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*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. Three levels of controlled and constant CO_2 concentrations were imposed, simulating past, present and future conditions at mean pCO_2 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 pCO_2 treatments. At the end of the experiment, 24-hr growth responses to pCO_2 levels varied as a function of cell size. The smallest size fraction (<5 μm) grew faster at the elevated pCO_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 altered pCO_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 suggests that extrapolation of species-specific responses or experiments with only present day and future pCO_2 treatments levels could produce misleading predictions of ocean acidification impacts on plankton production.

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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

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

Most studies of ocean acidification effects have focused on physiological responses of individual phytoplankton species. Elevated pCO_2 (CO_2 partial pressure) levels are theoretically expected to have a "fertilizer" effect on phytoplankton, given the low CO_2 affinity of the primary carboxylating enzyme RuBisCO (Badger et al., 1998) and the existence of other carbon concentrating mechanisms (Reinfelder, 2011). Indeed, several studies have observed such fertilizing effects for some species, while other species were intolerant of lowered pH levels and primary production rates decreased (e.g., Hutchins et al., 2007; Iglesias-Rodriguez et al., 2008; Rost et al., 2003; Sun et al., 2011; Wolf-Gladrow et al., 1999). Calcifying phytoplankton species have diverse and, at times, contradictory biological responses to altered carbonate chemistry (Iglesias-Rodriguez et al., 2008; Kroeker et al., 2010; Riebesell et al., 2000).

Attempts to scale up from the effects of increased pCO_2 on individual species to inferences about entire taxonomic groups, populations, or communities abound in the literature (e.g., Hendriks et al., 2009). However, strong biological sensitivity at the organismal level is neither a prerequisite for nor a predictor of strong sensitivity at higher levels of biological organization. In addition, the nature 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 pCO_2 have been examined in a number of experiments. Increased pCO_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 pCO_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 pCO_2 acted to increase overall productivity of the community through the growth of chain-forming diatoms (Tortell et al., 2008). Similarly, increased pCO_2 led to dramatic increases in diatom abundance in the North Atlantic (Feng et al., 2009). The variability in responses to increased pCO_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 pCO_2 manipulation. For example Gao et al. (2012) observed pH-dependent shifts in primary production that differed between haptophyte- and diatom-dominated communities. 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 (but see, 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 affected by light and temperature gradients that may or may not coincide with gradients in carbonate chemistry, there is an enormously complex array of potential species-specific outcomes. There are also likely to be differing degrees of genetic variation, phenotypic plasticity and adaptive potential among species (Collins et al., 2014). This may lead to evolutionary shifts in important physiological characteristics like growth rate as species respond to the selective pressure of increased pCO_2 (Scheinin et al., 2015). Thus, it is not surprising that opposing predictions about OA effects on size-abundance spectra have emerged and remain unresolved (e.g., Finkel et al., 2010; Wu et al., 2014). Given these considerations, more experiments are needed that include detailed monitoring of carbonate chemistry and consider the effects of both increases and decreases in CO_2 relative to modern-day concentrations.

Phytoplankton cell size is an easily measured and physiologically important characteristic that is often discussed in the context of responses to environmental change (Barton et al., 2013; Finkel et al., 2010), including OA (Schlüter et al., 2014). Using cell size abundance in the fossil record, Hannisdal et al. (2012) described periods of declining coccolithophore cell size during the pCO_2 decline in the Oligocene (34-23 million years before present). Within a few well-studied species, responses of mean cell size to temperature, pCO_2, and/or light perturbations have been reported (Xu et al., 2014). Gao and Campbell (2014) reviewed 17 studies of species-level responses to elevated pCO_2; results were roughly evenly split between positive, neutral and negative effects. Effects on the full size spectrum of observed phytoplankton communities are rarely reported (Engel et al., 2008), but may have greater predictive value for ecosystem science because of the biomass, energetic and carbon cycling implications of cell size (Menden-Deuer and Lessard, 2000).

In this study, we tested whether CO_2 enrichment could cause changes in community-level plankton community composition, size-abundance and production on scales large enough to motivate concern about effects on trophic transfer. We used outdoor untempered chemostat experiments to control and stabilize the carbonate chemistry while maintaining natural light and temperature cycles and inoculated the chemostats with natural seawater to retain complex phytoplankton community structure. We measured effects on specific taxa as well as community level metrics to assess linkages from species to communities and ecosystem function, such as trophic transfer and primary production rates. The pCO_2 level in our "future" treatment resembled levels that were uncommon in surface waters but already present in bottom waters during a recent study of Narragansett Bay and other northeast U.S. estuaries (Wallace et al., 2014).

2. Methods

2.1. Source water preparation and collection

Surface water samples containing source plankton were collected on 17 February 2014 as part of the Long Term Plankton Time Series station in the West Passage of Narragansett Bay (41° 34.5′ N, 71° 24.3′W); see Fig. 1 in Lawrence and Menden-Deuer

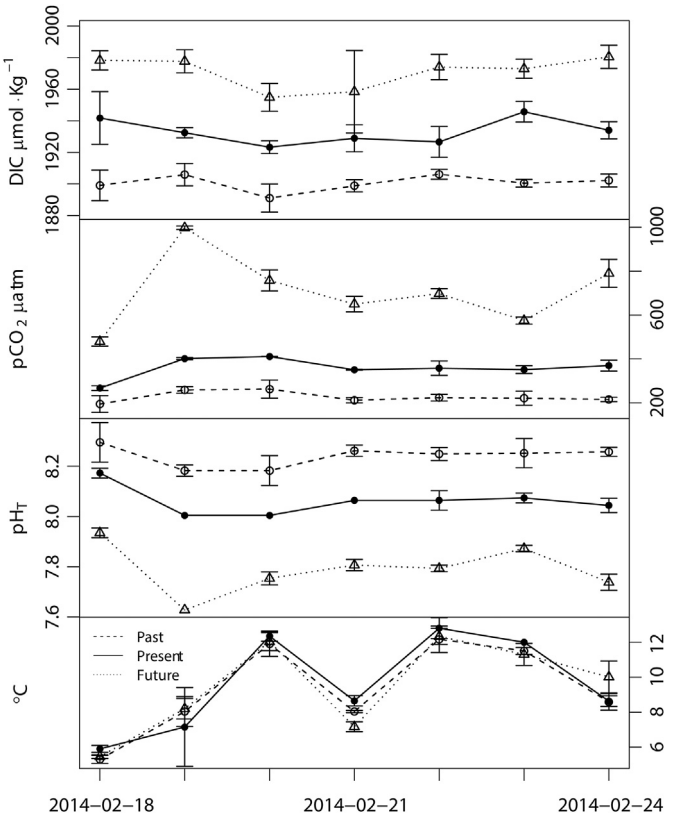

Fig. 1. Plots of daily treatment means and 95% confidence limits for water characteristics during the chemostat experiment. Dissolved inorganic carbon (DIC) and temperature were determined from water samples. Total pH (pH_T) was calculated from a spectrophotometrically calibrated glass electrode. pCO_2 was calculated stoichiometrically from pH_T, DIC, temperature and salinity (see text).

(2012). Stocks of diluent seawater with three pCO_2 levels were prepared in advance for the entire experiment using water collected from the seawater intake at the Graduate School of Oceanography, Narragansett, USA (41° 29′ 32N, 71° 25′ 8W). Manipulation of diluent seawater to achieve target pCO_2 was performed by aeration with custom CO_2 air mixtures simulating past (250 µatm), present (350 µatm) and future (750 µatm) pCO_2. Realized pCO_2 was monitored daily throughout the 7 day experiment. Gas mixtures were bubbled through a 2 µm ultra-fine gas sparger. Seawater used for pCO_2 manipulation was not autoclaved but gravity filtered with a 0.2 µm filter capsule before pCO_2 manipulation to avoid degassing and significant alteration of carbonate chemistry (Riebesell et al., 2010).

2.2. Altered pCO2 incubation experiments

The incubation experiments were started on the same day that source plankton were collected (17 February 2014). The setup for the incubation experiments was similar to that of a large chemostat experiment, following Hutchins et al. (2003). A total of eight 9.1 L polycarbonate bottles were incubated, representing triplicates for the Past and Future pCO_2 treatments and duplicates for the Present day treatment (due to 8 bottle limit of the chemostat). Each bottle received 10% whole seawater (0.91 L) and 90% filtered diluent seawater at the treatment-appropriate pCO_2. Although dilution can serve to reduce grazing effects, the primary purpose here was to keep the biomass in the bottles low enough to reduce effects of photosynthesis and respiration on target pCO_2. The ratio of whole seawater to diluent necessary to maintain stable seawater carbonate chemistry was determined in preliminary experiments (data not shown). Seawater for all replicate bottles for each pCO_2 treatment was supplied from a single diluent reservoir. The volume in the diluent reservoir was replenished regularly from a single source stock for each treatment. Diluent was pumped through 0.64 mm silicone tubing with a 16-channel peristaltic pump (ISMATEC IPC, IDEX H-S, GmbH) adjusted to a flow rate of 2 ml min^{-1}. All tubing, bottles and incubation materials were acid-cleaned in advance.

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

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

2.3. Growth rate experiments

Growth rate experiments were conducted using 24 h, closed bottle incubations at the beginning and end of the chemostat experiment. Growth rate measurements at the beginning used the same whole seawater used to initiate the chemostat experiment; post-chemostat growth rate experiments used seawater collected from the chambers at the end of the chemostat experiment. Chambers from each treatment were combined and then split into three 24 h incubations per treatment under the same conditions as the chemostat incubations. Growth rates were determined for the >GF/F (nominally >0.65 µm), >20 µm and >5 µm size fractions using Chl *a* extractions. These left-closed size fractions were converted to small (GF/F-5 µm), medium (5–20 µm), and large (>20 µm) intervals by subtracting the >5 µm fraction from the GF/F fraction and the >20 µm fraction from the >5 µm fraction to obtain the small and medium intervals, respectively. In several cases, this subtraction produced negative Chl *a* concentrations because the estimate of Chl *a* in the >20 µm fraction was slightly (by 0.04 µg L^{-1} or less) higher than in the >5 µm fraction. We attributed this to the precision of the method and thus replaced these numbers with the estimated detection limit (0.1 µg L^{-1}). Growth was calculated from the change in Chl *a* concentration as $k \cdot day^{-1} = \ln(Chl_{t=24}/Chl_{t=0})$ over the 24-hr growth rate experiments.

2.4. Carbonate chemistry

We determined total pH (pH_T) in the incubation chambers by measuring pH in the chamber effluent on an NBS-calibrated meter (Hanna Instruments, HI98150) and then transforming these to total pH by calibrating the same meter using the *m*-Cresol purple dye method on a Lambda 35 spectrophotometer (Dickson et al., 2007; Easley and Byrne, 2012). The resulting relationship was $pH_T = -7.999 + 1.981\ pH_{NBS}$ (adjusted R-sq. = 0.993), which likely was consistent through the experiment because of the short duration and stable source water conditions. We determined DIC by collecting chamber effluent using standard sample collection procedures (i.e., filling from the bottom; 1% head space) to fill 40 ml glass vials with Teflon-lined caps (Huang et al., 2012). These samples were preserved within 1 h of collection using mercuric chloride (0.05% by volume) and were subsequently analyzed on a

Shimadzu TOC-V in sample runs that included nine determinations of DIC in Certified Reference Material (CRM) for quality control (batch 128; http://cdiac.ornl.gov/oceans/Dickson_CRM/batches.html). All nine CRM determinations were within 2 SD of the measured CRM mean, which was 1.7% below the certified value. Thus, observed DIC values were converted to DIC*c* using a correction factor of 1.0174 (1/(1−0.017)). We calculated pCO_2 from DIC*c*, pH_T, temperature and salinity using the seacarb package (v.3) in R (R Core Team, 2014) with the Millero (2010) option selected for k_1 and k_2 constants, the Dickson and Riley (1979) option for k_f and the Dickson (1990) option for k_s.

2.5. Phytoplankton community analyses

Daily samples collected from each incubation replicate were fixed in 2% final concentration of acidified Lugol's solution (Menden-Deuer et al., 2001). Plankton species composition was determined at the beginning and end of the incubation using an inverted microscope (Nikon, Inc) following the methods of Utermöhl (1958). Ten-ml volumes were settled using Utermöhl chambers and cells larger than 5 μm were identified to class (e.g. diatoms, dinoflagellates) and genus, when possible. Plankton community structure and composition were compared among the three pCO_2 treatments using subroutines of Primer-E (V6; Clarke and Gorley, 2006). For univariate measures of diversity, unidentified taxa were removed from calculations of species richness, Pielou's evenness (J′), and Shannon's diversity ($H'_{\log\ e}$). For multivariate analyses of community composition, all taxa were included, but data were square-root transformed to even out the contributions of rare and abundant species. Similarity in community composition among replicates and among treatments was first analyzed with the SIMPER subroutine, and then a Bray-Curtis similarity matrix was calculated to compare community composition among treatments using the subroutine ANOSIM. All analyses were performed at the taxonomic levels of genus and class.

2.6. Statistical modeling of size-abundance

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

3. Results

3.1. Carbonate chemistry

Differences in carbonate chemistry imposed by the treatments were strong and consistent throughout the experiment (Fig. 1). DIC and pH_T, and hence the calculated pCO_2 in the Present day treatment ($\bar{x} = 361$ μatm, sd = 44) were intermediate between the Past ($\bar{x} = 224$ μatm, sd = 28) and Future ($\bar{x} = 724$ μatm, sd = 142) pCO_2 treatments. Incubation temperature, reflecting daily variations in surface temperatures of Narragansett Bay varied significantly over the incubation period, ranging from 5.1 to 13.1 deg. C ($\bar{x} = 9.5$). However, temperature changes did not differ among treatments (Fig. 1).

3.2. Chlorophyll a in the chemostats

Average, initial Chl *a* concentrations were ~0.30 μg Chl *a* L^{-1} and increased by 47% to ~0.44 μg Chl L^{-1} at the end of the chemostat experiment (Fig. 2). No significant differences in total Chl *a* concentration were detected among pCO_2 treatments (likelihood ratio test: $G^2 = 4.2$, df = 2, $Pr[\chi^2 \leq G^2] = 0.1207$), suggesting that the observed increases in Chl *a* over time ($G^2 = 28.0$, df = 1, $Pr[\chi^2 \leq G^2] < 0.0001$) were not related to pCO_2 level and remained roughly similar across treatments over the course of the incubation (Fig. 2). The relative stability and ultimate increase in Chl *a* concentrations in each treatment over time suggests that net phytoplankton growth slightly exceeded the 30% dilution rate per day from continuous flushing with treatment-specific seawater.

3.3. Instantaneous growth rates

Instantaneous growth rates were determined at the beginning and end of the chemostat experiment using 24 h, closed bottle incubations of either the source water collected from Narragansett Bay or water collected from bottles at the termination of the chemostat experiment. Phytoplankton growth rates in the Narragansett Bay source water (Fig. 3) were similar among the GF/F − 5 μm (0.08 d^{-1}, se = 0.09), 5–20 μm (0.09 d^{-1}, se = 0.19), and >20 μm (0.17 d^{-1}, se = 0.02) size fractions. After the chemostat incubation and thus after multi-day exposure to altered carbonate chemistry, phytoplankton growth rates differed among treatments depending on size fraction (Fig. 3). For the GF/F − 5 μm size fraction, mean growth rate was negative in the Past treatment ($k = -0.96$ d^{-1}) but was positive for both Present ($k = 0.47$ d^{-1}, se = 0.15) and Future treatments ($k = 0.48$ d^{-1}, se = 0.34). In the 5–20 μm fraction, growth rates in both the Past treatment ($k = -0.41$ d^{-1}, se = 0.39) and the Future treatment ($k = -0.91$ d^{-1}, se = 0.48) were lower

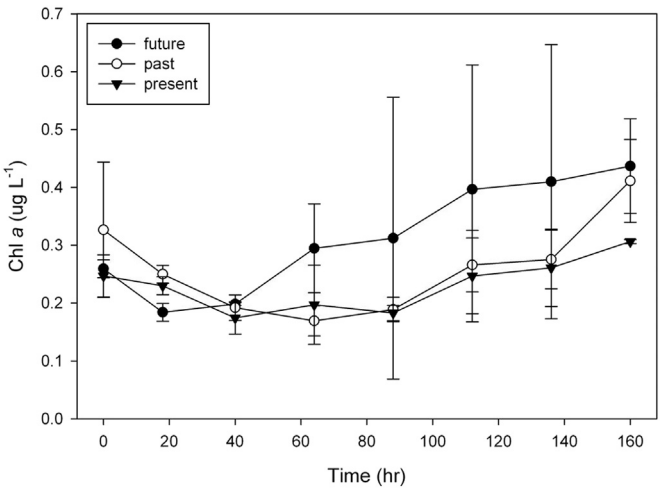

Fig. 2. Daily chlorophyll *a* concentrations (with standard deviations) in replicated phytoplankton incubations under three pCO_2 treatments.

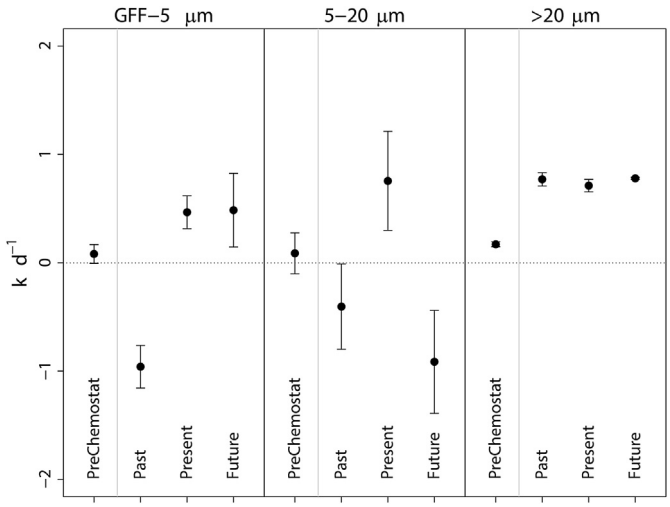

Fig. 3. Mean growth rates (k d^{-1} +/− s.e.) of Chl *a* size fractions estimated from 24 h closed bottle incubations of undiluted Narragansett Bay seawater before the chemostat experiment ("Pre-Chemostat) and from the treatment chambers at the end of the experiment.

than the Present treatment ($k = 0.75$ d^{-1}, se $= 0.46$). Between the beginning and end of the experiment, growth in the >20 μm size fraction increased by more than 4 fold and were similar among the three treatments.

3.4. Cell size-abundance

The size-abundance spectrum in the Present treatment remained invariant over the incubation period (Fig. 4). In contrast, Past and Future treatments caused a decrease in the frequency of 5–20 μm sized cells leading to a shift of the whole size spectrum towards an increase in relative abundance of 3–5 μm sized cells. This change is most visible in the comparison of size-abundance on the first and last days (Fig. 4), but statistical tests on the linearized spectra also revealed significant effects. Linear fits to the log-log and Box-Cox transformed data were initially similar across treatments but diverged by the end of the experiment (Fig. 5). The 3-way interaction among size, pCO_2 treatment, and time was statistically significant in the mixed effects model (likelihood ratio test: $G^2 = 207$, df $= 2$, $\Pr[\chi^2 \leq G^2] < 0.0001$), meaning that the temporal shift in size-abundance spectra differed between treatments. Based on equivalent spherical diameter measurements, mean cell volume at the beginning of the experiment was 147 μm^3, whereas final mean cell volumes in the Past, Present and Future treatments were 27, 92 and 17 μm^3, respectively (but note that distributions were left-skewed prior to the transformations). Increases in abundance were not consistently in the same size fractions for which instantaneous growth rates were highest (see Discussion).

3.5. Phytoplankton community composition

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

Taxonomic diversity appeared to increase from the beginning to

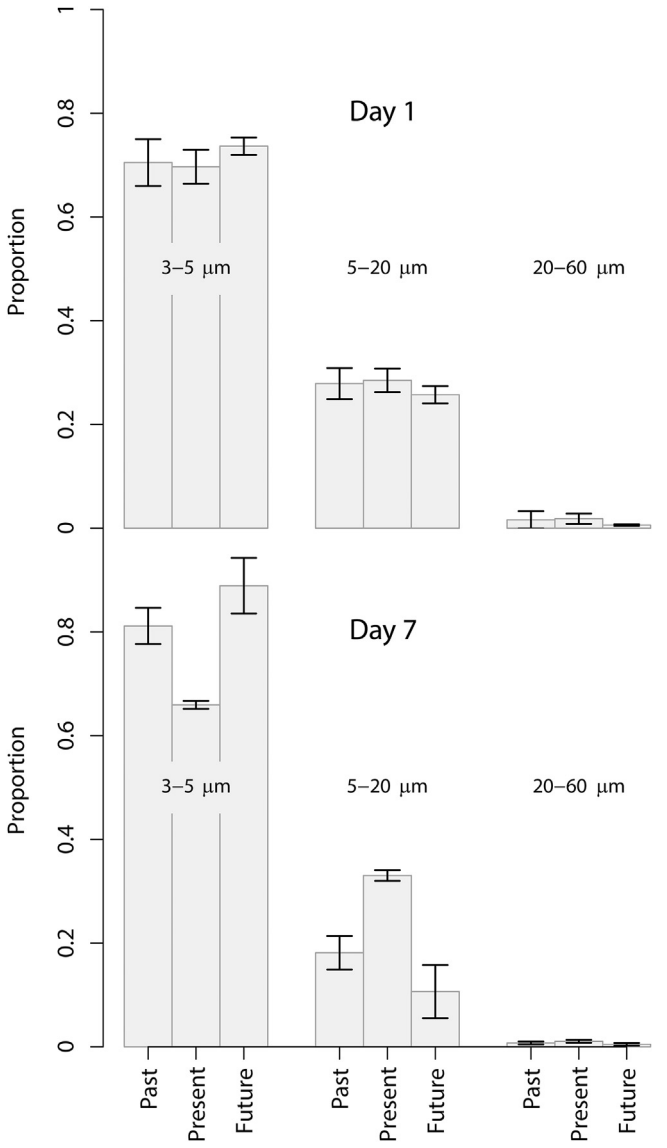

Fig. 4. Size abundance distributions (with standard deviations) binned into small (0–3 μm), medium (5–20 μm) and large (20–60 μm) size classes for Past, Present and Future treatments at day 1 (top) and day 7 (bottom).

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

There were no significant differences in phytoplankton community composition among pCO_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

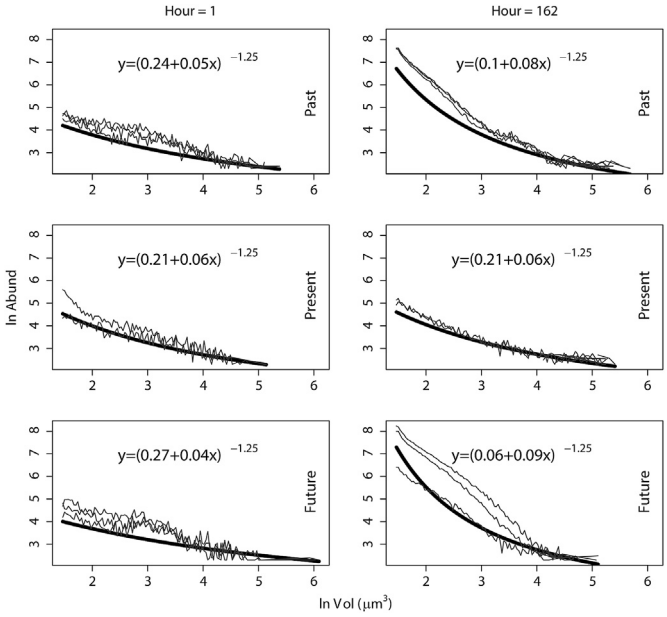

Fig. 5. Plots of ln *N* on ln cell volume at the beginning and end of the experiment. The thick line is the model prediction based on fixed effects only, which was fitted to a Box Cox transformation, $(\ln N)^k$, where $k = -0.8$. For plotting, the responses are back transformed to the original ln *N* 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.

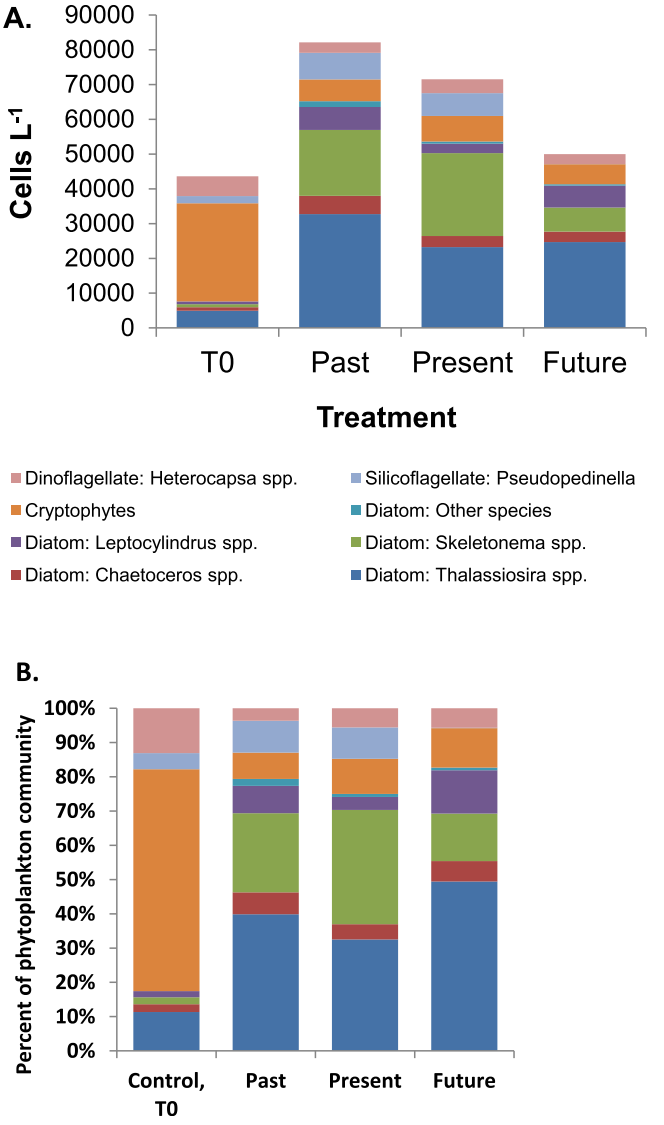

Fig. 6. Phytoplankton community composition of cells >5 μm at the beginning of the experiment (T_0) and in each of the pCO_2 treatments at the end of the experiment. a) Absolute abundance of cells in each treatment (cells L^{-1}); T_0 abundances were determined before dilution and then scaled to 10% of whole seawater; b) Relative abundance of cells in each treatment.

dissimilarity in community composition ranged from 16.5 to 25.5% at the genus level and 8.8–12.2% 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 would account for multiple hypothesis tests (one for each taxon), *Skeletonema* abundance in the Future treatment (mean = 6.927) was lower than in the Past treatment (mean = 18.967, $p = 0.0343$, Tukey's HSD) and the Present treatment (mean = 23.850, $p = 0.0140$).

4. Discussion

Increasing absorption of CO_2 into the surface ocean is anticipated to affect organismal physiology and ultimately ecosystem function. This has been confirmed by studies focused on single species responses to altered pCO_2, albeit with contradictory results (Feng et al., 2009; Fu et al., 2007; Hutchins et al., 2007; Iglesias-Rodriguez et al., 2008; Rost et al., 2003; Sun et al., 2011). In contrast, the implications of altered pCO_2 for diverse plankton communities in terms of composition and function such as primary production rates are less understood. Here we found that manipulating CO_2 partial pressure to mimic past, present and future anticipated pCO_2 levels in chemostat chambers resulted in whole-scale shifts in the size-abundance distributions of phytoplankton cells and in both decreases and increases in growth rates, depending on the treatment and size fraction. These results suggest that some predictions of pCO_2 effects on plankton community structure based on measurements in the North Atlantic (Moran et al., 2010) are applicable to this estuarine community. However, despite significant differences in chemistry among treatments, we did not observe a pCO_2 treatment-specific effect on the Chl *a* concentrations or taxonomic composition of the >5 μm fraction of manipulated communities. Ultimately, differences in seawater chemistry did not translate into measurable differences at the community level in terms of abundance of phytoplankton, which can be inferred from Chl *a* concentrations since there was an absence of grazing (data not shown). This suggests that in natural communities under similar conditions as measured here, altered pCO_2 would not drive changes in the availability of organic matter for either trophic transfer or export production.

This study utilized a modified chemostat approach (i.e., the "Ecostat," Pickell et al., 2009) for altering pCO_2. By imposing a steady dilution rate of each treatment, we were able to retain fine-scale control of carbonate chemistry throughout the experiment. This appears to be unusual among community-level phytoplankton

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

		T_0	Past	Present	Future
		Cells L^{-1}	Cells L^{-1}	Cells L^{-1}	Cells L^{-1}
Diatoms	*Thalassiosira*	49500	32733 ± 3828	23250 ± 9405	24707 ± 3985
	Chaetoceros	9900	5267 ± 2974	3200 ± 707	2987 ± 2372
	Skeletonema	8800	18967 ± 2250	23850 ± 8132	6927 ± 1866
	Leptocylindrus	7900	6567 ± 2899	2700 ± 1414	6327 ± 4467
	Corethron	0	133 ± 115	0	7 ± 12
	Cylindrotheca	0	500 ± 400	250 ± 212	113 ± 163
	Licmophora	0	167 ± 208	350 ± 495	273 ± 110
	Guinardia	0	333 ± 577	0	53 ± 50
	Lauderia	0	100 ± 173	0	0
	Rhizosolenia	0	67 ± 115	30 ± 42	7 ± 12
	Pleurosigma	0	33 ± 58	0	20 ± 20
	Odontella	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
	Pseudopedinella	20600	7633 ± 473	6550 ± 71	4400 ± 2381
	Other flagellates	38300	7433 ± 2212	8650 ± 71	3640 ± 1611
Dinoflagellates	*Heterocapsa*	57100	2994 ± 1988	3990 ± 14	2853 ± 1386
	Protoperidinium	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 T_0 where no replicate counts were available.

	Richness	Evenness	Shannon's diversity
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)

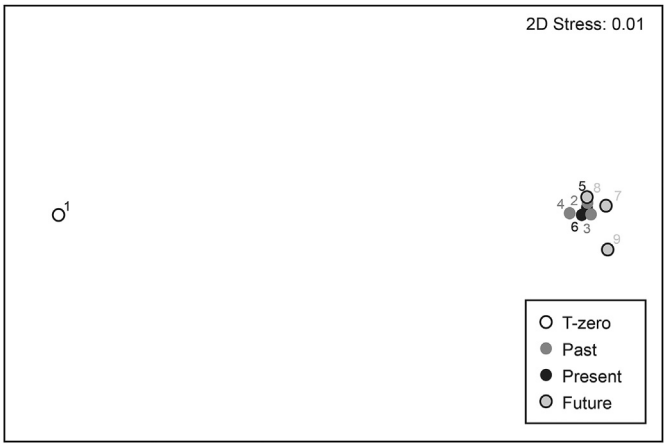

Fig. 7. Non-metric multidimensional scaling plot (nMDS) of Bray-Curtis similarity in square-root transformed data among T_0 (1) and the three treatments, Past (Past A = 2, Past B = 3, Past C = 4), Present (Present A = 5, Present B = 6), and Future (Future A = 7, Future B = 8, Future C = 9). No differences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

experiments involving CO_2 manipulation. For example, the range in DIC values observed during a closed mesocosm study by Engel et al. (2008) was approximately 58% greater than the range observed here. It is possible that the experiments by Maugendre et al. (2015) in an oligohaline system may have maintained stable carbonate chemistry, but chambers were sampled destructively at each time point so the within-chamber carbonate systems trends through time are not discernible. In their 113 day fjord mesocosm experiment, Bach et al. (2016) observed much higher CO_2 temporal variability in mesocosms where CO_2 was added than in the untreated controls.

Reduced variability in DIC and consistency among treatments is one of the benefits of using a chemostat rather than a mesocosm approach, but identifying the appropriate dilution rate is challenging. The dilution rate must be slow enough that plankton growth is equal to or greater than the dilution rate but fast enough so that build-up of phytoplankton biomass is avoided and stable carbonate chemistry is maintained. Here, two separate chemostat pre-experiments were conducted in order to determine the appropriate dilution rate (30% per day). Overall, the resulting stability made interpretation and identification of treatment effects associated with pCO_2 manipulations more straightforward.

Our use of three pCO_2 levels including a pre-industrial treatment led to different interpretations of OA effects on phytoplankton communities than would have been possible from a study of only present day and future pCO_2 levels. Although the pCO_2 treatments did not yield any statistically significant differences in community composition, interesting patterns in the data may shed light on results from this and other experiments. In our study, patterns in species composition did not exhibit simple relationships to pCO_2. This differs from previous studies where chemostat incubations at present day and future pCO_2 levels led to changes in the abundance of different taxa, including increases in diatoms and decreases in chrysophytes (Feng et al., 2009) and even to dramatic changes in species composition, including shifts in the relative abundance of pennate and centric diatoms (Feng et al., 2010). One reason for the difference between our study and past experiments from which only linear effects have been inferred is our addition of a third treatment reflecting pre-industrial pCO_2 levels. In general, treatment effects on abundances of specific groups were not statistically significant, but mean abundance estimates in 13 of the 21 groups were non-monotonically related to pCO_2 treatments (Table 1, Fig. 6). The underlying reasons for non-monotonic shifts in species composition, if they do indeed occur, are unknown but may have to do with the underlying complexity of natural plankton

communities both in terms of phylogenetic (e.g., physiological) and trophic diversity. In any case, such patterns can only be identified with three or more treatments, suggesting that future studies should include additional treatments in the experimental design.

Treatment effects on size-abundance spectra and size-specific growth rates were stronger than the response in community composition and were more clearly non-monotonic. For example, growth of the 5–20 μm size fraction was lower in both the Past and Future treatments compared to the Present treatment. Similarly, size spectra in both the Past and Future treatments showed a decrease in the relative abundance of mid-sized (~5–20 μm) cells and an increase in the relative abundance of small cells (<5 μm). In contrast, no changes in cell size-abundance spectra were detected in the Present treatment. It is not necessarily surprising that small cells became more abundant but exhibited lower growth rates at the end of the experiment. In a recent study of pCO_2 impacts on dinoflagellate species and assemblages, the species with the lowest standing stock exhibited the highest growth rates in at least one of the experiments (Tatters et al., 2013). Similarly, while size-abundance is a characteristic of the standing stock during continuous chemostat dilution, our 24 h post-chemostat incubations are measurements of instantaneous growth rate at the end of the experiment and should not necessarily correlate with abundance. Relationships between standing stocks and growth rate are often complex in natural systems, such as when competition or shading limit growth at high densities. In such cases, abundant taxa would have low growth rates. Conversely, high growth rates can coincide with low standing stock when selective grazing removes only part of the community. Species composition results are less easily compared with size-abundance results, since they exclude the smaller size fraction. However, it is noteworthy that pCO_2 treatments affected neither species composition nor aggregated growth rate in the >20 μm size class.

The prediction that growth and cell size distribution would be monotonically related to pCO_2 level was based in part on an assumed relationship between cell size, carbon transport and uptake, and cellular response to pCO_2 and pH (Wolf-Gladrow and Riebesell, 1997). However, other characteristics of phytoplankton physiology such as temperature responses and light thresholds and stress may affect responses to pCO_2 (reviewed in Gao and Campbell, 2014). Nutrient requirements may also affect community response and presumably the size-abundance response to pCO_2 manipulation. This was suggested by Engel et al. (2008) to explain differences between their study and the one by Engel et al. (2005), in which nutrient manipulations were used to favor *Emiliania huxleyi*. If such characteristics vary independently of cell size and are more strongly associated with particular taxa, for example, then the similar responses in size distribution for the low and high pCO_2 treatments (i.e., non-monotonic responses) may be driven by different underlying mechanisms. In the well-known EPOCA experiments, which is one of the few community-level phytoplankton studies that included detailed reporting on the stability of the carbonate system (Silyakova et al., 2013), there was an observed shift toward small phytoplankton (Brussaard et al., 2013). However, since there were no treatments mimicking pre-industrial conditions, it is unclear whether this size-abundance response to pCO_2 is monotonic in that arctic system. Similarly, Bach et al. (2016) observed an increase in picoeukaryotes under increased pCO_2, but no pre-industrial treatments were included. Our study is consistent with these results for ambient and high pCO_2 but raises the additional possibility that a reduction in pCO_2 would cause similar responses in aggregate characteristics of the community. Pre-industrial CO_2 treatments were included in the experiments of Endo et al. (2013). There were no clear CO_2 effects on total Chl *a*, but small eukaryotic phytoplankton (<10 μm) were positively affected by CO_2 in the incubations that did not receive iron amendments. However, their low CO_2 treatments experienced a steady decline in DIC, so results are not easily compared to our observation of shifts toward small cells when DIC was held steady in both pre-industrial and future CO_2 treatments.

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

Non-monotonic effects on species-specific instantaneous growth rates and the potential for optimization are easy to envision demographically. For example, suppose mortality (d) exhibits a logit-linear increase over a given range of pCO2 (e.g., $d \sim 1/(1 + \exp[-f_1(pCO_2)])$) due to pH effects. If there is a linear enrichment effect on the rate of cell division (b) over that same range of pCO_2 (i.e., $b \sim f_2(pCO_2)$), 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 pCO_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 pCO_2 level (based on Fig. 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 pCO_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) pCO_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 pCO_2 levels, then experiments with only Present and Future pCO_2 levels (i.e., without Past levels) may be misleading. Moreover, despite considerable documentation of species-specific effects of pCO_2 manipulation on phytoplankton in the literature, the subtle and non-monotonic community responses to pCO_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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