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Carbonic anhydrase regulation of plankton community structure in estuarine systems

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ABSTRACT: Carbon concentrating mechanisms (CCMs) are used by phytoplankton to concentrate dissolved inorganic carbon within their cells for use in photosynthesis. However, CCMs which involve carbonic anhydrase (CA) may become redundant in the future due to increasing surface water dissolved CO_2 ($CO_{2(aq)}$) concentrations. Most of our knowledge of the CA enzyme is based on single-species phytoplankton cultures or oligotrophic water samples. Few studies have examined the consequences of CA activity on competitive interactions in estuarine phytoplankton communities or measured the long-term effects on community composition. Using bioassays of natural phytoplankton communities, we explored 2 different estuarine systems and determined how community composition was altered when the CA enzyme was removed. Using the CA inhibitor ethoxyzolamide (EZ), our results demonstrate that communities are altered when the inhibitor is present and CA activity is suppressed. Diatoms were the dominant taxonomic group in all samples following a 3 d exposure of the community to EZ. However, our findings suggest that diatom growth was both stimulated and inhibited, depending on the salinity of the location where samples were collected. Furthermore, microscopy of the high salinity phytoplankton community indicated that centric diatom genera (e.g. *Skeletonema*, *Rhizosolenia*) were severely reduced in treatments that removed the competitive advantage of CA, while pennate diatom genera (e.g. *Asterionellopsis*, *Cylindrotheca*) dominated these same treatments. These shifts in community structure suggest that phytoplankton composition is affected by carbon acquisition using CA, and some diatom genera may depend on the competitive advantage of this CCM to maintain high abundances in estuarine environments.

KEY WORDS: Carbon acquisition · Carbon concentrating mechanisms · Community structure · Diatoms · Estuary · Phytoplankton

INTRODUCTION

Plants and phytoplankton both require inorganic carbon (C) for fixation in the Calvin cycle during photosynthesis. However, all photosynthetic organisms face the complication of RUBISCO's non-specific affinity for CO_2 over oxygen. To better understand the efficiency of carbon fixation and its uptake rates in photoautotrophs, much focus has been placed on understanding the carbon concentrating mechanisms (CCMs) in which photosynthesizers overcome possible limitations in carbon acquisition (Beardall et al. 1998, Raven et al. 2017). These mechanisms involve the ubiquitous enzyme carbonic anhydrase (CA) which is found in both terrestrial (e.g. plant leaves, Gillon & Yakir 2001) and aquatic organisms (e.g. phytoplankton, Reinfelder 2011). However, compared to model plants (e.g. millets, maize, sugarcane, switchgrass, Brutnell et al. 2010), CCMs in marine phytoplankton have been studied to a much lesser degree (Hopkinson et al. 2011).

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Marine microalgal communities comprise a rich diversity of photosynthetic characteristics that may reflect selection for a competitive ability to acquire limiting resources such as inorganic carbon. This diversity produces assemblages with different resource acquisition strategies (Tilman et al. 1982). Marine phytoplankton can acquire inorganic C by means of 3 different methods: diffusion of dissolved CO_2 (hereafter referred to as '$CO_{2(aq)}$'), dehydration of bicarbonate (HCO_3^-) into $CO_{2(aq)}$, or direct uptake of bicarbonate across the plasma membrane of the cell. While all of these methods incorporate dissolved inorganic carbon (DIC) inside the cell, inorganic C must be in the form of $CO_{2(aq)}$ to be used by the RUBISCO enzyme (Falkowski & Raven 2007). Currently, at normal seawater pH (ca. 8.0), most of the DIC is in the form of bicarbonate. Some phytoplankton with small radii (e.g. <10 µm) may support high specific carbon fixation through the reaction–diffusion supply rate of CO_2 across their membrane (Reinfelder 2011). However, factors such as larger cell radii or high photosynthetic rates could cause phytoplankton to become 'C-limited.' This may be because maximal $CO_{2(aq)}$ diffusion rates are not sufficient to support realized photosynthetic rates or very low concentrations of $CO_{2(aq)}$ (Falkowski & Raven 2007). As mentioned above, to accumulate carbon effectively, marine phytoplankton have developed CCMs to overcome these potential limitations (Falkowski & Raven 2007, Raven et al. 2017).

In an aqueous environment, CCMs act to overcome the scarcity of bioavailable CO_2 by utilizing CA to catalyze the reversible dehydration of HCO_3^- to $CO_{2(aq)}$ (Rost et al. 2003). Two types of biophysical mechanisms are used by marine phytoplankton in which this process potentially raises the concentration of $CO_{2(aq)}$ at the cell surface or internally at the site of fixation (Riebesell et al. 1993). The first form equilibrates CO_2/HCO_3^- to make cell surface CO_2 concentrations equivalent to bulk CO_2 concentrations by CA-catalyzed dehydration of HCO_3^-. This conversion allows passive diffusion through the membrane. Without this external CA, CO_2 concentrations at the cell surface would be lower than the surrounding bulk water. The second form involves the transportation of HCO_3^- across the membrane and then conversion of that ion to CO_2 by internal CA. Reinfelder (2011) reviewed these mechanisms in the 3 dominant groups of eukaryotic marine phytoplankton and how the cost of CCMs may affect primary production, nutrient fluxes, and species composition. Mercado et al. (2009) demonstrated that taxonomic groups of phytoplankton differed in their ability to uptake and efficiently use bicarbonate. This evidence of variation in the functional trait of DIC uptake suggests that there is a capacity for affecting the competitive hierarchy in phytoplankton communities.

At current low surface water $CO_{2(aq)}$ concentrations, larger phytoplankton with efficient CCMs (e.g. diatoms) may have the competitive advantage over smaller phytoplankton with less efficient CCMs (e.g. dinoflagellates, coccolithophores) (Reinfelder 2011). With raised $CO_{2(aq)}$ concentrations, this same active, energy-consuming process might be disadvantageous to the larger phytoplankton due to its high metabolic cost. Therefore, if environmental change such as ocean acidification allows for higher surface water $CO_{2(aq)}$, a higher proportion of primary production might be attributed to smaller, low-efficiency CCM species (Beardall & Raven 2004) because of their ability to fix carbon through diffusion across the membrane. The absence of a high-energy process would give smaller phytoplankton the advantage. However, these predictions have been countered by indications that down-regulation of CCMs in elevated $CO_{2(aq)}$ conditions may give an energy benefit to the larger algal species such as diatoms (Hopkinson et al. 2011). A reduction in energetic cost of CCMs could yield energetic savings, and therefore afford a competitive advantage to larger phytoplankton, that could be directed toward growth and photosynthesis (Shi et al. 2017).

To date, very few studies have looked at CA mechanistic effects on competition in estuarine phytoplankton communities. While CA can perform other functions such as gas exchange in lungs or pH homeostasis in macroscopic organisms, CA function in phytoplankton is mainly in CCMs. Gaining a mechanistic understanding of the impact of carbon acquisition on plankton assemblages may provide invaluable insights into competitive interactions that may determine phytoplankton community structure. The ecological success of a species is affected by its ability to obtain crucial resources (e.g. inorganic carbon) and to optimize the use of those resources between growth and loss processes (Riebesell 2004). As a response, phytoplankton have developed different pathways to maximize growth and reproduction to increase their fitness. Identifying the variability of successful uptake mechanisms among species represents a means for understanding the maintenance of diversity within communities (Keeley 1999). For example, knowledge of trait distributions for maximizing biochemical rates of fixation (e.g. through different concentrations of photosynthetic enzymes)

among species can help us to anticipate interactions between planktonic organisms and the environment under a variety of changing climate scenarios (Gillon & Yakir 2001). The ecological differences of these functional traits can be used to identify the capacity for future adaptation (Stepien et al. 2016).

Current predictions are that marine waters will experience increasing acidification in coming years (Guinotte & Fabry 2008). The increased concentration of $CO_{2(aq)}$ will minimize the importance of CA as a competitive advantage for some phytoplankton species. The purpose of this research was to experimentally evaluate phytoplankton community responses to the inhibition of CA activity in 2 different phytoplankton assemblages from high and low salinity sites. The primary hypothesis is that the removal of the competitive advantage of using CA by some species will result in significant alterations in phytoplankton community structure. From this, we can gain a better understanding on how CA activity regulates community composition and cell size distributions.

MATERIALS AND METHODS

Study site

Two separate sites were used during this study to determine if the CA enzyme regulates the phytoplankton community assemblage: North Inlet and Winyah Bay in South Carolina (USA). North Inlet (hereafter referred to as the 'high salinity site') is a *Spartina alterniflora*-dominated system strongly influenced by tidal exchange with the ocean and is considered essentially undisturbed with minimal anthropogenic impacts (Allen et al. 2014). Winyah Bay (the 'low salinity site') is a brackish river-dominated estuary which is exposed to high input from surrounding rivers (i.e. Waccamaw, Sampit, Black, and Pee Dee) that form a watershed exposed to agricultural and industrial development (Allen et al. 2014). The high salinity site typically exhibits salinity ranges from 29 to 34, while the low salinity site exhibits salinity ranges from 0.6 to 8.4 (South Carolina Sea Grant Consortium 1992, Allen et al. 2014). Both estuaries have microalgal communities mostly composed of diatoms with variable contributions of cryptophytes, cyanobacteria, chlorophytes, euglenophytes, dinoflagellates, and prasinophytes (Lawrenz et al. 2010, 2013, Allen et al. 2014). Chlorophyll *a* (chl *a*) measurements for phytoplankton concentrations are typically 4–12 µg l^{-1} (Allen et al. 2014) at the high salinity site, while the low salinity site has chl *a* measurements that are more variable, ranging from 4–80 µg l^{-1} (Allen et al. 2014).

Collection and experimental design

Water was collected in 10 l carboys during high tide at 2 separate sites — the high salinity site at Clambank Landing (33.3340° N, 79.1929° W) and the low salinity site at the Georgetown Marina (33.3652° N, 79.2663° W). The high salinity site was sampled in 2016 during the months of April, May, June, August, October, and November and in January 2017. The low salinity site was sampled in 2016 during the months of August, September, October, and November, as well as in January 2017. Mean salinities at the time of water collection for these experiments were ca. 31 and 3 for Clambank Landing and Georgetown Marina, respectively. These water collections were then transported on ice back to the lab and dispensed into 250 ml clear polystyrene cell culture flasks (n = 20 $site^{-1}$). These flasks were divided into 2 separate nutrient exposure conditions in this experiment. These conditions were included to observe how the removal of CA changed community composition without the influence of macronutrient limitation. In the first condition, no nutrients were added (ambient nutrient conditions). One set of quintuplet flasks in this condition contained the un-amended natural community that was identified as the control after the incubation. In the second condition, nutrients from sodium nitrate ($NaNO_3$) and potassium phosphate (KH_2PO_4) were added to achieve concentrations of 20 µM N and 10 µM P (nutrient-replete). Silica was not added to these bioassays because our samples consisted of a diverse phytoplankton community and silica additions would have biased diatom growth. For each nutrient treatment, the CA inhibitor, ethoxyzolamide (EZ, Sigma Aldrich, cat. no. 333328-1G), was added to 1 set of quintuplets at 100 µmol l^{-1} concentration. Initial stock solutions of EZ were prepared in 0.05 M NaOH (Mercado et al. 1998). EZ is a commonly used inhibitor that penetrates the cell and inhibits both external and internal CA in all evolutionary distinct classes found in marine phytoplankton (Mercado et al. 1998, 2009, Tortell et al. 2000, Capasso & Supuran 2015, Wu et al. 2015). It is important to use an inhibitor that is cell permeable because natural communities of phytoplankton can have CA performing an essential role in CCMs in different cellular locations such as in the cell walls and periplasmic spaces

(external) and/or localized in the carboxysome, chloroplasts, or thylakoid lumen (internal) (DiMario et al. 2018).

These assays were then incubated for 72 h on a bench under 12 h light:dark conditions at 100 µmol photons m^{-2} s^{-1} supplied by a fluorescent light (91 cm, 4 × 39 W Ocean Light T5 Hood, 10 000 K, 39 W, TRU fluorescent lamps) at 23°C. The flasks were gently inverted twice a day to ensure mixing of the natural phytoplankton communities that had settled. Every 24 h, cell counts and size distributions were taken using a Guava EasyCyte Plus Flow Cytometer system. The 2 cell size fractions, <20 µm and >20 µm, were established using red fluorescence and forward angle light scatter intensity measured using flow cytometry. At the end of the incubation, subsamples were collected for microscopy and photopigment analysis from all treatments (i.e. control, nutrient addition, EZ addition, and EZ plus nutrient addition).

Analyses

Samples of 40 ml were collected and preserved using Lugol's iodine solution, from which a subsample was placed in a 10 ml chamber for 24 h and allowed to settle. Enumerations were made using an inverted light microscope (Nikon Eclipse TS100), from which 400 cells were counted and identified at 200 and 400× magnification (Lund et al. 1958). Identifications were limited to microphytoplankton (cells >20 µm).

For the photopigment analysis, both whole water and the <20 µm size fraction were gently filtered onto separate Whatman GF/F filter papers and stored at −80°C. Photopigments were identified and quantified using high performance liquid chromatography (HPLC) (Pinckney et al. 2001, 2017, Roy et al. 2011). Briefly, the photopigments were extracted using 90% acetone (0.75 ml) and then stored again at −20°C for 24 h. Filter extract (250 µl) was injected into a Shimadzu HPLC with a monomeric column (Rainin Microsorb-MV, 0.46 cm × 10 cm, 3 µm) and a polymeric (Vydac 201TP54, 0.46 cm × 25 cm, 5 µm) reverse-phase C18 column in series. The mobile phase was composed of the solvents, 80% methanol:20% 0.5 M ammonium acetate and 80% methanol:20% acetone (Pinckney et al. 1996). Finally, pigment peaks were identified by comparing retention times and absorption spectra with pure standards (DHI). A synthetic carotenoid β-apo-8′carotenal was used as an internal standard.

These pigment concentrations were then analyzed with the program ChemTax (v. 1.95) to determine the absolute abundance of major phytoplankton groups (in µg chl *a* l^{-1}) (Mackey et al. 1996, Higgins et al. 2011). The initial pigment ratio matrix was derived from 2 different coastal phytoplankton matrices (Schlüter et al. 2000, Lewitus et al. 2005). The convergence procedure outlined by Latasa (2007) was used to minimize errors in algal group biomass due to inaccurate pigment ratio seed values. Photopigments from each month's treatment and location were analyzed separately and provided the relative abundance of major algal groups (cyanobacteria, euglenophytes, chlorophytes, prasinophytes, dinoflagellates, cryptophytes, and diatoms).

The percent change was calculated using the following equation:

$$\%\ \text{Change} = \left(\frac{a_{\text{treatment}} - a_{\text{control}}}{a_{\text{control}}} \right) \times 100 \qquad (1)$$

where a_{control} and $a_{\text{treatment}}$ are the algal group abundances in the control and the corresponding EZ and/or nutrient treatment, respectively.

Statistical analyses of phytoplankton group abundances were analyzed using multivariate ANOVAs to determine differences in community composition due to the inhibition of the CA enzyme activity and/or addition of nutrients using IBM SPSS Statistics, v.24. Discriminant analyses were performed following the multivariate analysis to predict group membership based on the observed characteristics of each treatment relative to the control. Additionally, 1-way ANOVAs were used to determine if there were significant shifts in the proportion of size fractions of the phytoplankton cells between the treatments and corresponding controls.

RESULTS

Community composition response to CA inhibition

From the CHEMTAX analyses, photopigment concentrations for ca. 80–90% of the total phytoplankton community were composed of diatoms, euglenophytes, and cryptophytes at the high salinity site, and ca. 80–90% of the total phytoplankton community was composed of diatoms, chlorophytes, and cryptophytes at the low salinity site. Phytoplankton community compositions were significantly altered when CA was inhibited with EZ and/or with the addition of nutrients for both the high salinity site (Pillai's trace = 0.896, $F = 11.237$, $p < 0.001$) and the low salinity site (Pillai's trace = 0.756, $F = 5.822$, $p < 0.001$). Discriminant analysis indicated that community composition

differed between all treatments (i.e. nutrient and/or inhibitor addition) for the high salinity site (Fig. 1). The analysis plot for the low salinity site showed that community composition differed in treatments with and without the CA inhibitor, EZ (Fig. 2). Additionally, the algal group of diatoms had the greatest impact on predicting group membership for both sites.

At the high salinity sites, diatoms were the primary contributor of chl *a* in the total phytoplankton biomass for all bioassays (mean ± SE, 83.9 ± 4.6%). This response was similar at the low salinity site (88.0 ± 2.3%). In response to nutrient additions, phytoplankton, as a community, increased in abundance, demonstrating a high positive percent change relative to the control. However, individual algal groups demonstrated variable responses in percent change depending on treatment type at both the high (Fig. 3)

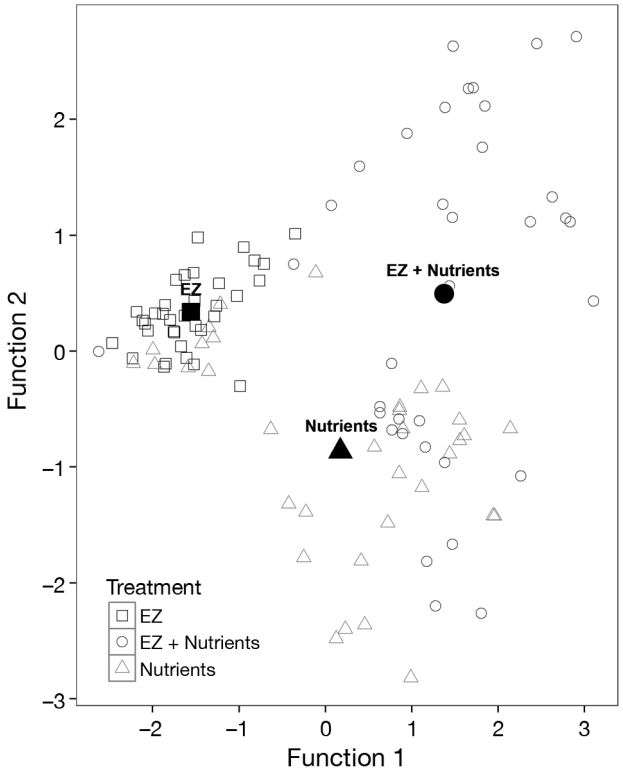

Fig. 1. Discriminant analysis plots for the percent change in the phytoplankton community relative to the control at the high salinity site. Individual points represent all replicates across all months. Black symbols indicate group centroids and are labeled with the 3 treatments of ethoxyzolamide (EZ) and/or nutrient additions. Matching shapes for individual points indicate group membership. Wilk's lambda = 0.294; $p < 0.001$; % variance explained = 100% by the first 2 functions; % classified correctly showing ability to classify membership = 71.4%

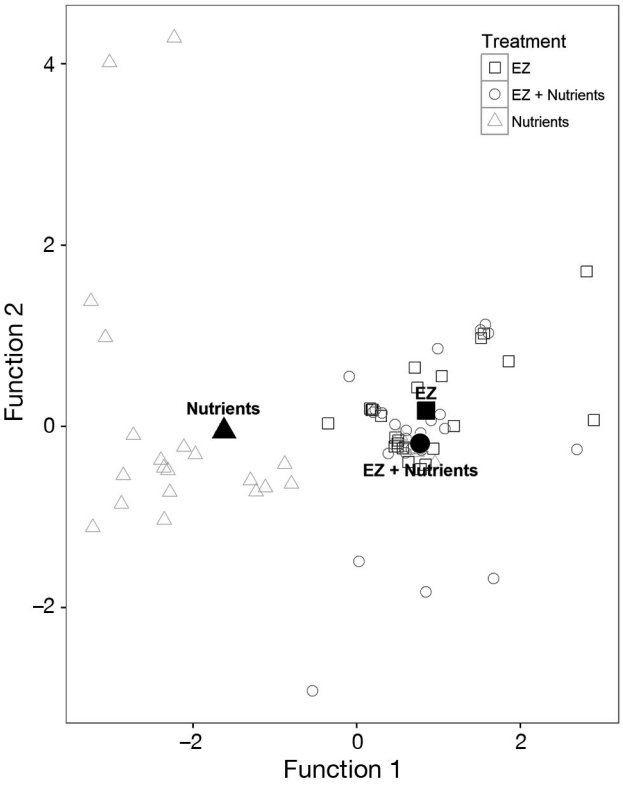

Fig. 2. As in Fig. 1, for the low salinity site. Wilk's lambda = 0.411; $p < 0.001$; % variance explained = 100% by the first 2 functions; % classified correctly showing ability to classify membership = 62.7%

and the low salinity site (Fig. 4). Due to their general low abundances, cryptophytes, dinoflagellates, prasinophytes, chlorophytes, euglenophytes, and cyanobacteria were excluded from further analysis.

Diatom growth response to CA inhibition

The treatments of inhibited CA activity and/or nutrient additions stimulated diatom abundance relative to the control at the high salinity site (Fig. 5). The percentage of stimulation varied across month but the principal response of increased quantity of diatoms was consistent across time and treatment. These results were contrasted in the low salinity site when the CA inhibitor, EZ, was present (Fig. 6; note different *y*-axis scale). Diatoms were inhibited across collection month with differing degrees of variation. The only exception of this general response was in August when the treatment with both EZ and nutrient additions demonstrated a 14.58 ± 10.66% (mean ± SE) positive increase relative to the control.

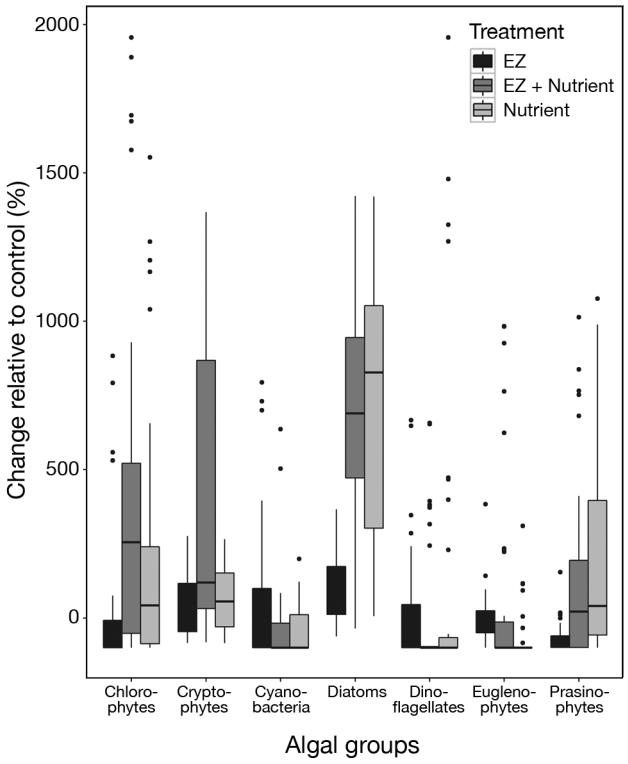

Fig. 3. Percent change for each algal group in the 3 treatments relative to the control at the high salinity site. The data represent all replicates across all months. These values are derived from ChemTax analyses. The horizontal line is the median, with the box including the upper and lower quartiles of the data. The whiskers encompass 95% of the data, and the individual data points indicate outliers. EZ: ethoxyzolamide

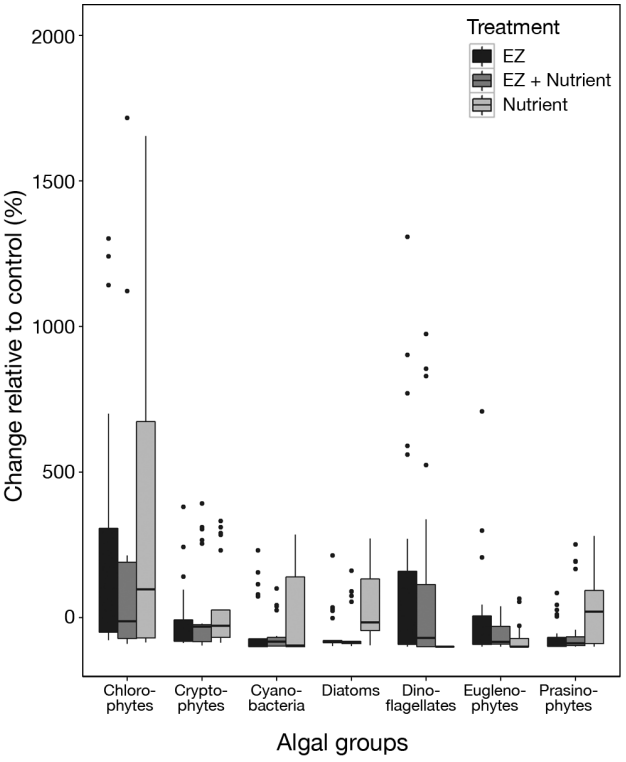

Fig. 4. As in Fig. 3, for the low salinity site

Cell size response to CA inhibition

Shifts in size fractions were examined using flow cytometry to determine if there was a shift in species composition. Relative to the control, shifts in cell size varied across month. For the high salinity site, the ambient nutrient condition assays containing the CA inhibitor had a significantly larger proportion of larger cells during April, June, and January and a significantly larger proportion of smaller cells during August (Fig. 7a, $p < 0.05$). When the treatments were nutrient-replete, the inhibitor assays had a significantly larger proportion of larger cells during November and January, and a significantly larger proportion of smaller cells during April, May, June, and August (Fig. 7b, $p < 0.05$).

The low salinity site demonstrated that size fractions shifted to significantly larger cells in the bioassays with the CA inhibitor relative to the control (Fig. 8a,b, nutrient ambient and nutrient-replete treatments, respectively). Similar trends in cell size shifts were seen in both nutrient treatments. Altogether, the collection months, except for October, had a significantly larger proportion of larger cells in the treatments inhibiting CA relative to the control ($p < 0.05$).

Qualitative microscopy response to CA inhibition

Due to visual constraints, only clearly identifiable diatoms were assigned to specific categories, while all other phytoplankton were assigned to the unknown cell category. The most common centric genera at the high salinity site were *Skeletonema*, *Rhizosolenia*, *Coscinodiscus*, *Chaetoceros*, and *Guinardia*. The most common pennate genera were *Thalassionema*, *Asterionellopsis*, *Pleurosigma*, and *Cylindrotheca*. Table 1 shows the results of 4 collection months across seasons. For all nutrient treatments and months except October, pennate diatoms exhibited stimulation in growth when CA was inhibited. Similarly, for centric diatoms, all nutrient treatments and months except April displayed inhibition when CA was inhibited. In control treatments, *Skeletonema*, *Guinardia*, and *Rhizosolenia* were the most abundant genera. In inhibitor treatments, the most

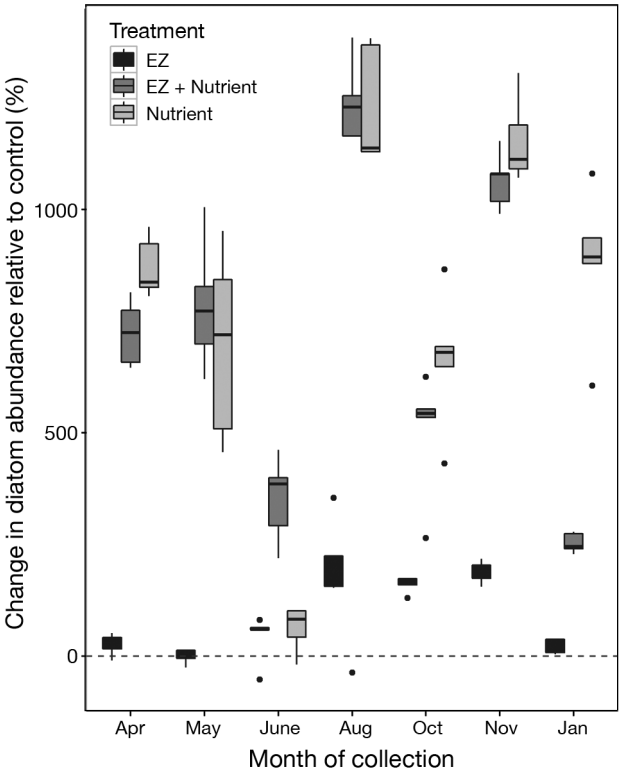

Fig. 5. Percent change for diatoms in the 3 treatments relative to the control at the high salinity site across collection months. These values are derived from ChemTax analyses. The dashed line represents no change. Box plot parameters as in Fig. 3

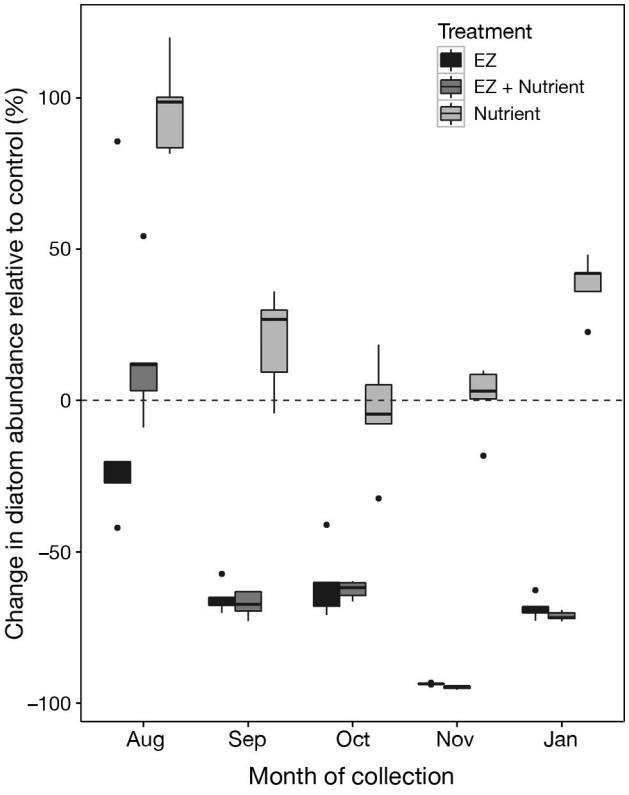

Fig. 6. As in Fig. 5, for the low salinity site

abundant genera became *Thalassionema* and *Asterionellopsis*. Likewise, *Cylindrotheca* increased in these treatments, but this genus was also larger in size (E. R. Knotts pers. obs.).

Microscopy of the samples from the high salinity site was limited to qualitative analysis because of constraints on identification. Out of the 400 cells counted and identified, only the diatom taxonomic groups were identified to genera. In the situation that identification was not possible, cells were categorized as unidentified cells <20 µm. These unidentified cells feasibly could have been attributed to the cryptophyte and chlorophyte taxonomic groups. No microscopy was completed for samples from the low salinity site because cell identification was inadequate based on cell sizes.

DISCUSSION

In this study, we investigated the impact of inhibiting CA activity on community composition and cell size in a natural phytoplankton assemblage. The 72 h incubations with inhibited CA altered the algal communities of both collection sites. Although there may be concern that CA inhibition alters physiological functions not accounted for (e.g. pH homeostasis),

Table 1. Effect of carbonic anhydrase inhibition on the 2 major groups of diatoms, i.e. centric diatoms (Coscinodiscophyceae) and pennate diatoms (Bacillariophyceae), at the high salinity site across 4 collections and 2 different nutrient conditions. '+': stimulation relative to the control, '−': inhibition relative to the control

Nutrient addition	Collection month/diatom group							
	April		August		October		January	
	Centric	Pennate	Centric	Pennate	Centric	Pennate	Centric	Pennate
None	+	+	−	+	−	−	−	+
20 µM N, 10 µM P	−	+	−	+	−	+	−	+

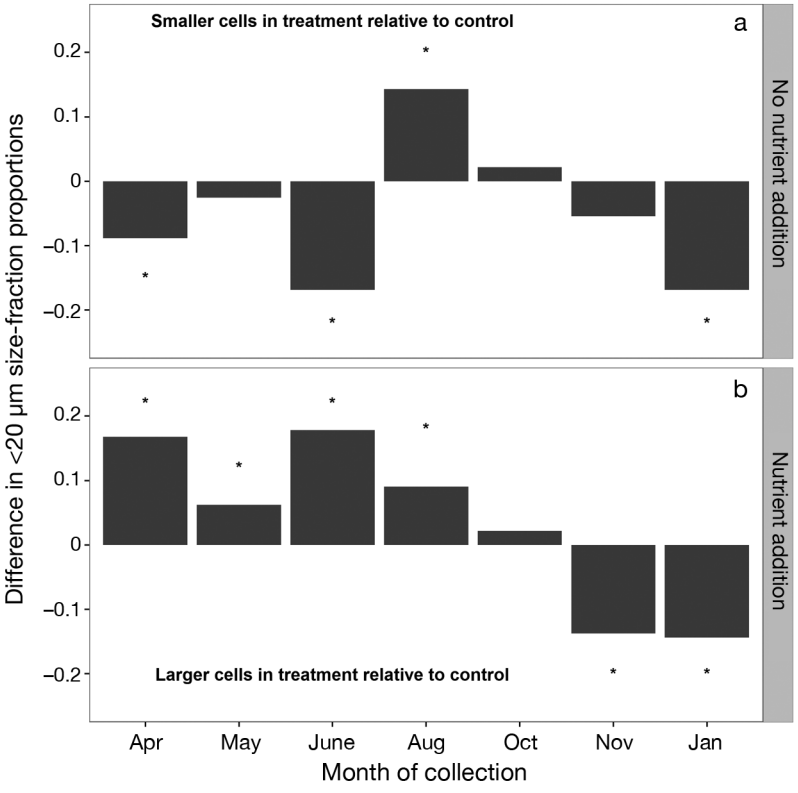

Fig. 7. Differences in the <20 µm cell size-fraction proportions between the inhibitor treatments and the controls at the high salinity site. If the difference was <0, there was a decreased proportion of smaller cells in the treatment incubation relative to the control, indicating larger cells in that treatment sample. Asterisks (*) represent a single ANOVA with $p < 0.05$

this concern is minor since in phytoplankton the most important function of CA is in CCMs. Previous studies have shown that community composition may not be significantly affected by varying levels of pCO_2 treatments (Tortell et al. 2000, Martin & Tortell 2006, Grear et al. 2017). However, Martin & Tortell (2006) commented that observing no large taxonomic differences of the dominant phytoplankton group in compositions containing diatoms, dinoflagellates, and nanoflagellates did not rule out species-level changes. Our results demonstrate that diatoms persistently have the competitive advantage in abundance, but there is variation in the amount of stimulation or inhibition that the taxonomic group experiences due to CA inhibition. Diatoms were mainly stimulated when CA was inhibited in samples from the high salinity site and were mainly inhibited in samples from the low salinity site. Regarding cell-size fractions, the general pattern of shifting toward larger cells when CA was inhibited was demonstrated in our experiments. However, at the high salinity site, cell size shifts seemed to be influenced by seasonal fluctuations. Finally, microscopic examinations of samples from the high salinity site showed a genus-level change when the competitive advantage of CA was removed. The overall implication of our study is that the competitive advantage of the CCM may influence phytoplankton community structure.

Community composition at the high salinity site, in terms of percent change relative to the control, was altered for each of the 3 treatments. The discriminant analysis grouped these treatments separately depending on what was added to each bioassay (Fig. 1). This indicates that both nutrients and CA actively structure the community and when levels of nitrate and phosphate or CA are adjusted, the community shifts as a response. For the bioassays collected from the low salinity site, the addition of EZ was the main determinant in shifting the community composition. Regardless of nutrient additions, the discriminant analysis grouped the 2 treatments containing the CA inhibitor together (Fig. 2). This result suggests that, despite nutrients shifting the community composition in one direction, active CA is important in determining community composition in another direction regardless of nutrient addition. The differences in high salinity vs. low salinity responses to the CA inhibitor may be related to the differences in DIC availability. Freshwater systems typically have low DIC, making inorganic carbon acquisition very important in low salinity sites, while seawater systems typically have much higher DIC levels (Clark & Flynn 2000, Oliveira et al. 2017). In April, the high salinity site had a DIC value of 1.995 mM (carbonate species: 1.904 mM HCO_3^-, 0.048 mM CO_2, 0.044 mM CO_3^{-2}) and the low salinity site had a DIC value of 0.327 mM (carbonate species: 0.308 mM HCO_3^-, 0.015 mM CO_2, 0.004 mM CO_3^{-2}). Therefore, CA may not be as important in high salinity estuaries as it is in low salinity estuaries.

Irrespective of stimulation or inhibition of abundances, diatoms remained the dominant taxonomic group at both collection sites in terms of biomass. This maintenance of dominance of the phytoplankton population is consistent with multiple studies that used elevated $CO_{2(aq)}$ manipulation to examine the response of phytoplankton communities to ocean

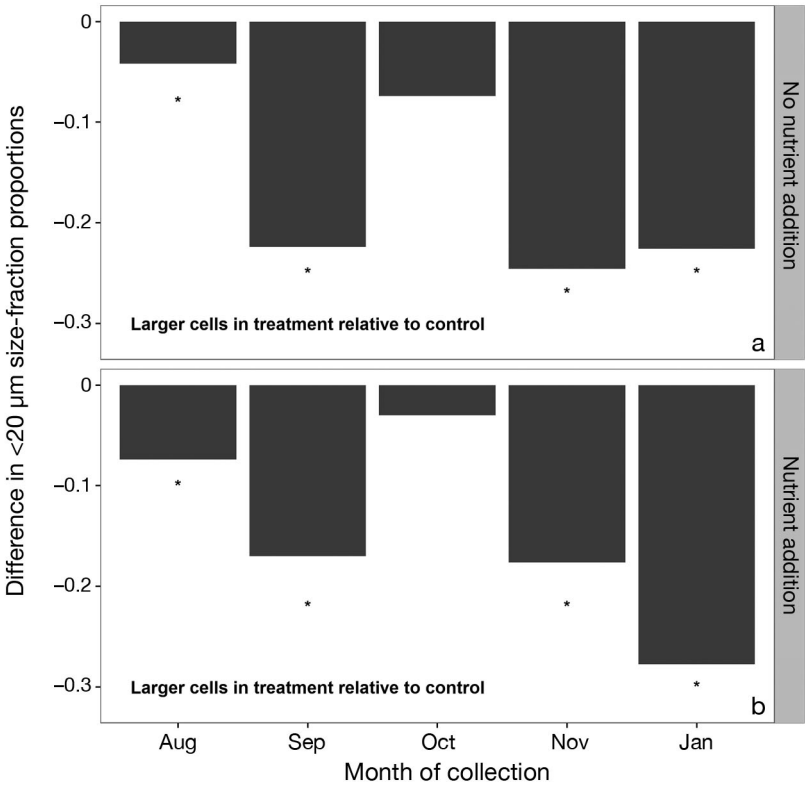

Fig. 8. As in Fig. 7, for the low salinity site

acidification (Tortell et al. 2008, Feng et al. 2009, Grear et al. 2017). The high abundance of diatoms is likely the main explanation why this taxonomic group has the greatest influence in predicting the group membership of communities between treatments. Therefore, we focused on this algal group to better understand the role CA played in structuring the community. This emphasis on diatoms has also been key for many culture studies measuring phytoplankton responses to elevated pH (Wu et al. 2015) and changes in pCO_2 (Shi et al. 2017), and characterization of CA activity (Satoh et al. 2001, Morant-Manceau et al. 2007, Martin & Tortell 2008). Diatoms are highly abundant, widely distributed, and are major primary producers accounting for ca. 40% of total marine primary production, thus contributing 20–25% of global net primary production (Nelson et al. 1995, Smetacek 1999, Finkel et al. 2010, Wu et al. 2015). This production plays a crucial role in carbon export and marine food webs.

High salinity site

Hopkinson et al. (2011) suggested that diatoms could allocate energy savings toward growth when CCMs are down-regulated. This down-regulation would free energy that is typically expended when transporting DIC into the cytoplasm and chloroplasts using these mechanisms. This is suggested in various studies that observed CCM down-regulation as a benefit in either cultured diatom species (Wu et al. 2010, Yang & Gao 2012, Trimborn et al. 2013, Shi et al. 2017) or natural populations (Johnson et al. 2013, Young et al. 2015) for optimizing resource allocation. Although our experiment did not down-regulate CCMs with elevated pCO_2, our results suggest that diatoms gained some energetic advantage at the high salinity site with stimulated abundance relative to the control. While the energy consumption in CCMs is mostly in HCO_3^- transport, possibly some energy and materials originally put into the construction of CAs might have been enough to benefit diatoms. It is important to note that, in general, when nutrients were included in the treatment that inhibited CA, there was also stimulation of small flagellates (i.e. cryptophytes, prasinophytes) along with chlorophytes (see Fig. 3). This increase in abundance of these algal groups exposed to EZ and nutrients suggests that small flagellates with lower nutrient uptake rates can successfully compete with diatoms in the community. For example, Lomas & Glibert (2000) demonstrated that cultured diatoms have greater nitrate uptake rate than flagellates. Under ambient nutrient conditions, diatoms have the physiological advantage for growth in low nutrient conditions such as the high salinity site. However, in nutrient-replete conditions, competition for nutrients may have been reduced, allowing cryptophytes and prasinophytes to flourish along with small chlorophytes.

Variation in the community composition of the phytoplankton in estuarine waters throughout the year is influenced by seasonal cycles, nutrient fluxes, and disturbances (Cloern & Jassby 2010). At North Inlet (i.e. the high salinity site), Lewitus et al. (1998) documented that, while diatoms comprise the greater part of the community throughout the year, diatoms are generally more dominant in the winter with occasional summer blooms of smaller nanoflagellates and picoplankton. Based on the starting community composition, differing abundances of diatoms relative to

other taxonomic groups may have influenced the variation seen in the amount of diatom stimulation across the monthly collections. However, since diatoms were consistently the most abundant taxon, with other taxa experiencing very low abundances, the majority of the variation could be connected to the disturbance we introduced to our treatments by inhibiting CA and/or adding nutrients triggering a shift in cell sizes. Overall, extreme stimulation of diatoms exhibited a shift in the cell size distribution toward a greater proportion of <20 µm cells. This shift in cell size toward smaller cells occurred during the spring and summer months when nutrients and DIC are rapidly depleted and smaller cells are more abundant. When abundance was lightly stimulated, cell sizes shifted toward a greater proportion of >20 µm cells. Yoshiyama & Klausmeier (2007) discussed how smaller cells should be more efficient at resource uptake primarily due to the greater surface:volume ratio and the diffusion limitation of resource transport. This concept is reversed when a high resource supply enables larger cells to become superior competitors. Perhaps high nutrient concentrations and DIC values free large cells from the constraint of surface:volume ratios and diffusion limitations. Further experiments in coastal waters should test whether seasonal fluctuations in resources influence the shift in cell size. We predict that when nutrients and DIC are in low concentrations, smaller cell sizes have the competitive advantage.

Flow cytometry determined cell size fractions using all algal groups as a collective and was not focused on diatoms as a specific group. Therefore, any comparison and conclusions drawn between diatom growth and cell-size shifts should be considered cautiously. Because the data from flow cytometry were limited, qualitative microscopy was used to determine if there were shifts occurring in the algal groups or in the diatom genera. When split into the categories of centric or pennate diatoms, the results showed a lower level community shift in the dominant diatom genera. Centric diatoms (e.g. *Skeletonema*, *Guinardia*, and *Rhizosolenia*) dominated control samples. This result suggests that they have a competitive advantage with an active CA enzyme. However, when CA was inhibited, pennate diatoms (e.g. *Asterionellopsis*, *Thalassionema*, and *Cylindrotheca*) became more abundant. These genera may not be dependent on CA activity to maintain a competitive advantage over other phytoplankton. Martin & Tortell (2008) demonstrated significant variability in CCM characteristics among phytoplankton species. Multiple culture studies have examined enzymatic activity to better understand which genera have the highest levels of, or the most efficient, enzymatic activity (4 genera: Morant-Manceau et al. 2007; 12 genera: Martin & Tortell 2008; *Thalassiosira*: Hopkinson et al. 2013, Wu et al. 2015, Shi et al. 2017). Although most studies have focused on diatoms because of high abundances and high efficiency CCMs, few of those investigations have studied pennate diatoms (*Cylindrotheca*: Hobson et al. 2001; *Asterionella, Pseudonitzschia, Cylindrotheca*: Martin & Tortell 2008). Further investigation is needed to determine why pennate diatoms such as *Asterionellopsis, Cylindrotheca*, and *Thalassionema* have an advantage over centric diatoms when CA is removed. We predict that these genera have higher RUBISCO content with lower K_ms for $CO_{2(aq)}$, which would require low $CO_{2(aq)}$ for saturation and therefore not require high resource allocation to CCMs (see Young et al. 2016).

Low salinity site

The phytoplankton community from the low salinity site responded differently to the inhibition of CA activity. In these assemblages, diatom abundances were inhibited across all treatments containing EZ and collection months except for the bioassay in August that contained the EZ and nutrient additions. It is important to note that the nutrient addition treatment also had a large increase in diatom abundance when compared to all other months. This may indicate that nutrients were lower during this collection period and the addition of nutrients benefited algal growth in general.

Reinfelder (2011) suggested that at low $CO_{2(aq)}$ concentrations, larger phytoplankton with efficient CCMs (e.g. diatoms) can outcompete smaller phytoplankton with less efficient CCMs because they have higher rates of carbon acquisition. As previously demonstrated in past studies (see Lawrenz et al. 2010, 2013, Pinckney et al. 2017), diatoms were a major fraction of the phytoplankton assemblage at this collection site. We show that the energy consuming process of CCMs was advantageous to commonly large phytoplankton and, with its removal, diatoms experienced strong inhibition. However, the inhibition of CA hindered the growth of all phytoplankton groups and therefore there was no major change in the higher-level taxonomic composition of the phytoplankton community. Nevertheless, the composition of the community shifted toward larger cell sizes relative to the control. Shi et al. (2017) suggested that the reduction in energetic cost of CCMs could yield

energetic savings that could be directed toward growth and photosynthesis. Cell size of individual taxa is expected to vary as a response to several environmental and biological factors (e.g. temperature, nutrient supply, cell cycle) (Barton et al. 2013, Svensson et al. 2014). Although no single algal group outcompeted the other, our results indicate that all resources were allocated towards increasing cellular size rather than a shift in community composition to species with larger cell sizes.

Falkowski & Oliver (2007) proposed that if the cells were exposed to high nutrient concentrations and had high maximum uptake rates for nutrients, the community would favor large cells. While the low salinity site contained high nutrient concentrations, it may be that lower DIC availability at this site maintained smaller cell sizes. This supports the concept put forward by Yoshiyama & Klausmeier (2007) that smaller cells should be more efficient at taking up resources like DIC. However, with the removal of CA and therefore the highly competitive diatoms, those small cells had the potential to grow into larger cells with slower nutrient uptake rates, but maintain strong competition due to high nutrient availability. This can be seen in the increased abundance of chlorophytes and dinoflagellates in treatments containing EZ (Fig. 4). Similar to Beardall & Raven (2004), our results imply that taxonomic groups with lower-efficiency CCMs did play a greater role in the community structure once the competitive advantage of CA was removed. Additionally, those cells that remained in our bioassays took advantage of the nutrients present to increase in size. This is evident in the larger proportion of >20 µm cells in our EZ treatments with and without nutrient additions relative to the control (Fig. 8).

CONCLUSION

Previous studies have emphasized the need to identify ecological variables that regulate phytoplankton community structure to better understand the environmental issues of modern stresses (Keeley 1999, Gillon & Yakir 2001, Noble et al. 2003, Stepien et al. 2016). Through recognition of carbon acquisition pathways such as CA, we can explain coexistence and dominance among taxonomic algal groups present in a community. Additionally, examining how CA functions to maintain community composition can help explain how carbon acquisition strategies may adapt in the future if environmental change encourages a shift in competitive resource acquisition processes. This research demonstrates that phytoplankton community composition is influenced by CA activity and that phytoplankton assemblages and cell size-classes respond to its removal. Future studies could examine whether this effect is removed with high pCO_2 or DIC controls to fully offset the loss of the CA aspect for CCMs. By identifying how the community assemblage is structured now and what changes could occur due to fluctuations in resources (e.g. surface water $CO_{2(aq)}$ concentrations), we can better understand and prepare for changes in the major processes (e.g. food web dynamics) that phytoplankton play a role in.

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