

1     **Pesticide-induced Alterations to Phytoplankton Abundance and Community Structure**  
2                     **Alter Ecosystem Respiration: Implications for the Carbon Cycle?**

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29

30 ABSTRACT

31 Current predictions of the effects of synthetic chemicals on freshwater ecosystems are  
32 hampered by the sheer number of chemical contaminants entering aquatic systems, the diversity  
33 of organisms inhabiting these systems, and uncertainties about how contaminants alter ecosystem  
34 metabolism. We conducted a mesocosm experiment that elucidated the responses of ponds  
35 composed of phytoplankton and zooplankton to standardized concentrations of 12 pesticides,  
36 nested within four pesticide classes and two pesticide types. We show that the effects of the  
37 pesticides on algae were consistent within herbicides and insecticides and responses of over 70  
38 phytoplankton species and genera were consistent within broad taxonomic groups. Insecticides  
39 generated top-down effects on phytoplankton community composition and abundance, which  
40 were associated with persistent increases in ecosystem respiration. Herbicides reduced  
41 phytoplankton abundance, which was associated with decreases in primary productivity and  
42 ecosystem respiration. These results suggest that widespread pesticide use could have  
43 underexplored implications for the global carbon cycle. While these effects on ecosystem  
44 respiration were mediated through complex effects on communities, taxonomic groups of  
45 organisms responded similarly to pesticide types, suggesting opportunities to simplify ecological  
46 risk assessment.

47

48 INTRODUCTION

49 The importance of aquatic systems to life on earth cannot be overstated. Humanity relies  
50 on freshwater as a precious natural resource for drinking, food production, and carbon capture  
51 (Meybeck 2003, Vörösmarty et al. 2005, World Water Assessment Programme 2009).  
52 Freshwater systems are hotspots for biodiversity (Dudgeon *et al.* 2006; Balian *et al.* 2008), and  
53 they play a crucial role in the capture, storage, and release of atmospheric carbon (Holgerson &  
54 Raymond 2016). Yet, the benefits of economic productivity, spurred by access to water, have  
55 been accompanied by impairments that threaten aquatic systems (Vörösmarty *et al.* 2010). In  
56 particular, the increasing rate of synthetic chemical pollution, which outpaces all other global  
57 change drivers, presents a major threat to freshwater systems globally (Bernhardt *et al.* 2017). In  
58 the United States alone, more than 500 million pounds of pesticide active ingredients are applied  
59 every year (Atwood & Paisley-Jones 2017). These applications have led to well-documented and  
60 widespread contamination of freshwater (Dudgeon *et al.* 2006; Gilliom & Hamilton 2006; Stone  
61 *et al.* 2014) and are implicated as a contributing factor to biodiversity declines in these systems  
62 (Malaj *et al.* 2014; Stehle & Schulz 2015).

63 Despite the magnitude of the threat that pesticide pollution imposes on freshwater  
64 systems, it is somewhat surprising that ecologists and their funding agencies have largely ignored  
65 the intricacies that these disturbances can yield on communities and ecosystems (Bernhardt *et al.*  
66 2017; Burton *et al.* 2017). For instance, while pesticides have the potential to alter carbon  
67 cycling in freshwater systems via changes in the abundance or composition of community  
68 members, few studies have evaluated this possibility (but see McMahon *et al.* 2012; Halstead *et*  
69 *al.* 2014; Rumschlag *et al.* 2020). Classic toxicological research has employed lab-based  
70 exposure experiments, documenting that scores of pesticides harm model organisms. While these

71 tests have been crucial in uncovering mechanisms of toxicity and are the basis of chemical  
72 regulation around the world, they often fail to predict the complex suite of effects that occur  
73 when pesticides enter freshwater systems (Gessner & Tlili 2016; Rohr *et al.* 2016; Bernhardt *et*  
74 *al.* 2017). For instance, traditional toxicological tests that focus on single pesticides and single  
75 organisms under controlled conditions ignore indirect effects of pesticides mediated through  
76 species interactions and food web structures (Kidd *et al.* 2014; Rumschlag *et al.* 2019). Instead,  
77 if the focus is on assessing the safety of pesticides on ecological systems, more realistic  
78 experimental conditions, like those of replicated field-based mesocosm experiments, should be  
79 incorporated into a framework of risk assessment. Mesocosms mimic well the complexity of  
80 multitrophic communities, so that both direct and indirect effects of pesticides can be evaluated  
81 (Rohr *et al.* 2006; Clements & Rohr 2009). In addition, testing that incorporates naturally  
82 complex communities allows for the evaluation of whether pesticide exposure can directly or  
83 indirectly alter ecosystem functions, including ecosystem metabolism, which is not possible with  
84 the traditional single species tests (Bernhardt *et al.* 2010; Halstead *et al.* 2014; Gessner & Tlili  
85 2016).

86         In the U.S. and Europe, tens of thousands of synthetic chemicals are registered for use,  
87 and in the U.S. alone more than 350 pesticides are applied annually in agriculture (Baker &  
88 Stone 2015). Further, freshwater systems are home to about 125,000 described species, even  
89 though they only occupy 0.8% of earth's surface (Dudgeon *et al.* 2006, Balian *et al.* 2008).  
90 Predicting the cumulative effects of pesticides on freshwater systems is enormously challenging  
91 because of the diverse array of pesticides to which ecosystems are exposed, combined with the  
92 diversity of organisms that exist in freshwater systems. Predicting these effects could be  
93 simplified if the effects of pesticides are similar within pesticide types (e.g. insecticides and

94 herbicides that are designed to target insect and plant pests, respectively) or pesticide classes (i.e.  
95 chemical classes of pesticides that share similar chemical structures and molecular targets within  
96 a pest). Further, predicting the effects of pesticides could be simplified if organisms that are  
97 taxonomically related or share similar functional roles within an ecosystem have similarities in  
98 their responses to pesticides, a trend that has been shown in previous toxicological research  
99 (Ippolito *et al.* 2012; Hua & Relyea 2014). For example, our previous research has shown  
100 consistency in the effects of pesticides by class and type on parasite transmission, ecosystem  
101 functions, and macroinvertebrate and amphibian communities (Rumschlag *et al.* 2019, 2020). In  
102 addition, organisms that share functional roles in a community have been shown to respond  
103 similarly to types and classes of pesticides (Rumschlag *et al.* 2020). But to date, no study has  
104 attempted to evaluate the consistency of responses of phytoplankton community members to  
105 pesticide classes and types.

106         In the current study, we conducted an outdoor, replicated mesocosm experiment focused  
107 on exploring the effects of a diverse array of pesticides on phytoplankton and zooplankton  
108 communities and their contributions to ecosystem respiration. Phytoplankton and zooplankton  
109 were chosen as focal communities because: both groups have relatively short generation times  
110 (e.g. upwards of a day for phytoplankton (Laws 2013) and weeks for zooplankton (Kalff 2002))  
111 allowing them to establish population dynamics within the duration of this experiment, both  
112 groups are taxonomically diverse allowing for the assembly of diverse communities in this  
113 experiment, and finally both groups are important contributors to ecosystem metabolism (Kalff  
114 2002). Algae and macrophytes convert sunlight into biomass via photosynthesis, and  
115 zooplankton are the primary consumers of algae. Our objectives were to: 1) determine the  
116 consistency in the effects of pesticides by type, class, and individual pesticide on freshwater

117 phytoplankton communities, 2) gauge the consistency of responses of phytoplankton community  
118 members to pesticides within five broad taxonomic groups (green algae, diatoms, cryptophytes,  
119 cyanobacteria, and euglenoids), 3) evaluate how herbicides and insecticides alter phytoplankton  
120 abundance and community composition via direct toxicity and indirect, top-down effects  
121 mediated by the zooplankton community, and 4) examine how respiration of aquatic systems is  
122 altered by pesticide-induced perturbations to aquatic communities over time.

123         We proposed four hypotheses. First, we hypothesized that the effects of pesticides would  
124 be consistent within pesticide types and classes; pesticides with similar taxonomic targets or  
125 similar chemical structures would have similar effects on phytoplankton communities. This  
126 hypothesis is motivated by the nestedness of the biologic activity of pesticides by class and by  
127 type. Pesticides of similar types (e.g. insecticides, herbicides) have similar targets in the  
128 environment (e.g. insects and plants, respectively), so pesticides within types likely have similar  
129 effects on non-target taxa (e.g. zooplankton and algae, respectively). Classes of pesticides share  
130 modes of action, meaning that they target the same biochemical and molecular pathways (e.g.  
131 triazine herbicides bind to the QB protein in the photosystem II reaction center blocking  
132 photosynthesis), which would drive similarity of observed effects within classes on  
133 phytoplankton communities. Second, we hypothesized that the responses of phytoplankton to  
134 pesticide exposures would be similar within broad taxonomic groups; a trend shown in other  
135 studies (Peterson *et al.* 1994; Ippolito *et al.* 2012; Hua & Relyea 2014). Third, we hypothesized  
136 that herbicides would cause direct reductions in abundance of phytoplankton across broad  
137 taxonomic groups, while insecticides would increase phytoplankton abundance and alter  
138 community composition through top-down effects on the zooplankton community. And finally,  
139 we hypothesized that pesticides would induce changes in ecosystem respiration; the duration of

140 these changes would be explained by the environmental persistence of the pesticides and the  
141 generation time of the organism to which the pesticide is directly toxic. For instance, we  
142 predicted that respiration in communities exposed to herbicides might recover quickly because  
143 photosynthetic phytoplankton have short generation times and thus might rebound rapidly from  
144 the direct toxicity of herbicides. In contrast, respiration in communities exposed to insecticides  
145 might recover more slowly because direct toxicity occurs to longer-lived zooplankton that might  
146 have top-down effects on algae. In addition, we predicted that pesticides that persist for short  
147 durations of time in the environment would cause short-term disruptions to respiration because  
148 as the pesticide degrades, the community would recover more quickly from the initial  
149 perturbation relative to more persistent pesticides.

150

## 151 METHODS

### 152 *Aquatic Communities and Experimental Design*

153 A randomized-block experiment was performed at the Russell E. Larsen Agricultural  
154 Research Center (Pennsylvania Furnace, PA, USA) using replicated mesocosm ponds.  
155 Mesocosms were 1,100 L cattle tanks covered with 60% shade cloth lids. The spatial block was  
156 distance from a tree line in the mesocosm field. We filled mesocosms with 800 L of water and  
157 300 g mixed hardwood leaves. Mesocosms were inoculated with zooplankton, phytoplankton,  
158 and periphyton that were homogenized from four local ponds. Three weeks later, after these  
159 additions, pesticides were applied. To mimic the complex food web structure of natural ponds,  
160 we also added two snail, three larval anuran, one larval dragonfly, one water bug, one water  
161 beetle, one larval salamander, and one backswimmer species to each mesocosm on the day of  
162 pesticide applications, just prior to application. More specifically, each mesocosm received 11



163 *Helisoma (Planorbella) trivolvis*, 10 *Physa gyrina*, 20 *Hyla versicolor*, 20 *Lithobates palustris*,  
164 20 *Lithobates clamitans*, 2 *Anax junius*, 2 *Belostoma flumineum*, 5 *Hydrochara* sp., 3 *Ambystoma*  
165 *maculatum*, and 6 *Nototeka undulata*. Responses of these community members to the established  
166 treatments are explored in Rumschlag *et al.* 2020 and are not a focus of the current study.

167 We randomly assigned 14 treatments (12 pesticides, 2 controls) with four replicate  
168 mesocosms of each treatment, which resulted in 56 total mesocosms (Fig. 1A). The 12 pesticide  
169 treatments were nested; treatments included two pesticide types (insecticide, herbicide), two  
170 classes within each pesticide type (organophosphate insecticide, carbamate insecticide,  
171 chloroacetanilide herbicide, triazine herbicide), and three different pesticides in each of four  
172 classes (Fig. 1A). Samples from two mesocosms were not processed for phytoplankton  
173 identification because of an error, so all analyses contain only 54 total mesocosms with three  
174 replicates for acetochlor and simazine treatments. At the start of the experiment, we applied a  
175 single dose of technical grade pesticides at environmentally relevant concentrations to mimic  
176 runoff of pesticides into freshwater systems following rainfall. To calculate environmentally  
177 relevant concentrations, we used U.S. Environmental Protection Agency's GENEEC v2 software  
178 to generate estimated environmental concentrations of pesticides. We acquired pesticides from  
179 ChemService (West Chester, PA, USA). Nominal concentrations of pesticides ( $\mu\text{g/L}$ ) were: 64  
180 chlorpyrifos, 101 malathion, 171 terbufos, 91 aldicarb, 219 carbaryl, 209 carbofuran, 123  
181 acetochlor, 127 alachlor, 105 metolachlor, 102 atrazine, 202 simazine, and 106 propazine. One  
182 hour after pesticides were applied, composite water samples were collected from mesocosms  
183 with the same pesticide treatment. These samples were shipped on ice to Mississippi State  
184 Chemical Laboratory to verify nominal concentrations. Measured concentrations of pesticides  
185 ( $\mu\text{g/L}$ ) were: 60 chlorpyrifos, 105 malathion, 174 terbufos, 84 aldicarb, 203 carbaryl, 227

186 carbofuran, 139 acetochlor, 113 alachlor, 114 metolachlor, 117 atrazine, 180 simazine, and 129  
187 propazine. The experimental design also included water and solvent (0.0001% acetone) controls  
188 (Fig. 1A). The experiment ran for four weeks, from June to July.

189

#### 190 *Measurement of Experimental Responses*

191 To characterize the algal community, we collected phytoplankton from the entire water  
192 column by inserting an upright PVC pipe, measuring 10 cm in diameter and 60 cm in height in  
193 the center of each mesocosm. A 1-liter subsample of the volume of water collected with the pipe  
194 was preserved with in a 1% solution of Lugol's. Phytoplankton samples were collected in week  
195 four of the experiment. All samples were analyzed using the Utermöhl technique at 200-400  
196 magnification (Lund *et al.* 1958). At least 400 natural units (colonies, filaments, and unicells)  
197 were enumerated using standard keys with taxonomy updated as necessary (Prescott 1962; Wehr  
198 & Kociolek 2015; Guiry & Guiry 2020). Densities per ml were calculated for a total of 74 genera  
199 and species of phytoplankton.

200 To characterize the zooplankton community, we collected zooplankton from the entire  
201 water column with a PVC pipe in the same manner as the phytoplankton samples; then, we  
202 capped the bottom and poured the water through 20  $\mu\text{m}$  Nitex mesh. Two zooplankton samples  
203 were collected from each mesocosm, and these samples were combined and preserved in 70%  
204 ethanol. We counted and identified zooplankton to genera in 5 mL subsamples for each  
205 mesocosm with a zooplankton counting wheel (Wildlife Supply Company, Yulee, FL, USA) and  
206 a dissecting microscope. Zooplankton sampling occurred in week two of the experiment.

207 To measure total abundance of phytoplankton, we took 10 mL water samples, filtered  
208 phytoplankton onto glass fiber filters (under low vacuum pressure, <10 psi; Whatman EPM

209 2000, 0.3  $\mu\text{m}$ , 47 mm), and measured chlorophyll-*a* concentrations of each sample. We used an  
210 organic extraction procedure with a 50:50 mixture of 90% acetone to DMSO and measured  
211 chlorophyll-*a* concentrations using a standard fluorometric technique (Carrick *et al.* 1993).  
212 Chlorophyll-*a* was measured from water samples taken in week two of the experiment.

213 To measure ecosystem respiration, we measured dissolved oxygen (DO) at dusk and  
214 dawn on subsequent days using hand-held meters (YSI, Yellow Springs, OH, USA) during  
215 weeks two and four of the experiment. Respiration was calculated as DO at dusk minus DO at  
216 dawn from the following day. Three previous manuscripts, which use the same design as the  
217 current manuscript, also describe this experimental design and methods in detail (Rohr *et al.*  
218 2008; Rumschlag *et al.* 2019, 2020).

219

### 220 *Statistical Analyses*

221 To evaluate the consistency of the effects of pesticides within type, class, and individual  
222 pesticide, we conducted a permutational multivariate analysis of variance (PERMANOVA,  
223 Table 1). This statistical model allowed us to attribute the variation explained in each pesticide  
224 level of organization (type, class, and individual pesticide), while accounting for the nested  
225 structure of our experimental design (Fig. 1A). The predictors were the following random  
226 categorical terms: type (insecticide, herbicide), class (carbamate, organophosphate,  
227 chloroacetanilide, triazine) nested with type, and pesticide (12 total) nested within class within  
228 type. No controls were included because they were not hierarchically nested (Fig. 1A). The  
229 multivariate response was a Bray Curtis similarity matrix based on a community matrix of  
230 square-root transformed densities of phytoplankton (abundance per ml) identified to genus or  
231 species at the end of the experiment. Across all treatments, 74 genera or species of

232 phytoplankton were identified. The nested PERMANOVA used 9999 permutations and residuals  
233 under a reduced model. In addition, we evaluated pairwise differences between controls,  
234 carbamates, organophosphates, chloroacetanilides, and triazines with a multivariate comparison  
235 test using PERMANOVA (Fig. 1B). In this statistical model, 9999 unrestricted permutations of  
236 raw data were used. In both the nested and pairwise PERMANOVAs, Type III partial sums of  
237 squares were evaluated.

238 To gauge how consistent the responses of phytoplankton community members are within  
239 broad taxonomic groups, we completed a second nested PERMANOVA on the algal community  
240 simplified to broad taxonomic groups (green algae, diatoms, cryptophytes, cyanobacteria, and  
241 euglenoids). (Table 1). This second nested PERMANOVA followed the same methods as the  
242 first, except that the multivariate response was based on densities of green algae, diatoms,  
243 cryptophytes, cyanobacteria, and euglenoids. This community matrix was generated by summing  
244 abundances of phytoplankton species and genera within their respective taxonomic groups. To  
245 determine how consistent the responses of phytoplankton were to pesticide exposures, we  
246 compared the relative amount of variation explained by the predictors and the residual variation  
247 in the two PERMANOVA models: the genera/species-level model and the model including the  
248 five taxonomic groups (Table 1).

249 To visualize 1) the consistency of the pesticide effects within type, class, and individual  
250 pesticide, 2) the consistency of responses of phytoplankton within broad taxonomic groups, and  
251 3) how pesticides altered community composition of phytoplankton, we used a distance-based  
252 redundancy analysis (dbRDA, Fig. 1B, C), which is an ordination technique that constrains the  
253 community response matrix by environmental variables, in this case the pesticide treatments. The  
254 dbRDA was based on Bray-Curtis similarities of the square root transformed densities of the

255 more than 70 genera or species of phytoplankton. The underlying categorical predictors were  
256 organophosphate, carbamate, chloroacetanilide, triazine, and control. In the dbRDA plot, we  
257 show the centroid values for the 14 experimental treatments. Both PERMANOVA models and  
258 the dbRDA were first executed using PERMANOVA+ for PRIMER version 7 (PRIMER-E Ltd,  
259 Plymouth, UK). Then, for ease of visualization of the dbRDA point and vector plots, data from  
260 PERMANOVA+ for PRIMER were exported, and plots were made using ‘*ggplot2*’ package in  
261 R.

262 Changes in zooplankton communities were evaluated using box-and-whisker plots (Fig.  
263 1D). Namely, we examined the changes in the relative abundance of two major taxonomic  
264 groups of zooplankton, cladocerans and copepods, in response to classes of pesticides and the  
265 controls. To evaluate the associations between the densities of zooplankton and phytoplankton  
266 groups, we conducted four simple generalized linear regressions with Poisson distributions using  
267 the variation produced in the controls and the insecticide treatments (Fig. 1E). In these models,  
268 the response was either density of green algae or diatoms and the predictors were either  
269 cladocerans or copepods. For each model, we evaluated Type II sums of squares, a Wald  $\chi^2$  test  
270 statistic, and a McFadden’s pseudo- $R^2$ .

271 To explore how exposure to pesticides altered abundance of broad taxonomic groups of  
272 phytoplankton, we examined box-and-whisker plots of densities of broad taxonomic groups of  
273 phytoplankton in response to classes of pesticides and the controls (Fig. 2A). In addition, we  
274 examined box-and-whisker plots of chlorophyll-*a*, a metric of total phytoplankton abundance  
275 (Fig. 2B). Since chlorophyll-*a* was measured soon after pesticide applications, we argue that  
276 chlorophyll-*a* measurements better reflect the immediate impacts of treatments on total

277 abundance of phytoplankton compared to total phytoplankton density calculated from physical  
278 counts, which were measured at the end of the experiment.

279 To examine how respiration of aquatic systems is altered by pesticides over time, we  
280 compared the effects of pesticides by class to controls on respiration at two- and four-weeks  
281 using log response ratios (Fig. 3A). For each time point, log response ratios of ecosystem  
282 respiration for individual mesocosms exposed to pesticides were calculated relative to the  
283 average of the control treatments (solvent and water controls) within a given spatial block. These  
284 calculations allow us to evaluate the magnitude of the difference in respiration of pesticide-  
285 treated mesocosms relative to the controls. We dropped observations in which DO at dawn was  
286 equal to DO at dusk, which generated an undefined value for the log response ratio ( $n = 2$ ).

287 Next, to test how the community composition of phytoplankton was related to respiration  
288 measured at two weeks, we used non-metric multidimensional scaling (NMDS) ordination of  
289 broad taxonomic groups of phytoplankton densities based on Bray-Curtis similarities  
290 ('metaMDS' function, vegan package, Fig. 3B). Following NMDS ordination, we fit respiration  
291 from week two, using generalized additive models with the 'ordisurf' function (vegan package),  
292 which is a test of how well the ordination of the phytoplankton community predicts respiration  
293 (Fig. 3B). Finally, we assessed how the abundance of phytoplankton, as measured by  
294 chlorophyll-*a*, was related to respiration at two and four weeks using simple linear regressions  
295 (Fig. 3C). For the two models, the response was respiration measured at either two or four  
296 weeks, and the predictor was log-transformed chlorophyll-*a*. We evaluated Type II sums of  
297 squares. All analyses and plots, with the exception of the PERMANOVA models, were  
298 completed in R version 3.6.1. Preliminary analyses revealed no significant influence of the  
299 spatial block, so the spatial block was not included in the analyses.

300

301

## RESULTS

302 *Pesticide types have consistent effects on broad taxonomic groups of phytoplankton*

303 To evaluate the consistency of the effects of pesticides, we conducted a PERMANOVA  
304 that allowed us to attribute the variation explained in the phytoplankton community by pesticide  
305 type, class, and individual pesticide. For phytoplankton communities composed of more than 70  
306 species and genera, pesticide type explained 24% of the variation in these communities (Table  
307 1), which shows that the effects of pesticides on phytoplankton communities are generalizable to  
308 pesticide type.

309 To gauge the consistency in the responses of phytoplankton community members within  
310 broad taxonomic groups, we compared the genera/species-level PERMANOVA model to a  
311 model with the phytoplankton community simplified to broad taxonomic groups. Similar to the  
312 genera/species-level model, pesticide type explained 22% of the variation in the taxonomic  
313 groups (Table 1). Further, the residual variation in the model including broad taxonomic groups  
314 of phytoplankton was less than the genera/species-level model (54% versus 63% of residual  
315 variation, respectively, Table 1). Together, these results suggest that pesticides generally have  
316 similar effects on community members from the same broad taxonomic groups.

317

318 *Insecticides generate top-down effects on algal community composition and abundance which is*  
319 *associated with persistent increases in ecosystem respiration*

320 We visualized the consistency in the effects of pesticides by type and the consistency in  
321 the responses of phytoplankton by broad taxonomic groups using dbRDA (Fig. 1B, C). The  
322 dbRDA showed that insecticide exposures significantly decreased green algae and modestly

323 increased diatoms (Fig. 1B, C). These changes in community composition were likely the result  
324 of top-down effects of the zooplankton community. Insecticides had direct toxic effects on  
325 cladocerans, which lead to a competitive release of copepods (Fig. 1D). Across insecticides and  
326 controls, density of green algae was positively associated with cladocerans ( $p < 0.001$ , Wald  $\chi^2 =$   
327 8495, pseudo- $R^2 = 0.28$ , Fig. 1E top-left) and negatively associated with copepods ( $p < 0.001$ ,  
328 Wald  $\chi^2 = 4378$ , pseudo- $R^2 = 0.19$ , Fig. 1E, top-right). At the same time, diatoms were  
329 negatively associated with cladocerans ( $p < 0.001$ , Wald  $\chi^2 = 1455$ , pseudo- $R^2 = 0.05$ , Fig 1E,  
330 bottom-left) and positively associated with copepods ( $p < 0.001$ , Wald  $\chi^2 = 8329$ , pseudo- $R^2 =$   
331 0.21, Fig.1E, bottom-right). In addition to changes in community composition, total abundance  
332 of phytoplankton, as indicated by chlorophyll-*a*, increased with insecticide exposure (Fig. 2B).

333         Comparisons of the effects of pesticides by class to controls using log-response ratios  
334 demonstrated that respiration of the entire aquatic community increased significantly in response  
335 to insecticide exposure, and this effect persisted throughout the experiment (Fig. 3A). The  
336 increase in ecosystem respiration was associated with both changes in community composition  
337 and total abundance of phytoplankton. For instance, the NMDS ordination of phytoplankton  
338 community composition and the vector overlay of ecosystem respiration at week two showed a  
339 significant association ( $p < 0.001$ ,  $F_{9,44} = 1.93$ ,  $R^2 = 0.25$ , Fig. 3B). The increase in ecosystem  
340 respiration in insecticide treatments was associated with more diatoms and euglenoids and less  
341 green algae relative to all other treatments (Fig. 3B). In addition, the greater total abundance of  
342 phytoplankton, represented by chlorophyll-*a*, in insecticide treatments was positively associated  
343 with ecosystem respiration in weeks two and four (week 2:  $p < 0.001$ ,  $F_{1,52} = 26.88$ ,  $R^2 = 0.34$ ;  
344 week 4:  $p < 0.001$ ,  $F_{9,44} = 48.34$ ,  $R^2 = 0.48$ , Fig. 3C).

345



346 *Herbicides decrease phytoplankton abundance which is associated with short-term decreases in*  
347 *ecosystem respiration*

348 In contrast to the effect of insecticides, the dbRDA and pairwise comparisons  
349 demonstrated no difference in community composition of phytoplankton between the herbicides  
350 and the controls (Fig. 1B, 2A). Instead, relative to controls, herbicide exposure resulted in a  
351 significant decrease in total abundance of phytoplankton, as indicated by chlorophyll-*a* (Fig. 2B).  
352 Log-response ratios showed that herbicides reduced ecosystem respiration, but these changes did  
353 not persist for the length of the experiment (Fig. 3A). In addition, the effects of herbicides on  
354 ecosystem respiration varied by class, with triazines having a greater magnitude of effect  
355 compared to chloroacetanilides (Fig. 3A). The NMDS ordination of the phytoplankton  
356 community composition and the vector overlay of ecosystem respiration at week two showed  
357 herbicides grouping with controls, suggesting no herbicide-induced changes in phytoplankton  
358 community composition could be linked to ecosystem respiration (Fig. 3B). Instead, the lower  
359 total abundance of phytoplankton (represented by chlorophyll-*a*) in herbicide treatments was  
360 associated with lower ecosystem respiration in weeks two and four (Fig. 3C).

361

362

## DISCUSSION

363 Synthetic chemicals represent a globally widespread disturbance that threatens freshwater  
364 ecosystems. Yet, what remains largely unknown is how consistent the responses of a diversity of  
365 community members are to an array of contaminants and how these changes in communities  
366 correspond to alterations in the retention and release of carbon in ponds. We evaluated the effects  
367 of 12 pesticides, nested in four pesticide classes and two pesticide types on pond ecosystems, and  
368 included diverse phytoplankton and zooplankton communities. Our results demonstrate that: 1)  
369 the effects of pesticides were consistent within herbicide and insecticide types and within broad

370 taxonomic groups of phytoplankton, 2) herbicides decreased total phytoplankton abundance but  
371 had no effect on phytoplankton composition, 3) by shifting dominant zooplankton from  
372 cladocerans to towards copepods, insecticides indirectly increased phytoplankton abundance and  
373 altered phytoplankton community composition via a top-down effect, and 4) herbicides lead to  
374 short-term decreases in ecosystem respiration that varied by herbicide classes, whereas  
375 insecticides lead to persistent increases in ecosystem respiration.

376 First, our results suggest that prediction of the staggering number of possible direct and  
377 indirect effects associated with freshwater communities being exposed to thousands of synthetic  
378 chemicals could be simplified to groups of chemicals with similar environmental targets and to  
379 broad groups of taxonomically related organisms. Given that only 0.36% of the more than 100  
380 million unique chemicals currently in existence have gone through regulation by a federal  
381 agency (Gessner & Tlili 2016) and that freshwater systems are home to more than 125,000  
382 described species (Dudgeon *et al.* 2006; Balian *et al.* 2008), society requires a risk assessment  
383 approach that can efficiently screen a vast number of chemicals against whole communities and  
384 ecosystems to accurately predict environmental safety; risk assessment using traditional  
385 approaches of testing a single compound against a single model organism could be extended to  
386 include tests of whole communities and ecosystems (Rohr *et al.* 2006, 2016; Clements & Rohr  
387 2009). Simplifying prediction to groups of chemicals with similar environmental targets and  
388 responses of related organisms would improve efficiency for federal regulating bodies around the  
389 world and allow for more resources to be devoted to looking for exceptions to general patterns  
390 (Rohr *et al.* 2006, 2016; Clements & Rohr 2009).

391 The generalizable effects of insecticides and herbicides on algal and zooplankton  
392 communities that we found were consistent with previous studies. In the literature, insecticide

393 exposure regularly reduce cladoceran zooplankton, which leads to a competitive release of  
394 copepods (Bridges & Boone 2003; Boone *et al.* 2005; Relyea & Diecks 2008; Relyea 2009; Hua  
395 & Relyea 2014). Additionally, these changes in the zooplankton community regularly have top-  
396 down effects on phytoplankton, consistent with the observed increase in total abundance of  
397 phytoplankton and a shift in the phytoplankton community observed in our study (Bridges &  
398 Boone 2003; Boone *et al.* 2005; Relyea & Diecks 2008; Relyea 2009; Hua & Relyea 2014).  
399 More specifically, we found increased density of copepods was negatively associated with the  
400 density of green algae and positively associated with diatom density. Total phytoplankton  
401 abundance might have increased because, compared to cladocerans, copepods are less efficient  
402 phytoplankton feeders and have broader diets encompassing non-algal food sources (Sommer &  
403 Sommer 2006). Changes in the phytoplankton community could have been the results of  
404 differences in feeding preferences of copepods versus cladocerans. Alternatively, insecticides  
405 may have indirectly altered the availability of nutrients for phytoplankton. Insecticides could  
406 have been directly toxic to insect predators, which in turn could have altered herbivory of snail  
407 and tadpoles and therefore, nutrient availability (Peacor & Werner 2000). Herbicides did not  
408 change phytoplankton composition but decreased total phytoplankton abundance; reduction in  
409 phytoplankton have been shown in other studies (Rohr & Crumrine 2005; Halstead *et al.* 2014,  
410 2018). Similar to other studies, the total abundance of zooplankton in herbicide-treated ponds  
411 likely indirectly decreased because of the decrease in phytoplankton, a common food source for  
412 all zooplankton (Noack *et al.* 2003; Relyea 2009).

413         Few studies have attempted to evaluate how synthetic chemical disturbances in  
414 communities are associated with changes in ecosystem functions (Bernhardt *et al.* 2010; Rosi-  
415 Marshall & Royer 2012; but see McMahon *et al.* 2012; Rosi-Marshall *et al.* 2013; Halstead *et al.*

416 2014; Rumschlag *et al.* 2020). Our study found that insecticides led to persistent, and non-trivial,  
417 increases in ecosystem respiration, while herbicides lead to short-term decreases in ecosystem  
418 respiration that varied with pesticide classes. Alterations in the abundance and composition of  
419 phytoplankton caused by exposure to insecticides and herbicides could have been linked to  
420 changes in ecosystem in three ways, either individually or in combination. First, phytoplankton  
421 may have contributed directly to ecosystem respiration. So, decreased phytoplankton abundance  
422 in herbicide treatments might have resulted in less total respiration, while increased  
423 phytoplankton abundance and altered composition in insecticide treatments might have resulted  
424 in more total respiration. Second, the abundance of phytoplankton may have driven the  
425 abundance of microbial decomposers, which in turn could have contributed to ecosystem  
426 respiration. Finally, the abundance of phytoplankton may have led to more and/or larger  
427 secondary and/or tertiary consumers, which contributed to ecosystem respiration.

428         The variation in the duration and magnitude of changes in ecosystem respiration by  
429 pesticide type and class could be explained by the environmental persistence of the pesticides  
430 and the generation time of the organism to which the pesticide is directly toxic. For instance,  
431 ecosystem respiration in ponds exposed to herbicides might have recovered more quickly  
432 because herbicides were directly toxic to phytoplankton communities, whose generation times  
433 are upwards of a day (Laws 2013), and likely were able to rebound from the direct toxicity of  
434 herbicides. In contrast, ecosystem respiration in ponds exposed to insecticides might have been  
435 slower because direct toxicity affected zooplankton that have generation times of several weeks  
436 (Kalff 2002). In addition to ecosystem respiration varying by pesticide type, ecosystem  
437 respiration also varied by herbicide class. Triazine herbicides had a greater and more persistent  
438 negative effect on ecosystem respiration than chloroacetanilide herbicides, likely because

439 triazines persist in the environment longer than chloroacetanilides (soil half-lives of 110–146  
440 days versus 14–26 days respectively [Pesticide Action Network Pesticide Database]).

441 Pesticide-induced alterations to respiration found in our study suggest that chemical  
442 contamination has the potential to alter the ecosystem metabolism in freshwater systems. While  
443 the influence of nutrient subsidies (nitrogen, phosphorus, and carbon) on metabolism in lakes  
444 and streams is well studied (Woodward *et al.* 2012; Stanley *et al.* 2016; Williamson *et al.* 2020),  
445 few studies have examined the influence of chemical contaminants, such as pesticides,  
446 pharmaceuticals (but see Rosi-Marshall *et al.* 2013; Robson *et al.* 2020), or heavy metals (but see  
447 Carlisle & Clements 2005), on components of the carbon cycle. In our study, while insecticides  
448 led to persistent increases in ecosystem respiration and a bloom in phytoplankton, insecticides  
449 could have led to carbon storage as biomass, release of atmospheric carbon, or no change in net  
450 primary productivity compared to controls. The outcome of the effect of insecticides on these  
451 patterns would depend on the size of the contribution of phytoplankton blooms to gross primary  
452 productivity. For instance, if gross primary productivity was greater or less than respiration, then  
453 carbon would have been stored as biomass or released, respectively. In contrast, we posit that  
454 herbicides could have led to a short-term release of atmospheric carbon because herbicides were  
455 associated with short-term decreases in respiration and phytoplankton abundance, which likely  
456 translated to reduced gross primary productivity. Future studies should investigate the effects of  
457 pesticides on the carbon cycle more holistically by directly evaluating changes to gross and net  
458 primary productivity and rates of carbon storage. These additional pieces of the carbon cycle  
459 would allow for the evaluation of how synthetic chemicals alter net fluxes and pools of carbon in  
460 aquatic systems. Runoff from spring application of pre-emergent herbicides on agricultural fields  
461 could possibly lead to short-term releases of atmospheric carbon in adjacent water bodies. Given

462 the enormous amount of herbicides released in the environment annually (1.3 billion kg  
463 worldwide in 2012; Atwood & Paisley-Jones 2017) and that inland freshwater systems account  
464 for 0.6 billion tons of carbon storage (more than all carbon buried in oceanic sediments, Battin *et*  
465 *al.* 2009; Aufdenkampe *et al.* 2011), we postulate that herbicide use could have profound  
466 impacts on the global carbon cycle.

467         Given that the production and application of synthetic chemicals has been increasing  
468 exponentially for decades (Gessner & Tlili 2016), understanding the generalizable mechanisms  
469 by which synthetic chemicals can alter aquatic ecosystems is critical if our goal is efficiency in  
470 environmental risk assessment (Rohr & Crumrine 2005; Rohr *et al.* 2006, 2016). Our results  
471 support the hypothesis that predicting the effects of synthetic chemicals on complex, diverse  
472 freshwater systems can be simplified by generalizing patterns to groups of chemicals that share  
473 similar targets and to groups of organisms that are taxonomically related. To better understand,  
474 regulate, and protect human and ecological health, policy makers should look to generalizable  
475 patterns so that efficiency in risk assessment can become a priority, which will free resources to  
476 look for exceptions to general patterns.

477

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482 **availability:** All data are available at Figshare under the **accession number** XXXXXXXX.

483

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621

622 **Table 1.** Results of PERMANOVA models evaluating the effects of pesticides on the densities  
 623 of algal communities. The multivariate response in the first model includes 74 species and  
 624 genera. The multivariate response in the second model includes five broad taxonomic groups,  
 625 including diatoms, green algae, cryptophytes, cyanobacteria, and euglenoids. *P* values were  
 626 generated by Monte Carlo sampling, and those less than 0.05 are bolded. Variation explained is  
 627 the proportion of the estimated component of variation for a given predictor relative to the  
 628 model's total variation.

Endpoints and Source of Variation	<i>df</i>	Pseudo <i>F</i>	<i>P</i>	Variation Explained
Algal community by species and genera				
Type	1	5.08	<b>0.001</b>	0.238
Class(Type)	2	0.68	0.845	0.000
Pesticide(Class(Type))	8	1.17	0.175	0.134
Residual	34			0.628
Algal community by taxonomic group				
Type	1	18.20	<b>0.009</b>	0.218
Class(Type)	2	0.12	0.971	0.000
Pesticide(Class(Type))	8	1.77	0.052	0.242
Residual	34			0.539

629

630 **Figure 1. A)** Experimental design highlighting the nested structure of pesticide treatments. Each  
631 treatment was replicated four times with mesocosm as the replicate. **B)** Distance-based  
632 redundancy analysis (dbRDA) plot of the phytoplankton community, showing differences among  
633 treatments by pesticide type. Points are the centroids of the 12 treatments. Treatments sharing the  
634 same letter are not different from each other in pairwise comparisons. **C)** Vector overlay of  
635 phytoplankton species or genera colored by broad taxonomic group, showing that insecticides  
636 were associated with a decrease in green algae and modest increase in diatoms. The gray circle  
637 corresponds to vector lengths that would have a correlation coefficient of one with a given axis.  
638 **D)** Cladoceran and copepod zooplankton densities in response to pesticide classes and the  
639 controls. Exposure to insecticides lead to copepods becoming more abundant compared to  
640 cladocerans. In contrast, herbicides reduced total zooplankton, but the relative amounts of  
641 cladocerans and copepods remained similar to the controls. **E)** Associations between densities of  
642 cladocerans or copepods and green algae or diatoms in insecticide and control treatments.  
643 Density of green algae was positively associated cladocerans and negatively associated with  
644 copepods, while diatoms were negatively associated with cladocerans and positively associated  
645 with copepods. In the plot of green algae and cladocerans, points have been jittered by 30 units  
646 in width and 50 units in height for ease of visualization.  $R^2$  values are McFadden pseudo- $R^2$   
647 values.

648

649 **Figure 2. A)** Densities of broad taxonomic groups of phytoplankton in response to pesticide  
650 classes and controls at the end of the experiment. Exposure to insecticides resulted in a decrease  
651 in green algae and an increase in the mean abundance of diatoms relative to controls. **B)** Total  
652 abundance of phytoplankton, as reflected by chlorophyll-*a* measurements, in response to

653 pesticide classes and controls midway through the experiment. Insecticides were associated with  
654 increases in phytoplankton, while triazine herbicides were associated with decreases in  
655 phytoplankton relative to the control.

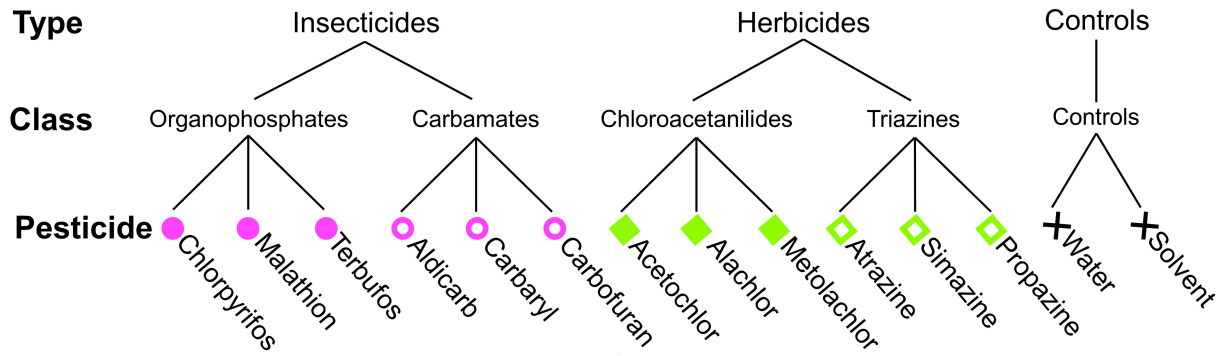
656

657 **Figure 3. A)** Log response ratios and 95% confidence intervals showing the effect of pesticides  
658 by class on ecosystem respiration at week two and four relative to the controls. Pairwise  
659 comparisons were conducted within time points. Classes sharing the same letter are not different  
660 from each other. Insecticides resulted in an increase in ecosystem respiration that persisted  
661 through week four. In contrast, herbicides were initially associated with a decrease in ecosystem  
662 respiration that did not persist to week four. **B)** NMDS of the phytoplankton community by  
663 broad taxonomic group ( $k = 3$ , stress = 0.10) and contour overlap of ecosystem respiration from  
664 week two. This plot shows that the increase in ecosystem respiration in insecticide treatments  
665 was associated with more diatoms and euglenoids and less green algae, relative to all other  
666 treatments. Herbicide treatments grouped with controls, suggesting herbicide-induced changes in  
667 algal community composition are not linked to ecosystem respiration. **C)** Regression plot  
668 showing a positive association between the abundance of phytoplankton, as measured by  
669 chlorophyll-*a*, and ecosystem respiration at weeks two and four suggesting that changes in the  
670 abundance of phytoplankton, as the result of herbicide and insecticide exposures, influenced  
671 ecosystem respiration. Associations at week two are shown with the solid line and opaque points.  
672 Associations at week four are shown with the dotted line and translucent points.

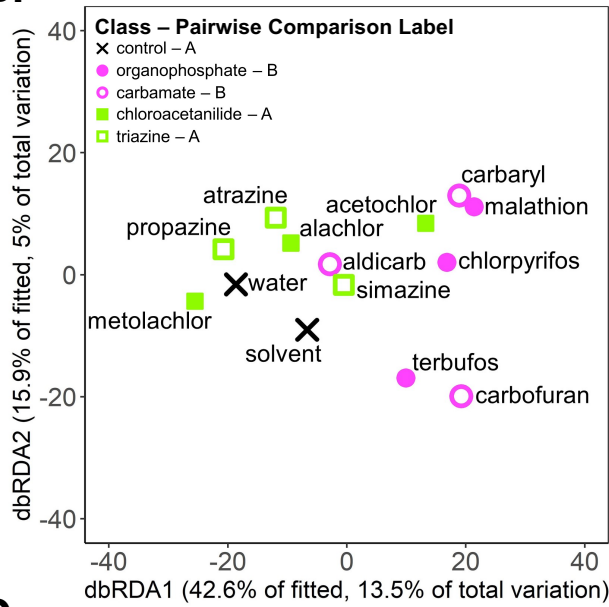
673

674 **Figure 1.**

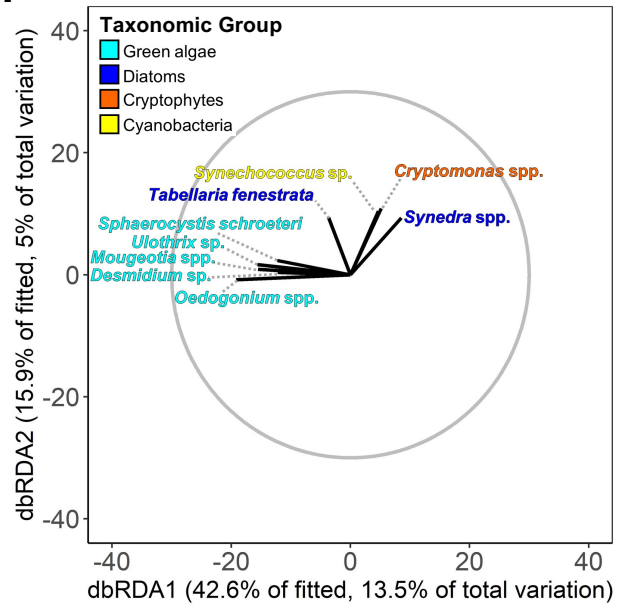
**A.**



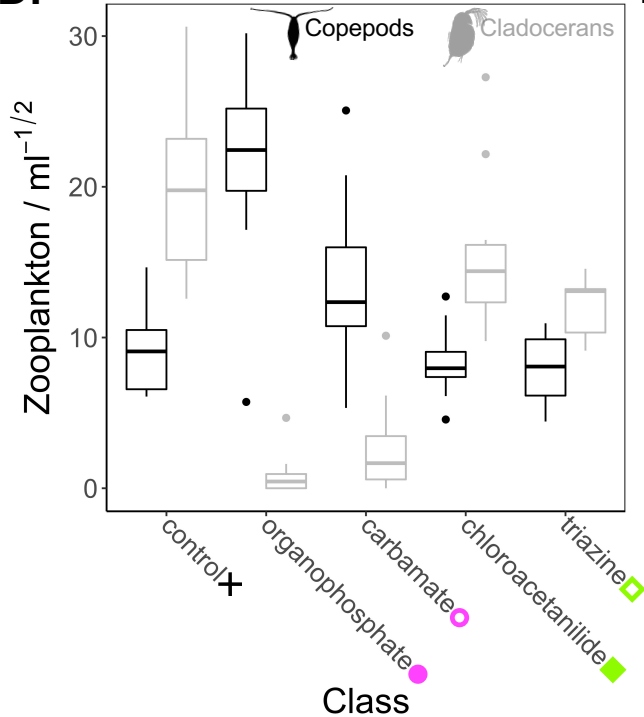
**B.**



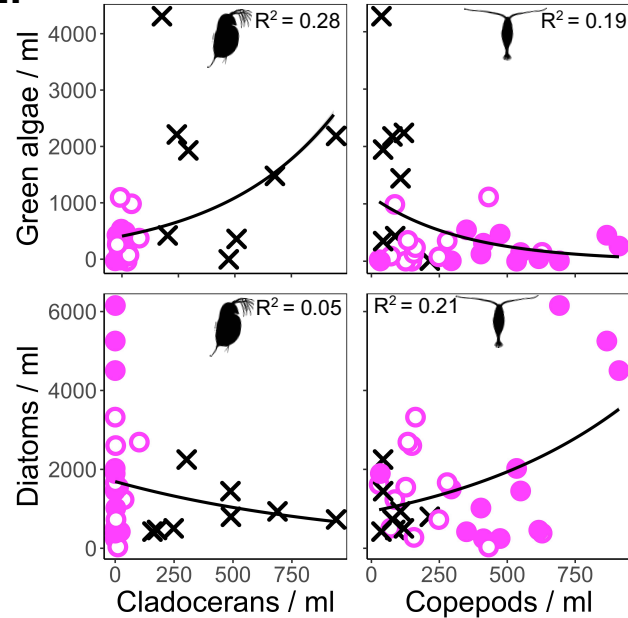
**C.**



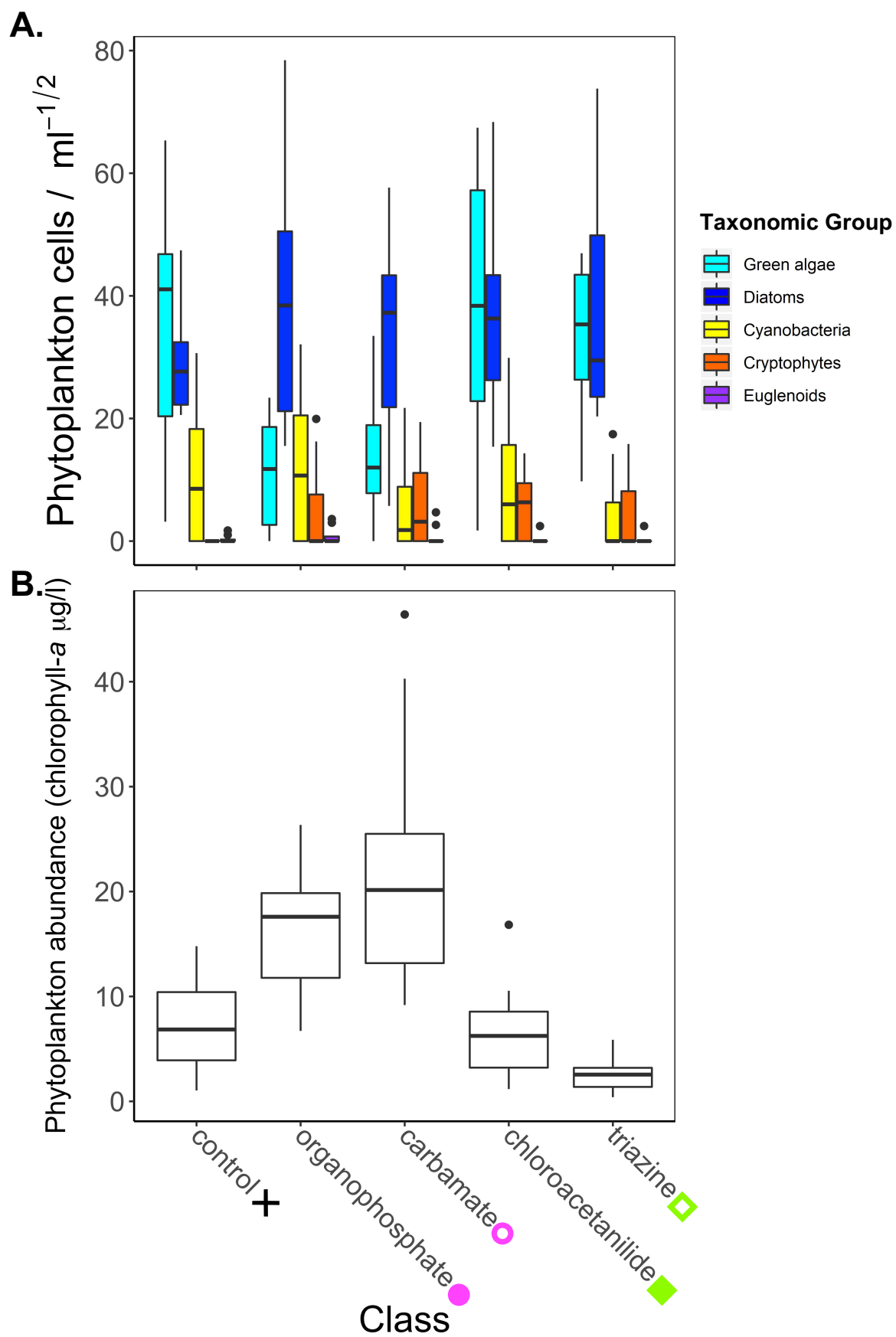
**D.**



**E.**



675 **Figure 2.**



676 **Figure 3.**

677

