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1 **Disentangling the direct and indirect effects of agricultural runoff on freshwater ecosystems**  
2 **subject to global warming: A microcosm study.**

3

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17 **Abstract**

18 Aquatic ecosystems are exposed to multiple stressors such as agricultural run-off (ARO) and climate-  
19 change related increase of temperature. We aimed to determine how ARO and the frequency of its  
20 input can affect shallow lake ecosystems through direct and indirect effects on primary producers  
21 and primary consumers, and whether warming can mitigate or reinforce the impact of ARO. We  
22 performed a set of microcosm experiments simulating ARO using a cocktail of three organic  
23 pesticides (terbuthylazine, tebuconazole, pirimicarb), copper and nitrate. Two experiments were  
24 performed to determine the direct effect of ARO on primary producers (submerged macrophytes,  
25 periphyton and phytoplankton) and on the grazing snail *Lymnaea stagnalis*, respectively. Three  
26 different ARO concentrations added as single doses or as multiple pulses at two different  
27 temperatures (22°C and 26°C) were applied. In a third experiment, primary producers and consumers  
28 were exposed together to allow trophic interactions. When functional groups were exposed alone,  
29 ARO had a direct positive effect on phytoplankton and a strong negative effect on *L. stagnalis*. When  
30 exposed together, primary producer responses were contrasting, as the negative effect of ARO on  
31 grazers led to an indirect positive effect on periphyton. Periphyton in turn exerted a strong control  
32 on phytoplankton, leading to an indirect negative effect of ARO on phytoplankton. Macrophytes  
33 showed little response to the stressors. Multiple pulse exposure increased the effect of ARO on *L.*  
34 *stagnalis* and periphyton when compared with the same quantity of ARO added as a single dose. The  
35 increase in temperature had only limited effects. Our results highlight the importance of indirect  
36 effects of stressors, here mediated by grazers and periphyton, and the frequency of the ARO input in  
37 aquatic ecosystems.

38

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

41           **1. Introduction**

42 Ecosystems are severely affected by anthropogenic stressors (Barnosky et al., 2011; Butchart et al.,  
43 2010) and aquatic ecosystems are particularly threatened (Reid et al., 2019). In this context, it is  
44 crucial to determine the mechanisms by which the impact of stressors on specific organisms can alter  
45 ecosystem state and functioning (Bruder et al., 2019). Aquatic ecosystems are exposed to multiple  
46 anthropogenic stressors (Jackson et al., 2016; Ormerod et al., 2010) partially originating from local  
47 sources such as agricultural activities (Ippolito et al., 2015; Malaj et al., 2014; Stehle and Schulz,  
48 2015). Agricultural run-off (ARO) is not only responsible for high nutrient input, but it also contains  
49 multiple xenobiotics such as herbicides, insecticides, fungicides and trace metals that might affect  
50 ecosystems and interact with other stressors in an unpredictable way (Schriever et al., 2007).  
51 Nutrient and pesticide input to adjacent waterbodies through ARO is highly dependent on the  
52 strength and frequency of rain events and agricultural practices. Therefore, aquatic ecosystems are  
53 frequently exposed to multiple pulses of nitrates and pesticides with variable intensity (Daouk et al.,  
54 2013; Liess et al., 1999; Reinert et al., 2002), further complicating the assessment and prediction of  
55 ARO effects. Existing studies on ARO revealed severe effects on aquatic ecosystem processes such as  
56 primary production and ecosystem respiration (Peters et al., 2013). Multiple pulse exposure to  
57 pollutants and nutrients can modulate the effects compared to a single or continuous exposure  
58 (Davis et al., 2019; Reinert et al., 2002; Varga et al., 2019). In addition to local stressors, global  
59 warming affects aquatic ecosystems by influencing the metabolic rates of organisms (Brown et al.,  
60 2004; Gillooly et al., 2001) as well as their sensitivity towards stressors (Cuco et al., 2016; Debecker  
61 et al., 2017; Delnat et al., 2019). All the different stressors may have interactive effects, but  
62 determining how multiple stressors interact is challenging (Jackson et al., 2016), especially in  
63 complex systems where biotic interactions lead to indirect effects (Bruder et al., 2019).

64 Shallow lakes are ideal ecosystems to address the aforementioned challenges, as they are the most  
65 abundant waterbodies worldwide (Verpoorter et al., 2014), often exposed to ARO (Hijosa-Valsero et

66 al., 2016) and prone to warming (Adrian et al., 2009). The state of a shallow lake ecosystem is shaped  
67 by biotic interactions among and between primary producers and higher trophic levels, and thus they  
68 respond non-linearly to nutrient loading with rapid shifts between two alternative stable states: a  
69 macrophyte-dominated clear-water state and a phytoplankton-dominated turbid state (Scheffer and  
70 van Nes, 2007; Scheffer et al., 1993). Such shifts have severe consequences for several ecosystem  
71 functions (Hilt et al., 2017) and services (Janssen et al., 2020). Both warming and exposure to ARO  
72 have been shown to affect macrophytes (Mckee et al., 2002), phytoplankton (Donald et al., 2011;  
73 Huisman et al., 2018; Paerl and Huisman, 2008; Zhao et al., 2019a), periphyton (Piggott et al., 2015)  
74 and primary consumers (Schaum et al., 2018; Zhang et al., 2018). Increasing temperature and  
75 nutrient loading were predicted to increase the probability of a shift toward the turbid state (Moss et  
76 al., 2011; Scheffer et al., 2015), while the effects of other pollutants including pesticides, trace metal  
77 elements, trace organics, pharmaceuticals and vanity products remained largely unexplored (Phillips  
78 et al., 2016). Combining expertise from ecology and ecotoxicology can help elucidating the  
79 complexity of stressor and biotic interactions in shallow lakes, e.g. by using microcosm experiments  
80 providing a higher ecological realism than single species exposed to stressors such as pollutants. Such  
81 experiments will reveal direct and indirect effects on interacting functional groups in aquatic  
82 communities, an essential prerequisite to predict and eventually mitigate the impact of global  
83 warming by controlling local stressors (Scheffer et al., 2015).

84 Here, we investigated in microcosms the combined effect of global warming and ARO on two trophic  
85 levels (primary producers and their consumers) representative of shallow lake ecosystems. We  
86 hypothesised that 1) ARO and warming will affect primary producer and consumer development.  
87 Particularly, we expected that ARO will cause a shift towards a phytoplankton dominance and that  
88 warming will increase this ARO effect. We also expected that 2) direct effects on some organisms will  
89 affect others indirectly through biotic interactions. We further hypothesise that 3) multiple pulses of  
90 ARO will favour the overall resistance of the ecosystem in comparison to a single pulse treatment  
91 with the same overall amount of pollutant added to the microcosms, because a more progressive

92 input of toxicants would allow organisms to acclimate to this stressor. The three microcosm  
93 experiments were performed mimicking ARO containing nitrate, copper and typical organic  
94 pesticides with different exposure modes (addition of ARO by a single or multiple pulses) and  
95 warming following the IPCC RCP 8.5 scenario (+4°C, IPCC, 2014). The effects of these stressors were  
96 evaluated on microcosms containing only the primary producers, only the grazers or both with the  
97 overall aim to gain a mechanistic understanding of direct and indirect effects of two major  
98 environmental stressors affecting shallow aquatic ecosystems. We used structural equation  
99 modelling to determine further causal linkages between these stressors and observed effects.

100

## 101 2. Material and methods

102 Three microcosm experiments were performed. In Experiments 1 and 2, respectively, primary  
103 producers and grazers were exposed independent of each other to ARO and warming, while in  
104 experiment 3, they were exposed together to allow for trophic interactions. The microcosms were  
105 incubated at two different temperatures and exposed to a cocktail of pesticides, copper and nitrate  
106 designed to mimic the effect of ARO (see Table 1 for ARO composition). Six different ARO treatments  
107 were used: a control without ARO; three treatments corresponding to the ARO at the lowest  
108 concentration (X), twice the lowest concentration (2X), and thrice the lowest concentration (3X) as a  
109 single addition at the beginning of the experiment; two treatments with two or three pulses of ARO  
110 at the lowest concentration (X) with four-day intervals between each addition (multiple pulse  
111 treatments XX and XXX, with the same final amount of ARO as in 2X and 3X, respectively. Target  
112 temperatures were set at 22°C (controls representing typical temperate lake summer temperatures  
113 e.g. Mckee et al., 2002; Sand-Jensen et al. 2019) and 26°C (representing the future climate warming  
114 scenario). We therefore used 12 different combinations of ARO and temperature: cold-control,  
115 heated-control, cold-1X, heated-1X, cold-2X, heated-2X, cold-3X, heated-3X, cold-XX, heated-XX,  
116 cold-XXX, heated-XXX with 5 replicates for each condition. In experiment 2, with grazers alone, only

117 the 1X and 3X treatments at both temperatures were used. In addition, the concentration of nitrates  
118 was reduced since nitrates would not be consumed in the absence of primary producers (Table 1).

119 The ARO cocktail contained terbuthylazine (CAS number 5915-41-3), a triazine photosynthesis-  
120 inhibiting herbicide (Lorente et al., 2015), tebuconazole (CAS number 107534-96-3), a triazole  
121 fungicide known to affect biofilm and phytoplankton (Artigas et al., 2014), pirimicarb (CAS number  
122 23103-98-2), a carbamate insecticide inhibiting acetylcholinesterase (Krieger, 2010), and copper  
123 ( $\text{CuSO}_4$ , CAS number 7758-98-7), a trace metal used in fungicidal, bactericidal and algicidal  
124 preparations (Lamichhane et al., 2018). All pesticides were PESTANAL<sup>®</sup> analytical standards (Merck,  
125 Darmstadt, Germany). Concentrations were based on published EC values (EC10, 20, 50) for each  
126 organism group, available SSD data and some unpublished data on the toxicity of all ARO  
127 components. From these data, we identified an effective concentration for each ARO pollutant  
128 (excluding nitrate) equivalent to the EC20 for chronic exposure for the most sensitive group, which  
129 was used as the highest concentration in the subsequent experiments (Table 1). Stock solutions of  
130 organic pesticides were prepared in Dimethyl sulfoxide (DMSO, CAS 67-68-5, Merck). The same  
131 amount of DMSO was added in all microcosms at a final concentration of 0.125%. Copper and nitrate  
132 ( $\text{KNO}_3$ ) solutions were prepared in MilliQ water.

133

#### 134 **2.1. Experiment 1 - primary producers alone**

135 In experiment 1, 8-L microcosms contained submerged macrophytes (*Myriophyllum spicatum* L.,  
136 *Elodea nuttallii* Michx., and *Potamogeton perfoliatus* L), planted in artificial sediments (OECD TG 239  
137 (2014) modified to replace nutrient salts by 5 g kg<sup>-1</sup> nettle powder as fertilizer), and ten strains of  
138 microalgae (SI table S1). Macrophytes were introduced first to allow rooting before being submitted  
139 to competition with phytoplankton and periphyton. Algae were introduced in the microcosms 9 days  
140 after macrophytes and 8 days before ARO addition and temperature increase, just after a complete  
141 water renewal to remove initial differences in algae and nutrient levels that had developed in the

142 microcosms. Achieved temperatures were  $22.5 \pm 0.4^\circ\text{C}$  (unheated) and  $25.7 \pm 0.4^\circ\text{C}$  (heated  
143 microcosms). Microcosms were maintained during 28 days after ARO addition with regular water and  
144 nutrient addition until final sampling. Air temperature was maintained at  $22^\circ\text{C}$ , light at  $70 \mu\text{mol m}^{-2} \text{s}^{-1}$   
145 <sup>1</sup> (16:8 h light:dark cycle). Translucent polypropylene strips were placed in each microcosm for  
146 periphyton development monitoring. More information on microcosm settings are provided in the  
147 supplementary material.

## 148 **2.2. Experiment 2 – grazers alone**

149 The pond snail *Lymnaea stagnalis* was exposed to ARO at  $22 \pm 0.2^\circ\text{C}$  and  $26 \pm 0.2^\circ\text{C}$  in the absence of  
150 any primary producer. This species was selected because it is commonly found in shallow freshwater  
151 ecosystems, feeding primarily on benthic algae and aquatic plants (Elger et al., 2009). It is also a good  
152 model in ecotoxicology (Amorim et al., 2019) recently normalized as OECD test guideline (OECD  
153 2016). *Lymnaea stagnalis* was obtained from a nearby pond and bred in the laboratory for several  
154 generations to obtain a parasite-free population. Individuals were selected to minimise size  
155 differences, measured and weighed (mean shell length  $\pm$  SD =  $13.7 \pm 1.3$  mm, mean total weight =  
156  $202 \pm 58$  mg). Each snail was placed in a 12-cm diameter, 30-cm high cylindrical glass aquarium filled  
157 with 1 L of Volvic® water with temperature control as in experiment 1. Snails were starved for 12 h  
158 before simultaneous addition of ARO and ten pre-weighed 12-mm diameter lettuce discs (organically  
159 grown). After 24 h, the remaining leaf discs were collected and freeze-dried. Additional lettuce discs  
160 were used to determine initial dry mass, and the dry mass of lettuce consumed by each snail was  
161 determined. The snails were then fed *ad libitum* with lettuce for 21 days. Water evaporation in the  
162 aquaria was compensated by addition of deionised water twice a week. The light cycle was 16:8  
163 light:dark with only  $5 \mu\text{mol m}^{-2} \text{s}^{-1}$  irradiance to control possible algal development. At the end of the  
164 experiment, snails were starved for 12 h before a new feeding test was performed with 10 pre-  
165 weighed 14-mm diameter lettuce discs and stopped after 16 h to avoid complete consumption of the  
166 food provided. The snails were then measured and weighed.



167

168 **2.3. Experiment 3 - primary producers and consumers**

169 We used the same experimental set up as in experiment 1 but added one individual of *L. stagnalis*,  
170 one individual of zebra mussel (*Dreissena polymorpha*) and twenty *Daphnia magna* neonates in each  
171 microcosm. Snail individuals collected as in experiment 2 had an initial mean ( $\pm$  SD) shell length of  
172 18.7 ( $\pm$  2.2) mm and a mean total fresh mass of 0.4 ( $\pm$  0.1) g including shell. Zebra mussels collected  
173 in a lake were placed in aquaria for 1 week before being transferred to the microcosms and  
174 measured using an electronic calliper (16.0  $\pm$  1.2 mm). Individuals were selected to be as  
175 homogenous in size as possible. *D. magna* were bred at the laboratory. Macrophytes, algae and  
176 primary consumers were introduced in the mesocosms 10, 7 and 3 days before addition of ARO,  
177 respectively, to allow for settlement without stressors.

178 In addition, several adjustments were made due to observations in the first experiment and changes  
179 in material availability. The organic pesticides were prepared as a 10-fold more concentrated  
180 solution, and therefore, the amount of DMSO added to the microcosms was ten times lower (0.01%).  
181 CaCO<sub>3</sub> at 7.5 g kg<sup>-1</sup> was added in the sediment of each microcosm as a buffer to limit potential effects  
182 of acidity on the primary consumers. An additional plastic strip was added. Mean water  
183 temperatures were 21.4  $\pm$  0.3°C and 23.7  $\pm$  0.3°C in the non-heated and heated microcosms,  
184 respectively. Final sampling was done 21 days after the addition of ARO as it corresponded to the  
185 maximal development of phytoplankton in experiment 1.

186

187 **2.4. Measured parameters**

188 At the end of each experiment, water was collected and filtered at 0.7  $\mu$ m (GF/F). In the filtered  
189 water, concentrations of nitrate and dissolved phosphorus were determined by ion chromatography  
190 (Dionex Ics-5000<sup>+</sup>, Sunnyvale, CA, USA) and by ammonium molybdate spectrophotometric method,

191 respectively (AFNOR 1990). The plastic strips were cut at 20 cm from the bottom, and all *L. stagnalis*  
192 eggs and juveniles present on the strips were collected and counted. Periphyton on these strips was  
193 scraped and collected with 20 mL of Volvic® water. From the resulting suspension, subsamples of 1.5  
194 mL were centrifuged (10000 g, 10 min) in 2 mL tubes. The filters and pellets were stored at -80°C  
195 until photosynthetic pigment analysis.

196 Phytoplankton and periphyton pigments were extracted to estimate total planktonic and periphytic  
197 algae biomass and the proportion of diatoms, green algae and cyanobacteria. Pigment extraction was  
198 carried out on periphyton and phytoplankton samples after freeze-drying by the addition of 2 mL  
199 methanol buffered with 2% ammonium acetate 1 M, followed by an initial sonication step (3 min at  
200 4°C) and 15 min of incubation at -20°C, and then a second sonication step. Pigment extracts were  
201 recovered after centrifugation (10000 g, 5 min, at 2°C). The extraction steps were repeated until  
202 obtained extracts were no longer visibly coloured. All the extracts of the same sample were pooled.  
203 Extracts were filtered through a 0.2-µm PTFE membrane syringe filter (VWR International, United  
204 States), and analysed by HPLC. HPLC analysis of pigments was performed as described by Capdeville  
205 et al. (2019). Pigments specific to a particular group of primary producer (diatoms: fucoxanthin,  
206 diatoxanthin and diadinoxanthin; green algae: chlorophyll *b*, lutein, violaxanthin and neoxanthin;  
207 cyanobacteria: zeaxanthin, echinenone and myxoxanthophyll) were used to assess variation of the  
208 different groups of microalgae (Mackey et al., 1996; Majdi et al., 2011). The total P concentration in  
209 periphyton biomass was determined following the ammonium molybdate spectrophotometric  
210 method after persulphate digestion in acidic conditions of freeze-dried periphyton subsamples  
211 following AFNOR guidelines (AFNOR 1990).

212 Macrophytes were handled as described in Gross et al. (2018). Macrophytes were removed from the  
213 sediments and rinsed in water to remove attached particles and epiphytes. All the plants of one  
214 species were spread in a large tray equipped with a ruler, and a picture was taken. Roots and  
215 aboveground parts were separated and rapidly dried. Aboveground parts were then weighed to

216 estimate macrophyte biomass. Visible signs of macrophyte consumption by grazers were recorded  
217 based on the pictures taken.

218 In experiment 3, adult *L. stagnalis* were collected, weighed and measured. Individuals of *D.*  
219 *polymorpha* were collected and measured. The presence of dead *L. stagnalis* or *D. polymorpha* was  
220 recorded. Due to a rapid disappearance of *D. magna* in all microcosms, no measurement was  
221 possible.

222

## 223 **2.5. Statistics**

224 ARO concentration, exposure mode (single or multiple pulses of ARO addition) and temperature  
225 effects on the different measured parameters were analysed using an ANCOVA with ARO  
226 concentration as a continuous variable and temperature and exposure mode as categorical  
227 independent variables. The effect of exposure mode was analysed using only 2X/XX and 3X/XXX data  
228 with contrast analysis. The magnitude of the effects was measured using Hedges'  $g$  (Hedges, 1981)  
229 effect size calculated from  $t$ -statistics derived from the ANCOVA according to Berben et al. (2012)  
230 using the R package {esc}.

231

## 232 **2.6. Structural equation modelling**

233 To explore the simultaneous effect of ARO and warming on the interactions between macrophytes,  
234 phytoplankton, periphyton and grazers, we built a path analysis subjected to structural equation  
235 modelling (SEM; Grace et al., 2010). SEM has been developed as a powerful tool for inferring cause-  
236 effect relationships among variables via proposed hypotheses tested against data (Grace et al., 2012;  
237 Grace and Irvine, 2020). Furthermore, SEM permits the evaluation of direct and indirect effects  
238 within a network of variables (Bruder et al., 2019). This approach has been successfully applied to  
239 identify the mechanistic pathway underlying the effect of different stressors on aquatic ecosystems  
240 (Alexander et al., 2016, 2013; Majdi et al., 2014; Rodrigues et al., 2018). Indeed, as a framework for

241 quantitative analyses, SEM is an extension of univariate mediation analyses taking into account the  
242 effect of variable X on variable Z through its effect on Y (Grace and Irvine, 2020; Reisner et al., 2013).  
243 Using *a priori* knowledge, we built a full model of cause-effect relationships based on hypothetical  
244 pathways supported by literature (Table 1). Notably, the full model included a direct pathway  
245 between ARO exposure and phytoplankton biomass, and several indirect pathways via competition  
246 with periphyton or macrophytes (See Supplementary Figure S6). The hypothetical relationships  
247 between variables (table 1) was then tested on the correlation matrix of experimental data (Grace  
248 and Irvine, 2020; Reisner et al., 2013). We considered all available variables to investigate the  
249 interactions among trophic levels. Using *a priori* knowledge, a full path analysis model was created  
250 including simultaneously several hypothetical pathways (Table 2) through which ARO and warming  
251 could affect macrophytes, phytoplankton, periphyton and grazers. We only used the biomasses of  
252 macrophytes, phytoplankton, periphyton and grazers from experiment 3 (with primary producers  
253 and consumers) to perform this analysis. For the primary consumers, only *L. stagnalis* size increase  
254 was used as *D. magna* did not develop and no growth was observed on *D. polymorpha*. The different  
255 directions and kind of interactions among trophic levels were tested. Then, successive full models  
256 were simplified by step-wise exclusion of variables and/or linkages with non-significant weights and  
257 non-significant co-variance, as estimated by Akaike Index Criterion (AIC), until a minimal adequate  
258 model showing specific linkages remained. The best model was selected based on its coherence with  
259 the direct effect observed in experiments 1 and 2. The adequacy of the final model was determined  
260 using quality indicators such as Chi<sup>2</sup>, root mean square error of approximation (RMSEA), standardised  
261 root mean square residual (SRMR), adjusted goodness of fit index (aGFI) and comparative fit index  
262 (CFI) (Fan et al., 2016). SEM was performed using the R package {sem}.

### 263 3. Results

#### 264 3.1. Experiment 1: Effects of stressors on primary producers without consumers

265 ARO exposure and elevated temperature had no effect on total macrophyte aboveground biomass  
266 (Fig. 1). The exposure mode did not change the effect on macrophytes (Fig. 1). The mean final

267 macrophyte dry mass per microcosm was  $0.61 \pm 0.24$  g with values for individual microcosms ranging  
268 from 0.11 to 1.23 g (Supplementary material Fig. S1).

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

275 ARO exposure and warming had no significant effect on periphyton biomass, with a mean value of  
276  $1.08 \pm 0.71 \mu\text{g cm}^{-2}$  of Chl *a*. However, we observed a trend for higher periphyton biomass ( $p = 0.060$ )  
277 with ARO and a higher periphyton biomass with multiple pulses as compared to single additions of  
278 the ARO ( $p = 0.046$ , Fig. 1). ARO induced an increase of the cyanobacterial pigment/Chl *a* ratio in  
279 both periphyton and phytoplankton (Fig. 2). ARO had no significant effect on the total amount of P  
280 contained in periphyton (Supplementary material Fig. S5).

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

286

### 287 **3.2. Experiment 2: Effects of stressors on grazers**

288 ARO exposure strongly reduced *L. stagnalis* growth with a significant effect on its increase in mass  
289 and length (Fig. 1). The snail mass increased by  $603 \pm 32$  mg without ARO and by only  $184 \pm 44$  mg at

290 the highest ARO concentration. Warming positively influenced length increase but had no effect on  
291 snail mass (Fig. 1).

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

297

### 298 **3.3. Experiment 3: Effects of stressors on interacting primary producers and consumers**

299 Macrophyte biomass did not respond to ARO addition (independent of the exposure mode) nor  
300 warming (Fig. 1). In this experiment, ARO concentration negatively affected phytoplankton biomass  
301 (Fig. 1), and these effects were stronger at the lower temperature indicating an interaction between  
302 ARO exposure and warming (Fig. 1). The highest phytoplankton biomasses were recorded in the cold  
303 control microcosms (cold:  $84.8 \pm 36.0$   $\mu\text{g L}^{-1}$  Chl *a*, warm:  $45.0 \pm 14.4$   $\mu\text{g L}^{-1}$  Chl *a*).

304 Conversely, ARO concentration caused a strong increase of periphyton biomass (Fig. 1). Periphyton  
305 biomass was very low in control conditions ( $0.22 \pm 0.07$   $\mu\text{g cm}^{-2}$  Chl *a* and  $0.38 \pm 0.19$   $\mu\text{g cm}^{-2}$  Chl *a* for  
306 cold and warm temperatures, respectively) but was significantly higher with ARO, especially when  
307 added as multiple pulses (Fig. 1). At the highest ARO concentration, periphyton biomass reached  $2.90$   
308  $\pm 0.61$   $\mu\text{g cm}^{-2}$  Chl *a* (cold) and  $3.51 \pm 0.67$   $\mu\text{g cm}^{-2}$  Chl *a* ( ) when ARO was added as multiple pulses  
309 (XXX), while it reached only  $2.05 \pm 0.60$   $\mu\text{g Chl } a \text{ cm}^{-2}$  (in cold) and  $2.11 \pm 0.78$   $\mu\text{g Chl } a \text{ cm}^{-2}$  (in warm)  
310 when ARO was added as a single dose (3X). ARO, and particularly exposure to multiple pulses,  
311 increased the P content of periphyton ( $p < 0.001$  and  $p = 0.019$ ). Warming did not significantly affect  
312 final periphyton biomass ( $p = 0.48$ ).

313 *D. magna* neonates disappeared in all microcosms except six microcosms where *D. magna*  
314 population develop but also disappeared before the end of the experiment. ARO strongly affected  
315 the mussels, all of which died at the higher ARO concentration (independent of frequency of  
316 addition), while they survived in all control microcosms. No clear increase in mussel size was  
317 observed in any of the microcosms where they remained alive.

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

328 ARO and warming induced a decrease of the proportion of green algae and an increase of diatoms in  
329 the periphyton (Fig. 2). ARO also caused an increase of the diatom pigment in the phytoplankton.  
330 Warming negatively affected the growth of *M. spicatum* and *E. nuttallii* but not of *P. perfoliatus*.  
331 Repeated addition of ARO had a negative effect on macrophytes when compared with a single  
332 addition (Fig. 2).

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

338

### 339 **3.4. Structural equation modelling**

340 A first SEM model was constructed including all possible relationships among measured variables and  
341 between the measured variables and experimental parameters (Table 2, supplementary material Fig.  
342 S6). We did not find a significant relationship between warming and any measured parameter in the  
343 SEM performed, and therefore removed warming effects in the model. In the resulting model  
344 (supplementary material Fig. S6), only periphyton biomass and snail growth were directly affected by  
345 ARO. To explore further the direct and indirect effects of ARO on macrophyte or phytoplankton  
346 dominance, we used a third model considering only the effect of periphyton on phytoplankton and of  
347 periphyton on macrophytes without the respective reciprocal effects (Fig. 3).

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

356

## 357 **4. Discussion**

358 The combination of experiments with one and two trophic levels allowed us to assess direct and  
359 indirect effects of two major environmental stressors, climate warming and agricultural run-off (ARO)  
360 on the functioning of shallow aquatic systems. Confirming our first hypothesis, we showed direct  
361 effects of ARO on primary producers and primary consumers. We further revealed the structuring  
362 role of periphyton and their grazers (*L. stagnalis*) in these systems often described only by the



363 alternative dominance of submerged macrophytes or phytoplankton (Scheffer and van Nes, 2007;  
364 Scheffer et al., 1993). Repeated exposure to ARO, as can be expected for shallow aquatic systems in  
365 an agricultural landscape, enhanced these effects. In contrast to our expectations, warming by  
366 approx. 3.5 °C did not enhance the negative effects, probably because our tested temperature  
367 increase did not affect primary producers directly. Our results underline the importance to increase  
368 ecological complexity when evaluating the effect of agricultural run-off including pesticides on  
369 aquatic systems, as single species tests would not be able to reveal all direct and indirect effects. In  
370 the following, we will detail how the results matched with our initial hypotheses.

#### 371 ***4.1. Direct effects of ARO and warming on single trophic levels***

372 To test our first hypothesis, experiments 1 and 2 were designed to determine the effect of the  
373 stressors without trophic interactions. We observed a positive effect of ARO on phytoplankton  
374 growth. This positive effect may be linked to the presence of nitrate in the ARO because it is  
375 consistent with what can be expected with nitrate addition in P-rich environments (Donald et al.,  
376 2011; Leavitt et al., 2006; Paerl and Scott, 2010). The low amount of remaining nitrate at the end of  
377 the experiment further supports a possible effect of nitrate. This increase in phytoplankton biomass  
378 did not lead to a strong negative competitive effect on macrophytes, as could be expected, and there  
379 was no shift to a typical phytoplankton-dominated state. However, such shifts can be expected under  
380 *in situ* conditions, when higher water depth and longer duration result in significant shading of  
381 macrophytes by phytoplankton.

382 The most remarkable direct effect of ARO was a strong inhibition of growth and feeding of the snail *L.*  
383 *stagnalis*. This is consistent with the known effect of pesticides and copper on this species (Amorim  
384 et al., 2019; Tufi et al., 2016). Indeed, Tufi et al. (2016) observed acetylcholinesterase inhibition on *L.*  
385 *stagnalis* exposed to 3.62 µg L<sup>-1</sup> of pirimicarb. In our experiment, pirimicarb concentration ranged  
386 from 5 to 15 µg L<sup>-1</sup>. Tebuconazole and copper are linked to embryotoxicity in terrestrial snails (*Helix*  
387 *aspersa*; Druart et al., 2012). The highest concentration of Cu used in our study (42 µg L<sup>-1</sup>) is in the

388 range of the estimated EC50 on juvenile *L. stagnalis* growth rate in a 28-day experiment (Vlaeminck  
389 et al., 2019). Nitrate cannot be responsible for the negative effect observed because the  
390 concentration in our experiment was more than 50-fold below the toxic concentration for any  
391 freshwater mollusc (Soucek and Dickinson, 2012).

392 The hypothesis that warming will promote phytoplankton dominance could not be confirmed.  
393 Warming did not affect primary producers nor snail feeding activity. This result is surprising because  
394 warming in the range of our experiments brings both chlorophytes and cyanobacteria closer to their  
395 optimum temperature (Lürling et al., 2013), and snail activity was expected to increase with warming  
396 in the tested range (Schaum et al., 2018; Zhang et al., 2018). However, a lack of warming effects on  
397 macrophytes was also reported in other studies, for instance, McKee et al., (2002) observed no  
398 temperature-induced change in the development of macrophytes in a 2-year mesocosm experiment  
399 with a 3°C warming above ambient temperature. Similarly, Feuchtmayr et al. (2019) found no effect  
400 on primary producers using a 4°C temperature elevation, in a 1-year experiment where temperature  
401 followed natural variations. Primary producers may have been driven more by nutrient limitation  
402 (although we regularly added nutrients) than by temperature. Indeed, an antagonist between  
403 warming and nutrient limitation have been observed in several studies (Birk et al., 2020; Richardson  
404 et al., 2019). Also, studies with an impact of temperature on grazers (Schaum et al., 2018; Zhang et  
405 al., 2018) used a higher temperature difference than our study.

406

#### 407 ***4.2. Importance of indirect effects mediated by biotic interactions in a more complex system***

408 In experiment 3, including both primary producers and grazers, the net effect of ARO on  
409 phytoplankton development was negative and thus opposite to the single trophic level experiment,  
410 probably due to the toxic effects of ARO on periphyton grazers that released periphyton from grazing  
411 pressure. By this mechanism, ARO had an indirect positive effect on periphyton, in addition to the  
412 direct positive effect of ARO on periphyton indicated by the SEM model. A strong negative

413 relationship was also observed between periphyton and phytoplankton probably due to competition  
414 between the two functional groups for nutrients (more specifically for phosphorus because a  
415 decrease in phytoplankton biomass was observed while nitrate was added with the ARO).

416 The negative effect of ARO on *L. stagnalis* was reduced in experiment 3 (with primary producers and  
417 grazers), where the microcosms also included primary producers, compared to experiment 2 (grazers  
418 alone). Different factors may explain this reduced effect. Notably, the other organisms present may  
419 also have led to a reduced exposure of snails due to increased adsorption and degradation of  
420 toxicants. Periphyton also represents a higher quality food than lettuce, leading to a decrease in the  
421 sensitivity to toxicants (Fidder et al., 2018; Vlaeminck et al., 2019). Beside effects of food quality, the  
422 available food in terms of periphyton biomass in the control microcosms was very low. Snail growth  
423 may have been food limited, thus reducing the magnitude of the observed effect of ARO. This is  
424 supported by the fact that *L. stagnalis* grazing on *P. perfoliatus* was more frequently observed in the  
425 control microcosms than in treatments with higher periphyton biomass, indicating a limitation in the  
426 availability of periphyton that they preferentially graze. This effect of resource limitation on snail  
427 growth may have led to an underestimation of ARO effects on *L. stagnalis*.

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

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

437 The role of periphyton and their grazers is not considered in classical models of alternative stable  
438 states in shallow eutrophic lakes (Scheffer and van Nes, 2007; Scheffer et al., 1993). Yet, shading  
439 effects of periphyton on macrophytes (Jones and Sayer, 2003; Phillips et al., 2016; Roberts et al.,  
440 2003) and positive effects of snail grazing on macrophytes are well known (Mormul et al., 2018). Our  
441 results contrasted with theory predicting the increase of phytoplankton biomass and the regression  
442 of macrophytes in shallow lakes as a result of eutrophication and warming (Kosten et al., 2012; Mooij  
443 et al., 2007; Moss et al., 2011; Scheffer and van Nes, 2007). However, they support modelling studies  
444 (Genkai-Kato et al., 2012; Jäger and Diehl, 2014) indicating that also periphyton can control the  
445 development of phytoplankton. This suggests a key role of periphyton and their grazers in  
446 macrophyte-phytoplankton interactions, as already outlined by Phillips et al. (1978), reinforced in  
447 Phillips et al. (2016). We therefore consider that periphyton should receive more attention among  
448 the steering factors controlling alternative stable states in shallow lakes.

449 We show here that while some stressors are expected leading to higher phytoplankton abundance  
450 due to direct effects, complex interactions can lead to completely different results. Generally, the  
451 monitoring of lake ecological quality is based on an indicator of eutrophication, including for example  
452 measurement of phytoplankton Chl *a* (Laplace-Treytore and Feret, 2016). The complex effect of ARO  
453 observed in this study can remain undetected by lake monitoring programs although the functioning  
454 of the system has been truly altered. Similarly, while different environmental parameters are used to  
455 predict the occurrence of cyanobacterial blooms (Zhao et al., 2019a), biotic interactions are not  
456 included. Such indirect effects must be take into account, and therefore multiple components of the  
457 ecosystems should be used for ecosystem health assessment (Zhao et al., 2019b), together with a  
458 good understanding of mechanistic pathways linking stressors to observed effects.

459

#### 460 ***4.3. ARO arriving in multiple pulses increases effects***

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

## 482 5. Conclusion

483 Shallow lakes are complex ecosystems influenced by a strong benthic-pelagic coupling. They are  
484 considered in a good ecological status when dominated by submerged macrophytes, while  
485 dominance of phytoplankton or a massive development of periphytic algae are signs of degradation.

486 A few studies have already addressed of the potential of eutrophication and/or climate warming for  
487 shifts between alternative stable states typical for these systems. Yet, our study is the first to  
488 investigate the impact of single or multiple pulses of agricultural run-off composed of organic and  
489 inorganic pesticides and nitrates in the context of climate warming. We tested the first two trophic  
490 levels, primary producers and primary consumers, and show that negative effects can foster either  
491 phytoplankton or periphyton development. Our findings underline the importance to go beyond  
492 simple laboratory systems using only single species or functional groups. Single species tests cannot  
493 account for the complexity in these systems due to multiple direct and indirect effects acting in  
494 parallel. Our series of microcosm experiments based on individual and combined trophic levels  
495 coupled with SEM allowed us to disentangle direct and indirect effects of two major global stressors,  
496 climate warming and agricultural run-off (ARO) on the development of macrophytes, phytoplankton  
497 and periphytic algae. We showed that direct positive effects of ARO on phytoplankton were  
498 completely reversed by indirect effects mediated by biotic interactions such as the competition  
499 between periphyton and phytoplankton or herbivory by grazers on periphyton. Effect of ARO on  
500 grazers such as *L. stagnalis* strongly affected the balance between functional groups of primary  
501 producers, and this effect was enhanced when ARO arrived in multiple pulses. Experiments with  
502 more complex systems allowing to take into account biotic interactions and different scenarios of  
503 stressor exposure are a more reliable basis for ecosystem assessment and management.

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- 793

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

	Single addition			Multiple pulses	
	1X	2X	3X	XX	XXX
797 Terbutylazine ( $\mu\text{g L}^{-1}$ )	1	2	3	2	3
798 Tebuconazole ( $\mu\text{g L}^{-1}$ )	30	60	90	60	90
799 Pirimicarb ( $\mu\text{g L}^{-1}$ )	5	10	15	10	15
800 Cu ( $\mu\text{g L}^{-1}$ )	14	28	42	28	42
801 N-NO <sub>3</sub> (mg L <sup>-1</sup> )	3	6	9	6	9

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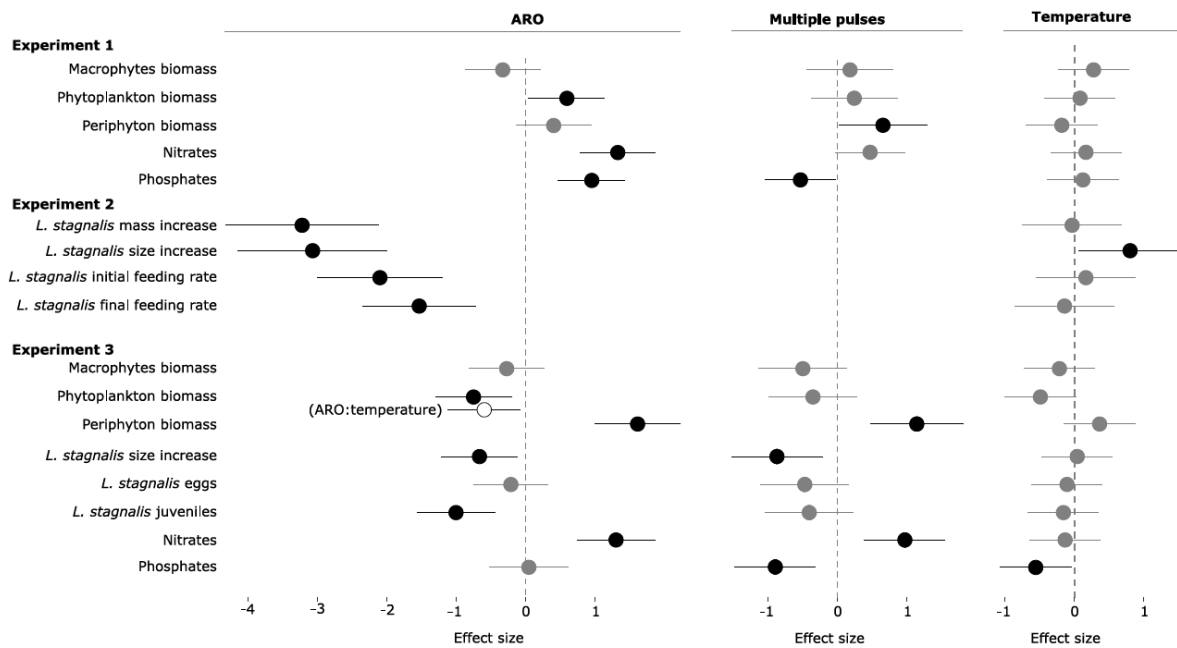
803 Table 2. Links tested in the SEM. Experiment 1: primary producers alone, experiment 2: grazers  
 804 alone.

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

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806 Figures:

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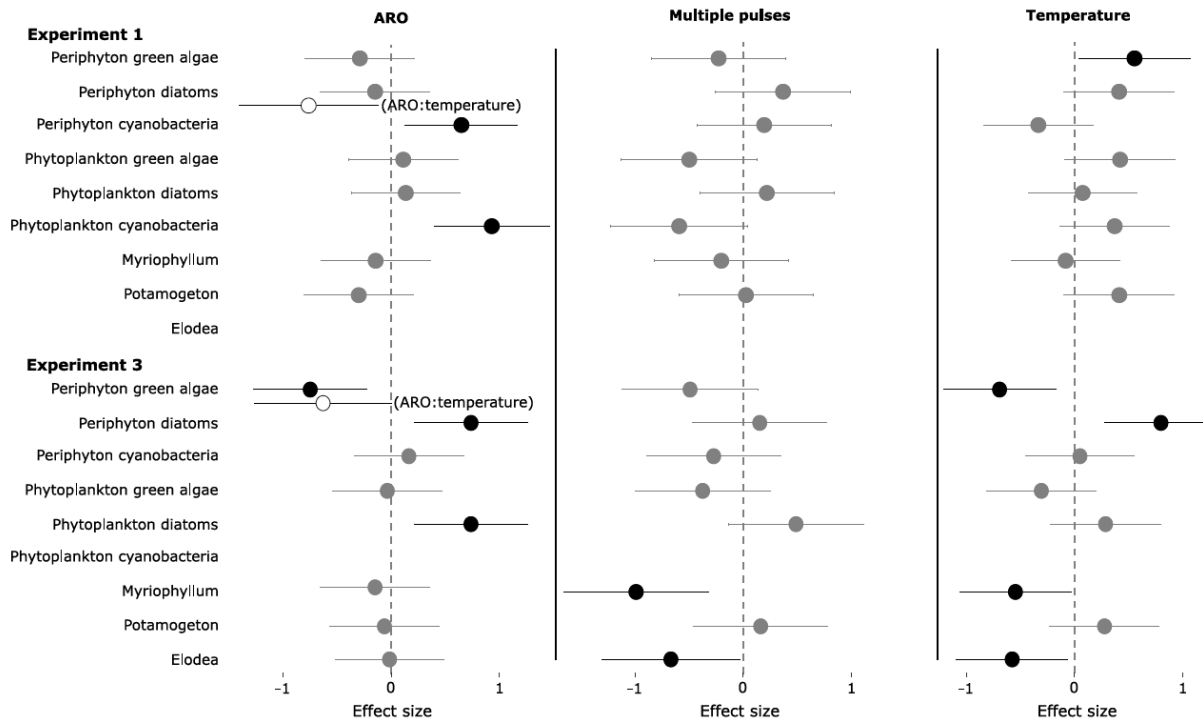


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809 Fig. 1. Effect size (Hedges'  $g \pm 95\%$  confidence interval) on biomass or growth parameters in each  
810 experiment for the three factors tested: ARO total concentration, exposure mode (*i.e.* single vs.  
811 multiple pulses for a similar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

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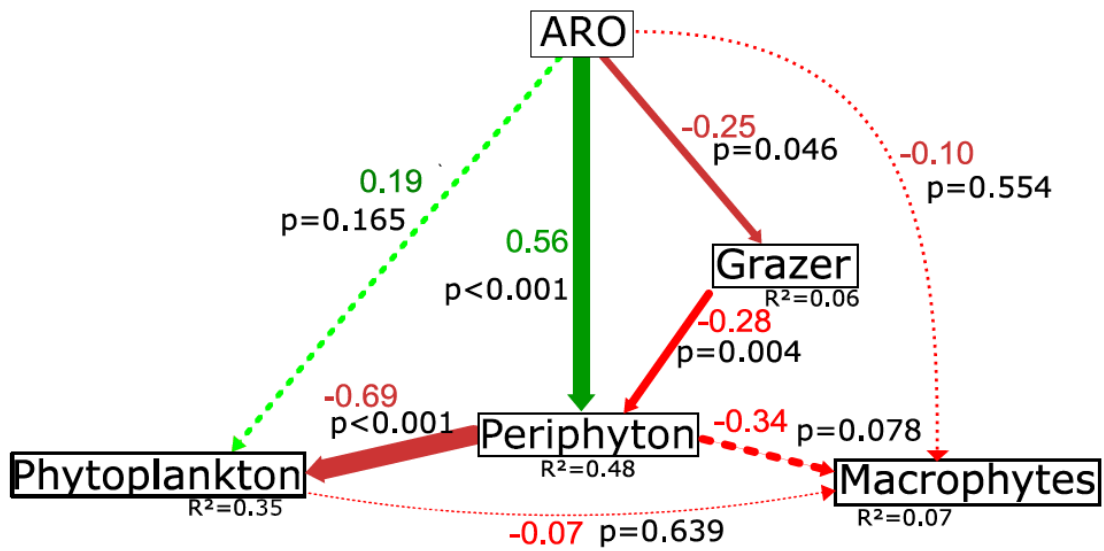
815

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

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827 Fig. 3. Path diagram of the selected SEM model. Red arrows indicate negative relations between  
 828 variables. Green arrows indicate positive relations between variables. Dashed arrows represent non-  
 829 significant relations between variables. Significant threshold was set at  $p = 0.05$ .  $R^2$  are the  
 830 proportion of variation explained by the model for each response variable.

831