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Evaluating Multiple Stressor Effects on Benthic–Pelagic Freshwater Communities in Systems of Different Complexities: Challenges in Upscaling

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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. Additionally, 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 the 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. The 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; terbuthylazine; pirimicarb; tebuconazole; climate warming

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1. Introduction

Alternative stable states between phytoplankton and macrophytes commonly occur in shallow lakes, and state shifts are primarily driven by eutrophication [1]. The likelihood for such shifts may be enhanced by warming, e.g., through an increased release of nutrients from the sediment [2]. While the focus of many eutrophication studies in shallow lakes has been on phosphorus, recent studies show that high nitrate concentrations may also facilitate to regime shifts [3–5]. 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* [6,7], algae and the OECD test plant *Myriophyllum spicatum* [8], 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 [9,10], 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 for the comparison of results among research groups [11,12]. 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 [13–16]. 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 the 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 [17], 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 [18–20].

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 [21]. 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 [22,23]. 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 increases 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 the overall responses of the system to nutrient enrichment [24]. 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), Martinsried, Germany). 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;
3. A gradient design will allow for 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 and 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. [22], 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 an inorganic fungicide, three organic pesticides (a herbicide, an insecticide and 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 S1 and Allen et al. [22]. 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 the 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 × 2 pesticide levels × 2 temperatures × 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, Thermo Fisher Scientific France, Illkirch-Graffenstaden, France), ammonium (spectrophotometry) and alkali elements (Ca, K, Mg, Na; flame ionisation, Thermo Scientific ICE 3300, Thermo Fisher Scientific France, Illkirch-Graffenstaden, France).

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 [8], but by replacing inorganic nutrient salts with nettle powder (0.5% *w*/*w*). Each dish was placed within a glass cylinder (height 40 cm, Ø 19 cm; Sandra Rich GmbH, Ebernhahn, Germany) filled with 8 L of the respective water. The microcosms were then distributed evenly into 4 glass tanks, consisting of circulating, temperature controlled water baths, 2 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 2 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 [25] or WC [26] medium and adapted to Vw before the start of the experiment. Four polypropylene sheets measuring 29.7 cm × 2.6 cm length × 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 fertilised 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; RS Components, Beauvais Oise, France).

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; Champagne-au-Mont-d'Or, France). 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 the 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 (Varian GmbH, Frankfurt, Germany).

Depending on the final density of phytoplankton, between 40 and 100 mL per sample was filtered (25 mm GF/F filters, 0.7 µm pore size, Whatman France Sarl, Versailles, France) for the analysis of carbon. The periphyton on the 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; Thermo Fisher Scientific France, Illkirch-Graffenstaden, France).

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.

The 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; Bois-Colombes, France): $B = 0.01 \times L^{2.62}$, where B represents biomass in mg and L = length in mm [27].

The snails and mussels were removed and their lengths measured.

The macrophytes were removed, including the roots, rinsed and separated into aboveground and belowground parts. The carbon content of the apical 10 cm dry plant shoot was measured. The carbon content of primary producers was measured using a CHNS elemental analyser (Carlo-ERBA Na 2100 CE; Carlo Erba, Val de Reuil, France).

2.1.3. Data Analysis

A three-way ANOVA was performed using R (v4.1.0; [28]) 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 test and the Bartlett test, respectively. Log or square root transformations were performed if the data did not fit these assumptions. When the data could not be normalised, 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; [29]). 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 [30] 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 [30] and factoextra packages [31] 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 16 enclosures and volumes of 640 L each using Munich well water and exposed to a gradient of ARO to determine the concentration-dependent effects on the primary producers. See Figure 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 S2), with two trophic levels, but with the higher biodiversity (at both taxonomic and functional levels) provided by natural periphyton and plankton communities (see Table S3 for a comparison of morphotypes between micro- and mesocosms). 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). Since 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 as similar to the microcosms, and then diluted in 6 steps at a 1:1 dilution factor (Table S1). 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 µmol L^{-1} N and 1 µmol L^{-1} P as KNO_3 and KH_2PO_4, respectively) to ensure the growth of the primary producers.

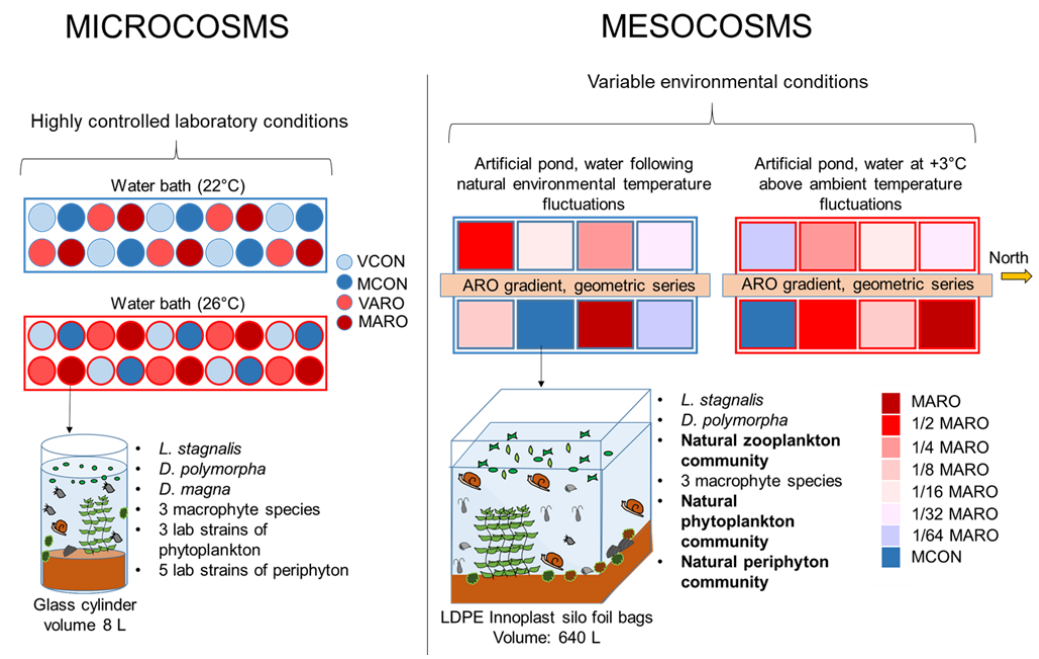

Figure 1. Comparison of the design between the indoor controlled microcosms and the outdoor variable mesocosms. The concentrations of the pesticides and nitrate comprising the agricultural runoff (ARO) in the microcosms were comparable to the highest ARO in the mesocosms. VCON = Volvic water control, MCON = Munich water control, VARO = Volvic water ARO and MARO = Munich water ARO.

Each of the sixteen enclosures consisted of an inner watertight layer (Innoplast silo foil; BayWa, Munich, 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. The 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. The average irradiance ranged from 300 to 2000 µmol photons m^{-2} s^{-1}.

Before starting the experimental manipulations (T0), all the 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 0/5 sieved, Bernhard Glück Kies-Sand-Hartsteinsplitt GmbH, Gräfelfing, Germany). Without agitating the sediment layers, 590 L of well water was 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 µmol L^{-1} P to adjust the N:P ratio in Mw was added.

On days 16 and 15, the 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, 12 transparent polypropylene strips as support for periphyton (2.5 × 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.

2.2.2. Sampling and Measured Parameters

Integrated water samples were taken with a tube sampler (1 L). The phytoplankton chlorophyll-*a* concentration was measured with a multispectral fluorometer (AlgaeLab-Analyser, bbe Moldaenke GmbH, Schwentinental, Germany). The phytoplankton carbon and nitrogen content were measured with an elemental analyser (varioMICRO Cube, Elementar Analysensysteme GmbH, Langenselbold, Germany). For continuous light intensity and temperature measurements, HOBOs (HOBO MX2202Pendant, Onset Computer Corporation, Bourne MA, USA) 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. [32] and described in Schmitt-Jansen and Altenburger [33]), and at the end of the experiment, the dry weights were measured. The carbon equivalents were estimated as 60% dry weight based on the 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 the 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 the macrophytes were taken out by gently releasing the roots from the sediment until the plants were free but intact. The 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. The carbon content was determined similar to the microcosms.

For both the experiments, copper was analysed in LIEC, Metz, France, by atomic absorption spectroscopy (Varian SpectrAA 800 Zeeman; Thermo Fisher Scientific France, Illkirch-Graffenstaden, France) 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 Fisher Scientific, Karlsruhe, Germany).

2.2.3. Data Analysis

Simple linear and quadratic regressions were tested on the primary producers using R [28] to identify any relationships between the stressors and their biomass.

2.3. Comparison of the Primary Producer Community Structure in Micro- and Mesocosms

The primary producer community structures in the micro- and mesocosms were compared using the biomasses of each primary producer group expressed as total carbon. For the phytoplankton, the measured carbon was extrapolated to the total volume per microcosm (8 L) or mesocosm (640 L) to obtain total carbon. For the periphyton, the 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, the total carbon was calculated from the total macrophyte aboveground biomass per micro- and mesocosm.

3. Results

3.1. Microcosm-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 the higher contents in calcium and magnesium in Mw by 611% and 160%, respectively (Table S4). After a 4-week exposure, the two water types were quite similar in nutrient concentration and pH. The conductivity remained

consistently higher in the Mw treatments during all 4 weeks, but the difference between the 2 water types was reduced from 440 µS cm^{-1} at the start of the experiment to 70 µS cm^{-1} after 4 weeks (Figure S1). 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). The decline of pesticides, including copper, did not differ between the two temperatures.

Overall, the water type did not strongly affect the 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 (Figure 2C). The water type, however, caused a small though significant increase in biomass of *E. nuttallii* in Mw compared with Vw (Figures S2 and S3, Table S5). This resulted in only a marginal effect on total macrophytes ($p = 0.07$). An interaction between the water type and ARO was also observed for periphyton (Figure 3). The water type had no effect on the other primary producers or consumers, nor did it modify the temperature or ARO toxicity towards them. Primary producer proportions were also highly comparable in both water types for the same treatment (Figure S4).

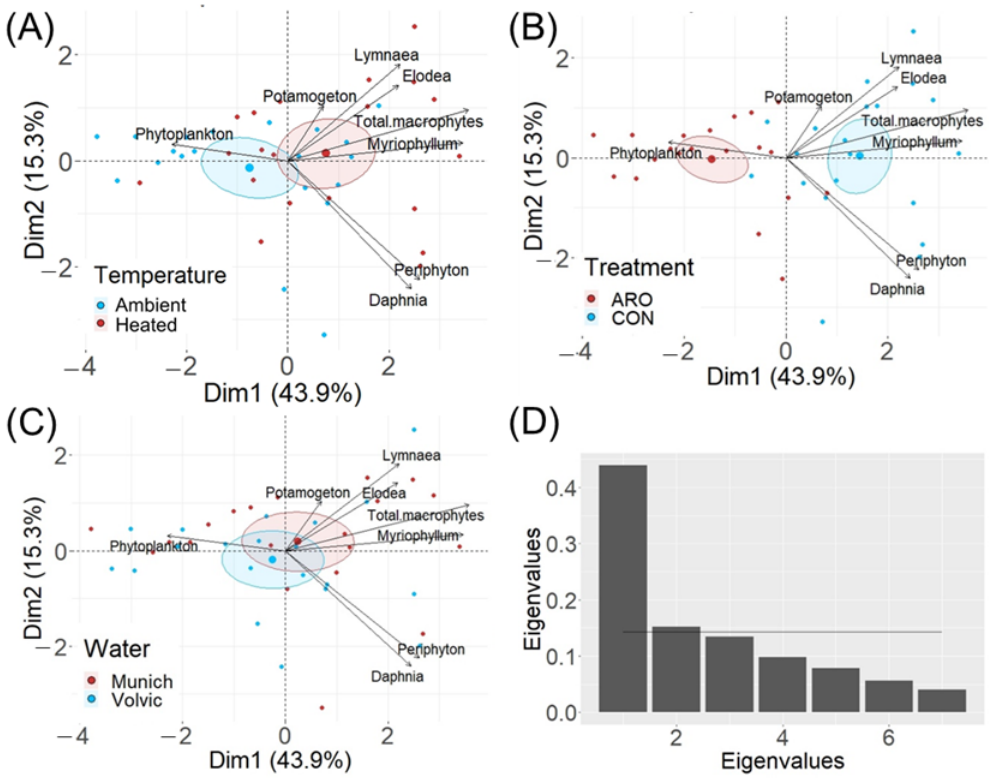

Figure 2. Microcosms. Principal components analysis plot showing the most significant axes and all response variables. The temperature, treatment and water effects are projected as supplementary data: (**A**) ambient (blue) or heated (red), (**B**) CON (blue) or ARO (red) and (**C**) Volvic (blue) or Munich well water (red). (**D**) Eigenvalue scores of the 7 main axes.

3.2. Stressor Effects on the Primary Producers

3.2.1. Microcosms

Both the macrophytes and periphyton respond negatively to the ARO, whereas phytoplankton responds positively (Figure 3). The total macrophyte dry aboveground biomass in the ARO treatments decreases by 60% compared with the control (Figure 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. The pesticide effects on phytoplankton were much stronger in the cold treatments, accounting

for the overall strong pesticide effect (Figure 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.

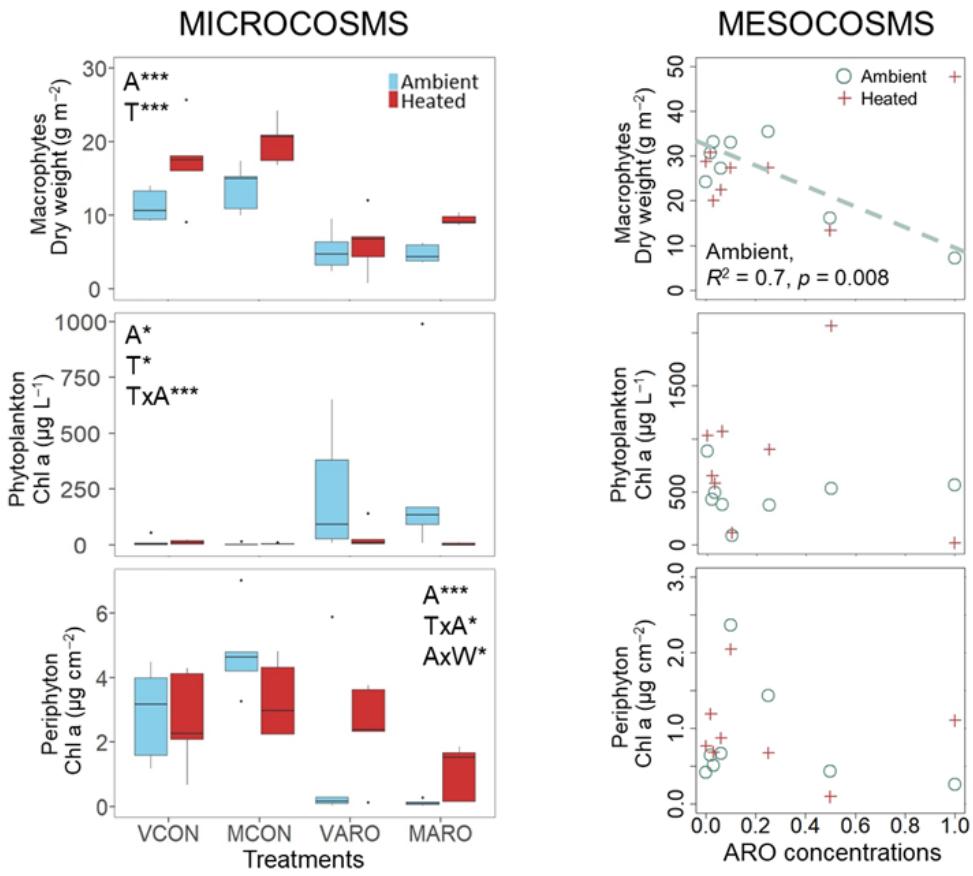

Figure 3. Stressor effects on the final aboveground biomass of all macrophytes, chl-*a* concentration of phytoplankton and periphyton in the microcosms (**left**) and mesocosms (**right**). Microcosms: VCON = Volvic control; MCON = Munich well water control. ARO; VARO = Volvic ARO; MARO = Munich well water ARO. Box plots of 5 replicates showing the 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 and W = Water. Mesocosms: Regression plots with the ARO concentration gradient along the *x*-axis. Mesocosms: R^2 and *p*-value from the linear regression. For the microcosm experiment, the significance levels are shown as * $p < 0.05$; *** $p < 0.001$.

3.2.2. Mesocosms

Not all the 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, the total macrophyte biomass is negatively related to the ARO level with a decrease of 70% in the highest ARO compared to the control (Figure 3). However, the highest macrophyte biomass can be observed in the 1.0-H (ARO concentration in the heated mesocosms) treatment, showing no clear overall response pattern to the stressors (Figure 3). Both *M. spicatum* and *E. nuttallii* reach their maxima in the 1.0-H treatment and mimima in the 1.0-A treatment (Figure S3). *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 reaches its highest biomass in the 0.5-H mesocosm and the lowest in the the 1.0-H concentration (Figure 3). 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 (Figure 3).

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 the Primary Consumers

3.3.1. Microcosms

The grazers were negatively affected by the pesticides. *L. stagnalis* lengths are on average 20% smaller in the ARO compared with the control (Figure 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.

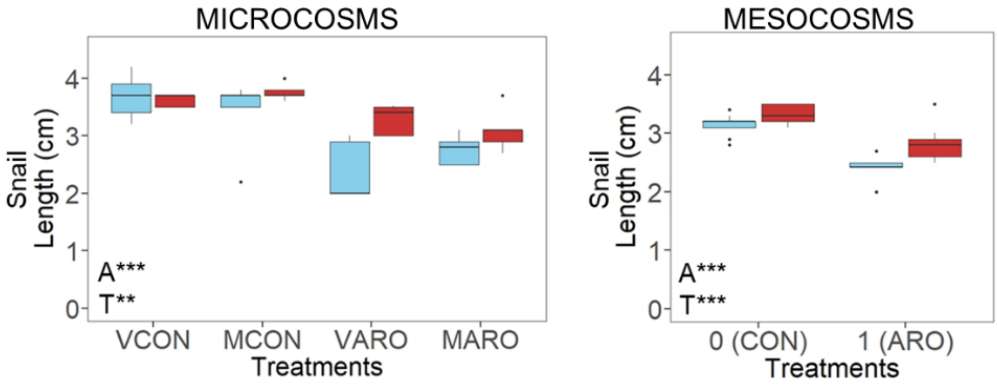

Figure 4. Stressor effects on the final length of snails in the microcosms (**left**) and mesocosms (**right**). Box plots of 5 replicates in the microcosms and 10 replicates in the mesocosms showing the median, 25 and 75% percentiles, lowest and highest whiskers (as Q1 − [1.5 × IQR] and Q3 + [1.5 × 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. ** $p < 0.01$; *** $p < 0.001$.

The highest *Daphnia* numbers are reached during week 2 with the controls showing 82% more individuals L^{-1} than the ARO (Figure 5). By the end of the 4-week exposure, the numbers in the controls reduced from 1213 ± 883 to 345 ± 329. *Daphnia* were controlling the 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 in both *Daphnia* numbers (Figure 5) and phytoplankton (Figure S5).

3.3.2. Mesocosms

Similar to the microcosms, the snails are affected negatively by ARO with a 16% reduced length in the 1.0 ARO compared with the control (Figure 4).

In contrast to the microcosms, *D. magna* was not part of our zooplankton community in the mesocosms. The natural zooplankton community comprises nine functional groups (Figure 5). The most prevalent morphotypes were calanoid copepods, *Daphnia longispina* and *Bosmina*, together representing over 80% of the community. During the

experiment, the community structure shifts in all enclosures (Figure 5). 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.

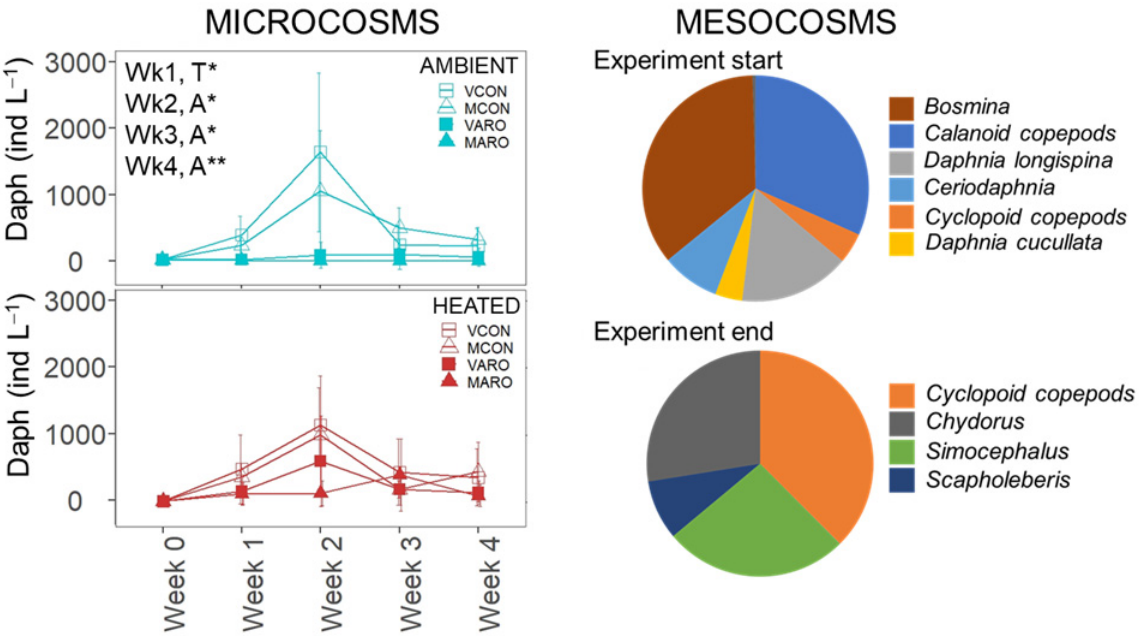

Figure 5. Impact of the stressors on the zooplankton in the microcosms (**left**) and mesocosms (**right**). In the microcosms, the temporal effects of the stressor on *Daphnia* numbers in the ambient (blue) and heated (red) microcosms are shown. Microcosms: VCON = Volvic control; MCON = Munich well water control. ARO; VARO = Volvic ARO; MARO = Munich well water ARO. Means ± SD, $n = 5$. A = ARO, T = Temperature and Wk = week. Mesocosms: zooplankton population at the start and end of experiment, pooled from all enclosures. Daph = *Daphnia*. * $p < 0.05$; ** $p < 0.01$.

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* (Figure 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 the enclosures. The total insect larvae biomass was very similar between the ambient and heated enclosures. Ephemeroptera density, however, was halved by heating and chironomid density was even reduced by 70%. By contrast, the biomass of the predatory odonate larvae was increased by 700% due to heating.

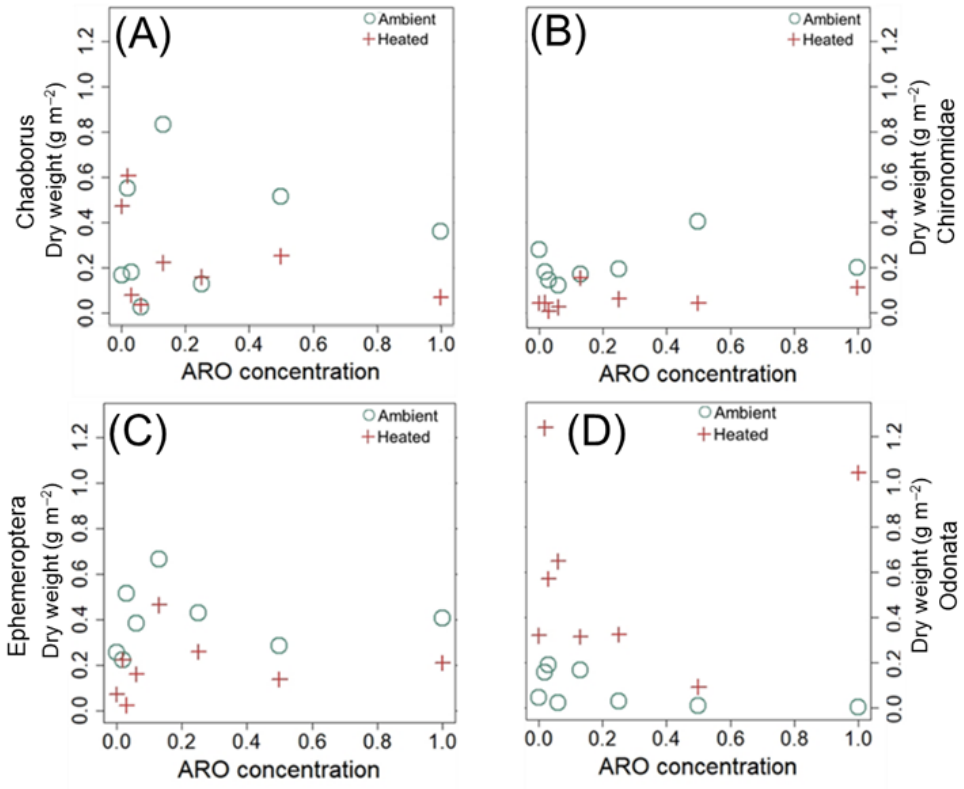

Figure 6. Mesocosms. Biomass of the four main groups of invasive invertebrates at the final sampling: (**A**) *Chaoborus*, (**B**) Chironomidae, (**C**) Ephemeroptera and (**D**) Odonata.

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 (Figure S6), 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 cold ARO treatments (750% increase in phytoplankton proportion), compared with the warm ARO (171% increase in phytoplankton proportion) (Figure 7). As the mesocosms were more biologically complex, either macrophyte or phytoplankton dominance was observed among the enclosures. Intermediate ARO concentrations of the ambient mesocosms tended to be more macrophyte-dominated (Figure 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 shows the 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 are the most phytoplankton-dominated accompanied by a low macrophyte biomass, both with ~70% phytoplankton and 25% macrophytes (Figure 7). The biovolume of macrophytes remains comparable between the micro- and mesocosms, but the zooplankton:phytoplankton ratio is relatively lower in the mesocosms (Figure S7).

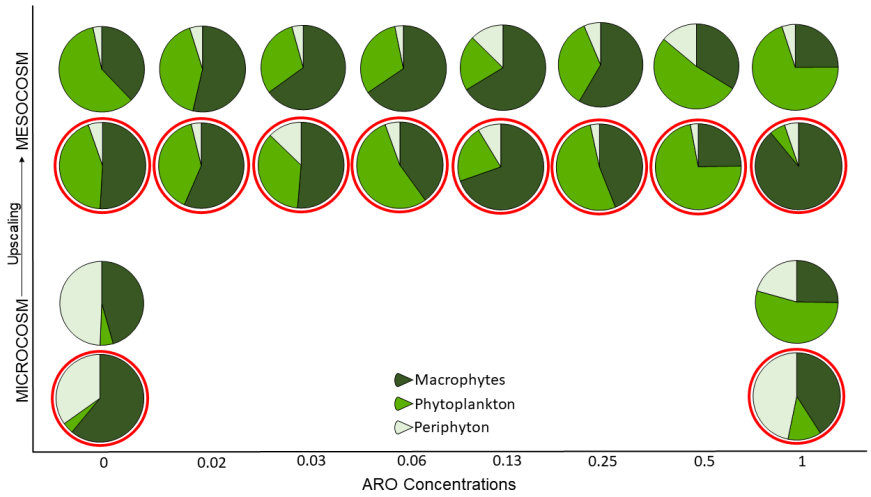

Figure 7. Microcosm–mesocosm comparison. Pie charts showing the relative proportion of primary producers in the microcosms (Munich well water treatments) and mesocosms when exposed to the ARO and the two temperatures. Heated treatments are represented by a red circle around the pie charts.

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 show to affect aquatic organisms [9,10], adapting the water physiochemistry to suit the organism's needs can alleviate significant differences in effects due to water type. In our experiment, the nutrient levels in Mw were adjusted to more closely match the standard Vw, and, as a result, the water type did not affect the 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 the causal understanding and prediction of effects in real-world scenarios.

4.1. The 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 the Munich well water (Mw) in promoting the growth of *E. nuttallii* may be related to the differences in the mineral status between the two waters, particularly the higher calcium and carbonate (higher alkalinity) concentrations in Mw compared to Vw, which can influence the photosynthesis or nutrient uptake capacity of this plant and thereby its growth [34,35]. *Elodea nuttallii* was much more efficient at phosphorus uptake in water that had higher calcium concentrations [34]. 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 the 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 [36]. 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 cold microcosms. Similar phytoplankton blooms were observed in the experiments with single or pulsed ARO exposure [22]. Since ARO and temperature effects were the same in both water types and the 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. The 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, [22]), 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 the pesticides, it may show either antagonistic or synergistic effects [37]. Pesticides have been shown to degrade faster at higher temperatures [38], 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. The 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 [39] 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 [40,41]. 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. The observed changes in zooplankton assemblages may have influenced the strength of top-down and even bottom-up control [42,43]. One likely reason for the 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 show that *Daphnia* are strongly affected by the pesticides, which may explain their disappearance in the mesocosm experiments. Additionally, nutrient levels and the presence of alternative food sources may have also influenced the zooplankton assemblages. Du et al. [42] 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 [44]. 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 [45]. 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 [46]. 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. The 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. The densities of these invading insects were comparable to natural densities [47]. 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 [48]. In the microcosms, the snails were the only key grazers of periphyton, and ARO effects on periphyton could be linked to the snails [22]. In the mesocosms, both the 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 [49] 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 normalised OECD test guideline for *Lymnaea*

stagnalis reproduction has been adopted recently [50], showing their relevance as indicators of toxicant effects.

The stressors may also affect the primary producers differently, when embedded in branched or looped trophic chains with even or uneven trophic levels [24,51,52]. 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 [24]. Since 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 [53] and feeding behaviour of the second trophic level [54], 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 [55]. The foraging activities by *Chaoborus* and Odonata on other insects and zooplankton may therefore have increased, but the temperature may also have strongly regulated microplankton assemblages [42]. 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.

5. Conclusions

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 [56]. 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, the highest temperature reversed the effect of the highest ARO treatment on primary producers. This modified effect may be due to changes in community structure, increased metabolic activity and strong top-down control by consumers at higher temperatures. Depending on the number of trophic levels and the feeding habits of the consumer community within the ecosystem studied, the temperature may modify the direction of ARO effects. Strong positive and negative effects observed in the 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 the stressor effects at different scales and complexity is therefore a promising direction for risk evaluation studies in aquatic ecology and ecotoxicology.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/w14040581/s1: Figure S1: Microcosms. Weekly measures of water physico-chemistry; Figure S2: Hedges' g effect sizes calculated from the F-statistic; Figure S3: Stressor effects on macrophytes in the micro- and mesocosms; Figure S4: Microcosms. Relative proportions of primary producers; Figure S5: Microcosms. Weekly measures of the absorbance of water at 663 nm; Figure S6: Mesocosms. Pesticide concentrations measured over 8 weeks; Figure S7: Biomass per volume of macrophytes and zooplankton:phytoplankton ratios in the micro- and mesocosms; Table S1: ARO concentrations and temperatures of micro- and mesocosms; Table S2: Starting size of biotic community in micro- and mesocosms; Table S3: Morphotypes used in the micro- and mesocosms; Table S4: Comparison of physico-chemistry between Volvic and Munich well water before and after exposure; and Table S5: Statistical details for individual and combined stressor effects on the different functional groups.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not yet publicly available, but will be deposited in local repositories (Université de Lorraine and Ludwig-Maximilians University), and are available upon request once the CLIMSHIFT project is finished.

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