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

3

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16 **ABSTRACT**

17 Shallow lakes provide essential ecological and environmental services but are exposed to
18 multiple stressors, including agricultural runoff (ARO) and climate warming, which may act on
19 different target receptors disrupting their normal functioning. We performed a microcosm
20 experiment to determine the individual and combined effects of three stressors—pesticides,
21 nitrate and climate warming—on two trophic levels representative of communities found in
22 shallow lakes. We used three submerged macrophyte species (*Myriophyllum spicatum*,
23 *Potamogeton perfoliatus*, *Elodea nuttallii*), eight benthic or pelagic microalgal species and three
24 primary consumer species (*Daphnia magna*, *Lymnaea stagnalis*, *Dreissena polymorpha*) with
25 different feeding preferences for benthic and pelagic primary producers. Eight different
26 treatments consisted of a control, only nitrate, a pesticide cocktail, and a combination of nitrate
27 and pesticides representing ARO, each replicated at ambient temperature and +3.5°C, mimicking
28 climate warming. Pesticides negatively affected all functional groups except phytoplankton,
29 which increased. Warming and nitrate modified these effects. Strong but opposite pesticide and
30 warming effects on *Myriophyllum* drove the response of the total macrophyte biomass. Nitrate
31 significantly suppressed *Myriophyllum* final biomass, but not overall macrophyte and microalgal
32 biomass. Nitrate and pesticides in combination caused a macrophyte decline, and the system
33 tipped towards phytoplankton dominance. Strong synergistic or even reversed stressor
34 interaction effects were observed for macrophytes or periphyton. We emphasize the need for
35 more complex community- and ecosystem-level studies incorporating multiple stressor
36 scenarios to define safe operating spaces.

37

38 **KEYWORDS**

39 agricultural runoff, benthic–pelagic coupling, microcosm, multiple stressors, regime shifts,
40 stressor interactions

41

42 1. Introduction

43 Multiple stressors affecting aquatic systems do not spare shallow lakes, the most
44 abundant type of freshwater systems worldwide (Meerhoff & Jeppesen, 2009). These lakes,
45 including fishless water bodies such as kettle holes and peatland ditches, are common in
46 agricultural landscapes, and are hubs of biodiversity (Pätzig et al., 2012; Peeters, 2005; Whatley
47 et al., 2014). Typical lakes may exhibit either the more desirable clear-water macrophyte-
48 dominated state providing a variety of ecosystem services or the degraded turbid
49 phytoplankton-dominated state (Janssen et al., 2021). In addition, periphyton shading on
50 macrophytes can play an important role in the transition from the clear-water to the turbid state
51 (Phillips et al., 1978). These state shifts have been largely ascribed to nutrient loading to aquatic
52 systems, which has significantly increased since the agricultural revolution (Moss et al., 2013).
53 Yet, the role of nitrogen in regime shifts has received less attention than phosphorus,
54 particularly due to the presence of nitrogen fixation as a compensation mechanism for nitrogen
55 shortage (Moss et al., 2013; Schindler et al., 2008). Increasing nitrogen concentrations may lead
56 to reduced macrophyte abundance and diversity due to phytoplankton shading (Moss, 1976;
57 Phillips et al., 2016). Higher nitrogen concentrations are consistently measured close to
58 agricultural sites (~ 1.5 to 1.8 mg L^{-1}) compared with non-agricultural watersheds ($\sim 1.0 \text{ mg L}^{-1}$)
59 (Xu et al., 2014). Actual nitrogen input concentrations may be masked by quick assimilation by
60 phytoplankton or periphyton. Reduced macrophyte biomass and species richness as well as
61 increased phytoplankton and chlorophyll-*a* concentrations have been observed at nitrogen
62 concentrations between 2 and 10 mg L^{-1} (Barker et al., 2008; Sagrario et al., 2005). These shifts
63 occurred irrespective of total phosphorus concentrations, ranging between 0.03 and 1.2 mg L^{-1}
64 (Sagrario et al., 2005). High nitrogen loading can therefore increase phytoplankton and
65 periphyton biomass in nitrogen-limited or co-limited lakes (Smith & Lee, 2006; Zhang & Mei,
66 2013). Nitrates are easily leached into groundwater or carried away in agricultural runoff (ARO
67 in the following). Concentrations in shallow lakes may thus rise proportionally with increasing
68 fertilizer applications, underlining the need for nitrogen control around shallow lakes. While

69 alternative states are less common in smaller lakes such as ditches and kettle holes, where
70 macrophytes and periphyton are more abundant (Kazanjian et al., 2018), they are still possible
71 under high eutrophication (Whatley et al., 2014).

72 The primary source of nitrogen in these lakes is ARO (Rücker et al., 2019), which may also
73 contain organic and inorganic pesticides. Global production and use of pesticides has risen
74 significantly after the 1950s (Schäfer et al., 2011; Sharma et al., 2019). Pesticides, varying in
75 concentrations in aquatic systems based on rain events and surface or subsurface runoff
76 (Graymore et al., 2001), can directly affect all types of aquatic primary producers through, e.g.,
77 inhibition of photosynthesis, carotenoid biosynthesis, or growth regulation (Mohaupt et al.,
78 2020; Vonk & Kraak, 2020). They may also indirectly alter their development by affecting top-
79 down control (Cuenca Cambronero et al., 2018). Macrophytes are affected directly by
80 phytoplankton and periphyton shading, and indirectly through top-down control of microalgae
81 by invertebrate grazers (Phillips et al., 2016). Zooplankton efficiently controls phytoplankton,
82 leading to high transparency in lakes (Lampert et al., 1986), and invertebrate grazing on
83 periphyton may alleviate shading on macrophytes (Jones et al., 2003). Pesticides may affect not
84 only these primary consumers (Allen et al., 2021; Hanazato, 2001) but also predators such as
85 fish (Ali et al., 2020) or other higher trophic levels if present. Depending on their modes of
86 action, pesticides may therefore alter macrophyte abundance directly or indirectly through
87 trophic interactions (Yamamuro et al., 2019), reducing the resilience of the system (Scheffer et
88 al., 1993).

89 In addition to local chemical stressors, shallow lakes are increasingly subjected to climate
90 warming. The International Panel on Climate Change (IPCC) has projected a +4°C increase in
91 global temperatures in its RCP 8.5 scenario (IPCC, 2014). Such an increase may have negative
92 effects for shallow lakes particularly in combination with chemical pollution entering these
93 systems. Modelling studies propose that rising temperatures will cause an increase in
94 phytoplankton biomass (Trolle et al., 2014). Yet, warming may also reduce phytoplankton
95 biomass due to enhanced grazing in fishless lakes (Hansson et al., 2012). Further, filtering rates

96 of *Daphnia*, key grazers in fishless ditches, may be reduced at higher temperatures (Müller et al.,
97 2018). Climate warming leading to increased nutrient release from sediments (Jeppesen et al.,
98 2009), or increased herbivory on macrophytes (Zhang et al., 2019) may contribute to
99 phytoplankton dominance and ultimately cause regime shifts in shallow eutrophic lakes
100 (Scheffer, 2001).

101 These multiple stressors may affect shallow aquatic lakes in several ways. Nitrate or other
102 nutrients could act antagonistic to pesticide effects, e.g., dampening negative effects of
103 herbicides on primary producers (Halstead et al., 2014; Dalton et al., 2015). High nutrient
104 availability for microalgae provide a better food quality for grazers (Guo et al., 2016) but might
105 interact with grazer sensitivity towards pesticides. Climate warming may further complicate the
106 prediction of combined nitrate and pesticide effects. The resilience of the clear-water,
107 macrophyte-dominated state may be reduced, and tipping points for state shifts more easily
108 reached (Scheffer et al., 1993) as a result of the combined effects of multiple stressors (nitrate,
109 pesticides and warming) on community- and ecosystem processes in shallow lakes or fishless
110 ditches or kettle holes. We combined this complex scenario in our microcosms to test the
111 interactive effects of nitrate, pesticides and warming, on a benthic–pelagic system whose
112 respective response to the stressors can induce state shifts of the system. To the best of our
113 knowledge, this type of complex integrative study, including a top–down consumer control of
114 both phytoplankton and periphyton, has not been performed before.

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

123 2. Materials and methods

124 2.1 *Experimental design*

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

134 Three functional groups each of primary producers and primary consumers,
135 representative of the first two trophic levels in shallow freshwater ecosystems, were used,
136 adapted from Allen et al. (2021). Details of each species and their maintenance are provided in
137 the supplementary information (SI).

138

139 2.2 *Set-up of the microcosms*

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

147 For the ARO cocktail, we mixed three widely used organic pesticides, a herbicide
148 (terbuthylazine), an insecticide (pirimicarb) and a fungicide (tebuconazole), copper as an
149 ingredient of inorganic pesticide mixtures and nitrate (SI; Table S1). Concentrations of the
150 pesticides were based on experimental evidence at UFZ, Leipzig (BHP and MSJ, unpublished
151 data), as well as some published EC values and species sensitivity distribution data.

152 On day -6, the cylinders were inoculated with four phytoplankton and four periphyton
153 strains. A similar biovolume per strain was used, yielding $\sim 0.5 \text{ mg L}^{-1}$ and 1 mg L^{-1} of organic
154 carbon (Behl et al., 2011) for phytoplankton and periphyton, respectively, per microcosm.
155 Periphyton development was followed using four vertically placed polypropylene strips (rough
156 surface; 29.7 cm x 2.6 cm length x 300 μm thick; PolyClearView, GBC, Chicago, USA). On day -4,
157 each two 10-cm apical shoots of three macrophyte species were planted in the sediment,
158 ensuring homogenous distribution of fresh weights among treatments. These were
159 *Myriophyllum spicatum*, *Potamogeton perfoliatus* and *Elodea nuttallii* (hereafter *Myriophyllum*,
160 *Potamogeton* and *Elodea*, respectively), commonly found in meso- to eutrophic, still or slow-
161 moving waters. On day -3, twenty *Daphnia magna* (hereafter *Daphnia*) neonates, one pond snail
162 (*Lymnaea stagnalis*, hereafter *Lymnaea*) and three zebra mussels (*Dreissena polymorpha*,
163 hereafter *Dreissena*) were introduced per microcosm, ensuring homogeneous mean size among
164 treatments. *Daphnia* is a typical pond species efficiently grazing on phytoplankton and
165 sometimes on periphyton, *Lymnaea* feeds primarily on periphyton and sometimes on
166 macrophytes, and *Dreissena* feeds on phytoplankton. On day 0, the chemical stressors were
167 added, and the set temperature of half of the water tanks was increased by $+3.5^\circ\text{C}$. The exposure
168 period was four weeks with regular macronutrient dosage to prevent nutrient limitation and
169 deionized water addition to account for evaporation and keep the water volume constant. For
170 more details on settings and organisms mentioned in this paragraph, please see SI, S2.2.

171

172 2.3. *Sampling and measured response variables*

173 2.3.1. *Sampling scheme*

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

179

180 2.3.2. *Water*

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

189

190 2.3.3. *Plankton and periphyton*

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

196 During the experiment, 50 mL water was collected weekly, and *Daphnia* individuals
197 counted. All *Daphnia* were returned to their respective microcosms after counting. Numbers
198 were extrapolated to total volume for number of individuals per mesocosm. At the end of the
199 experiment, sampled *Daphnia* were fixed in sugar ethanol to avoid size changes (Haney & Hall,
200 1973) and their length measured using a digital microscope (VHX-6000; Keyence, Bois-
201 Colombes, France). Length measures were converted into dry weight using the formula $B =$
202 $0.01 \times L^{2.62}$, where B represents biomass in mg and L = length in mm ($r = 0.99$; Pauw et al.,
203 1981).

204 The development of periphyton was followed weekly on one of the plastic strips taken out
205 of the microcosm to measure the minimum fluorescence (F0) by Pulse Amplitude Modulation
206 (PAM). The strip was immediately placed back in the cylinder. At the end of the experiment,
207 periphyton from all four strips (400 cm²) per microcosm was brushed off into 20 mL Volvic
208 water using a soft toothbrush. Following homogenization and centrifugation, pellets were stored
209 at -80°C, then lyophilised for the analysis of organic carbon.

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

212

213 2.3.4. *Macrophytes*

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

219

220 *2.3.5. Chemical analyses of microalgae and macrophytes*

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

228

229 *2.3.6. Benthic consumers*

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

232

233 *2.4. Statistical analyses*

234 A three-way analysis of variance (ANOVA) was applied using R (R Core Team, 2018) to
235 test Pest, Nitr, Tmp (fixed factors) and their interaction effects on all response factors. A
236 Kruskal–Wallis test by ranks was performed for non-parametric data. The Hedges' *g* effect sizes
237 and 90% confidence intervals (CI) were calculated based on Hedges and Olkin (1985). When the
238 effect sizes were large (>0.8 ; Cohen, 2013) and their CIs did not touch the 0 line, they were
239 considered meaningful. Additive effects of the stressors—Pest+Nitr, Pest+Tmp, Nitr+Tmp and
240 Pest+Nitr+Tmp—were predicted from the Hedges' *g* for individual stressors. Interactions were
241 considered to be additive, synergistic, antagonistic or reversed if they were similar to, higher
242 than, lower than, or opposite in direction to the calculated additions, respectively, respecting CI
243 margins. To analyse shifts in the relative dominance among the three macrophyte species and
244 among the three primary producer groups, a permutational multivariate analysis of variance

245 (PERMANOVA) was performed using the vegan package (Oksanen et al., 2020). To visualize
246 significant patterns in the variability of the final sampling data, and to reduce the confounding
247 influence of multiple highly correlated variables, principal components analyses (PCA) were
248 performed using the vegan (Oksanen et al., 2020) and factoextra packages (Kassambara &
249 Mundt, 2020). To decipher direct and indirect stressor effects on the organisms, as well as
250 species interactions, a path analysis subjected to structural equation modelling (SEM) was
251 created (package sem; Fox et al., 2021) using several hypothetical pathways similar to Allen et
252 al. (2021) (Table S2). Further details of statistical approaches are presented in the supplemental
253 material.

254

255 **3. Results**

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

261

262 *3.1 Water analyses*

263 The average temperatures remained consistent throughout the experiment and measured
264 $24.6 \pm 0.3^\circ\text{C}$ in the warm microcosms, approximately 3.5°C higher than the ambient microcosms
265 ($21.2 \pm 0.1^\circ\text{C}$). Distinct temporal changes were observed for water conductivity, pH and O₂
266 (Table S4, Fig. S1) and are presented in the supplemental material.

267 High nitrate additions to the NO₃ and ARO treatments were no longer measurable at the
268 end of the experiment. Continuous fertilisation by nitrate and phosphate at a 16:1 μM ratio did
269 not result in an accumulation of dissolved nutrients compared to initial values of the Volvic

270 water ($1.71 \text{ mg L}^{-1} \text{ N-NO}_3$, $0.176 \text{ mg L}^{-1} \text{ P-PO}_4$). Remaining orthophosphate was lower in ambient
271 treatments ($58 \pm 36 \text{ } \mu\text{g L}^{-1}$) compared with warm ($119 \pm 97 \text{ } \mu\text{g L}^{-1}$) and variable in the CON and
272 NO₃ treatments. We observed strong Tmp x Pest interactions for P-PO₄ and the NP molar ratio
273 (Fig. S2B). Particulate phosphorus was low at the end of the experiment (range $5.6 - 31.5 \text{ } \mu\text{g L}^{-1}$),
274 with significantly higher levels in the PST and ARO treatments (Fig. S2E).

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

277

278 3.2 Response of primary producers

279 Macrophyte biomass was lower in the PST and ARO treatments, with a concomitant
280 increase in phytoplankton (Fig. 1, Table S3). Total macrophyte aboveground biomass reduced
281 by 55% in the ambient ARO ($0.09 \pm 0.05 \text{ g}$) compared with ambient CON ($0.2 \pm 0.04 \text{ g}$). All three
282 stressors influenced the relative abundance of macrophyte species (Table S3, Fig. 2A).
283 *Myriophyllum* dominated and showed the strongest response to all stressors. Aboveground
284 biomass significantly decreased with pesticides and nitrate, and increased with warming.
285 Pesticides or nitrate did not affect the other species. Warming significantly reduced
286 *Potamogeton* aboveground biomass. Due to the opposite temperature response of *Myriophyllum*
287 and *Potamogeton*, warming only marginally positively influenced total macrophyte
288 aboveground biomass ($p = 0.06$).

289 Final periphyton biomass was significantly lower in PST and ARO treatments but only in
290 the ambient-temperature treatments (significant Pest and Tmp x Pest effect) (Fig. 1). Distinct
291 temporal dynamics were observed between weeks 1 and 3 (Fig. 3), with initial positive Nitr and
292 negative Tmp effects, followed by positive Pest effects (Table S3).

293 By the end of the experiment, the phytoplankton comprised predominantly green algae.
294 Their biomass in the ambient ARO treatment was high at the end of the experiment (Fig. 3),
295 reflected in both the chlorophyll-*a* (chl-*a*) and water OD measurements. Phytoplankton,

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

306 This reduction of total macrophyte biomass in the ARO treatments resulted in significant
307 changes in the relative abundance of primary producers (Fig. 2B). Whereas the controls were
308 dominated by macrophytes, the ARO treatments were dominated by phytoplankton. Pesticides
309 caused declining shares of macrophytes and increasing dominance of phytoplankton (Table S3,
310 PERMANOVA). When pesticides and nitrate were present together (ARO), effects were amplified
311 (Fig. 2B). Warming had a positive influence on macrophyte dominance.

312

313 3.3 *Response of primary consumers*

314 Three of the 40 initial *Lymnaea* individuals did not survive. All dead snails belonged to
315 treatments containing pesticides. Growth rate was significantly reduced with pesticides but
316 increased under warming (Fig. 1). Nitrate significantly increased snail growth only at a higher
317 temperature. Only five snails had produced clutches, two from ambient CON, one each from
318 ambient and warm NO₃, and one from warm ARO. The NO₃ treatments had the highest number
319 of eggs per clutch (144 ± 23), while the ARO had the least (9). Mussels exhibited high mortality
320 irrespective of treatment type, and have not been included further in the analyses.

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

328

329 3.4 Integrative assessment of stressor effects and interactions

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

335 Mostly, the observed and predicted effect sizes were similar, showing that stressor effects
336 were additive. Three exceptions exist (Fig. 4): The observed combined effect of pesticides and
337 nitrate on total macrophyte biomass and periphyton biomass was negative, while the predicted
338 effect was neutral. This indicates a synergistic interaction of both stressors. When increased
339 temperature was applied with this stressor combination, a reversed effect was observed for
340 total macrophyte biomass.

341 PCAs provided a global view of the strongest patterns in the data. Distinct Pest and Tmp
342 effects were visible along axis 1 of the PCA (Fig. S4A-C). Axes 1 and 2 contributed to 33.9% and
343 17.8% of the variation in the data, respectively. Although the combined contribution of these
344 two principal components to explain the variability of our observations is admittedly low
345 (51.7%), the contribution of PC1 is clear. It separated total macrophyte biomass and
346 *Myriophyllum* biomass on the right from phytoplankton on the left, and is explained by

347 temperature and pesticides. The effect of nitrate is weak. The warm microcosms without
348 pesticides explained the high total macrophyte and *Myriophyllum* biomass. Along axis 2,
349 periphyton and *Lymnaea* are placed on opposite sides, suggesting a top-down control.

350 SEMs constructed based on hypothesized interactions between stressors and functional
351 groups allowed us to trace food web effects. Using final sampling data, the three stressors
352 affected long-lived organisms such as the dominant macrophyte *Myriophyllum* or *Lymnaea*, but
353 not, or only marginally, organisms with shorter generation times, like phytoplankton, *Daphnia*
354 and periphyton (Fig. 5). *Myriophyllum* hindered phytoplankton development possibly also
355 through allelopathy (Hilt & Gross, 2008), and *Lymnaea* controlled periphyton. Temporal
356 dynamics likely influenced this outcome (Fig. S5).

357 Strong direct positive nitrate effects were observed on periphyton during the first week of
358 exposure (Fig. S5A). Phytoplankton on the other hand was directly or indirectly affected by
359 nitrate, depending on the exposure phase. Pesticides directly affected the number of *Daphnia* in
360 the first two weeks (Fig. S5B). During the intermediate exposure, phytoplankton was indirectly
361 affected by pesticides through *Daphnia* grazing. Warming had a direct positive influence on
362 *Daphnia*, but only in the first week. The preliminary SEMs using all expected pathways are
363 presented in Fig. S6 and S7. The models created with *Potamogeton* and *Elodea* are not included
364 as no significant stressor or biotic interactions were observed. All SEMs with the final sampling
365 data (Fig. 5, S6, & S7) fit the quality indicators. The SEMs with the temporal data (Fig. S5),
366 however, did not fit these quality indicators, and should therefore be interpreted as a hypothesis
367 for further experimental research.

368

369 **4. Discussion**

370 Our results confirm the first hypothesis: The aquatic primary producers were affected
371 through negative pesticide effects on the primary consumers, which promoted phytoplankton

372 development. The second hypothesis is also confirmed: Nitrate and pesticides interacted
373 synergistically to reduce macrophyte-dominance. Warming together with pesticides and
374 nitrates even reverted the expected outcome on macrophytes based on individual stressor
375 effects, shown by a significant negative effect of the combined stressors on total macrophyte
376 abundance. This means that a typical agricultural runoff (ARO) containing a mixture of
377 pesticides and nitrate can be more detrimental to macrophyte-dominated fishless water bodies
378 than the individual stressors alone and climate warming might even enhance these effects.

379

380 4.1 *Effect of pesticides*

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

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

395 The sensitivity of phytoplankton to pesticides may be species-dependent. Some species
396 were inhibited already at 1 $\mu\text{g L}^{-1}$ and others not even at 25 $\mu\text{g L}^{-1}$ (Fiori et al., 2013). Green algae
397 exhibited EC50s for copper between 30 $\mu\text{g L}^{-1}$ (De Schamphelaere et al., 2005) and 9200 $\mu\text{g L}^{-1}$

398 (Gatidou & Thomaidis, 2007). Less sensitive species therefore probably developed fast in the
399 PST or ARO treatments. The positive indirect effect of pesticides on phytoplankton, as suggested
400 by the SEM, is likely related to the temporal effects of *Daphnia* feeding on it. In fact, *Daphnia*
401 numbers showed a similar dynamic to phytoplankton with a lag of one week, and a strong peak
402 in week 2 in the CON and NO3 treatments.

403 The direct negative effect of pesticides on *Daphnia* abundances observed during the first
404 two weeks may be due to pirimicarb, exhibiting EC50 values for *D. magna* of 16-24 $\mu\text{g L}^{-1}$ (Kusk,
405 1996). Modelling studies show that copper concentrations comparable to those applied in our
406 experiment (42 $\mu\text{g L}^{-1}$) affected top-down control of *Daphnia* on green algae (Prosnier et al.,
407 2015) and should thus have been sufficient to cause a significant decrease in phytoplankton
408 filtration by *Daphnia*. One potential reason for the decline in *Daphnia* numbers at the end of the
409 experiment could be food shortage as no further phytoplankton peak emerged in CON and NO3,
410 although other reasons, such as fungal infections cannot be excluded. The decline in *Lymnaea*
411 growth when exposed to pesticides is in line with Allen et al. (2021) and may be related to
412 copper (Brix et al., 2011) or pirimicarb (Tufi et al., 2015).

413

414 4.2 Modulation of pesticide effects by nitrate

415 Remarkably, nitrate or pesticides alone did not affect the proportion of the different
416 functional groups of primary producers. In pesticide-free treatments, nitrate effects on
417 periphyton and phytoplankton biomass were compensated by snail and zooplankton grazing
418 pressure and thus did not affect the total macrophyte biomass. Pesticides alone hampered
419 *Daphnia* and thus promoted phytoplankton, but the relative proportion of phytoplankton or
420 macrophytes did not change significantly compared to CON or NO3 treatments. Synergistic
421 stressor interactions observed for the combination of pesticides and nitrate on total macrophyte
422 biomass and periphyton, and for the combination of all three stressors on total macrophyte
423 biomass highlight the importance of better understanding multiple stressor effects. Further,

424 only the combination of pesticides and nitrate affected the overall proportion of primary
425 producers. This is likely due to a facilitation of periphyton growth by nitrate in the early phase
426 of exposure and negative effects of pesticides on *Daphnia* promoting phytoplankton. These
427 findings may have direct relevance for small fishless lakes with high levels of resource
428 abundance, where *Daphnia* are dominant filter feeders (Romanovsky & Feniova, 1985). The fact
429 that the system moves towards a much lower resilience is indicated by both the general increase
430 and the wide variability in the phytoplankton biomass in the combined treatments, although
431 PERMANOVA indicated no significant interactions between pesticides and nitrate. In fact, such
432 an increase in variability can also be used as an indicator that a system is shifting from one
433 stable state to the other (Scheffer et al., 2015).

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

443

444 4.3 Warming and its modification of pesticide effects

445 Global warming is generally expected to stimulate macrophyte growth in temperate
446 (Zhang et al., 2019) and arctic (Lauridsen et al., 2020) lakes unless critical thresholds in nutrient
447 loading that result in phytoplankton dominance have been crossed (Mooij et al., 2008). One
448 explanation for the increased biomass of *Myriophyllum* in our warm treatments is its higher
449 optimum temperature range of up to 35°C (Grace & Wetzel, 1978). However, indirect effects, for

450 example, on snail activity by increased grazing on periphyton or by accelerated plant growth as
451 a compensatory mechanism to leaf loss by grazing are likely. Allen et al., (2021) whose
452 microcosms did not have a top-down control on phytoplankton showed no effects of warming
453 on *Myriophyllum* or the other two macrophyte species. In our experiment, two non-exclusive
454 explanations are possible for the modulation of pesticide effects by warming. The first is that
455 top-down control of microalgae was higher in the warm ARO treatments, reflected by increased
456 snail growth rate and an increase in *Daphnia* numbers in the early phase of the experiment,
457 increasing light and nutrient availability to macrophytes. Herbivore-plant interactions can in
458 fact be strengthened by warming (Zhang et al., 2019). The second explanation is that the warm
459 treatments promoted the development of bacteria, which may also have been influenced by a
460 higher nutrient cycling by consumers, thereby increasing competition for resources with
461 phytoplankton (Joint et al., 2002).

462 The effect of warming on phytoplankton and periphyton, which we expected to be positive
463 (Mahdy et al., 2015; Rasconi et al., 2015), remained context-specific and was likely quickly
464 counterbalanced by grazing effects of *Daphnia* and *Lymnaea*, similar to previous findings
465 (Kazanjian et al., 2018; Velthuis et al., 2017). The increased phytoplankton and decreased
466 periphyton in the pesticide treatments were significant only in the ambient treatments, and may
467 be linked to the lower consumer activity in these treatments. Warming was therefore mitigating
468 pesticide toxicity. Elevated temperature in the form of heatwaves applied with pesticides have
469 shown antagonistic or synergistic effects depending on the type of pesticide used (Polazzo et al.,
470 2021). The type of pesticides present and the community composition, including number of
471 trophic levels and their feeding behaviours, will all determine how warming affects such shallow
472 lake ecosystems. Several studies have included warming as a stressor along with nitrate (Bourai
473 et al., 2020; Mckee et al., 2002) or pesticides (Wijewardene et al., 2021; Knillmann et al., 2013),
474 often using simplified food webs or communities. Yet, the literature is lacking on experiments
475 combining all three stressors, which is a more realistic scenario, and incorporating more
476 complex food web structures to disentangle direct and indirect effects. Such a mechanistic

477 understanding is of paramount importance if safe operating spaces are to be defined in the
478 context of rising global temperatures.

479

480 **5. Conclusion**

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

501

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515

516 **Author contributions**

517 The concept of the CLIMSHIFT project was developed by EMG, SH, HS, JL, MSJ, and FH. This
518 experiment was carried out by VV, EMG and ML with major contributions by JA and NA. The
519 paper was written by VV. Formal analyses was performed by VV and supported by ML, JA and
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522

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Figures (main manuscript)

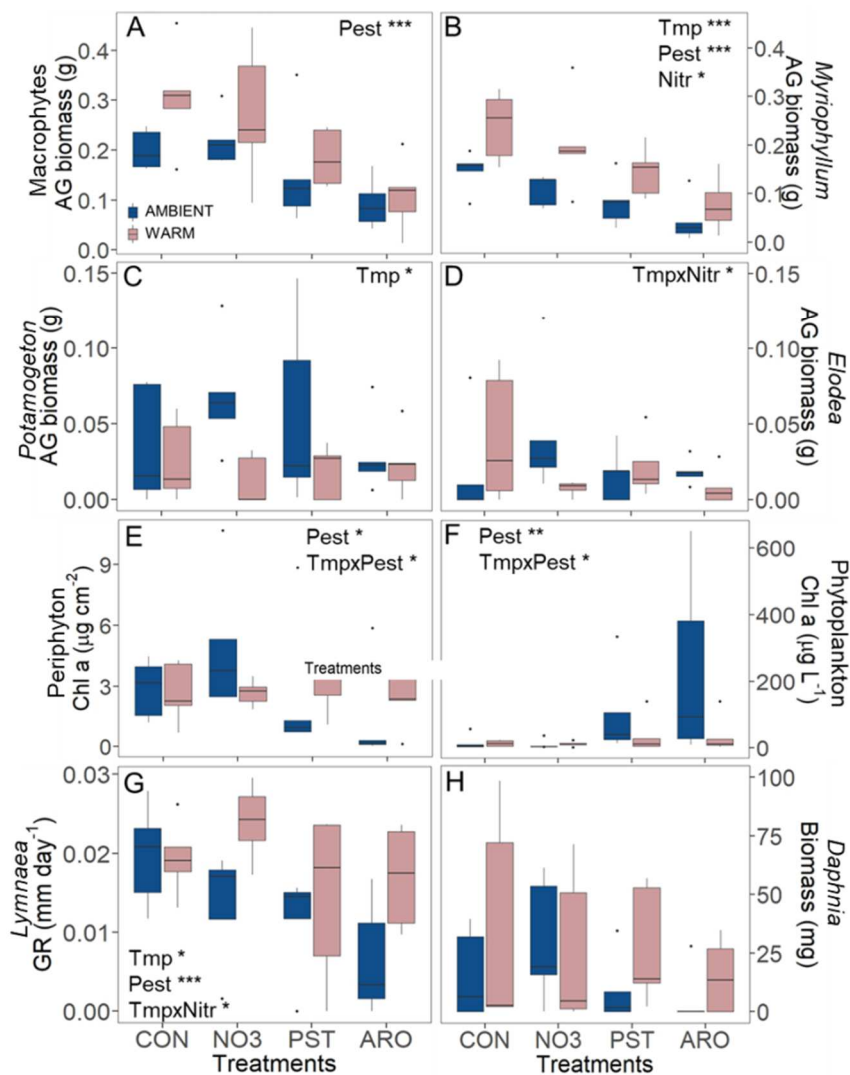


Fig. 1. Temperature and treatment (CON = control; NO3 = nitrates; PST = organic pesticides and copper; ARO = PST + NO3) effects on primary producers and consumers. Final aboveground biomass (dry mass) of (A) All macrophytes, (B) *M. spicatum*, (C) *P. perfoliatus*, and (D) *E. nuttallii*. Chl a content 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 $Q1 - [1.5 \cdot IQR]$ and $Q3 + [1.5 \cdot IQR]$, respectively, IQR – interquartile range), and outliers (dots). Asterisks represent significant effects observed with the ANOVA ($p < 0.05$): using 3 fixed factors: Tmp = temperature effect; Pest = pesticide effect, Nitr = nitrate effect. Blue and mauve represent ambient and warm microcosms, respectively.

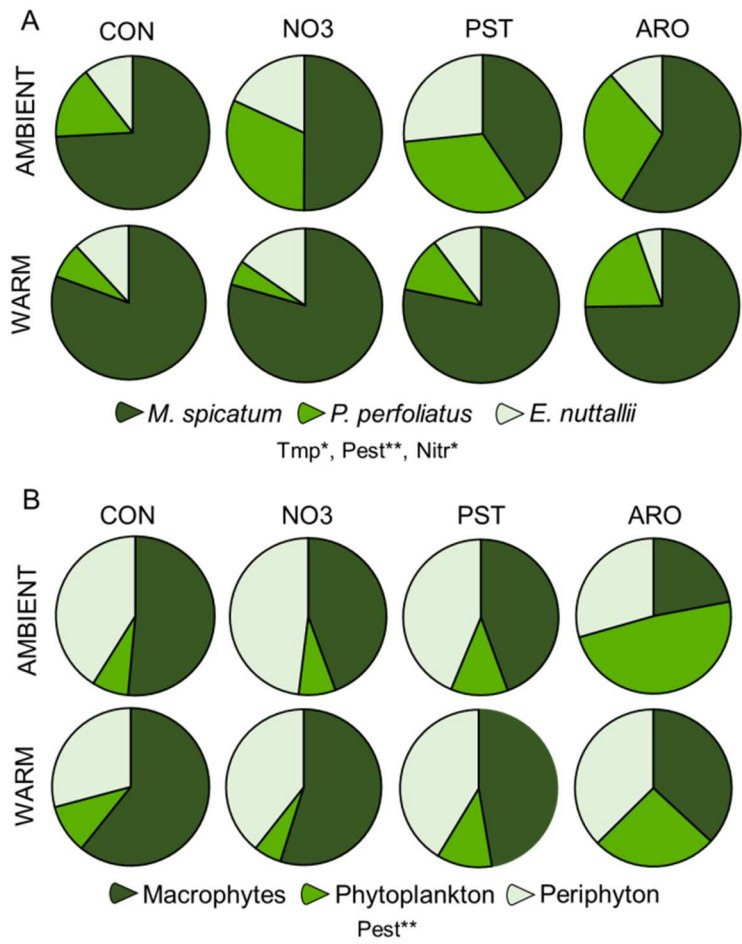


Fig. 2. Relative abundances of (A) macrophyte species and (B) primary producer groups. Abundance was measured as a function of total carbon content per microcosm at the end of the experiment. CON = control; NO3 = nitrates; PST = organic pesticides and copper; ARO = PST + NO3. Asterisks represent significant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

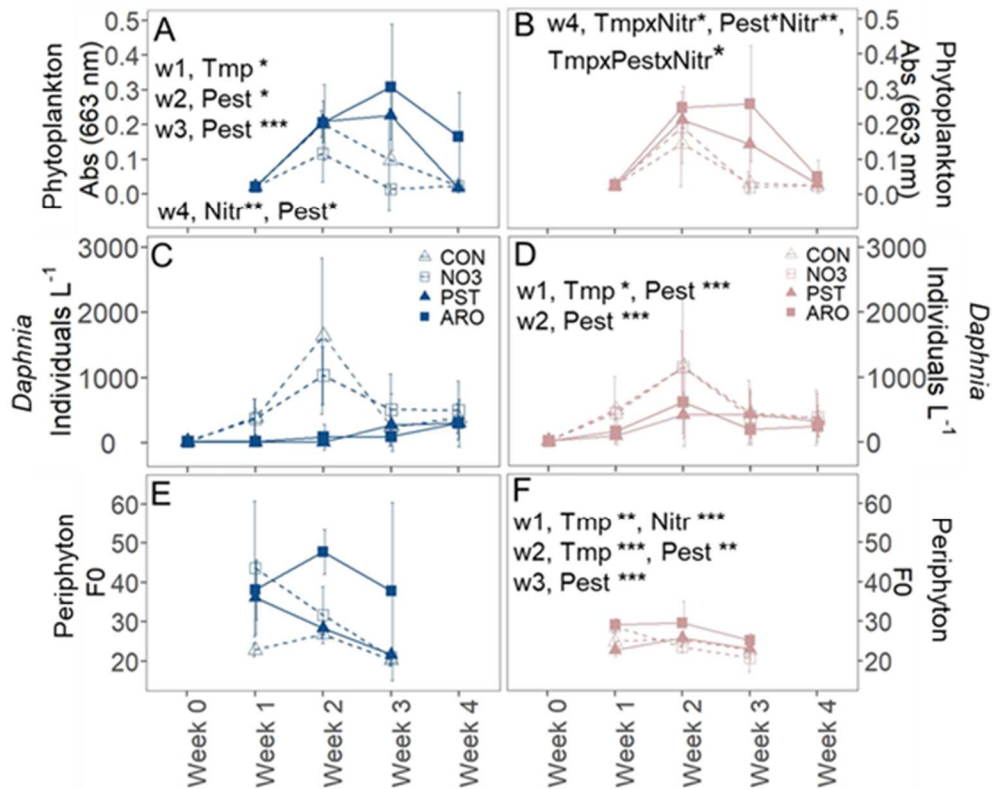


Fig. 3. Weekly measures of (A,B) phytoplankton biomass (absorbance at 663 nm), (C,D) numbers of *D. magna* adults, and (E,F) periphyton biomass (as F0 fluorescence) in the ambient (blue) and warm (mauve) microcosms. Means \pm SD, $n = 5$. CON = control; NO3 = nitrates; PST = organic pesticides and copper;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

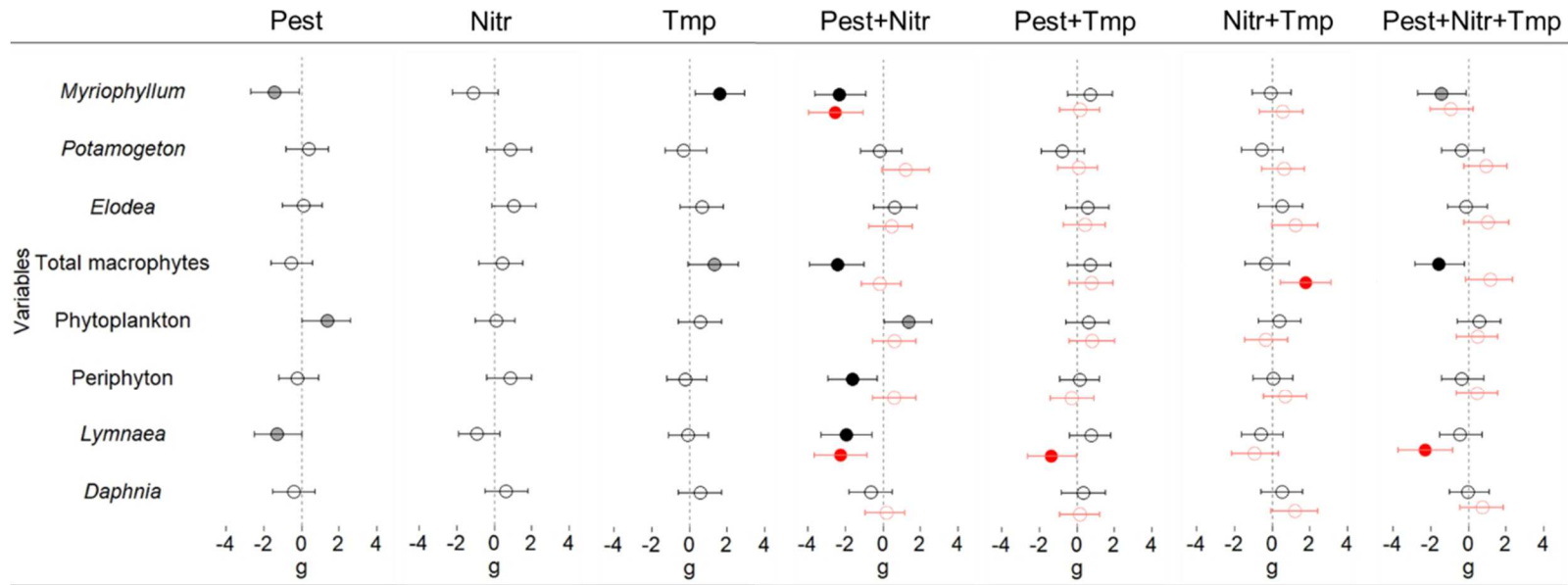


Fig. 4. Effect sizes (Hedges' $g \pm 90\%$ confidence interval) of individual and combined stressor effects on the key functional groups at the end of the experiment. No effects are inferred when confidence intervals cross the zero line. Grey symbols: non-significant effects (>0.09), black symbols: significant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

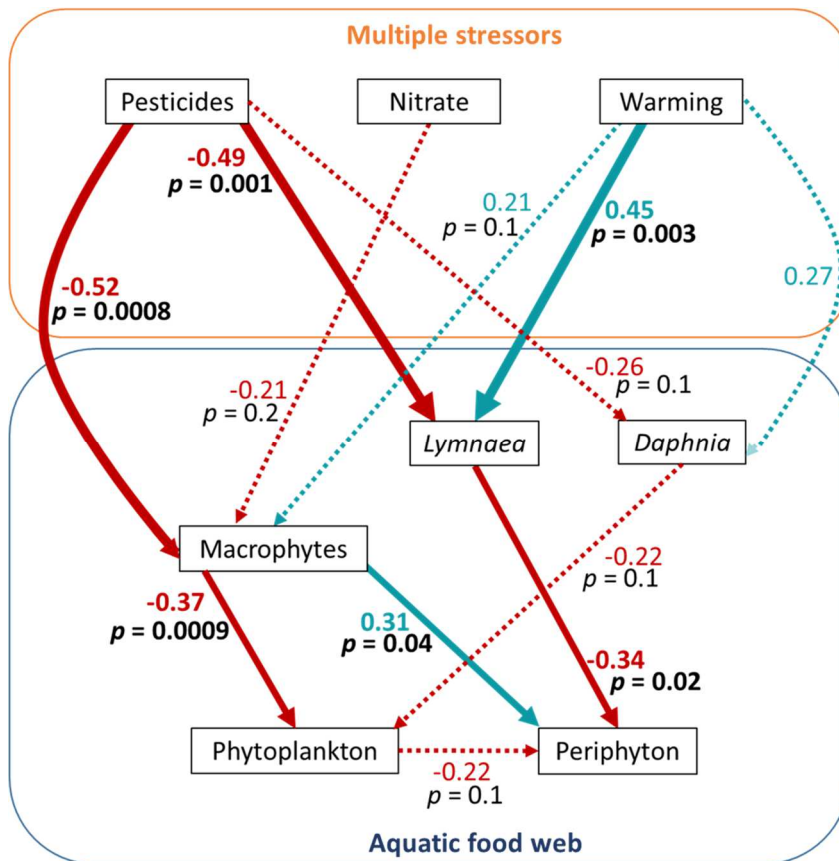


Fig. 5. Path diagram of the selected structural equation model based on final sampling data. Red, green and dashed arrows indicate negative, positive and nonsignificant relations between variables, respectively. Significant threshold was set at $p = 0.05$. The proportions of variation explained by the model for each response variable are represented by R -values in blue and red for positive and negative effects, respectively. $\text{Chi}^2 = 17.4$, root mean square error of approximation = 0.05, standardised root mean square residual = 0.1, adjusted goodness of fit index = 0.8, and comparative fit index = 0.1.

