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## Warming and CO2 effects under oligotrophication on temperate phytoplankton communities

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1 Warming and CO<sub>2</sub> effects under oligotrophication on temperate phytoplankton  
2 communities

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41 **Abstract**

42 Eutrophication, global warming, and rising carbon dioxide (CO<sub>2</sub>) levels are the three  
43 most prevalent pressures impacting the biosphere. Despite their individual effects are  
44 well-known, it remains untested how oligotrophication (i.e. nutrients reduction) can  
45 alter the planktonic community responses to warming and elevated CO<sub>2</sub> levels. Here,  
46 we performed an indoor mesocosm experiment to investigate the warming×CO<sub>2</sub>  
47 interaction under a nutrient reduction scenario (40%) mediated by an in-lake  
48 management strategy (i.e. addition of a commercial solid-phase phosphorus sorbent -  
49 Phoslock<sup>®</sup>) on a natural freshwater plankton community. Biomass production increased  
50 under warming×CO<sub>2</sub> relative to present-day conditions; however, a Phoslock<sup>®</sup>-mediated  
51 oligotrophication reduced such values by 30-70%. Conversely, the  
52 warming×CO<sub>2</sub>×oligotrophication interaction stimulated the photosynthesis by 20%  
53 compared to ambient nutrient conditions, and matched with higher resource use  
54 efficiency (RUE) and nutrient demand. Surprisingly, at a group level, we found that the  
55 multi-stressors scenario increased the photosynthesis in eukaryotes by 25%, but greatly  
56 impaired in cyanobacteria (ca. -25%). This higher cyanobacterial sensitivity was  
57 coupled with a reduced light harvesting efficiency and compensation point. Since  
58 Phoslock<sup>®</sup>-induced oligotrophication unmasked a strong negative warming×CO<sub>2</sub> effect  
59 on cyanobacteria, it becomes crucial to understand how the interplay between climate  
60 change and nutrient abatement actions may alter the, ecosystems functioning. With an  
61 integrative understanding of these processes, policy makers will design more  
62 appropriate management strategies to improve the ecological status of aquatic  
63 ecosystems without compromising their ecological attributes and functioning.

64

## 65 **1.- Introduction**

66 Eutrophication constitutes the most pervasive global problem of the last decades  
67 affecting all types of aquatic ecosystems (Rabalais et al. 2009). Together global  
68 warming and rising atmospheric carbon dioxide (CO<sub>2</sub>) levels, these pressures are  
69 altering life on Earth in unpredictable ways, with severe consequences for the goods and  
70 services that ecosystems provide for humanity (Steffen et al. 2015). In aquatic  
71 ecosystems, these stressors influence biogeochemical cycles, carbon-, oxygen- and  
72 nutrient-availability, as well as, species physiology and community dynamics (Basu &  
73 Mackey 2018). Moreover, freshwater ecosystems, which cover ~4% of the total Earth's,  
74 and are more active in terms of C-sequestration, processing and burial than terrestrial  
75 and marine ecosystems (Downing 2010; Tranvik et al. 2009), are particularly vulnerable  
76 to environmental changes as they are comparably smaller, and often undergo faster  
77 changes in ecosystem functions (Yvon-Durocher et al. 2017).

78 Despite the fact that warming and increasing CO<sub>2</sub> levels are intimately linked,  
79 with recent reports claiming the need to study their impacts jointly due to  
80 synergistic/antagonistic effects (Kim et al. 2013; Paul et al. 2015; Sett et al. 2018), most  
81 of available evidence come from studies: (1) performed in marine ecosystems and (2)  
82 with impacts assessed in isolation. On one hand, warming has been reported to exert  
83 stimulatory (O'Beirne et al. 2017) as well as an inhibitory (Boyce et al. 2010; Lassen et  
84 al. 2009; Osman et al. 2019) effect on the phytoplankton productivity. Also, warming  
85 can increase the biodiversity (Yvon-Durocher et al. 2015) and the C-use efficiency  
86 (Padfield et al. 2016), reduce species size and shift the community composition  
87 (Daufresne et al. 2009), alter the partitioning of the organic matter pools (Wohlers et al.  
88 2009), and ultimately, weaken the CO<sub>2</sub>-sink capacity of the ecosystems (Yvon-

89 Durocher et al. 2010; Yvon-Durocher et al. 2017). On the other hand, increased CO<sub>2</sub>  
90 concentrations have a fertilizing effect that stimulates primary production (Tortell et al.  
91 2008), phytoplankton growth and biomass (Egge et al. 2009), and the efficiency of  
92 phytoplankton in using limiting nutrients (Paul et al. 2015). Nevertheless, other reports  
93 have also showed neither a stimulatory nor inhibitory effect of both stressors acting  
94 separately (Li et al. 2019; Strecker et al. 2004; Verschoor et al. 2013) or in combination  
95 (Keys et al. 2018) on different phytoplanktonic physiological and metabolic processes  
96 (e.g. photosystem II efficiency, growth rates, carbon assimilation rates).

97         The above described changes exerted by temperature (T) and CO<sub>2</sub> on the biotic  
98 environment are also affecting the abiotic environment of ecosystems i.e. nutrient  
99 availability. Thus, an increased T changes the physiological requirements of  
100 phytoplankton toward elevated carbon:nutrient content (De Senerpont Domis et al.  
101 2014), and triggers a higher production of prokaryotic (i.e. cyanobacteria) vs. other  
102 eukaryotic phytoplankton groups due to higher competitive advantage in nutrient  
103 acquisition (Lüring et al. 2018) and resource use efficiency (RUE; Escalas et al. 2019;  
104 Filstrup et al. 2014). High CO<sub>2</sub> concentrations favor the production of pico compared to  
105 microphytoplankton (Hernández-Hernández et al. 2018; Ji et al. 2017), and do not seem  
106 to alter the relative proportion of cyanobacteria (~25%) in phytoplankton communities  
107 (Ullah et al. 2018). Surprisingly, most of these studies addressing the interaction  
108 between warming (or high CO<sub>2</sub>) focused on high nutrient conditions, revealing positive  
109 synergistic effects (Schulhof et al. 2019; Villar-Argaiz et al. 2018; Zingel et al. 2018).  
110 However, the net impact of the warming (or CO<sub>2</sub>) × nutrients interaction may differ  
111 when nutrient levels are naturally low (i.e. oligotrophic ecosystems), or when they are  
112 reduced through bottom-up (i.e. biological activity) and / or top-down [i.e. management

113 process - (re)-oligotrophication] controls. In fact, a recent study by Verbeek et al.  
114 (2018a) reported that the interaction between warming and re-oligotrophication reduced  
115 biomass and diversity but increased RUE. By contrast, such negative effects on  
116 community structure, and positive on the RUE disappeared when nutrient  
117 concentrations were high.

118 Thus, as eutrophication constitutes a severe global economic and health problem  
119 (i.e. toxic cyanobacterial blooms, Glibert 2019; Jeppesen et al. 2007; Smith 2003), as  
120 well as one of the main pressures on water quality (Teurlincx et al. 2019), efforts for  
121 reducing the excessive nutrient loading in ecosystems have become a great challenge for  
122 water managers. In fact, it is well-known that the first step in reducing high nutrient  
123 concentrations is to tackle direct input of phosphorus (P) (Hilt et al. 2006). Based on  
124 this, the installment of the EU-Water Framework Directives (e.g. European Union  
125 WFD, Union 2000) with the aim to maintain and restore good water quality, may  
126 modify the magnitude and direction of the effects that the T×CO<sub>2</sub> interaction could have  
127 on ecosystems in the future.

128 To better understand these multiple pressures at play in aquatic systems, we have  
129 tested how the warming×CO<sub>2</sub> interaction under nutrient reduction modulates the  
130 responses of a natural plankton community from a highly productive shallow lake. To  
131 this end, we performed a controlled indoor mesocosm experiment comprising 9 so-  
132 called Limnotrons (Verschoor et al. 2013), in which we manipulated T (ambient or  
133 ambient + 3°C), CO<sub>2</sub> (400 or 1000 ppm) and nutrient availability (ambient  
134 concentrations or reduced concentrations by 40% respect to ambient conditions) by  
135 adding Phoslock<sup>®</sup> in a cluster scenarios design (Valiñas et al. 2018; Xu et al. 2014).  
136 Throughout the experiment, we monitored the performance of the photosynthetic

137 apparatus of the phytoplankton, its photosynthetic activity, total biomass, and RUE to  
138 test the following questions:

139 (1) Will an oligotrophication process under a warming and high CO<sub>2</sub> scenario  
140 produce an earlier and stronger reduction in phytoplankton biomass by a lower RUE to  
141 sustain the algal growth?; (2) Will the warming and high CO<sub>2</sub> interaction produce  
142 higher (and maximum) photosynthetic rates during the bloom and under ambient  
143 nutrient concentrations by an increased RUE?; and (3): Will a future environmental  
144 scenario under oligotrophication unmask a higher sensitivity of prokaryotic (Prok, i.e.  
145 cyanobacteria) compared to eukaryotic (Euk) phytoplankton groups due to their higher  
146 nutritional requirements to sustain the growth and functioning of the photosynthetic  
147 apparatus?.

## 148 **2. Material and Methods**

149 *Study area:* This study was carried out with water collected at Lake Ertveldplas  
150 (mean depth 10.9 m) in s'Hertogenbosch (Hertogenbosch, The Netherlands, 51.71° N;  
151 5.29° E). The study area (42.5 ha) is a turbid (mean light extinction,  $Kd_{PAR} = \sim 3.5 \text{ m}^{-1}$ )  
152 and eutrophic ecosystem with recurrent phytoplankton blooms (Seelen et al. 2019). It is  
153 located close to an area of intense industrial activity and has a harbor used for  
154 commercial shipping from the Meuse River and the city of s'Hertogenbosch. Currently,  
155 almost all agricultural land surrounding the lake has been converted into a protected  
156 nature area (Maas 2014).

157 *2.1. Experimental setup:* Surface water (~10000 L, from the upper 1 m of the  
158 water column) was collected using a hydraulic pump mounted on a 20000L tank truck  
159 on the 17<sup>th</sup> of July 2018. Sediment (~150 first cm-depth of the sediment layer) samples



160 were collected in large opaque containers on July 16<sup>th</sup>, 2018 using an Ekman grab  
161 sampler at 10-15 m depth. Upon sampling, water and sediments were immediately  
162 transported to laboratory facilities of the Aquatic Ecology Department at the Dutch  
163 Institute of Ecology (NIOO-KNAW) and used to inoculate the Limnotrons.

164           Nine Limnotrons ( $\varnothing = 0.97$  m;  $h = 1.35$  m) were inoculated with natural  
165 sediment from the lake ( $\sim 0.04$  m<sup>3</sup> per Limnotron), and filled with Lake Ertveldplas water  
166 (900 L per Limnotron), thus mimicking both the natural organismal composition, as  
167 the physical structure of shallow lake ecosystems. We homogenized both water and  
168 sediment prior to inoculating the Limnotrons, to ensure similar starting conditions. The  
169 biota was left to establish and acclimatize for fifteen days prior to the experiment (July  
170 17<sup>th</sup> – August 1<sup>st</sup>, 2018).

171           The Limnotrons represented three clusters of environmental scenarios (in  
172 triplicate) as follows: (A) **Present**: Limnotrons filled with water maintained under  
173 similar conditions as prevailing in Lake Ertveldplas during time of sampling  
174 (Temperature =  $23 \pm 0.7$  °C; carbon dioxide [CO<sub>2</sub>] = 400 ppm; pH = 7.40), (B) **Future**:  
175 Limnotrons filled with water warmed 3°C above ambient temperature (Supplementary  
176 information Fig. S1A-C), and aerated using a mix of CO<sub>2</sub>-enriched air (1000 ppm; pH =  
177 7.20); and (C) **Future + Phoslock**<sup>®</sup> (Future<sub>Phos</sub>): Limnotrons filled with water that was  
178 warmed (Supplementary information Fig. S1A-C) and CO<sub>2</sub>-enriched similar to the  
179 Future scenario, but here, Phoslock<sup>®</sup> was added, as a restoration tool, to lock the  
180 sedimentary mobile P (resulting in a dose of 1.02 g Phoslock<sup>®</sup> L<sup>-1</sup> final concentration).  
181 The rationale for evaluating the combined effect of T and CO<sub>2</sub> underlies in the positive  
182 feedback found among both drivers over the past climate (Scheffer et al. 2006), and the  
183 fact that recent results suggest that much care must be taken in making inferences

184 related with the T and CO<sub>2</sub> effects to an ecosystem level from single-driver studies  
185 (Harvey et al. 2013). Phoslock<sup>®</sup> is a lanthanum (La) modified clay, that has successfully  
186 been applied to reduce the dissolved P concentrations in reservoirs, lakes and ponds,  
187 and controlling eutrophication (Epe et al. 2017; Lürling and Van Oosterhout 2013).  
188 Phoslock<sup>®</sup> contains 5% La by weight, and P is adsorbed by La at a molar ratio of 1:1  
189 (Ross et al. 2008). For that, in dosage calculations, only the freely available P  
190 components were considered (i.e. soluble reactive P in the water and mobile P in the  
191 sediment; Meis et al. 2013). We chose this sorbent because it has the advantage of  
192 stripping dissolved P from the water column, and blocking the P-release from the  
193 sediment after settling in the lake bottom (Yuan et al. 2009). Using this chemical solid  
194 phase sorbent, we try to simulate an oligotrophication process through a human-induced  
195 management strategy (see below), decreasing the P-availability for plankton  
196 communities under the above mentioned future global change conditions expected for  
197 the end of the 21<sup>st</sup> Century (IPCC 2013).

198         Experimental warming was achieved by an external electronic element (AKO-  
199 71025- 230V-600W) connected to a thermocouple with dual temperature forcing,  
200 keeping the target temperature constant at  $\pm 0.5^{\circ}\text{C}$  (Supporting Information Fig. S1A).  
201 Temperature at 0.5 and 1 m depth was continuously logged using the Farex SR mini  
202 system with PT100 electrodes. CO<sub>2</sub> concentrations were achieved using a Witt-  
203 Gasetechnik device (model KM60-2ME) that aerated both CO<sub>2</sub>-enriched and ambient  
204 into the Limnotrons at a constant flux rate of  $0.5 \text{ L min}^{-1}$ . In each Limnotron an impeller  
205 was set at 0.54 rotations per min (rpm) to simulate comparable mixing conditions to  
206 those that usually occur in the water column of shallow lakes. The illumination  
207 (photosynthetically active radiation [PAR]) was provided by cool white-light tubes

208 (surface irradiances:  $710.17 \pm 81.73 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ; Agrilight AL2007 400;  
209 Monster, The Netherlands), and had a 12h L:12h D cycle in order to mimic the daily  
210 radiation doses received by communities in Dutch surface waters during the  
211 experimental period. Phoslock<sup>®</sup> addition was implemented by creating a slurry with  
212 897 g in a subsample (5 L of water coming from the corresponding Limnotron). Once  
213 added, Phoslock<sup>®</sup> was allowed to settle for 24 h (i.e. August 2<sup>nd</sup>) prior to starting the  
214 experiment. Based on the temporal evolution of the chlorophyll *a* (Chl *a*) over the  
215 experimental period, we divided phytoplankton succession into three distinct phases; a  
216 pre-bloom, bloom and a post-bloom period (see a description in results).

## 217 *2.2. Sampling and analysis:*

218 *2.2.1. Inorganic macronutrients:* 50 mL-samples for phosphate [ $\text{PO}_4^{3-}$ ], nitrate +  
219 nitrite [ $\text{NO}_3^- + \text{NO}_2^-$ ] and ammonia [ $\text{NH}_4^+$ ] were taken using a cylindrical sampler ( $\emptyset =$   
220 0.04 m;  $h = 1.20$  m) that allows for depth-integrated samples throughout the water  
221 column of the Limnotrons. Once taken, all samples were frozen and stored at  $-20^\circ\text{C}$   
222 until analysis. Nutrient concentrations were determined using a Seal QuAAtro39  
223 continuous segmented flow analyzer (SEAL Analytical, Inc., USA).

224 *2.2.2. Dissolved organic and inorganic carbon (DOC):* 50 mL-samples were  
225 pre-filtered through pre-combusted (2 h at  $500^\circ\text{C}$ ) glass-fiber filters (GF/F Whatman;  
226 Whatman®, Sanford, ME, USA), and collected in combusted vials (3 h at  $500^\circ\text{C}$ ).  
227 Samples were acidified with HCl 1N (2%), and measured by high-temperature catalytic  
228 oxidation using a total organic carbon (TOC) analyzer (model TOC-V CSH/CSN;  
229 Shimadzu, Kyoto, Japan).

230 2.2.3. *Photosystem II* ( $\Phi_{\text{PSII}}$ ) *photochemical activity and total chlorophyll a* (Chl  
231 *a*): *In vivo* Chl *a* fluorescence was measured with a pulse amplitude modulated (PAM)  
232 fluorometer (Phyto-PAM, Walz, Effeltrich, Germany) equipped with an optical unit  
233 (ED101-US). For excitation of the Chl *a* fluorescence, the Phyto-PAM fluorometer  
234 applied an array of four different types of light emitting diodes (LED) with emission  
235 wavelengths peaking at 470, 520, 645, and 665 nm (Phyto-ML). We used blue, green  
236 and brown signals, respectively, as a proxy to differentiate the Chl *a* fluorescence of  
237 cyanobacteria (Prok), green algae and diatoms/dinoflagellates (Euk), but also to detect  
238 other eukaryotic phytoplankton in the water. For this end, we used an in-built reference  
239 spectra stored in the Phyto-PAM and routinely used by our group, which is constituted  
240 by three representative phytoplankton species of the above mentioned groups (i.e.  
241 *Synechococcus leopoliensis* [a cyanobacteria], *Chlorella vulgaris* [a green algae], and  
242 *Phaeodactylum tricorutum* [a diatom]) which allow us properly differentiate blue,  
243 green and brown *in vivo* Chl *a* fluorescence signals, respectively.

244 10-mL samples were taken from each Limnotron nine times (days 0, 3, 5, 7, 10,  
245 13, 17, 21 and 24) over the experimental period. For each sampling day, we took  
246 samples at the beginning of the diel cycle, every 1.5-2 h during the light exposure period  
247 (12 h in total), every 30 min during the first 1.5-2 h of darkness and at the end of the  
248 darkness period (12 h in total) (10-12 measurements over each diel cycle). The samples  
249 were placed in a quartz cuvette and directly measured after sampling without any dark-  
250 adaptation. The effective photochemical quantum yield of photosystem II ( $\Phi_{\text{PSII}}$ ), a  
251 measurement of the cellular fitness, was measured following the equation of Maxwell &  
252 Johnson (2000):

253 
$$\Phi_{\text{PSII}} = \Delta F / F'_{\text{m}} = (F'_{\text{m}} - F_{\text{t}}) / F'_{\text{m}} \quad (1)$$

254 where  $F'm$  is the maximum fluorescence induced by a saturating light pulse (3832  $\mu\text{mol}$   
255 photons  $\text{m}^{-2} \text{s}^{-1}$  in 0.2 s) and  $F_t$  is the current steady-state fluorescence induced by a  
256 weak actinic light pulse (704  $\mu\text{mol}$  photons  $\text{m}^{-2} \text{s}^{-1}$ ) in light-adapted cells. All  
257 measurements were performed without any pre-concentration of the samples and at the  
258 same T and light intensity to which they were exposed in the Limnotrons.

259 The rate of electrons transported through the PSII (rETR, in  
260  $\mu\text{mol}$  photons  $\text{m}^{-2} \text{s}^{-1}$ ) i.e., an estimation of the photosynthetic rate, was calculated for  
261 each measurement over the experimental period from the values of  $\Phi_{\text{PSII}}$  as:

$$262 \quad \text{rETR} = \Phi_{\text{PSII}} \times E_{\text{PAR}} \times 0.5 \quad (2)$$

263 where  $\Phi_{\text{PSII}}$  is the effective photochemical quantum yield,  $E_{\text{PAR}}$  is the PAR  
264 energy received by the phytoplankton cells (see above), and 0.5 is a correction factor as  
265 half of the absorbed light energy is diverted to the PSII (Kromkamp et al. 1998; Suggett  
266 et al. 2003). Daily integrated rETRs for each experimental condition were calculated  
267 integrating the rates measured each day vs. time.

268 Phyto-PAM was also used to measure other photosynthetic properties of the  
269 studied communities (Ralph and Gademann 2005). Rapid light curves (RLC) were  
270 measured at day 0 (hereafter, Initial) and the last experimental day. RLCs were run with  
271 actinic light intensities up to 1619  $\mu\text{mol}$  photons  $\text{m}^{-2} \text{s}^{-1}$ , with each actinic light  
272 exposure lasting 10 s, in dark-adapted cells. Photosynthetic parameters such as the  
273 photoadaptive index ( $E_k$ , a measure of the light intensity for saturation of electron  
274 transport), maximum rates of electron transport ( $\text{rETR}_{\text{max}}$ ), and light harvesting  
275 efficiency ( $\alpha$ ) were determined according to Ralph & Gademann (2005), and are related  
276 through the  $E_k$  equation (i.e.  $\text{rETR}_{\text{max}} / \alpha$ ).

277           2.2.4. *Chl a*: 10 mL-samples were taken at the beginning of each sampling day  
278 of the experiment and measured using the Phyto-PAM (Phyto-PAM, Walz, Effeltrich,  
279 Germany). The total *Chl a* fluorescence-derived concentration was used as a proxy of  
280 the total biomass in the community because recent results by Lüring et al. (2018)  
281 evidenced a very high correlation between *Chl a* concentrations determined by classic  
282 spectrophotometric techniques and by Phyto-PAM for natural communities from Dutch  
283 lakes.

284           The growth rates ( $\mu$ ) in each environmental scenario were calculated based on  
285 *Chl a* concentrations as:

$$286 \qquad \qquad \qquad \mu = \text{Ln} (N_{t+1} / N_t) / (t_{+1} - t) \quad (3)$$

287           where  $N_{t+1}$  and  $N_t$  represent *Chl a* concentrations at the end ( $t_{+1}$ ) and at the initial  
288 ( $t$ ), respectively, of each phase of the phytoplankton succession.

289           2.3. *Data treatment and statistical analyses:*

290           As the biomass of the communities differed over the pre-bloom, bloom and post-  
291 bloom periods, the effect of each environmental scenario on Euk and Prok was  
292 compared using log response ratios (LRRs). LRRs are commonly used as effect size  
293 metric in ecological research (Lajeunesse 2011), as well as, on the response of  
294 phytoplankton communities to global change stressors (Galic et al. 2018; Lüring et al.  
295 2018). Thus, the effect of each environment scenario on the response variable  
296 considered was assessed as:

$$297 \qquad \qquad \qquad \text{LRR Future} = \text{Ln} (\text{Future} / \text{Present}) \quad (4)$$

$$298 \qquad \qquad \qquad \text{LRR Future}_{\text{Phos}} = \text{Ln} (\text{Future}_{\text{Phos}} / \text{Present}) \quad (5)$$

299 
$$\text{LRR Phoslock}^{\text{®}} = \text{Ln} (\text{Future}_{\text{Phos}} / \text{Future}) \text{ (6)}$$

300 where LRR Future refers to the effect of warming and high CO<sub>2</sub>, and LRR Future<sub>Phos</sub>  
301 represents the interactive effect of future conditions under oligotrophication (by  
302 Phoslock<sup>®</sup> addition) respect to a present scenario. LRR Phoslock<sup>®</sup> represents the single  
303 effect of oligotrophication by Phoslock<sup>®</sup> addition. Using LRR reveals the magnitude as  
304 well as the direction of the responses relative to control conditions (i.e. Present  
305 scenario).

306 RUE was used as a proxy for ecosystem functioning because it allows tracking  
307 the functional change in relation or reaction to species change, and was defined as unit  
308 biomass production in Chl *a* (μg L<sup>-1</sup>) per unit total phosphorus (μg L<sup>-1</sup>).

309 We used one-way analysis of the variance (ANOVA) to test significant  
310 differences between environmental scenarios on T, macro-nutrients (PO<sub>4</sub><sup>3-</sup>, NO<sub>2</sub><sup>-</sup>+NO<sub>3</sub><sup>-</sup>,  
311 and NH<sub>4</sub><sup>+</sup>), and DOC concentrations over the entire experimental period; and one-way  
312 repeated measures (RM) ANOVA to test significant differences between environmental  
313 scenarios over the different stages of the phytoplankton succession (i.e. pre-bloom,  
314 bloom and post-bloom) on Chl *a*, RUE and growth rates. A two-way ANOVA was used  
315 to test interactions between functional group (Euk and / or Prok) and scenario on the  
316 performance of the photosynthetic apparatus (i.e. α, rETRmax and E<sub>k</sub>), and on the LRR  
317 of such properties. A two-way RM-ANOVA was used to test interactions between  
318 functional group (Euk and / or Prok) and environmental scenario on rETR and LRR  
319 rETR over the different stages of the phytoplankton succession. Prior to ANOVA  
320 analysis, assumptions of Normality (by Q-Q plot residual analysis and Shapiro-Wilk's  
321 test) homoscedasticity (by Levene's Equal Variance test) and sphericity (by Mauchly's  
322 test) were checked. When the interaction was significant, differences between and

323 among groups were detected using a Least Significant Differences (LSD) *post hoc* test.  
324 All data were analyzed using R Core Team Environment (2014) and the *physcho*, and  
325 *car* packages.

### 326 **3. Results**

327 The changes observed in Chl *a* over the experimental period show the general  
328 evolution of phytoplankton blooms: an *initiation* period (or pre-bloom; days 0 – 10) in  
329 which biomass remains more or less stable, *succession* (or bloom; days 11 – 17) period  
330 in which biomass increases until reach the carrying capacity, and *decay* (or post-bloom;  
331 days 18 – 24) period from which the phytoplankton biomass begin to decay (Fig. 1A).  
332 Total Chl *a* exhibited a slight increase during the pre-bloom, from 10.9 to 14.1  $\mu\text{g L}^{-1}$   
333 but without significant differences among environmental scenarios (LSD *post hoc* test,  
334  $p > 0.1$ ). During the bloom phase, Chl *a*, showed a strong increase, and peaked on day  
335 14. Growth rates showed a similar increase with significant higher rates under a Future  
336 ( $255.20 \pm 21.30 \mu\text{g L}^{-1} - 0.44 \pm 0.08 \text{ d}^{-1}$ ) than under a Future<sub>Phos</sub> ( $167.20 \pm 5.60 \mu\text{g L}^{-1} -$   
337  $0.32 \pm 0.03 \text{ d}^{-1}$ ) and Present ( $57.80 \pm 7.50 \mu\text{g L}^{-1} - 0.20 \pm 0.01 \text{ d}^{-1}$ ) scenarios  
338 (Supplementary information Fig. S1D). In contrast, after the bloom phase, a consistent  
339 decline in Chl *a* occurred in all scenarios. The initial positive effect of a Future and  
340 Future<sub>phos</sub> scenario on Chl *a* production turned later into a negative effect, resulting in a  
341 reduction both in biomass as well as growth rates, which was reflected by a significant  
342 interactive Scenario $\times$ Time effect on both variables (Chl *a* =  $F_{103,22}$ ,  $p < 0.001$ ;  $\mu$  =  $F_{18,01}$ ,  
343  $p < 0.001$ ) (Fig. 1A; Table S1). However, such reductions in growth were significantly  
344 higher in the Future than in the Future<sub>Phos</sub> scenario ( $-0.61$  vs.  $-0.32 \text{ d}^{-1}$ ; Supplementary  
345 information Fig. S1D). Nevertheless, the response pattern found during the bloom



346 (Future > Future<sub>Phos</sub> > Present values) was maintained until the end of the experimental  
347 period (Fig. 1A).

348 RUE significantly changed over time under the different environmental  
349 scenarios tested (Scenario×Time =  $F_{1392.15}$ ;  $p < 0.001$ ; Table S1). During the pre-bloom,  
350 RUE was significantly higher (LSD *post hoc* test,  $p < 0.05$ ) under a Future<sub>Phos</sub> than  
351 under Future and Present scenarios, where no significant differences were detected  
352 (LSD *post hoc* test,  $p > 0.05$ ). Maximum RUE values were reached during the bloom  
353 phase (day 13 for Future<sub>Phos</sub> and Present scenarios and day 17 for Future scenario),  
354 being significantly higher for Future<sub>Phos</sub> at the beginning of the phase, but decreasing  
355 below the levels of Future scenario during the late bloom period. At the end of the  
356 experimental period, maximum RUE was newly (and significantly) higher under a  
357 Future<sub>Phos</sub> scenario (LSD *post hoc* test,  $p < 0.001$ ).

358 Daily cycles of the rETR, a measurement of the photosynthetic activity, for each  
359 group of the main components of the phytoplankton community are shown in figure 2.  
360 In all scenarios and during the bloom and post-bloom, we observed an expected trough-  
361 shaped pattern in which rETR decreased during the light exposure period, followed by a  
362 slight recovery during darkness. However, during the pre-bloom the response pattern  
363 found was opposite. Regarding the absolute rETR values, these were mostly higher at  
364 the beginning of the exposure period of each individual day than at the end, excepting  
365 during the post-bloom period where such rates were similar or higher at the end of the  
366 diel cycle regardless of the scenario considered. For group-specific responses, our  
367 findings show apparent similar (or slightly higher) rETR in Prok compared to Euk  
368 during the pre-bloom period under the three environmental scenarios (Fig. 2A-C).

369 Noticeably, such rates were similar (i. e. bloom period) or even higher (e.g. Future<sub>Phos</sub>)  
370 in Euk as the succession progressed.

371           Based on the previously shown daily responses, we calculated the daily  
372 integrated rETRs for each period of the phytoplankton succession (i.e. pre-bloom,  
373 bloom and post-bloom), and grouped them into Euk and Prok. Then, we used these  
374 values as a proxy of daily production in both compartments of the community (Fig. 3).  
375 In all scenarios, we observed that the maximum rates occurred during the bloom for  
376 Euk, and during the pre-bloom in Prok. In addition, whereas Euk generally has higher  
377 rETR during the succession, Prok showed a significant consistent decline over this  
378 period. The observed decrease during the experimental period on Prok was, however,  
379 most pronounced in the Future<sub>Phos</sub> scenario. It was reflected by a significant  
380 Scenario×Group (rETR =  $F_{13,29}$ ,  $p < 0.001$ ; Table S2) and a Scenario×Group×Time  
381 (rETR =  $F_{5,31}$ ,  $p < 0.01$ ; Table S2) interaction.

382           Overall, the future scenario exerted a greater stimulatory effect than the present  
383 scenario did (assessed as LRR) on daily production of Euk during pre-bloom and  
384 bloom, and a lower inhibitory effect during the post-bloom period than those found on  
385 Prok (Fig. 3B). This opposite effect on both groups was even stronger under the  
386 oligotrophication conditions derived from the Phoslock<sup>®</sup> addition. Thus, whereas the  
387 daily production was enhanced 12-25% in Euk, it was reduced 5-42% in Prok relative to  
388 the Present scenario. Oligotrophication derived from Phoslock<sup>®</sup> addition exerted a  
389 stimulatory effect in Euk over the phytoplankton succession, and negative on Prok  
390 (except during the bloom phase).

391           Finally, the reduction observed in the rETR mentioned above in Prok was also  
392 coupled with a significantly reduced light harvesting efficiency ( $\alpha$ ), rETR<sub>max</sub> and light

393 compensation point ( $E_k$ ; i.e. light intensity where photosynthesis and respiration rates  
394 are equal) (Fig. 4A-C and Table S3) over the experiment. In fact, when we quantified  
395 the net effect of the environmental scenarios tested, a Future<sub>Phos</sub> scenario reduced such  
396 parameters between -40 and -60% on Prok (Fig. 4D-F). Conversely, they were  
397 stimulated (e.g.  $\alpha$ ; 10-20%; Fig. 4D) or mildly impacted ( $\sim$ -15%; Fig. 4 E, F) in Euk,  
398 regardless of the scenario considered.

#### 399 **4. Discussion**

400         This study constitutes the first empirical evidence showing that under a future  
401 warming and high CO<sub>2</sub> scenario, oligotrophication through an in-lake management  
402 strategy (i.e. Phoslock<sup>®</sup> application) may greatly impair cyanobacterial photosynthesis  
403 compared to other eukaryotic phytoplankton groups. The potential reductions in the  
404 carbon uptake were consequence of an impaired functioning of the photosynthetic  
405 apparatus due to a higher sensitivity of the cyanobacteria to the environmental drivers  
406 assayed. These findings are relevant for understanding how the ecosystem functioning  
407 could be impacted by reductions in nutrients availability in a warmer and more CO<sub>2</sub>-  
408 enriched environment. Even though the duration of our experimental precluded  
409 communities from evolving under the prevailing conditions, we consider that our future  
410 warming and CO<sub>2</sub> conditions were quite realistic because: (1) the communities were  
411 gradually exposed to the future conditions, avoiding shock response which could  
412 exacerbate the susceptibility of them to the drivers tested. (2) Communities were  
413 exposed to realistic environmental scenarios as predicted by the IPCC (2019) for the  
414 end of this century; and (3) we quantified the phytoplankton responses at short- (i.e. diel  
415 cycles) and at generational time spans (i.e. weeks) which give us an idea about their  
416 acclimation capacity to the future expected conditions.

417 *4.1. Warming×CO<sub>2</sub> impacts on biomass and ecosystem functioning*

418           The faster increases in biomass reported under a future scenario agrees with  
419 recent evidences reported in micro/mesocosm experiments investigating warming  
420 (Yvon-Durocher et al. 2017; Wilken et al. 2018) and CO<sub>2</sub> (Low-Décarie et al. 2015;  
421 Paquette and Beisner 2018) effects in isolation, and their combined impacts but only in  
422 marine ecosystems (Sett et al. 2018; Sommer et al. 2015). This result suggests that the  
423 patterns observed in marine systems may also apply to other aquatic biomes. This  
424 stimulatory effect is likely due to enhanced cellular activities by warming, as stated by  
425 the Metabolic Theory of Ecology (Brown et al. 2004), and high CO<sub>2</sub> concentrations  
426 prompted a down-regulation of the CO<sub>2</sub>-concentrating mechanisms, reducing the  
427 cellular energetic expenditure (Raven et al. 2012).

428           After two weeks of incubation, a negative synergistic  
429 warming×CO<sub>2</sub>×oligotrophication interaction reduced between 30-70% the biomass  
430 respect to observed under a future scenario. The observed trend is in support of recent  
431 observational results by Verbeek et al. (2018b) showing a 71.5% decrease in  
432 phytoplankton biomass over a 10-year period after reductions in the phosphorus  
433 availability comparable to our experimental conditions. It is plausible that the  
434 warming×CO<sub>2</sub> interaction boosted the nutritional requirements of phytoplankton to  
435 grow, hence if such demands were not met by the nutrient availability, this would likely  
436 lead to altered RUE and ultimately, to impaired biomass production. In fact, and despite  
437 previous results have shown that global change stressors may decrease RUE when  
438 nutrients availability does (De Senerpont Domis et al. 2014; Verbeek et al. 2018a), our  
439 findings do not support neither reject our own expectations nor previous published  
440 results, as maxima RUE was found under the future<sub>Phos</sub> scenario. This maximum RUE

441 evidenced could lead to potential stoichiometric trophic constraints (De Senerpont  
442 Domis et al. 2014). Nevertheless, it is also shown that the RUE was variable as it  
443 depended on the phase of the phytoplankton succession. Therefore, it could be plausible  
444 that the different cellular status, loss of productive species or buffering of rare species  
445 over this period have contributed to accentuate this decay process observed (Corcoran  
446 and Boeing 2012) although high RUE values were found. Additionally, considering that  
447 an isolated ecosystem, as assessed here, cannot gain species through immigration to  
448 maintain the functional redundancy of the community, it is clear that the interaction  
449 between several stressors could destabilize key functions in these ecosystems in the  
450 future. Notwithstanding, based on our results, we can discard that the changes in  
451 biomass reported were influenced by the zooplankton grazing, as we did not observed  
452 differences neither in total abundances ( $\sim 128.10 \pm 35.35$  individuals  $L^{-1}$ ) nor in the  
453 relative abundance (18.10, 60.80 and 21.10% for cladocerans, copepods, and rotifers,  
454 respectively) of the main contributors to the zooplankton community between  
455 environmental scenarios over the experiment (Álvarez-Manzaneda et al., unpub. data).

#### 456 *4.2. Warming $\times$ CO<sub>2</sub> impacts on photosynthesis over the phytoplankton succession*

457 Overall and partially confirming our initial expectations, the positive LRR-effect  
458 reported under future conditions on photosynthesis was  $\sim 20\%$  higher under  
459 oligotrophication than ambient nutrients concentrations. This higher photosynthetic  
460 activity found under these conditions could be supported by two processes: (1) the  
461 higher RUE highlighted above; and (2) an increased nitrogen-demand by cells under  
462 warming (Yu et al. 2018). Both processes would allow phytoplankton to invest extra  
463 energy and resources in protein synthesis and assembly (Cotner et al. 2006). Although  
464 we did not quantify neither nitrogen uptake nor denitrification (i.e. important ecological

465 processes that reduce nitrogen concentration in water bodies, Chen et al. 2012), we  
466 hypothesize that the decreased  $\text{NO}_2^- + \text{NO}_3^-$  (5-fold) coupled with the increased  $\text{NH}_4^+$  (4-  
467 fold) concentrations only found under a Future<sub>Phos</sub> scenario would support an increased  
468 uptake and / or a dissimilatory nitrate reduction to ammonium (DNRA; Kamp et al.  
469 2011). Thus, a higher N and P-cellular content joint to simultaneous increase in C  
470 derived from the biomass production already mentioned could trigger a boosting of the  
471 biological pump mediated by photosynthesis.

472 *4.3. Sensitivity of cyanobacteria versus other eukaryotic phytoplankton groups to*  
473 *warming × CO<sub>2</sub> under oligotrophication*

474 According to the results shown so far, climate change would be a single-edged  
475 sword, as it would result in large increases of community biomass, RUE and production  
476 (i.e. positive effect); however, when we zoomed into other organization levels, such as  
477 the community structure, the second edge appears (i.e. negative effects). Our results  
478 show an opposite impact of the scenarios tested on the main contributors of the  
479 phytoplankton community. In fact, the warming × CO<sub>2</sub> interaction exerted a consistent  
480 negative synergistic effect on Prok (i.e. cyanobacteria), and mostly positive on other  
481 Euk phytoplankton. Moreover, these negative and positive effects found on Prok and  
482 Euk, respectively, under *in situ* nutrient conditions were accentuated ~25% under  
483 oligotrophication.

484 This result constitute, to our knowledge, the first empirical evidence showing a  
485 higher sensitivity of Prok- compared to Eukaryotic phytoplankton to a warming and  
486 high CO<sub>2</sub> scenario. This higher sensitivity is consistent with the impaired photochemical  
487 performance i.e. a reduced light harvesting efficiency (ca. -65%), rETR<sub>max</sub> (ca. -50%)  
488 and light compensation point (ca. -20%) found in this study. An impaired

489 cyanobacterial performance under such conditions contrasts with previous studies that  
490 shown a cyanobacterial dominance when N:P ratios in the water column decreased  
491 (Havens et al. 2003; Smith 1983). Thus, considering that: (1) nutrient concentrations  
492 (Rabalais et al. 2009; van Loosdrecht and Brdjanovic 2014), and (2) global climate  
493 change (Paerl and Huisman 2008; Yang et al. 2017) are the two main causes of the  
494 cyanobacterial blooms intensification, we propose that a displacement toward a higher  
495 competitive advantage of eukaryotic phytoplankton could potentially reduce the  
496 incidence of these blooms that increasingly threaten human and animal health, as many  
497 of them produce a suite of potent toxins (e.g. microcystin, Lüring et al. 2017).  
498 Additionally, a potential weakening of the cyanobacterial dominance could  
499 subsequently attenuate the strong disruption of the structure, and functioning of  
500 phytoplankton communities in conditions (i.e. high temperature or CO<sub>2</sub> and low N:P  
501 ratios) in which this group dominates, increasing their richness (Escalas et al. 2019;  
502 Visser et al. 2016).

## 503 **5. Conclusion**

504 This study revealed an unprecedented role of the  
505 warming×CO<sub>2</sub>×oligotrophication interaction on production and functioning of a model  
506 freshwater ecosystem. As the positive effects of the warming×CO<sub>2</sub> became strongly  
507 negative on cyanobacterial (and positive on eukaryotes) photosynthesis when nutrient  
508 availability was lowered by an in-lake management strategy (i.e. Phoslock<sup>®</sup> addition), it  
509 is necessary to know how the interplay between climate change-derived problems and  
510 current management strategies alter the equilibrium and the dominance in  
511 phytoplankton communities. With a robust knowledge of these processes, policy makers  
512 could design more appropriate future management strategies to improve the ecological

513 status of the aquatic ecosystems (i.e. reduced cyanobacterial blooms, good water  
514 quality) without compromising neither their ecological attributes (i.e. high total primary  
515 production) nor functioning.

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535



536 **7. Author's contribution**

537 Conceived the idea: MJC, MIA-M, EL-P, GG-J, LNdSD, ST, JMG-O. Developed and  
538 performed the research: MJC, MIA-M, EL-P, GG-J, LNdSD, ST, JMG-O. Analyzed  
539 the data, and drafted the manuscript: MJC. Discussed the presentation of the results and  
540 approved the final version of the manuscript: MJC, MIA-M, EL-P, GG-J, LNdSD, ST,  
541 JMG-O.

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788 **Figure captions**

789 Figure 1.- Mean ( $\pm$ SD) (A) Total chlorophyll *a* (Chl *a*) concentrations and (B) resource  
 790 use efficiency (RUE) of the phytoplankton community exposed to three environmental  
 791 scenarios (Present, Future and Future + Phoslock<sup>®</sup>) during the experiment.

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793 Figure 2.- Mean ( $\pm$ SD) diel relative electron transport rates (rETR) of eukaryotes (i.e.  
 794 green algae, diatoms and dinoflagellates) and prokaryotes (i.e. cyanobacteria) exposed  
 795 to three environmental scenarios (Present, Future and Future + Phoslock<sup>®</sup>) during the  
 796 experiment.

797 Figure 3.- Mean ( $\pm$ SD) (A) Integrated relative electron transport rates (rETR) in  
 798 eukaryotes (Euk, green algae, diatoms and dinoflagellates) and prokaryotes (Prok,  
 799 cyanobacteria) exposed to three environmental scenarios (Present, Future and Future +  
 800 Phoslock<sup>®</sup>) during the experiment (pre-bloom, bloom and post-bloom phases). (B) Log-  
 801 response ratios (LRR) of the rETR in Euk and Prok under the above mentioned  
 802 scenarios. Letters on the top of bars represent represent significant differences by the

803 Least Significant Differences (LSD) *post hoc* test. Positive and negative values of LRR  
804 indicate a stimulatory and an inhibitory effect, respectively.

805 Figure 4.- Mean ( $\pm$ SD) (A) Light harvesting efficiency ( $\alpha$ ), (B) relative maxima electron  
806 transport rates ( $rETR_{max}$ ), and (C) light compensation point ( $E_k$ ) in eukaryotes (Euk,  
807 green algae, diatoms and dinoflagellates) and prokaryotes (Prok, cyanobacteria)  
808 exposed to three environmental scenarios (Present, Future and Future + Phoslock<sup>®</sup>) at  
809 the initial and at the end of the experiment (pre-bloom, bloom and post-bloom phases).  
810 Log-response ratios (LRR) of  $\alpha$  (D),  $rETR_{max}$  (E) and  $E_k$  (F) in Euk and Prok under the  
811 above mentioned scenarios. Letters on the top of bars represent significant differences  
812 by the Least Significant Differences (LSD) *post hoc* test. Positive and negative values  
813 of LRR indicate a stimulatory and an inhibitory effect, respectively.

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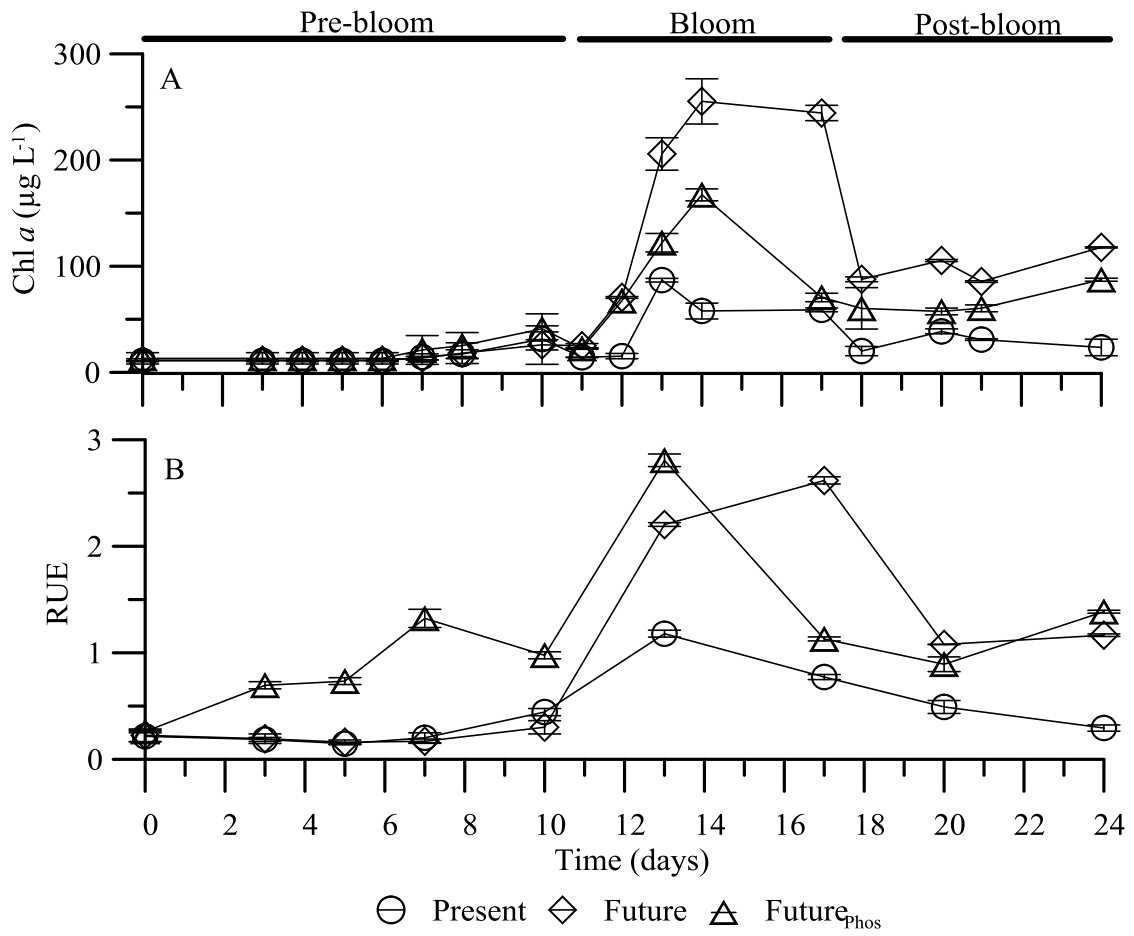
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Figure 1

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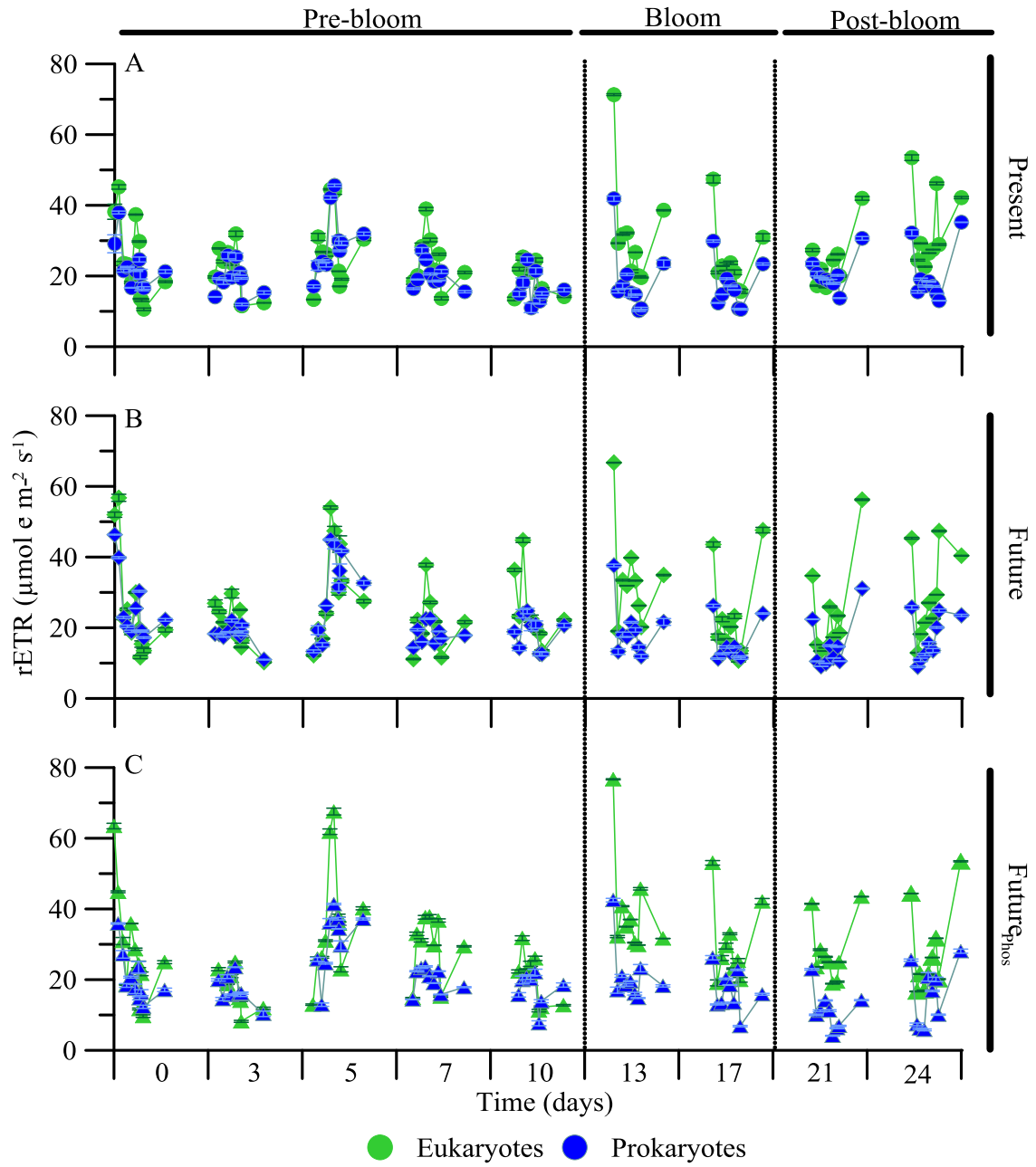
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Figure 2

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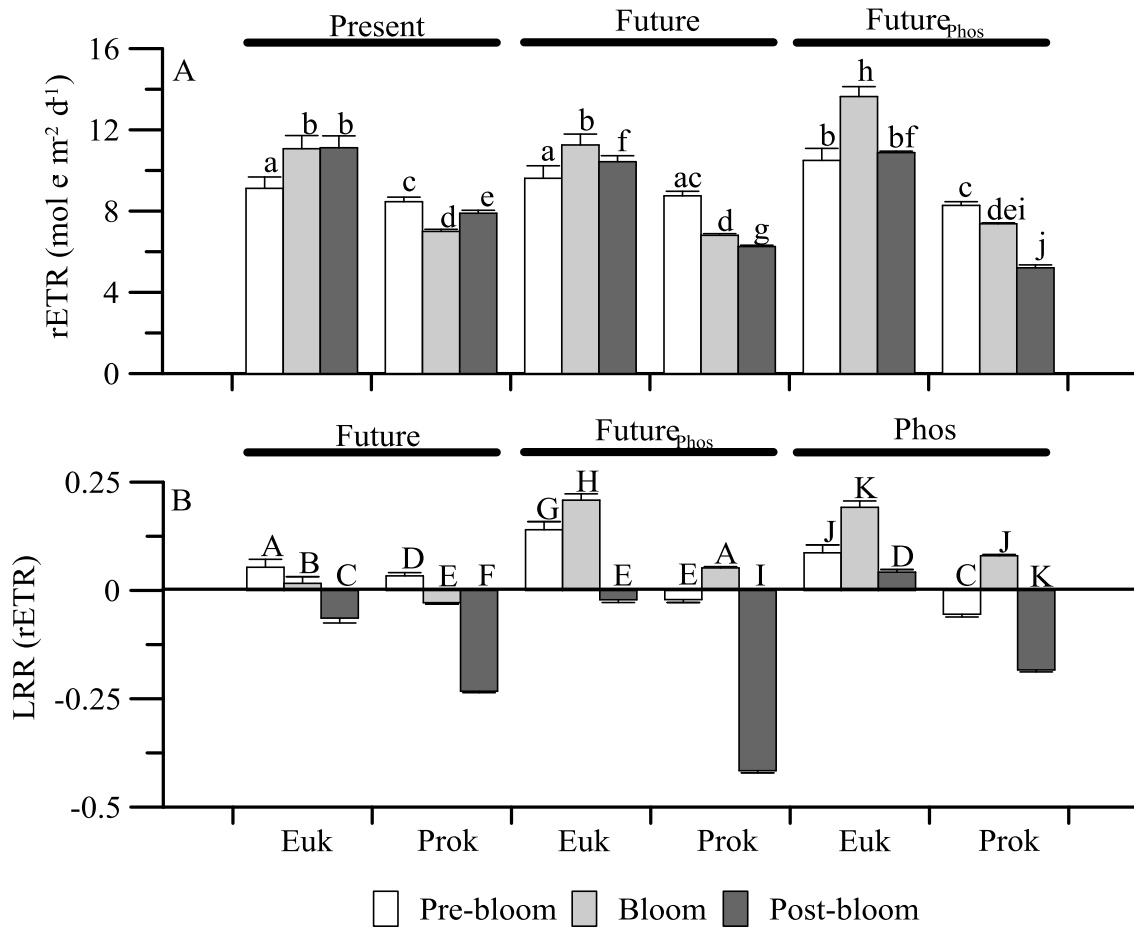
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Figure 3

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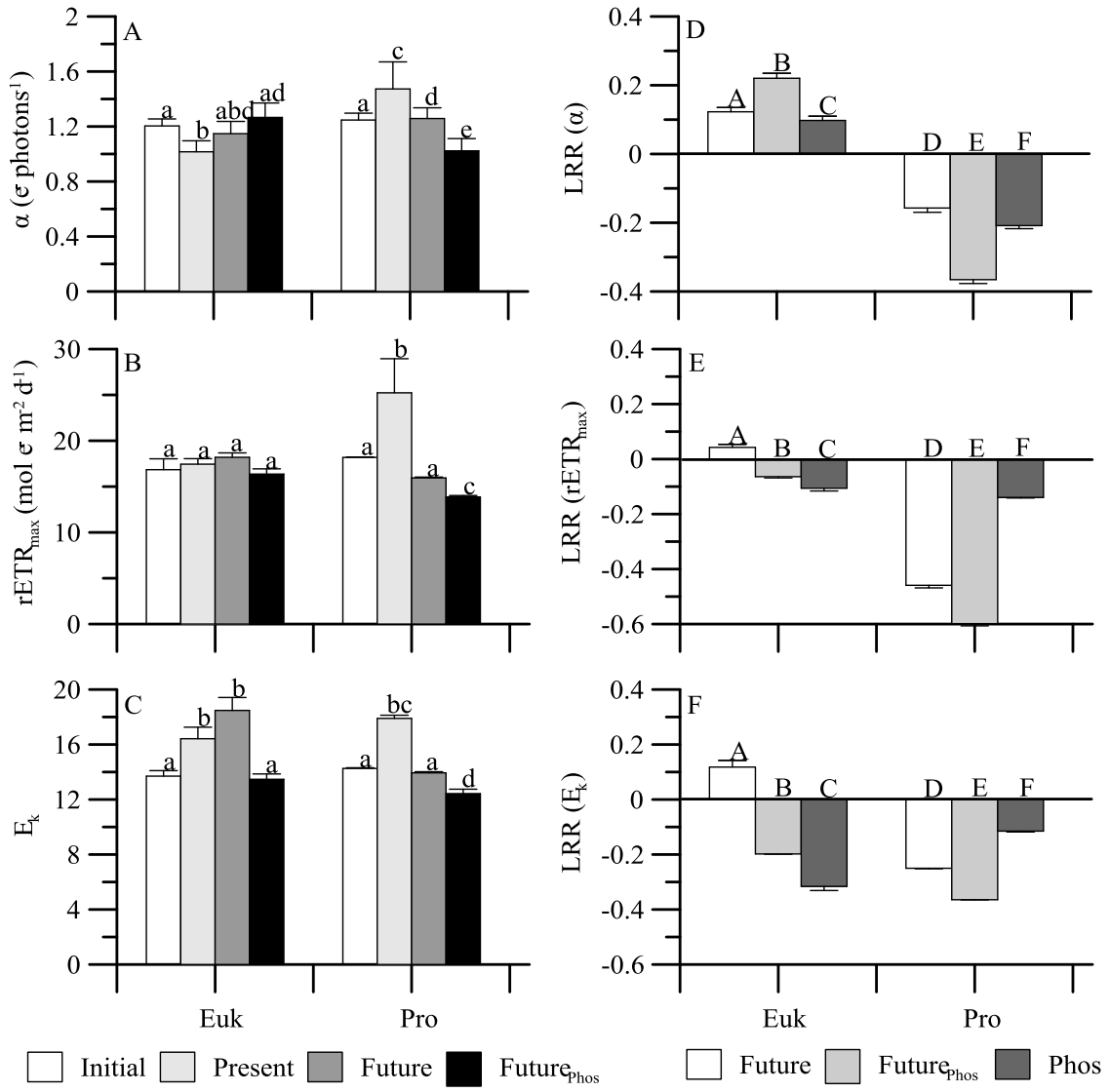
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Figure 4