*p*CO₂ effects on species composition and growth of an estuarine phytoplankton community

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ABSTRACT

The effects of ongoing changes in ocean carbonate chemistry on plankton ecology have important implications for food webs and biogeochemical cycling. However, conflicting results have emerged regarding species-specific responses to *p*CO₂ enrichment and thus community responses have been difficult to predict. To assess community level effects (e.g., production) of altered carbonate chemistry, studies are needed that capitalize on the benefits of controlled experiments but also retain features of intact ecosystems that may exacerbate or ameliorate the effects observed in single-species or single cohort experiments. We performed incubations of natural plankton communities from Narragansett Bay, RI, USA in winter at ambient bay temperatures (5-13 °C), light and nutrient concentrations under three levels of controlled and constant CO₂ concentrations, simulating past, present and future conditions at mean *p*CO₂ levels of 224, 361, and 724 µatm respectively. Samples for carbonate analysis, chlorophyll *a*, plankton size-abundance, and plankton species composition were collected

daily and phytoplankton growth rates in three different size fractions (<5, 5-20, and >20 μm) were measured at the end of the 7-day incubation period. Community composition changed during the incubation period with major increases in relative diatom abundance, which were similar across $p\text{CO}_2$ treatments. At the end of the experiment, 24-hr growth responses to $p\text{CO}_2$ levels varied as a function of cell size. The smallest size fraction (<5 μm) grew faster at the elevated $p\text{CO}_2$ level. In contrast, the 5-20 μm size fraction grew fastest in the Present treatment and there were no significant differences in growth rate among treatments in the > 20 μm size fraction. Cell size distribution shifted toward smaller cells in both the Past and Future treatments but remained unchanged in the Present treatment. Similarity in Past and Future treatments for cell size distribution and growth rate (5-20 μm size fraction) illustrate non-monotonic effects of increasing $p\text{CO}_2$ on ecological indicators and may be related to opposing physiological effects of high CO_2 and low pH both within and among species. Interaction of these effects with other factors (e.g., nutrients, light, temperature, grazing, initial species composition) may explain variability among published studies. The absence of clear treatment-specific effects at the community level suggest that extrapolation of species-specific responses or experiments with only present day and future $p\text{CO}_2$ treatments levels would produce misleading predictions of ocean acidification impacts on plankton production.

KEYWORDS plankton, phytoplankton, pH, ocean acidification, carbon dioxide, incubation, ecostat, community, composition, mesocosm, carbonate chemistry, cell size, chemostat

1. INTRODUCTION

Ocean chemistry is changing due to absorption of anthropogenic carbon dioxide (CO_2) from the atmosphere (Caldeira and Wickett, 2003; Sabine et al., 2004). This change in chemistry is anticipated to have significant effects on ocean biology (Riebesell, 2008). According to recent meta-analyses, the effects of lowered pH on traits such as calcification, growth, and early life stage survival in diverse marine organisms are highly variable, but on balance, are generally negative (Kroeker et al., 2013).

49 Although *in situ* effects have been reported from a few field experiments and in natural gradients (Hall-
50 Spencer et al., 2008), the extent to which laboratory results are predictive of ecological effects in
51 marine environments is one of the major unknowns in ocean acidification science (Fabry et al., 2008).
52 Despite the predictable effects of ocean acidification on biological processes (Kroeker et al., 2013), it is
53 not yet clear how often these biological responses would persist in populations, communities, or
54 ecosystems in ways that scientists, environmental advocates, or society would consider important. To
55 address this gap, experiments are needed that capitalize on the error control of manipulative
56 experiments and yet retain features of intact ecosystems that may exacerbate or ameliorate the effects
57 observed in single-species or single cohort experiments.

58 Most studies of ocean acidification effects have focused on physiological responses of
59 individual phytoplankton species. Elevated $p\text{CO}_2$ (CO_2 partial pressure) levels are theoretically
60 expected to have a “fertilizer” effect on phytoplankton, given the low CO_2 affinity of the primary
61 carboxylating enzyme RuBisCO (Badger et al. 1998) and the existence of other carbon concentrating
62 mechanisms (Reinfelder, 2011). Indeed, several studies have observed such fertilizing effects for some
63 species, while other species were intolerant of lowered pH levels and primary production rates
64 decreased (e.g., Hutchins et al., 2007; Iglesias-Rodriguez et al., 2008; Rost et al., 2003; Sun et al.,
65 2011; Wolf-Gladrow et al., 1999). Calcifying phytoplankton species have diverse and, at times,
66 contradictory biological responses to altered carbonate chemistry (Iglesias-Rodriguez et al., 2008;
67 Kroeker et al., 2010; Riebesell et al., 2000).

68 Attempts to scale up from the effects of increased $p\text{CO}_2$ on individual species to inferences
69 about entire taxonomic groups, populations, or communities abound in the literature (e.g., Hendriks et
70 al., 2009). However, strong biological sensitivity at the organismal level is neither a prerequisite for
71 nor a predictor of strong sensitivity at higher levels of biological organization. In addition, the nature
72 and magnitude of ecological changes that would be considered negative is often poorly defined,

creating fertile ground for intractable speculation (Duarte et al., 2015).

The responses of whole communities to elevated $p\text{CO}_2$ have been examined in a number of experiments. Increased $p\text{CO}_2$ has been shown to drive shifts in abundance or composition of primary producers and their consumers (Aberle et al., 2006; Hare et al., 2007; Rost et al., 2003; Tortell et al., 2002) as well as community rates of photosynthesis and productivity (Egge et al., 2009; Feng et al., 2009; Fu et al., 2007). For instance, increased $p\text{CO}_2$ had little effect on primary production in a coastal sub-arctic system (e.g., Egge et al., 2009) whereas in Southern Ocean waters, increased $p\text{CO}_2$ acted to increase overall productivity of the community, through the growth of chain-forming diatoms (Tortell et al., 2008). Similarly, increased $p\text{CO}_2$ led to dramatic increases in diatom abundance in the North Atlantic (Feng et al., 2009). The variability in responses to increased $p\text{CO}_2$ observed thus far does not allow responses to be extrapolated from one phytoplankton species, community or functional group to another. Moreover, responses are dependent on plankton community composition and environmental conditions at the time of $p\text{CO}_2$ manipulation. For example Gao et al. (2012) observed pH-dependent shifts in primary production that differed between haptophyte- and diatom-dominated communities. Moreover, it seems likely that experiments with highly unstable carbonate chemistry, as would be expected when large blooms and crashes occur during incubations, would exacerbate these contributors to variability. Although many experiments have been conducted, they span a variety of environments (estuaries, fjords, coasts, open ocean) and very few have reported measurements of carbonate chemistry with sufficient temporal detail to assess stability (e.g., Bach et al., 2016; Brussaard et al., 2013; Endo et al., 2013; Silyakova et al., 2013).

Ocean acidification (OA) alters the carbonate system and results in both increased CO_2 and decreased pH, the responses to which are expected to have opposing energetic costs. The net sum of these opposing costs is likely to vary with a number of traits, one of the most important of which is cell size (Gao and Campbell, 2014; Wolf-Gladrow et al., 1999; Wu et al., 2014). Since these net costs are

97 affected by light and temperature gradients that may or may not coincide with gradients in carbonate
98 chemistry, there is an enormously complex array of potential species-specific outcomes. There are also
99 likely to be differing degrees of genetic variation, phenotypic plasticity and adaptive potential among
100 species (Collins et al., 2014). This may lead to evolutionary shifts in important physiological
101 characteristics like growth rate as species respond to the selective pressure of increased $p\text{CO}_2$ (Scheinin
102 et al., 2015). Thus, it is not surprising that opposing predictions about OA effects on size-abundance
103 spectra have emerged and remain unresolved (e.g., Finkel et al., 2010; Wu et al., 2014). Given these
104 considerations, more experiments are needed that include detailed monitoring of carbonate chemistry
105 and consider the effects of both increases and decreases in CO_2 relative to modern-day concentrations.

106 Phytoplankton cell size is an easily measured and physiologically important characteristic that
107 is often discussed in the context of responses to environmental change (Barton et al., 2013; Finkel et
108 al., 2010), including OA (Schlüter et al., 2014). Using cell size abundance in the fossil record,
109 Hannisdal et al. (2012) described periods of declining coccolithophore cell size during the $p\text{CO}_2$
110 decline in the Oligocene (34-23 million years before present). Within a few well-studied species,
111 responses of mean cell size to temperature, $p\text{CO}_2$, and/or light perturbations have been reported (Xu et
112 al., 2014). Gao and Campbell (2014) reviewed 17 studies of species-level responses to elevated $p\text{CO}_2$;
113 results were roughly evenly split between positive, neutral and negative effects. Effects on the full size
114 spectrum of observed phytoplankton communities are rarely reported (Engel et al., 2008), but may have
115 greater predictive value for ecosystem science because of the biomass, energetic and carbon cycling
116 implications of cell size (Menden-Deuer and Lessard, 2000).

117 In this study, we tested whether CO_2 enrichment could cause changes in community-level
118 plankton community composition, size-abundance and production on scales large enough to motivate
119 concern about effects on trophic transfer. We used outdoor un-tempered chemostat experiments to
120 control and stabilize the carbonate chemistry while maintaining natural light and temperature cycles

121 and inoculated the chemostats with natural seawater to retain complex phytoplankton community
122 structure. We measured effects on specific taxa as well as community level metrics to assess linkages
123 from species to communities and ecosystem function, such as trophic transfer and primary production
124 rates. The $p\text{CO}_2$ level in our “future” treatment resembled levels that were uncommon in surface
125 waters but already present in bottom waters during a recent study of Narragansett Bay and other
126 northeast estuaries (Wallace et al., 2014).

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128 2. METHODS

129 2.1 Source water preparation and collection

130 Surface water samples containing source plankton were collected on 17 February 2014 as part
131 of the Long Term Plankton Time Series station in the West Passage of Narragansett Bay ($41^\circ 34.5' \text{ N}$,
132 $71^\circ 24.3' \text{ W}$), see Fig. 1 in Lawrence and Menden-Deuer (2012)). Stocks of diluent seawater with three
133 $p\text{CO}_2$ levels were prepared in advance for the entire experiment using water collected from the
134 seawater intake at the Graduate School of Oceanography, Narragansett, USA ($41^\circ 29' 32\text{N}$, $71^\circ 25'$
135 8W). Manipulation of diluent seawater to achieve target $p\text{CO}_2$ was performed by aeration with custom
136 CO_2 air mixtures simulating past ($250 \mu\text{atm}$), present (ambient air, $350 \mu\text{atm}$) and future ($750 \mu\text{atm}$)
137 $p\text{CO}_2$. Realized $p\text{CO}_2$ was monitored daily throughout the 7 day experiment. Gas mixtures were
138 bubbled through a $2 \mu\text{m}$ ultra-fine gas sparger. Seawater used for $p\text{CO}_2$ manipulation was not
139 autoclaved but gravity filtered with a $0.2 \mu\text{m}$ filter capsule before $p\text{CO}_2$ manipulation to avoid
140 degassing and significant alteration of carbonate chemistry (Riebesell et al., 2010).

141 2.2 Altered $p\text{CO}_2$ incubation experiments

142 The incubation experiments were started on the same day that source plankton were collected
143 (17 February 2014). The setup for the incubation experiments was similar to that of a large chemostat
144 experiment, following Hutchins et al. (2003). A total of eight 9.1 L polycarbonate bottles were

145 incubated, representing triplicates for the Past and Future $p\text{CO}_2$ treatments and duplicates for the
146 Present day treatment (due to 8 bottle limit of the chemostat). Each bottle received 10% whole seawater
147 (0.91 L) and 90% filtered diluent seawater at the treatment-appropriate $p\text{CO}_2$. Although dilution can
148 serve to reduce grazing effects, the primary purpose here was to keep the biomass in the bottles low
149 enough to reduce effects of photosynthesis and respiration on target $p\text{CO}_2$. The ratio of whole seawater
150 to diluent necessary to maintain stable seawater carbonate chemistry was determined in preliminary
151 experiments (data not shown). Seawater for all replicate bottles for each $p\text{CO}_2$ treatment was supplied
152 from a single diluent reservoir. The volume in the diluent reservoir was replenished regularly from a
153 single source stock for each treatment. Diluent was pumped through 0.64 mm silicone tubing with a
154 16-channel peristaltic pump (ISMATEC IPC, IDEX H-S, GmbH) adjusted to a flow rate of 2 ml min^{-1} .
155 All tubing, bottles and incubation materials were acid-cleaned in advance.

156 The incubator was set up shore-side to Narragansett Bay and was thus subject to the same air
157 temperature and irradiance variations as the source water collected from the surface of Narragansett
158 Bay for the experiments. Un-tempered Narragansett Bay surface water was circulated through the
159 incubator bath to maintain surface water temperature.

160 Daily sampling from each of the eight bottles included removal of 230 ml of whole water
161 samples for determination of chlorophyll *a* (Chl *a*) concentrations, dissolved inorganic carbon (DIC),
162 species composition and pH. Chl *a* measurements were made in triplicate from each carboy, following
163 Graff and Rynearson (2011) using 20, 5 μm and GF/F filters. Size abundance spectra of plankton were
164 determined using a Coulter Counter Multisizer 3 (Beckman Coulter, Inc) following Kim and Menden-
165 Deuer (2013).

166 2.3. *Growth rate experiments*

167 Growth rate experiments were conducted using 24 hr, closed bottle incubations at the beginning
168 and end of the chemostat experiment. Growth rate measurements at the beginning used the same whole

169 seawater used to initiate the chemostat experiment; post-chemostat growth rate experiments used
170 seawater collected from the chambers at the end of the chemostat experiment. Chambers from each
171 treatment were combined and then split into three 24 hr incubations per treatment under the same
172 conditions as the chemostat incubations. Growth rates were determined for the >GF/F (nominally
173 >0.65 μm), >20 μm and >5 μm size fractions using Chl *a* extractions. These left-closed size fractions
174 were converted to small (GF/F-5 μm), medium (5 -20 μm), and large (>20 μm) intervals by subtracting
175 the >5 μm fraction from the GF/F fraction and the >20 μm fraction from the >5 μm fraction to obtain the
176 small and medium intervals, respectively. In several cases, this subtraction produced negative Chl *a*
177 concentrations because the estimate of Chl *a* in the >20 μm fraction was slightly (by .04 $\mu\text{g L}^{-1}$ or less)
178 higher than in the >5 μm fraction. We attributed this to the precision of the method and thus replaced
179 these numbers with the estimated detection limit (0.1 $\mu\text{g L}^{-1}$). Growth was calculated from the change
180 in Chl *a* concentration as $k \cdot \text{day}^{-1} = \ln (\text{Chl}_{t=24}/\text{Chl}_{t=0})$ over the 24-hr growth rate experiments.

181 2.4 Carbonate chemistry

182 We determined total pH (pH_T) in the incubation chambers by measuring pH in the chamber
183 effluent on an NBS-calibrated meter (Hanna Instruments, HI98150) and then transforming these to
184 total pH by calibrating the same meter using the *m*-Cresol purple dye method on a Lambda 35
185 spectrophotometer (Dickson et al., 2007; Easley and Byrne, 2012). The resulting relationship was pH_T
186 $= -7.999 + 1.981 \text{ pH}_{\text{NBS}}$ (adjusted R-sq. = 0.993), which likely was consistent through the experiment
187 because of the short duration and stable source water conditions. We determined DIC by collecting
188 chamber effluent using standard sample collection procedures (i.e., filling from the bottom; 1% head
189 space) to fill 40 ml glass vials with Teflon-lined caps (Huang et al., 2012). These samples were
190 preserved within 1 hour of collection using mercuric chloride and were subsequently analyzed on a
191 Shimadzu TOC-V in sample runs that included nine determinations of DIC in Certified Reference
192 Material (CRM) for quality control (batch 128;

193 http://cdiac.ornl.gov/oceans/Dickson_CRM/batches.html). All nine CRM determinations were within
194 2 SD of the measured CRM mean, which was 1.7% below the certified value. Thus, observed DIC
195 values were converted to DIC_c using a correction factor of 1.0174 (1/(1-0.017)). We calculated $p\text{CO}_2$
196 from DIC_c, pH_T, temperature and salinity using the seacarb package (v.3) in R (R Core Team, 2014)
197 with the Millero (2010) option selected for k_1 and k_2 constants, the Dickson and Riley (1979) option for
198 k_f and the Dickson (1990) option for k_s .

199 2.5 *Phytoplankton community analyses*

200 Daily samples collected from each incubation replicate were fixed in 2% final concentration of
201 acidified Lugol's solution (Menden-Deuer et al., 2001). Plankton species composition was determined
202 at the beginning and end of the incubation using an inverted microscope (Nikon, Inc) following the
203 methods of Utermöhl (1958). Ten-ml volumes were settled using Utermöhl chambers and cells larger
204 than 5 μm were identified to class (e.g. diatoms, dinoflagellates) and genus, when possible. Plankton
205 community structure and composition were compared among the three $p\text{CO}_2$ treatments using
206 subroutines of Primer-E (V6; Clarke and Gorley, 2006). For univariate measures of diversity,
207 unidentified taxa were removed from calculations of species richness, Pielou's evenness (J'), and
208 Shannon's diversity ($H'_{\log e}$). For multivariate analyses of community composition, all taxa were
209 included, but data were square-root transformed to even out the contributions of rare and abundant
210 species. Similarity in community composition among replicates and among treatments was first
211 analyzed with the SIMPER subroutine, and then a Bray-Curtis similarity matrix was calculated to
212 compare community composition among treatments using the subroutine ANOSIM. All analyses were
213 performed at the taxonomic levels of genus and class.

214 2.6 *Statistical modeling of size-abundance*

215 We used a power law approach for analyzing size-abundance distributions in the Coulter
216 Counter data. We computed cell volume (V) from the equivalent spherical diameters reported by the

217 Coulter Counter and then log-transformed cell volume and cell counts (N) to linearize the analysis. A
 218 Box-Cox transformation allowed analysis using normal probability distributions, which could not be
 219 suitably fitted to $\ln N$. Thus, the fixed effects part of the statistical model was $\ln N = (\mathbf{X}\beta)k$, where N is
 220 the count, $\mathbf{X}$ is the design matrix (treatment, time, $\ln V$, plus two-way and three-way interactions), β is
 221 the matrix of linear coefficients, and k is the Box-Cox coefficient (k was selected using the profile
 222 likelihood method, Venables and Ripley, 2002). This produced a complex relationship between the
 223 raw data and the statistical response variable but allowed the use of standard statistical methods. Since
 224 we used repeated observations of each chamber to examine interactions between time and treatment,
 225 we included random effects for each chamber to capture the expected correlation between these
 226 repeated measurements. We performed these statistical analyses using the linear mixed effects package
 227 **lme4** in R (R Core Team, 2014). All statistical analyses were deemed significant at $p < 0.05$.

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229 3. RESULTS

230 3.1 *Carbonate chemistry*

231 Differences in carbonate chemistry imposed by the treatments were strong and consistent
 232 throughout the experiment (Fig 1). DIC and pH_T , and hence the calculated $p\text{CO}_2$ in the Present day
 233 treatment ($x = 361 \mu\text{atm}$, $\text{sd} = 44$) were intermediate between the Past ($x = 224 \mu\text{atm}$, $\text{sd} = 28$) and
 234 Future ($x = 724 \mu\text{atm}$, $\text{sd} = 142$) $p\text{CO}_2$ treatments. Incubation temperature, reflecting daily variations
 235 in surface temperatures of Narragansett Bay varied significantly over the incubation period, ranging
 236 from 5.1 to 13.1 deg. C ($x=9.5$). However, temperature changes did not differ among treatments (Fig.
 237 1).

238 3.2 *Chlorophyll a in the chemostats*

239 Average, initial Chl *a* concentrations were $\sim 0.30 \mu\text{g Chl } a \text{ L}^{-1}$ and increased by 47% to ~ 0.44

240 $\mu\text{g Chl L}^{-1}$ at the end of the chemostat experiment. No significant differences in total Chl *a*
 241 concentration were detected among $p\text{CO}_2$ treatments (likelihood ratio test: $G^2 = 4.2$, $\text{df} = 2$, $\text{Pr}[\chi^2 \leq G^2]$
 242 $= 0.1207$), suggesting that the observed increases in Chl *a* over time ($G^2 = 28.0$, $\text{df} = 1$, $\text{Pr}[\chi^2 \leq G^2] <$
 243 0.0001) were not related to $p\text{CO}_2$ level and remained roughly similar across treatments over the course
 244 of the incubation (Fig 2). The relative stability and ultimate increase in Chl *a* concentrations in each
 245 treatment over time suggests that net phytoplankton growth slightly exceeded the 30% dilution rate per
 246 day from continuous flushing with treatment-specific seawater.

247 3.3 *Instantaneous growth rates*

248 Instantaneous growth rates were determined at the beginning and end of the chemostat
 249 experiment using 24 hr, closed bottle incubations of either the source water collected from Narragansett
 250 Bay or water collected from bottles at the termination of the chemostat experiment. Phytoplankton
 251 growth rates in the Narragansett Bay source water (Fig. 3) were similar among the GF/F – 5 μm (0.08
 252 d^{-1} , $\text{se} = 0.09$), 5 -20 μm (0.09 d^{-1} , $\text{se} = 0.19$), and $>20\mu\text{m}$ (0.17 d^{-1} , $\text{se} = 0.02$) size fractions. After the
 253 chemostat incubation and thus after multi-day exposure to altered carbonate chemistry, phytoplankton
 254 growth rates differed among treatments depending on size fraction (Fig 3). For the GF/F – 5 μm size
 255 fraction, mean growth rate was negative in the Past treatment ($k = -0.96 \text{ d}^{-1}$) but was positive for both
 256 Present ($k = 0.47 \text{ d}^{-1}$, $\text{se} = 0.15$) and Future treatments ($k = 0.48 \text{ d}^{-1}$, $\text{se} = 0.34$). In the 5 -20 μm
 257 fraction, growth rates in both the Past treatment ($k = -0.41 \text{ d}^{-1}$, $\text{se} = 0.39$) and the Future treatment ($k = -$
 258 0.91 d^{-1} , $\text{se} = 0.48$) were lower than the Present treatment ($k = 0.75 \text{ d}^{-1}$, $\text{se} = 0.46$). Between the
 259 beginning and end of the experiment, growth in the $>20\mu\text{m}$ size fraction increased by more than 4 fold
 260 and were similar among the three treatments.

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Figure 1. Plots of daily treatment means and 95% confidence limits for water characteristics during the chemostat experiment. Dissolved inorganic carbon (DIC) and temperature were determined from water samples. Total pH (pH_T) was calculated from a spectrophotometrically calibrated glass electrode. $p\text{CO}_2$ was calculated stoichiometrically from pH_T , DIC, temperature and salinity (see text).

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Figure 2. Daily chlorophyll *a* concentrations (with standard deviations) in replicated phytoplankton incubations under three *p*CO₂ treatments.

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275 Figure 3. Mean growth rates ($k \cdot d^{-1} \pm$ s.e.) of Chl *a* size fractions estimated from 24 hr closed bottle
276 incubations of undiluted Narragansett Bay seawater before the chemostat experiment (“Pre-Chemostat”) and from the treatment chambers at the end of the experiment.

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3.4 *Cell size-abundance*

281 The size-abundance spectrum in the Present treatment remained invariant over the incubation
282 period (Fig 4). In contrast, Past and Future treatments caused a decrease in the frequency of 5-20 μm
283 sized cells leading to a shift of the whole size spectrum towards an increase in relative abundance of 3
284 -5 μm sized cells. This change is most visible in the comparison of size-abundance on the first and last
285 days (Fig. 4), but statistical tests on the linearized spectra also revealed significant effects. Linear fits
286 to the log-log and Box-Cox transformed data were initially similar across treatments but diverged by
287 the end of the experiment (Fig. 5). The 3-way interaction among size, $p\text{CO}_2$ treatment, and time was
288 statistically significant in the mixed effects model (likelihood ratio test: $G^2 = 207$, $\text{df} = 2$, $\text{Pr}[\chi^2 \leq G^2] <$
289 0.0001), meaning that the temporal shift in size-abundance spectra differed between treatments. Based
290 on equivalent spherical diameter measurements, mean cell volume at the beginning of the experiment
291 was $147 \mu\text{m}^3$, whereas final mean cell volumes in the Past, Present and Future treatments were 27, 92
292 and $17 \mu\text{m}^3$, respectively (but note that distributions were left-skewed prior to the transformations).
293 Increases in abundance were not consistently in the same size fractions for which instantaneous growth
294 rates were highest (see Discussion).

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296 Figure 4. Size abundance distributions (with standard deviations) binned into small (0 – 3 μm),
 297 medium (5 - 20 μm) and large (20 – 60 μm) size classes for Past, Present and Future treatments at day
 298 1 (top) and day 7 (bottom).

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Figure 5. Plots of $\ln N$ on $\ln$ cell volume at the beginning and end of the experiment. The thick line is the model prediction based on fixed effects only, which was fitted to a Box Cox transformation, $(\ln N)^k$, where $k = -0.8$. For plotting, the responses are back transformed to the original $\ln N$ scale using $1/k = -1.25$. Thin lines are the data for each chamber which, at $t = 162$, are the means of several Coulter counts for each chamber. Offsets between the fixed effects line and each chamber are due in part to the random effects that were estimated in the model from the repeated measures design.

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310 Figure 6. Phytoplankton community composition of cells > 5 μm at the beginning of the
311 experiment (T_0) and in each of the $p\text{CO}_2$ treatments at the end of the experiment. a) Absolute
312 abundance of cells in each treatment (cells L^{-1}); T_0 abundances were determined before dilution and

313 then scaled to 10% of whole seawater; b) Relative abundance of cells in each treatment.

314 3.5 *Phytoplankton community composition*

315 At the start of the experiment (T_0), the phytoplankton community was dominated by cryptophytes,
316 which comprised 64.7% of cells (Fig. 6, Table 1). Diatoms contributed 17.5% of the cells, dominated
317 by the genus *Thalassiosira* (11.4%). The dinoflagellate genus *Heterocapsa* contributed 13.1%. By the
318 end of the experiment, species composition changed significantly in all treatments (genus: $R = 0.34$, p
319 $= 0.03$; class: $R = 0.46$, $p = 0.02$) (Fig. 6). Dissimilarity in community composition between the initial
320 and final time points varied from 29 - 42% at both the genus and class level.

321 Taxonomic diversity appeared to increase from the beginning to the end of the experiment (Table
322 2) and was most likely due to the appearance of several diatom genera that were below the limit of
323 detection in the T_0 sample (Table 1). Between the beginning and end of the experiment, cryptophytes
324 decreased from 65 to 8-10% of the community (Fig. 6, 7). The number of diatom taxa increased and the
325 total relative contribution of diatoms increased by over four-fold during the course of the experiment,
326 although actual cell abundance changed by less than a factor of two (Table 1). The largest increase
327 within the diatoms over this time period was the genus *Skeletonema*, whose relative abundance in the
328 community increased by 17-fold. While absolute and relative abundance increased for some diatom
329 groups that were initially less abundant, (e.g., *Thalassiosira* spp; Table 1), they decreased substantially
330 for chryptophytes. Microzooplankton also decreased between the beginning and end of the experiment,
331 with ciliates decreasing to 28 – 48% from their original abundance (Table 1).

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333 Table 1. Average abundance of phyto- and microzoo-plankton at the beginning of the experiment (T_0 ,
334 prior to dilution) and in each of the treatments at the end of the experiment. Standard deviation
335 shown for counts of two replicate bottles for the Present treatment and of three replicate bottles for
336 the Past and Future treatments. Replicated counts could not be obtained for T_0 .

		T₀ Cells L ⁻¹	Past Cells L ⁻¹	Present Cells L ⁻¹	Future Cells L ⁻¹
Diatoms	<i>Thalassiosira</i>	49500	32733 ± 3828	23250 ± 9405	24707 ± 3985
	<i>Chaetoceros</i>	9900	5267 ± 2974	3200 ± 707	2987 ± 2372
	<i>Skeletonema</i>	8800	18967 ± 2250	23850 ± 8132	6927 ± 1866
	<i>Leptocylindrus</i>	7900	6567 ± 2899	2700 ± 1414	6327 ± 4467
	<i>Corethron</i>	0	133 ± 115	0	7 ± 12
	<i>Cylindrotheca</i>	0	500 ± 400	250 ± 212	113 ± 163
	<i>Licmophora</i>	0	167 ± 208	350 ± 495	273 ± 110
	<i>Guinardia</i>	0	333 ± 577	0	53 ± 50
	<i>Lauderia</i>	0	100 ± 173	0	0
	<i>Rhizosolenia</i>	0	67 ± 115	30 ± 42	7 ± 12
	<i>Pleurosigma</i>	0	33 ± 58	0	20 ± 20
	<i>Odontella</i>	0	267 ± 462	0	0
	Unidentified pennate diatoms	0	67 ± 115	0	167 ± 208
	Unidentified diatom chains	0	0	0	3200 ± 5543
Flagellates	Cryptophytes	282400	6300 ± 4187	7350 ± 2333	5753 ± 3284
	<i>Pseudopedinella</i>	20600	7633 ± 473	6550 ± 71	4400 ± 2381
	Other flagellates	38300	7433 ± 2212	8650 ± 71	3640 ± 1611
Dinoflagellates	<i>Heterocapsa</i>	57100	2994 ± 1988	3990 ± 14	2853 ± 1386
	<i>Protoperidinium</i>	0	0	0	7 ± 0
Ciliates	aloricate ciliates	9900	273 ± 155	480 ± 170	240 ± 212
	tintinnids	1700	39 ± 67	190 ± 156	133 ± 31

Table 2: Univariate measures of plankton taxonomic diversity; average richness (S), evenness (J') and Shannon's diversity ($H'_{\log e}$) for each treatment. Standard deviation shown in parentheses for each treatment except T₀ where no replicate counts were available.

	<i>Richness</i>	<i>Evenness</i>	<i>Shannon's diversity</i>
T ₀	10	0.631	1.452
Past	13.67 (2.08)	0.710 (0.01)	1.850 (0.01)
Present	12.00 (1.41)	0.737 (0.03)	1.828 (0.02)
Future	14.67 (2.31)	0.691 (0.05)	1.846 (0.03)

Figure 7. Non-metric multidimensional scaling plot (nMDS) of Bray-Curtis similarity in square-root transformed data among T_0 (1) and the three treatments, Past (Past A = 2, Past B = 3, Past C = 4), Present (Present A = 5, Present B = 6), and Future (Future A = 7, Future B = 8, Future C = 9). No differences among treatments were found, whether or not the T_0 counts were included.

There were no significant differences in phytoplankton community composition among $p\text{CO}_2$ treatments at the end of the incubation (Fig. 7; genus: $R = 0.12$, $p > 0.1$; class: $R = 0.25$, $p = 0.1$) and dissimilarity in community composition ranged from 16.5 to 25.5% at the genus level and 8.8 to 12.2% at the class level. The level of dissimilarity among treatments was about half as large as the dissimilarity between initial and final composition. Univariate measures of taxonomic diversity did not vary considerably among treatments (Table 2). The mean abundances at the end of the experiment were non-monotonic for 13 of the 21 groups in Table 1 (i.e., the Present treatment had either the highest or lowest mean abundance). However, there were no statistically significant treatment effects on abundance of specific genera, except for *Skeletonema*. If a Bonferroni correction is not used, which

360 would account for multiple hypothesis tests (one for each taxon), *Skeletonema* abundance in the Future
361 treatment (mean = 6.927) was lower than in the Past treatment (mean = 18.967, $p = 0.0343$, Tukey's
362 HSD) and the Present treatment (mean = 23.850, $p = 0.0140$).

363

364 4. DISCUSSION

365 Increasing absorption of CO₂ into the surface ocean is anticipated to affect organismal
366 physiology and ultimately ecosystem function. This has been confirmed by studies focused on single
367 species responses to altered $p\text{CO}_2$, albeit with contradictory results (Feng et al., 2009; Fu et al., 2007;
368 Hutchins et al., 2007; Iglesias-Rodriguez et al., 2008; Rost et al., 2003; Sun et al., 2011). In contrast,
369 the implications of altered $p\text{CO}_2$ for diverse plankton communities in terms of composition and
370 function such as primary production rates are less understood. Here we found that manipulating CO₂
371 partial pressure to mimic past, present and future anticipated $p\text{CO}_2$ levels in chemostat chambers
372 resulted in wholesale shifts in the size-abundance distributions of phytoplankton cells and in both
373 decreases and increases in growth rates, depending on the treatment and size fraction. These results
374 suggest that some predictions of $p\text{CO}_2$ effects on plankton community structure based on
375 measurements in the North Atlantic (Moran et al., 2010) are applicable to this estuarine community.
376 However, despite significant differences in chemistry among treatments, we did not observe a $p\text{CO}_2$
377 treatment-specific effect on the Chl *a* concentrations or taxonomic composition of the >5 μm fraction
378 of manipulated communities. Ultimately, differences in seawater chemistry did not translate into
379 measurable differences at the community level in terms of abundance of phytoplankton, which can be
380 inferred from Chl *a* concentrations since there was an absence of grazing (data not shown). This
381 suggests that in natural communities under similar conditions as measured here, altered $p\text{CO}_2$ would
382 not drive changes in the availability of organic matter for either trophic transfer or export production.

383 This study utilized a modified chemostat approach (i.e., the "Ecostat," Pickell et al., 2009) for

384 altering $p\text{CO}_2$. By imposing a steady dilution rate of each treatment, we were able to retain fine-scale
385 control of carbonate chemistry throughout the experiment. This appears to be unusual among
386 community-level phytoplankton experiments involving CO_2 manipulation. For example, the range in
387 DIC values observed during a closed mesocosm study by Engel et al. (2008) was approximately 58%
388 greater than the range observed here. It is possible that the experiments by Maugendre et al. (2015) in
389 an oligohaline system may have maintained stable carbonate chemistry, but chambers were sampled
390 destructively at each time point so the within-chamber carbonate systems trends through time are not
391 discernible. In their 113 day fjord mesocosm experiment, Bach et al. (2016) observed much higher
392 CO_2 temporal variability in mesocosms where CO_2 was added than in the untreated controls.

393 Reduced variability in DIC and consistency among treatments is one of the benefits of using a
394 chemostat rather than a mesocosm approach, but identifying the appropriate dilution rate is
395 challenging. The dilution rate must be slow enough that plankton growth is equal to or greater than the
396 dilution rate but fast enough so that build-up of phytoplankton biomass is avoided and stable carbonate
397 chemistry is maintained. Here, two separate chemostat pre-experiments were conducted in order to
398 determine the appropriate dilution rate (30% per day). Overall, the resulting stability made
399 interpretation and identification of treatment effects associated with $p\text{CO}_2$ manipulations more
400 straightforward.

401 Our use of three $p\text{CO}_2$ levels including a pre-industrial treatment led to different interpretations
402 of OA effects on phytoplankton communities than would have been possible from a study of only
403 present day and future $p\text{CO}_2$ levels. Although the $p\text{CO}_2$ treatments did not yield any statistically
404 significant differences in community composition, interesting patterns in the data may shed light on
405 results from this and other experiments. In our study, patterns in species composition did not exhibit
406 simple relationships to $p\text{CO}_2$. This differs from previous studies where chemostat incubations at
407 present day and future $p\text{CO}_2$ levels led to changes in the abundance of different taxa, including

408 increases in diatoms and decreases in chrysophytes (Feng et al., 2009) and even to dramatic changes in
409 species composition, including shifts in the relative abundance of pennate and centric diatoms (Feng et
410 al., 2010). One reason for the difference between our study and past experiments from which only
411 linear effects have been inferred is our addition of a third treatment reflecting pre-industrial $p\text{CO}_2$
412 levels. In general, treatment effects on abundances of specific groups were not statistically significant,
413 but mean abundance estimates in 13 of the 21 groups were non-monotonically related to $p\text{CO}_2$
414 treatments (Table 1, Fig. 6). The underlying reasons for non-monotonic shifts in species composition,
415 if they do indeed occur, are unknown but may have to do with the underlying complexity of natural
416 plankton communities both in terms of phylogenetic (e.g., physiological) and trophic diversity. In any
417 case, such patterns can only be identified with three or more treatments, suggesting that future studies
418 should include additional treatments in the experimental design.

419 Treatment effects on size-abundance spectra and size-specific growth rates were stronger than
420 the response in community composition and were more clearly non-monotonic. For example, growth of
421 the 5-20 μm size fraction was lower in both the Past and Future treatments compared to the Present
422 treatment. Similarly, size spectra in both the Past and Future treatments showed a decrease in the
423 relative abundance of mid-sized ($\sim$ 5-20 μm) cells and an increase in the relative abundance of small
424 cells ($<5\mu\text{m}$). In contrast, no changes in cell size-abundance spectra were detected in the Present
425 treatment. It is not necessarily surprising that small cells became more abundant but exhibited lower
426 growth rates at the end of the experiment. In a recent study of $p\text{CO}_2$ impacts on dinoflagellate species
427 and assemblages, the species with the lowest standing stock exhibited the highest growth rates in at
428 least one of the experiments (Tatters et al., 2013). Similarly, while size-abundance is a characteristic of
429 the standing stock during continuous chemostat dilution, our 24 hr post-chemostat incubations are
430 measurements of instantaneous growth rate at the end of the experiment and should not necessarily
431 correlate with abundance. Relationships between standing stocks and growth rate are often complex in

432 natural systems, such as when competition or shading limit growth at high densities. In such cases,
433 abundant taxa would have low growth rates. Conversely, high growth rates can coincide with low
434 standing stock when selective grazing removes only part of the community. Species composition
435 results are less easily compared with size-abundance results, since they exclude the smaller size
436 fraction. However, it is noteworthy that $p\text{CO}_2$ treatments affected neither species composition nor
437 aggregated growth rate in the $>20\ \mu\text{m}$ size class.

438 The prediction that growth and cell size distribution would be monotonically related to $p\text{CO}_2$
439 level was based in part on an assumed relationship between cell size, carbon transport and uptake, and
440 cellular response to $p\text{CO}_2$ and pH (Wolf-Gladrow and Riebesell, 1997). However, other characteristics
441 of phytoplankton physiology such as temperature responses and light thresholds and stress may affect
442 responses to $p\text{CO}_2$ (reviewed in Gao and Campbell, 2014). Nutrient requirements may also affect
443 community response and presumably the size-abundance response to $p\text{CO}_2$ manipulation. This was
444 suggested by Engel et al. (2008) to explain differences between their study and the one by Engel et al.
445 (2005), in which nutrient manipulations were used to favor *Emiliania huxleyi*. If such characteristics
446 vary independently of cell size and are more strongly associated with particular taxa, for example, then
447 the similar responses in size distribution for the low and high $p\text{CO}_2$ treatments (i.e., non-monotonic
448 responses) may be driven by different underlying mechanisms. In the well-known EPOCA
449 experiments, which is one of the few community-level phytoplankton studies that included detailed
450 reporting on the stability of the carbonate system (Silyakova et al., 2013), there was an observed shift
451 toward small phytoplankton (Brussaard et al., 2013). However, since there were no treatments
452 mimicking pre-industrial conditions, it is unclear whether this size-abundance response to $p\text{CO}_2$ is
453 monotonic in that arctic system. Similarly, Bach et al. (2016) observed an increase in picoeukaryotes
454 under increased $p\text{CO}_2$, but no pre-industrial treatments were included. Our study is consistent with
455 these results for ambient and high $p\text{CO}_2$ but raises the additional possibility that a reduction in $p\text{CO}_2$

456 would cause similar responses in aggregate characteristics of the community. Pre-industrial CO₂
457 treatments were included in the experiments of Endo et al. (2013). There were no clear CO₂ effects on
458 total Chl *a*, but small eukaryotic phytoplankton (< 10 µm) were positively affected by CO₂ in the
459 incubations that did not receive iron amendments. However, their low CO₂ treatments experienced a
460 steady decline in DIC, so results are not easily compared to our observation of shifts toward small cells
461 when DIC was held steady in both pre-industrial and future CO₂ treatments.

462 As is typical in phytoplankton incubations, our experiments ran continuously without an
463 acclimation period. Acclimation of mixed plankton communities to target treatments poses a particular
464 challenge (Tatters et al., 2013). The difficulties of examining long-term responses to gradually
465 changing environmental conditions experimentally on a single species are multiplied in a mixed-
466 species assemblage due to species interactions and differences in autecology. For mono-specific
467 phytoplankton laboratory cultures Brand and Guillard (1981) found that in order to achieve stability of
468 a single metric (growth rate), the required acclimation period was 1-3 weeks and depended on species.
469 Thus, diverse plankton communities would require a prolonged incubation where stable species
470 composition and abundance and carbonate chemistry are difficult to maintain and assess. If
471 acclimation is defined to include a species' adjustment to interactions with its competitors, predators,
472 and prey, then recombination from acclimated unialgal cultures may itself produce a perturbation
473 response that needs to be addressed. Nonetheless, results from Tatters et al. (2013) suggest that the
474 non-monotonic responses we observed are insensitive to acclimation.

475 Non-monotonic effects on species-specific instantaneous growth rates and the potential for
476 optimization are easy to envision demographically. For example, suppose mortality (*d*) exhibits a
477 logit-linear increase over a given range of *p*CO₂ (e.g., $d \sim 1/(1+\exp[-f_1(p\text{CO}_2)])$) due to pH effects. If
478 there is a linear enrichment effect on the rate of cell division (*b*) over that same range of *p*CO₂ (i.e., $b \sim$
479 $f_2(p\text{CO}_2)$), with *f*₁ and *f*₂ both being linear functions, then the expected growth rate (i.e., $b - d$) will

vary non-monotonically over the given range. Such community- and species-level scenarios are biologically plausible and possibly common over $p\text{CO}_2$ levels that range over a few hundred μatm .

The dinoflagellate experiments in Tatters et al. (2013) support this possibility that the non-monotonic responses we observed are common. Mean growth rate among the four species appears to have been non-monotonic in their 2 week initial incubation of samples from the natural community since the lowest rates were at the medium $p\text{CO}_2$ level (based on Figure 4 in Tatters et al., 2013). Similarly, mean growth rate appeared highest in the medium treatment for incubations of the communities that were recombined after 12 months of $p\text{CO}_2$ pre-conditioning. At the species level, mean responses for *Prorocentrum micans* and *Lingulodinium polyedrum* appeared to be non-monotonic in initial and post-conditioning experiments, respectively. Some of these differences were not tested or were not statistically significant, but the mean pattern is at least consistent with our claim that non-monotonic responses may be common, even when sophisticated methods to address acclimation have been included. Further, in agreement with our finding of a non-monotonic response, Heiden et al. (2016) found significantly lower growth rates at both pre-industrial (180 μatm) and future (1000 μatm) $p\text{CO}_2$ levels compared to present day (380 μatm) for two prominent Antarctic diatoms (*Fragilariopsis curta* and *Odontella weissflogii*).

If non-monotonic responses of species or communities are centered with their maxima or minima at modern day $p\text{CO}_2$ levels, then experiments with only Present and Future $p\text{CO}_2$ levels (i.e., without Past levels) may be misleading. Moreover, despite considerable documentation of species-specific effects of $p\text{CO}_2$ manipulation on phytoplankton in the literature, the subtle and non-monotonic community responses to $p\text{CO}_2$ enrichment that we observed indicate that effects on marine food webs and carbon fixation will be difficult to predict from experiments focused on single-species responses.

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513

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