



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

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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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## Abstract

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

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

## 1. Introduction

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

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

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

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

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

## 2. Material and methods

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

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

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

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

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

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

## 2.2. Experiment 2 – grazers alone

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



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### 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

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

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

## 2.5. Statistics

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

## 2.6. Structural equation modelling

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

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

### 3. Results

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

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

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

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

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

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

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

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

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

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

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

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

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



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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

## 5. Conclusion

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

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

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

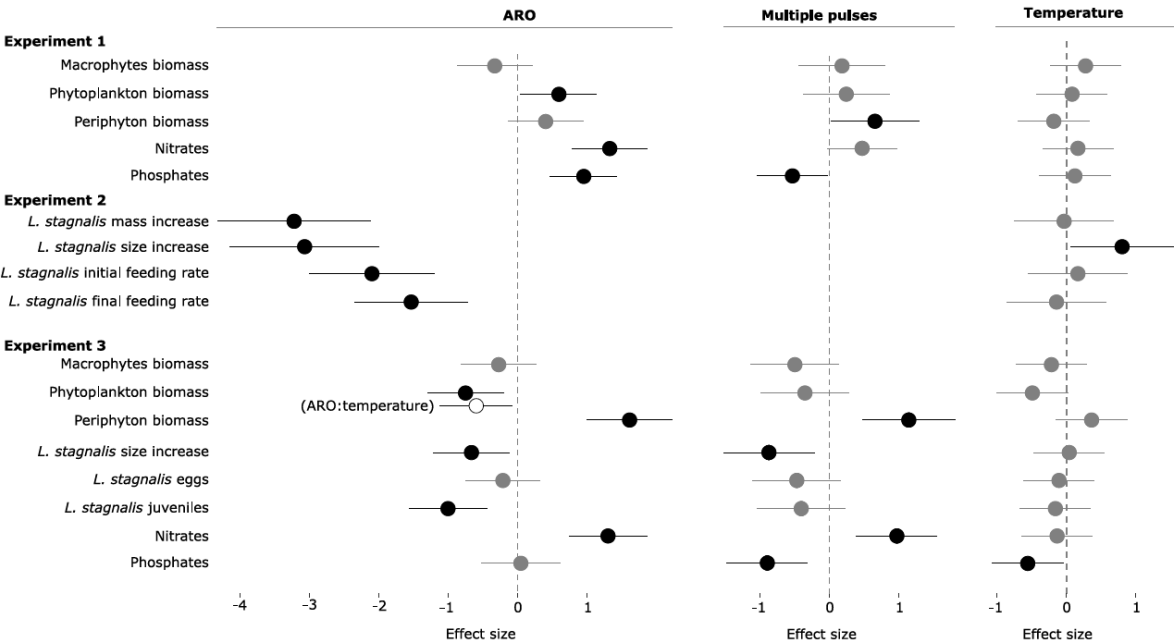
	Single addition			Multiple pulses	
	1X	2X	3X	XX	XXX
797 Terbutylazine ( $\mu\text{g L}^{-1}$ )	1	2	3	2	3
798 Tebuconazole ( $\mu\text{g L}^{-1}$ )	30	60	90	60	90
Pirimicarb ( $\mu\text{g L}^{-1}$ )	5	10	15	10	15
799 Cu ( $\mu\text{g L}^{-1}$ )	14	28	42	28	42
800 N-NO <sub>3</sub> (mg L <sup>-1</sup>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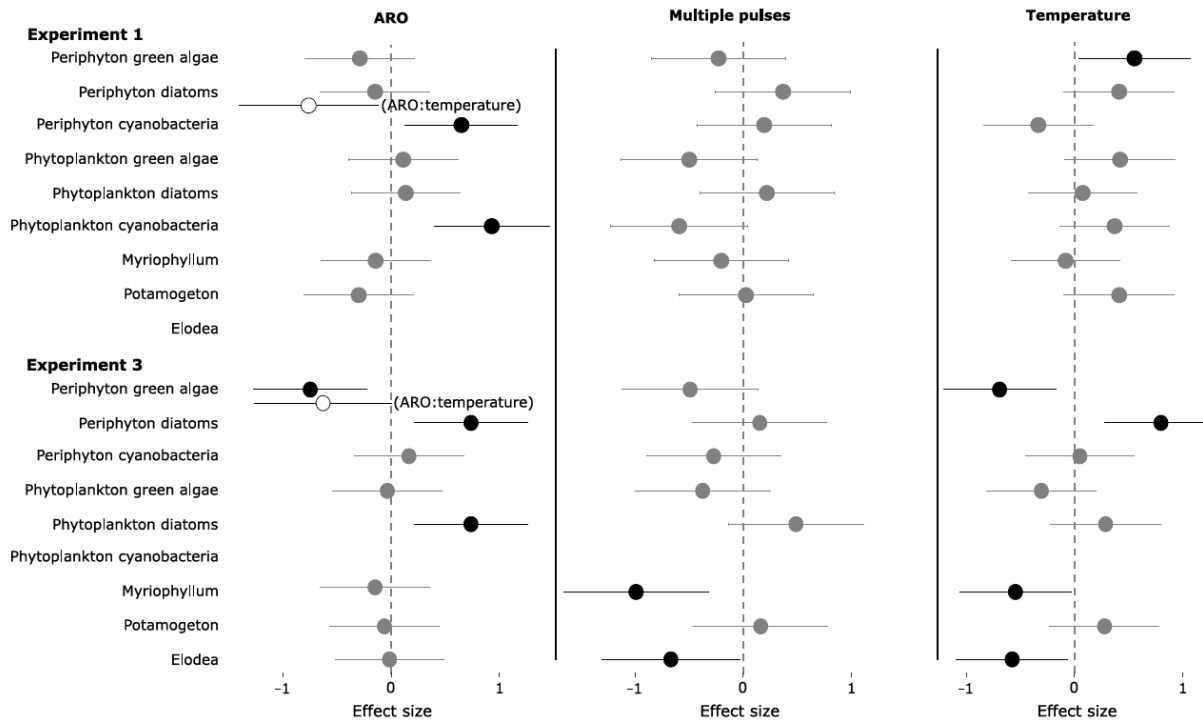


Fig. 2. Effect size (Hedges'  $g \pm 95\%$  confidence interval) on the composition of periphyton, phytoplankton and macrophyte communities for the three factors tested: ARO total concentration, exposure mode (*i.e.* single vs. multiple pulses for a similar ARO concentration) and temperature. Variations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

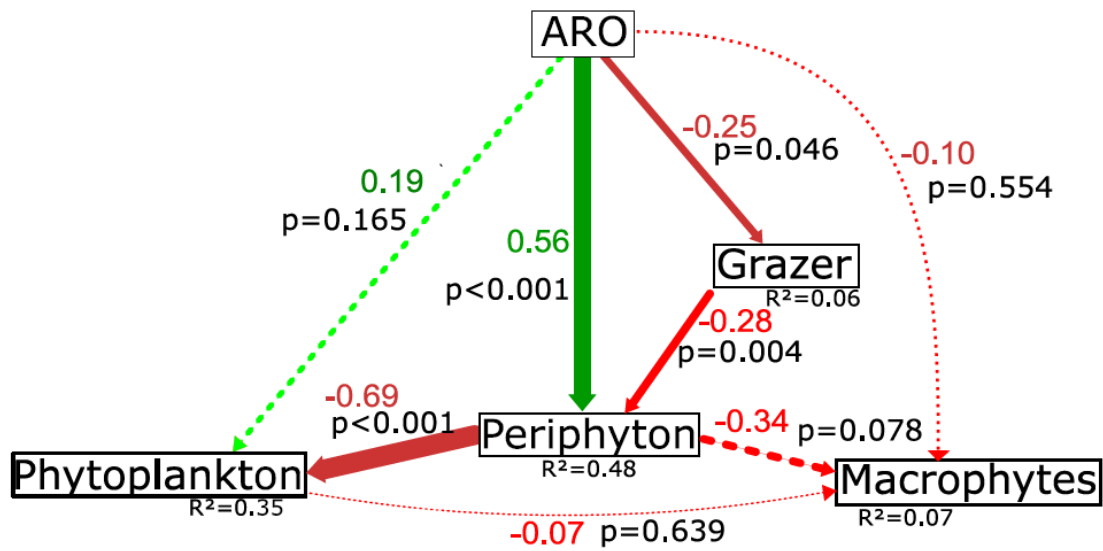


Fig. 3. Path diagram of the selected SEM model. Red arrows indicate negative relations between variables. Green arrows indicate positive relations between variables. Dashed arrows represent non-significant relations between variables. Significant threshold was set at  $p = 0.05$ .  $R^2$  are the proportion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