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Understanding regime shifts in shallow lakes subject to multiple stresses : Individual and combined effects of agricultural runoff and climate warming on submerged macrophytes

Vinita Vijayaraj

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Understanding regime shifts in shallow lakes subject to multiple stresses: Individual and combined effects of agricultural runoff and climate warming on submerged macrophytes

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*Dedicated to freshwater ecology
and all fascinating hubs of
biodiversity*

Acknowledgments

A PhD is not just about performing complex experiments (that sometimes never work) or working hard to get those interesting results (that you don't always have). It isn't just about figuring out how to do complicated statistical analyses or about writing a long literature review. It is also and maybe much more about personal growth. A PhD shows you what your weaknesses are and forces you to either better them or come to terms with them. It shows you your strengths and teaches you to make use of them. It guides you when you are lost and looking for directions, and it humbles you when you make mistakes. Most of all, it shows you that it doesn't take only intelligence to do a PhD. It takes a lot of motivation and a crazy amount of support.

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Scientific publications and communication

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Allen, J., Gross, E. M., Courcou, C., Bouletreau, S., Compin, A., Elger, A., Ferriol, J., Hilt, S., Jassey, V., Laviale, M., Polst, B. H., Schmitt-Jansen, M., Stibor, H., **Vijayaraj, V.**, & Leflaive, J. (2021). Disentangling the direct and indirect effects of agricultural runoff on freshwater ecosystems subject to global warming: A microcosm study. *Water Research*, 190, 116713. <https://doi.org/10.1016/j.watres.2020.116713>

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Polst, B. H., Hilt, S., Stibor, H., Hölker, F., **Vijayaraj, V.**, Kipferler, N., Leflaive, J., Gross, E. M., Schmitt-Jansen, M. Warming lowers critical thresholds for multiple stressor–induced shifts between aquatic primary producers. Submitted to *STOTEN*.

Conference presentations:

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Gross, E. M., **Vijayaraj, V.**, Amoussou, N., Kipferler, N., Allen, J., et al. A question of complexity: Investigating regime shifts caused by agricultural run-off and climate warming between aquatic plants and microalgae in micro- and mesocosms. SETAC SciCON, SETAC.org, May 2020, Dublin, Ireland

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Posters:

Gross, E. M., **Vijayaraj, V.**, Allen, J., Polst, B. H., Kipferler, N. et al. Regime shifts in freshwater ecosystems exposed to multiple stressors by increasing temperature, fertilizers and pesticides. SETAC Helsinki, May 2019, Helsinki, Finland

Gross, E. M., **Vijayaraj, V.**, Leflaive, J., Polst, B. H., Allen, J., et al. Impact of agricultural run-off and climate warming on shallow aquatic ecosystems—Possible effects on a regime shift between aquatic primary producers. SETAC Helsinki, May 2019, Helsinki, Finland.

Abstract in English

Submerged macrophytes stabilize the clear-water status in shallow lakes, supporting many ecosystem services. Shallow lakes are exposed to agricultural runoff (ARO) containing pesticides and nutrients, which can affect macrophytes and threaten the clear-water state. Recent studies show that nitrate may affect macrophyte growth and species richness. Pesticides can also affect macrophytes directly or indirectly by disrupting biotic interactions. Climate warming is an additional stress to shallow lakes. Increased temperatures may promote phytoplankton growth and nutrient release or can modify top-down control. The combined stressors may have additive, synergistic or reversed effects, and these effects are difficult to predict. The objectives of this thesis were to investigate (1) the individual and combined effects of nitrate, pesticides and climate warming on macrophytes; (2) whether upscaling from micro- to mesocosms would preserve the response of macrophytes; and (3) whether the stressor effects could be detected in the macrophyte physiology.

Several experiments integrating different functional groups (macrophytes, phytoplankton, periphyton and their respective consumers) were performed under controlled conditions (laboratory microcosms). These ecosystem models were exposed for several weeks to a simplified cocktail simulating an ARO (nitrates alone or mixed with 4 pesticides) at two temperatures (ambient and +4°C). Following this, more realistic ecosystems were studied using outdoor mesocosms exposed to ARO and simulating the same type of heating (semi-controlled conditions). In all these experiments, the response of the macrophytes was documented by measuring their growth (biomass, size) and physiology (photosynthetic pigments, phenolic compounds, anthocyanins, elemental stoichiometry).

Results from the microcosms showed that all functional groups were affected directly or indirectly by the tested stress factors. Pesticides alone but not nitrate reduced macrophyte biomass. Nitrate and pesticides in combination acted synergistically reducing the resilience of the macrophyte-dominated state. Warming marginally promoted macrophyte growth but together with ARO reduced macrophyte growth (objective 1). Results from the mesocosms showed that the strong negative effect of ARO on the growth of macrophytes was preserved in the outdoor mesocosms but only at ambient temperature. Warming enhanced macrophyte growth, similar to the microcosms, but also significantly buffered the ARO effect, highlighting different responses compared with the microcosms (objective 2). Complex biotic interactions along with invasions of invertebrate larvae may have

reversed responses to the stressors. Besides their effects on growth, ARO and warming had a significant effect on the content of carotenoids, phenolic compounds and stoichiometry. These results need to be confirmed to determine their relevance as early warning signals of stress (objective 3).

This thesis shows that the exposure of shallow lakes to ARO can induce regime shifts by tipping the system towards a degraded state dominated by phytoplankton. In particular, the role of nitrate, which is much less studied compared to phosphorus, is clearly highlighted. Despite the positive effects of warming on macrophyte growth, the three stressors combined (pesticides, nitrates and warming) reversed the expected effect and led to a sharp decline in macrophyte biomass. The change in scale achieved confirms the need to better take into account the complexity of biotic interactions for a more reliable assessment of the environmental risks to which aquatic ecosystems are exposed.

Résumé en français

Les macrophytes immergés stabilisent les lacs peu profonds dans un état d'eau claire, leur permettant ainsi de fournir de nombreux services écosystémiques. Ce régime écologique ordinairement stable est menacé par l'apport d'eaux de ruissellement agricole (RA) contenant des pesticides et des nutriments pouvant affecter les macrophytes directement ou indirectement en perturbant les interactions biotiques. Combinés à une augmentation de la température de l'eau sous l'effet du changement climatique, les effets de ces facteurs de stress sur les écosystèmes aquatiques sont difficiles à prédire. Dans ce contexte, les objectifs de cette thèse étaient de développer une approche expérimentale pour 1) étudier les effets individuels et combinés des nitrates, des pesticides et du réchauffement climatique sur la réponse des macrophytes ; 2) comparer ces réponses dans le cadre d'expérimentations de complexité croissante (changement d'échelle) et 3) identifier des biomarqueurs physiologiques témoin de ces effets.

Pour ce faire, plusieurs expérimentations intégrant différents groupes fonctionnels (macrophytes, phytoplancton, périphyton et leur consommateurs respectifs) ont été réalisées en conditions contrôlées (microcosmes au laboratoire). Ces écosystèmes simplifiés ont été exposés plusieurs semaines à un cocktail simplifié simulant un RA (nitrates seuls ou en mélange avec 4 pesticides), à deux températures (ambiante et +4°C). Puis des écosystèmes plus réalistes ont été étudiés en utilisant des mésocosmes placés en extérieur qui ont été exposés au RA tout en simulant le même type de réchauffement (conditions semi-contrôlées). Dans toutes ces expériences, la réponse des macrophytes a pu être documentée du point de vue de leur croissance (biomasse, taille) et de leur physiologie (pigments photosynthétiques, composés phénoliques, anthocyanes, composition élémentaire).

Nos résultats obtenus en microcosmes montrent que tous les groupes fonctionnels ont été affectés par les facteurs de stress testés. Les pesticides seuls ont affecté la biomasse des macrophytes, et de manière plus forte en présence de nitrates (effets synergiques), tandis que les nitrates seuls n'ont pas eu d'effet significatif. La croissance des macrophytes a été marginalement favorisée par le réchauffement en l'absence de RA, tandis que l'effet inverse a été observé en présence de RA, mettant ainsi en évidence ainsi des effets indirects liés aux interactions biotiques entre groupes fonctionnels (objectif 1). Nos résultats obtenus en mésocosmes montrent un fort effet négatif du RA sur la croissance des macrophytes, mais uniquement à température ambiante. A température plus

élevée, cet effet négatif a été compensée par un effet positif du réchauffement (effets antagonistes), mettant ainsi en évidence des réponses différentes par rapport à ceux obtenus dans des conditions plus simples (objectif 2). Outre leurs effets sur la croissance, le RA et le réchauffement ont eu un effet significatif sur le contenu en caroténoïdes, en composés phénoliques ainsi que sur la stœchiométrie. Ces résultats nécessitent d'être confirmés pour déterminer leur pertinence en tant que signaux d'alerte précoce de stress (objectif 3).

Cette thèse confirme que l'exposition des lacs peu profonds aux RA peut induire un changement de régime en faisant basculer le système vers un état dégradé dominé par le phytoplancton. En particulier, le rôle du nitrate, jusqu'ici peu étudié par rapport au phosphore, est clairement mis en évidence. Malgré les effets positifs du réchauffement sur la croissance des macrophytes, les trois facteurs de stress combinés (pesticides, nitrates et réchauffement) ont inversé l'effet attendu et entraîné une forte baisse de la biomasse des macrophytes. Le changement d'échelle réalisé confirme la nécessité de mieux prendre en compte la complexité des interactions biotiques pour une évaluation plus fiable des risques environnementaux auxquels les écosystèmes aquatiques sont soumis.

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Introduction

Water is an essential environmental resource. Humans depend on sources such as freshwater lakes and seas for drinking water, for nutrition and recreation, and for environmental services such as climate control and waste management. All other terrestrial and aquatic organisms also rely on water for hydration and nutrition, and aquatic organisms for habitat. Among these sources, freshwater systems comprise only about 3% of earth's water, out of which half is present in the form of ice, and only half actually available for human use, emphasizing the importance of preserving them. Since the agricultural and industrial revolutions, the widespread use of pesticides and fertilizers has led to the chemical contamination of freshwater systems through runoff from these sites. Chemical pollution is not the sole type of stress that these systems are exposed to. Since the pre-industrial period, when humans began their quest to harness energy through the burning of fossil fuels, the rise in greenhouse gases led to an increase in global average temperatures by about 1°C. This temperature is currently estimated to increase by about 0.2°C per decade (PNAS, n.d.). The effects of both these local and global stressors in the form of chemicals and warming may have as yet unknown but far-reaching consequences for the status of these aquatic systems.

Shallow lakes are a hub of biodiversity and the most abundant type of freshwater systems on earth, providing a variety of ecosystem, ecological and environmental services. They are usually present in fertile lowland areas close to agricultural sites and exposed to multiple environmental stressors, including runoff containing fertilizers and pesticides as well as elevated temperatures due to global warming. The exposure of shallow lakes to multiple environmental stressors places them at risk for a degradation of their status. The shallow lake clear-water state is stabilized by aquatic plants (macrophytes) and other key biotic interactions, and provides the numerous services that are important for humans and all other life. A loss of macrophytes through direct or indirect stressor effects may lead to a shift from the clear-water state to a degraded state dominated by phytoplankton, reducing the quality and number of services that they provide.

Agricultural runoff containing nutrients and pesticides may affect macrophytes directly or indirectly by modifying biotic interactions or abiotic conditions. Along with rising environmental temperatures through climate warming, their combined effect may be synergistic (an effect stronger than the addition of the individual effects), additive (an effect equal to the addition of individual effects), antagonistic (an effect lower than the

addition of the individual stressors) or reversed (an effect in a direction opposite of the individual stressor additions). These effects are challenging to predict but are still possible with the use of well-defined ecosystem models. Identifying threshold concentrations of the chemicals in the context of rising environmental temperatures may provide a foundation for policy makers to define safe operating spaces for shallow lakes.

The focus of this thesis is the effect of agricultural runoff and climate warming on macrophytes and what this may mean for the status of shallow lakes. The thesis is divided into six chapters. This first chapter begins with the state of the art, describing shallow lake ecology and the multiple stressors affecting it. A short discussion of tipping points for regime shifts followed by the potential of macrophyte biomarkers as indicators of shallow lake status is provided. At the end of this chapter, the objectives of the project CLIMSHIFT, followed by the aims and objectives of this thesis, which are based within the framework of CLIMSHIFT are presented.

In the second chapter, an outline of the approach, including the design of microcosms and mesocosms as part of the project CLIMSHIFT, and the general methodology adopted to study the main objectives of this thesis, is provided.

In the third to fifth chapters, I present the main results of my study. The third chapter focuses on the individual and combined stressor effects on macrophyte growth, and the fourth chapter on the challenges of upscaling ecosystem models from the laboratory to outdoor systems. The fifth chapter focuses on the effects of the stressors on macrophyte physiology. The third and fourth chapters include two of my papers that are published in international scientific journals (Chapter 3: *Water Research*, Chapter 4: *Water*). The fifth chapter provides results on which a third publication will be based.

The sixth chapter is the general discussion, beginning with an outlook of our approach followed by an integration and evaluation of all results.

Supplementary data containing two additional papers that I co-authored as part of CLIMSHIFT, as well as a summary of my thesis in French are provided at the end of this document.

1. Shallow lake ecology

A shallow lake has a depth small enough to allow sufficient sunlight to reach the lake bottom. These lakes are typically characterised by a large surface-to-volume ratio. Most shallow lakes have a mean depth of up to 3 m, although some may be deeper. At these low depths, constant mixing of water creates relatively homogeneous water bodies, rather than stratified layers with different light, temperature and nutritional environments typical of deeper lakes (Lemmons, 2021; Wetzel, 2001). Because of the availability of sufficient sunlight, primary productivity tends to be high in shallow lakes compared to deep lakes (Nixdorf & Deneke, 1997). In addition, the exchange of energy and nutrients between the sediment and water column promotes a higher coupling between the benthic (lake bottom) and pelagic (open water) zones (Cooke et al., 2001).

Shallow lakes may exist in one of two equilibrium states: a clear water state dominated by macrophytes or a turbid state dominated by phytoplankton (pelagic algae) (Mitchell, 1989). The specific regime of the lake at any given time depends on several biotic and abiotic factors. Although both states may occur in nature, the clear water state dominated by macrophytes is considered the more desired state in terms of ecological and ecosystem services, as it supports a larger biodiversity, and is maintained by stabilizing negative feedback loops (Janssen et al., 2021; Scheffer & Jeppesen, 2007). However, under certain circumstances, a shift may occur from this desirable clear-water state to the degraded turbid state, which is amplified by positive feedbacks, commonly triggered by loss of keystone species populations or an overamplification of other species (Scheffer et al., 1993; Virginia & Wall, 2013). This shift to a phytoplankton-dominated state is considered a degraded state, as the ecological, environmental and social services provided by the lakes are largely diminished (Janssen et al., 2021; Scheffer et al., 1993).

Macrophytes, which include angiosperms, mosses, ferns and macroalgae, perform numerous functions that enable them to maintain clear-water conditions in shallow lakes. They maintain low nutrient levels in the water column by sequestering excess nutrients (Barko & James, 1998; Tang et al., 2017). By limiting water nutrient content, they can control the growth of both phytoplankton (microalgae developing in the water column) and periphyton (microalgae developing on sediment and other surfaces)/epiphyton (microalgae developing on plants), which they compete with for resources. Further, they

play a strong structuring role by allowing phytoplankton feeders to take refuge from predators and produce allelopathic chemicals, which inhibit phytoplankton growth (Hilt & Gross, 2008; van Donk & van de Bund, 2002). Submerged rooted macrophytes prevent sediment resuspension otherwise caused by fish and wave movement. While macrophytes are regarded to have a central role in maintaining clear water conditions in shallow lakes, other organisms directly or indirectly support macrophyte growth and help in stabilizing this clear-water state.

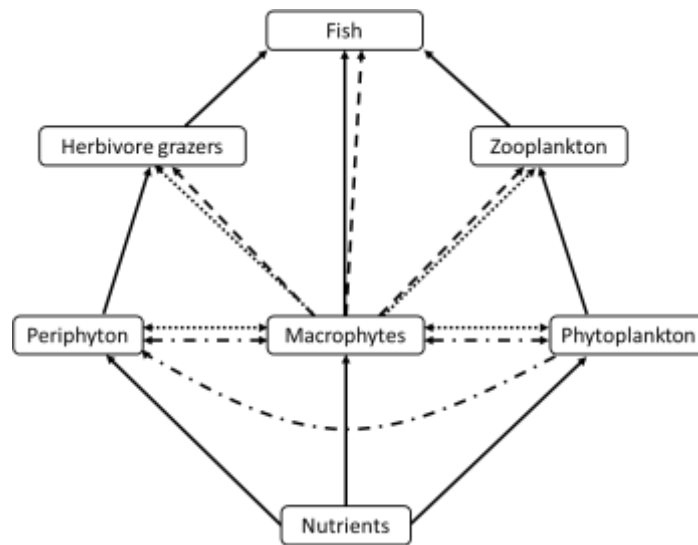


Fig. 1.1. Interactions between key functional groups of an aquatic food web. Solid lines = food web links, dotted lines = allelopathy, dashed lines = spatial refuge, dashed and dotted lines = competition for light and nutrients. Modified from van Donk and van de Bund (2002).

Shallow lake communities are structured based on several biotic interactions between different functional groups. Macrophytes compete with phytoplankton and periphyton/epiphyton for nutrients and light. Competition among primary producers is influenced by resource availability. The presence of top-down grazers also influence this competition, as outlined by (Phillips et al., 2016). These include, for example, snail grazing on periphyton/epiphyton or *Daphnia* grazing on phytoplankton (Jones et al., 2002; Moreno-Ostos et al., 2007) and predation (e.g., consumption of invertebrate grazers by fish) (De Bernardi, 1981) (see Fig. 1.1). Some studies show that periphyton react even more strongly than phytoplankton to nutrient additions (Sand-Jensen & Søndergaard, 1981). Some suggest that they are controlled more by fish (Mazumder et al., 1989) or snails (Jones et al., 2002) than by nutrients. Similar observations were made for phytoplankton, with some showing a stronger nutrient control (Zhang et al., 2007) and others a stronger zooplankton control (Severiano et al., 2012). Consumers can also affect the development

of microalgae indirectly through nutrient cycling (excretion). The rate of recycling will depend on the stoichiometry of consumers as well as that of the food source. For example, if their food source has nutrient concentrations much higher than required by the consumers, they will excrete higher concentrations of these nutrients back into the water (DeMott et al., 1998; Elser et al., 1988; Elser & Urabe, 1999; Grimm, 1988; Urabe, 1993). All of these factors will define the trophic structure of the system.

The implications of such biotic interactions is that shallow lake ecosystems are interconnected. Although two species may not directly interact, they can influence each other indirectly “through a series of intermediaries” (Preston, 2002). For example, fish may not directly interact with periphyton, but by controlling herbivore grazers, they can promote periphyton growth (Fig. 1.1). The same principal can apply to environmental stressors. If a stressor affects a certain species, a restructuring of the community and ecosystem may result from indirect consequences on biotic interactions (Fig. 1.2). These ecosystem effects may be more pronounced when keystone species are affected.

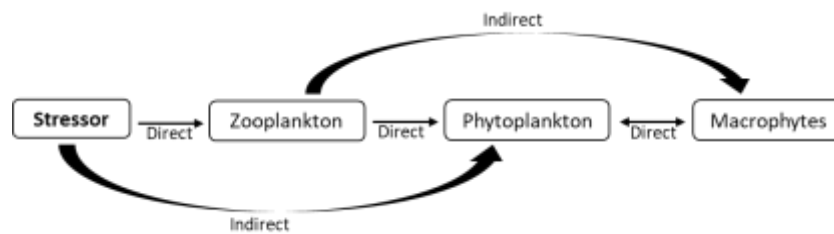


Fig. 1.2. Conceptual model of direct and indirect species interaction and stressor effects. Indirect ecological effects may result from the interactions among interdependent species or from the effects of stressors on such interactions. Adapted from Preston (2002).

Shallow lakes are exposed to multiple environmental stressors or pressures, which can cause abrupt, non-linear changes to the relatively stable macrophyte-dominated state (Biggs et al., 2018; Scheffer et al., 2001). The stable equilibrium of shallow lakes may be affected by stressors such as eutrophication (Scheffer et al., 1993), habitat or biodiversity loss (Hilt et al., 2017), increased salinity (Sim et al., 2006) and presence of invasive species (Gaertner et al., 2014) (Fig. 1.3). Multiple other stressors, such as pesticides and climate warming are also acting on shallow lakes. These stressors and their effects on shallow lakes are discussed in the following sections.

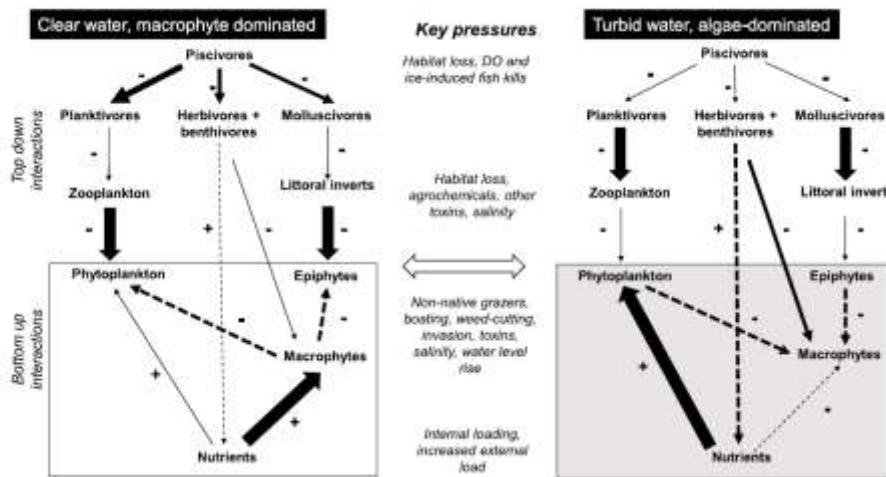


Fig. 1.3. Major bottom–up and top–down interactions in shallow lake ecosystems. “+” indicates a positive effect and “-” indicates a negative effect. The thickness of the arrows indicates the magnitude of the effects. The dashed arrows indicate allelopathy, nutrient sequestration, shading, bioturbation and physiological constraints. This is the updated version of the model first proposed by Phillips et al. (1978) which focused on bottom–up effects. Source: Phillips et al. (2016).

2. Stressors acting on shallow lakes

Nutrients reach shallow lakes in the form of agricultural runoff also containing pesticides and metals. In addition to this, climate warming is simultaneously acting on shallow lakes (Fig. 1.4). Predicting the effects of nutrients alongside these multiple other co-stressors on a system stabilized by complex biotic interactions remains a challenge. The following sections discuss the literature on nutrient, pesticide and climate warming effects in shallow lakes and on macrophytes, followed by some literature on combined effects of nutrients with warming or with pesticides.

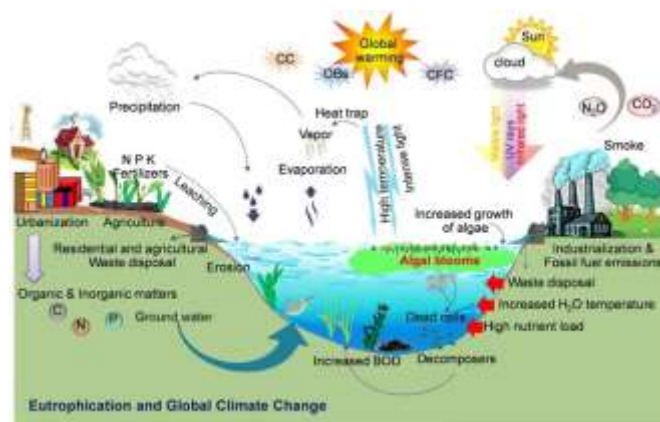


Fig. 1.4. Factors promoting cyanobacterial blooms. Warming and nutrient loading promote high cyanobacterial chlorophyll-*a* and algal chlorophyll-*a* concentrations in freshwater systems (Lüring et al., 2018; Rastogi et al., 2015). Source: Rastogi et al. (2015).

2.1. Nutrients

Eutrophication in shallow lakes may occur naturally or by industrial and agricultural activity (Conley et al., 2009), as well as sewage disposal (Schindler, 1974). Eutrophication occurs naturally over centuries with allochthonous input of organic matter made visible by a slow deposition of sediments (Carpenter, 1981). Anthropogenic activity has accelerated this process, with large inputs of nutrients reaching lakes and marine systems through spray drift or surface and subsurface run-off after rain events. Shallow lakes are abundant in fertile lowland regions, where farming and agriculture are based. Their proximity to these regions make them highly prone to the inflow of agricultural runoff transporting fertilizers rich in nitrogen (N) and phosphorus (P).

Phosphorus exists in the aquatic and terrestrial environments as dissolved and as particulate forms, and both types may exist in organic or inorganic forms. Phosphorus is less easily leached into soils, and most of its contribution to eutrophication of shallow lakes is through surface water flow, carrying both dissolved and particulate forms (Sharpley et al., 1999; Sharpley & Rekolainen, 1997). Nitrogen also exists as organic and inorganic forms. The main inorganic forms, nitrate (NO_3^-) and ammonium (NH_4^+), are highly mobile in water and are the forms readily taken up by plants (Follet, 1995; Nolan et al., 2002). Because of their highly mobile nature, they are both easily leached into groundwater or carried away in agricultural runoff.

Before human activity, aquatic systems were most likely both nitrogen (N) and phosphorus (P) limited. The geological supply to biological need ratio of P is low compared to all elements derived from the lithosphere (Hutchinson, 1973; Moss et al., 2013). P may be partially lost as iron phosphide in the earth's metallic core and therefore not easily available for terrestrial and aquatic primary productivity (Hutchinson, 1973). Natural levels of N in the environment are controlled largely by microorganisms through biological nitrogen fixation (Fowler et al., 2013). The N:P molar ratios of shallow lakes may also have tended to remain close to the Redfield ratio (16:1) (Redfield, 1934, 1958), with the growth rates of organisms being adapted to the natural mean concentrations of N and P, and their excretory and recycling mechanisms maintaining this ratio (Moss et al., 2013). With the advent of farming and agricultural practices, however, the concentrations of N and P in shallow lakes has been rising (Peñuelas et al., 2013).

Despite the rising concentrations of both N and P shallow lakes, eutrophication has mainly been linked to P, with N being side-lined (Moss et al., 2013; Schindler, 1974). The reasoning behind this was that submerged macrophytes are able to access P from the sediment. Large input of P would be required to promote the growth of microalgae. Nitrogen shortage, however, could be made up for by nitrogen fixers (Moss et al., 2013). This belief that phosphorus rather than nitrate plays a role in eutrophication has culminated in a more stable global consumption of phosphate fertilizers (FAOSTAT, 2014), but a continuous rise in nitrogenous fertilizers (Yu et al., 2015). In fact, recent studies are showing that N:P ratios in aquatic systems is increasing, probably linked to the enhanced use of N fertilizers (Peñuelas et al., 2013).

Recent studies, however, indicate that macrophyte biomass, density and diversity are affected by increasing N concentrations (2 to 10 mg L⁻¹), irrespective of phosphorus concentrations (Barker et al., 2008; Sagrario et al., 2005). The mechanism of nitrogen effects on macrophytes remain unclear (Phillips et al., 2016). Some studies indicate direct toxic stress in the form of NH₄⁺ on plant metabolism. For example, NH₄⁺ may reduce macrophyte growth (Cao et al., 2004; Yu et al., 2015), affect carbon and nitrogen metabolism (Cao et al., 2004), and inhibit photosynthesis at concentrations exceeding 10 mg L⁻¹ (Su et al., 2012). Nitrate is non-toxic to macrophytes and can be sequestered in their biomass (Melzer & Kaiser, 1986). Nitrogen may also indirectly affect macrophytes by promoting either phytoplankton or periphyton/epiphyton development, similar to phosphorus, leading to increased competition and shading (Barker et al., 2008; Kilroy et al., 2020; Olsen et al., 2015; Phillips et al., 1978; Sagrario et al., 2005).

The negative effects of eutrophication were initially believed to be due to increasing phytoplankton biomass, which absorb sufficient light and thereby prevent net photosynthesis in macrophytes (Mason & Bryant, 1975; Phillips et al., 1978). However, as early as 1978, Phillips et al. had proposed a more complex hypothesis, on grounds that shallow lakes are not deep enough for macrophytes to be light limited by phytoplankton development. This hypothesis stated that periphyton played an intermediate role in regime shifts by blanketing (as epiphytes) and subsequently suppressing macrophyte growth. This reduced growth of macrophytes leads to a decreased production of allelopathically active substances that would normally suppress phytoplankton growth. In the absence of competition and inhibition by macrophytes, phytoplankton are able to thrive (Fig. 1.5)

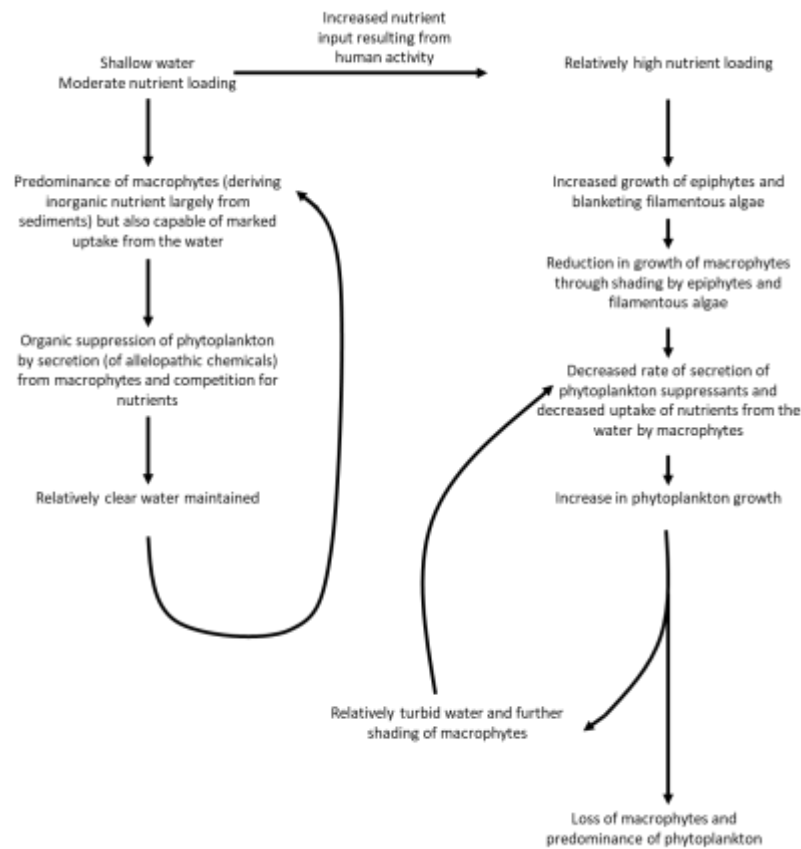


Fig. 1.5. Conceptual model for macrophyte decline in shallow lakes exposed to eutrophication. Phytoplankton blooms may be the consequence of a decline in macrophyte growth and allelopathy. The decline of macrophytes may be a consequence of blanketing by periphyton, whose growth is prompted by eutrophication. Adapted from Phillips et al. (1978).

In a mesocosm study, Phillips et al. (1978) observed a clear divergence in the growth of the submerged macrophyte species *Najas marina* in response to nutrient fertilisation. After 2 weeks, the nutrient-enriched tanks showed half the macrophyte biomass and a significant increase in epiphyte biomass compared to control tanks. After day 14, the epiphytic biomass still remained higher than the controls. This increased periphyton biomass was linked to the decrease in macrophyte growth through epiphyte shading. This shading potential by epiphytes was confirmed in subsequent studies (Hilt et al., 2018; Sand-Jensen & Borum, 1991). In the experiment by Phillips et al. (1978), the tanks were enriched with both N and P. However, epiphyte development has strongly been linked to N availability (Fitzgerald, 2007; Shacklock et al., 1973) highlighting the potential indirect N effects on macrophytes and regime shifts.

While regime shifts may occur at a range of nutrient concentrations, external perturbations may act as tipping points favouring one state over the other (Scheffer et al., 1993). The source of nutrients in shallow lakes is primarily through agricultural runoff, which contains multiple other chemicals such as organic and inorganic pesticides. While nutrients are more likely to affect the system through an increasing bottom–up control, pesticides may affect both bottom–up and top–down control, depending on the modes of action of pesticides present. The next section outlines our current knowledge on pesticide effects on macrophytes and shallow lake status.

2.2. Pesticides

Global pesticide production increased significantly after World War II, from 500,000 tonnes to more than 3 million tonnes per annum currently (Schäfer et al., 2011; Sharma et al., 2019). With the increase in human population and consequent demand for food, arguing for a reduction in pesticide use becomes challenging. The primary concern of pesticides in ecology is that they are designed to act against specific functions or processes of the target organisms, but at the same time have an unavoidable impact on other organisms in the environment (Mohaupt et al., 2020). Pesticides sprayed in agricultural sites finally reach shallow lakes through runoff, where they may affect macrophytes directly or indirectly.

There are several categories of pesticides; those used in agriculture may generally fall into three groups - herbicides, insecticides and fungicides (Oerke & Dehne, 2004). In Europe, the most widely used pesticides are fungicides and bactericides (46%), followed by herbicides (29%) and insecticides and acaricides (11%) (Sales of Pesticides in the EU, 2020). These pesticides may affect different organisms depending on their mode of action (MoA) (Table 1.1).

Bacteria, microalgae, macrophytes, filter feeders, grazers and fish can be affected directly through physiological action or physical damage. Herbicides, for example, are designed to inhibit plant growth by affecting photosynthesis, modifying cell permeability or regulating growth of certain plant parts (Duke, 1990; Mohaupt et al., 2020). Insecticides can act by targeting the nervous system of insects (Mohaupt et al., 2020; Table 1.1). Fungi can be affected through inhibition of sterols or essential proteins. Apart from the direct effect of these pesticides, they may also affect non-target organisms indirectly due to ecological interactions (Preston, 2002). However, pesticide effects at the organismic level

are better studied, but those at the community or ecosystem level are much less understood.

Despite ongoing efforts to replace the more toxic pesticides by less toxic pesticides, the physiological similarity of some aquatic species to target terrestrial organisms means that pesticides will continue to threaten the healthy structure and functioning of shallow lakes. In fact, effects on non-target organisms in freshwater systems, as well as their indirect effects on the community structure are still reported. Some of these pesticides are persistent in the environment even years after application. For example, although the first used insecticides in the 1950s to 1970s mainly comprising organochlorines have been replaced by less persistent pyrethroids and organophosphates, effects of these organochlorines even on non-target organisms are still detected (Schäfer et al., 2011).

The literature pertaining to toxic herbicide effects on macrophyte growth in general provides conflicting results, such as no effects (Jones et al., 2012), reductions in macrophyte biomass (Parsons et al., 2009) or changes in macrophyte biomass depending on the species composition (Wagner et al., 2007). However, at the species level, *Lemna gibba* (free-floating macrophyte species) and *Myriophyllum spicatum* (submerged macrophyte species), both OECD test plants, are among the most sensitive macrophyte species for 50% and 25%, respectively, of 14 tested pesticides (11 herbicides and 3 fungicides) (Giddings et al., 2013). Triazines are a range of widely used herbicides for weed control in sugarcane, corn, sorghum and cotton cultivation. Atrazine, a type of triazine, is effective, inexpensive and the most used herbicide in the world (Graymore et al., 2001), although it is forbidden in several European countries. Concentrations in freshwaters range from as low as $0.2 \mu\text{g L}^{-1}$ to as high as $1000 \mu\text{g L}^{-1}$ soon after heavy rain events (Pereira & Hostettler, 1993; Pereira & Rostad, 1990; Readman et al., 1993), with higher concentrations correlating with shifts from macrophyte- to phytoplankton-dominated lakes (Dewey, 1986). In a mesocosm study, atrazine affected periphyton production at concentrations of $15 \mu\text{g L}^{-1}$ but also filter feeders and grazers such as daphnids and tadpoles, modifying community structure (Detenbeck et al., 1996). Other triazines such as terbuthylazine, which are found in surface waters at concentrations less than $1 \mu\text{g L}^{-1}$, are unlikely to affect the macrophyte community up to concentrations of $3 \mu\text{g L}^{-1}$ (Cedergreen et al., 2004). In fact, in some cases, a hormetic effect with growth stimulation was observed for some of the macrophyte species exposed to these herbicides.

Table 1.1. Mode of action of pesticides (MoA).

MoA – group	MoA – effects	Target organisms
Photosynthesis inhibition ¹	Photosynthesis is inhibited by pesticide (herbicide) binding to proteins in the photosystem II complex of plants (Duke, 1990)	Plants
Plant growth regulator ¹	Plant growth is regulated through the phytohormones that make individual plant parts grow stronger. Some substances inhibit the plant's longitudinal growth and promote fruit growth. Other substances promote the growth of the green parts of the plant, while at the same time root growth stagnates. As a result, the plant dies due to the lack of nutrients in the plant.	Plants
Cell membrane disruption ^{1,3}	The selectivity of the cell membrane is disturbed so that it becomes more or less permeable.	Plants and fungi
Mitosis, cell cycle ^{1,2}	Inhibition of growth by preventing cell division.	Plants and insects
Respiratory action ^{1,2}	Various processes prevent the exchange of oxygen in membranes or the chemical binding of oxygen.	
Neurotoxic ²	Prevents the transmission of stimuli or the reaction in nerves.	Plants and insects
Lipid metabolism ^{1,2,3}	Inhibition of the enzyme “acetyl-CoA-carboxylase”, which is responsible for controlling fatty acid metabolism and degradation. By inhibiting lipid biosynthesis, the development of the immature stages (larvae and nymphs) of certain insects and mites will be stopped, thus reducing fertility.	Plants, insects and fungi
Multi-site activity ^{1,2,3}	Inhibition can be caused by membrane damage to specific modulation of receptors, or inhibition of enzymes, or a mixture of several effects.	Plants, insects and fungi
Signal transduction ^{2,3}	Inhibition of transmission of molecular signals from a cell's exterior to its interior in fungi and plants.	Insects and fungi
Fungal spore inhibitor ³	The reproduction of fungi is disturbed.	Fungi
Sterol biosynthesis inhibition ³	Inhibition of the important cell membrane component of the sterol type (= typical MoA for fungicides).	Fungi
Protein denaturation ³	Essential proteins are destroyed in fungi so that the metabolism is disturbed.	Fungi

¹MoA to plants; ²MoA to insects; ³MoA to fungi. MoA = mode of action. Note: The MOA-effects for photosynthesis inhibition has been adapted and is not part of the original table from (Mohaupt et al., 2020). Source: Adapted from Mohaupt et al. (2020).

These herbicides are not present alone, and even though some of them do not exhibit individual toxicity to macrophyte species at environmental concentrations, a combination of different herbicides may result in concentrations that exceed toxic levels (Faust et al., 2003). Because of the varying concentrations in aquatic systems based on rain events, surface or subsurface runoff and sensitivity of the aquatic environment, (Graymore et al., 2001) suggest that a single maximum limit cannot be proposed that will protect aquatic ecosystems. Yet despite legal provisions have ensuring that the use of triazine herbicides such as atrazine are limited (Eurostat, 2007), they are still being used and their possible effects on the environment in combination with co-occurring stressors need to be evaluated.

Among insecticides, organophosphates and carbamates are the two most used within the European Union. Annually, 4645 tonnes of organophosphates and 854 tonnes of carbamates are used (Eurostat, 2007). Although both types of insecticides are structured differently, their mode of action is the same, that is they affect the nervous system by inhibiting the neurotransmitter acetylcholinesterase (O'Malley & O'Malley, 2020). This can lead to death due to respiratory failure (Ray & Ghosh, 2006). Because of their mode of action, insecticides may affect both aquatic invertebrates and vertebrates, which include filter feeders such as zooplankton species and molluscs, as well as other grazers and fish. These effects may indirectly affect primary producers through modified top–down control, restructuring the community of aquatic systems. In some cases, direct effects of these insecticides on macrophytes have also been observed. For example, (Boonyawanich et al., 2001) have indicated that macrophyte leaf development and chlorophyll content may be affected by carbamate insecticides through absorption of these chemicals into their xylem and phloem.

Compared to herbicides and insecticides, the effects of fungicides in aquatic ecosystems have received much less attention, even though they occur in aquatic systems and can be toxic to a broad range of organisms (Zubrod et al., 2019). Unlike herbicides and insecticides, the mode of action of fungicides is broad. They affect processes that are common in all living organisms, such as blocking electron transfer in mitochondria, binding to sulphhydryl bonds, inhibiting sterol biosynthesis or disrupting energy production (Maltby et al., 2009; Mohaupt et al., 2020; Stenersen, 2004). Fungicides are categorized into different classes such as carbamates, benzimidazoles and triazoles. Triazoles comprise one of the largest groups of fungicides, first produced by Bayer in 1973. This group of

fungicides is used widely for wheat crops, with more active compounds being produced compared with the earlier ones (Thabit et al., 2021). Certain triazoles such as tebuconazole and epoxiconazole inhibit respiration and photosynthesis in bacterial and microalgal communities at concentrations of $2 \mu\text{g L}^{-1}$ (Artigas et al., 2014). Fungicides also hamper litter decomposition by inhibiting hyphomycetes and other microorganisms playing important roles as decomposers. In combination with insecticides, reduction in litter decomposition may be even stronger as both detritivores and decomposers are affected (Cornejo et al., 2021). Even if direct effects are not observed on macrophytes, invertebrates and vertebrates (Zubrod et al., 2019), highly toxic effects on the microbial community can also result in a degradation of important ecosystem processes that may reduce the resilience of the system.

In addition to organic pesticides, inorganic pesticides such as metals may also affect shallow lakes. In a study ranking the risk of several metals to aquatic wildlife, copper, aluminium and zinc were at the top, with copper having the highest risk. The Bordeaux mixture comprises copper sulphate and quicklime for vineyards and fruit-farms. The sale and use of these chemicals are illegal in several European countries, but are still use in others such as France and Belgium (Lewis et al., 2016). Copper can be harmful to aquatic organisms. In freshwater systems, concentrations of $2.5 \mu\text{g L}^{-1}$ lead to a 57 to 81% reduction in periphyton productivity and those at $2.8 \mu\text{g L}^{-1}$ affect the rainbow trout *Oncorhynchus mykiss* (Donnachie et al., 2014). Other aquatic life such as macrophytes (Tc & Rolli, 2017) and fish (Gharendaashi et al., 2013) are also inhibited by copper. For macrophytes, the specific genotype of the species may also influence its sensitivity to copper, as was seen for *M. spicatum*, with EC50s ranging from 42 to $296 \mu\text{g L}^{-1}$ (Roubeau Dumont, 2019).

Owing to their different modes of action, a combination of these pesticides can have complex effects on shallow lake ecosystems by targeting different functional groups or organisms. However, pesticides are acting on shallow lakes systems in combination with other stressors such as nutrients and climate warming. Nutrients have already been discussed. An increase in water temperatures may affect lake function. The following section discusses the literature available on effects of elevated temperature on macrophytes and the consequences for shallow lakes.

2.3. Elevated temperature

Although the Earth's climate has never remained constant throughout the measurable geological timescale, temperatures at the surface of the earth have been rising rapidly in the past century, compared to the pre-industrial period. Based on a report by the Intergovernmental Panel on Climate Change (IPCC, 2014), global mean surface temperatures may increase by 3 to 4°C by 2100 relative to the period from 1986 to 2005, given the current rate of carbon emissions. Climate affects the distribution of Earth's water through evaporation, vapour transport and precipitation among freshwater lakes, rivers, oceans and the atmosphere, as well as the distribution of major biomes (Carpenter et al., 2003). In shallow lakes, a rise in average water temperatures due to increasing global temperatures will affect primary producer growth and fitness (Haag & Gorham, 1977; Olkeba et al., 2020). Warmer temperatures can also promote trophic cascades from fish to phytoplankton (Scheffer & van Nes, 2007) and can cause regime shifts in lake ecosystems (Scheffer et al., 2001).

Temperature affects macrophyte physiology, such as photosynthesis (Barko & Smart, 1981) and respiration (Anderson, 1969), and depending on species sensitivity, macrophyte diversity or richness may change. In extreme cases, the overall macrophyte biomass may be affected. As a general finding, studies show that higher temperatures increase macrophyte biomass. While small increases in temperature do not create large divergences in total macrophyte biomass, year-long investigation of macrophytes in lakes showed that it took differences of ~6°C to significantly affect macrophyte biomass (Rooney & Kalff, 2000). Studying the effects of temperature among three submerged macrophytes, (*Elodea canadensis*, *Potamogeton nodosus*, *Vallisneria americana*), Barko et al. (2011) found a high optimum temperature range of 28 to 32°C. Similarly, Zhang et al. (2019) exposed three submerged macrophyte species, *Potamogeton lucens*, *Vallisneria spiralis* and *Elodea nuttallii*, to 15, 20, and 25°C, and arrived at the same conclusion that higher temperatures significantly increased macrophyte growth (Fig. 1.6). A study by Hussner and Jahns (2015), comparing two rooted submerged macrophyte species *Myriophyllum spicatum* and *M. heterophyllum*, showed highest growth of these macrophytes at both 21 and 26°C compared to 16°C. Mckee et al. (2002) performed a microcosm study to determine the effects of warming on three macrophyte species with different growth forms compared with Barko et al. (2011) and Zhang et al. (2019). They studied effects on *Lagarosiphon major*, a free-floating macrophyte, *Elodea nuttallii*, a rooted submerged macrophyte, and

Potamogeton natans a rooted, floating and submerged leaved macrophyte under ambient temperature, summer-only warming at 3°C above ambient, and continuous warming at 3°C above ambient in outdoor microcosms. The entire macrophyte species responded little to warming, but the proportions of macrophyte species making up the community changed, with *L. major* increasing in the continuously warmed treatments, indicating that specific macrophyte growth forms and adaptations/sensitivities to different temperatures may affect macrophyte community response to warming. This increase in proportion of *L. major* in the continuously warmed treatments resulted in a decrease in *E. nuttallii*.

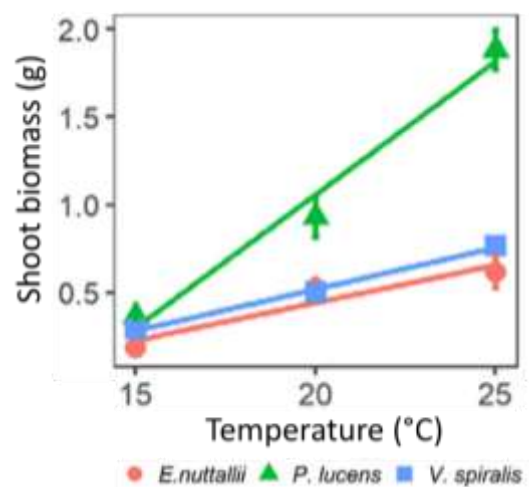


Fig. 1.6. Response of three aquatic macrophytes to elevated temperatures. All three macrophytes showed an increased growth at higher temperatures. Mean \pm SE ($n = 15$), $p < 0.05$. Source: Zhang et al. (2019).

Macrophytes are also affected indirectly by warming through changes in nutrient availability and food web dynamics. Current climate models propose that rising temperatures will hasten the switch from macrophyte-dominated to phytoplankton dominance (Trolle et al., 2014). Because macrophytes are influenced by turbidity, understanding how warming affects the phytoplankton abundance also provides some insight into indirect effects on macrophytes. Based on the metabolic theory (Brown et al., 2004), warming increases metabolic rate as well as the nutrient demand of phytoplankton, and will support less phytoplankton biomass if limiting resources remain constant in lakes (Daufresne et al., 2009; Yvon-Durocher et al., 2011). However, higher temperatures can lead to increased nutrient release from sediments (Jeppesen et al., 2009), thereby increasing resource availability in the water column and promoting phytoplankton development. The conducive warming effects on both macrophytes and phytoplankton complicates the

understanding of the role of temperature as a driver of regime shifts. Kraemer et al. (2017) suggested that the reduction in phytoplankton size and abundance due to warming as proposed by the metabolic theory (Brown et al., 2004) did not always hold true, and that phytoplankton response to warming depended on the trophic status of the lake. Their study indicated that warming decreased chlorophyll-*a* in phytoplankton-poor lakes ($\text{chl-}a < \sim 3 \mu\text{g L}^{-1}$) whereas it increased in phytoplankton-rich lakes ($\text{chl-}a > \sim 20 \mu\text{g L}^{-1}$). The combination of warming and high nutrient concentrations may therefore promote phytoplankton development, but the rapid and earlier proliferation of phytoplankton due to temperature increase would mean faster depletion of the available nutrient pool, subsequently promoting growth of macrophytes (Asaeda et al., 2001), whose biomass increases at higher temperatures as exemplified in the previous paragraph.

Rising temperatures may further strengthen top-down herbivore-plant interactions (Zhang et al., 2019), adding another dimension of complexity in determining temperature effects on macrophytes. Several studies show that periphyton biomass is negatively influenced by rising temperatures (Hao et al., 2020; Zhang et al., 2019), and linked to the strong top-down control of periphyton under warming (Hao et al., 2020; Zhang et al., 2019). Warming may also lead to increased respiration compared to photosynthesis, another explanation for a decrease in periphyton biomass at higher temperatures (O'Connor et al., 2009). Because periphyton is capable of reducing light availability to plants when growing epiphytically, an impact of warming on periphyton may indirectly affect macrophyte development.

Although warming has been shown to promote phytoplankton biomass, the switch from macrophyte to phytoplankton dominance as temperatures rise does not always occur. The role of elevated temperature on shallow lake dynamics and dominance of either macrophytes or phytoplankton is very complex as the response depends on numerous factors such as trophic status, top-down control and community structure. In combination with nutrients, pesticides or both, the prediction of effects on macrophytes becomes very challenging. In the following section, I discuss the literature on combined stressor effects in shallow lakes.

2.4. Potential combined stressor effects

None of these described stressors, that is, nutrients (here nitrate), pesticides or climate warming, are acting in an isolated manner within shallow lakes. Many studies have

focused on single stressor effects on target single-species organisms or on microcosms comprising simplified food webs. Combined effects of multiple commonly co-occurring stressors are still scarce. In combination, the stressors may have synergistic, additive, antagonistic or reversed effects. Synergistic effects occur when the combined effects of the stressors is higher than the sum of the individual stressor effects, additive effects occur when the combined effect of the stressors is equal to the sum of the individual stressor effects, antagonistic effects occur when the combined effect of the stressors is lower than sum of the individual stressor effects and reversed effects occur when the combined stressors produce an effect in a direction opposite to the individual stressors (Côté et al., 2016; Jackson et al., 2016).

A few recent studies have focused on the effects of nutrients with either pesticides or warming. For example, nutrient enrichment ameliorated or mitigated destabilizing effects of pesticides such as atrazine (Starr et al., 2017) or copper (Prosnier et al., 2015) on phytoplankton development. Dalton et al. (2015) showed that significant reductions in submerged macrophyte biomass in streams were linked to high nitrate concentrations rather than the herbicide atrazine. Although the herbicide was present at concentrations toxic to amphibians and fish, no significant changes were observed in community structure. In a mesocosm study of shallow lake communities, Feuchtmayr et al. (2009) assessed the effects of eutrophication and warming by 4°C on primary producers. Their study suggested that warming plus increased nutrient loading caused a decrease in species richness by increasing the floating plant biomass. Contrary to what is predicted for global warming and eutrophication, the switch to a phytoplankton-dominated state was not observed, with warming increasing macrophyte biomass and nutrients increasing floating plant biomass but reducing total macrophyte biomass. A recent review of the literature on individual and combined effects of heatwaves and chemicals suggested that elevated temperature had a positive effect on primary producers at the species and population level, but in combination with herbicides, the effect was antagonistic. Elevated temperature also

had a positive effect on consumers at the species and population level, but in combination with insecticides, synergistic effects were observed (Polazzo et al., 2022; Fig. 1.7).

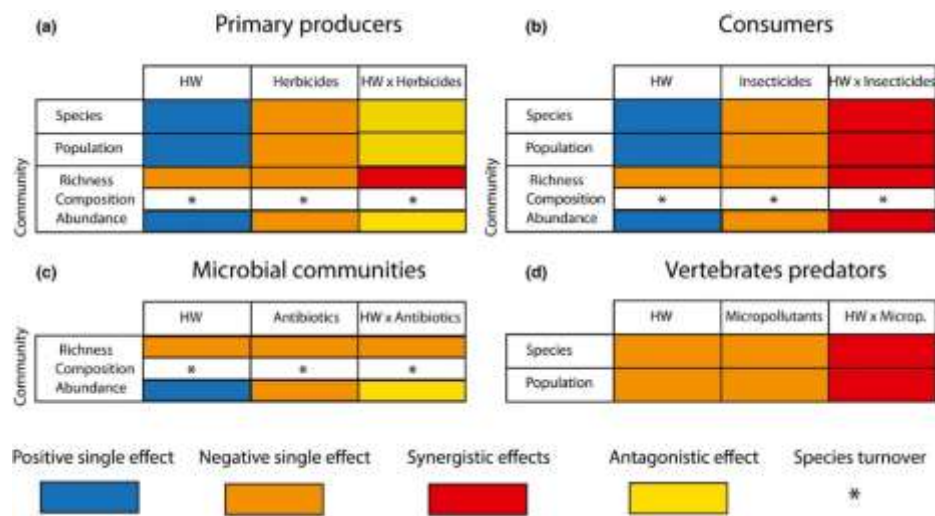


Fig. 1.7. Interactions between heatwaves and chemical pollutants on different aquatic communities. The effect of heatwaves depends the type of interacting chemical (herbicide, insecticide, antibiotic, etc.) and the target community (primary producers, consumers, microbial communities, vertebrate predators). Source: Polazzo et al. (2022).

Predicting the effect of two stressors is already complex, and the effect of three stressors is much more challenging. But identifying how the three stressors (nutrients, pesticides and warming, each of whose effect can vary between communities and species) affect the overall resilience of the macrophyte-dominated state when acting in combination will provide some insight on the current state of stress faced by shallow lakes and a foundation for maintaining good ecological status in shallow eutrophic lakes.

3. Tipping points for regime shifts

When shallow lakes are exposed to multiple stressors simultaneously, tipping points may be more easily reached (Scheffer et al., 1993), leading to earlier and more abrupt regime shifts. In the following sections, the role of nutrients (in particular nitrate), pesticides and warming as drivers of regime shifts are presented.

Under the influence of internal or external stressors, complex systems such as natural ecosystems and the climate may reach “tipping points” at which sudden shifts to an alternative regime state occur (Scheffer et al., 2009). Identifying these tipping points is not straightforward as complex systems usually show no signs or indications of change

before a shift occurs (Scheffer et al., 2009). One of the most commonly used indicators that a system is reaching a tipping point is the “critical slowing down” (Dakos et al., 2015). Based on this concept, as a system arrives at a critical threshold (Fig. 1.8), its resilience decreases, meaning that the system stays longer in an unstable condition closer to a bifurcation point, and does not easily revert to the original stable state. A well-known consequence of this critical slowing down is an increase in variance in the pattern of fluctuations within the system (Dakos et al., 2015; Scheffer et al., 2009). As the variance in response to a certain stressor or a combination of stressors increases, the possibility of a shift to an alternative state also increases. Other early warning signals have been proposed, such as flickering. Flickering occurs when the system moves back and forth between the basins of attraction of the two alternative attractors long enough before permanently shifting to the alternative state (Berglund & Gentz, 2002; Horsthemke & Lefever, 2007).

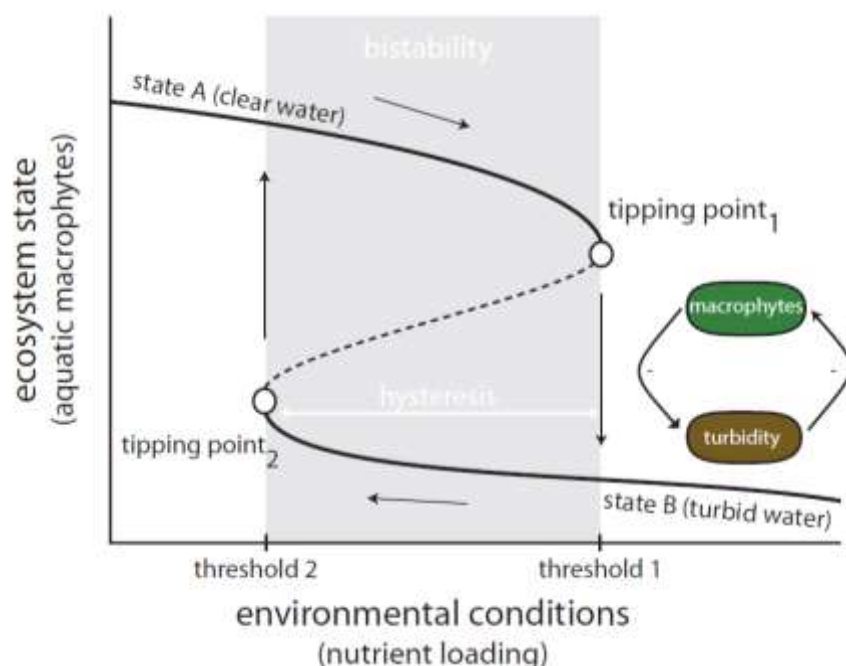


Fig. 1.8. Tipping points for regime shifts in an ecosystem. The ecosystem remains stable until it reaches the first threshold value or tipping point (for example, high nutrient loading). After this point, the ecosystem switches to an alternative regime state (e.g. moving from the macrophyte-dominated clear-water state to the phytoplankton-dominated turbid state). By modifying conditions of the ecosystem (e.g., reducing the nutrient concentrations, usually much lower than present before the shift), it may reach the second threshold point or tipping point, allowing it to switch back to the original regime state. Between the two stable states is a condition called the bi-stability, where the system may exist in either the macrophyte-dominated or turbid state, and this depends on the hysteresis of the system. Solid lines = stable equilibria. Dotted line = border between the basins of attraction. Source: Dakos et al. (2020).

Identifying early warning signals leading to a forward switch (macrophyte to phytoplankton dominated state) or a backward switch (phytoplankton to macrophyte dominated state) is an emerging topic for shallow lakes exposed to multiple environmental stressors. Most existing studies have focused on shifts induced by single environmental drivers, such as nutrients or warming. In addition, there is very little available research in terms of what biological parameters can be used as early warning signals. Some examples of possible early warnings signals, exist, such as increased fluctuations in fish populations that signal a deterioration following exploitation of fish stocks (Hsieh et al., 2006) and high impact of herbivore grazing in shallow lakes, which signals reduced resilience of vegetation to eutrophication (Hidding et al., 2016), leading to a sudden shift to turbid conditions. Haojie et al. (2020) have shown that changes in lower trophic level abundance and size, such as those of phytoplankton and zooplankton, may act as early warning signals indicating an imminent negative effect on macrophyte abundance. Because macrophytes are regarded as central to the good status and functioning of shallow lakes, studying their responses to multiple environmental stressors and linking changes in their development/physiology to direct and indirect stressor effects could also provide a potential tool as early warnings signalling a regime shift.

4. Macrophyte chemical fingerprint

The majority of hydrophytes ... show definite preferences and aversions in the matter of the soil underlying the water, in which they grow, and of the resulting differences in the nature of the solution in which they are immersed.

—(Arber, 1920), cited in Hutchinson (1970)

Kohler and Schneider (2003) cited the definition of bioindicators as provided by Arndt et al. (1996) at the first Hohenheim Workshop on Bioindication in 1995: “Bioindicators are organisms or communities of organisms, which react to environmental influences by alterations of their life functions and/or by their chemical composition. Thereby, it is possible to draw conclusions concerning their environmental conditions. In addition, Kohler and Schneider (2003) state that “indicator plants may be individual species, groups of species or communities, which provide information on the status of an ecosystem”.

Macrophyte community composition and abundance have been used widely in ecology as a measure of the general development of macrophytes in response to environmental changes as well as for environmental monitoring of the ecological state of water bodies (EU WFD, 2000/60/EC). A reduction in macrophyte biomass could signify a transition to a new regime state, or a reduction in species richness may signify high nutrient loading. Several macrophyte indices exist and are well-established that provide information on environmental status, most often trophic status, based on the morphological forms and types of species thriving in these systems. The Macrophyte Biological Index for Rivers (IBMR; Haury et al., 2006) was developed as one of the requirements by the European Water Framework Directive (EU WFD; 2000). This method uses the nutrient requirements of macrophyte species to identify trophic degradation in rivers. Changes in absence or presence of species or relative abundance are not the only response factors possible to analyse. At the individual species level, more specific macrophyte growth factors may be used, such as growth rate and root:shoot ratio. The specific response factor used may depend on the question. Macrophyte leaf, stem and root mass fractions are also a very relevant indicator of stress (Gross et al., 2018). Changes in the environment, such as light, temperature and nutrient availability may alter general plant traits such as the leaf area, plant height and reproduction (Fu et al., 2013). In addition to morphological traits, physiological traits can also be considered when evaluating a response to stressors.

Among these are changes in dry matter content and stoichiometry, photosynthetic pigments and secondary metabolites. Riedl et al. (2012) studied the metabolic fingerprint of the submerged macrophyte *M. spicatum* from different lakes exposed to varying environmental conditions and concluded not only that the metabolic fingerprints of the macrophytes growing in the fields can be replicated but also that their biological responses to the environment could be detected. In their study, the metabolic fingerprint of the macrophytes responded to the nutrient status of the lakes in which they were present. While metabolomics is a powerful tool to study changes in plant chemical composition, it is also a complex and expensive method for large sample sizes. Simpler methods may be used to study macrophyte physiology (Gross et al., 2018; Nuttens et al., 2016). In this section, I present different physiological response factors that can be used as indicators of macrophyte response to stressors.

The *dry matter content (DMC)* of macrophytes is the ratio of dry weight to fresh weight and may be affected abiotic and biotic factors in the environment. An increase in DMC is related to higher plant toughness. Higher temperatures may lead to increased DMC and plant toughness through more carbon accumulation and may result in lower physiological activity (Pennings et al., 1998; Richter & Gross, 2013). Eutrophication may reduce plant toughness by an increased nutrient uptake by plants (Zhang et al., 2019), and nutrient limitation can lead to high ash content of plants (Richter & Gross 2013), all affecting DMC. Such changes have implications for palatability of macrophytes by herbivores. Plants with higher dry DMC tend to be less palatable due to their toughness (Cronin et al., 2002; Elger & Willby, 2003), and plants react to herbivory with an increase in DMC (Fornoff & Gross 2014).

Stoichiometry refers to the concentrations of micro- and macroelements within a system. Among these, the macroelements carbon (C), nitrogen (N) and phosphorus (P) are most important for the growth of living organisms. Nitrogen and P are major nutrients, and C enables production of organic biomass in primary producers. The uptake of these elements from the environment is therefore a fundamental necessity. Primary producers, in particular, are considered non-homeostatic, meaning their elemental composition can vary widely depending on concentrations in their environment and other biotic and abiotic factors (Persson et al., 2010; Sterner & Elser, 2002). Heterotrophs, on the other hand tend to be homeostatic, and metabolism allows them to maintain their stoichiometric signature through assimilation and excretion (Persson et al., 2010; Sterner & Elser, 2002). By studying the ratios of these macro-elements, ecological stoichiometry provides an understanding of the flow of energy resulting from biological interactions and nutrient cycling within ecosystems (Elser et al., 2010; Sardans et al., 2012).

Macrophyte stoichiometry affects both decomposition as well as palatability for grazers, influencing energy flow within aquatic systems (Sterner & Elser, 2002). The literature to date has proposed several factors as affecting macrophyte stoichiometry, such as spatial scale, habitat, plant type (Li et al., 2010; Van de Waal et al., 2010; Wu et al., 2012) and temperature (He et al., 2008; Zhang et al., 2019). Eutrophication also affects macrophyte stoichiometry (Güsewell & Koerselman, 2002; Sardans et al., 2012). Very high N and P concentrations in water for example cause a decrease in CN or CP ratios (Velthuis et al., 2017).

Changes in the stoichiometry of macrophyte leaves have implications for their palatability to herbivores (Bakker & Nolet, 2014; Cronin et al., 2002; Fornoff & Gross, 2014; Hay, 1996; Sterner & Elser, 2002), with an increase in N or a decrease in the C:N ratios leading to increased grazing (Sterner & Elser, 2002; Zhang et al., 2019). When environmental nutrient concentrations are low, the expected increase in C:N ratio results in higher plant toughness and lower nutrient value for herbivore grazers (Sterner & Elser, 2002), leading them to seek other more nutritive sources. On the other hand, higher nutrient content or lower C:N ratios may be linked with higher herbivory (Bakker et al., 2016). In fact, Hidding et al. (2016) proposed that an increased top–down control on macrophytes by grazers due to the higher nutritive value may be the reason for loss of macrophytes from eutrophic lakes.



Fig. 1.9. Conceptual model of temperature and nutrient effects on stoichiometry, palatability and consequent herbivory in shallow lakes. Upward triangles (▲) indicate an increase whereas downward triangles (▼) indicate a decrease. Contribution to primary production refers to the percentage of macrophyte biomass relative to total primary producer biomass. C:N = carbon:nitrogen, C:P = carbon:phosphorus, DMC = dry matter content.

Temperature can also affect macrophyte stoichiometry. Among three tested submerged macrophyte species, two of them (*Potamogeton lucens* and *Vallisneria spiralis*) had lower N and P concentrations and higher C:N and C:P ratios when exposed to higher temperatures (Zhang et al., 2019). This response is in line with the temperature–biogeochemistry hypothesis, which proposes that reduced microbial activity occurring at lower temperatures would result in lower nutrient availability and hence reduced nitrogen and phosphorus concentrations in plant leaves (Aerts & Chapin, 1999). A combination of rising temperatures and eutrophication would therefore be expected to result in an additive rise in macrophyte leaf nutrient concentrations, rendering them more palatable to grazers, with warmer temperatures amplifying this condition through accelerated top–down control (Fig. 1.9). However, in the study by Zhang et al. (2019), the stoichiometry of *Elodea nuttallii* was not affected, indicating that similar to what is observed for eutrophication, the effects of warming on macrophyte stoichiometry may also be species-specific.

Table 1.2. Macrophyte leaf stoichiometry.

Measured parameter	Mean \pm SD ($n = 220$)
C%	36.48 \pm 5.48
N%	2.05 \pm 0.79
P%	0.15 \pm 0.07
C:N	20.54 \pm 8.58
C:P	296.08 \pm 134.53
N:P	14.99 \pm 5.16

Leaf carbon, nitrogen, phosphorus percentages, and their ratios in 220 submerged macrophytes sampled from the arid zone of northwestern China. Source: Gong et al. (2018).

Gong et al. (2018) summarized the leaf carbon, nitrogen, phosphorus and their ratios in percentage of 220 submerged macrophytes samples from the arid zone of northwestern China (Table 1.2). As suggested in the previous sections, the response of macrophyte stoichiometry to environmental stressors may vary between species (Choi et al., 2001; Güsewell & Koerselman, 2002). Identifying how macrophyte stoichiometry in general (all macrophytes) and in particular (depending on species) is changing in response to the combined presence of multiple stressors, here warming, nitrate and pesticides, may thus be challenging. Nevertheless, it is important to include these physiological response factors in the evaluation of macrophyte performance in shallow lakes.

Photosynthetic pigments are lipophilic molecules present in the thylakoid membranes of the chloroplasts in plant cells. In macrophytes, chlorophylls *a* (chl-*a*) and *b* (chl-*b*) are the most important pigments, accompanied by carotenoids. Chl-*a* is the main pigment involved in photosynthesis and absorbs light at the orange–red, violet–blue range of the electromagnetic spectrum (Fig. 1.10), with absorption maxima at ~ 661 and ~ 428 nm (Lichtenthaler & Buschmann, 2001). Exact maxima depend on the solvent used for extraction of these pigments. Chl-*b* is an accessory pigment not directly involved in photosynthesis but produced as an adaptive mechanism when exposed to shaded or low light conditions. The absorption maxima for chl-*b* is at the blue range of the electromagnetic spectrum (~ 647 and ~ 453 nm; Lichtenthaler & Buschmann, 2001). In other words, chl-*b* fills the space between both maxima for chl-*a* (Fig. 1.10), expanding the wavelength useful for photosynthesis.

When light is limited, or plants are shaded, the production of chl-*b* may begin to increase in relation to chl-*a*. However, this physiological shade adaptation usually observed in terrestrial plants and algae involving an increase in the chl *b/a* ratio has not always been observed for macrophytes (Barko & Filbin, 1983).

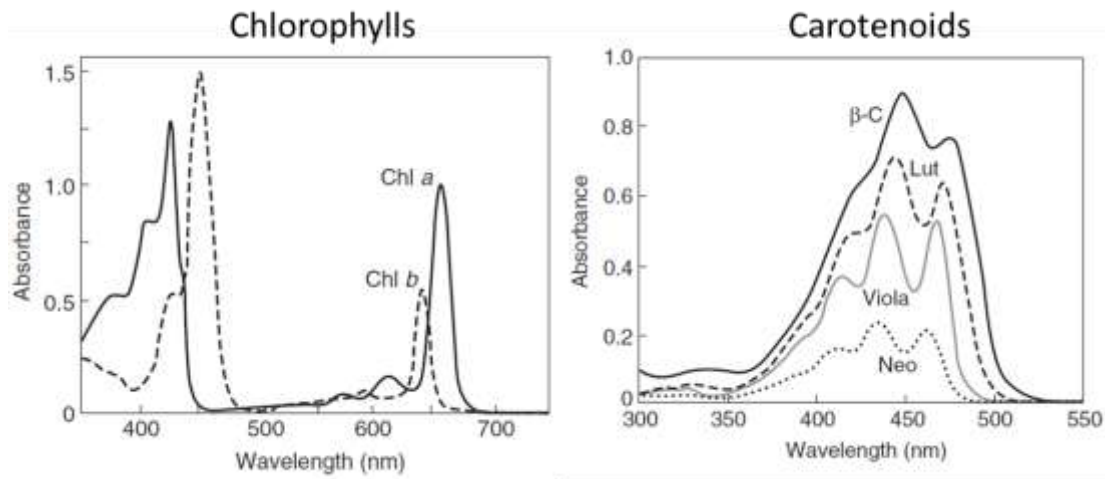


Fig. 1.10. Absorption spectra for chlorophyll-*a*, chlorophyll-*b* and carotenoids. Source: Lichtenthaler and Buschmann (2001).

In shallow lakes, the light environment is influenced by biotic and abiotic factors. Low light stress to macrophytes may result from increasing periphyton and phytoplankton development, shading by neighbouring macrophytes, or resuspension of sediment particles. Changes in the light environment can be induced by eutrophication through increased turbidity, or even by warming through increased top-down control of microalgae by herbivores (Cao et al., 2014; Zhang et al., 2019).

In a study using three submerged macrophytes, *Elodea canadensis*, *Potamogeton nodosus* and *Vallisneria americana*, to determine the influence of light and temperature on their chlorophyll composition, Barko and Filbin (1983) showed that the total chlorophyll concentrations increased with decreasing irradiance and sometimes with increasing temperature. This inverse relationship between total chlorophyll concentrations and irradiance was only observed above the minimum photon flux densities required by macrophytes, which is species-specific (Barko & Filbin, 1983). Although in general, Barko and Filbin (1983) defined low, mid and high photon flux densities as around 100, 600 and 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$, species of *Myriophyllum*, *Egeria* and *Hydrilla* are able to grow at an irradiance below 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Barko & Smart, 1981). Sand-Jensen and Madsen (1991)

showed that the chlorophyll concentrations in relation to light formed an inverted U with the highest chlorophyll concentrations measured at intermediate light levels. An increase in chlorophyll concentrations in macrophytes may signify an optimization of photosynthetic activity in response to ideal light conditions, while a decrease may signify high or low light stress. This may explain the contradictory results of some studies that report a decrease in total chlorophyll concentrations with decreasing irradiance (Marcus, 1980; Van et al., 1977).

Modifications in the photosynthetic pigment concentrations, however, may not only be a direct response to changes in the light environment. Nuttens et al. (2016) observed a decrease in chlorophyll concentrations when exposed to high nitrate levels. They also observed a decrease when exposed to the herbicide mesosulfuron-methyl and an increase with isoproturon suggesting compound-specific responses to these chemicals. Photosynthetic pigments may also reflect a trade-off in response to herbivory, with macrophytes investing in defense rather than in growth. Fornoff and Gross (2014), for example, identified a 34% decrease in photosynthetic pigments in shoots induced by herbivory from the Lepidoptera larvae *Acentria ephemerella*. By reducing the chlorophyll content of their leaves, they may avoid loss of valuable tissue for carbon assimilation.

Carotenoids, like chl-*b*, are accessory pigments that expand the wavelength range of light used to drive photosynthesis. These pigments absorb light in the blue–green region of the visible light spectrum (Hashimoto et al., 2016) and have a broad spectral range of absorption between ~400 and ~500 nm (Lichtenthaler & Buschmann, 2001) (Fig. 1.10). When exposed to harmful levels of excess light, carotenoids may provide a photoprotective effect by receiving energy from chlorophylls (Demmig-Adams, 1990; Hashimoto et al., 2016). Further, they can act as markers of oxidative stress defence to toxic chemicals (Flores-Rojas et al., 2019). When excess light is present, photoprotection processes are set up, through increased carotenoid content on the long term or xanthophyll cycle functioning in the short term (Chalanika De Silva & Asaeda, 2017; Demmig-Adams, 1990). The ratios of chlorophylls to carotenoids may also describe the light stress for the plants. For example, under high irradiance, an increase in carotenoids compared to chlorophylls is observed compared with that observed under low irradiance (Lichtenthaler et al., 2007). In addition to providing information about light stress, carotenoids may be used as a measure of heat, light or, chemical stress (Chalanika De Silva & Asaeda, 2017; Gross et al., 2018; Nuttens et al., 2016; Zhang et al., 2019). Chalanika De Silva and Asaeda

(2017) performed a study to determine the effects of heat stress on the photosynthetic pigments of three submerged macrophytes, *Elodea nuttallii*, *Potamogeton crispus*, *Vallisneria asiatica*. With increasing temperatures (25, 30 and 35°C), the carotenoid production increased in all three macrophyte species. Nuttens et al. (2016) showed changes in carotenoids production at herbicide concentrations above 5 µg L⁻¹, with an increase or decrease depending on the herbicide used.

To summarise, photosynthetic pigments such as chlorophylls and carotenoids as well as their ratios may provide information about the light environments for the macrophytes, potential competition with microalgae or evasion of herbivory, and stress responses to chemicals or warming.

Plants produce a vast array of *secondary metabolites*. Secondary metabolites are derived from primary metabolites or from intermediate compounds produced in the biosynthetic pathway of primary metabolites (Kessler & Kalske, 2018; Pang et al., 2021). They play a supporting role in plant developmental processes, innate immunity (Piasecka et al., 2015; Yang et al., 2018, cited in Pang et al., 2021), response to environmental stresses (Yang et al., 2018) and defence response signalling (Isah, 2019). The four major classes of secondary metabolites in plants are the terpenoids, phenolic compounds, alkaloids, and sulphur-containing compounds (Guerriero et al., 2018).

The most widespread class of secondary metabolites are phenolic compounds. They are carbon based molecules, generally not involving N or other elements, and have been widely studied for their role in allelopathy and as response factors to biotic and abiotic stressors (Dixon & Paiva, 1995). Allelopathy was defined by Rice (1984) as a direct or indirect beneficial or harmful effect of one plant on the other through release of chemicals into the environment (Inderjit, 1996). For example, macrophytes may inhibit the photosynthetic activity of phytoplankton and periphyton, through the production of allelopathic chemicals that form a complex with and inactivate algal extracellular enzymes (Gross, 1999). Gross (1999) hypothesized that the effectiveness of allelopathy in defence strategies against microalgae would be especially high under competition for nutrients and light due to interaction effects observed between resource availability and production of allelopathic chemicals. An important resource for the production of phenolic compounds is phenylalanine, which as an amino acid requires N for its biosynthesis. When the plants are growing under favourable nutrient conditions, particularly when N is available, phenylalanine is used for protein synthesis, while under limiting N levels, phenylalanine is

used for production of phenolics, thereby also reducing competition from other primary producers for these nutrients (Březinová & Vymazal, 2018; Gross, 2003).

While both light and nutrients can affect the production of phenolic compounds by macrophytes, the effect they have on these compounds (increase/decrease) may differ. For example, low light intensities lead to low plant phenolic content, while low nutrient levels lead to high phenolic content (Cronin & Lodge, 2003). This effect of nutrients is in line with the carbon nutrient balance hypothesis, which states that high nutrient concentrations lead to a reduction in the production of carbon-based defence compounds. While phenolic content of macrophytes can provide clues as to the nutrient and light status of lakes, it is still unclear whether they can tell us about direct or indirect stress due to other chemicals such as pesticides. Nuttens et al. (2016) recently showed that total phenolic concentrations were reduced in *M. spicatum* when exposed to the herbicide mesosulfuron-methyl and suggested a link with carbon metabolism.

The presence of grazers can modify the effects of light and nutrients on phenolic concentrations. Cronin and Lodge (2003) linked an increase in phenolic content under optimum conditions of light and nutrients to macrophyte defence against herbivory. This reasoning was based on the plant stress theory predicting that plants are more vulnerable to herbivory under stressful light and nutrient conditions, including N, resulting from lower levels of defences (such as phenolic compounds) or higher foliar nutrient content (more details on carbon/nitrogen balance in section on stoichiometry) (Joern & Mole, 2005; Rhoades, 1983; White, 1984). Similarly, Fornoff and Gross (2014) showed that *Myriophyllum spicatum* when induced by the insect herbivore *Acentria ephemerella* increased production of phenolic content by 20%. Lemoine et al. (2009) studied the effects of snail grazing on three macrophyte species, *M. spicatum*, *E. nuttallii* and *E. canadensis*, and observed an increase in the phenolic content of *M. spicatum* and not of *E. nuttallii* when snails were present, indicating that the chemical defences of macrophyte species against herbivory may vary. On the other hand, Elger and Lemoine (2005) showed that although no direct relationship was observed between phenolic content and plant palatability to the herbivore grazers *Lymnaea stagnalis*, the most palatable macrophyte species consistently exhibited lower phenolic content. Macrophytes with higher phenolic concentrations may therefore better deter herbivores from grazing on them.

The effect of warming on macrophyte phenolic content is unclear, but a recent study showed that two macrophyte species (*Potamogeton lucens* and *Vallisneria spiralis*) had

high phenolic content in relation to N (or low N:phenolic content) at higher temperatures (20 and 25°C compared to 15°C), indicating that higher temperatures may reduce macrophyte palatability. There is still some controversy on the mechanisms behind the defence strategy of macrophytes, but it seems that an increase in phenolic content occurs in response to competition with microalgae and a defence against herbivory. This increase, however, is dependent on the nutrient and light conditions of the environment. The effects of warming on phenolic content would benefit from further studies.

Table 1.3. Total phenolic content of macrophyte leaves.

Species	Mean	SD	Min.	Max.	N
<i>Hottonia palustris</i> L.	12	9	2	31	20
<i>Potamogeton acutiflorus</i> Link	14	4	6	20	23
<i>Potamogeton pectinatus</i> L.	14	11	1	46	19
<i>Elodea nuttallii</i> (Planchon) St. John	18	7	4	36	30
<i>Callitriche platycarpa</i> Kutz.	19	17	83	51	22
<i>Potamogeton gramineus</i> L.	19	9	4	39	19
<i>Potamogeton perfoliatus</i> L.	19	12	26	3	42
<i>Lemna trisulca</i> L.	22	8	6	47	22
<i>Ranunculus circinatus</i> Sibth.	27	9	17	43	13
<i>Ceratophyllum demersum</i> L.	29	16	8	69	30
<i>Utricularia minor</i> L.	30	11	13	52	24
<i>Potamogeton obtusifolius</i> Mert. & Koch	41	10	20	61	35
<i>Potamogeton lucens</i> L.	41	67	18	92	22
<i>Myriophyllum spicatum</i> L.	84	26	41	133	31

Mean values of phenolic content are provided as mg g⁻¹ dry weight. N = number of stands from which each species was sampled. Source: Smolders et al. (2000).

The phenolic concentrations present in macrophytes may vary between species. Table 1.3 presents the phenolic concentrations measured per dry weight for several submerged macrophyte species sampled at different stands (Smolders et al., 2000). As is evident from the table, the submerged macrophyte *M. spicatum* has the highest concentrations of phenolic compounds compared to the other macrophytes. *Myriophyllum spicatum* has in fact been established as a highly competitive species with strong allelopathic potential. Their total phenolic content including its major active allelochemical, tellimagrandin II, comprise up to 10% of the dry weight of *M. spicatum* (Leu et al., 2002).

These concentrations, however, may be higher in the apical region of the plant. Smolders et al. (2000), for example, recorded a range for *M. spicatum* leaves between 84 and 133 mg g⁻¹ dry weight, whereas Hempel et al. (2009) recorded a range for *M. spicatum* apical leaves between 200 and 240 mg g⁻¹ dry weight.

Anthocyanins are a type of phenolic compound that help plants respond to biotic and abiotic stress (Dixon & Paiva, 1995; Fineblum & Rausher, 1997; Winkel-Shirley, 2002). They give flowers their red-to-purple colour and act as a deterrent to herbivory (Kovinich et al., 2015). Similar to carotenoids, these compounds may respond to oxidative stress (Juszczuk et al., 2004). In fact, increases in anthocyanins in the submerged macrophyte *M. spicatum* and the free-floating macrophyte *Lemna gibba* when exposed to arsenic was proposed to be a protective strategy against oxidative stress (Gross et al., 2018; Leão et al., 2013). Anthocyanins have well-known implications for the bright colours they impart to different plant parts as a defence against herbivory (Schaefer & Rolshausen, 2006). However, most literature has focused on terrestrial plants and trees (Juszczuk et al., 2004; Kovinich et al., 2015; Zheng et al., 2020). The literature in terms of anthocyanin production by macrophytes in freshwater systems is limited, and may benefit from an understanding of the role of these pigments in macrophyte response to multiple stressors such as nutrients, warming and pesticides.

4.1. Use as early warning signals of stress

The macrophyte biomarkers described have potential for use as early warning signals because they respond to biotic and abiotic changes in the environment. Macrophyte stoichiometry may be used as an indicator of light and nutrient stress, and may also respond to changes in temperature. Changes in the elemental ratios can provide information on the quality of the macrophyte and their potential palatability to herbivores. An increased palatability, induced for example by high N concentrations, may increase its susceptibility to herbivore grazing, potentially reducing the resilience of the macrophyte-dominated state. Photosynthetic pigments are indicators of light, heat and oxidative stress. An increase or decrease in the production of these pigments may signal a direct or indirect response to the chemical and temperature stress. They can signal changes in competition with microalgae, whose growth may also be affected by the multiple stressors. Phenolic compounds can be used as indicators of nutrient, light or herbivory stress. A decrease in the production of total phenolic compounds can indicate high nutrient concentrations or an increase may indicate a response to stress induced by grazers. The chemical fingerprint as a whole may provide an understanding of macrophyte responses to multiple environmental stressors.

5. The project CLIMSHIFT

This thesis is embedded in a large French–German project called CLIMSHIFT (ANR-DFG) focusing on the impact of multiple stressors (climate warming and agricultural run-off) on freshwater systems composed of different types of primary producers and their consumers. Project partners provide complementary expertise in different areas of freshwater ecology and ecotoxicology, and include the Laboratoire Interdisciplinaire des Environnements Continentaux (LIEC), Metz and the Laboratoire Ecologie Fonctionnelle et Environnement (ECOLAB), Toulouse in France, and Ludwig–Maximilians University Munich; the Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Berlin; and the Helmholtz-Centre for Environmental Research (UFZ), Leipzig in Germany. The project is part of the plan Ecophyto II, and has been additionally funded by Aquacosm, a European network of mesocosm facilities for research on aquatic ecosystems with global collaboration (H2020-INFRIA-Project No. 731065; aquacosm.eu).

6. Description and objectives

The aim of CLIMSHIFT is to identify shifts in the dominance between macrophytes and phytoplankton induced by multiple environmental stressors. As seen in the literature, periphyton may play an intermediary role in the shift between the macrophyte- and phytoplankton-dominated states (Phillips et al., 1978). Benthic and pelagic grazers also indirectly affect macrophytes through top–down control of periphyton and phytoplankton, respectively. To obtain a mechanistic understanding of regime shifts between the primary producer groups, macrophytes and phytoplankton, key functional groups of a two-trophic-level benthic–pelagic community were used.

Shallow lakes are currently exposed to both local stressors such as agricultural runoff comprising nutrients and pesticides and to global stressors in the form of climate warming. Each of these stressors can act on different target organisms affecting either top–down or bottom–up control, directly or indirectly affecting macrophytes. The role of nitrate in regime shifts is beginning to gain attention in the scientific field, but its influence in combination with multiple other stressors such as pesticides and warming is not known. While nitrate may lead to eutrophication, in combination with multiple other co-occurring stressors tipping points may be more easily reached, leading to sudden regime shifts (Scheffer et al., 1993). The main hypothesis proposed by CLIMSHIFT is that climate warming will enhance the negative effects of agricultural run-off (ARO) containing nitrate,

copper and organic pesticides (a mixture of a herbicide, a fungicide and an insecticide) in freshwater systems.

To study this hypothesis, several indoor microcosm experiments and outdoor mesocosm experiments were used. Model ecosystems mimicking miniature systems of natural processes were created by including key functional groups of a two-trophic level benthic–pelagic community that play pivotal roles within shallow lakes. Microcosm experiments were conducted in all partner laboratories, each of which focused on different end points, exposures, or functional groups. Based on the results obtained, these microcosms were then upscaled to a large outdoor mesocosm experiment held at LMU, Munich, and LIEC, Metz, to bridge the gap between the controlled laboratory and variable outdoor conditions. Possible thresholds at which regime shifts may occur between the clear-water state dominated by submerged macrophytes and the turbid state dominated by phytoplankton were investigated. The overall aim of the project was to define safe operating spaces for shallow exposed to multiple stressors by identifying the maximum tolerable ARO threshold concentration in the context of climate warming to prevent regime shifts.

7. Choice of model organisms

Model ecosystems comprised the first two trophic levels typical of shallow lakes, including small fishless ditches and kettle holes, that are common in agricultural landscapes. The first trophic level included the three major primary producer groups, macrophytes, periphyton (benthic microalgae) and phytoplankton (pelagic microalgae). The macrophytes comprised three submerged species often co-occurring in lakes and slow-flowing rivers with a medium ecological amplitude and ability to thrive in eutrophic conditions (Haury et al., 2006; Robach et al., 1996): *Myriophyllum spicatum*, *Potamogeton perfoliatus* and *Elodea nuttallii*. All three primary producer groups, important in shallow lakes, compete with each other for nutrients and light, and are controlled by primary consumers. Benthic filter-feeding mussels and pelagic filter-feeding zooplankton control phytoplankton top–down. In this study, *Dreissena polymorpha* was used representative of the benthic filter feeders, and *Daphnia magna* was used as representative of the pelagic filter feeder. Benthic grazers such as snails control periphyton, but *D. magna* also feeds on this resource. The pond snail *Lymnaea stagnalis* was used as representative of benthic grazers. Most of the chosen organisms are approved test organisms in normalized ecotoxicity assays, chlorophyte algae, *D. magna*, *M. spicatum* and *L. stagnalis*, respectively (OECD, 2006,

2012, 2014, 2016). This is a simplified food web excluding higher trophic levels such as planktivorous or piscivorous fish. Each of these model organisms is described in detail in the following sections:

7.1. Macrophytes

7.1.1. Myriophyllum spicatum

Myriophyllum spicatum otherwise known as Eurasian milfoil is a highly competitive submerged rooted aquatic angiosperm capable of quickly outgrowing other macrophyte species under competitive environmental conditions (Grace & Tilly, 1976; Lind & Cottam, 1969; Smith et al., 1967) (Fig. 1.11). It is found in still or slow-flowing freshwater lakes and rivers, as well as in brackish waters. It has a medium coefficient of ecological amplitude, meaning it is able to survive in different environmental conditions, including very eutrophic conditions (Haury et al., 2006; Robach et al., 1996). It is a perennial plant that can overwinter, as well as perpetuate itself through both sexual (production of pollen from monoecious flowering spikes) and asexual methods (vegetative fragmentation) (Grace & Wetzel, 1978). Its leaves are arranged in whorls of four, with 14 to 20 pairs of leaflets, and shoots can reach up to 4 m long (Grace & Wetzel, 1978). At the surface of the water, it begins to branch profusely, forming a canopy (Grace & Wetzel, 1978), and can shade other slower growing submerged macrophytes and phytoplankton. The optimum temperature for its net photosynthetic activity ranges between 30°C and 35°C (Titus, 1977; Stanley & Naylor, 1972; Van et al., 1976). While it is capable of nutrient uptake by both roots and shoots, the type of inorganic nitrogen in the water determines the route of N assimilation to the plant. For example, when NH₄-N is present in the water, foliar uptake and supply of N to the plant is much higher than from the roots (D. Nichols & Keeney, 2006). *Myriophyllum spicatum* thrives well in slightly alkaline environments (Hutchinson, 1970), which may be due to its ability to use bicarbonate (HCO₃⁻) as a source of carbon for photosynthesis, but it grows better when CO₂ is available (Hussner & Jahns, 2015). Its growth form, vegetative reproductive capacity, efficient use of nitrogen and phosphorus and ability to thrive in a wide amplitude of environmental conditions all render *M. spicatum* a competitive success among other macrophyte species (Grace & Wetzel, 1978). In addition, because of its potential role in allelopathy, it is an interesting model to study in terms of competition among primary producers (Gross, 1999; Gross, 2003; Leu et al., 2002).



Fig. 1.11. *Myriophyllum spicatum*. (A) Full sampled plant, showing stem, leaves and roots, Own picture; (B) Apical stem and leaves on surface of the water, ©Fleur; (C) Flowers produced above the surface of water, ©NatureSpot.

7.1.2. Potamogeton perfoliatus

Potamogeton perfoliatus also called perfoliate pondweed is a perennial submerged macrophyte common in Central European lakes that overwinters through the formation of turions (hardy underground vegetative buds). Similar to *M. spicatum*, this species is found in still or slow-flowing freshwater lakes and rivers. It has a medium ecological amplitude, allowing it to grow in very eutrophic conditions. It can proliferate at temperatures up to 35°C and is capable of physiological adaptation even at 40°C (Anderson, 1969). It can grow in slightly alkaline waters with an optimum pH range between 7.9 and 9.0 (Bayindir & İkinçi, 2020). It has polar leaves, utilizing HCO_3^- from the lower side and releasing OH^- from the upper side (Prins et al., 1982). This utilization of HCO_3^- results in significant increases in free CO_2 concentrations near the lower epidermis (Prins et al., 1982). Its leaves are laminate, arranged alternately on the stem, with one leaf per node. *Potamogeton perfoliatus* may sometimes form a canopy, but unlike *M. spicatum*, its flowers do not always emerge above water (Wolfer, 2008; see Fig. 1.12). This species is sensitive to high nutrient loads but recover when conditions return to the optimum (Wolfer, 2008). It has a clonal structure, with an underground rhizome running horizontally in different directions and ramets emerging from its nodes. It has leafy shoots, which can reach lengths of 4.5 m (Wolfer, 2008; Wolfer et al., 2006; Wolfer & Straile, 2004). Its clonal structure allows an increased phenotypic plasticity by changing morphological structures in response to changes in environmental conditions (Tóth & Vári, 2013; Wolfer et al., 2006; Wolfer & Straile, 2004).

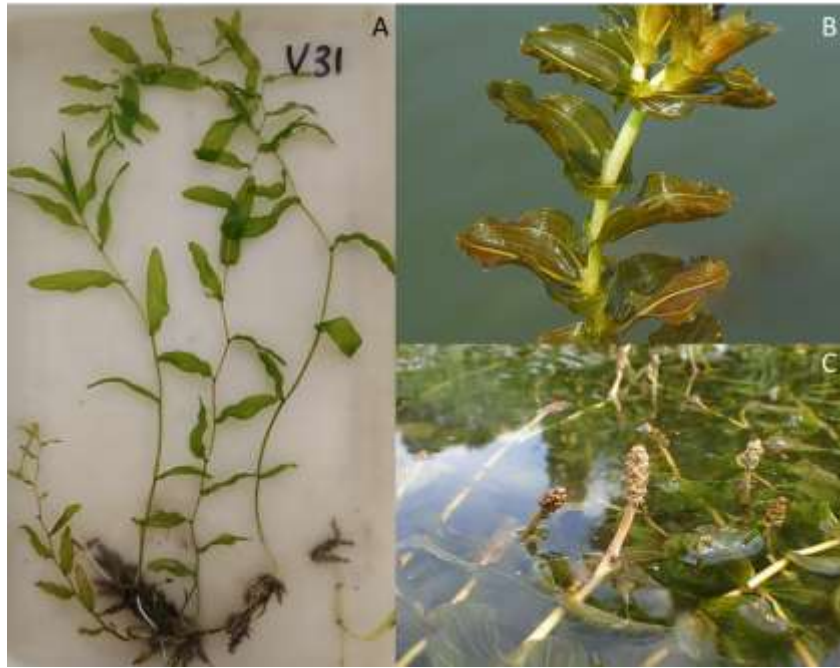


Fig. 1.12. *Potamogeton perfoliatus*. (A) Full sampled plant, showing ramets emerging from rhizome, Own picture; (B) Apical stem and leaves, © [Flora](#) of Cyprus; (C) Flowers produced above the surface of water, ©WikimediaCommons.

7.1.3. *Elodea nuttallii*

Elodea nuttallii also known as Nuttall’s waterweed is a perennial submerged rooted macrophyte native to North America and introduced into Europe in 1939 (Cabi.org, n.d.). This species is found in still or slow-flowing freshwater lakes and rivers. Similar to *M. spicatum* and *P. perfoliatus*, it has a medium coefficient of ecological amplitude and can grow in very eutrophic conditions. *Elodea nuttallii* is also able to use bicarbonate as a source of carbon when CO₂ is limiting (Jones et al., 1993), and has a similar mechanism of HCO₃⁻ utilization as *P. perfoliatus*. Leaves are small measuring between 4 and 15.5 mm with typically three leaves per whorl/node (NativePlantTrust, n.d.). This species grows profusely forming dense monocultures and can reproduce vegetatively through fragmentation similar to *M. spicatum* (see Fig. 1.13). As a result, it is a highly competitive species and can quickly outcompete other macrophyte species (Cook & Urmi-König, 1985; S. Nichols & Shaw, 1986). It overwinters by production of vegetative buds at the stem tips that sink into the sediment (Preston & Croft, 2001). It can reach heights of 3 to 5 m, but shoot growth generally stops when it reaches the water surface, after which it forms a canopy. *Elodea nuttallii* has a great phenotypic plasticity and can thrive in eutrophic conditions, especially when concentrations of nitrogen and ammonia are high (Dendène et al., 1993). Uptake of phosphorus is higher from the shoot than from the roots (Angelstein & Schubert, 2008).



Fig. 1.13. *Elodea nuttallii*. (A) Full sampled plant, showing stem, leaves and roots, Own picture; (B) Source: Canopy and flowers on the surface of the water, © Plantes Invasives des Gardons.

7.2. Microalgae

7.2.1. Phytoplankton

Phytoplankton are microalgae present in the water column, and comprise different groups such as chlorophytes, cyanobacteria and diatoms. Because of their presence in the water column, phytoplankton have better access to sunlight compared with periphyton (Tilman, 1982). The growth of phytoplankton is limited by several micro- and macro nutrients. The most important macronutrients required by phytoplankton are nitrate and phosphate, with diatoms also requiring silicate. The actual community composition present may depend on several abiotic and biotic factors, including limiting nutrients and grazing control (Barcelos e Ramos et al., 2017). Cyanobacteria and dinoflagellates are high in eutrophic lakes, but dinoflagellates are not common in nonstratified lakes (Sommer et al., 1986). The presence of diatoms, on the other hand, has not been consistently linked with trophic status (Sommer et al., 1986). Phytoplankton usually develop rapidly at the end of winter when nutrient and light availability are high, but are grazed by planktonic herbivores, whose populations increase exponentially enabling a high community filtration rate exceeding the reproduction rate of phytoplankton (PEG model; (Sommer et al., 1986)). Grazing control by planktonic herbivores is therefore linked to a clear-water phase in early spring. For the microcosms, four phytoplankton species were chosen: the cyanobacteria *Anabaena* sp. and *Chroococcus minutum*, and the chlorophytes *Scenedesmus obliquus* and

Desmodesmus subspicatus. For the mesocosms, a natural phytoplankton community comprising between 30 to 50 different species was used

7.2.2. Periphyton

Periphyton are microalgae that colonize surfaces, and depending on the type of surface they colonize have different names. Epiphytes are periphyton that grow on plant surfaces, epipsammic are those growing on sand, and epilithic or endolithic grow on or in stones. Similar to phytoplankton, periphyton communities comprise groups such as chlorophytes, cyanobacteria and diatoms and can be limited by nutrients. Because they grow closer to the benthic floor, they may have more access to nutrients and less access to sunlight compared with phytoplankton (Tilman, 1982). For the microcosms, six periphyton species were chosen: the cyanobacteria *Phormidium autumnale* and *Komvophoron* sp., the diatoms *Nitzschia palea* and *Gomphonema parvulum*, and the chlorophytes *Oedogonium* sp. and *Uronema confervicolum*. For the mesocosms, a natural periphyton community was used.

7.3. Primary consumers

7.3.1. Lymnaea stagnalis

Lymnaea stagnalis (see Fig. 1.14A) is commonly known as the pond snail and is found at the margins of shallow, still or slow-moving lakes or rivers where vegetation is dense. It often feeds on periphytic or epiphytic microalgae, decaying plants and detritus, but may also feed on living plants (Kuroda & Abe, 2020; Lance et al., 2006). Its optimum temperature for growth and reproduction is 20°C (Van der Schalie & Berry, 1973), and its rate of consumption increases with rising temperatures (Zhang et al., 2018). Due to its feeding preferences, *L. stagnalis* may play a significant role in promoting macrophyte growth through top-down control of epiphytes, as has been shown for other snail species (Mormul et al., 2018). This species is able to survive under low oxygen environments as it can breathe both through its body wall and through its lungs (Lukowiak et al., 1996). *L. stagnalis* has been used as a biomarker in several toxicological studies where mortality indicators as well as sublethal and sensitive endpoints have been measured (Amorim et al., 2019). It is a sensitive and reliable indicator of ecosystem health particularly because of its well-studied developmental and reproductive biology (Amorim et al., 2019; Fodor et al., 2020). It has also been used to study effects of toxicity resulting from insecticides (Tufi et al., 2015; Vehovszky et al., 2015) and climate warming (Leicht et al., 2017; Teskey et al.,

2012) in addition to other stressor conditions arising from metal and nano-pollution. *Lymnaea stagnalis* has been suggested as a reliable test species by the (OECD, 2016).

7.3.2. Dreissena polymorpha

Dreissena polymorpha (see Fig. 1.14B) also known as the zebra mussel is a freshwater bivalve found in mesotrophic waters, and surviving in a wide amplitude of environmental conditions, including variable dissolved oxygen levels and nutrient conditions. *Dreissena polymorpha* is a filter feeder. It feeds on phytoplankton measuring less than 53 μm (Lori, 2006), and can control lake turbidity. Although its optimum minimum and maximum water temperatures are 17 and 25°C, it has been found tolerating up to 32°C (cabi.org, n.d.). While *D. polymorpha* is considered one of the most invasive freshwater species, its ability to withstand varying environmental conditions and to bioaccumulate or bioconcentrate toxic elements in the water make it a very useful bioindicator and model organism of environmental stress (Benito et al., 2017). Tolerance of *D. polymorpha* has previously been linked to Nickel, and other metals, but this tolerance varies greatly due to a high inter-population variability (Potet et al., 2016). Its sensitivity to multiple stressors such as agricultural runoff and climate warming could provide more information on its usefulness as a bio-indicator.

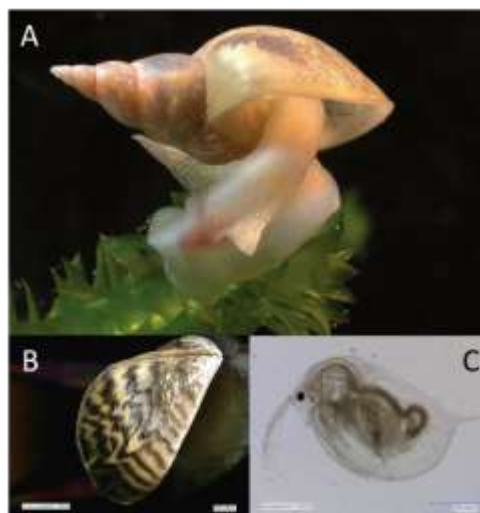


Fig. 1.14. Chosen primary consumers. (A) *Lymnaea stagnalis*, © (Ito et al., 2013); (B) *Dreissena polymorpha*, Own picture; (C) *Daphnia magna*, Own picture.

7.3.3. Daphnia magna

These pelagic water fleas (see Fig. 1.14C) are zooplankton that play a central role in aquatic food webs. They are strongly controlled both top-down and bottom-up (Lampert, 1997). They are found in freshwater and brackish lakes or rivers with

temperatures ranging between 18 and 22°C, although they can tolerate a wider range of temperatures (Ebert, 2005; Vanoverbeke et al., 2007). Higher temperatures and unstable environmental conditions shorten their lifespans, which may average between 40 and 56 days (Animal Diversity Web, n.d.). They usually reproduce in spring but sometimes in summer too, with an average of 6 to 10 eggs per brood, an average of 6 times throughout the female's life span. However, they can also reproduce asexually (Ebert, 2005; Enserink et al., 1995; Ignace et al., 2011). Like *D. polymorpha*, they are filter feeders primarily grazing on phytoplankton and small zooplankton with sizes smaller than 50 µm in diameter, but also on bacteria, detritus and fungal spores (Ebert, 2005; Hanski & Ranta, 1983; Roozen & Lüring, 2001). Because of their strong top-down control on phytoplankton, clear water phases in lakes are often associated with high densities of *D. magna* (Lampert, 1991; Moreno-Ostos et al., 2007). In the absence of fish, in smaller lakes such as kettle holes and peatland ditches, they play an important role in phytoplankton control. These water fleas have been used in the study of toxicity from a range of environmental stressors such as metals (Dave, 1984; Tomasiks & Warren, 1996), detergents (Lal et al., 1984), pesticides (Brausch & Salice, 2011; Shahid et al., 2019; Takács et al., 2017), as well as temperature (Khan & Khan, 2008). Their sensitivity to these environmental stressors, short average lifespan, quick reproduction cycle and easy cultivation make them suitable model organisms in ecotoxicology and ecological research. They have also been suggested as a reliable test organism by the OECD (2012).

8. Choice of stressors

Shallow lakes are exposed to multiple stressors, including local chemical exposure such as nutrients and pesticides and the global stressor climate warming. The Water Framework Directive (WFD) and Nitrates Directive (ND), which is also part of the WFD, have established certain strategies to ensure that the pollution of aquatic ecosystems is minimized and to “achieve good ecological and chemical status at European level” (European Commission, 2016). The EU has set environmental quality standards (EQS) for priority substances, among which are the three typical pesticides used in agriculture (herbicides, insecticides and fungicides). The ND identifies surface waters with nitrate concentrations of more than 50 mg L⁻¹ (~11.2 mg L⁻¹ N-NO₃) to be polluted or at risk of pollution, and puts in place strategies and action programs for good agricultural practice to ensure these limits are not exceeded. In addition to the concern from these local chemical stressors is the global stress from rising environmental temperatures. These

multiple stressors acting simultaneously on shallow lakes may have potential effects for the resilience of the macrophyte-dominated state.

8.1. Simulation of agricultural runoff

Agricultural runoff may enter shallow lakes following rainfall events. They contain nutrients, metals and organic pesticides depending on the specific phytoprotective regime applied in nearby agricultural sites. For this study, a combination of three types of organic pesticides, one inorganic pesticide and nitrate was used as representative of agricultural runoff. The organic pesticides comprised the herbicide terbuthylazine, the insecticide pirimicarb and the fungicide tebuconazole, and the inorganic pesticide was the metal copper added as copper sulphate. Concentrations of the pesticides were defined based on experimental evidence at UFZ, Leipzig (BHP and MSJ, unpublished data), and published species sensitivity distribution data and effective concentration (EC) values. The chemical cocktail was designed based on its potential to affect different organism groups based on the mode of action of each component: terbuthylazine can affect primary producers through photosynthesis inhibition (Busch et al., 2016), pirimicarb can affect invertebrate grazers through acetylcholinesterase inhibition (Ishimota & Tomiyama, 2020), tebuconazole can affect aquatic microbial communities and thereby important ecosystem function such as litter degradation (Artigas et al., 2014; Bundschuh et al., 2011; Rasmussen et al., 2012). Copper may affect all groups of organisms (Brix et al., 2011; Dave, 1984; Le et al., 2020; Pistocchi et al., 1997; Roubeau Dumont, 2019). High concentrations of nitrate may lead to eutrophication, promoting strong periphyton development or phytoplankton blooms (Barker et al., 2008; Sagrario et al., 2005). Such complex mixtures are commonly found in agricultural waters, but studying their effects at the population or ecosystem level is challenging, and results from controlled laboratory conditions cannot directly be extrapolated to real lakes. Binary mixtures provide predictable outcomes, but tertiary mixtures and higher may produce unpredictable outcomes. In fact, when agrochemicals were tested as binary mixtures on macroinvertebrate populations in small ditches, the outcomes were usually predictable from single stressor exposure experiments, but tertiary mixtures produced outcomes that deviated from effects produced by the three individual stressors (Barmantlo et al., 2018). Therefore, potential synergistic, additive, antagonistic or reversed effects (Côté et al., 2016; Jackson et al., 2016) of three or more stressors may call for a re-evaluation of the concentration limits identified by single-stressor studies.

8.1.1. Terbuthylazine

Terbuthylazine is a triazine herbicide (see Fig. 1.15A) structurally similar to atrazine and used widely in farming for broad-spectrum weed control. In Europe, it is used especially for corn and sorghum cultivation. Terbuthylazine is regarded an environmental contaminant and xenobiotic (ChEBI, n.d.), and is one of the most frequently detected herbicides across several countries in the European Union (Vonk & Kraak, 2020). Although it has been detected in rain and snow samples, it more easily absorbs onto soil particles. Its downward mobility is limited and as a result, mobilization of this herbicide from agricultural sites into aquatic systems is usually by surface runoff (Ulrich et al., 2013). Here, it may pose a threat to aquatic life. In particular, they may affect macrophytes and microalgae by inhibiting electron transport at the photosystem II receptor site. Photodegradation may occur both on soil-attached particles as well as in water exposed to sunlight, although volatilisation is unlikely. Environmental concentrations of terbuthylazine are estimated below $1 \mu\text{g L}^{-1}$ (Cedergreen et al., 2004; Dörfler et al., 1997; Mikulíková et al., 2011; Pereira et al., 2017) but sometimes concentrations as high as $13 \mu\text{g L}^{-1}$ are measured (Cedergreen et al., 2004). Macrophyte communities have not been shown to respond to terbuthylazine at the low environmental concentrations of 1 to $3 \mu\text{g L}^{-1}$ but may respond when co-occurring with other herbicides (Cedergreen et al., 2004). Other aquatic organisms may be affected at much higher concentrations of terbuthylazine. In a chronic toxicity test to evaluate the effect of terbuthylazine on the common carp *Cyprinus carpio*, the general growth and development negatively responded to concentrations above $520 \mu\text{g L}^{-1}$ and no effects observed at $160 \mu\text{g L}^{-1}$ (Štěpánová et al., 2012). In another study, concentrations of $60 \mu\text{g L}^{-1}$ affected fish physiology by reducing their mean corpuscular volume and mean corpuscular haemoglobin (Mikulíková et al., 2011). Therefore, although its mode of action targets the photosynthetic processes in plants, it can have negative effects on other non-target organisms as well.

8.1.2. Pirimicarb

Pirimicarb is a carbamate insecticide (see Fig. 1.15B) whose mode of action is inhibition of acetylcholinesterase. It is used to control aphids in vegetable and cereal crops. Similar to terbuthylazine, it is considered an environmental contaminant and a xenobiotic. Its use as a spray causes direct release into the environment including into water sources. It has a variable mobility in soil but absorbs better onto clayey soils and those low in organic matter. Although volatilization is not expected from water, it is photolysed with a short half-life of 1 day in water receiving sunlight (PubChem, n.d.). Pirimicarb has been

detected in groundwater in Denmark at concentrations of $0.1 \mu\text{g L}^{-1}$ (Spliid & Køppen, 1998), and up to $0.22 \mu\text{g L}^{-1}$ in stream water in Sweden (Kreuger et al., 1999). In an experiment without the inclusion of sediments, Kusk (1996) found new-born *D. magna* to survive for 72-h at pirimicarb concentrations of $20 \mu\text{g L}^{-1}$. Concentrations as high as $100 \mu\text{g L}^{-1}$, however, affected number of offspring and weight of *D. magna*, and concentrations of $70 \mu\text{g L}^{-1}$ resulted in reduced number and weight of offspring (Andersen et al., 2006). In combination with other chemical stressors and nutrient stresses, toxic effects of pirimicarb may occur at much lower concentrations.

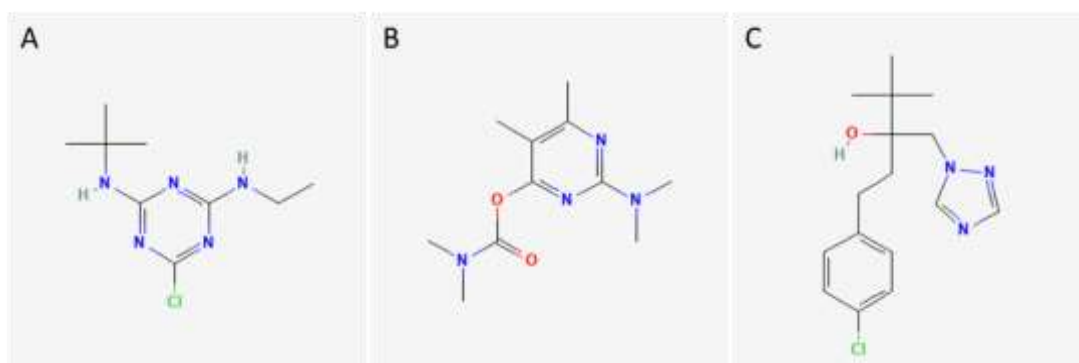


Fig. 1.15. Structure of chosen organic pesticides. (A) Terbutylazine, ©PubChem; (B) Pirimicarb, ©PubChem; (C) Tebuconazole, ©PubChem.

8.1.3. Tebuconazole

Tebuconazole is a triazole fungicide (see Fig. 1.15C) used to inhibit fungal growth in agricultural sites as well as on woods and plastic (NCBI, n.d.). In agriculture, they are used for cereals, peanuts, oilseed rape, and fruits (Meister & Sine, 2009). They are used for seed treatment as well as a foliar spray. Tebuconazole is not very mobile in soil, and may attach to sediment particles when released into water. Studies on toxicity of tebuconazole on microbial communities or degradation activity have shown no effects at concentrations of $20 \mu\text{g L}^{-1}$ (Pesce et al., 2016) but some effects at concentrations of about $33 \mu\text{g L}^{-1}$ (Artigas et al., 2014). In another study, Zubrod et al. (2019) showed that the aquatic decomposer-detrivore system was affected at $65 \mu\text{g L}^{-1}$ tebuconazole, which resulted from a reduced physiological fitness of the leaf-shredding gammarid *Gammarus fossarum* as well as reduced fungal biomass, both of which affect leaf-litter breakdown and therefore have negative effects for ecosystem health.

8.1.4. Copper

Copper is an essential trace metal that functions as an enzyme cofactor and catalyst for several biological functions, but when present at concentrations exceeding the needs of the organisms, it can become toxic (Mahamoud Ahmed et al., 2018). Copper is widely used as a fungicide. In a 2018 study gathering data from 25 EU member states, 16 states had water bodies that measured copper concentrations exceeding the environmental quality standard. This accounted for 808 water bodies among 111,105, that is, 0.7% of all water bodies (European Environment Agency, 2018). Copper is toxic to macrophytes and can inhibit photosynthesis, decrease growth and even cause chlorosis and necrosis (Costa et al., 2018). However, the toxicity of copper to macrophytes may vary between different species and even genotypes. For example, depending on the genotype, *M. spicatum* responded to concentrations between 42 and 296 $\mu\text{g L}^{-1}$ (Roubeau Dumont, 2019). Copper is toxic to microalgae at low concentrations. In fact, copper is often used as an algacide (Effler et al., 1980). Aquatic invertebrates, including gastropods, crustaceans and rotifers, are also sensitive to copper and may respond at concentrations of $\sim 25 \mu\text{g L}^{-1}$ (Joachim et al., 2017). Finally, copper affects microbial communities by modifying their structure and most of their functions (Mahamoud Ahmed et al., 2018). An effect of copper on microbial communities can have negative consequences for ecological and ecosystem functioning of shallow lakes.

8.1.5. Nitrate

While small increases in nutrients may promote growth of macrophytes, submerged rooted macrophytes are generally not nutrient-limited, as they can take up nutrients from the sediment (Moss, 1998). However, high nutrient concentrations can have negative indirect effects on macrophyte by promoting the growth of microalgae, which are limited by nutrients (Jeppesen, 1998). Among the most important macronutrients limiting microalgae are N and P. The role of N has only recently begun to gain interest as a risk factor for eutrophication. High levels of N in shallow lakes may affect macrophyte species richness (Barker et al., 2008; Moss, 1976; Phillips et al., 2016; Phillips et al., 1978; SAGRARIO et al., 2005). The most common form of N, nitrate, is not directly toxic to macrophytes. However, at high concentrations, periphyton or phytoplankton development may be promoted, indirectly affecting macrophyte growth (Barker et al., 2008). This influence of nitrate on microalgae can modify the overall community structure including that of upper trophic levels by, for example, modifying the zooplankton feeding behaviour (Carpenter & Kitchell, 1984). Therefore, while the bottom-up effects of nitrate may be strongest on

the primary producers, increased nitrate eutrophication will also affect higher trophic levels.

8.2. Elevated temperature

Elevated temperatures can affect organisms differently, depending on their optimum temperature requirements as well temperatures to which they are acclimated. Higher temperatures promote primary producer growth (Barko & Filbin, 1983; Zhang et al., 2019), and metabolic activity for most poikilotherms (Levinton, 1995). However, these effects may be counteracted at temperatures that exceed the respective species optimum (Maceda-Veiga et al., 2015; Rooney & Kalff, 2000), and the overall effect on the ecosystem will depend on each species or functional group's sensitivity to warming. Based on the reports by the Intergovernmental Panel on Climate Change (IPCC) that average global temperatures could rise 4°C above pre-industrial levels (RCP 8.5 scenario, IPCC, 2014), we compared the effects of current average summer temperatures (22°C) with a projected 4°C increase (26°C).

The described organic and inorganic chemicals are pertinent in agricultural sites and eventually find their way into aquatic ecosystems through surface runoff or groundwater leaching. Studying their individual and combined effects on macrophytes provides a more in-depth understanding of the potential risk for regime shifts in shallow lakes.

9. Objectives of the thesis

The objectives of my thesis have been developed within the framework of the project CLIMSHIFT. As part of the project, my main objective was to identify how ARO and warming would affect macrophyte development. The focus of this thesis is therefore the macrophytes, although taking into account other species and functional groups within the model ecosystem is integral to understanding the effects of the stressors on the macrophytes. First, the effects of nitrate and pesticides were disentangled from the overall effects of ARO on the macrophytes. A mechanistic understanding of the stressor effects was sought by identifying direct and indirect interactions between the different functional groups. This was followed by a study to evaluate whether the ARO effects observed in the controlled laboratory could be replicated in variable outdoor conditions. Finally, the physiological response of macrophytes to the multiple stressors was studied by measuring

their main biomarkers (stoichiometry, photosynthetic pigments, and secondary metabolites). In summary, the three main objectives of my study were:

- (1) To determine the individual and combined effects of nitrate, pesticides and climate warming on macrophytes
- (2) To determine whether upscaling from micro- to mesocosms would preserve the macrophyte response
- (3) To determine whether the stressor effects could be detected in the macrophyte physiology

CHAPTER 2: APPROACH

The CLIMSHIFT project combines ecotoxicological and ecological knowledge to identify and disentangle direct and indirect effects of the multiple stressors. Several controlled laboratory microcosm experiments were performed by the different project partners, aiming to answer specific questions, such as dose–response effects of the ARO (the Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Berlin); effects of single ARO exposures versus pulsed exposures (Laboratoire Ecologie Fonctionnelle et Environnement, Toulouse); nitrate and pesticide effects on primary producers (Laboratoire Interdisciplinaire des Environnements Continentaux [LIEC], Université de Lorraine, Metz); direct ARO effects on macrophytes, zooplankton and snails (Université de Lorraine, Ludwig-Maximilians University Munich (LMU), Laboratoire Ecologie Fonctionnelle et Environnement); and effects of ARO on the primary producers when exposed via water column or sediment (Leibniz-Institute of Freshwater Ecology and Inland Fisheries), to name a few. Following these microcosms, data were analysed, including both biotic (growth, physiology) and abiotic end points (water chemistry). These data were then used for modelling (Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Berlin) (see Fig. 2.1 for the general approach). Discussions of these results and models between the project partners then enabled tailored design of the two large outdoor mesocosm experiments, which were conducted at LMU and LIEC. The mesocosms in LMU were performed to identify a threshold concentration of the ARO in the context of climate warming above which regime shifts would occur, and the type of freshwater system modelled was a shallow lake. The mesocosms in LIEC were conducted to study the effects of ARO and warming in slow-flowing streams. Although the mesocosm experiment in LIEC was scheduled to be a part of this thesis, the experiment was delayed by a year due to the COVID-19 pandemic, and therefore could not be included.

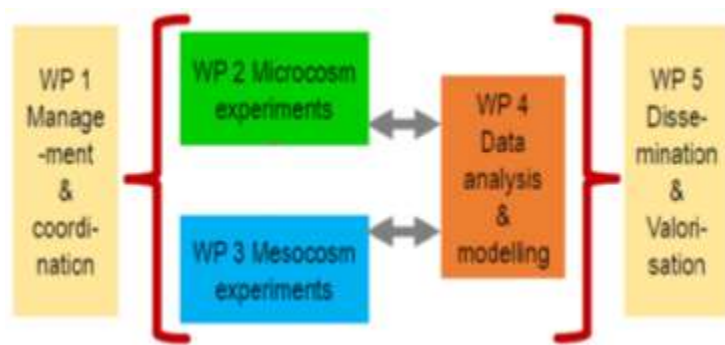


Fig. 2.1. General approach of the project CLIMSHIFT. Several microcosm and mesocosm experiments were conducted, modelling studies was performed on the obtained data, followed by communication of and valorisation of the results.

This thesis focuses on the data obtained from three microcosm experiments and one mesocosm experiment (details follow).

In short, the microcosms comprised cylindrical glass vases filled with 8L of Volvic water and a crystallizing dish insert filled with sediment. The mesocosms comprised LDPE Innoplast silo foil bags filled with 640 L of Munich well water with the base filled with a 10 cm sediment layer (details of the set-up are presented in Chapter 3) (see Fig. 2.2). Polystyrene strips were inserted into the microcosms and mesocosms to measure the development of periphyton. Based on the question of each study, the complexity of the benthic–pelagic community varied (only primary producers or both primary producers and primary consumers) between the microcosms, but always included only lab-cultured strains/species. The mesocosms comprised both primary producers and primary consumers. Since they were built for higher complexity, natural benthic microalgae and plankton communities were used, rather than lab-cultured strains/species.

Macrophyte data were sampled and analysed from the two microcosm experiments performed at Laboratoire Ecologie Fonctionnelle et Environnement in Toulouse to determine how single and pulsed doses of the ARO affect macrophyte growth and physiology. The third microcosm experiment was performed at LIEC to disentangle the effects of nitrate and pesticides from the combined ARO, and the effects on the macrophytes were studied. The mesocosm experiment was then conducted at Ludwig–Maximilians University Munich (where I participated for a 3-month period when the experiment was being run), and the potential effects of ARO on the macrophytes were studied. A more detailed description of each of these microcosm and the mesocosm

experiments is provided in the next sections. This is followed by a general overview of the sampling strategy and studied end points. Fig. 2.3 provides a graphic overview of the four experiments.

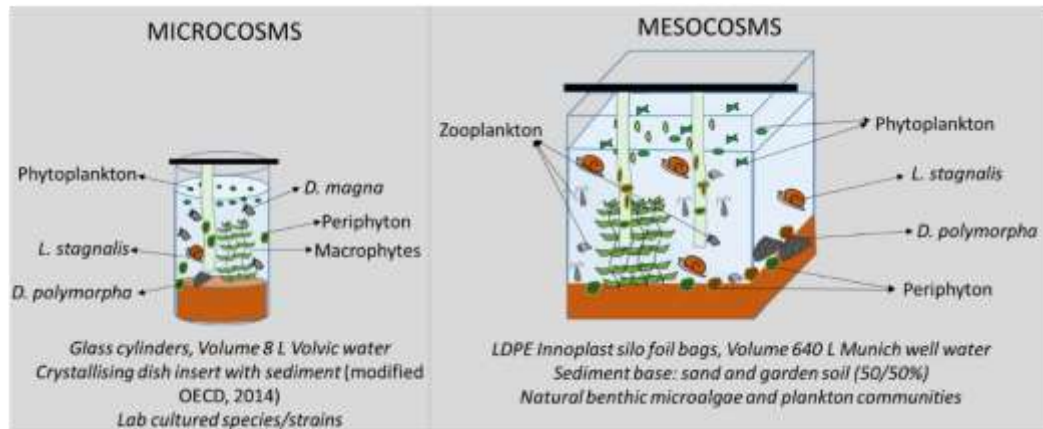


Fig. 2.2. Comparison of ecosystem models between the microcosm and mesocosm experiments.

L. stagnalis = *Lymnaea stagnalis*, *D. polymorpha* = *Dreissena polymorpha*, *D. magna* = *Daphnia magna*.

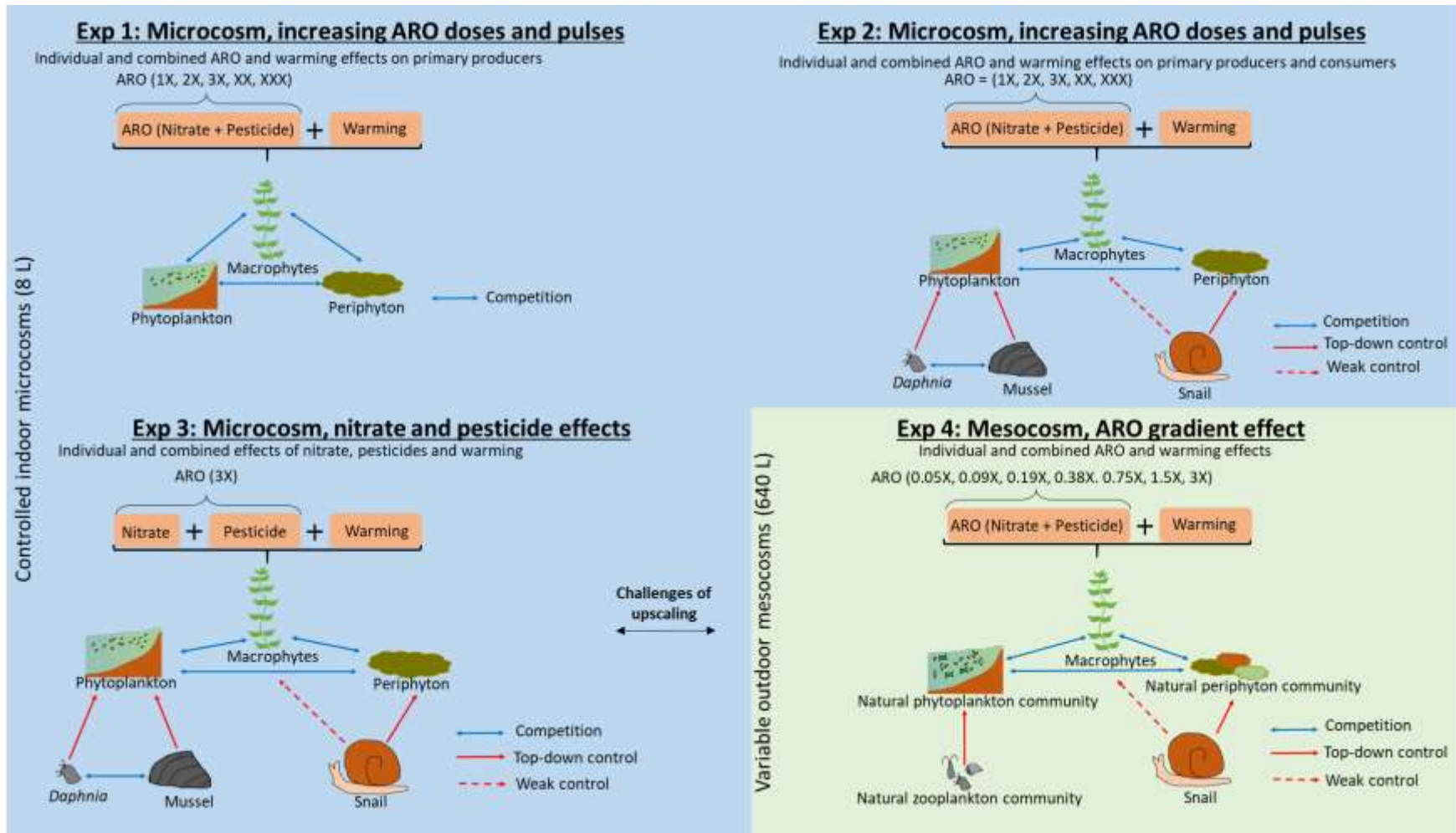


Fig. 2.3. Overview of the microcosm and mesocosm experiments conducted within the project CLIMSHIFT.

1. Experiment 1: Increasing doses and pulses of agricultural runoff on benthic–pelagic systems without consumers

Primary producers are in competition with each other for resources. An impact of the stressors on one of the three major primary producer groups, that is, macrophytes, phytoplankton or periphyton, can indirectly affect the other primary producer groups. In terms of ARO, the herbicides such as terbuthylazine and copper, as well as nitrate, may have a larger role to play in affecting primary producers through their mode of action and bottom–up control, respectively. To identify how the stressors, ARO and warming, affect the primary producer groups and the competition between them, a microcosm experiment was designed using only these three major groups. The exposure of ARO in shallow lakes depends on rainfall events. Single ARO exposures or several pulsed exposures can occur, depending on the intensity and frequency of rainfall. To identify the effects of these exposure scenarios, the primary producers were exposed to several increasing single doses and pulses of the ARO (C, X, 2X and 3X, XX and XXX). In total, 60 microcosms were set-up, with five replicates per treatment (see Fig. 2.4). The temperature of half the microcosms were maintained at an ambient summer temperature of 22°C, while the other half were subjected to a 3.5°C temperature increase from the ambient. The macrophytes were introduced first into the microcosms, allowing them to acclimate for 9 days before adding the microalgae. The stressors were applied 17 days after the macrophytes were introduced into the system.



Fig. 2.4. Photo of microcosms in Experiment 1. Plastic strips were hung vertically by attaching to a pipette placed horizontally over the rim of the glass cylinders. The lower ends of the cylinders were covered in black plastic to mimic light exposure in real lakes.

2. Experiment 2: Increasing doses of agricultural runoff on benthic–pelagic systems with consumers

In addition to the interactions and competition for resources, primary producers are also controlled top–down by grazers. Benthic grazers such as snails feed on periphyton and epiphyton. Benthic filter-feeders such as mussels control phytoplankton, as do the pelagic filter-feeders such as *Daphnia* and other zooplankton. These consumers may also be affected by ARO and warming, and an impact on them will indirectly affect the primary producers through trophic interactions. In this case, the insecticide present in the ARO are expected to affect the primary consumer growth through their mode of action, and likely indirect impact the macrophytes. This microcosm experiment was similar to Experiment 1, except for the inclusion of a second trophic level with the benthic and pelagic grazers or filter-feeders (see Fig. 2.5). The macrophytes were introduced into the system first. The microalgae and consumers were added 3 and 7 days after the macrophytes, and the stressors 10 days after macrophyte introduction. In this experiment as well, the half of the microcosms were heated to 3.5°C above the ambient 22°C. In the first two experiments, the temperature of the heated treatments was performed using heating mats regulated with a thermostat maintaining a 3.5°C temperature difference between the heated and unheated microcosms.



Fig. 2.5. Photo of microcosms in Experiment 2. Microcosms on the left and right are placed above heating mats and represent the heated treatments. Phytoplankton development is visible. Plastic strips were hung vertically by attaching to a pipette placed horizontally over the rim of the glass cylinders. The lower ends of the cylinders were covered in black plastic to mimic light exposure in real lakes.

3. Experiment 3: Disentangling the nitrate and pesticide effects from the full ARO cocktail

The ARO defined in our experiments comprised a pesticide mixture (organic pesticides and copper) and nitrate. Both these major ARO components can affect primary producers in different ways. Pesticides for example can directly affect primary producers by inhibiting photosynthesis (herbicides), or they may indirectly affect them through modified top-down control (insecticides). Nitrate on the other hand may more strongly influence bottom-up control. To disentangle the pesticide and nitrate effect, a benthic-pelagic community was exposed to each of these ARO components separately and together. In this experiment, the microalgae were introduced first into the system, followed by the macrophytes 2 days later, the consumers 3 days after the microalgae, and the ARO 4 days after the microalgae were introduced into the system. A total of 40 microcosms were set-up with five replicates per treatment (Control, Nitrate, Pesticide and ARO). Each treatment was replicated at two temperatures, an ambient 22°C and a +3.5°C increase. Heating was performed by placing the glass cylinders in large temperature-controlled water baths (see Fig. 2.6.).

For all three microcosms, the exposure period was between 3 and 4 weeks. Light intensities, measured as photosynthetically active radiation (PAR), were maintained in the range of 60 to 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The highest ARO concentration (3X) from Experiments 1 and 2 was identical to the ARO concentration used in Experiment 3.

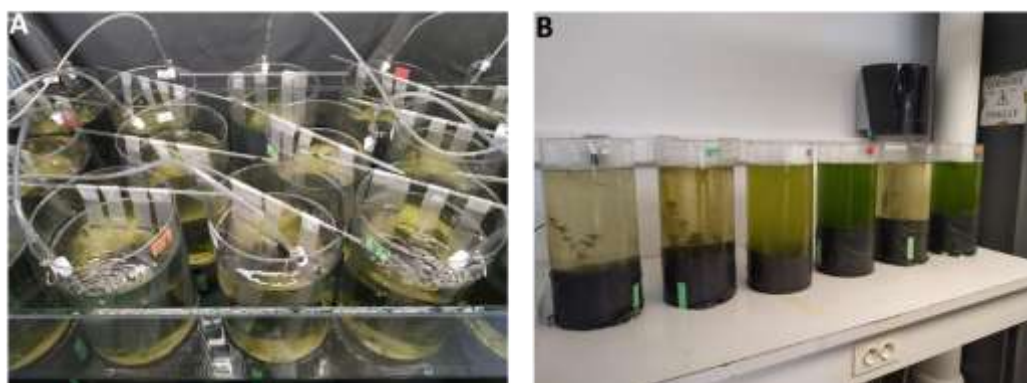


Fig. 2.6. Photos of microcosms in Experiment 3. (A) Set-up of the microcosms placed within the water bath. Plastic strips were hung vertically by attaching to a pipette placed horizontally over the rim of the glass cylinders. Tubes fitted with Pasteur pipettes and inserted into the vases at one end, and attached to an air pump at the other end allow aeration of the system. (B) Microcosms during the mid-exposure period. Back plastic covers the base of the cylinders to mimic light exposure in real lakes. Some clear-water and some turbid-water states, depending on treatment, are visible.

4. Experiment 4: Upscaling to large outdoor mesocosm using an ARO gradient approach

Outdoor mesocosms allow for a higher complexity and therefore more closely reflects a real ecosystem. Because they are performed outdoors, there are several variable environmental and biological parameters, not to mention confounding factors, that can affect the outcome of the experiment. Making causal relationships is not very straightforward. However, if a stressor effect on a benthic–pelagic community identified under controlled conditions is replicated in large outdoor experiments, despite the higher stability provided by the increased biological complexity, a strong indication of the risk for real ecosystems may be provided. To identify whether effects observed in the microcosms could be replicated in mesocosms with higher biological complexity, a benthic–pelagic community comprising primary producers and primary consumers were exposed to the ARO. To increase the biological complexity, natural benthic microalgae and plankton communities were collected from eutrophic lakes rather than using lab-cultured strains. A gradient approach was used for the mesocosms, to identify whether ARO concentration-dependent community effects could be observed. The highest concentration was set as 3X, similar to those from the microcosms, and was then diluted in 6 steps at a 1:1 factor. Altogether, 16 mesocosms were set-up, with a control and 7 ARO treatments, and each replicated at ambient environmental temperature and a +3°C increase. The exposure period was 8 weeks. This experiment enabled the investigation of macrophyte development under more natural conditions but also provided certain challenges due to the variable and complex outdoor conditions. See Fig. 2.7 for examples of the mesocosms.

The mesocosms were subjected to natural variations in light and temperature. The light and temperature environment of each mesocosm enclosure also varied based on the development of the organisms, specifically the primary producers. The minimum temperature in the ambient microcosms was ~15°C in mid-May during the set-up and the maximum reached was ~31°C in mid-June during the heatwave in summer 2019. However, the difference between the ambient and the warm mesocosms was always maintained at a constant 3°C by a powerful heating system (see supplementary data, Chapter S1). The maximum light intensity at the surface of the mesocosms may have ranged between 1000 to 2000 $\mu\text{mol m}^{-2} \text{s}^{-1}$, as they were exposed to natural light.

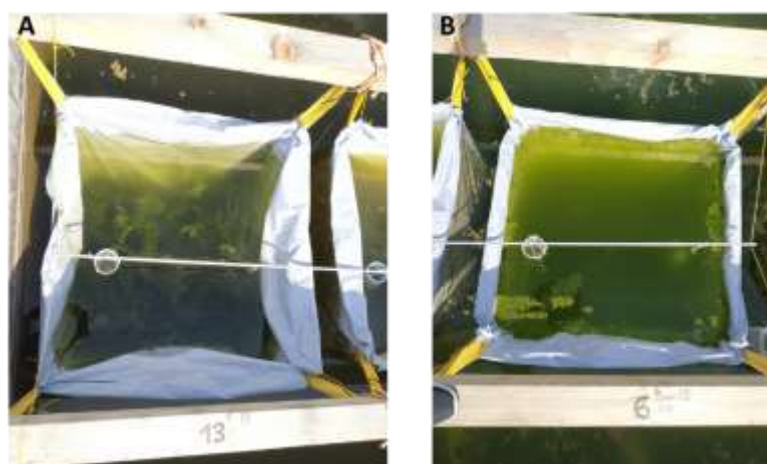


Fig. 2.7. Photos of the mesocosms in Experiment 4. Ambient and heated mesocosms were submerged in two large artificial ponds each, one of which was heated to obtain a 3°C increase above ambient temperature in the mesocosms. (A) Clear-water macrophyte-dominated mesocosm enclosure. (B) Turbid, phytoplankton-dominated mesocosm enclosure.

5. Overview of Sampling and Data Analyses

In this section, a general overview of end-of-experiment sampling for the microcosms and mesocosms are provided. Temporal sampling varied between experiments. Further details on the specific sampling scheme for each experiment can be found in Chapters 3 to 5.

The sampling scheme was designed to ensure minimum destruction of the system while enabling complete sampling/measurement of biological and physico-chemical parameters. In the microcosms, water chemistry was first measured, and depending on the particular microcosm, this included pH, conductivity and saturated O₂ (multi-parameter analyser; WTW Multiline 3410) phytoplankton optical density at 663 nm (Cary 50, Varian® Agilent, USA), nutrient (ion chromatography; Dionex ICS 1100), copper (atomic absorption spectroscopy; Varian SpectrAA 800 Zeeman) and pesticide analytics (liquid chromatography–mass spectrometry; Thermo-Scientific, USA [performed by project partners at UFZ, Leipzig]). This was followed by filtration of water over GF/F filters to use for phytoplankton pigment analyses and stoichiometry. The water was filtered over a 500-µm beaker to remove the *Daphnia* if included in the study. *Daphnia* were counted and their lengths measured (digital microscope; VHX-6000; Keyence, Bois-Colombes, France). The polystyrene strips were removed and the periphyton brushed into Volvic water, after which it was also filtered over GF/F filters for periphyton pigment analysis and stoichiometry.

For both periphyton and phytoplankton, pigment analyses were performed using high performance liquid chromatography-diode array detector (HPLC-DAD, UHPLC Ultimate 3000 Rs THERMO; following the method by Capdeville et al. 2019), and carbon and nitrogen using a CHNS elemental analyser (Carlo-ERBA Na 2100 CE Instrument). Phosphorus was measured following alkaline digestion of the samples and subsequent colorimetric analysis using an automated analyser (Konelab 420-XTi, Thermo Fisher Analytics).

When present, the consumers (snails and mussels) were removed, counted and measured for length and/or biomass.

The macrophytes were sampled in the end. They were removed gently with the roots from the sediment, rinsed with water and placed on trays. Pictures were taken (Fig. 2.8A). Shoot and root lengths were measured with a ruler. The apical 10 cm portions were separated from the plant, weighed and then lyophilized. For *M. spicatum* and *P. perfoliatus*, leaves and stems were separated. For *E. nuttalli*, separation into leaves and stems was not feasible within the available time, due to the large number and density of leaves. The apical portion is the more metabolically active part of these canopy-forming macrophytes and was chosen for analyses of plant physiology (See Fig. 2.8B). For *M. spicatum* and *P. perfoliatus*, only apical leaves were used for physiological analyses, while for *E. nuttalli* the whole apical portion (stem and leaves) was used. Before chemical analyses, their dry weights were also measured. Pigment and secondary metabolites were analysed using a spectrophotometer (Cary 50, Varian® Agilent, USA). Carbon, nitrogen and phosphorus were analysed from aliquots of the apical leaves using the same methods as described for microalgae. The remaining aboveground and belowground plant material were weighed separately per species and vase. These samples were then dried in an oven for 48 h at 60°C, and their dry weights noted.

At the end of the mesocosm experiment, individual mesocosms were retrieved by a gantry crane and placed upright using a scaffold to facilitate sampling of the different compartments (Fig. 2.9). The sampling scheme for the mesocosms followed a similar order as in the microcosms, beginning with the water and phytoplankton analyses, periphyton, invertebrates and finally the macrophytes. However, phytoplankton chl-*a* was measured using a multispectral fluorometer (AlgaeLab-Analyser, bbe Moldaenke GmbH, Schwentingen, Germany) and carbon and nitrogen using an elemental analyser (varioMICRO Cube, Elementar Analysensysteme GmbH, Langenselbold, Germany).

Macrophytes were much taller than in the microcosms and were placed in large plastic sheets to enable measurement of their heights (Fig. 2.10).

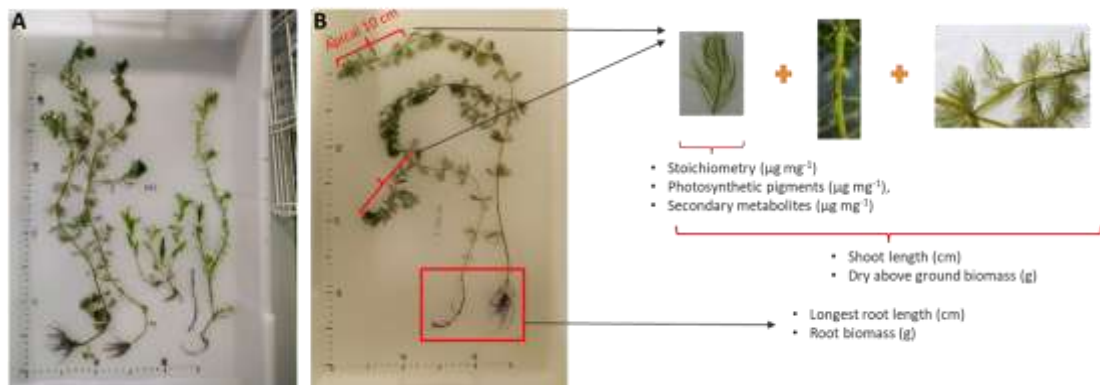


Fig. 2.8. Sampling of macrophytes. (A) *Myriophyllum spicatum*, *Potamogeton perfoliatus* and *Elodea nuttallii* sampled from a single microcosm unit and placed on a white tray for measurement of shoot and root lengths. (B) Measurement of the different plant parts and sampling of the apical portion for analyses of macrophyte chemistry.



Fig. 2.9. Mesocosm retrieval and sampling. (A) Group photo taken after retrieval of the last mesocosm enclosure using a gantry crane. (B) Image of a mesocosm placed upright using a scaffold. In image, project head Elisabeth Maria Gross (Laboratoire Interdisciplinaire des Environnements Continentaux, Metz, France) watering the outer wall of the mesocosm enclosure. In the back, Nora Kipferler from Ludwig–Maximilians University Munich, Germany.

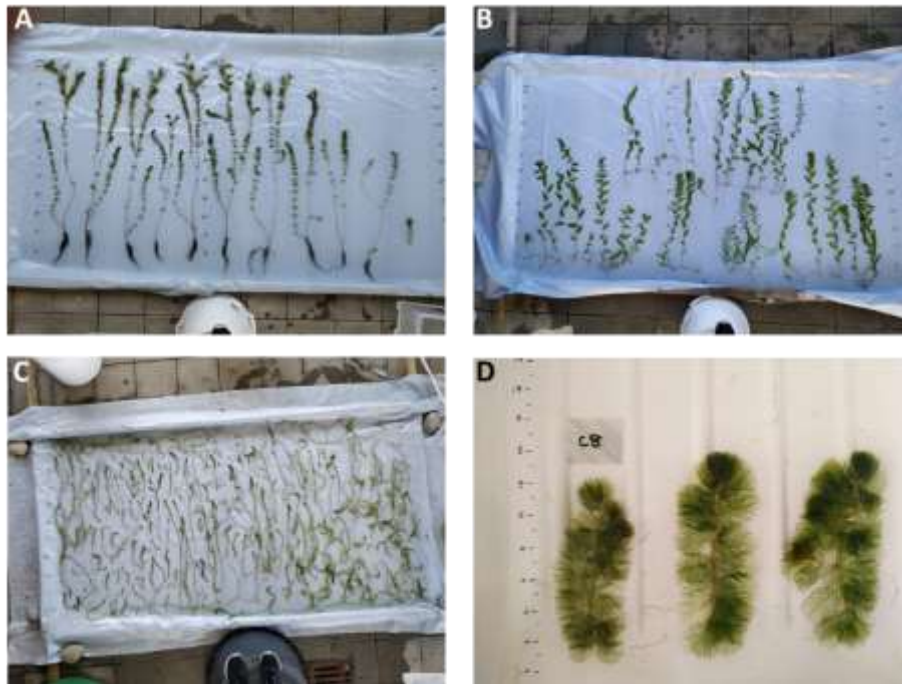


Fig. 2.10. Macrophytes sampled from the mesocosms. (A) *Myriophyllum spicatum*, (B) *Potamogeton perfoliatus*, (C) *Elodea nuttallii*, (D) Apical shoots of *M. spicatum*.

CHAPTER 3: MULTIPLE-STRESSOR EXPOSURE OF AQUATIC FOOD WEBS: NITRATE AND WARMING MODULATE THE EFFECT OF PESTICIDES

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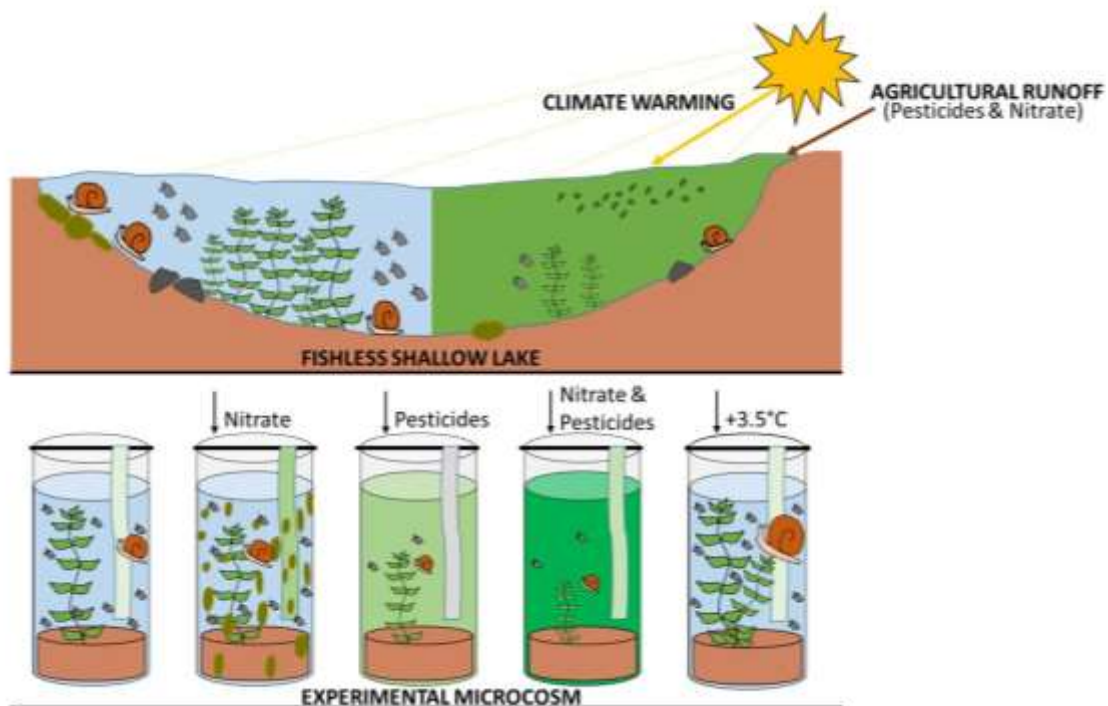
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Abstract

Shallow lakes provide essential ecological and environmental services but are exposed to multiple stressors, including agricultural runoff (ARO) and climate warming, which may act on different target receptors disrupting their normal functioning. We performed a microcosm experiment to determine the individual and combined effects of three stressors—pesticides, nitrate and climate warming—on two trophic levels representative of communities found in shallow lakes. We used three submerged macrophyte species (*Myriophyllum spicatum*, *Potamogeton perfoliatus*, *Elodea nuttallii*), eight benthic or pelagic microalgal species and three primary consumer species (*Daphnia magna*, *Lymnaea stagnalis*, *Dreissena polymorpha*) with different feeding preferences for benthic and pelagic primary producers. Eight different treatments consisted of a control, only nitrate, a pesticide cocktail, and a combination of nitrate and pesticides representing ARO, each replicated at ambient temperature and +3.5°C, mimicking climate warming. Pesticides negatively affected all functional groups except phytoplankton, which increased. Warming and nitrate modified these effects. Strong but opposite pesticide and warming effects on *Myriophyllum* drove the response of the total macrophyte biomass. Nitrate significantly suppressed *Myriophyllum* final biomass, but not overall macrophyte and microalgal biomass. Nitrate

and pesticides in combination caused a macrophyte decline, and the system tipped towards phytoplankton dominance. Strong synergistic or even reversed stressor interaction effects were observed for macrophytes or periphyton. We emphasize the need for more complex community- and ecosystem-level studies incorporating multiple stressor scenarios to define safe operating spaces.

Keywords: agricultural runoff, benthic–pelagic coupling, microcosm, multiple stressors, regime shifts, stressor interactions



1. Introduction

Multiple stressors affecting aquatic systems do not spare shallow lakes, the most abundant type of freshwater systems worldwide (Meerhoff & Jeppesen, 2009). These lakes, including fishless water bodies such as kettle holes and peatland ditches, are common in agricultural landscapes, and are hubs of biodiversity (Pätzig et al., 2012; Peeters, 2005; Whatley et al., 2014). Typical lakes may exhibit either the more desirable clear-water macrophyte-dominated state providing a variety of ecosystem services or the degraded turbid phytoplankton-dominated state (Janssen et al., 2021). In addition, periphyton shading on macrophytes can play an important role in the transition from the clear-water to the turbid state (Phillips et al., 1978). These state shifts have been largely ascribed to nutrient loading to aquatic systems, which has significantly increased since the agricultural revolution (Moss et al., 2013). Yet, the role of nitrogen in regime shifts has received less attention than phosphorus, particularly due to the presence of nitrogen fixation as a compensation mechanism for nitrogen shortage (Moss et al., 2013; Schindler et al., 2008). Increasing nitrogen concentrations may lead to reduced macrophyte abundance and diversity due to phytoplankton shading (Moss, 1976; Phillips et al., 2016). Higher nitrogen concentrations are consistently measured close to agricultural sites (~ 1.5 to 1.8 mg L^{-1}) compared with non-agricultural watersheds ($\sim 1.0 \text{ mg L}^{-1}$) (Xu et al., 2014). Actual nitrogen input concentrations may be masked by quick assimilation by phytoplankton or periphyton. Reduced macrophyte biomass and species richness as well as increased phytoplankton and chlorophyll-*a* concentrations have been observed at nitrogen concentrations between 2 and 10 mg L^{-1} (Barker et al., 2008; Sagrario et al., 2005). These shifts occurred irrespective of total phosphorus concentrations, ranging between 0.03 and 1.2 mg L^{-1} (Sagrario et al., 2005). High nitrogen loading can therefore increase phytoplankton and periphyton biomass in nitrogen-limited or co-limited lakes (Smith & Lee, 2006; X. Zhang & Mei, 2013). Nitrates are easily leached into groundwater or carried away in agricultural runoff (ARO in the following). Concentrations in shallow lakes may thus rise proportionally with increasing fertilizer applications, underlining the need for nitrogen control around shallow lakes. While alternative states are less common in smaller lakes such as ditches and kettle holes, where macrophytes and periphyton are more abundant (Kazanjian et al., 2018), they are still possible under high eutrophication (Whatley et al., 2014).

The primary source of nitrogen in these lakes is ARO (Rücker et al., 2019), which may also contain organic and inorganic pesticides. Global production and use of pesticides has risen significantly after the 1950s (Schäfer et al., 2011; Sharma et al., 2019). Pesticides, varying in concentrations in aquatic systems based on rain events and surface or subsurface runoff (Graymore et al., 2001), can directly affect all types of aquatic primary producers through, e.g., inhibition of photosynthesis, carotenoid biosynthesis, or growth regulation (Mohaupt et al., 2020; Vonk & Kraak, 2020). They may also indirectly alter their development by affecting top-down control (Cuenca Cambronero et al., 2018). Macrophytes are affected directly by phytoplankton and periphyton shading, and indirectly through top-down control of microalgae by invertebrate grazers (Phillips et al., 2016). Zooplankton efficiently controls phytoplankton, leading to high transparency in lakes (Lampert et al., 1986), and invertebrate grazing on periphyton may alleviate shading on macrophytes (Jones et al., 2003). Pesticides may affect not only these primary consumers (Allen et al., 2021; Hanazato, 2001) but also predators such as fish (Ali et al., 2020) or other higher trophic levels if present. Depending on their modes of action, pesticides may therefore alter macrophyte abundance directly or indirectly through trophic interactions (Yamamuro et al., 2019), reducing the resilience of the system (Scheffer et al., 1993).

In addition to local chemical stressors, shallow lakes are increasingly subjected to climate warming. The International Panel on Climate Change (IPCC) has projected a +4°C increase in global temperatures in its RCP 8.5 scenario (IPCC, 2014). Such an increase may have negative effects for shallow lakes particularly in combination with chemical pollution entering these systems. Modelling studies propose that rising temperatures will cause an increase in phytoplankton biomass (Trolle et al., 2014). Yet, warming may also reduce phytoplankton biomass due to enhanced grazing in fishless lakes (Hansson et al., 2012). Further, filtering rates of *Daphnia*, key grazers in fishless ditches, may be reduced at higher temperatures (Müller et al., 2018). Climate warming leading to increased nutrient release from sediments (Jeppesen et al., 2009), or increased herbivory on macrophytes (Zhang et al., 2019) may contribute to phytoplankton dominance and ultimately cause regime shifts in shallow eutrophic lakes (Scheffer, 2001).

These multiple stressors may affect shallow aquatic lakes in several ways. Nitrate or other nutrients could act antagonistic to pesticide effects, e.g., dampening negative effects of herbicides on primary producers (Halstead et al., 2014; Dalton et al., 2015). High nutrient availability for microalgae provide a better food quality for grazers (Guo et al.,

2016) but might interact with grazer sensitivity towards pesticides. Climate warming may further complicate the prediction of combined nitrate and pesticide effects. The resilience of the clear-water, macrophyte-dominated state may be reduced, and tipping points for state shifts more easily reached (Scheffer et al., 1993) as a result of the combined effects of multiple stressors (nitrate, pesticides and warming) on community- and ecosystem processes in shallow lakes or fishless ditches or kettle holes. We combined this complex scenario in our microcosms to test the interactive effects of nitrate, pesticides and elevated temperature, on a benthic–pelagic system whose respective response to the stressors can induce state shifts of the system. To the best of our knowledge, this type of complex integrative study, including a top–down consumer control of both phytoplankton and periphyton, has not been performed before.

The objective of our study is to determine the individual and combined effects of nitrate, pesticides and climate warming in microcosms using key functional groups representative of the first two trophic levels in shallow aquatic ecosystems. The organisms we chose are used in normalized ecotoxicity assays (OECD, 2011, 2012, 2014, 2016). To disentangle the nitrate, pesticide and warming effects, we exposed our microcosms to these stressors individually and in combination. We hypothesized that (1) pesticides will affect the primary producers through effects on trophic interactions, and (2) nitrate and warming will interact synergistically with pesticides to weaken macrophyte dominance.

2. Material and methods

2.1. Experimental design

To determine the individual and combined effects of nitrate, pesticides and temperature on shallow aquatic systems, a full three-factorial microcosm experiment was performed. The three factors were nitrate (Nitr, 2 levels: addition or no addition), pesticide (Pest, 2 levels: addition or no addition) and temperature (Tmp, 2 levels: ambient or warm). The treatments were control (CON), nitrate addition (NO₃), pesticides (PST) and pesticides and nitrate combined (ARO). All treatments were replicated 5 times (40 microcosms in total). The targeted temperatures were 22°C characteristic of present central European summer, and a +4°C climate-warming scenario as projected in the RCP 8.5 (IPCC, 2014). With our heating system, we achieved a difference of 3.5°C.

Three functional groups each of primary producers and primary consumers, representative of the first two trophic levels in shallow freshwater ecosystems, were used,

adapted from Allen et al. (2021). Details of each species and their maintenance are provided in the supplementary information at the end of this chapter.

2.2. Set-up of the microcosms

Each microcosm consisted of a glass cylinder (Sandra Rich GmbH, height 40 cm, Ø 19 cm) with a crystallizing dish insert (height 8 cm, Ø 15 cm) filled with 750 g of sediment (modified OECD TG 239, 2014; see Allen et al., 2021 for sediment preparation). Each cylinder was filled with 8 L of Volvic® water. Microcosms were randomly distributed to four large, temperature-regulated water tanks. The microcosms were maintained under a 16:8 h day:night cycle at an irradiance of $77.2 \pm 6.8 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ photosynthetically active radiation (PAR) at the water surface (ToLEDo LED fluorescent tubes, cool white, 150 cm, 27 W, Sylvania).

For the ARO cocktail, we mixed three widely used organic pesticides, a herbicide (terbuthylazine), an insecticide (pirimicarb) and a fungicide (tebuconazole), copper as an ingredient of inorganic pesticide mixtures and nitrate (SI; Table S3.1). Concentrations of the pesticides were based on experimental evidence at UFZ, Leipzig (BHP and MSJ, unpublished data), as well as some published EC values and species sensitivity distribution data.

On day -6, the cylinders were inoculated with four phytoplankton and four periphyton strains. A similar biovolume per strain was used, yielding $\sim 0.5 \text{ mg L}^{-1}$ and 1 mg L^{-1} of organic carbon (Behl et al., 2011) for phytoplankton and periphyton, respectively, per microcosm. Periphyton development was followed using four vertically placed polypropylene strips (rough surface; 29.7 cm x 2.6 cm length x 300 μm thick; PolyClearView, GBC, Chicago, USA). On day -4, each two 10-cm apical shoots of three macrophyte species were planted in the sediment, ensuring homogenous distribution of fresh weights among treatments. These were *Myriophyllum spicatum*, *Potamogeton perfoliatus* and *Elodea nuttallii* (hereafter *Myriophyllum*, *Potamogeton* and *Elodea*, respectively), commonly found in meso- to eutrophic, still or slow-moving waters. On day -3, twenty *Daphnia magna* (hereafter *Daphnia*) neonates, one pond snail (*Lymnaea stagnalis*, hereafter *Lymnaea*) and three zebra mussels (*Dreissena polymorpha*, hereafter *Dreissena*) were introduced per microcosm, ensuring homogeneous mean size among treatments. *Daphnia* is a typical pond species efficiently grazing on phytoplankton and sometimes on periphyton, *Lymnaea* feeds primarily on periphyton and sometimes on macrophytes, and *Dreissena* feeds on

phytoplankton. On day 0, the chemical stressors were added, and the set temperature of half of the water tanks was increased by +3.5°C. The exposure period was four weeks with regular macronutrient dosage to prevent nutrient limitation and deionized water addition to account for evaporation and keep the water volume constant. For more details on settings and organisms mentioned in this paragraph, please see SI, S2.2.

2.3. Sampling and measured response variables

2.3.1. Sampling scheme

Weekly measures of water physico-chemistry and the development of phytoplankton, *Daphnia* and periphyton were made. Dissolved nutrients, organic pesticide and copper concentrations were measured at the start and end of the experiment. Final sampling was performed over four days to allow sampling of all compartments. Water and plankton were sampled first, followed by periphyton, benthic grazers and macrophytes.

2.3.2. Water

Water pH, conductivity and dissolved oxygen were measured using a multi-parameter analyser (WTW Multiline 3410) directly in the cylinders. Dissolved inorganic nutrients as nitrate, nitrite, ammonium and orthophosphate were determined using ion chromatography (Dionex ICS 1100). Dissolved organic carbon was measured in water filtered over combusted (carbon-free) GF/F filters (Whatman, 0.7 µm; Shimadzu TOC-V_{CSH} Analyser). Dissolved copper was analysed by atomic absorption spectroscopy (Varian SpectrAA 800 Zeeman). Organic pesticides were analysed from filtered water samples (0.22 µm PVDF syringe filters) by liquid chromatography–mass spectrometry using an LTQ Orbitrap XL (Thermo-Scientific, USA).

2.3.3. Plankton and periphyton

Phytoplankton development was followed by measuring the optical density (OD; as a proxy of biomass) at 663 nm of water passed over a 500-µm mesh beaker to remove *Daphnia*. OD measurements were taken with a spectrophotometer (Cary 50, Varian® Agilent, USA). At the end of the experiment, between 40 and 100 mL (depending on density) was filtered on GF/F filters to determine particulate carbon concentration.

During the experiment, 50 mL water was collected weekly, and *Daphnia* individuals counted. All *Daphnia* were returned to their respective microcosms after counting. Numbers were extrapolated to total volume for number of individuals per mesocosm. At

the end of the experiment, sampled *Daphnia* were fixed in sugar ethanol to avoid size changes (Haney & Hall, 1973) and their length measured using a digital microscope (VHX-6000; Keyence, Bois-Colombes, France). Length measures were converted into dry weight using the formula $B = 0.01 \times L^{2.62}$, where B represents biomass in mg and L = length in mm ($r = 0.99$; Pauw et al., 1981).

The development of periphyton was followed weekly on one of the plastic strips taken out of the microcosm to measure the minimum fluorescence (F_0) by Pulse Amplitude Modulation (PAM). The strip was immediately placed back in the cylinder. At the end of the experiment, periphyton from all four strips (400 cm^2) per microcosm was brushed off into 20 mL Volvic water using a soft toothbrush. Following homogenization and centrifugation, pellets were stored at -80°C , then lyophilised for the analysis of carbon.

From both the water and periphyton suspensions, 1.5 ml aliquots were fixed in formaldehyde (3%) for analysis by cytometry to determine number of bacterial cells.

2.3.4. Macrophytes

After four weeks, macrophytes were sampled, cleaned and separated by species into aboveground and belowground parts. The carbon content (based on dry mass) was measured in apical 10 cm shoot sections. Total carbon content of each species per microcosm was calculated from their respective aboveground biomass. Total macrophyte dry mass and carbon content reflect the sums for all three macrophyte species per microcosm.

2.3.5. Chemical analyses of microalgae and macrophyt

Photosynthetic pigments of phytoplankton and periphyton were analysed by HPLC-DAD (high performance liquid chromatography-diode array detector) (UHPLC Ultimate 3000 Rs THERMO; Capdeville et al. 2019). Chlorophyll-*a* concentrations were used as a proxy for biomass of phytoplankton ($\mu\text{g L}^{-1}$) and periphyton ($\mu\text{g cm}^{-2}$). The carbon content of all primary producers was determined based on dry biomass for macrophytes and periphyton, and on filtered volume for phytoplankton, using a CHNS elemental analyser (Carlo-ERBA Na 2100 CE Instrument).

2.3.6. Benthic consumers

After four weeks, all snails and mussels were retrieved, live individuals counted, their lengths measured, and growth rate calculated (see SI, S2.3.6).

2.4. Statistical analyses

A three-way analysis of variance (ANOVA) was applied using R (R Core Team, 2018) to test Pest, Nitr, Tmp (fixed factors) and their interaction effects on all response factors. A Kruskal–Wallis test by ranks was performed for non-parametric data. The Hedges’ *g* effect sizes and 90% confidence intervals (CI) were calculated based on Hedges and Olkin (1985). When the effect sizes were large (>0.8 ; Cohen, 1988) and their CIs did not touch the 0 line, they were considered meaningful. Additive effects of the stressors—Pest+Nitr, Pest+Tmp, Nitr+Tmp and Pest+Nitr+Tmp—were predicted from the Hedges’ *g* for individual stressors. Interactions were considered to be additive, synergistic, antagonistic or reversed if they were similar to, higher than, lower than, or opposite in direction to the calculated additions, respectively, respecting CI margins. To analyse shifts in the relative dominance among the three macrophyte species and among the three primary producer groups, a permutational multivariate analysis of variance (PERMANOVA) was performed using the *vegan* package (Oksanen et al., 2020). To visualize significant patterns in the variability of the final sampling data, and to reduce the confounding influence of multiple highly correlated variables, principal components analyses (PCA) were performed using the *vegan* (Oksanen et al., 2020) and *factoextra* packages (Kassambara & Mundt, 2020). To decipher direct and indirect stressor effects on the organisms, as well as species interactions, a path analysis subjected to structural equation modelling (SEM) was created (package *sem*; Fox et al., 2021) using several hypothetical pathways similar to Allen et al. (2021) (Table S3.2). Further details of statistical approaches are presented in the supplemental material.

3. Results

The factorial design of our experiment allowed us to identify individual and combined effects of pesticides, nitrate and warming in the different treatments (CON, NO₃, PST, ARO) on the development of the different functional groups, and on water chemistry. Table S3.3 shows the statistical outputs of either three-way ANOVA, Kruskal–Wallis test by ranks and PERMANOVA, supporting the following results.

3.1. Water analyses

The average temperatures remained consistent throughout the experiment and measured $24.6 \pm 0.3^\circ\text{C}$ in the warm microcosms, approximately 3.5°C higher than the ambient microcosms ($21.2 \pm 0.1^\circ\text{C}$). Distinct temporal changes were observed for water conductivity, pH and O_2 (Table S3.4, Fig. S3.1) and are presented in the supplemental material.

High nitrate additions to the NO_3 and ARO treatments were no longer measurable at the end of the experiment. Continuous fertilisation by nitrate and phosphate at a 16:1 μM ratio did not result in an accumulation of dissolved nutrients compared to initial values of the Volvic water ($1.71 \text{ mg L}^{-1} \text{ N-NO}_3$, $0.176 \text{ mg L}^{-1} \text{ P-PO}_4$). Remaining orthophosphate was lower in ambient treatments ($58 \pm 36 \mu\text{g L}^{-1}$) compared with warm ($119 \pm 97 \mu\text{g L}^{-1}$) and variable in the CON and NO_3 treatments. We observed strong Tmp x Pest interactions for P- PO_4 and the NP molar ratio (Fig. S3.2B). Particulate phosphorus was low at the end of the experiment (range $5.6 - 31.5 \mu\text{g L}^{-1}$), with significantly higher levels in the PST and ARO treatments (Fig. S3.2E).

Pesticides were still present at the end of the experiment (30-78%, see supplementary S3.1) except for terbuthylazine, which was not found in any of the samples.

3.2. Response of primary producers

Macrophyte biomass was lower in the PST and ARO treatments, with a concomitant increase in phytoplankton (Fig. 3.1, Table S3.3). Total macrophyte aboveground biomass reduced by 55% in the ambient ARO ($0.09 \pm 0.05 \text{ g}$) compared with ambient CON ($0.2 \pm 0.04 \text{ g}$). All three stressors influenced the relative abundance of macrophyte species (Table S3.3, Fig. 3.2A). *Myriophyllum* dominated and showed the strongest response to all stressors. Aboveground biomass significantly decreased with pesticides and nitrate, and increased with warming. Pesticides or nitrate did not affect the other species. Warming significantly reduced *Potamogeton* aboveground biomass. Due to the opposite temperature response of *Myriophyllum* and *Potamogeton*, warming only marginally positively influenced total macrophyte aboveground biomass ($p = 0.06$).

Final periphyton biomass was significantly lower in PST and ARO treatments but only in the ambient-temperature treatments (significant Pest and Tmp x Pest effect) (Fig.

3.1). Distinct temporal dynamics were observed between weeks 1 and 3 (Fig. 3.3), with initial positive Nitr and negative Tmp effects, followed by positive Pest effects (Table S3.3).

By the end of the experiment, the phytoplankton comprised predominantly green algae. Their biomass in the ambient ARO treatment was high at the end of the experiment (Fig. 3.3), reflected in both the chlorophyll-*a* (chl-*a*) and water OD measurements. Phytoplankton, measured as water OD, first responded positively to temperature (week 1) and then to pesticides (weeks 2, 3 and 4). Nitrate strongly affected water OD at the time of sampling, but did not affect phytoplankton chl-*a*, and was possibly driven by an underestimation of water OD in the ambient PST. Spectrophotometric methods are in general less specific, reporting 1.4 times lower chl-*a* compared with the HPLC method (Ward et al., 1994). Final biomass (chl-*a*) responded strongly to pesticides and showed a Tmp x Pest interaction (Table S3.3, Fig. 3.1). The highest and lowest mean chl-*a* concentrations were present in the ambient microcosms for the ARO ($231.9 \pm 277 \mu\text{g L}^{-1}$) and NO₃ ($9.6 \pm 14.6 \mu\text{g L}^{-1}$) treatments, respectively, opposite of what was observed for periphyton. The number of bacterial cells measured in the water and the biofilm significantly increased in the warm treatments compared with the ambient (Fig. S3.3).

This reduction of total macrophyte biomass in the ARO treatments resulted in significant changes in the relative abundance of primary producers (Fig. 3.2B). Whereas the controls were dominated by macrophytes, the ARO treatments were dominated by phytoplankton. Pesticides caused declining shares of macrophytes and increasing dominance of phytoplankton (Table S3.3, PERMANOVA). When pesticides and nitrate were present together (ARO), effects were amplified (Fig. 3.2B). Warming had a positive influence on macrophyte dominance.

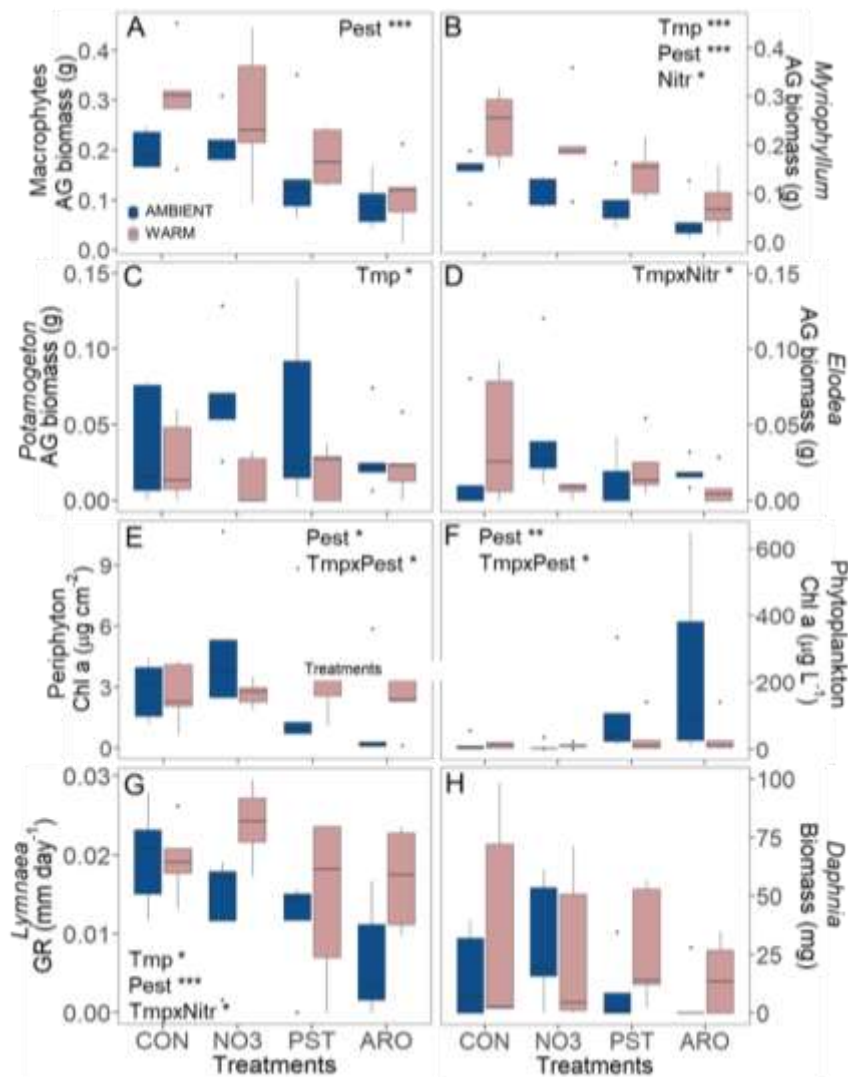


Fig. 3.1. Temperature and treatment (CON = control; NO3 = nitrates; PST = organic pesticides and copper; ARO = PST + NO3) effects on primary producers and consumers. Final aboveground biomass (dry mass) of (A) All macrophytes, (B) *M. spicatum*, (C) *P. perfoliatus*, and (D) *E. nuttallii*. Chl a content of (E) Periphyton, and (F) Phytoplankton. (G) Growth rate (GR) of *L. stagnalis*, (H) Final biomass of *D. magna*. Box plots of 5 replicates showing median, 25 and 75% percentiles, lowest and highest whiskers (as $Q1 - [1.5 \cdot IQR]$ and $Q3 + [1.5 \cdot IQR]$, respectively, IQR – interquartile range), and outliers (dots). Asterisks represent significant effects observed with the ANOVA ($p < 0.05$): using 3 fixed factors: Tmp = temperature effect; Pest = pesticide effect, Nitr = nitrate effect. Blue and mauve represent ambient and warm microcosms, respectively.

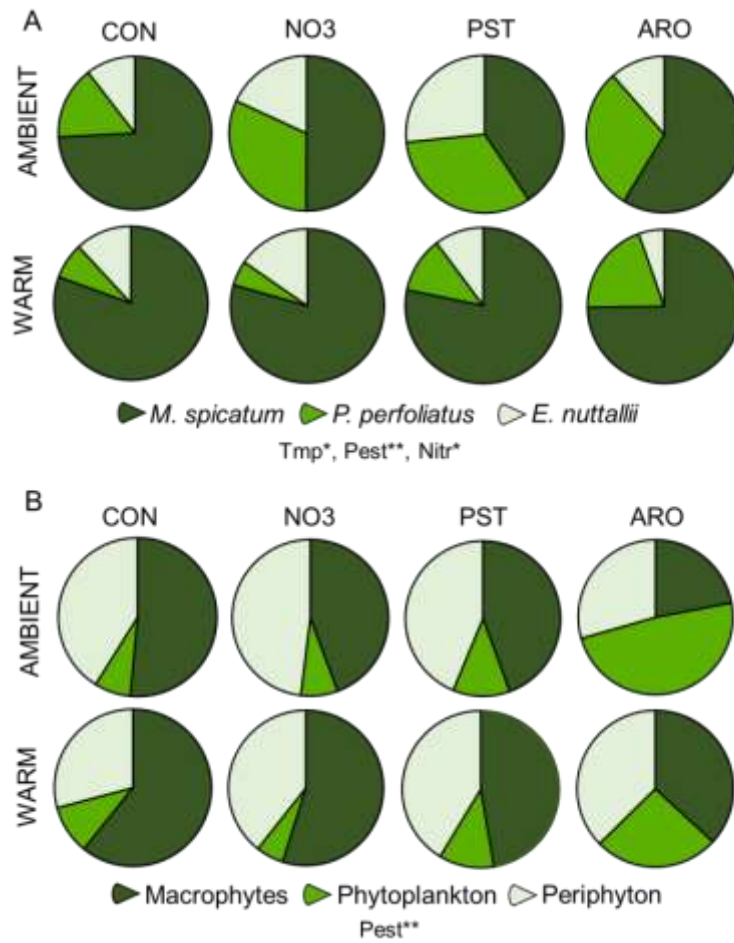


Fig. 3.2. Relative abundances of (A) macrophyte species and (B) primary producer groups. Abundance was measured as a function of total carbon content per microcosm at the end of the experiment. CON = control; NO3 = nitrates; PST = organic pesticides and copper; ARO = PST + NO3. Asterisks represent significant effects observed with the PERMANOVA ($p < 0.05$): using 3 fixed factors: Tmp = temperature effect; Pest = pesticide effect, Nitr = nitrate effect.

3.3. Response of primary consumers

Three of the 40 initial *Lymnaea* individuals did not survive. All dead snails belonged to treatments containing pesticides. Growth rate was significantly reduced with pesticides but increased under warming (Fig. 3.1). Nitrate significantly increased snail growth only at a higher temperature. Only five snails had produced clutches, two from ambient CON, one each from ambient and warm NO3, and one from warm ARO. The NO3 treatments had the highest number of eggs per clutch (144 ± 23), while the ARO had the least (9). Mussels exhibited high mortality irrespective of treatment type, and have not been included further in the analyses.

A strong negative effect of pesticides on *Daphnia* abundance was found in the first two weeks and a positive effect of temperature in the first week (Table S3.3, Fig. 3.3). In the second week, the percentage decrease in *Daphnia* numbers in the ambient and warm ARO treatments was 95% (88 ± 196 individuals L^{-1}) and 55% (620 ± 673 individuals L^{-1}) compared with the ambient and warm CON (1636 ± 1197 and 1152 ± 554 individuals L^{-1}), respectively. Final biomass of *Daphnia* did not reflect any response to the stressors compared with the ambient CON.

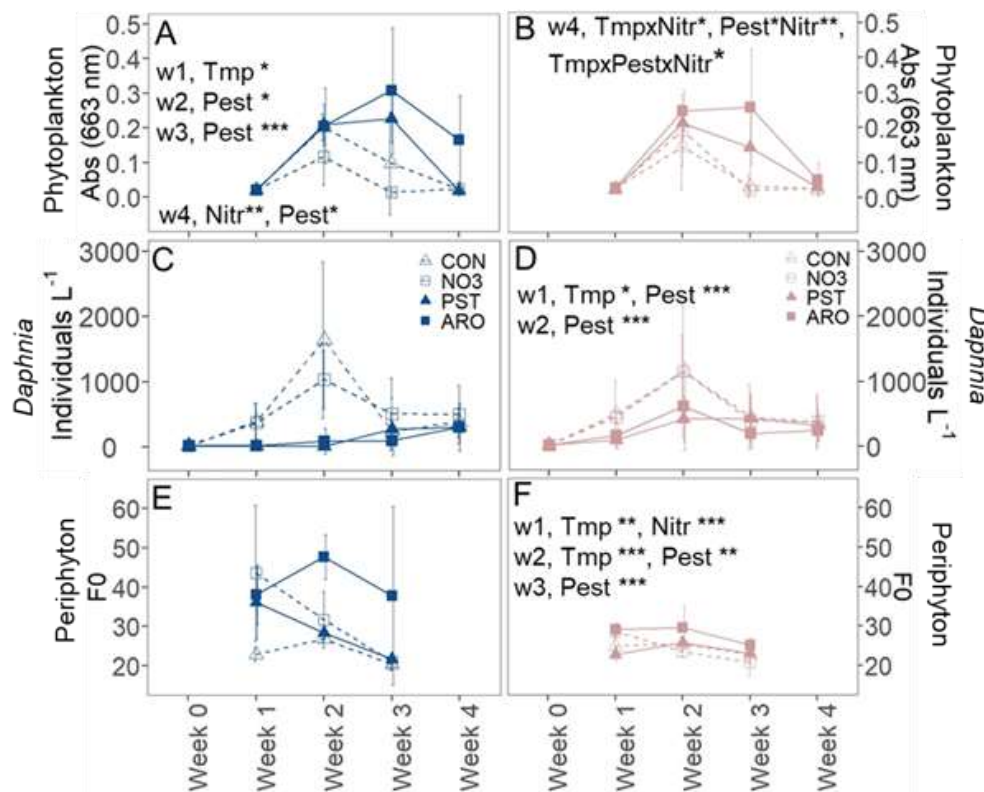


Fig. 3.3. Weekly measures of (A,B) phytoplankton biomass (absorbance at 663 nm), (C,D) numbers of *D. magna* adults, and (E,F) periphyton biomass (as F0 fluorescence) in the ambient (blue) and warm (mauve) microcosms. Means \pm SD, $n = 5$. CON = control; NO3 = nitrates; PST = organic pesticides and copper; ARO = PST + NO3. Asterisks represent significant effects observed with the ANOVA ($p < 0.05$): using 3 fixed factors: Tmp = temperature; Pest = pesticide, Nitr = nitrate.

3.4. Integrative assessment of stressor effects and interactions

Effect sizes allowed us to compare the observed and predicted effects of individual and combined stressors. Effect sizes for individual stressors were calculated based on pairwise comparisons between each experimental condition and the control ($n = 5$). Therefore, some effects observed in the three-way ANOVA, based on the data of the forty microcosms, were not observed with this analysis.

Mostly, the observed and predicted effect sizes were similar, showing that stressor effects were additive. Three exceptions exist (Fig. 3.4): The observed combined effect of pesticides and nitrate on total macrophyte biomass and periphyton biomass was negative, while the predicted effect was neutral. This indicates a synergistic interaction of both stressors. When increased temperature was applied with this stressor combination, a reversed effect was observed for total macrophyte biomass.

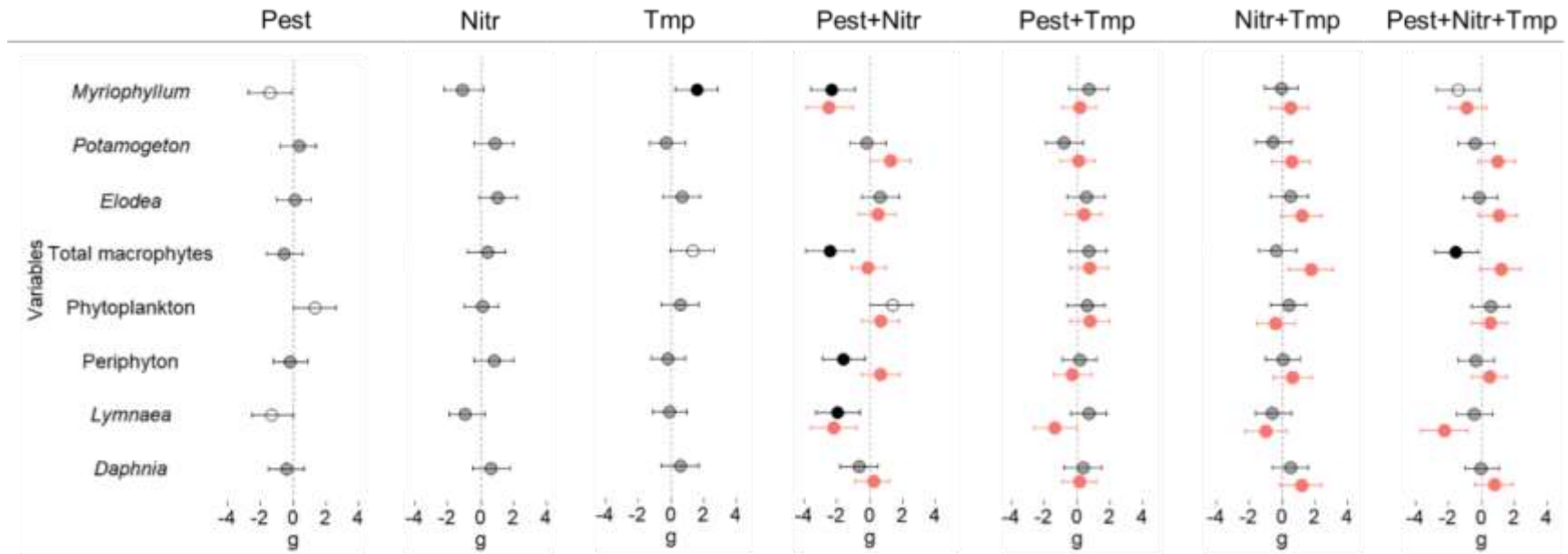


Fig. 3.4. Effect sizes (Hedges' $g \pm 90\%$ confidence interval) of individual and combined stressor effects on the key functional groups at the end of the experiment, calculated from the F-statistics derived from the ANOVA. No effects are inferred when confidence intervals cross the zero line. Grey symbols: non-significant effects (>0.09), black symbols: significant effects (<0.05), white symbols: marginal effects ($0.05-0.09$), calculated using 3 fixed factors: Tmp = temperature; Pest = pesticide, Nitr = nitrate. Red symbols: predicted additive effects calculated from the Hedges' g of individual stressors.

PCAs provided a global view of the strongest patterns in the data. Distinct Pest and Tmp effects were visible along axis 1 of the PCA (Fig. S3.4A-C). Axes 1 and 2 contributed to 33.9% and 17.8% of the variation in the data, respectively. Although the combined contribution of these two principal components to explain the variability of our observations is admittedly low (51.7%), the contribution of PC1 is clear. It separated total macrophyte biomass and *Myriophyllum* biomass on the right from phytoplankton on the left, and is explained by temperature and pesticides. The effect of nitrate is weak. The warm microcosms without pesticides explained the high total macrophyte and *Myriophyllum* biomass. Along axis 2, periphyton and *Lymnaea* were placed on opposite sides, suggesting a top-down control.

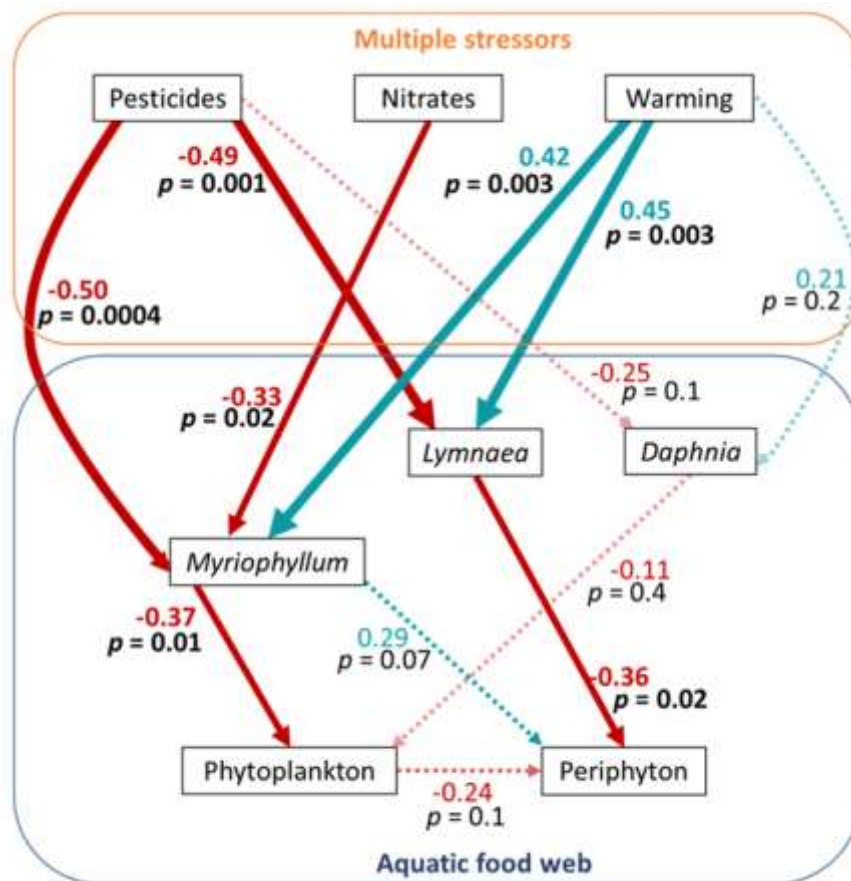


Fig. 3.5. Path diagram of the selected structural equation model based on final sampling data. Red, green and dashed arrows indicate negative, positive and nonsignificant relations between variables, respectively. Significant threshold was set at $p = 0.05$. The proportions of variation explained by the model for each response variable are represented by R-values in blue and red for positive and negative effects, respectively. $\text{Chi}^2 = 17.4$, root mean square error of approximation = 0.05, standardised root mean square residual = 0.1, adjusted goodness of fit index = 0.8, and comparative fit index = 0.1.

SEMs constructed based on hypothesized interactions between stressors and functional groups allowed us to trace food web effects. Using final sampling data, the three stressors affected long-lived organisms such as the dominant macrophyte *Myriophyllum* or *Lymnaea*, but not, or only marginally, organisms with shorter generation times, like phytoplankton, *Daphnia* and periphyton (Fig. 3.5). *Myriophyllum* hindered phytoplankton development possibly also through allelopathy (Hilt & Gross, 2008), and *Lymnaea* controlled periphyton. Temporal dynamics likely influenced this outcome (Fig. S3.5).

Strong direct positive nitrate effects were observed on periphyton during the first week of exposure (Fig. S3.5A). Phytoplankton on the other hand was directly or indirectly affected by nitrate, depending on the exposure phase. Pesticides directly affected the number of *Daphnia* in the first two weeks (Fig. S3.5B). During the intermediate exposure, phytoplankton was indirectly affected by pesticides through *Daphnia* grazing. Warming had a direct positive influence on *Daphnia*, but only in the first week. The preliminary SEMs using all expected pathways are presented in Fig. S3.6 and S3.7. The models created with *Potamogeton* and *Elodea* are not included as no significant stressor or biotic interactions were observed. All SEMs with the final sampling data (Fig. 3.5, S3.6, & S3.7) fit the quality indicators. The SEMs with the temporal data (Fig. S3.5), however, did not fit these quality indicators, and should therefore be interpreted as a hypothesis for further experimental research.

4. Discussion

Our results confirm the first hypothesis: The aquatic primary producers were affected through negative pesticide effects on the primary consumers, which promoted phytoplankton development. The second hypothesis is also confirmed: Nitrate and pesticides interacted synergistically to reduce macrophyte-dominance. Warming together with pesticides and nitrates even reverted the expected outcome on macrophytes based on individual stressor effects, shown by a significant negative effect of the combined stressors on total macrophyte abundance. This means that a typical agricultural runoff (ARO) containing a mixture of pesticides and nitrate can be more detrimental to macrophyte-dominated fishless water bodies than the individual stressors alone and climate warming might even enhance these effects.

4.1. Effect of pesticides

The different primary producer groups and the species in each group compete for light and nutrients. Stressor effects on one group or species may thus affect other primary producers. The dominance of *Myriophyllum* among the tested macrophyte species is not surprising, as it is fast growing and able to successfully outcompete other species, especially under eutrophic conditions (Grace & Wetzel, 1978).

While it remains unclear whether terbuthylazine was present in the samples, the other pesticides including copper might have affected the growth of macrophytes. Yet neither terbuthylazine nor copper showed effects on macrophytes in comparable studies and in the same range of concentrations (BHP, submitted; VV, unpublished data). In different genotypes of *Myriophyllum*, EC50s ranging from 42-296 $\mu\text{g L}^{-1}$ copper have been identified (Roubeau Dumont et al., 2019). The presumed negative effect of pesticides as seen in the SEM might therefore be primarily indirect, resulting from shading by phytoplankton or periphyton at the early or intermediate exposure period. The lack of strong direct or indirect stressor effects on *Potamogeton* or *Elodea* might be related to the dominance of *Myriophyllum*.

The sensitivity of phytoplankton to pesticides may be species-dependent. Some species were inhibited already at 1 $\mu\text{g L}^{-1}$ and others not even at 25 $\mu\text{g L}^{-1}$ (Fiori et al., 2013). Green algae exhibited EC50s for copper between 30 $\mu\text{g L}^{-1}$ (De Schamphelaere et al., 2005) and 9200 $\mu\text{g L}^{-1}$ (Gatidou & Thomaidis, 2007). Less sensitive species therefore probably developed fast in the PST or ARO treatments. The positive indirect effect of pesticides on phytoplankton, as suggested by the SEM, is likely related to the temporal effects of *Daphnia* feeding on it. In fact, *Daphnia* numbers showed a similar dynamic to phytoplankton with a lag of one week, and a strong peak in week 2 in the CON and NO₃ treatments.

The direct negative effect of pesticides on *Daphnia* abundances observed during the first two weeks may be due to pirimicarb, exhibiting EC50 values for *D. magna* of 16-24 $\mu\text{g L}^{-1}$ (Kusk, 1996). Modelling studies show that copper concentrations comparable to those applied in our experiment (42 $\mu\text{g L}^{-1}$) affected top-down control of *Daphnia* on green algae (Prosnier et al., 2015) and should thus have been sufficient to cause a significant decrease in phytoplankton filtration by *Daphnia*. One potential reason for the decline in *Daphnia* numbers at the end of the experiment could be food shortage as no further phytoplankton peak emerged in CON and NO₃, although other reasons, such as fungal

infections cannot be excluded. The decline in *Lymnaea* growth when exposed to pesticides is in line with Allen et al. (2021) and may be related to copper (Brix et al., 2011) or pirimicarb (Tufi et al., 2015).

4.2. Modulation of pesticide effects by nitrate

Remarkably, nitrate or pesticides alone did not affect the proportion of the different functional groups of primary producers. In pesticide-free treatments, nitrate effects on periphyton and phytoplankton biomass were compensated by snail and zooplankton grazing pressure and thus did not affect the total macrophyte biomass. Pesticides alone hampered *Daphnia* and thus promoted phytoplankton, but the relative proportion of phytoplankton or macrophytes did not change significantly compared to CON or NO₃ treatments. Synergistic stressor interactions observed for the combination of pesticides and nitrate on total macrophyte biomass and periphyton, and for the combination of all three stressors on total macrophyte biomass highlight the importance of better understanding multiple stressor effects. Further, only the combination of pesticides and nitrate affected the overall proportion of primary producers. This is likely due to a facilitation of periphyton growth by nitrate in the early phase of exposure and negative effects of pesticides on *Daphnia* promoting phytoplankton. These findings may have direct relevance for small fishless lakes with high levels of resource abundance, where *Daphnia* are dominant filter feeders (Romanovsky & Feniova, 1985). The fact that the system moves towards a much lower resilience is indicated by both the general increase and the wide variability in the phytoplankton biomass in the combined treatments, although PERMANOVA indicated no significant interactions between pesticides and nitrate. In fact, such an increase in variability can also be used as an indicator that a system is shifting from one stable state to the other (Scheffer et al., 2015).

Although we saw strong direct negative nitrate effects on *Myriophyllum*, controlled laboratory studies showed that *Myriophyllum* could tolerate nitrogen levels up to ~14 mg L⁻¹ (Palove-Balang et al., 2016). Field studies on the other hand show that *Myriophyllum* growth is reduced after a week if concentrations exceed ~0.5 mg L⁻¹ (Palove-Balang et al., 2016), suggesting that macrophytes are affected by other nitrate-related factors, for example, through competition with phytoplankton or periphyton. In our study, periphyton growth in the nitrate treatments significantly increased in the first week causing shading of macrophytes. This highlights the relevance of including epiphytes in studies concerning regime shifts between primary producers (Phillips et al., 2016).

4.3. Warming and its modification of pesticide effects

Global warming is generally expected to stimulate macrophyte growth in temperate (Zhang et al., 2019) and arctic (Lauridsen et al., 2020) lakes unless critical thresholds in nutrient loading that result in phytoplankton dominance have been crossed (Mooij et al., 2008). One explanation for the increased biomass of *Myriophyllum* in our warm treatments is its higher optimum temperature range of up to 35°C (Grace & Wetzel, 1978). However, indirect effects, for example, on snail activity by increased grazing on periphyton or by accelerated plant growth as a compensatory mechanism to leaf loss by grazing are likely. Allen et al., (2021) whose microcosms did not have a top–down control on phytoplankton showed no effects of warming on *Myriophyllum* or the other two macrophyte species. In our experiment, two non-exclusive explanations are possible for the modulation of pesticide effects by warming. The first is that top–down control of microalgae was higher in the warm ARO treatments, reflected by increased snail growth rate and an increase in *Daphnia* numbers in the early phase of the experiment, increasing light and nutrient availability to macrophytes. Herbivore–plant interactions can in fact be strengthened by warming (Zhang et al., 2019). The second explanation is that the warm treatments promoted the development of bacteria, which may also have been influenced by a higher nutrient cycling by consumers, thereby increasing competition for resources with phytoplankton (Joint et al., 2002).

The effect of warming on phytoplankton and periphyton, which we expected to be positive (Mahdy et al., 2015; Rasconi et al., 2015), remained context-specific and was likely quickly counterbalanced by grazing effects of *Daphnia* and *Lymnaea*, similar to previous findings (Kazanjian et al., 2018; Velthuis et al., 2017). The increased phytoplankton and decreased periphyton in the pesticide treatments were significant only in the ambient treatments, and may be linked to the lower consumer activity in these treatments. Warming was therefore mitigating pesticide toxicity. Elevated temperature in the form of heatwaves applied with pesticides have shown antagonistic or synergistic effects depending on the type of pesticide used (Polazzo et al., 2021). The type of pesticides present and the community composition, including number of trophic levels and their feeding behaviours, will all determine how warming affects such shallow lake ecosystems. Several studies have included warming as a stressor along with nitrate (Bourai et al., 2020; Mckee et al., 2002) or pesticides (Wijewardene et al., 2021; Knillmann et al., 2013), often using simplified food webs or communities. Yet, the literature is lacking on experiments

combining all three stressors, which is a more realistic scenario, and incorporating more complex food web structures to disentangle direct and indirect effects. Such a mechanistic understanding is of paramount importance if safe operating spaces are to be defined in the context of rising global temperatures.

5. Conclusion

Our findings have implications for decision-making on alleviating stressor effects not only in shallow lakes but also in peatland ditches and kettle holes. In our study, the applied agricultural run-off (ARO) strongly reduced phytoplankton and periphyton control by primary consumers (*Daphnia*, *Lymnaea*). Alone, pesticides and nitrate caused low or no effects at the individual or community level, but when present in combination, the macrophyte-dominated state was threatened, especially at ambient temperature, as highlighted by the synergistic stressor interaction effects on macrophytes and periphyton. We highlight the importance of reducing both nitrate and pesticide use in agriculture to positively influence the water quality of adjacent aquatic ecosystems by enhancing potential for macrophyte dominance. Reducing just one of these chemical stressor types may be insufficient. The situation even gets more complex when adding warming to these stressors. Despite the apparent buffering effect of temperature on nitrate and pesticide effects for phytoplankton and *Daphnia*, total macrophyte biomass was significantly reduced. The significant reversed stressor interaction found for nitrate, pesticides and warming on submerged macrophytes highlights the risk in predicting multiple stressor effects. A better understanding of such complex benthic–pelagic interactions as outlined in our study is necessary for policymakers to develop strategies that enable the achievement of the “good” ecological status of shallow lakes as defined by the Water Framework Directive. Future studies can build on our study by considering e.g., larger scales and a longer duration of the experiment or the implementation of more complex food webs including higher trophic levels.

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Supplementary information

The supplementary information provides additional detailed information on the “Material and Methods” and the “Results”. The numbering follows the numbering in the main text.

S2. Material and methods

S2.1. Experimental design

Periphyton and phytoplankton strains were used as in Allen et al. (2021), except *Uronema confervicolum* and *Gomphonema parvulum*, which did not grow in our cultures. As primary consumers, we introduced *Daphnia magna* (hereafter *Daphnia*), a typical pond species efficiently grazing on phytoplankton and sometimes on periphyton, the pond snail *Lymnaea stagnalis* (hereafter *Lymnaea*) feeding primarily on periphyton and sometimes on macrophytes, and the zebra mussel *Dreissena polymorpha* feeding on phytoplankton.

S2.2. Set-up of the microcosms – species acclimations

All species were acclimated in the laboratory to experimental conditions for at least four weeks before use. Macrophytes were from the eutrophic shallow lake Lac de Madine (48°55'N, 5°44' E). The initial fresh weight per shoot was comparable for all species, and for *Myriophyllum* 0.26 ± 0.12 g, for *Potamogeton* 0.30 ± 0.13 g and for *Elodea* 0.23 ± 0.07 g. Algal and cyanobacterial strains were cultured first in BG11 (Rippka et al., 1979) or WC medium (Guillard & Lorenzen, 1972), and acclimated to Volvic water one week before the start. Biovolumes of each strain used, that is, $8.35 \times 10^5 \mu\text{m}^3 \text{mL}^{-1}$ for periphyton and $7.5 \times 10^5 \mu\text{m}^3 \text{mL}^{-1}$ for phytoplankton species, were equivalent to $\sim 0.5 \text{mg L}^{-1}$ and 1mg L^{-1} of organic carbon (Behl et al., 2011) per microcosm, respectively.

Daphnia were obtained from LMU Munich, Germany. All neonates used in the experiment were from the same cohort. At the time of introduction, they were less than 24 h old and measured $1391 \pm 17 \mu\text{m}$ (mean \pm standard deviation [SD], $n = 10$). *Lymnaea stagnalis* was initially obtained from the Laboratory of Functional Ecology (UMR 5245 CNRS), Toulouse, France, and have been cultivated multiple generations in our laboratory. Starting size was $19.02 \pm 2.4 \text{mm}$ (mean \pm SD, $n = 40$). *D. polymorpha* were obtained from Lac de Madine, and measured $9.3 \pm 1.3 \text{mm}$ (mean \pm SD, $n = 120$). Twenty *Daphnia* neonates, one snail and three mussels were introduced per microcosm.

Concentrations of the pesticides were based on experimental evidence at UFZ, Leipzig (BHP and MSJ, unpublished data), as well as some published EC values and species sensitivity distribution data (see Table S3.1 for concentrations).

During the four-week exposure period, thrice weekly, macronutrients were added at the Redfield ratio 16:1 (16 $\mu\text{mol L}^{-1}$ N and 1 $\mu\text{mol L}^{-1}$ P as KNO_3 and KH_2PO_4 , respectively) to prevent nutrient limitation of the primary producers. Evaporated water was replaced with deionized water three times a week to keep the 8-L volume.

S2.3. Sampling and measured response variables

S2.3.1. Periphyton

For weekly dynamics, each polystyrene strip was placed in a tray with a thin water layer, and dark adapted for 20 minutes. Using a MINI-PAM-II fluorometer (Heinz Walz GmbH, Germany), the minimal fluorescence (F_0) was measured by Pulse Amplitude Modulation (PAM) at a position of the strip 5 cm below the surface of the water as a proxy for chlorophyll a. Plastic strips were then returned to the respective microcosm.

S2.3.2. Chemical analyses of microalgae and macrophytes

Total carbon content of phytoplankton was calculated by using total volume (8 L) for the phytoplankton, and total surface area of all 4 polystyrene strips (both sides, 400 cm^2) plus inner surface of the glass wall (2013 cm^2) of the microcosm for the periphyton.

S2.3.3. Benthic consumers

Snail growth rate was calculated using the formula, $GR = (\ln_{L_f} - \ln_{L_i})/N_{\text{days}}$, where L_f and L_i are the final and initial shell sizes (in mm), respectively, and N_{days} is the exposure period (28 days).

S2.4. Statistical analyses

For the ANOVA, residuals were first tested for normality and homoscedasticity using the Shapiro–Wilk’s test and the Bartlett test, respectively. When required, log or square root transformations were performed to ensure the data fit these assumptions. Significance was considered at $p < 0.05$. When normality or homoscedasticity could not be reached, the non-parametric Kruskal–Wallis test by ranks was used to determine Pest, Nitr or Tmp effects.

To calculate the effect sizes, all treatment and control groups were tested individually for normality and homoscedasticity, and log or square root transformations applied whenever necessary. The Hedges' g effect sizes were calculated using the formula based on Hedges and Olkin (1985):

$$\frac{Y^E - Y^C}{s^*},$$

where Y^E and Y^C are the respective experimental and control means and s^* is the pooled standard deviation, given by:

$$s^* = \sqrt{\frac{(n^E - 1)(s^E)^2 + (n^C - 1)(s^C)^2}{n^E + n^C - 2}}.$$

Unpaired two-sample *t*-tests were performed using R studio to test the significance of effects.

Based on nomographs provided in Appendix F of Hedges and Olkin (1985), 90% confidence intervals (CIs) were obtained by tracing the ordinate values of the intersections between Hedges' g and the upper and lower curves provided for a sample size of 5 (p. 91 in Hedges & Olkin 1985). We used 90% CIs rather than 95% due to our small sample size.

The PERMANOVA was performed with pesticides, nitrate and temperature as explanatory variables, and proportions (values from 0 to 1) of carbon content per macrophyte species or per primary producer group. Total carbon content was used as it allowed a common proxy for biomass for all primary producers.

The PCA was performed based on the eight main response variables (total macrophytes, each macrophyte species, phytoplankton, periphyton, *Lymnaea* and *Daphnia*) and seven supplementary quantitative variables (water physico-chemistry). The stressors were displayed as supplementary categorical data (ambient or warm treatments, and presence or absence of pesticides and nitrate) with their barycentres and 95% confidence ellipses in three separate graphs. All combinations of the first four significant axes were first tested against each other. The final PCA plots presented are created with axes 1 and 2. No significant patterns were identified with axes 3 and 4. Only the top five variables with the highest contributions to the extracted principal components were plotted.

For the SEMs, biomass data for the primary producers and *Daphnia*, and growth rates for the snails were used. Preliminary models were created including all expected relationships between the stressors and main response variables measured at the final sampling. The weakest links were eliminated step-by-step, and various expected direct and indirect interactions tested. While reciprocal biotic interactions were tested, the final models included only directions in which the link was strongest. The adequacy of the model was tested using quality indicators such as Chi^2 , root mean square error of approximation (RMSEA), standardised root mean square residual (SRMR), adjusted goodness of fit index (aGFI) and comparative fit index (CFI) (Fan et al., 2016). To determine how the dominant macrophyte species would affect the model, similar models were also created using each macrophyte species separately in place of the total macrophytes. The model with the dominant macrophyte species was finally chosen for discussion. Two additional SEM models were developed to study temporal effects of pesticides and warming on *Daphnia* and phytoplankton and temporal effects of nitrate on periphyton F0 and phytoplankton OD.

S3. Results

S3.1. Water analyses

The conductivity was higher in the treatments with nitrate. At the end of the experiment, conductivity was $398 \pm 34 \mu\text{S cm}^{-1}$ in the nitrate treatments and $366 \pm 31 \mu\text{S cm}^{-1}$ in treatments without nitrate. Both pH and O_2 were lower in the warm treatments and treatments without pesticides. After four weeks, however, only the temperature effect on pH remained (ambient = 8.0 ± 0.4 , warm = 7.7 ± 0.3). Effects on O_2 were no longer measurable. Dissolved organic carbon was significantly higher in the pesticide treatments ($10.8 \pm 5.6 \text{ mg L}^{-1}$) compared with treatments without pesticides ($7.2 \pm 0.89 \text{ mg L}^{-1}$). This difference cannot be explained by the addition of the organic pesticides, but is likely due to a higher exudation of organic carbon by organisms, likely macrophytes.

Pesticides declined over time but were mostly still detectable at the end of the experiment. Final copper concentrations ranged between 45 and 87% (average $12.6 \pm 5.5 \mu\text{g L}^{-1}$) of the initially applied concentration ($42 \mu\text{g L}^{-1}$). The copper present in the control treatment reflects background values of the water and sediment applied and/or might be due to aerosol drift from treated microcosms. For pirimicarb, 73 to 78% were still present at the end, and for tebuconazole 30 to 46%. We found traces of pirimicarb and

tebuconazole in the controls, probably due to aerosols caused by bubbling. Terbutylazine was not detected in the system probably due to a co-elution during analysis or signal suppression caused by matrix effects (Choi et al., 2001).

Table S3.1. Concentrations of chemical stressors added to the different treatments.

Substance	CON	NO3	PST	ARO
Nitrates^a	-	9000 µg L ⁻¹	-	9000 µg L ⁻¹
Copper^b	-	-	42 µg L ⁻¹	42 µg L ⁻¹
Terbuthylazine^c	-	-	3 µg L ⁻¹	3 µg L ⁻¹
Pirimicarb^d	-	-	15 µg L ⁻¹	15 µg L ⁻¹
Tebuconazole^e	-	-	90 µg L ⁻¹	90 µg L ⁻¹
DMSO^f	10 µl L ⁻¹	10 µl L ⁻¹	10 µl L ⁻¹	10 µl L ⁻¹

^a as KNO₃; CAS number 7757-79-1, Merck, analytical grade; ^b as CuSO₄; CAS number 7758-98-7, Merck, analytical grade; ^c CAS number 5915-41-3, Sigma-Aldrich, purity ≥ 98.0%; ^d CAS number 23103-98-2, Sigma-Aldrich, purity ≥ 98.5%; ^e CAS number 107534-96-3, Sigma-Aldrich, purity > 99% ^f CAS 67-68-5, Merck, analytical grade. All organic pesticides were Pestanal standard quality. CON = control; NO₃ = only nitrates; PST = organic and inorganic pesticides; ARO = agricultural run-off.

Table S3.2. Hypothesized interactions between stressors and functional groups used for the SEMs.

Interactions	Response	Hypothesis	Observed direct effect
Stressor effects			
Pest → Total macrophytes	No effect	Low toxic concentrations of organic and inorganic pesticides (Lewis & Thursby, 2018; Vonk & Kraak, 2020, unpublished data, VV)	Direct negative (W4)
Pest → <i>M. spicatum</i>	No effect	Low toxic concentrations of organic and inorganic pesticides	Direct negative (W4)
Pest → <i>P. perfoliatus</i>	No effect	Low toxic concentrations of organic and inorganic pesticides	No effect
Pest → <i>E. nuttallii</i>	No effect	Low toxic concentrations of organic and inorganic pesticides	No effect
Pest → Periphyton	No effect	Pesticide toxicity	No effect
Pest → Phytoplankton	-	Pesticide toxicity	No effect
Pest → <i>L. stagnalis</i>	-	Pesticide toxicity	Direct negative (W4)
Pest → <i>D. magna</i>	-	Pesticide toxicity	Direct negative (W1 and W2)
Nitr → Total macrophytes	No effect	Low toxic NO ₃ concentrations (Palove-Balang et al., 2016)	No effect
Nitr → <i>M. spicatum</i>	No effect	Low toxic NO ₃ concentrations	Direct negative
Nitr → <i>P. perfoliatus</i>	No effect	Low toxic NO ₃ concentrations	Direct negative
Nitr → <i>E. nuttallii</i>	No effect	Low toxic NO ₃ concentrations	Direct negative
Nitr → Periphyton	+	Fertilisation by nitrates	Direct positive (W1)
Nitr → Phytoplankton	+	Fertilisation by nitrates	Direct positive (W4)
Temp → Total macrophytes	+/-	Enhanced metabolism/heat stress	No effect
Temp → <i>M. spicatum</i>	+/-	Enhanced metabolism/heat stress	Direct positive (W4)
Temp → <i>P. perfoliatus</i>	+/-	Enhanced metabolism/heat stress	No effect
Temp → <i>E. nuttallii</i>	+/-	Enhanced metabolism/heat stress	No effect
Temp → Periphyton	+/-	Enhanced metabolism/heat stress	No effect
Temp → Phytoplankton	+/-	Enhanced metabolism/heat stress	No effect
Temp → <i>L. stagnalis</i>	+/-	Enhanced metabolism/heat stress	Direct positive (W4)
Temp → <i>D. magna</i>	+/-	Enhanced metabolism/heat stress	Direct positive (W1)
Biotic interactions			

Snails → Total macrophytes	-	Grazing	No effect
<i>L. stagnalis</i> → <i>M. spicatum</i>	-	Grazing	No effect
<i>L. stagnalis</i> → <i>P. perfoliatus</i>	-	Grazing	No effect
<i>L. stagnalis</i> → <i>E. nuttallii</i>	-	Grazing	No effect
<i>L. stagnalis</i> → Periphyton	-	Grazing	Direct negative (W4)
<i>D. magna</i> → Phytoplankton	-	Grazing	Direct negative (marginally between W1&2 and significantly between W2&3)
Phytoplankton → macrophytes	-	Light & nutrient competition	No effect
Macrophytes → phytoplankton	-	Light & nutrient competition	Direct negative (W4)
Phytoplankton → periphyton	-	Light & nutrient competition	No effect
Periphyton → phytoplankton	-	Light & nutrient competition	Direct negative (significantly between W2&3 and W3&4)
Periphyton → macrophytes	-	Light & nutrient competition	No effect
Macrophytes → periphyton	-	Light & nutrient competition	Direct negative (W4)

Tmp = temperature effect; Pest = pesticide effect, Nitr = nitrate effect, W = week.

Table S3.3. Statistical analysis of individual and interaction effects for abiotic and biotic response factors. Significance values for response variables obtained using parametric and nonparametric tests

Response factor	Analysis	Individual effects			Interactions				
		Tmp	Pest	Nitr	Tmp*Pest	Tmp*Nitr	Pest*Nitr	Tmp*Pest*Nitr	
Physicochemistry									
Dissolved organic carbon	KW rank sum test	0.4	0.04	0.6					
Dissolved oxygen w0 (%)	ANOVA	0.6	0.8	0.8	0.7	0.6	0.9	0.7	
Dissolved oxygen w1 (%)	ANOVA	0.2	0.1	0.6	0.5	0.6	0.5	0.5	
Dissolved oxygen w2 (%)	ANOVA	0.6	0.0005	0.1	0.5	0.1	0.009	0.05	
Dissolved oxygen w3 (%)		0.1	0.5	0.001	0.004	0.4	0.09	0.9	
Dissolved oxygen w4 (%)	KW rank sum test	0.07	0.16	0.2					
pH w0	ANOVA	0.01	0.8	0.6	0.7	0.7	0.2	0.4	
pH w1	ANOVA	0.006	0.01	0.2	0.7	0.3	0.3	0.07	
pH w2	KW rank sum test	0.07	7.39e-07	0.5					
pH w3	KW rank sum test	0.05	0.02	0.03					
pH w4	ANOVA	0.003	0.7	0.9	0.7	0.6	0.9	0.8	
Conductivity w0	KW rank sum test	0.3	0.1	0.7					
Conductivity w1	KW rank sum test	0.7	0.5	9.686e-07					
Conductivity w2	ANOVA	0.9	0.5	2.15e-05	0.0006	0.06	0.002	0.01	

Conductivity w3	KW rank sum test	0.003	0.03	0.002					
Conductivity w4	KW rank sum test	0.004	0.5	0.001					
P-PO4	ANOVA	0.04	0.5	0.5	0.0004	0.3	0.9	0.8	
N-NO2	ANOVA	0.04	0.6	0.7	0.4	0.01	0.7	0.4	
N-NO3	ANOVA	0.9	0.05	0.7	0.5	0.6	0.8	0.4	
N-NH4	ANOVA	0.03	0.07	1.0	0.2	0.1	0.5	0.3	
Total N	ANOVA	0.06	0.07	0.8	0.9	0.09	0.6	0.3	
N:P molar ratio	ANOVA	0.9	0.2	0.4	0.001	0.5	0.8	0.5	
Macrophytes									
Total macrophyte AG	ANOVA	0.06	0.0001	0.2	0.3	0.5	0.3	0.6	
<i>M. spicatum</i> AG	ANOVA	0.0004	3.05e-05	0.02	0.2	0.7	0.7	0.7	
<i>P. perfoliatus</i> AG	ANOVA	0.01	0.7	1	0.6	0.7	0.4	0.07	
<i>E. nuttallii</i> AG	ANOVA	0.9	0.8	0.4	0.6	0.01	0.3	1	
<i>M. spicatum</i> BG	KW rank sum test					0.7	0.1	0.07	
<i>P. perfoliatus</i> BG	KW rank sum test					0.2	0.6	0.4	
<i>E. nuttallii</i> BG	KW rank sum test					0.2	0.7	0.7	
Relative abundance	PERMANOVA	0.01	0.002	0.03	0.5	0.2	0.5	0.5	
Biofilm									
Biofilm Chl a	ANOVA	0.6	0.05	0.7	0.05	0.9	0.09	0.4	
Biofilm F0 w1	KW rank sum test	0.005	0.2	0.0001					

Biofilm F0 w2	KW rank sum test	0.0005	0.004	0.18				
Biofilm F0 w3	KW rank sum test	0.6	0.0008	0.07				
Phytoplankton								
Phytoplankton Chl a	ANOVA	0.2	0.002	0.9	0.02	0.8	0.6	0.8
OD663 w1	ANOVA	0.01	0.5	0.5	0.8	0.9	0.4	0.7
OD663 w2	ANOVA	0.5	0.04	0.9	0.7	0.1	0.5	0.4
OD663 w3	ANOVA	0.5	1.27e-06	0.4	0.7	0.1	0.1	1
OD663 w4	KW rank sum test	0.7	0.02	0.002	0.3	0.02	0.001	0.05
No. of bacteria								
In water (per ml)	ANOVA	0.0005	0.3	0.3	0.4	0.6	0.8	0.9
In biofilm (per cm ²)	ANOVA	0.003	0.07	0.9	0.1	0.9	0.2	0.7
Primary producers								
Relative abundance	PERMANOVA	0.4	0.005	0.3	0.3	0.3	0.2	0.8
<i>L. stagnalis</i>								
Growth rate	ANOVA	0.01	0.0005	0.6	0.6	0.04	0.9	0.8
Death rate		0.6	0.3	0.6				
<i>D. polymorpha</i>								
Growth rate	ANOVA	0.06	0.1	0.2	0.5	0.1	0.5	0.7
<i>D. magna</i>								
Biomass	ANOVA	0.07	0.1	0.3	0.3	0.4	0.2	0.5
Individuals L ⁻¹ (w1)	ANOVA	0.01	5.18e-07	0.4	0.2	0.9	0.8	1
Individuals L ⁻¹ (w2)	ANOVA	0.1	8.32e-06	0.9	0.06	0.9	0.6	0.9
Individuals L ⁻¹ (w3)	ANOVA	0.2	0.1	0.6	0.5	0.4	0.1	0.6
Individuals L ⁻¹ (w4)	ANOVA	0.4	0.8	0.8	0.5	0.8	0.9	0.8

Table S3.4. Physicochemical parameters measured after 4-week exposure, means \pm *SD*, *n* = 5

	CON		NO3		PST		ARO	
	22	26	22	26	22	26	22	26
Temperature (°C)	21.2±0.09	24.8±0.2	21.2±0.05	24.8±0.3	21.1±0.1	24.6±0.3	21.2±0.04	24.7±0.2
pH	8.0±0.2	7.7±0.2	8.1±0.3	7.6±0.3	8.0±0.3	7.8±0.4	8.1±0.7	7.8±0.1
Conductivity (μ S cm ⁻¹)	354.8±14.0	367.6±15.4	393.6±12.5	417.6±17.5	350.4±4.6	392±55.0	363.6±44.6	418.2±23.8
O ₂ (%)	113.3±12.6	96.4±15.48	106.82±20.5	86.14±27.4	104.12±9.49	86.8±30.2	82.94±51.4	90.26±9.47
O ₂ (mg L ⁻¹)	10.04±1.0	8.0±1.3	9.3±1.8	8.2±1.0	9.2±0.8	7.2±2.5	7.4±4.5	7.4±0.8
DOC (mg L ⁻¹)	7.2±0.6	6.8±0.6	7.3±1.1	7.5±1.3	12.5±8.3	8.0±1.8	13.5±7.9	9.4±4.4
N-NO ₂ (mg L ⁻¹)	0.009±0.007	0.09±0.10	0.05±0.03	0.04±0.07	0.007±0.002	0.15±0.2	0.03±0.04	0.03±0.02
N-NO ₃ (mg L ⁻¹)	0.166±0.10	0.226±0.08	0.244±0.19	0.162±0.04	0.132±0.07	0.114±0.07	0.210±0.22	0.126±0.04
N-NH ₄ (mg L ⁻¹)	0.09±0.12	0.90±0.90	0.40±0.50	0.23±0.31	0.01±0.01	0.50±0.70	0.13±0.15	0.24±0.30
Total N (mg L ⁻¹)	0.27±0.22	1.21±0.90	0.70±0.60	0.44±0.30	0.15±0.08	0.74±0.82	0.40±0.34	0.40±0.30
P-PO ₄ (μ g L ⁻¹)	72.3±33.4	128.7±134.8	115.6±86.1	54.8±72.2	22.4±19.8	176.41±123.8	20.0±6.7	117.0±57.1
N:P (molar ratio)	9.89±11.1	62.44±78.1	14.26±5.8	44.50±48.4	23.17±21.4	6.90±5.5	36.65±27.6	11.50±11.0
Cu (μ g L ⁻¹)	6.5 \pm 2.1	ND	ND	ND	10±3.7	14±5.9	12±5	15±7.3
Terbutylazine (μ g L ⁻¹)	-	-	ND	ND	-	-	-	-
Pirimicarb (μ g L ⁻¹)	0.14	0.03	ND	ND	11.8±0.3	11.0 \pm 0.4	11.1±0.8	10.9±0.4
Tebuconazole (μ g L ⁻¹)	0.20	0.09	ND	ND	41.3±3.2	35.4 \pm 1.3	27.3 \pm 5.3	39.4 \pm 6.9

Supplementary figures

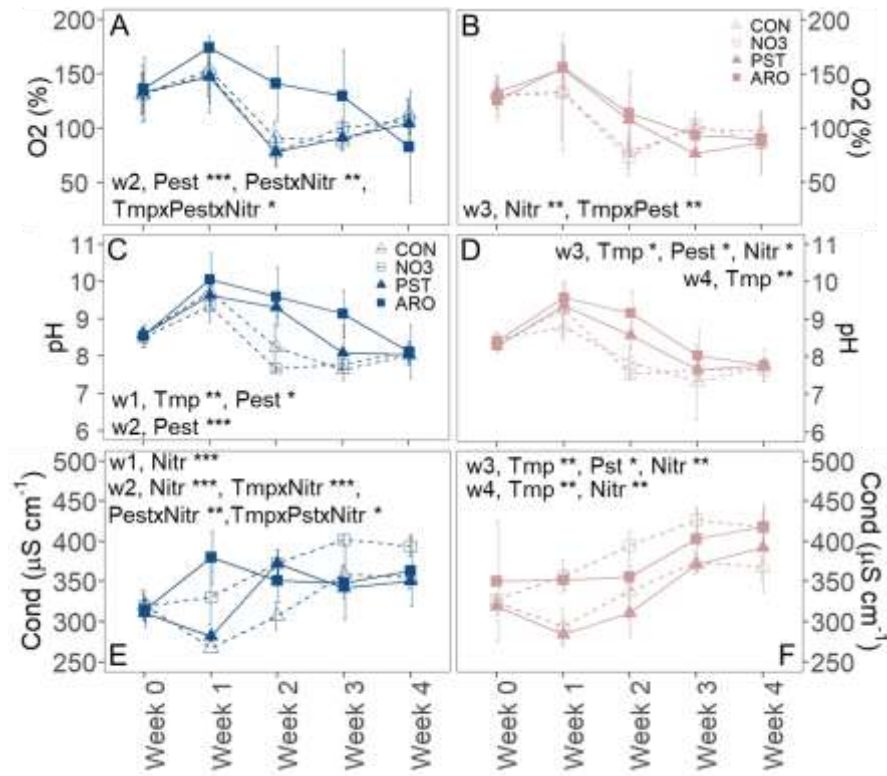


Fig. S3.1. Weekly dynamics of physico-chemical parameters in the ambient (blue) and warm (mauve) microcosms. Means \pm SD, $n = 5$. CON = control; NO₃ = nitrates; PST = organic pesticides and copper; ARO = PST + NO₃. Asterisks represent significant effects observed with the ANOVA ($p < 0.05$) using three fixed factors: Tmp = temperature; Pest = pesticide, Nitr = nitrate at different time points of the experiment (weeks (w) 1-4). Cond = conductivity.

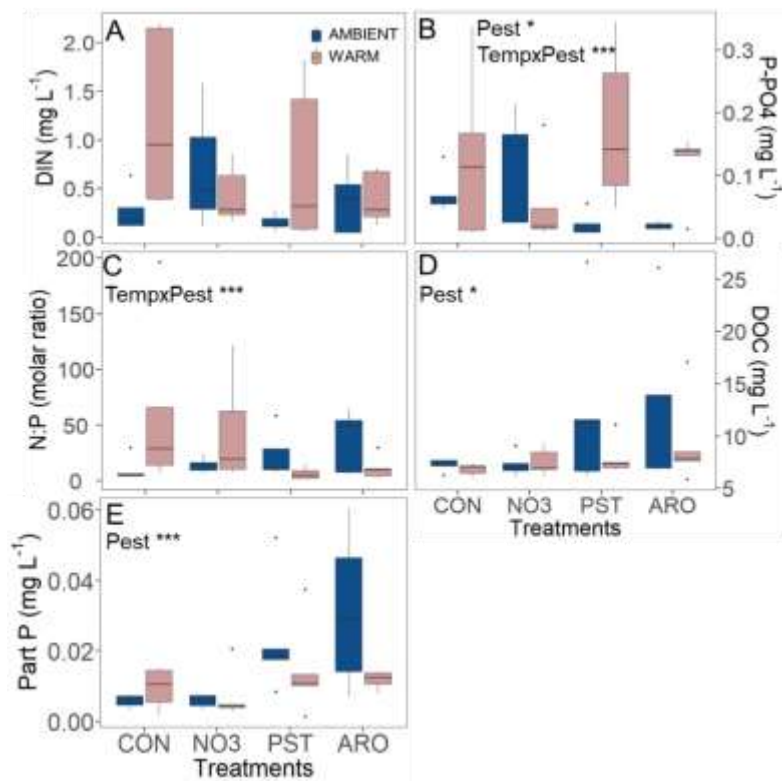


Fig. S3.2. Box plots displaying temperature and treatment effects. (A) dissolved inorganic nitrogen (DIN); (B) orthophosphate (P-PO₄) concentrations in the water; (C) N:P molar ratios; (D) dissolved organic carbon (DOC) and (E) particulate phosphorus (Part P). CON = control; NO₃ = nitrates; PST = organic pesticides and copper; ARO = PST + NO₃. Asterisks represent significant effects observed with the ANOVA ($p < 0.05$): using 3 fixed factors: Tmp = temperature; Pest = pesticide, Nitr = nitrate. Details for box plots as shown for Fig. 1. Blue and mauve represent ambient and warm microcosms, respectively.

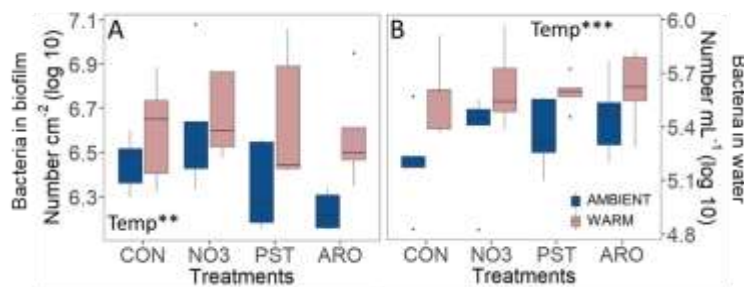


Fig. S3.3. Box plots displaying temperature and treatment effects on the number of bacterial cells in (A) biofilm and (B) water. CON = control; NO₃ = nitrates; PST = organic pesticides and copper; ARO = PST + NO₃. Asterisks represent significant effects observed with the ANOVA ($p < 0.05$): using 3 fixed factors: Tmp = temperature; Pest = pesticide, Nitr = nitrate. Details for box plots as shown for Fig. 1. Blue and mauve represent ambient and warm microcosms, respectively.

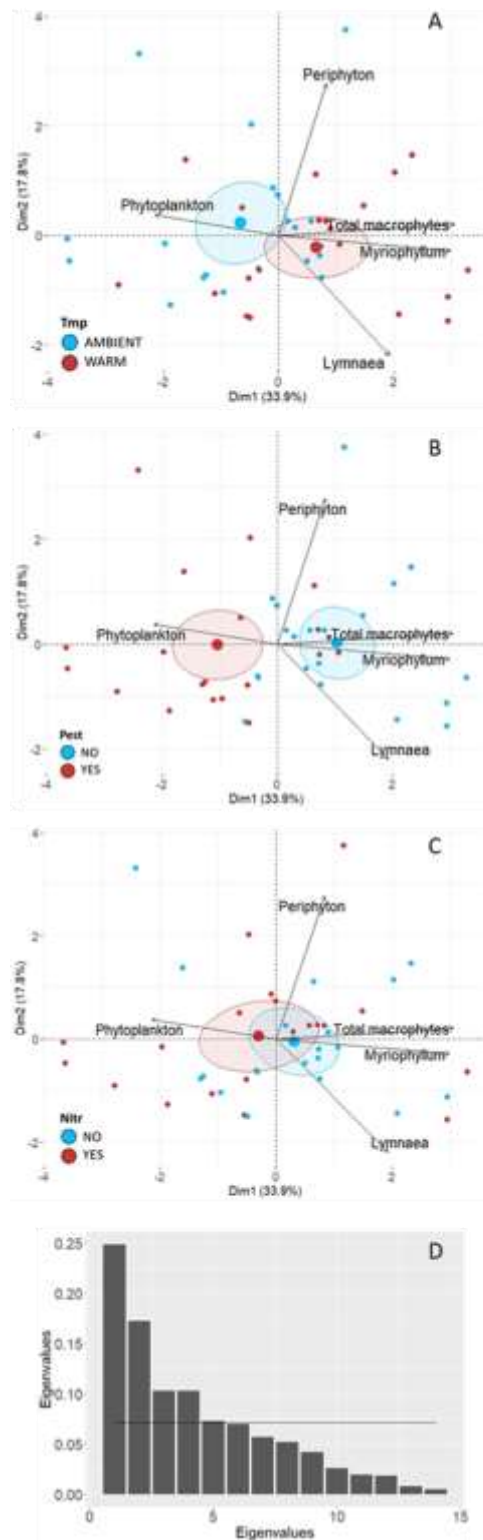


Fig. S3.4. Principal components analyses of 8 response variables, plotted using axes 1 and 2, with projection of supplementary data including continuous variables (7 physicochemical variables, in blue) and categorical variables: (A) cold and warm treatments, (B) presence and absence of pesticides, and (C) presence and absence of nitrates. Eigenvalues are presented in the barplot (D), and the most significant axes represented as values higher than the horizontal line based on the Kaiser-Guttman criterion (Guttman, 1954; Kaiser, 2011; Kaiser, 1960).

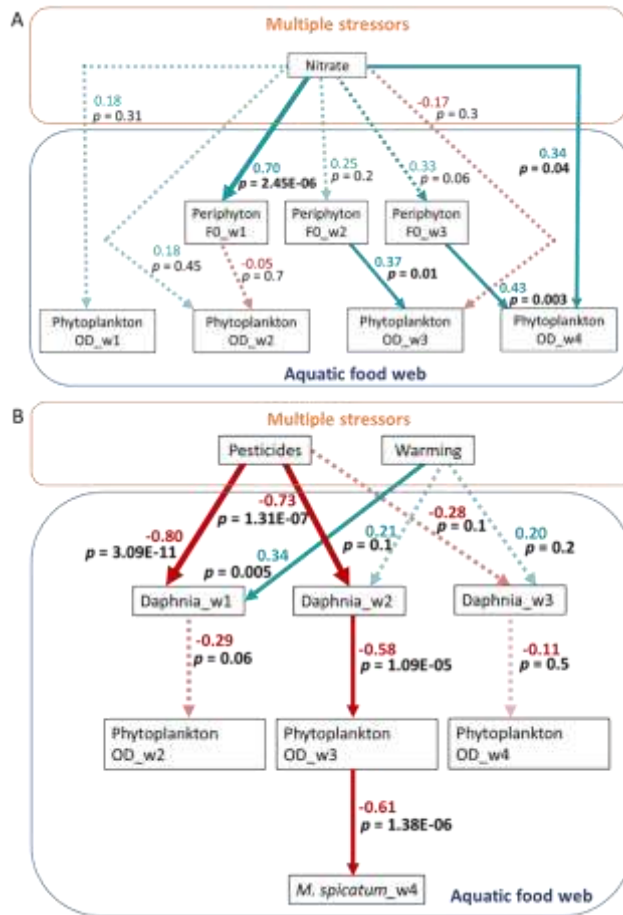


Fig. S3.5. SEMs created using temporal data. (A) Effect of nitrate on periphyton and phytoplankton. $\text{Chi}^2 = 62$, root mean square error of approximation = 0.3, standardised root mean square residual = 0.2, adjusted goodness of fit index = 0.5, and comparative fit index = 0.4. (B) Effect of pesticide and warming on Daphnia, phytoplankton and macrophytes. $\text{Chi}^2 = 85$, root mean square error of approximation = 0.2, standardised root mean square residual = 0.2, adjusted goodness of fit index = 0.6, and comparative fit index = 0.7. Red, green and dashed arrows indicate negative, positive and nonsignificant relations between variables, respectively. Significant threshold was set at $p = 0.05$. R values, which are the proportion of variation explained by the model for each response variable, are represented in blue and red for positive and negative effects, respectively.

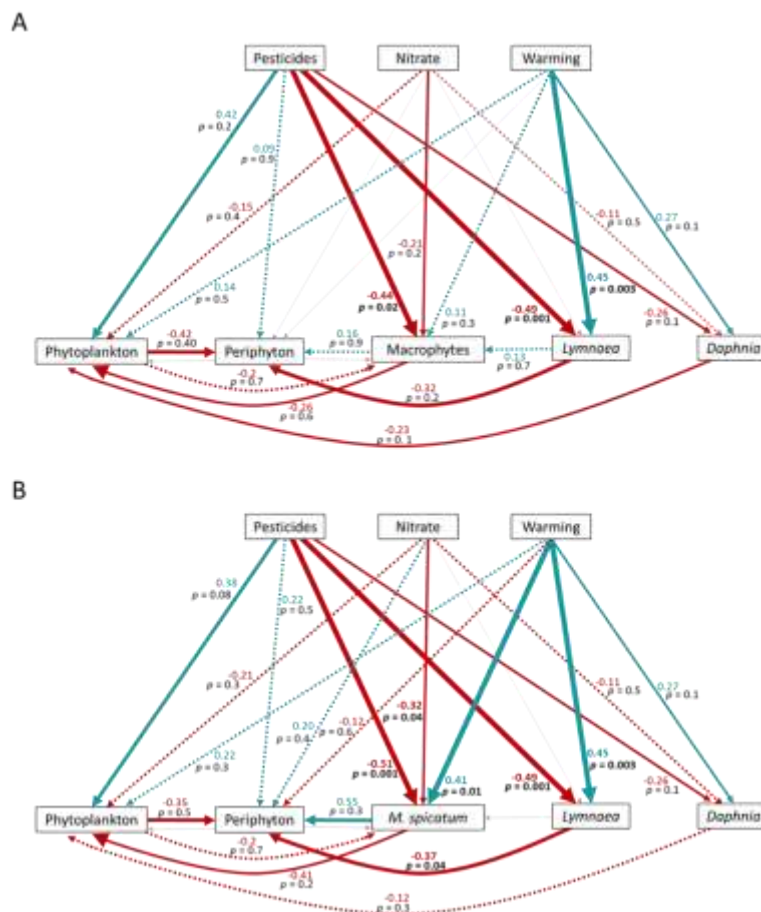


Fig. S3.6. Preliminary SEMs using all expected pathways. (A) based on all macrophytes, (B) based only on the dominant MS. Red, green and dashed arrows indicate negative, positive and nonsignificant relations between variables, respectively. Significant threshold was set at $p < 0.05$. R values, which are the proportion of variation explained by the model for each response variable, are represented in blue and red for positive and negative effects, respectively. (A) $\text{Chi}^2 = 1.8$, root mean square error of approximation = 0.00, standardised root mean square residual = 0.03, adjusted goodness of fit index = 0.9, and comparative fit index = 1. (B) $\text{Chi}^2 = 6.6$, root mean square error of approximation = 0.05, standardised root mean square residual = 0.05, adjusted goodness of fit index = 0.8, and comparative fit index = 1.

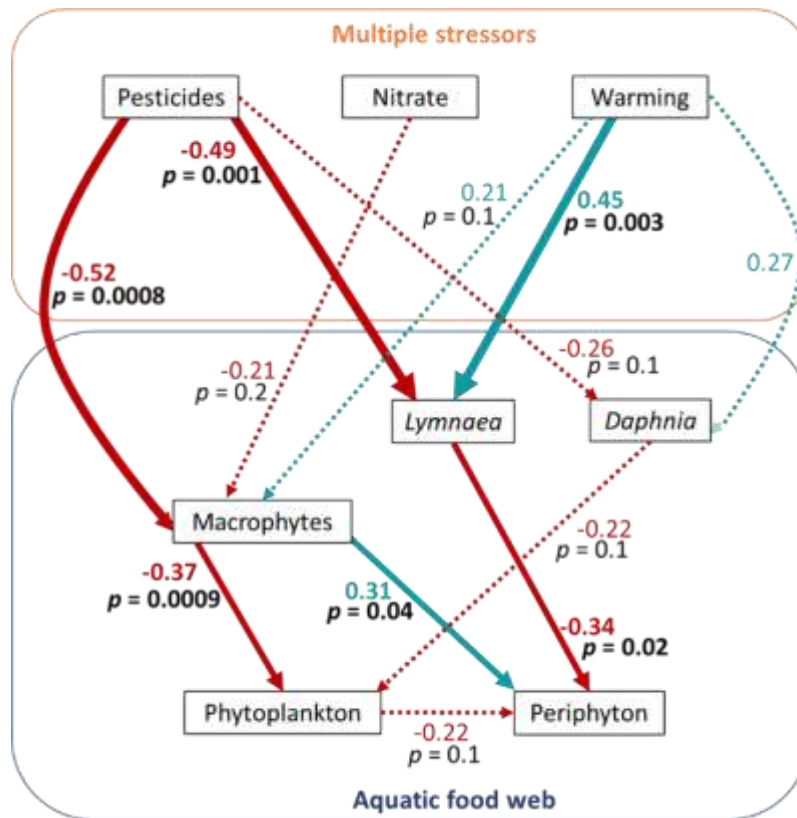


Fig. S3.7. Preliminary SEM using total macrophyte biomass. Red, green and dashed arrows indicate negative, positive and nonsignificant relations between variables, respectively. Significant threshold was set at $p = 0.05$. R values, which are the proportion of variation explained by the model for each response variable, are represented in blue and red for positive and negative effects, respectively. $\chi^2 = 9.5$, root mean square error of approximation = 0.00, standardised root mean square residual = 0.09, adjusted goodness of fit index = 0.9, and comparative fit index = 1.

CHAPTER 4: EVALUATING MULTIPLE STRESSOR EFFECTS ON BENTHIC–PELAGIC FRESHWATER COMMUNITIES IN SYSTEMS OF DIFFERENT COMPLEXITY: CHALLENGES IN UPSCALING

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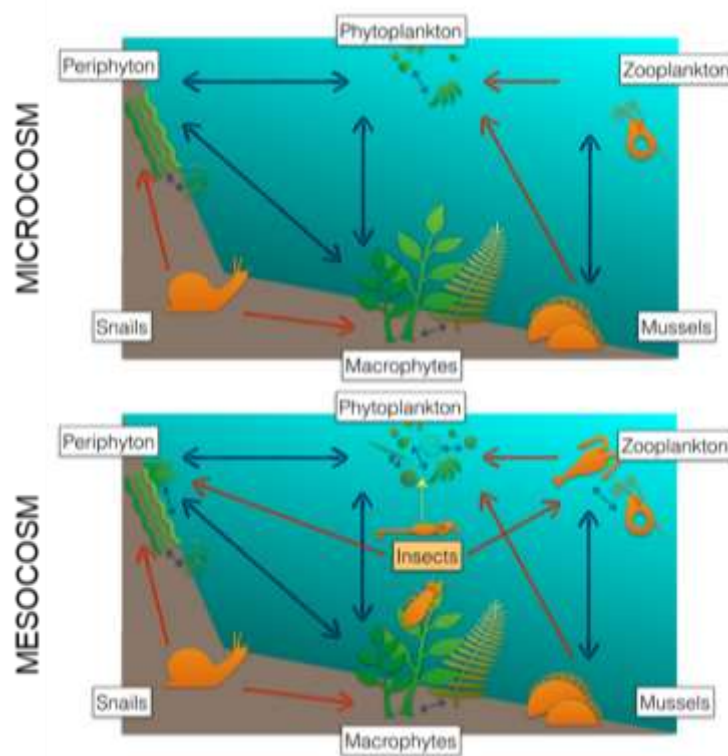
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Abstract

Upscaling of ecological effects from indoor microcosms to outdoor mesocosms bridging the gap between controlled laboratory conditions and highly complex natural environments poses several challenges: Typical standard water types used in laboratory experiments are not feasible in large outdoor experiments. Also, moving from the micro- to meso-scale, biodiversity is enhanced. We performed an indoor microcosm experiment to determine the effects of agricultural run-off (ARO) on a defined benthic–pelagic community comprising primary producers and primary consumers, exposed to ambient summer temperature and +3.5°C. Treatments were replicated in two water types (standard Volvic and Munich well water). We then scaled up to outdoor mesocosms using an ARO concentration gradient and +3°C warming above ambient temperature, using Munich well water. We included the same benthic macroorganisms but more complex periphyton and plankton communities. All functional groups were affected by stressors in the microcosms, and a shift from macrophyte to phytoplankton dominance was observed. While effects were present, they were less pronounced in the mesocosms, where a higher biodiversity may have modified the responses of the system to the stressors. Stressor effects observed

in controlled experiments may thus be masked in more complex outdoor experiments, but should not be interpreted as “no effects”.

Keywords: microcosms, mesocosms, community complexity, stressor interactions, biotic interactions, copper, terbutylazine, pirimicarb, tebuconazole, climate warming



1. Introduction

Alternative stable states between phytoplankton and macrophytes commonly occur in shallow lakes, and state shifts are primarily driven by eutrophication (Scheffer et al., 1993). The likelihood for such shifts may be enhanced by warming, e.g., through an increased release of nutrients from the sediment (Jeppesen et al., 2009). While the focus of many eutrophication studies in shallow lakes has been on phosphorus, recent studies show that high nitrate concentrations may also facilitate regime shifts (Barker et al., 2008; Moss et al., 2013; Sagrario et al., 2005). High nutrient input into lakes occurs after heavy rainfall events through surface run-off from agricultural sites also containing pesticides. The ecotoxicological effects of these pesticides are well studied at the organismic level, but the community- and ecosystem-level effects are less understood. A combination of nutrient and pesticide stressors may further facilitate the shift to turbid conditions.

With the growing use of pesticides and fertilisers, and rising global temperatures, identifying safety margins to preserve the stability of the macrophyte-dominated state in these systems exposed to such multiple stressors is essential. Starting with laboratory-controlled microcosms comprising less complex benthic–pelagic communities and upscaling to more complex and variable communities in outdoor mesocosms might allow for the identification of stressor patterns or effects as they would occur in natural ecosystems, even in the presence of confounding factors.

Indoor microcosms allow a better control of environmental factors and can be set up with key functional groups representative of shallow lake benthic–pelagic communities, such as macrophytes, phytoplankton, periphyton, benthic and pelagic filter feeders and grazers. They allow the use of organisms common in standardised ecotoxicity tests, such as *Daphnia* (Colbourne et al., 2011; OECD, 2012), algae and the OECD test plant *Myriophyllum spicatum* (OECD, 2014) all typically playing key roles in natural systems. Most importantly, microcosms can be designed to couple benthic and pelagic organisms. Upscaling to larger outdoor mesocosms will further enable the inclusion of natural environmental factors such as irradiance and temperature fluctuations, more diverse communities with more species representative of each functional group and/or ecosystem type and more trophic levels.

Upscaling, however, can pose several challenges due to the size of experimental units and natural variability in abiotic and biotic factors. The chemistry of the water used

in the experimental set-up is important (Ebenebe et al., 2019; Rameshkumar et al., 2019), as is the technical availability in terms of costs and logistics. In controlled laboratory experiments, defined culture media or widely available bottled natural mineral waters (e.g., Volvic®) are often used, allowing comparison of results among research groups (Gross et al., 2018; Potet et al., 2016). However, using this for large outdoor mesocosms at the cubic metre scale is not feasible. Most mesocosms are usually filled with surrounding lake/sea water or tap/groundwater, depending on availability (Davidson et al., 2018; Havens, 1994; Landkildehus et al., 2014; Riis et al., 2018). While these waters vary in their physico-chemical properties, it may still be reasonable to use them in mesocosms, provided precautions are made based on the organisms' needs. For example, adapting the concentration and ratio of initial macronutrients, conductivity range, alkalinity, pH, and dissolved organic and inorganic carbon of the water at the start of the experiment may allow optimum conditions and better comparability between experiments. In addition to the central parameter “water type”, other abiotic parameters such as photoperiod, temperature, pH, dissolved oxygen and light intensities vary over time (Caquet et al., 2001), making them less controllable than laboratory microcosms. Such varying natural abiotic conditions can play an important role in influencing the response of organisms to stressors, and should be well monitored during the experiments (Campbell et al., 1999; Pansch & Hiebenthal, 2019; Watts & Bigg, 2001).

Most studied mesocosms also suffer from large temporal and spatial variations between replicates, in some cases, with more than 50% of the variation being observed on biological variables (Kermoyan et al., 2012). By increasing community complexity in mesocosms, several direct and indirect effects may modify the response of the system to stressors compared with microcosms where biotic interactions are less complex (Allen et al., 2021; Carrier-Belleau et al., 2021). Culturing enough organisms for stocking large mesocosms is challenging and sometimes impossible. To solve this issue, mesocosm studies usually incorporate organisms from natural communities, e.g., by using natural plankton or benthic communities from field sites versus relying on specific cultured organisms in laboratory experiments. While this will increase the complexity of the community, it may also be the aim of the study, as a higher biodiversity allows more closely mimicking stressor effects in a real-world habitat.

Outdoor experiments are also prone to species invasions into the experimental units. These may not always be easy to control, and in some cases may lead to new trophic

levels, which may reverse overall responses of the system to nutrient enrichment (Wollrab et al., 2012). Some species invasions can be prevented using specific measures (e.g., netting against birds), but open outdoor mesocosms cannot be easily protected from invasions by smaller organisms such as aquatic insects without interfering, e.g., with light climate and gas exchange. Therefore, decision-making on which invasions can be informative and allowed, and which might be disruptive to the data analyses is fundamental to answering specific research questions.

This article focuses on some of the challenges of upscaling research on the effects of multiple stressors on shallow aquatic systems by combining small-scale laboratory studies to large-scale outdoor experiments. Microcosms were designed to analyse the effects of ARO and temperature on a predefined less complex and *a priori* assembled benthic–pelagic community. Mesocosms were planned to be more complex, with the aim of studying the responses of natural and diverse communities to a gradual increase in ARO associated with elevated temperature. Microcosms were run with a well-defined mineral water (Volvic water) whereas the large water volume needed for mesocosms could only be met by using Munich well water available at the site where the mesocosm experiment was conducted (Ludwig Maximilian University of Munich [LMU], Munich). To compare the effects of using either standard Volvic or Munich well water, the controlled microcosm experiments were performed with both water sources. We hypothesised that,

- (1) Water type will not modify effects of the stressors,
- (2) Response of model (laboratory) communities to the stressors can be mirrored in more complex field (mesocosm) communities, and
- (3) A gradient design will allow the detection of concentration-dependent community effects in more complex systems

2. Materials and methods

2.1. Microcosm experiment

2.1.1. Set-up and design

We performed experiments beginning with smaller controlled indoor microcosms each with a volume of 8 L exposed to ARO and warming. Aquatic organisms were selected as representative of fishless ponds, and included two trophic levels (primary producers and consumers) comprising key functional groups: macrophytes, phytoplankton, periphyton as the three major groups of primary producers in aquatic systems, *D. magna* as representative

of pelagic herbivore filter feeders (food source: phytoplankton), and *L. stagnalis* as representative of benthic grazers (food source: periphyton). The macrophyte community consisted of three submerged macrophytes, *Myriophyllum spicatum*, *Potamogeton perfoliatus*, and *Elodea nuttallii*. The microalgae community was based on eight cultured strains of periphyton or phytoplankton similar to Allen et al. (2021), except *Uronema confervicolum* and *Gomphonema parvulum*, which did not grow in our cultures. The second trophic level included three primary consumers: snails (*Lymnaea stagnalis*) feeding on periphyton and sometimes macrophytes, and mussels (*Dreissena polymorpha*) and zooplankton (*Daphnia magna*) feeding on phytoplankton. Half of the microcosms were exposed to ARO, comprising copper as inorganic fungicide, three organic pesticides (an herbicide, an insecticide, a fungicide) and nitrate (as KNO_3), and the other half were controls. The chosen pesticides are widely used in European agriculture, and concentrations of all chemicals are based on sensitivity data, as well as background environmental concentrations. Further details of the ARO can be found in Table S4.1 and Allen et al. (2021). The treatments were replicated at two temperatures, aiming for a +4°C increase above ambient temperature in the heated microcosms, as projected by the IPCC RCP 8.5.

To determine whether water type can affect the community response to applied stressors (temperature and ARO), we performed a microcosm experiment using a full-factorial design with 2 water types x 2 pesticide levels x 2 temperatures x 5 replicates, for a total of 40 microcosms. We used two water types: Volvic water (Vw), as a frequently used standard in experimental research, and Munich well water (Mw), which was our choice in the mesocosm experiments. Our treatments were the controls in Vw (VCON) and Mw (MCON), and those exposed to ARO in Vw (VARO) and Mw (MARO), each replicated at two temperatures. The chemistry of both water types was analysed using standard methods before the experiment. This included alkalinity (Gran titration), major anions (nitrate, nitrite, orthophosphate, chloride, sulphate; ion chromatography, Dionex ICS 1100), ammonium (spectrophotometry) and alkali elements (Ca, K, Mg, Na; flame ionisation, Thermo Scientific ICE 3300).

The microcosms comprised a crystallizing dish insert (height 8 cm, Ø 15 cm) filled with a layer of sediment according to the OECD TG 239 (OECD, 2014) but by replacing inorganic nutrient salts with nettle powder (0.5% w/w). Each dish was placed within a glass cylinder (Sandra Rich GmbH, height 40 cm, Ø 19 cm) filled with 8 L of the respective water. The microcosms were then distributed evenly into four glass tanks, consisting of

circulating, temperature controlled water baths, two each at 22°C and 26°C. However, the average minimum and maximum temperatures reached with the heating system were 21.1°C ± 0.2°C and 24.6°C ± 0.3°C, for a difference of ~3.5°C between the two temperature treatments.

All species were acclimated to experimental conditions for at least 4 weeks and sorted for size before addition to the microcosms, to ensure homogeneous distribution among the treatments. The microalgae were cultured in BG11 (Rippka et al., 1979) or WC (Guillard & Lorenzen, 1972) medium and adapted to Vw before start of the experiment. Four polypropylene sheets measuring 29.7 cm x 2.6 cm length x width were hung vertically into each microcosm for follow-up of periphyton development (cf. section 2.2.2). Before the microalgae were added to the microcosms, the Mw treatments were fertilized with phosphorus to achieve the Redfield molar ratio (16:1 N:P) similar to that present in Vw. Similar biovolumes of both the periphyton and phytoplankton were added to the microcosms six days before the start (T-6). Two days later, two 10 cm shoots of each macrophyte species were planted into the sediment (T-4). Twenty *Daphnia* neonates, one snail and three mussels per microcosm were added on day T-2. The ARO was added at T0, and at the same time, the temperature of half the treatments was set at 26°C. The microcosms were exposed to a 16:8 h day:night cycle with irradiance at an average of 76.01 ± 7.9 μmol photons m⁻² s⁻¹ photosynthetically active radiation (PAR) at the water surface (ToLEDo LED fluorescent tubes, cool white, 150 cm, 27 W, Sylvania).

2.1.2. Sampling and measured parameters

Weekly measures of pH, conductivity and oxygen saturation of the water were made using a multi-parameter analyser (WTW Multiline 3410). Dissolved inorganic nutrients were measured after inoculation with organisms but before exposure.

At the end of the experiment (4 weeks), water was first sampled for chemistry, followed by plankton and periphyton, and then the benthic consumers. The macrophytes were sampled at the end to ensure minimum disruption to the system.

During the final sampling, the dissolved inorganic nutrients were again measured in all microcosms. The optical density of the water at 663 nm, used as a proxy for the development of phytoplankton over time, was measured weekly with a Varian Cary® UV-VIS spectrophotometer.

Depending on the final density of phytoplankton, between 40 and 100 mL per sample were filtered (25 mm GF/F filters, Whatman, 0.7 µm pore size) for analysis of carbon. The periphyton on polypropylene strips was brushed gently into 20 mL Vw. Pellets were homogenised, centrifuged, frozen and lyophilised, and the dry powder used for carbon analyses. Photosynthetic pigments of microalgae were analysed by HPLC-DAD (high performance liquid chromatography-diode array detector) (UHPLC Ultimate 3000 Rs THERMO).

Weekly *Daphnia* counts were made by collecting 50 mL of water after gentle stirring of the water column. This number was extrapolated to the total volume per microcosm. Counted *Daphnia* were returned to the microcosms. At the end of the experiment, all *Daphnia* were collected. Their biomass was estimated from length measures, obtained using a numerical microscope (VHX-6000; Keyence): $B = 0.01 * L^{2.62}$, where B represents biomass in mg and L = length in mm (Pauw et al., 1981).

The snails and mussels were removed and their lengths measured.

Macrophytes were removed including the roots, rinsed and separated into aboveground and belowground parts. Carbon content of the apical 10 cm dry plant shoot was measured. Carbon content of primary producers was measured using a CHNS elemental analyser (Carlo-ERBA Na 2100 CE).

2.1.3. Data analyses

A three-way ANOVA was performed using R (v4.1.0; R Core Team, 2018) to test the individual and combined effects of water type, ARO and temperature on all end points. Residuals were first tested for normality and homoscedasticity using the Shapiro-Wilk's test and the Bartlett test, respectively. Log or square root transformations were performed if data did not fit these assumptions. When data could not be normalized, a Kruskal–Wallis test by ranks was applied. Significance was considered at $p < 0.05$. The Hedges' g (Hedges, 1981) were also calculated from the *F*-statistics derived from the three-way ANOVA to determine the size of individual and interaction effects on the biomass of primary producer groups, and on the length or biomass of consumers (using the *esc* package in R; Lüdecke, 2019). To determine significant ARO, water or temperature effects on the distribution of the three primary producer groups, a PERMANOVA was performed using the *vegan* package (J. Oksanen et al., 2020) in R, with proportions (values from 0 to 1) of macrophyte, phytoplankton and periphyton carbon content as the dependent variables. Principal

components analyses (PCAs) were performed using the *vegan* (J. Oksanen et al., 2020) and *factoextra* packages (Kassambara & Mundt, 2020) in R to determine any strong patterns in the data.

2.2. Mesocosms

2.2.1. Set-up and design

Following the microcosm experiment, we scaled up to larger outdoor mesocosms set up at LMU (48° 6' 31.961" N 11° 27' 26.896" E) with a total sixteen enclosures and volumes of 640 L each using Munich well water and exposed to a gradient of ARO to determine concentration-dependent effects on primary producers. See Fig. 4.1 for a comparison of the design of the indoor microcosms and outdoor mesocosms. We aimed to have a similar trophic structure as in the microcosms (Table S4.2), with two trophic levels, but with the higher biodiversity (at both taxonomic and functional levels) provided by natural periphyton and plankton communities (see Table S4.3 for a comparison of morphotypes between micro- and meosocosms). We included the same three macrophyte species, snails and mussels, and obtained the periphyton and plankton community inoculum from nearby eutrophic and mesotrophic lakes (Lake Bannsee, 47°57'52.4"N 12°26'25.1"E, and Lake Klostersee, 47°58'21.5"N 12°27'25.6"E). Because the mussels had a high mortality rate in both experiments, irrespective of treatment type, they are not discussed further. The highest ARO concentration was set similar to the microcosms, and then diluted in 6 steps at a 1:1 dilution factor (Table S4.1). This gradient was applied to two series of mesocosms differing in temperature (eight at ambient environmental and eight at a +3°C increase, including daily temperature fluctuations). The +3°C difference was chosen to stay similar to the actual temperature difference obtained in the microcosms (+3.5°C). For both experiments, thrice weekly, macronutrients were added at the Redfield ratio (16 $\mu\text{mol L}^{-1}$ N and 1 $\mu\text{mol L}^{-1}$ P as KNO_3 and KH_2PO_4 , respectively) to ensure the growth of the primary producers.

Each of the sixteen enclosures consisted of an inner watertight layer (Innoplast silo foil; BayWa, Germany) and an outer weight-bearing layer. They were suspended from wooden beams in two concrete ponds. This way, all enclosures in the same pond were kept at the same temperature and the heated enclosures could be heated from the outside using two industrial heating elements (48 KW, ISA-Heinrich-Industrietechnik, Falkensee, Germany). Each mesocosm contained 640 L well water resulting in a water column of 70 cm above a sediment layer of 10 cm. Mesocosms stayed without cover and open to full

solar radiation, precipitation and evaporation. Average temperatures throughout the experiment ranged from 16 to 22°C with a peak of 27°C in the ambient mesocosms and from 19 to 25°C with a peak of 31°C in the heated. The difference of 3°C remained consistent between the temperature treatments. Average irradiance ranged from 300 to 2000 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$.

Before starting the experimental manipulations (T0), all organisms were given time to establish in the new environment. Setup started one month before (T-31) with the deployment of the mesocosms. Each one was filled with 80 L of sediment consisting of two layers. An even mixture of sand and soil constituted the bottom layer. This was covered by another layer of sand (Spielsand 0-1 mm and Fortunat Humus 0/5sieved, Bernhard Glück Kies-Sand-Hartsteinsplitt GmbH, Germany). Without agitating the sediment layers, 590 L of well water were pumped into each mesocosm.

A natural phytoplankton community was introduced by adding 50 L of water from the eutrophic lake Bannsee to each mesocosm on the next day. Natural periphyton collected from Lake Klostersee and resuspended in lake water was added to all mesocosms. An initial pulse of 9.8 $\mu\text{mol L}^{-1}$ P to adjust the N:P ratio in Mw was added.

On day -16 and -15, macrophytes were planted. Ten stems of *M. spicatum* and *P. perfoliatus* and fifteen stems of *E. nuttallii* were planted as three distinct patches at the same location in each mesocosm. Along the middle north-south axis, twelve transparent polypropylene strips as support for periphyton (2.5 x 60 cm) were hung in each mesocosm. Snails and mussels were released to the mesocosms on day -4 after homogenisation for size. Zooplankton was collected from Lake Klostersee with a 250 μm net and introduced into the mesocosms on day -2.

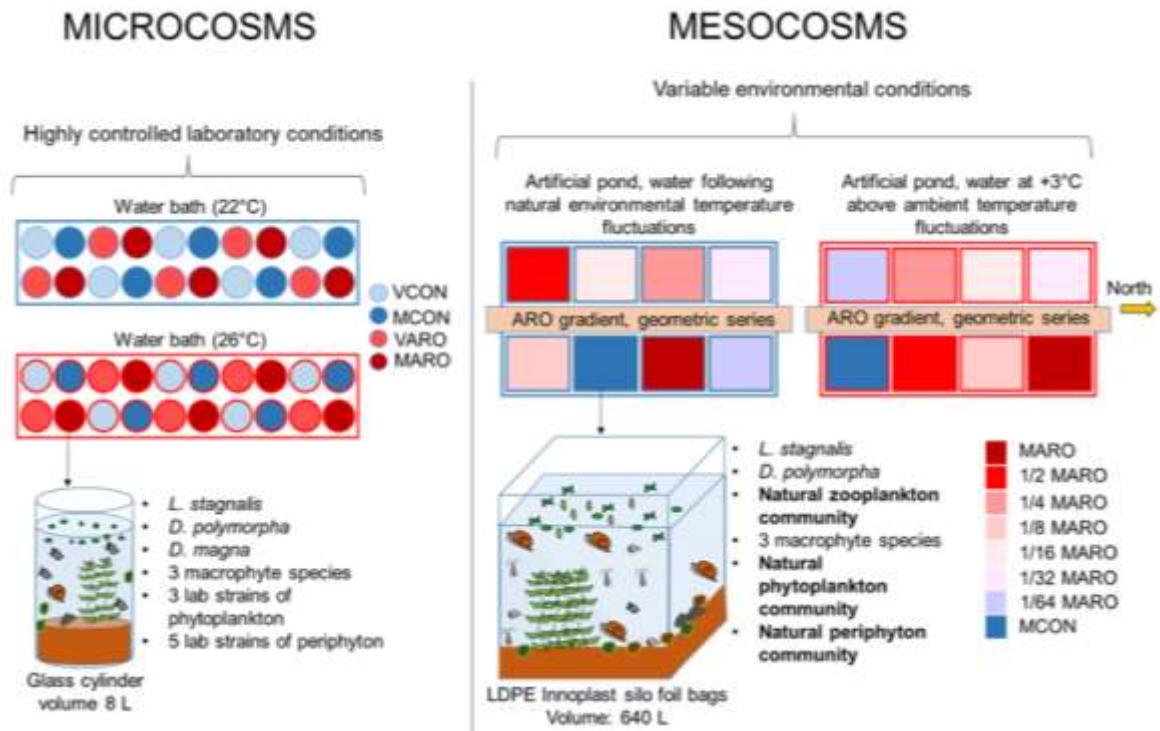


Fig. 4.1. Comparison of design between the indoor controlled microcosms and the outdoor variable mesocosms. The concentrations of the pesticides and nitrate comprising the agricultural runoff (ARO) in the microcosms were comparable to the highest ARO in the mesocosms. VCON = Volvic water control, MCON = Munich water control, VARO = Volvic water ARO, MARO = Munich water ARO.

2.2.2. Sampling and measured parameters

Integrated water samples were taken with a tube sampler (1 L). Phytoplankton chlorophyll-*a* concentration was measured with a multispectral fluorometer (AlgaeLabAnalyser, bbe Moldaenke GmbH). Phytoplankton carbon and nitrogen content were measured with an elemental analyser (varioMICRO Cube, elemental). For continuous light intensity and temperature measurements, HOBOS (HOBO MX2202Pendant, onset) were placed on top of the sediment in the middle of the macrophyte-free quarter.

Periphyton was sampled bi-weekly for chl-*a* measurements (adapted from Woitke et al., 1994 and described in Schmitt and Altenburger, 2005), and at the end of the experiment, dry weights were measured. Carbon equivalents were estimated as 60% dry weight based on average percentages calculated in a previous experiment (VV, unpublished).

Snails were collected before the mesocosms were dismantled, and their lengths measured.

After eight weeks of exposure, the mesocosms were dismantled one by one. The whole water column was passed through a 250 µm net and zooplankton and insect larvae were collected. Additionally, animals clinging to plants and the mesocosm wall or the sediment were collected. All samples were fixed in 96% ethanol. Individual species groups were counted using a stereo microscope. The samples were then dried and weighed.

Once all the water was removed from the mesocosms, all macrophytes were taken out by gently releasing the roots from the sediment until the plants were free but intact. Total dry biomass per species and mesocosm was determined after gently rinsing and removing debris and/or insects, and drying for at least 48 h at 80°C. Carbon content was determined similar to the microcosms.

For both experiments, copper was analysed in LIEC, Metz, France, by atomic absorption spectroscopy (Varian SpectrAA 800 Zeeman) and the organic pesticides were analysed in UFZ, Leipzig, Germany, after filtering water samples (0.22 µm PVDF syringe filters), by liquid chromatography–mass spectrometry using an LTQ Orbitrap XL (Thermo-Scientific, USA).

Simple linear and quadratic regressions were tested on the primary producers using R (R Core Team, 2018) to identify any relationships between the stressors and their biomass.

2.3. Comparison of primary producer community structure in micro- and mesocosms

The primary producer community structures in the micro- and mesocosms were compared using biomasses of each primary producer group expressed as total carbon. For the phytoplankton, measured carbon was extrapolated to the total volume per microcosm (8 L) or mesocosm (640 L) to obtain total carbon. For the periphyton, carbon values were roughly estimated by extrapolating to the surface area of the polystyrene strips plus inner surface of either the microcosm glass walls or the mesocosm enclosure walls. For the macrophytes, total carbon was calculated from the total macrophyte aboveground biomass per micro- and mesocosm.

3. Results

3.1. Microcosms - Effects of water type

Before the start of the experiment, Munich well water (Mw) had a conductivity 197% higher than Volvic water (Vw), likely due to higher contents in calcium and magnesium in Mw by 611% and 160%, respectively (Table S4.4). After a 4-week exposure, the two water types were quite similar in nutrient concentration and pH. Conductivity remained consistently higher in the Mw treatments during all 4 weeks, but the difference between the two water types was reduced from $440 \mu\text{S cm}^{-1}$ at the start of the experiment to $70 \mu\text{S cm}^{-1}$ after 4 weeks (Fig. S4.1). Pirimicarb and tebuconazole declined but were still found at the end of the experiment (30–90%) and did not differ between Vw and Mw, but terbuthylazine was not found in any of the samples (Table S4.4). The decline of pesticides, including copper, did not differ between the two temperatures.

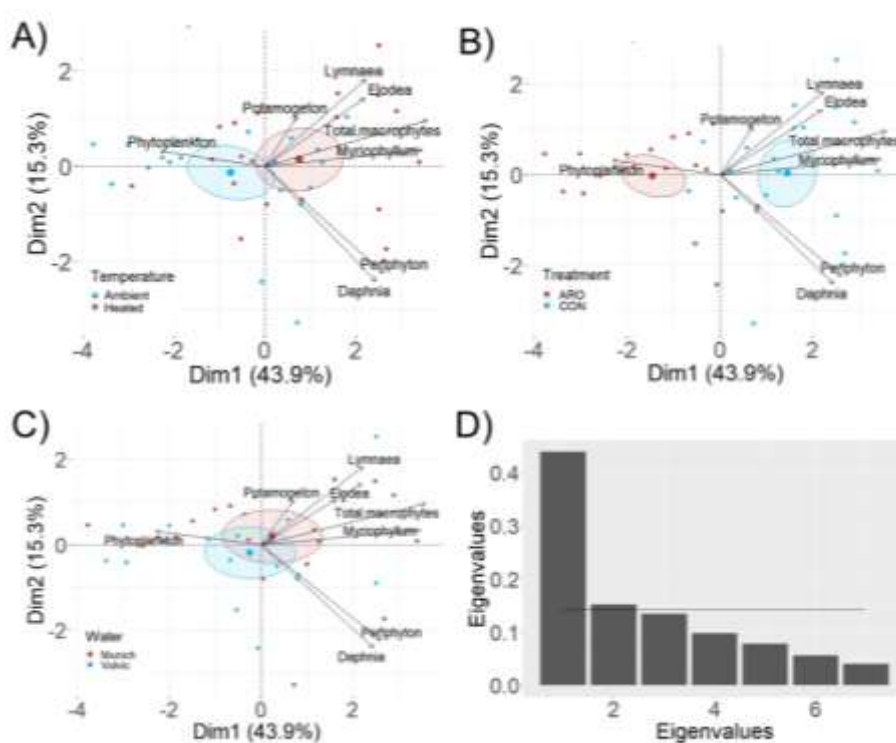


Fig. 4.2. MICROCOSMS. Principal components analysis plot showing the most significant axes and all response variables. Temperature, treatment and water effects are projected as supplementary data: (A) ambient (blue) or heated (red), (B) CON (blue) or ARO (red), (C) Volvic (blue) or Munich well water (red). (D) Eigenvalue scores of the 7 main axes.

Overall, water type did not strongly affect stressor toxicity, nor did it significantly affect the growth of the different functional groups. The 95% ellipses for Mw and Vw overlapped in the PCAs, confirming this (Fig. 4.2C). Water type, however, caused a small though significant increase in biomass of *E. nuttallii* in Mw compared with Vw (Fig. S4.2, Fig. S4.3, Table S4.5). This resulted in only a marginal effect on total macrophytes ($p = 0.07$). An interaction between water type and ARO was also observed for periphyton (Fig. 4.3). Water type had no effect on the other primary producers or consumers, nor did it modify temperature or ARO toxicity towards them. Primary producer proportions were also highly comparable in both water types for the same treatment (Fig S4.4).

3.2. Stressor effects on primary producers

Both the macrophytes and periphyton responded negatively to the ARO, whereas phytoplankton responded positively (Fig. 4.3). The total macrophyte dry aboveground biomass in the ARO treatments decreased by 60% compared with the control (Fig. 4.3). Periphyton chl-*a* also decreased by 60% in the ARO treatment. The reduction in macrophyte and periphyton biomass was accompanied by an increase in phytoplankton biomass, which measured on average 1338% higher in the ARO treatments compared with the controls. Pesticide effects on phytoplankton were much stronger in the ambient treatments, accounting for the overall strong pesticide effect (Fig. 4.2). Warming on the other hand promoted total macrophyte biomass. The dominant but also most sensitive macrophyte species was *M. spicatum*, which significantly decreased in the ARO treatments by 65% compared with the control and increased by 70% in the heated treatments compared with the ambient. The other two macrophyte species grew little and were not strongly affected by the stressors.

Not all effects observed in the microcosms could be seen in the mesocosms. ARO effects on the primary producers were more comparable with the microcosms at ambient temperature. At ambient temperature, total macrophyte biomass was negatively related to ARO level with a decrease of 70% in the highest ARO compared to the control (Fig. 4.3). However, the highest macrophyte biomass was observed in the 1.0-H (ARO concentration in the heated mesocosms) treatment, showing no clear overall response pattern to the stressors (Fig. 4.3B). Both *M. spicatum* and *E. nuttallii* reached their maxima in the 1.0-H treatment and minima in the 1.0-A treatment (Fig. S4.3). *M. spicatum* produced the most biomass (181.4 g) among the macrophytes, followed by *E. nuttallii* (131.9 g) and *P. perfoliatus*

(111.7 g). Similar to the microcosms, *M. spicatum* grew better in the heated microcosms compared with the ambient.

Phytoplankton reached its highest biomass in the 0.5-H mesocosm and the lowest in the the 1.0-H concentration (Fig. 4.3B). Periphyton was not abundant in the mesocosms. The highest and lowest chl-*a* content were measured in the 0.1-A and the 0.5-H treatments, respectively (Fig. S4.3B).

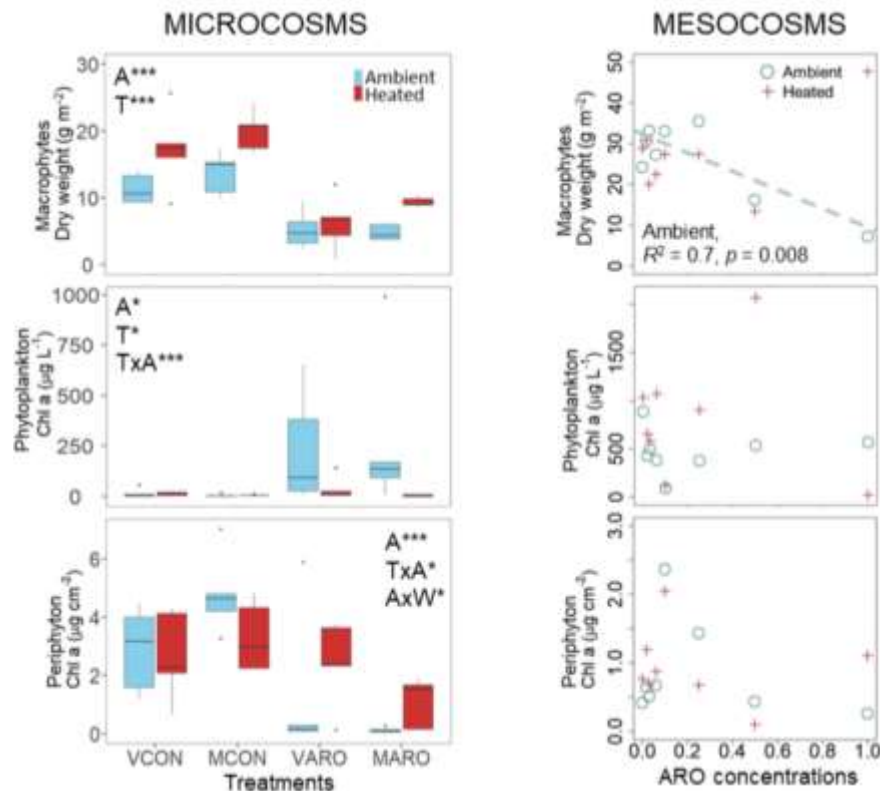


Fig. 4.3. Stressor effects on final aboveground biomass of all macrophytes, chl-*a* concentration of phytoplankton and periphyton in the microcosms (left) and mesocosms (right). Microcosms: VCON = Volvic control; MCON = Munich well water control. ARO; VARO = Volvic ARO; MARO = Munich well water ARO. Box plots of 5 replicates showing median, 25 and 75% percentiles, lowest and highest whiskers (as Q1-[1.5*IQR] and Q3+[1.5*IQR], respectively), and outliers (dots). A = ARO, T = Temperature, W = Water. Mesocosms: Regression plots with ARO concentration gradient along the x-axis. Mesocosms: R² and p value from the linear regression. For the microcosm experiment, significance levels shown as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Again, comparing only the ambient mesocosms, the 0.1 ARO treatment had the highest periphyton and the lowest phytoplankton biomass, similar to the microcosms. Additionally, although linear or quadratic models showed no correlation, the graphs point

toward possible inverse relationships between phytoplankton and periphyton at intermediate ARO concentrations

3.3. Stressor effects on primary consumers

The grazers were negatively affected by pesticides. *L. stagnalis* lengths were on average 20% smaller in the ARO compared with the control (Fig. 4.4). Warming on the other hand promoted their growth by 11%. A positive correlation was observed between *L. stagnalis* growth rate and *M. spicatum* biomass (Pearson, $r = 0.72, p < 0.0001$), but between *L. stagnalis* and the other macrophytes.

The highest *Daphnia* numbers were reached during week 2 with the controls showing 82% more individuals L^{-1} than the ARO (Fig. 4.5). By the end of the 4-week exposure, the numbers in the controls reduced from 1213 ± 883 to 345 ± 329 . *Daphnia* were controlling phytoplankton top-down, over time in the controls. While ARO significantly suppressed *D. magna* development during all 4 weeks, it significantly promoted phytoplankton development from weeks 2 to 4. The total average biomass of *Daphnia* in the controls at the end of the experiment was 73% higher compared with the ARO. Temperature effects on plankton were measurable only in week 1, with an increase of both *Daphnia* numbers (Fig. 4.5) and phytoplankton (Fig. S4.5).

Similar to the microcosms, the snails were affected negatively by ARO with a 16% reduced length in the 1.0 ARO compared with the control (Fig. 4.4B).

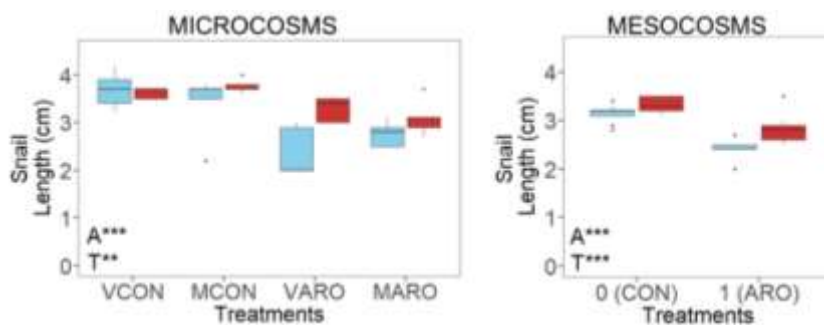


Fig. 4.4. Stressor effects on final length of snails in the microcosms (left) and mesocosms (right).

Box plots of 5 replicates in the microcosms and 10 replicates in the mesocosms showing median, 25 and 75% percentiles, lowest and highest whiskers (as $Q1 - [1.5 * IQR]$ and $Q3 + [1.5 * IQR]$, respectively), and outliers (dots). Microcosms: VCON = Volvic control; MCON = Munich well water control. ARO; VARO = Volvic ARO; MARO = Munich well water ARO. Mesocosms: 0 (CON) = control in Munich well water; 1 (ARO) = highest ARO concentration in Munich well water.

In contrast to the microcosms, *D. magna* was not part of our zooplankton community in the mesocosms. The natural zooplankton community comprised nine functional groups (Fig. 4.5B). The most prevalent morphotypes were calanoid copepods, *Daphnia longispina* and *Bosmina*, together representing over 80% of the community. During the experiment, the community structure shifted in all enclosures (Fig. 4.5B). The dominant groups at the end of the experiment were cyclopoid copepods, *Simocephalus* and *Chydorus*, together representing over 90% of the community. On average, the total zooplankton abundance rose from 8 individuals L⁻¹ at the start to 130 individuals L⁻¹ at the final sampling, but behaved very differently in each of the enclosures. The lowest density with 0.2 individuals L⁻¹ was reached in the 1.0-A concentration. The highest density was reached at 711 individuals L⁻¹ in the 0.25-A treatment. There was no clear correlation with ARO concentration or temperature.

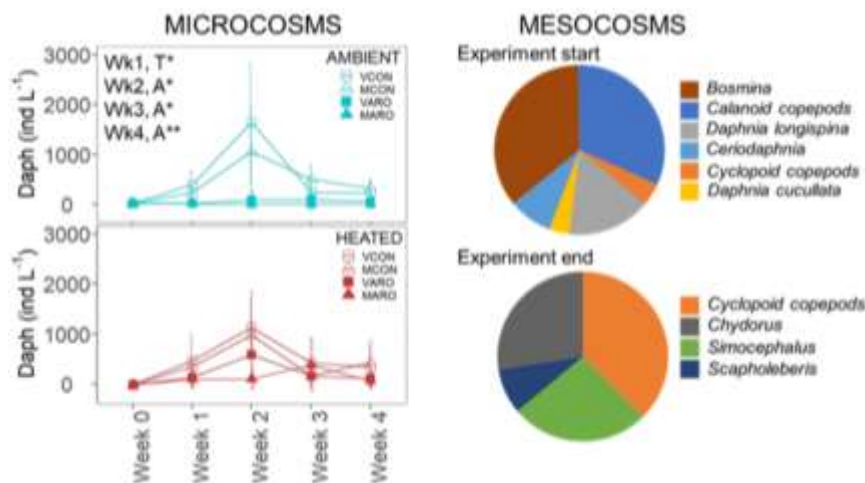


Fig. 4.5. Impact of stressors on the zooplankton in the microcosms (left) and mesocosms (right). In the microcosms, temporal effects of stressor on *Daphnia* numbers in the ambient (blue) and heated (red) microcosms are shown. Microcosms: VCON = Volvic control; MCON = Munich well water control. ARO; VARO = Volvic ARO; MARO = Munich well water ARO. Means \pm SD, n = 5. A = ARO, T = Temperature, Wk = week. Mesocosms: zooplankton population at the start and end of experiment, pooled from all enclosures. Daph = *Daphnia*.

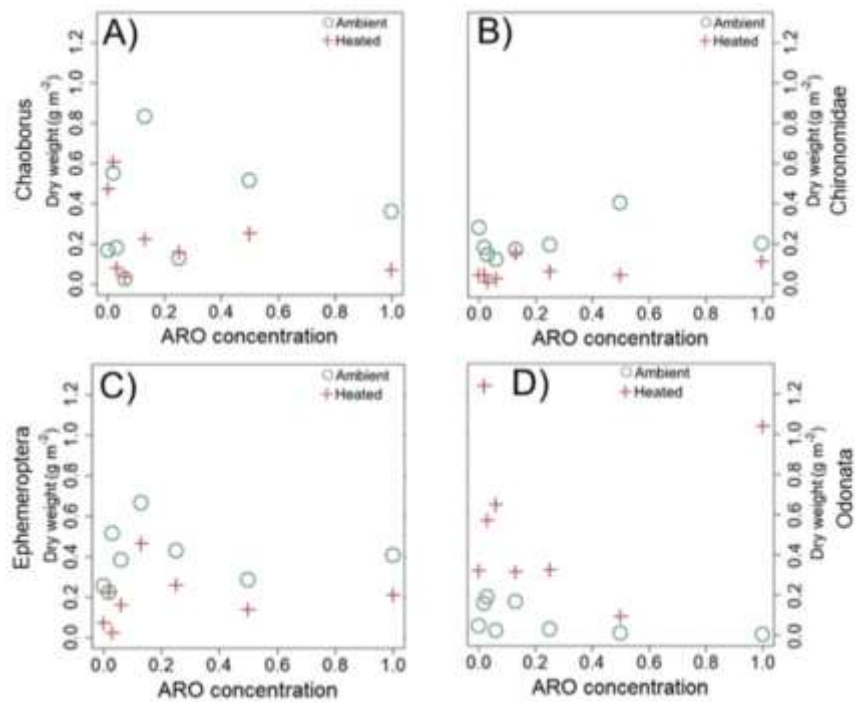


Fig. 4.6. MESOCOSMS. Biomass of the four main groups of invasive invertebrates at the final sampling. (a) *Chaoborus*, (B) Chironomidae, (C) Ephemeroptera, (D) Odonata.

In addition to zooplankton and snails added as experimental organisms, there were other invertebrates invading the mesocosms from early on. Eleven functional groups (based on families or genera) of insect larvae were distinguished, including Chironomidae, Aeschnidae, Libellulidae, Coenagrionidae, *Cleon*, *Dysticus*, Canidae, Culicidae, Hydrachnidia, Pleidae, and Trichoptera (mostly *Hydropsyche*). Most of them belonged to four main groups: Ephemeroptera, Chironomidae, Odonata and *Chaoborus* (Fig. 4.6). Among these groups, Odonata larvae had the highest average dry biomass (0.32 g m^{-2}), followed by Ephemeroptera and *Chaoborus*, each averaging 0.29 g m^{-2} . Chironomidae showed the least biomass (0.14 g m^{-2}) among these four groups. Odonata larvae were positively correlated with biomass of *M. spicatum* ($r = 0.5$, $p = 0.03$) but not with total macrophyte biomass ($r = 0.4$, $p = 0.1$). ARO did not affect the insect larvae. The average total insect larvae density stayed between 0.52 and 2.11 g m^{-2} in all enclosures. Total insect larvae biomass was very similar between ambient and heated enclosures. Ephemeroptera density, however, was halved by heating and chironomid density was even reduced by 70%. By contrast, biomass of the predatory odonate larvae was increased by 700% due to heating.

3.4. Regime shifts in micro- and mesocosms

The microcosms and mesocosms were compared to determine whether similar patterns could be observed in the proportion of different primary producer groups. Despite not finding terbuthylazine in the microcosms and the rapid decline of pirimicarb in the mesocosms (Fig. S4.6), among ambient treatments, the highest ARO of the mesocosms was the most phytoplankton dominated similar to the ARO in the microcosms. In the microcosms, the control treatments were dominated by macrophytes (average proportion to standard stock organic carbon, macrophytes = 54%, average proportion of phytoplankton = 6.5%) while the ARO treatments had a comparable proportion of phytoplankton and macrophytes (average proportion of macrophytes = 31%, average proportion of phytoplankton = 35%). However, the apparent reduced resilience of the macrophyte-dominated state was significantly more pronounced in the ambient ARO treatments (750% increase in phytoplankton proportion) compared with the warm ARO (171% increase in phytoplankton proportion) (Fig. 4.7). As the mesocosms were more biologically complex, either macrophyte or phytoplankton dominance was observed among enclosures. Intermediate ARO concentrations of the ambient mesocosms tended to be more macrophyte-dominated (Fig. 4.7), while the control and highest ARO had comparable proportions of both primary producer groups. Trends of dominance in the heated mesocosms were less clear. The 1.0-H showed strongest dominance of macrophytes, with 89% macrophytes and only 5% of both periphyton and phytoplankton, while the 1.0-A and the 0.5-H were the most phytoplankton-dominated accompanied by low macrophyte biomass, both with ~70% phytoplankton and 25% macrophytes (Fig. 4.7). The biovolume of macrophytes remained comparable between the micro- and mesocosms, but the zooplankton:phytoplankton ratio was relatively lower in the mesocosms (Fig. S4.7).

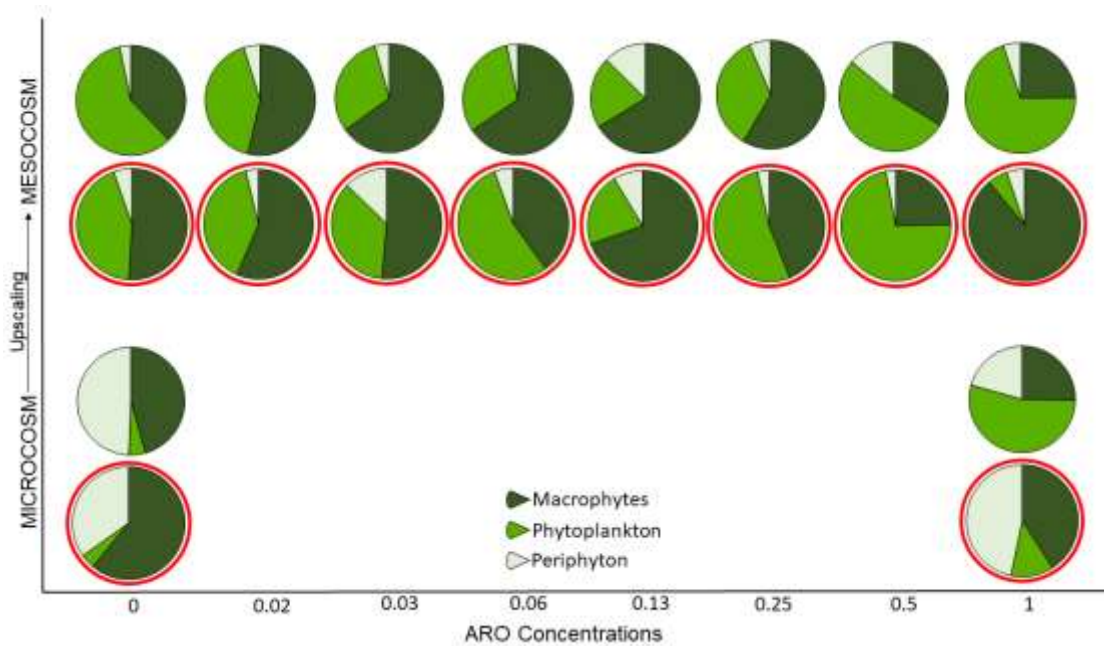


Fig. 4.7. MICROCOSM–MESOCOSM COMPARISON. Pie charts showing the relative proportion of primary producers in the microcosms (Munich well water treatments) and mesocosms when exposed to the ARO and the two temperatures. Heated treatments are represented by a red circle around the pie charts.

4. Discussion

Our experimental set-up and approach in upscaling from the highly controlled laboratory to the more complex outdoor conditions allowed us to confirm our first two hypotheses. First, while physiochemical properties of water have been shown to affect aquatic organisms (Ebenebe et al., 2019; Rameshkumar et al., 2019), adapting the water physiochemistry to suit the organism’s needs can alleviate significant differences in effects due to water type. In our experiment, nutrient levels in Mw were adjusted to more closely match the standard Vw, and as a result, water type did not affect overall stressor effects and only showed some minor species-specific effects. Second, some stressor effects on the primary producers and snails could be replicated in the mesocosms. Our third hypothesis, however, was more complex. Some correlations between ARO and macrophytes could be found, and speculations on nutrient effects on the primary producers at intermediate ARO concentrations are possible. The lack of strong observable effects in the mesocosms shows that upscaling is challenging. However, an understanding of the role of enhanced species diversity and complexity, including more direct and indirect interaction effects and invasions, in modifying or even reversing strong responses observed in the microcosms,

helps to evaluate how both experimental “scales” can be combined to increase causal understanding and prediction of effects in real-world scenarios.

4.1. Role of water type in upscaling experiments

Although water type had a species-specific effect on the macrophytes and modulated the impact of ARO to periphyton, the overall effects on the system did not differ between the two water types. The effect of Munich well water (Mw) in promoting growth of *E. nuttallii* may be related to the differences in mineral status between the two waters, particularly the higher calcium and carbonate (higher alkalinity) concentrations in Mw compared to Vw, which can influence photosynthesis or nutrient uptake capacity of this plant and thereby its growth (Baldy et al., 2015; Jones et al., 1993). *Elodea nuttallii* was much more efficient at phosphorus uptake in water that had higher calcium concentrations (Baldy et al., 2015). This effect on *E. nuttallii* only marginally affected the total macrophyte biomass, and thus did not significantly modify the overall response of the system. The interaction effect between ARO and water type on periphyton growth may be due to compensatory feeding by snails linked to changes in the quality of the periphyton between Vw and Mw. Such compensatory feeding can make up for the low quality of some food sources, even if their growth is not affected (Wacker & Baur, 2004). Despite the conductivity remaining significantly higher in Mw treatments and the minor effects on *E. nuttallii* and periphyton, irrespective of water type, both controls were always dominated by macrophytes, both ARO treatments always dominated by phytoplankton, and a much stronger effect was observed in the ambient microcosms. Similar phytoplankton blooms were observed in experiments with single or pulsed ARO exposure (Allen et al., 2021). Because ARO and temperature effects were the same in both water types and water type itself did not significantly affect any of the main functional groups, that is, the macrophytes, phytoplankton, periphyton, *Daphnia*, and snails, we were able to justify the use of the easily available Mw for our large outdoor mesocosms. Our results show that micro- and mesocosms can be performed using different water types and still allow comparability among research groups provided the water type is chemically tested and adjusted for important physicochemical parameters that influence the studied biotic community.

4.2. Role of community complexity in upscaling

When we scaled up to the large outdoor mesocosms, some effects of the highest ARO on the primary producer groups at ambient temperature and on snails at both

temperatures were similar to the microcosms. Although it is unclear whether terbuthylazine was present in the microcosms, this likely did not strongly affect the primary producers in both the micro- and mesocosms. Previous studies at similar concentrations have shown no negative effects of terbuthylazine on the primary producers (Polst et al., submitted; Vijayaraj, unpublished). In addition, other studies have shown similar algal blooms as in our microcosms when exposed to a combination of the same pesticides and nitrate (BPH, submitted, Allen et al., 2021), indicating that the resilience of the system is reduced when exposed to multiple stressors. In the mesocosms, at intermediate ARO concentrations, however, the positive effects of nitrate may have prevailed, thereby resulting in no observable negative effects by pesticides on the primary producer proportions. The effects on the primary producer proportion at the highest ARO of the ambient treatments in both experiments highlight a preservation of net negative effects in the mesocosms despite the increase in biological complexity. Warming on the other hand showed less clear effects and may not always threaten the macrophyte-dominated state. In fact, in combination with pesticides, it may show either antagonistic or synergistic effects (Polazzo et al., 2022). Pesticides have been shown to degrade faster at higher temperatures (Op de Beeck et al., 2017), but in both our micro- and mesocosms, pesticide decline did not differ between the two temperatures. The reduced effect of the ARO in the microcosm and the reversed effect in the mesocosm are most likely linked to increased or modified top-down control by the primary consumers in the heated treatments. Biotic structure and multiple stressor interactions can therefore complicate the prediction of warming effects on shallow lakes.

The lack of clearly observable net negative effects does not mean that no effects occurred. Our mesocosms had a rich biodiversity compared to the microcosms. Enhanced biodiversity offers a higher system stability (Loreau & de Mazancourt, 2013) and potentially more direct and indirect biotic interactions, which probably masked clear direct stressor effects. This can potentially produce an outcome in a direction opposite of the direct effects. In the microcosms, direct or indirect stressor effects could be linked to individual species or interactions, as we included only the key organisms per trophic level. For example, the strong positive effect of the ARO on phytoplankton was possibly linked to reduced top-down control by *Daphnia*, which is also a key grazer in natural systems (Cottingham et al., 1997; Mazumder, 1994). However, in the mesocosms, *Daphnia*, or large filter feeding cladocerans in general, were either absent or much fewer, and instead a complex zooplankton community existed. Observed changes in zooplankton assemblages

may have influenced the strength of top–down and even bottom-up control (Du et al., 2015; Rose et al., 2021). One likely reason for changes in the community is that the calanoids and large filter feeding cladocerans were more sensitive to the ARO and replaced by other more resistant groups. Our microcosm experiments showed that *Daphnia* were strongly affected by the pesticides, which may explain their disappearance in the mesocosm experiments. Besides, nutrient levels and the presence of alternative food sources may have also influenced the zooplankton assemblages. Du et al. (Du et al., 2015) reported that at high nutrient concentrations, both an increase in chl a and the presence of food sources other than phytoplankton, such as detritus and bacteria, promoted the biomass of cyclopoids and rotifers. This may be an explanation for why cladocerans and calanoids were eventually replaced by cyclopoids and other more competitive zooplankton species.

Phytoplankton community composition may in addition have been affected by selective grazing by zooplankton (Carpenter & Kitchell, 1984). Such selective grazing may also allow certain zooplankton species to evade the effect of ARO on phytoplankton, with the non-selective feeders being more susceptible. Different sensitivities/responses of the zooplankton species to the ARO may also drive the phytoplankton response, for example, through changes in the zooplankton community size structure (Carpenter et al., 1985). The complex community structure in the mesocosms also meant that the trophic cascade was more complex than in the microcosms. There was a high chance of omnivores feeding from more than a single trophic level (e.g., mixotrophic phytoplankton, or zooplankton, e.g. copepods, feeding on both algae and microzooplankton), thereby dampening potential strong direct top–down effects. Community structure may therefore play a defining role in modifying shallow lake responses to multiple pressures (Kuiper et al., 2015). Allowing for a higher biodiversity in mesocosms is necessary to predict more closely real-world effects, especially in aquatic ecological risk studies with the aim of defining safe operating spaces. Such complexity allows us to follow the succession of species as well as determine the most sensitive and the fittest organisms under various stressor conditions. At the same time, controlled microcosm experiments that use key test organisms are fundamental in disentangling stressor effects on biotic interactions. Incorporating more natural communities in controlled laboratory conditions may provide a means of bridging this gap between the experimental scales.

4.3. Role of invasions in upscaling

An additional trophic complexity was introduced through invasions in our mesocosms, which was not part of our microcosms. For many insects, freshly set-up mesocosms with dense macrophyte stands appeared an ideal opportunity for egg deposition. Soon, insect larvae belonging to many different functional groups appeared in all enclosures. Benthic Chironomid larvae, for example, feed on detritus, algae and protozoans, as do Ephemeroptera larvae. *Chaoborus* and Odonate larvae are ambush predators feeding on zooplankton and insect larvae. By direct or indirect top-down control, these invading species likely affected planktonic primary producers and primary consumers, as well as benthic primary producers. Densities of these invading insects were comparable to natural densities (Benke, 1978). They probably had serious consequences for our mesocosms, as they fed not only on zooplankton, thereby comprising a more-advanced third trophic level compared to the copepods, but also on periphyton and may thus affect water quality and trophic state (Hölker et al., 2015). In the microcosms, the snails were the only key grazers of periphyton, and ARO effects on periphyton could be linked to the snails (Allen et al., 2021). In the mesocosms, both snails and the other invading invertebrates may have influenced periphyton biomass. Apart from the possible role of nitrate at low ARO concentrations, the lack of any strong effects on periphyton could be attributed to the compensation of snail feeding by that of invading insect larvae, which were less affected by ARO. Despite the invasions, however, both experiments point toward a general relationship between *M. spicatum* and consumers, with snails being influential in the microcosms through periphyton grazing (Phillips et al., 1978) and odonate larvae in the mesocosms possibly by modifying top-down control of periphyton. In both cases, the primary consumers and/or odonate larvae developed better in the heated mesocosms and tended to buffer ARO effects. The very high biomass of odonate larvae in the highest ARO treatment of the heated mesocosm, which also happened to be the most macrophyte-dominated system, indicates the extent of the influence consumers may have in system response to stressors.

An interesting implication is that because the ARO effects on snails remained the same at both scales, they should be considered a sensitive bio-indicator in freshwater ecosystems exposed to agricultural run-off. In fact, a normalized OECD test guideline for *Lymnaea stagnalis* reproduction has been adopted recently (OECD, 2016), showing their relevance as indicators of toxicant effects.

The stressors may also affect primary producers differently when embedded in branched or looped trophic chains with even or uneven trophic levels (Eriksson et al., 2012; L. Oksanen et al., 1981; Wollrab et al., 2012). There was no top–down control on the snails, and the food web in the mesocosms was therefore branched rather than looped. The invasion by additional invertebrates resulted in a change from branched even–even food web as in the microcosms to an unintentional branched odd–even food web (Wollrab et al., 2012). Because the top consumers are expected to control their prey or resources at an odd distance from themselves, any change in food web structure should have large consequences. The establishment of a strong carnivorous trophic level in our mesocosms, which can be influenced by resource availability (L. Oksanen & Oksanen, 2000) and feeding behaviour of the second trophic level (Abrams, 1984), could therefore significantly have modified the direction of effects that we observed in the microcosms where only two trophic levels were included.

In warmer environments, poikilotherm organisms have a higher metabolic activity (Levinton, 1995). Foraging activities by *Chaoborus* and Odonata on other insects and zooplankton may therefore have increased, but temperature may also have strongly regulated microplankton assemblages (Du et al., 2015). Therefore, the strong temperature effects observed on odonates and sometimes Ephemeroptera might have indirectly reversed the response of primary producers to the ARO, without observable interaction effects of temperature with ARO on the food web dynamics. Invasions by predators in large outdoor mesocosm experiments can therefore change net effects of stressors on primary producers and should also be well-monitored.

ARO lowered the resilience of alternative stable states in our model lake ecosystems, increasing the potential for regime shifts towards a phytoplankton-dominated state that supports fewer ecosystem services than macrophyte dominance (Janssen et al., 2021). These ARO effects were conserved at both scales at ambient environmental temperature, suggesting that ecosystems exposed to a combination of nitrate and pesticides are at risk for shifts to a degraded turbid state, and further action should be taken to reduce the use of these chemicals to protect shallow lakes. Increased temperature modified the effect of ARO, and the direction of this effect varied based on the ecological complexity of the system. At the microcosm scale, the negative ARO effect on macrophytes was still present at higher temperature but was buffered, while at the mesocosm scale, at the highest temperature reversed the effect of the highest ARO treatment on primary producers. This

modified effect may be due to the changes in community structures, increased metabolic activity and strong top–down control by consumers at higher temperatures. Depending on the number of trophic levels and feeding habits of the consumer community within the ecosystem studied, temperature may modify the direction of ARO effects. Strong positive and negative effects observed in controlled experiments can cancel out in complex outdoor studies to a zero net effect. No visible net ARO effects do not mean that there were no effects. While ARO effects may have been hidden within complexity in the mesocosms, our microcosms clearly indicated that ARO affects the biotic community. Controlled microcosm experiments are therefore important to disentangle the “effect pathways” of ARO within communities potentially important for real world scenarios. They may result in important hypotheses and a related design for testing when aiming to upscale the experimental system. Variable outdoor mesocosms allow us to identify gaps, and then improve the design and set-up of experiments at both scales to strengthen their complementarity. A comparison of stressor effects at different scales and complexity is therefore a promising direction for risk evaluation studies in aquatic ecology and ecotoxicology.

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Supplementary information

Table S4.1. ARO concentrations and temperature of micro- and mesocosms.

ARO	Microcosms	Mesocosms
Terbutylazine ($\mu\text{g L}^{-1}$)	0, 3	Gradient: 0, 0.02, 0.03, 0.06, 0.13, 0.75, 1.5, 3
Pirimicarb ($\mu\text{g L}^{-1}$)	0, 15	Gradient: 0, 0.2, 0.5, 0.9, 1.9, 3.75, 7.5, 15
Tebuconazole ($\mu\text{g L}^{-1}$)	0, 90	Gradient: 0, 1.4, 2.8, 5.6, 11.3, 22.5, 45, 90
Copper (Cu, $\mu\text{g L}^{-1}$)	0, 42	Gradient: 0, 0.7, 1.3, 2.6, 5.25, 10.5, 21, 42
Nitrate (N-NO ₃ , mg L^{-1})	0, 9	Gradient: 0, 0.1, 0.3, 0.6, 1.1, 2.25, 4.5, 9

Table S4.2. Starting size of biotic community in micro- and mesocosms.

Species	Microcosm Starting size, Average \pm SD	Mesocosm
<i>M. spicatum</i>	10 cm (2 shoots)	10 cm (10 shoots)
<i>P. perfoliatus</i>	10 cm (2 shoots)	10 cm (10 shoots)
<i>E. nuttallii</i>	10 cm (2 shoots)	10 cm (15 shoots)
Phytoplankton	$7.5 \times 10^5 \mu\text{m}^3 \text{mL}^{-1}$	-
Periphyton	$8.35 \times 10^5 \mu\text{m}^3 \text{mL}^{-1}$	-
<i>L. stagnalis</i>	18.9 \pm 2.4 mm (1 individual per microcosm)	10.7 \pm 3.7 mm (10 individuals per mesocosm)
<i>D. polymorpha</i>	9.1 \pm 1.3 mm (3 individuals per microcosm)	19.7 \pm 1.9 mm (10 individuals per mesocosm)
<i>D. magna</i> /zooplankton	1391 \pm 17 mm (2.5 individuals L ⁻¹)	(8 individuals L ⁻¹)

Biovolume was not measured for the mesocosms as natural benthic and pelagic communities were mixed, but may have been lower in the mesocosms due to a longer incubation within the enclosures.

Table S4.3. Morphotypes used in the micro- and mesocosms.

	Species richness		
	Community	Initial inoculum	Final sample
Microcosms			
Macrophytes	Chosen species	3 species	3 species
Phytoplankton	Chosen culture strains	3 strains	Mostly green algae
Periphyton	Chosen culture strains	5 strains	Mostly diatoms
Zooplankton	Chosen culture strains	1 species	1 species
Mussels	Chosen species	1 species	1 species
Snails	Chosen species	1 species	1 species
Mesocosms			
Macrophytes	Chosen species	3 species	3 species
Phytoplankton	Natural community	30-50 species	9 species
Periphyton	Natural community		
Zooplankton	Natural community	9 species	5 species
Mussels	Chosen species	1 species	0 species
Snails	Chosen species	1 species	2 species
Insect larvae (non-predatory)	Invasive	0	5 species
Insect larvae (predatory)	Invasive	0	6 species

Table S4.4. Comparison of physicochemistry between Volvic and Munich well water before and after exposure.

Before exposure	Volvic water	Munich well water
Cond ($\mu\text{S cm}^{-1}$)	223	663
pH	7.8	8.2
Alk. (mM)	0.64	2.9
Cl (mg L^{-1})	14.9	14.7
SO ₄ (mg L^{-1})	9.08	10.7
Ca (mg L^{-1})	12.8	91.1
Mg (mg L^{-1})	8	20.8
Na (mg L^{-1})	11.7	8.9
K (mg L^{-1})	6.1	1.5
N-NO ₃ (mg L^{-1})	1.7	2.96
N-NO ₂ (mg L^{-1})	0.08 \pm 0.04	0.07 \pm 0.01
N-NH ₄ (mg L^{-1})	0.002 \pm 0.001	0.005 \pm 0.005
DIN ($\mu\text{mol L}^{-1}$)	210.7 \pm 20.8	310.6 \pm 26.1
P-PO ₄ (mg L^{-1})	0.07 \pm 0.06	0.12 \pm 0.1*
P-PO ₄ ($\mu\text{mol L}^{-1}$)	2.4 \pm 2.01	3.73 \pm 3.12
NP molar ratio	269.8 \pm 291.4	332.3 \pm 381.7
1h after exposure (ARO treatments)		
Terbutylazine	-	-
Pirimicarb	15.51 \pm 0.63	15.91 \pm 0.48
Tebuconazole	79 \pm 13.25	65 \pm 7.7
Copper	42.9 \pm 5.9	42.3 \pm 3.0
After 4-week exposure (control treatments)		
	Volvic water	Munich well water
Cond ($\mu\text{S cm}^{-1}$)	354 \pm 14.09	424 \pm 18.8
pH	8.0 \pm 0.24	8.16 \pm 0.05
Cl (mg L^{-1})	21.49 \pm 0.62	21.30 \pm 0.48
SO ₄ (mg L^{-1})	14.5 \pm 1.6	15.8 \pm 2.0

N-NO ₃ (mg L ⁻¹)	0.16±0.10	0.2±0.1
N-NO ₂ (mg L ⁻¹)	0.01±0.01	0.01±0.01
N-NH ₄ (mg L ⁻¹)	0.09±0.12	0.08±0.09
DIN (μmol L ⁻¹)	19.13±15.72	21.30±15.07
P-PO ₄ (mg L ⁻¹)	0.07±0.03	0.05±0.03
P-PO ₄ (μmol L ⁻¹)	2.33±1.08	1.58±1.03
NP molar ratio	9.89±11.13	15.14±7.83
After 4-week exposure (ARO treatments)	Volvic water	Munich well water
Terbuthylazine	-	-
Pirimicarb	12.4±0.7	13.7±0.7
Tebuconazole	31±8.1	34±2.5
Copper	13.1±6.1	13.5±7.6

* 0.013 mg L⁻¹ before P fertilization. See text for details. Cond = conductivity, Alk. = Alkalinity, DIN = dissolved inorganic nitrogen.

Table S4.5. Statistical details for individual and combined stressor effects on the different functional groups.

Endpoints	Test	Individual effects	Interactions					
		T	A	W	T*A	T*W	A*W	T*A*W
		<i>p-value</i>						
Macrophytes								
MS biomass	3-way ANOVA	1.25E-05	2.66E-10	0.467	0.1	0.5	0.7	0.6
PF biomass	3-way ANOVA	0.1	0.5	0.4	0.5	0.5	0.4	0.4
EN biomass	3-way ANOVA	0.1	0.05	0.01	0.04	0.1	0.2	0.4
Total macro-phyte biomass	3-way ANOVA	0.0001	1.59E-10	0.07	0.1	0.3	0.6	0.4
Microalgae								
Biofilm_chla	3-way ANOVA	0.2	3.90E-06	0.7378	0.01	0.6	0.02	0.7
Phytoplankton chla	3-way ANOVA	0.01	0.02	0.1	0.0007	0.2	0.6	0.1
Shifts in primary producer abundance								
Macrophyte, phytoplankton and periphyton C	PERMANOVA	0.01	0.001	0.5	0.01	0.5	0.5	0.6
Consumers								
LS_length	3-way ANOVA	0.005	2.08e-06	0.9	0.06	0.8	0.5	0.05
DM_number_w1	3-way ANOVA	0.02	2.56E-05	0.4	0.4	0.9	0.9	0.7
DM_number_w2	3-way ANOVA	0.1	0.01	0.7	0.08	0.6	0.7	0.06
DM_number_w3	3-way ANOVA	0.1	0.01	0.8	0.08	0.6	0.8	0.06
DM_number_w4	Kruskal-Wallis rank sum	0.2	3.00E-03	0.11				
DM_biomass	3-way ANOVA	0.09	3.00E-04	0.9	0.2	0.4	0.2	0.4
Optical density								
OD_663_w1	Kruskal-Wallis rank sum	0.03	0.9	0.4				
OD_663_w2	3-way ANOVA	0.7	0.04	0.5	0.08	0.5	1	0.9
OD_663_w3	Kruskal-Wallis rank sum	0.5	9.15E-06	0.2				
OD_663_w4	Kruskal-Wallis rank sum	0.2	2.17E-05	0.2				

Table S4.5. Statistical details for individual and combined stressor effects on the different functional groups (contd).

Endpoints	Test	Individual effects		Interactions				
		T	A	W	T*A	T*W	A*W	T*A*W
		<i>p-value</i>						
Conductivity								
Conductivity_w0	Kruskal-Wallis rank sum	0.3	0.6	8.47E-08				
Conductivity_w1	3-way ANOVA	0.2	6.21E-08	1.09E-05	0.02	0.1	0.009	0.4
Conductivity_w2	3-way ANOVA	0.01	0.4	8.65E-10	0.002	0.9	0.001	0.2
Conductivity_w3	3-way ANOVA	0.0006	0.2	2.53E-06	0.09	0.8	0.03	0.8
Conductivity_w4	3-way ANOVA	6.94E-05	0.1	1.80E-07	0.02	0.4	0.09	0.9
Oxygen saturation								
O2_w0	3-way ANOVA	0.4	0.8	0.1	0.9	0.1	0.8	0.6
O2_w1	3-way ANOVA	0.7	0.03	0.5	0.3	0.08	0.9	0.4
O2_w2	3-way ANOVA	0.04	5.03E-09	0.2	0.9	0.6	0.03	0.4
O2_w3	Kruskal-Wallis rank sum	0.2	0.2	0.2				
O2_w4	Kruskal-Wallis rank sum	0.2	0.2	0.2				
pH								
pH_w0	3-way ANOVA	0.4	0.5	0.02	0.4	0.2	0.8	0.4
pH_w1	3-way ANOVA	0.0003	0.001	0.03	0.5	0.09	0.3	0.2
pH_w2	3-way ANOVA	0.01	1.85E-12	0.5	0.5	0.9	0.1	0.6
pH_w3	Kruskal-Wallis rank sum	0.05	0.0003	0.04				
pH_w4	Kruskal-Wallis rank sum	0.002	0.5	0.2				

Note: MS = *M. spicatum*, PF = *Potamogeton perfoliatus*, EN = *E. nuttallii*, IS = *L. stagnalis*, DM = *D. magna*, OD = optical density. T = temperature, A = ARO, W = water.

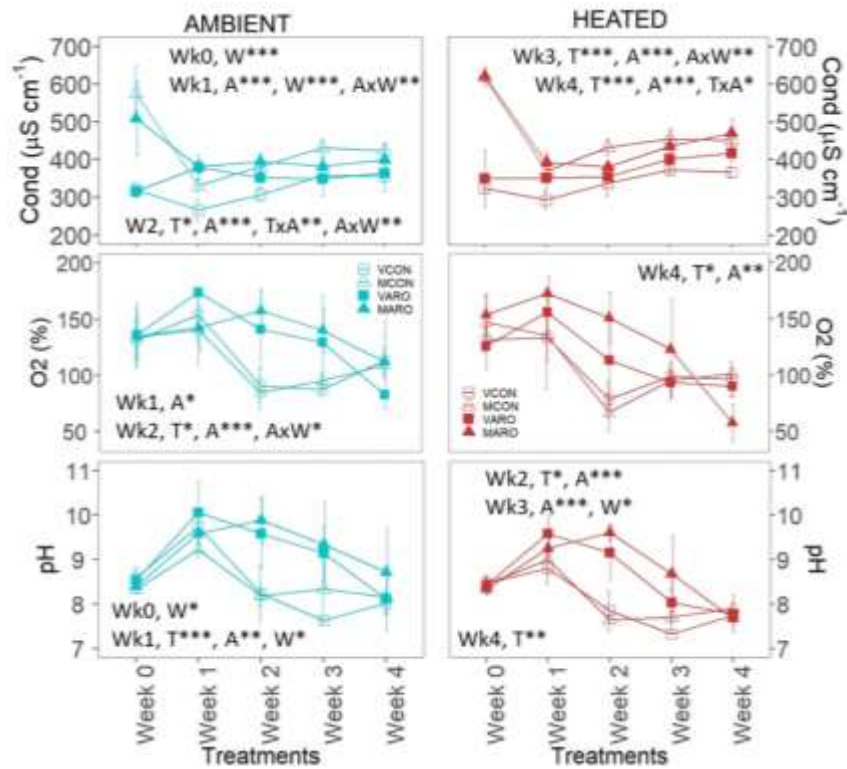


Fig. S4.1. MICROCOSMS. Weekly measures of water physico-chemistry in the ambient (left, blue lines and symbols) and heated (right, red lines and symbols) microcosms. VCON = Volvic control; MCON = Munich well water control. ARO; VARO = Volvic ARO; MARO = Munich well water ARO. A = ARO, T = Temperature, W = Water. Means \pm SD, $n = 5$. Statistical differences between treatments are shown per week (Wk1, 2, 3, or 4) and factor (A, T or W) or interactions between factors. Significance levels shown as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

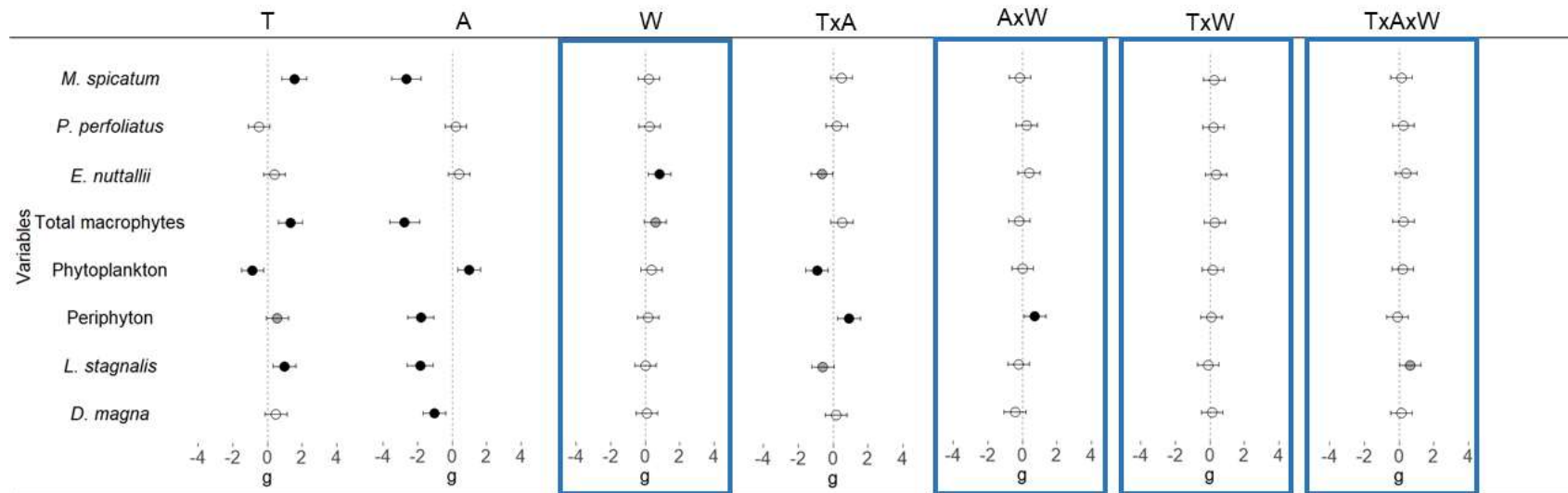


Fig. S4.2. Effect sizes (Hedges' $g \pm 95\%$ confidence interval) of individual stressors and stressor interactions on the key functional groups at the end of the experiment, calculated from the F-statistics derived from the ANOVA. No effects are inferred when confidence intervals cross the zero line. White symbols: non-significant effects ($p > 0.09$), grey symbols: marginal effects ($p = 0.05-0.09$), black symbols: significant effects ($p < 0.05$). A = ARO, T = Temperature, W = Water. Water effects and its interactions with temperature and ARO or both are outlined in blue.

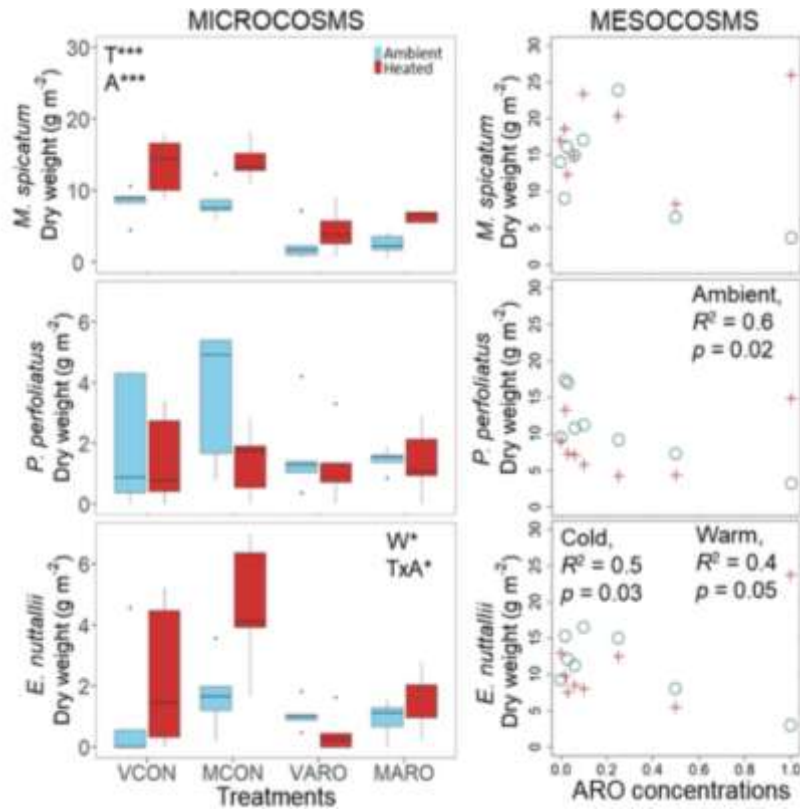


Fig. S4.3. Stressor effects on macrophytes in the micro- and mesocosms. Microcosms: VCON = Volvic control; MCON = Munich well water control. ARO; VARO = Volvic ARO; MARO = Munich well water ARO. A = ARO, T = Temperature, W = Water. Box plots of 5 replicates showing median, 25 and 75% percentiles, lowest and highest whiskers (as $Q1 - [1.5 \cdot IQR]$ and $Q3 + [1.5 \cdot IQR]$, respectively), and outliers (dots). Mesocosms: R^2 and p values from linear regressions. For both experiments, significance levels shown as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

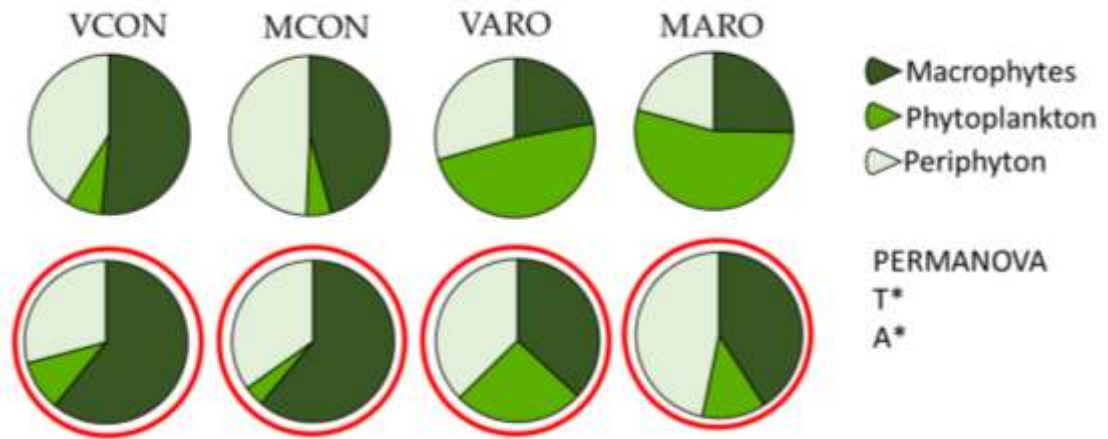


Fig. S4.4. MICROCOSMS. Relative proportion of primary producers when exposed to the ARO in Volvic and Munich well water at the two temperatures. VCON = Volvic control; MCON = Munich well water control. ARO; VARO = Volvic ARO; MARO = Munich well water ARO. A = ARO, T = Temperature, W = Water. Significance levels shown as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Heated treatments are represented by a red circle around the pie charts.

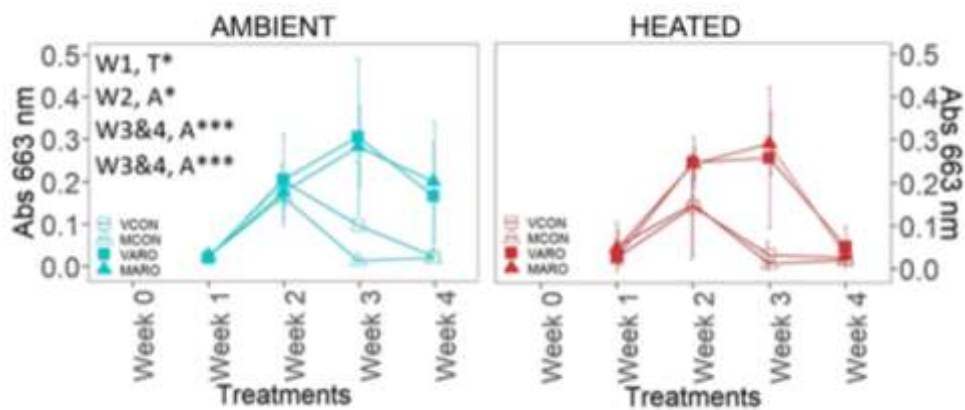


Fig. S4.5. MICROCOSMS. Weekly measures of absorbance of water at 663 nm in the ambient (blue) and heated (red) mesocosms. Blue represents the unheated microcosms and red indicates the heated. ARO; VARO = Volvic ARO; MARO = Munich well water ARO. A = ARO, T = Temperature, W = Water. Means \pm SD, $n = 5$ Statistical differences between treatments are shown per week (Wk1, 2, 3, or 4) and factor (A, T or W) or interactions between factors. Significance levels shown as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

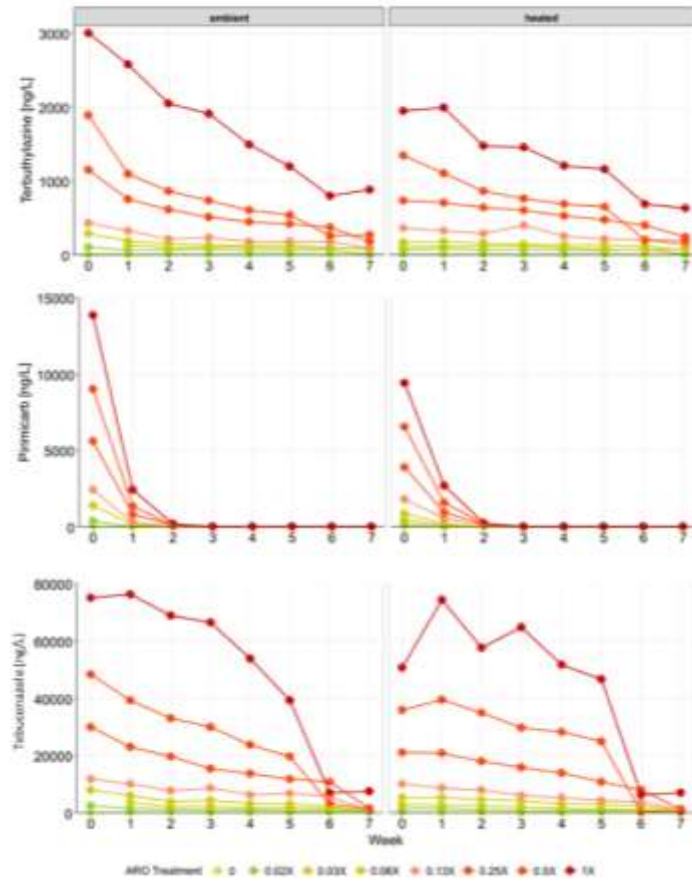


Fig. S4.6. MESOCOSMS. Pesticide concentrations measured over 8 weeks.

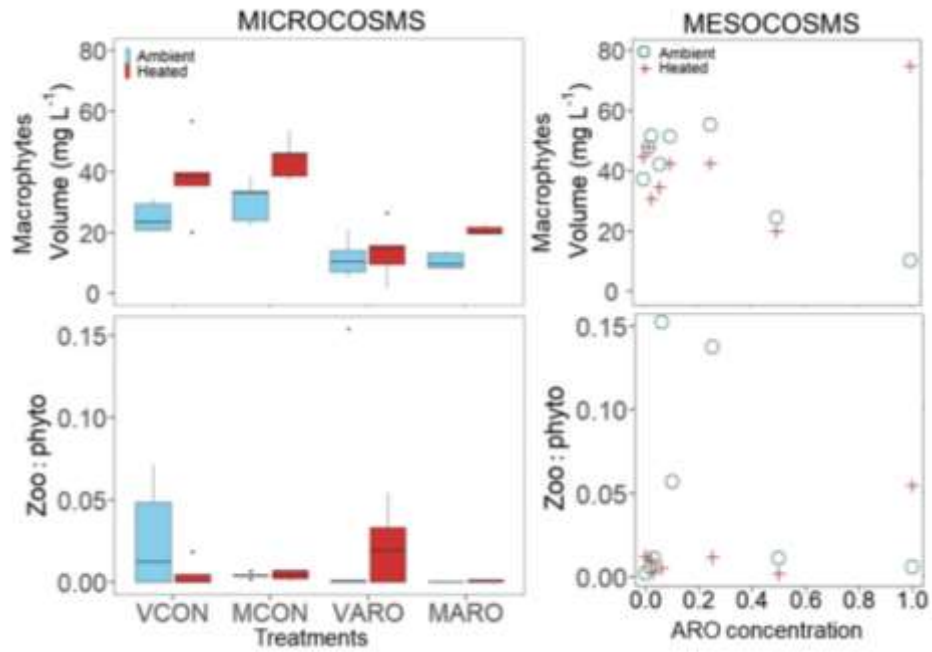


Fig. S4.7. Biomass per volume of macrophytes and zooplankton:phytoplankton ratios (zoo:phyto; mass ratios) in the microcosms (left) and mesocosms (right). Microcosms: VCON = Volvic control; MCON = Munich well water control. ARO; VARO = Volvic ARO; MARO = Munich well water ARO. Box plots of 5 replicates showing median, 25 and 75% percentiles, lowest and highest whiskers (as $Q1 - [1.5 \cdot IQR]$ and $Q3 + [1.5 \cdot IQR]$, respectively).

CHAPTER 5: GROWTH AND PHYSIOLOGY OF MACROPHYTES EXPOSED TO AGRICULTURAL RUNOFF AND TEMPERATURE

1. Introduction

Shallow lakes are common in fertile lowlands, and are exposed to agricultural runoff containing nutrients and pesticides and climate warming. The role of phosphorus (P) in eutrophication has been widely studied, whereas nitrate has been given less attention (Moss et al., 2013; Schindler et al., 2008). Recent studies show that nitrate can also affect lake status (Barker et al., 2008; Sagrario et al., 2005). Agricultural runoff enters these lakes from nearby agricultural sites, and the input through runoff depends not only on pesticide and fertilizer application but also on weather conditions (Pereira et al., 2017; Pereira & Hostettler, 1993). Exposure of lakes may occur as a sole event or in the form of multiple pulses depending on rainfall frequency. Elevated water temperatures through rising climate warming further affect shallow lakes by promoting phytoplankton blooms and/or macrophyte growth, or by strengthening top-down grazer control of microalgae (Trolle et al., 2014; Zhang et al., 2019).

Within shallow lakes, these multiple stressors can affect macrophytes directly. Depending on the mode of action, herbicides present in agricultural runoff may inhibit photosynthetic activity, cause mitosis or cell membrane disruption or affect plant growth regulators, among others (Mohaupt et al., 2020). Increased nutrient loading through agricultural runoff may promote macrophyte nutrient uptake (Dorenbosch & Bakker, 2011; Dülger et al., 2017). Pesticides and nutrients may also affect the production of macrophyte chemical defences such as phenolic compounds (Nuttens et al., 2016). A reduction in macrophyte growth and the production of allelopathic compounds may provide conducive conditions for phytoplankton blooms (Phillips et al., 2016; Phillips et al., 1978). Warming may promote macrophyte growth, but enhanced growth may lead to changes in the macrophyte elemental stoichiometry (Zhang et al., 2019, 2020). Such changes in physiology can affect macrophyte quality, and this has implications for the status of shallow lakes.

These stressors can also affect macrophytes indirectly by modifying their abiotic and biotic conditions. Very high nutrient concentrations may promote phytoplankton blooms, which will modify nutrient and light availability for macrophytes. Biotic interactions are part of the stabilizing mechanism in shallow lakes, and any effect on them

may cause regime shifts. Grazers may control the development of microalgae (Cao et al., 2004; Zhang et al., 2019), but pesticides such as insecticides may inhibit the growth and metabolism of these invertebrate grazers. Warming on the other hand may strengthen top-down control of grazers or filter feeders on periphyton or phytoplankton. Such changes in microalgae control will influence the competition among primary producers for light and nutrients.

These direct and indirect stressor effects may be reflected not only in growth responses but also, much earlier, in the macrophyte physiology. The main physiological biomarkers of macrophytes studied in the literature are the photosynthetic pigments, secondary metabolites and stoichiometry (Elger & Lemoine, 2005; Fornoff & Gross, 2014; He et al., 2008; Nuttens et al., 2016; Zhang et al., 2019). In response to changes in the light and nutrient environment, macrophytes make photosynthetic adjustments, which may be measured by changes in their chlorophyll concentrations (Barko & Filbin, 1983; Chen et al., 2016; Nuttens et al., 2016). When irradiance decreases, for example, some macrophytes may increase their production of total chlorophylls (Barko & Filbin, 1983) to enhance photosynthetic carbon (C) assimilation. Although an inverse relationship is observed between irradiance and total chlorophylls, this holds true only above the minimum photon flux density required by the plant species (Barko & Filbin, 1983). While nitrogen (N) is needed for chlorophyll biosynthesis, total chlorophylls have been found to be inversely related to nitrate levels but this effect may be more prominent at concentrations that inhibit macrophyte growth (Nuttens et al., 2016). If high nitrate concentrations promote phytoplankton blooms, this may indirectly influence the macrophyte production of total chlorophylls through competition for nutrients and light. Herbicides can also cause changes in the production of chlorophylls, but the direction of effects are not always the same and depend on the mode of action. Glyphosate and mesosulfuron-methyl, for example, reduced the chlorophyll content for the waterweed *Egeria densa* Planch (Diagonal et al., 2022) and the milfoil *Myriophyllum spicatum*, whereas isoproturon increased the chlorophyll content in *M. spicatum* (Nuttens et al., 2016).

Secondary metabolites, among which phenolic compounds are the most widespread class, provide information about biotic or abiotic stress in the environment, including herbivory and changes in nutrient and light availability (Cronin & Lodge, 2003; Fornoff & Gross, 2014; Gross, 1999; Muzika, 1993). When competition for nutrients and light is high, macrophytes may increase their production of phenolic compounds, which

have allelopathic activity and can inhibit phytoplankton photosynthetic activity (Gross, 1999). Low light may cause a decrease in phenolic content while low nutrients induce a higher production (Cronin & Lodge, 2003). The effect of pesticides and warming on phenolic compounds is not well understood. Nuttens et al. (2016) showed a reduction of anthocyanins and total phenolic compounds in the milfoil *M. spicatum*, but this was observed only for the herbicide mesosulfuron-methyl and not for isoproturon. In terms of warming, Zhang et al. (2019) showed no effects of warming on total phenolic compounds.

Plant stoichiometry (or elemental concentrations) and their ratios provide information about the quality of the macrophyte species and their palatability to herbivores (Elger & Lemoine, 2005; Zhang et al., 2019). Macrophyte stoichiometry can reflect nutrient conditions in the environment (Demars & Edwards, 2007). High N or P concentrations in the sediment or water may result in decreased C:N or C:P ratios in the macrophytes (Su et al., 2016). A nutrient depletion by macrophytes or phytoplankton at elevated temperatures may result in higher C:N ratios (Zhang et al., 2015). The higher the macrophyte nutrient content or the lower the C:N or C:P ratios the higher its quality for consumption by grazers (Bakker et al., 2016; Dorenbosch & Bakker, 2011; Sterner & Elser, 2002). Macrophyte stoichiometry therefore has implications for their loss through herbivory, but also for other ecosystem processes such as decomposition and biogeochemical cycles (Carvalho et al., 2015; König et al., 2014; Menéndez et al., 2004).

The European Water Framework Directive has identified macrophytes as suitable bioindicators for the quality and ecological status of freshwaters. Parameters such as macrophyte community composition and abundance have been used widely in ecology as a measure of the general development of macrophytes in response to environmental changes as well as for environmental monitoring of the ecological state of water bodies (EU WFD, 2000/60/EC). For example, a reduction in macrophyte biomass could signify a transition to a new regime state, or a reduction in species richness may signify high nutrient loading (Moss, 1976; Phillips et al., 2016). Other individual macrophyte growth characteristics / traits such as the root:shoot ratio or height, which might differ depending on macrophyte species, have been considered as possible indicators of nutrient status or as a response to warming (Bloom et al., 1985; Zhang et al., 2019). Macrophyte physiological response to environmental conditions such as nutrients, light, pesticides, and temperature may be an additional tool to determine macrophyte quality and the quality of their environment (Grime et al., 1986). If specific physiological changes could be linked to the

chemical stressors, these might have potential for use as early warning signals for regime shifts.

We performed three indoor microcosm experiments representing shallow lakes and exposed them to agricultural runoff at different exposure scenarios. All treatments were replicated at two temperatures, ambient summer and a +4°C increase as projected by the Intergovernmental Panel on Climate Change (IPCC; 2014). In the first two experiments (Experiments 1 and 2), single increasing doses (X, 2X 3X) or pulses (XX and XXX) of the ARO were applied. In the third experiment, the individual and combined main compartments of ARO (nitrate and pesticide) were applied to disentangle their effects (see Fig. 2.3 in the “Approach”). At the end of the experimental exposure (3-4 weeks), the macrophytes were sampled and analysed for their growth, stoichiometry, photosynthetic pigments and phenolic compounds. Details of the set-up for Experiments 1 and 2 are presented in Allen et al. (2021; see Chapter S2) and Vijayaraj et al. (2022; see Chapter 3). A detailed description of the chemical analyses is provided in the following section. The objectives of this study were to determine whether:

- a. ARO and temperature would affect macrophyte physiology
- b. The ARO exposure mode would cause specific physiological responses
- c. Nitrate and pesticides would elicit different physiological responses in the macrophytes

2. Material and methods

For each of the three experiments, details of the set-up are given in the supplementary (Allen et al., 2021; Chapter S2) and in Chapter 3. Experiments 1 and 2 in this chapter refer to Experiments 1 and 3 in Allen et al. (2021). Experiment 3 in this chapter refers to the experiment presented in Chapter 3.

2.1. Macrophyte growth

After experimental exposure of the macrophytes to ARO and elevated temperature for 3 to 4 weeks, the macrophytes were sampled by gently removing them from the sediment including the root. Photographs were taken, and the lengths of aboveground main and side shoots for all plants were measured. The apical stems were then cut at 10 cm, and the leaves were separated, (shock) frozen and lyophilized.

2.2. Macrophyte chemical analyses

To prepare for the chemical analyses, the lyophilized leaves were crushed into a powder using a bead beater (Retsch MM300) for 2 min at 30 Hz. Depending on the phytochemical or stoichiometric element to be analysed, between 0.5 mg and 3 mg of the crushed plant material were weighted on a balance of high precision (0.01 mg) and then analysed.

Photosynthetic pigments were extracted from ~0.5 mg plant powder with 1 mL extraction solvent (20% 0.1 M Tris-HCl, pH 7.8 and 80% acetone (v/v); adapted from Lichtenthaler and Buschmann, 2001). Anthocyanins were extracted from ~ 1 mg powder with 1 mL extraction solvent, prepared with 80% methanol, 19% ultrapure water and 1% HCl 37% (v/v/v). Plant total phenolic compounds were extracted using 100 µl of the extraction solvent per mg plant material. The extraction solvent was prepared using 50% acetone and 50% ultrapure (Milli-Q®) water. Extractions were performed for 2h at 16°C in an Eppendorf Thermo-Mix. After centrifugation, the absorption of the supernatant was measured using a spectrophotometer (Varian Cary 50). Wavelength used were 470 nm for carotenoids, and 647 and 644 nm for chlorophylls *a* and *b*, respectively. For anthocyanins, absorption was measured at 532 and 653 nm. The concentrations of chlorophyll *a*, chlorophyll *b*, carotenoids and anthocyanins were calculated based on equations established in the literature (Lichtenthaler & Buschmann, 2001; Lichtenthaler & Wellburn, 1983; Murray & Hackett, 1991). The total phenolic compounds were determined using the Folin-Ciocalteu reagent and tannic acid as a standard (Gross et al., 1996).

For the measurement of elemental C and N content, up to 2 mg of the plant material were weighed and compacted in a tin capsule. Elemental C and N content were determined using a CHNS elemental analyser (Carlo-ERBA Na 2100 CE Instrument). Phosphorus content was measured from 0.5 mg powder by spectrophotometric quantification (AFNOR, 1990) using an automated analyser (Konelab 420X Ti, Thermo Fisher Analytics) after alkaline digestion of the plant material.

2.3. Microalgal chemistry

Water samples from the microcosms (between 40 and 100 mL depending on phytoplankton density) were passed through GF/F filters. Periphyton on the polystyrene plastic strips were brushed into 20 mL Volvic water. Periphyton subsamples were centrifuged (Chapter S1) or the phytoplankton suspension was passed through GF/F

filters. Pigment extraction was performed using the GF/F filters or pellets, and chlorophyll *a* of the microalgae was measured using HPLC analyses. Refer to Chapter S4 (Allen et al., 2021) and Chapter 3 for further details on the pigment analyses for the microalgae. A comparison of the chlorophyll *a* (used as a proxy for biomass) for the phytoplankton and periphyton among the three experiments was made to determine how competition for nutrients and light may indirectly have affected plant response to the ARO. For the microalgae, stoichiometry was measured similar to the macrophytes but using GF/F filters through which water samples (between 40 and 100 mL) from the microcosms were filtered.

2.4. Statistical analyses

All statistical analyses were performed in R (v4.1.0, R Core Team, 2018). The macrophyte data were first visualized using principal components analyses (PCA) to identify main stressor patterns and effects. This was followed by pairwise comparisons between the control and the 3X ARO as well as the ambient and warm treatments for all three experiments to identify the magnitude of the effects. To identify whether there were any correlations between macrophyte growth and the microalgae development, Pearson's or Spearman's correlation tests were performed. Finally, two-way (Experiments 1 and 2) or three-way (Experiment 3) analyses of variance (ANOVA) were performed to test for the individual and combined treatment effects.

2.4.1. Principal components analyses

Principal components analyses (PCA) using the *factoextra* (Kassambara & Mundt, 2020) and *vegan* (Oksanen et al., 2020) packages in R were performed to identify the main responses of the three macrophyte species to ARO and temperature. Eight types of response variables were considered: shoot height as a growth response and seven physiological biomarkers (total chlorophylls as chl-*a* + chl-*b*, carotenoids, total phenolic compounds, anthocyanins, C, N and P content) measured in the three macrophyte species (*M. spicatum*, *P. perfoliatus* and *E. nuttallii*). This resulted in a total of 24 variables for all three macrophyte species. Only the 10 most significant responses were plotted using the “select.var = list(contrib=10)” from the *fviz* function in R. For each of the three experiments, first, the controls and ARO (3X) were projected as supplementary data. This was followed by a second plot with the ambient and heated treatments projected as supplementary data. These analyses were performed for each of the three experiments and allowed a comparison of effects among the experimental designs. For Experiment 3,

additional PCAs were created, performing the same analyses, but projecting the nitrate-only and pesticides-only treatments as supplementary data.

2.4.2. Effect size

Pairwise comparisons of the control and 3X ARO treatments as well as the ambient control and warm control treatments for all plant responses were performed using a *t*-test for parametric data or a Wilcoxon Pair test for non-parametric data. The size of the effects and their 95% CIs were then calculated based on (Hedges & Olkin, 1985). When the effects were both large (>0.8, Cohen, 1988) and significant, they were considered meaningful.

2.4.3. Correlation tests

Periphyton and phytoplankton can compete with the macrophytes for nutrients and light, and might thus influence plant physiology. Therefore, Pearson's or Spearman's correlation tests were performed with total chlorophylls and carotenoids of all macrophyte species versus the phytoplankton and periphyton chl-*a*. Effects of ARO or warming were analysed by pairwise comparisons of either the control and 3X ARO treatments or the ambient control and warm control treatments. This was done for all plant responses, using a *t*-test for parametric data or a Wilcoxon Pair test for non-parametric data.

2.4.4. Exposure scenario

For Experiments 1 and 2, a two-way analyses of variance (ANOVA) was applied to test treatment and temperature effects, and their interactions. Treatments included the control, single low dose (X), single application of a double ARO dose (2X) or the application of double dose as a pulses (XX), single application of a triple ARO dose (3X) or the application of triple dose as a pulses (XXX), all at two temperatures. This was followed by a post-hoc test, which included a Tukey test for parametric data or a Dunn's test for nonparametric data to determine whether there were any differences in effects between the double single dose (2X) or pulse (XX) or between the triple single dose (3X) or pulse (XXX).

2.4.5. Individual versus combined effects of pesticides and nitrate

For Experiment 3, a three-way ANOVA was applied to test for individual and combined Pest (pesticide), Nitr (nitrate), Tmp (temperature) (fixed factors) on all response factors.

For both the two-way and three-way ANOVA, the Shapiro–Wilk’s test and the Bartlett test were applied to test for normality and heteroscedasticity, respectively. Data were transformed using log or square root when required. When normality or homoscedasticity could not be reached, the non-parametric Kruskal–Wallis test by ranks was used. Significance was considered at $p < 0.05$.

3. Results

The results of the PCA, effect sizes, correlation tests and ANOVA are presented as subchapters for each experiment. Because the treatments selected for the PCA and effect sizes were limited (see “Material and Methods”) compared to the two-way or three-way ANOVA, which included all treatments, the results vary slightly depending on the analyses. The PCA plots (Fig. 5.1 & 5.2), their eigenvalues (Fig. 5.3) and effect sizes (Fig. 5.4) for all three experiments are first provided, followed by the subchapters describing the results. The first two subchapters (Chapters 5.1 & 5.2) describe the results from Experiments 1 and 2, respectively, focusing on the effects of ARO exposure mode on the macrophyte chemistry. The third subchapter (Chapter 5.3) describes the results from Experiment 3, focusing on the nitrate and pesticide effects on the macrophyte chemistry. This is followed by the fourth subchapter (Chapter 5.4) providing a comparison of the microalgal biomass across all three experiments. The last subchapter (Chapter 5.5) is a discussion of results from all three experiments. A figure is provided at the start of each subchapter as a reminder of the general experimental design.

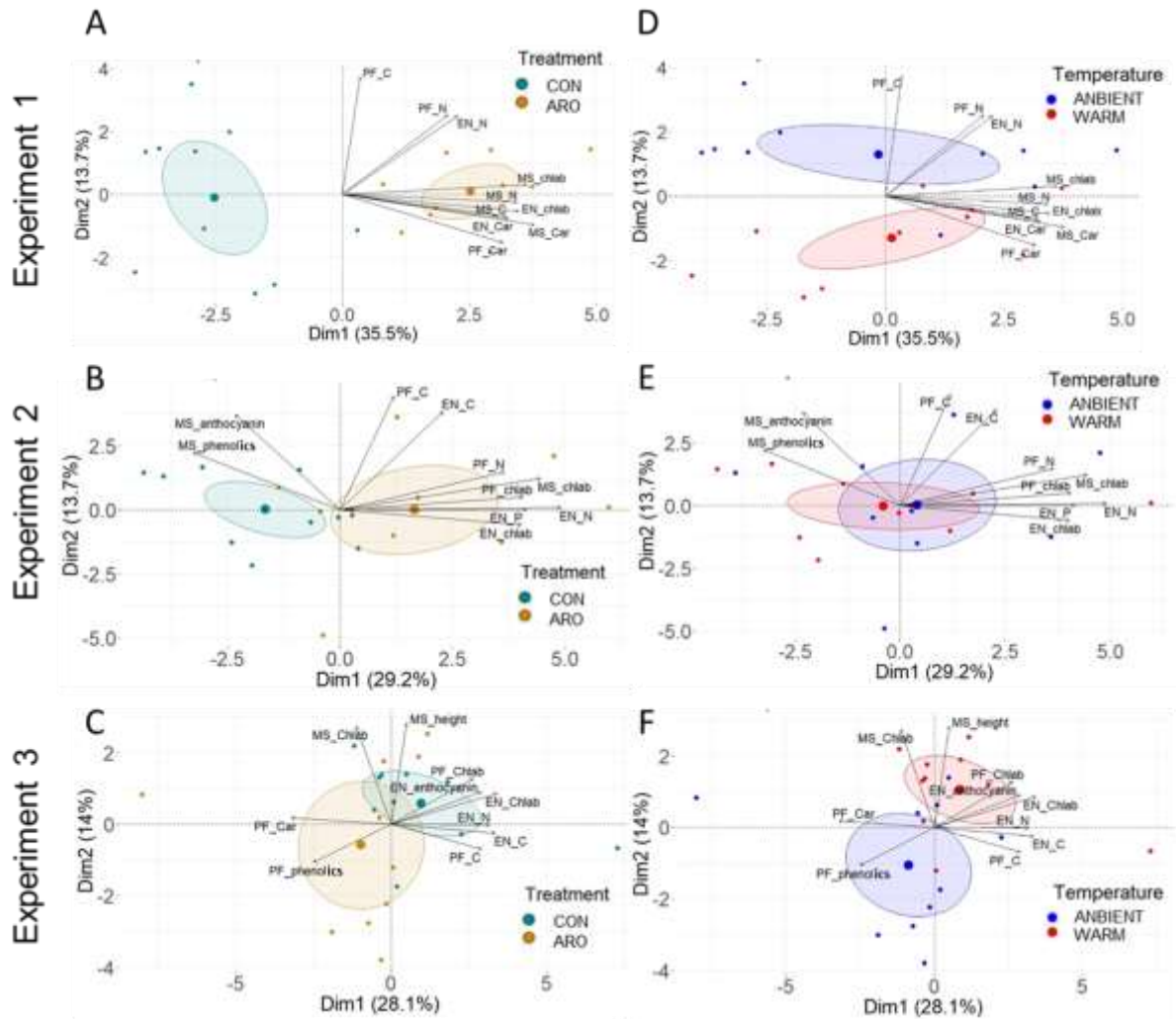


Fig. 5.1. Principal components analyses (PCA) showing ARO and temperature effects in Experiments 1 to 3, performed with axes 1 and 2. Eight response factors for all three macrophyte species were used in the analyses, including growth and physiology (see “Material and Methods”). Either ARO (A-C) or temperature (D-F) treatments were projected as supplementary data. For each experiment, only the 10 most significant variables were plotted (using “select.var = list(contrib=10” from the fviz function using factoextra)). Note: Phosphorus concentrations for *P. perfoliatus* and *E. nuttallii* are not available for Experiment 1 and not included in the analyses. MS = *M. spicatum*, PF = *P. perfoliatus*, EN = *E. nuttallii*, C =carbon, N = nitrogen, P = phosphorus, Car = carotenoids, chlab = chlorophyll-*a* + chlorophyll-*b*, Experiment 1 = only primary producers, Experiment 2 = primary producers and consumers (only snails), Experiment 3 = primary producers and consumers (snails and *Daphnia*).

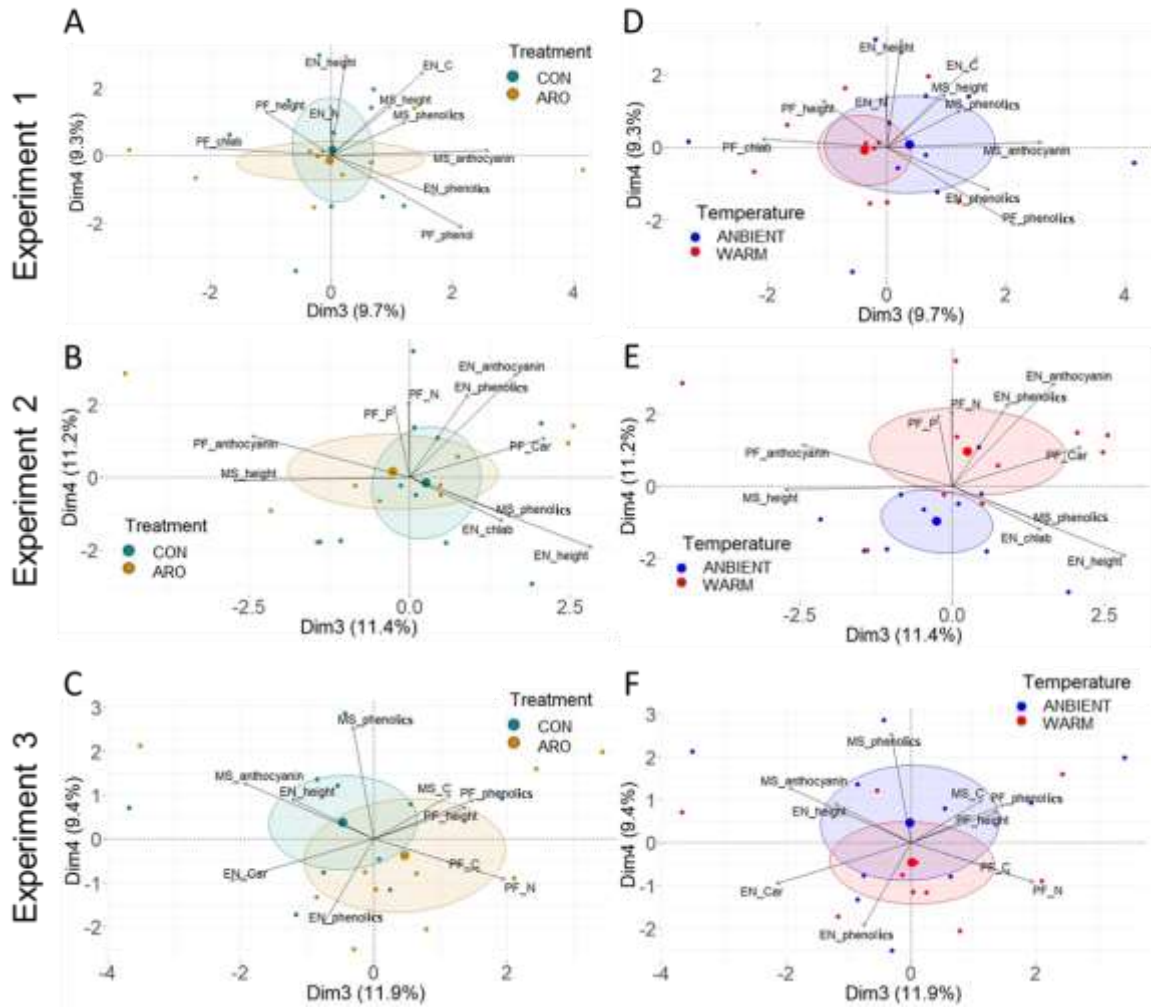


Fig. 5.2. Principal components analyses (PCA) showing ARO and temperature effects in Experiments 1 to 3, performed with axes 3 and 4. Eight response factors for all three macrophyte species were used in the analyses, including growth and physiology (see “Material and Methods”). Either ARO (A-C) or temperature (D-F) treatments were projected as supplementary data. For each experiment, only the 10 most significant variables were plotted (using “select.var = list(contrib=10)” from the fviz function using factoextra). Note: Phosphorus concentrations for *P. perfoliatus* and *E. nuttallii* are not available for Experiment 1 and not included in the analyses. MS = *M. spicatum*, PF = *P. perfoliatus*, EN = *E. nuttallii*, C = carbon, N = nitrogen, P = phosphorus, Car = carotenoids, chl_{ab} = chlorophyll-*a* + chlorophyll-*b*. Experiment 1 = only primary producers, Experiment 2 = primary producers and consumers (only snails), Experiment 3 = primary producers and consumers (snails and *Daphnia*).

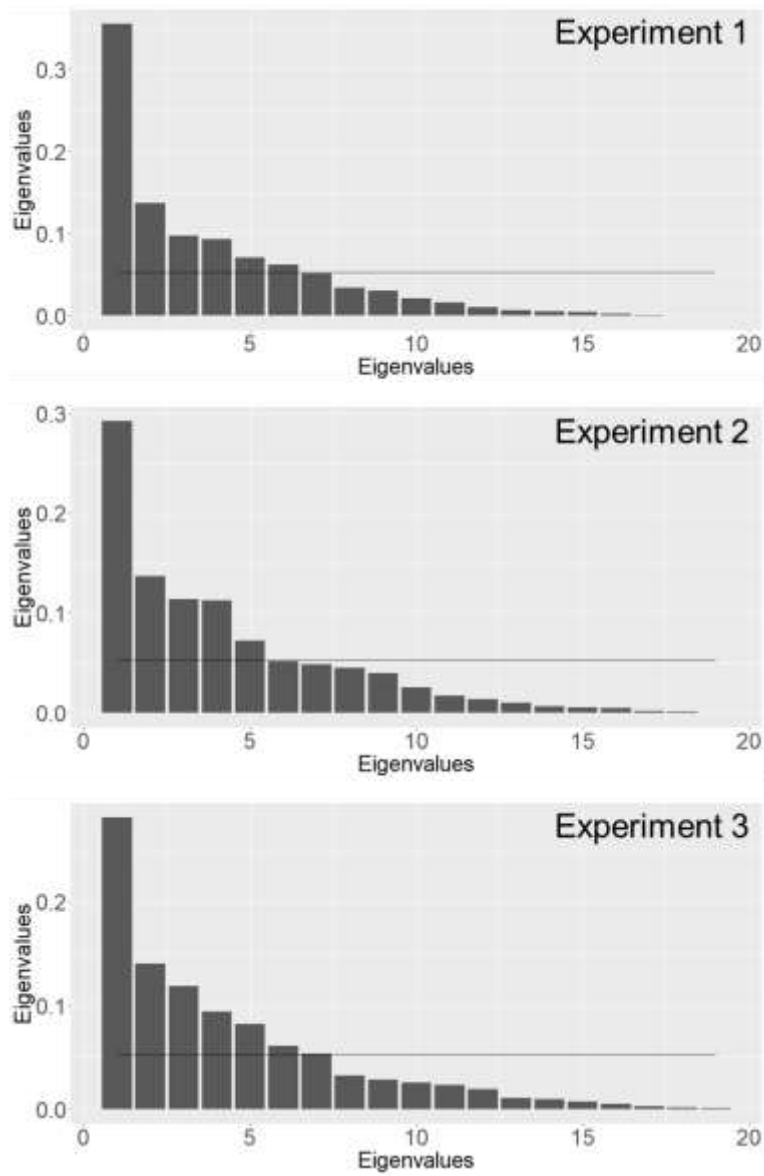


Fig. 5.3. Eigenvalues of the principal components.

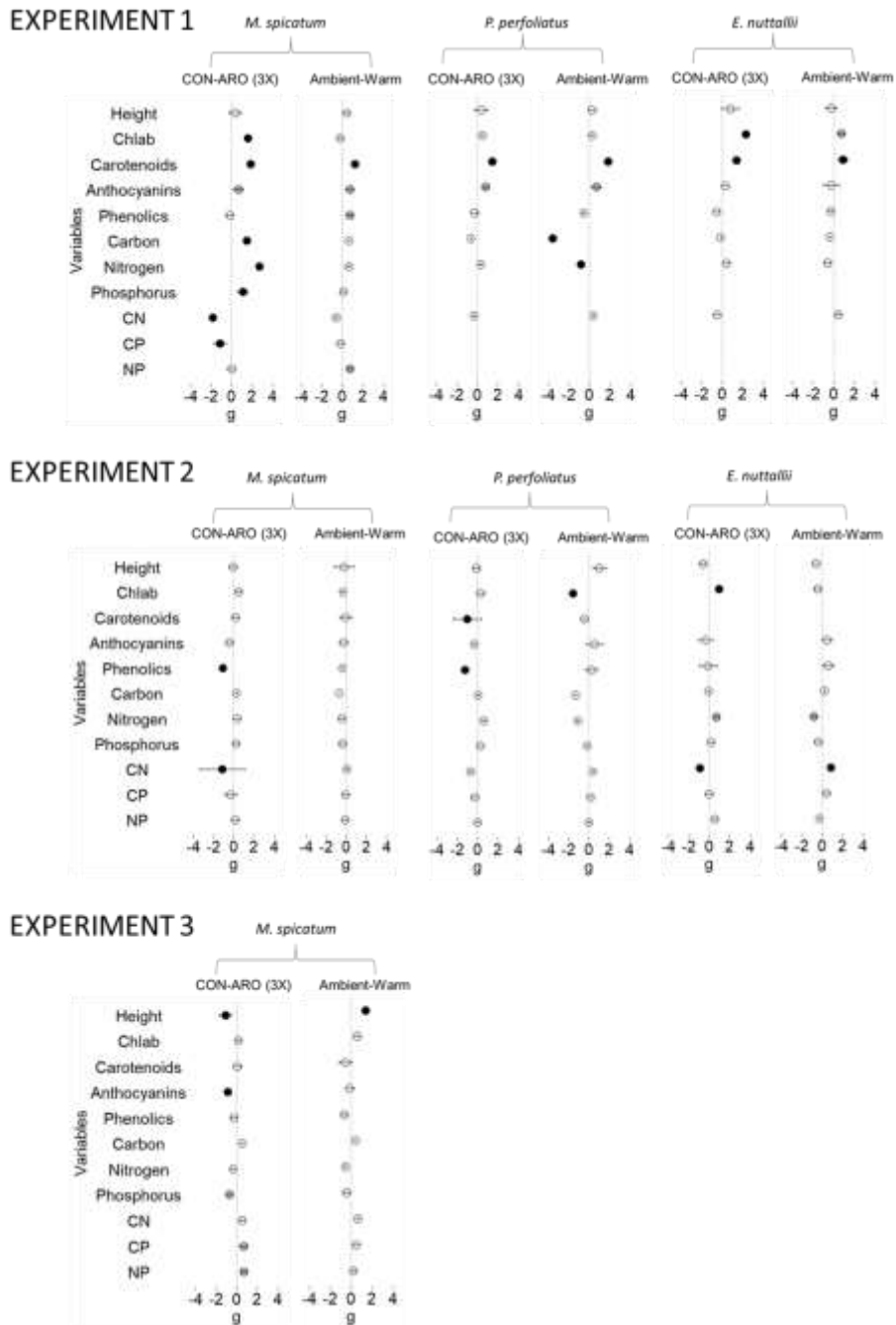
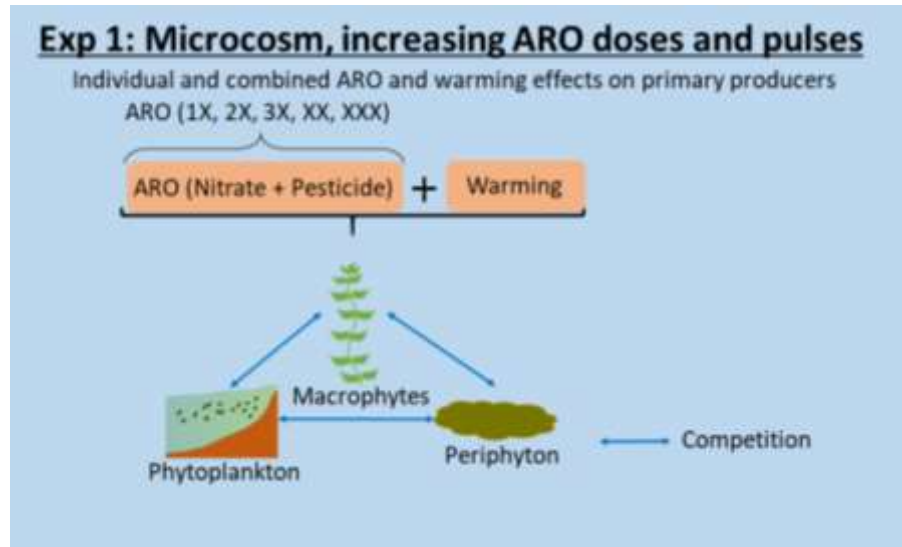


Fig. 5.4. Hedge's *g* effect sizes calculated from standardized means and pooled standard deviations (Hedges & Olkin, 1985) of the control and the highest ARO treatments (3X), and of the ambient and warm microcosms for all three experiments for macrophyte growth and physiology. Black symbols represent significant effects ($p < 0.05$), grey symbols represent marginal effects ($p \leq 0.10$) and white symbols represent non-significant effects ($p \geq 0.10$). Significance values derived from the unpaired t-test. Experiment 1 = only primary producers, Experiment 2 = primary producers and consumers (only snails), Experiment 3 = primary producers and consumers (snails and *Daphnia*). Phosphorus content for *P. perfoliatus* and *E. nuttallii* are not available for Experiment 2. Due to the overall low biomass of *P. perfoliatus* and *E. nuttallii* in Experiment 3, effect sizes are not provided for these two species.

Chapter 5.1. Experiment 1: Effect of ARO exposure mode on macrophyte growth and chemistry



Experiment 1: Primary producers exposed to increasing single ARO doses and pulses.

1. Principal components analyses (PCA): Effect of ARO and temperature on macrophyte growth and physiology

The first two axes contributed to 49.2% of the variation, while the third and fourth axes together contributed to 19% (Fig. 5.1 & 5.2). Both temperature and ARO effects are visible on the first biplot (PCA 1 and 2).

1.1. ARO effect

The principal components analyses (PCA) was performed on growth and seven physiological biomarkers of the macrophytes, with the ARO (3X) treatments and controls projected as supplementary data (Fig. 5.1). The ARO effect was explained by Axis 1, with the controls on the left separated from the ARO treatments on the right. The most significant response variables were the photosynthetic pigments and stoichiometry. The photosynthetic pigments (chl-*a* + chl-*b* and/or carotenoids) of all three macrophyte species and the C and N content of *M. spicatum* all increased in response to the ARO. The most responsive species was *M. spicatum*, also increasing the uptake of N and C when exposed to ARO, followed by *E. nuttallii*. Carbon content of *P. perfoliatus* was explained by axis 2. Axis 2 was not explained by the ARO or temperature.

1.2. Temperature effect

To visualize the temperature effects, the same analyses were performed as for the ARO effect, but in this case, the ambient and heated treatments were projected as supplementary data. The second axis explained the temperature effect (Fig. 5.1). The C content of *P. perfoliatus* increased at ambient temperature. None of the other physiological biomarker responses were clearly explained by temperature.

2. Stressor effect sizes

2.1. ARO effect

The calculation of effect sizes enables us to determine the magnitude of the stressor effects (Fig. 5.4). *Myriophyllum spicatum* responded significantly to ARO with large effect sizes (>0.8 , Cohen, 1988) for total chlorophylls, carotenoids, C, N, as well as stoichiometry (C:N and C:P ratios). For *P. perfoliatus* and *E. nuttallii*, only the photosynthetic pigments (chl-*a* + chl-*b* and/or carotenoids) showed large effect sizes.

2.2. Temperature effect

The largest temperature effect was observed for *P. perfoliatus*, with carotenoids significantly increasing, and C and N significantly reducing at elevated temperature. Both *M. spicatum* and *E. nuttallii* also exhibited large temperature effects for the carotenoids, which increased at elevated temperature.

3. Correlation analyses

Correlation analyses between the microalgal chl-*a* and macrophyte total chlorophylls indicated that phytoplankton was driving macrophyte response. Significant positive correlations were observed both between *M. spicatum* chl-*ab* and phytoplankton chl-*a* (Spearman's $r = 0.5$, $p = 0.01$), and between *E. nuttallii* chl-*ab* and phytoplankton chl-*a* (Spearman's $r = 0.6$, $p = 0.01$). No correlations were found between macrophyte chl-*ab* and periphyton chl-*a*. In addition, *M. spicatum* and *E. nuttallii* showed stress responses to phytoplankton indicated by positive correlations between phytoplankton chl-*a* and carotenoids ($r = 0.5$, $p = 0.03$ and $r = 0.5$, $p = 0.04$, respectively).

4. Effect of increasing single versus pulse ARO exposure on macrophytes

The two-way ANOVA revealed significant ARO treatment effects on almost all physiological response factors in the three macrophytes, while effects of temperature or ARO × Temperature effects were visible only for a few. Detailed results of the statistical analyses are provided in Table 5.1.1.

Macrophyte height was neither affected by single or pulsed doses of ARO. The tallest macrophyte species (sum of total shoot length per vase) was *P. perfoliatus* (172 ± 68 cm, mean \pm standard deviation), followed by *M. spicatum* (127 ± 41 cm). *E. nuttalli* was the smallest (69 ± 53 cm). The total chlorophyll contents were higher for all three macrophytes in this experiment compared with the Experiments 2 and 3, ranging in the controls from $17.3 \pm 1.4 \mu\text{g mg}^{-1}$, $24.5 \pm 10.4 \mu\text{g mg}^{-1}$ and $10.7 \pm 1.6 \mu\text{g mg}^{-1}$ for *M. spicatum*, *P. perfoliatus* and *E. nuttalli*, respectively. For the three species, total chlorophylls increased significantly when exposed to the double and triple ARO doses irrespective of the exposure mode. The highest increase was observed for both *M. spicatum* and *E. nuttalli* in the triple dose treatments (Fig. 5.1.1, 5.1.2 & 5.1.3).

Table 5.1.1. Statistical results from the 2-way ANOVA or Kruskal–Wallis test by ranks for Experiment 1.

Macrophyte response	Test	Treatment	Post hoc (Between-treatment)	Temperature	TreatmentxTemperature
<i>M. spicatum</i>					
Height	2-way ANOVA	0.5		0.9	0.7
Chl- <i>a</i>	2-way ANOVA	3.45E-06	C-2X, <i>p</i> = 6.00E-04 C-3X, <i>p</i> < 0.0001 C-XX, <i>p</i> = 8.00E-04 C-XXX, <i>p</i> = 0.005 X-2X, <i>p</i> = 0.03 X-XX, <i>p</i> = 0.03 X-3X, <i>p</i> = 0.002	0.6	0.3
Chl- <i>a</i> + Chl- <i>b</i>	2-way ANOVA	0.001	C-2X, <i>p</i> = 0.0004 C-3X, <i>p</i> < 0.0001 C-XX, <i>p</i> = 0.0002 C-XXX, <i>p</i> = 0.002 X-2X, <i>p</i> = 0.02 X-3X, <i>p</i> = 0.001 X-XX, <i>p</i> = 0.01	0.1	0.1
Chl- <i>a</i> /Chl- <i>b</i>	2-way ANOVA	0.08		0.4	0.06
Carotenoids	2-way ANOVA	0.07		0.04	0.07
Carotenoids/(Chl- <i>a</i> + Chl- <i>b</i>)	2-way ANOVA	0.07		0.05	0.07
Anthocyanins	2-way ANOVA	0.3		0.4	0.2
Total phenols	2-way ANOVA	0.01		0.8	0.1

Carbon	2-way ANOVA	2.48E-07	C-X, $p = 0.002$ C-2X, $p = 0.0003$ C-3X, $p < 0.0001$ C-XX, $p < 0.0001$ C-XXX, $p = 0.0001$	0.3	0.3
Nitrogen	2-way ANOVA	1.01E-07	C-2X, $p = 0.01$ C-3X, $p = 0.001$ C-XX, $p = 0.005$ C-XXX, $p = 0.006$ X-2X, $p = 0.002$ X-3X, $p = 0.0002$ X-XX, $p = 0.001$ X-XXX, $p = 0.001$	0.8	0.4
Phosphorus	2-way ANOVA	1.00E-04	C-XXX, $p = 0.0003$ X-XXX, $p = 0.0006$ 2X-XXX, $p = 0.004$ 3X-XXX, $p = 0.001$ XX-XXX, $p = 0.001$	1.00E-04	1.00E-04
C:N	2-way ANOVA	7.05E-07	C-2X, $p = 0.01$ C-3X, $p = 0.001$ C-XX, $p = 0.005$ C-XXX, $p = 0.006$ X-2X, $p = 0.002$ X-3X, $p = 0.0002$ X-XX, $p = 0.001$ X-XXX, $p = 0.001$	1	0.5
C:P	2-way ANOVA	5.76E-05	C-XXX, $p = 0.06$	1.08E-04	2.02E-04

			X-XXX, $p = 0.04$	
N:P	2-way ANOVA	0.1		9.00E-04 0.007
<i>P. perfoliatus</i>				
Height	2-way ANOVA	0.5		0.2 0.7
Chl- <i>a</i>	Kruskal-Wallis	0.001	C-2X, $p = 0.01$ C-XX, $p = 0.0007$ C-3X, $p = 0.002$ C-XXX, $p < 0.0001$ X-XX, $p = 0.03$ X-XXX, $p = 0.004$ 2X-XXX, $p = 0.04$	0.07
Chl- <i>a</i> + Chl- <i>b</i>	Kruskal-Wallis	1.54E-05	C-2X, $p = 0.01$ C-3X, $p = 0.0003$ C-XX, $p = 0.0002$ C-XXX, $p < 0.0001$ X-3X, $p = 0.02$ X-XX, $p = 0.01$ X-XXX, $p = 0.0002$ 2X-XXX, $p = 0.006$	0.3
Chl- <i>a</i> /Chl- <i>b</i>	2-way ANOVA	0.06		0.6
Carotenoids	Kruskal-Wallis	0.02	C-X, $p = 0.01$ C-2X, $p = 0.003$ C-3X, $p = 0.0003$ C-XX, $p = 0.03$ C-XXX, $p = 0.01$	0.8

Carotenoids/(Chl- <i>a</i> + Chl- <i>b</i>)	2-way ANOVA	0.07		0.9	
Anthocyanins	Kruskal-Wallis	1.56E-04	X-3X, <i>p</i> = 0.02 X-2X, <i>p</i> = 0.004 X-3X, <i>p</i> = 0.0004 X-XX, <i>p</i> = 0.0007 2X-XXX, <i>p</i> = 0.05 C-XXX, <i>p</i> = 0.001 X-XXX, <i>p</i> < 0.0001	0.4	
Total phenols	2-way ANOVA	0.1		5.00E-04	1
Carbon	2-way ANOVA	0.04	2X-XXX, <i>p</i> = 0.02	2.15E-11	1.15E-04
Nitrogen	2-way ANOVA	3.00E-04	C-XXX, <i>p</i> = 0.004 X-3X, <i>p</i> = 0.05 X-XXX, <i>p</i> = 0.002 2X-XXX, <i>p</i> = 0.02	0.4	0.08
Phosphorus					
C:N	2-way ANOVA	6.00E-04	C-XXX, <i>p</i> = 0.006 X-XXX, <i>p</i> = 0.003 2X-XXX, <i>p</i> = 0.02	0.5	0.2
C:P					
N:P					
<i>E. nuttallii</i>					
Height	2-way ANOVA	0.7		0.5	0.8
Chl- <i>a</i>	2-way ANOVA	1.00E-06	C-2X, <i>p</i> = 0.0004 C-XX, <i>p</i> < 0.0001	0.2	0.6

			C-3X, $p = 0.0004$ C-XXX, $p < 0.0001$ X-XX, $p = 0.01$ X-XXX, $p = 0.006$		
Chl- <i>a</i> + Chl- <i>b</i>	2-way ANOVA	2.56E-07	C-2X, $p = 0.0002$ C-3X, $p = 0.0002$ C-XX, $p < 0.0001$ C-XXX, $p < 0.0001$ X-XX, $p = 0.009$ X-XXX, $p = 0.002$	0.2	0.7
Chl- <i>a</i> /Chl- <i>b</i>	2-way ANOVA	0.04	C-XX, $p = 0.04$	0.5	0.2
Carotenoids	2-way ANOVA	0.3		0.3	0.02
Carotenoids/(Chl- <i>a</i> + Chl- <i>b</i>)	2-way ANOVA	0.3		0.3	0.02
Anthocyanins	2-way ANOVA	0.1		0.1	0.1
Total phenols	2-way ANOVA	0.4		0.07	0.02
Carbon	Kruskal-Wallis	0.1		0.9	
Nitrogen	Kruskal-Wallis	0.003	C-2X, $p = 0.02$ C-3X, $p = 0.01$ C-XX, $p = 0.03$ C-XXX, $p = 0.0005$ X-2X, $p = 0.03$ X-3X, $p = 0.01$ X-XXX, $p = 0.0004$	0.4	

Phosphorus				
C:N	Kruskal-Wallis	0.002	C-2X, $p = 0.009$ C-3X, $p = 0.003$ C-XX, $p = 0.04$ C-XXX, $p = 0.0002$ X-2X, $p = 0.03$ X-3X, $p = 0.01$ X-XXX, $p = 0.001$	0.4
C:P				
N:P				

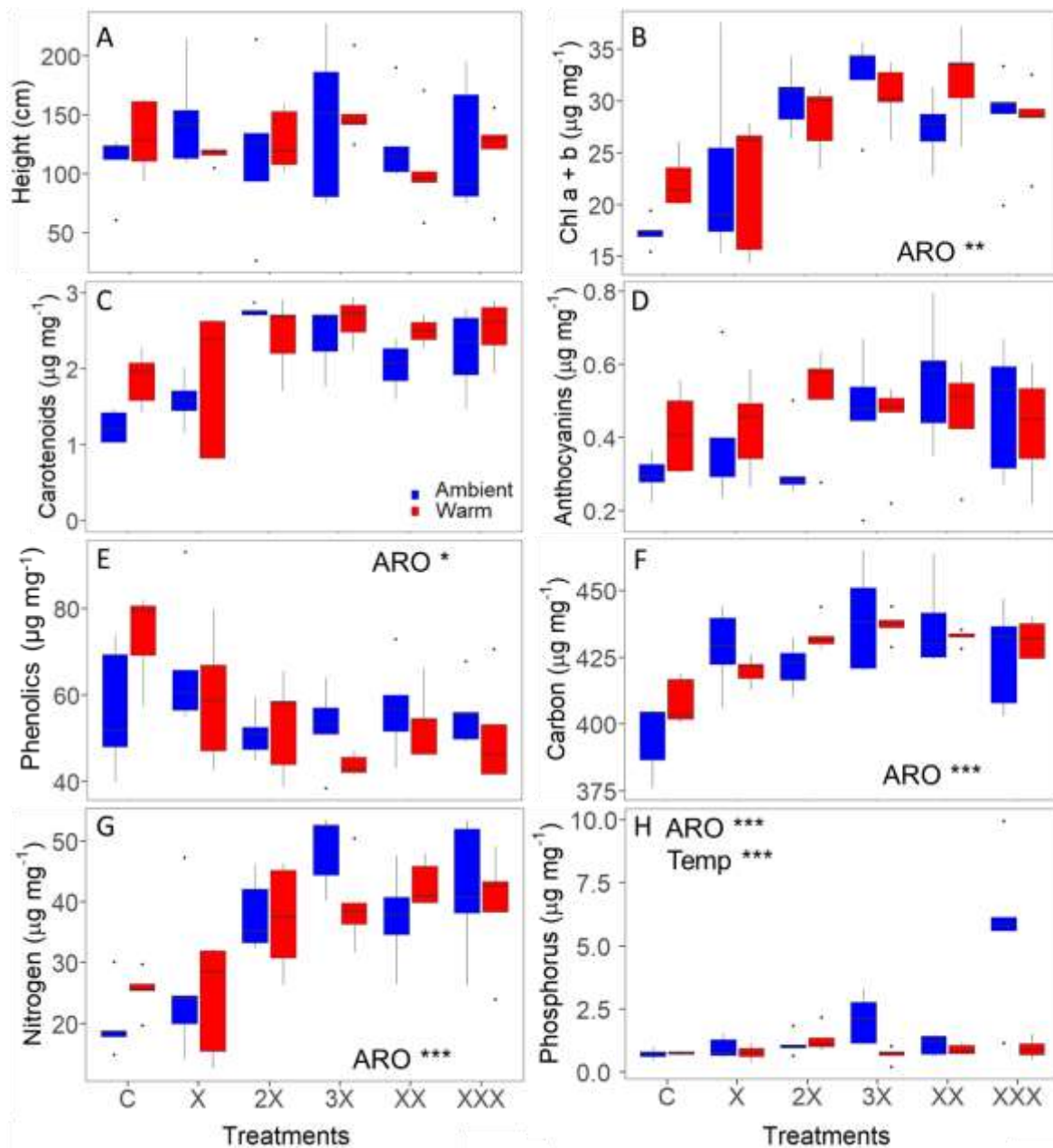


Fig. 5.1.1. Box plots showing ARO and temperature (Temp) effects on *M. spicatum* growth and physiology in Exp 1 (experiment with only primary producers). C = control, X = single low dose, 2X = double ARO dose applied once, 3X = triple ARO dose applied once, XX = double ARO dose applied as a pulse, XXX = triple ARO dose applied as a pulse. Blue = ambient microcosms, red = warm microcosms. Box plots of 5 replicates showing median, 25 and 75% percentiles, lowest and highest whiskers (as $Q1 - [1.5 \times IQR]$ and $Q3 + [1.5 \times IQR]$, respectively), and outliers (dots). Significant temperature or ARO effect is indicated as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

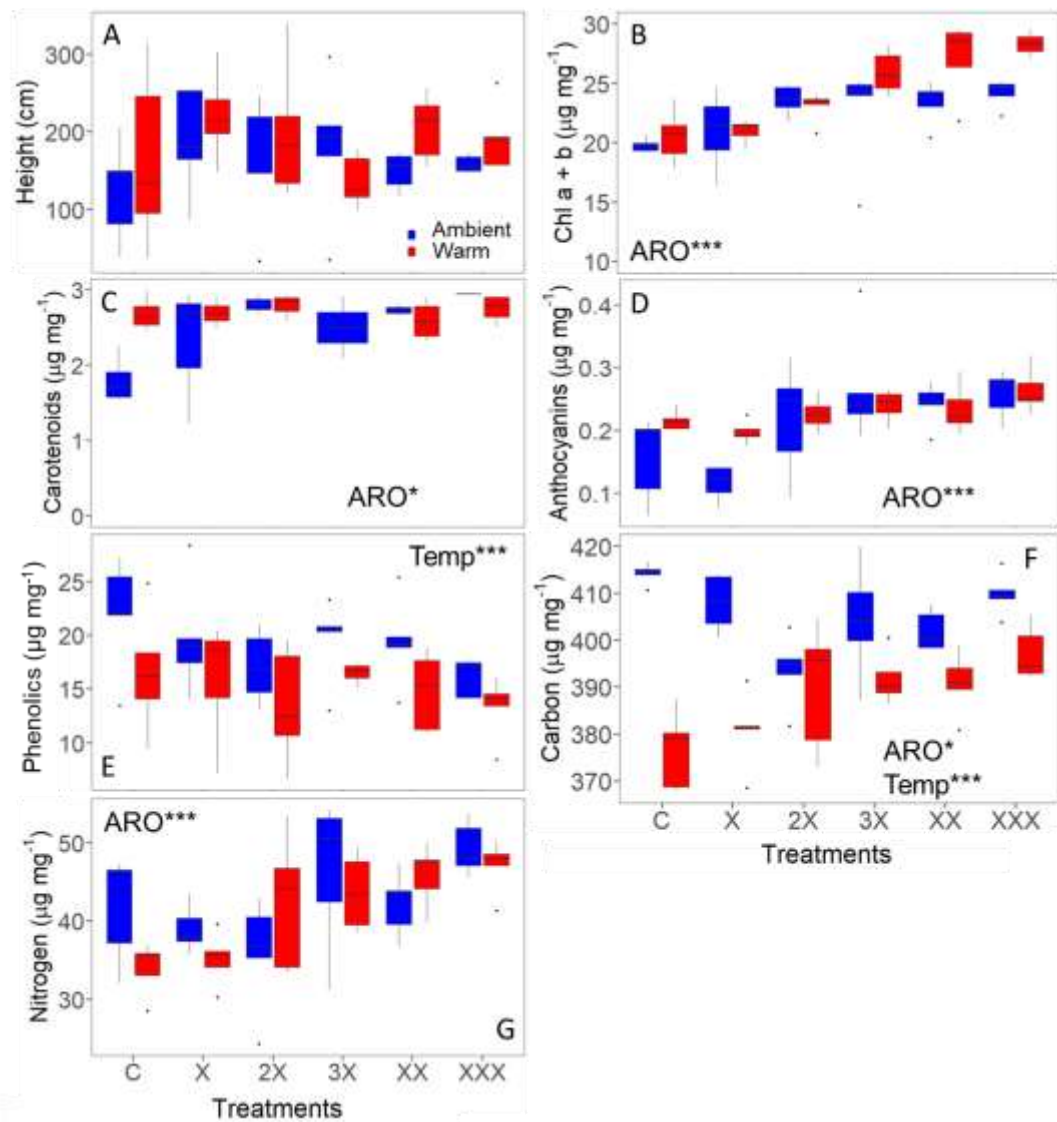


Fig. 5.1.2. Box plots showing ARO and temperature (Temp) effects on *P. perfoliatus* growth and physiology in Experiment 1 (only primary producers). C = control, X = single low dose, 2X = double ARO dose applied once, 3X = triple ARO dose applied once, XX = double ARO dose applied as a pulse, XXX = triple ARO dose applied as a pulse. Blue = ambient microcosms, red = warm microcosms. Box plots of 5 replicates showing median, 25 and 75% percentiles, lowest and highest whiskers (as $Q1 - [1.5 \cdot IQR]$ and $Q3 + [1.5 \cdot IQR]$, respectively), and outliers (dots). Significance levels shown as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

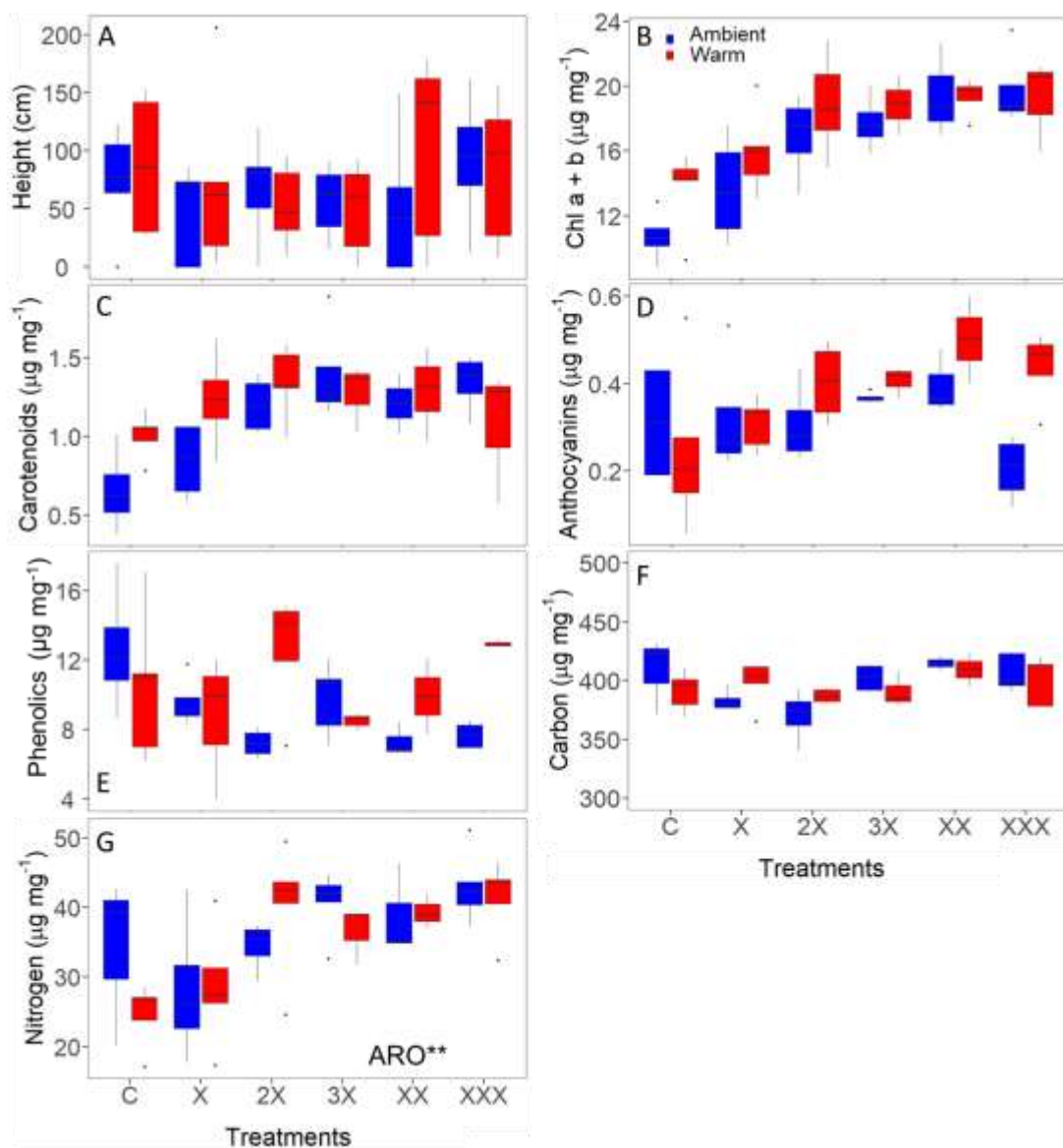


Fig. 5.1.3. Box plots showing ARO and temperature (Temp) effects on *E. nuttallii* growth and physiology in PP (experiment with only primary producers). C = control, X = single low dose, 2X = double ARO dose applied once, 3X = triple ARO dose applied once, XX = double ARO dose applied as a pulse, XXX = triple ARO dose applied as a pulse. Blue = ambient microcosms, red = warm microcosms. Box plots of 5 replicates showing median, 25 and 75% percentiles, lowest and highest whiskers (as Q1-[1.5*IQR] and Q3+[1.5*IQR], respectively), and outliers (dots). Significance levels shown as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

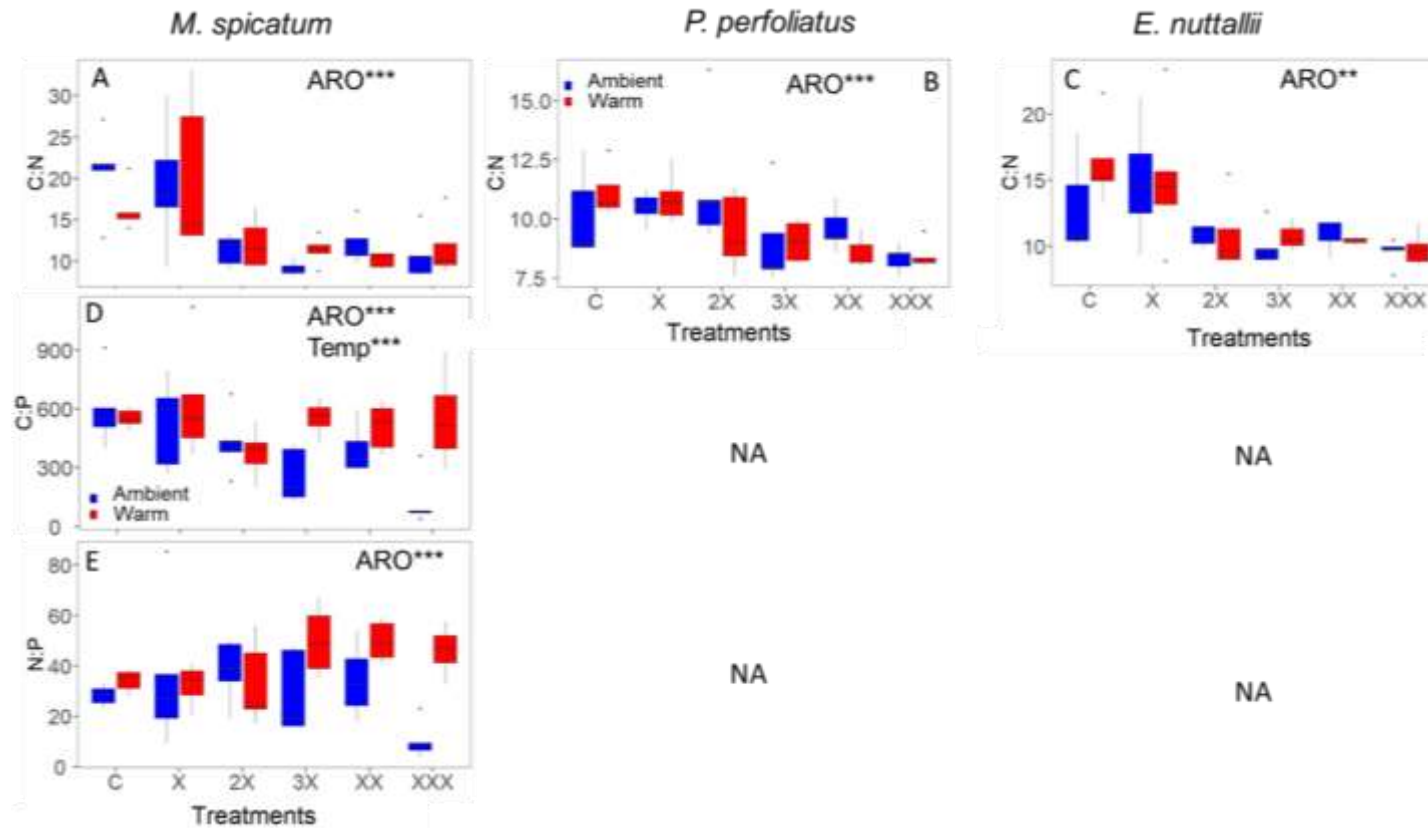
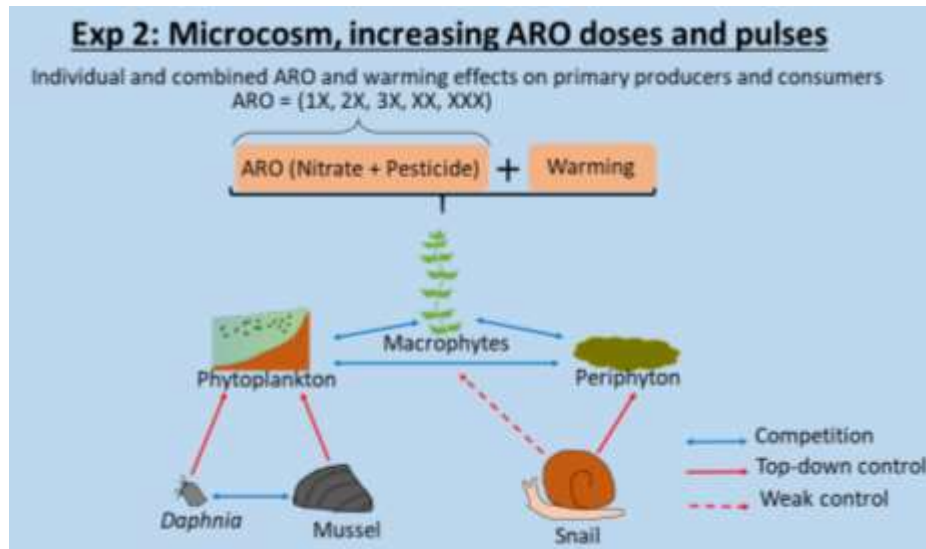


Fig. 5.1.4. Box plots showing ARO and temperature (Temp) effects on macrophyte elemental stoichiometry in Experiment 1 (only primary producers). C = control, X = single low dose, 2X = double ARO dose applied once, 3X = triple ARO dose applied once, XX = double ARO dose applied as a pulse, XXX = triple ARO dose applied as a pulse. Blue = ambient microcosms, red = warm microcosms. Box plots of 5 replicates showing median, 25 and 75% percentiles, lowest and highest whiskers (as Q1-[1.5*IQR] and Q3+[1.5*IQR], respectively), and outliers (dots). Significance levels shown as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Phosphorus contents for *P. perfoliatus* and *E. nuttallii* are not available (NA) and therefore CP and NP are not reported.

In the controls, total phenolic content measured an average $57 \pm 14 \mu\text{g mg}^{-1}$ for the *M. spicatum*, $22 \pm 5 \mu\text{g mg}^{-1}$ for *P. perfoliatus* and $13 \pm 4 \mu\text{g mg}^{-1}$ for *E. nuttallii*. The phenolic content of *M. spicatum* decreased with ARO, but exposure mode did not affect the response. For anthocyanins, the concentrations ranged from 0.1 to $0.32 \mu\text{g mg}^{-1}$ for all macrophytes. Anthocyanin content in *P. perfoliatus* significantly increased with ARO, e.g., the XXX treatment showed a 67% increase compared with the control. In addition, all the higher ARO doses and pulses showed significantly higher anthocyanin content compared with the lowest ARO dose. However, ARO exposure mode did not influence this response. No significant single ARO dose or ARO pulse effects were observed on the anthocyanins or phenolic content of *E. nuttallii*.

Carbon and nitrogen content in the controls for all three macrophytes ranged between ~ 370 and $415 \mu\text{g mg}^{-1}$. For *M. spicatum*, N content was slightly lower ($20 \pm 5 \mu\text{g mg}^{-1}$) compared to the other two macrophytes ($42 \pm 7 \mu\text{g mg}^{-1}$ for *P. perfoliatus*, $34 \pm 12 \mu\text{g mg}^{-1}$ for *E. nuttallii*) in controls. For *M. spicatum*, P measured between 0.4 and $1.0 \mu\text{g mg}^{-1}$. Elemental stoichiometry of all three species was significantly affected by ARO, but exposure mode did not affect this response. Carbon increased significantly for *M. spicatum* in all ARO exposure scenarios compared to the control, and N increased in the double and triple dose and pulses compared with the control and the lowest ARO. The increase of *M. spicatum* C and N in 3X compared with the control was 11% and 145%, respectively. *E. nuttallii* N content showed similar responses to the ARO, while *P. perfoliatus* showed increases in N mainly in the triple dose and pulse scenarios. *M. spicatum* showed a very significant increase in P content in the triple pulse ARO conditions compared with all other treatments, but this was driven by the strong effect in the ambient microcosm. This effects on elemental concentrations caused significant decreases in the macrophyte stoichiometric ratios (Fig. 5.1.4).

Chapter 5.2. Experiment 2: Effect of ARO exposure mode on macrophyte growth and chemistry



Experiment 2: Primary producers and consumers exposed to increasing single ARO doses and pulses. In Experiment 2, top-down control of phytoplankton was not present, as both *Daphnia* and mussels did not survive.

1. Principal components analyses (PCA): Effect of ARO and temperature on macrophyte growth and physiology

The first two axes contributed to 42.9% of the variation, while the third and fourth axes together contributed to 22.6%. The ARO effect was visible on the first biplot (PCA 1 and 2), and the temperature effect was visible on the second biplot (PCA 3 and 4) (Fig. 5.1 & 5.2).

1.1. ARO effect

As with Experiment 1, the PCA was performed on growth and seven physiological biomarkers of the macrophytes. The ARO (3X) treatments and controls were projected as supplementary data (Fig. 5.1 & 5.2). The ARO effect was explained by Axis 1, with the controls on the left separated from the ARO treatments on the right. The most significant response variables were the photosynthetic pigments and elemental concentrations. The photosynthetic pigments (chl-*a* + chl-*b*) of all three macrophyte species, the N content of *P. perfoliatus* and *E. nuttallii* and the P content of *E. nuttallii* all increased in response to the ARO. Unlike Experiment 1, in Experiment 2, *E. nuttallii* was the most responsive species to the ARO. When ARO was not present, the phenolic content of *M. spicatum* was marginally higher. Carbon content of *P. perfoliatus* was slightly explained by axis 2.

1.2. Temperature effect

To visualize the temperature effect, the analyses of the same variables as previously described was performed, with ambient and heated treatments projected as supplementary data. The first PCA biplot (Fig. 5.1) with axes 1 and 2 did not explain the temperature effect. However, the second biplot (Fig. 5.2) using axes 3 and 4 showed a separation of ambient and heated treatments. The effect was more related to axis 4. The total phenolic content as well as the anthocyanins of *E. nuttallii* were higher at elevated temperature. The carbon content of *P. perfoliatus* was higher at ambient temperature. None of the other physiological biomarkers was clearly explained by temperature. Although not explained by temperature, this plot also shows a correlation between *E. nuttallii* height and photosynthetic pigments (Fig. 5.2).

2. Stressor effect sizes

2.1. ARO effect

In Experiment 2, *M. spicatum* did not respond as strongly to the ARO. For both *M. spicatum* and *P. perfoliatus*, only the total phenolic compounds exhibited a large effect size (>0.8 , Cohen, 1988). Although significant effects were observed for C:N and carotenoids for these two species, they were not considered important as their 95% CIs were very wide (likely due to the small sample size), crossing the “0” line. For *E. nuttallii*, large effect sizes were observed for total chlorophylls and C:N, which significantly increased and decreased, respectively, when exposed to ARO (Fig. 5.4).

2.2. Temperature effect

Effect sizes for *M. spicatum* response to warming were small (<0.8 , Cohen, 1988), and no significant effects were observed on either the growth or physiology. For *P. perfoliatus*, the temperature effect on the total chlorophylls was large (<0.8 , Cohen, 1988), showing a significant reduction at higher temperature. Although the effect sizes for carbon and nitrogen were also large, the effect was not significant and therefore not considered important. Opposite to the ARO effect, temperature significantly increased the C:N ratios for *E. nuttallii*, with a large effect size for this response.

3. Correlation analyses

As seen in the PCAs, the ARO treatments were represented by higher total chlorophyll content of all three macrophyte species. Interestingly, however, contrary to what was observed in Experiment 1, correlations were not observed between the macrophyte total chlorophylls and phytoplankton chl-*a*. Instead, the analyses indicated that periphyton was driving the increased macrophyte total chlorophylls. In particular, significant correlations were found between *M. spicatum* total chlorophylls and periphyton chl-*a* (Spearman's $r = 0.6$, $p = 0.006$). The other macrophytes did not respond to periphyton or phytoplankton, but their elevated photosynthetic pigments may have resulted from competition between all macrophyte species for nutrients and light.

4. Effect of increasing single versus pulse ARO exposure on macrophytes

The heights of all three macrophyte species were neither affected by ARO nor by exposure mode (single vs. pulse). Overall, heights of all three macrophyte species were smaller compared to those in Experiment 1. *M. spicatum* and *P. perfoliatus* measured more or less the same (50 ± 24 cm and 46 ± 25 cm, respectively), while *E. nuttallii* was much smaller (19 ± 8 cm). Total chlorophylls were lower in Experiment 2 compared to Experiment 1, with $12 \pm 1 \mu\text{g mg}^{-1}$, $16 \pm 1 \mu\text{g mg}^{-1}$ and $8 \pm 1 \mu\text{g mg}^{-1}$ for *M. spicatum*, *P. perfoliatus* and *E. nuttallii* in controls, respectively. Both *P. perfoliatus* and *E. nuttallii* showed a significant increase in total chlorophylls when exposed to ARO, both at the highest single dose (3X) and pulse (XXX) compared with the control, but with no differences between these two exposure modes (Fig. 5.2.1, 5.2.2 & 5.2.3). For *P. perfoliatus*, a small but significant increase (11%) was found in the XX compared to 2X. Results of the statistical analyses are provided in Table 5.2.1.

Table 5.2.1. Statistical results from the 2-way ANOVA or Kruskal–Wallis test by ranks for Experiment 2.

Macrophyte response	Test	Treatment	Post hoc (Between-treatment)	Temperature	TreatmentxTemperature
<i>M. spicatum</i>					
Height	2-way ANOVA	0.2		0.2	0.8
Chl- <i>a</i>	2-way ANOVA	0.3		0.3	0.2
Chl- <i>a</i> + Chl- <i>b</i>	2-way ANOVA	0.08		0.1	0.1
Chl- <i>a</i> /Chl- <i>b</i>	2-way ANOVA	0.3		0.3	0.6
Carotenoids	2-way ANOVA	0.7		0.6	0.9
Carotenoids/(Chl- <i>a</i> + Chl- <i>b</i>)	Kruskal-Wallis	0.6		0.9	
Anthocyanins	2-way ANOVA	0.5		0.01	0.3
Total phenols	2-way ANOVA	0.008	C-X, <i>p</i> = 0.03 C-2X, <i>p</i> = 0.004	0.5	0.1
Carbon	Kruskal-Wallis	0.3		0.5	
Nitrogen	2-way ANOVA	0.03		0.8	0.1
Phosphorus	2-way ANOVA	0.8		0.4	0.1

C:N	2-way ANOVA	1.87E-05	C-X, $p = 0.0002$ C-2X, $p = 0.0003$ C-3X, $p = 0.0001$ C-XX, $p = 0.0005$ C-XXX, $p = 0.0001$	0.6	0.2
C:P	2-way ANOVA	0.3		0.4	0.9
N:P	2-way ANOVA	0.2		0.3	0.9
<i>P. perfoliatus</i>					
Height	2-way ANOVA	0.6		0.05	0.8
Chl- <i>a</i>	2-way ANOVA	0.02	C-XXX, $p = 0.03$	0.3	1
Chl- <i>a</i> + Chl- <i>b</i>	Kruskal-Wallis	9.00E-04	C-3X, $p = 0.004$ C-XXX, $p = 0.0001$ C-XX, $p = 0.0002$ X-XX, $p = 0.02$ X-XXX, $p = 0.01$ 2X-XX, $p = 0.02$ 2X-XXX, $p = 0.01$	0.3	
Chl- <i>a</i> /Chl- <i>b</i>	2-way ANOVA	0.7		0.4	0.5
Carotenoids	2-way ANOVA	0.09		0.05	0.3
Carotenoids/(Chl- <i>a</i> + Chl- <i>b</i>)	2-way ANOVA	0.09		0.05	0.3

Anthocyanins	2-way ANOVA	0.5		0.3	0.6
Total phenols	2-way ANOVA	0.02	C-3X, $p = 0.02$ C-XXX, $p = 0.03$	0.8	0.8
Carbon	Kruskal-Wallis	0.02	C-XXX, $p = 0.003$ X-2X, $p = 0.05$ XX-2X, $p = 0.02$ 2X-XXX, $p = 0.0007$	0.6	
Nitrogen	2-way ANOVA	6.97E-07	C-XX, $p = 0.0001$ C-3X, $p = 0.007$ C-XXX, $p < 0.0001$ 2X-XXX, $p = 0.0008$ X-XXX, $p = 0.002$ XX-2X, $p = 0.05$	0.8	0.4
Phosphorus	2-way ANOVA	0.06		1	0.5
C:N	2-way ANOVA	3.25E-06	C-3X, $p = 0.002$ C-XX, $p < 0.0001$ C-XXX, $p < 0.0001$ X-XXX, $p = 0.03$	0.8	0.7
C:P	2-way ANOVA	0.1		0.08	0.8
N:P	2-way ANOVA	0.5		0.8	0.7
<i>E. nuttallii</i>					
Height	2-way ANOVA	0.9		0.2	0.2
Chl- <i>a</i>	Kruskal-Wallis	0.07		0.9	

Chl- <i>a</i> + Chl- <i>b</i>	Kruskal-Wallis	0.02	C-3X, <i>p</i> = 0.003 C-XXX, <i>p</i> = 0.0005 2X-XXX, <i>p</i> = 0.04 XX-XXX, <i>p</i> = 0.03	0.7	
Chl- <i>a</i> /Chl- <i>b</i>	Kruskal-Wallis	0.02	C-X, <i>p</i> = 0.05 C-3X, <i>p</i> = 0.003 C-XXX, <i>p</i> = 0.0005 2X-XXX, <i>p</i> = 0.04 XX-XXX, <i>p</i> = 0.03	0.7	
Carotenoids	2-way ANOVA	0.2		0.03	0.7
Carotenoids/(Chl- <i>a</i> + Chl- <i>b</i>)	2-way ANOVA	0.2		0.03	0.7
Anthocyanins	2-way ANOVA	0.5		6.41E-05	0.3
Total phenols	2-way ANOVA	0.6		4.00E-04	0.6
Carbon	Kruskal-Wallis	0.2		0.3	
Nitrogen	2-way ANOVA	9.00E-04	C-3X, <i>p</i> = 0.02 C-XX, <i>p</i> = 0.002 C-XXX, <i>p</i> = 0.0006	0.8	0.3
Phosphorus	2-way ANOVA	0.2		0.8	0.7
C:N	2-way ANOVA	0.001	C-2X, <i>p</i> = 0.03 C-3X, <i>p</i> = 0.02 C-XX, <i>p</i> = 0.003 C-XXX, <i>p</i> = 0.002	0.3	0.2

C:P	2-way ANOVA	0.3	0.9	0.6
N:P	2-way ANOVA	0.6	0.5	1

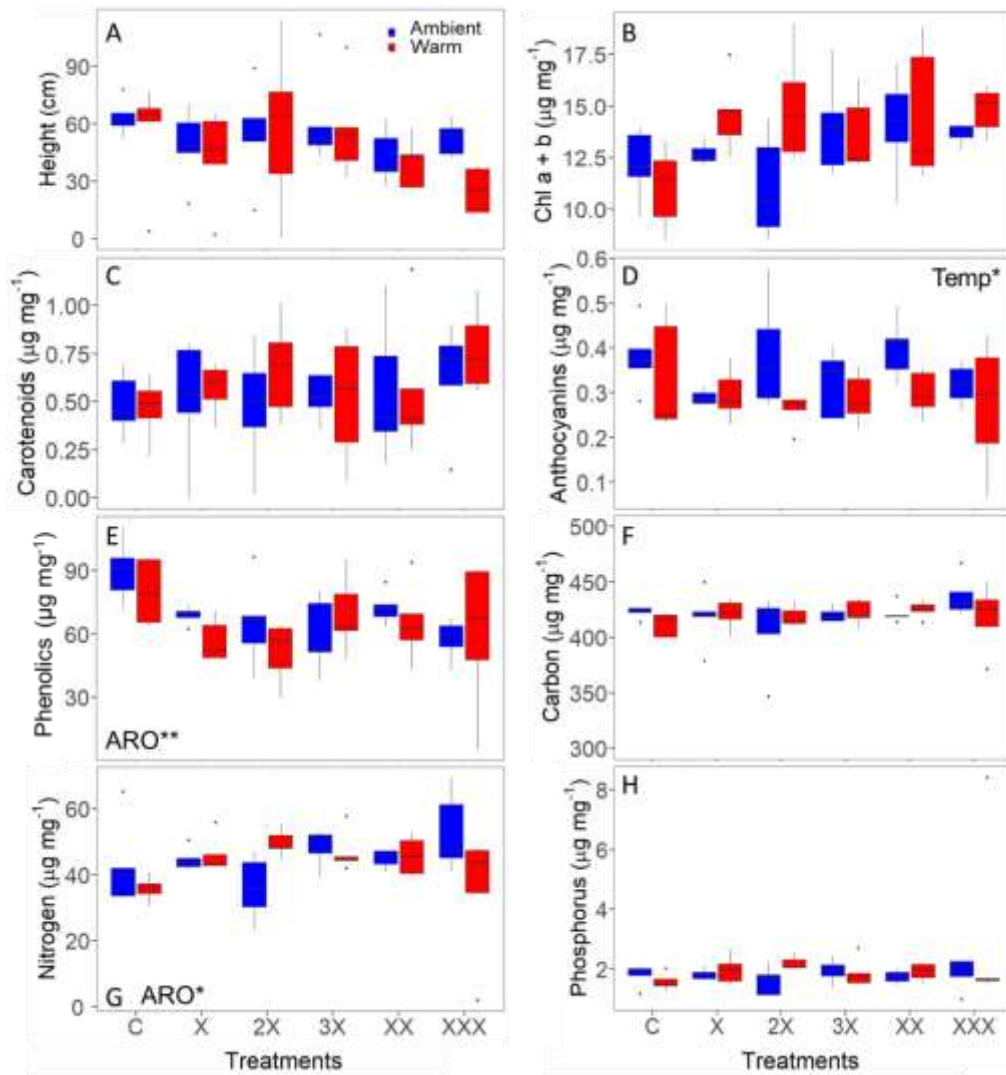


Fig. 5.2.1. Box plots showing ARO and temperature (Temp) effects on *M. spicatum* growth and physiology in Experiment 2 (primary producers and consumers – snails). C = control, X = single low dose, 2X = double ARO dose applied once, 3X = triple ARO dose applied once, XX = double ARO dose applied as a pulse, XXX = triple ARO dose applied as a pulse. Blue = ambient microcosms, red = warm microcosms. Box plots of 5 replicates showing median, 25 and 75% percentiles, lowest and highest whiskers (as $Q1 - [1.5 * IQR]$ and $Q3 + [1.5 * IQR]$, respectively), and outliers (dots). Significant ARO or temperature effect is indicated as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

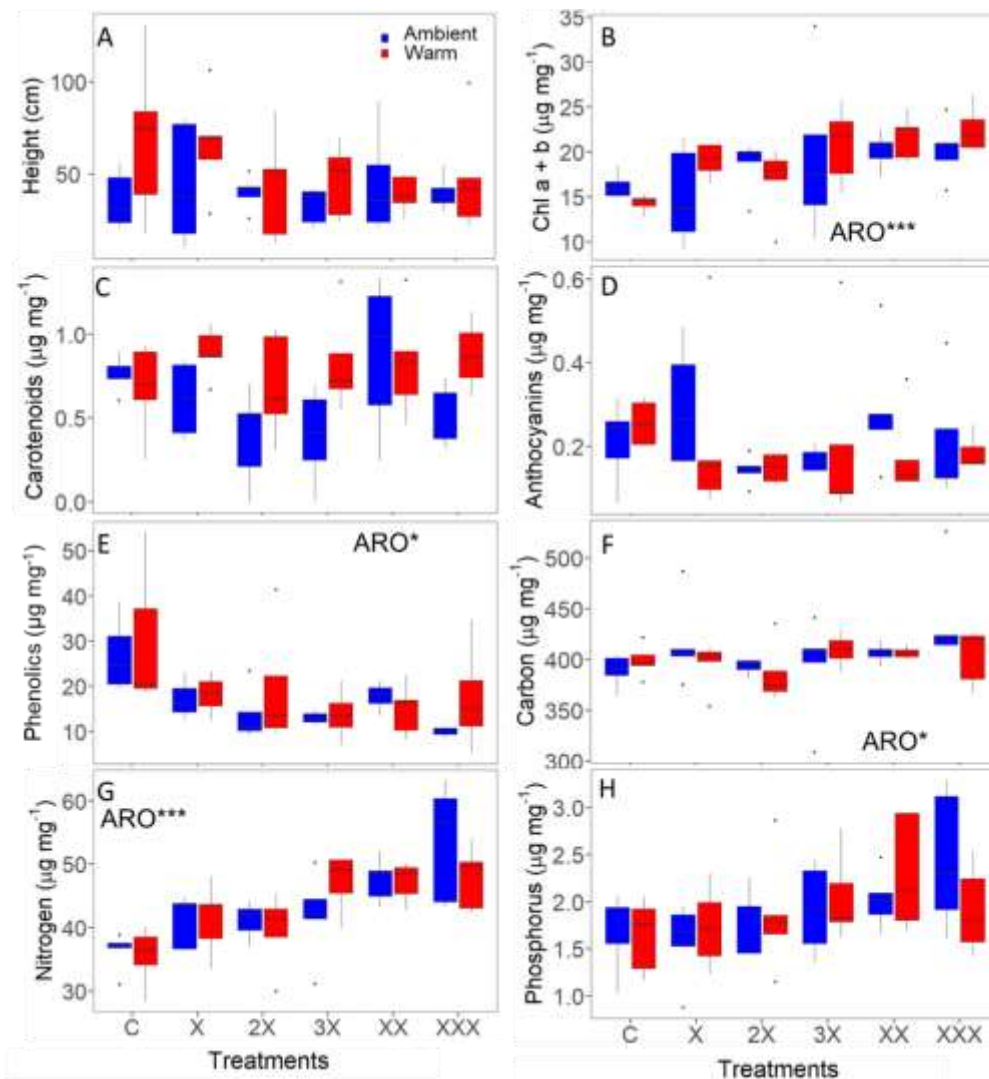


Fig. 5.2.2. Box plots showing ARO and temperature (Temp) effects on *P. perfoliatus* growth and physiology in Experiment 2 (primary producers and consumers – snails). C = control, X = single low dose, 2X = double ARO dose applied once, 3X = triple ARO dose applied once, XX = double ARO dose applied as a pulse, XXX = triple ARO dose applied as a pulse. Blue = ambient microcosms, red = warm microcosms. Box plots of 5 replicates showing median, 25 and 75% percentiles, lowest and highest whiskers (as $Q1 - [1.5 \times IQR]$ and $Q3 + [1.5 \times IQR]$, respectively), and outliers (dots). Significance levels shown as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

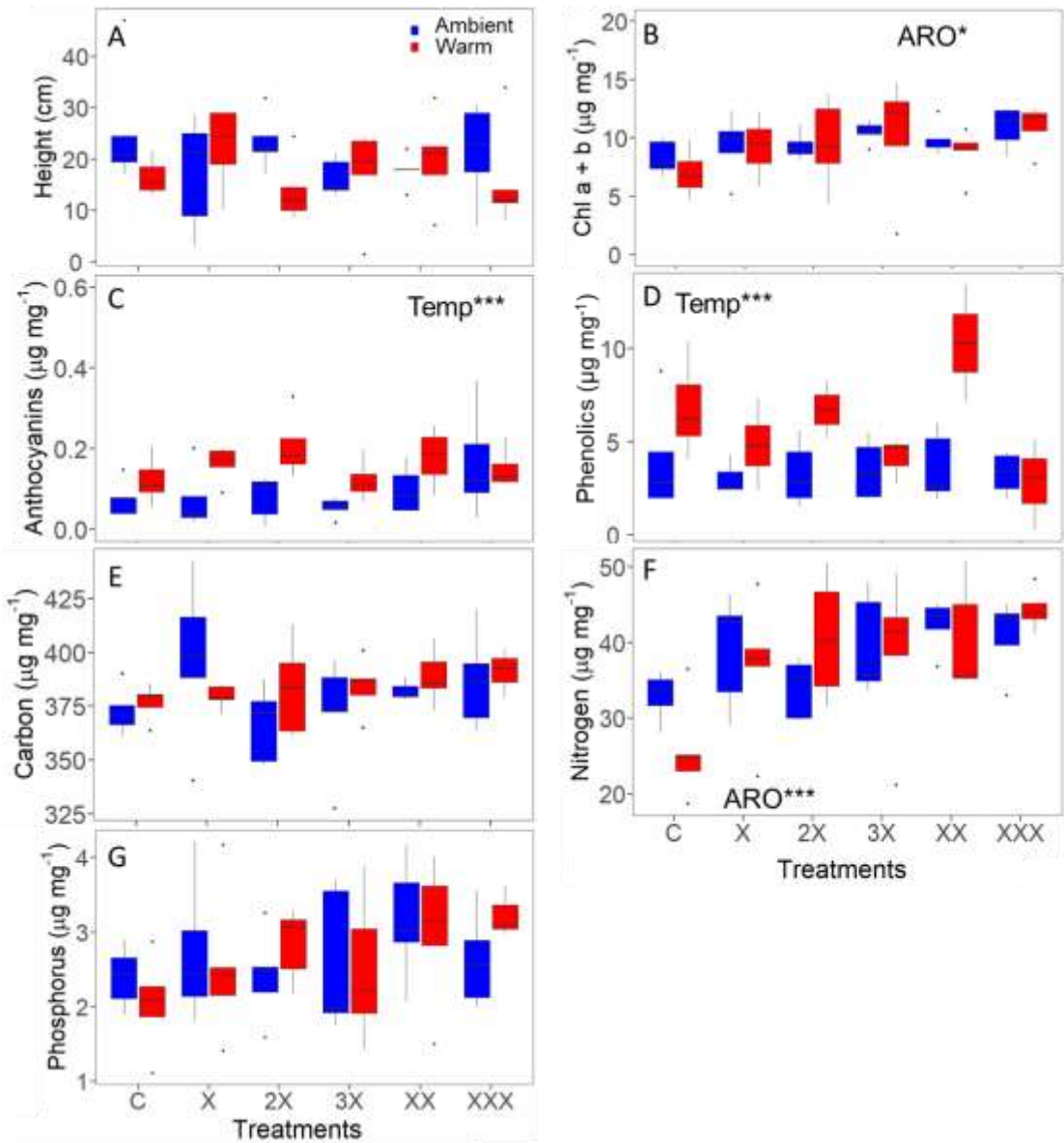


Fig. 5.2.3. Box plots showing ARO and temperature (Temp) effects on *E. nuttallii* growth and physiology in Experiment 2 (primary producers and consumers – snails). The concentrations of carotenoids were very low or negligible and therefore not reported. C = control, X = single low dose, 2X = double ARO dose applied once, 3X = triple ARO dose applied once, XX = double ARO dose applied as a pulse, XXX = triple ARO dose applied as a pulse. Blue = ambient microcosms, red = warm microcosms. Box plots of 5 replicates showing median, 25 and 75% percentiles, lowest and highest whiskers (as $Q1 - [1.5 \times IQR]$ and $Q3 + [1.5 \times IQR]$, respectively), and outliers (dots). Significance levels shown as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

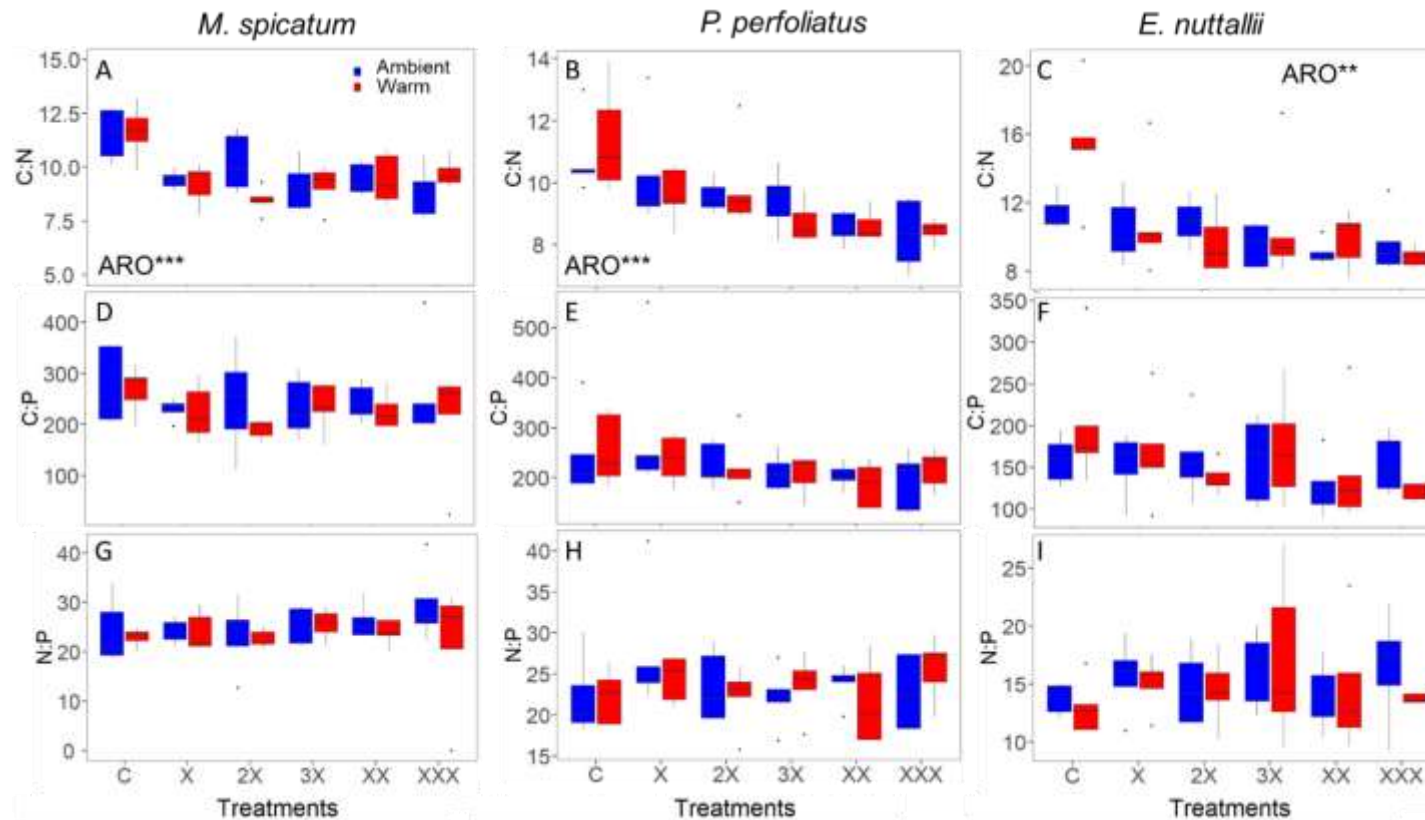
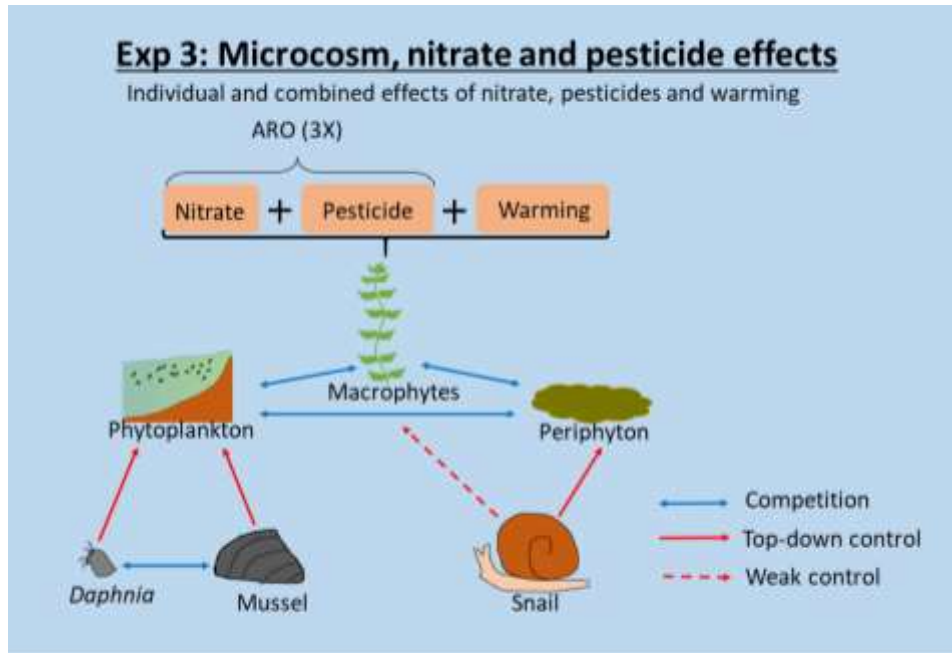


Fig. 5.2.4. Box plots showing ARO and temperature (Temp) effects on elemental stoichiometric ratios in *M. spicatum* (A,D,G), *P. perfoliatus* (B,E,H) and *E. nuttallii* (C,F,I), in Experiment 2 (primary producers and consumers – snails). C = control, X = single low dose, 2X = double ARO dose applied once, 3X = triple ARO dose applied once, XX = double ARO dose applied as a pulse, XXX = triple ARO dose applied as a pulse. Blue = ambient microcosms, red = warm microcosms. Box plots of 5 replicates showing median, 25 and 75% percentiles, lowest and highest whiskers (as $Q1 - [1.5 * IQR]$ and $Q3 + [1.5 * IQR]$, respectively), and outliers (dots). Significance levels shown as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Total phenolic content was much higher in this experiment than in Experiment 1 for *M. spicatum* with an average $90 \pm 15 \mu\text{g mg}^{-1}$. Concentrations for *P. perfoliatus* ($27 \pm 9 \mu\text{g mg}^{-1}$) remained comparable with Experiment 1, but those for *E. nuttallii* were much lower ($4 \pm 3 \mu\text{g mg}^{-1}$). Anthocyanins were also lowest for *E. nuttallii*, measuring on average $0.09 \pm 0.05 \mu\text{g mg}^{-1}$, while for *M. spicatum* this was highest among the three macrophytes ($0.3 \pm 0.09 \mu\text{g mg}^{-1}$). Although effects of ARO were observed on the total phenolic content of *M. spicatum* and *P. perfoliatus*, no differences were observed between exposure mode. For *P. perfoliatus*, the reduction was significant at the triple ARO dose (50%) and pulse exposures (57%) compared with the control, whereas for *M. spicatum*, the decrease was seen only at X and 2X. *E. nuttallii* phenolic content was not affected by ARO.

Carbon content for *M. spicatum* measured on average 423.5 ± 21.7 , while that for both *P. perfoliatus* ($397.8 \pm 27.3 \mu\text{g mg}^{-1}$) and *E. nuttallii* ($376.3 \pm 15.8 \mu\text{g mg}^{-1}$) was slightly lower. Nitrogen was higher for *M. spicatum* ($43 \pm 13 \mu\text{g mg}^{-1}$) and slightly lower for both *P. perfoliatus* and *E. nuttallii*, both averaging $35 \pm 3 \mu\text{g mg}^{-1}$. Overall, this led to a decline in the C:N ratio with ARO for all species. Phosphorus measured on average $1.8 \pm 0.4 \mu\text{g mg}^{-1}$ for both *M. spicatum* and *P. perfoliatus* but was higher for *E. nuttallii* ($2.3 \pm 0.7 \mu\text{g mg}^{-1}$). This elemental stoichiometry was affected by ARO, but not as strongly as in the experiment with only primary producers. Carbon content of *P. perfoliatus* was only marginally affected by ARO, showing a decrease in the double ARO dose and an increase in the triple ARO pulse compared with the control. Similar to total chlorophylls, significant differences between the double single dose (2X) and pulse (XX) were observed for carbon. Carbon content of the other two macrophytes were not affected by ARO. Nitrogen content, however, was affected in all three macrophyte species, but most significantly in *P. perfoliatus* and *E. nuttallii*, where increases in both the triple ARO dose (17% and 21%, respectively) and pulse exposures (47% and 21%, respectively) were observed compared to the control. Significant increases in nitrogen were found in the double pulse exposure (XX) compared to the corresponding single dose (2X) for *P. perfoliatus*. The significant increase in nitrogen in *E. nuttallii* and *P. perfoliatus* with ARO exposure resulted in a lower C:N ratio (Fig. 5.2.4). No AR effects were observed on phosphorus content of the macrophytes.

Chapter 5.3. Experiment 3: Effect of nitrate, pesticides and warming on macrophyte growth and chemistry



Experiment 3: Primary producers and consumers exposed to nitrate, pesticides and warming. In Experiment 3, mussels did not survive.

1. Principal components analyses (PCA): Effect of ARO and temperature on macrophyte growth and physiology

The first two axes contributed to 42.1% of the variation, while the third and fourth axes together contributed to 21.3%. Both ARO and temperature effects were visible on the first biplot (PCA 1 and 2) (Fig. 5.1 & 5.2).

1.1. ARO effect

In Experiment 3, the ARO effect on macrophyte chemistry differed from Experiments 1 and 2. ARO caused an increase in the phenolic content and carotenoids of *P. perfoliatus*. The photosynthetic pigments of both *E. nuttalli* and *P. perfoliatus* were higher in the absence of ARO rather than in the presence.

1.2. Temperature effect

The effect of temperature was also not comparable to Experiments 1 or 2. The photosynthetic pigments of *P. perfoliatus* and *E. nuttalli*, and the height of *M. spicatum*, increased due to warming. An increase in anthocyanins by *E. nuttalli* was observed at

higher temperature. The total phenolic content of *P. perfoliatus* increased at ambient temperature.

2. PCA: Effect of nitrate and pesticides on macrophyte growth and physiology

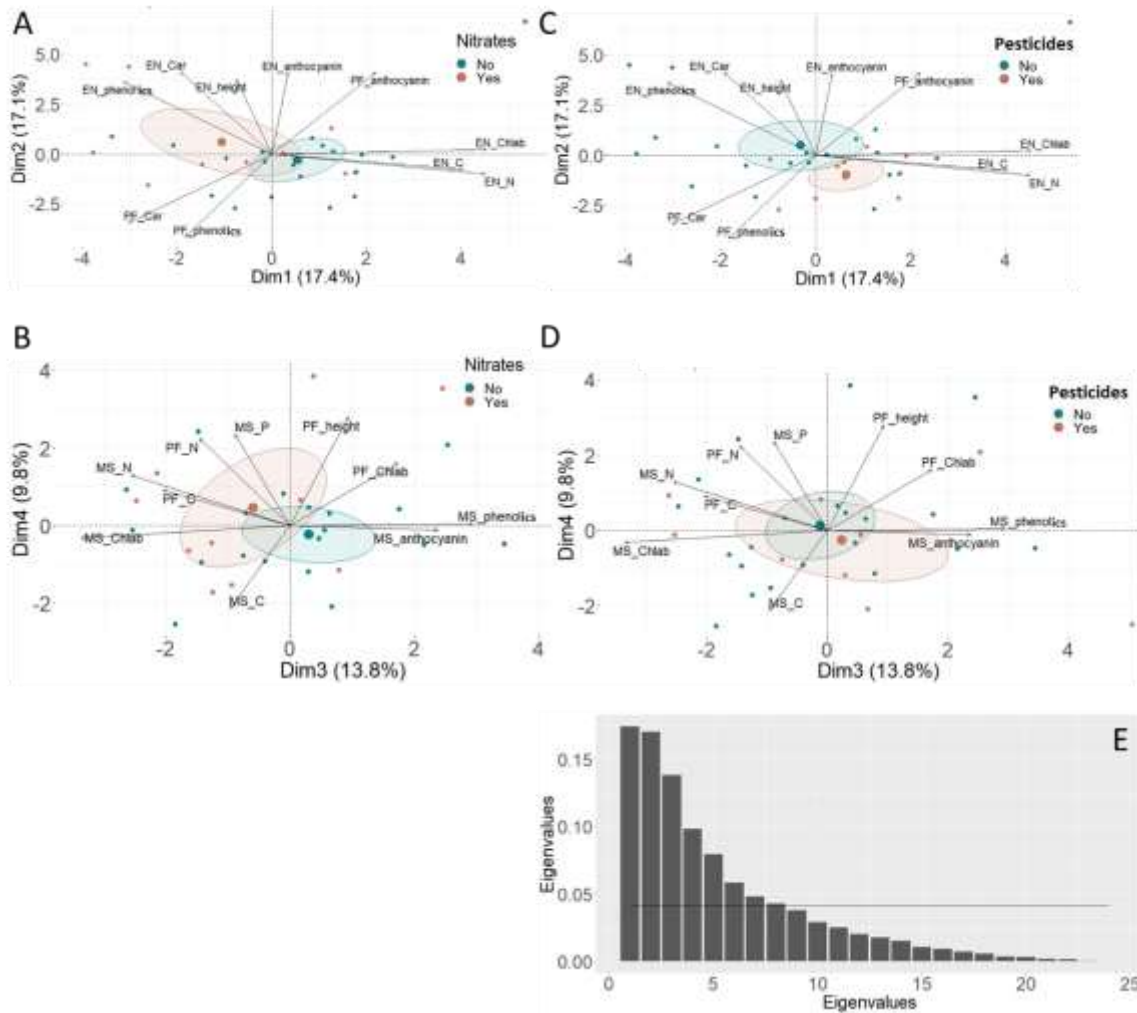


Fig. 5.3.1. Principal components analyses (PCA) performed with axes 1, 2, 3 and 4. Eight response factors for all three macrophyte species were used in the analyses, including growth and physiology (see “Material and Methods”). Either nitrate (A-B) or pesticides (C-D) treatments were projected as supplementary data. For each experiment, only the 10 most significant variables were plotted (using “select.var = list(contrib=10” from the fviz function using factoextra). (E) Eigenvalues for the principal components. Note: Phosphorus concentrations for *P. perfoliatus* and *E. nuttallii* are not available for Experiment 1 and not included in the analyses. MS = *M. spicatum*, PF = *P. perfoliatus*, EN = *E. nuttallii*, C = carbon, N = nitrogen, P = phosphorus, Car = carotenoids, Chlab = chlorophyll-*a* + chlorophyll-*b*. Experiment 1 = only primary producers, Experiment 2 = primary producers and consumers (only snails), Experiment 3 = primary producers and consumers (snails and *Daphnia*).

The first two axes contributed to 34.5% of the variation, while the third and fourth axes together contributed to 23.6%. The nitrate and pesticide effects were visible on both biplots (PCA 1 and 2, and PCA 3 and 4) (Fig. 5.3.1).

2.1. Nitrate effect

A nitrate effect was observed on the first axis, with nitrate on the left and controls on the right (Fig. 5.3.1; PCA biplot axes 1 and 2). *E. nuttallii* phenolic content was related to presence of nitrate. In the absence of nitrate, *E. nuttallii* produced high total chlorophylls as well as C and N. The nitrate effect was more clearly observed in the third axis (PCA biplot axes 3 and 4). Here, the total phenolic content and anthocyanins of *M. spicatum* were higher in the absence of nitrate. *M. spicatum* nitrate and total chlorophylls were higher in the presence of nitrate. *P. perfoliatus* carbon content increased in the presence of nitrate.

2.2. Pesticide effect

Although the pesticide effect could not be described clearly by either the first or second axes, there was a clear separation between the presence and absence of pesticides (Fig. 5.3.1; PCA biplot axes 1 and 2), with the absence of pesticides present at the top left of the biplot and the presence of pesticides at the bottom right. In the absence of pesticides, *E. nuttallii* carotenoids and phenolic content increased. Higher *E. nuttallii* total chlorophylls as well as C and N were observed in the presence of pesticides.

3. Stressor effect sizes

3.1. ARO effect

In Experiment 3, responses of the macrophytes to the ARO were different from that in Experiments 1 and 2. The height of *M. spicatum* was significantly affected by the ARO, with a large effect size (>0.8 ; Cohen, 1988). This effect on growth was not observed in Experiments 1 and 2. Anthocyanins also significantly reduced with a large ARO effect size (Fig. 5.4). Marginal ARO effects were observed on elemental stoichiometry as well. *M. spicatum* was the most dominant species, with the other two species growing very little. Due to very low biomass and subsequent lack of sufficient replicates for chemical analyses, effect sizes for *P. perfoliatus* and *E. nuttallii* could not be reported in this experiment.

3.2. Temperature effect

In Experiment 3, only the height of *M. spicatum* had a large and significant positive temperature effect (Fig. 5.4).

4. Correlation analyses

Correlation analyses showed no significant effects of macrophyte photosynthetic pigments in relation to phytoplankton or periphyton, but the height of *M. spicatum* strongly negatively correlated with phytoplankton (Spearman's $r = -0.5$, $p = 0.02$) and positively with periphyton (Spearman's $r = 0.5$, $p = 0.01$).

5. Effect of nitrate versus pesticide on the macrophytes

Experiment 3 was conducted to disentangle the nitrate and pesticide effect from the combined ARO. A three-way ANOVA was performed to determine individual pesticide (Pest), nitrate (Nitr) or temperature (Temp) effects, and their interactions, on the macrophyte growth and physiology. For non-parametric data, a Kruskal–Wallis test by ranks was performed. Results of the statistical analyses are presented in Table 5.3.1.

Table 5.3.1. Statistical results from the 3-way ANOVA or Kruskal–Wallis test by ranks for Experiment 3.

Macrophyte response	Test	Pest	Nitr	Temp	PestxNitr	PestxTemp	NitrxFem P	PestxNitrxFem P
<i>M. spicatum</i>								
Height	2-way ANOVA	1.35E-05	0.2	1.52E-05	0.2	0.6	0.2	0.6
Chl- <i>a</i>	2-way ANOVA	0.9	0.7	0.004	0.2	0.7	0.5	0.8
Chl- <i>a</i> + Chl- <i>b</i>	2-way ANOVA	0.8	0.7	0.002	0.1	0.8	0.5	0.9
Chl- <i>a</i> /Chl- <i>b</i>	2-way ANOVA	0.1	0.9	0.6	0.3	0.2	0.6	0.06
Carotenoids	2-way ANOVA	0.8	0.5	0.4	0.2	0.6	0.6	0.01
Carotenoids/ (Chl- <i>a</i> + Chl- <i>b</i>)	2-way ANOVA	0.8	0.7	0.2	0.05	0.6	0.5	0.02
Anthocyanin s	2-way ANOVA	0.5	0.02	0.4	0.2	0.6	0.3	0.6
Total phenols	2-way ANOVA	0.4	0.017	0.016	0.8	0.7	0.6	0.8
Carbon	2-way ANOVA	0.02	0.7	0.1	0.9	0.3	0.8	0.5
Nitrogen	2-way ANOVA	0.1	0.4	0.4	0.3	0.4	0.1	0.04
Phosphorus	2-way ANOVA	0.2	0.7	0.6	0.1	0.01	0.2	0.03

C:N	2-way ANOVA	0.4	0.7	0.4	0.1	0.0001	0.6	0.4	
C:P	2-way ANOVA	0.1	0.7	0.5	0.1	0.03	0.2	0.04	
N:P	2-way ANOVA	0.2	0.7	0.3	0.3	0.6	0.2	0.05	
<i>P. perfoliatus</i>									
Height	2-way ANOVA	0.4	0.08	0.05	0.2	0.2	0.08	0.07	
Chl- <i>a</i>	Kruskal-Wallis	0.06	0.7	1					
Chl- <i>a</i> + Chl- <i>b</i>	Kruskal-Wallis	0.03	0.9	0.6					
Chl- <i>a</i> /Chl- <i>b</i>	Kruskal-Wallis	1	0.8	0.03					
Carotenoids	Kruskal-Wallis	0.4	0.7	0.02					
Carotenoids/ (Chl- <i>a</i> + Chl- <i>b</i>)	Kruskal-Wallis	0.3	0.4	0.006					
Anthocyanins	2-way ANOVA	0.8	0.4	0.2	0.1	0.3	0.4	0.9	
Total phenols	2-way ANOVA	0.1	0.8	0.03	0.9	0.7	0.8	0.09	
Carbon	Kruskal-Wallis	0.7	0.5	0.1					
Nitrogen	Kruskal-Wallis	0.5	0.6	0.6					

Phosphorus	2-way ANOVA	0.7	0.2	0.4	0.6	0.6	0.9	0.8
C:N	Kruskal-Wallis	0.5	0.7	0.6				
C:P	Kruskal-Wallis	0.2	0.5	0.1				
N:P	Kruskal-Wallis	0.3	0.6	0.2				
<i>E. nuttallii</i>								
Height	2-way ANOVA	0.6	0.8	0.7	0.4	0.6	0.05	0.7
Chl- <i>a</i>	Kruskal-Wallis	0.7	0.03	0.1				
Chl- <i>a</i> + Chl- <i>b</i>	Kruskal-Wallis	0.9	0.01	0.04				
Chl- <i>a</i> /Chl- <i>b</i>	Kruskal-Wallis	0.3	0.9	0.7				
Carotenoids	Kruskal-Wallis	0.6	0.2	0.5				
Carotenoids/ (Chl- <i>a</i> + Chl- <i>b</i>)	Kruskal-Wallis	0.4	0.3	0.6				
Anthocyanin s	2-way ANOVA	0.2	0.8	0.04	0.9	0.9	0.9	0.8
Total phenols	2-way ANOVA	0.5	0.06	0.5	0.4	0.04	0.5	0.2
Carbon	Kruskal-Wallis	0.3	0.07	0.05				

Nitrogen	Kruskal-Wallis	0.8	0.2	0.9				
Phosphorus	2-way ANOVA	0.1	0.3	0.7	0.9	0.6	0.8	0.9
Carbon : nitrogen	2-way ANOVA	0.8	0.1	0.6	0.9	0.07	0.1	0.4
Carbon : phosphorus	Kruskal-Wallis	0.04	0.8	0.2				
Nitrogen: phosphorus	Kruskal-Wallis	0.007	0.6	0.3				

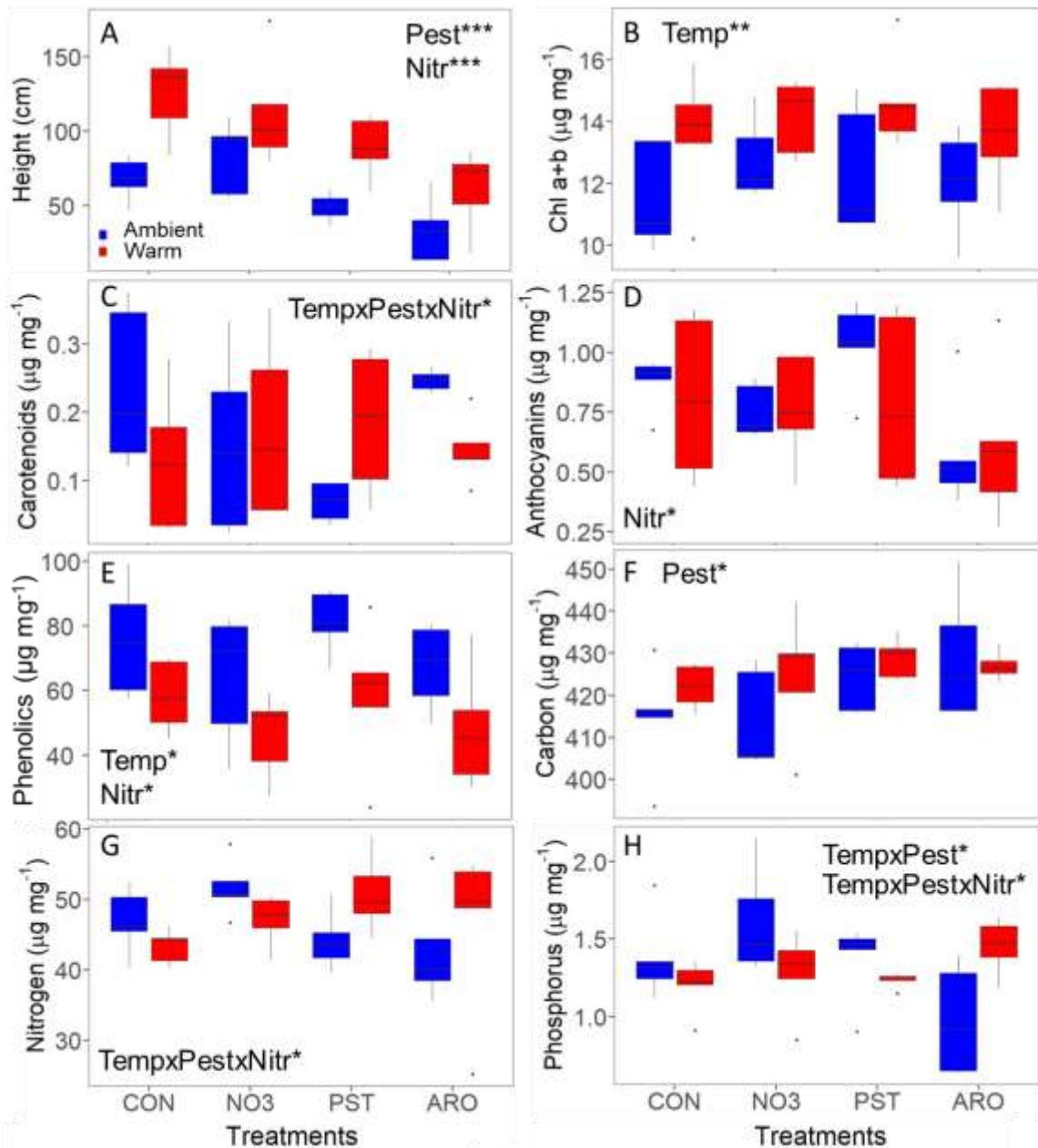


Fig. 5.3.2. Box plots showing pesticide (Pest), nitrate (Nitr) and temperature (Temp) effects on *M. spicatum* growth and physiology in Exp 3 (primary producers and consumers – snails and *Daphnia*). Blue = ambient microcosms, red = warm microcosms. Significance was set at $p < 0.05$. Box plots of 5 replicates showing median, 25 and 75% percentiles, lowest and highest whiskers (as $Q1 - [1.5 \times IQR]$ and $Q3 + [1.5 \times IQR]$, respectively), and outliers (dots). Significant effect of temperature, pesticide, nitrate and/or their interaction are indicated as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

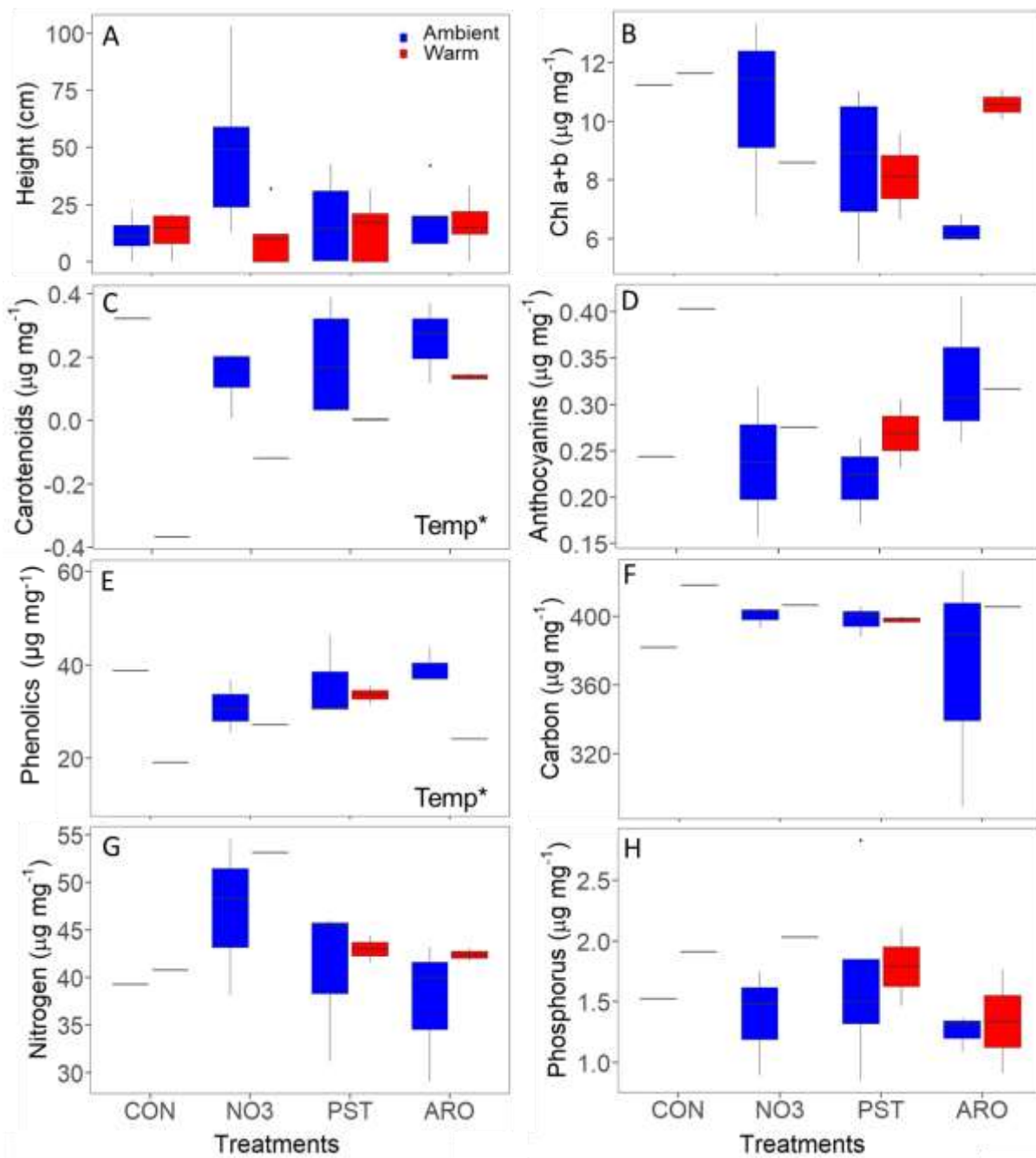


Fig. 5.3.3. Box plots showing pesticide (Pest), nitrate (Nitr) and temperature (Temp) effects on *P. perfoliatus* growth and physiology in Experiment 3 (primary producers and consumers – snails and *Daphnia*). Because of the low final biomass, sufficient replicates were not available for some treatments. Blue = ambient microcosms, red = warm microcosms. Significance was set at $p < 0.05$. Box plots of 5 replicates showing median, 25 and 75% percentiles, lowest and highest whiskers (as $Q1 - [1.5 \cdot IQR]$ and $Q3 + [1.5 \cdot IQR]$, respectively), and outliers (dots). Significance levels shown as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

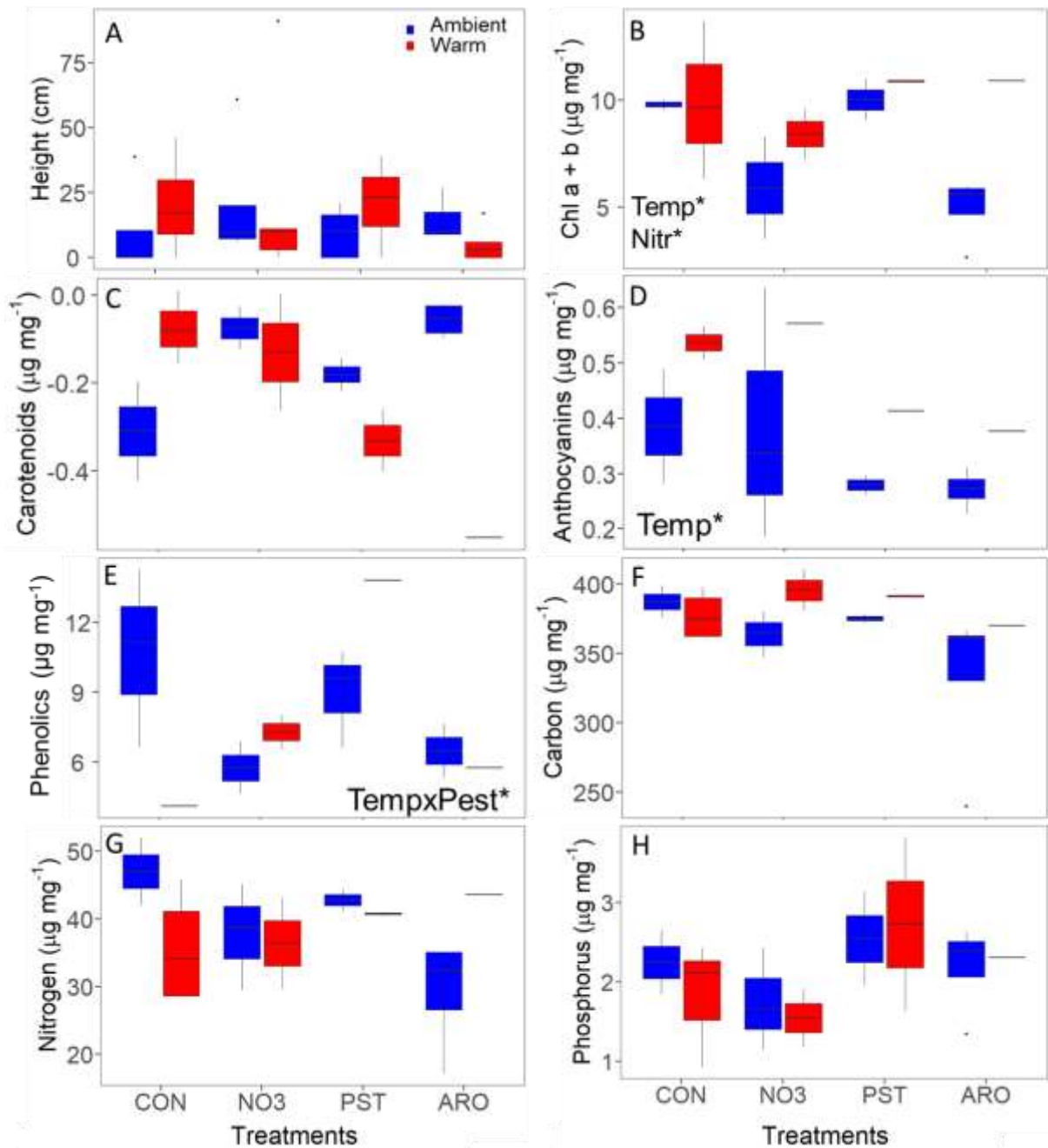


Fig. 5.3.4. Box plots showing pesticide (Pest), nitrate (Nitr) and temperature (Temp) effects on *E. nuttallii* growth and physiology in Experiment 3 (primary producers and consumers – snails and *Daphnia*). Because of the low final biomass, sufficient replicates were not available for some treatments. Blue = ambient microcosms, red = warm microcosms. Significance was set at $p < 0.05$. Box plots of 5 replicates showing median, 25 and 75% percentiles, lowest and highest whiskers (as $Q1 - [1.5 \times IQR]$ and $Q3 + [1.5 \times IQR]$, respectively), and outliers (dots). Significance levels shown as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

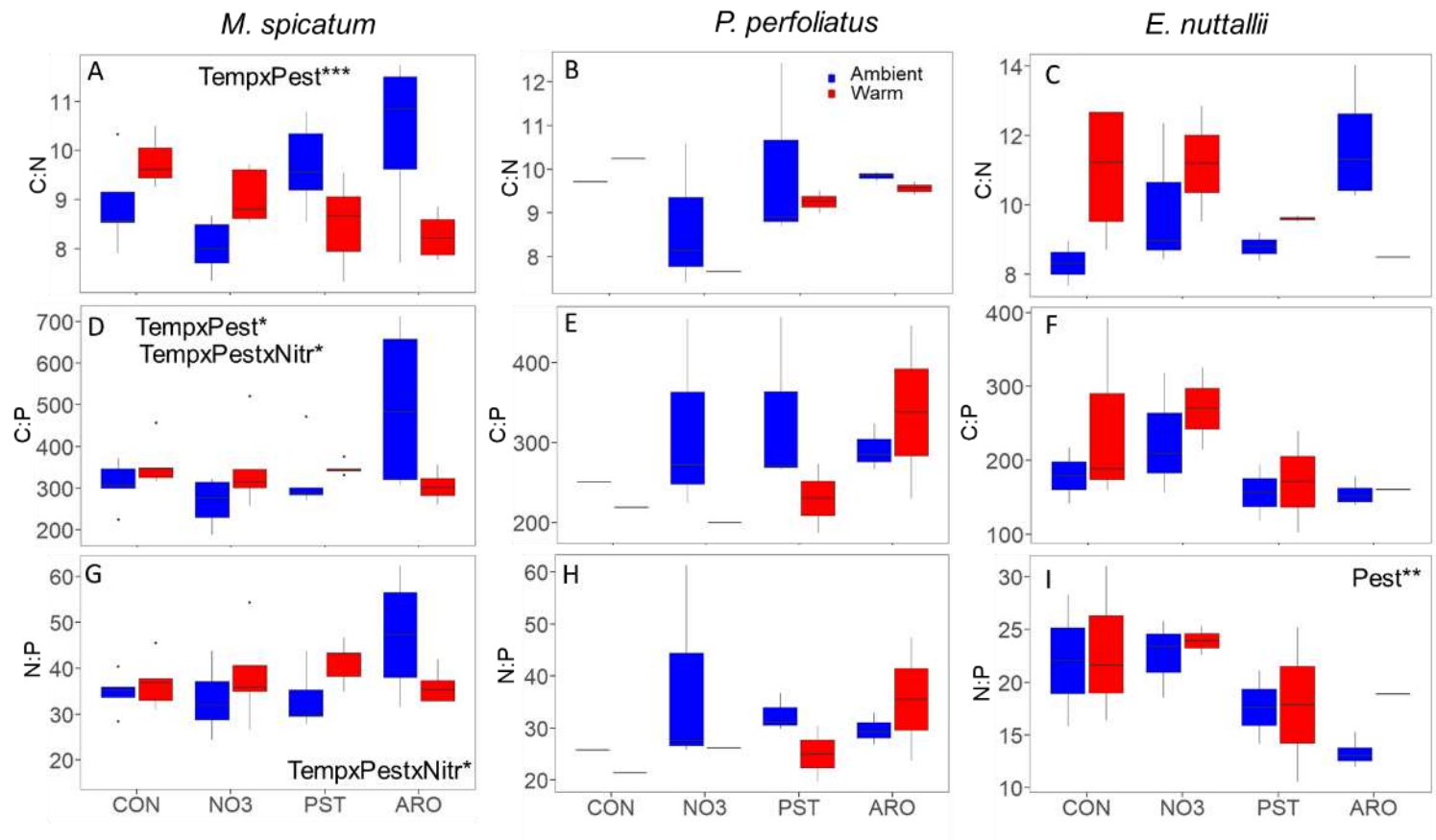


Fig. 5.3.5. Box plots showing pesticide (Pest), nitrate (Nitr) and temperature (Nitr) effects on elemental stoichiometry ratios in *M. spicatum* (A,D,G), *P. perfoliatus* (B,E,H) and *E. nuttallii* (C,F,I) in Experiment 3 (primary producers and consumers – snails and *Daphnia*). Blue = ambient microcosms, red = warm microcosms. Significance was set at $p < 0.05$. Box plots of 5 replicates showing median, 25 and 75% percentiles, lowest and highest whiskers (as $Q1 - [1.5 * IQR]$ and $Q3 + [1.5 * IQR]$, respectively), and outliers (dots). Significance levels shown as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Myriophyllum spicatum dominated the other two macrophytes in this experiment, measuring 70 ± 15 cm in the controls. *P. perfoliatus* measured 11 ± 9 cm, and *E. nuttallii* measured 10 ± 17 cm. The height of *M. spicatum* was significantly reduced in the treatments with pesticides (Pest) compared with the control, and significantly increased in the heated treatments compared with the ambient (Fig. 5.3.2). *M. spicatum* height was reduced by 51% in the ARO compared to the control and increased by 88% in the warm control compared with the ambient control. The other two macrophyte species grew very little, and their growth was not strongly affected by the stressors (Fig. 5.3.3 & 5.3.4).

Nitrate and pesticides affected the macrophyte physiology, although differently. Nitrate sometimes affected the photosynthetic pigments and more often the phenolic compounds/anthocyanins, while interaction effects between pesticides and the other stressors were more often observed for the stoichiometry. Total chlorophylls in the controls measured on average 12 ± 2 , 9 ± 3 and 8 ± 3 $\mu\text{g mg}^{-1}$ for *M. spicatum*, *P. perfoliatus* and *E. nuttallii*, respectively. Total phenolics were highest for *M. spicatum*, measuring on average 62.2 ± 18 $\mu\text{g mg}^{-1}$ and were lower for both *P. perfoliatus* (33.3 ± 9.5 $\mu\text{g mg}^{-1}$) and *E. nuttallii* (7.8 ± 3.4 $\mu\text{g mg}^{-1}$). Anthocyanins were the highest for *M. spicatum* (0.9 ± 0.1) and were lower for *P. perfoliatus* (~ 0.2 $\mu\text{g mg}^{-1}$) and *E. nuttallii* (0.4 ± 0.1 $\mu\text{g mg}^{-1}$). *E. nuttallii* produced significantly lower total chlorophylls when exposed to nitrate, and *P. perfoliatus* produced less when exposed to pesticides.

Only for *M. spicatum* did nitrate significantly reduce production of anthocyanins (14% decrease in NO₃ compared with CON, Fig. 5.3.2).

Carbon content was higher for *M. spicatum* (422.6 ± 11.6 $\mu\text{g mg}^{-1}$) compared to the other two macrophyte species (387.9 ± 46.1 $\mu\text{g mg}^{-1}$ for *P. perfoliatus* and 362 ± 42.9 for *E. nuttallii*). Nitrogen content measured on average 40.2 ± 7.3 for all three macrophytes. Phosphorus content was higher for *E. nuttallii* (2.2 ± 0.6 $\mu\text{g mg}^{-1}$) compared to those for *M. spicatum* (1.2 ± 0.3 $\mu\text{g mg}^{-1}$) and *P. perfoliatus* (1.4 ± 0.4 $\mu\text{g mg}^{-1}$). In terms of stressor effects on stoichiometry, *M. spicatum* produced slightly more carbon per biomass when exposed to pesticides, with a 2.5 to 3.5% increase in the PST and ARO compared with the control. Both nitrogen and phosphorus content were significantly affected by interactions between temperature and pesticides, and sometimes between all three stressors. These effects of the stressor interactions on the elemental concentrations affected all the stoichiometry ratios in *M. spicatum*. *E. nuttallii* elemental concentrations were not affected strongly by the

pesticides, but showed significant reductions in their C:P and N:P ratios when exposed to pesticides (Fig. 5.3.5).

Chapter 5.4. Comparison of microalgae biomass between the three experiments

For a better interpretation of macrophyte growth and physiological responses, the final concentrations of periphyton and phytoplankton in all experiments are shown in Fig. 5.4.1. Periphyton chlorophyll-*a* concentration remained comparable in the controls of Experiment 1 ($0.9 \pm 0.3 \mu\text{g cm}^{-2}$) and Experiment 3 ($1.2 \pm 0.6 \mu\text{g cm}^{-2}$), but were lower in the controls of Experiment 2 ($0.1 \pm 0.08 \mu\text{g cm}^{-2}$). This pattern remained more or less the same at both temperatures. Phytoplankton concentration also was comparable in the controls of both Experiment 1 ($21 \pm 27 \mu\text{g L}^{-1}$) and Experiment 3 ($15 \pm 23 \mu\text{g L}^{-1}$), but was higher in the control of Experiment 2 ($85 \pm 36 \mu\text{g L}^{-1}$). This pattern was also similar at both temperatures.

In Experiment 1, periphyton was significantly higher (67%) in the ARO compared with the control, and in Experiment 2, periphyton concentration was ~11-fold higher in the ARO compared with the respective control. These significant effects were observed only in the ambient microcosms. For Experiment 3, most vases had lower periphyton biomass in the ARO than in the control, but no significant effect was observed due to the variation in the data. Phytoplankton concentration was about 6-fold higher in Experiment 1 of the heated ARO microcosms compared with the controls. In Experiment 2, phytoplankton concentration was reduced by ~70% in the ambient ARO compared with the ambient control. In Experiment 3, a large variation in phytoplankton concentration was seen among replicates of the ambient ARO, with the two highest phytoplankton concentrations driven by two microcosms in which no *Daphnia* survived following ARO exposure. Although the ARO effect was not significant due to the large variation, only two ARO vases had comparable concentrations to the controls ($\sim 50 \mu\text{g L}^{-1}$), while the remaining ones were significantly higher ($>90 \mu\text{g L}^{-1}$). Overall, a 15-fold increase in phytoplankton concentration was observed in the ambient ARO compared with the ambient control. This difference was much smaller in the warmed treatments.

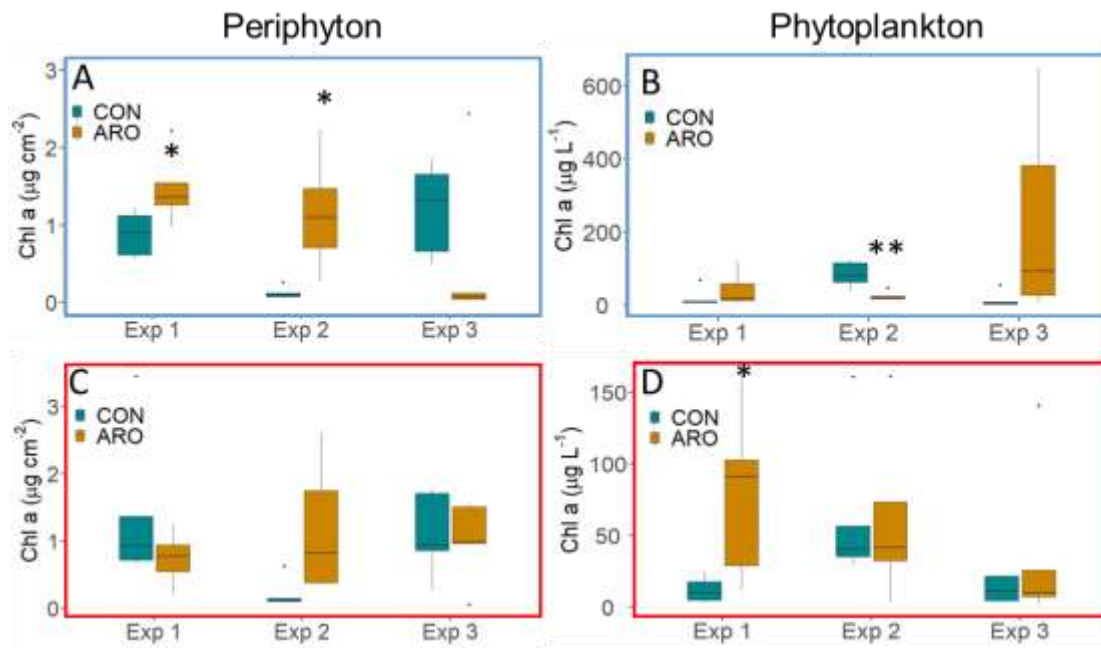


Fig. 5.4.1. Chlorophyll-*a* concentrations for periphyton (A, C) and phytoplankton (B, D) in the ambient (blue outline) and warm (red outline) microcosms for all three experiments. Experiment 1 = only primary producers, Experiment 2 = primary producers and consumers (only snails), Experiment 3 = primary producers and consumers (snails and *Daphnia*).

Chapter 5.5. Discussion

This study exposing macrophytes to ARO and elevated temperature using three different microcosm approaches allowed us to evaluate macrophyte developmental and physiological responses to the chosen stressors. The discussion is divided into three sections, each discussing one of the three objectives proposed in the introduction to this chapter. The main objectives of this study was to evaluate whether (a) ARO and temperature would affect macrophyte physiology, (b) The ARO exposure mode would cause specific physiological responses, (c) Nitrate and pesticides would elicit different physiological responses in the macrophytes

1. Effects of ARO and temperature on macrophyte growth and physiology

ARO and temperature both affected macrophyte growth and physiology. However, these effects differed among macrophyte species. The OECD test plant *M. spicatum* (OECD, 2014) was the most responsive to ARO stress in all three experiments. The other two species, *P. perfoliatus* and *E. nuttallii*, also responded to stress by ARO, but these responses were less strong and not always present. Therefore, I will focus on *M. spicatum*, with few references to the other two species.

Responses of *M. spicatum* to ARO were strongest in Experiment 1 where only primary producers were present. ARO significantly affected 7 out of 10 (including stoichiometric ratios) physiological response factors and one marginally (see Fig. 5.4). The increase in photosynthetic pigments in response to ARO may have been a direct response particularly to the herbicides copper and terbuthylazine. However, based on available data, direct effects of these two herbicides on *M. spicatum* at the concentrations used are unlikely (BHP, submitted; VV, unpublished data). Phytoplankton biomass was higher in the ARO treatments compared with the control, and *M. spicatum* might have responded by increased production of photosynthetic pigments. Increases in plant photosynthetic pigments is a typical response to decreasing irradiance (Barko & Filbin, 1983). Our experiments showed that both phytoplankton and periphyton can lead to shading of the macrophytes, supporting the early claim that not only phytoplankton but also periphyton may play an important and possibly intermediary role in macrophyte shading (Phillips et al., 1978). Carotenoids aid in deactivation of hydrogen peroxide (H₂O₂), and its increase may have resulted from oxidative stress (Chalanika De Silva & Asaeda, 2017) produced directly or indirectly by the pesticides. Both *P. perfoliatus* and *E. nuttallii* also responded by increased production of photosynthetic pigments when phytoplankton and/or periphyton biomass

was high, indicating that macrophyte photosynthetic pigments are a good indicator of nutrient and light stress produced by ARO. Increase in macrophyte concentrations of carbon, nitrogen and phosphorus have previously been linked to trophic status (Xing et al., 2015). In our study, increased uptake of these elements, and especially nitrogen, by *M. spicatum* occurred in response to ARO, but only in Experiment 1, which exhibited high phytoplankton biomass. This was not observed in Experiment 2, where ARO caused a high periphyton biomass. One likely explanation is that macrophyte competition for nitrate is much higher with periphyton than with phytoplankton. In fact, in a whole-lake enrichment experiment, (Axler & Reuter, 1996) showed that periphyton rather than phytoplankton accounted for nutrient depletion from the water column. The assimilation of N may have also increased carbon fixation and phosphorus uptake. Despite the increased growth of phytoplankton and competition for light, *M. spicatum* fixed higher carbon in its biomass. This may have resulted from its ability to reallocate resources and/or to use bicarbonate as a source of carbon (Hussner & Jahns, 2015). The stoichiometry of the other two macrophyte species was not significantly affected by the ARO and therefore may not be ideal indicators of stress.

While increasing concentrations of macronutrients in *M. spicatum* may seem like a positive effect on the plants, there are negative implications of the reduced C:N and C:P ratios, which render these species increasingly palatable or nutritious to herbivores (Sterner & Elser, 2002). An increased grazing in lakes by herbivores may eventually lead to a reduction or total loss of biomass in shallow lakes (Hidding et al., 2016).

Marginal increases in anthocyanins were observed for both *M. spicatum* and *P. perfoliatus* in response to ARO in Experiment 1. In Experiment 2, with only snails as consumers, the total phenolic content of both *M. spicatum* and *P. perfoliatus* decreased, while in Experiment 3, with both snails and *Daphnia* as consumers, the anthocyanins of *M. spicatum* decreased. Phenolic compounds are carbon-based with implications for allelopathy. They have been shown to increase under high competition for nutrients and light or under nutrient limitation (Březinová & Vymazal, 2018; Gross, 1999). In our experiment, a decrease in total phenolic content was observed, in line with the high nutrient availability, which is also supported by the significant reduction in the C:N ratio. However, a possible link exists between herbivory and phenolic content, with macrophyte defences being much lower under stressful conditions, which can render them more susceptible to grazing by herbivores (Cronin & Lodge, 2003; Fornoff & Gross, 2014). Elger & Lemoine

(2005) showed that the most palatable macrophytes usually had a higher phenolic content. Much less is known about anthocyanins, but the decrease of these phenolic compounds in *M. spicatum* may be related to the presence of consumers or their influence on the light and nutrient environment, since such effects were not observed in their absence.

In Experiment 1, temperature induced an increased production of carotenoids for all three macrophyte species. A similar effect of carotenoid production in macrophytes, including *E. nuttallii*, in response to higher temperatures was observed in a previous study (Chalanika De Silva & Asaeda (2017). *M. spicatum* has a high temperature optimum of up to 35°C (Grace & Wetzel, 1978). However, they were maintained at a lower temperature (~22°C) before applying the temperature treatment (~26°C), and this may have acted as a heat stress. The increase in carotenoids was strongest in *P. perfoliatus* in response to increased temperature. At the same time, *P. perfoliatus* accumulated less carbon and nitrogen. Similar reduced carbon and nitrogen concentrations in response to increased temperature was observed in the leaves of *Potamogeton crispus* (Zhang et al., 2015). According to the temperature–plant physiological hypothesis, at higher temperatures plants require less nutrients to carry out biochemical reactions (Reich & Oleksyn, 2004). However, in our experiment, corresponding increases in the C:N ratio of *P. perfoliatus*, as observed by (Zhang et al., 2015), indicating changes in the efficiency of nutrient use were not observed, even though the temperature difference (~4°C) was similar between our study and theirs. While this may result from differences in the plant part used (leaves from the whole aboveground biomass in Zhang et al. (2015) versus leaves from the apical 10 cm in our study), our results indicate that elemental concentrations rather than elemental ratios might be a better indicator of heat stress in macrophytes. Similarly, in Experiment 2, only *P. perfoliatus* responded strongly to the temperature increase by reduced production of total chlorophylls.

In Experiment 3, temperature promoted the growth *M. spicatum*, which was much taller in the heated vases than in the ambient vases. However, no effects of temperature were observed on their physiology. Because of their high temperature optima (Grace & Wetzel, 1978), *M. spicatum* may have quickly acclimated to the higher temperature optima, and the presence of periphyton grazers may have promoted nutrient and light availability by strong top–down control, which also usually increases at higher temperature (Cao et al., 2004).

The set-up and design of the three experiments differed, and macrophyte physiology was not consistently the main indicator of stress. For example, the macrophyte physiological response was very strong in Experiment 1, particularly for *M. spicatum* in response to ARO and for *P. perfoliatus* in response to temperature. However, the physiological response was either not present or reduced in Experiment 2, and in Experiment 3, stronger effects were observed on growth rather than physiology.

One explanation for these different responses may be related to the community composition. However, despite the range of phytoplankton and periphyton being similar between Experiment 1, with only primary producers, and Experiments 2 and 3, with primary producers and consumers, the response of the macrophytes differed between the experiments. Another more likely explanation for the different responses of macrophytes between experiments may be the period of establishment by macrophytes within the microcosms before exposure to the stressors.

For example, in Experiment 3, the strong effect of the stressors on *M. spicatum* height rather than physiology may be due to the early exposure of the macrophytes to the ARO (4 days after planting) compared with Experiments 1 and 2 (17 days and 10 days after planting, respectively). Macrophytes in Experiment 1 may therefore have been more established making them less susceptible to the effects of ARO and temperature but capable of acclimating, whereas in Experiment 3, the shorter period for macrophyte establishment may have been detrimental to the regulation of their physiology to prevent adverse effects on growth.

Therefore, while we have shown that photosynthetic pigments, particularly total chlorophylls and carotenoids, are good indicators of stress induced by ARO, this effect clearly does not hold if the macrophytes are exposed during their early growth phase. In our microcosms, we planted apical macrophyte shoots, which would establish roots over the experiment (~ 1 week; VV, personal observation). The chosen macrophytes usually overwinter by reduced aboveground stems and conserved belowground biomass (rhizomes or turions), and they begin to produce new shoots in spring. This means that they will be much more susceptible to the effects of ARO and/or temperature if exposed during this growing period when fresh leaves are beginning to form again.

Our results, therefore, suggest that the growth stage of the macrophytes may modify the ability of macrophytes to acclimate to stressors and thereby affect their usefulness as early warning signals of stress.

2. Effect of ARO exposure mode (increasing single doses or pulses)

Exposure mode did not play a strong role in growth or physiological response of macrophytes. In most cases, all ARO treatments elicited similar increases or decreases in macrophyte biomarkers. *P. perfoliatus* was likely a more sensitive species among the three tested macrophyte species to exposure mode, exhibiting higher chlorophyll or carbon concentrations in the double pulse (XX) vs the double dose (2X). This may be related to competition with periphyton for nutrients and light. There was in fact a trend for increase of these biomarkers in the pulse exposures compared with the single doses, and the vases exposed to pulses of ARO happened to have higher periphyton biomass.

3. Effect of nitrate and pesticides individually or in combination on macrophytes

Pesticides and nitrate may affect the physiological response of macrophytes directly through their mode of action or indirectly by modifying the biotic or abiotic conditions in their environment. No nitrate and pesticide effects on *M. spicatum* photosynthetic pigments could be detected in Experiment 3, although their heights were significantly reduced. Yet, our study suggests that the reduced phenolic compounds or anthocyanins in Experiments 2 and 3 in the ARO treatments were likely influenced by nitrate rather than the pesticides in the ARO. Changes in phenolic content depending on nitrate concentrations have already been shown in the literature, indicating changes in the quality of the macrophyte species (Nuttens et al., 2016). Carbon increased in *M. spicatum* (Experiment 3) only in response to ARO, but not to nitrate alone. A similar response in carbon content for *M. spicatum* was shown with isoproturon (Nuttens et al., 2016). However, direct comparisons between the experiments cannot be made, as Nuttens et al. (2016) used axenic cultures, and the pesticide effects in our system might have been an indirect response to phytoplankton. Rao et al. (2020) showed that macrophyte leaf carbon increased with higher water nitrogen concentrations; however, he attributed this to changes in light and nutrient availability. Because pesticides or pesticides in combination with nitrate promoted phytoplankton biomass rather than nitrate alone, in our experiment, the subsequent changes in nutrient and light environment for macrophytes may have affected macrophyte carbon content and the reallocation of their resources. It is more likely that these effects, therefore, of either

pesticide or nitrate on the macrophyte physiology are indirect rather than direct effects, as the effects were not the same in all experiments. ARO caused different effects on the systems, likely depending on the community complexity present.

4. Conclusion

Macrophytes play an important role in stabilizing shallow lake status, and their loss can be detrimental to the ecosystem, and to the ecological and environmental services that these lakes provide. Their high phenotypic plasticity suggests their use as indicators of stress. The presence of high phytoplankton or periphyton biomass in lakes can induce an increased production of photosynthetic pigments by macrophytes. However, this physiological response of macrophytes to the stressors was not always consistent in our experiments and likely depended on several environmental factors. Community composition, macrophyte species used and their growth stage may modify their response to stressors and thus question their potential for use as early warning signals. Nevertheless, the OECD test species *M. spicatum* was most responsive to the ARO effects, and *P. perfoliatus* was most responsive to temperature effects. Increased temperature may cause changes in both elemental concentrations and their ratios (Zhang et al., 2015), but based on our study, elemental concentrations rather than ratios proved a more robust biomarker. Nitrate or pesticides induced different physiological responses in macrophytes, with nitrates affecting phenolic compounds/anthocyanins and pesticides affecting macrophyte stoichiometry, but this effect may have varied with changes in the environment, such as nutrient and light availability, influenced directly or indirectly by the stressors present. Therefore, while some of the physiological responses of macrophytes could be mapped to the stressors, the high variability between species, experimental set-up and conditions means that further research is needed to confirm the usefulness of the macrophyte chemical fingerprint as early warning signals of environmental stress.

CHAPTER 6: GENERAL DISCUSSION

Macrophytes play a central role in stabilization of the clear-water lake status. They also have a high phenotypic plasticity enabling them to adapt to environmental stressors. Shallow lakes are exposed to multiple stressors in the form of agricultural runoff (ARO) (containing nutrients and pesticides) and climate warming, which can affect macrophytes directly or indirectly. Identifying the effects of ARO and climate warming on macrophyte growth and physiology enables us to predict the potential risk for shallow aquatic ecosystems exposed to these multiple environmental stressors. The main objectives of this study were:

- (1) To determine the individual and combined effects of nitrate, pesticides and climate warming on macrophytes
- (2) To determine whether upscaling from micro- to mesocosms would preserve the macrophyte response
- (3) To determine whether the stressor effects could be detected in the macrophyte physiology

In this chapter, I discuss how the experimental approach enabled the achievement of these three thesis objectives, beginning by an introduction of risk assessment in aquatic ecotoxicology. I summarise the key results obtained from the microcosms, particularly the individual and combined nitrate, pesticide, and warming effects (Objective 1: Individual and combined effects of nitrate, pesticides and climate warming) followed by the effect of ARO exposure mode on the macrophytes. I then discuss the mesocosm experiment, describing how results from outdoor variable conditions compared with those obtained in controlled laboratory conditions, with a focus on the macrophytes (Objective 2: Preservation of effects in the mesocosms?). Following this, I discuss the key bio-indicators we used in our study and provide some suggestions for future studies. After briefly discussing the limitations of our study, I discuss the implications for the stressor effects on the macrophyte chemical fingerprint and their usefulness as early warning signals of regime shifts (Objective 3: Detection of stressor effects in the macrophyte chemical fingerprint?). Finally, I provide some examples on how ecosystem function and services may also be affected by the stressors.

1. Risk assessment in ecotoxicology

Aquatic ecotoxicology evolved to meet the needs for a merger between toxicology and aquatic ecology (Relyea & Hoverman, 2006), and our study was designed to fit within this context. Toxicology focuses on the effect of chemical/physical substances on single species, using response biomarkers ranging in scale from the genetic level up to the individual level. Regulatory studies assessing environmental quality or even the safety of new chemical products are based on such single-species studies (Boudou & Ribeyre, 1997). Not only are these single-species studies necessary to obtain a direct causal link between chemical stress and response, but they also have a high level of reproducibility and replicability. Until recently, environmental risk assessment has largely focused on data obtained from single-species studies (Connon et al., 2012; Relyea & Hoverman, 2006). The choice of species used may vary between regions, but typically, standardised test organisms or model species such as the cladoceran *Daphnia magna* and some phytoplankton species such as *Chlorella* and *Scenedesmus* are used (Boudou & Ribeyre, 1997). Internationally, OECD provides guidelines for risk assessment tests particularly focusing on single-species and single-substance testing. Among their suggested model organisms as suitable for risk assessment in freshwater aquatic systems are *Daphnia magna* (OECD, 2012), *Lymnaea stagnalis* (OECD, 2016), *Lemna* spp. (OECD, 2006), and *Myriophyllum spicatum* (OECD, 2014).

The shortcoming of single-species tests is that they cannot be extrapolated to real-world aquatic systems that are characterized by direct and indirect interactions between different organisms and their environment (Connon et al., 2012). Combining results of several single-species tests would also overlook possible stressor outcomes mediated by these direct and indirect species interactions. Modelling ecosystems that resemble those of the real world enables us to make better predictions, but this requires a systemic analysis of key biotic interactions within these ecosystems. Depending on the study, this can be achieved by using functional units that vary in size and complexity, for example, whole lake ecosystems (including benthic–pelagic coupling) versus micro-habitats (sediment microbiology) (Boudou & Ribeyre, 1997). Such studies are designed as miniature ecosystems in the form of microcosms.

2. Microcosm experiments

Within a shallow lake ecosystem, organisms or organism groups have different functional roles. It is not possible to replicate with precision all species interacting with each other in a shallow lake. However, incorporating “keystone” species in ecosystem-level studies allows us to better predict possible outcomes in real shallow lakes. *Daphnia magna* is a key grazer of phytoplankton in fishless shallow lakes, and their presence has been linked to clear-water phases (Scheffer et al., 1997). Grazers such as snails control periphyton (Jones et al., 2002), which can indirectly influence phytoplankton development (Allen et al., 2021). Submerged macrophytes, particularly canopy formers, such as *M. spicatum* and *P. perfoliatus* can shade out other vegetation. *M. spicatum* is particularly known to have strong alleopathic potential and can limit phytoplankton development (Gross et al., 1996). A loss of these species may lead to a reduced the resilience of the clear-water lake status. Designing controlled microcosm experiments with key species representative of important functional roles and typical of the ecosystem in question allows us to obtain a better understanding of the actual environmental risk for systems exposed to these multiple stressors. One of the constraints with such multiple-species/community-level studies is that because of their complexity, a retrospective and descriptive analysis of effects is almost unavoidable (Connon et al., 2012).

The use of microcosms has developed a lot of interest in the recent years. Simplified food webs have been adopted to study environmental stressor effects. Most of these studies have included a single trophic level or a single trophic chain. Some studies have used only zooplankton or phytoplankton communities (Carter et al., 2005; Stampfli et al., 2013). Other studies have attempted to understand the snail–periphyton–macrophyte interactions (Cao et al., 2014) or the zooplankton–phytoplankton–macrophyte (Rocha et al., 2020; Mulderij et al., 2007; van Donk & van de Bund, 2002).

In our study, we designed a much more complex food web, including not only one- and two-trophic-level systems (only primary producers vs. primary producers and consumers) to disentangle direct and indirect effects, but also two trophic chains (snail–periphyton and *Daphnia*–phytoplankton) (Fig. 6.1). The primary producers included three macrophyte species (*Myriophyllum spicatum*, *Potamogeton perfoliatus* and *Elodea nuttalli*), four to six strains of benthic algae (cyanobacteria, chlorophytes and diatoms) and four strains of pelagic algae (cyanobacteria and chlorophytes). The primary consumers comprised the benthic and pelagic filter feeders *D. polymorpha* and *D. magna*, and the snail *Lymnaea stagnalis*.

Our benthic–pelagic ecosystem model therefore included OECD test species that play important functional roles in stabilising lake status: *M. spicatum* (OECD, 2014), *D. magna* (OECD, 2012), *Lymnaea stagnalis* (OECD, 2016). Using a microcosm design, we exposed these communities to the stressors, agricultural runoff (nitrate and/or pesticides) and warming. The volume of the microcosms (8 L) allowed for suitable replication (5) and robust statistical power. It also provided ample space for the establishment of the different functional groups at realistic densities. With the use of specific statistical analyses (structural equation model), the microcosms allowed us to determine the direct and indirect ARO and temperature effects on the macrophytes (Chapter 3; Allen et al., 2021). Our microcosm design allowed us to identify the individual and combined pesticide, nitrate and warming effects or to test the effect of ARO exposure mode on the macrophytes (Chapter 3, Chapter 5). Finally, by performing weekly samplings of water, phytoplankton, periphyton and *Daphnia*, we were able to follow the temporal dynamics of the system, which influence the effects observed at the end of the experiment. Based on the question, three different microcosm experiments were conducted. The first two experiments (Experiments 1 and 2) were designed to study the effects of single ARO doses versus pulses on the macrophytes. Microcosms were exposed to single increasing ARO doses (1X, 2X, 3X) or pulses (XX and XXX) (see supplementary Chapter S2). The third microcosm experiment (Experiment 3) was designed to disentangle the nitrate and pesticide effects from the full ARO. The microcosms were exposed to either nitrate or pesticide or to both combined.

2.1. Objective 1: Individual and combined effects of nitrate, pesticides and climate warming

From the microcosm experiment designed to disentangle pesticide and nitrate from the ARO (Experiment 3), we were able to show how nitrate affected the macrophytes. Although the total macrophyte biomass was not affected, nitrate did cause a significant decrease in the biomass of *M. spicatum*. Temporal sampling data indicated that the reduced *M. spicatum* growth was likely a result of competition with periphyton during the intermediate exposure period. Phillips et al. (1978) suggested that shading of macrophytes would lead to reduced allelopathy and a subsequent bloom of phytoplankton through elimination of competition. The question whether the system would have eventually shifted towards a phytoplankton-dominated state if the exposure period were extended remains relevant, and is discussed later in this chapter. While the dichotomy

between macrophytes and phytoplankton is well known, our study highlights that periphyton can also affect macrophyte development.

The significant reduction of *M. spicatum* when exposed to nitrate at 9 mg L⁻¹ provides a strong case for reducing nitrate loading in shallow lakes. Other studies have also shown that high nitrate concentrations can cause reductions in macrophyte biomass and species richness (Barker et al., 2008; SAGRARIO et al., 2005). Contrary to Schindler et al.'s (2008) argument that reducing nitrogen (N) input cannot control regime shifts, our study suggests that N control may be just as important as phosphorus (P) control in shallow lakes. Increasing nitrate concentrations in shallow lakes may also affect the quality of macrophytes. With a higher nutritive value (or lower C:N and C:P ratios), macrophytes may become more susceptible to grazing by consumers (Sternner & Elser, 2002; Zhang et al., 2019), and this can reduce the resilience of shallow lakes (see section 6). Higher input of nitrate may also affect the NP ratios in shallow lakes. In recent decades, agriculture has seen a precarious rise in the use of nitrate fertilizers whereas phosphate fertilizers have remained stable or low (FAOSTAT, 2014; Moss et al., 2013; Q. Yu et al., 2015). This widely disproportionate use of phosphorus and nitrate fertilizers is resulting in high N:P ratios in aquatic systems (Peñuelas et al., 2013) deviating from the Redfield ratio (Redfield, 1934, 1958). Such changes may have implications for species richness and biodiversity as the growth rate of organisms is related to their N:P ratios (Hall, 2009; Seastedt & Vaccaro, 2001). Changes in the community structure may further push the stabilizing feedback mechanisms of clear water lakes towards the turbid state.

In experiment 3, we showed that the effect of the pesticides on the macrophytes was much stronger than the effect of nitrate. Rather than a direct effect, our study indicated that the effect was linked to competition with phytoplankton (Chapter 3). Preliminary microcosms using only *M. spicatum* as the test species indicated that the pesticides, including the herbicides terbuthylazine and copper, did not directly affect its growth (VV, unpublished). Yet in Experiment 3, we saw that the pesticide cocktail (containing an organic herbicide, insecticide and fungicide and copper as inorganic herbicide and fungicide) significantly reduced total macrophyte biomass, and specifically *M. spicatum*, biomass. Therefore, the direct effect of the herbicides on macrophytes seemed less important than the much stronger indirect effects through insecticide or fungicide inhibition of the primary consumers. Such indirect effects suggest that basing

environmental concentration limits on data derived from single-species or single-toxicant studies will underestimate the actual toxic concentrations to non-target species.

Whether the individual stressors had no effect (nitrate) or a negative effect (pesticides) on the total macrophyte biomass, their combined presence had significant negative consequences. Nitrate and pesticides acted synergistically, resulting in a higher reduction in the total macrophyte biomass than expected by the addition of individual effects. Our study highlights the risk of co-occurring multiple stressor exposure of shallow lakes. Even if nitrate on its own did not pose a significant threat to the macrophytes, it is clear that in combination with other stressors, the resilience of the macrophyte-dominated state is threatened. Both nitrate and pesticide use should be reduced to ensure that macrophyte dominance in shallow lakes is not challenged by significant disruptions in biotic interactions.

However, the negative effect of the combined ARO cocktail containing nitrate and pesticides on the macrophytes was only observed in Experiment 3. Despite increases in either phytoplankton or periphyton which compete with macrophytes for resources, and despite comparable ranges of phytoplankton or periphyton biomass (chl-*a*) across all three experiments (Chapter 3.3), the macrophytes were not affected by ARO in Experiments 1 and 2. The most likely explanation for this is the growth stage of the macrophytes. In both Experiments 1 and 2, the macrophytes were allowed to establish for 10 days or longer compared with Experiment 3, where macrophytes were exposed to ARO after 4 days (Fig. 6.1). Different sequence of introduction of the species (e.g., Macrophytes-microalgae-consumers-ARO+Temperature *or* Microalgae-macrophytes-consumers-ARO+Temperature) and different timelines of macrophyte exposure to the stressors (e.g., 17, 10 or 4 days) were tested to consider various scenarios, for example, clear macrophyte-dominated lakes or turbid phytoplankton-dominated systems. This allowed us to determine the ability of the system to revert to an alternative stable state when exposed to the stressors. However, it also presented several challenges in terms analysing the outcome of our microcosm studies. This is an example of the type of retrospective analyses that are often required when performing complex-community experiments. The implication of these results is that macrophytes exposed to the ARO early during its growth stage, that is, in early spring, may be more vulnerable than if exposed much later, such as late spring or early summer when they are already established.

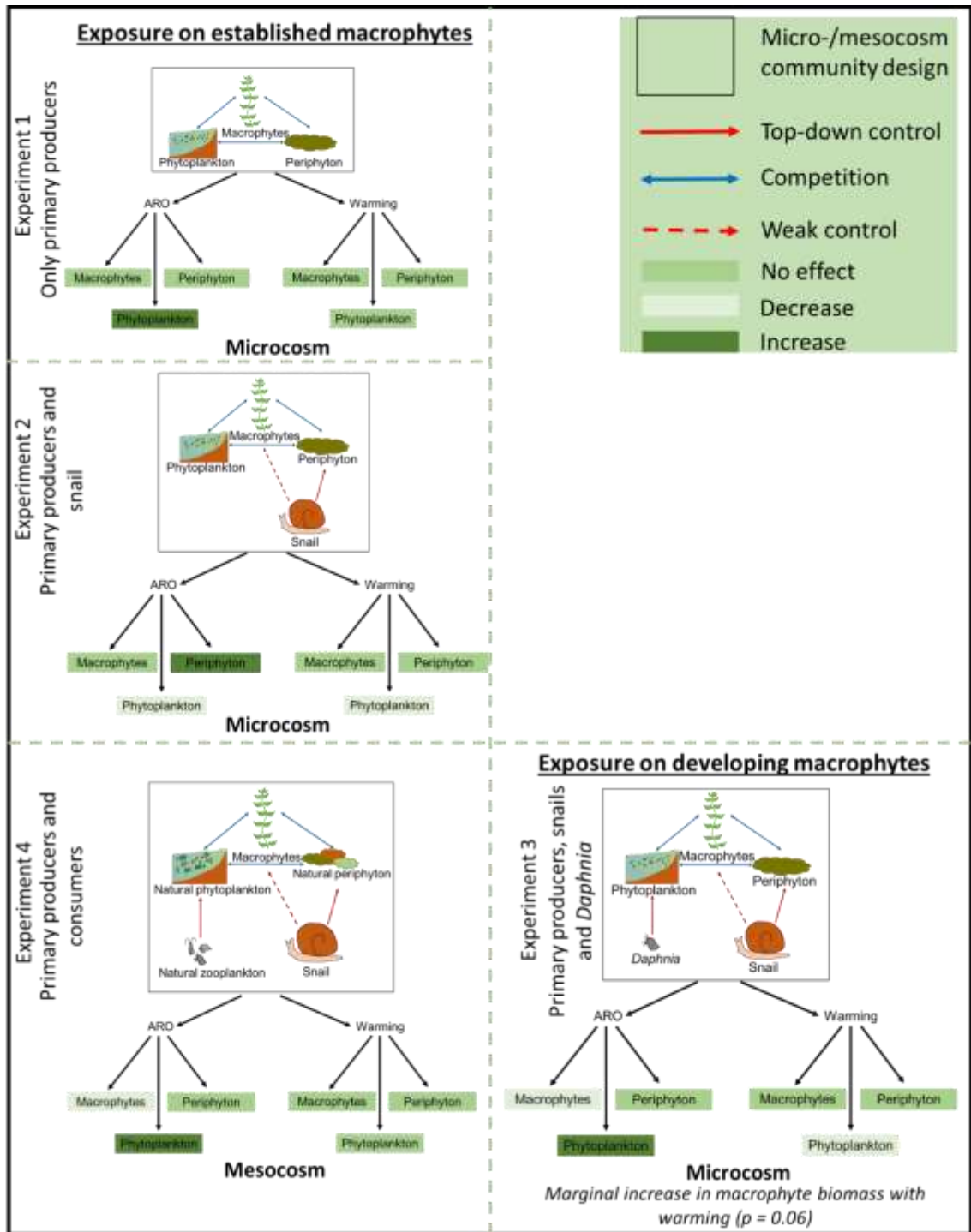


Fig. 6.1. Comparison of ARO and warming effects on the three main primary producer groups across all four experiments (three microcosms and one mesocosm) conducted in the framework of CLIMSHIFT. Microcosms refer to controlled indoor laboratory experiments, and the mesocosm refers to the outdoor experiment with variable environmental conditions.

In Experiments 1 and 2, different modes of ARO exposure did not affect the growth of macrophytes. Previous studies have shown that multiple pulse exposures of pesticides, particularly herbicides, can inhibit macrophyte growth (Vallotton et al., 2008; Varga et al., 2019). This effect may depend on the type of species and their ability to recover (Varga et al., 2019). The herbicide in our study did not seem to directly affect the macrophytes, and even the highest concentration used (single dose: 3 $\mu\text{g L}^{-1}$; triple pulse: 1 $\mu\text{g L}^{-1}$ three times with a gap of 4 days), although environmentally relevant, may not have been sufficient to see significant effects on the macrophytes (Cedergreen et al., 2004). However, Allen et al. (2021) showed significant negative effects of ARO pulses on the snails, and positive effects on periphyton. In Experiment 1, using only primary producers, the increase in phytoplankton may reflect a reaction to the nitrate present in ARO and the development of herbicide-resistant microalgae. In Experiment 2, the increase in periphyton in response to ARO may be explained by effects on trophic interactions (Allen et al. 2021/supplementary chapter S2). Constant exposure of shallow lakes to various mixtures of pesticides could pose an indirect threat to the macrophyte-dominated state depending on species sensitivities and strength of biotic interactions.

Contrary to the chemical stressors, warming had a marginal positive effect on the total macrophyte growth in Experiment 3. Within an optimum temperature range for macrophytes, an increase in water temperature can in fact promote macrophyte growth (Barko & Smart, 1981; Hussner & Jahns, 2015; Mckee et al., 2002; Zhang et al., 2019). Yet, in our study, the effect of temperature varied between macrophyte species. Despite the ability of both *M. spicatum* and *P. perfoliatus* to proliferate at temperatures as high as 35°C, *M. spicatum* growth was significantly promoted by warming, whereas *P. perfoliatus* growth was hampered. The decline of *P. perfoliatus* in the heated treatments may be linked to the increased competition not only with the dominant *M. spicatum* but also with phytoplankton, which showed an early-phase increase in the heated treatments compared with the ambient. In Experiments 1 and 2, warming had no effect on the macrophytes (see chapter 5; Allen et al., 2021/supplementary Chapter S2). Globally, the temperature difference of $\sim 3.5^\circ\text{C}$ in the microcosms did not cause significant changes in the growth of the macrophytes during the exposure period. The response may have differed for a longer exposure period. The large variability in the response of the phytoplankton and periphyton to warming also make the response of macrophytes very context-specific. It may in fact take large temperature increases ($>6^\circ\text{C}$) to see a consistent decline in macrophyte growth (Rooney & Kalff, 2000).

However, in combination with the pesticides, the effect of warming may be different (Polazzo et al., 2022).

In Experiment 3, the reduced biomass of macrophytes in the treatments with both nitrate and pesticides (ARO) was accompanied by an increase in phytoplankton biomass. Although this increased phytoplankton biomass was slightly buffered by warming due to the increased top–down control, the decrease in macrophyte biomass was much stronger when warming was added as an additional stressor to the ARO. Such synergistic or reversed effects of the stressors need to be considered in the context of rising global temperatures. The projection by the IPCC (2014) is that air temperatures could rise as much as 4°C above the average preindustrial level, and this could affect shallow aquatic systems. We already experience extreme temperatures, for example, the summer of 2019. During our outdoor mesocosm experiment at Ludwig Maximilian University of Munich, Germany, the ambient mesocosms exposed to natural environmental temperatures reached as high as 27°C. In our microcosms, we had aimed for a temperature of 26°C but reached a maximum of 24.5°C due to the capacity of our heating system. Studies have shown that temperatures as high as 26°C drastically increase the mortality of *D. magna* (Maceda-Veiga et al., 2015), key grazers in these types of systems. At these temperatures, the negative ARO effect observed on the macrophytes, which was linked to reduced phytoplankton control by *D. magna*, would be even stronger. In lakes where fish are present, warming may significantly increase the predation on zooplankton. Hansson et al. (2012) showed that the length of the food chain would define community response to climate warming, and that the top predator in the ecosystem and every second trophic level from the top would benefit from warming. This would mean that when planktivorous fish are present, they can indirectly promote phytoplankton biomass at higher temperatures through increased feeding on zooplankton. If this were the case, we should expect much severe effects of warming in combination with nitrate and pesticides. In shallow lakes, where both piscivorous and planktivorous fish are present, we may expect similar effects of warming as seen in our study because of the increased grazing expected by both piscivorous fish and zooplankton at higher temperatures that fall within the optimum range for metabolic activity. Yet despite the increased grazing control of phytoplankton at a higher temperature, the significant reversed effect of warming on macrophyte growth when applied in combination with ARO, compared to what was predicted, highlights the risk for shallow lakes exposed to ARO in the context of rising temperatures.

The microcosm experiments provided an understanding of the individual and combined stressor effects on the macrophytes (Objective 1). They warn us about the potential dangers, particularly of pesticides and of pesticides in combination with other stressors such as nitrate and warming. Yet how reliable are these results considering that the microcosms were carried out under controlled conditions that are not representative of the natural environment?

3. Mesocosm experiment

Aiming at higher environmental realism in aquatic ecotoxicology, experimental designs have been moving away from highly controlled indoor microcosms towards variable and complex outdoor mesocosms (e.g., Davidson et al., 2018; Finnegan et al., 2018; Riis et al., 2018). Mesocosms are more realistic in that they allow the inclusion of natural biotic and abiotic variability, stochastic events and confounding factors in the evaluation of stressor effects on communities. Because of the higher realism, extrapolation of stressor effects to the real lakes are more dependable.

The challenge with upscaling is that with increased size, material and community structure, it is not always easy to achieve a sufficient number of replicated systems (Fig. 6.2). If sufficient replication is not possible, incorporating a chemical gradient design may be more suitable. For non-linear systems such as shallow lakes, the gradient system has in fact been suggested over the replication design (Kreyling et al., 2018; also Polst et al., submitted). A gradient design might allow the identification of threshold values. As part of CLIMSHIFT, the identification of a lower threshold under warming was hypothesised and was the main aim of the chosen design for the outdoor mesocosms.

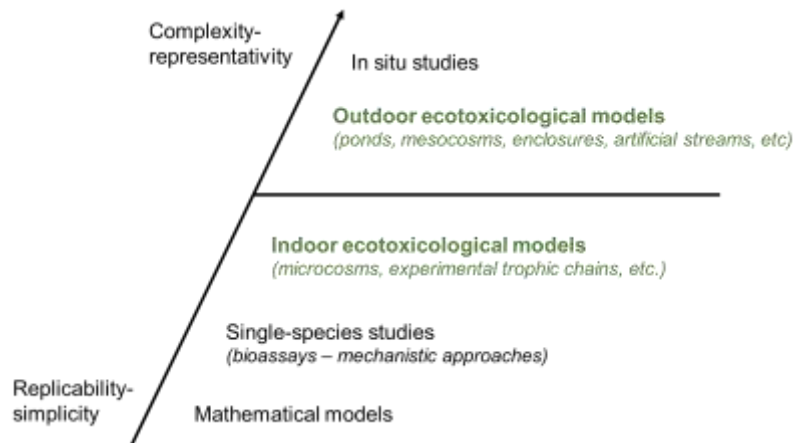


Fig. 6.2. Representation of methods used in aquatic ecotoxicology. Simple methods such as single-species assays allow for high replicability, whereas complex multiple species models enable closer representation of real systems. Source: Boudou and Ribeyre (1997).

Outdoor mesocosms also require more resources in terms of money, material and labour. At the end of the experiment, the disposal of the used material can be a challenge, especially when chemical pollutants are present. It is therefore important that mesocosms are conducted only after evaluating whether the costs particularly in terms of the environmental burden do not exceed the need for conducting such an experiment. Crossland and La Point (1992) suggested in their review that mesocosms be performed only after obtaining evidence of persistence and toxicity of a chemical at the estimated exposure concentration. They concluded that in the event that toxicity, persistence and exposure concentrations are very low compared to estimated toxic concentrations, mesocosms might not prove useful. However, if environmentally relevant concentrations of the stressors pose a risk, outdoor mesocosms may be of significant value. The potency of a chemical present in the environment may be measured by whether a strong toxic effect observed in the laboratory is still observed under highly variable conditions in the environment. It should also not be excluded that low chemical exposure concentrations in situ have a much stronger effect than observed in single-species single-toxicant standardized laboratory test systems. Synergistic effects of pollutant mixtures or combined stressors might exacerbate effects.

3.1. Objective 2: Preservation of effects in the mesocosms?

The strong effects of the combined stressors observed in our microcosm experiments encouraged us to upscale to the large outdoor mesocosms with a higher realism (Experiment 4). Although interpreting the stressor effects on the macrophytes was

made much more complicated because of variable environmental conditions acting on these outdoor systems (as shown in the following sections), we were able to provide evidence for the risk of multiple-stressor exposure to shallow lakes.

Clear ARO gradient effects were not observed. In the low to intermediate ARO concentration levels, nitrate concentrations may have been beneficial to the macrophytes and pesticide concentrations were likely too low to affect them, leading to an overall better macrophyte stability. However, in the two highest ARO treatments (the highest being comparable to the concentrations used in microcosms), a significant reduction in macrophyte biomass accompanied by an increase in phytoplankton biomass was observed. This effect is comparable to that observed in the microcosms (Experiment 3) and highlights a conservation of effects despite the high variability of responses observed along the gradient, and despite the presence of stochastic events and confounding factors. Although a clear ARO threshold could not be identified with our gradient study, we were able to provide sufficient evidence that concentrations above our defined 1.5X of the ARO (see chapter 4)—which reflect environmentally relevant nitrate and pesticides concentrations—pose a huge risk for the resilience of the macrophyte-dominated state of shallow lakes.

When warming was applied alone, the contribution of total macrophytes to the primary producer biomass increased. This positive effect of warming on the macrophytes may be compared with that observed in Experiment 3, where warming marginally promoted macrophyte growth. Also interesting was that the macrophyte species-specific responses were similar to those observed in the microcosm. *M. spicatum* grew better at higher temperature and *P. perfoliatus* grew less (Mesocosms, Fig. 6.3; also Chapter 3, Fig. 3.1). In fact, for *M. spicatum*, warming also induced an early flowering (see supplementary Chapter S1).

However, contrary to the preservation of ARO-alone or even warming-alone effects in the mesocosms, when ARO and warming were applied together, the system reacted very unpredictably. Numerous ARO and temperature interaction effects may have occurred depending on the particular ARO concentration level, environmental factors and the direct and indirect biotic interactions, the latter made more complex with invasions by invertebrate larvae (Chapter 4). In the heated mesocosm at highest ARO concentration (comparable to the highest ARO concentration used in the microcosms), the macrophyte growth was significantly promoted. This mesocosm was the most macrophyte-dominated

enclosure, despite the highest ARO level. The contribution of phytoplankton to the total primary producer biomass also significantly reduced. Whether this effect is due to the complex biotic interactions as discussed in Chapter 4, or due to a random phenomenon, requires further investigation. However, it confirms what (Polazzo et al., 2022) have shown in their study, that the effects of warming may vary depending on the type of pesticides and the response of the community. It also warns us that warming may complicate the understanding of chemical stressor effects, and calls for more studies to understand how chemical stressors in the presence of warming will affect shallow lakes.

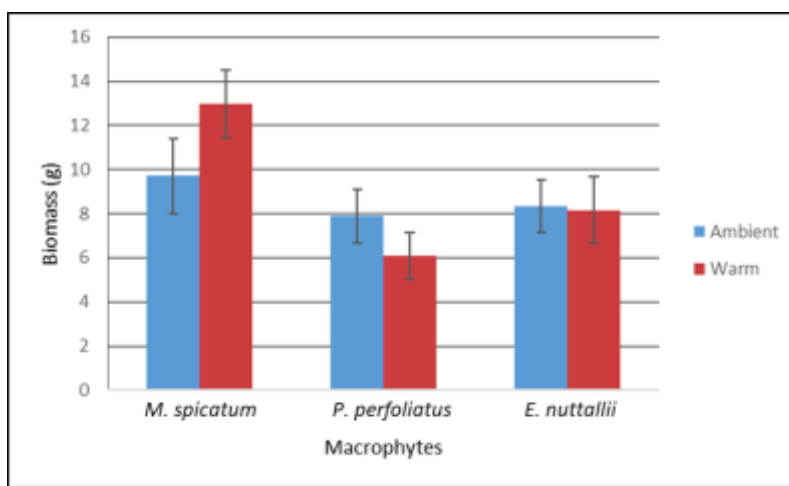


Fig. 6.3. Effect of temperature on the three macrophyte species in the mesocosm experiment. The experiment was conducted in summer 2019 at the mesocosms facility of Ludwig Maximilian University at Martinsried. Average \pm *SD*, *n* = 8 (average and *SD* were obtained from 8 mesocosms each for ambient and warm).

4. Key biological indicators in freshwater ecotoxicology

Our experiments highlight the advantage of using key functional groups and OECD test species in micro- or mesocosms studies. Under combined stressor conditions, *M. spicatum* tended to have a competitive advantage over both *P. perfoliatus* and *E. nuttallii*. This is in line with other studies that also suggest a high competitive advantage of *M. spicatum* (Grace & Tilly, 1976; Huang et al., 2021; Lind & Cottam, 1969; Zefferman, 2015). This has been attributed to its wide ecological amplitude and ability for nutrient assimilation, allowing it to thrive in different trophic environments, as well as its low palatability (Bosch et al., 2009; Feng et al., 2016; J. Yu et al., 2016). *Myriophyllum spicatum* was also the most responsive macrophyte species to the stressors, highlighting its importance for use in studies assessing environmental risk. The other two macrophyte species were also important indicators of stress, although their responses were subtler and

tended to be stressor-specific. *Potamogeton perfoliatus*, for example, responded more often negatively to a temperature increase, while *E. nuttallii* showed stronger responses to higher levels of nitrate (Chapter 3, Chapter 5). All three species are canopy-forming macrophytes (Gustafsson & Norkko, 2019; Madsen et al., 2001; Xu et al., 2016), which play an important role as submerged macrophytes in preventing sediment resuspension and in nutrient cycling between the sediment and water column. As canopy formers, they also shade out phytoplankton, and are common in freshwater lakes and rivers. Among the consumers used in our microcosms, both *Lymnaea stagnalis* and *Daphnia magna* are OECD test organisms (OECD 2012, 2016). In our microcosm experiments, we showed that they have implications for the clear-water status of shallow lakes and were sensitive to both ARO and temperature. To summarise, the species we used proved important biological indicators of stress in freshwater systems. Using these model organisms also allow us to compare responses in the complex micro- or mesocosms studies with those observed in single-species single-pollutant studies.

In small ditches and kettle holes, typical shallow, fishless aquatic systems, species such as the free-floating macrophytes like *Lemna* species (OECD, 2006) and unrooted submerged macrophytes like *Ceratophyllum* (only rhizoids) species are also common (Pätzig et al., 2012). Including these species in future studies might be interesting as it may provide a more comprehensive and global understanding of the response of and competition between these macrophytes communities to the stressors and the threat for these types of shallow lakes. In fact, dominance by floating plants may also be considered a type of alternative stable state (Scheffer et al., 2003). Their competitive advantage may promote anoxic conditions and modify the light environment for the other plants, reducing species richness (Scheffer et al., 2003). Their use might be justified if highly eutrophic or even hypertrophic systems are the subject of investigation.

5. Limitations of the experimental set-up

Yet our study also has certain limitations. With the rampant use of chemicals that eventually find their way into water bodies all around the globe, there is likely no aquatic system that exists in pristine conditions. Pesticide and nitrate concentrations may in fact be higher than actual measured concentrations. Nitrate levels are usually much higher than actual input due to the rapid accumulation by primary producers. Pesticides may also be adsorbed onto soil or bio-accumulated. The control conditions that we used in our experiments may therefore not be representative of natural lakes. Also, in our experiment

we used standard Volvic water or well water. These conditions are not representative of natural lakes. However, within the project, we wanted to be able to compare results to identify recurrent themes or directions of effects. Using natural lake water may not have enabled us to make these comparisons as project partners were located in different geographical regions. Lake systems, their eutrophic status and chemical pollutants would have varied vastly, increasing the number of confounding factors acting on the system.

Some important limitations of the microcosms should also be discussed. Natural systems are exposed to irradiance much higher than those used in our labs. For example, in the mesocosms, we measured between 300 to 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$, whereas in the microcosms, we reached a maximum of 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Even though species such as *M. spicatum* can grow at irradiance less than 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Barko & Smart, 1981), there is insufficient evidence whether the irradiance in our microcosms may itself have been limiting for the primary producers.

Smaller systems are generally more conducive to rapid periphyton development compared with larger systems (Hill et al., 2018). This was in fact observed in our study. In the control microcosm, periphyton contributed a larger percentage ($\sim 50\%$) of the primary production compared with the phytoplankton ($\sim 5\%$) whereas in the mesocosms, the periphyton contribution was very low ($\sim 5\%$) compared with the phytoplankton ($\sim 60\%$) (Chapter 4, Fig. 4.7). In our microcosms, the development of periphyton may have been much higher than would be expected in real lakes, preventing the development of phytoplankton where in natural lakes this might have occurred (e.g., in the NO_3 treatment, see also Fig. 3.2 in Chapter 3).

6. Predicting regime shifts

Predicting regime shifts is not easy because of the number of environmental drivers that influence shallow lakes. Shifts occur in different directions (forward switch from macrophyte to phytoplankton dominance or backward switch from phytoplankton to macrophyte dominance). Identifying the different drivers causing shifts is difficult, but reliable predictions are highly essential for the management of shallow lakes. Once shifts have occurred, returning these lakes to their original clear-water state may be expensive and difficult, because of hysteresis effects, and may require significant reductions in nutrient levels, far below what was present before the shift occurred (Carpenter et al., 1999; Scheffer, 2001). Signals of regime shifts may even be detectable several generations before

a shift actually occurs (Drake & Griffen, 2010), and any indications of such changes should be well studied. Early warning signals should allow us to detect changes that indicate a heightened vulnerability of shallow lakes to further environmental perturbations, alerting us about potential regime shifts (Haojie et al., 2020).

Because of the short experimental exposure period (3 to 8 weeks), none of our experiments showed a clear regime shift (phytoplankton bloom at complete loss of macrophytes). However, when exposed to ARO, we observed several indicators of a reduced resilience of the macrophyte-dominated state, most apparent of which was an increased phytoplankton biomass, a consistent result in all our experiments (see Fig. 6.1). Further, in Experiment 3, the high variability in the phytoplankton biomass when exposed to ARO (Chapter 3) may be an indicator of a reduced resilience, as the system fluctuates widely around its equilibrium state (Dakos et al., 2015; Scheffer et al., 2009). In order to identify whether we could detect other signs of an imminent regime shift, we studied the macrophyte physiology.

6.1. Objective 3: Detection of stressor effects in the macrophyte chemical fingerprint?

Macrophytes have a high phenotypic plasticity, not only in morphology but also physiology. They may prove useful as early warning signals if changes in their chemical fingerprint can be linked to the presence of stressors. In our study, we looked at whether ARO or warming induced changes in the macrophyte stoichiometry, photosynthetic pigments and secondary metabolites. Stoichiometry or changes in the elemental concentrations and ratios can provide indications about the quality of macrophytes and of their environment (Demars & Edwards, 2007; Elger & Lemoine, 2005; Zhang et al., 2019). An increase or decrease in the photosynthetic pigments of macrophytes may indicate competition with phytoplankton or periphyton for nutrients and light (Barko & Filbin, 1983; Chen et al., 2016; Nuttens et al., 2016). An increase in secondary metabolites can signal a defence against biotic stress, and a decrease can lead to increased grazing by herbivores or indicate a rise in nitrate levels in the water (Cronin & Lodge, 2003; Fornoff & Gross, 2014; Gross, 1999; Nuttens et al., 2016).

In our study, the macrophytes in all three microcosm experiments did show changes in physiology when exposed to the stressors, but the responses varied between stressors, species, set-up and environmental conditions (Chapter 3). Macrophyte

physiological response to ARO was much more explicit when phytoplankton biomass increased rather than when periphyton biomass increased (Chapter 5). Among the macrophyte species, *M. spicatum* exhibited the most physiological markers of ARO stress compared to *P. perfoliatus* or *E. nuttallii*. Compared with ARO, warming induced fewer physiological responses, with the most physiological responses observed in *P. perfoliatus* compared with the other two species. The physiological responses of macrophytes also largely depended on the exposure period. When macrophytes were provided more time to establish themselves before exposing them to the ARO, they showed stronger physiological responses but no effects on growth. When they were exposed soon after planting, their growth was significantly reduced but few physiological responses were observed.

The most significant physiological response to ARO was an increase in photosynthetic pigments (total chl and/or carotenoids) of the macrophytes. The increase in photosynthetic pigments is most likely an indirect effect, resulting from the high competition with phytoplankton or periphyton (both promoted when ARO was present) for resources, mainly light and nutrients. The most robust indicator among all physiological biomarkers tested were the carotenoids. In Experiment 1, all three macrophyte species responded to both ARO and warming by increasing their production of carotenoids. In response to the stress by ARO and stress, an accumulation of hydrogen peroxidase (H_2O_2) in the macrophytes may have occurred, leading to an increased production of carotenoids to detoxify H_2O_2 (Chalanika De Silva & Asaeda, 2017).

Another meaningful indicator of stress was the change in elemental ratios. Both C:N and C:P of *M. spicatum* significantly reduced when exposed to ARO. The low C:N ratio is likely linked to the nitrate in the ARO. While the higher N observed in macrophytes may suggest that they successfully competed with phytoplankton and periphyton for this resource, a higher nutritive value or lower C:N or C:P ratios can also increase the palatability of the macrophytes for herbivore grazers (Elger & Lemoine, 2005; Sterner & Elser, 2002; Zhang et al., 2019). If grazing is high enough to reduce the macrophyte biomass significantly, this may promote conditions for phytoplankton blooms (Hidding et al., 2016). Our system included snails, which feed primarily on periphyton but also on macrophytes (Brönmark et al., 1991; Elger & Barrat-Segretain, 2002). In real lakes, macrophytes are exposed to other herbivores, whose impact by increased grazing may lead to significant losses of macrophytes.

Secondary metabolites were also affected by ARO. Total phenolics and the more specific anthocyanins both decreased when *M. spicatum* was exposed to ARO, but these effects were only observed in Experiments 2 and 3, where consumers were present (Chapter 5). This may indicate changes in the nutrient environment mediated by consumer behaviour, but what is more important is that a lower phenolic content may also be linked to increased palatability by grazers (Elger & Lemoine, 2005).

While globally, our study shows that reduced C:N and C:P ratios, increased photosynthetic pigments, and reduced phenolic content reflect an enhanced vulnerability of the macrophyte-dominated state, particularly linked to stress and a potential for higher grazing, the variability of these responses means that more studies are needed to validate the use of these biomarkers as potential indicators of regime shifts. In addition, the usefulness of these indicators in combination with other available indicators such as changes in fish populations and herbivore grazing (Hidding et al., 2016; Hsieh et al., 2006), and increases in variance and autocorrelation time series data (correlations between observations at different points in time) in these systems (Haojie et al., 2020) should also be studied.

7. Implications of the stressors for ecosystem function

In addition to the potential risk for regime shifts in shallow lakes exposed to agricultural runoff and climate warming, the lake ecosystem function may also be affected (Polazzo et al., 2022; Spaak et al., 2017). Ecosystem functions are challenged when the abundance of a species or its function is affected, or by changes in community composition (Fox & Kerr, 2012). Decomposition of organic matter is an important ecosystem function that makes available nutrients and minerals for uptake by macrophytes and other primary producers (Gessner et al., 2010). A disturbance of organic matter degradation and subsequent changes in biogeochemical cycles can have strong consequences for aquatic systems (Gessner et al., 2010; Graça & Canhoto, 2006; Shilla et al., 2006), by reducing nutrient cycling and leaving nutrients largely unavailable for the macrophytes.

We therefore chose to investigate cotton band degradation in our experiments. Fig. 6.4 shows the effect of ARO and warming on degradation of cotton strips. In the pesticide only treatments, the cotton bands were significantly less degraded, but not in the treatment with both pesticides and nitrate. The higher degradation in the warmer treatments is expected as microbial activity is strongly influenced by temperature changes (Cheung et

al., 2018; Zhao et al., 2021). In our system, warming and pesticides/ARO had about the same degradation rates as the ambient control or nitrate only treatments, highlighting the complex stressor interaction effects sometimes observed. However, the strong reduction in the degradation rate observed in the pesticide treatment calls for further investigation to better understand the implications of these stressors for ecosystem function.

Ecosystem services may also be affected by these stressors and should be considered in future studies. Reduced availability of clean drinking water, loss of biodiversity and changes to carbon burial and global greenhouse gas emissions (Hilt et al., 2017) may all be affected by the presence of the stressors. The potential stressor effects on ecosystem function and implications for ecosystem services underline the need for further studies on warning signals that can aid in the management of shallow lakes and in defining safe threshold concentrations of the chemical stressors within the context of rising global temperatures.

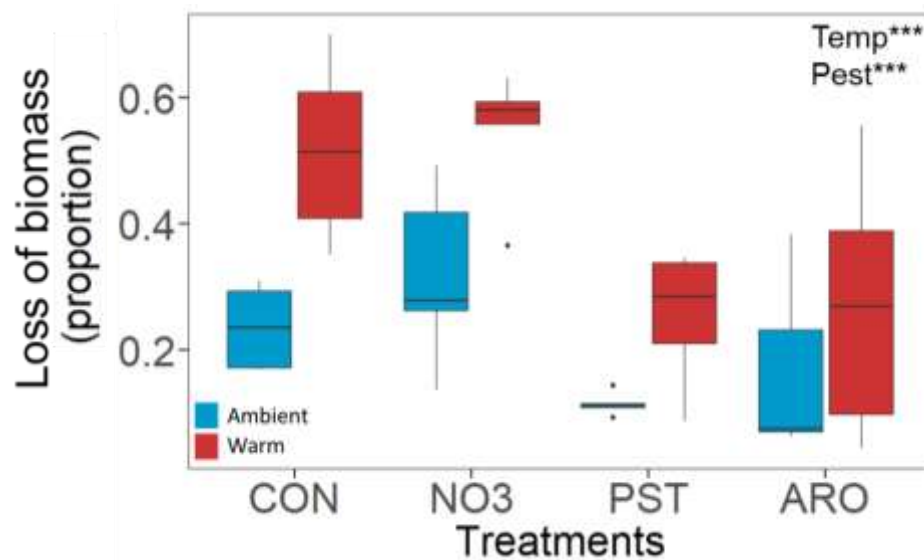


Fig. 6.4. Box plots showing the temperature, pesticide and nitrate effect on the loss of cotton band biomass in Experiment 3. Five replicates showing median, 25 and 75% percentiles, lowest and highest whiskers (as $Q1 - [1.5 \times IQR]$ and $Q3 + [1.5 \times IQR]$, respectively), and outliers (dots). Temp = temperature, Pest = pesticide. Significance level shown as *** $p < 0.001$.

8. Conclusion

Positive or negative effects depend on “for whom.” Most of our advancement and policy-making has largely focused on humans as the beneficiaries, and oftentimes this has come at a cost of other life. This has particularly been the case with the use of pesticides

and fertilizers. While the challenge of food security for a growing population is understandably a complex one, there is simultaneously an urgent need for humans to foresee issues in the context of the long-term. It has been evident seventy years ago, from the time Rachel Carson spoke up in *Silent Spring*, that the devastating effects of pesticides and fertilizers will affect the ecological balance that ironically is just as important as, and for, human life. Although the EU has developed legislation against the pollution of these lakes by agricultural chemicals, their objectives of water quality have not been met and continues to be a challenge (Wuijts et al., 2021). The research continues to suggest dire long-term consequences of these agricultural chemicals in our environment, and there is an important role for policy makers to put into action regulations to reduce their use, particularly when a very fundamental resource such as water is being destroyed. Our study has stressed the risk for multiple stressor exposure. Particularly, we have shown, contrary to what has been believed for a long time, that nitrate also has implications for shallow lake status, and this is especially true when present in combination with pesticides. As long as these chemicals continue to be used, concentrations within shallow lakes will rise and the status of these lakes as well as the ecological and environmental services will continue to fall. Warming may complicate the understanding of multiple stressor effects, but the significant negative effect on our model systems when warming was applied with the ARO should be enough reason to ensure that environmental chemical concentrations are not exceeded, particularly in the context of rising global temperatures.

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CHAPTER S1: DESIGN OF A SUSPENDED BENTHIC–PELAGIC SHALLOW LAKE MESOCOSM SYSTEM FOR MULTIPLE STRESSOR AND GLOBAL WARMING RESEARCH

Submitted to *L&O Methods*

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Abstract

Mesocosm experiments have a long tradition in aquatic research, and several different designs have been developed. Still, optimisations are possible depending on the target ecosystems simulated and the stressors tested. Here we describe a new mesocosm setup developed to mimic small and shallow lentic freshwater habitats with benthic–pelagic coupling and test the effect of warming along with a combination of chemical stressors typically found in agricultural runoff. The mesocosms of the described setup are suspended in artificial ponds and have closed and strengthened bottoms to support the sediment. All mesocosms followed the same natural day–night cycles of temperature typical for small ponds. Moreover, mesocosms simulating climate warming were heated from the outside by regulating the temperature of the surrounding pond, thus avoiding local small-scale heating artefacts in individual mesocosms. Over the 8-week experiment (mid-May to mid-July), the heating system reliably maintained the targeted +3°C temperature difference between the control and warmed pond. The differences between mesocosms of the same temperature treatment were maintained within a narrow range of 0.30 ± 0.26 °C.

Benthic and pelagic organisms introduced into the mesocosms developed well and could be conveniently sampled due to easy accessibility during the experiment and the extraction of entire mesocosms from the ponds with a gantry crane at the end of the experiment.

Versatility and cost-effectiveness further increase the application potential of this approach for investigating stressors in benthic–pelagic mesocosm systems.

Keywords: suspended mesocosms, benthic–pelagic coupling, mesocosm heating, climate change research

1. Introduction

Mesocosm experiments fill an important gap between highly controllable microcosm experiments and observational field studies (Riebesell et al. 2010, Stewart et al. 2013). Whereas microcosm experiments are usually stripped of the complexity of natural ecosystems using mainly key test organisms within simple food webs, field observations are usually not capable of establishing unambiguous cause and effect relationships and replications. Hence, mesocosm experiments can in best case combine the strength of modern experimental designs, allowing the use of either gradient approaches or sufficient replication depending on available resources, simultaneously capturing a large part of the complexity of natural systems (Cottingham et al. 2005). Nevertheless, planning experimental mesocosm research poses many challenges and needs to be done meticulously. Mesocosm experiments are often costly and labour intensive, especially with increasing mesocosm size and complexity. To perform such experiments, more organisms and materials are needed than for a microcosm experiment, resulting in longer preparation and setup times.

Aquatic mesocosms are implemented to simulate marine and freshwater systems. They have largely focused on effects of experimental manipulation of stressors at the population, community and ecosystem levels (Boyle & Fairchild 1997, Riebesell et al. 2010, Stewart et al. 2013). Whereas they often include pelagic communities, systems suitable for studying benthic–pelagic coupling are rare (Dimitriou et al. 2017). Mesocosms can accommodate natural and/or artificial ecosystems with multiple species per trophic level and, depending on mesocosm size, multiple trophic levels. Including complex community structures is important especially in ecological risk studies (Simmons et al. 2021) as most stressors will affect not only an organism by itself but also its interactions with other organisms (competition, predation, parasitism) within the same system. This results in a large number of direct and indirect effects, which ultimately modulate the ecological state of the entire system (Allen et al. 2021). By allowing for complex communities as test subjects, mesocosms facilitate the research of these indirect effects (Moe et al. 2013). Furthermore, by using outdoor mesocosms instead of indoor experiments, typical natural dynamics are included in the experimental design. Most of the time, outdoor mesocosms are exposed to natural irradiation, temperature fluctuation, precipitation and invasion by additional species. Thus, they can mimic real-life scenarios of stressor exposure much better and allow extrapolating results to potential responses of natural systems to the

chosen stressor combinations. Mesocosms therefore play a vital role in multiple stressor research and are a useful element in the available experimental toolbox.

Mesocosms are particularly useful for mimicking small water bodies. Small shallow ponds and lakes are globally abundant (Verpoorter et al. 2014), and in Northern Europe and North America, kettle holes (also referred to as prairie potholes in North America) are particularly common in agriculturally used landscapes (Van der Valk 2005, Lischeid & Kalettka 2012, Kazanjian et al. 2018). These water bodies are often exposed to multiples stressors, namely agricultural runoff containing pesticides and nutrients. In addition, they are particularly vulnerable to climate warming (Moss et al. 2011).

As there are many interaction effects operating between benthic and pelagic communities in shallow lakes, they should not be investigated separately (Vadebonceur et al. 2002, Hölker et al. 2015). For example, benthic primary producers can compete for nutrients with phytoplankton. Phytoplankton and periphyton can limit macrophyte growth through shading (Moss 1976, Phillips et al. 2016). Benthic grazers can remobilize nutrients bound in periphyton and thereby facilitate phytoplankton blooms (Rober et al. 2011). Filter feeders can control phytoplankton growth and cause clear water phases (Nicholls & Hopkins 1993), while periphyton grazers similarly can control periphyton biomass and release macrophytes from shading (Jones & Sayer 2003). An increase in temperature can affect filtration and grazing rates of consumers (Zhang et al. 2020) and growth rates of primary producers (Velthuis et al. 2018). Also, it can change the resuspension of nutrients from the sediment (Jeppesen et al. 2009, Aben et al. 2017). Therefore, mesocosms designed to closely match small shallow freshwater systems need to incorporate both benthic and pelagic habitats to generate meaningful results and understand the effect of stressors in shallow waters. However, this task also includes several critical challenges.

Mesocosms containing a benthic compartment are often built on land, to support the weight of the sediment. The temperature fluctuations in these experimental units are strongly influenced by the comparably small volume of mesocosms and the thermal conductivity of the surrounding medium. During the day, the sun can heat up the mesocosm from multiple sides and during the night the surrounding air will cool down the mesocosms quickly. To reduce these fluctuations, such land based mesocosms can be wrapped with insulating material (Hansson et al. 2012) or buried in the ground with the water surface close to the ground surface level (Liboriussen et al. 2005, Polazzo et al. 2021). The surrounding soil thereby dampens rapid temperature fluctuations.

Instead of using terrestrial environments to buffer the temperature dynamics, mesocosms can be suspended in larger water bodies at a scale close to the water body to be mimicked. As an additional benefit, all mesocosms suspended in the same body of water keep the same temperature, and the variation between individual mesocosms is reduced.

Such submerged mesocosms, incorporating a benthic habitat, are often set up in shallow lakes and the lake bottom itself becomes part of the experimental unit (Baulch et al. 2003). The mesocosms are open at the bottom and the mesocosm wall is then pushed into the sediment or held at the lake bottom with weights. This creates an enclosed space, but slow water exchange between the mesocosm and lake is still possible. However, this forecloses the use of important experimental stressors, such as pesticides.

There are several methods for heating mesocosms, whose advantages and disadvantages are presented by Netten et al. (2008). Electric heating elements are often positioned near the bottom of the enclosures (Liboriussen et al. 2005). Even with the heating close to the sediment, it will not introduce much warmth into the sediment, due to the warm water rising upwards. Additionally, differences in the performance and calibration of heating elements between the individual mesocosms may further affect the “background noise” of experiments.

In many experiments, air bubbling by pumps is used to mix the water layers. However, this has the potential to seriously disturb and alter planktonic communities. For this reason, paddles or discs are often used for mixing in plankton experiments. These would however most likely damage the macrophyte community in a benthic habitat.

To avoid temperature stratification and local heating artefacts that can arise in individually heated mesocosms, suspended mesocosms can be heated by regulating the temperature of the surrounding water. This method avoids the need for individual heating elements within enclosures and for artificial mixing.

For our study, we combined different methods, tailoring the design to mimic shallow freshwater habitats including benthic–pelagic coupling and allow for the safe, precise and reliable exposure to multiple stressors.

Here, we present the design of this new mesocosm system developed to test the influence of warming and agricultural runoff, composed of nutrients and pesticides, on small shallow water bodies. We explain details on the construction and show selected

results of an 8-week experiment running from May to July 2019. Based on this, we discuss the main advantages and limitations of our setup.

2. Methods

We constructed 16 benthic–pelagic mesocosms that were suspended in two artificial concrete ponds. One pond was left at ambient temperature and the other was continuously heated to +3°C above ambient temperature. Each mesocosm contained a complex community of primary producers and consumers. Each set of eight mesocosms was exposed to a gradient of a mixture of substances representing agricultural runoff. The gradient approach might provide quantitative output that can be incorporated into ecological models more effectively than ANOVA output (Cottingham et al. 2005), in particular when observing non-linear systems with sudden shifts (Kreyling et al. 2018).

The experiment took place from April until July 2019 using the outdoor mesocosm facility at the Biocenter Planegg-Martinsried of Ludwig-Maximilian-University Munich. Part of the offered infrastructure are two artificial concrete ponds, each with an overflow drainage connected to the sewage system and a size of 6 x 4.5 m and 1.5 m depth (40.000 L).

We used two industrial heating elements (48 KW, ISA-Heinrich-Industrietechnik, Falkensee, Germany) to warm one of the two ponds. A temperature probe was installed in each pond within the upper third of the water column and connected to a computer controller. The temperature in the heated pond was regulated in a 5-minute frequency to stay +3°C above the temperature of the ambient pond at all times. To evenly distribute temperature across the whole pond and circulate water around the mesocosms, a submersible pump (12.000 L/h) was installed in each pond. The heating setup was already successfully used for a pelagic mesocosm experiment (Diehl et al. 2021, in press).

2.1. Construction

The mesocosms themselves (Fig. S1.1a) were welded from LDPE Innoplast silo foil 125 µm (BayWa, Germany). This makes them completely sealed to the surrounding water of the heating ponds but enables fast temperature adjustment between the inside and outside water.

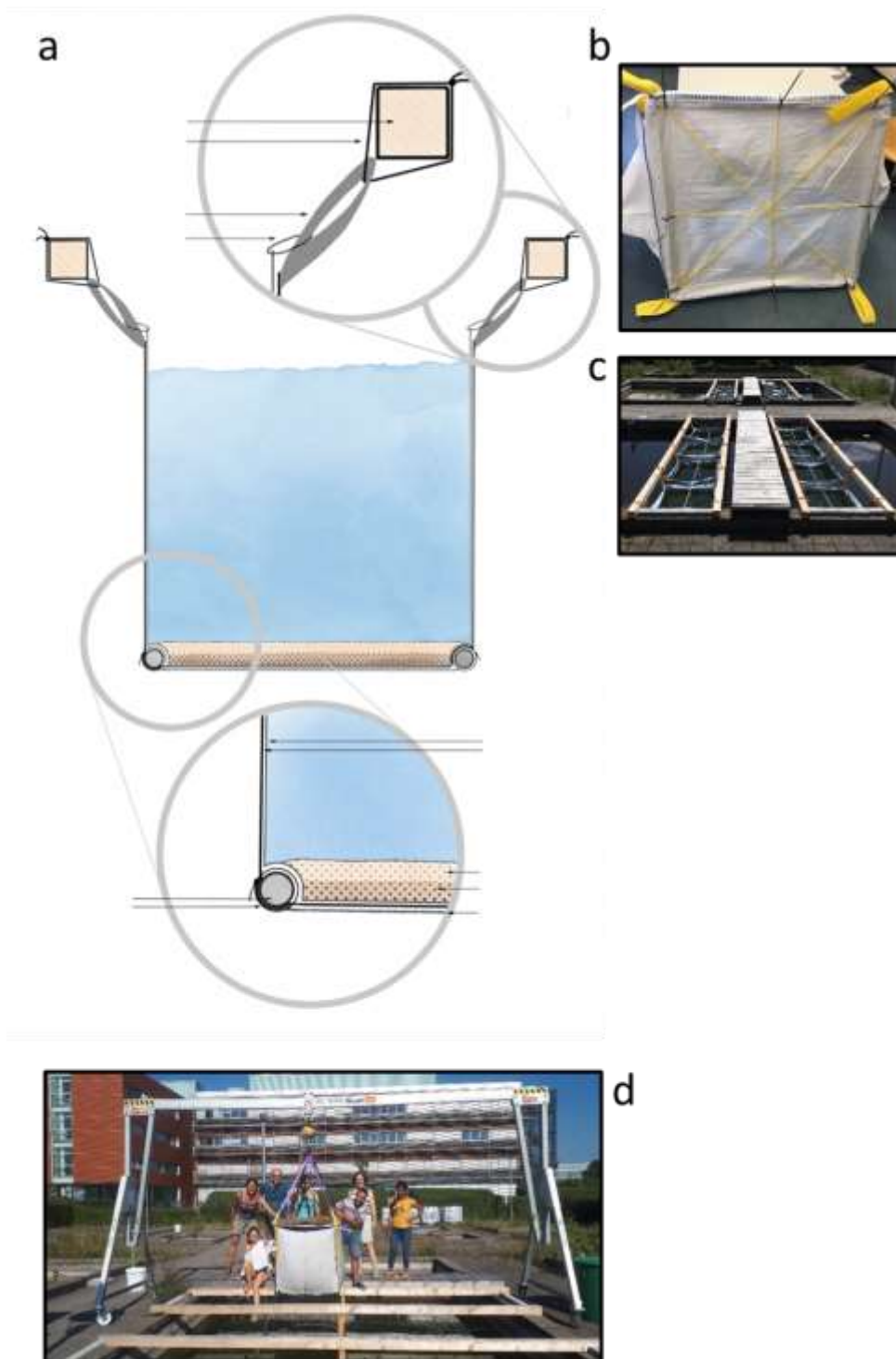


Fig. S1.1. Mesocosm construction. (a) illustration of an enclosure with the materials labelled: 1 - inner layer (Silofoil); 2 - outer layer (BigBag); 3 - PVC pipe; 4 - cable tie; 5 - plastic string; 6 - garden soil and sand mixture; 7 - sand layer; 8 - BigBag carrying loop; 9 - plastic rope; 10 - wooden beam; (b): string and PVC construction stabilizing the mesocosm bottom; (c): picture of all mesocosms in the concrete ponds; (d) the gantry crane taking out an enclosure at the end of the experiment.

The weight of the sediment necessary to incorporate a functional benthic habitat into this mesocosm experiment needed additional support. The cubic silo foil bags were

therefore built with an edge length of 86 cm. These fitted perfectly into customary normed “BigBags” (ISO 21898:2004), which are constructed to bear a weight of up to 1000 kg and have carrying loops which can be used to hang them from a support structure.

Wooden beams with 10 cm cross-section traversed the two concrete ponds and held the rim of the BigBags above the water surface. For additional support, a brace was added in the middle of the pond under each beam. A wooden bridge crossing each pond was used for easier access to the mesocosm.

We chose not to cover the mesocosms because we did not want to impede natural sunlight. They were open to the atmosphere, natural precipitation and evaporation, hence also invasion by aquatic insects.

To follow evaporation and precipitation, buckets filled with water and equipped with a scale calculated relative to the surface area of the enclosures were hung in the heated and unheated ponds. The same amount of fresh water from a deep groundwater well was added to all enclosures relative to the water loss observed in the buckets. This way, the stronger evaporation in the heated pond was still in effect.

The sediment was added into the enclosures before they were lowered carefully into the water using tension straps. An additional support at the bottom of the enclosures made from a square frame of PVC pipes and plastic string (Fig. S1.1b) kept the sediment layer flat and equally deep across the plane (Fig. S1.1a). A total of 80 L sediment were used per enclosure, resulting in a layer of 10 cm thickness. It consisted of a bottom layer of well mixed sand and garden soil (50/50 [v/v]) covered by a top layer of sand (Spielsand 0-1 mm and Fortunat Humus 0/5 gesiebt, Bernhard Glück Kies-Sand-Hartsteinsplitt GmbH, Germany).

2.2. Setup schedule

The enclosures were filled with 590 L well water each, resulting in a 70 cm water column. During the filling process, the tension straps were loosened regularly to lower the bottom of the enclosure and keep it from tipping to one side. This was done one month (day -31) before the start of the experimental manipulations (day 0). In the meantime, all organisms were introduced to the mesocosms with enough time to acclimate and stabilize.

A natural phytoplankton community was introduced on day -30 by adding 50 L of water from the eutrophic Lake Bannsee (47°57'52.4"N 12°26'25.1"E) per mesocosm. Mesozooplankton was excluded by filtering through a 250 µm sieve. An area of 1 m² of gravel from Lake Klostersee (47°58'21.5"N 12°27'25.6"E) was scrubbed to gain a natural periphyton community inoculum. This was added in equal shares to all mesocosms the same day. Choice of the inoculum sources was based on diversity at that time point. We aimed for a diverse starting community, to prevent the exclusion of certain species from the beginning.

To compensate for the unbalanced N:P dissolved nutrient ratio (1500:1) of the well water, a single pulse of 9.8 µmol/L P was given to all enclosures to reach a ratio (22:1) more suited for fast initial phytoplankton growth. Regular fertilization by 1 µmol/L P and 16 µmol/L N was done three times per week to simulate nutrient influx from the catchment area.

Three macrophyte species (*Myriophyllum spicatum*, *Elodea nuttallii* and *Potamogeton perfoliatus*) were added to each mesocosm using a total of 35 stems. Each apical shoot was cut to 10 cm stem length, and side shoots were removed. Using a soft brush, they were cleaned of snails and other invertebrates and planted in the sediment on days -16 and -15 with the help of 70 cm long forceps (Eheim, Germany). Ten stems of *M. spicatum* (6 g fresh weight (FW)), 10 stems *P. perfoliatus* (11 g FW) and, due to lower biomass per shoot, 15 stems of *E. nuttallii* (7.5 g FW) were planted as patches in the same quadrant in each mesocosm to ensure comparability between treatments.

The primary producers had 12 days to acclimate before the consumers were introduced. On day -4, 10 individuals each of *Lymnaea stagnalis* and *Dreissena polymorpha* were added per enclosure. Beforehand, their shell length had been measured and the groups sorted by size, so that each mesocosm had the same average size of molluscs (10.7 mm for *L. stagnalis* and 20 mm for *D. polymorpha*). On day -2, a natural zooplankton community was introduced into all enclosures. They were caught at Lake Klostersee using a 250 µm net.

2.3. Sampling

Additional to the organisms, a variety of sampling equipment was installed in each mesocosm. Transparent polypropylene strips with a rough surface, each 2.5 x 60 cm (GBC, USA), were hung along the middle axis (north–south) as growing substrate for periphyton,

which also facilitated and standardized periphyton sampling. For high temporal resolution (5 min) of temperature and oxygen measurements, miniDOTs (miniDOT, PME) were suspended at half height of the water column. On top of the sediment in the middle of the macrophyte-free quarter, HOBOs (HOBO MX2202Pendant, onset) were positioned for continuous light intensity and additional temperature measurements. Before and after the experiment, all data loggers were checked for synchronicity. Net cages containing cotton strips to compare degradation of organic matter among mesocosms were placed on the sediment.

Water samples integrating most of the water column were taken with a tube sampler twice a week, and phytoplankton chlorophyll-*a* content was measured using a multispectral fluorometer (AlgaeLabAnalyser, bbe Moldaenke GmbH, Germany), beforehand calibrated with selected laboratory strains of different algal groups. Phytoplankton C, N and P content, pH and water chemistry parameters were also measured. One of the bi-weekly water samples was used for pesticide analysis. To detect any leakage from the mesocosm, additional water samples were taken from the surrounding water after one week and at the end of the experiment. Periphyton biomass, macrophyte length and zooplankton abundance were evaluated regularly during the experiment.

For the final sampling, each mesocosm was taken out of the ponds with a gantry crane, and all organisms of interest were sampled carefully and as completely as possible. To sample zooplankton and invertebrates, the whole water column was drained through a 250 µm sieve. Remaining invertebrates were picked from the walls, plants and sediment. All samples were then fixed in 96% ethanol for later counting and identification of species.

All macrophytes were gently removed including the roots, rinsed and evenly spread on large white plastic sheets for visual inspection. To determine how warming affected the plant reproduction, the number of flowers from ambient and warmed mesocosms was counted. The flowering stage was also noted as beginning, when the buds just appeared, intermediate, during the development, and complete, when the full flower had developed. Finally, all plants were dried and weighted.

3. Assessment and Discussion

3.1. Abiotic parameters and temperature control

Our measurements showed that the heating system worked reliably during the whole experiment. The desired temperature difference was reached within one day and was held reliably at $3.1 \pm 0.27^\circ\text{C}$ throughout the experimental duration of 8 weeks (Fig. S1.2). Even during a heat wave at the end of June, with ambient water temperatures reaching up to 27°C , the temperature difference between the treatments was maintained. With our design, we were able to circumvent two of the main disadvantages of an electrical heating system mentioned by Netten et al. (2008), namely local heating artifacts and no diel or seasonal temperature profile. Seasonal as well as diurnal temperature fluctuations were followed well within the mesocosms of both temperature treatments (Fig. S1.3).

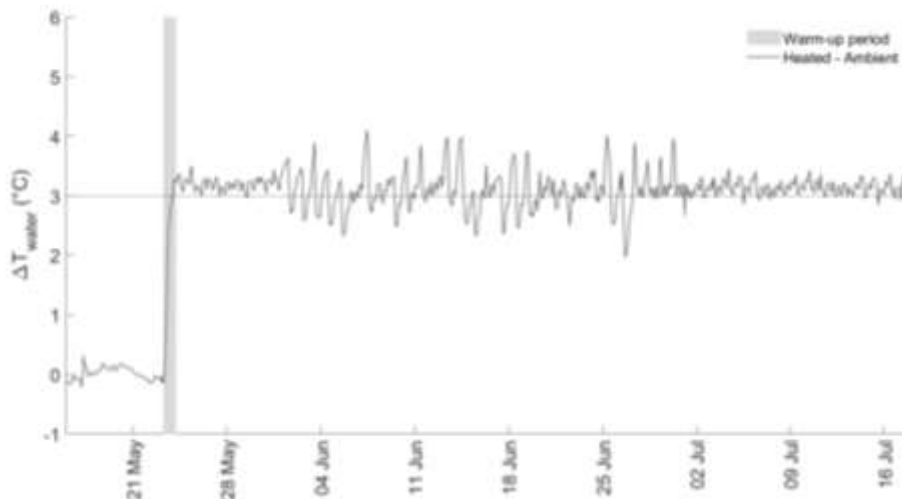


Fig. S1.2. Temperature difference between ambient and heated enclosures shown for the entire experiment.

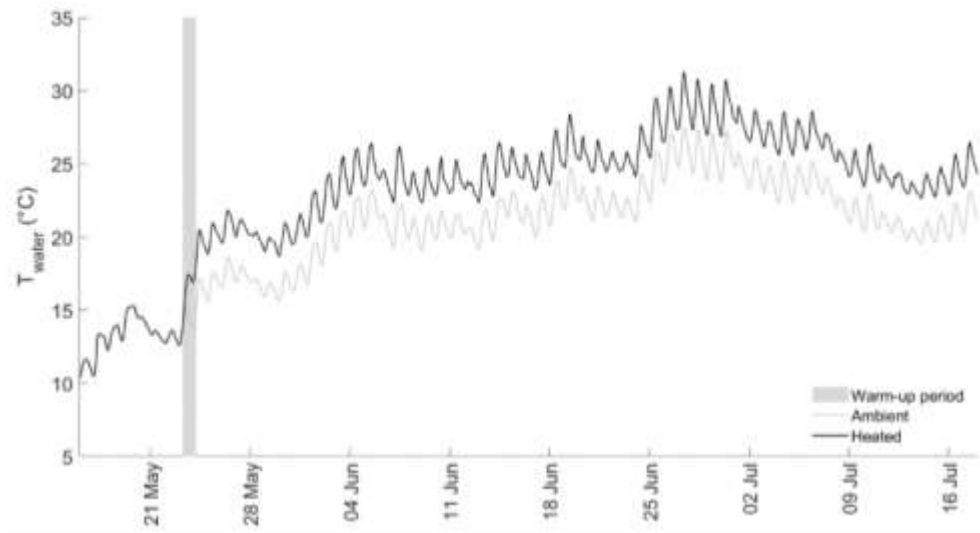


Fig. S1.3. Temperature profile of all 16 mesocosms. Treatment application started on 24. May 2019. Area shaded in red shows the warm up time needed by the heating system to reach the desired temperature difference.

Day to night temperature difference manifested at 2.17 ± 0.83 °C in ambient and at 2.40 ± 1.06 °C in heated mesocosms. At the same time, the temperature differences between mesocosms of the same treatment at the same point in time were maintained within a narrow range of 0.30 ± 0.26 °C in the ambient and 0.23 ± 0.16 °C in the heated mesocosms.

Including the natural temperature fluctuations of the pond into the mesocosm design is an important step in meaningful mesocosm research (Wahl et al. 2015). It also vastly improves the possibilities of creating realistic models incorporating respiration and productivity calculations.

A complete record of water temperature measured at least once an hour, ideally at finer time scales, is fundamental to estimate daily whole-ecosystem metabolic rates. For this purpose, characterising diel fluctuations is necessary, as respiration rates are estimated using night-time values whereas daytime data is used to estimate gross primary productivity assuming daytime respiration levels are similar to those occurring during the night (Staehr et al. 2010). However, the need of high resolution oxygen measurements also comes with a need for fast temperature control without much delay between heated and unheated treatments as temperature has a large effect on oxygen levels. If, for example, increasing daytime temperatures in unheated treatments are only followed slowly by heated treatments, production and respiration measurement comparisons between treatments will

be biased. Our results show that our system was capable of transferring temperature changes in unheated controls very fast and accurately to the heated mesocosms (Fig. S1.3).

We performed high temporal (5 min) resolution measurements of oxygen saturation levels within each mesocosm. Notably, oxygen saturation oscillated between 100% to 200% most of the time. This over-saturation is common in water bodies with strong photosynthetic activity (Higgins 2014). During naturally occurring high temperatures end of June, oxygen concentrations dropped in many mesocosms, but oxygen minima never lasted long and did not reach unnatural levels (G. A. López Moreira M., unpublished).

Over the whole experiment, the evaporation per heated enclosure was roughly 40 L higher than in the ambient ones. This equals 6% of the total enclosure volume.

Pesticide concentrations in the water of the mesocosm decreased throughout the experiment, as chemicals were absorbed and degraded. At the end of the experiment, levels had reached approximately 10-20% of the original concentration. No pesticides in quantifiable amounts were found in the surrounding pond water. The omnipresence of pesticides in agricultural landscapes requires better understanding of their effects and therefore mesocosm designs that are suited for pesticide application. Mesocosms suspended in artificial bodies of water are a safer choice than those located in natural lakes or ponds.

3.2. Performance of biota

Primary producers were able to thrive in this mesocosm design. Right before treatment addition (day 0), the total chl-*a* had reached $40 \pm 11 \mu\text{g/L}$, and the chl-*a* levels did not differ considerably between mesocosms (Welch's t-test, $p=0.12$). Future heated and non-heated mesocosms were also not significantly different in their chl-*a* levels before start of the heating treatment. Mesocosm phytoplankton communities diverged in their species composition following treatment application. Overall, many different functional phytoplankton groups were present, including motile and non-motile, large and small, autotrophic and mixotrophic and colonial and single-cell species.

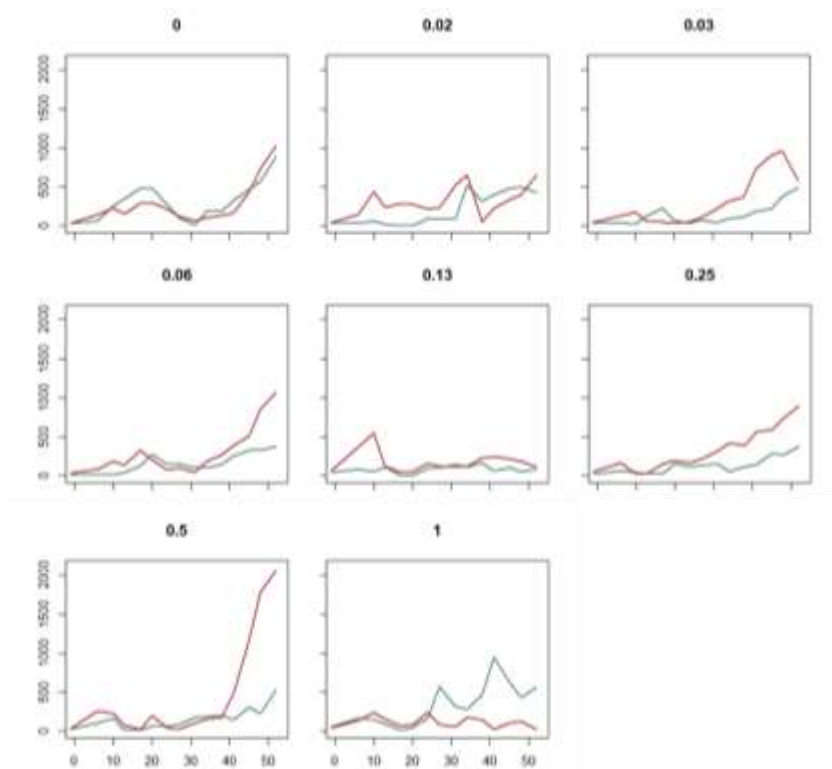


Fig. S1.4. Total phytoplankton (chl. a/L) over the course of the mesocosm experiment (experimental days on x-axis) for ambient (blue) and heated treatments (red) along the ARO gradient (0 to 1).

Multiple phytoplankton blooms and clear water stages could be observed, and high phytoplankton densities were reached (Fig. S1.4). Without the regular nutrient input, this would most likely not have been possible. Mesocosms offer a large surface for periphyton growth relative to the water volume. Consequently, nutrients often get locked in the periphyton which can dominate primary production in fishless mesocosms subject to strong zooplankton grazing (Kazanjian et al. 2018). Still, competition for nutrients with other primary producers cannot be excluded. Competition for light was most likely. Both phytoplankton-dominated and macrophyte-dominated states were reached in mesocosms within the experimental period (Fig. S1.5).



Fig. S1.5. Picture of two mesocosms, a macrophyte dominated on the left and a phytoplankton dominated on the right.

All three macrophyte species, which are commonly found in still or slow-flowing water, grew well in the mesocosms, indicating that they had sufficient nutrients in the water and sediment. Sampling of each macrophyte plant intact and from the root was possible following the dismantling of the mesocosm units, removal of water up to the sediment surface, and gentle uprooting. The mesocosm design allowed ample space for *P. perfoliatus* to spread its underground rhizome and develop ramets. Total lengths of the rhizome measured in all directions reached an average of 313.9 ± 113.7 cm, and the number of ramets averaged 62 ± 18 . *M. spicatum* dominated and formed a canopy on the surface (tallest shoots, ~110-120 cm). This was followed by *E. nuttallii*, whose growth was dense and spread horizontally but did not reach the surface (averaging ~60 cm), and *P. perfoliatus* whose ramets reached the water surface (tallest shoots, ~70-80 cm). Only *M. spicatum* developed flowers. They started to bloom 6 weeks post-exposure in all mesocosms. However, the higher temperature in the warm mesocosms affected macrophyte reproduction, with 77% more flowers developing in the warmed mesocosms compared with the ambient. In particular, the total number of flowers in the starting and middle

stages were higher in the warmed microcosms, with increases of 275% and 70%, respectively, compared with the ambient (Fig. S1.6). Our heating system therefore allowed us to observe changes in flowering phenology, which have been indicated to occur due to warming (Sparks et al. 2000; Gillespie et al. 2016).

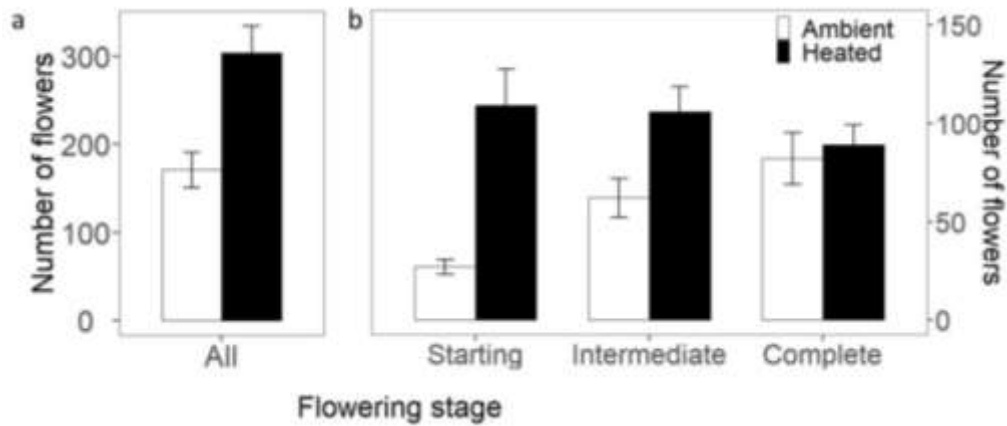


Fig. S1.6. Effect of temperature on *M. spicatum* flower development. (a) Total number of flowers, and (b) number of flowers in starting, intermediate and completed stages. The bar plots were created using the number of flowers across all ambient and heated mesocosms, Mean \pm SD, $n = 8$.

Leaving the mesocosms uncovered enabled multiple species of insects to lay their eggs and invade the system. Predatory insect larvae resulted in further food web complexity and thereby affected our primary producer and primary consumer system. We were able to sample all macroinvertebrates in detail during the final sampling, when the enclosures were removed from the ponds.

3.3. Costs

Due to the pre-existing infrastructure at the experimental location, this mesocosm setup was very cost-effective. The mesocosms can be self-assembled from affordable materials. Renting the gantry crane accounted for a large portion of the total material resulting in costs of roughly 2500€.

Probes for continuous measurements, sampling and analysis equipment as well as costs for waste disposal are not included in this calculation, as they will vary depending on the experimental goal.

4. Conclusion

The described mesocosm design distinguishes itself from other setups by combining a unique array of advantages to provide a suitable tool in shallow lake research for multiple stressor studies combining climate warming or heat wave scenarios with chemical stressor application. The inclusion of benthic–pelagic coupling in the mesocosm ecosystem increases the validity of experimental results in studies where high complexity is needed. Constant heating of the mesocosms to a set temperature above ambient by a heating system with quick reaction time preserves natural day–night fluctuations as well as the seasonal temperature profile in all treatments. Heating of the suspended mesocosms by adjusting the temperature of the surrounding water allows even heating of all mesocosms without local heating artifacts and no need for artificial mixing, which makes the inclusion of fragile species possible. The water-tight mesocosms suspended in artificial ponds allow for the application of chemicals and also allochthonous or invasive species without contaminating natural water bodies. Good accessibility during the experiments and the removal of the entire mesocosms from the ponds at the end of the experiment facilitate meticulous sampling. The cost-effectiveness and easy assembly of the mesocosms themselves make this setup easily replicable and a valuable tool in mesocosm research. The setup is flexible, and the shape and size of the BigBags used can be adjusted to match different experimental needs. Hence, new possibilities are opened for multiple stressor impact mesocosm studies including manipulations such as chemical contaminants, global warming or invasion scenarios, while including complex benthic–pelagic coupling.

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CHAPTER S2: DISENTANGLING THE DIRECT AND INDIRECT EFFECTS OF AGRICULTURAL RUNOFF ON FRESHWATER ECOSYSTEMS SUBJECT TO GLOBAL WARMING: A MICROCOSM STUDY

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Abstract

Aquatic ecosystems are subject to multiple stressors such as agricultural run-off (ARO) and climatechange-related increase of temperature. We aimed to determine how ARO and the frequency of its input can affect shallow lake ecosystems through direct and indirect effects on primary producers and primary consumers, and whether warming can mitigate or reinforce the impact of ARO. We performed a set of microcosm experiments simulating ARO using a cocktail of three organic pesticides (terbuthylazine, tebuconazole, pirimicarb), copper and nitrate. Two experiments were performed to determine the direct effect of ARO on primary producers (submerged macrophytes, periphyton and phytoplankton) and on the grazing snail *Lymnaea stagnalis*, respectively. Three different ARO concentrations added as single doses or as multiple pulses at two different temperatures (22°C and 26°C) were applied. In a third experiment, primary producers and consumers were exposed together to allow trophic interactions. When the functional groups were exposed alone, ARO had a direct positive effect on phytoplankton and a strong negative effect on *L. stagnalis*. When exposed together, primary producer responses were contrasting, as the negative effect of ARO on grazers led to an indirect positive effect on periphyton. Periphyton in turn exerted a strong control on phytoplankton, leading to an indirect

negative effect of ARO on phytoplankton. Macrophytes showed little response to the stressors. Multiple pulse exposure increased the effect of ARO on *L. stagnalis* and periphyton when compared with the same quantity of ARO added as a single dose. The increase in temperature had only limited effects. Our results highlight the importance of indirect effects of stressors, here mediated by grazers and periphyton, and the frequency of the ARO input in aquatic ecosystems.

Keywords: Multiple stressors, aquatic ecosystems, agricultural run-off, global warming, biotic interactions

1. Introduction

Aquatic ecosystems are exposed to multiple anthropogenic stressors (Jackson et al., 2016; Ormerod et al., 2010) partially originating from local sources such as agricultural activities (Ippolito et al., 2015; Malaj et al., 2014; Stehle and Schulz, 2015). Agricultural runoff (ARO) is not only responsible for high nutrient input, but it also contains multiple xenobiotics such as herbicides, insecticides, fungicides and trace metals that might affect ecosystems and interact with other stressors in an unpredictable way (Schriever et al., 2007). Nutrient and pesticide input to adjacent waterbodies through ARO is highly dependent on the strength and frequency of rain events and agricultural practices. Therefore, aquatic ecosystems can be exposed to multiple pulses of nitrates and pesticides with variable intensity (Daouk et al., 2013; Liess et al., 1999; Reinert et al., 2002), further complicating the assessment and prediction of ARO effects. Existing studies on ARO revealed severe effects on aquatic ecosystem processes such as primary production and ecosystem respiration (Peters et al., 2013). Multiple pulse exposure to toxicants and nutrients has been suggested to modulate the effects compared to a single or continuous exposure (Davis et al., 2019; Reinert et al., 2002; Varga et al., 2019). In addition to local stressors, global warming also affects aquatic ecosystems by influencing the metabolic rates of organisms (Brown et al., 2004; Gillooly et al., 2001) as well as their sensitivity towards stressors (Cuco et al., 2016; Debecker et al., 2017; Delnat et al., 2019). All the different stressors may have interactive effects, but determining how multiple stressors interact is challenging (Jackson et al., 2016), especially in complex systems where biotic interactions lead to indirect effects (Bruder et al., 2019).

Shallow lakes are ideal ecosystems to address the aforementioned challenges, as they are the most abundant waterbodies worldwide (Verpoorter et al., 2014), often exposed to ARO (Hijosa-Valsero et al., 2016) and prone to warming (Adrian et al., 2009). The state of a shallow lake ecosystem is shaped by biotic interactions among and between primary producers and higher trophic levels, and thus they respond non-linearly to nutrient loading with rapid shifts between two alternative stable states: a macrophyte-dominated clear-water state and a phytoplankton-dominated turbid state (Scheffer and van Nes, 2007; Scheffer et al., 1993). Such shifts have severe consequences for several ecosystem functions (Hilt et al., 2017) and services (Janssen et al., 2020). Both warming and exposure to ARO have been shown to affect macrophytes (Mckee et al., 2002), phytoplankton (Donald et al., 2011; Huisman et al., 2018; Paerl and Huisman, 2008), periphyton (Piggott et al., 2015) and primary consumers (Schaum et al., 2018; Zhang et al., 2018). Increasing temperature and

nutrient loading were predicted to increase the probability of a shift toward the turbid state (Moss et al., 2011; Scheffer et al., 2015), while the effects of other pollutants including pesticides, heavy metals, trace organics, pharmaceuticals and vanity products remained largely unexplored (Phillips et al., 2016). Considering the complexity of stressor and biotic interactions in shallow lakes, more experiments are thus warranted to understand the combined effects of warming and mixtures of pollutants on interacting communities, an essential prerequisite to mitigate the impact of global warming by controlling local stressors (Scheffer et al., 2015).

Here, we investigated in microcosms the combined effect of global warming and ARO on two trophic levels (primary producers and their consumers) representative of shallow lake ecosystems to determine the direct and indirect effects of these stressors. The effects of these stressors were evaluated on microcosms containing only the primary producers, only the grazers or both with the overall aim to gain a mechanistic understanding of direct and indirect effects of two major environmental stressors affecting shallow aquatic ecosystems. The three microcosm experiments were performed mimicking ARO containing nitrate, copper and typical organic pesticides with different exposure modes (addition of ARO by a single or multiple pulses) and warming following the IPCC RCP 8.5 scenario (+4°C, IPCC, 2014). We hypothesised that 1) ARO and warming will affect primary producer and consumer development. Particularly, we expected that ARO will cause a shift towards a phytoplankton dominance and that warming will increase this ARO effect. We also expected that 2) direct effects on some organisms will affect others indirectly through biotic interactions. We further hypothesise that 3) multiple pulses of ARO will favour the overall resistance of the ecosystem in comparison to a single pulse treatment with the same overall amount of pollutant added to the microcosms, because a more progressive input of toxicants would allow organisms to acclimate to this stressor.

2. Material and methods

Three microcosm experiments were performed. In Experiments 1 and 2, respectively, primary producers and grazers were exposed independent of each other to ARO and warming, while in experiment 3, they were exposed together to allow for trophic interactions. The microcosms were incubated at two different temperatures and exposed to a cocktail of pesticides, copper and nitrate designed to mimic the effect of ARO (see Table S2.1 for ARO composition). Six different ARO treatments were used: a control without ARO; three treatments corresponding to the ARO at the lowest concentration

(X), twice the lowest concentration (2X), and thrice the lowest concentration (3X) as a single addition at the beginning of the experiment; two treatments with two or three pulses of ARO at the lowest concentration (X) with four-day intervals between each addition (multiple pulse treatments XX and XXX, with the same final amount of ARO as in 2X and 3X, respectively). In experiment 2, with grazers alone, only the 1X and 3X treatments were used. In addition, the concentration of nitrates was reduced since nitrates would not be consumed in the absence of primary producers (Table S2.1).

Table S2.1. Final concentration of the contaminants in the microcosms. For the experiment 2, only 1X and 3X treatments were used.

	Single addition			Fractionated addition	
	1X	2X	3X	XX	XXX
Terbutylazine ($\mu\text{g L}^{-1}$)	1	2	3	2	3
Tebuconazole ($\mu\text{g L}^{-1}$)	30	60	90	60	90
Pirimicarb ($\mu\text{g L}^{-1}$)	5	10	15	10	15
Cu ($\mu\text{g L}^{-1}$)	14	28	42	28	42
N-NO ₃ (mg L ⁻¹)	3	6	9	6	9

The ARO cocktail was composed of terbuthylazine (CAS number 5915-41-3), a triazine photosynthesisinhibiting herbicide (Lorente et al., 2015); tebuconazole (CAS number 107534-96-3), a triazole fungicide known to affect biofilm and phytoplankton (Artigas et al., 2014); pirimicarb (CAS number 23103-98-2), a carbamate insecticide inhibiting acetylcholinesterase (Krieger, 2010); and copper (CuSO₄, CAS number 7758-98-7), a trace metal used in fungicidal and bactericidal and algicidal preparations (Lamichhane et al., 2018). All pesticides were PESTANAL® analytical standards (Merck, Darmstadt, Germany). Concentrations were based on published EC values (EC10, 20, 50) for each organism group, available SSD data and some unpublished data on the toxicity of all ARO components. From these data, we identified an effective concentration for each ARO pollutant (excluding nitrate) equivalent to the EC20 for chronic exposure for the most sensitive group, which was used as the highest concentration in the subsequent experiments (Table S2.1). Stock solutions of organic pesticides were prepared in Dimethyl sulfoxide (DMSO, CAS 67-68-5, Merck). The same amount of DMSO was added in all microcosms at a final concentration of 0.125%. Copper and nitrate (KNO₃) solutions were prepared in MilliQ water.

2.1. Experiment 1: Primary producers alone

In experiment 1, the microcosms contained submerged macrophytes, planted in artificial sediments, and microalgae. Macrophytes were introduced first to allow rooting before being submitted to competition with phytoplankton and periphyton. Algae were introduced in the microcosms 8 days before ARO addition and temperature increase, just after a complete water renewal to remove initial differences in algae and nutrient levels that had developed in the microcosms.

The microcosms were 10 L cylindrical glass aquaria (19 cm diameter, 40 cm height) filled with 8 L of Volvic® water and placed in a temperature-controlled room at 22°C with $70 \mu\text{mol m}^{-2} \text{s}^{-1}$ light at the water surface and a 16:8 h light:dark cycle. Sediments were placed in crystallising dishes (14 cm diameter, 10 cm height) inside the aquaria. Half of the microcosms were placed on heating mats regulated by a self-made thermostat programmed to maintain a 4°C temperature difference between the heated and unheated microcosms. Achieved temperatures were $22.5 \pm 0.4^\circ\text{C}$ (unheated) and $25.7 \pm 0.4^\circ\text{C}$ (heated microcosms). Deionised water was added three times a week to compensate for evaporation and to keep the volume constant. Nutrients were added three times a week ($224 \mu\text{g L}^{-1}$ N and $31 \mu\text{g L}^{-1}$ P, as NaNO_3 and KH_2PO_4 based on the Redfield ratio at $16 \mu\text{M}$ N and $1 \mu\text{M}$ P). Microcosm water was constantly aerated by slight air bubbling.

Three submerged macrophyte species common in freshwaters were used: *Myriophyllum spicatum* L., *Elodea nuttallii* Michx., and *Potamogeton perfoliatus* L. Macrophytes were collected from lakes and ponds without known important pollution sources (supplementary material Table S1). Two apical 10 cm shoot segments of each species (1.26 ± 0.16 g total macrophyte fresh mass per microcosm), made free of attached animals, were then planted in crystallising dishes filled with sediment 16 days before the addition of ARO. Sediments were prepared according to the OECD TG 239 (2014). As a source of nutrients for the plants, 5 g kg^{-1} of dry nettle powder was added to the sediments instead of watersoluble N and P salts. Each dish was filled with 1050 g of sediment mix covered by a 10 mm layer of sand.

Cultures of six strains of benthic microalgae (two cyanobacteria, two diatoms, and two green algae) and four strains of planktonic algae (two cyanobacteria and two green algae, supplementary material Table S1) were established in Volvic® water at 22°C under constant aeration with a 16:8 light:dark cycle and a $70 \mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance during the

light phase. Planktonic diatoms were not available for the experiment. Each microcosm was inoculated with a total biovolume of $1.0 \times 10^6 \mu\text{m}^3 \text{mL}^{-1}$ for benthic as well as planktonic strains, ensuring an equal total biovolume of $1.67 \times 10^5 \mu\text{m}^3 \text{mL}^{-1}$ for each benthic strain and $2.50 \times 10^5 \mu\text{m}^3 \text{mL}^{-1}$ for each planktonic strain. The microcosms were inoculated with only these ten species, but other species may have been introduced in the microcosms via plant epiphytes.

Three translucent polypropylene strips (297 x 25.4 mm, 300 μm thick, rough surface; PolyClearView, GBC, Chicago, USA) were suspended in the middle of each microcosm to monitor periphyton development. Approximately 21 cm of the plastic strip were constantly immersed in the water column.

Final sampling of all organisms and compartments was realised 28 days after ARO addition.

2.2. Experiment 2: grazers alone

The pond snail *Lymnaea stagnalis* was exposed to ARO at $22 \pm 0.2^\circ\text{C}$ and $26 \pm 0.2^\circ\text{C}$ in the absence of any primary producer. *Lymnaea stagnalis* was obtained from a nearby pond and bred in the laboratory for several generations to obtain a parasite-free population. Individuals were selected to minimise size differences, measured and weighed (mean shell length \pm SD = 13.7 ± 1.3 mm, mean total weight = 202 ± 58 mg). Each snail was placed in a 12-cm diameter, 30-cm high cylindrical glass aquarium filled with 1 L of Volvic® water with temperature control as in experiment 1. Snails were starved for 12 h before simultaneous addition of ARO and ten pre-weighed 12-mm diameter lettuce discs (organically grown). After 24 h, the remaining leaf discs were collected and freeze-dried. Additional lettuce discs were used to determine initial dry mass, and the dry mass of lettuce consumed by each snail was determined. The snails were then fed *ad libitum* with lettuce for 21 days. Water evaporation in the aquaria was compensated by addition of deionised water twice a week. The light cycle was 16:8 light:dark with only $5 \mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance to control possible algal development. At the end of the experiment, snails were starved for 12 h before a new feeding test was performed with 10 pre-weighed 14-mm diameter lettuce discs and stopped after 16 h to avoid complete consumption of the food provided. The snails were then measured and weighed.

2.3. Experiment 3: Primary producers and consumers

We used the same experimental set up as in experiment 1 but added one individual of *L. stagnalis*, one individual of zebra mussel (*Dreissena polymorpha*) and twenty *Daphnia magna* neonates in each microcosm. Snail individuals collected as in experiment 2 had an initial mean (\pm SD) shell length of 18.7 (\pm 2.2) mm and a mean total fresh mass of 0.4 (\pm 0.1) g including shell. Zebra mussels collected in a lake were placed in aquaria for 1 week before being transferred to the microcosms and measured using an electronic calliper (16.0 \pm 1.2 mm). Individuals were selected to be as homogenous in size as possible. *D. magna* were bred at the laboratory. Macrophytes, algae and primary consumers were introduced in the mesocosms 10, 7 and 3 days before addition of ARO, respectively, to allow for settlement without stressors.

In addition, several adjustments were made due to observations in the first experiment and changes in material availability. The organic pesticides were prepared as a 10-fold more concentrated solution, and therefore, the amount of DMSO added to the microcosms was ten times lower (0.01%). CaCO₃ at 7.5 g kg⁻¹ was added in the sediment of each microcosm as a buffer to limit potential effects of acidity on the primary consumers. An additional plastic strip was added. Mean water temperatures were 21.4 \pm 0.3°C and 23.7 \pm 0.3°C in the non-heated and heated microcosms, respectively. Final sampling was done 21 days after the addition of ARO as it corresponded to the maximal development of phytoplankton in experiment 1.

2.4. Measured parameters

At the end of each experiment, water was collected and filtered at 0.7 μ m (GF/F). In the filtered water, concentrations of nitrate and dissolved phosphorus were determined by ion chromatography (Dionex Ics-5000⁺, Sunnyvale, CA, USA) and by ammonium molybdate spectrophotometric method, respectively. The plastic strips were cut at 20 cm from the bottom, and all *L. stagnalis* eggs and juveniles present on the strips were collected and counted. Periphyton on these strips was scraped and collected with 20 mL of Volvic® water. From the resulting suspension, subsamples of 1.5 mL were centrifuged (10000 g, 10 min) in 2 mL tubes. The filters and pellets were stored at -80°C until photosynthetic pigment analysis.

Phytoplankton and periphyton pigments were extracted to estimate total planktonic and periphytic algae biomass and the proportion of diatoms, green algae and

cyanobacteria. Pigment extraction was carried out on periphyton and phytoplankton samples after freeze-drying by the addition of 2 mL methanol buffered with 2% ammonium acetate 1 M, followed by an initial sonication step (3 min at 4°C) and 15 min of incubation at -20°C, and then a second sonication step. Pigment extracts were recovered after centrifugation (10000 g, 5 min, at 2°C). The extraction steps were repeated until obtained extracts were not visibly coloured. All the extracts of the same sample were pooled. Extracts were filtered through a 0.2-µm PTFE membrane syringe filter (VWR International, United States) and analysed on HPLC. HPLC analysis of pigments was performed as described by Capdeville et al. (2019). Pigments specific to a particular group of primary producer (diatoms: fucoxanthin, diatinoxanthin and diadinoxanthin; green algae: chlorophyll *b*, lutein, violaxanthin and neoxanthin; cyanobacteria: zeaxanthin, echinenone and myxoxanthophyll) were used to assess variation of the different groups of microalgae. The total P concentration in periphyton biomass was determined following the ammonium molybdate spectrophotometric method after persulphate digestion in acidic conditions of freeze-dried periphyton subsamples.

Macrophytes were removed from the sediments and rinsed in water to remove attached particles and epiphytes. All the plants of one species were spread in a large tray equipped with a ruler, and a picture was taken. Roots and aboveground parts were separated and rapidly dried. Aboveground parts were then weighed to estimate macrophyte biomass. Visible signs of macrophyte consumption by grazers were recorded based on the pictures taken.

In experiment 3, the adult *L. stagnalis* were collected, weighed and measured. The *D. polymorpha* were collected and measured. The presence of dead *L. stagnalis* or *D. polymorpha* was recorded. Due to a rapid disappearance of *D. magna* in all microcosms, no measurement was made on *D. magna*.

2.5. Statistics

ARO concentration, exposure mode (single or multiple pulses of ARO addition) and temperature effects on the different measured parameters were analysed using an ANCOVA with ARO concentration as a continuous variable. The effect of exposure mode was analysed using only 2X/XX and 3X/XXX data with contrast analysis. The magnitude of the effects was measured using Hedges' *g* (Hedges, 1981) effect size calculated from *t*-

statistics derived from the ANCOVA according to Berben et al. (2012) using the R package {esc}.

To explore the simultaneous effect of ARO and warming on the interactions between macrophytes, phytoplankton, periphyton and grazers, we built a path analysis subjected to structural equation modelling (SEM; Grace et al., 2010). We considered all available variables to investigate the interactions among trophic levels. Using *a priori* knowledge, a full path analysis model was created including simultaneously several hypothetical pathways (Table S2.2) through which ARO and warming could affect macrophytes, phytoplankton, periphyton and grazers. We only used the biomasses of macrophytes, phytoplankton, periphyton and grazers from experiment 3 to perform this analysis. The different directions and kind of interactions among trophic levels were tested. Then, successive full models were simplified by step-wise exclusion of variables and/or linkages with non-significant weights and non-significant co-variance, as estimated by Akaike Index Criterion (AIC), until a minimal adequate model showing specific linkages remained. The best model was selected based on its coherence with the direct effect observed in experiments 1 and 2. The adequacy of the final model was determined using quality indicators such as Chi^2 , root mean square error of approximation (RMSEA), standardised root mean square residual (SRMR), adjusted goodness of fit index (aGFI) and comparative fit index (CFI) (Fan et al., 2016). SEM was performed using the R package {sem}.

Table S2.2. Links tested in the SEM.

Interaction	Effect	Hypothesis	Known direct effect
ARO → Macrophytes	+/-	Fertilisation by nitrates/toxicity of pesticides and Cu	
ARO → periphyton	+/-	Fertilisation by nitrates/toxicity of pesticides and Cu	Non-significant effect but slight positive trend observed in experiment 1
ARO → phytoplankton	+	Fertilisation by nitrates/toxicity of pesticides and Cu	Positive effect observed in experiment 1
ARO → Grazers	-	Toxicity of pesticides and Cu	Negative effect observed in experiment 2
Temperature → Macrophytes	+/-	Enhanced metabolisms/heat stress	No effect in experiment 1
Temperature → periphyton	+/-	Enhanced metabolisms/heat stress	No effect in experiment 1
Temperature → phytoplankton	+/-	Enhanced metabolisms/heat stress	No effect in experiment 1
Temperature → Grazers	+/-	Enhanced metabolisms/heat stress	Very limited effect in experiment 2
Grazer → biofilm	-	grazing	
Grazer → macrophytes	-	grazing	Few damaged macrophytes
Biofilm → phytoplankton	-	Light & nutrient competition	
Phytoplankton → periphyton	-	Light & nutrient competition	
Phytoplankton → macrophytes	-	Light & nutrient competition	

Macrophytes → phytoplankton - Light & nutrient competition

Macrophytes → periphyton - Light & nutrient competition

Biofilm → Macrophytes - Light & nutrient competition

3. Results

3.1. Experiment 1: Effects of stressors on primary producers without consumers

ARO exposure and elevated temperature had no effect on total macrophyte aboveground biomass (Fig. S2.1). The exposure mode did not change the effect on macrophytes (Fig. S2.1). The mean final macrophyte dry mass per microcosm was 0.61 ± 0.24 g with values for individual microcosms ranging from 0.11 to 1.23 g (Supplementary material Fig. S1).

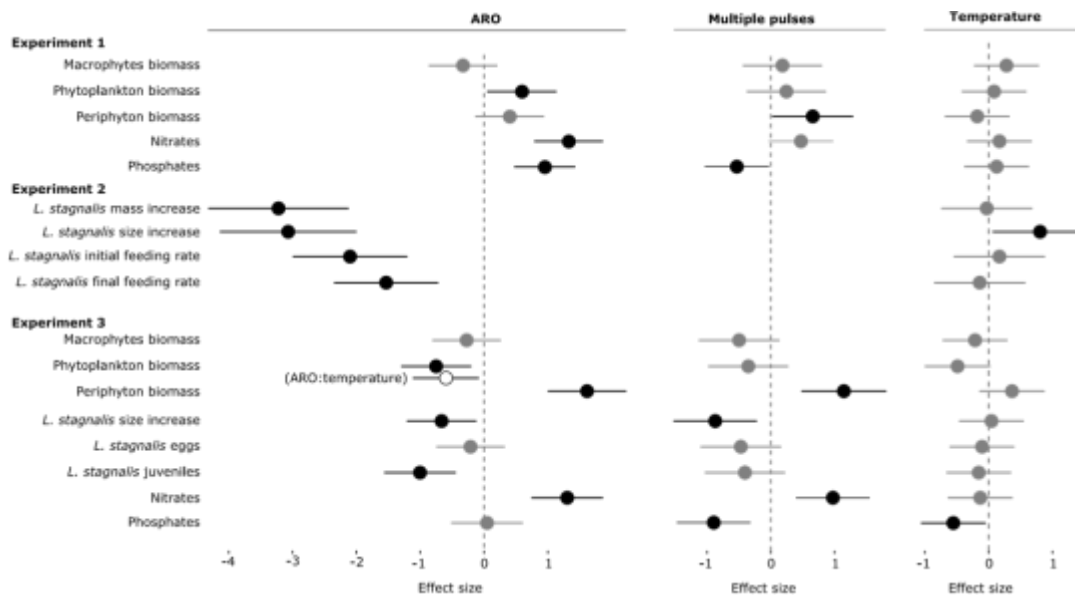


Fig. S2.1. Effect size (Hedges' $g \pm 95\%$ confidence interval) on biomass or growth parameters in each experiment for the three factors tested: ARO total concentration, exposure mode (i.e., single vs. multiple pulses for a similar ARO concentration) and temperature. Open symbols show interaction effect when significant. Grey symbols: non-significant effects, black symbols: significant effects.

Phytoplankton biomass varied between 2 and $238 \mu\text{g L}^{-1}$ Chl a and increased with increasing concentrations of ARO ($p = 0.041$; Fig. S2.1). Clear-water states with low phytoplankton ($< 50 \mu\text{g L}^{-1}$ of Chl a) were observed in all treatments, but highly turbid water with high phytoplankton ($> 100 \mu\text{g L}^{-1}$ of Chl a) was observed only at the two higher ARO concentrations. The exposure mode did not change the effect of ARO on phytoplankton. Warming had no significant effect on phytoplankton (Fig. S2.1) but favoured green algae (Fig. S2.2).

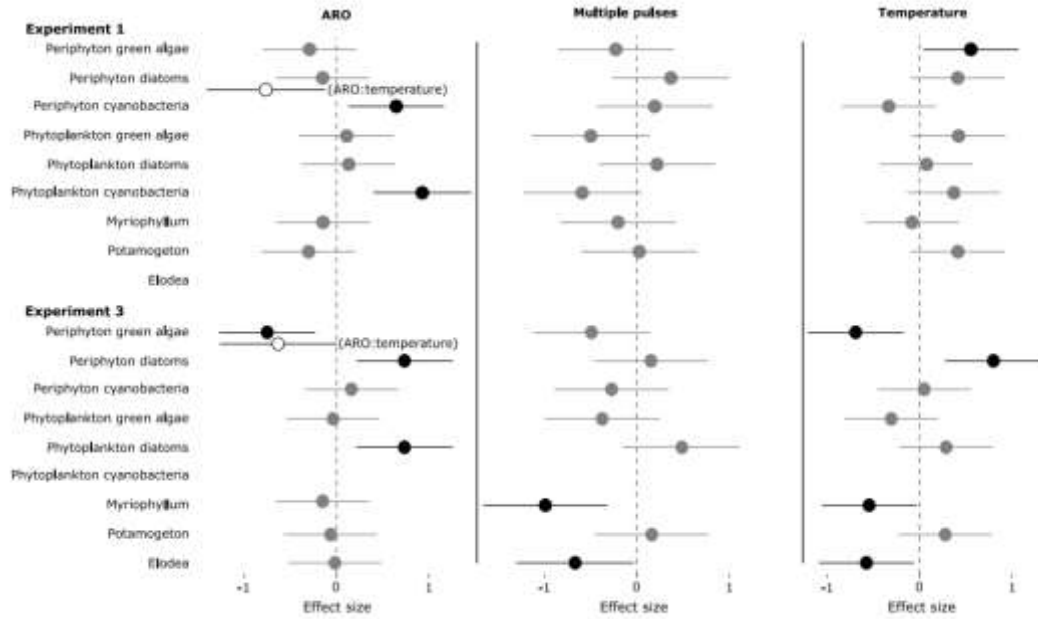


Fig. S2.2. Effect size (Hedges' $g \pm 95\%$ confidence interval) on the composition of periphyton, phytoplankton and macrophyte communities for the three factors tested: ARO total concentration, exposure mode (i.e. single vs. pulses for a similar ARO concentration) and temperature. Variations of green algae, diatom and cyanobacteria biomass was assessed by taxa specific pigment/Chl-*a* ratio. Macrophyte biomass was separated into *M. spicatum*, *P. perfoliatus* and *E. nuttallii* biomass. Open symbols show interaction effect when significant. Grey symbols: non-significant effects, black symbols: significant effects.

ARO exposure and warming had no significant effect on periphyton biomass, with a mean value of $1.08 \pm 0.71 \mu\text{g cm}^{-2}$ of Chl *a*. However, we observed a trend for higher periphyton biomass ($p = 0.060$) with ARO and a higher periphyton biomass with multiple pulses as compared to single additions of the ARO ($p = 0.046$, Fig. S2.1). ARO induced an increase of the cyanobacterial pigment/Chl *a* ratio in both periphyton and phytoplankton (Fig. S2.2). ARO had no significant effect on the total amount of P contained in periphyton (Supplementary material Fig. S5).

At the end of the experiment, concentrations of dissolved phosphate ($p < 0.001$) and nitrates ($p < 0.001$) were significantly higher in microcosm water with higher ARO additions. Repeated addition of ARO resulted in significantly lower dissolved phosphate concentrations compared to treatments with single ARO additions ($p = 0.04$), while only a marginal difference was recorded for nitrate concentrations ($p = 0.07$, Fig. S2.1).

3.2. Experiment 2: Effects of stressors on grazers

ARO exposure strongly reduced *L. stagnalis* growth with a significant effect on its increase in mass and length (Fig. S2.1). The snail mass increased by 603 ± 32 mg without ARO and by only 184 ± 44 mg at the highest ARO concentration. Warming positively influenced length increase but had no effect on snail mass (Fig. S2.1).

ARO induced a significant reduction ($p < 0.001$) of the consumption rates of *L. stagnalis* at the beginning of the exposure (from 8.14 ± 0.79 mg day⁻¹ in the controls to 2.68 ± 0.62 mg day⁻¹ in the 3X treatment). Similarly, after 3 weeks of exposure, consumption rates of *L. stagnalis* were 35% lower when exposed to the highest ARO dose (from 17.9 ± 1.9 mg day⁻¹ in the control to 11.6 ± 2.4 mg day⁻¹ at the 3X ARO concentration). No effect of warming on consumption rate was observed (Fig. S2.1).

3.3. Experiment 3: Effects of stressors on interacting primary producers and consumers

Macrophyte biomass did not respond to ARO addition (independent of the exposure mode) nor warming (Fig. S2.1). In this experiment, ARO concentration negatively affected phytoplankton biomass (Fig. S2.1), and these effects were stronger at the lower temperature indicating an interaction between ARO exposure and warming (Fig. S2.1). The highest phytoplankton biomasses were recorded in the cold control microcosms (cold: 84.8 ± 36.0 µg L⁻¹ Chl *a*, warm: 45.0 ± 14.4 µg L⁻¹ Chl *a*).

Conversely, ARO concentration was linked to a strong increase of periphyton biomass (Fig. S2.1). Periphyton biomass was very low in control conditions (0.22 ± 0.07 µg cm⁻² Chl *a* and 0.38 ± 0.19 µg cm⁻² Chl *a* for cold and warm temperatures, respectively) but was significantly higher with ARO, especially when added as multiple pulses (Fig. S2.1). At the highest ARO concentration, periphyton biomass reached 2.90 ± 0.61 µg cm⁻² Chl *a* and 3.51 ± 0.67 µg cm⁻² Chl *a* (in cold and warm microcosms, respectively) when ARO was added as multiple pulses (XXX), while it reached only 2.05 ± 0.60 µg Chl *a* cm⁻² (in cold) and 2.11 ± 0.78 µg Chl *a* cm⁻² (in warm) when ARO was added as a single dose (3X). ARO, and particularly exposure to multiple pulses, increased the amount of P contained on surfaces covered by periphyton ($p < 0.001$ and $p = 0.019$). Warming did not significantly affect final periphyton biomass ($p = 0.48$).

D. magna neonates only developed in six microcosms. ARO strongly affected the mussels, all of which died at the higher ARO concentration (independent of frequency of addition), while they survived in all control microcosms. No clear increase in mussel size was observed in any of the microcosms.

The snails survived in all the microcosms except one. A noticeable size increase ($65.8 \pm 35.4\%$) and the production of a high number of eggs (up to 489 eggs were counted in one microcosm, while several microcosms had no visible eggs) were observed. Juvenile snails were also observed, principally in the controls (supplementary material). ARO strongly reduced the growth of *L. stagnalis* (Fig. S2.1). Multiple pulses led to a higher reduction of growth than a single addition of ARO (Fig. S2.1). Warming had no significant effect on snail growth or reproduction. The number of snail eggs was not affected by ARO treatments, but the number of juveniles was strongly reduced by ARO concentration independent of the exposure mode (Fig. S2.1). Damage due to snail feeding on *P. perfoliatus* was observed in 80% of the controls; in 44%, 20% and 30% of the microcosms in the 1X, 2X and 3X treatments; and in 0% of the microcosms in the multiple pulses (XX and XXX) treatments.

ARO and warming induced a decrease of the proportion of green algae and an increase of diatoms in the periphyton (Fig. S2.2). ARO also caused an increase of the diatom pigment in the phytoplankton.

Warming negatively affected the growth of *M. spicatum* and *E. nuttallii* but not of *P. perfoliatus*. Repeated addition of ARO had a negative effect on macrophytes when compared with a single addition (Fig. S2.2).

Nitrate concentrations at the end of the experiment increased significantly with increasing ARO concentrations ($p < 0.001$). This effect was stronger in treatments with repeated ARO addition as compared to single additions ($p < 0.001$, Fig. S2.1). Concentrations of dissolved phosphate were lower in treatments with multiple pulses as compared to single additions. Warming led to significantly lower dissolved phosphate concentrations ($p = 0.02$, Fig. S2.1).

3.4. Structural equation modelling

A first SEM model was constructed including all possible relationships among measured variables and between the measured variables and experimental parameters

(Table S2.2, supplementary material Fig. S6). We did not find a significant relationship between warming and any measured parameter in the SEM performed, and therefore removed warming effects in the model. In the resulting model (supplementary material Fig. S6), only periphyton biomass and snail growth were directly affected by ARO. To explore further the direct and indirect effects of ARO on macrophyte or phytoplankton dominance, we used a third model considering only the effect of periphyton on phytoplankton and of periphyton on macrophytes without the respective reciprocal effects (Fig. S2.3).

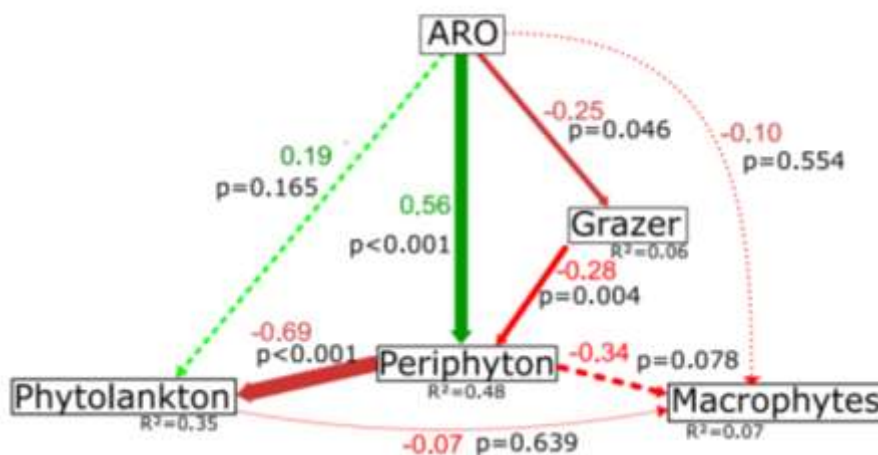


Fig. S2.3. Path diagram of the selected SEM model. Red arrows indicate negative relations between variables. Green arrows indicated positive relations between variables. Dashed arrows represent nonsignificant relations between variables.

Our model showed a strong positive effect of ARO concentration on periphyton development not only directly (path = 0.59, $p < 0.001$; Fig. S2.3) but also indirectly through the negative effect of ARO on periphyton grazers (Fig. S2.3). Indeed, ARO decreased grazer growth, which in turn reduced grazer consumption of periphyton leading finally to an increased periphyton development (Fig. S2.3). We also found a strong negative effect of periphyton on phytoplankton development (path = -0.69, $p < 0.001$; Fig. S2.3). Therefore, phytoplankton development was indirectly affected by ARO through its effect on periphyton. Our model further indicated no significant relationship between macrophyte biomass and other parameters.

4. Discussion

Our experiments with single trophic levels revealed that ARO facilitated phytoplankton growth probably due to a prevailing effect of nitrate, while it hampered

growth of the grazing snail *L. stagnalis*. This supports the initial hypothesis on direct effects of ARO on primary producers and consumers. However, when testing two trophic levels together, ARO effects on periphyton grazers changed the interactions among primary producers resulting in flourishing periphyton at the cost of phytoplankton. Repeated ARO addition at the same total amount increased these effects, while warming had no major impact.

4.1. Direct effects of ARO and warming on single trophic levels

To test our first hypothesis, experiments 1 and 2 were designed to determine the effect of the stressors without trophic interactions. We observed a positive effect of ARO on phytoplankton growth. This positive effect may be linked to the presence of nitrate in the ARO because it is consistent with what can be expected with nitrate addition in P-rich environments (Donald et al., 2011; Leavitt et al., 2006; Paerl and Scott, 2010). The low amount of remaining nitrate at the end of the experiment further supports a possible effect of nitrate. This increase in phytoplankton biomass did not lead to a strong negative competitive effect on macrophytes, as could be expected, and there was no shift to a typical phytoplankton-dominated state. However, such shifts can be expected under *in situ* conditions, when higher water depth and longer duration result in significant shading of macrophytes by phytoplankton.

The most remarkable direct effect of ARO was a strong inhibition of growth and feeding of the snail *L. stagnalis*. This is consistent with the known effect of pesticides and copper on this species (Amorim et al., 2019; Tufi et al., 2016). Indeed, Tufi et al. (2016) observed acetylcholinesterase inhibition on *L. stagnalis* exposed to 3.62 $\mu\text{g L}^{-1}$ of pirimicarb. In our experiment, pirimicarb concentration ranged from 5 to 15 $\mu\text{g L}^{-1}$. Tebuconazole and copper are linked to embryotoxicity in terrestrial snails (*Helix aspersa*; Druart et al., 2012). The highest concentration of Cu used in our study (42 $\mu\text{g L}^{-1}$) is in the range of the estimated EC50 on juvenile *L. stagnalis* growth rate in a 28-day experiment (Vlaeminck et al., 2019). Nitrate cannot be responsible for the negative effect observed because the concentration in our experiment was more than 50-fold below the toxic concentration for any freshwater mollusc (Soucek and Dickinson, 2012).

The hypothesis that warming will promote phytoplankton dominance could not be confirmed: warming did not affect primary producers nor snail feeding activity. This result is surprising because warming in the range of our experiments brings both chlorophytes and cyanobacteria closer to their optimum temperature (Lürling et al., 2013), and snail activity was expected to increase with warming in the tested range (Schaum et al., 2018; Zhang et al., 2018). However, a lack of warming effects on macrophytes was also reported in other studies, for instance, McKee et al., (2002) observed no temperature-induced change in the development of macrophytes in a 2-year mesocosm experiment with a 3°C warming above ambient temperature. Similarly, Feuchtmayr et al. (2019) found no effect on primary producers using a 4°C temperature elevation, in a 1-year experiment where temperature followed natural variations.

4.2. Importance of indirect effects mediated by biotic interactions in a more complex system

In experiment 3, including both primary producers and grazers, the net effect of ARO on phytoplankton development was negative and thus opposite to the single trophic level experiment, probably mainly due to the toxic effects of ARO on periphyton grazers that released periphyton from grazing pressure. By this mechanism, ARO had an indirect positive effect on periphyton, in addition to the direct positive effect of ARO on periphyton indicated by the SEM model. A strong negative relationship was also observed between periphyton and phytoplankton probably due to competition between the two functional groups for nutrients (more specifically for phosphorus because a decrease in phytoplankton biomass was observed while nitrate was added with the ARO).

The negative effect of ARO on *L. stagnalis* was reduced in experiment 3, where the microcosms also included primary producers, compared to experiment 2, where snails were exposed alone. Different factors may explain this reduced effect, notably, the other organisms present may also have led to a reduced exposure of snails due to increased adsorption and degradation of toxicants. Periphyton also represents a higher quality food than lettuce, leading to a decrease in the sensitivity to toxicants (Fidder et al., 2018; Vlaeminck et al., 2019). Beside effects of food quality, in the control microcosms, the available food in terms of periphyton biomass was very low. Snail growth may have been food limited, thus reducing the magnitude of the observed effect of ARO. This is supported by the fact that *L. stagnalis* grazing on *P. perfoliatus* was more frequently observed in the control microcosms than in treatments with higher periphyton biomass, indicating

a limitation in the availability of periphyton that they preferentially graze. This effect of resource limitation on snail growth may have led to an underestimation of ARO effects on *L. stagnalis*.

This may have also resulted in an underestimation of the indirect effect of ARO on periphyton mediated by snail grazing as well as a lower direct effect than estimated with the SEM. Moreover, this is supported by the fact that ARO did not strongly affect periphyton in experiment 1, so a strong direct effect is unlikely.

The inhibition of phytoplankton development by periphyton that we observed is consistent with other mesocosm studies that attributed this inhibition to allelopathic effects of filamentous algae (Trochine et al., 2011) or to nutrient competition (Kazanjian et al., 2018). Indeed, a higher periphyton biomass in ARO treatments retained more P, which was not available for phytoplankton development.

Theory predicts the increase of phytoplankton biomass and the regression of macrophytes in shallow lakes with a combination of eutrophication and warming (Kosten et al., 2012; Mooij et al., 2007; Moss et al., 2011; Scheffer and van Nes, 2007). In the present study, we did not observe direct or indirect effects of ARO or warming on macrophytes, and the effect of ARO on phytoplankton, although indirect, was negative. These discrepancies may have partly arisen from the specificities of our microcosms. Indeed, the low depth of the water column limits the ability of phytoplankton to exert a significant shading pressure that would have impeded macrophyte growth. In addition, while filter-feeding organisms were initially included in the microcosms, they did not develop. Therefore, no effective filter feeders were present, while in lakes, filter feeders are known to be able to reduce phytoplankton (Higgins et al., 2014). Inherent limitations of microcosm experiments, no predation pressure on primary consumers and the high surface-to-volume ratio of the microcosms promoting the development of periphyton have to be taken into account when extrapolating these results to shallow lakes.

However, these limitations have also reduced the complexity of the system and thereby the number of indirect effects but allowed to highlight the important role of periphyton and its grazers in controlling phytoplankton development. The role of periphyton and their grazers is not considered in classical models of alternative stable states in shallow lakes (Scheffer and van Nes, 2007; Scheffer et al., 1993), although shading effects of periphyton on macrophytes (Jones and Sayer, 2003; Phillips et al., 2016; Roberts

et al., 2003) and positive effects of snail grazing on macrophytes are known (Mormul et al., 2018). Our results support modelling studies (Genkai-Kato et al., 2012; Jäger and Diehl, 2014) indicating that periphyton can also control the development of phytoplankton. This suggests a key role of periphyton and their grazers in macrophyte-phytoplankton interactions that should be considered among the important factors controlling the alternative stable states in shallow lakes.

4.3. ARO additions by multiple pulses increase effects

Towards our third hypothesis, we expected that exposure to multiple pulses, compared to a single addition of the same quantity of compounds, would allow the system to remain below a critical threshold, thus allowing the organisms to acclimate and reducing the possibility of a shift. The negative effect of multiple pulses on snail growth may be attributed to a delayed recovery due to the multiple exposures. A positive effect on periphyton development was also observed, this latter effect probably being mostly due to the effect on grazers. However, a similar nearly significant trend was observed in the first experiment. Therefore, it might be, partially, a direct effect of ARO (particularly of the nitrate). A few studies have considered single or repeated additions of toxicants. Pulses of herbicide have been suggested to modulate the impact on macrophytes and algae of a subsequent exposure, while it depends strongly on the ability of the organisms to recover from previous exposure (Valloton et al., 2008, 2009; Varga et al., 2019). The stronger effect of the multiple-pulse exposure on the grazers and periphyton can be due to a rapid degradation of the pesticides or an interaction between pesticides or copper and compounds present in the microcosms (e.g. extracellular polymeric substances produced by biofilm) or changes in copper redox states (Coutaud et al., 2018) that limit the ARO effect. In an experiment with similar microcosms, using ARO concentrations equivalent to our 3X treatment but with only the primary producers present, 54% of terbuthylazine, 67% of pirimicarb and 88% of tebuconazole were recovered in the water after 20 days (data not shown). It is therefore important to consider that ARO input to lakes as multiple pulses can possibly increase the effect of agricultural runoff. Indeed, multiple pulses can better reflect the real contamination patterns of aquatic ecosystems (Daouk et al., 2013; Reinert et al., 2002).

5. Conclusion

Direct positive effects of ARO on phytoplankton were completely reversed by indirect effects mediated by biotic interactions involving periphyton and grazers. Beside suggesting a major influence of periphyton and grazers on macrophyte-phytoplankton competition in shallow lakes, this study highlights the importance of taking into account biotic interactions when studying the effect of multiple stressors in ecosystems. Moreover, the exposure to ARO through multiple pulses had an increased effect compared to single additions, supporting the use of more realistic exposure modes in aquatic stress ecology.

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Introduction

Les lacs peu profonds constituent le type le plus abondant de systèmes d'eau douce. Ils se caractérisent par un état dit « d'eau claire » dominé par les macrophytes, synonyme de bon état écologique permettant d'assurer de nombreux services écosystémiques et environnementaux, ou bien un état dégradé dit « d'eau turbide » dominé par le phytoplancton. Les macrophytes submergés jouent un rôle central dans la stabilisation de l'état de l'eau claire en séquestrant les nutriments en excès (Barko & James, 1998 ; Tang et al., 2016), en limitant la remise en suspension des sédiments, en offrant un refuge au zooplancton, en augmentant la sédimentation du phytoplancton peu mobile et en produisant des composés chimiques allélopathiques, qui sont des inhibiteurs de la croissance du phytoplancton (Hilt & Gross, 2008 ; van Donk & van de Bund, 2002). Différents facteurs peuvent altérer le développement des macrophytes et entraîner leur disparition au profit du phytoplancton. Le périphyton peut aussi jouer un rôle clé dans ce basculement vers la dominance du phytoplancton en recouvrant les macrophytes ou en limitant la disponibilité des nutriments (Phillips et al., 1978).

Les lacs peu profonds sont exposés aux pesticides et nutriments à la suite du ruissellement agricole (RA). Ces produits chimiques atteignent les lacs en fonction de l'intensité et de la fréquence des événements pluvieux. Ils peuvent être nocifs pour les macrophytes en agissant sur les plantes directement ou indirectement. Concernant les nutriments, le rôle du phosphore dans l'eutrophisation a été largement étudié, comparativement au nitrate dont le rôle a longtemps été négligé (Moss et al., 2013). Des études récentes montrent que le nitrate peut affecter la croissance des macrophytes et la richesse en espèces (Barker et al., 2008 ; Sagrario et al., 2005). De plus, il a également été démontré que le nitrate favorise le développement du périphyton, limitant la disponibilité de la lumière et des nutriments pour les macrophytes (Phillips et al., 1978). Concernant les pesticides, ils peuvent affecter les macrophytes directement ou indirectement selon leur mode d'action. Par exemple, les herbicides vont agir directement en inhibant la photosynthèse, modifier la perméabilité cellulaire ou réguler la croissance de certaines parties de la plante (Mohaupt et al., 2020) tandis que les insecticides peuvent perturber les interactions biotiques, induisant un changement dans la pression de broutage contrôlant le développement du périphyton ou du phytoplancton (Cuenca Cambronero et al., 2018 ; Schäfer et al., 2011), ou encore les

fongicides peuvent affecter des fonctions écosystémiques clés telles que la dégradation de la litière (Münze et al., 2015). Enfin, le réchauffement climatique agit également comme un stress pour les lacs peu profonds. Des températures élevées peuvent affecter les macrophytes directement ou indirectement en favorisant la croissance des macrophytes ou du phytoplancton, en induisant la libération des nutriments contenus dans les sédiments ou en modifiant la pression de broutage contrôlant le développement des microalgues (Hansson et al., 2012 ; Jeppesen et al., 2009 ; Müller et al., 2018). Les pesticides, les nitrates et le réchauffement peuvent agir tous simultanément dans les lacs peu profonds. Ensemble, ils peuvent avoir des effets additifs, synergiques ou inverses sur les macrophytes, qui ne sont pas faciles à prédire.

En raison de leur rôle dans la stabilisation des lacs, les macrophytes ont été utilisés dans le suivi de l'état environnemental et écologique des masses d'eau (EU WFD, 2000/60/EC). Par exemple, les réductions de la biomasse et/ou de la diversité des macrophytes sont des indicateurs d'une résilience réduite de l'état dominé par les macrophytes, par exemple, induit par des concentrations élevées de nutriments dans le système aquatique. Un indice basé sur l'abondance de certaines plantes aquatiques et le recouvrement total a été développé pour les rivières (IBMR, Haury et al., 2006) et les lacs (IBML, Boutry, 2015). Au-delà de ces indicateurs, les macrophytes sont également connus pour répondre au stress nutritif, lumineux, chimique et biotique en modifiant leur physiologie (Barko & James, 1998 ; Fornoff & Gross, 2014 ; Gross, 1999 ; Nuttens et al., 2016 ; Sterner & Elser, 2002). Cette grande plasticité phénotypique leur permettant de réagir rapidement aux changements de l'environnement, elle offre des perspectives intéressantes pour le diagnostic écologique des milieux aquatiques, en particulier la disponibilité des nutriments et de la lumière, ou le stress biotique et abiotique. Ainsi la stœchiométrie élémentaire, les pigments photosynthétiques et les métabolites secondaires pourraient être utilisés en tant que signaux d'alerte précoce des changements de régime.

Cette thèse s'inscrit dans le cadre du projet franco-allemand CLIMSHIFT (financement ANR [Agence Nationale de Recherche, France] & DFG [Deutsche Forschungsgemeinschaft, Allemagne] ; 2018-2022) dont l'objectif a été de définir des espaces de fonctionnement sécurisés pour les lacs peu profonds exposés à de multiples facteurs de stress. Pour atteindre cet objectif, plusieurs expériences ont été réalisées en microcosme et mésocosme pour répondre à des questions concernant l'effet des facteurs de stress, y compris les effets individuels et combinés, les effets dose-réponse, le mode

d'exposition aux contaminants (à travers les sédiments par rapport à la colonne d'eau) et les effets directs et indirects de ces facteurs de stress. Ces expérimentations ont été le fruit d'une collaboration entre cinq partenaires en France (Metz et Toulouse) et en Allemagne (Leipzig, Berlin et Munich), chacun ayant une expertise spécifique en écologie ou écotoxicologie des systèmes aquatiques.

Au sein du projet CLIMSHIFT, le but de cette thèse a été d'étudier la réponse des macrophytes à l'effet combiné du ruissellement agricole et du réchauffement climatique. Les principaux objectifs étaient d'étudier (1) les effets individuels et combinés des nitrates, des pesticides et du réchauffement climatique sur les macrophytes ; (2) si un changement d'échelle opéré des microcosmes vers les mésocosmes préserverait la réponse des macrophytes ; et (3) si les effets de ces facteurs de stress pouvaient être détectés dans la physiologie des macrophytes.

Matériel et méthodes

Dans le cadre de CLIMSHIFT, des microcosmes contrôlés intégrant une communauté benthopélagique ont été conçus. Dans les microcosmes, les communautés comprenaient trois espèces de macrophytes submergés, des souches de culture de phytoplancton et de périphyton avec ou sans leurs brouteurs respectifs (daphnies, limnées et moules). Les macrophytes ont été *Myriophyllum spicatum*, *Potamogeton perfoliatus* et *Elodea nuttallii*, trois espèces qui coexistent communément dans des eaux stagnantes ou à faible courant où elles forment des canopées denses limitant la disponibilité en lumière pour le phytoplancton. Les communautés de microalgues comprenaient des espèces de cyanobactéries, de chlorophytes et de diatomées. Des biovolumes similaires de périphyton et de phytoplancton ont été ajoutés, correspondant à environ 0,5 mg L⁻¹ et 1 mg L⁻¹ de carbone organique par microcosme. Le choix des consommateurs primaires a été effectué selon le raisonnement suivant : les filtreurs *Daphnia magna* (crustacé) et *Dreissena polymorpha* (bivalve) peuvent contrôler le phytoplancton, *D. magna* se nourrit parfois de périphyton et le gastéropode *Lymnaea stagnalis* peut contrôler le périphyton et se nourrit parfois de macrophytes. Chaque unité expérimentale était constituée d'un contenant cylindrique en verre d'un volume de 8 L, contenant un insert de 900 mL rempli d'un sédiment artificiel (ligne directrice d'essai de l'OCDE pour *M. spicatum*, OCDE 2014). Les microcosmes ont été exposés à un ruissellement agricole prédéfini (RA : nitrate + un mélange de pesticides) pendant 3 à 4 semaines selon différents scénarios d'exposition dépendant de l'objectif de l'étude (voir paragraphe suivant). Le mélange de pesticides comprenait un fongicide

inorganique et organique, un herbicide et un insecticide. Les traitements ont été répliqués à température ambiante et +3 ou 4°C, selon la puissance calorifique. Chaque traitement, y compris le contrôle, a été répété 5 fois.

L'exposition aux RA dans les lacs peu profonds dépendant des événements pluvieux, différents scénarios d'exposition aux RA ont été testés : les producteurs primaires uniquement (Expérience 1) ou les producteurs primaires et leurs brouteurs (Expérience 2) ont été exposés à des doses uniques croissantes du RA (X, 2X et 3X) ou à des pulses appliquées avec un intervalle de 4 jours (XX et XXX). Pour démêler les effets des nitrates et des pesticides au sein du RA, les producteurs primaires et leurs consommateurs ont été exposés uniquement aux nitrates (NO₃), uniquement aux pesticides (PST) ou à la combinaison des deux (NO₃+PST = RA).

Les mésocosmes extérieurs permettent une plus grande complexité, reflétant plus fidèlement un scénario environnemental réel avec des facteurs biotiques et abiotiques variables. Les forts effets de stress observés dans les microcosmes peuvent être testés dans les mésocosmes pour déterminer la fiabilité des effets et fournir un meilleur moyen d'extrapolation. Pour ce faire, une expérience en mésocosme a été réalisée pendant 8 semaines sur la plateforme expérimentale de Martinsried (LMU, Munich). Une communauté benthopélagique a été utilisée, mais avec des inoculums naturels de périphyton et de plancton. Chaque unité expérimentale comprenait un sac à silo (LDPE Innoplast) rempli de 640 L d'eau avec une base de sédiments de 10 cm. Seize mésocosmes ont été utilisés pour exposer les communautés à une concentration unique de RA (gradient de 8 concentrations) à 2 températures (ambiante et +3°C). L'objectif était de déterminer si les effets observés dans des conditions contrôlées de laboratoire seraient conservés dans les conditions extérieures variables (Expérience 4).

À la fin de chacune de ces expériences, les macrophytes ont été délicatement retirés et leurs longueurs mesurées. Leur biomasse aérienne fraîche et sèche a été mesurée. Les feuilles de la partie apicale de la plante (10 premiers cm), connue pour être la plus active du point de vue métabolique, ont été utilisées pour des analyses de biomarqueurs physiologiques. Pour les microalgues, la chlorophylle-*a* a été utilisée comme proxy de la biomasse. La longueur ou le taux de croissance des limnées et la biomasse sèche des daphnies et du zooplancton ont également été mesurés. Le nombre de cellules bactériennes dans l'eau et le biofilm ont été comptés par cytométrie. Pour étudier la dynamique temporelle du système, un échantillonnage hebdomadaire de la chimie de l'eau (pH, saturation en oxygène et

conductivité), du phytoplancton (densité optique comme proxy de la biomasse) et du périphyton (fluorescence de la chlorophylle) a été effectué. Le nombre d'individus de daphnies a également été compté chaque semaine.

Selon l'expérience considérée, des ANOVA à 2 ou 3 voies ont été réalisées pour déterminer les effets individuels et combinés du RA et de la température (TMP) dans les expériences 1 et 2 ; et du NO₃, des PST et de TMP dans l'expérience 3. Un test post hoc utilisant les tests de Dunn ou de Tukey a permis d'identifier des différences de mode d'exposition (dose unique du RA vs impulsion). Une PERMANOVA a été appliquée pour déterminer les effets des facteurs de stress sur la distribution des groupes de producteurs primaires. Les tailles d'effet ont été calculées pour déterminer l'ampleur des effets. Des effets de stress synergiques, contraires ou additifs ont été identifiés sur la base des effets additifs prédits des facteurs de stress individuels. Des analyses en composantes principales ont été effectuées pour visualiser les principaux modèles de facteurs de stress. La modélisation par équation structurelle a permis d'identifier les effets directs et indirects des facteurs de stress et les interactions biotiques. Toutes les analyses ont été réalisées avec R (R Core Team, 2018).

Résultats

Tous les producteurs et consommateurs primaires ont été affectés par le RA et la température, mais la réponse dépendait de la configuration et des conditions expérimentales. Dans l'ensemble, le RA a soit réduit la biomasse des macrophytes, soit induit des changements physiologiques chez les macrophytes pour s'acclimater au stress. Le RA a toujours favorisé le développement des microalgues, soit du phytoplancton ou du périphyton. Le réchauffement a parfois favorisé la croissance des macrophytes. En présence du RA, cependant, les effets du réchauffement variaient d'une expérience à l'autre.

Les expériences 1 et 2 ont montré que l'ajout unique ou sous forme de pulses du RA ne modifiait pas significativement la réponse des macrophytes (Allen et al. 2021). Cependant, la présence de brouteurs (uniquement des limnées) a eu un effet sur les interactions entre producteurs primaires : en présence du RA, dans l'expérience 1 avec uniquement des producteurs primaires, le phytoplancton dominait sous RA, tandis que dans l'expérience 2 avec des producteurs primaires et des limnées, le périphyton dominait.

Dans l'expérience 3, *M. spicatum* a été l'espèce de macrophyte la plus dominante, *P. perfoliatus* et *E. nuttallii* poussant moins. Les pesticides seuls ont réduit la biomasse totale de macrophytes, du fait des effets importants sur *M. spicatum*. La biomasse de daphnies et le taux de croissance de *L. stagnalis* ont également été considérablement réduits dans les traitements contenant des pesticides (PST, RA). Le nitrate seul (NO₃) a réduit la biomasse de *M. spicatum* mais pas celle des macrophytes totaux. Le NO₃ a également induit une augmentation précoce de la biomasse du périphyton. En combinaison avec les pesticides, le nitrate a agi en synergie, faisant basculer le système vers une dominance phytoplanctonique avec une biomasse macrophytique réduite (Vijayaraj et al. 2022a ; voir chapitre 3).

Le réchauffement a légèrement favorisé la croissance des macrophytes et a considérablement favorisé la croissance de *M. spicatum*. La biomasse de *P. perfoliatus* a diminué lorsqu'elle était exposée au réchauffement, du fait de la compétition avec *M. spicatum*. Malgré l'effet positif marginal du réchauffement sur la biomasse totale des macrophytes, lorsqu'il est appliqué avec le RA, le réchauffement a inversé l'effet attendu en réduisant considérablement la croissance des macrophytes. Le taux de croissance de *L. stagnalis* et le nombre de bactéries dans l'eau ont également augmenté à des températures plus élevées.

Lorsqu'une communauté benthopélagique plus complexe a été exposée au RA et au réchauffement dans les mésocosmes extérieurs, certains effets observés dans les microcosmes ont été préservés. Le fort effet négatif des RA sur la biomasse de macrophytes avec une augmentation conséquente de la biomasse de phytoplancton a été observé dans les mésocosmes extérieurs mais seulement à température ambiante (Vijayaraj et al. 2022b ; voir chapitre 4). Le réchauffement a amélioré la croissance des macrophytes, semblable aux microcosmes. Cependant, le réchauffement a également considérablement atténué l'effet du RA, augmentant la biomasse de macrophytes et réduisant la biomasse de phytoplancton. Les interactions biotiques complexes dues à la plus grande richesse spécifique des communautés de microalgues benthique et de plancton ainsi que les invasions de larves d'invertébrés formant un troisième niveau trophique peuvent avoir inversées les réponses du système aux facteurs de stress. Dans cette expérience, la forte dominance des espèces de macrophytes n'était pas présente, les trois montrant une biomasse comparable. Cependant, comme dans l'expérience 3, la croissance de *M. spicatum* a été favorisée à une température plus élevée tandis que celle de *P. perfoliatus* a été réduite.

Les biomarqueurs physiologiques des macrophytes ont été mesurés pour déterminer leur pertinence en tant que signaux d'alerte précoce du stress. L'expérience avec uniquement des producteurs primaires (expérience 1) a montré la réponse la plus forte aux facteurs de stress, *M. spicatum* affichant le plus grand nombre de changements physiologiques au RA et *P. perfoliatus* au réchauffement. Les pigments photosynthétiques étaient probablement l'indicateur le plus fiable du stress, en particulier les caroténoïdes, qui ont augmenté chez les trois espèces de macrophytes en réponse à la fois au RA et au réchauffement. La stœchiométrie modifiée de *M. spicatum*, en particulier l'augmentation du carbone (C) et de l'azote (N) et la diminution du C:N et du C:P dans les traitements RA, ont indiqué une valeur nutritive plus élevée des macrophytes, les rendant potentiellement plus attractifs pour les herbivores. Dans les expériences 2 et 3, où des brouteurs étaient présents, les concentrations de métabolites secondaires ont diminué en réponse au RA, probablement en raison de la modification de la disponibilité des nutriments influencée par les brouteurs ou en tant que stratégie de défense contre le stress biotique tel que le broutage (détails voir chapitre 5).

Discussion

L'écotoxicologie aquatique fusionne les domaines de la toxicologie et de l'écologie aquatique. La toxicologie se concentre sur l'effet des substances chimiques sur une seule espèce. Les études réglementaires évaluant la qualité de l'environnement ou encore la sécurité des nouveaux produits chimiques s'appuient sur des études mono-espèces (Boudou & Ribeyre, 1997) car des liens de causalité directe peuvent être établis entre stress chimique et réponse. Cependant, les études portant sur une seule espèce ne permettent pas une extrapolation directe des effets aux systèmes aquatiques du monde réel qui sont caractérisés par des interactions directes et indirectes entre différents organismes et leur environnement, comme montré dans le projet CLIMSHIFT et proposé dans des études antérieures (Allen et al., 2021 ; Cambronerero et al., 2018 ; Zhang et al., 2019). La mise en place d'écosystèmes modèles qui ressemblent au monde réel nous permet de faire de meilleures prédictions ; ceci peut être réalisé par l'utilisation de micro- ou mésocosmes contrôlés.

De telles études au niveau communautaire ont suscité beaucoup d'intérêt ces dernières années, la plupart des études précédentes utilisant des réseaux trophiques simplifiés pour étudier les effets des facteurs de stress environnementaux. La plupart de ces études incluaient un seul niveau trophique ou une seule chaîne trophique, certaines uniquement

des communautés de zooplancton ou de phytoplancton (Carter et al., 2005 ; Stampfli et al., 2013), d'autres une communauté de gastéropodes–périphyton–macrophytes (Cao et al., 2014) ou une communauté zooplancton–phytoplancton–macrophyte (Rocha et al., 2020 ; Mulderij et al., 2007 ; van Donk & van de Bund, 2002).

Dans notre étude, nous avons conçu un réseau trophique beaucoup plus complexe, comprenant des systèmes à un et deux niveaux trophiques (uniquement les producteurs primaires ou bien les producteurs primaires et les consommateurs) pour démêler les effets directs et indirects. Dans le système à deux niveaux trophiques, nous avons inclus à la fois la chaîne trophique benthique et pélagique en ajoutant des consommateurs tels que les limnées et les daphnies. Les daphnies sont bien connues pour leur rôle de brouteurs clés du phytoplancton. Les limnées peuvent affecter la communauté des producteurs primaires en contrôlant le périphyton (Allen et al., 2021). Dans nos expériences, nous avons utilisé des espèces tests de l'OCDE qui jouent un rôle fonctionnel important dans l'état du lac (*M. spicatum*, *D. magna*, *L. stagnalis*).

A partir des résultats de nos expériences en microcosme, nous avons pu montrer que le RA et la température affectaient les macrophytes (Objectif 1). La biomasse réduite de macrophytes dans l'expérience 3 était liée à une modification de la pression de broutage du phytoplancton par *D. magna*. L'effet négatif du nitrate sur *M. spicatum* a été probablement lié à une augmentation précoce de la biomasse de périphyton. L'effet positif du réchauffement sur *M. spicatum* a pu être expliquée par une pression accrue des consommateurs sur les microalgues ou à l'augmentation des bactéries, qui entrent également en compétition avec le phytoplancton pour les nutriments.

Une telle compréhension systémique des effets des facteurs de stress au sein des écosystèmes est nécessaire pour prédire comment les systèmes réels peuvent réagir. Par exemple, bien que nous nous attendions à ce que les herbicides affectent directement les macrophytes en inhibant la photosynthèse, nos expériences montrent que les effets indirects de l'insecticide et/ou du fongicide étaient probablement plus importants pour expliquer l'importance relative des principaux groupes de producteurs primaires observée à la fin de l'expérience, en modifiant le contrôle trophique des microalgues par les brouteurs.

Pour atteindre un réalisme encore plus élevé, les approches expérimentales doivent s'éloigner des microcosmes intérieurs hautement contrôlés pour se rapprocher de

mésocosmes extérieurs plus variables et complexes (Davidson et al., 2018 ; Finnegan et al., 2018 ; Riis et al., 2018). Ces expériences permettent d'inclure la variabilité biotique et abiotique naturelle, les événements stochastiques et les facteurs de confusion dans l'évaluation des effets des facteurs de stress sur les communautés. En raison du plus grand réalisme de ces systèmes, les extrapolations des résultats pour faire des prédictions sur les effets des facteurs de stress dans les vrais lacs sont plus fiables. Dans notre expérience en mésocosmes, les effets du RA et du réchauffement observé dans les microcosmes contrôlés ont été préservés dans les conditions variables extérieures, soulignant non seulement la robustesse de nos modèles d'écosystème mais également le risque potentiel d'effets de stress dans les systèmes réels (Objectif 2). Le rôle joué par la communauté biotique complexe dans l'inversion des réponses du système aux facteurs de stress combinés souligne l'importance des études au niveau communautaire pour l'évaluation des risques en écotoxicologie aquatique ainsi que les défis liés à la prédiction des effets de plusieurs facteurs de stress sur des systèmes complexes.

Enfin, les effets des facteurs de stress ont pu être détectés dans l'empreinte chimique des macrophytes (Objectif 3). Il y avait une grande variabilité dans les réponses physiologiques des macrophytes aux facteurs de stress en fonction de la configuration expérimentale. Cependant, les fortes réponses physiologiques observées chez les macrophytes dans l'expérience 1 suggèrent qu'il existe un potentiel pour leur utilisation comme signaux d'alerte précoce. En outre, la réponse physiologique spécifique à l'espèce au stress (une réponse plus élevée de *M. spicatum* au RA par rapport à une réponse plus élevée de *P. perfoliatus* au réchauffement), indiquent des orientations futures potentielles de la recherche sur la réponse physiologique des macrophytes au stress.

Conclusion

Cette thèse démontre que l'exposition des lacs peu profonds au RA peut induire des changements de régime en faisant basculer le système vers un état dégradé dominé par le phytoplancton. En particulier, le rôle du nitrate, beaucoup moins étudié par rapport au phosphore, est clairement mis en évidence. Malgré les effets positifs du réchauffement sur la croissance des macrophytes, les trois facteurs de stress combinés (pesticides, nitrates et réchauffement) ont inversé l'effet attendu et entraîné une forte baisse de la biomasse des macrophytes. Le changement d'échelle réalisé en passant des microcosmes intérieurs aux mésocosmes extérieurs souligne la nécessité de mieux prendre en compte la complexité

des interactions biotiques pour une évaluation plus fiable des risques environnementaux pour lesquels les écosystèmes aquatiques sont exposés.