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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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15

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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 (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[®] 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 (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⁻¹ 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°C , light at $70 \mu\text{mol m}^{-2} \text{s}^{-1}$
145 ¹ (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 ± 1.3 mm, mean total weight =
156 202 ± 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 CaCO₃ at 7.5 g kg⁻¹ 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 μ m (GF/F). In the filtered
189 water, concentrations of nitrate and dissolved phosphorus were determined by ion chromatography
190 (Dionex Ics-5000⁺, 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 Chi², 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 ± 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 ± 32 mg without ARO and by only 184 ± 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 ± 0.79 mg day⁻¹ in the controls to 2.68 ± 0.62 mg day⁻¹ 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 ± 1.9 mg day⁻¹ in the control to 11.6 ± 2.4 mg day⁻¹
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 ± 36.0 $\mu\text{g L}^{-1}$ Chl *a*, warm: 45.0 ± 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 ± 0.07 $\mu\text{g cm}^{-2}$ Chl *a* and 0.38 ± 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 ± 0.61 $\mu\text{g cm}^{-2}$ Chl *a* (cold) and 3.51 ± 0.67 $\mu\text{g cm}^{-2}$ Chl *a* () when ARO was added as multiple pulses
309 (XXX), while it reached only 2.05 ± 0.60 $\mu\text{g Chl } a \text{ cm}^{-2}$ (in cold) and 2.11 ± 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⁻¹ of pirimicarb. In our experiment, pirimicarb concentration ranged
386 from 5 to 15 µg L⁻¹. 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⁻¹) 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 taken 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
	N-NO ₃ (mg L ⁻¹)	3	6	9	6	9

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802

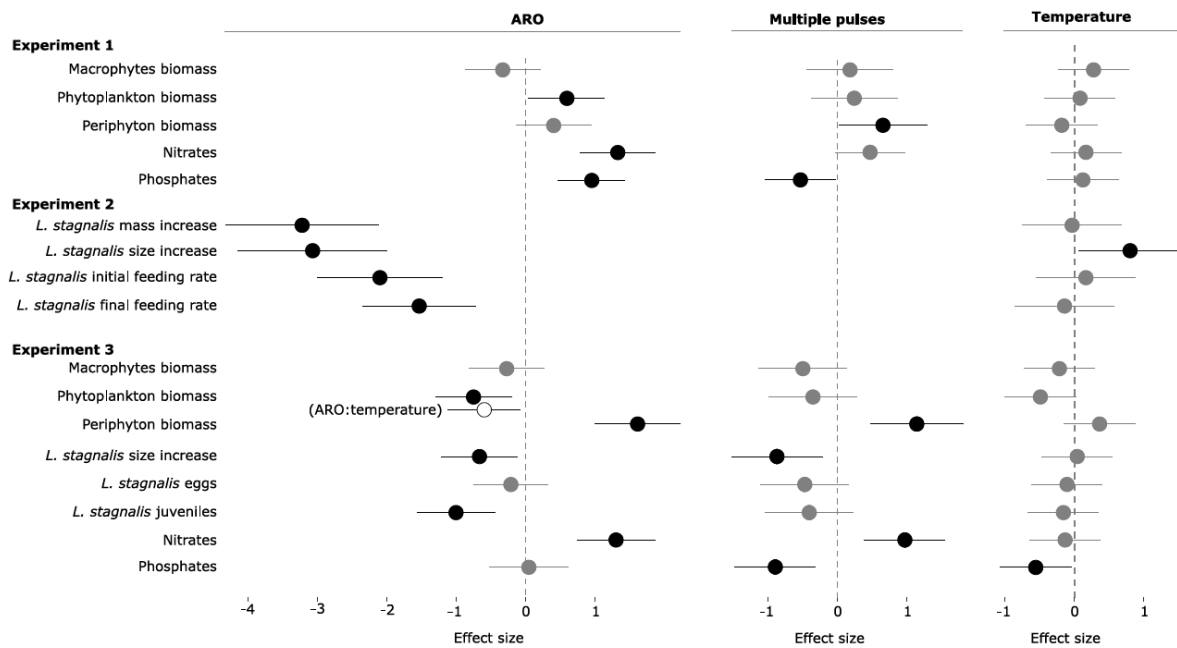
803 Table 2. Links tested in the SEM. Experiment 1: primary producers alone, experiment 2: grazers
 804 alone.

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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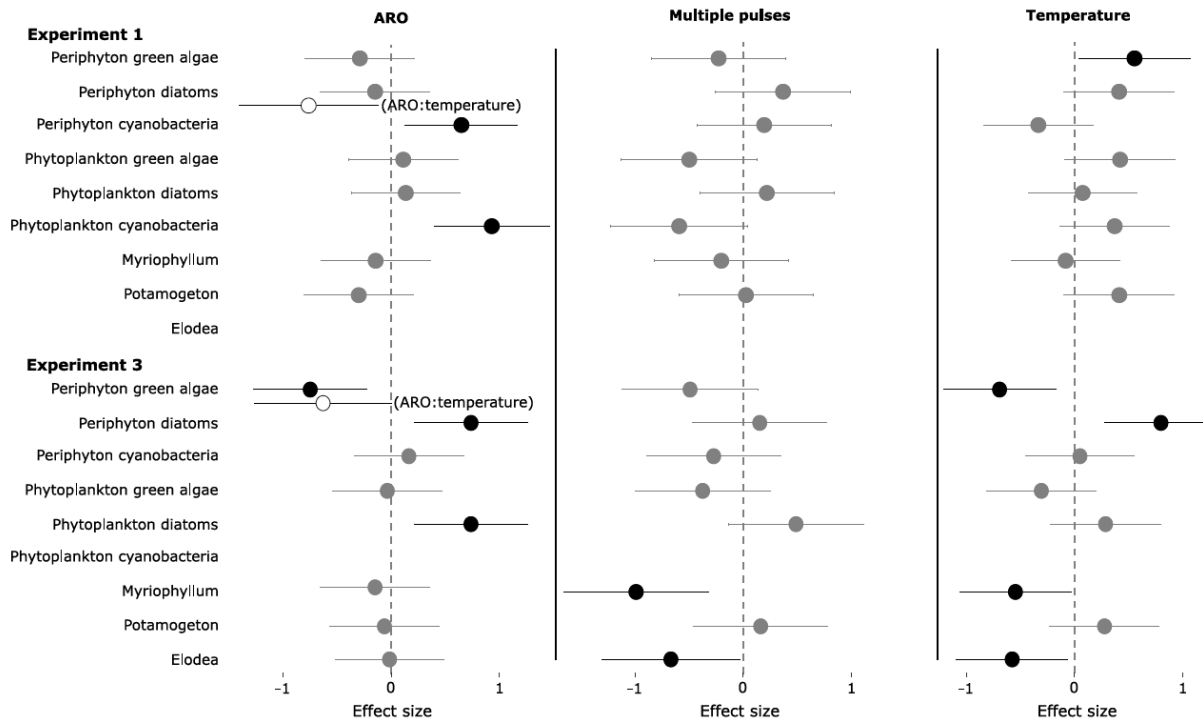


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809 Fig. 1. Effect size (Hedges' $g \pm 95\%$ confidence interval) on biomass or growth parameters in each
810 experiment for the three factors tested: ARO total concentration, exposure mode (*i.e.* single vs.
811 multiple pulses for a similar ARO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

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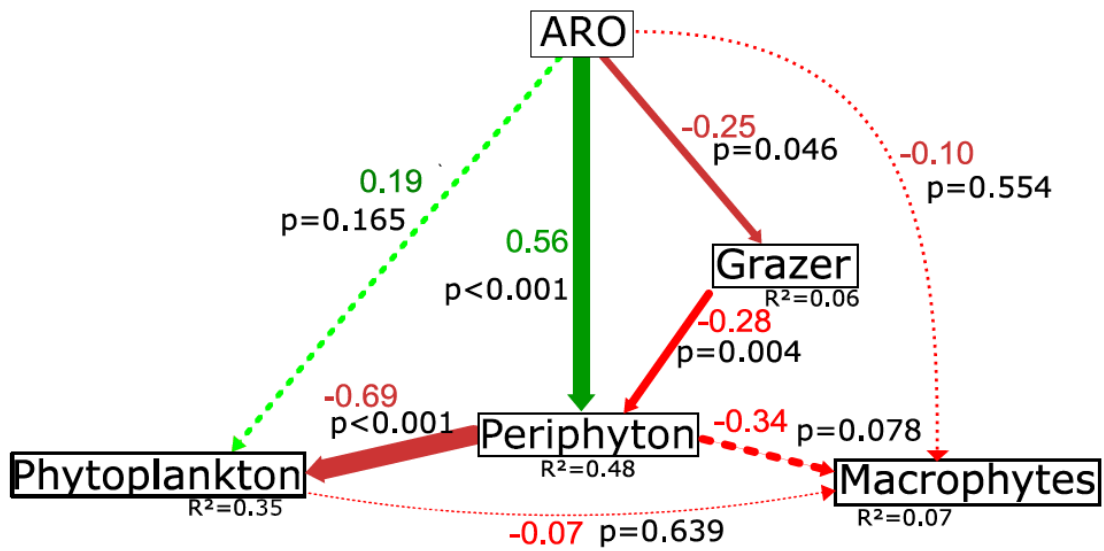
815

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