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Multiple-stressor exposure of aquatic food webs: nitrate and warming modulate the effect of pesticides

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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 microalgae. 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 *M. spicatum* drove the response of the total macrophyte biomass. Nitrate significantly suppressed *M. spicatum* 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 observed for macrophytes or periphyton alert us about multiple stressor effects. 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, which are the most abundant type of freshwater systems worldwide (Meerhoff & Jeppesen, 2009). These lakes may exhibit 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). While the dichotomy between phytoplankton and macrophytes is well acknowledged for these systems, periphyton shading on macrophytes also plays 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). More studies are showing that increasing nitrogen concentrations lead not only to reduced macrophyte abundance due to phytoplankton shading but also to a decrease in their diversity (Moss, 1976; Phillips et al., 2016). While nitrogen concentrations vary between lakes, higher concentrations are consistently measured close to agricultural sites (~1.5 to 1.8 mg L^{-1}) compared with non-agricultural watersheds (~1.0 mg L^{-1}) (Xu et al., 2014). Natural lake concentrations measuring ~2 mg L^{-1} account for a moderately eutrophic status (Sagrario et al., 2005). Actual nitrogen input concentrations may be masked by quick assimilation by microalgae. 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 to lakes can therefore result in increased phytoplankton and periphyton biomass in nitrogen-limited or co-limited lakes (Bergström et al., 2005; Goldman, 1988; Smith & Lee, 2006; Zhang & Mei, 2013). Nitrates are easily leached into groundwater or carried away in agricultural runoff

(ARO). Concentrations in shallow lakes may thus rise proportionally with increasing fertilizer applications, underlining the need for nitrogen control around shallow lakes.

The primary source of nitrogen in shallow lakes is ARO (Rücker et al., 2019), which contains multiple other chemicals such as organic and inorganic pesticides. Pesticides are of specific concern as their use is increasing. Global production has risen significantly after the 1950s, from 500,000 to more than 3 million tons annually (Schäfer et al., 2011; Sharma et al., 2019). These pesticides, varying in concentrations in aquatic systems based on rain events and surface or subsurface runoff (Graymore et al., 2001), may directly affect primary producer growth (Vonk & Kraak, 2020). They may also indirectly alter their development by affecting top–down control (Cuenca Cambronero et al., 2018). Depending on their modes of action, pesticides may alter macrophyte abundance on which the resilience of the clear-water state depends (Scheffer et al., 1993). Macrophytes are directly affected 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). These primary consumers may also be strongly affected by pesticides (Allen et al., 2021; Hanazato, 2001).

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 other chemicals entering these systems. State-of-the-art modelling studies propose that rising temperatures will cause an increase in phytoplankton biomass (Trolle et al., 2014). This could be due to increased nutrient release from the sediments (Jeppesen et al., 2009), a change in metabolic rates of organisms (Brown et al., 2004), or a reinforcement of top–down herbivore–plant interactions (Zhang et al., 2019). These various direct and indirect effects of climate warming may contribute to regime shifts in shallow eutrophic lakes (Scheffer, 2001).

These multiple stressors may affect shallow aquatic lakes in several ways. Nitrate could act antagonistic to pesticide effects, e.g., dampening negative effects of herbicides on primary producers (Halstead et al., 2014). Nutrients overriding negative effects of atrazine in freshwater wetlands has been shown (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 complicate the prediction of combined nitrate and pesticide effects. A good ecological status with abundant submerged macrophytes in shallow lakes can be challenged by strong phytoplankton and periphyton development. This can be limited by reduced nutrient loads, efficient grazing by filter-feeding zooplankton on phytoplankton (Lampert, 2006; Sommer et al., 1986), or invertebrate grazing on periphyton (Allen et al., 2021; Jones et al., 2003).

Owing to the potential combined effects of these multiple stressors (nitrate, pesticides and warming) within a shallow lake, the resilience of the clear-water state may be reduced, and tipping points for state shifts more easily reached (Scheffer et al., 1993). Experimental studies tackling these questions should include community- and ecosystem-level scenarios. Micro- or mesocosm studies may generate such results and provide policymakers with a practical foundation to define environmentally safe thresholds of chemical stressors under global warming. Studying the potential effects of stressors in a complex benthic–pelagic system is challenging but possible in appropriately designed microcosm experiments.

The objective of our study was to determine the individual and combined effects of nitrate, pesticides and climate warming in microcosms with two trophic levels mimicking fishless lentic shallow aquatic ecosystems. To disentangle the nitrate and pesticide effects, we exposed our microcosms to these stressors individually and in combination. Our microcosms contained macrophytes, periphyton and phytoplankton, and their respective grazers. We hypothesized that (1) the development of all producers and consumers will be affected by pesticides, and (2) both nitrate and warming will reinforce pesticide effects, facilitating phytoplankton dominance.

2. Materials 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: presence or absence), pesticide (Pest, 2 levels: presence or absence) and temperature (Tmp, 2 levels: presence or absence). The treatments were control (CON), only nitrate (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, typical for fishless shallow freshwater ecosystems, were used, adapted from Allen et al. (2021). Details of each species and their maintenance are provided in the supplementary material.

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 ± 6.8 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).

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 (Supplementary Table S1).

On day −6, the cylinders were inoculated with the corresponding volumes of the periphyton and phytoplankton. Periphyton development was followed using four polypropylene strips (rough surface; 29.7 cm x 2.6 cm length x 300 µm thick; PolyClearView, GBC, Chicago, USA) hanging vertically in each microcosm. On day −4, two 10-cm apical shoots of each macrophyte species were planted in the sediment, ensuring homogenous distribution based on fresh weights among treatments. On day −3, consumers were introduced, ensuring homogeneous mean size among treatments. 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.

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

168 analysed from filtered water samples (0.22 µm PVDF syringe filters) by liquid chromatography–
169 mass spectrometry using an LTQ Orbitrap XL (Thermo-Scientific, USA).

170

171 *2.3.3. Plankton and periphyton*

172 Phytoplankton development was followed by measuring the optical density (OD) at 663 nm
173 of water passed over a 500-µm mesh beaker to remove *Daphnia*. OD measurements were taken
174 with a spectrophotometer (Cary 50, Varian® Agilent, USA). At the end of the experiment, between
175 40 and 100 mL (depending on density) was filtered (GF/F) to determine carbon concentration.

176 During the experiment, 50 mL water was collected weekly, and *Daphnia* individuals
177 counted. All *Daphnia* were returned to their respective microcosms after counting. Numbers were
178 extrapolated to total volume for number of individuals per mesocosm. At the end of the
179 experiment, sampled *Daphnia* were fixed in sugar ethanol to avoid size changes (Haney & Hall,
180 1973) and their length measured using a digital microscope (VHX-6000; Keyence, Bois-Colombes,
181 France). Length measures were converted into biomass using the formula $B = 0.01 \times L^{2.62}$, where
182 B represents biomass in mg and L = length in mm (r = 0.99; Pauw et al., 1981).

183 The development of periphyton was followed weekly on one of the plastic strips taken out
184 of the microcosm to measure the minimum fluorescence (F0) by Pulse Amplitude Modulation
185 (PAM). The strip was immediately placed back in the cylinder. At the end of the experiment,
186 periphyton from all four strips (400 cm^2) per microcosm was brushed off into 20 mL Volvic water
187 using a soft toothbrush. Following homogenization and centrifugation, pellets were stored at -
188 80°C, then lyophilised for the analysis of carbon.

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

191

192 *2.3.4. Macrophytes*

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

198

199 *2.3.5. Chemical analyses of microalgae and macrophytes*

200 Photosynthetic pigments of phytoplankton and periphyton were analysed by HPLC-DAD
201 (high performance liquid chromatography-diode array detector) (UHPLC Ultimate 3000 Rs
202 THERMO; Capdeville et al. 2019). Chlorophyll a concentrations were used as a proxy for biomass
203 of phytoplankton (µg L^{-1}) and periphyton (µg cm^{-2}).

204 The carbon content of all primary producers was determined using a CHNS elemental
205 analyser (Carlo-ERBA Na 2100 CE Instrument).

206

207 *2.3.6. Benthic consumers*

208 After four weeks, all snails and mussels were retrieved, live individuals counted, their
209 lengths measured, and growth rate calculated. Number of snail clutches and eggs per clutch were
210 counted.

211

212 ## *2.4. Statistical analyses*

213 A three-way analysis of variance (ANOVA) was applied using R (R Core Team, 2018) to test
214 Pest, Nitr, Tmp (fixed factors) and their interaction effects on all response factors. A Kruskal–
215 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, 2013) 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 synergistic, antagonistic or reversed if they were higher or 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 S2). 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 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 ± 0.3°C in the warm microcosms, approximately 3.5°C higher than the ambient microcosms (21.2 ± 0.1°C). Distinct temporal changes were observed for water conductivity, pH and O_2 (Table S4, Fig. S1) and are presented in the supplemental material.

High nitrate additions to the NO3 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 mg L^{-1} $N\text{-}NO_3$, 0.176 mg L^{-1} $P\text{-}PO_4$). Remaining orthophosphate was lower in ambient treatments (58 ± 36 µg L^{-1}) compared with warm (119 ± 97 µg L^{-1}) and variable in the CON and NO3 treatments. We observed strong Tmp x Pest interactions for P-PO4 and the NP molar ratio (Fig. S2).

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. 1, Table S3). Total macrophyte aboveground biomass reduced by 55% in the ambient ARO (0.09 ± 0.05 g) compared with ambient CON (0.2 ± 0.04 g). All three stressors influenced the relative abundance of macrophyte species (Table S3, Fig. 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. 1). Distinct temporal dynamics were observed between weeks 1 and 3 (Fig. 3), with initial positive Nitr and negative Tmp effects, followed by positive Pest effects (Table S3).

The phytoplankton biomass in the ambient ARO treatment was high at the end of the experiment (Fig. 3), and was 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, Fig. 1). The highest and lowest mean chl a concentrations were present in the ambient microcosms for the ARO (231.9 ± 277 μg L^{-1}) and NO3 (9.6 ± 14.6 μg 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).

This reduction of total macrophyte biomass in the ARO treatments resulted in significant changes in the relative abundance of primary producers (Fig. 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, PERMANOVA). When pesticides and nitrate were present together (ARO), effects were augmented (Fig. 2B). Warming had a positive influence on macrophyte dominance.

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. 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, Fig. 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.

3.4 Global 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. 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

314 temperature was applied with this stressor combination, a reversed effect was observed for total
315 macrophyte biomass.

316 PCAs provided a global view of the strongest patterns in the data. Distinct Pest and Tmp
317 effects were visible along axis 1 of the PCA (Fig. S4A-C). Axes 1 and 2 contributed to 33.9% and
318 17.8% of the variation in the data, respectively. Although the combined contribution of these two
319 principal components to explain the variability of our observations is admittedly low (51.7%), the
320 contribution of PC1 is clear. It separated total macrophyte biomass and *Myriophyllum* biomass on
321 the right from phytoplankton on the left, and is explained by temperature and pesticides. The
322 effect of nitrate is weak. The warm microcosms without pesticides explained the high total
323 macrophyte and *Myriophyllum* biomass. Along axis 2, periphyton and *Lymnaea* were placed on
324 opposite sides, suggesting a top–down control.

325 SEMs constructed based on hypothesized interactions between stressors and functional
326 groups allowed us to trace food web effects. Using final sampling data, the three stressors affected
327 long-lived organisms such as the dominant macrophyte *Myriophyllum* or *Lymnaea,* but not, or only
328 marginally, organisms with shorter generation times, like phytoplankton, *Daphnia* and
329 periphyton (Fig. 5). *Myriophyllum* hindered phytoplankton development likely through
330 allelopathy (Hilt & Gross, 2008), and *Lymnaea* controlled periphyton. This outcome was likely
331 strongly influenced by temporal dynamics (Fig. S5).

332 Strong direct positive nitrate effects were observed on periphyton during the first week of
333 exposure (Fig. S5A). Phytoplankton on the other hand was directly or indirectly affected by
334 nitrate, depending on the exposure phase. Pesticides directly affected the number of *Daphnia* in
335 the first two weeks (Fig. S5B). During the intermediate exposure, phytoplankton was indirectly
336 affected by pesticides through *Daphnia* grazing. Warming had a direct positive influence on
337 *Daphnia*, but only in the first week. The preliminary SEMs using all expected pathways are
338 presented in Fig. S6 and S7. The models created with *Potamogeton* and *Elodea* are not included as
339 no significant stressor or biotic interactions were observed. All SEMs with the final sampling data

(Fig. 5, S6, & S7) fit the quality indicators. The SEMs with the temporal data (Fig. S5), however, did not fit these quality indicators, and should therefore be interpreted as a hypothesis for further experimental research.

4. Discussion

Our results confirmed the first hypothesis: all tested aquatic producer and consumer groups were negatively affected by pesticides except for phytoplankton, which was promoted. The second hypothesis can be confirmed in part: When both nitrate and pesticides were present together, the resilience of a macrophyte-dominated state was reduced. Yet, elevated temperature attenuated partially the effects of pesticides. This means that typical agricultural runoff (ARO) containing a mixture of pesticides and nitrate can be more detrimental to macrophyte-dominated shallow water bodies than can the individual stressors, whereas climate warming might counterbalance part of 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 µg L^{-1} to 296 µg L^{-1} copper have been identified (Roubeau

365 Dumont et al., 2019). The presumed negative effect of pesticides as seen in the SEM might
366 therefore be primarily indirect, resulting from shading by phytoplankton or periphyton at the
367 early or intermediate exposure period. We conclude that effects observed at the end of the
368 experiment need to be interpreted in the context of temporal dynamics. The lack of strong direct
369 or indirect stressor effects on *Potamogeton* or *Elodea* might be related to the dominance of
370 *Myriophyllum*.

371 The sensitivity of phytoplankton to pesticides may be species-dependent. Marine
372 microalgae have shown different sensitivities to terbuthylazine, with some species such as
373 *Fibrocapsa japonica* and *Gonyaulax spinifera* undergoing photosystem II inhibition at 1 µg L^{-1} and
374 others such as *Prorocentrum minimum* not affected even at 25 µg L^{-1} (Fiori et al., 2013). Green
375 algae such as *Chlorella spp.* have shown an EC50 of 30 µg L^{-1} for copper (De Schamphelaere et al.,
376 2005), while others such as *Dunaliella tertiolecta* had an EC50 as high as 9200 µg L^{-1} (Gatidou &
377 Thomaidis, 2007) when exposed for up to four days. Less sensitive species therefore probably
378 developed fast in the PST or ARO treatments. The positive indirect effect of pesticides on
379 phytoplankton, as suggested by the SEM, is likely related to the temporal effects of zooplankton
380 (*Daphnia*) feeding on it. In fact, *Daphnia* numbers showed a similar dynamic to phytoplankton
381 with a lag of one week, and a strong peak in week 2 in the CON and NO3 treatments.

382 The direct negative effect of pesticides on *Daphnia* abundances observed during the first
383 two weeks may be due to pirimicarb, which can be toxic to a large range of vertebrates and
384 invertebrates (Sánchez-Bayo, 2012). The EC50 value for pirimicarb is 21-24 µg L^{-1} for neonates
385 and 16 µg L^{-1} for adults (Kusk, 1996). Modelling studies also show that copper concentrations
386 comparable to those applied in our experiment affected top–down control of *Daphnia* on green
387 algae (Prosnier et al., 2015). Our initial copper exposure concentration of 42 µg L^{-1} should thus
388 have been sufficient to cause a significant decrease in phytoplankton filtration by *Daphnia* in the
389 pesticide treatments. *Daphnia* declined at the end of the experiment probably because of food
390 shortage as no further phytoplankton peak emerged in CON and NO3. The decline in *Lymnaea*

391 growth when exposed to pesticides is in line with Allen et al. (2021) and may be related to copper
392 (Brix et al., 2011) or pirimicarb (Tufi et al., 2015).

393

394 *4.2 Modulation of pesticide effects by nitrate*

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

414 Although we saw strong direct negative nitrate effects on *Myriophyllum*, controlled
415 laboratory studies showed that *Myriophyllum* could tolerate nitrogen levels up to ~14 mg L^{-1}
416 (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 also possible. 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, 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 probably 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.

5. Conclusion

Our findings have implications for decision-making on alleviating stressor effects on shallow aquatic systems. 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. Although no effects of warming could be observed on phytoplankton, this should not be translated as "no effects": Early-phase dynamics clearly showed effects that were quickly counteracted by grazing and competition. 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 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.

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Author contributions

The concept of the CLIMSHIFT project was developed by EMG, SH, HS, JL, MSJ, and FH. This experiment was carried out by VV, EMG and ML with major contributions by JA and NA. The paper was written by VV. GLM and JA supported the formal analysis, BP performed pesticide analytics, and NK provided validation of *Daphnia* data. All authors contributed to planning and discussions of this experiment and the manuscript.

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Figure

Figures (main manuscript)

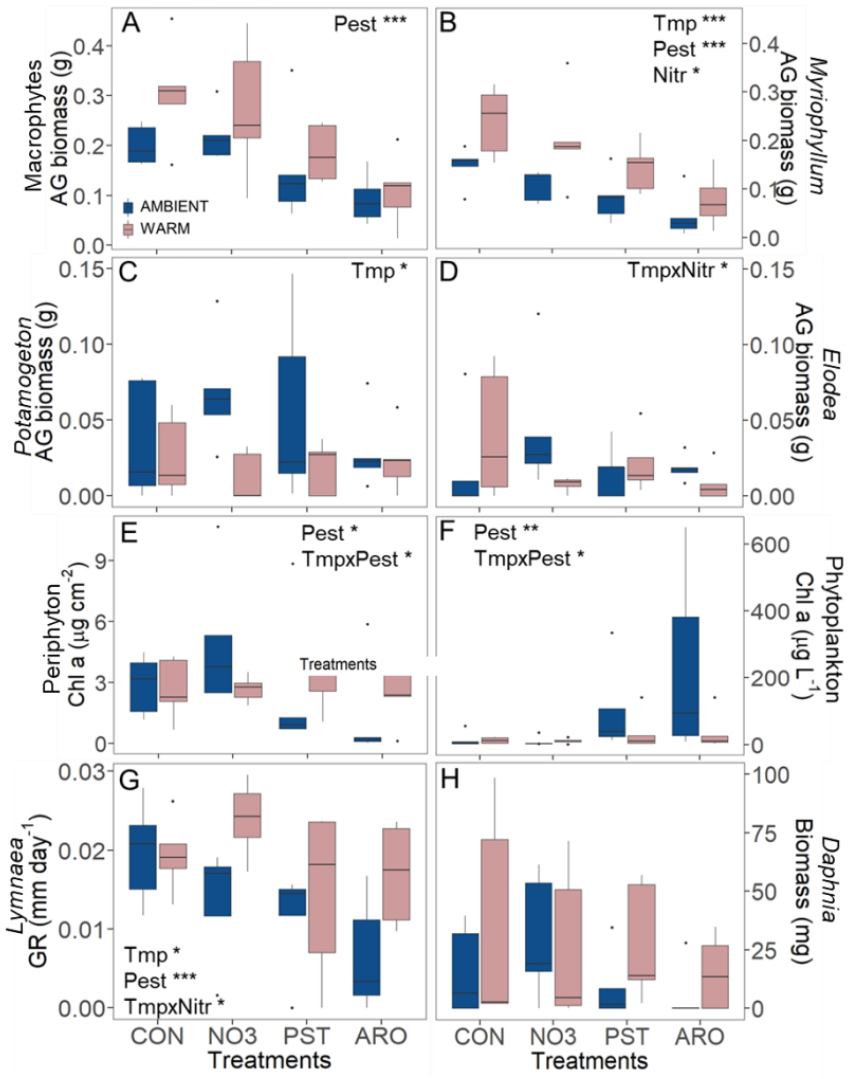

Fig. 1. Temperature and treatment (CON = control; NO3 = nitrates; PST = organic pesticides and copper; ARO = PST + NO3) effects on primary producers and consumers. Final aboveground biomass (dry mass) of (A) All macrophytes, (B) *M. spicatum*, (C) *P. perfoliatus,* and (D) *E. nuttallii.* Chl a content 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*IQR] and Q3+[1.5*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.

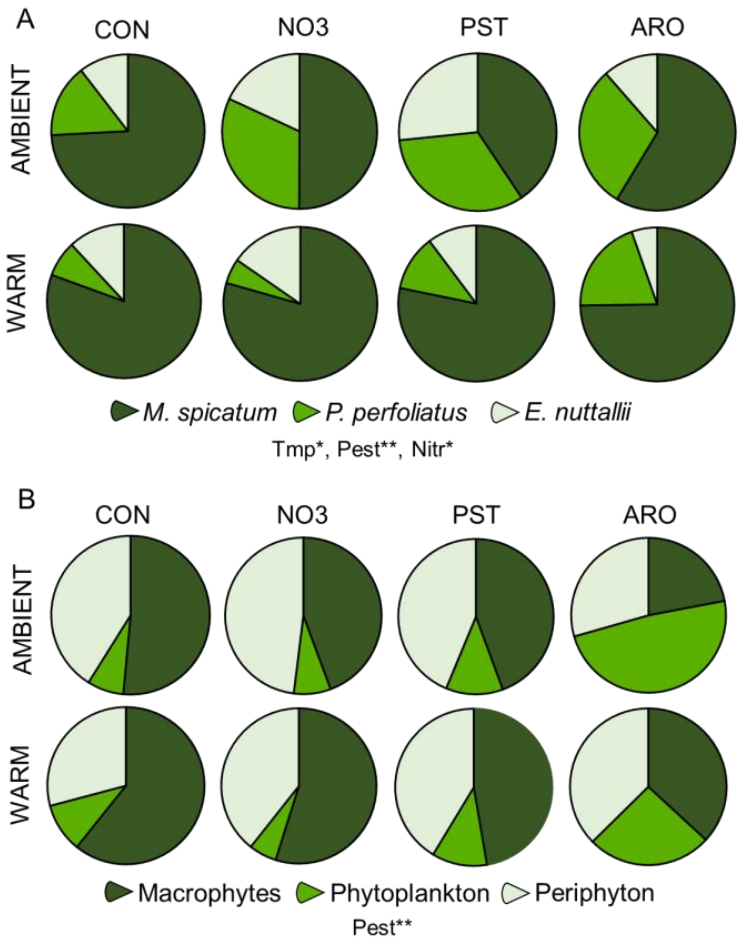

Fig. 2. Relative abundances of (A) macrophyte species and (B) primary producer groups. Abundance was measured as a function of total carbon content per microcosm at the end of the experiment. CON = control; NO3 = nitrates; PST = organic pesticides and copper; ARO = PST + NO3. Asterisks represent significant effects observed with the PERMANOVA ($p < 0.05$): using 3 fixed factors: Tmp = temperature effect; Pest = pesticide effect, Nitr = nitrate effect.

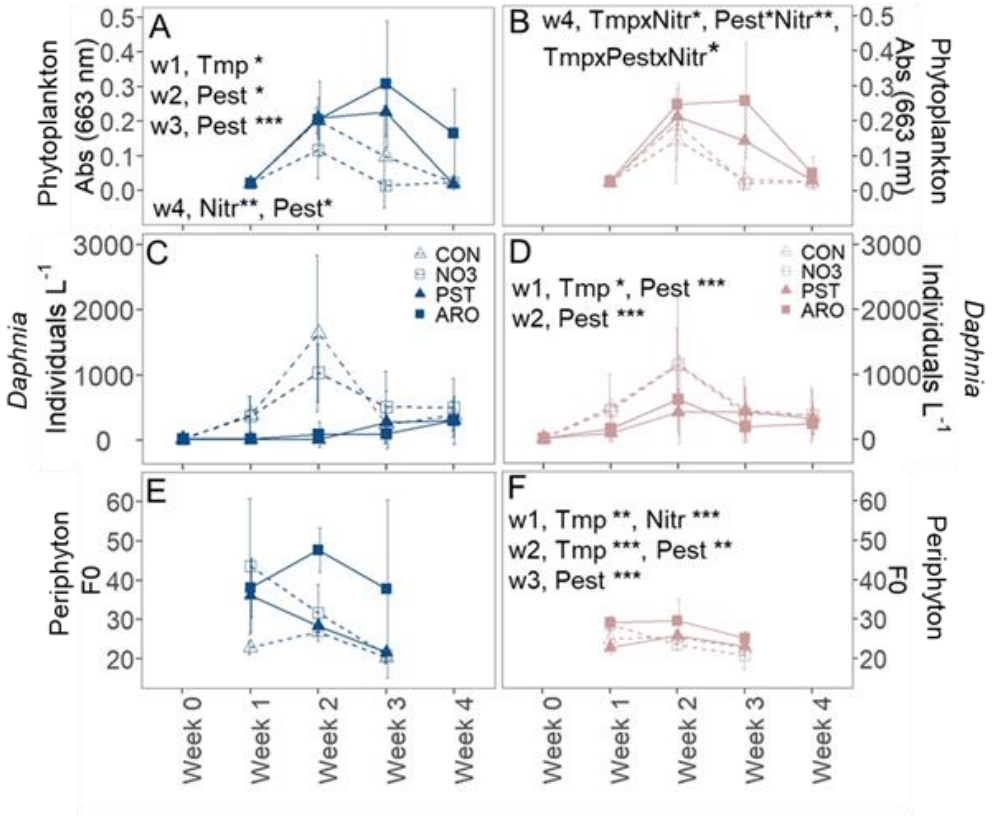

Fig. 3. Weekly measures of (A,B) phytoplankton biomass (absorbance at 663 nm), (C,D) numbers of *D. magna* adults, and (E,F) periphyton biomass (as F0 fluorescence) in the ambient (blue) and warm (mauve) microcosms. Means ± *SD*, *n* = 5. CON = control; NO3 = nitrates; PST = organic pesticides and copper; ARO = PST + NO3. Asterisks represent significant effects observed with the ANOVA ($p < 0.05$), using 3 fixed factors: Tmp = temperature; Pest = pesticide, Nitr = nitrate at different time points of the experiment (weeks (w) 1-4).

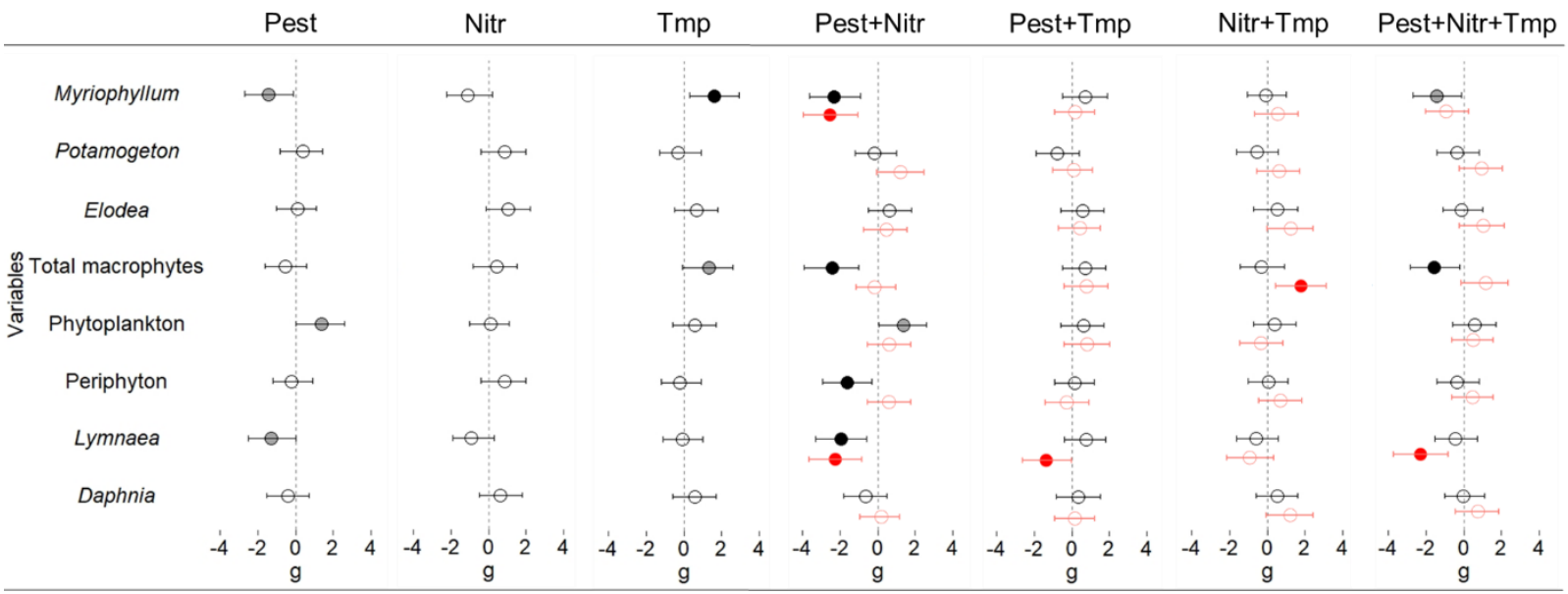

Fig. 4. Effect sizes (Hedges' g ± 90% confidence interval) of individual and combined stressor effects on the key functional groups at the end of the experiment. No effects are inferred when confidence intervals cross the zero line. White symbols: non-significant effects (>0.09), grey symbols: marginal effects (0.05-0.09), black symbols: significant effects (<0.05), calculated using 3 fixed factors: Tmp = temperature; Pest = pesticide, Nitr = nitrate. Red symbols: predicted additive effects calculated from the Hedges' g of individual stressors.

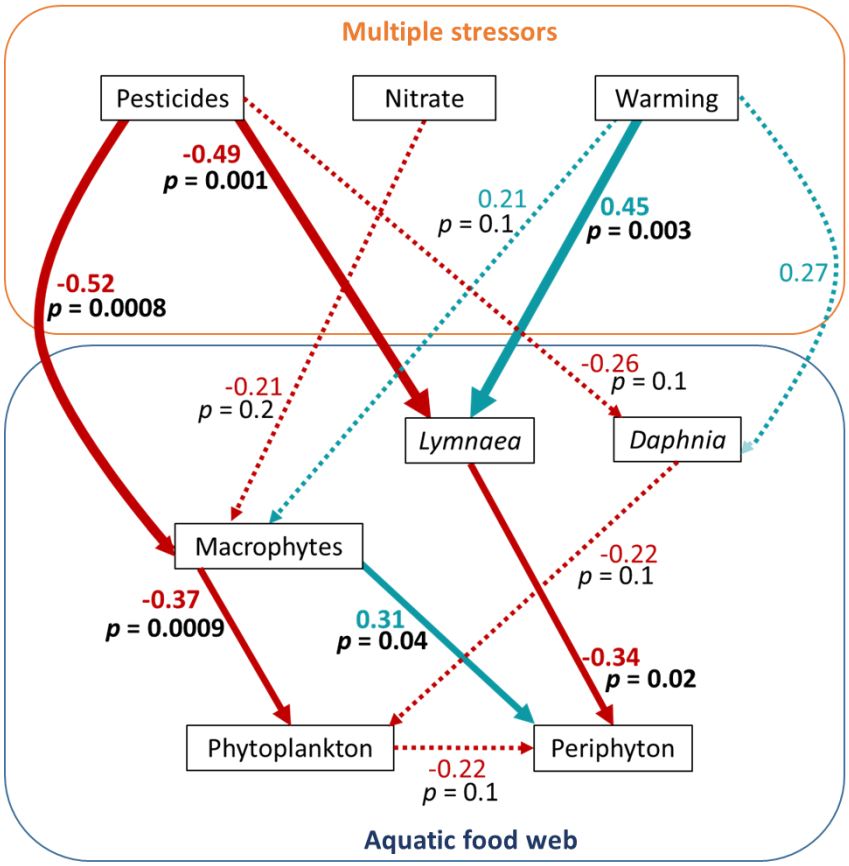

Fig. 5. Path diagram of the selected structural equation model based on final sampling data. Red, green and dashed arrows indicate negative, positive and nonsignificant relations between variables, respectively. Significant threshold was set at $p = 0.05$. The proportions of variation explained by the model for each response variable are represented by R-values in blue and red for positive and negative effects, respectively. $Chi^2 = 17.4$, 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.

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