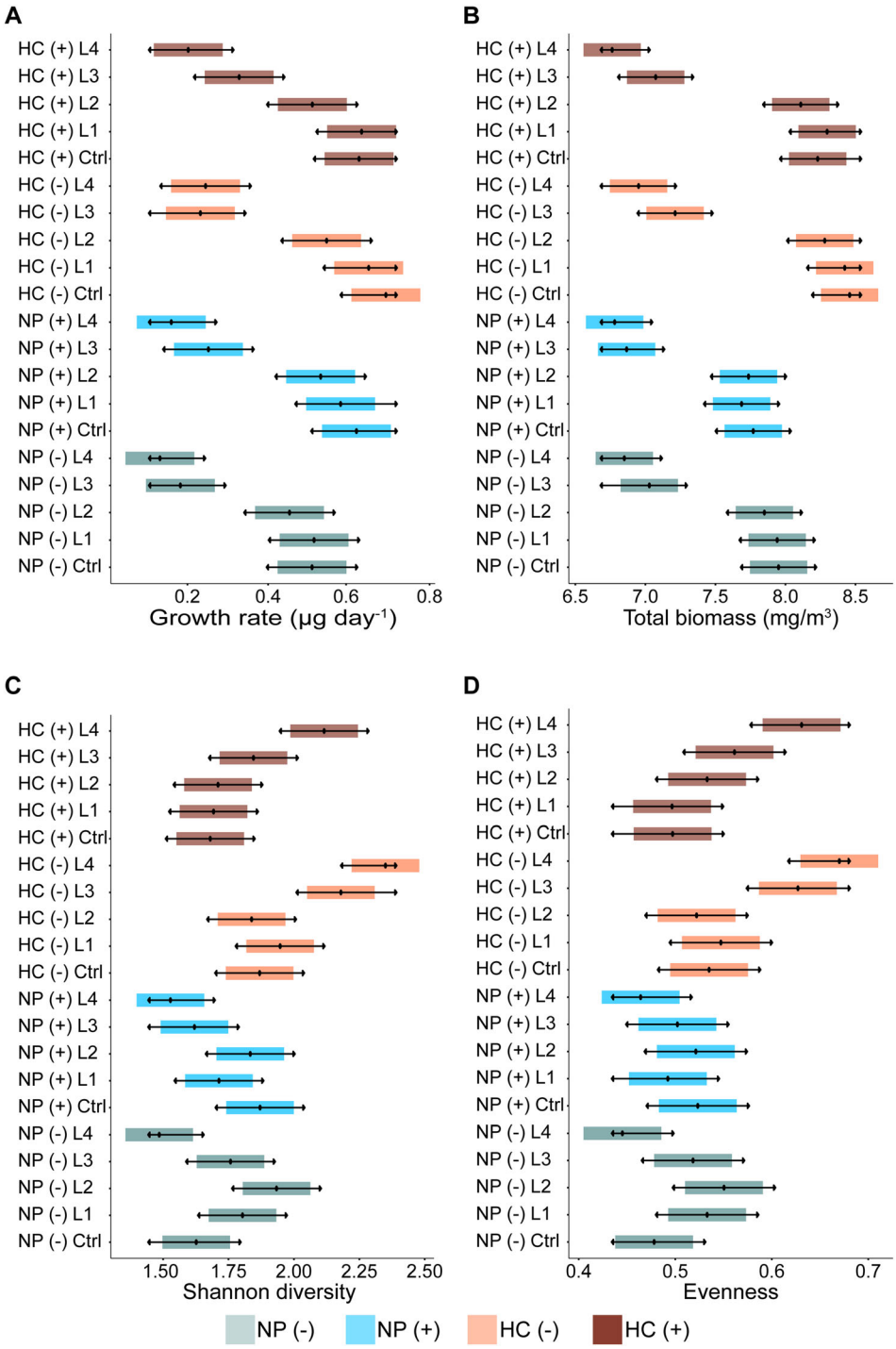


Figure 4. Pairwise comparison based on estimated marginal means for the growth rate (**A**), total biomass (**B**), Shannon diversity index (**C**) and evenness (**D**) of phytoplankton observed during the second phase of the experiment. The central points in the figure indicate the mean response with 95% confidence interval for the combined main effects (contamination history, conditioning, Isoproturon exposure) for the near pristine (NP) lake and the historically contaminated (HC) lake that were conditioned with (+) and without (−) Isoproturon during germination.

mant stages in the sediment of the historically contaminated lake. The experiment with phytoplankton communities from the near-pristine lake should therefore not be considered as a direct term of comparison but as a means to refute H1, and therefore test its robustness. Phase I of the experi-

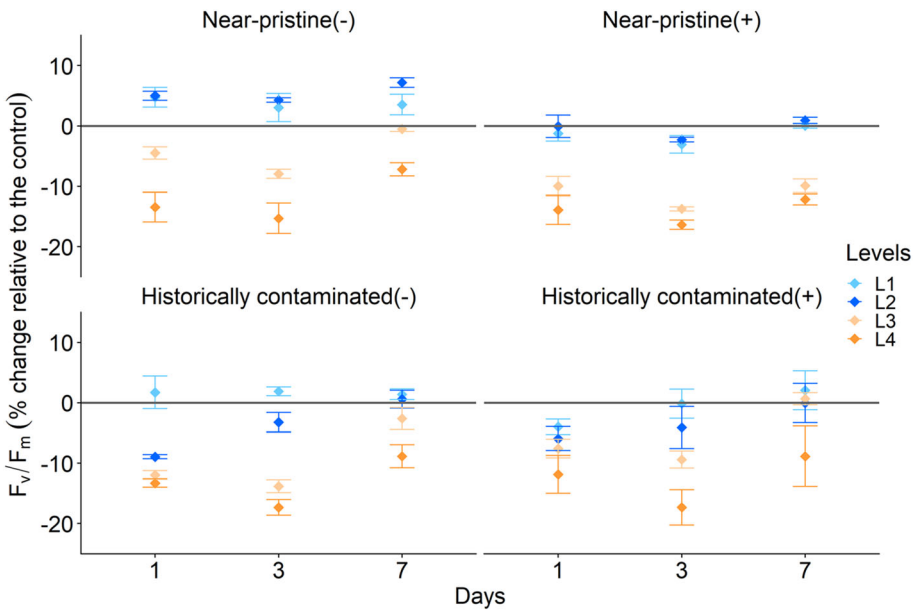


Figure 5. Photosynthetic efficiency (Fv/Fm): Percentage of change relative to control in the near-pristine and historically contaminated phytoplankton communities that were conditioned with (+) and without (−) Isoproturon during germination. Error bars indicate standard error.

mental design was conceived to assemble natural communities harboring different genotypes from two lakes that differed in contamination histories. During phase II, EM is evaluated by assessing the ability of phytoplankton assemblages from the historically contaminated lake to maintain high production and stable composition under the Isoproturon gradient.

The results from phase II of the experiment support our expectations that conditioning of the community from the historically contaminated lake facilitates the recruitment of tolerant species that had acquired adaptation in the past. Phytoplankton assemblages from the historically contaminated lake, upon conditioning, were in fact able to restore photosynthetic efficiency (Figure 5) and proved to be structurally (Figure 6 and Table 3) more resistant when exposed to an increasing gradient of Isoproturon. At the same time, conditioning did not lead to the same effect in the near pristine lake (Figures 5, 6 and Table 3).

Implications of EM for Production: Total Biomass and Photosynthetic Efficiency

Total biomass and photosynthetic efficiency were monitored during the second phase of the experiment. The behavior of the two parameters differed markedly and was dependent on the lake contamination background and conditioning. Conditioning did not increase the total phytoplankton biomass (Figure 3B) irrespective of the lakes' contamination history. In contrast, photosynthetic efficiency was restored within a few days, despite being impacted in the earlier stage of the second phase of the experiment (Figure 5), while Isoproturon was still present (Table 1). However, such an improvement in restoring photosynthetic efficiency was only observed in phytoplankton assemblages that originated from the historically contaminated lake following conditioning during gemination (Figure 5). The contrasting responses between photosynthetic efficiency and biomass development can reflect differences in the temporal scales of these processes (Kriegman and others 2018). Physiological responses, such as photosynthetic efficiency, occur more rapidly and most likely reflect changes in the regulation of photosystem reaction centers (Antonacci and others 2018). This type of response is expected for organisms exposed to Isoproturon, as this herbicide is a photosystem II inhibitor (Antonacci and others 2018). Herbicide-resistance has been shown to occur following a substitution mutation that altered structure of the targeted intracellular site, for example, the D1 protein of Photosystem II (Antonacci and others 2018). The improvement in photosynthetic efficiency might be linked to an increase in the prevalence of species/strains that express a mutation of the D1 protein of the photosystem reaction center to counteract the inhibitory effects of Isoproturon (Antonacci and others 2018). In contrast

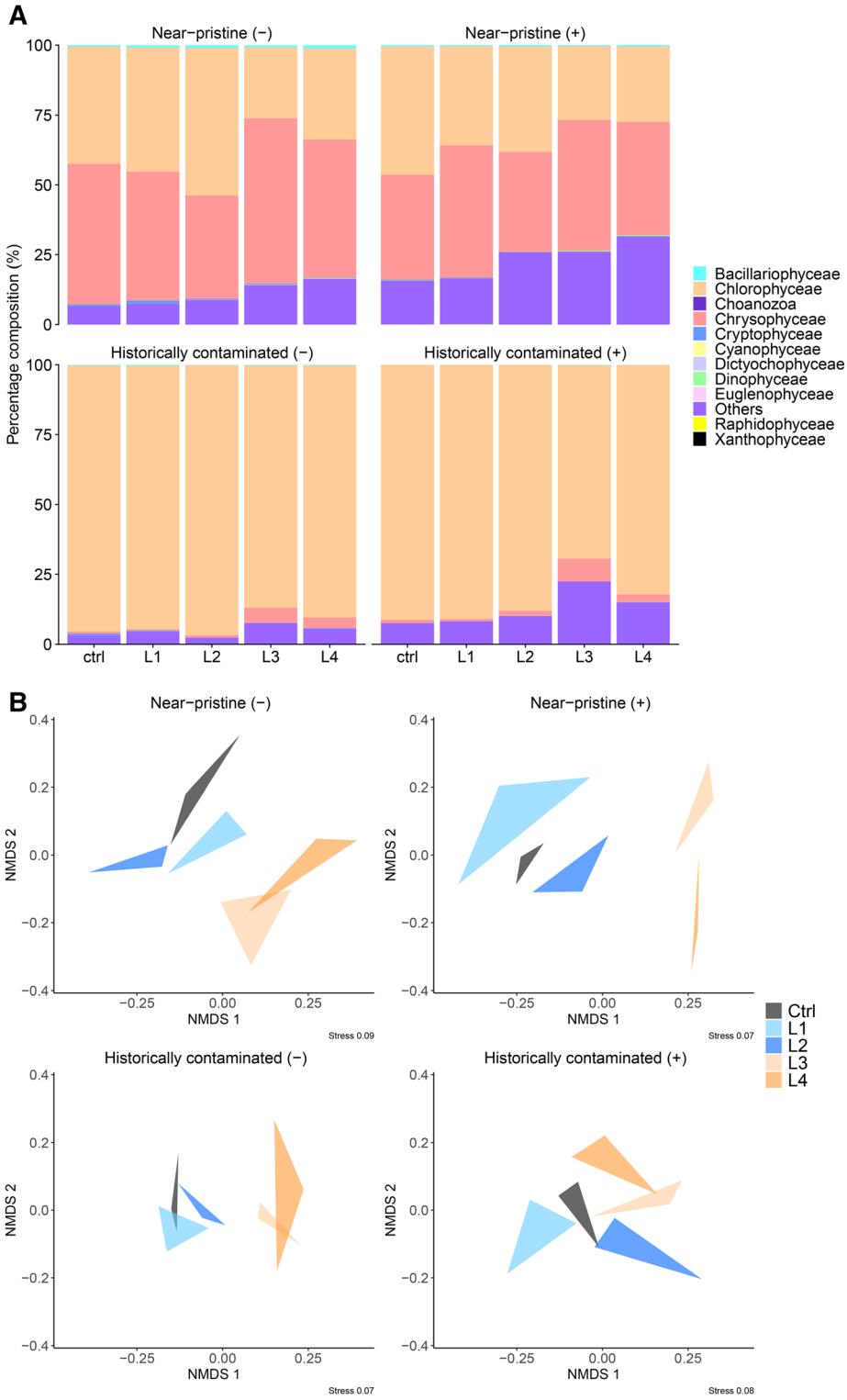


Figure 6. Changes in community structure observed during the exposure phase. (**A**) Percentage composition (relative abundances) of the major phytoplankton groups identified in the two lake (near-pristine and historically contaminated) conditioned without (−) and with (+) Isoproturon (**B**) non-metric multidimensional scaling plot (NMDS) from the four experimental units. The corners of the polygons represent the replicates.

to photosynthetic efficiency, biomass development may reflect changes in energy and resource allocation within organisms that might occur at a slower pace (van Straalen and Hoffmann 2000). This time lag might explain the lack of higher total biomass mediated by EM (Tilmon 2008; Hertz and others 2013), perhaps also due to the relatively short duration (7 days) of the second phase the experiment.

Implications of Ecological Memory for Structural Resistance

Following conditioning, the community composition of phytoplankton from the historically contaminated lake displayed a higher structural resistance (Figure 6 and Table 3), compared to the non-conditioned ones. Here, we observed that the distance between the centroids of the control and Isoproturon exposure levels (especially L4) for the phytoplankton communities decreased when conditioning was applied to phytoplankton from the historically contaminated lake. These results support our initial hypothesis (H1) concerning the structural stability aspects and suggest that phytoplankton communities that have been previously exposed to a stressor can boost their capacity to cope with subsequent encounters to the same stressor (Johnstone and others 2016). Such an increase in structural resistance was not observed in either the conditioned or the non-conditioned communities from the near-pristine lake, thus supporting H2. Regardless of conditioning, phytoplankton from the near-pristine lake showed weaker structural resistance (Figure 6B, Table 3). In this case, the distance between the centroids of controls and the two highest treatment levels (L3 and L4; Figure 6B, Table 3) actually increased as a consequence of conditioning, suggesting an increase in community sensitivity.

Moreover, the benefits of ecological memory in promoting structural resistance might involve a fundamental trade-off with the ability to maintain high biomass production (Vinebrooke and others 2004). Our results only provide some evidence of such a trade-off under a short-term designed experiment. For instance, the communities from the historically contaminated lake had a higher growth rate than the near-pristine lake (Figure 3) for the two highest treatment levels, but this did not result in larger biomass; this may relate to the occurrence of a trade-off. Similar trade-offs (that is the negative relationship between acquiring tolerance and building biomass) were observed by others (Coley and others 1985; Strauss and others 2002; Boivin and others 2003; Vila-Aiub and others 2009) as the most tolerant species might not necessarily be the most productive ones (Moe and others 2013; Rizzuto and others 2020). Further research is needed to fully elucidate trade-offs in the context of EM and over longer time scales.

Long-term herbicide exposure can favor the selection of tolerant strains (Schäfer and others 2011) or species that could be stored and eventually retrieved from seed banks. The selection of tolerant species can occur through ecological adaptation. Ecological adaptation acknowledges the replacement of sensitive species with tolerant ones (that is, pollution-induced community tolerance [PICT]; Blanck 2002), thereby helping to maintain key processes and structures. Previous empirical evidence showed that certain herbicides can shift the distribution of sensitive species toward more tolerant species and thereby increase the community tolerance (Bérard and Benninghoff 2001; Seguin and others 2002). In the present study, the hypothesis of an increase in the prevalence of tolerant species after conditioning was supported by the observed increase in Shannon diversity and evenness along the herbicide exposure gradient, which only occurred in phytoplankton assemblages of the historically contaminated lake (Figures 3C, D, 4C, D). Changes in evenness have previously been shown to be a more robust indicator of ecological change than species richness (Hillebrand and others 2008), which is in line with our findings.

Altogether, these results indicate that: (a) acquired structural resistance by the community is a consequence of retrieving EM through conditioning; and (b) EM is an attribute only of the systems that had historically been exposed to Isoproturon or other herbicides with a similar mode of toxic action. Since EM was retained in dormant stages, the role of conditioning was crucial in facilitating the recruitment of tolerant species, which were selectively favored by previous stress episodes.

These results complement previous findings and provide new insights on the concept of EM. Hughes and others (2019) described beneficial effects of EM in coral reefs exposed to two successive heat wave events causing bleaching. The authors showed that the mortality of coral reefs decreased after the second heat episode (in 2017) compared to the first one (in 2016), where the first event increased the proportion of resistant species (Hughes and others 2019). In another study, Feckler and others (2018) showed that the performance of microbial communities in decomposing leaf-litter from an agricultural stream was enhanced under exposure to

pesticides compared to a community from a near-pristine stream. In addition to earlier assessments, our results strengthen the perception that previous encounters with a stressor matter (Samani and Bell 2016) and can increase structural resistance, even when these adaptations are not present or prevalent in the standing crop community.

Conclusion

We used an experimental approach to test an ecological concept (EM) that has previously remained elusive to empirical testing. We showed that adaptations from past experience that are present in dormant phytoplankton stages in lake sediments can be readily expressed when stressors reappear. Our results show the beneficial effects of EM in restoring certain processes (for example, photosynthetic efficiency) and increasing structural resistance of the phytoplankton communities. However, they also indicate a trade-off between resistance and other processes related to biomass production. The lack of EM to herbicide in the phytoplankton from the near-pristine lake system did not measurably yield stress tolerant species and structurally resistant communities. In addition, other processes related to spatial distribution and dispersal of species (for example, dispersal of metacommunities: Leibold and others 2004) can also influence ecosystems and contribute to stability toward the stressors. Better knowledge of these temporal (EM) and spatial (dispersal of metacommunities) processes are essential to gain a deeper understanding of the abilities of ecosystems to cope with recurrent stress.

Acknowledgements

The authors wish to thank Per Færøvig from the Section of Aquatic Biology and Ecotoxicology, University of Oslo for helping with the experimental setup. The authors thank: Vladyslava Hostyeva and Petra Mutinova from Culture Collection of Algae (NIVA-CCA) of the Norwegian Institute for Water Research (NIVA) for preparing the nutrient medium used during the experiment; and the personnel from the visitor centre of Tåkern Naturum for lending us their boat during sampling. This study was co-funded by the Research Council of Norway (contract 244460) for the PEACE (Pollution and Ecosystem Adaptation to Changes in the Environment) project and an internal grant from NIVA under the Institute Strategic Priorities (SIS) on ecosystem remediation (TRADE-OFF). SR was supported by the Lancaster University Faculty of Science and Technology and a Lancaster Environment Centre PhD scholarship.

Data Availability

Data are available at: https://doi.org/10.6084/m9.figshare.13554689.v1.

References

Antonacci A, Lambreva MD, Margonelli A, Sobolev AP, Pastorelli S, Bertalan I, Johanningmeier U, Sobolev V, Samish I, Edelman M, Havurinne V, Tyystjärvi E, Giardi MT, Mattoo AK, Rea G. 2018. Photosystem-II D1 protein mutants of Chlamydomonas reinhardtii in relation to metabolic rewiring and remodelling of H-bond network at QB site. Sci Rep 8:14745.

Arnaud L, Taillandier G, Kaouadji M, Ravanel P, Tissut M. 1994. Photosynthesis Inhibition by Phenylureas: A QSAR Approach. Ecotoxicol Environ Saf 28:121–133.

Beketov MA, Liess M. 2008. Potential of 11 pesticides to initiate downstream drift of stream macroinvertebrates. Arch Environ Contam Toxicol 55:247–253.

Bell G. 2017. Evolutionary rescue. Annu Rev Ecol Evol Syst 48:605–627.

Bérard A, Benninghoff C. 2001. Pollution-induced community tolerance (PICT) and seasonal variations in the sensitivity of phytoplankton to atrazine in nanocosms. Chemosphere 45:427–437.

Blanck H. 2002. A critical review of procedures and approaches used for assessing pollution-induced community tolerance (PICT) in biotic communities. Hum Ecol Risk Assess Int J 8:1003–1034.

Boivin T, Bouvier JC, Chadœuf J, Beslay D, Sauphanor B. 2003. Constraints on adaptive mutations in the codling moth *Cydia pomonella* (L.): measuring fitness trade-offs and natural selection. Heredity 90:107–113.

Brock TCM, Lahr J, Van den Brink PJ. 2000. Ecological risks of pesticides in freshwater ecosystems; Part 1: herbicides. Alterra

Cochran WG, Chambers SP. 1965. The planning of observational studies of human populations. J R Stat Soc Ser Gen 128:234–266.

Cohen J. 2013. Statistical power analysis for the behavioral sciences, Revised. New York: Academic Press.

Coley PD, Bryant JP, Chapin FS. 1985. Resource availability and plant antiherbivore defense. Science 230:895.

Collins S, Gardner A. 2009. Integrating physiological, ecological and evolutionary change: a Price equation approach. Ecol Lett 12:744–757.

Donohue I, Hillebrand H, Montoya JM, Petchey OL, Pimm SL, Fowler MS, Healy K, Jackson AL, Lurgi M, McClean D, O'Connor NE, O'Gorman EJ, Yang Q. 2016. Navigating the complexity of ecological stability. Ecol Lett 19:1172–1185.

Ellegaard M, Godhe A, Ribeiro S. 2018. Time capsules in natural sediment archives—tracking phytoplankton population genetic diversity and adaptation over multidecadal timescales in the face of environmental change. Evol Appl 11:11–16.

Feckler A, Goedkoop W, Konschak M, Bundschuh R, Kenngott KGJ, Schulz R, Zubrod JP, Bundschuh M. 2018. History matters: Heterotrophic microbial community structure and function adapt to multiple stressors. Glob Change Biol 24:e402–e415.

Fölster J, Johnson RK, Futter MN, Wilander A. 2014. The Swedish monitoring of surface waters: 50 years of adaptive monitoring. AMBIO 43:3–18.

Hagman CHC, Skjelbred B, Thrane J-E, Andersen T, de Wit HA. 2019. Growth responses of the nuisance algae Gonyostomum semen (Raphidophyceae) to DOC and associated alterations of light quality and quantity. Aquat Microb Ecol 82:241–251.

Hanelt D. 1998. Capability of dynamic photoinhibition in Arctic macroalgae is related to their depth distribution. Mar Biol 131:361–369.

Hertz PE, Arima Y, Harrison A, Huey RB, Losos JB, Glor RE. 2013. Asynchronous evolution of physiology and morphology in anolis lizards. Evolution 67:2101–2113.

Hillebrand H, Bennett DM, Cadotte MW. 2008. Consequences of dominance: a review of evenness effects on local and regional ecosystem processes. Ecology 89:1510–1520.

Hillebrand H, Langenheder S, Lebret K, Lindström E, Östman Ö, Striebel M. 2018. Decomposing multiple dimensions of stability in global change experiments. Ecol Lett 21:21–30.

Hughes TP, Kerry JT, Connolly SR, Baird AH, Eakin CM, Heron SF, Hoey AS, Hoogenboom MO, Jacobson M, Liu G, Pratchett MS, Skirving W, Torda G. 2019. Ecological memory modifies the cumulative impact of recurrent climate extremes. Nat Clim Change 9:40–43.

Johnstone JF, Allen CD, Franklin JF, Frelich LE, Harvey BJ, Higuera PE, Mack MC, Meentemeyer RK, Metz MR, Perry GL, Schoennagel T, Turner MG. 2016. Changing disturbance regimes, ecological memory, and forest resilience. Front Ecol Environ 14:369–378.

Kraft NJB, Adler PB, Godoy O, James EC, Fuller S, Levine JM. 2015. Community assembly, coexistence and the environmental filtering metaphor. Funct Ecol 29:592–599.

Kriegman S, Cheney N, Bongard J. 2018. How morphological development can guide evolution. Sci Rep 8:13934.

Leibold MA, Holyoak M, Mouquet N, Amarasekare P, Chase JM, Hoopes MF, Holt RD, Shurin JB, Law R, Tilman D, Loreau M, Gonzalez A. 2004. The metacommunity concept: a framework for multi-scale community ecology. Ecol Lett 7:601–613.

Medina MH, Correa JA, Barata C. 2007. Micro-evolution due to pollution: possible consequences for ecosystem responses to toxic stress. Chemosphere 67:2105–2114.

Moe SJ, De Schamphelaere K, Clements WH, Sorensen MT, Van den Brink PJ, Liess M. 2013. Combined and interactive effects of global climate change and toxicants on populations and communities. Environ Toxicol Chem Setac 32:49–61.

Ogle K, Barber JJ, Barron-Gafford GA, Bentley LP, Young JM, Huxman TE, Loik ME, Tissue DT. 2015. Quantifying ecological memory in plant and ecosystem processes. Ecol Lett 18:221–235.

Orsini L, Schwenk K, De Meester L, Colbourne JK, Pfrender ME, Weider LJ. 2013. The evolutionary time machine: using dormant propagules to forecast how populations can adapt to changing environments. Trends Ecol Evol 28:274–282.

Padisak J. 1992. Seasonal succession of phytoplankton in a Large Shallow Lake (Balaton, Hungary)—a dynamic approach to ecological memory, its possible role and mechanisms. J Ecol 80:217–230.

Peterson GD. 2002. Contagious disturbance, ecological memory, and the emergence of landscape pattern. Ecosystems 5:329–338.

R Core Development Team. 2015. R: A Language and Environment for Statistical Computing.

Rizzuto S, Thrane J-E, Baho DL, Jones KC, Zhang H, Hessen DO, Nizzetto L, Leu E. 2020. Water browning controls adaptation and associated trade-offs in phytoplankton stressed by chemical pollution. Environ Sci Technol 54:5569–5579.

Rohr JR, Crumrine PW. 2005. Effects of an herbicide and an insecticide on pond community structure and processeS. Ecol Appl 15:1135–1147.

Samani P, Bell G. 2016. The ghosts of selection past reduces the probability of plastic rescue but increases the likelihood of evolutionary rescue to novel stressors in experimental populations of wild yeast. Ecol Lett 19:289–298.

Schäfer RB, van den Brink PJ, Liess M. 2011. Impacts of pesticides on freshwater ecosystems. Ecol Impacts Toxic Chem:111–37.

Scheffer M, Carpenter SR. 2003. Catastrophic regime shifts in ecosystems: linking theory to observation. Trends Ecol Evol 18:648–656.

Schroer AFW, Belgers JDM, Brock CM, Matser AM, Maund SJ, Van den Brink PJ. 2004. Comparison of laboratory single species and field population-level effects of the pyrethroid insecticide λ-cyhalothrin on freshwater invertebrates. Arch Environ Contam Toxicol 46:324–335.

Seguin F, Le Bihan F, Leboulanger C, Bérard A. 2002. A risk assessment of pollution: induction of atrazine tolerance in phytoplankton communities in freshwater outdoor mesocosms, using chlorophyll fluorescence as an endpoint. Water Res 36:3227–3236.

Strauss SY, Rudgers JA, Lau JA, Irwin RE. 2002. Direct and ecological costs of resistance to herbivory. Trends Ecol Evol 17:278–285.

Sturm A, Radau TS, Hahn T, Schulz R. 2007. Inhibition of rainbow trout acetylcholinesterase by aqueous and suspended particle-associated organophosphorous insecticides. Chemosphere 68:605–612.

Suzuki S, Yamaguchi H, Nakajima N, Kawachi M. 2018. *Raphidocelis subcapitata* (=*Pseudokirchneriella subcapitata*) provides an insight into genome evolution and environmental adaptations in the Sphaeropleales. Sci Rep 8:8058.

Thompson JN. 1998. Rapid evolution as an ecological process. Trends Ecol Evol 13:329–332.

Tilman D. 1982. Resource competition and community structure. Princeton University Press.

Tilman D, Cassman KG, Matson PA, Naylor R, Polasky S. 2002. Agricultural sustainability and intensive production practices. Nature 418:671–677.

Tilmon KJ. 2008. Specialization, Speciation, and Radiation. 1st ed. University of California Press. https://doi.org/10.1525/j.ctt1pnq3k

Turner MG. 2010. Disturbance and landscape dynamics in a changing world. Ecology 91:2833–2849.

van Straalen NM, Hoffmann AA. 2000. Review of experimental evidence for physiological costs of tolerance to toxicants. In: Kammenga JE, Laskowski R, editors. Demography in Ecotoxicology. John Wiley and Sons. pp 147–61. https://research.vu.nl/en/publications/ccdfbf36-ee1c-4050-a0ef-e6ed63169b91

Vila-Aiub MM, Neve P, Powles SB. 2009. Evidence for an ecological cost of enhanced herbicide metabolism in *Lolium rigidum*. J Ecol 97:772–780.

Vinebrooke RD, Cottingham KL, Norberg J, Scheffer M, Dodson SI, Maberly SC, Sommer U. 2004. Impacts of multiple stressors on biodiversity and ecosystem functioning: the role of species co-tolerance. Oikos 104:451–457.

Vörösmarty CJ, Léveque C, Revenga C, Bos R, Caudill C, Chilton J, Douglas EM, Meybeck M, Prager D, Balvanera P. 2005. Fresh water. Millenn Ecosyst Assess 1:165–207.

Vörösmarty CJ, McIntyre PB, Gessner MO, Dudgeon D, Prusevich A, Green P, Glidden S, Bunn SE, Sullivan CA, Liermann CR, Davies PM. 2010. Global threats to human water security and river biodiversity. Nature 467:555.

Weatherhead EK, Howden NJK. 2009. The relationship between land use and surface water resources in the UK. Land Use Futur 26:S243–S250.

Chemosphere 285 (2021) 131524

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Critical assessment of an equilibrium-based method to study the binding of waterborne organic contaminants to natural dissolved organic matter (DOM)

Simone Rizzuto [a], Kevin C. Jones [a,*], Hao Zhang [a,**], Didier L. Baho [b], Eva Leu [c], Luca Nizzetto [b,d]

[a] Lancaster Environment Centre, Lancaster University, Lancaster, LA1 4YQ, UK
[b] Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, 0349, Oslo, Norway
[c] Akvaplan-niva, CIENS, Science Park, Gaustadalléen 21, 0349, Oslo, Norway
[d] RECETOX, Masaryk University, Kamenice 753/5, 625 00, Brno, Czech Republic

ARTICLE INFO

Handling Editor: Willie Peijnenburg

Keywords:
Natural dissolved organic matter
Isoproturon
Equilibrium-based method
DOM-contaminants binding

ABSTRACT

Dissolved organic matter (DOM) can play a major role in determining availability of pollutants to aquatic biota. Equilibrium dialysis is the most commonly used method to assess the interaction between DOM and organic contaminants. However, results obtained through this method can be affected by confounding factors linked to the diffusion of DOM through the membrane or the interaction of DOM and/or the compounds with the membrane itself. In this study, we propose an improved experimental approach, where highly hydrophilic cellulose-ester membranes with small molecular cut-off (100–500 Da) were used to overcome some of these hindrances. The performance of the method to determine the binding of a commonly used moderately hydrophobic herbicide (Isoproturon - ISU) with natural DOM was critically evaluated through a set of quality assurance criteria, across a range of DOM concentrations and pH conditions. DOM *trans*-membrane diffusion was prevented by the smaller pore size of the dialysis membrane. Good measurement reproducibility, mass balance closure, and successful *trans*-membrane equilibrium of ISU were obtained. ISU showed relatively low affinity with DOM (log K_{DOC} 1–2 L g^{-1}), which was significantly influenced by varying pH and DOM concentration. An alternative membrane may be needed for higher pH conditions as the greater adsorption effect blurred the observation of *trans*-membrane equilibrium and confounding mass balance closure. The paper makes recommendations on how to avoid measurement artefacts, while considering criteria for the expected mass distribution of compounds at equilibrium and for sorption onto the membrane and surfaces of the experimental units.

1. Introduction

Large amounts of chemical contaminants are continuously discharged to freshwaters from anthropogenic activities (FAO, 2017), threatening the integrity of ecosystems (Pal et al., 2010; Schulz et al., 2021). The effects of chemical contaminants on aquatic biota do not only depend on their concentrations and inherent toxicological properties, but can also be influenced by environmental factors that can alter their bioavailability (Fischer et al., 2013). Assessments of the effects of waterborne contaminants on aquatic biota typically overlook environmental determinants, considering simplified standard exposure scenarios (OECD, 2009). This leads to assessments of chemical risk that can be poorly representative of real environmental conditions (Rowett et al., 2016). Understanding the interactions of key environmental factors (including the role of constituents of natural freshwaters and the water chemistry) with chemical contaminants is crucial for a correct prediction of ecological risk.

Dissolved organic matter (DOM) can play a major role in affecting the form and effects of waterborne contaminants. DOM is an heterogeneous mixture of a wide range of natural organic substances that can be found in all aquatic environments (Leenheer and Croue', 2003). It has the ability to bind, adsorb and/or transform contaminants by forming complexes that are too large or too polar to cross biological membranes (Lipnick, 1995), thereby reducing the bioavailability and toxic outcome of contaminants (Lin et al., 2018a; Rowett et al., 2016). The affinity of DOM with contaminants (generally expressed as a distribution

* Corresponding author.
** Corresponding author.
E-mail addresses: k.c.jones@lancaster.ac.uk (K.C. Jones), h.zhang@lancaster.ac.uk (H. Zhang).

https://doi.org/10.1016/j.chemosphere.2021.131524
Received 28 May 2021; Received in revised form 8 July 2021; Accepted 9 July 2021
Available online 12 July 2021

coefficient K_{DOC}) can be affected by the water chemistry (e.g. pH), temperature, physicochemical properties of both the contaminants (i.e. hydrophobicity – presence of functional groups), and DOM (e.g. molecular size, aromaticity, type of functional groups in the DOM molecules and concentration) (Lin et al., 2018b; Pokrovsky et al., 2016; Xu and Guo, 2017). For instance, higher molecular size DOM constituents (i.e. humic acids) generally show higher affinity for binding with chemical compounds (Bai et al., 2019; Xu et al., 2019), while the opposite can occur for lower molecular size constituents (Ding et al., 2011; Pokrovsky et al., 2016). This is usually related to the molecular size altering the physicochemical properties of DOM, where larger size fractions of DOM are usually connected with more condensed structure, stronger hydrophobicity, abundant aromaticity and therefore higher affinity with organic chemicals (Bai et al., 2019; Ma and Yates, 2018). A change in water pH may affect the complexation of DOM and contaminant, since many chemicals, including pesticides, exists simultaneously as ionic and neutral forms n the aquatic environment (Ashauer and Escher, 2010; Rozman and Doull, 2000). Neutral species dominate at water pH lower than the compound's acid dissociation constant (pKa) and tend to be more toxic, possibly because the organisms' lipid membranes are often more permeable to non-polar molecules (Lipnick, 1995). Neutrality in the molecular charge can in turn increase the likelihood of hydrophobic interactions with DOM (Rowett et al., 2016), possibly resulting in lower bioavailability and toxicity. Water pH can also alter the physicochemical properties of the DOM, by altering its chemical configuration and therefore its binding affinity with the contaminants (Longstaffe et al., 2013; Myeni et al., 1999). The binding capacity of the DOM is generally dependent on its concentration (Burkhard, 2000; Krop et al., 2001), however higher concentrations of DOM could also influence the supramolecular configuration of the DOM itself (Vialykh et al., 2020), preventing contaminants from accessing binding domains in the DOM (Akkanen and Kukkonen, 2003; Cao et al., 2018). These complex behaviours cannot be easily predicted. Addressing the role of DOM on chemical risk assessment therefore requires dedicated studies and reliable experimental approaches.

Several techniques have been used to investigate DOM and contaminants interactions. These include fluorescence quenching (Hong et al., 2021), solubility enhancement (Wei-Hass et al., 2014), purging or sparging (Hassett and Milicic, 1985), solid-phase micro-extraction (Ren et al., 2020; Ripszam and Haglund, 2015), reverse-phase HPLC separation (Hsieh et al., 2015; Laundrum et al., 1984), size-exclusion chromatography (Jota and Hassett, 1991; Lee and Hur, 2017), liquid-liquid extraction (Johnsen, 1987) and equilibrium dialysis (Akkanen et al., 2001; Yamada and Katoh, 2020; Zhao et al., 2014). Equilibrium dialysis has been the most common approach due to its apparent simplicity and the possibility of easily providing confirmation of measurement validity through mass balance closure. This method exploits the osmosis process where a solute moves from an area at higher concentration to one at lower concentration until it reaches equilibrium between two sides of a semipermeable membrane. Contaminants and DOM are usually spiked inside a dialysis membrane ideally made of an inert material, and with pores of a defined molecular cut-off (e.g. 1000 Da). In principle, this setup allows the contaminant molecules to freely diffuse across the membrane, whereas the larger-sized DOM complexes are confined inside the dialysis bag (Akkanen and Kukkonen, 2003; Yamada and Katoh, 2020). If binding occurs, the concentration of contaminant inside the bag is higher than that outside, as it represents the sum of the freely dissolved fraction (at *trans*-membrane equilibrium) and the amount bound to DOM. In this case calculation of distribution coefficients is, in principle, possible.

While conceptually simple, several confounding factors can blur experimental results and potentially produce artefacts and errors. One technical hindrance is that the pore size typically used in these experiments (1000 Da) does not completely prevent the crossing of smaller DOM molecules from inside the dialysis bag, leading to an underestimation of the binding effect of DOM (Akkanen et al., 2004; Akkanen and Kukkonen, 2001, 2003). The use of membrane with a smaller molecular cut-off could solve this issue, but it may also present other challenges, such as an amplified interaction of the membrane with both DOM and contaminants (Thevenot et al., 2009). Membranes made of polymers with different compositions and properties can be used (Dias and Duarte, 2013). The choice of material is key for preventing interactions of DOM and/or contaminants with the membrane. Polymers used in dialysis include polycarbonate, polysulfones, polyacrylonitrile and - more commonly - a range of cellulose-based membranes (Dias and Duarte, 2013; Tolkoff-Rubin, 2011). Electrochemical properties or hydrophobicity of the membranes can therefore vary markedly with their composition. This can affect the interaction of both contaminants and DOM with the membrane, which may alter permeability and mass recovery, and therefore affecting mass balance results, invalidating the measurements.

Dialysis equilibrium experiments usually overlook adsorption of the investigated chemicals on the experimental units (e.g. glassware, membrane and any material in contact with the solution), under the assumption that such an interaction would not interfere with the *trans*-membrane equilibrium (Akkanen and Kukkonen, 2003). Nevertheless, significant adsorption could negatively affect the quality of the experiment (Thevenot et al., 2009).

To stress the performance and viability of the equilibrium dialysis method to measure binding between contaminants and DOM, we introduce an approach based on the following improvements:

- Reduced membrane pore size (100–500 Da) to prevent DOM crossing the membrane.
- Use of cellulose ester membrane to reduce hydrophobic interactions between membrane and DOM/contaminants.
- Measurements of test compound concentrations by direct injection to LC-MS/MS;
- Adoption of a rigorous set of quality assurance criteria, aimed at preventing leakage of DOM through dialysis membrane, verifying equilibrium conditions, ensuring system mass-balance closure, assessing the sorption of the contaminants on the membrane and experimental unit components.

The herbicide Isoproturon (ISU) was used as a model compound. It was selected for its chemical structure, with hydrophobic and partly charged molecular domains, providing possibilities for both hydrophobic and weak electrostatic interactions between the chemical and the functional groups of the DOM. The binding was tested with DOM isolated from a natural lake (Gjessing et al., 1999) to ensure a natural complex spectrum of DOM constituents from boreal ecosystems (Leenheer and Croue', 2003). The experiments were carried out for a range of DOM concentrations and water pH, to challenge the method's ability to provide consistent and reliable results and elucidate its possible limitations.

2. Materials and methods

2.1. Selection and settings of experimental conditions

Chemicals: ISU (CAS 34123-59-6) is a 1,1-dimethyl-3-(4-isopropylphenyl)-urea substituted by a p-cumenyl group at position 3, and is an herbicide acting as a photosynthesis inhibitor for weed control (Tomlin, 2000). Its molecular formula is $C_{12}H_{18}N_{20}$, and its molecular weight of 206.82 g mol^{-1}. It is non-ionic and relatively hydrophilic (octanol-water partition coefficient log K_{ow} 2.87 - Hansch et al., 1995). Other important physicochemical properties are: water solubility of 65 mg L^{-1} at 22 °C (MacBean, 2008); Henry's Law constant of 1.9×10^{-9} atm-cu m/mole (MacBean, 2008); melting point of 158.0 °C (O'Neil, 2013); flash point of 100 °C (212 °F) (MacBean, 2008); density of 1.2 at 20 °C (MacBean, 2008); vapour pressure of 9.1×10^{-3} mPa/2.47×10^{-8} mm Hg/at 25 °C (MacBean, 2008); atmospheric OH rate constant: 1.20e-^{11}

cm^3/molecule × sec (Palm et al., 1998). ISU's chemical structure is shown in Fig. 1. The interaction of ISU and DOM has been studied previously, but focusing on soil organic matter (Beck and Jones, 1996; Ertli et al., 2004). Beyond hydrophobic interaction driven by the moderate K_{ow} value, the ability of ISU to weakly bind with O, N and H atoms of the DOM (Ertli et al., 2004), and possibly the membrane, makes this compound a useful and challenging molecular model to test the experimental method. ISU standard reference material (99.21% HPLC purity) was purchased from Sigma-Aldrich (US). The compound was prepared with methanol to create a 1 mg mL^{-1} stock solution. Two serial dilution procedures were carried out to create a 10 μg mL^{-1} ISU working solution.

Natural DOM and water pH: The DOM used in this study was previously isolated with reversed osmosis from the water of Hellerudmyra (Norway), a small catchment (0.08 km^2) used for most natural DOM research at the Norwegian Institute for Water Research (NIVA, Norway), providing samples for the (IHSS) Nordic Fulvic and Humic Reference Material (Gjessing et al., 1999). The most relevant physicochemical properties of this natural DOM are reported in Table 1. The levels of DOM used in this experiment were 0, 5, 10 and 20 mg L^{-1} DOC, which represent low to mid-high concentrations typically found in boreal lakes (Henriksen et al., 1998). The DOM working solution was prepared by weighing the dry DOM and dissolving in equivalent MQ water to reach 1 mg mL^{-1} concentration. The solution pH used (4, 5, 6, 7 and 8) represent the range expected within boreal freshwater systems (Bååth and Kritzberg, 2015). The pH was monitored daily during the experiment. No significant changes were observed (data not shown).

Dialysis experiment: Cellulose ester dialysis bags, with a flat-width of 31 mm and molecular cut-off 100–500 Da (Spectra/Por, Spectrum Europe, Breda, The Netherlands) were used; 10 cm-long sections were cut and prepared by thoroughly washing with Milli-Q water to remove any excess of the preserving agents and were maintained soaked in Milli-Q water overnight before the experiment. Ten litres of soft artificial freshwater (SAF) were prepared by adding 1.17 g NaCl to Milli-Q water to reach 0.01 M (0.58 g L^{-1}), to give an ionic strength commonly detected in boreal freshwaters. SAF was then split into five bottles (2 L each) where the pH was adjusted by titration with HCl or NaOH to reach pH 4, 5, 6, 7 and 8 respectively. At the start of the experiment, each bag was spiked with 200 μL of ISU 10 μg mL^{-1} working solution. The control bag with no DOM was topped up with SAF to reach 10 mL total volume. The other bags were spiked with 170 μL, 340 μL and 640 μL of DOM working solutions to reach 5, 10 and 20 mg L^{-1} DOC respectively, and then SAF was added to each bag to reach 10 mL total volume. Each dialysis bag was then sealed with standard closures (Spectra/Por, Spectrum Europe, Breda, The Netherlands), before being placed in a 250 mL glass beaker, containing 200 mL of SAF (Fig. 2). This procedure was repeated for each pH level, in triplicates, for a total of 60 units. After adding glass-coated metal stirrer bars (20 mm), the experimental units were closed at the top with Teflon linen plugs, and placed on a magnetic stirrer (Multistirrer15, Progen Scientific, UK) in the dark at 18 ± 1.6 °C for 48 h. Samples (2.0 mL) were collected from the beakers (external to the dialysis bag) at 6-time intervals (t0 = 0 h, t1 = 4 h s, t2 = 8 h, t3 = 12 h, t4 = 24 h, t5 = 48 h) and split into two 1 mL aliquots for the determination of ISU and to check for potential leakage of DOM through the dialysis membrane, respectively. At 48 h, 1 mL sample was also collected from inside the bag of each unit (Fig. 2). Samples for chemical analyses were filtered through a 0.2 μm filter (Whatman, UK) placed on a syringe, transferred into a 2.5 mL amber glass vial, and stored at −20 °C until analyses. Expected concentration of the herbicide at the end of the experiment at equilibrium conditions was 9.5 μg L^{-1}. This concentration was selected *a priori* through a standard ecotoxicological test based on OECD guidelines of 2009 causing sub-lethal effects on phytoplankton (Text S1). Mass recovery was determined by following the method reported in Text S2. Mass loss due to adsorption of ISU was quantified in each experimental unit inside and outside the dialysis bag. Each experimental unit was rinsed with a total amount of 10 mL of methanol. 5 mL were used to rinse the internal part of the unit (inside the bag). The other 5 mL were used to rinse the external part (outside wall of the dialysis bag, internal wall of the beaker, clips and stirrer bars). The respective solvents were collected in amber vials, to be later gently dried with nitrogen stream, and re-suspended in 1 mL methanol, than filtered and stored at −20 °C in 2.5 mL amber glass vials. The results were further checked by mass balance.

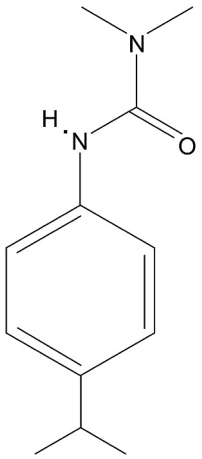


Fig. 1. Chemical structure of Isoproturon.

2.2. Chemical analyses

ISU was quantified by direct injection liquid chromatography mass spectrometry (Shimadzu, 8040), using an XBridge BEH C18 column (2.1 mm × 100 mm, 3.5 μm). The mobile media were A, 0.2% ammonium formate in Milli-Q water, and B, acetonitrile. The gradient procedure was optimized at: 0–1 min 20% B, then increased to 100% within 8 min, held at 100% for 5 min, after that decreased to the initial conditions (20% B) within 1 min. Finally, 6 min of post-run ensured re-equilibration of the column before the next injection. The injection volume was 10 μL, while the column and the tray temperature were set to 25 °C. The quantification of ISU was based on internal standard method (Isoproturon-d6, Sigma Aldrich), and the instrument detection limit of 0.96 ng mL^{-1}. To check on possible ion suppression, standard ISU samples were analysed after every sample injection. Samples with quantification accuracy below 98% were discarded.

2.3. Assessment of method performance and quality of the measurements

A set of criteria concerned with system functionality, equilibrium condition and mass recovery were used to critically assess the method performance under varying pH and DOM concentration. These were:

DOM trans-membrane leakage: The absence of traces of DOM outside the dialysis bag was a quality assurance criterion to exclude artefacts linked to DOM *trans*-membrane leakage. DOM concentration outside the dialysis bag was analysed immediately after each experiment using a plate reader coupled with a spectrophotometer (BioTek Synergy MX; Winosky, VT, US). Triplicates of solution samples from outside the bag of each experimental unit were directly loaded on clear flat-bottom 96-well black microplates (300 μL in each well) (Corning, US). Samples from standard DOM solutions of 5 and 20 mg L^{-1} DOC were also loaded for comparison. Absorbance wavelengths between 250 and 280 nm were measured, in accordance with other studies (Hagman et al., 2018; Rizzuto et al., 2020; Thrane et al., 2014).

Reproducibility: The quality of replicate measurement of ISU concentration in and outside the bags was checked for the presence of outliers. The total variability among each set of triplicates was considered acceptable when it was below 25% (of which 10% attributable to

Table 1
Physicochemical properties of the natural DOM used in this study when prepared at typical concentration found in the source lake (see Gjessing et al., 1999 for details).

pH	Conductivity (mS m^{-1})	Colour (mg Pt L^{-1})	UV absorbance 254 nm	($SUVA_{254}$)[a] (mgC x 10^2)	%Corg[b]	molecular weight (Da)	C_{ar}/C_{al}[c]
5.17	2.49	166	0.813	4.59	50.3	3900	0.22

Lake DOC concentration typically of 17.7 mg L^{-1}.
[a] Specific UV – Absorbance at 254 nm.
[b] Percent of Carbon content.
[c] Ratio of aromatic to aliphatic carbon.

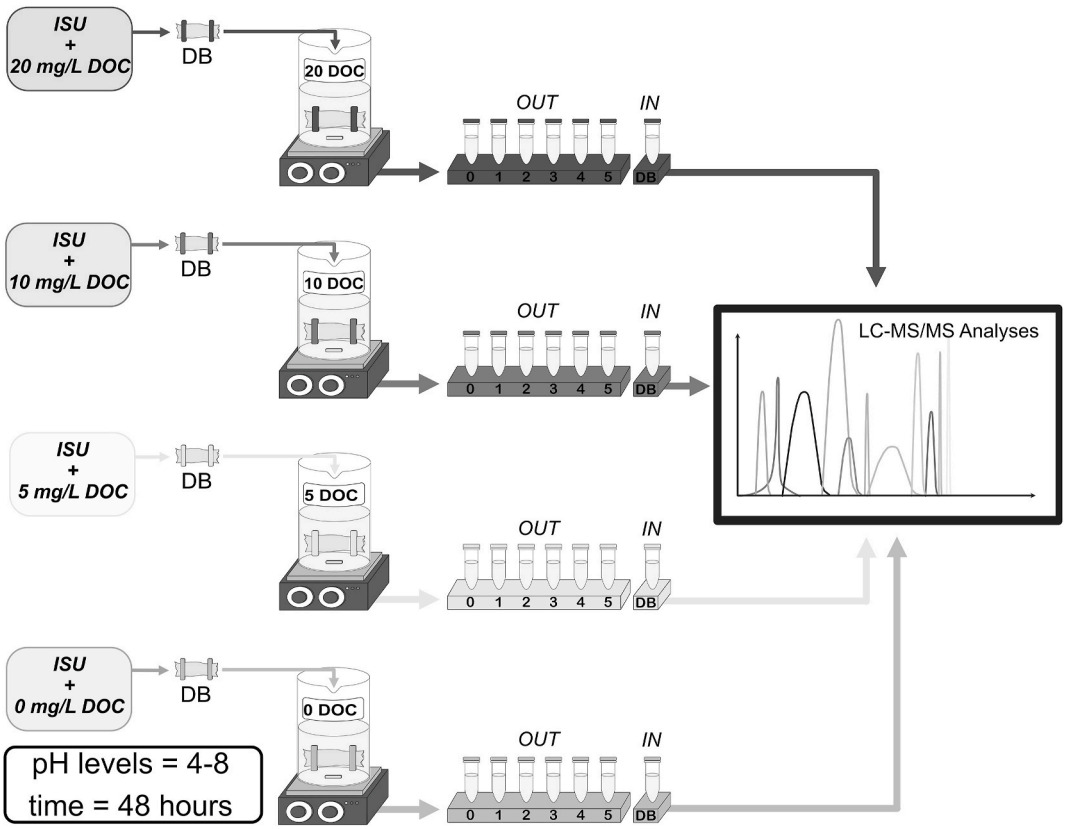


Fig. 2. Experimental design. The association of ISU to DOM (0, 5, 10 and 20 mg L^{-1} DOC) was tested through equilibrium dialysis technique at 5 pH levels (4, 5, 6, 7 and 8). DB; dialysis bags. OUT; sampled collected at six time intervals (0 = 0 h, 1 = 4 h, 2 = 8 h, 3 = 12 h, 4 = 24 h, 5 = 48 h) outside the dialysis bag. IN; sampled collected inside the dialysis bag 48 h after the start of the experiment.

analytical variability of the direct injection analysis method and 15% of acceptable system results variability).

Mass recovery and adsorption: Total mass recovery of the herbicide (i.e. mass balance closure) was tested by comparing ISU masses recovered inside and outside the bag. In addition, the mass of ISU adsorbed to components of the experimental units were analysed (both on the internal wall of the membrane, from here referred as A_{in} (μg)) and on the outside surfaces (i.e. sum of external wall of the bag, glassware, clips and stirring bars, A_{out} (μg)). These recovered masses were summed together and compared with the spiked mass to calculate recovery and mass balance closure.

Assessment of trans-membrane equilibrium of ISU: The fundamental criterion for checking *trans*-membrane equilibrium was that in the control units (e.g. the experimental units with no added DOM), the concentration of ISU inside (C_{in}, μg L^{-1}) and outside (C_{out}, μg L^{-1}) the dialysis bag was the same. However, because the solution volumes inside and outside the dialysis bags were very different (nominally 10 mL inside vs. 200 mL outside), an additional criterion based on comparing experimental observation with theoretical expectation of mass distribution inside and outside the bag was introduced. For instance, the mass (μg) of the herbicide was calculated inside (mass detected inside - $M_{D/in}$) and outside (mass detected outside - $M_{D/out}$) the bag by multiplying measured C_{in} and C_{out} (respectively) with the volumes of the solution measured in the control units in the respective compartments at the end of the experiments. $M_{D/in}$ and $M_{D/out}$ were then compared with the masses inside and outside the dialysis bag of the control units theoretically expected at equilibrium ($M_{E/in}$ and $M_{E/out}$, respectively), considering the experimentally measured adsorption on the membrane and glassware. Only when all equilibrium criteria were met, the DOM-ISU binding was assessed.

2.4. DOM-ISU binding

When the equilibrium conditions and all other quality criteria were verified, the DOM-ISU binding was assessed in the experimental units containing DOM. Theoretically, if DOM bound the herbicide, a significantly higher concentration will be detected inside the dialysis bags than outside, because complexation by the DOM prevents a certain amount of compound from crossing the membrane due the larger molecular size of the DOM. Hence, significantly higher C_{in} than C_{out} was deemed as required evidence for partial or complete binding of ISU to DOM. A second criterion was also considered, whereby C_{in} in the experimental

units with DOM should be significantly higher than C_{in} in the control units. Furthermore, to rule out the confounding factor of different dilutions inside and outside the bag, another condition to verify ISU binding to DOM was that the mass $M_{D/in}$ in the experimental units containing DOM should be higher than the mass $M_{D/in}$ of the control units.

In summary, three scenarios were considered to determine complexation in the units containing DOM:

- No binding: $C_{in} \sim C_{out}$; $M_{D/in} \sim M_{E/in,}$ and $M_{D/out} \sim M_{E/out}$.
- Complete binding: $M_{D/in}$ represented 100% of the ISU mass spiked in the experimental unit, $C_{in} \gg C_{out}$ and $C_{out} \sim 0$, $M_{D/in}$ DOM units > $M_{D/in}$ control units, while $M_{D/out} = 0$.
- Partial binding: $C_{in} > C_{out}$, $M_{D/in}$ DOM units > $M_{D/in}$ control units.

In the units where partial or complete binding was observed, the magnitude of the complexation of ISU with DOM was also expressed by calculating the conditional distribution coefficient (K_{DOC}, L g^{-1}). K_{DOC} was calculated as follows (from Buschmann et al., 2006) assuming at equilibrium:

$$= \frac{[\quad] - [\quad]}{[\quad]\ [\quad]} * 1000 \tag{1}$$

where the term $[C_{in}]$-$[C_{out}]$ is the concentration of compound complexed with DOM and $[C_{out}]$ is the concentration of free (unbound) compound. DOC and DOM values can be inter-converted in the equation by using the DOM's % of C content value of 50.3 (Table 1).

The percentage of bound compound (B_{DOM}), defined by mass of bound compound divided by the total mass of the compound inside the bag, was calculated as follows:

$$= \frac{- \quad *}{} * 100 \tag{2}$$

where V_{in} is the solution volume inside the dialysis bag and $C_{out} \times V_{in}$ is the mass of unbound inside the bag. M_{in} is the total mass of compound inside the bag.

2.5. Statistical analyses

Data analyses and statistics were conducted using R (version 3.5.1) statistical software (R Core Development Team, 2015). The single comparison analyses between C_{in} and C_{out}, K_{DOC} and B_{DOM} between different DOM levels were tested by one-way ANOVA. The graphs were prepared by using the R package "ggplot 2" (Wickham, 2006).

3. Results and discussions

3.1. Quality of measurements: trans-membrane diffusion of DOM

The absorbance measurements (A_{280}) made after 24 and 48 h showed no differences between the samples collected outside the dialysis bag from the DOM units with 5 and 20 mg L^{-1} DOC and the control unit in the absence of DOM, at all pH levels (Fig. S1B, $p = 0.76$). Samples from standard DOM solutions at 5 and 20 mg L^{-1} DOC are also reported for comparison in Fig. S1A. These results proved that the low molecular cut-off membrane used in this experiment (100–500 Da) confined all the DOM inside the bag throughout the duration of the experiment. The efficacy of smaller pore size membrane (also previously reported by others - Buschmann et al., 2006), represents a substantial improvement. For instance, Thevenot et al. (2009) reported DOM losses during their experiments with dialysis membrane with 1000 Da molecular weight cut-off. Akkanen et al. (2003) reported a 15% decrease in the DOM content (20 mg L^{-1} DOC) inside the dialysis bag after 4 days of dialysis using a membrane with 1000 Da pore size. Williams and others had similar results (1999). Carter and Suffet (1982) also observed 5% losses with Aldrich humic acids. Those inappropriate pore sizes have led to the misestimation of the interaction between DOM and contaminants due to the leakage of DOM-contaminants complexes from the dialysis bags (Thevenot et al., 2009; Williams et al., 1999; Yamada and Katoh, 2020). While *trans*-membrane transfer of DOM molecules smaller than 100 Da cannot be excluded in principle, no measurable amounts were detected outside the membrane, suggesting that - if present - these artefacts had negligible influence on the measurement quality. It must be also noted that there is preferential binding of organic chemicals to the high molecular weight fraction of DOM (Vialykh et al., 2020). In addition, while the reduced pore size of the membrane used in this study prevented the diffusion of DOM through the dialysis membrane, the results may also vary with other types of DOM with different molecular size.

3.2. Quality of measurements: reproducibility, mass balance and adsorption

The variability of the replicates was ≤25%, which was the threshold set *a priori* for measurement acceptability. The total mass recovery of the herbicide, which combined the mass measured in the experimental solutions and the one adsorbed to the different compartments of the experimental units (dialysis membranes, glassware and bag closures), yielded an overall 100 ± 0.3% at all DOM concentrations and pH levels (Table 2, S1). Such optimal mass balance closure of the herbicide indicated negligible ISU degradation/volatilisation during the experiment, in agreement with expectation from data on ISU degradation half-life (Bi et al., 2012; Böttcher and Schroll, 2007).

The mass of the herbicide recovered from adsorption to the different experimental compartments ranged from 5% to 42% and differed across pH and DOM levels (Table 2, S1). Two distinct patterns were observed at pH 4–6 and 7–8, respectively. At lower pH levels (4–6), the A_{in} was below 20% in the control units, ranging between 10 and 17% (Table 2, S1). Notably, a significant increasing pattern of A_{in} was observed across the DOM levels, reaching values of 30–38% at the highest levels of DOM (20 mg L^{-1} DOC, Table 2). The A_{out} remained consistently below 20% in all units, ranging between 5% and 17%, with no significant differences between controls and units with DOM (Table 2). At the higher pH levels (7–8) in contrast, the A_{in} values of the control units were significantly higher ($p < 0.01$) compared to those observed at pH 4–6, ranging between 37% and 40% (Table 2, S1). No differences in A_{in} were observed between the control and the DOM units. In addition, A_{out} at the higher pH levels was significantly higher compared to those at pH 4–6 ($p < 0.01$), yielding values between 30% and 42%, with no significant differences between the control and DOM units (Table 2, S1).

The different adsorption observed across the pH levels could be attributed to the influence of water pH on the speciation/form of the herbicide, and/or the dialysis membrane, which could vary their ionic configuration, modify their physical-chemical properties, and in turn affect their interactions (Ashauer and Escher, 2010; Rizzuto et al, 2020, 2021). A modification in the properties of ISU is the least likely, due to its non-ionic characteristics at the pH values considered here. Instead, several cellulose esters of the dialysis membranes are carriers of carboxylic or phthalates groups (such as trimellitate and phthalates-functionalized cellulose) with pKa values in the range of conditions studied here (Dias and Duarte, 2013). Changes in pH levels can confer wettability and hydrophilic character to the membrane (which limit hydrophobic interactions) and likely promote pH-dependent weak interactions with the herbicide (Dias and Duarte, 2013). Hence, higher pH values (7–8) could have modified the membrane properties, leading to higher adsorption of the herbicide to both the inner and outer walls of the dialysis membrane in all experimental units compared to the lowest pH levels (Table 2, S1). High degrees of adsorption may affect the quality of the experiments and the accuracy of the binding results. As the binding of ISU with DOM is not significant at high pH conditions (C_{in} and C_{out} are similar), the adsorption issue is irrelevant for this work at high pH. However, an alternative membrane

Table 2
Percentages of ISU mass recovered (mean ± standard deviation) a) inside, b) outside the bag, adsorbed c) inside (A_{in}) and d) outside the bag (A_{out}), and e) total recovery.

pH	DOM	% recovery in (a)		% recovery out (b)		% A_{in} (c)		%A_{out} (d)		%total recovery (e)	
		Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd
4	0	89.0	0.6	89.7	2.8	10.5	2.7	10.8	1.0	100.2	0.1
	5	102.4	0.6	74.4	1.4	25.6	1.4	10.8	0.5	99.9	0.0
	10	107.7	2.0	72.2	1.0	27.8	1.0	7.7	1.8	99.9	0.01
	20	103.9	2.6	69.9	1.5	30.0	1.5	11.9	2.8	99.9	0.1
5	0	84.8	1.3	80.5	4.7	17.3	1.5	15.7	0.9	100.3	0.5
	5	133.3	2.8	73.9	2.0	16.0	2.0	5.9	0.5	101.8	0.1
	10	108.7	0.6	66.4	1.5	13.5	1.6	5.6	0.5	100.6	0.1
	20	88.6	0.7	78.4	3.6	22.0	3.9	15.1	0.4	100.4	0.5
6	0	86.3	0.7	73.3	1.6	15.7	1.1	12.9	0.5	99.9	0.01
	5	96.9	0.9	73.9	2.0	26.0	2.0	11.5	0.9	99.9	0.0
	10	94.2	0.3	64.6	0.8	35.3	0.8	13.6	0.1	99.9	0.0
	20	77.7	1.2	61.9	0.4	38.0	0.4	23.9	1.0	99.9	0.0
7	0	74.9	5.8	61.5	4.7	38.5	4.7	24.5	5.5	99.9	0.0
	5	68.7	1.9	59.9	2.4	40.2	2.4	30.8	1.8	100.1	0.2
	10	67.6	1.4	62.9	1.2	37.3	1.2	32.2	1.3	100.1	0.2
	20	63.9	1.4	61.9	1.9	38.0	1.9	35.3	1.3	99.9	0.0
8	0	62.0	0.8	62.8	1.3	37.2	1.3	37.4	1.0	99.9	0.0
	5	63.6	1.1	62.6	0.8	37.3	0.8	36.0	1.3	99.9	0.0
	10	62.2	0.4	62.6	0.8	37.5	1.0	37.4	0.5	100.1	0.1
	20	57.1	3.6	59.8	0.9	40.1	0.8	42.3	3.5	99.9	0.3

with lower adsorption may be needed for assessing binding accurately at higher pH conditions as the greater adsorption effect blurred the observation of *trans*-membrane equilibrium and confounding mass balance closure.

The increasing pattern of A_{in} across the DOM levels observed only at the lower pH levels can be explained with the interaction between the herbicide, the dialysis membrane, and the DOM itself. For instance, DOM includes humic and fulvic acids rich in carboxylic groups and electronegative domains such as oxygen atoms in alcohols and ketones functional groups, whose dipolar moment and interaction with partly charged domains of the ISU or the dialysis bag can be strongly influenced by water pH (Dias and Duarte, 2013; Pace et al., 2012; Tanaka et al., 2005). Hence, the lower pH may have changed the chemical configuration of the DOM, increased its binding affinity with both the herbicide and the dialysis membrane. This hypothesis is corroborated by the fact that the A_{in} in the control units and the A_{out} in all experimental units is consistently below 20%. Hence, while the influence of A_{out} on the quality of measurements can be considered negligible, the increasing pattern of A_{in} in the DOM units at pH 4–6 should be taken in consideration during the evaluation of the method performance to avoid blurring the true effect of the DOM and cause artefacts in the analysis of DOM-ISU binding. Some previous studies of DOM-contaminant binding have overlooked adsorption to glassware and the membrane, leading to uncertainties over mass balance closure and measurement validity (Lee and Farmer, 1989; Thevenot et al., 2009). These results showed that assessing mass recovery and adsorption to the experimental unit surfaces is crucial for ensuring high confidence in the measurement quality, as also suggested in other studies (Rizzuto et al., 2021).

3.3. Method performance: trans-membrane equilibrium

Equilibration across the membrane was checked in the control units after 48 h, based on the criteria enunciated above (for C_{out} during the 48 h experiment see Fig. 3). C_{out} and C_{in} measured after 48 h are shown in Fig. 4 for the control units, the different DOM levels, and pH conditions. The control units displayed no significant differences between ISU C_{in} and C_{out} after 48 h at all pH levels (Table S2) indicating equilibrium had been attained by this time. Furthermore, in compliance with the evaluation parameters, the herbicide $M_{D/in}$ values in the control units were not significantly higher than $M_{E/in}$, confirming that the equilibrium conditions were met at all pH levels. These results therefore confirm that the smaller pore size membrane allowed ISU *trans*-membrane diffusion while preventing DOM diffusion through the membrane pores. In addition, these results indicate that the adsorption losses reported in the previous section had no observable effect on the *trans*-membrane equilibrium of the herbicide in the control units.

3.4. Method performance: DOM-ISU binding

The final step of the critical evaluation was to test the degree of ISU binding with DOM. The first criterion of the framework was that $C_{in} > C_{out}$ in the experimental units containing DOM, which was fulfilled under most of the pH and DOM conditions. For instance, results showed that C_{in} was significantly higher than C_{out} in all the experimental units containing DOM at pH 4, 5, 6 ($p < 0.001$, Tables S3-S5) and 7 ($p < 0.05$, Tables S3-S5), while no significant differences were observed at pH 8 (Tables S3-S5). However, while the results observed at pH 4–7 may be interpreted as evidence of DOM-ISU binding, the high A_{in} observed in the control units and the high A_{out} reported in all experimental units at pH 7 (ca. 38%) could not allow a high level of confidence in the evaluation of the DOM-ISU binding in the DOM units. It is plausible that such high levels of A_{out} measured at this pH level could have driven the equilibrium between the two sides of the membranes, causing the higher C_{in} compared to C_{out} (Table S1). Hence, to avoid the occurrence of false positive DOM-ISU binding, the data at pH 7 were not analysed further. In contrast, for the other three levels of pH where C_{in} was found higher than C_{out} (pH 4–6), the low A_{out} in all experimental units (<17%) and the minor A_{in} in the controls (<17%) ensured high confidence in the quality of measurements and therefore in the evaluation of the binding of DOM with the herbicide. Hence, the experimental units at pH 4–6 were admitted for evaluation to the second criterion of the framework, where C_{in} in the DOM units must be significantly higher than the control ones. Since the A_{in} was higher in the DOM units compared to the controls at pH 4–6 (Table 2, S1), the extra ISU adsorbed inside the dialysis bag was assumed to be complexed with the DOM (for more information on this process please refer to paragraph 'Quality of measurements: reproducibility, mass balance and adsorption'). By applying this assumption, C_{in} in the units treated with DOM yielded significantly higher values compared to the C_{in} of the control units at pH 4, 5 and 6 ($p < 0.001$) (Fig. 4). Thus, all the units at pH 4–6 satisfied the second criterion and were admitted to the last part of the framework for the evaluation of the DOM-ISU binding, where the $M_{D/in}$ of the experimental units must be higher than the $M_{D/in}$ in the control units. Results showed that by accounting for the extra ISU adsorbed inside the bag as

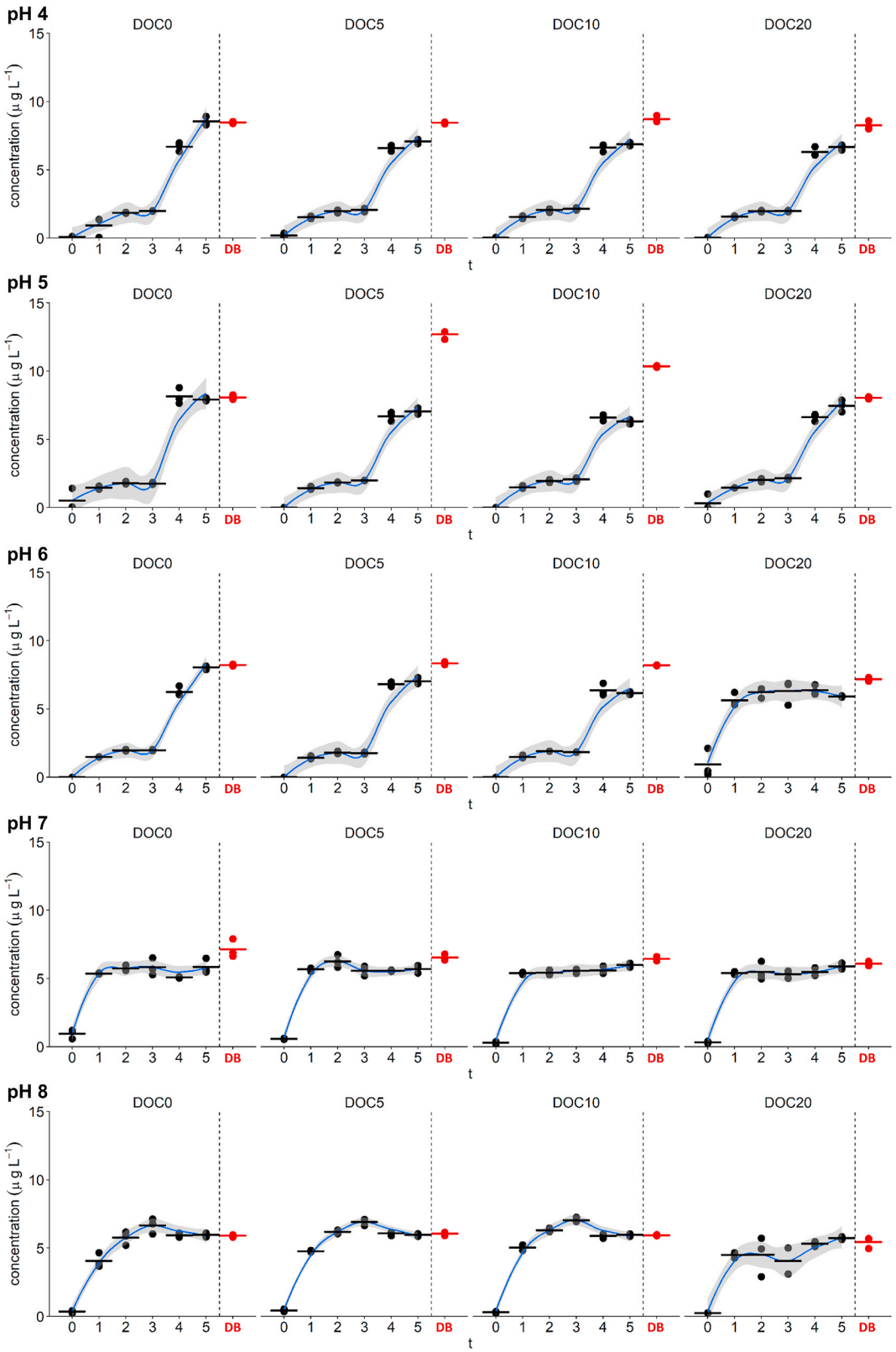


Fig. 3. ISU concentrations detected outside the dialysis bag at six time intervals (0 = 0 h, 1 = 4 h, 2 = 8 h, 3 = 12 h, 4 = 24 h, 5 = 48 h) and inside the bag at 48 h (DB), at 4 levels of DOM (0, 5, 10, 20 mg L^{-1} DOC) and 5 levels pH (4–8). Horizontal bars represent the mean values. Locally estimated scatterplot smoothing (LOESS) was fitted to the data points to generate line plots. Grey areas indicate 95% confidence interval.

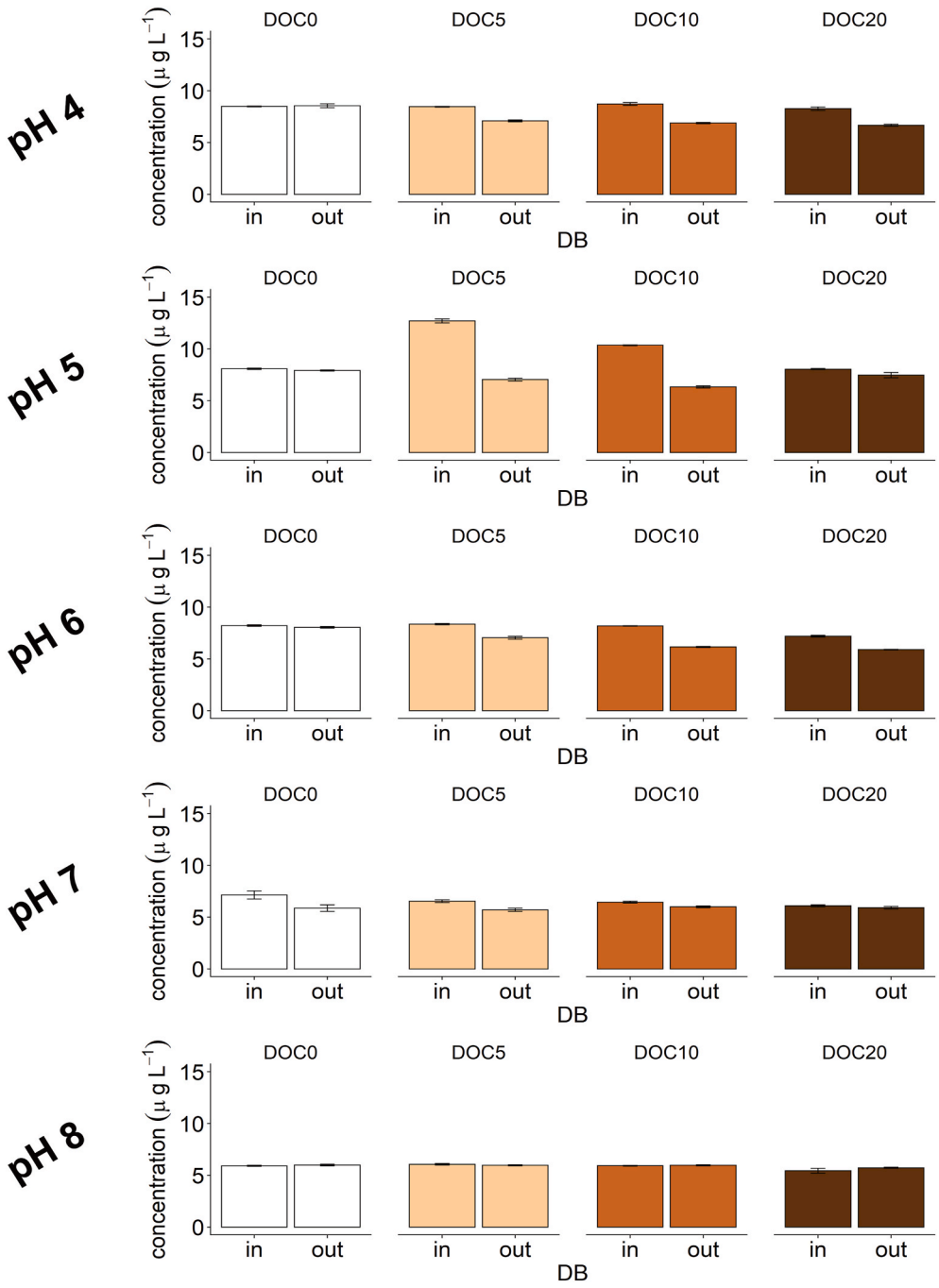


Fig. 4. ISU concentration inside (C_{in}) and outside (C_{out}) the dialysis bag (DB) after 48 h of the experiment, at 4 levels of DOM (0, 5, 10 and 20 mg L^{-1} DOC) and 5 levels of pH (4, 5, 6, 7 and 8). Black error bars represent standard deviation.

complexed with DOM, the ISU $M_{D/in}$ of the DOM units yielded significantly higher values than $M_{D/in}$ of the controls at all concentrations of DOM at pH 4–6 (Table 2, S1). In addition, to further corroborate the choice to exclude the data at pH 7 from further analyses, results showed that ISU $M_{D/in}$ of the DOM units yielded significantly lower values than $M_{D/in}$ of the controls, which infringes the assumption set in the quality criteria. In contrast, results observed at pH 4, 5 and 6 fulfilled all the quality assurance criteria, indicating partial DOM-ISU binding. The complete binding scenario was not observed for any of the pH levels investigated.

The results presented here highlight that the dialysis method can yield potential false positives, and that quality assurance criteria based on mass distribution, rather than only on concentration comparison across the membrane is key to avoid significant errors in assessing binding of DOM with contaminants.

3.5. *Method performance: K_{DOC} and B_{DOM}*

In general, the hydrophobicity of a compound is considered as a determinant of its affinity for DOM (Pan et al., 2008). However, our experiments showed that an herbicide with relatively low hydrophobicity such as ISU (log K_{ow} 2.87) can partially bind with natural DOM possibly due to pH-dependent weak electrostatic interactions. Eqs (1) and (2) were used to calculate K_{DOC} and B_{DOM} respectively, using the data obtained for the treatment conducted at pH 4, 5 and 6. The results are shown in Table 3. For instance, log K_{DOC} and B_{DOM} were 1.6 L g^{-1}

Table 3
Log conditional distribution coefficient (log K_{DOC}), and percentage of bound compound (B_{DOM}) for DOM-ISU binding at three different levels of DOM (5, 10 and 20 mg L^{-1} DOC), and water pH 4, 5 and 6.

pH	DOM	K_{DOC}	Log K_{DOC}	B_{DOM}
4	5	74.6	1.9	16.2
	10	48.8	1.7	21.1
	20	24.1	1.4	19.4
5	5	161	2.2	44.6
	10	63.8	1.8	38.9
	20	6.4	0.8	11.2
6	5	61.6	1.8	15.7
	10	45.5	1.7	24.8
	20	12.6	1.1	18.0

and 18.9%, 1.6 L g^{-1} and 30.2%, 1.5 L g^{-1} and 19.5% respectively at pH 4, 5 and 6 (average among DOM levels, Table 3). The association of ISU with DOM was already reported for soil-water systems (Beck and Jones, 1996; Ertli et al., 2004), but – to the best of our knowledge – there are no other studies reporting the direct DOM-ISU binding in natural water environments. In another study, Akkanen et al. (2001) tested the binding of natural DOM with atrazine, a phenylurea herbicide with very similar physical-chemical properties to ISU, using the equilibrium-dialysis method. The authors reported that the binding between the herbicide and DOM was too low or too weak to be detected (Akkanen and Kukkonen, 2001, 2003). The larger cut-off of the membranes used in that study (e.g. > 1000 Da) could result in DOM breakthrough, leading to an underestimation of DOM binding (Thevenot et al., 2009). In our study, the smaller pore size used (100–500 Da) prevented DOM molecules from crossing the dialysis membrane (Figure S1) and ensured a better assessment of natural DOM interaction with a phenylurea herbicide.

The K_{DOC} values of ISU previously measured for the interaction with organic matter in soil leachates varied greatly, and this variation may be caused by the different properties of organic matter, soil chemistry, as well as the different methods used (Burkhard, 2000; Krop et al., 2001). Cook et al. (2004) reported log K_{DOC} values of ISU ranging 1.96–5.75 in UK agricultural soil using standard batch adsorption procedures, suggesting that the interaction of ISU with DOM was directly related to soil organic carbon content. Other authors observed ISU log K_{OC} values ranging between 1 and 3 in experiments of sorption/desorption of the herbicide to DOM originated from compost (Barriuso et al., 2011). These assessments are generally consistent with the present study results.

3.6. Influence of pH and DOM concentration on DOM-ISU binding

The partial binding between ISU and DOM was observed only at the lower levels of pH (4, 5 and 6) across all DOM levels (Table S3-S5), while no binding occurred at the higher pH levels (7–8) according to the quality criteria. The effect of water pH on the speciation/form of both the herbicide and the DOM could have played a key role in this process, as also reported in the previous paragraph ('3.2 Quality of measurements: reproducibility, mass balance and adsorption'). Most likely, the binding of ISU with DOM could have been favoured at the lower pH levels, where the alteration of the configuration of ionizable groups operated by the lower water pH can in turn form hydrogen bonds with electronegative domains of the ISU molecules or other types of weak electrostatic interactions (e.g. with the induced dipole of the phenylurea group in ISU). This hypothesis is supported by other studies, which report evidence of the effect of lower water pH on the ISU association with DOM. For instance, Beck and Jones (1996) showed that the affinity between ISU and DOM increased in more acidic soils compared to more alkaline ones. In another study on the effect of pH on association of ISU with natural DOM, Ertli et al. (2004) showed a strong pH dependence, where adsorption of ISU was higher at lower pH in acidic soils.

The binding of ISU with DOM did not show significant differences between pH levels 4, 5 and 6. For instance, the average log K_{DOC} and B_{DOM} calculated among the different levels of DOM were of 1.7 ± 0.2, 1.6 ± 0.6 and 1.5 ± 0.3 L g^{-1}, and of 18.9 ± 2.1%, 30.2 ± 16.5% and 19.5 ± 3.9% at pH 4, 5 and 6, respectively. At the same time, the binding of ISU across the DOM concentrations differed between the pH levels. While no significant differences were observed between pH 4 and 6 (Table 3), at pH 5 the association of ISU with the lower levels of DOM (5 and 10 mg L^{-1}) was significantly higher ($p < 0.01$) than that measured at pH 4 and 6, as reported by the K_{DOC} and B_{DOM} values (Table 3). The influence of pH in altering the configuration of ISU, DOM and the dialysis membrane at different degrees for different pH could be the explanation for the increased binding between DOM and ISU at pH 5. Most likely, pH 5 represented an ideal condition for both the herbicide and DOM to interact, which resulted in the higher binding (Table 3). Preferred interaction of the herbicide ISU with natural DOM at pH 5 was also reported elsewhere (Ertli et al., 2004). Also, pH 5 gave the best experimental conditions, with the lowest adsorption of the herbicide compared to the other pH levels (Table 2). At the same time, the exceptionally low binding data at pH 5 for the DOM concentration of 20 mg L^{-1} cannot be explained logically. It is possible that the concentration of ISU in the sample taken from outside of the dialysis bag, C_{out}, was contaminated during sample treatment or analysis, which will result in incorrect binding data when using Eqs (1) and (2).

Notably, at all the pH levels investigated in this study, the association of ISU with DOM decreased ($p < 0.001$) with increasing concentration of DOM. For instance, the log K_{DOC} values were significantly higher at the lower levels of DOM (5 mg L^{-1} DOC), and lower at the higher levels of DOM (20 mg L^{-1} DOC) at pH 4, 5 and 6 (Table 3). The effect of DOM concentration on the percentage of bound compound (B_{DOM}) is less clear (Table 3). However, there is a general trend of lower B_{DOM} at 5 mg L^{-1} DOC. The lack of linear relationship between the DOM concentration and both the K_{DOC} and B_{DOM} results may stem from cross interaction between DOM constituents at higher DOM concentrations (Carter and Suffet, 1982; Deng et al., 2021), affecting the density of available binding sites for ISU on the DOM. Previous studies have discussed that higher activity of DOM in a solution may alter the DOM macromolecular configuration (Engebretson and von Wandruszka, 1994; Jiang et al., 2020), hindering access of ISU to the more reactive binding molecular domains. Behaviour similar to what was observed here was described in previous studies using humic acids (Akkanen et al., 2004; Akkanen and Kukkonen, 2001), and natural DOM extracted from soils (Ling et al., 2005; Wang et al., 2018).

4. Conclusions

In this study, the performance of an optimized dialysis equilibrium-based method for measuring the binding of organic contaminants to natural dissolved organic matter (DOM) was improved using:

- reduced membrane pore size (100–500 Da) to prevent DOM crossing the membrane;
- highly hydrophilic cellulose-ester membrane to reduce hydrophobic interactions between DOM and the contaminants;
- direct injection to LC-MS/MS to quantify DOM-contaminant binding;

It was assessed against a set of quality assurance criteria based on: the evaluation of the mass distribution of the compounds in the system; the assessment of adsorption of the compounds on components of the experimental unit; and evaluation of DOM *trans*-membrane leakage. This assessment was carried out while measuring the binding between natural DOM and the herbicide Isoproturon. The diffusion of DOM through the dialysis membrane was successfully prevented by the reduced pore size of the dialysis membrane. The method demonstrated good reproducibility, optimal mass balance closure, and successful fulfilment of *trans*-membrane equilibrium at all pH levels. Our results also suggest that water pH influenced the physical-chemical properties

of the dialysis membrane, driving to substantially different levels of adsorption of the herbicide between the lower pH (4–6) compared to the higher (7–8). Therefore, the conditional distribution coefficient of the herbicide was successfully measured at the lower pH levels (4–6), while the high levels of adsorption observed inside and outside the walls of the dialysis membrane at the higher pH (7–8) did not allow high confidence in the method performance, as could potentially lead towards false positive of the DOM-ISU binding. This study demonstrated that without the implementation of strict quality assurance criteria (e.g. reproducibility, mass balance and adsorption values), rather than only through comparing concentrations at the two sides of the membrane, artefacts and false results could be produced. In addition, by providing an optimization of the experimental set-up, the present results demonstrate that the resolution and sensitivity of the equilibrium dialysis method could be substantially improved, even for challenging compounds such as phenylurea herbicides, characterized by low hydrophobicity but capable of engaging into weak-electrostatic interactions with the DOM. The approach used in this study may not be suitable for all classes of chemical compounds. This study tested a compound with moderate aqueous solubility, relatively low volatility and slow biodegradation such as ISU. More volatile/semi-volatile compounds would need controls to avoid compound volatilisation, while compounds with lower aqueous solubility could have stronger interactions with cellulose-ester dialysis membranes or the glassware, requiring further/different QA/QC procedures and checks. However, the current procedure is currently suitable for other compounds in the intermediate range of solubility and low volatility. One technical improvement in this study was to use natural DOM extracted from the relevant waters to ensure a natural complex spectrum of DOM constituents. However, as the physicochemical properties of DOM are known to vary greatly among different ecosystems (i.e. molecular size, functional groups), it would be useful to compare the binding capacity over a selection of DOM, isolated from different locations to understand the binding variation in relationship with different physicochemical properties of DOM.

The present study confirms the binding of ISU to natural DOM, highlighting a controlling effect operated both by water pH and DOM concentrations. Our results also corroborate other studies indicating preferential binding of ISU with DOM at lower pH, and a relationship between the amount of ISU bound to the DOM that was not dependent on the DOM concentration. These results add information to earlier assessments of DOM-organic chemicals interactions and highlight the importance of considering more realistic environmental conditions when estimating availability of organic substances to biota during risk assessment, through the use of reliable methods. In particular, it is shown that despite being hydrophilic, phenylurea pesticides such as ISU, can establish complex interactions with naturally occurring DOM. Hence, the equilibrium-based approach used in this study can be used not only as a useful research tool for the investigation of the DOM-contaminants binding, but also as a systematic assessment of new/existing contaminants for risk assessment purposes.

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.

Acknowledgements

The authors thank Rolf Vogt from the Department of Chemistry, University of Oslo for providing the DOM used in this study. We also thank David Rochester from the Department of Chemistry, Yanying Li and Runmei Wang from Lancaster Environment Centre, Lancaster University (UK), for helping with the setup of the LC/MS-MS. The authors also wish to thank the anonymous reviewers whose comments helped improving the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2021.131524.

Funding

This study was financed by the Research Council of Norway, project grant no. 244460, Pollution and Ecosystem Adaptation to Changes in the Environment (PEACE). The research was co-funded by a discretional internal grant from the Norwegian Institute for Water Research (NIVA), under the Institute Strategic Priorities (SIS) on ecosystem remediation (TRADE-OFF). SR was also supported by the Lancaster University Faculty of Science and Technology and a Lancaster Environment Centre PhD Scholarship.

Credit author statement

SR, LN, EL, DLB, KCJ and HZ conceived the idea. SR, LN, KCJ and HZ designed the experiment. SR collected and analysed the data. SR took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analyses and manuscript.

References

Akkanen, J., Kukkonen, J.V.K., 2003. Measuring the bioavailability of two hydrophobic organic compounds in the presence of dissolved organic matter. Environ. Toxicol. Chem. 22, 518–524. https://doi.org/10.1897/1551-5028(2003)022<0518:MTBOTH>2.0.CO;2.

Akkanen, J., Kukkonen, J.V.K., 2001. Effects of water hardness and dissolved organic material on bioavailability of selected organic chemicals. Environ. Toxicol. Chem. 20, 2303–2308.

Akkanen, J., Penttinen, S., Haitzer, M., Kukkonen, J.V.K., 2001. Bioavailability of atrazine, pyrene and benzo[a]pyrene in European river waters. Chemosphere 45, 453–462. https://doi.org/10.1016/S0045-6535(01)00038-8.

Akkanen, J., Vogt, R.D., Kukkonen, J.V.K., 2004. Essential characteristics of natural dissolved organic matter affecting the sorption of hydrophobic organic contaminants. Aquat. Sci. 66, 171–177. https://doi.org/10.1007/s00027-004-0705-x.

Ashauer, R., Escher, B.I., 2010. Advantages of toxicokinetic and toxicodynamic modelling in aquatic ecotoxicology and risk assessment. J. Environ. Monit. 12, 2056–2061. https://doi.org/10.1039/c0em00234h.

Bååth, E., Kritzberg, E., 2015. pH tolerance in freshwater bacterioplankton: trait variation of the community as measured by leucine incorporation. Appl. Environ. Microbiol. 81, 7411–7419.

Bai, L., Zhang, Q., Wang, C., Yao, X., Zhang, H., Jiang, H., 2019. Effects of natural dissolved organic matter on the complexation and biodegradation of 17A-ethinylestradiol in freshwater lakes. Environ. Pollut. 246, 782–789. https://doi.org/10.1016/j.envpol.2018.12.098.

Barriuso, E., Andrades, M.S., Benoit, P., Houot, S., 2011. Pesticide desorption from soils facilitated by dissolved organic matter coming from composts: experimental data and modelling approach. Biogeochemistry 106, 117–133.

Beck, A.J., Jones, K.C., 1996. The effects of particle size, organic matter content, crop residues and dissolved organic matter on the sorption kinetics of atrazine and isoproturon by clay soil. Chemosphere 32, 2345–2358. https://doi.org/10.1016/0045-6535(96)00147-6.

Bi, Y.F., Miao, S.S., Lu, Y.C., Qiu, C. Bin, Zhou, Y., Yang, H., 2012. Phytotoxicity, bioaccumulation and degradation of isoproturon in green algae. J. Hazard Mater. 243, 242–249. https://doi.org/10.1016/j.jhazmat.2012.10.021.

Böttcher, T., Schroll, R., 2007. The fate of isoproturon in a freshwater microcosm with Lemna minor as a model organism. Chemosphere 66, 684–689. https://doi.org/10.1016/j.chemosphere.2006.07.087.

Burkhard, L.P., 2000. Estimating dissolved organic carbon partition coefficients for nonionic organic chemicals. Environ. Sci. Technol. 34, 4663–4668. https://doi.org/10.1021/es001269l.

Buschmann, J., Kappeler, A., Lindauer, U., Kistler, D., Berg, M., Sigg, L., 2006. Arsenite and arsenate binding to dissolved humic acids: influence of pH, type of humicacid, and aluminum. Environ. Sci. Technol. 40, 6015–6020.

Cao, B., Jiang, Z., Li, J., Zhang, X., Hu, Y., Chen, J., Zhang, Y., 2018. Different dissolved organic matter (DOM) characteristics lead to diverse atrazine adsorption traits on the non-rhizosphere and rhizosphere soil of Pennisetum americanum (L.) K. Schum. Chemosphere 209, 608–616. https://doi.org/10.1016/j.chemosphere.2018.06.069.

Carter, C.W., Suffet, I.H., 1982. Binding of DDT to dissolved humic materials. American Chemical Society Environ. Sel. Technol.

Cooke, C.M., Shaw, G., Collins, C.D., 2004. Determination of solid–liquid partition coefficients (Kd)for the herbicides isoproturon and trifluralin infive UK agricultural soils. Environ. Pollut. 132, 541–552.

Deng, Y., Yan, C., Nie, M., Ding, M., 2021. Bisphenol A adsorption behavior on soil and biochar: impact of dissolved organic matter. Environ. Sci. Pollut. Res. 1–12. https://doi.org/10.1007/s11356-021-12723-1.
Dias, F., Duarte, C., 2013. Cellulose and its derivatives use in the pharmaceutical compounding practice. In: Cellulose - Medical, Pharmaceutical and Electronic Applications. InTech. https://doi.org/10.5772/56637.
Ding, Q., Wu, H.L., Xu, Y., Guo, L.J., Liu, K., Gao, H.M., Yang, H., 2011. Impact of low molecular weight organic acids and dissolved organic matter on sorption and mobility of isoproturon in two soils. J. Hazard Mater. 190, 823–832. https://doi.org/10.1016/j.jhazmat.2011.04.003.
Engebretson, R.R., von Wandruszka, R., 1994. Microorganization in dissolved humic acids. Environ. Sci. Technol. 28, 1934–1941.
Ertli, T., Marton, A., Földényi, R., 2004. Effect of pH and the role of organic matter in the adsorption of isoproturon on soils. Chemosphere 57, 771–779. https://doi.org/10.1016/j.chemosphere.2004.07.009.
FAO, 2017. Water Pollution from Agriculture: a Global Review. The Food and Agriculture Organization of the United Nations (FAO) and the International Water Management Institute (IWMI) on behalf of the Water Land and Ecosystems research program, Rome & Colombo.
Fischer, B.B., Pomati, F., Eggen, R.I.L., 2013. The toxicity of chemical pollutants in dynamic natural systems: the challenge of integrating environmental factors and biological complexity. Sci. Total Environ. 449, 253–259. https://doi.org/10.1016/j.scitotenv.2013.01.066.
Gjessing, E.T., Egeberg, P.K., Håkedal, J., 1999. Natural organic matter in drinking water - the "NOM-typing project", background and basic characteristics of original water samples and NOM isolates. Environ. Int. https://doi.org/10.1016/S0160-4120(98)00119-6.
Hagman, C., Skjelbred, B., Thrane, J., Andersen, T., de Wit, H., 2018. Growth responses of the nuisance algae Gonyostomum semen (Raphidophyceae) to DOC and associated alterations of light quality and quantity. Aquat. Microb. Ecol. 82, 241–251. https://doi.org/10.3354/ame01894.
Hansch, C., Leo, A., Hoekman, D., 1995. Exploring QSAR: Hydrophobic, Electronic, and Steric Constants. American Chemical Society, Washington, DC.
Hassett, J.P., Milicic, E., 1985. Determination of equilibrium and rate constant for binding of a polychlorinated biphenyl congener by dissolved humic substances. Environ. Sci. Technol. 19, 638–648.
Henriksen, A., Skjelvåle, B.L., Mannio, J., Wilander, A., Harriman, R., Curtis, C., Jensen, J.P., Fjeld, E., Moiseenkon, T., 1998. Northern European lake survey, 1995. Ambio 27, 80–91.
Hong, H., Wu, S., Wang, Q., Dai, M., Qian, L., Zhu, H., Li, Junwei, Zhang, J., Liu, J., Li, Jian, Lu, H., Yan, C., 2021. Fluorescent dissolved organic matter facilitates the phytoavailability of copper in the coastal wetlands influenced by artificial topography. Sci. Total Environ. 790, 147855. https://doi.org/10.1016/j.scitotenv.2021.147855.
Hsieh, P.C., Lee, C.L., Jen, J.F., Chang, K.C., 2015. Complexation-flocculation combined with microwave-assisted headspace solid-phase microextraction in determining the binding constants of hydrophobic organic pollutants to dissolved humic substances. Analyst 140, 1275–1280. https://doi.org/10.1039/c4an01923g.
Jiang, T., Wang, D., Meng, B., Chi, J., Laudon, H., Liu, J., 2020. The concentrations and characteristics of dissolved organic matter in high-latitude lakes determine its ambient reducing capacity. Water Res. 169, 115217. https://doi.org/10.1016/j.watres.2019.115217.
Johnsen, S., 1987. Interactions between polycyclic aromatic hydrocarbons and natural aquatic humic substances. Contact time relationship. Sci. Total Environ. 67, 269–278.
Jota, M.A.T., Hassett, J.P., 1991. Effects of environmental variables on binding of a PCB congener by dissolved humic substances. Environ. Toxicol. Chem. 10.
Krop, H.B., van Noort, P.C., Govers, H.A., 2001. Determination and theoretical aspects of the equilibrium between dissolved organic matter and hydrophobic organic micropollutants in water (Kdoc). Rev. Environ. Contam. Toxicol. https://doi.org/10.1007/978-1-4613-0107-3_1.
Laundrum, P.F., Nihart, S.R., Eadie, B.J., Gardner, W.S., 1984. Reverse phase separation method for determining pollutant binding to Aldrich humic acid and dissolved organic carbon of natural waters. Environ. Sci. Technol. 19, 187–192.
Lee, D.Y., Farmer, W.J., 1989. Dissolved organic matter interaction with napropamide and four other nonionic pesticides. J. Environ. Qual. 18, 468.
Lee, Y.K., Hur, J., 2017. Using two-dimensional correlation size exclusion chromatography (2D-CoSEC) to explore the size-dependent heterogeneity of humic substances for copper binding. Environ. Pollut. 227, 490–497. https://doi.org/10.1016/j.envpol.2017.04.099.
Leenheer, J., Croue', J., 2003. Characterizing dissolved aquatic organic matter. Environ. Sci. Technol.
Lin, H., Xia, X., Bi, S., Jiang, X., Wang, H., Zhai, Y., Wen, W., 2018a. Quantifying bioavailability of pyrene associated with dissolved organic matter of various molecular weights to Daphnia magna. Environ. Sci. Technol. 52, 644–653. https://doi.org/10.1021/acs.est.7b05520.
Lin, H., Xia, X., Jiang, X., Bi, S., Wang, H., Zhai, Y., Wen, W., Guo, X., 2018b. Bioavailability of pyrene associated with different types of protein compounds: direct evidence for its uptake by Daphnia magna. https://doi.org/10.1021/acs.est.8b03349.
Ling, W., Xu, J., Gao, Y., 2005. Effects of dissolved organic matter from sewage sludge on the atrazine sorption by soils. Sci. China Ser. C Life Sci. 48, 57–66. https://doi.org/10.1360/04yc0129.
Lipnick, R.L., 1995. Structure-activity relationships. In: Rand, G.M. (Ed.), Fundamentals of Aquatic Toxicology - Effects, Environmental Fate, and Risk Assessment. Taylor & Francis, Washington, pp. 609–655.
Longstaffe, J.G., Courtier-Murias, D., Simpson, A.J., 2013. The pH-dependence of organofluorine binding domain preference in dissolved humic acid. Chemosphere 90, 270–275. https://doi.org/10.1016/j.chemosphere.2012.07.004.
Ma, L., Yates, S.R., 2018. Dissolved organic matter and estrogen interactions regulate estrogen removal in the aqueous environment: a review. Sci. Total Environ. https://doi.org/10.1016/j.scitotenv.2018.05.301.
MacBean, C., 2008. Isoproturon (34123-59-6). In: E-pesticide Manual. British Crop Protection Council, Alton, UK.
Myeni, S.C.B., Brown, J.T., Martinez, G.A., Meyer-Ilse, W., 1999. Imaging of humic substance macromolecular structures in water and soils. Science 286 (80), 1335–1337.
O'Neil, M.J., 2013. The Merck Index: an Encyclopedia of Chemicals, Drugs, and Biologicals. CamRoyal Society of Chemistry, Cambridge, UK.
OECD, 2009. OECD Guidelines for the testing of chemicals. Proposal for updating guideline 201, Freshwater alga and cyanobacteria. Growth Inhibition Test 1–21.
Pace, M.L., Reche, I., Cole, J.J., Fernández-Barbero, A., Mazuecos, I.P., Prairie, Y.T., 2012. pH change induces shifts in the size and light absorption of dissolved organic matter. Biogeochemistry 108, 109–118. https://doi.org/10.1007/S10533-01.
Pal, A., Gin, K.Y.H., Lin, A.Y.C., Reinhard, M., 2010. Impacts of emerging organic contaminants on freshwater resources: review of recent occurrences, sources, fate and effects. Sci. Total Environ. https://doi.org/10.1016/j.scitotenv.2010.09.026.
Palm, W.U., Millet, M., Zetzsch, C., 1998. OH radical reactivity of pesticides adsorbed on aerosol materials: first results of experiments with filter samples. In: Ecotoxicology and Environmental Safety. Academic Press Inc., pp. 36–43. https://doi.org/10.1006/eesa.1998.1664
Pan, B., Ning, P., Xing, B., 2008. Part IV - sorption of hydrophobic organic contaminants. Environ. Sci. Pollut. Res. 15, 554–564. https://doi.org/10.1007/s11356-008-0051-y.
Pokrovsky, O.S., Manasypov, R.M., Loiko, S.V., Shirokova, L.S., 2016. Organic and organo-mineral colloids in discontinuous permafrost zone. Geochem. Cosmochim. Acta 188, 1–20. https://doi.org/10.1016/j.gca.2016.05.035.
R Core Development Team, 2015. R: a language and environment for statistical computing. https://doi.org/10.1007/978-3-540-74686-7.
Ren, H., Ma, F., Yao, X., Shao, K., Yang, L., 2020. Multi-spectroscopic investigation on the spatial distribution and copper binding ability of sediment dissolved organic matter in Nansi Lake, China. J. Hydrol. 591, 125289. https://doi.org/10.1016/j.jhydrol.2020.125289.
Ripszam, M., Haglund, P., 2015. Automated method for determination of dissolved organic carbon-water distribution constants of structurally diverse pollutants using pre-equilibrium solid-phase microextraction. Environ. Toxicol. Chem. 34, 266–274. https://doi.org/10.1002/etc.2805.
Rizzuto, S., Baho, D.L., Jones, K.C., Zhang, H., Leu, E., Nizzetto, L., 2021. Binding of waterborne pharmaceutical and personal care products to natural dissolved organic matter. Sci. Total Environ. 784, 147208. https://doi.org/10.1016/j.scitotenv.2021.147208.
Rizzuto, S., Thrane, J., Baho, D.L., Jones, K.C., Zhang, H., Hessen, D.O., Nizzetto, L., Leu, E., 2020. Water browning controls adaptation and associated trade-offs in phytoplankton stressed by chemical pollution. Environ. Sci. Technol. X, acs.est c00548. https://doi.org/10.1021/acs.est.0c00548.
Rowett, C.J., Hutchinson, T.H., Comber, S.D.W., 2016. The impact of natural and anthropogenic Dissolved Organic Carbon (DOC), and pH on the toxicity of triclosan to the crustacean Gammarus pulex (L.). Sci. Total Environ. 565, 222–231. https://doi.org/10.1016/j.scitotenv.2016.04.170.
Rozman, K.K., Doull, J., 2000. Dose and time as variables of toxicity. Toxicology. https://doi.org/10.1016/S0300-483X(99)00204-8.
Schulz, R., Bub, S., Petschick, L.L., Stehle, S., Wolfram, J., 2021. Applied pesticide toxicity shifts toward plants and invertebrates, even in GM crops. Science (80-.) 372, 81–84. https://doi.org/10.1126/science.abe1148.
Tanaka, F., Fukushima, M., Kikuchi, A., Yabuta, H., Ichikawa, H., Tatsumi, K., 2005. Influence of chemical characteristics of humic substances on the partition coefficient of a chlorinated dioxin. Chemosphere 58, 1319–1326. https://doi.org/10.1016/j.chemosphere.2004.10.008.
Thevenot, M., Dousset, S., Hertkorn, N., Schmitt-Kopplin, P., Andreux, F., 2009. Interactions of diuron with dissolved organic matter from organic amendments. Sci. Total Environ. 407, 4297–4302. https://doi.org/10.1016/j.scitotenv.2009.04.021.
Thrane, J.E., Hessen, D.O., Andersen, T., 2014. The absorption of light in lakes: negative impact of dissolved organic carbon on primary productivity. Ecosystems 17, 1040–1052. https://doi.org/10.1007/s10021-014-9776-2.
Tolkoff-Rubin, N., 2011. Treatment of irreversible renal failure. In: Goldman's Cecil Medicine: Twenty, fourth ed. Elsevier Inc., pp. 818–826. https://doi.org/10.1016/B978-1-4377-1604-7.00133-0
Tomlin, C.S.D., 2000. Isoproturon (464). In: The E-Pesticide Manual, twelfth ed. British Crop Protection Council, Farnham, UK3. Version 2.0.
Vialykh, E.A., Mckay, G., Rosario-Ortiz, F.L., 2020. Computational assessment of the three-dimensional configuration of dissolved organic matter chromophores and influence on absorption spectra. Environ. Sci. Technol. 54, 15904–15913. https://doi.org/10.1021/acs.est.0c05860.
Wang, Z., Jiang, Q., Wang, R., Yuan, X., Yang, S., Wang, W., Zhao, Y., 2018. Effects of dissolved organic matter on sorption of oxytetracycline to sediments. Geofluids 2018. https://doi.org/10.1155/2018/1254529.
Wei-Hass, M.L., Hageman, K.J., Chin, Y.P., 2014. Partitioning of polybrominated diphenyl ethers to dissolved organic matter isolated from arctic surface waters. Environ. Sci. Technol. 48, 4825–4859.
Wickham, H., 2006. ggplot2: Elegant Graphics for Data Analysis 35, 2006.
Williams, C.F., Farmer, W.J., Letey, J., Nelson, S.D., 1999. Design and characterization of a new dialysis chamber for investigating dissolved organic matter-organic

complexes. J. Environ. Qual. 28, 1757–1760. https://doi.org/10.2134/jeq1999.00472425002800060010x.

Xu, H., Guo, L., 2017. Molecular size-dependent abundance and composition of dissolved organic matter in river, lake and sea waters. Water Res. 117, 115–126. https://doi.org/10.1016/j.watres.2017.04.006.

Xu, H., Zou, L., Guan, D., Li, W., Jiang, H., 2019. Molecular weight-dependent spectral and metal binding properties of sediment dissolved organic matter from different origins. Sci. Total Environ. 665, 828–835. https://doi.org/10.1016/j.scitotenv.2019.02.186.

Yamada, N., Katoh, M., 2020. Feature of lead complexed with dissolved organic matter on lead immobilization by hydroxyapatite in aqueous solutions and soils. Chemosphere 249, 126122. https://doi.org/10.1016/j.chemosphere.2020.126122.

Zhao, J., Wang, Z., Ghosh, S., Xing, B., 2014. Phenanthrene binding by humic acid-protein complexes as studied by passive dosing technique. Environ. Pollut. 184, 145–153. https://doi.org/10.1016/j.envpol.2013.08.028.

Science of the Total Environment 784 (2021) 147208

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Binding of waterborne pharmaceutical and personal care products to natural dissolved organic matter

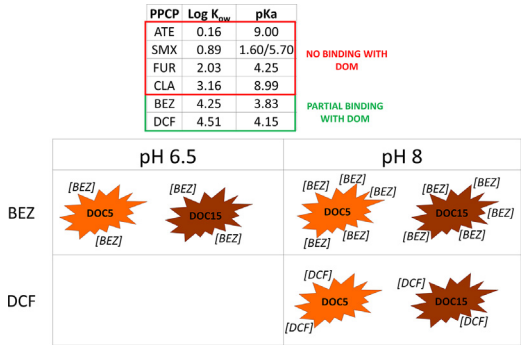
Simone Rizzuto [a], Didier L. Baho [b,c], Kevin C. Jones [a,*], Hao Zhang [a,*], Eva Leu [d], Luca Nizzetto [c,e]

[a] Lancaster Environment Centre, Lancaster University, Lancaster LA1 4YQ, UK
[b] Department of Aquatic Sciences and Assessment, Swedish University of Agricultural Sciences, Uppsala, Sweden
[c] Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, 0349 Oslo, Norway
[d] Akvaplan-niva, CIENS, Science Park, Gaustadalléen 21, 0349 Oslo, Norway
[e] RECETOX, Masaryk University, Kamenice 753/5, 625 00 Brno, Czech Republic

HIGHLIGHTS

- Natural DOM partially bind to PPCPs with carboxylic functional groups.
- DOM-PPCPs binding dependent on water pH
- DOM concentration does not influence the binding

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 2 February 2021
Received in revised form 13 April 2021
Accepted 13 April 2021
Available online 20 April 2021

Editor: Jay Gan

Keywords:
Natural dissolved organic matter
Pharmaceuticals and personal care products
Water chemistry
Binding

ABSTRACT

Information on how key environmental conditions such as natural dissolved organic matter (DOM) and water pH alter the possible risks posed by pharmaceuticals (PPCPs) is still scarce. In our previous study, the presence of natural DOM at high pH reduced the toxicity of a mix of waterborne PPCPs to algae. DOM-complexation and pH effect on speciation of the more hydrophobic and neutral compounds of the mix was suggested to be driving this behaviour. However, the study design did not allow the verification of this hypothesis. Here, the DOM- PPCPs interaction at different pH was investigated for 6 PPCPs through equilibrium dialysis, under the same conditions of DOM and pH as our previous study. Association with DOM was confirmed for the more hydrophobic PPCPs at high pH. The results suggest the binding was driven by i) the presence of carboxylic groups of PPCPs, ii) high pH shifting the structural configuration of DOM, making it more suited to bind some of the PPCPs. A non-linear change of binding capacity with increasing DOM concentration was also observed among the tested PPCPs.

1. Introduction

The widespread occurrence of anthropogenic contaminants in freshwater ecosystems is a major concern (Pal et al., 2010). Emerging contaminants such as the group of pharmaceutical and personal care products (PPCPs (Boxall et al., 2012)), widely used and continuously discharged in treated and untreated wastewater (Schwarzenbach

* Corresponding authors.
E-mail addresses: k.c.jones@lancaster.ac.uk (K.C. Jones), h.zhang@lancaster.ac.uk (H. Zhang).

https://doi.org/10.1016/j.scitotenv.2021.147208

et al., 2006), are particularly worrisome as they are biologically active at low concentration. In the environment, PPCPs can interfere with fundamental metabolic pathways (e.g. chlorophyll-a and lipid synthesis) in non-target organisms such as freshwater phytoplankton (Zhang et al., 2012, 2019); this could possibly translate into severe repercussions for the functioning of freshwater ecosystems (Arnold et al., 2013), considering the key ecological functions underpinned by microalgae. Knowledge of freshwater biota sensitivity to these stressors is mostly derived from experiments conducted under controlled laboratory conditions (OECD, 2009). There is a paucity of information on how the risk posed by these contaminants is modulated by the prevailing environmental conditions (Holmstrup et al., 2010; Laskowski et al., 2010). Dissolved organic matter (DOM) is present in all surface freshwaters (Leenheer and Croue', 2003) and has the ability to bind, adsorb and/or transform contaminants by forming complexes that are too large or too polar to cross biological membranes (Lipnick, 1995). Through this process, DOM (generally analyzed as concentration of dissolved organic carbon – DOC) may reduce the bioavailable fraction and the toxicity of contaminants (Chen et al., 2017; Pan et al., 2009; Rowett et al., 2016).

The DOM binding affinity (usually expressed as distribution coefficient K_{DOC} or K_d) can be controlled by several factors, such as the water chemistry (i.e. pH), the physicochemical properties of the compounds (i.e. hydrophobicity, presence of functional groups) (Ashauer and Escher, 2010; Behera et al., 2010; Sun et al., 2020) and of the DOM (i.e. molecular size, aromaticity, presence of functional groups and concentration; Gu et al., 2007; Tanaka et al., 2005). For example, a change in water pH can affect the compound-DOM complexation by altering the compound and/or the DOM molecular configuration (Engebretson and von Wandruszka, 1994; Ghosh and Schnitzer, 1980; Myeni et al., 1999). The relationship between DOM binding capacity and its concentration is also unclear. For instance, while linear correlations are generally reported (Burkhard, 2000; Krop et al., 2001), other studies observed that increasing concentrations of DOM could generate tighter molecular rearrangements, preventing the chemical compounds accessing the more hydrophobic areas of the DOM where binding generally takes place (Akkanen et al., 2001; Akkanen and Kukkonen, 2003). Hence, the effect of these complex interactions on toxicity of contaminants cannot be predicted easily. This is particularly important in boreal freshwater ecosystems. Here, climate and land-use changes - together with recovery from past acidification - have caused an increase in the levels of DOM and altered the water pH over recent decades, a process also known as water browning (Monteith et al., 2007; Williamson et al., 2015).

The influence of the interaction between DOM and water pH on the toxicity of PPCPs has been a subject of research (Pan et al., 2009). In a recent study (Rizzuto et al., 2020), we found that the toxicity of an environmentally realistic mix of 12 PPCPs to algae was reduced in water with low levels of natural DOM and high pH. We suggested that high pH conditions increased the DOM-PPCPs binding affinity by controlling the physicochemical properties of both PPCPs and DOM. In addition, a direct effect of DOM in hindering algal growth was observed with a non-linear dependence on DOM concentration (Rizzuto et al., 2020). However, that study design did not allow evaluation of the hypothesis on the mechanisms of interaction between PPCPs and DOM.

In this study we therefore explicitly addressed the interaction between PPCPs and the natural DOM at two different pH. Six PPCPs with demonstrated toxic effects on phytoplankton, with a range of different physicochemical properties were selected from the original mix; the same pH values, natural DOM (extracted from the same lake, Gjessing et al., 1999) and concentrations were used as in the previous study (Rizzuto et al., 2020). An equilibrium dialysis technique was used to investigate the chemical-DOM binding (Akkanen et al., 2001; Akkanen and Kukkonen, 2001). With this method, the contaminant molecules can freely diffuse across the dialysis membrane, whereas the larger-sized DOM complexes are restricted to one side of the membrane. This method has been commonly used, but usually with a commercially available DOM only (i.e. Suwannee River, isolated humic or fulvic acids, Böhm et al., 2016). However, natural DOM is polydisperse, and may contain different constituents with varying levels of hydrophilicity, as well as several functional groups with different chemistry (Leenheer and Croue', 2003). Hence, while it is important to have a standardized DOM and/or to investigate the influence of different functional groups, it is also crucial to test the effects arising from DOM naturally occurring in local freshwater ecosystems that is relevant to the investigation (Akkanen et al., 2001). In addition, one technical hindrance recognized by several authors concerns the pore size of the membrane generally used in these experiments (>1000 Da); this could cause leakage of smaller molecular size DOM across the dialysis membrane, leading to an over-underestimation of the binding effect of DOM (Akkanen and Kukkonen, 2003). In the present study we overcame these limitations by using: i) natural DOM isolated from a boreal catchment (Gjessing et al., 1999), containing virtually the full spectrum of constituents native of boreal systems; ii) hydrophilic semipermeable membranes with a smaller pore size (100–500 Da), enabling only relatively small and free chemicals (e.g. in the range of the PPCPs used here) to permeate.

Results from the equilibrium dialysis studies are used to test the hypotheses arising from our previous study, considering the implications for the potential effects on freshwater ecosystems.

2. Materials and methods

The association of a mix of 6 PPCPs to three levels of natural DOM (0, 5 and 15 mg L^{-1} DOC), at two water pH values (6.5, 8) was tested using the equilibrium dialysis technique (Fig. 1).

2.1. Selection of experimental conditions

2.1.1. Chemical compounds

Six PPCPs were selected from the mix used in our previous study (Rizzuto et al., 2020), namely: Atenolol (ATE), Sulfamethoxazole (SMX), Furosemide (FUR), Clarithromycin (CLA), Bezafibrate (BEZ), and Diclofenac (DCF) (chemical structures in Fig. S1). They are among the most commonly detected PPCPs in European wastewaters and freshwaters (concentrations in natural freshwaters ranging from 0.1 to 380 μg L^{-1}, Table S1). This selection provides: i) a broad range of hydrophobicity (measured as the octanol-water partition coefficient - log K_{ow}); ii) a range of acid dissociation constant (pKa) values; and iii) presence of different functional groups on the analytes (Table 1). Expected concentrations of each chemical at the end of the experiment at equilibrium conditions (C_E, Table 1) are within the range of those detected in European freshwaters (Table S1).

2.1.2. DOM and pH

The DOM used in this experiment was previously isolated through reverse osmosis from the water of the Hellerudmyra tarn (Norway) (Gjessing et al., 1999), and donated by the University of Oslo (Norway). Hellerudmyra tarn is a small catchment (0.08 km^2) that provided DOM samples for the IHSS Nordic Fulvic and Humic Reference Material (Gjessing et al., 1999). The most relevant physicochemical properties of this natural DOM are reported in Table 2. Other properties of the DOM were detailed in Gjessing et al. (1999). The levels of DOM applied here represent low to medium-high concentrations typically found in Northern European lakes (Henriksen et al., 1998). The pH levels were used to represent the range typically found in Northern European Lakes (Henriksen et al., 1998). pH was monitored in all experimental units. No significant change in pH occurred in any of the units during the experiment (data not shown).

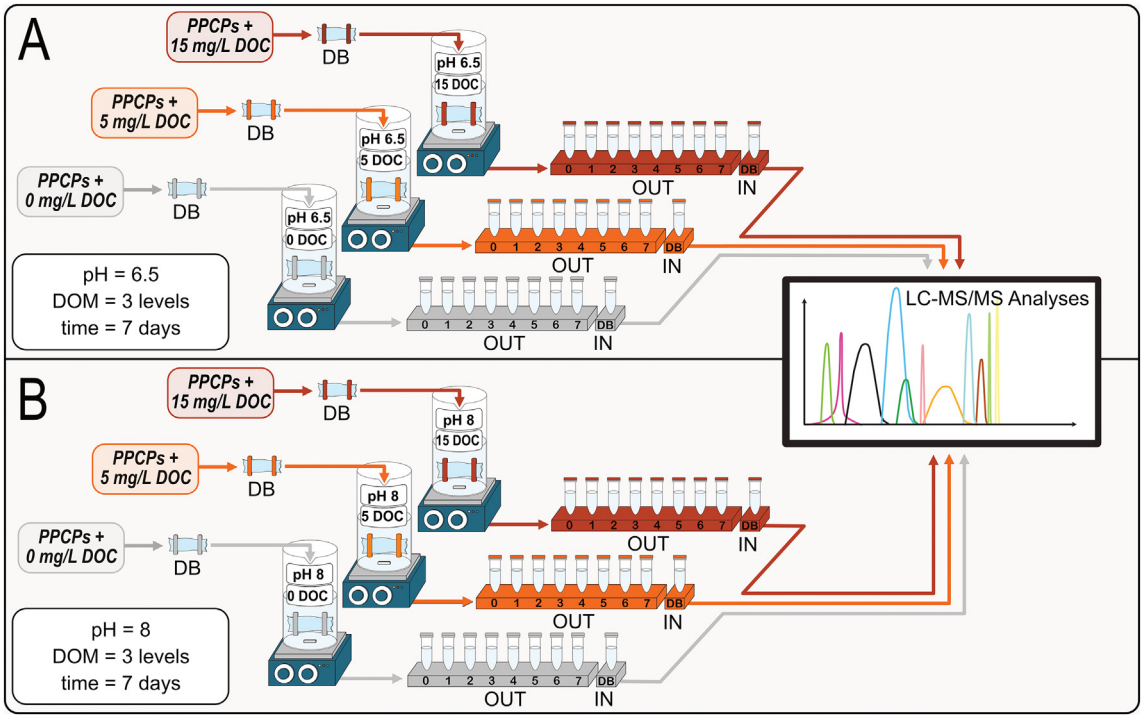


Fig. 1. Experimental design. The association of the mix of 6 PPCPs to DOM (0, 5 and 15 mg L^{-1} DOC) was tested through equilibrium dialysis technique at pH 6.5(A) and 8(B). DB; dialysis bags, OUT; samples collected each day over 7 days outside the dialysis bag, IN; samples collected inside the dialysis bag on the seventh day of the experiment.

2.2. Experimental setting

2.2.1. Preparation of PPCPs and DOM working solutions

Analytical standards of the 6 compounds were purchased from Sigma-Aldrich (USA) and individually diluted in methanol (Sigma-Aldrich) to create 4 stock solutions of 1 mg mL^{-1} for ATE, CLA, BEZ and DCF, and 2 stock solutions of 100 μg mL^{-1} for SMX and FUR. To prepare the working solution of the mix, 100 μL from the ATE, SMX, FUR, CLA and BEZ stock solutions and 200 μL from DCF stock solution was spiked into an amber glass vial, to reach the following concentrations in 10 mL total volume: ATE, CLA and BEZ 10 μg mL^{-1}, DCF 20 μg mL^{-1} and SMX and FUR 1 μg mL^{-1}. The DOM working solution was prepared by weighing 10 mg of dry DOM and adding 10 mL of MQ water to reach a concentration of 1 mg DOM mL^{-1}.

2.2.2. Preparation of artificial water solutions at two different pH

Four litres of soft artificial water (SAF) was prepared by adding MQ water with 1.17 g NaCl to reach 0.01 M (0.58 g L^{-1}) ionic strength, commonly detected in boreal freshwaters. The solution was then split in two 2 L bottles, and then adjusted by titration with HCl or NaOH to reach 6.5 and 8 respectively. The SAF solution was used inside and outside the dialysis bags.

2.2.3. The experimental units

Cellulose ester dialysis bags, with a flat-width of 31 mm and molecular cut-off of 100–500 Da (Spectra/por, Spectrum Europe, Breda, The Netherlands), were cut to 10 cm lengths and thoroughly washed with Milli-Q water. 460 μL aliquots of the PPCPs mix working solution were spiked in each dialysis bag. For the control units with no DOM, SAF

Table 1
Physicochemical properties of the 6 investigated compounds.

PPCPs	Use	Log K_{ow}	Functional groups	Molecular weight (g mol^{-1})	Water solubility (mg L^{-1})	pKa	C_E (μg L^{-1})
Atenolol (ATE)	Beta-blocker	0.16 (a)	Amine	266.34	13,300	9 (f)	22
Sulfamethoxazole (SMX)	Antibiotic	0.89 (a)	Isoxazole	253.28	610	1.6/5.7 (g)	2.2
Furosemide (FUR)	Diuretic	2.03 (b)	Chlorobenzoic	330.74	73.1	3.9 (h)	2.2
Clarithromycin (CLA)	Antibiotic	3.16 (c)	Amine	747.96	1.63	8.99 (c)	22
Bezafibrate (BEZ)	Lipid-lowering	4.25 (d)	Carboxylic	361.82	1.55	3.83 (i)	22
Diclofenac (DCF)	Non-steroidal anti-inflammatory	4.51 (e)	Carboxylic	296.14	2.37	4.15 (b)	44

For reference on Log K_{ow} and pKa values:
a. (Hansch et al., 1995),
b. (Sangster, 1997),
c. (McFarland et al., 1997),
d. (Tang et al., 2014),
e. (Avdeef, 2005),
f. (O'Neil, 2013),
g. (Boreen et al., 2004),
h. (Khan and Ongerth, 2004),
i. (ChemAxon, 2021).

Table 2
Physicochemical properties of the natural DOM used in this study when prepared at typical concentration found in the source lake (see Gjessing et al., 1999 for details).

pH	Conductivity ($mS\ m^{-1}$)	Colour ($mg\ Pt\ L^{-1}$)	UV absorbance 254 nm	$(SUVA_{254})^a$ ($mgC \times 10^2$)	%Corg[b]	molecular weight (Da)	C_{ar}/C_{al}[c]
5.17	2.49	166	0.813	4.59	50.3	3900	0.22

Lake DOC concentration typically of 17.7 $mg\ L^{-1}$.
[a] Specific UV – Absorbance at 254 nm.
[b] Percentage of Carbon content.
[c] Ratio of aromatic to aliphatic carbon.

was then added to each bag, to reach the final volume of 10 mL. For the 5 $mg\ L^{-1}$ and 15 $mg\ L^{-1}$ DOC units, 170 μL and 510 μL of DOM working solution were spiked into the respective bags, before adding SAF to reach the final volume of 10 mL. Dialysis bags were sealed with standard closures (Spectra/por, Spectrum Europe, Breda, The Netherlands) and placed into a 200 mL of SAF solution (Fig. 1). After adding glass-coated metal stirrer bars (20 mm) the experimental units were closed on top with Teflon linen plugs, and placed on a magnetic stirrer (Multistirrer15, Progen Scientific, UK) in the dark at 19 ± 1.2 °C for 1 week. The experimental procedure was repeated at both pH levels (6.5, 8), in triplicates (Fig. 1). Every day for 7 days, 2 mL samples were collected from the units (externally from the dialysis bag) and split into two 1 mL aliquots for the determination of PPCPs and to check for a potential break-through of DOM through the dialysis membrane, respectively. On the last day of the experiment, 1 mL sample was also collected from each unit, from inside the bags. Samples for chemical analyses were filtered through a 0.2 μm syringe, placed into a 2.5 mL amber glass vial, and stored at −20 °C until analyses. Chemical analyses were carried out through direct injection in LC-MS/MS (Shimadzu, 8081) following the method reported in Text S1. MS/MS acquisition parameters are reported in Table S2. The samples for DOM loss were processed immediately following the method reported in Text S2. No detectable release of DOM from the dialysis bags was observed at either pH level (Fig. S2). Mass recovery (Text S3) and mass loss due to adsorption of the PPCPs to all the components of the experimental units (dialysis bag, glassware, clips and stirrer bars; Text S4) was also tested.

2.3. Data management scheme and quality assurance criteria

Data were treated according to the scheme presented in Fig. 2. In summary, quality thresholds for reproducibility, mass balance closure in the experimental units, adsorption of PPCPs on the glassware and membranes and quality criteria to verify transmembrane equilibrium were considered as described in the following sections:

2.3.1. Reproducibility, mass balance and adsorption to glassware and membrane

The quality of replicates, mass recovery of the compounds (i.e. mass balance closure), and their mass loss due to adsorption to components of the experiment unit were checked both inside (internal wall of the bag, hereon refereed as A_{in} (μg)) and outside the bag (sum of external wall of the bag, glassware, clips and stirring bars, A_{out} (μg)). Acceptable mass recovery was set to be at 100 ± 10% of the mass of the compounds. Equilibrium dialysis studies do not generally report adsorption parameters, because they are based on the assumption that any chemical adsorbed to the bag, the glassware or other components should not interfere with the equilibrium between the compound inside and outside the bag (Akkanen et al., 2001; Akkanen and Kukkonen, 2003). However, excessive adsorption could impose a gradient preventing the compound from reaching equilibrium, such that the true effect of DOM may not be observable. In addition, our experimental setup differs from the others since we used a smaller pore size of the dialysis membrane (100–500 Da) to prevent DOM leakage, which can potentially influence the adsorption of the compounds by slowing their diffusion. Hence, mass loss due to adsorption (A_{in}, A_{out}) was included in the QA/QC parameters and set as acceptable in the range of 0–35% of the total added mass of individual PPCPs.

2.3.2. Equilibrium

A condition for the data to be considered for the next steps of the assessment of DOM complexation was that the concentration of PPCP in the control units reached equilibrium across the dialysis membrane by the end of the experiment. The criteria used to establish equilibrium conditions were:

- No significant differences were found between the concentration of the compound inside the control dialysis bag, C_{in} ($\mu g\ L^{-1}$), and concentration outside the control bag, C_{out} ($\mu g\ L^{-1}$). C_{in} could include both complexed and free compound, while C_{out} only represents free compound. Because the environments inside and outside the dialysis bags had very different volumes (10 mL inside vs. 200 mL outside), the mass (μg) of each chemical was calculated inside ($M_{D/in}$) and outside ($M_{D/out}$) the bag to avoid any potential under-overestimation induced by dilution. The mass was calculated from the concentrations

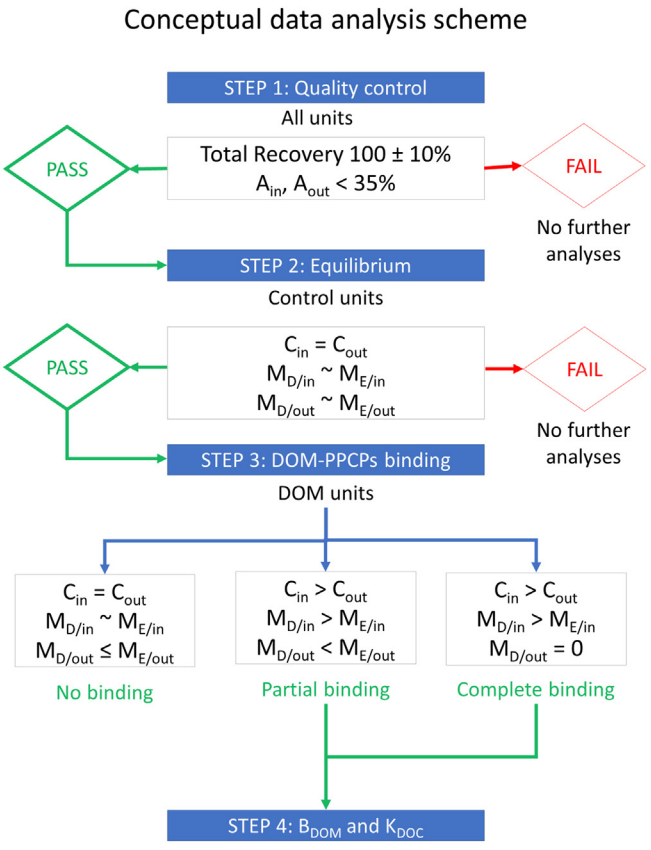


Fig. 2. Conceptual data analysis scheme. C_{in} and C_{out} are the concentrations of each PPCP inside/outside the dialysis bags. $M_{D/in}$ is the mass of each PPCP detected inside the dialysis bag. $M_{D/out}$ is the mass of each PPCP detected outside the dialysis bag. $M_{E/in}$ is the mass of each PPCP expected inside the bag of the control units at equilibrium.

detected inside and outside the bag, not including any associated with the glassware and dialysis bag.
- The value of $M_{D/in}$ and $M_{D/out}$ should not be significantly different from the mass expected inside and outside the dialysis bag of the control units at the end of the experiment ($M_{E/in}$ and $M_{E/out}$, respectively). The values of $M_{E/in}$ and $M_{E/out}$ were calculated from the values of the final concentration expected inside and outside the dialysis bag of the control units at the end of the experiment (C_E), assuming that C_{in} equilibrated with C_{out}, and allowing A_{in} and A_{out} values ranging 0–35%.

Failure to meet these conditions for the control units prompted the exclusion of the compound at that pH level from the study.

2.3.3. DOM-PPCPs binding

When the PPCPs in the control units reached equilibrium and all the quality criteria were fulfilled, the DOM-PPCPs binding was also assessed for the units containing DOM. Theoretically, if DOM bound the compounds, a significantly higher concentration will be detected inside the dialysis bags than outside, because complexation by the DOM prevents the compound crossing the membrane of the dialysis bag due the larger size. Analogously to the comparison of $M_{D/in}$ and $M_{D/out}$ performed on the control units to assess equilibrium conditions, a difference of $M_{D/in}$ and $M_{D/out}$ whereby $M_{D/in}$ being in significant excess of $M_{D/out}$ was deemed as one required piece of evidence for partial or complete binding of PPCPs to DOM. Furthermore, to rule out the confounding factor of different dilution between the bag inner and outer environment (and following the conceptual scheme of Fig. 2), another condition for PPCP binding to DOM was that $M_{D/in}$ should be higher than $M_{E/in}$ for the experimental units with DOM and, concurrently, $M_{D/out}$ should be lower than $M_{E/out}$.

$M_{E/out}$ was calculated on the same assumptions used for $M_{E/in}$ (see paragraph *2.3.2 Equilibrium*). In summary, after checking for equilibration in the control units, three scenarios were considered to determine complexation in the units containing DOM:

- No binding: $C_{in} \sim C_{out}$; $M_{D/in} \sim M_{E/in}$, and $M_{D/out} \leq M_{E/out}$.
- Complete binding: $M_{D/in}$ represented 100% of the PPCP mass spiked in the experimental unit, $M_{D/in} > M_{E/in}$, while $M_{D/out} = 0$.
- Partial binding: $M_{D/in} > M_{E/in}$ and $M_{D/out} < M_{E/out}$.

The magnitude of the effect of DOM was also tested by calculating the conditional distribution coefficient (K_{DOC}, L g^{-1}) at both levels of DOM. K_{DOC} was calculated as follows (from (Buschmann et al., 2006)):

$$K_{DOC} = \frac{[C_{in}] - [C_{out}]}{[C_{out}]\ x\ [DOC]} * 1000$$

where the term $[C_{in}]$-$[C_{out}]$ is the concentration of compound complexed with DOM and $[C_{out}]$ is the concentration of free (unbound) compound. DOC and DOM values can be inter-converted in the equation by using the DOM's % of C content value of 50.3 (see Table 2).

The percentage of bound compound (B_{DOM}), defined by mass of bound compound divided by the total mass of the compound inside the bag, was calculated as follows:

$$B_{DOM} = \frac{M_{in} - C_{out} * V_{in}}{M_{in}} * 100$$

where V_{in} is the solution volume inside the dialysis bag and $C_{out}{}^{*}V_{in}$ is the mass of unbound inside the bag. M_{in} is the total mass of compound inside the bag.

2.4. Statistical analyses

Data analyses and statistics were conducted using R (version 3.5.1) statistical software (R Core Development Team, 2015). The single comparison analyses between C_{in} and C_{out}, K_{DOC}, and B_{DOM} between different DOM levels were tested by one-way ANOVA test. The graphs were prepared using the R package "ggplot2" (Wickham, 2006).

3. Results and discussions

3.1. Step 1: quality control

All the compounds had 100 ± 10% total mass recovery at all pH and DOM levels in all units, apart from 3 outliers (Tables S3-S9). The optimal recovery rates shown by each targeted PPCP (Table S3-S9) indicated that ion suppression, a technical hindrance that can affect the accuracy of LC-MS/MS analyses using direct injection (Antignac et al., 2005; George et al., 2018), was negligible. Results from ATE, SMX, FUR, BEZ and DCF showed that A_{in} and A_{out} were minor or acceptable (2–35% mass loss (Tables S3-S8). In contrast, CLA showed higher A_{in} and A_{out} than the other compounds (Table S6), yielding values of ca. 32% at pH 6.5, and ca. 70% at pH 8 (Table S6). Higher adsorption of this compound at pH 8 probably links to its relative hydrophobicity (log K_{ow} 3.16 in the associated form with a pKa of 8.99). At pH 8, 91% of CLA was expected to be present in the solution in the associated form, compared to 99.7% at pH 6.5. The presence of more neutral (and consequently more hydrophobic) forms of the compound available at pH 8 may have enhanced its adsorption to the glassware, plastic clips or stirrer bars. The chemical configuration of CLA is very complex (see Fig. S1) and offers the presence of different domains that can create H-bonds. At the same time, H-bonds are also sensitive to pH, as a consequence of the pH-induced charge density and the conformation change of CLA or of the dialysis membrane. Variable adsorption of CLA to glassware has been reported previously (Wibawa et al., 2003). Daily monitoring of water pH reported no significant changes (data not shown), which excluded possible confounding effects induced by water pH drifting during the experiment.

3.2. Step 2: equilibrium

Secondly, we checked transmembrane equilibration in the control units, based on the criteria mentioned above (Fig. 2). C_{out} and C_{in} measured on the seventh day are depicted in Fig. 3 for the different DOM and pH conditions (for C_{out} during the seven-day experiment see also Fig. S3). The control units displayed no significant differences between C_{in} and C_{out} on day 7 at pH 6.5 or pH 8 for ATE, SMX, CLA (at pH 6.5 only), BEZ and DCF (Fig. 3, Table S10). Furthermore, in compliance with the set quality assurance criteria, the $M_{D/in}$ values of these compounds observed in the control units were not higher than $M_{E/in}$ (Table S3-S4, S6-S8). This confirms that the equilibrium conditions were met for free chemical compounds (not complexed) at both pH levels. In contrast, significant differences were observed for FUR at pH 8, where C_{in} in the control unit was significantly higher than C_{out} (Fig. 3, Table S10), while no differences were observed at pH 6.5. In addition, $M_{D/in}$ of this compound was approximatively 20% higher than $M_{E/in}$ (Table S5). These results indicate that FUR did not reach equilibrium before the end of the experiment at pH 8. Water pH was found to significantly influence the behaviour of FUR ($F = 5.66$, $p < 0.05$). This is not surprising because FUR is a relatively large molecule, with MW close to the lower boundary of the molecular cut off of the membrane and includes a carboxylic group with an estimated pKa = 4.25. Beyond this ionizable functional group, FUR contains two domains capable of forming N-H···O hydrogen bonds. These domains can obviously form hydrogen bonds with groups of the cellulose ester, making FUR-membrane interactions sensitive to pH. Furthermore, the sulphamoil group at position 2 of the chlorobenzoic acid domain of FUR and the amine group in position 5 interacting with the oxygen of the furan group will both affect steric configuration and intramolecular interaction of this compound, depending on solution pH. Because of these characteristics FUR is an example of a conformational polymorph (Thakuria et al., 2017). Examples of the effect of pH on steric

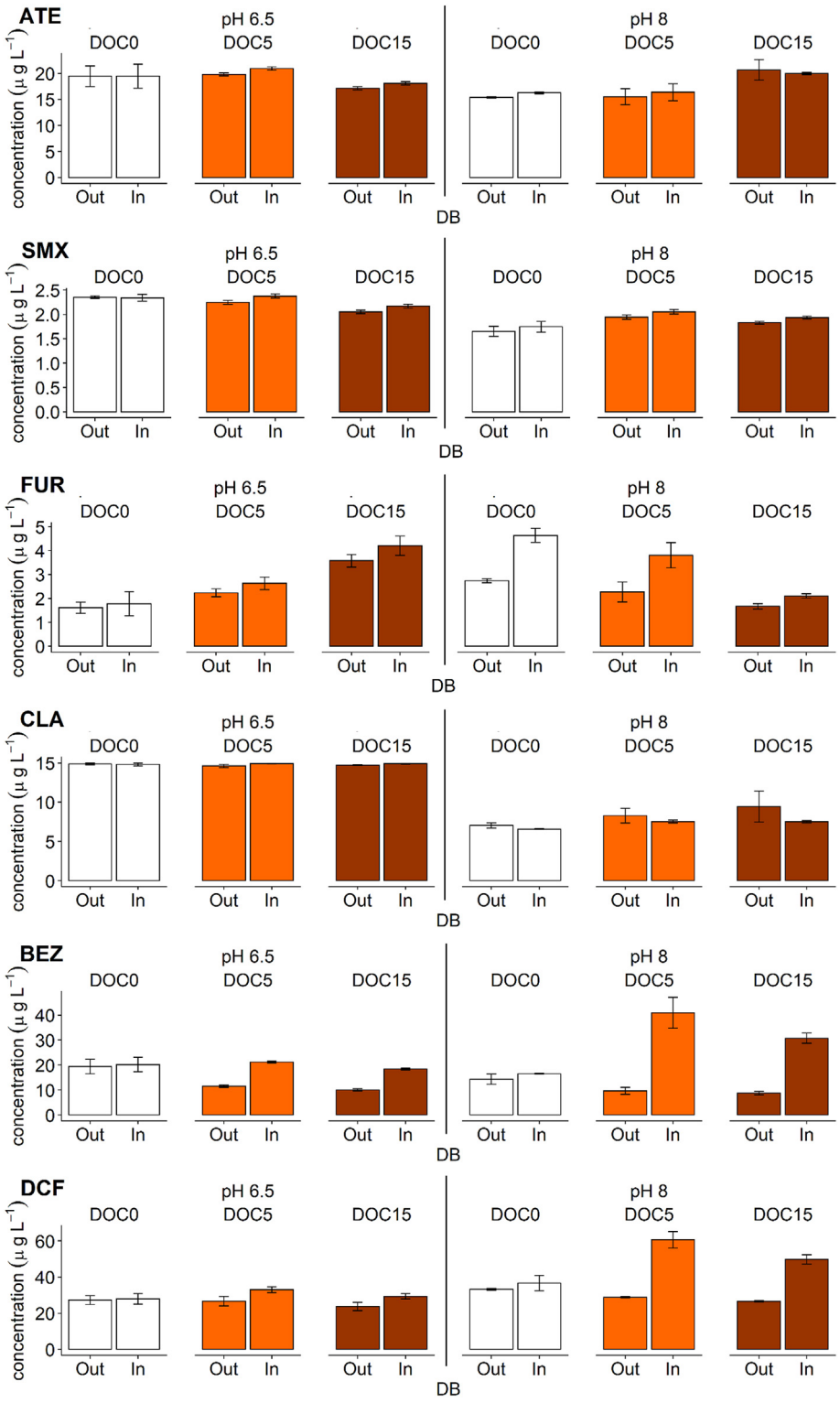


Fig. 3. Concentration outside (C_{out}) and inside (C_{in}) the dialysis bag (DB) on the last day of the experiment for the six investigated PPCPs, at three levels of DOM (0, 5 and 15 mg L^{-1} DOC) and two levels of pH (6.5, 8). Error bars represent standard deviation.

arrangements of compounds used in drugs formulations are illustrated elsewhere (Frenkel et al., 2005). FUR at pH 8 failed to meet the equilibrium conditions and was therefore excluded from further analysis and discussion. The compounds reaching equilibrium (ATE, SMX, CLA at pH 6.5, FUR at pH 6.5, BEZ and DCF) were admitted to the next procedural steps, where the DOM-PPCP binding was assessed.

3.3. Step 3 DOM-PPCPs binding

The third step of the data processing scheme was to assess the degree of binding with DOM for those chemicals that passed steps 1 and 2. ATE, SMX, CLA (pH 6.5) and FUR (pH 6.5) did not show significant differences between C_{in} and C_{out} for different DOM levels (Table S11–12),

which indicates no binding according to the experimental design criteria and conceptual analysis scheme in Fig. 2. In contrast, BEZ and DCF showed significantly higher C_{in} than C_{out} in the water containing DOM (Fig. 3, Table S11–12). $M_{D/in}$ of the DOM units was significantly higher than $M_{E/in}$ for both the compounds (Tables S7-S8). In addition, the $M_{D/out}$ was >0 for both compounds at both DOM levels (Table S7-S8), indicating partial binding with DOM, in line with the conceptual analysis scheme (Fig. 2). Complete binding was not observed for any of the investigated compounds, according to the conceptual analysis scheme.

3.4. Factors influencing DOM-PPCPs binding affinity

3.4.1. Physicochemical properties of PPCPs and the role of DOM composition

Generally, the interaction between organic contaminants and DOM can be driven by different processes which may act both individually or synergistically. These may be hydrophobic interactions, weak H-bond interactions and covalent bonds.

The linear free energy concept, whereby the DOM-contaminant affinity – K_{DOC} or K_d - is positively correlated with the degree of hydrophobicity of contaminants – K_{ow} - (Pan et al., 2009) describes hydrophobic interactions. The results broadly indicate an influence of this interaction, with the higher hydrophobicity compounds (DCF and BEZ, log K_{ow} of 4.5 and 4.25, respectively – Table 1) showing association/partial binding with the DOM. In contrast, no DOM-binding was observed for the 4 compounds with lower hydrophobicity (ATE, SMX, FUR (pH 6.5) and CLA (pH 6.5), log K_{ow} of 0.16, 0.89, 2.03 and 3.16, respectively – Table 1). Nevertheless, interpretation only based on hydrophobic interaction is over simplistic. Firstly, because these compounds all have weak acid groups which can interact with electron donors in the DOM and functional groups (such as amines) that can form N-H···O hydrogen bonds with oxidized groups in the DOM. Furthermore, all these PPCPs have aromatic rings which may engage in ion-dipole induced or dipole-dipole induced interactions with a range of DOM domains (especially aromatic and carboxylic groups as well as ketones, abundant in DOM) (Holbrook et al., 2004). In principal, cation exchange, cation bridging, surface complexation, and hydrogen binding can represent different forms of interaction between these PPCPs and DOM (Hernandez-Ruiz et al., 2012; Kwon and Armbrust, 2008; Pan et al., 2009). This is indicated by the B_{DOM} and K_{DOC} results (Table S13), that show BEZ having a higher degree of association with DOM than DCF, despite the latter having greater hydrophobicity. BEZ showed average B_{DOM} and log K_{DOC} ranging 45–74% and 1.98–2.52 respectively, while DCF indicated average values of 49% for B_{DOM} and 2.05 for log K_{DOC} (Table S13). The association of DCF and BEZ with DOM has been reported previously (Lu et al., 2018). Yamamoto et al. (2003) reported that other moderately lipophilic pharmaceuticals - 17β-estradiol, estriol and 17β ethynylestradiol (log K_{ow} of 3.94, 2.45 and 3.67, respectively) - with carboxyl groups similar to DCF - bound to DOM with sorption energy comparable to that of hydrogen bonding. Other studies report evidence of BEZ interacting with DOM, under experimental conditions designed to study degradation or removal of PPCPs from drinking water (Maeng et al., 2012; Vieno et al., 2010). To the best of our knowledge, the results presented here are the first on the interactions of naturally occurring DOM with BEZ.

Our study differs from others in reporting evidence of weak interactions for the other 4 compounds with DOM. Yamamoto et al. (2005) showed very low sorption of ATE to DOM (0.2% bound fraction, through fluorescence quenching observations) probably due to specific sorption forces other than hydrophobic interactions, or π- π interactions with Gibbs free energy contributions as an alternative (Delgado et al., 2015; Keiluweit and Kleber, 2009; Zeng et al., 2012). Some other evidence suggested that binding mechanisms of sulphonamide antibiotics such as SMX tended to be hydrophobic partitioning, despite their low K_{ow} values (Chen et al., 2017). FUR was found to form complexes with fulvic acids, even though the mechanism behind the complexation remains unclear (Prakash Agarwal et al., 2008). Interactions of CLA with terrestrial humic acids (Elliot soil humic acid) through cationic species – low proton affinity sites of humic acids have also been reported (Christl et al., 2016). Despite their relevance for the topic treated in the present studies, none of these cited works used the equilibrium dialysis membrane method or were directly focused on investigating DOM-PPCPs binding. Rather, they were aimed at investigating the photodegradation of the compounds (Zeng et al., 2012), or chemical removal from drinking waters (Delgado et al., 2015; Zeng et al., 2012). Hence the present study introduces novel elements compared to previous assessments. There is no claim that the present approach and results can shed full mechanistic light on the physicochemical processes controlling the interaction between PPCPs and DOM. The focus here was primarily on assessing the occurrence of binding and the potential relevance for exposure and risk assessment, while providing some fundamental evidence of the role of environmental conditions (e.g. by means of varying pH and DOM levels) and of the complexity of such interactions.

Previous assessments of the PPCPs-DOM interactions utilized commercial DOM standards such as Suwannee River Fulvic Acid, Suwannee River Humic Acid, Nordic Lake Fulvic Acid and Nordic Lake Humic Acid (Chen et al., 2004; Delgado et al., 2015; Keiluweit and Kleber, 2009; Zeng et al., 2012; Zhu and Pignatello, 2005). In the present study natural DOM extracted from a boreal lake was utilized. Chemical composition and the molecular configuration of DOM are key to determine modalities and level of interaction with other chemical species in the solution (Chin et al., 1997; Tanaka et al., 2005). The binding affinity of DOM was previously shown to positively correlate with its aromaticity and/or molecular weight (Akkanen et al., 2004; Ripszam et al., 2015; Tanaka et al., 2005). The natural DOM used in this experiment has a medium-high degree of aromaticity ($SUVA_{254}$ 4.59) and molecular weight (3900 Da) compared to that used in previous studies (Akkanen et al., 2004; Ripszam et al., 2015). Relatively high molecular weight DOM in humic-rich lakes was also reported in other studies (Tulonen et al., 1992; Wang et al., 2020).

3.5. Effects of pH on DOM-PPCPs binding affinity

The results indicated that the association of DOM with BEZ and DCF was influenced by the water pH. BEZ had higher C_{in} than C_{out} at both levels of DOM, at pH 6.5 (ANOVA, $F = 227.9$, $p < 0.001$, Table S11, S12) and 8 (ANOVA, $F = 101.8$, $p < 0.001$, Table S11, S12). However, the results showed that the water pH affected the binding of the DOM and BEZ differently. While at pH 6.5 the $M_{D/out}$ was lower than $M_{E/out}$ (which is in line with our experimental criteria), the $M_{D/in}$ value was lower than the $M_{E/in}$ (Table S9, S7). This could be caused by the higher adsorption to the dialysis bag observed at lower pH, as indicated by the higher A_{in} at pH 6.5 compared to pH 8 (Table S9, S7). In contrast, at pH 8 the $M_{D/in}$ yielded significantly higher values than $M_{E/in}$ at both DOM levels, and the $M_{D/out}$ was lower than $M_{E/out}$ (Table S9, S7). BEZ yielded significantly lower B_{DOM} and log K_{DOC} values at pH 6.5 (45% and 1.98, respectively; average values between the two DOM levels) compared to pH 8 (74% and 2.52 respectively; average values between the two DOM levels) (Table S13). Hence, according to the BEZ results at pH 6.5 and 8, and criteria for binding (Fig. 2), BEZ associated with DOM at both pH levels, but the interaction was stronger at pH 8.

DCF showed significantly higher C_{in} compared to C_{out} at pH 8 ($F = 82.53$, $p < 0.001$), while the difference between C_{in} and C_{out} was too small to be significant at pH 6.5 (Table S11). The primary criterion for DOM-association was not met at pH 6.5, which means that no binding occurred. Significant binding with DOM at pH 8 was instead confirmed by $M_{D/in}$ yielding higher values than $M_{E/in}$ at both DOM levels, and by the $M_{D/out}$ lower than $M_{E/out}$ (Table S9, S8). Losses (ca. 35%) were observed from the solution outside the bag due to adsorption of the compound onto to the beaker for both BEZ and DCF (Tables S7-S8).

The effect of high pH increasing the binding with DOM observed for both compounds could be driven by two different processes. The first is a direct effect of water pH altering the ionic configuration and speciation of chemicals (Ashauer and Escher, 2010), changing their chemical properties and/or association with DOM. At higher pH weak acid groups in both PPCPs and DOM will be more in the dissociated form. While this may reduce the contribution of hydrophobic interaction on the binding process, it may simultaneously promote ionic or dipole-dipole interactions. This can explain the results for BEZ. However, BEZ and DCF are weak carboxylic acids (pKa = 3.83 and 4.15, respectively) and the difference in their speciation between pH 6.5 and 8 is negligible. For instance, both BEZ and DCF show 99% of ionized form at both pH 6.5 and 8 (data not shown). Hence, a more likely driving process will be the effect of pH on DOM physicochemical properties and molecular configuration (Engebretson and von Wandruszka, 1994; Ghosh and Schnitzer, 1980; Myeni et al., 1999). For example, a change in pH may modulate the speciation of DOM functional groups (Tanaka et al., 2005), altering the fraction of protonated carboxylic groups, modulating the intra- and intermolecular H-bonding and leading to a different binding affinity (Gu et al., 2007; Pace et al., 2012). More acidic environments generally induce a more tightly condensed structure of DOM polymers and colloids, while a more alkaline environment usually causes an expansion of these structures (Pace et al., 2012). It was suggested that the primary mechanism responsible of this shift may not be the change in pH as in H^+ concentration, but a modulation in base cation concentration promoting the expansion and stabilisation of DOM structures (Engebretson and von Wandruszka, 1994; Ghosh and Schnitzer, 1980; Myeni et al., 1999).

3.6. Effects of DOM concentrations on B_{DOM} and K_{DOC}

The binding of BEZ and DCF with the DOM was not substantially different between the two different concentrations of DOM. The results of the percentage of bound compound, B_{DOM}, showed that the effect of increasing DOM concentration on them was negligible. For instance, B_{DOM} values yielded 76% at the lower level of DOM, and 72% at the higher level for BEZ. For DCF, B_{DOM} was 46% at 5 mg L^{-1} DOC, and 52% at 15 mg L^{-1} DOC (Table S13). These results indicate that the maximum capacity to bind PPCPs by the natural DOM was already reached at 5 mg L^{-1} DOC. The results of the conditional distribution coefficient, $K_{DOC,}$ showed higher values at lower DOM concentrations. For instance, BEZ log K_{DOC} was 2.22 at 5 mg L^{-1} DOC and 1.74 at 15 mg L^{-1} DOC at pH 6.5, and 2.81 at 5 mg L^{-1} DOC and 2.22 at 15 mg L^{-1} DOC at pH 8, while for DCF log K_{DOC} was 2.34 at the lower level of DOM, and 1.76 at the higher one, at pH 8 (Table S13). Generally, log K_{DOC} results should not be affected by the DOM concentration, unless the binding capacity has reached its limit with excessive amount of DOM. The fact that higher amounts of DOM in the solution did not bind more PPCP might be because higher cross interaction of DOM constituents at higher DOM concentrations (Carter and Suffet, 1982) can cause changes in the DOM macromolecular structure (Engebretson and von Wandruszka, 1994; Ghosh and Schnitzer, 1980), hindering contaminant access to binding domains of the DOM. A non-linear binding pattern of DOM has been reported previously, showing decreasing K_{DOC} with increasing concentrations of humic acids (Carter and Suffet, 1982). These results were in line with earlier results reporting lower distribution coefficients with increasing DOM concentrations (Akkanen and Kukkonen, 2003).

The K_{DOC} values for PPCPs vary greatly in literature, depending on the different composition and source of OM and the different methodologies used. Our results were in the range of previously reported studies (Carballa et al., 2008; Lobo et al., 2014; Maeng et al., 2012). Nevertheless, it was not possible to directly compare our results with others because, to the best of our knowledge, no other paper reported K_{DOC} values for BEZ and DCF in water. Many K_{DOC} values have been obtained for the association of BEZ and DCF to DOM in soil, using different methodologies. For example, Lyman et al. (1990) reported a K_{DOC} for BEZ of 2.62 through estimation from K_{OW} values. Barron et al. (2009) reported DCF K_{OC} values of 2.39 in agricultural soil by using combined pressurised liquid extraction and solid phase extraction methods prior to LC-MS/MS. These values are similar to those reported in this paper and also indicate that BEZ may have higher affinity for DOM compared to DCF. Rewitt et al. (2015) reported values of K_{DOC} for BEZ and DCF from batch adsorption experiments with DOM extracted from two different soils, where both compounds had higher K_{DOC} values in the soil with higher pH (4.22 ± 0.01; 7.22 ± 0.05) and higher carbon content (83 ± 0.3; 36.5 ± 2.7 g kg-1). DCF also indicated higher K_{OC} values than BEZ (BEZ; K_{OC} 1.6–2.80, DCF; K_{OC} 2.11–3.33). This is further evidence that organic matter originating from different sources can have different level of interaction with chemical pollutants. The use of different methodologies presented in the above-mentioned studies such as fluorescence quenching, equilibrium dialysis, or batch adsorption can also yield different results.

3.7. Implications for PPCP toxicity assessments

Toxicity tests for informing risk assessments of pollutants are conducted under standardized conditions, which typically do not include an analysis of key environmental variables such as the level of DOM in the solution. Hence, more detailed ecological risk assessments for waterborne PPCPs will benefit from a better understanding of how interaction with DOM under a range of environmentally relevant pH levels influences availability of these compounds. In a previous study (Rizzuto et al., 2020), we found that the inhibition effect of a mix of PPCPs on the growth of a micro-algae population was reduced by the presence of natural DOM at pH 8. We hypothesized that decreased toxicity could be attributed to the formation of less bioavailable/toxic DOM-complexes. As several of the PPCPs in the tested mix were weak acids with pKa ranging from 7.99 to 13, we postulated that pH could have a significant influence in determining the interaction and lead to the reduced toxic effects. The study also showed that the effect of DOM in hindering PPCP toxicity was not linearly dependent on its concentration.

Here, by using the same DOM and pH conditions, we empirically demonstrated that the combined effect of low concentrations of natural DOM (5 mg L^{-1} DOC) and high water pH (8) can control availability of some compounds, especially those that are more hydrophobic and more likely to diffuse through cell walls and biological membranes of algae - such as DCF and BEZ (Del Vento and Dachs, 2009). The present results also help explain the observed toxicological outcomes, in that they show a complexing ability of DOM that is not dependent on DOM concentration and is dependent on pH. Since the binding of DOM with PPCPs results in the formation of complexes which are too large or too polar to cross cell membranes (Chalew and Halden, 2009; Rowett et al., 2016), such interactions can reduce the bioavailability and toxic effect of PPCP mixtures (Alsop and Wilson, 2019; Maeng et al., 2012; Rizzuto et al., 2020), especially for compounds such as DCF, which has a demonstrated toxic effect on algae (Doležalová Weissmannová et al., 2018).

3.8. Conclusions

In summary, the present study empirically confirms the hypothesis emerging from our previous study on the complexing role of DOM for PPCPs, even at low DOM concentrations and at pH typical of freshwater environments during the development of algal blooms (Doležalová Weissmannová et al., 2018; Isidori et al., 2007). These results highlight the importance of considering more realistic environmental conditions when addressing the toxicological effects of PPCPs micro-pollutants, by showing that despite their relatively hydrophilic character, they can establish complex interactions with DOM that occurs in all-natural waterbodies. We believe there is a need to expand knowledge on

micro-pollutants effects on biota in waters, through a better understanding of the influence of the key environmental variables.

CRediT authorship contribution statement

SR, LN, EL, DLB, KCJ and HZ conceived the idea. SR, LN, KCJ and HZ designed the experiment. SR collected and analyzed the data. SR took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analyses and manuscript.

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.

Acknowledgements

The authors thank Rolf Vogt from the Department of Chemistry, University of Oslo for providing the DOM used in this study. The authors wish to thank David Rochester from the Department of Chemistry and Runmei Wang from Lancaster Environment Centre, Lancaster University (UK), for helping with the setup of the LC/MSMS. The authors also want to thank the anonymous reviewers, whose comments and suggestions helped to improve the manuscript.

Funding

This study was financed by the Research Council of Norway, project grant no. 244460, Pollution and Ecosystem Adaptation to Changes in the Environment (PEACE). The research was co-funded by a discretional internal grant from the Norwegian Institute for Water Research (NIVA), under the Institute Strategic Priorities (SIS) on ecosystem remediation (TRADE-OFF). SR was also supported by the Lancaster University Faculty of Science and Technology and a Lancaster Environment Centre PhD Scholarship.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.147208.

References

Akkanen, J., Kukkonen, J.V.K., 2001. Effects of water hardness and dissolved organic material on bioavailability of selected organic chemicals. Environ. Toxicol. Chem. 20, 2303–2308.

Akkanen, J., Kukkonen, J.V.K., 2003. Measuring the bioavailability of two hydrophobic organic compounds in the presence of dissolved organic matter. Environ. Toxicol. Chem. 22, 518–524. https://doi.org/10.1897/1551-5028(2003)022<0518:MTBOTH>2.0.CO;2.

Akkanen, J., Penttinen, S., Haitzer, M., Kukkonen, J.V.K., 2001. Bioavailability of atrazine, pyrene and benzo[a]pyrene in European river waters. Chemosphere 45, 453–462. https://doi.org/10.1016/S0045-6535(01)00038-8.

Akkanen, J., Vogt, R.D., Kukkonen, J.V.K., 2004. Essential characteristics of natural dissolved organic matter affecting the sorption of hydrophobic organic contaminants. Aquat. Sci. 66, 171–177. https://doi.org/10.1007/s00027-004-0705-x.

Alsop, D., Wilson, J.Y., 2019. Waterborne pharmaceutical uptake and toxicity is modified by pH and dissolved organic carbon in zebrafish. Aquat. Toxicol. 210, 11–18. https://doi.org/10.1016/j.aquatox.2019.02.008.

Antignac, J.P., De Wasch, K., Monteau, F., De Brabander, H., Andre, F., Le Bizec, B., 2005. The ion suppression phenomenon in liquid chromatography-mass spectrometry and its consequences in the field of residue analysis. Analytica Chimica Acta. Elsevier, pp. 129–136 https://doi.org/10.1016/j.aca.2004.08.055.

Arnold, K.E., Boxall, A., Brown, A.R., Cuthbert, R., Gaw, S., Hutchinson, T.H., Jobling, S., Madden, J.C., Metcalfe, C.D., Naidoo, V., Shore, R.F., Smits, J.E., Taggart, M.A., Thompson, H.M., 2013. Assessing the exposure risk and impacts of pharmaceuticals in the environment on individuals and ecosystems. Biol. Lett. 9, 20130492. https://doi.org/10.1098/rsbl.2013.0492.

Ashauer, R., Escher, B.I., 2010. Advantages of toxicokinetic and toxicodynamic modelling in aquatic ecotoxicology and risk assessment. J. Environ. Monit. 12, 2056–2061. https://doi.org/10.1039/c0em00234h.

Avdeef, A., 2005. Physicochemical profiling (solubility, permeability and charge state). Curr. Top. Med. Chem. 1, 277–351. https://doi.org/10.2174/1568026013395100.

Barron, L., Havel, J., Purcell, M., Szpak, M., Keller, B., Paull, B., 2009. Predicting sorption of pharmaceuticals and personal care products onto soil and digested sludge using artificial neural networks. Analyst 134, 663–670. https://doi.org/10.1039/b817822d.

Behera, S.K., Oh, S.Y., Park, H.S., 2010. Sorption of triclosan onto activated carbon, kaolinite and montmorillonite: effects of pH, ionic strength, and humic acid. J. Hazard. Mater. 179, 684–691. https://doi.org/10.1016/j.jhazmat.2010.03.056.

Böhm, L., Schlechtriem, C., Düring, R.A., 2016. Sorption of highly hydrophobic organic chemicals to organic matter relevant for fish bioconcentration studies. Environ. Sci. Technol. 50, 8316–8323. https://doi.org/10.1021/acs.est.6b01778.

Boreen, A.L., Arnold, W.A., McNeill, K., 2004. Photochemical fate of sulfa drugs in then aquatic environment: sulfa drugs containing five-membered heterocyclic groups. Environ. Sci. Technol. 38, 3933–3940. https://doi.org/10.1021/es0353053.

Boxall, A., Rudd, M., Brooks, B., Hickmann, S., Snape, J., Parrott, J., Tetreault, G., Innes, E., McLaughlin, A., Choi, K., Ostapyk, K., Berninger, J., Szabo, N., Trudeau, V., Moore, R., Hallstrom, L., Ericson, J., Dyer, S., Verslycke, T., Coors, A., Belanger, S., Servos, M., Larsson, D., McMaster, M., Karlsson, M., Straub, J., Sibley, P., Staveley, J., Meyerhoff, R., Carriquiriborde, P., DeLeo, P., Giesy, J., Beazley, K., Gagné, F., Ankley, G., Caldwell, D., Van Der Kraak, G., Murray-Smith, R., Mastrocco, F., Topp, E., Gouin, T., Lazorchak, J., 2012. Pharmaceuticals and personal care products in the environment: what are the big questions? Environ. Health Perspect. 120, 1221–1229. https://doi.org/10.1289/ehp.1104477.

Burkhard, L.P., 2000. Estimating dissolved organic carbon partition coefficients for nonionic organic chemicals. Environ. Sci. Technol. 34, 4663–4668. https://doi.org/10.1021/es001269l.

Buschmann, J., Kappeler, A., Lindauer, U., Kistler, D., Berg, M., Sigg, L., 2006. Arsenite and arsenate binding to dissolved humic acids: influence of pH, type of humicacid, and aluminum. Environ. Sci. Technol 40, 6015–6020.

Carballa, M., Fink, G., Omil, F., Lema, J.M., Ternes, T., 2008. Determination of the solid–water distribution coefficient(Kd) for pharmaceuticals, estrogens and musk fragrances indigested sludge. Water Res. 42, 287–295.

Carter, C.W., Suffet, I.H., 1982. Binding of DDT to Dissolved Humic Materials. American Chemical Society Environ. Sel, Technol.

Chalew, T.E.A., Halden, R.U., 2009. Environmental exposure of aquatic and terrestrial biota to triclosan and triclocarban. J. Am. Water Resour. Assoc. 45, 4–13. https://doi.org/10.1111/j.1752-1688.2008.00284.x.

ChemAxon, 2021. Calculators and Predictors | ChemAxon [WWW Document]. URL https://chemaxon.com/products/calculators-and-predictors#pka (accessed 1.27.21).

Chen, H., Zhou, W., Zhu, K., Zhan, H., Jiang, M., 2004. Sorption of ionizable organic compounds on HDTMA-modified loess soil. Sci. Total Environ. 326, 217–223. https://doi.org/10.1016/j.scitotenv.2003.12.011.

Chen, K.L., Liu, L.C., Chen, W.R., 2017. Adsorption of sulfamethoxazole and sulfapyridine antibiotics in highorganic content soils. Environ. Pollut. 1163–1171. Doi:https://doi.org/10.1016/j.envpol.2017.08.011.

Chin, Y.-P., Aiken, G.R., Danielsen, K.M., 1997. Binding of pyrene to aquatic and commercial humic substances: the role of molecular weight and aromaticity. Environ. Sci. Technol 31, 1630–1635.

Christl, I., Ruiz, M., Schmidt, J.R., Pedersen, J.A., 2016. Clarithromycin and tetracycline binding to soil humic acid in the absence and presence of calcium. Environ. Sci. Technol. 50, 9933–9942. https://doi.org/10.1021/acs.est.5b04693.

Del Vento, S., Dachs, J., 2009. Prediction of uptake dynamics of persistent organic pollutants by bacteria and phytoplankton. Environ. Toxicol. Chem. 21, 2099–2107. https://doi.org/10.1002/etc.5620211013.

Delgado, L.F., Charles, P., Glucina, K., Morlay, C., 2015. Adsorption of ibuprofen and atenolol at trace concentration on activated carbon. Sep. Sci. Technol. 50. https://doi.org/10.1080/01496395.2014.975360 14887-1496.

Doležalová Weissmannová, H., Pavlovský, J., Fišerová, L., Kosárová, H., 2018. Toxicity of diclofenac: cadmium binary mixtures to algae desmodesmus subspicatus using normalization method. Bull. Environ. Contam. Toxicol. 101, 205–213. https://doi.org/10.1007/s00128-018-2384-7.

Engebretson, R.R., von Wandruszka, R., 1994. Microorganization in dissolved humic acids. Environ. Sci. Technol 28, 1934–1941.

Frenkel, Y.V., Clark, A.D., Das, K., Wang, Y.-H., Lewi, P.J., Janssen, P.A.J., Arnold, E., 2005. Concentration and pH Dependent Aggregation of Hydrophobic Drug Molecules and Relevance to Oral Bioavailability. https://doi.org/10.1021/jm049439i.

George, R., Haywood, A., Khan, S., Radovanovic, M., Simmonds, J., Norris, R., 2018. Enhancement and suppression of ionization in drug analysis using HPLC-MS/MS in support of therapeutic drug monitoring: a review of current knowledge of its minimization and assessment. Ther. Drug Monit. 40, 1–8. https://doi.org/10.1097/FTD.0000000000000471.

Ghosh, K., Schnitzer, M., 1980. Macromolecular structures of humic substances. Soil Sci. 129, 266–276.

Gjessing, E.T., Egeberg, P.K., Håkedal, J., 1999. Natural organic matter in drinking water - the "NOM-typing project", background and basic characteristics of original water samples and NOM isolates. Environ. Int. 25, 145–159. https://doi.org/10.1016/S0160-4120(98)00119-6.

Gu, C., Karthikeyan, K.G., Sibley, S.D., Pedersen, J.A., 2007. Complexation of the antibiotic tetracycline with humic acid. Chemosphere https://doi.org/10.1016/j.chemosphere.2006.08.028.

Hansch, C., Leo, A., Hoekman, D., 1995. Exploring QSAR: Hydrophobic, Electronic, and Steric Constants. American Chemical Society, Washington, DC.

Henriksen, A., Skjelvåle, B.L., Mannio, J., Wilander, A., Harriman, R., Curtis, C., Jensen, J.P., Fjeld, E., Moiseenkon, T., 1998. Northern European lake survey, 1995. Ambio 27, 80–91.

Hernandez-Ruiz, S., Abrell, L., Wickramasekara, S., Chefetz, B., Chorover, J., 2012. Quantifying PPCP interaction with dissolved organic matter in aqueous solution:

combined use of fluorescence quenching and tandem mass spectrometry. Water Res. 46, 943–954. https://doi.org/10.1016/j.watres.2011.11.061.

Holbrook, R.D., Love, N.G., Novak, J.T., 2004. Sorption of 17β-estradiol and 17α-ethinylestradiol by colloidal organic carbon derived from biological wastewater treatment systems. Environ. Sci. Technol. 38, 3322–3329. https://doi.org/10.1021/es035122g.

Holmstrup, M., Bindesbøl, A.M., Oostingh, G.J., Duschl, A., Scheil, V., Köhler, H.R., Loureiro, S., Soares, A.M.V.M., Ferreira, A.L.G., Kienle, C., Gerhardt, A., Laskowski, R., Kramarz, P.E., Bayley, M., Svendsen, C., Spurgeon, D.J., 2010. Interactions between effects of environmental chemicals and natural stressors: a review. Sci. Total Environ. 408, 3746–3762. https://doi.org/10.1016/j.scitotenv.2009.10.067.

Isidori, M., Nardelli, A., Pascarella, L., Rubino, M., Parrella, A., 2007. Toxic and genotoxic impact of fibrates and their photoproducts on non-target organisms. Environ. Int. 33, 635–641. https://doi.org/10.1016/j.envint.2007.01.006.

Keiluweit, M., Kleber, M., 2009. Molecular-level interactions in soils and sediments: the role of aromatic π-systems. Environ. Sci. Technol 43.

Khan, S.J., Ongerth, J.E., 2004. Modelling of pharmaceutical residues in Australian sewage by quantities of use and fugacity calculations. Chemosphere 54, 355–367. https://doi.org/10.1016/j.chemosphere.2003.07.001.

Krop, H.B., van Noort, P.C., Govers, H.A., 2001. Determination and theoretical aspects of the equilibrium between dissolved organic matter and hydrophobic organic micropollutants in water (Kdoc). Rev. Environ. Contam. Toxicol. https://doi.org/10.1007/978-1-4613-0107-3_1.

Kwon, J.W., Armbrust, K.L., 2008. Aqueous solubility, n-octanol-water partition coefficient, and sorption of five selective serotonin reuptake inhibitors to sediments and soils. Bull. Environ. Contam. Toxicol. 81, 128–135.

Laskowski, R., Bednarska, A.J., Kramarz, P.E., Loureiro, S., Scheil, V., Kudłek, J., Holmstrup, M., 2010. Interactions between toxic chemicals and natural environmental factors - a meta-analysis and case studies. Sci. Total Environ. 408, 3763–3774. https://doi.org/10.1016/j.scitotenv.2010.01.043.

Leenheer, J., Croue', J., 2003. Characterizing dissolved aquatic organic matter. Environ. Sci. Technol. 37 (1), 18A–26A.

Lipnick, R.L., 1995. Structure-activity relationships. In: Rand, G.M. (Ed.), Fundamentals of Aquatic Toxicology - Effects, Environmental Fate, and Risk Assessment. Taylor & Francis, Washington, pp. 609–655.

Lobo, S., Li, H., Farhan, N., Yan, G., 2014. Evaluation of diclofenac prodrugs for enhancing transdermal delivery. Drug Dev. Ind. Pharm. 40, 425–432. https://doi.org/10.3109/03639045.2013.767828.

Lu, G., Xie, Z., Zhang, Z., 2018. Effects of dissolved organic matter, feeding, and water flow on the bioconcentration of diclofenac in crucian carp (Carassius auratus). Environ. Sci. Pollut. Res. 25, 7776–7784. https://doi.org/10.1007/s11356-017-1081-0.

Lyman, W.J., Reehl, W.F., Rosenblatt, D.H., 1990. Handbook of Chemical Property Estimation Methods: Environmental Behaviour of Organic Compounds. American Chemical Society, American Chemical Society.

Maeng, S.K., Sharma, S.K., Abel, C.D.T., Magic-Knezev, A., Song, K.G., Amy, G.L., 2012. Effects of effluent organic matter characteristics on the removal of bulk organic matter and selected pharmaceutically active compounds during managed aquifer recharge: column study. J. Contam. Hydrol. https://doi.org/10.1016/j.jconhyd.2012.08.005.

McFarland, J.W., Berger, C.M., Froshauer, S.A., Hayashi, S.F., Hecker, S.J., Jaynes, B.H., Jefson, M.R., Kamicker, B.J., Lipinski, C.A., Lundy, K.M., Reese, C.P., Vu, C.B., 1997. Quantitative structure-activity relationships among macrolide antibacterial agents: in vitro and in vivo potency against Pasteurella multocida. J. Med. Chem. 40, 1340–1346. https://doi.org/10.1021/jm960436i.

Monteith, D.T., Stoddard, J.L., Evans, C.D., De Wit, H.A., Forsius, M., Høgåsen, T., Wilander, A., Skjelkvåle, B.L., Jeffries, D.S., Vuorenmaa, J., Keller, B., Kopácek, J., Vesely, J., 2007. Dissolved organic carbon trends resulting from changes in atmospheric deposition chemistry. Nature 450, 537–541. https://doi.org/10.1038/nature06316.

Myeni, S.C.B., Brown, J.T., Matinez, G.A., Meyer-Ilse, W., 1999. Imaging of humic substance macromolecular structures in water and soils. Science (80-.) 286, 1335–1337.

OECD, 2009. OECD guidelines for the testing of chemicals. Proposal for Updating Guideline 201, Freshwater Alga and Cyanobacteria, Growth Inhibition Test, pp. 1–21.

O'Neil, M.J., 2013. The Merck Index: An Encyclopedia of Chemicals, Drugs, and Biologicals. CamRoyal Society of Chemistry, Cambridge, UK.

Pace, M.L., Reche, I., Cole, J.J., Fernández-Barbero, A., Mazuecos, I.P., Prairie, Y.T., 2012. pH change induces shifts in the size and light absorption of dissolved organic matter. Biogeochemistry 108, 109–118. https://doi.org/10.1007/S10533-01.

Pal, A., Gin, K.Y.H., Lin, A.Y.C., Reinhard, M., 2010. Impacts of emerging organic contaminants on freshwater resources: review of recent occurrences, sources, fate and effects. Sci. Total Environ. https://doi.org/10.1016/j.scitotenv.2010.09.026.

Pan, B., Ning, P., Xing, B., 2009. Part V - sorption of pharmaceuticals and personal care products. Environ. Sci. Pollut. Res. 16, 106–116. https://doi.org/10.1007/s11356-008-0052-x.

Prakash Agarwal, S., Khalid Anwer, M., Aqil, M., 2008. Complexation of furosemide with fulvic acid extracted from Shilajit: a novel approach. Drug Dev. Ind. Pharm. 34, 506–511. https://doi.org/10.1080/03639040701744053.

R Core Development Team, 2015. R: A Language and Environment for Statistical Computing. https://doi.org/10.1007/978-3-540-74686-7.

Rewitt, D.M., Balogh, T., Jones, H., 2015. Sorption behaviours and transport potentials for selected pharmaceuticals and triclosan in two sterilised soils. Remediat. Manag. Contam. or Degrad. Lands 15, 594–606.

Ripszam, M., Paczkowska, J., Figueira, J., Veenaas, C., Haglund, P., 2015. Dissolved organic carbon quality and sorption of organic pollutants in the Baltic Sea in light of future climate change. Environ. Sci. Technol. 49, 1445–1452. https://doi.org/10.1021/es504437s.

Rizzuto, S., Thrane, J.E., Baho, D.L., Jones, K.C., Zhang, H., Hessen, D.O., Nizzetto, L., Leu, E., 2020. Water Browning controls adaptation and associated trade-offs in phytoplankton stressed by chemical pollution. Environ. Sci. Technol. 54, 5569–5579. https://doi.org/10.1021/acs.est.0c00548.

Rowett, C.J., Hutchinson, T.H., Comber, S.D.W., 2016. The impact of natural and anthropogenic Dissolved Organic Carbon (DOC), and pH on the toxicity of triclosan to the crustacean Gammarus pulex (L.). Sci. Total Environ. 565, 222–231. https://doi.org/10.1016/j.scitotenv.2016.04.170.

Sangster, J., 1997. LOGKOW A Databank of Evaluated Octanol-Water Partition Coefficients.

Schwarzenbach, R., Escher, B.I., Fenner, K., Hofstetter, R., Johnson, C., Von Gunten, U., Wehrli, B., 2006. The challenge of micropollutants in aquatic system. Science (80-.) 313, 1072–1077. https://doi.org/10.1126/science.1127291.

Sun, M., Duker, R.Q., Gillissen, F., Van den Brink, P.J., Focks, A., Rico, A., 2020. Influence of pH on the toxicity of ionisable pharmaceuticals and personal care products to freshwater invertebrates. Ecotoxicol. Environ. Saf. 191, 110172. https://doi.org/10.1016/j.ecoenv.2020.110172.

Tanaka, F., Fukushima, M., Kikuchi, A., Yabuta, H., Ichikawa, H., Tatsumi, K., 2005. Influence of chemical characteristics of humic substances on the partition coefficient of a chlorinated dioxin. Chemosphere 58, 1319–1326. https://doi.org/10.1016/j.chemosphere.2004.10.008.

Tang, Y., Li, X.-M., Xu, Z.-C., Guo, Q.-W., Hong, C.-Y., Bing, Y.-X., 2014. Removal of naproxen and bezafibrate by activated sludge under aerobic conditions: kinetics and effect of substrates. Biotechnol. Appl. Biochem. 61. https://doi.org/10.1002/bab.1168 (n/a-n/a).

Thakuria, R., Sarma, B., Nangia, A., 2017. Hydrogen Bonding in Molecular Crystals, in: Comprehensive Supramolecular Chemistry II. Elsevier Inc., pp. 25–48 https://doi.org/10.1016/B978-0-12-409547-2.12598-3.

Tulonen, T., Salonen, K., Arvola, L., 1992. Effects of different molecular weight fractions of dissolved organic matter on the growth of bacteria, algae and protozoa from a highly humic lake. Hydrobiologia 229, 239–252. https://doi.org/10.1007/BF00007003.

Vieno, N., Tuhkanen, T., Kronberg, L., 2010. Removal of pharmaceuticals in drinking water treatment: effect of chemical coagulation. Environ. Technol. 27, 183–192.

Wang, W., Zheng, B., Jiang, X., Chen, J., Wang, S., 2020. Characteristics and source of dissolved organic matter in Lake Hulun, a large shallow eutrophic steppe lake in northern China. Water 12. Doi:doi:https://doi.org/10.3390/w12040953.

Wibawa, J.I.D., Shaw, P.N., Barrett, D.A., 2003. Quantification of clarithromycin, its 14-hydroxy and decladinose metabolites in rat plasma, gastric juice and gastric tissue using high-performance liquid chromatography with electrochemical detection. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 783, 359–366. https://doi.org/10.1016/S1570-0232(02)00765-1.

Wickham, H., 2006. ggplot2: elegant graphics for data. Analysis 35, 2006.

Williamson, C.E., Overholt, E.P., Pilla, R.M., Leach, T.H., Brentrup, J.A., Knoll, L.B., Mette, E.M., Moeller, R.E., 2015. Ecological consequences of long-term browning in lakes. Sci. Rep. https://doi.org/10.1038/srep18666.

Yamamoto, H., Liljestrand, H.M., Shimizu, Y., Morita, M., 2003. Effects of physical−chemical characteristics on the sorption of selected endocrine disruptors by dissolved organic matter surrogates. Environ. Sci. Technol 37, 2646–2657.

Yamamoto, H., Yamamoto, Hiroshi, Hayashi, A., Nakamura, Y., Sekizawa, J., 2005. Fate and Partitioning of Selected Pharmaceuticals in Aquatic Environment, Environmental Sciences.

Zeng, C., Ji, Y., Zhou, L., Zhang, Y., Yang, X., 2012. The role of dissolved organic matters in the aquatic photodegradation of atenolol. J. Hazard. Mater. 239–240, 340–347. https://doi.org/10.1016/j.jhazmat.2012.09.005.

Zhang, W., Zhang, M., Lin, K., Sun, W., Xiong, B., Guo, M., Cui, X., Fu, R., 2012. Ecotoxicological effect of carbamazepine on Scenedesmus obliquus and chlorella pyrenoidosa. Environ. Toxicol. Pharmacol. 33, 344–352. https://doi.org/10.1016/j.etap.2011.12.024.

Zhang, Yonggang, Guo, J., Yao, T., Zhang, Yalei, Zhou, X., Chu, H., 2019. The influence of four pharmaceuticals on Chlorellapyrenoidosa culture. Sci. Rep. 9 (1624), 1–10. https://doi.org/10.1038/s41598-018-36609-4.

Zhu, D., Pignatello, J., 2005. Characterizatiion of aromatic compound sorptive interactions with black carbon (charcoal) assisted by graphite as a model. Environ. Sci. Technol 35.

Lancaster Environment Centre

Lancaster University

Responses of freshwater phytoplankton exposed to chemical contaminants: tolerance acquisition, physiological trade-offs and environmental controls

(Appendices and Supplementary Material)

Simone Rizzuto, MSc

Submitted for the degree of

Doctor of Philosophy in Environmental Science

November 2021

Table of Contents – Appendices and Supplementary Material

Supplementary Information Chapter II: Water browning controls adaptation an associated trade-offs in phytoplankton stressed by chemical pollution.

Simone Rizzuto[1], Jan-Erik Thrane[2], Didier L. Baho[2,3], Kevin C. Jones[2], Hao Zhang[2], Dag Hessen[4,5], Luca Nizzetto[*2,6], Eva Leu[7]

[1] Lancaster Environment Centre, Lancaster University, Lancaster, LA1 4YQ, UK.

[2] Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, 0349, Oslo, Norway.

[3] Department of Aquatic Sciences and Assessment, Swedish University of Agricultural Sciences, P.O. Box 7050, SE-750-07 Uppsala, Sweden.

[4] Section for Aquatic Biology and Ecotoxicology, University of Oslo, Post box 1066, Blindern, 0316 Oslo, Norway.

[5] Center of Biogeochemistry in the Anthropocene (CAB), University of Oslo, Post box 1066, Blindern, 0316 Oslo, Norway.

[6] RECETOX, Masaryk University, Kamenice 753/5, 625 00 Brno, Czech Republic.

[7] Akvaplan-niva, CIENS, Science Park, Gaustadalléen 21, 0349, Oslo, Norway.

* Corresponding author. Email: luca.nizzetto@niva.no

List of Tables

List of Figures

List of Text:

Table S2.1. Summary data on the occurrence and concentration (ng/L) of PPCPs used in this study found in European freshwaters (lakes and rivers). The data was obtained from the Norman database. Norman is the Network of reference laboratories, research centres and related organisations for monitoring of emerging environmental substances (www.Norman-network.net). This table was modified from the paper published by Pomati et al. (2017) and Baho et al. (2019).

Chemical	Time analyzed	Times detected	Percentage detection (%)	Min conc. (ng/L)	Max conc. (ng/L)	Mean conc. (ng/L)	standard deviation (ng/L)	Q1 conc. (ng/L)	Median conc. (ng/L)	Q3 conc. (ng/L)
Atenolol	977	723	74	0.1	900	26.3	70.7	6	11	19
Bezafibrate	1384	764	55.2	0.3	21200	108.5	1162.7	8	13	28
Carbamazepine	22270	19361	86.9	0.8	7600	158.3	295.8	33	70	160
Clarithromycin	945	730	77.2	0.9	1100	21	44.7	10	13	21
Diclofenac	6320	4439	70.2	0.2	110000	785	5977.4	23	57	130
Furosemide	507	84	16.6	0.5	283000	9253.7	44732.1	12.25	35	76
Hydrochlorothiazide	484	235	48.6	4	389000	4425	36594.8	22	41	85.5
Ibuprofen	5154	3668	71.2	1.2	303000	214.5	5167.9	15	32	70
Ranitidine	50	29	58	1.3	200	33.4	55.1	2.3	5.4	40
Sulfamethoxazole	2616	2133	81.5	0.7	700	33.3	46	12	20	40
Benzophenone-4	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Triclosan	11565	9053	78.3	1	3060	20.4	56.9	8	12	20

Table S2.2. Chemical properties (acid dissociation constant – pKa, and octanol/water partition coefficient – log Kow), spiked concentrations (µg/L), and reported effective concentrations (µg/L) inhibiting 50% of growth (EC50) in phytoplankton species for the 12 studied chemical compounds. Toxicity values were obtained from the U.S. Environmental Protection Agency ECOTOXicology Database System (2015, Version 4.0, www.epa.gov/ecotox/). This table was modified from the papers published by Baho et al. (2019) and Pomati et al. (2017).

chemical	CAS ID	mm (g mol^{-1})	pKa	Log K_{ow}	water solubility (mg L^{-1})	Spiked conc. (µg L^{-1})	Mean EC50 (µg L^{-1})	SD (µg L^{-1})	Num. studies
Atenolol	29122-68-7	266.34	9	0.16	13300	22	3.18E+05	2.63E+05	3
Bezafibrate	42859-67-0	361.82	3.83	4.25	1.55	2.2	3.50E+04	2.63E+03	3
Carbamazepine	298-46-4	236.27	13.9	2.45	18	22	1.37E+05	2.83E+05	24
Clarithromycin	81103-11-9	747.96	8.99	3.16	1.63	22	1.97E+01	2.33E+01	3
Diclofenac	15307-86-5	296.14	4.15	4.51	2.37	22	6.27E+04	6.73E+04	6
Furosemide	54-31-5	330.74	4.25	2.03	73.1	2.2	> 7.000E+04	NA	1
Hydrochlorothiazide	58-93-5	297.73	7.9	-0.07	722	22	NA	NA	NA
Ibuprofen	15867-27-1	206.28	4.91	3.97	21	22	3.29E+05	1.92E+04	2
Ranitidine	66357-35-5	314.4	7.8	0.08	660000	2.2	2.70E+04	4.87E+04	2
Sulfamethoxazole	723-46-6	253.28	1.6/5.7	0.89	610	2.2	2.15E+03	3.10E+03	7
Benzophenone-4	4065-45-6	308.3	7.6	0.37	249.7	22	1.00E+04	NA	1
Triclosan	3380-34-5	289.53	7.9	4.76	10	2.2	5.86E+02	7.82E+02	24

Table S2.3. Growth inhibition test of *Chlamydomonas reinhardtii* exposed to the mix of PPCPs. The exposure levels used in our study were based on a preliminary test conducted on *C. reinhardtii* following the OECD guidelines (OECD, 2007). Eight levels of exposure were applied following a factorial increase (0, 1, 3, 10, 30, 100, 300, 1000). The concentrations used in this study were the one from level 5 (in bold), causing 28.6% growth inhibition.

	Concentrations (µg/L)							
Chemical	**Ctrl**	**L1**	**L2**	**L3**	**L4**	**L5**	**L6**	**L7**
Atenolol	0	0.22	0.66	2.2	6.6	**22**	66	220
Bezafibrate	0	0.022	0.066	0.22	0.66	**2.2**	6.6	22
Carbamazepine	0	0.22	0.66	2.2	6.6	**22**	66	220
Clarithromycin	0	0.22	0.66	2.2	6.6	**22**	66	220
Diclofenac	0	0.22	0.66	2.2	6.6	**22**	66	220
Furosemide	0	0.022	0.066	0.22	0.66	**2.2**	6.6	22
Hydrochlorothiazide	0	0.22	0.66	2.2	6.6	**22**	66	220
Ibuprofen	0	0.22	0.66	2.2	6.6	**22**	66	220
Ranitidine	0	0.022	0.066	0.22	0.66	**2.2**	6.6	22
Sulfamethoxazole	0	0.022	0.066	0.22	0.66	**2.2**	6.6	22
Benzophenone-3	0	0.22	0.66	2.2	6.6	**22**	66	220
Triclosan	0	0.022	0.066	0.22	0.66	**2.2**	6.6	22
n	6	3	3	3	3	**3**	3	3
mean growth rate µ (d^{-1})	1.62	1.79	1.61	1.64	1.66	**1.16**	0.90	0.55
SD	0.05	0.05	0.05	0.09	0.02	**0.01**	0.06	0.01
% growth inhibition		-10.7	0.8	-1.6	-2.8	**28.6**	44.4	65.7

Text S2.2. PPCPs stability test

In order to check for degradation of the mix of PPCPs, the experimental units exposed to the contaminants were sampled during both phases of the experiment as follows. 1 mL samples were collected in triplicates, stored in 2 mL GC amber glass vials at -20°C in the dark. The compounds were extracted through SPE extraction using HLB cartridges (Oasis) in 5 mL of MeOH. The extract was blown down to dryness with a gentle N^2 flow, reconstituted in 1 mL MeOH, and filtered through 0.2 µm PP syringes filters (Pall, UK) into a 2 mL GC vial. The samples were analysed by HPLC-MS (Shimadzu, 8040), using an XBridge BEH C18 column (2.1 mm x 100 mm, 3.5 µm) to separate the compounds. The mobile phases were A, 0.2% Ammonium hydroxide in MQ water, and B, 50% Methanol and Acetonitrile. The gradient procedure was optimized at: 0-1 min 20% B, then increased to 100% within 8 min, held at 100% for 5 min, after that decreased to the initial conditions (20% B) within 1 min. Finally, 6 minutes of post-run ensured re-equilibration of the column before the next injection. The injection volume was 15 µL and the column and the tray temperature were set to 35°C. The quantification of the compounds was based on internal standard method (Atenolol d7 and Ibuprofen d3, Sigma Aldrich), and the instrument detection limit was 3.87 ng/mL.

Table S2.4. Percentage of recovery (± standard deviation) of the mix of PPCPs at different levels of DOM and pH at the end of phase I and phase II.

	Chemical	Spiked conc. (ng/L)	Recovery DOC 0 (% ± sd)	Recovery DOC 5 (% ± sd)	Recovery DOC 15 (% ± sd)	
phase I	Atenolol	22	100 ± 0.3	94.3 ± 6.3	99.4 ± 0.1	pH 6.5
	Bezafibrate	2.2	99.3 ± 2.2	100.3 ± 1.0	97.6 ± 1.0	
	Carbamazepine	22	102.9 ± 1.2	101.2 ± 2.3	104.3 ± 3.3	
	Clarithromycin	22	98.2 ± 1.9	104.3 ± 2.3	105.2 ± 4.2	
	Diclofenac	22	99.4 ± 2.1	102.2 ± 2.0	100.3 ± 1.1	
	Furosemide	2.22	96.4 ± 4.1	99.3 ± 2.4	98.2 ± 2.4	
	Hydrochlorothiazide	22	103.7 ± 1.3	98.4 ± 2.5	98.8± 6.7	
	Ibuprofen	22	100.3 ± 0.3	99.4 ± 4.1	96.8 ± 7.4	
	Ranitidine	2.2	99.4 ± 2.8	98.7 ± 4.4	102.3 ± 2.3	
	Sulfamethoxazole	2.2	97.3 ± 2.1	95.6 ± 6.3	104.2 ± 4.0	
	Benzophenone-4	22	104.4 ± 3.4	99.2 ± 4.4	105.3 ± 4.0	
	Triclosan	2.2	99.2 ± 2.1	104.3 ± 5.4	99.7 ± 1.0	
	Atenolol	22	99.3 ± 0.9	100.4 ± 0.8	100.9 ± 1.0	pH 8
	Bezafibrate	2.2	102.4 ± 1.0	102.9 ± 3.2	101.7 ± 0.4	
	Carbamazepine	22	100.2 ± 2.0	98.2 ± 2.2	100.3 ± 0.8	
	Clarithromycin	22	103.3 ± 0.4	99.2 ± 4.2	105.3 ± 5.7	
	Diclofenac	22	98.4 ± 2.4	101.0 ± 1.2	104.5 ± 0.4	
	Furosemide	2.22	99.7 ± 2.4	104.2 ± 5.0	101.5 ± 6.3	
	Hydrochlorothiazide	22	95.4 ± 5.2	100.4 ± 1.0	98.5 ± 3.7	
	Ibuprofen	22	100.9 ± 1.3	99.8 ± 1.4	100.0 ± 1.2	
	Ranitidine	2.2	102.5 ± 3.3	98.9 ± 0.2	100.2 ± 3.2	
	Sulfamethoxazole	2.2	101.0 ± 3.0	96.2 ± 5.0	104.7 ± 7.0	
	Benzophenone-4	22	97.6 ± 2.2	102.8 ± 2.0	98.5 ± 0.3	
	Triclosan	2.2	96.6 ± 4.4	101.2 ± 0.2	99.3 ± 3.3	
phase II	Atenolol	22	99.7 ± 2.8	104.3 ± 6.4	100.0 ± 1.0	pH 8
	Bezafibrate	2.2	104.2 ± 1.5	102.3 ± 2.6	100.2 ± 1.0	
	Carbamazepine	22	100.3 ± 2.2	103.2 ± 3.7	102.0 ± 2.4	
	Clarithromycin	22	97.8 ± 2.5	99.8 ± 1.1	102.4 ± 1.0	
	Diclofenac	22	98.3 ± 1.1	98.8 ± 2.0	101.3 ± 0.2	
	Furosemide	2.22	99.1 ± 1.4	97.3 ± 4.0	99.2 ± 0.2	
	Hydrochlorothiazide	22	102.2 ± 2.7	98.6 ± 2.1	100.8 ± 1.0	
	Ibuprofen	22	101.3 ± 3.5	100.2 ± 2.4	101.2 ± 1.0	
	Ranitidine	2.2	98.8 ± 4.4	101.2 ± 2.3	99.6 ± 2.0	
	Sulfamethoxazole	2.2	102.4 ± 0.3	101.0 ± 2.1	99.3 ± 3.2	
	Benzophenone-4	22	101.0 ± 1.1	95.6 ± 4.2	96.6 ± 3.1	
	Triclosan	2.2	97.1 ± 3.0	99.0 ± 1.5	100.2 ± 2.0	

Table S2.5. Pairwise comparison post-hoc Tukey test on the gap between the growth rate μ (d^{-1}) in the absence/presence of the PPCPs in phase II in the non-adapted population, and in the population adapted to PPCPs at different levels of DOM. Significant values are reported in bold.

Population	DOC (mg L^{-1})	contrast PPCPs	estimate	df	t ratio	*p*
non-adapted	0	(-) vs (+)	**1.05**	**12**	**7.38**	**< 0.001**
adapted	0		**0.28**	**18**	**2.39**	**0.03**
	5		**0.39**	**18**	**3.35**	**0.04**
	15		**0.55**	**18**	**4.71**	**< 0.001**

Table S2.6. Pairwise comparison post-hoc Tukey test between the populations adapted in presence of PPCPs at different levels of DOC, in the absence/presence of PPCPs in phase II. In the table are reported the growth rate μ (d^{-1}), cell size (μm^3) and recruitment rate μ (d^{-1}). Significant values are reported in bold.

Variable	PPCPs	contrast (DOC levels)	estimate	df	t ratio	*p*
growth rate μ (d^{-1})	**(-)**	0-5	-0.0125	18	-0.107	0.994
		0-15	-0.1	18	-0.853	0.675
	(+)	0-5	0.1	18	0.853	0.675
		0-15	0.172	18	1.472	0.327
cell size (μm^3)	**(-)**	**0-5**	**-0.174**	**18**	**-3.451**	**< 0.05**
		0-15	0.038	18	0.755	0.735
	(+)	**0-5**	**0.266**	**18**	**5.264**	**< 0.001**
		0-15	0.018	18	0.359	0.932
recruitment rate μ (d^{-1})	**(-)**	0-5	0.022	18	0.257	0.964
		0-15	-0.003	18	-0.04	0.999
	(+)	0-5	0.126	18	1.496	0.316
		0-15	**0.267**	**18**	**3.168**	**0.014**

Table S2.7. Pairwise comparison post-hoc Tukey test between the populations adapted in presence of PPCPs at different levels of DOM (mg L^{-1} DOC) and the non-adapted population, in the absence/presence of PPCPs in phase II. In the table are reported growth rate, cell size and recruitment rate. Significant values are reported in bold.

Variable	Contrast	PPCPS	df	estimated mean difference	t ratio	*p*
growth rate μ (d^{-1})	adapted with 0 mg L^{-1} DOC vs. Non-adapted	(+)	**12**	**0.51**	**3.53**	**<0.05**
		(-)	**12**	**-0.27**	**-1.88**	**<0.05**
cell size (μm^3)		(+)	**12**	**0.35**	**8.7**	**<0.001**
		(-)	**12**	**-0.19**	**-4.79**	**<0.001**
recruitment rate μ (d^{-1})		(+)	**12**	**0.19**	**2.08**	**<0.05**
		(-)	**12**	**-0.25**	**-2.63**	**<0.05**
Variable	**Contrast**	**PPCPS**	**df**	**estimated mean difference**	**t ratio**	***p***
growth rate μ (d^{-1})	adapted with 5 mg L^{-1} DOC vs. Non-adapted	(+)	**18**	**0.4**	**2.99**	**<0.05**
		(-)	18	-0.25	-1.89	0.08
cell size (μm^3)		(+)	**18**	**0.09**	**0.69**	**<0.01**
		(-)	18	-0.02	-2.81	0.5
recruitment rate μ (d^{-1})		(+)	18	0.06	1.43	0.18
		(-)	**18**	**-0.27**	**-5.52**	**<0.001**
Variable	**Contrast**	**PPCPS**	**df**	**estimated mean difference**	**t ratio**	***p***
growth rate μ (d^{-1})	adapted with 15 mg L^{-1} DOC vs. Non-adapted	(+)	**18**	**0.33**	**2.39**	**<0.05**
		(-)	18	-0.17	-1.21	0.24
cell size (μm^3)		(+)	**18**	**0.33**	**7.81**	**<0.001**
		(-)	**18**	**0.23**	**-5.42**	**<0.001**
recruitment rate μ (d^{-1})		(+)	18	-0.07	1.06	0.3
		(-)	**18**	**-0.24**	**-3.62**	**<0.05**

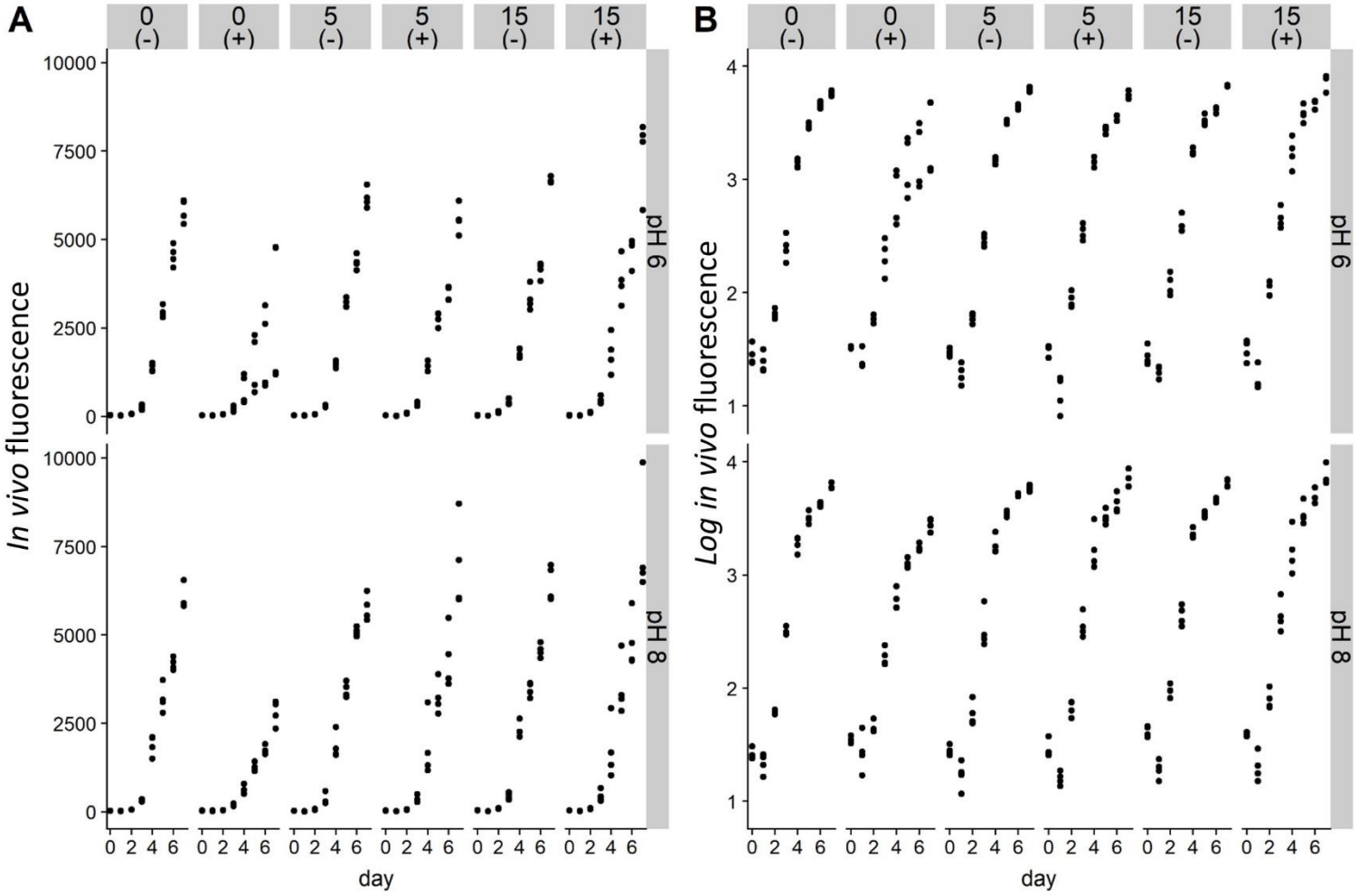


Figure S2.1. (A) Daily biomass development measured as the in vivo fluorescence and (B) log in vivo fluorescence data of the phytoplankton population under different DOM (DOC 0, 5, 15 mg L^{-1}) and pH levels (6.5, 8), in the absence (-) and the presence (+) of PPCPs, during phase I.

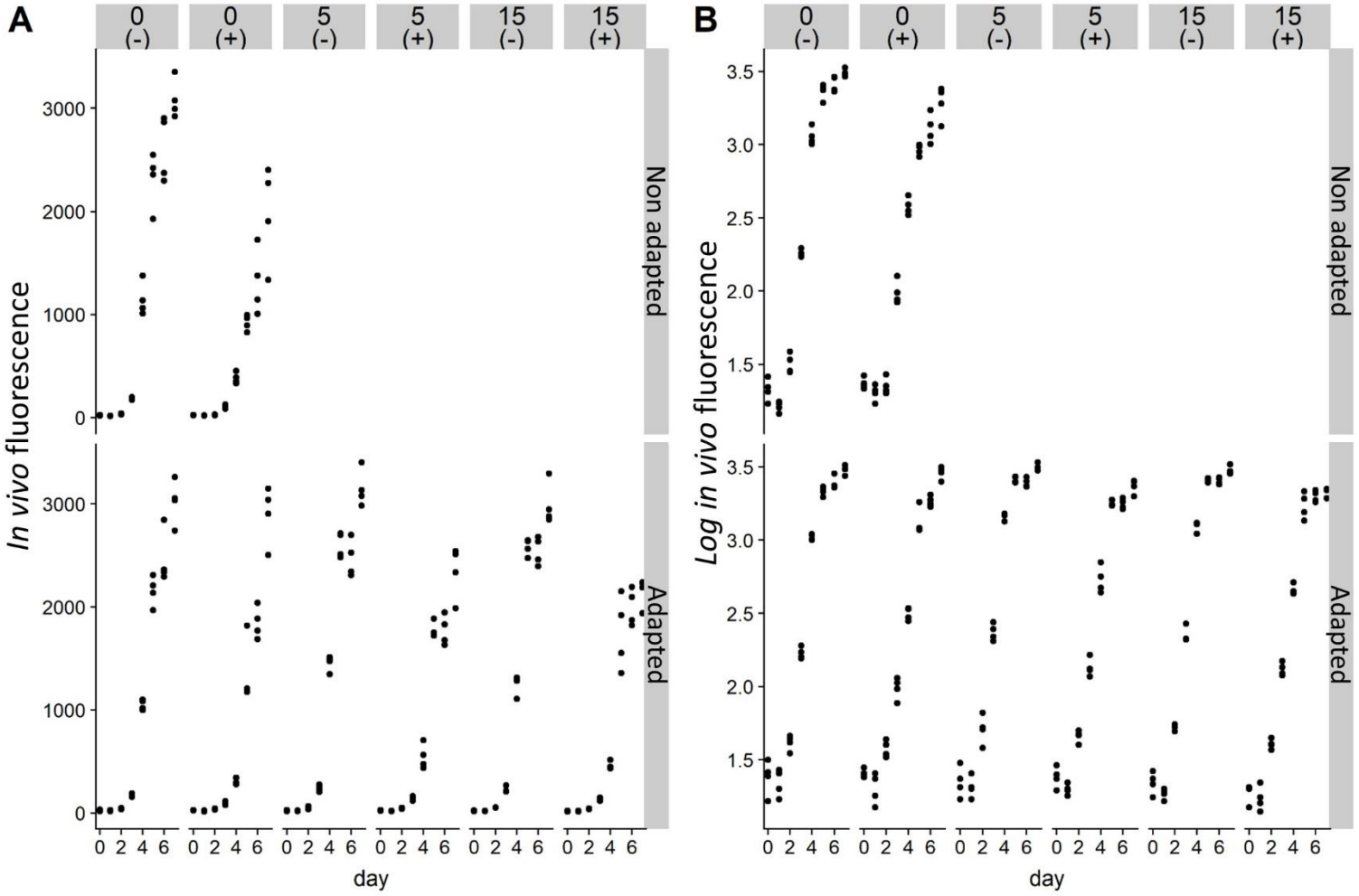


Figure S2.2. (A) Daily biomass development measured as the in vivo fluorescence and (B) log in vivo fluorescence data of the phytoplankton populations under different DOM levels (DOC 0, 5, 15 mg L^{-1}), in the absence (-) and the presence (+) of PPCPs, in the non-adapted and adapted populations during phase II.

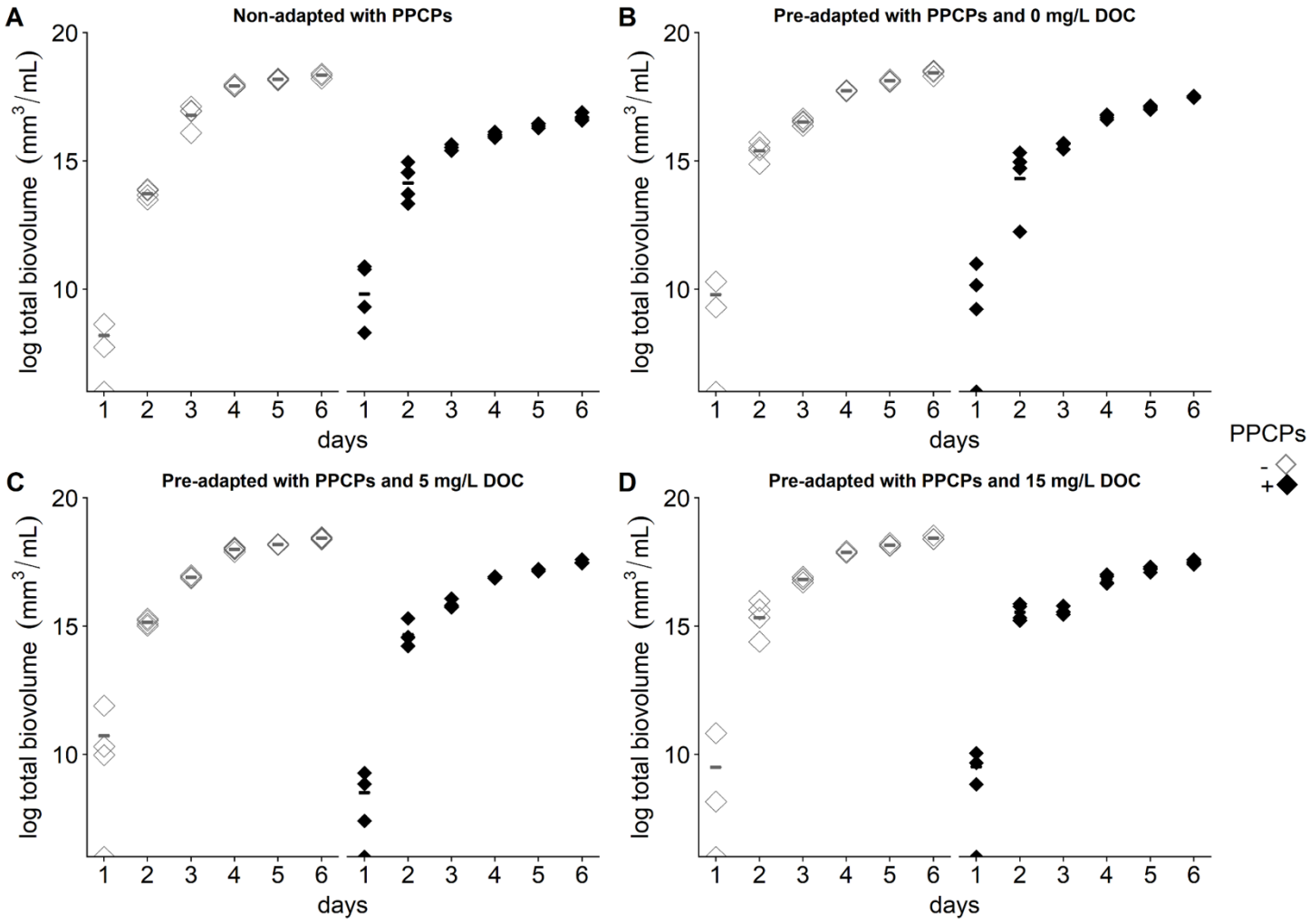


Figure S2.3. Log daily biovolume development (mm3/mL) of C. reinhardtii in the non-adapted (A), and adapted to PPCPs at 0 mg L^{-1} DOC (B), 5 mg L^{-1} DOC (C) and 15 mg L^{-1} DOC (D), in the absence (-) and the presence (+) of PPCPs during phase II. Short horizontal bars represent the mean of each group.

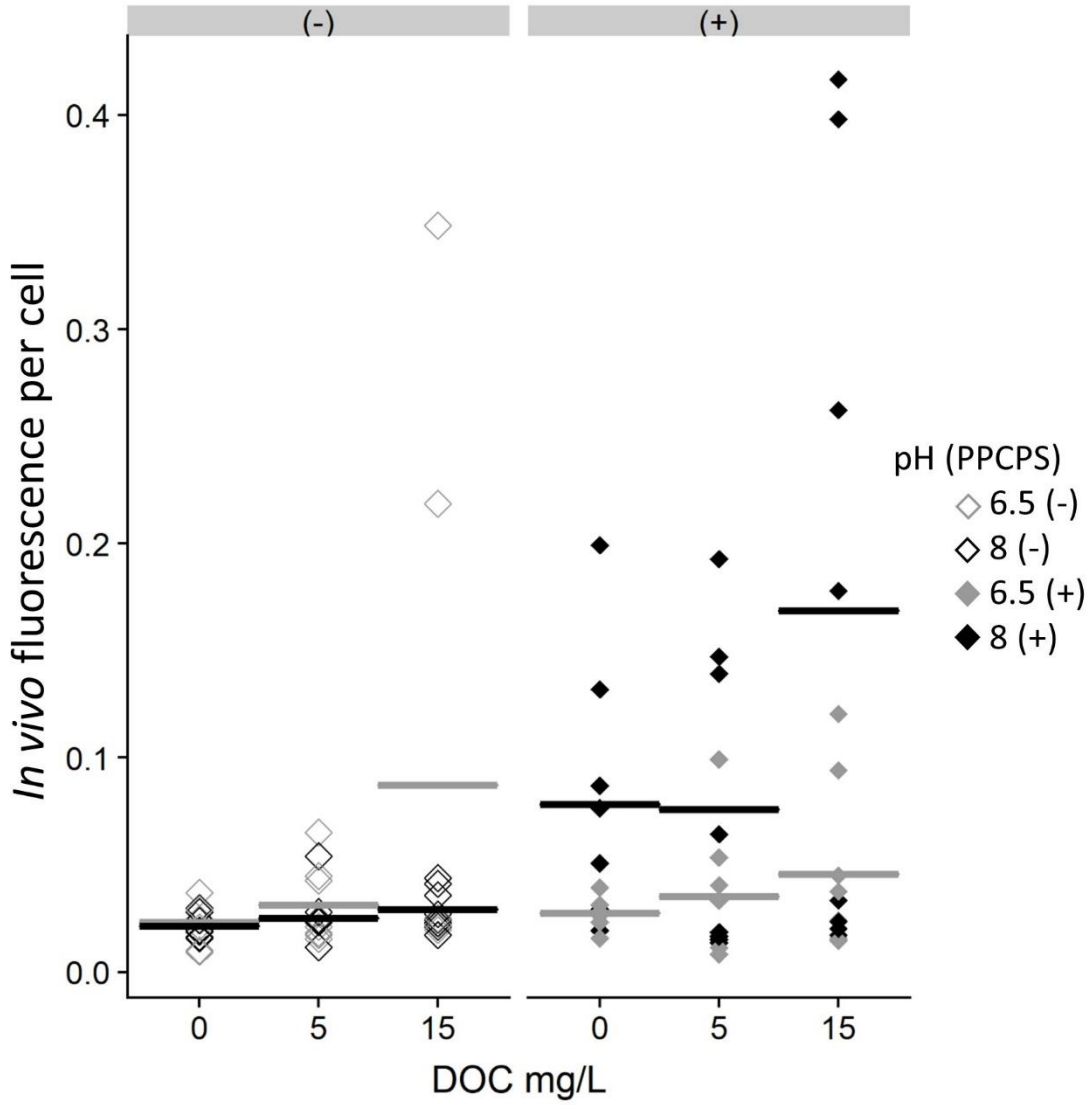
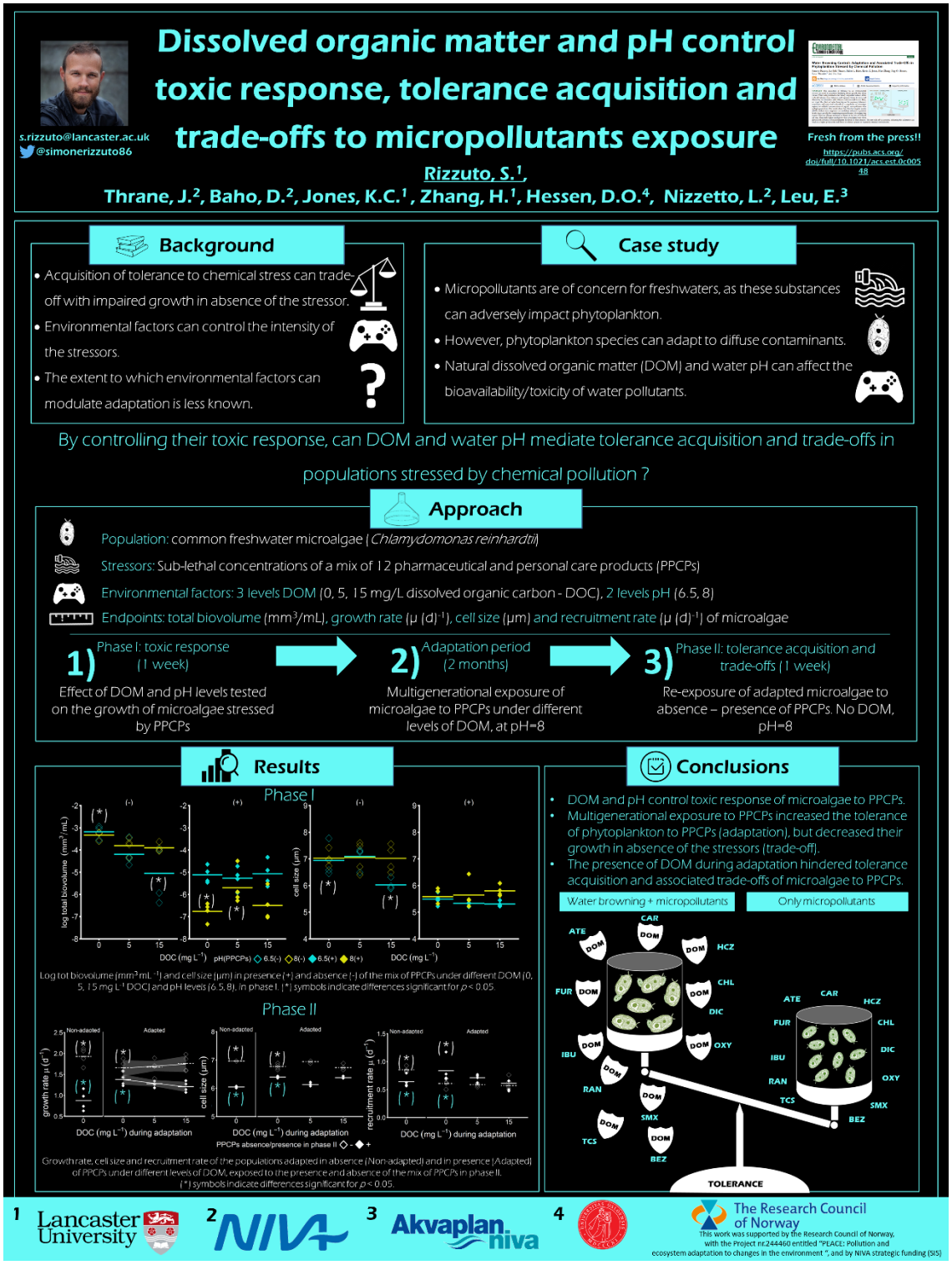


Figure S2.4. In vivo fluorescence per unit of biomass data of C. reinhardtii under different DOM (0, 5, 15 mg L^{-1} DOC) and pH (6.5, 8) levels, in the absence (-) and the presence (+) of PPCPs, during phase I.

Appendix Chapter II: Dissolved Organic Matter and pH control toxic response, tolerance acquisition and fitness trade-offs to micropollutants exposure.

Poster presentation at SETAC SciCon 2020



Supplementary Information Chapter III: Ecological Memory of historical contamination influences the response of phytoplankton communities.

Didier L. Baho[a,b*], Simone Rizzuto[c], Luca Nizzetto[a,d*], Dag O. Hessen[e], Jon Norberg[f], Birger Skjelbred[a], Kevin C. Jones[c], Hao Zhang[c] and Eva Leu[g]

[a] Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, 0349, Oslo, Norway

[b] Department of Aquatic Sciences and Assessment, Swedish University of Agricultural Sciences, Uppsala, Sweden

[c] Lancaster University, Lancaster Environment Centre, Lancaster, United Kingdom

[d] RECETOX, Masarik University, Kamenice 753/5, 625 00 Brno, Czech Republic

[e] University of Oslo, Dept. Biosciences, Box 1066 Blindern, 0316, Oslo, Norway

[f] Stockholm Resilience Centre, Stockholm University, Kräftriket 2B, SE-10691, Stockholm, Sweden

[g] Akvaplan-niva, CIENS, Science Park, Gaustadalléen 21, 0349, Oslo, Norway

* Corresponding authors. Email: didier.baho@slu.se, luca.nizzetto@niva.no

List of Tables:

List of Figures:

List of Text:

Text S3.1. Selection of study sites and their characterization

The lakes were selected using information from the Swedish monitoring programs on inland freshwater ecosystems. These data are freely accessible from the host webpage: https://www.slu.se/institutioner/vatten-miljo/datavardskap/. Both lakes shared similar physical-chemical characteristics including water depth (ca. 1m depth), pH (ca. 7.0), similar dominance of submerged aquatic macrophytes (*Myriophyllum* genus), and total phosphorus above 25 µg/L (eutrophic condition). The lakes differed mainly in their contamination history. Lake Finnsjön is a near-pristine lake located in a forested catchment in Uppland (Fig. S1). Lake Tårkern (Fig. S1) is located in the County of Östergötland and has an been exposed to many pesticides (Boström and others 2016) including Isoproturon (Table S1) for a relatively long time (decades). The herbicide (Isoproturon) was not detected in the water column of the two selected lakes when the sediments were sampled.

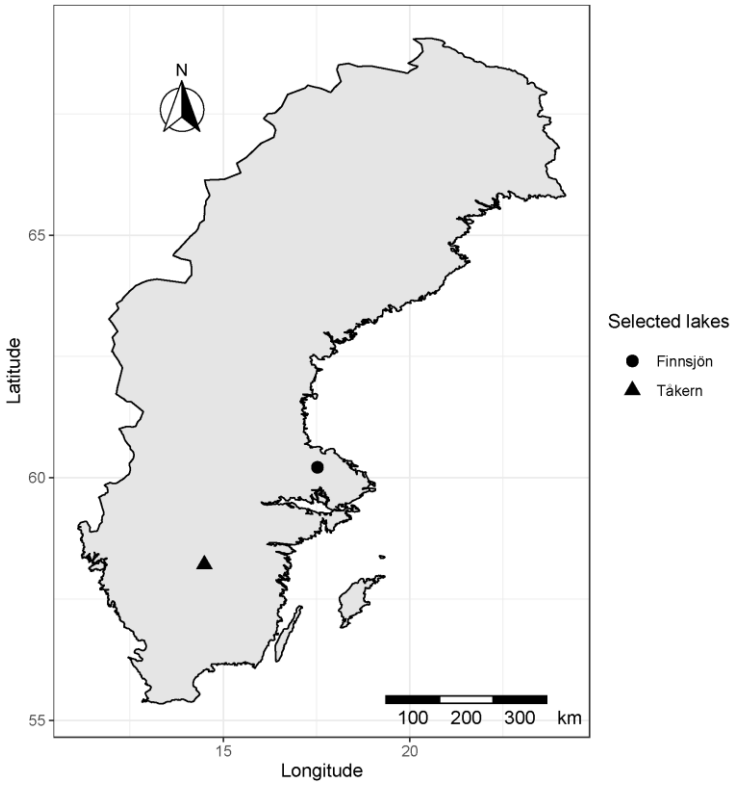


Figure S3.1. Location of the selected sampling sites: Lake Finnsjön (60°21'45.1"N, 17°52'56.1"E) is a near-pristine lake and Lake Tåkern is impacted by herbicides runoff (58°21'07.0"N, 14°49'42.7"E).

Table S3.1. List of selected herbicides (including some secondary metabolites; AMPA and Terbuthylazine) found in the catchment of Lake Tårkern, located in the County of Östergötland, between 2002–2015. The data was downloaded from the database (http://jordbruksvatten.slu.se/pesticider_start.cfm) on 25th July 2017.

Substances	Times detected	Min conc. (µg/L)	Median conc. (µg/L)	90th Percent. conc. (µg/L)	Max conc. (µg/L)
Aminomethylphosphonic acid (AMPA)	92	0.05	0.12	0.78	3
2, 6-Dichlorobenzamide (BAM)	34	0.01	0.01	0.03	0.04
Bentazon	295	0.02	0.17	0.56	21
Cyprodinil	6	0.01	0.04	0.07	0.07
Glyphosate	169	0.03	0.08	0.24	2.27
Isoproturon	119	0.002	0.01	0.35	5
Clopyralid	246	0.01	0.10	0.44	2.2
2-Methyl-4-chlorophenoxyacetic acid (MCPA)	141	0.01	0.09	2.6	28
Metribuzin	119	0.01	0.06	0.3	2.6
Terbutylazindesetyl	10	0.002	0.003	0.004	0.01

Text S3.2. **Isoproturon growth inhibition.**

The choice of the Isoproturon concentrations applied during the germination and exposure phase of the experiment was conceived to encompass the wide range of concentrations usually recorded in the field. Isoproturon runoff concentrations has been found to reach up to 60 mg/L in freshwater systems (Lecomte and others 2001). Moreover, Nitsche and Schlüsser (1998) showed that concentrations of the herbicide up to 42 µg/L were observed in rural-effluent wastewater in the spring months from April to May, which are the periods when the herbicide is applied, and runoff events are more likely to occur. For this purpose, different Isoproturon levels (L1=0.06, L2=0.12, L3=0.24, L4=0.48, L5=0.96, L6=1.92, L7=3.84, L8=7.68, L9=15.36, L10=30.72, L11=61.44 µg/L) were tested for 96 hours on the growth of a model phytoplankton species (*Pseudokirchneriella subcapitata* that has recently revised and renamed to *Raphidocelis subcapitata*), and of phytoplankton communities from both lakes. The results

from the three test cultures (*Pseudokirchneriella subcapitata*, phytoplankton communities from the two lakes obtained after germination from sediments) showed no effects from the first 7 exposure levels, whereas L8 (7.68 µg/L) caused 5-10%, L9 (15.36 µg/L) 20-25%, L10 (30.72 µg/L) 45-50% and L11 (61.44 µg/L) 70-75% growth inhibition on the model species and both the communities (Fig. S2).

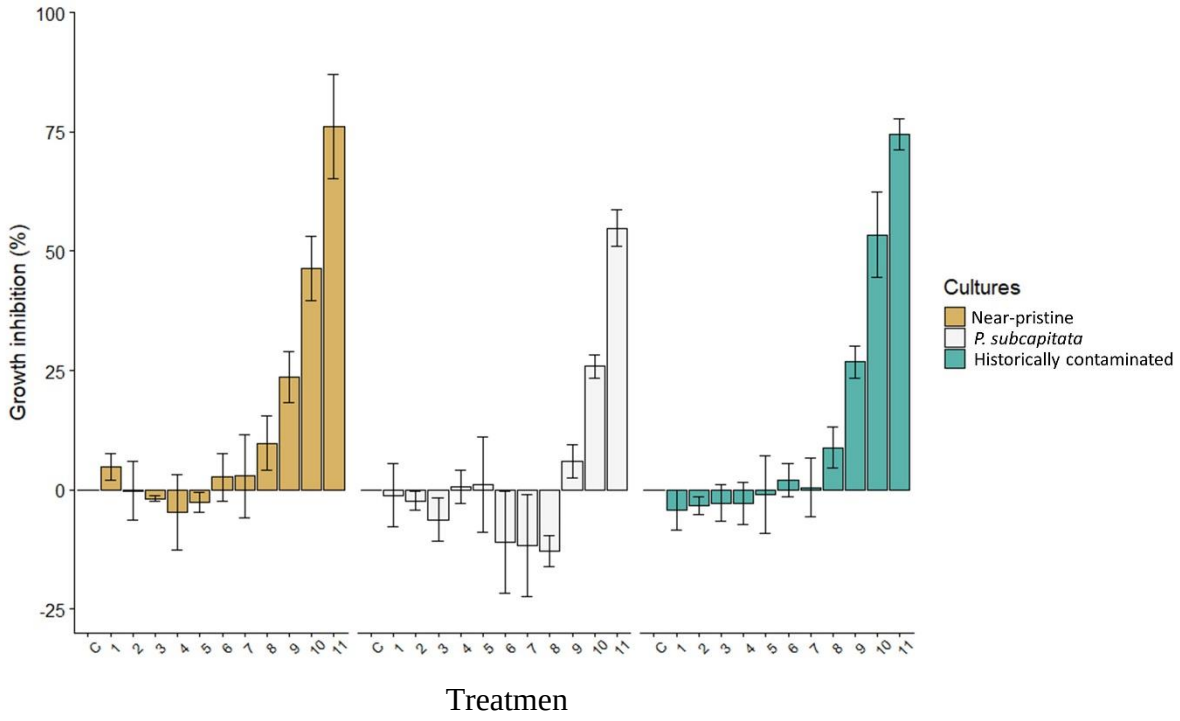


Figure S3.2. Growth inhibition (%) of the near-pristine, the historically contaminated community and the model species Pseudokirchneriella subcapitata along an increasing Isoproturon concentration that summed up to 11 exposure levels (0.06, 0.12, 0.24, 0.48, 0.96, 1.92, 3.84, 7.68, 15.36, 30.72, 61.44 µg/L) and the control.

Text S3.3. Measuring Isoproturon concentration during the experiment

In order to check for degradation of the herbicide, samples were taken during both phases of the experiment. Water samples of 20 mL were collected from the bioreactors with 12 µg/L Isoproturon from the germination phase, and from the experimental two experiment units; L1 (7 µg/L) and L3 (61 µg/L), from the exposure phase and stored in amber glass bottles at -20°C in the dark. The herbicide was extracted through SPE extraction using HLB cartridges (Oasis) in 5 mL of MeOH. The extract was dried with a gentle N_2 flow, reconstituted in 1 mL MeOH, and filtered through 0.2 µm PP syringes filters (Pall, UK) into a 2 mL GC vial. The samples were analyzed using liquid chromatography mass spectrometry (Shimadzu, 8040), using an XBridge BEH C18 column (2.1 mm x 100 mm, 3.5 µm) to separate the compounds. The mobile media used were A, 0.2% Ammonium formate in MQ water, and B, acetonitrile. The gradient procedure was optimized at: 0-1 min 20% B, then increased to 100% within 8 min, held at 100% for 5 min, after that decreased to the initial conditions (20% B) within 1 min. Finally, 6 minutes of post-run ensured re-equilibration of the column before the next injection. The injection volume was 10 µL, while the column and the tray temperature were set to 25°C. The quantification of ISU was based on internal standard method (Isoproturon-d6, Sigma Aldrich), and the instrument detection limit of 1.82 ng/mL.

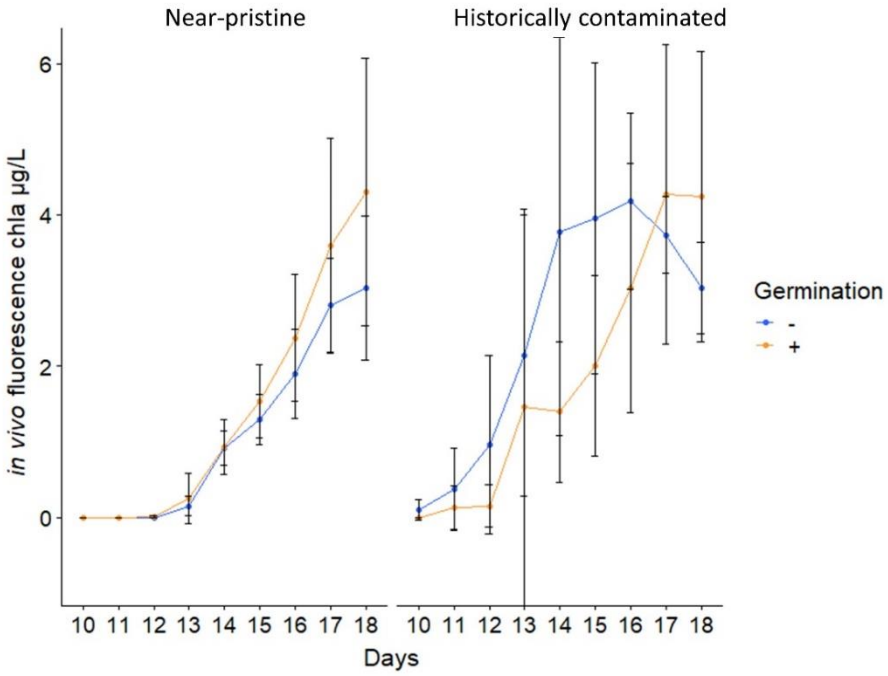


Figure S3.3. Growth of phytoplankton monitored on a daily basis during phase I (gemination and conditioning phase) measured as the in vivo fluorescence emission in the near-pristine and historically contaminated communities germinated without (-) and with (+) Isoproturon in the germination phase. Bars represent standard deviation.

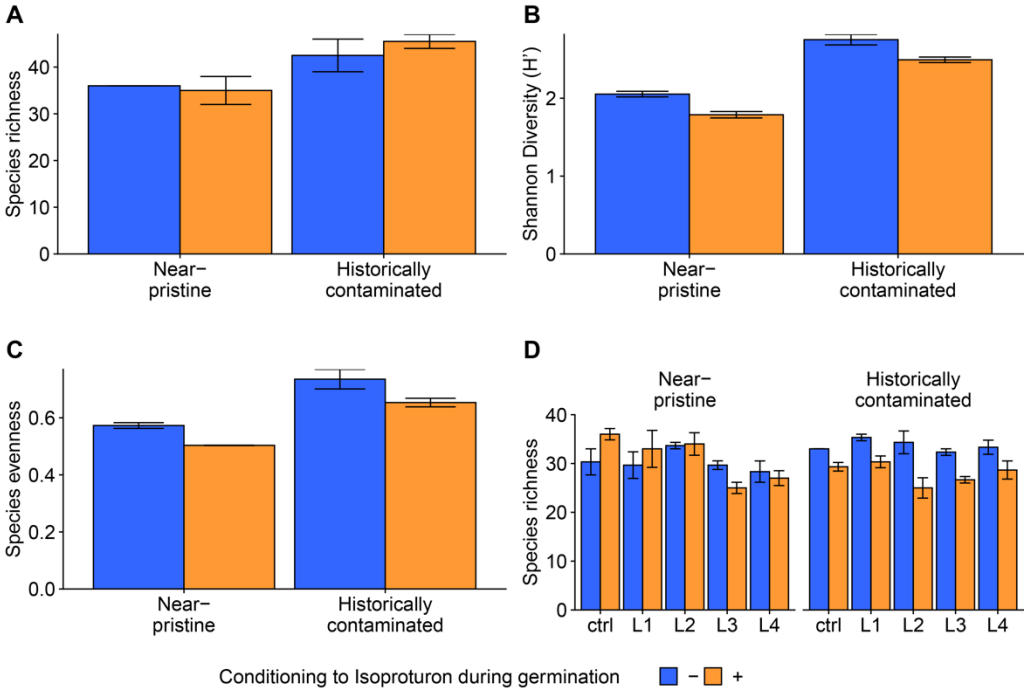


Figure S3.4. Comparison of (a) Species richness, (b) Shannon diversity and (c) evenness of the near-pristine and historically contaminated phytoplankton germinated without (-) and with (+) herbicide in the phase I. Panel (d) shows the species richness that was observed during phase II. The error bars represent standard error. Shannon diversity (b) is also presented in the main text.

Table S3.2. Summary of the effects of the contamination history, conditioning and their interaction on; growth rate, total biomass, species richness, Shannon diversity and evenness of phytoplankton recorded during phase I. Significant values are reported in bold.

Endpoints	Effects	df	SS	F	p
Growth rate	Contamination history	1, 20	0.003	0.13	0.73
	Conditioning	1, 20	0.03	1.52	0.24
	Contamination history: Conditioning	1, 20	0.0009	0.04	0.85
Total biomass	**Contamination history**	**1, 8**	**1.99**	**230.08**	**< 0.001**
	Conditioning	1, 8	0.01	1.70	0.26
	Contamination history: Conditioning	**1, 8**	**0.08**	**9.68**	**< 0.05**
Species richness	**Contamination history**	**1, 8**	**144.5**	**12.30**	**<0.05**
	Conditioning	1, 8	2.0	0.17	0.70
	Contamination history: Conditioning	1, 8	8.0	0.68	0.46
Shannon diversity	**Contamination history**	**1, 8**	**0.11**	**234.10**	**< 0.001**
	Conditioning	**1, 8**	**0.02**	**32.90**	**< 0.01**
	Contamination history: Conditioning	1, 8	0.0001	0.27	0.63
Evenness	**Contamination history**	**1, 8**	**0.05**	**65.31**	**< 0.01**
	Conditioning	**1, 8**	**0.01**	**15.35**	**< 0.05**
	Contamination history: Conditioning	1, 8	0.00008	0.11	0.76

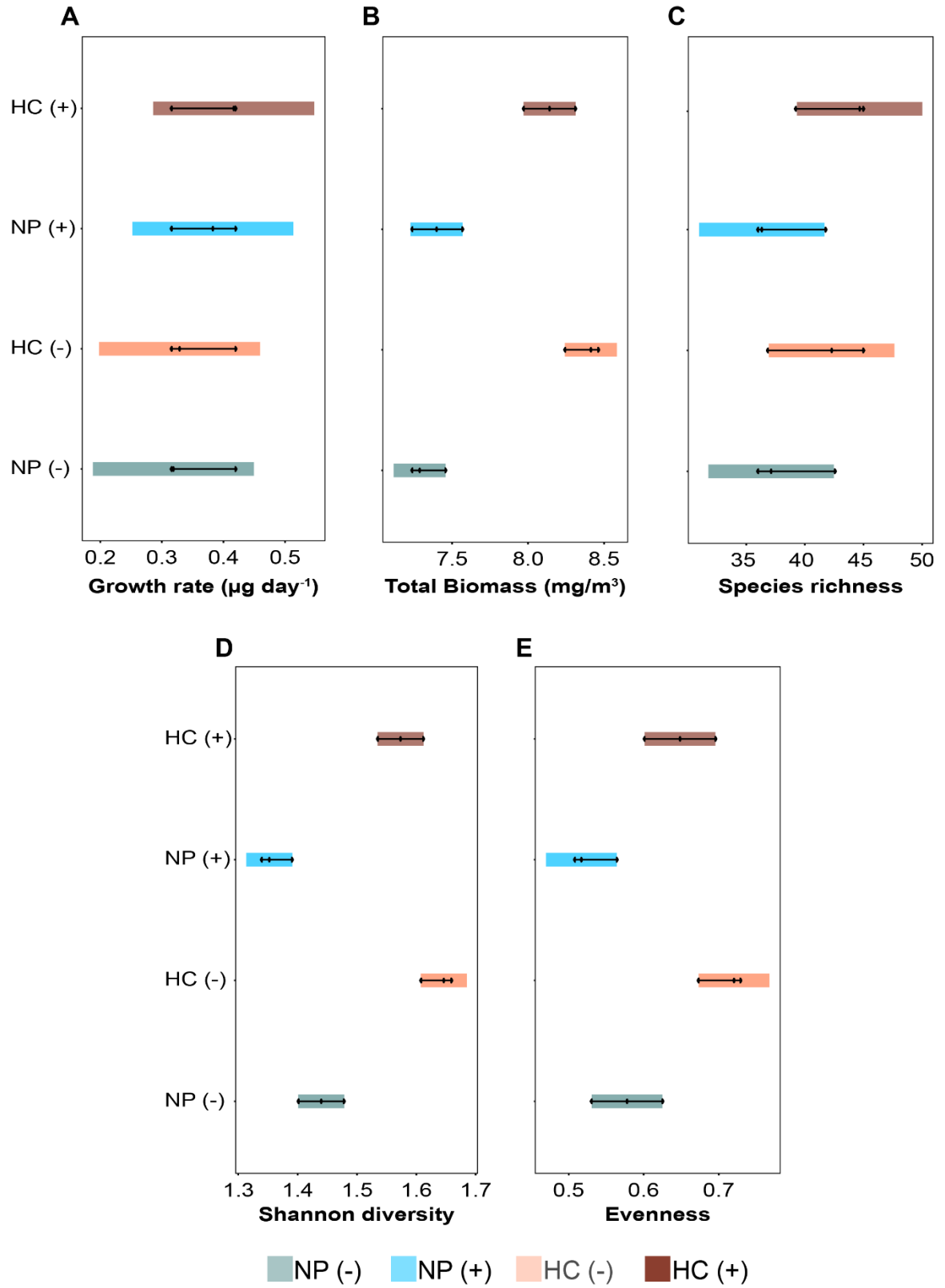


Figure S3.5. Estimated marginal means for (a) growth rate, (b) total biomass, (c) species richness, (d) Shannon diversity and (e) evenness of phytoplankton observed at the end phase I. The central points in the figure indicate the mean response with 95 % confidence interval for the combined main effects (contamination history, germination treatment) for the historically contaminated (HC) lake and near-pristine (NP) conditioned with (+) and without (-) Isoproturon during.

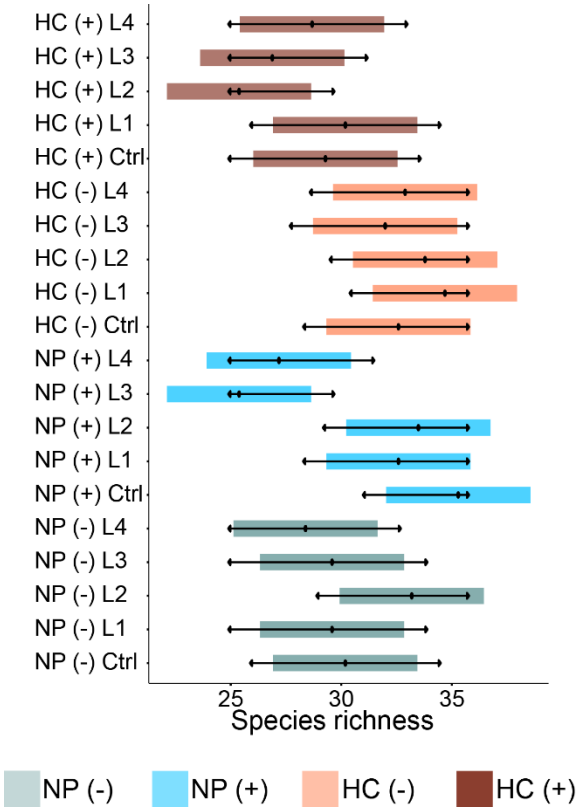


Figure S3.6. Estimated marginal means for species richness phytoplankton observed at the end phase II. The central points in the figure indicate the mean response with 95 % confidence interval for the combined main effects (contamination history, conditioning, Isoproturon exposure) for the historically contaminated (HC) lake and near-pristine (NP) conditioned with (+) and without (-) Isoproturon during germination and the five different Isoproturon exposure levels used during phase II.

Table S3.3. Summarizing the results of size Cohen's d effect size, comparing the control and four different exposure levels (L1-L4), for growth rate and total biomass of phytoplankton from phase II.

			Cohen's d	
Contamination History	**Conditioning**	**Comparisons**	**Growth rate**	**Total biomass**
Near-pristine	(-)	Ctrl vs L1	0.13	0.05
		Ctrl vs L2	2.11	0.51
		Ctrl vs L3	24.32	4.13
		Ctrl vs L4	46.73	5.50
Historically contaminated	(-)	Ctrl vs L1	0.5	0.41
		Ctrl vs L2	2.31	1.51
		Ctrl vs L3	5.37	11.52
		Ctrl vs L4	6.55	17.22
Near-pristine	(+)	Ctrl vs L1	1.12	0.81
		Ctrl vs L2	8.42	0.32
		Ctrl vs L3	30.89	7.67
		Ctrl vs L4	19.8	8.59
Historically contaminated	(+)	Ctrl vs L1	0.16	0.50
		Ctrl vs L2	0.89	0.41
		Ctrl vs L3	12.96	9.20
		Ctrl vs L4	8.97	10.57

Table S3.4. Summarizing the results of the repeated measures analysis of variance during phase II. The main effects included: herbicide exposure, germination treatment, time and the interaction terms on the photosynthetic efficiency of the phytoplankton communities across the two lakes that differed in contamination histories. Huynd-Feldt correction was applied when assumption of sphericity was breached, significant values are reported in bold.

Contamination History	Effects	df	F	p
Near-pristine	Isoproturon exposure	**4, 20**	**51.44**	**< 0.01**
	Conditioning	1, 20	1.43	0.24
	Day	**2, 40**	**30.18**	**< 0.01**
	Isoproturon exposure: Conditioning	**4, 20**	**3.23**	**0.03**
	Isoproturon exposure: Day	**8, 40**	**17.91**	**< 0.01**
	Germination treatment: Day	**2, 40**	**35.32**	**< 0.01**
	Isoproturon exposure: Conditioning: Day	8, 40	1.92	0.08
Historically contaminated	Isoproturon exposure	**4, 20**	**15.19**	**< 0.01**
	Conditioning	1, 20	0.14	0.71
	Day	**2, 40**	**119.4**	**< 0.01**
	Isoproturon exposure: Conditioning	4, 20	0.71	0.59
	Isoproturon exposure: Day	**8, 40**	**21.35**	**< 0.01**
	Germination treatment: Day	**2, 40**	**14.67**	**< 0.01**
	Isoproturon exposure: Conditioning: Day	8, 40	1.73	0.12

Table S3.5. Summary of the effects of Isoproturon exposure on the day 7 of the phase II on the photosynthetic efficiency of the phytoplankton assemblages germinated without (-) and with (+) herbicide from the sediments of the near-pristine and historically contaminated catchments. Significant values are reported in bold.

Contamination History	Conditioning	Effect	df	SS	F	p
Near-pristine	(-)	Treatment	**4, 15**	**0.01**	**15.32**	**< 0.001**
	(+)		**4, 15**	**0.02**	**74.15**	**< 0.001**
Historically contaminated	(-)		**4, 15**	**0.01**	**7.91**	**< 0.01**
	(+)		4, 15	0.01	1.96	0.17

Table S3.6. PERMANOVA test showing significant effect of the Isoproturon exposure gradient during the phase II. Shown are: df: degrees of freedom, SS: sum of squares, MS: mean of squares, F-statistic. Significant results ($p < 0.01$) are reported in bold.

Contamination History	Conditioning	df	SS	MS	F	P
Near-pristine	(-)	**4, 14**	**3537.36**	**884.34**	**8.25**	**<0.001**
	(+)	**4, 14**	**3968.25**	**992.06**	**8.75**	**<0.001**
Historically contaminated	(-)	**4, 14**	**3784.00**	**946.00**	**12.81**	**<0.001**
	(+)	**4, 14**	**3842.44**	**960.61**	**6.73**	**<0.001**

Appendix Chapter III: Freshwater phytoplankton community response across different historical contamination backgrounds.

Oral presentation at SETAC Helsinki 2019 – Winner Best Presentation Award. https://awards.setac.org/past-europe-award-winners/

Supplementary Information Chapter IV: Influence of ecological memory on phytoplankton early assemblages: a trait-based approach

Simone Rizzuto[1], Didier Baho[2], Kevin C. Jones[1], Hao Zhang[1], Francesco Pomati[3], Eva Leu[4], Luca Nizzetto[2,5]

[1] Lancaster Environment Centre, Lancaster University, Lancaster, LA1 4YQ, UK.

[2] Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, 0349, Oslo, Norway.

[3] Department of Aquatic Ecology, Eawag: Swiss Federal Institute of Aquatic Science and Technology, Überlandstrasse 133, 8600 Dübendorf, Switzerland.

[4] Akvaplan-niva, CIENS, Science Park, Gaustadalléen 21, 0349, Oslo, Norway.

[5] RECETOX, Masaryk University, Kamenice 753/5, 625 00 Brno, Czech Republic.

List of Figure:

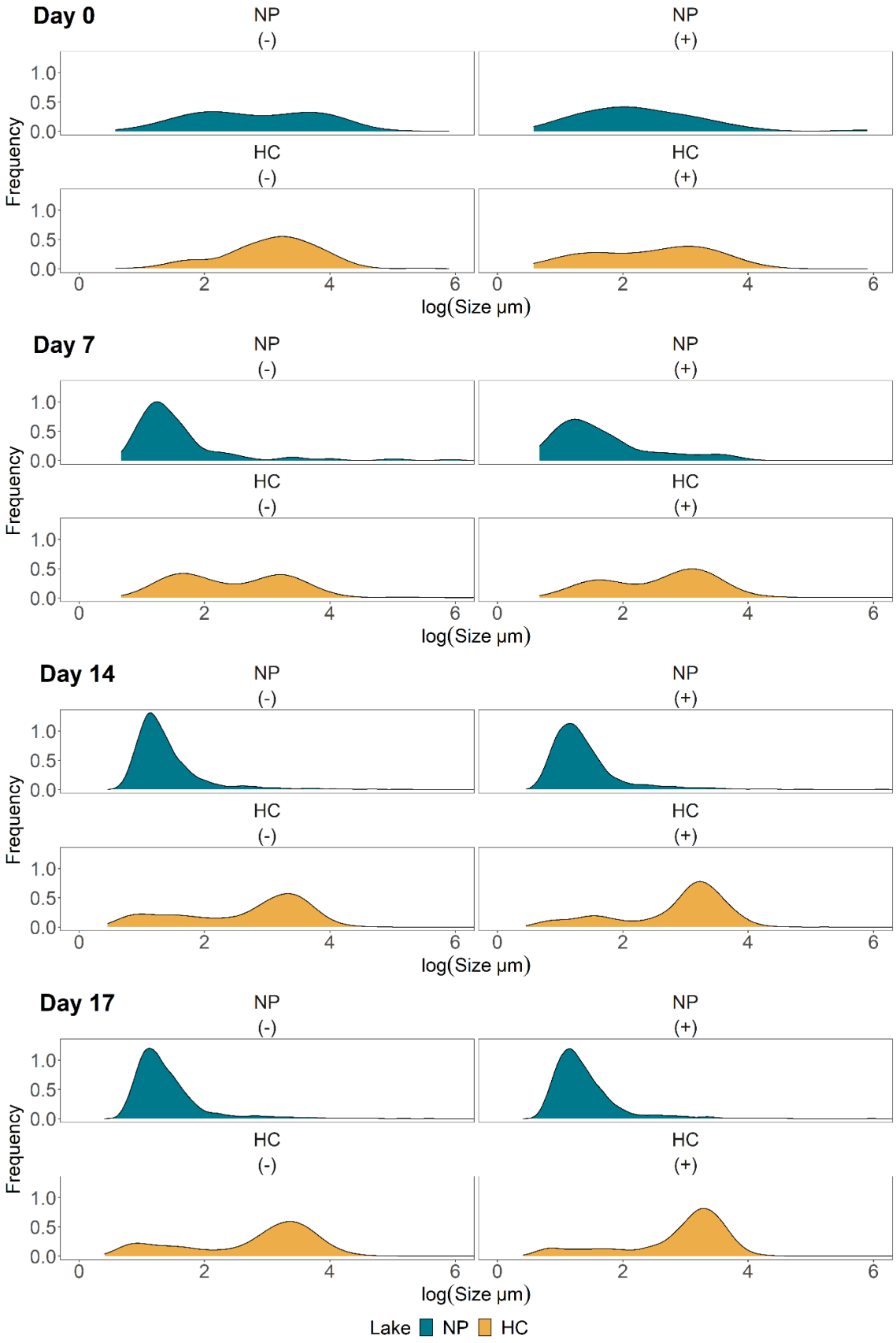


Figure S4.1. Size distribution of the phytoplankton communities from the NP and HC catchments germinates with and without herbicide.

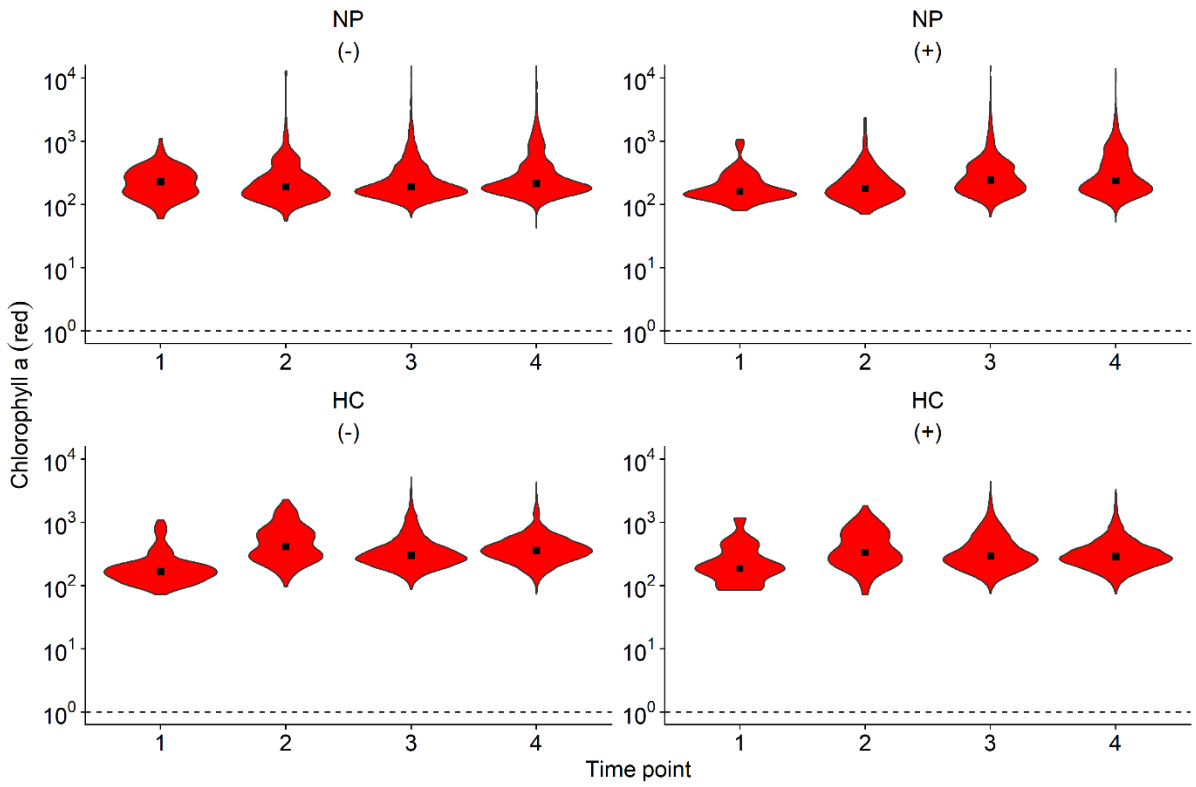


Figure S4.2. Violin plot of chlorophyll a of the phytoplankton communities from the NP and HC catchments germinates with and without herbicide.

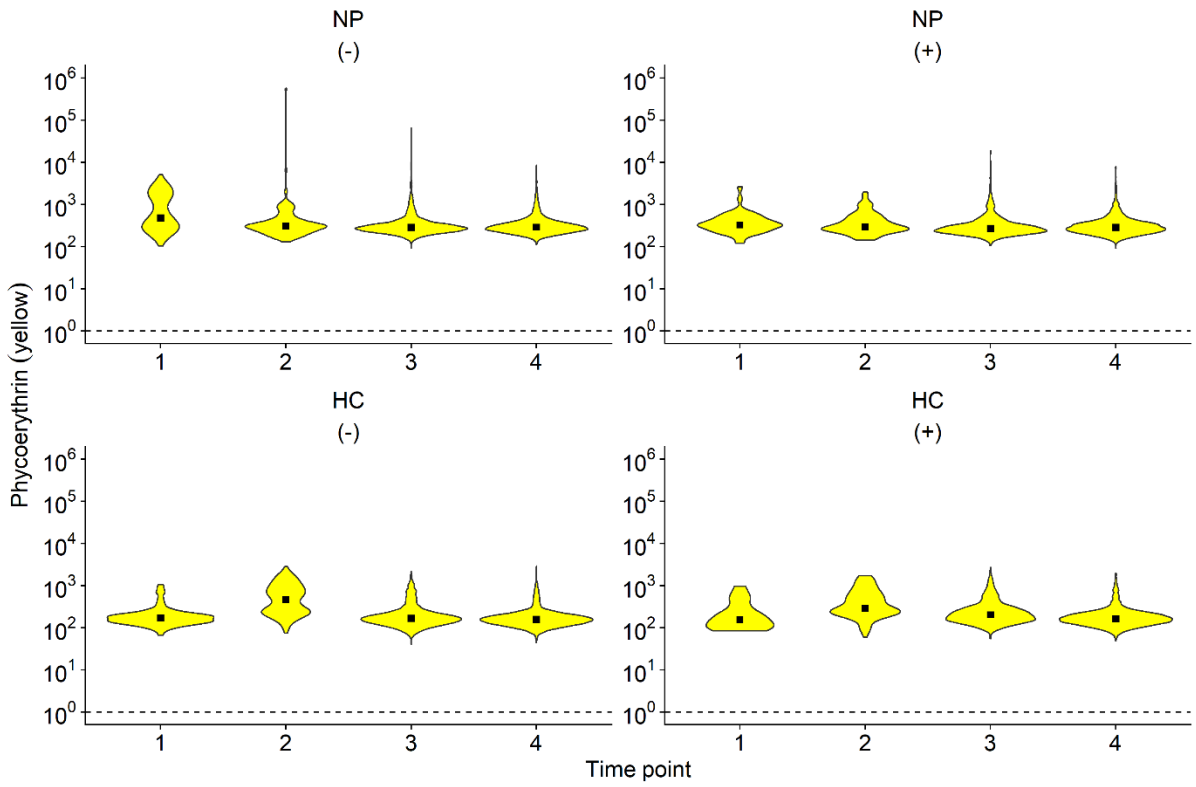


Figure S4.3. Violin plot of phycoerythrin of the phytoplankton communities from the NP and HC catchments germinates with and without herbicide.

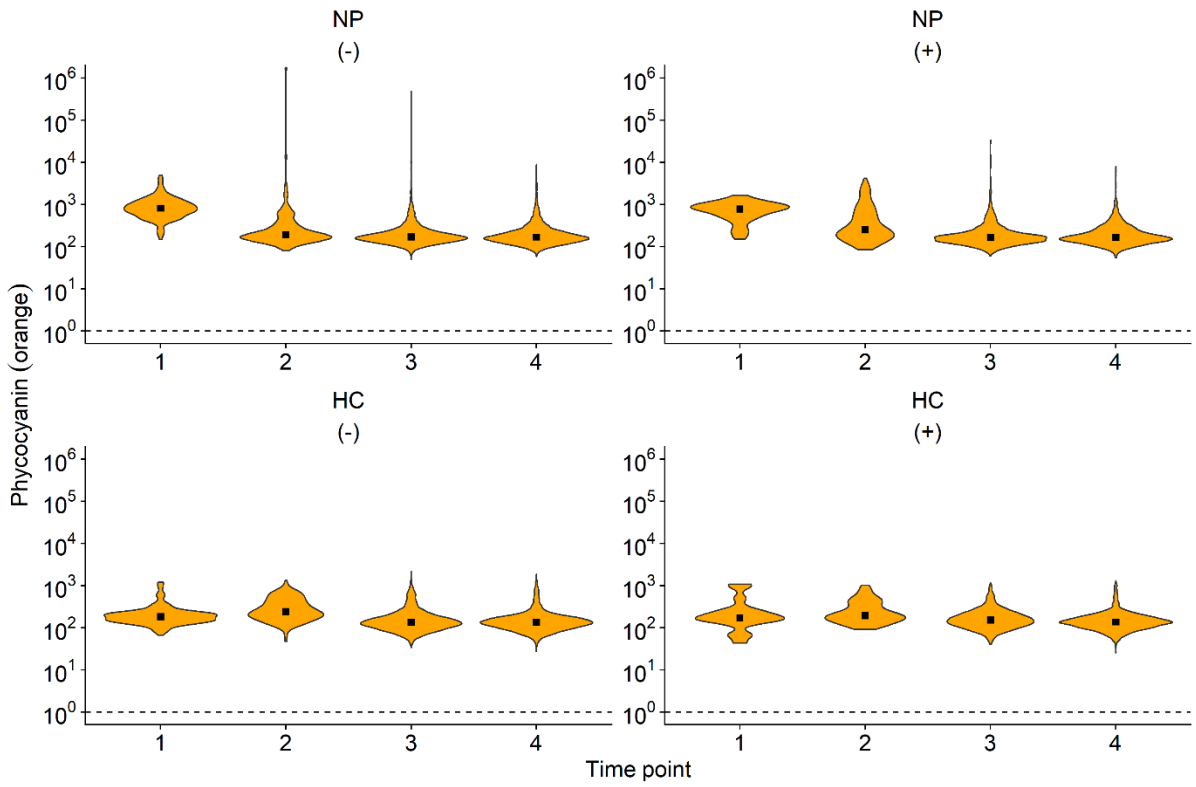


Figure S4.4. Violin plot of phycocyanin of the phytoplankton communities from the NP and HC catchments germinates with and without herbicide.

Appendix Chapter IV: Trait diversity calculation.

TOP Richness Index

The richness index TOP (Figure) was calculated as the sum of all successive areas touching all the points in the trait distribution, as reported by Fontana et al. (2016). The calculation is performed as follows: after the first minimum convex hull containing the outermost points has been built and its area has been measured, these points are deleted from the trait distribution and a second convex hull is calculated with the new outermost points (Figure A1).

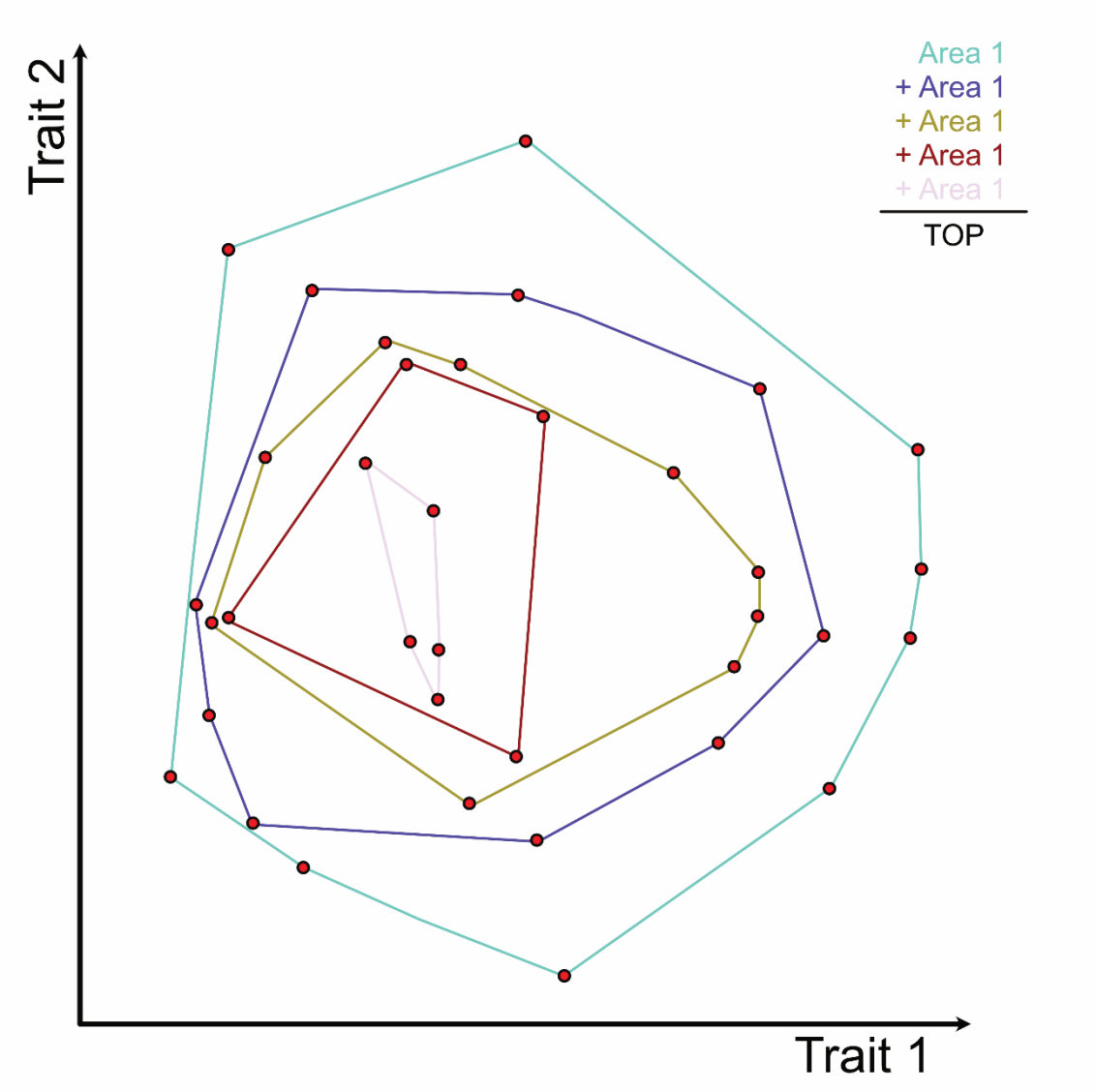


Figure A1. Simplified exemplification (2D) of the calculation of the TOP richness index, modified from Fontana et al. (2016). Red points represent an individual organism and its position is defined by

different traits (axes). The perimeters of the five convex polygons (indicated by the different colours) would represent areas of convex hulls in a multidimensional space. The TOP index of trait richness is then calculated as the sum of all areas.

This process carries on, similarly to peeling off layers of an onion, until the number of remaining points is insufficient for a convex hull (at least $n + 1$ points are needed to build a convex hull in an n-dimensional trait space). Since the number of individuals is generally much bigger than the number of traits considered, the influence of the remaining points (besides the ones accounting for the smallest area) can be considered negligible. The sum of all areas obtained this way represents the TOP index, which is more sensitive to the loss of individuals at the edges of the distribution, but also consider changes in the middle of the cloud of data points. Although TOP is conceptually similar to the convex onion-peeling approach proposed by Chazelle (1985) and Abellanas and others (1996), it has been developed independently and for a different application in a multidimensional trait space.

TED Evenness Index

The TED evenness index is a measure of how evenly distributed individuals are within the trait space (Fontana et al., 2016). It uses a reference distribution obtained starting from equidistant (evenly distributed) points in a n-dimensional space, where n is the number of traits considered. Here, a n-dimensional sphere with evenly distributed points is used as model reference (*geozoo* R-package), but it is also possible to use any n-dimensional geometric shape, provided that the same reference distribution is used for all communities for comparison. Since the number of points forming an n-dimensional sphere cannot be varied at will, the sphere with the lowest number of excess points relative to the test sample is automatically selected. Then, the most distant points from the centroid of the distribution (outermost points) are deleted, in order to obtain a cloud of evenly distributed points that is as similar as possible to a sphere and has exactly the same number of data points as in the test sample. Distance matrices among all

individual data points in the test sample and in the (even) reference distribution are calculated. The Kullback-Leibler divergence (KLdiv) (Kullback and Leibler, 1951) between the two probability distributions of distances (default settings of density functions are used in R) is inversely proportional to the evenness of the test sample. Hence, TED is calculated as:

$$1 - log_{10}(KLdiv + 1)$$

TED maximum value is therefore 1 (minimum *KLdiv* being 0). Graphical representation can be found below in Figure A2.

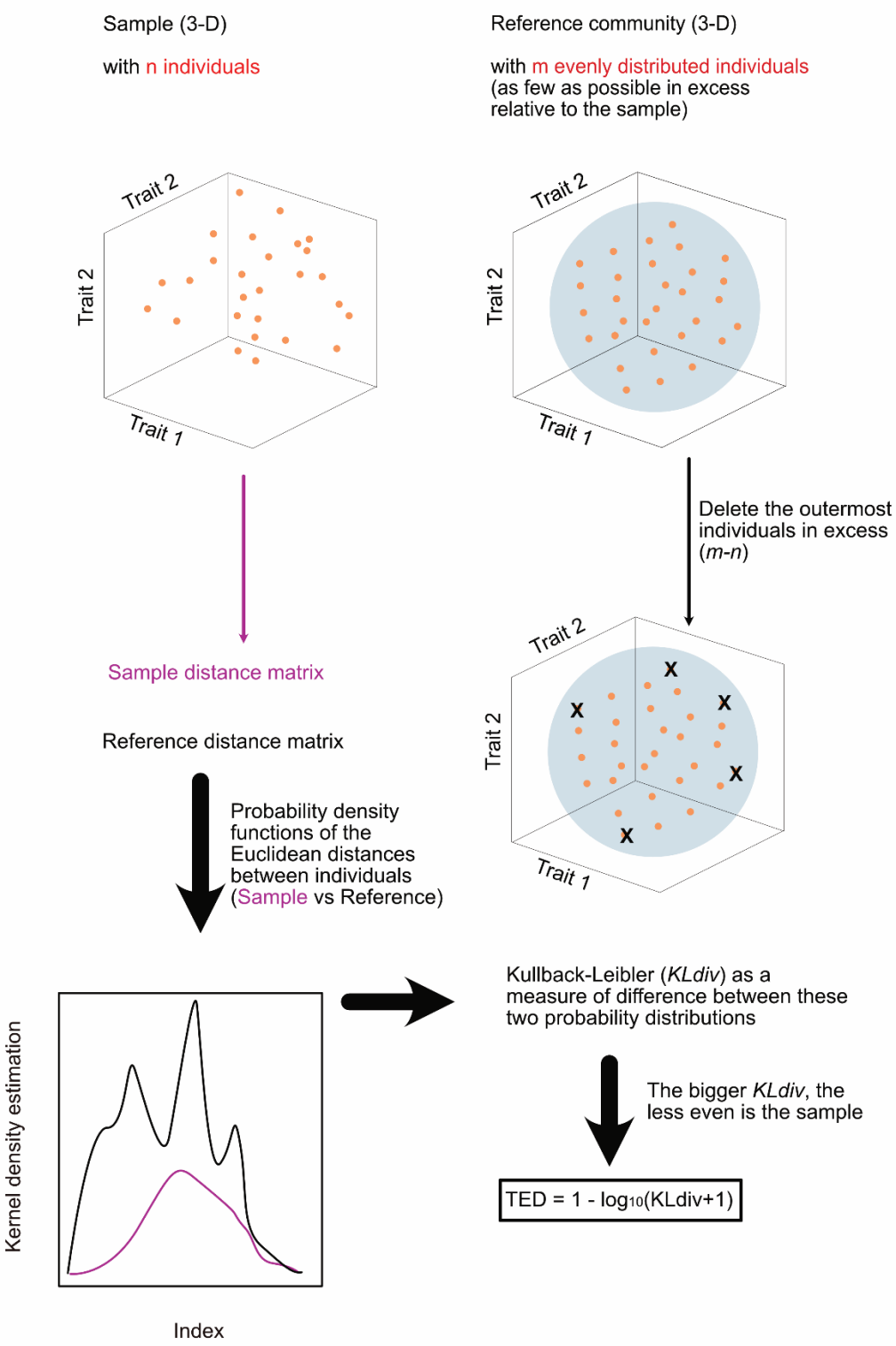


Figure A2. Representation of the steps required to calculate the TED index of trait evenness, modified from Fontana et al. (2016). The proposed example shows a community of 27 individuals distributed in a 3D trait space. Each orange point represent an individual organism, and its position is defined by different traits (axes).

FDis divergence index

According to its developers (Laliberte and Legendre, 2010), FDis represents the mean distance in a multidimensional trait space of individuals species to the centroid of all species; it accounts for species abundances by shifting the position of the centroid toward the more abundant species and weighting distances of individual species by their relative abundances. FDis is the multivariate analogue of the weighted mean absolute deviation, which makes the index unaffected by species richness.

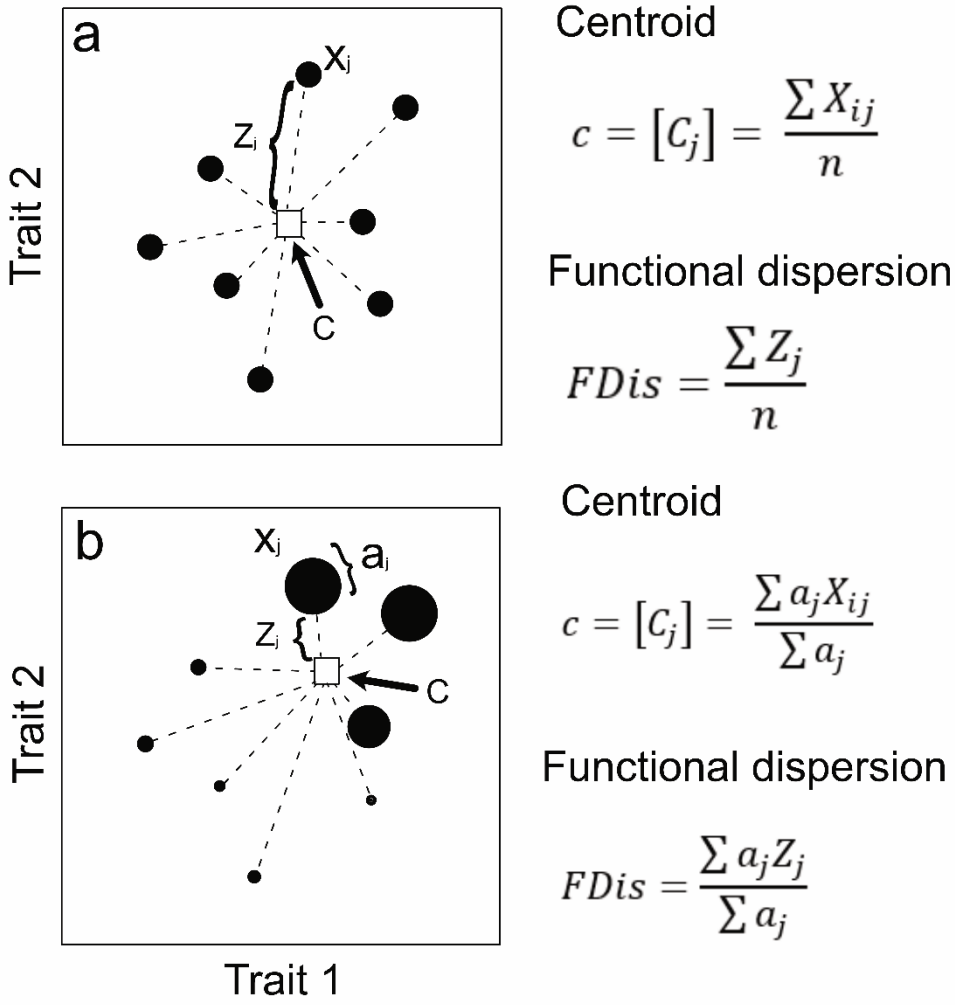


Figure A3. Example showing the computation of FDis (modified from Laliberte & Legendre, 2010).

An example of how FDis is computed is reported in Figure A3. The *n* individual species in a 2D trait space are represented by black circles whose sizes are proportional to their abundances.

Vector $\boldsymbol{X}_j$ represents the position of species j, vector c is the centroid of the n species (white square), Z_j is the distance of species *j* to the centroid **c**, and a_j is the abundance of species *j*. In panel (a), all species have equal abundances (i.e. presence – absence data). In that case, $\mathbf{c} = [c_i]$, where c_i is the mean value of trait *i*, and FDis is the mean of distances *Z* of individual species to **c**. In panel (b), species have different abundances. In that case, the position of **c** is weighted by the species relative abundances, such as it shifts towards the more abundant species. Individual distances *Z* of species to **c** are weighted by their relative abundances to compute FDis.

Supplementary Information Chapter V: Critical assessment of an equilibrium-based method to study the binding of waterborne contaminants to natural dissolved organic matter (DOM).

Simone Rizzuto [1], Kevin C. Jones [1*], Hao Zhang [1*], Didier L. Baho [2], Eva Leu [3], Luca Nizzetto [2, 4]

[1] Lancaster Environment Centre, Lancaster University, Lancaster, LA1 4YQ, UK.

[2] Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, 0349, Oslo, Norway.

[3] Akvaplan-niva, CIENS, Science Park, Gaustadalléen 21, 0349, Oslo, Norway.

[4] RECETOX, Masaryk University, Kamenice 753/5, 625 00 Brno, Czech Republic.

List of Tables:

List of Figures:

List of Text:

Text S5.1. Ecotoxicological test

Different Isoproturon levels (L1=0.06, L2=0.12, L3=0.24, L4=0.48, L5=0.96, L6=1.92, L7=3.84, L8=7.68, L9=15.36, L10=30.72, L11=61.44 µg/L) were tested for 96 hours on the growth of a model phytoplankton species (*Pseudokirchneriella subcapitata*), following a standard eco-toxicological test based on the OECD guidelines of 2009. The results showed no effects from the first 7 exposure levels, whereas L8 (7.68 µg/L) caused 5-10%, L9 (15.36 µg/L) 20-25%, L10 (30.72 µg/L) 45-50% and L11 (61.44 µg/L) 70-75% growth inhibition on the model species and both the communities (Figure S5.2). Therefore, a sub-lethal concentration of 9.5 µg L^{-1} was selected as final concentration.

Text S5.2. Mass recovery

The mass recovery was calculated to account for adsorption, volatilization or degradation of the compounds, as the difference in percentage between the mass expected and the mass detected in each experimental compartment.

$$Mass\ recovery\ (\%) = \left[\frac{Mass_{measured} * 100}{Mass_{expected}}\right] - 100$$

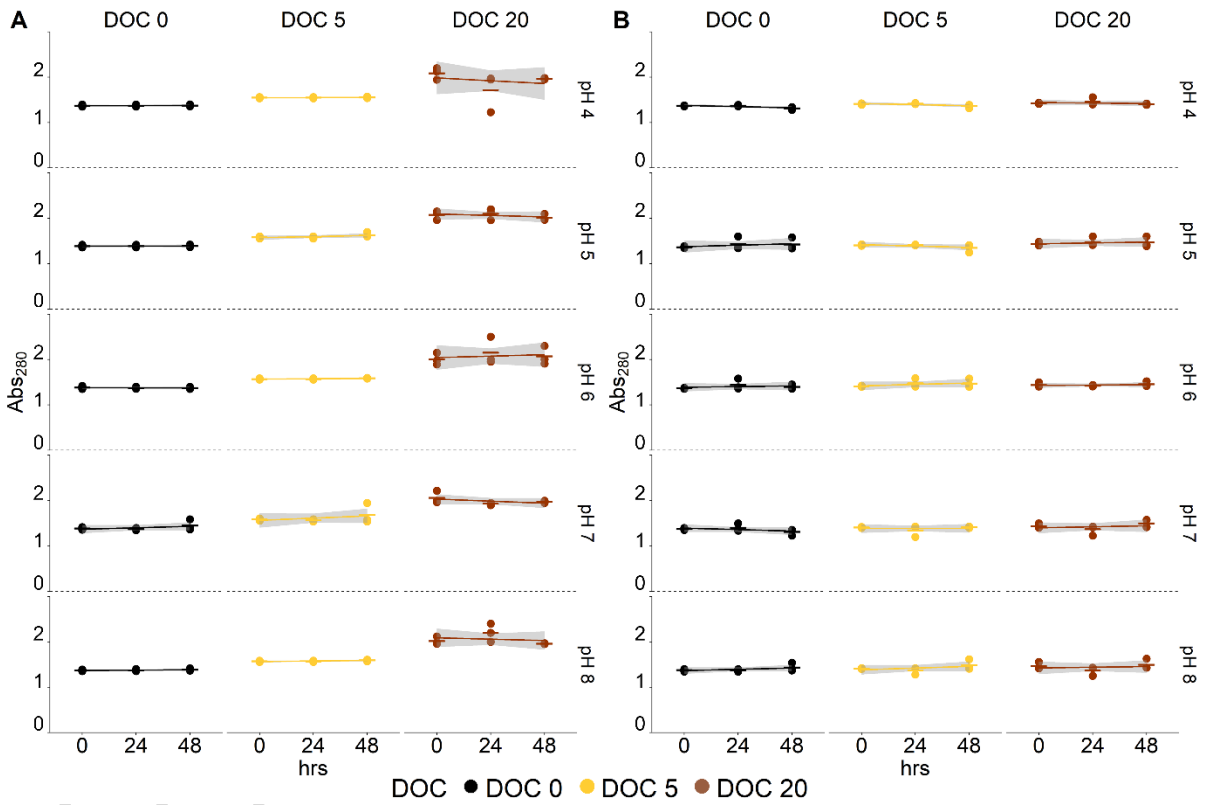


Figure S5.1. Absorbance (280 nm) of the lower (DOC5 = 5 mg L-1 DOC) and higher (DOC20 = 20 mg L-1 DOC) DOM levels measured on (A) the DOM standards and (B) the samples outside the bag at 0, 24 and 48 hours of the experiment at all pH levels. Locally estimated scatterplot smoothing (LOESS) was fitted to the data points to generate line plots. The grey area represents the 95% confidence interval.

Table S5.1. Summary table reporting mean and standard values of; Total recovery (%), Cin, Cout, MD/in and MD/out of ISU at 4 levels of DOM (0, 5, 10, 20 mg L-1 DOC) and 5 levels of pH (4, 5, 6, 7, 8). CE was 9.5 µg L-1. ME/in and ME/out were 0.95 and 0.05 µg.

pH	DOC (mg L^{-1})	Tot recovery (%)		C_{in} (µg L^{-1})		C_{out} (µg L^{-1})		$M_{det/in}$ (µg)		$M_{det/out}$ (µg)	
		mean	sd	mean	sd	mean	sd	mean	sd	mean	sd
4	0	100.2	0.1	8.5	0.1	8.6	0.3	0.1	0.0	1.7	0.1
	5	99.9	0.0	9.7	0.1	7.1	0.1	0.1	0.0	1.4	0.0
	10	99.9	0.0	10.2	0.2	6.9	0.1	0.1	0.0	1.4	0.0
	20	99.9	0.1	9.9	0.2	6.7	0.2	0.1	0.0	1.3	0.0
5	0	100.5	0.6	8.0	0.1	7.9	0.1	0.1	0.0	1.6	0.0
	5	101.8	0.1	12.7	0.3	7.0	0.2	0.1	0.0	1.4	0.0
	10	100.6	0.1	10.4	0.1	6.3	0.1	0.1	0.0	1.3	0.0
	20	100.4	0.5	8.4	0.1	7.5	0.3	0.1	0.0	1.5	0.1
6	0	100.2	0.2	8.2	0.1	8.1	0.1	0.1	0.0	1.6	0.0
	5	99.9	0.0	9.2	0.1	7.0	0.2	0.1	0.0	1.4	0.0
	10	99.9	0.0	9.0	0.0	6.2	0.1	0.1	0.0	1.2	0.0
	20	99.9	0.0	7.4	0.1	5.9	0.0	0.1	0.0	1.2	0.0
7	0	99.9	0.0	7.1	0.6	5.9	0.4	0.1	0.0	1.2	0.1
	5	100.1	0.2	6.5	0.2	5.7	0.2	0.1	0.0	1.1	0.1
	10	100.2	0.2	6.4	0.1	6.0	0.1	0.1	0.0	1.2	0.0
	20	99.9	0.0	6.1	0.1	5.9	0.2	0.1	0.0	1.2	0.0
8	0	99.9	0.0	5.9	0.1	6.0	0.1	0.1	0.0	1.2	0.0
	5	100.0	0.0	6.1	0.1	6.0	0.1	0.1	0.0	1.2	0.0
	10	100.1	0.1	5.9	0.0	6.0	0.1	0.1	0.0	1.2	0.0
	20	99.9	0.3	5.4	0.3	5.7	0.1	0.1	0.0	1.1	0.0

Table S5.2. Equilibrium conditions. One-way ANOVA testing of the difference between the concentration of ISU inside (Cin) and outside (Cout) the bag of the control units at the end of the experiment.

pH	variable	df	SS	F	p
4	**C_{in}-C_{out}**	1	0.007	0.133	0.734
	Residuals	4	0.22		
5	**C_{in}-C_{out}**	1	0.043	2.551	0.171
	Residuals	4	0.086		
6	**C_{in}-C_{out}**	1	0.034	2.251	0.231
	Residuals	4	0.045		
7	**C_{in}-C_{out}**	1	2.458	6.557	0.062
	Residuals	4	1.499		
8	**C_{in}-C_{out}**	1	0.008	0.522	0.51
	Residuals	4	0.06187		

Table S5.3. ANOVA table reporting the differences between the concentration of the compounds detected inside (Cin) and outside the dialysis bag (Cout), at the lower level of DOM (5 mg L-1 DOC), at pH 4-8.

pH	variable	df	SS	F	p
4	**C_{in}-C_{out}**	1	2.8	187.8	**<0.001**
	Residuals	4	0.06		
5	**C_{in}-C_{out}**	1	48	597.2	**<0.001**
	Residuals	4	0.32		
6	**C_{in}-C_{out}**	1	2.57	78.72	**<0.001**
	Residuals	4	0.13		
7	**C_{in}-C_{out}**	1	1.05	16.18	**<0.05**
	Residuals	4	0.26		
8	**C_{in}-C_{out}**	1	0.01	1.01	0.37
	Residuals	4	0.05		

Table S5.4. ANOVA table reporting the differences between the concentration of the compounds detected inside (Cin) and outside the dialysis bag (Cout), at the 10 mg L-1 DOC, at pH 4-8.

pH	variable	df	SS	F	p
4	**C_{in}-C_{out}**	1	5.1	148.5	**<0.001**
	Residuals	4	0.14		
5	**C_{in}-C_{out}**	1	24.36	1384	**<0.001**
	Residuals	4	0.07		
6	**C_{in}-C_{out}**	1	6.2	1244	**<0.001**
	Residuals	4	0.02		
7	**C_{in}-C_{out}**	1	0.3	13.36	**<0.05**
	Residuals	4	0.09		
8	**Cin-Cout**	1	0.01	0.39	0.57
	Residuals	4	0.02		

Table S5.5. ANOVA table reporting the differences between the concentration of the compounds detected inside (Cin) and outside the dialysis bag (Cout), at the higher level of DOM (20 mg L-1 DOC), at pH 4- 8.

pH	variable	df	SS	F	p
4	**C_{in}-C_{out}**	1	3.86	63.4	**<0.01**
	Residuals	4	0.24		
5	**C_{in}-C_{out}**	1	0.5	5.39	0.08
	Residuals	4	0.37		
6	**C_{in}-C_{out}**	1	2.51	214.1	**<0.001**
	Residuals	4	0.05		
7	**C_{in}-C_{out}**	1	0.05	1.48	0.29
	Residuals	4	0.15		
8	**C_{in}-C_{out}**	1	0.14	1.82	0.24
	Residuals	4	0.38		

Supplementary Information Chapter VI: Binding of Waterborne Pharmaceutical and Personal Care Products to Natural Dissolved Organic Matter

Simone Rizzuto [1], Didier L. Baho [2, 3], Kevin C. Jones [1*], Hao Zhang [1*], Eva Leu [4], Luca Nizzetto [3, 5]

[1] Lancaster Environment Centre, Lancaster University, Lancaster, LA1 4YQ, UK.

[2] Department of Aquatic Sciences and Assessment, Swedish University of Agricultural Sciences, Uppsala, Sweden.

[3] Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, 0349, Oslo, Norway.

[4] Akvaplan-niva, CIENS, Science Park, Gaustadalléen 21, 0349, Oslo, Norway.

[5] RECETOX, Masaryk University, Kamenice 753/5, 625 00 Brno, Czech Republic.

*corresponding authors: k.c.jones@lancaster.ac.uk; h.zhang@lancaster.ac.uk

List of Tables:

List of Figures:

List of Text:

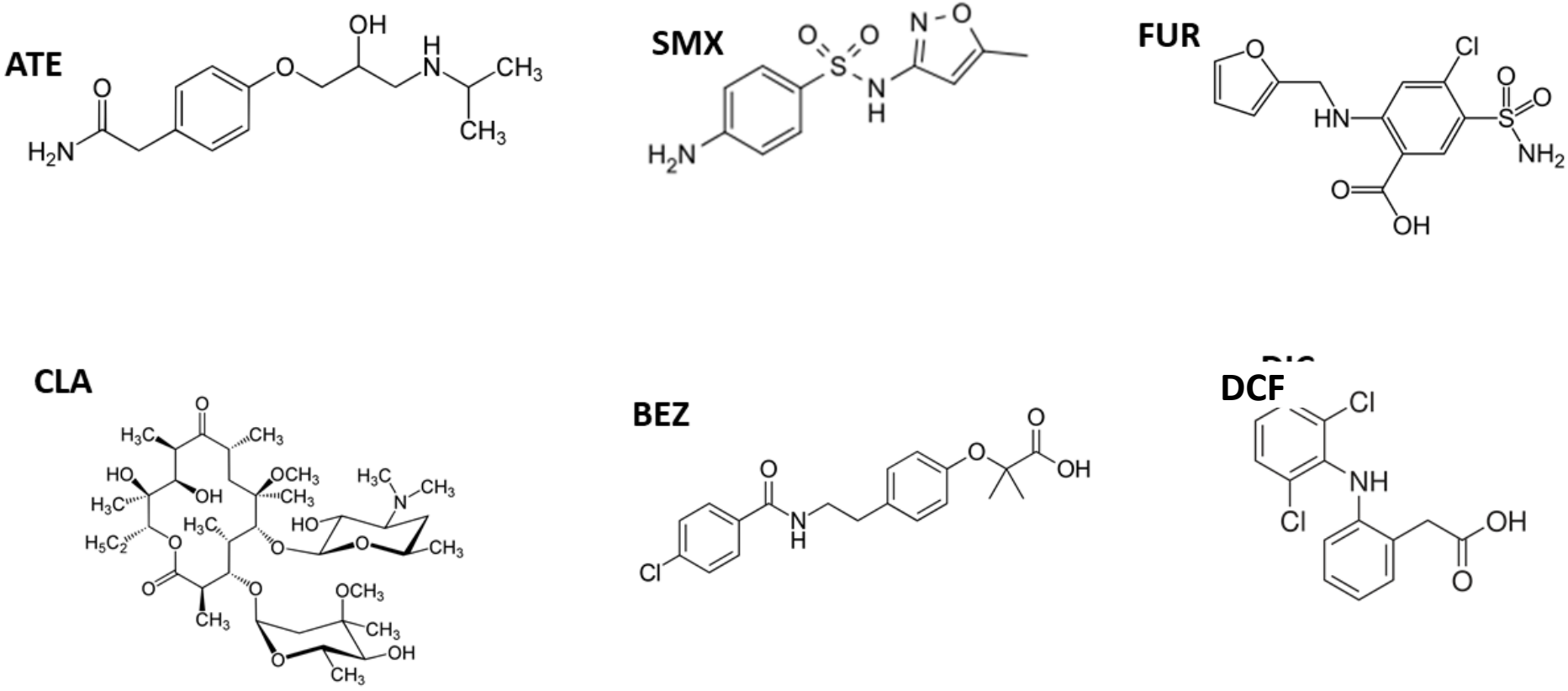


Figure S6.1. Chemical structures of the six investigated compounds.

Table S6.1. Summary data on the occurrence and concentration (ng/L) of PPCPs used in this study found in European freshwaters (lakes and rivers). The data was obtained from the Norman database. Norman is the Network of reference laboratories, research centres and related organisations for monitoring of emerging environmental substances (www.Norman-network.net). This table was modified from the paper published by Baho et al. (2019). Modified from Rizzuto et al. (2021).

Chemical	Time analyzed	Times detected	Percentage detection (%)	Min conc. (ng/L)	Max conc. (ng/L)	Mean conc. (ng/L)	standard deviation (ng/L)	Q1 conc. (ng/L)	Median conc. (ng/L)	Q3 conc. (ng/L)
Atenolol	977	723	74	0.1	900	26.3	70.7	6	11	19
Sulfamethoxazole	2616	2133	81.5	0.7	700	33.3	46	12	20	40
Furosemide	507	84	16.6	0.5	283000	9253.7	44732.1	12.25	35	76
Clarithromycin	945	730	77.2	0.9	1100	21	44.7	10	13	21
Bezafibrate	1384	764	55.2	0.3	21200	108.5	1162.7	8	13	28
Diclofenac	6320	4439	70.2	0.2	110000	785	5977.4	23	57	130

Text S6.1. Chemical analyses

PPCPs were analysed by LC-MS/MS (Shimadzu, 8040), using an XBridge BEH C18 column (2.1 mm x 100 mm, 3.5 μm, Waters) to separate the compounds. Samples were directly injected into the LC system. The mobile phases were A; 0.1% ammonium hydroxide in MQ water, and B; 50% methanol and 50% acetonitrile. The gradient procedure was optimized at: 0-1 min 15% B, increased to 90% within 5 min, then increased to 100% within 3 min, held at 100% for 3 min, after that decreased to the initial conditions (15% B) within 1 min. Finally, 6 minutes of post-run ensured re-equilibration of the column before the next injection. Acquisition parameters were both positive and negative ionization, dynamic MRM (Table S6.2). Gas temperature was 250°C, heat block temperature 400°C, drying gas flow 15 L/min, nebulizing gas flow 2 L/min. The injection volume was 15 μL and the column and the tray temperature were set to 35°C. The quantification of the compounds was based on internal standard method (Atenolol d7 for the positive ionization and Ibuprofen d3 for the negative, Sigma Aldrich); the instrument detection limit ranged between 0.56 - 0.87 ng/mL.

Table S6.2. MS/MS acquisition parameters of the investigated compounds.

Compound	ESI mode	precursor ion	product ion	retention time
ATE	(+)	267.1	145.10, 190.10	4.89
SMX	(+)	254.3	156.00, 92.15, 65.10	1.05
CLA	(+)	748.95	158.05, 591.10, 159.25	8.67
FUR	(-)	329.00	285.00, 205.00	3.57
BEZ	(-)	360.10	274.05, 154.10, 42.20	4.17
DCF	(-)	294.00	250.55, 214.00, 178.10	4.48

Text S6.2. Testing DOM's loss from dialysis bags

Other studies reported consistent loss of DOM at high concentration of DOM in the dialysis bags (Akkanen and Kukkonen, 2003, 2001). This issue recurred by using dialysis bags with 1000 Da pore size. In order to prevent this issue, we used smaller pore size (100-500 Da) and monitored on day 0, 1, 5 and 7 the loss of DOM from the dialysis bags, using a plate reader coupled with a spectrophotometer (BioTek Synergy MX; Winosky, VT, US). Triplicates of solution samples from outside the bag of each experimental unit were loaded on clear flat bottom 96 well black microplates (300 µL in each well) (Corning, US). Samples from standard DOM solutions of 5 and 15 mg L^{-1} DOC were also loaded for comparison. Absorbance wave lengths between 250-280 nm were measured, accordingly to other studies (Hagman et al., 2018). No loss was observed from either of the DOM levels over the 1-week period of the experiment (Figure S6.2).

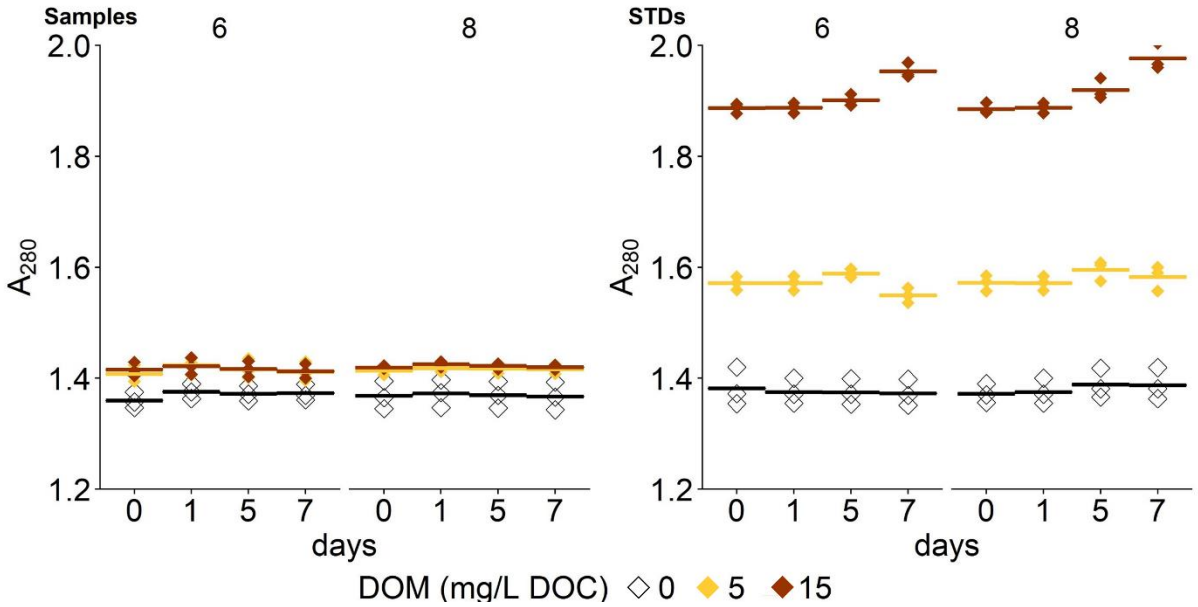


Figure S6.2. Absorbance (280 nm) of DOM levels measured on the samples and on standards on day 0, 1, 5 and 7 of the experiment, at pH 6.5 and 8.

Text S6.3. Mass recovery

The mass recovery was calculated to account for adsorption, volatilization or degradation of the compounds, as the difference in percentage between the mass expected (Table 6.1) and the mass detected in each experimental compartment.

$$Mass\ recovery\ (\%) = \left[\frac{Mass_{measured} * 100}{Mass_{expected}}\right] - 100$$

Text S6.4. Testing adsorption of PPCPs to experimental unit's components

During the experiment, more hydrophobic chemicals may adsorb to the glassware or to the bag. Hence, adsorption of the compounds to either the bag, the glassware, or other component of each experimental unit (clips, stirrer bars) was also tested. This was carried out by rinsing each experimental unit with a total amount of 10 mL of methanol. 5 mL were used to rinse the internal part of the unit (inside the bag). The other 5 mL to rinse the external part (outside the bag, internal wall of the beaker, stirrer bar and clips). The respective solvents were collected in amber vials, to be later gently dried with nitrogen stream, and re-suspended in 1 mL methanol, before being filtered and stored at -20° C in 2.5 mL amber glass vials. Chemical analyses using LC-MS/MS reported a recovery ranging from 98 – 110%.

Table S6.3. Atenolol. Table reporting the mean and standard deviation values of: percentage of mass loss compared to expected mass inside (a) and outside (b) the dialysis bag; mass recovered through adsorption inside (c) and outside (d) the bag; recovery percentages of the compound inside (e) and outside (f) the bag; total recovery percentage of the compound (g).

pH	DOM	(a) %Mass lost in		(b) %Mass lost out		(c) % A_{in}		(d) % A_{out}		(e) %Tot mass rec in		(f) %Tot mass rec out		(g) %Tot mass recovered	
		mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	Mean	sd	mean	sd
6.5	0	11.54	14.86	11.48	12.79	4.26	3.72	11.48	12.79	92.72	14.91	99.87	1.25	99.65	0.71
	5	4.83	2.03	9.95	1.93	4.67	1.80	9.95	1.93	99.83	0.54	101.25	0.85	99.99	0.03
	15	17.50	2.04	22.05	1.94	17.27	2.23	22.05	1.94	99.77	0.19	99.84	0.98	99.99	0.01
8	0	26.06	1.19	30.04	1.13	26.52	1.07	30.04	1.13	100.45	2.05	101.12	1.25	100.02	0.10
	5	25.44	10.49	29.45	9.93	18.03	13.54	29.45	9.93	92.59	19.94	99.74	0.97	99.65	0.95
	15	9.12	1.47	6.00	12.57	10.91	3.86	6.00	12.57	101.79	2.68	100.81	1.12	100.09	0.13

Table S6.4. Sulfamethoxazole. Table reporting the mean and standard deviation values of: percentage of mass loss compared to expected mass inside (a) and outside (b) the dialysis bag; mass recovered through adsorption inside (c) and outside (d) the bag; recovery percentages of the compound inside (e) and outside (f) the bag; total recovery percentage of the compound (g).

pH	DOM	(a) %Mass lost in		(b) %Mass lost out		(c) % A_{in}		(d) % A_{out}		(e) %Tot mass rec in		(f) %Tot mass rec out		(g) %Tot mass recovered	
		mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd
6.5	0	6.52	4.79	6.86	1.72	6.45	3.12	6.02	1.84	106.97	4.79	102.88	1.72	101.89	1.87
	5	7.85	2.60	2.19	2.54	8.45	2.25	7.32	2.42	108.31	2.60	102.51	2.19	102.78	2.21
	15	1.06	2.41	6.73	2.32	1.06	0.57	6.73	2.32	100.00	2.32	101.33	3.12	100.00	0.11
8	0	20.61	6.54	24.86	6.60	12.73	7.71	24.86	6.60	92.12	1.19	99.45	1.23	99.62	0.06
	5	6.76	2.93	11.72	2.83	13.64	12.86	11.72	2.83	106.87	15.46	100.64	0.98	100.33	0.74
	15	11.97	1.87	16.82	1.69	8.98	5.73	16.82	1.69	97.02	7.39	101.16	1.12	99.86	0.35

Table S6.5. Furosemide. Table reporting the mean and standard deviation values of: percentage of mass loss compared to expected mass inside (a) and outside (b) the dialysis bag; mass recovered through adsorption inside (c) and outside (d) the bag; recovery percentages of the compound inside (e) and outside (f) the bag; total recovery percentage of the compound (g).

pH	DOM	(a) %Mass lost in		(b) %Mass lost out		(c) % A_{in}		(d) % A_{out}		(e) %Tot mass rec in		(f) %Tot mass rec out		(g) %Tot mass recovered	
		mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd
6.5	0	19.24	2.30	26.86	4.86	18.21	2.22	26.86	4.86	101.97	1.12	100.00	1.01	100.57	1.48
	5	19.45	6.51	26.47	0.50	18.45	3.32	23.93	3.13	99.90	3.51	102.40	2.63	101.09	3.04
	15	19.12	6.41	26.35	6.80	20.45	6.00	24.02	6.30	98.57	3.41	102.37	1.80	101.76	2.26
8	0	-20.77	8.69	24.53	5.13	21.45	5.99	24.02	5.89	125.22	3.69	95.55	4.13	101.68	3.22
	5	22.96	6.78	23.31	7.03	20.45	3.88	22.60	4.96	102.42	3.78	103.91	4.57	103.75	3.34
	15	24.65	5.80	24.05	7.39	24.09	3.66	24.05	7.39	99.44	2.14	100.00	3.28	99.97	0.10

Table S6.6. Clarithromycin. Table reporting the mean and standard deviation values of: percentage of mass loss compared to expected mass inside (a) and outside (b) the dialysis bag; mass recovered through adsorption inside (c) and outside (d) the bag; recovery percentages of the compound inside (e) and outside (f) the bag; total recovery percentage of the compound (g).

pH	DOM	(a) %Mass lost in		(b) %Mass lost out		(c) % A_{in}		(d) % A_{out}		(e) %Tot mass rec in		(f) %Tot mass rec out		(g) %Tot mass recovered	
		mean	**sd**	**mean**	**sd**	**mean**	**sd**	**mean**	**sd**	**mean**	**sd**	**mean**	**sd**	**mean**	**sd**
7	**0**	32.66	0.42	32.41	0.83	33.12	0.75	32.18	0.79	100.46	0.52	101.20	0.80	100.02	0.02
	5	32.35	0.20	32.94	0.04	27.27	0.00	32.64	0.06	94.92	0.20	99.88	0.05	99.76	0.01
	15	32.35	0.26	33.05	0.28	30.92	1.00	34.05	0.34	98.57	0.77	98.12	0.38	99.93	0.04
8	**0**	70.42	0.44	67.99	2.17	64.18	4.39	66.16	2.16	93.75	4.76	99.14	2.15	99.70	0.23
	5	66.57	0.08	62.37	6.01	61.60	3.55	62.34	5.98	95.04	3.51	99.97	4.89	99.76	0.17
	15	66.76	0.14	57.12	12.65	66.67	2.14	59.16	11.65	99.90	2.11	102.16	12.16	100.00	0.10

Table S6.7. Bezafibrate. Table reporting the mean and standard deviation values of: percentage of mass loss compared to expected mass inside (a) and outside (b) the dialysis bag; mass recovered through adsorption inside (c) and outside (d) the bag; recovery percentages of the compound inside (e) and outside (f) the bag; total recovery percentage of the compound (g).

pH	DOM	(a) %Mass lost in		(b) %Mass lost out		(c) % A_{in}		(d) % A_{out}		(e) %Tot mass rec in		(f) %Tot mass rec out		(g) %Tot mass recovered	
		mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd
6.5	0	8.32	18.84	11.82	18.90	12.23	15.34	11.64	15.39	103.92	5.54	102.82	4.51	102.87	4.56
	5	3.78	2.65	37.45	3.12	8.86	6.56	37.12	3.64	105.09	7.66	99.67	0.52	99.92	0.85
	15	16.29	2.31	34.29	2.71	14.09	2.95	34.17	1.21	97.80	4.33	99.88	1.51	99.78	1.29
8	0	24.89	0.63	34.79	13.29	23.60	1.29	35.08	13.46	99.71	1.82	100.29	0.19	100.21	0.25
	5	-85.95	39.95	56.00	8.76	28.05	0.02	34.24	23.96	186.00	39.95	98.24	5.22	98.85	3.67
	15	-39.66	13.20	60.17	4.65	25.05	0.03	33.95	4.82	139.70	13.20	97.79	0.17	99.78	0.56

Table S6.8. Diclofenac. Table reporting the mean and standard deviation values of: percentage of mass loss compared to expected mass inside (a) and outside (b) the dialysis bag; mass recovered through adsorption inside (c) and outside (d) the bag; recovery percentages of the compound inside (e) and outside (f) the bag; total recovery percentage of the compound (g).

pH	DOM	(a) %Mass lost in		(b) %Mass lost out		(c) % A_{in}		(d) % A_{out}		(e) %Tot mass rec in		(f) %Tot mass rec out		(g) %Tot mass recovered	
		mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd
6.5	0	30.23	10.49	31.72	8.55	27.50	3.48	33.25	9.25	97.27	10.49	101.53	1.22	100.85	1.01
	5	17.46	5.77	33.31	9.21	19.17	5.89	34.58	9.26	101.70	0.49	101.27	0.17	101.29	0.16
	15	26.54	5.14	30.65	8.19	26.67	5.89	31.17	9.47	100.13	0.88	100.52	1.34	100.50	1.31
8	0	8.26	14.83	16.96	1.57	18.17	0.94	16.79	1.59	109.91	14.25	99.83	0.03	100.31	0.71
	5	-51.60	16.10	27.88	1.67	17.52	0.02	17.96	1.66	169.10	16.10	100.08	0.07	103.37	0.70
	15	-24.58	8.96	33.49	1.20	18.40	0.05	13.58	1.25	142.08	8.96	100.09	0.05	102.09	0.47

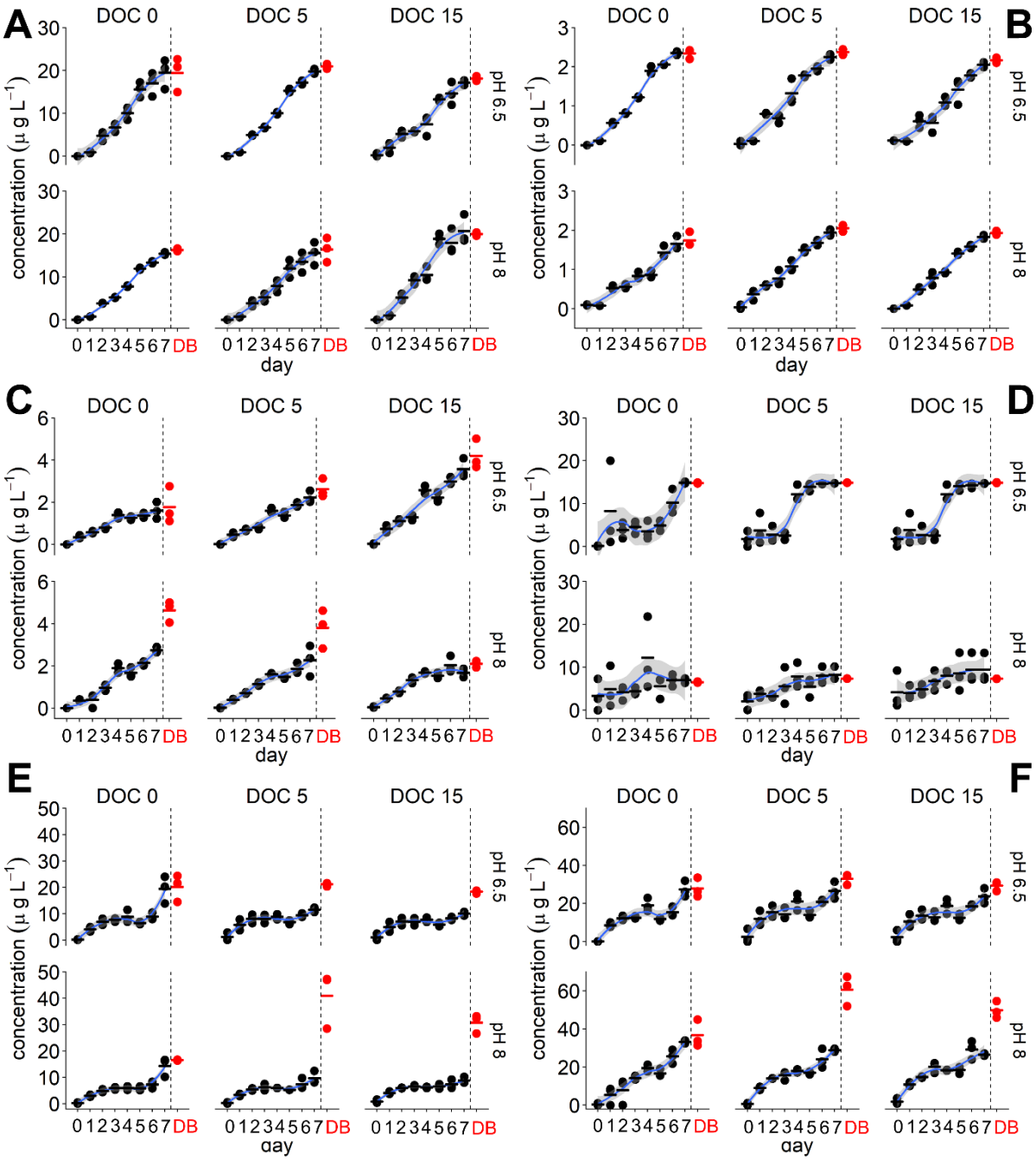


Figure S6.3. Daily concentrations (µg L-1) of (A) Atenolol, (B) Sulfamethoxazole, (C) Furosemide, (D) Clarithromycin, (E) Bezafibrate, and (F) Diclofenac outside the bag during the 7 days experiment, and inside the bag on the 7th day (DB) at 3 levels of DOM (0, 5, 15 mg L-1 DOC) and 2 levels of water pH (6.5, 8).

Table S6.9. Summary table of the 6 investigated compounds reporting mean and standard deviation values of; Total Recovery (%), concentration detected inside the bag (Cin), concentration detected outside the bag (Cout), the mass detected inside the bag (Mdet/in), and the mass detected outside the bag (Mdet/out) on the last day of the experiment, at the three levels of DOM (0, 5 and 15 mg L-1 DOC) and two levels of pH (6.5, 8). Mexp/in (µg) was 0.022 for SMX and FUR, 0.22 for ATE, CLA and BEZ, and 0.44 for DCF. Mexp/out (µg) was 0.44 for SMX and FUR, 4.4 for ATE, CH and BEZ, and 8.8 for DCF

Chem	pH	DOM (mg L^{-1} DOC)	Tot Recovery %		C_{in} (µg L^{-1})		C_{out} (µg L^{-1})		$M_{D/in}$ (µg)		$M_{D/out}$ (µg)	
			mean	sd	mean	sd	mean	sd	mean	sd	mean	sd
ATE	6.5	0	99.65	0.71	19.46	3.27	19.47	2.81	0.19	0.03	3.89	0.56
		5	99.99	0.03	20.94	0.45	19.81	0.42	0.21	0.00	3.96	0.08
		15	99.99	0.01	18.15	0.45	17.15	0.43	0.18	0.00	3.43	0.09
	8	0	100.02	0.10	16.27	0.26	15.39	0.25	0.16	0.00	3.08	0.05
		5	99.65	0.95	16.40	2.31	15.52	2.19	0.16	0.02	3.10	0.44
		15	100.09	0.13	19.99	0.32	20.68	2.77	0.20	0.00	4.14	0.55
SMX	6.5	0	106.89	1.87	2.34	0.11	2.35	0.04	0.02	0.00	0.47	0.01
		5	102.78	2.21	2.37	0.06	2.25	0.06	0.02	0.00	0.45	0.01
		15	100.00	0.11	2.18	0.05	2.05	0.05	0.02	0.00	0.41	0.01
	8	0	99.62	0.06	1.75	0.14	1.65	0.15	0.02	0.00	0.33	0.03
		5	100.33	0.74	2.05	0.06	1.94	0.06	0.02	0.00	0.39	0.01
		15	99.86	0.35	1.94	0.04	1.83	0.04	0.02	0.00	0.37	0.01
FUR	6.5	0	100.57	1.48	1.78	0.71	1.61	0.33	0.02	0.01	0.32	0.07
		5	106.09	8.04	2.63	0.36	2.23	0.23	0.03	0.00	0.45	0.05
		15	163.76	17.26	4.20	0.58	3.57	0.37	0.04	0.01	0.71	0.07
	8	0	128.68	5.22	4.64	0.41	2.74	0.11	0.05	0.00	0.55	0.02
		5	116.75	15.34	3.81	0.74	2.27	0.59	0.04	0.01	0.45	0.12
		15	99.97	0.10	2.10	0.13	1.67	0.16	0.02	0.00	0.33	0.03
CLA	6.5	0	100.02	0.02	14.82	0.09	14.87	0.18	0.15	0.00	2.97	0.04
		5	99.76	0.01	14.88	0.04	14.75	0.01	0.15	0.00	2.95	0.00
		15	99.93	0.04	14.88	0.06	14.73	0.06	0.15	0.00	2.95	0.01
	8	0	99.70	0.23	6.51	0.10	7.04	0.48	0.07	0.00	1.41	0.10
		5	99.76	0.17	7.36	0.02	8.28	1.32	0.07	0.00	1.66	0.26
		15	100.00	0.10	7.31	0.03	9.43	2.78	0.07	0.00	1.89	0.56
BEZ	6.5	0	102.87	4.56	20.17	4.14	19.40	4.16	0.20	0.04	3.88	0.83
		5	99.92	0.85	21.17	0.58	11.56	0.69	0.21	0.01	2.31	0.14
		15	99.78	1.29	18.42	0.51	10.06	0.60	0.18	0.01	2.01	0.12
	8	0	100.21	0.25	16.52	0.14	14.35	2.92	0.17	0.00	2.87	0.58
		5	93.85	13.67	40.91	8.79	9.68	1.93	0.41	0.09	1.94	0.39
		15	99.78	0.56	30.72	2.90	8.76	1.02	0.31	0.03	1.75	0.20
DCF	6.5	0	100.85	1.01	27.91	4.19	27.31	3.42	0.28	0.04	5.46	0.68
		5	101.29	0.16	33.02	2.31	26.67	3.68	0.33	0.02	5.33	0.74
		15	100.50	1.31	29.38	2.06	23.74	3.28	0.29	0.02	4.75	0.66
	8	0	100.31	0.71	36.70	5.93	33.22	0.63	0.37	0.06	6.64	0.13
		5	103.37	0.70	60.64	6.44	28.85	0.67	0.61	0.06	5.77	0.13

		15	102.09	0.47	49.83	3.58	26.60	0.48	0.50	0.04	5.32	0.10

Table S6.10. Equilibrium conditions. One-way ANOVA testing of the difference between the concentration of PPCPs inside (Cin) and outside (Cout) the bag of the control units at the end of the experiment. df; degree of freedom, F; F-statistic, SS; sum of squares, p; p-value.

PPCPs	variable	pH	df	SS	F	p
ATE	**C_{in} - C_{out}**	**6.5**	1	0.0002	0.00014	0.99
	Residuals		4	55.81		
	C_{in} - C_{out}	**8**	1	1.14	11.71	0.45
	Residuals		4	0.34		
SMX	**C_{in} - C_{out}**	**6.5**	1	0.0001	0.016	0.91
	Residuals		4	0.03	0.01	
	C_{in} - C_{out}	**8**	1	0.01	0.39	0.56
	Residuals		4	0.13		
FUR	**C_{in} - C_{out}**	**6.5**	1	0.04	0.09	0.77
	Residuals		4	1.83		
	C_{in} - C_{out}	**8**	**1**	**5.4**	**39.62**	**<0.05**
	Residuals		**4**	**0.54**		
CLA	**C_{in} - C_{out}**	**6.5**	1	0.004	0.15	0.72
	Residuals		4	0.13		
	C_{in} - C_{out}	**8**	1	0.43	2.4	0.2
	Residuals		4	0.71		
BEZ	**C_{in} - C_{out}**	**6.5**	1	0.89	0.03	0.86
	Residuals		4	103.4		
	C_{in} - C_{out}	**8**	1	7.12	1.11	0.35
	Residuals		4	25.69		
DCF	**C_{in} - C_{out}**	**6.5**	1	0.53	0.02	0.88
	Residuals		4	87.9		
	C_{in} - C_{out}	**8**	1	18.19	0.68	0.46
	Residuals		4	106.8	26.7	

Table S6.11. ANOVA table reporting the differences between the concentration of the compounds detected inside (Cin) and outside the dialysis bag (Cout), at the lower level of DOM (5 mg L-1 DOC), at pH 6.5 and 8.

PPCPs	variable	pH	df	SS	F	p
ATE	**C_{in} - C_{out}**	**6.5**	1	1.89	6.59	0.06
	Residuals		4	1.15		
	C_{in} - C_{out}	**8**	1	1.16	0.15	0.72
	Residuals		4	30.32		
SMX	**C_{in} - C_{out}**	**6.5**	1	0.02	4.9	0.09
	Residuals		4	0.02		
	C_{in} - C_{out}	**8**	1	0.02	2.96	0.16
	Residuals		4	0.02		
FUR	**C_{in} - C_{out}**	**6.5**	1	0.23	1.69	0.26
	Residuals		4	0.56		
CLA	**C_{in} - C_{out}**	**6.5**	1	0.11	1.88	0.24
	Residuals		4	0.24		
BEZ	**C_{in} - C_{out}**	**6.5**	**1**	**138.5**	**227.9**	**<0.001**
	Residuals		**4**	**2.43**		
	C_{in} - C_{out}	**8**	**1**	**1426.9**	**24.09**	**<0.01**
	Residuals		**4**	**242.9**		
DCF	**C_{in} - C_{out}**	**6.5**	1	60.31	4.26	0.11
	Residuals		4	56.69		
	C_{in} - C_{out}	**8**	**1**	**1516**	**48.24**	**<0.01**
	Residuals		**4**	**125.7**		

Table S6.12. ANOVA table reporting the differences between the concentration of the compounds detected inside (Cin) and outside the dialysis bag (Cout), at the higher level of DOM (15 mg L-1 DOC), at pH 6.5 and 8.

PPCPs	variable	pH	df	SS	F	p
ATE	**C_{in} - C_{out}**	**6.5**	1	1.42	4.9	0.09
	Residuals		4	1.16		
	C_{in} - C_{out}	**8**	1	0.72	0.12	0.74
	Residuals		4	23.27		
SMX	**C_{in} - C_{out}**	**6.5**	1	0.02	4.91	0.09
	Residuals		4	0.02		
	C_{in} - C_{out}	**8**	1	0.02	7.34	0.06
	Residuals		4	0.01		
FUR	**C_{in} - C_{out}**	**6.5**	1	0.6	1.69	0.26
	Residuals		4	1.42		
CLA	**C_{in} - C_{out}**	**6.5**	1	0.04	6.79	0.06
	Residuals		4	0.02		
BEZ	**C_{in} - C_{out}**	**6.5**	**1**	**104.83**	**227.9**	**<0.001**
	Residuals		**4**	**1.84**		
	C_{in} - C_{out}	**8**	**1**	**723.4**	**101.8**	**<0.001**
	Residuals		**4**	**28.4**		
DCF	**C_{in} - C_{out}**	**6.5**	1	47.77	4.26	0.11
	Residuals		4	44.9		
	C_{in} - C_{out}	**8**	**1**	**809.2**	**82.53**	**<0.001**
	Residuals		**4**	**39.2**		

Table S6.13. Log conditional distribution coefficient (log KDOC), and percentage of bound compound to DOM (BDOM) for the two PPCPs with binding to DOM, at two different levels of DOM (5, 15 mg L-1 DOC), and two water pH (6.5, 8).

PPCP	pH	DOM (mg L^{-1} DOC)	$logK_{DOC}$	B_{DOM}
BEZ	6.5	5	2.22	45
		15	1.74	45
	8	5	2.81	76
		15	2.22	71
DCF	8	5	2.34	52
		15	1.76	46

Appendix – Published Papers