Title:

Subtropical zooplankton assemblage promotes the harmful cyanobacterium *Cylindrospermopsis raciborskii* in a mesocosm experiment

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Running head: Zooplankton promote freshwater HABs

ABSTRACT

Harmful algal blooms (HAB) with public health impacts threaten freshwater ecosystems, including drinking water reservoirs, globally. Subtropical systems are often dominated by filamentous and colonial cyanobacteria, algae that are potentially less accessible for consumption by resident meso-zooplankton grazers. Less understood than selective grazing is the role of zooplankton in regenerating nutrients and facilitating growth of algae with efficient uptake strategies, such as the toxin-producing cyanobacterium, *Cylindrospermopsis raciborskii*. Using ~800 L bags suspended in the upper 3 m of the water column, we examined the growth of *C. raciborskii* under four treatments: 3x ambient zooplankton biomass, 10x zooplankton, 10x zooplankton plus inorganic P addition and a no amendment control (3Z, 10Z, 10ZP, control, respectively). After 4 days, *C. raciborskii* relative abundance doubled in the 10Z and 10ZP treatments compared to the control and 3Z treatments, and after 7 days P addition resulted in ~20 % higher relative *C. raciborskii* biomass compared to other treatments, and an order of magnitude increase in N-fixing phytoplankton. The particulate C:P ratio declined in the 10Z and 10ZP mesocosms, indicating that meso-zooplankton facilitated P transfer to algae. Overall, meso-zooplankton promoted *C. raciborskii* abundance and biomass in this subtropical plankton assemblage over the short-term, demonstrating their facilitation of subtropical freshwater HAB formation.

Keywords: Freshwater HABs, cyanobacteria, selective grazing, nutrient regeneration, copepods.

INTRODUCTION

Cyanobacteria are a prominent group in many freshwater planktonic communities, with some strains forming harmful algal blooms (HABs). Such taxa can have harmful effects through toxin production, oxygen depletion on decomposition, shading or smothering of benthic habitats, and disruption of energy flow through aquatic food webs (Havens, 2008; Paerl and Huisman, 2008). Apart from being a threat to potable water supply, toxin-producing cyanobacteria remain an important research priority due to rising concern over their increased competitiveness under climate change (Davis et al., 2008; O'Neil et al., 2011; Paerl and Otten 2013).

Meso-zooplankton play a significant role in aquatic food webs, and influence phytoplankton directly through grazing, and indirectly through nutrient regeneration (Sterner, 1990). While small-volume laboratory experiments have been valuable in assessing the outcomes of herbivore-prey interactions, including prey choice, stoichiometric constraints and algal defences (DeMott and Van Donk, 2013), *in situ* mesocosms (1-3 orders of magnitude larger in volume) more closely mimic the natural variability in aquatic habitats and incorporate a wider spectrum of plankton behaviour, and can thus more accurately assess cyanobacteria-zooplankton interactions. Most mesocosm studies to understand regulation of cyanobacteria by zooplankton have been carried out in temperate lakes (Sommer and Sommer, 2006). However, patterns of phytoplankton-zooplankton interactions in tropical and subtropical lakes may deviate from those in temperate lakes due to differences in physical, chemical, and biological characteristics (Havens et al., 1996; Low and Ng, 2010). In terms of the biological characteristics, temperate lakes generally have higher biomass of relatively large crustacean zooplankton such as *Daphnia spp*., whereas this niche is typically filled by smaller cladocerans or copepods in tropical and subtropical lakes (Bayly, 1992; Jeppesen et al., 2005; Sommer and Sommer, 2006; Lacerot et al. 2013). With respect to phytoplankton, cyanobacteria dominance in temperate regions is generally limited to the summer season by prevailing temperature, while in the tropics, cyanobacteria can dominate year round due to warm water temperatures, vertical stratification and their ability to access light through buoyancy regulation,

68 which suppresses algal competitors (Lei et al. 2011). Some cyanobacteria (e.g. *Anabaena*) have the
69 potential to fix nitrogen (N) and this strategy allows them to alternate between different N sources
70 when dissolved N is limiting (Briand et al., 2002; Burford et al. 2006a). Some cyanobacteria are
71 also able to grow well under low phosphate concentrations (Branco et al. 1994; Briand et al. 2002)
72 due to a high phosphate uptake affinity and storage capacity for phosphorus (P) relative to other
73 algal groups (Istvánovics et al. 2000; Shafik et al. 2001; Aubriot and Bonilla 2012).

74 In addition to differences between temperate and tropical systems, zooplankton interactions
75 with cyanobacteria may have different outcomes under low nutrient conditions, such that the
76 indirect benefits of nutrient regeneration are greater than the direct negative consequences of
77 consumption (Persson et al., 1988; Elser et al., 1990). Zooplankton may also transfer nutrients to
78 toxic cyanobacteria with high nutrient affinity at the expense of competing algal species (Mitra and
79 Flynn, 2006). Furthermore, under nutrient enrichment, grazing losses may be offset by allowing
80 previously limited phytoplankton to grow faster (Hunt and Matveev, 2005).

81 C*ylindrospermopsis raciborskii* is a filamentous cyanobacterium which blooms in lakes and
82 reservoirs in the southern and northern hemisphere including Australia (Harris and Baxter, 1996;
83 Saker et al., 1999; McGregor and Fabbro, 2000), South America (Brazil; Bouvy et al., 2001;
84 Figueredo and Giani, 2009), North America (Chapman and Schelske, 2008), and Asia (Thailand; Li
85 et al., 2001). Direct effects on *C. raciborskii* abundance due to zooplankton grazing may be modest,
86 based on relatively low clearance rates observed in small-scale laboratory experiments (Panosso et
87 al., 2003; Kâ et al. 2012; Hong et al., 2013). However, copepods cut *C. raciborskii* filaments,
88 effectively shortening them to an edible size for other zooplankton (Bouvy et al. 2001). In addition,
89 *C. raciborskii*'s ability to efficiently take up and store P (Padisák, 1997), suggests that its
90 abundance may be affected indirectly by attracting a larger proportion of zooplankton-derived
91 nutrients than co-existing algae.

92 In this study, we tested whether *C. raciborskii* in a reservoir dominated by this species would
93 increase in abundance under two different levels of zooplankton biomass, and whether additional P
94 enrichment would stimulate growth of other algae and reduce *C. raciborskii* relative abundance.
95 Our hypothesis was that selective zooplankton grazing and rapid uptake of regenerated nutrients by
96 *C. raciborskii* would act synergistically to facilitate its accumulation within a natural plankton
97 assemblage, and that enrichment with P would decrease *C. raciborskii*'s competitiveness.

98

99 METHOD

100 **Study site and experimental design**

101 The mesocosm experiment was carried out at Lake Wivenhoe (27.386 °S, 152.597 °E) in southeast
102 Queensland, Australia, a subtropical, oligotrophic reservoir with 0.50 ± 0.08 mg L^{-1} total N (36 ± 6
103 µmol L^{-1}) and 0.01 ± 0.00 mg L^{-1} total P (~0.3 µmol L^{-1}) across surface and bottom waters (Burford
104 et al., 2007*)*. The abundance of the toxic cyanobacterium *C. raciborskii* typically increases in
105 austral spring and peaks in summer (Burford and O'Donohue 2006), so the experiment was
106 conducted from 19 – 25 January, 2010 when *C. raciborskii* abundance was ~ 2.0 x 10^4 cells mL^{-1}
107 (pre-bloom). The deployment site was in the lower reservoir close to the dam wall distant from
108 littoral macrophytes and in approximately 10 m of water. It was therefore considered representative
109 of pelagic open water planktonic communities where *C. raciborskii* can dominate phytoplankton
110 communities in the summer. The mesocosms consisted of clear 150 µm thick bags (Redblade Pty
111 Ltd, Albion, Australia) made of 0.5 W x 0.5 D x 3 H m polyethylene sheeting with ~800 L capacity.
112 Each was sealed with a heat sealer at the lower end and had its top end sewed onto a square frame,
113 keeping it open to the atmosphere but cut off from the sediments. The final configuration involved
114 fitting four randomly allocated mesocosm bags onto a floating PVC framework for support. On
115 deployment, bags sat in the upper 3 m of the water and netting was put on top of the frames to
116 prevent birds from disturbing the experiment. The experiment had four treatments including an un-

117 amended control (surface water with ambient zooplankton), a 3Z zooplankton treatment (addition of
118 ~ 60 zooplankton individuals L^{-1} to 3 times ambient concentration), a 10Z zooplankton treatment
119 (addition of ~ 280 individuals L^{-1}). The fourth treatment (10ZP) had 10Z zooplankton with added P,
120 spiked daily in the form of inorganic KH_2PO_4 (4 µg L^{-1}) to maintain dissolved N:P concentrations
121 close to the Redfield (1958) ratio (16:1), and were mixed as in Muhid et al. (2013). Each treatment
122 had triplicate bags. The mesocosms were filled with surface reservoir water (unscreened) using
123 bilge pumps. Zooplankton was collected from the mesocosm site with vertical net tows (to 12 m)
124 using a 75 µm net (20 cm diameter, 0.5 m length). Zooplankton were pooled into a 20 L container,
125 gently mixed and subsamples taken for species composition and elemental analyses. Samples to
126 examine species composition were preserved in 70% ethanol, and those for elemental analysis were
127 filtered onto pre-combusted glass fibre filters (GF/F, Whatman), for later C and N analysis.
128 Zooplankton were then added into each treatment bag accordingly. The zooplankton assemblage
129 was dominated numerically by rotifers (43 % of total abundance) and copepods (14% of total
130 abundance), although copepods comprised most of the biomass based on size, with cladocerans
131 contributing only 1% of total counts (remaining animals were juveniles). The biomass added to the
132 3Z treatment was 1.42 ± 0.20 mg C L^{-1} and 7.09 ± 1.01 mg C L^{-1} for both 10Z and 10ZP treatments.
133 Bags were left overnight before sampling began on day 1.

134 **Physico-chemical measurements and sample collection**

135 Daily measurements of temperature, dissolved oxygen concentration (DO), pH, conductivity and
136 turbidity were made in mesocosm bags and adjacent reservoir water at the surface and at 1 m with a
137 multiparameter sonde with automated logger (handheld YSI 650 and YSI 6600; Yellow Springs,
138 Ohio, USA). Additionally, vertical profiles of irradiance were measured daily through the
139 mesocosms from 0 to 2 m using a 4-pi PAR sensor (LiCor, NB, USA) and the Secchi depth was
140 also recorded.

141 Water samples were collected from each mesocosm bag and the adjacent reservoir water
142 using a 3 m long depth-integrated sampler (volume = 5 L) approximately 10 hours after the
143 mesocosms were filled (day 1), then daily to day 5 and at the end of the experiment on day 7. To
144 assess phytoplankton biomass (chlorophyll-*a*), 50 mL was filtered under low vacuum (e.g. ≤ 100
145 mm Hg) onto 25 mm GF/F filters in low light (< 10 µmol photons m^{-2} s^{-1}). Filters were folded in
146 half, blotted dry on absorbent paper, placed into screw-capped cryovials and stored frozen at minus
147 80 °C until HPLC pigment analysis. Subsamples (200 ml) were fixed with Lugols solution (1%)
148 and stored for phytoplankton counts. Total particulate organic carbon and nitrogen (TPC and TPN,
149 respectively) were prepared on site by filtering water samples onto pre-combusted GF/F filters
150 (Whatman, USA) with a hand pump. TPP samples were prepared in a similar manner by filtering
151 water samples onto 0.45 µm membrane filters (Millipore, Ireland), with the filtrate collected for
152 DIN and DIP analyses. All nutrient samples were immediately stored on ice in the dark and on
153 return to the laboratory were frozen at –20°C until analysis.

154 To assess zooplankton biomass and assemblage structure, zooplankton were collected from
155 each mesocosm at the end of the experiment (day 7) by repeated vertical tows using a 75 µm, 20 cm
156 diameter (1 m long) net. Zooplankton samples were divided into three equal parts for identification
157 (preserved in ethanol, 70% final concentration), biovolume determination (preserved in
158 formaldehyde, 4% final concentration) and elemental analyses (stored frozen at -20 °C).
159 Zooplankton collected from adjacent reservoir water at the beginning of the experiment were
160 processed using the same method.

161 **Sample analyses**

162 *Nutrients.* Samples for TPP were digested using a persulfate digestion procedure. After
163 digestion, TPP was analysed based on the ascorbic acid reduction of phosphomolybdate (Towns
164 1986). The carbon content of phytoplankton was estimated using biovolume conversion factors
165 described in Hendrickson (2011) after TPC and TPN samples were lost during sample processing.

166 *Phytoplankton.* Phytoplankton samples from day 1 (initial), 2, 4 and 7 were identified and
167 counted using a Lund cell under 400x magnification on a compound microscope (Olympus BX50,
168 Hamburg, Germany). One short traverse, with more than 400 units (single cells or filaments) was
169 counted for each sample. For colonial and filamentous cyanobacteria, cell numbers in each filament
170 or colony were estimated by counting cells in an average of at least 30 units. The number of *C.*
171 *raciborskii* cells was determined by multiplying the number of filaments by 14. This value was
172 previously determined by counting cells in an average of 400 filaments from Lake Samsonvale
173 (Glenn McGregor, pers. comm.). Size classes were defined as nanoalgae (2 – 20 µm) and
174 microalgae (20 - 200 µm; i.e. filaments and colonies) (Sommer and Sommer, 2006). Two further
175 functional groups were identified: species with heterocysts (N_2-fixers, *Anabaena* and
176 *Aphanizomenon*) and potentially toxic genera (*Anabaena*, *Aphanizomenon*, *Cylindrospermopsis,*
177 *Planktothrix* and *Geitlerinema).* In a second series of counts, phytoplankton biovolume was
178 determined on an individual cell basis, where length and width were used to estimate the basic
179 shape and calculate the biovolume according to Hillebrand et al. (1999). We also measured the
180 length of *C. raciborskii* filaments at the beginning and during the experiment, to determine whether
181 they had been shortened through zooplankton activity (Bouvy et al. 2001).

182 Phytoplankton pigment concentrations were estimated using High Performance Liquid
183 Chromatography (HPLC). In the laboratory, pigments were extracted at 4 °C in the dark over 15–
184 18 h in 3 mL acetone (100%, diluted to 90% for analysis, Mallinkrodt, HPLC grade) then sonicated
185 on ice for 15 minutes. Samples were recovered using filtration (0.45 µm, Whatmann) and
186 centrifugation (2500 rpm, 5 min at 4°C). The samples were analysed by HPLC (Waters – Alliance
187 comprising a 2695XE separations module with column heater and refrigerated autosampler) using a
188 C_8 column (Zorbax Eclipse XDB-C8, Agilent Technologies) and binary gradient system with an
189 elevated column temperature (55° C) following a modified version of the Van Heukelem and
190 Thomas (2001) method. Pigments were identified by their retention time and absorption spectrum
191 from a photo-diode array detector (Waters – Alliance 2996 PDA). Concentrations of pigments were

192 determined from commercial and international standards (Sigma; DHI, Denmark). The HPLC
193 system was also calibrated using phytoplankton reference cultures (Australian National Algae
194 Culture Collection) whose pigment composition has been documented in the literature (Mantoura
195 and Llewellyn, 1983; Barlow et al., 1993; Jeffrey et al., 1997).

196 *Zooplankton.* Zooplankton were enumerated into functional groups (cladocerans, copepods,
197 rotifers and juveniles which included immature forms of cladocerans and copepods) using a
198 compound microscope (Olympus BX50, Hamburg, Germany). To assess their elemental content,
199 zooplankton were dried at 50 °C overnight, and analysed using an Elemental Analyser with 20-20
200 IRMS (Europa Scientific). Zooplankton biovolume was determined using an Optical Plankton
201 Counter (Focal Technologies, Inc., Dartmouth, Canada) configured as described by Moore and
202 Suthers (2006). The concentration of particles (zooplankton) is expressed as number per litre. The
203 zooplankton biovolume (mm^3 L^{-1}) was calculated as the sum of the products of volume (mm^3 $ind.^{-1}$)
204 and concentration (ind. L^{-1}) of particles over all equivalent spherical diameter (ESD) values
205 between 240 and 3000 µm ESD. The volume of water sampled for zooplankton was calculated
206 using the following equation: Volume = Π * (diameter of net/2)^2 * depth of tow (12 m in ambient
207 water and 3 m in mesocosm).

208 **Data Analysis**

209 Repeated measures analysis of variance (RM-ANOVA) was used to compare differences in
210 phytoplankton abundance (total and functional group, i.e., nanoalgae, microalgae, potentially toxic
211 algae, N_2-fixers) between treatments over time. One-way ANOVA was also performed to test the
212 different abundance of individual phytoplankton species among four treatments (Control, 3Z,
213 10Zand 10Z) on day 4 and day 7. ANOVAs were performed in SPSS version 8.0 ® (1997 SPSS
214 Inc.) and the significance level for all tests was p = 0.05. Tukey's multiple comparison tests were
215 used when significant differences were found between treatments. Data were examined for
216 normality and were ln (x + 1) transformed when normalization was required. When data did not

217 meet the assumptions of normality even after transformation, they were analysed using a non-
218 parametric Kruskal-Wallis one-way analysis of variance by ranks to test for the difference between
219 population medians. Results are expressed as X^2 (df, n = number of replicates) (Green and Salkind,
220 2008).

221 Multivariate analyses of phytoplankton composition data were undertaken with PRIMER
222 5.2.9 software ® (2002 PRIMER-E Ltd., Plymouth, UK). Transformed abundance data (ln (x + 1))
223 were used to generate a Bray-Curtis similarity matrix. The Bray-Curtis similarity matrix was then
224 used to ordinate samples (visualised using an nMDS plot) and undertake analysis of similarity
225 (ANOSIM). The ANOSIM test statistic, R, is based on the ratio of the between treatment to within-
226 treatment similarity ranking and ranges from 0 to 1, with the value indicating the degree of
227 dissimilarity (1 = completely dissimilar; 0 = completely similar). When a significant difference ($p <$
228 0.05) was detected, a similarity percentage breakdown (SIMPER) was conducted to determine
229 which taxa were primarily responsible for the observed differences.

230 RESULTS

231 **Physico-chemical characteristics**

232 The physical and chemical factors including light, temperature, conductivity, turbidity and pH were
233 similar among mesocosm treatments and relatively constant during the experiment (Table 1).
234 Despite inorganic P addition to the 10ZP treatment, DIP concentrations at day 7 were similar across
235 treatments ($F_{3,8} = 3.176$, $p = 0.085$), averaging 0.01 ± 0.00 mg L^{-1} (and 0.41 ± 0.01 mg L^{-1}, for
236 DIN). The molar DIN:DIP ratios averaged 60 ± 5 (SE), substantially higher than the Redfield ratio
237 (16), and were the same amongst treatments on day 7 ($F_{3,8} = 0.685$, $p = 0.601$). In contrast, the
238 particulate C:P ratio differed among treatments ($F_{3,6} = 0.202$, $p = 0.012$, Fig. 1A), and was lower in
239 10Z and 10ZP (128 ± 16 and 95 ± 16, respectively) compared to the control (212 ± 7) and 3Z (197
240 ± 48). However, zooplankton C:P ratio did not differ significantly amongst treatments ($p = 0.101$;
241 Fig. 1B).

242 **Phytoplankton**

243 Total phytoplankton abundance diverged among treatments on day 4 ($F_{3,12} = 7.41$, $p = 0.011$), being
244 lowest in the 3Z and highest in the 10ZP treatment ($0.90 \pm 0.10 \times 10^5$ versus $1.46 \pm 0.21 \times 10^5$ cells
245 mL^{-1}, respectively). At the end of the experiment (day 7), total phytoplankton abundance was
246 similar amongst control, 3Z and 10Z treatments ($F_{3,7} = 1.780$, $p = 0.238$; Fig. 2), but was greater in
247 the 10ZP treatment ($1.26 \pm 0.20 \times 10^5$ cells mL^{-1}; Fig. 2D). In addition, the chlorophyll-a (Chl-a)
248 concentration was twice as high in the 10ZP treatment (8.4 ± 1.0 μg L^{-1}) compared to all the others
249 ($F_{4,10} = 5.674$, $p = 0.012$; Fig. 3A), which were not significantly different from the initial (4.2 ± 0.4
250 μg L^{-1}).

251 Cyanobacteria comprised most of the phytoplankton assemblage biovolume in the
252 mesocosms on day 1 (84 ± 5%), with *Planktolyngbya, Cylindrospermopsis* and *Limnothrix* being
253 the dominant taxa (Table 2). However, the abundance of different phytoplankton functional groups
254 varied during the experiment. On day 4, the abundance of potentially toxic algae was greater in the
255 10Z and 10ZP treatments than in the 3Z and the control ($F_{3.7} = 12.608$, $p = 0.002$). By the end of the
256 experiment, the 10ZP treatment had the greatest abundance of potentially toxic algae (9.42 ± 1.00
257 $\times 10^4$ cells mL^{-1}) and the control treatment the least ($5.23 \pm 1.72 \times 10^4$ cells mL^{-1}) ($F_{3.7} = 4.80$, $p =$
258 0.049). N_2-fixers increased steadily in the 10ZP treatment (Fig. 3D) and were most abundant
259 amongst all other treatments on day 7 ($2.04 \pm 0.32 \times 10^4$ cells mL^{-1}; $F_{3,6} = 18.736$, $p = 0.008$).
260 Filamentous (and colonial) algae decreased until day 4 ($F_{3,7} = 6.732$, $p = 0.017$) and then steadied.

261 The abundance of *C. raciborskii* (approximately 22% of the total biovolume) was similar in
262 all bags at the start of the experiment, ranging from 1.82 to 2.44×10^4 cells mL^{-1}. However, by day
263 4, *C. raciborskii* abundance had more than doubled in the 10Z ($3.55 \pm 0.19 \times 10^4$ cells mL^{-1}) and
264 10ZP ($3.98 \pm 0.43 \times 10^4$ cells mL^{-1}) treatments ($F_{3,8} = 14.763$, $p = 0.001$, Fig. 4A) and increased its
265 relative abundance from 15% to 37% ($F_{3,8} = 8.235$, $p = 0.008$). On day 7, *C. raciborskii* relative
266 biomass was still greater in the 10ZP compared to other treatments (marginally significant, $n = 2$;

267 $F_{3,7} =$ 4.054, $p = 0.058$, Fig. 4B), but its absolute abundance was the same amongst treatments ($F_{3,7} =$

268 1.523, $p = 0.291$). Furthermore, *C. raciborskii* filaments were of similar length in the control and

269 10Z treatment (83 ± 39 µm versus 86 ± 36 µm, respectively) on day 4, providing little evidence that

270 copepods cut *C. raciborskii* filaments during our study, potentially shortening them to an edible size

271 for other zooplankton (Bouvy et al. 2001).

272 Considering the phytoplankton species composition as a whole, the ANOSIM showed

273 strong differences between treatments on day 4 (Global R: 0.429, $p = 0.019$). SIMPER analysis

274 revealed three filamentous species including *Cylindrospermopsis*, *Geitlerinema and Limnothrix*

275 were the major contributors to the dissimilarity amongst treatments. While morphologically similar,

276 these taxa showed different patterns over time. Potentially toxic *Geitlerinema* was more abundant in

277 the 10Z and 10ZP treatments *(*1.40 ± 0.54 and 2.31 ± 0.27 x 10^4 cells mL^{-1}, respectively) compared

278 to the control and 3Z on day 7 (0.45 ± 0.45 and 0.48 ± 0.40 x 10^4 cells mL^{-1}, respectively, $F_{3,8} =$

279 0.532 $p = 0.045$), while non-toxic *Limnothrix* cell abundance was greater in the control (2.4 ± 0.5 x

280 10^4 cells mL^{-1}) than in the 10Z (0.7 ± 0.6 x 10^4 cells mL^{-1}) and 10ZP treatments (0.1 ± 0.1 x 10^4

281 cells mL^{-1}; $F_{3,8} =$ 14.763, $p = 0.001$).

282 **Zooplankton**

283 As expected, zooplankton abundance ($F_{3,7} = 9.458$, $p = 0.007$, Fig. 5A) was higher in the 10Z and

284 10ZP treatments (albeit somewhat variable in the 10Z treatment) compared to the control and 3Z

285 treatments at the end of the experiment ($F_{3,7} = 9.458$, $p = 0.007$, Fig. 3B). Similarly total

286 zooplankton biovolume was higher in the 3Z treatment compared to the control and was greatest in

287 the 10Z and 10ZP treatments ($F_{3,7} = 9.394$, $p = 0.008$; Fig. 3C). Microscope counts revealed that

288 copepods (37 ± 3%) and rotifers (39 ± 4%) dominated the zooplankton assemblage on day 7 (Fig.

289 5), with copepods contributing the majority of the zooplankton biovolume, and did not differ

290 significantly between treatments. However, the proportion of juveniles decreased at least 20%

291 during the experiment ($F_{3,7} = 26.197$, $p < 0.001$) in all mesocosms, and the predator midge larva

292 *Chaoborus sp.* comprised 16 ± 3% of zooplankton abundance on day 7 when it was virtually absent

293 on day 0 ($F_{3,7}$ = 7.819, p = 0.010; Fig. 5).

294

295 DISCUSSION

296 This study determined that the abundance and relative biomass of *C. raciborskii* increased in the
297 presence of meso-zooplankton in a subtropical water storage during summer. Although the
298 experiment was relatively short-term (occurring over several days), the rotifer and copepod
299 dominated meso-zooplankton assemblage had an indirect effect of transferring P to phytoplankton
300 and favouring *C. raciborskii* growth.

301

302 While grazing was not quantified directly through gut-contents analysis, this mesocosm experiment
303 demonstrated that meso-zooplankton can have a short-term net positive impact on *C. raciborskii*
304 dominance *in situ*. *C. raciborskii* has been documented to inhibit feeding by cladocerans (Filho Ada
305 et al. 2009; Soares et al. 2009), and the copepod *Boeckella* sp. has very low clearance rates (<0.3
306 mL ind $^{-1}$ h^{-1}) on *C. raciborskii*, particularly when total prey abundance exceeds 1.0 mg C L^{-1} (Hong
307 et al. 2013), which was the case in this study. In the field, Fabbro and Duivenvoorden (1996)
308 reported the absence of zooplankton grazing on coiled *C. raciborskii* filaments, but noted that two
309 rotifer species (*Brachionus calyciflorus* and *B. angularis*) could ingest *C. raciborskii* following
310 breakage of larger filaments by *Daphnia lumholtzi*. Bouvy et al. (2001) made similar observations,
311 showing that cladocerans, copepods and rotifers can cut *C. raciborskii* filaments for ingestion.
312 However, in this study, there was no evidence of filament shortening, and the decline in abundance
313 of other filamentous algae such as *Limnothrix* indicated that prey morphology did not preclude
314 grazing. More recently Kâ et al. (2012) determined that zooplankton communities could clear
315 between 0.04 and 12.80 % *C. raciborskii* per day, even under relatively high cyanobacterial
316 dominance. Micro-zooplankton such as ciliates also have the capacity to consume toxin-producing

317 *C. raciborskii* (Fabbro et al. 2001), but because consumption was not measured in this study, we
318 cannot be certain that the increase in *C. raciborskii* abundance and biomass in the mesocosms was
319 due to grazing avoidance.
320
321 There is however, more evidence that zooplankton had an indirect effect on the phytoplankton
322 assemblage in this study through transfer of nutrients. Concurrent with the potential direct effects of
323 grazing, meso-zooplankton can influence phytoplankton through nutrient regeneration (Elser et al.,
324 1988; Sterner, 1990), and this study clearly demonstrated that addition of zooplankton lead to a
325 decline in the particulate C:P ratio. In the absence of horizontal advection and sedimentary
326 processes in the mesocosms, zooplankton were the only other potential source of P to
327 phytoplankton, through processes such as excretion and sloppy feeding.
328
329 We hypothesized that under low nutrient conditions, zooplankton interactions with cyanobacteria
330 would have a different outcome compared to enriched conditions, and for taxa such as *C.*
331 *raciborskii*, the indirect effects of zooplankton nutrient regeneration would be more beneficial than
332 for other algae, because of this cyanobacterium's high affinity and storage capacity for phosphorus
333 (P) (Istvánovics et al. 2000). Although it is unclear whether *C. raciborskii* acquired a greater share
334 of regenerated nutrients compared to other phytoplankton, *C. raciborskii* increased in abundance in
335 the presence of a copepod and rotifer dominated meso-zooplankton assemblage when there was no
336 external addition of nutrients. Inorganic P treatment addition also increased the numerical
337 abundance of *C. raciborskii* indicating that P was limiting its growth. Under similar P enrichment
338 (PO_4^{3-} added alone, not with N) in the same reservoir, Muhid et al. (2013) demonstrated that *C.*
339 *raciborskii* growth was preferentially promoted over other species, and in another reservoir in the
340 same region, daily pulses of DIP also favoured *C. raciborskii* accumulation (Posselt et al. (2009).
341 Additionally, P enrichment (10ZP treatment) caused a doubling of Chl-a relative to all other
342 treatments, a 60% increase in total cell abundance, and an order of magnitude increase in the cell

343 abundance of N-fixing taxa. The 100% increase in Chl-a with only 60% increase in cell abundance
344 suggests that cell size increased or cells produced more pigment or both. *C. raciborskii* was not
345 included in the N_2-fixing category in Figure 2, but it also has the capacity to switch between fixed
346 and atmospheric sources of N (Burford et al. 2006). Growth of N-fixing algae can be limited under
347 low external P concentrations due to a decrease in intracellular pools of nucleotides and low nucleic
348 acid content, especially RNA (Karl et al., 2002) and thus P addition has previously been shown to
349 benefit N-fixing cyanobacteria species (Vahtera et al. 2010).
350
351 There have been few mesocosm experiments to examine zooplankton-phytoplankton interactions in
352 subtropical freshwater systems where filamentous cyanobacteria are the major primary producers
353 and the zooplankton assemblage is dominated by copepods and rotifers. Enclosures have the
354 advantage of controlling experimental conditions and making them more ecologically-relevant
355 compared to lab-based studies, but also have their limitations. The bags used in this study limited
356 horizontal exchange, excluded fish and didn't include the sediment-water interface, but contained
357 vertical gradients in light which are important in the ecology of *C. raciborskii* (O'Brien et al. 2009).
358 The 3 m long bags were designed to capture processes in the surface mixed layer in summer when
359 the thermocline limits the vertical delivery of nutrients from deeper waters and the benthos.
360 Furthermore, the relatively large volume of the enclosures provided far less spatial constraints on
361 zooplankton swimming and feeding behaviour than previous laboratory studies. This allowed a
362 more realistic evaluation of the effect of zooplankton on *C. raciborskii* abundance, but provided
363 limited information about the relative importance of such interactions amongst other loss processes
364 including sedimentation, advection/dispersion, mixing, infection and parasitism. With respect to
365 how representative these observations are of what happens in other locations at other times, such
366 zooplankton–phytoplankton interactions occur in the epilimnion throughout the reservoir. During
367 summer when the water column is stratified (Burford et al. 2012), nutrient regeneration processes in
368 the surface mixed layer, such as those observed in this study, would be prevalent. Some Australian

369 strains of *C. raciborskii* produce cylindrospermopsins, and although we did not measure the
370 concentration of harmful metabolites in this study, similar enclosure experiments dominated by *C.*
371 *raciborskii* suggest that toxic strains became more prevalent with P enrichment (Burford et al.
372 2014). Given that copepods have very low clearance rates on toxic *C. raciborskii* strains compared
373 to a non-toxic strain (Hong et al. 2013), the accumulation of *C. raciborskii* observed in this study
374 would potentially be amplified if strains were producing toxins.

375

376 An additional consideration on the outcome of this experiment is whether zooplankton stocking
377 densities were similar in the 10Z and 10ZP treatments at the end of the experiment. We assessed
378 zooplankton abundance and biovolume using two different methods – microscope counts for
379 individuals >75 µm in size and biovolume estimates with an Optical Plankton Counter for
380 individuals at least 165 µm in equivalent spherical diameter. Based on microscope counts there was
381 no significant difference between the zooplankton abundance in the 10Z compared with the 10ZP
382 treatment due to variability amongst the replicates. Given the relatively consistent zooplankton
383 assemblage structure in the 10Z and 10ZP treatments, we suggest that the addition of P allowed
384 some of the resident zooplankton to grow and move into larger size groups (resulting in constant
385 biovolume) at the expense of smaller species, rather than cause mortality and potential release of
386 nutrients.

387

388 In summary, this study scaled-up previous laboratory observations of copepod interactions with a
389 freshwater HAB species and demonstrated that meso-zooplankton facilitate the accumulation of *C.*
390 *raciborskii*s in an oligotrophic subtropical reservoir over the short-term (~4 days). Meso-
391 zooplankton increased P transfer to phytoplankton, suggesting indirect effects of nutrient recycling
392 rather than direct effects of grazing could drive *C. raciborskii* abundance in this system. These
393 results are potentially novel and worth further investigation over larger space and time scales,
394 particularly with regards to the P content of *C. raciborskii* in comparison to the rest of the

395 phytoplankton assemblage.

396

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398

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410

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566

567 TABLE AND FIGURE LEGENDS

568

569 Table I: Water quality parameters mean (± SE) in mesocosms and adjacent ambient water at the
570 beginning and end of the mesocosm experiment.

571

572 Table II: Phytoplankton species composition at the beginning of the mesocosm experiment; N =
573 non-N-fixing algae, F = N-fixing algae, T = potentially toxic algae, Microalgae 20-200 µm,
574 Nanoalgae 2-20 µm.

575

576 Fig. 1: Particulate (A) and zooplankton (B) elemental ratios in treatments on day 7. Treatments
577 include: 3x ambient zooplankton abundance (3Z), 10x zooplankton abundance (10Z), 10x
578 zooplankton with inorganic P addition (10ZP) and a no amendment control. Data are means ±
579 standard error (SE; n = 3). Statistical comparisons indicated by letters above columns: a is different
580 from b, ab is not different from both a and b.

581

582 Fig. 2: Phytoplankton functional group abundance in different treatments during the mesocosm
583 experiment. Treatments include: A: no amendment control; B: 3x ambient zooplankton abundance
584 (3Z), C: 10x zooplankton abundance (10Z), and D: 10x zooplankton with inorganic P addition
585 (10ZP). Data are means ± SE (n = 3).

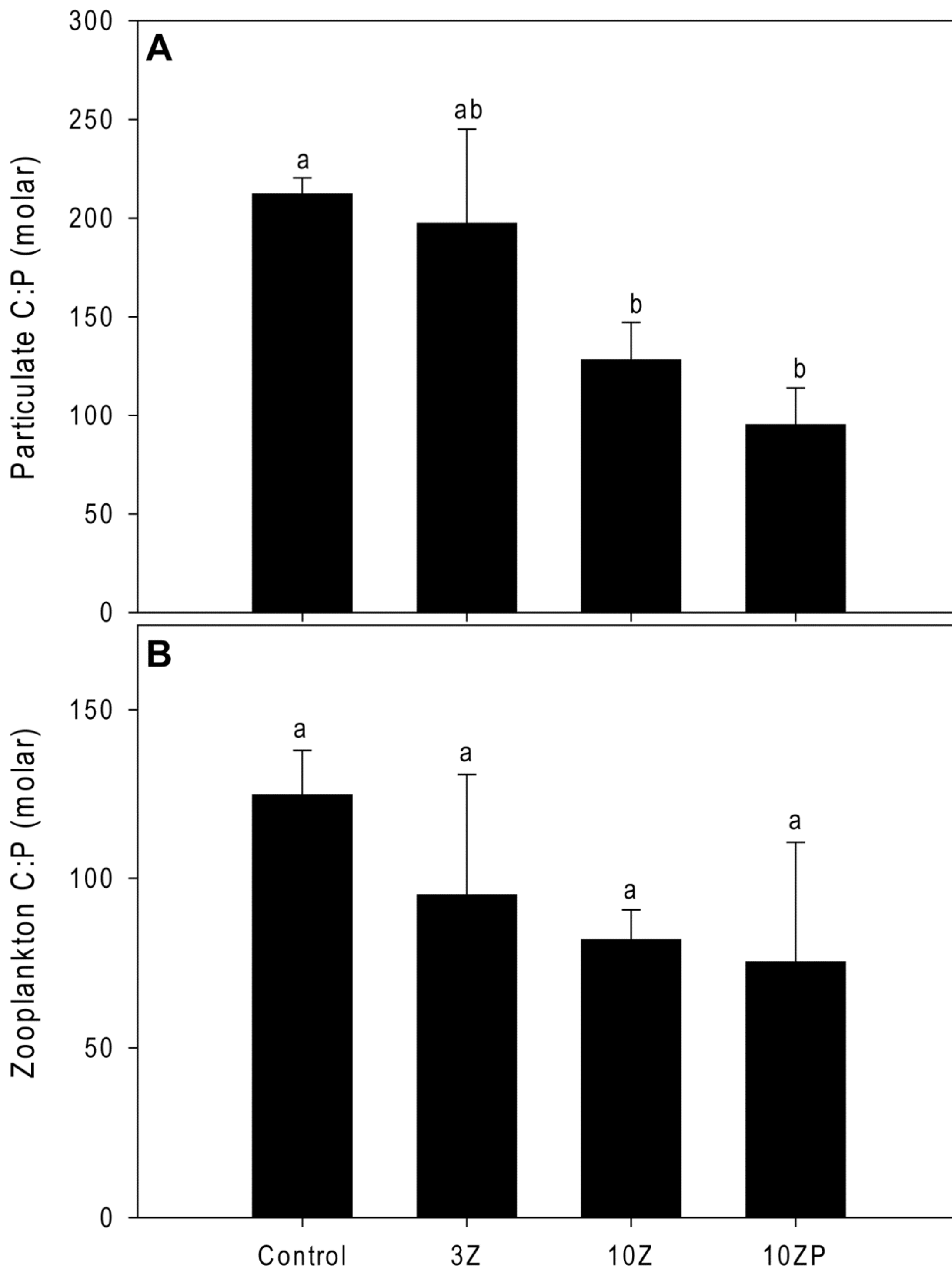
586

587 Fig. 3: Abundance of *C. raciborskii* (A) and the % of total phytoplankton biovolume comprised of
588 *C. raciborskii* (B) in different treatments during the mesocosm experiment. Treatments include: 3x
589 ambient zooplankton abundance (3Z), 10x zooplankton abundance (10Z), 10x zooplankton with

590 inorganic P addition (10ZP) and a no amendment control. Data are means ± SE (n = 3). Asterisk *
591 next to data point indicates significant difference between treatment and control at that time point;
592 ms means marginally significant (p = 0.058).
593

594 Fig. 4: Chlorophyll-a concentration (A), zooplankton abundance (B) and zooplankton biovolume
595 (C) in each treatment at the end of experiment (day 7) as estimated by microscope counts and the
596 Optical Plankton Counter, respectively. Treatments include: 3x ambient zooplankton abundance
597 (3Z), 10x zooplankton abundance (10Z), 10x zooplankton with inorganic P addition (10ZP) and a
598 no amendment control (Con). Data are means ± SE (n = 3). Statistical comparisons indicated by
599 letters above columns: a is different from b, ab is not different from both a and b, bc is not different
600 from both b and c.
601

602 Fig. 5: The proportion of zooplankton functional groups in mesocosm treatments at the beginning
603 (day 1) and end of the experiment (day 7). Treatments include: 3x ambient zooplankton abundance
604 (3Z), 10x zooplankton abundance (10Z), 10x zooplankton with inorganic P addition (10ZP) and a
605 no amendment control (Con). Data are means ± SE (n = 3).
606



607

608 Fig. 1

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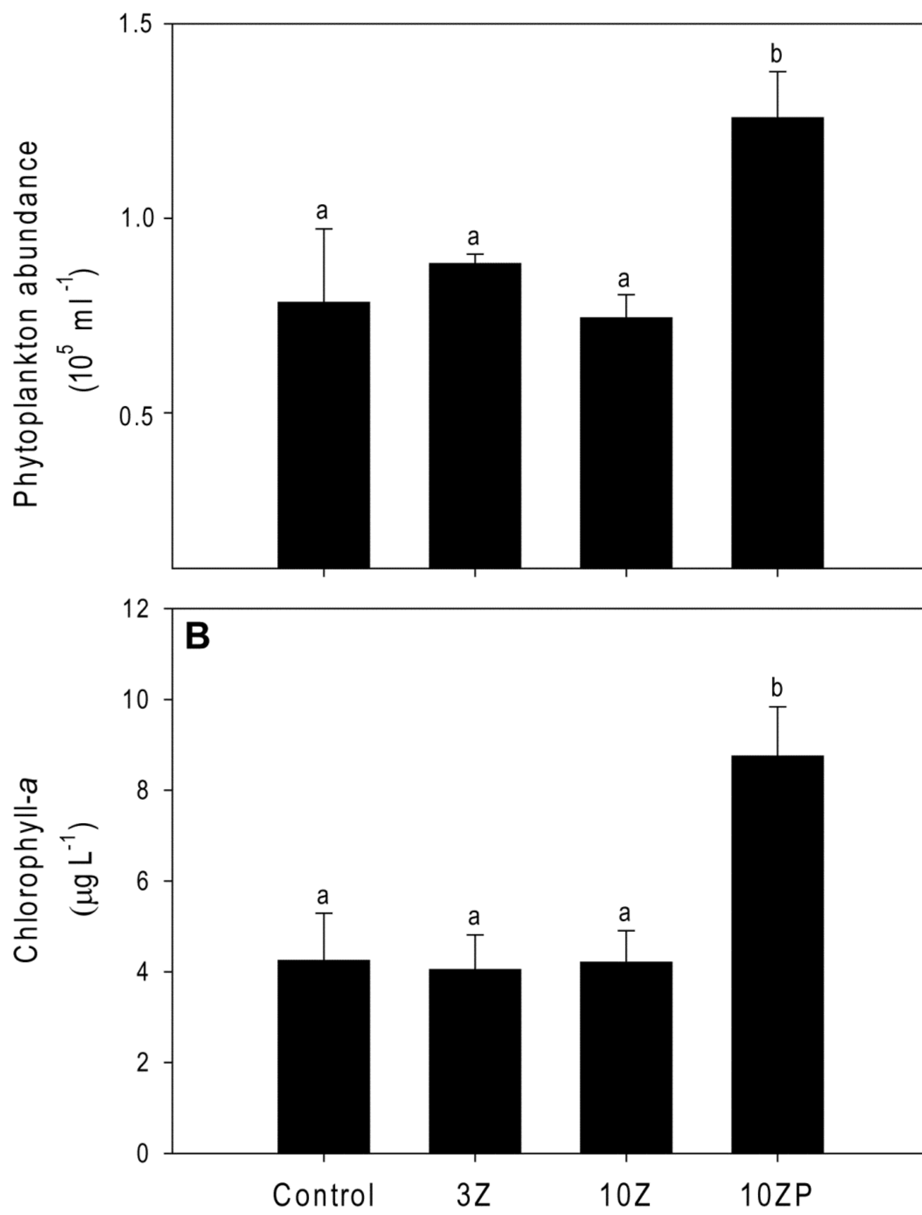

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612 Fig. 2

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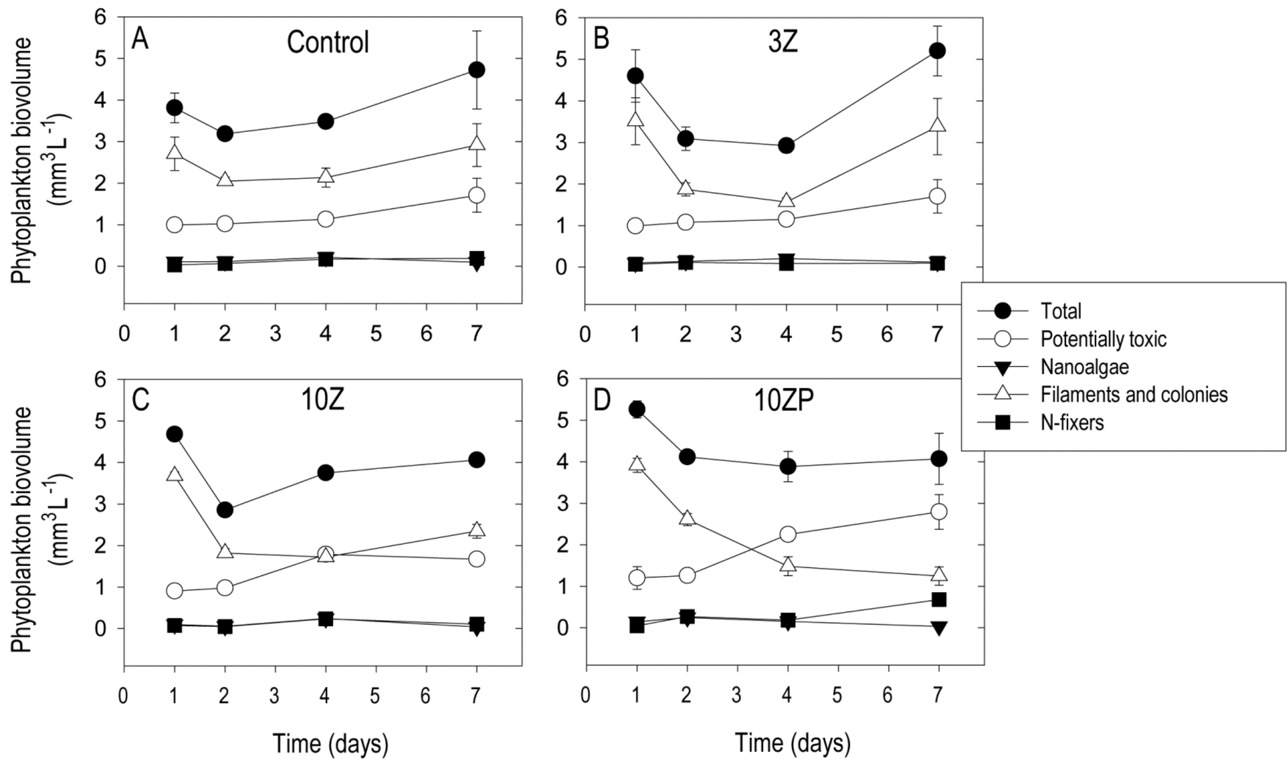

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615 Fig. 3

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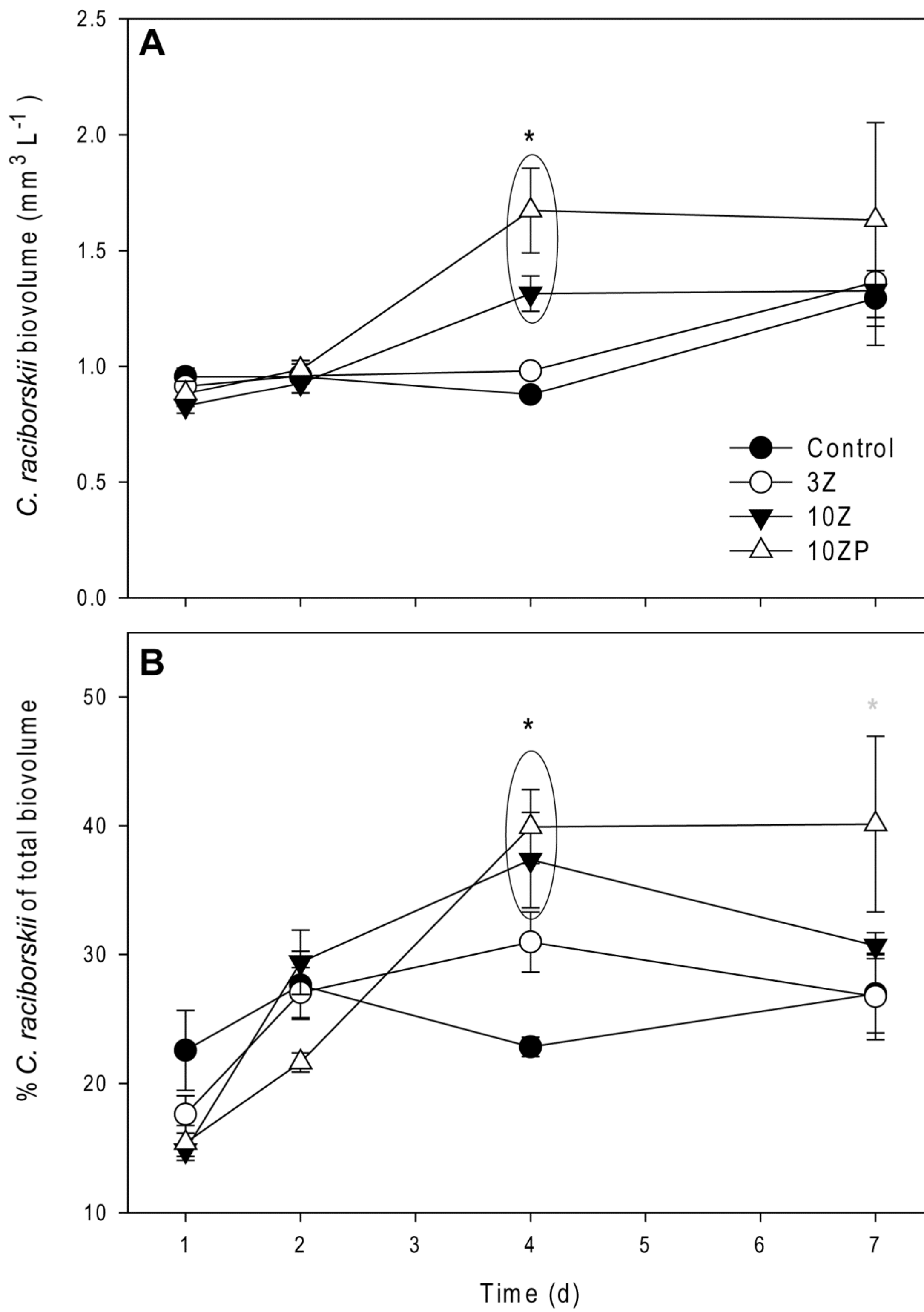

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618 Fig. 4

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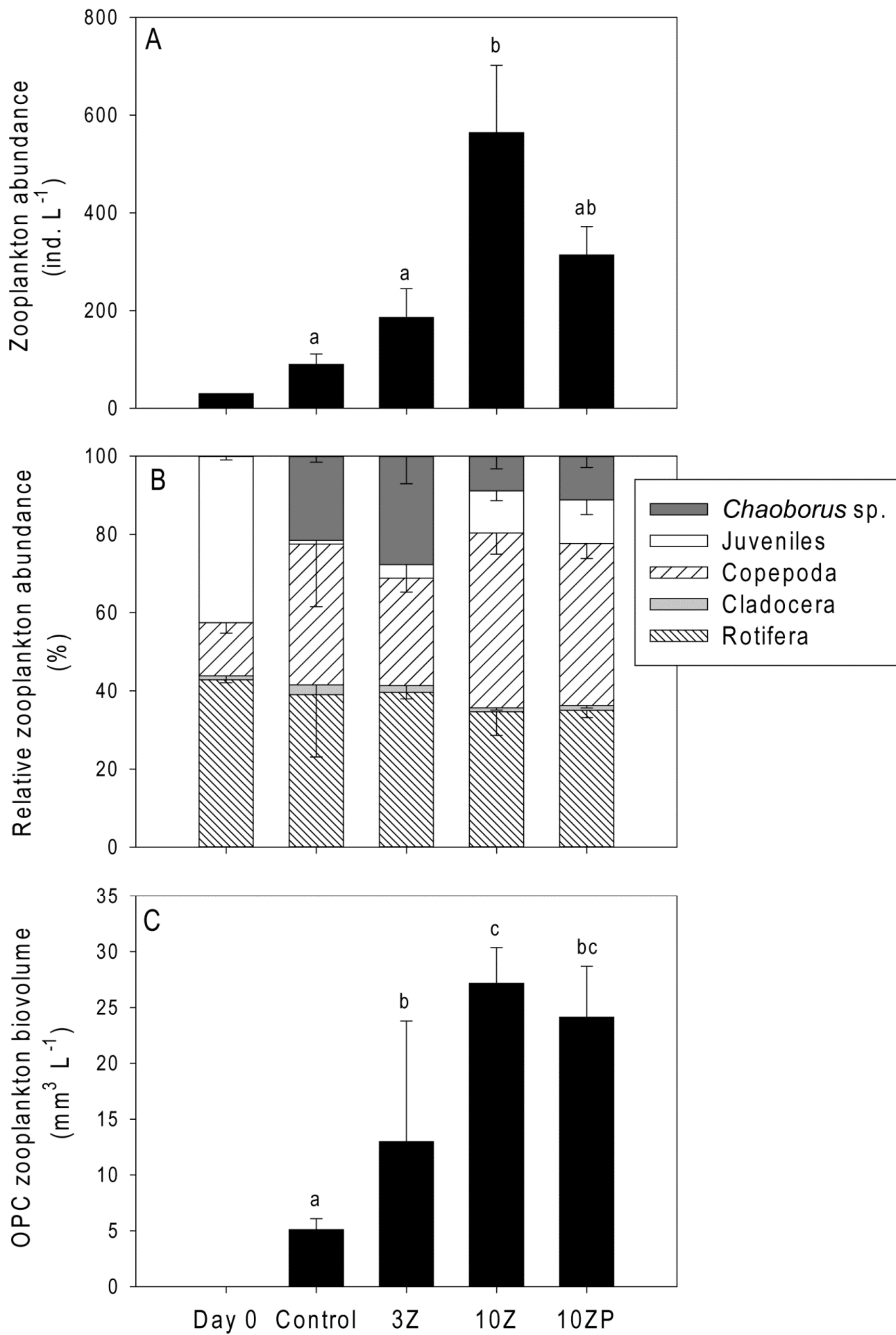

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621 Fig. 5

622

623 TABLES

624 Table I

	Ambient Day 0 (n=3)	Ambient Day 7 (n=1)	Mesocosm Day 0 (n=12)	Mesocosm day 7 (n=12)
Temperature surface (ºC)	29.6 ± 0.4	32.1	28.5 ± 0.2	29.8 ± 0.4
Temperature 1m (°C)	29.4 ± 0.4	29.6	28.4 ± 0.2	29.3 ± 0.2
Conductivity surface (µS/cm)	0.3 ± 0.0	0.3	0.3 ± 0.0	0.3 ± 0.0
Conductivity 1m (µS/cm)	0.3 ± 0.0	0.3	0.3 ± 0.2	0.3 ± 0.0
Turbidity surface (NTU)	2.3 ± 0.3	3.0	2.0 ± 0.1	2.9 ± 0.1
Turbidity 1m (NTU)	2.2 ± 0.0	3.2	1.8 ± 0.0	2.7 ± 0.1
pH surface	8.6 ± 0.1	8.7	8.5 ± 0.1	8.6 ± 0.1
pH 1m	8.7 ± 0.0	8.7	8.3 ± 0.2	8.3 ± 0.0
DO% surface	102.9 ± 1.8	101.9	94.6 ± 1.2	96.6 ± 1.4
DO% 1m	102.1 ± 2.3	100.5	91.5 ± 1.9	95.8 ± 1.7
Secchi depth (m)	1.8 ± 0.1	1.8	1.7 ± 0.0	1.7 ± 0.0

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626

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629

630 Table II

Species name	Functional group	Size	Class	Species abundance (cells mL^{-1})	% Biovolume
Anabaena sp.	F, T	Micro	Cyanophyta	1491	0.9
Aphanocapsa sp.	N	Micro	Cyanophyta	4830	0.0
Aphanizomenon sp	F, T	Micro	Cyanophyta	0	0.0
Cyanodictyon imperfectum	N	Micro	Cyanophyta	1344	0.0
Cylindrospermopsis raciborskii	F, T	Micro	Cyanophyta	25200	22.6
Geitlerinema sp.	T	Micro	Cyanophyta	378	0.1
Limnothrix sp.	N	Micro	Cyanophyta	27468	18.1
Merismopedia sp.	N	Micro	Cyanophyta	588	0.0
Planktolyngbya limnetica	N	Micro	Cyanophyta	12852	12.5
Planktolyngbya microspira	N	Micro	Cyanophyta	31983	23.2
Pseudanabaena limnetica	N	Micro	Cyanophyta	126	0.2
Merismopedia punctata	N	Micro	Cyanophyta	252	0.1
Cryptomonas sp.	N	Nano	Cryptophyta	42	1.2
Cyclotella sp.	N	Nano	Bacillariophyta	252	2.6
Synedra sp.	N	Micro	Bacillariophyta	84	0.0
Cosmarium sp.	N	Nano	Chlorophyta	42	0.2
Oocystis sp.	N	Micro	Chlorophyta	42	0.2
Scenedesmus sp.	N	Micro	Chlorophyta	42	0.0
Peridinium sp.	N	Nano	Dinophyta	105	7.5
Other					10