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Assessing the Competitive Advantage of Carbonic Anhydrase in Estuarine Microalgae Through Removed Enzymatic Activity

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ASSESSING THE COMPETITIVE ADVANTAGE OF CARBONIC ANHYDRASE IN ESTUARINE MICROALGAE THROUGH REMOVED ENZYMATIC ACTIVITY

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DEDICATION

This work is dedicated to my loving parents, Timothy and Bethelena, and my amazing siblings, Jake, Sierra, and Kira, without the support and encouragement of whom I would not be where I am today. Laughing with you all gave me the emotional strength needed to survive graduate school. I love you all.

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ABSTRACT

Carbon concentrating mechanisms (CCMs) are used by photoautotrophs to overcome possible limitations in carbon acquisition but the competitive strategies and efficiencies of these mechanisms among photosynthesizers can be variable. The diversity in carbon acquisition abilities establishes the potential for alterations in community structure with shifting carbon concentrations. Given the role of phytoplankton and benthic microalgae (BMA) in the trophodynamics of estuaries, understanding the mechanisms of carbon acquisition in these systems is important in predicting how primary productivity and nutrient cycling might change in response to increasing concentrations of atmospheric CO_2. Our approach to investigate whether induced carbon limitation would show predictable shifts in microalgal community structure and production was conducted through the inhibition of an enzyme used in CCMs, carbonic anhydrase (CA). CA catalyzes the rates of interconversion between CO_2 and HCO_3^- to facilitate transport of inorganic carbon into the cell and trap that carbon there. Although CA has the potential to help mitigate increasing CO_2 levels in the atmosphere, evaluations on how different species use CA and the physiological roles it may perform in microalgae are needed. We show phytoplankton communities from different environments are altered when a CA inhibitor (i.e. ethoxyzolamide, EZ) is present and CA activity is suppressed. Diatoms remained the dominant taxonomic group in all samples following a 3-day inhibition of CA but there were lower-level community shifts. 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 enzyme for their CCMs to maintain high abundances in estuarine environments. Most of the diatom genera had strong growth limitation and cell mortality without active CA, however, some pennate diatoms like *Cylindrotheca* persisted with positive growth rates. All four of our cultured diatoms experienced a decrease in gross primary production (GPP) and relative electron transport rate at high irradiance levels indicating that some other physiological traits were giving *Cylindrotheca* the competitive benefit. Decreased GPP was similarly observed in the BMA communities as well with CA inhibition. However, this limitation in carbon acquisition drove motile benthic microalgae to make use of a smaller vertical profile closer to the sediment surface rather than exhibit the mortality seen in most of our cultured diatom genera. Predicting marine microalgal responses to changes in CO_2 availability requires further characterization of other physiological traits across a higher diversity of growth conditions and taxa. Our research demonstrates that there can be wide variability in carbon acquisition strategies within the diatom genera and that the competitive advantage provided by CA and efficiency of their CCMs may be dependent on the environment's carbon availability. Continued mechanistic approaches are needed to recognize the impacts of CA activity on microalgal communities with respect to their assemblage, cell-size fractions, primary production rates, and physiological performance.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

Over the past century, anthropogenic activities have generated a large amount of carbon dioxide (CO_2) in the atmosphere bringing about the global climate change experienced today. As a result, questions regarding the carbon cycle (e.g. where CO_2 molecules are sequestered, how is it removed from the atmosphere) have become a priority. Most of the recent attention has been on marine organisms which play a critical role in the global carbon cycle via the biological carbon pump. The biological components of the ocean, specifically the microalgal (phytoplankton and benthic microalgae) members, play a major role in the sequestration of carbon, oxygen production, and other processes of nutrient and biogeochemical cycling. The photosynthetic ability of all microalgae allows for the consumption of CO_2 and release of oxygen and organic carbon to be passed to high consumers via the food web. Understanding the photosynthetic responses to increased CO_2 availability is required to ultimately predict future atmospheric CO_2 concentrations and changes in marine ecosystem services.

The carbonate system involves three main dissolved inorganic carbon (DIC) species, dissolved carbon dioxide ions ($CO_{2(aq)}$), bicarbonate ions (HCO_3^-), and carbonate ions (CO_3^{2-}). The term, ocean acidification, describes the event of increased uptake of

atmospheric CO_2 triggering a shift in the ocean's pH. Increased ocean acidity is caused by $CO_{2(aq)}$ ions reacting with water, some to remain as dissolved CO_2, while the rest form carbonic acid ions to then dissociate into HCO_3^- and hydrogen ions. Changes in this ocean chemistry with increased HCO_3^- and $CO_{2(aq)}$ concentrations, and decreased CO_3^{2-} ions can have many direct and indirect effects on marine organisms. These implications are still being documented, but there is substantial research demonstrating ecosystem impacts. While calcifying organisms experience the negative consequences associated with unsaturated carbonate concentrations, algae and seagrasses can benefit from increased CO_2 availability with higher photosynthetic activity.

Atmospheric CO_2 has been a driver shaping the history of algal evolution (Beerling 2012; Raven et al. 2012). In the past, CO_2 varied with a general downward trend, with lowest concentrations generally occurring during glaciation periods. Physiological adaptions in algae to these events provided different mechanisms to increase CO_2 assimilation in times of low CO_2 availability. These adaptations included higher CO_2 affinity by ribulose bisphosphate carboxylase-oxygenase (RubisCO) enzymes in the Calvin cycle of photosynthesis and carbon-concentrating mechanisms (CCMs) that provide a higher concentration of inorganic carbon in the compartment containing RubisCO than in the surrounding water, thereby increasing RubisCO's efficiency.

CCMs that have gained a considerable amount of attention recently. The mechanism's operation has an energetic cost to the organisms. Yet, elevated $CO_{2(aq)}$ can result in energetic savings associated with downregulation of the CCM (Wu et al. 2010; Gao et al. 2012). Most of the energetic costs of CCMs is associated with the active transport of HCO_3^- across the cellular membrane. However, carbonic anhydrase (CA) is

another enzyme that is built and maintained for use in CCMs. This enzyme readily speeds up the interconversion of CO_2 and HCO_3^- to facilitate transport of inorganic carbon into the cell and trap that carbon there for photosynthesis.

While microalgae use this enzyme for the physiological purpose of carbon acquisition, CA is ubiquitous in nature and can be found in all forms of life. It's essential role in facilitating the transport of CO_2 and protons across membranes provides a method for pH homeostasis, secretion of electrolytes, biosynthetic processes, and photosynthesis. This enzyme is grouped into seven genetically distinct families, named α-, β-, γ-, δ-, ζ-, η-, and θ-CAs, with different folds and structures but maintaining a common function in CO_2-HCO_3^- interconversion activity (Supuran and Capasso 2017). Among organisms, encoded CAs can range from belonging to only one family to two or more different genetic families. For example, Young and Hopkinson (2017) reviewed the diversity in CA expression and localization within different diatoms genera. *Thalassiosira pseudonana* had five different families of CAs spread throughout the cell's compartments while *Phaeodactylum tricornutum* had three different CAs localized internally.

As a major component of CCMs in microalgal species, CA's capacity to aid the fixation of atmosphere CO_2 and conversion to bicarbonate has gained great attention in recent years. According to Mondal et al. (2016), research into genetically modified algal strains for direct CO_2 bio-fixation and mass productivity has gained traction as a method for countering global warming. Recombinant CA has been used for converting atmospheric CO_2 into calcite for long term storage (Kim et al. 2012). However, understanding the functional role of CA needs more research before we can use these methods to mitigate increasing CO_2 levels in the atmosphere. Focus should be placed on

acquiring insights about how different species use CA in changing environmental conditions and the physiological roles it may have in these microalgae.

In this dissertation, we seek to contribute to the carbon acquisition literature through an examination of natural microalgal community responses to reduced carbon concentrating activity. We achieve this by inhibiting CA activity in our microalgae. Understanding the mechanisms of carbon acquisition and how they help structure microalgal communities in these systems is important if we are to predict how phytoplankton and benthic microalgae might react to future climate regimes. Primary productivity and nutrient cycling might change in response to increasing concentrations of atmospheric CO_2. However, the responses to higher CO_2 may be tied to environmental conditions and initial starting communities. Eggers et al. (2013) demonstrated how initial community composition had a greater impact than elevated CO_2 on phytoplankton biomass and final community. It is thus necessary to examine changes in phytoplankton community structure beyond physiological differences of carbon acquisition strategies in unialgal cultures. Instead, community-level investigations into structuring mechanisms are needed that consider properties like initial composition and competition.

This dissertation furthers our understanding of the competitive interactions via the physiological traits of active CA in different phytoplankton groups, as well as community structuring processes in microalgal communities both in the water column and sediment. It does so by focusing on the following:

CHAPTER 2 explores how CA structures the communities of a high and low salinity site. Specifically, this chapter investigates how important active CA is to maintain the community structure and cell size distribution. We examine whether specific

carbon acquisition strategies may be more important in sites that experience different levels of limited DIC. Mercado et al. (2009) demonstrated that taxonomic groups of phytoplankton differed in their ability to uptake and efficiently use bicarbonate. Variability in CA dependence in CCMs as a carbon acquisition strategy are explored through bioassay observations on community composition and size. Further, insight on how this enzyme regulates lower-level community shifts is established.

CHAPTER 3 investigates how benthic microalgal communities use CA to maintain vertical depth profiles, productivity, and biomass. It does so by exploring the impacts of removed CA activity on the oxygen production activity throughout the sediment's top 1 mm. By comparing the vertical production profiles between the control and CA inhibited treatment, this study establishes the importance of active CA for larger vertical profiles and higher productivity.

CHAPTER 4 reviews the carbon acquisition literature that addressed the gaps identified by Tortell (2000) review of the evolutionary and ecological perspectives on carbon acquisition in phytoplankton. Specifically, we evaluate how resource limitation (e.g. nitrogen, phosphorus, and iron) impacts CCM activity and how changing CO_2 concentrations shape species composition and succession of phytoplankton due to competition. We also examine the difficulties of predicting species dominance hierarchies using monoculture assays since these unialgal communities are commonly used to determine the physiological responses of CCMs to CO_2. Finally, we explore how controlling initial communities in microcosm studies that allow for interactions might be useful as a standard procedure in future investigations.

CHAPTER 5 expands on the importance of initial community composition explored in CHAPTER 4 through controlled microcosms of unialgal cultures, pairwise mixtures, and full community assemblages. Our methodology was adapted from Low-Décarie et al. (2011) and Pardew et al. (2018). This study establishes the differences in carbon acquisition strategies among a group of similar diatoms. We investigate whether CA activity is crucial for carbon uptake and growth in four diatom genera across two functional shapes. These genera were selected based on the results from CHAPTER 2. Our work helps determine whether these diatoms are dependent on CA activity to maintain a competitive advantage or whether other processes controlled the shift in community structure. We also examine other physiological processes that are impacted by CA inhibition such as oxygen production and electron transport rates.

CHAPTER 6 serves as a general conclusion to the dissertation.

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CHAPTER 2

CARBONIC ANHYDRASE REGULATION OF PLANKTON COMMUNITY STRUCTURE IN ESTUARINE SYSTEMS[1]

[1] Knotts ER, Pinckney JL. (2018). Carbonic anhydrase regulation of phytoplankton community structure in estuarine systems. Aquatic Microbial Ecology 82(1), 73-85. doi:10.3354/ame01879

2.1 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.

2.2 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).

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 re source 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 com munities.

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.

2.3 METHODS

2.3.1 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 in fluenced 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 $\mu g\ l^{-1}$ (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 $\mu g\ l^{-1}$ (Allen et al. 2014).

2.3.2 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, 10000 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).

2.3.3. 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:

$$\% \, Change = \left(\frac{a_{treatment} - a_{control}}{a_{control}}\right) * 100 \quad (1)$$

where $a_{control}$ and $a_{treatment}$ 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.

2.4 RESULTS

2.4.1 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. 2.1). The analysis plot for the low salinity site showed that community composition differed in treatments with and without the CA inhibitor, EZ (Fig. 2.2). 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. 2.3) and the low salinity site (Fig. 2.4). Due to their general low abundances, cryptophytes, dinoflagellates, pra sino phytes, chlorophytes, euglenophytes, and cyanobacteria were excluded from further analysis.

2.4.2 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. 2.5). 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. 2.6; 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.

2.4.3 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. 2.7a, 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. 2.7b, $p < 0.05$).

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. 2.8a,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 ($p< 0.05$).

2.4.4 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 un known 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 2.1 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 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.

2.5 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), 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 pCO2 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 de pending on what was added to each bioassay (Fig. 2.1). 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. 2.2). 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 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.

2.5.1 HIGH SALINITY SITE

Hopkinson et al. (2011) suggested that diatoms could allocate energy savings toward growth when CCMs are down-regulated. This downregulation 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. 2.3). 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. 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: MorantManceau 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 re moved. 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).

2.5.2 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. 2.4). 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. 2.8).

2.5.3 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 $CO2_{(aq)}$ concentrations), we can better understand and prepare for changes in the major processes (e.g. food web dynamics) that phytoplankton play a role in.

2.6 ACKNOWLEDGEMENTS

We thank the Baruch Marine Field Lab for access to the North Inlet Estuary; K. N. Demitrus, J. P. Everhart, and A. Ripley for their assistance with the bioassays and with photopigment analyses; and B. Hopkinson for proving valuable feedback and comments during the preparation of this manuscript. This is publication #1866 from the Belle W. Baruch Institute for Marine and Coastal Sciences. We declare that we have no conflicts of interest.

2.7 TABLES

Table 2.1: 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	-	+	-	+	-	+	-	+

2.8 FIGURES

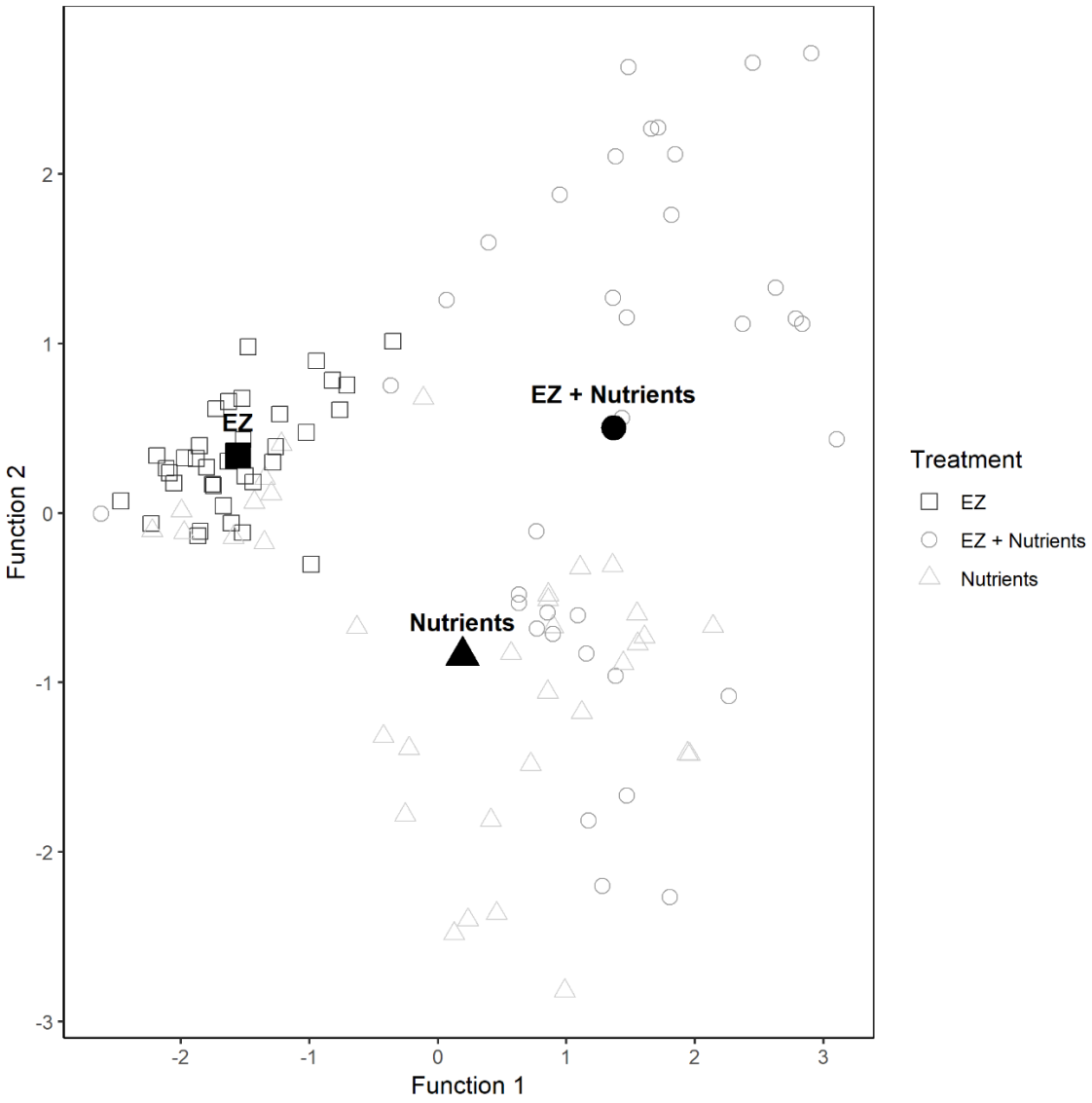

Figure 2.1: Discriminant analysis plots for the percent change in the phytoplankton community relative to the control at the high salinity site. Individual points represent all replicates across all months. Black symbols indicate group centroids and are labeled with the 3 treatments of ethoxyzolamide (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%

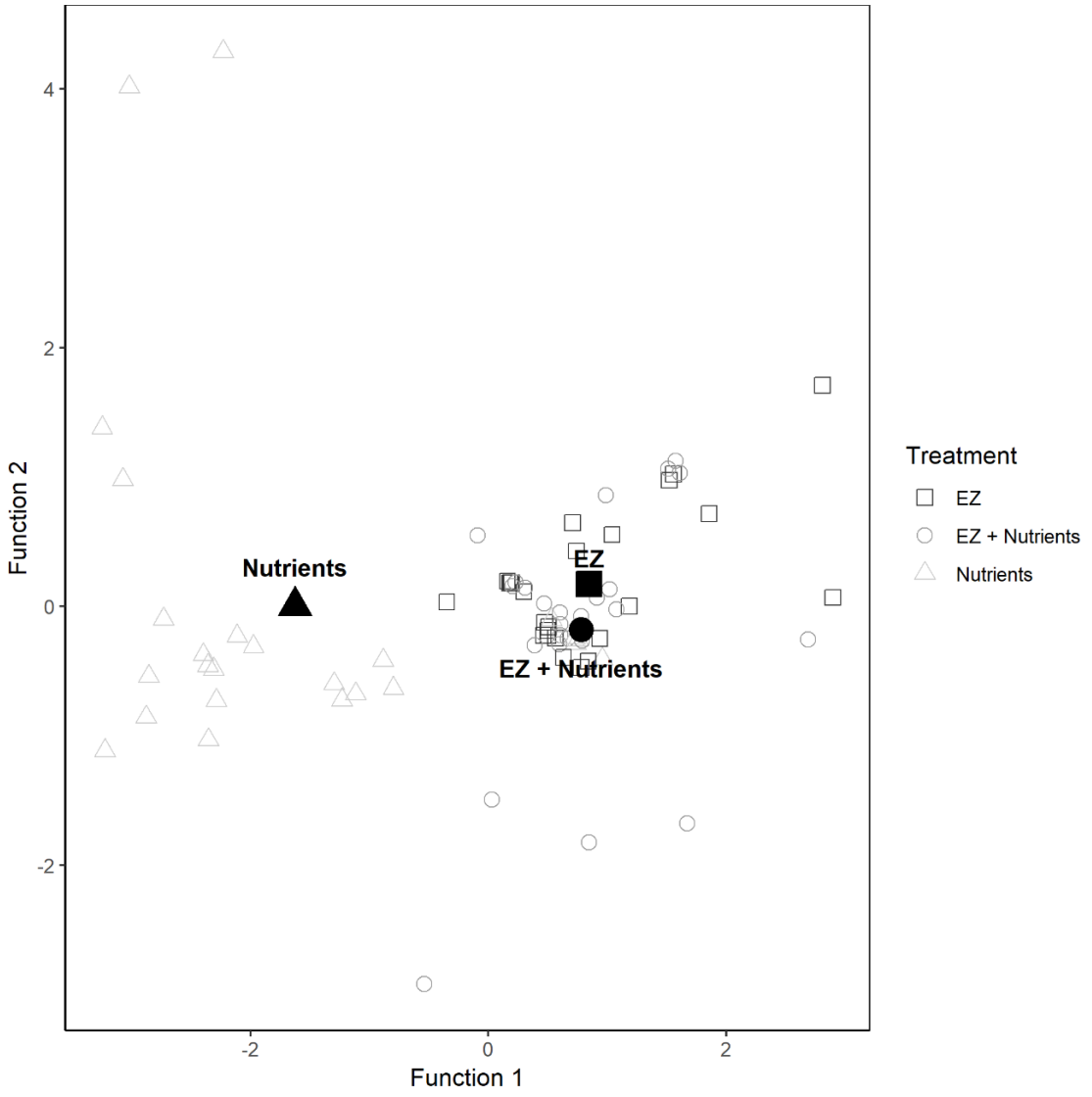

Figure 2.2: As in Fig. 2.1, for the low salinity site. Wilk's lambda = 0.411; $p < 0.001$; % variance explained = 100% by the first 2 functions; % classified correctly showing ability to classify membership = 62.7%

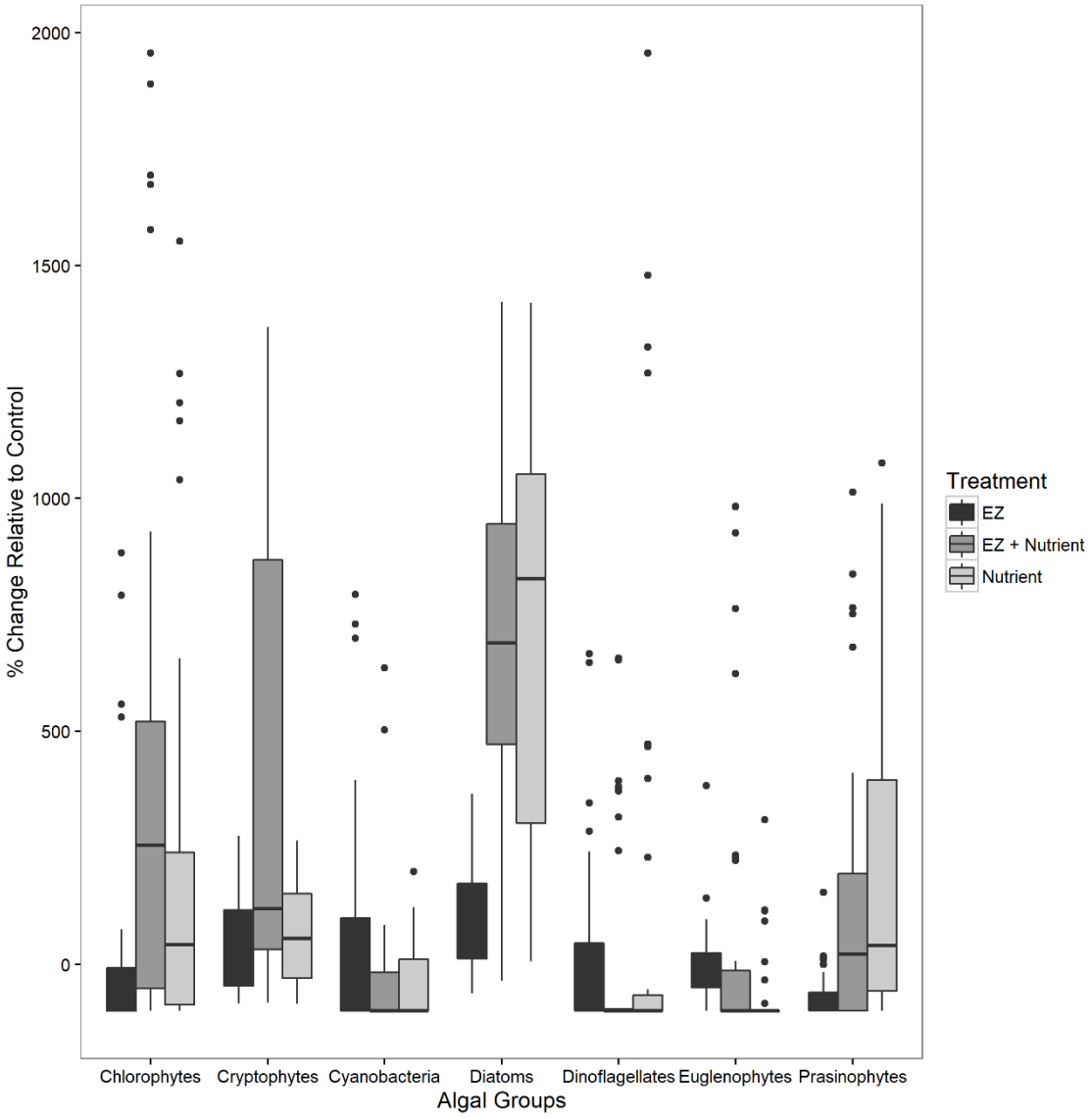

Figure 2.3: Percent change for each algal group in the 3 treatments relative to the control at the high salinity site. The data represent all replicates across all months. These values are derived from ChemTax analyses. The horizontal line is the median, with the box including the upper and lower quartiles of the data. The whiskers encompass 95% of the data, and the individual data points indicate outliers. EZ:ethoxyzolamide

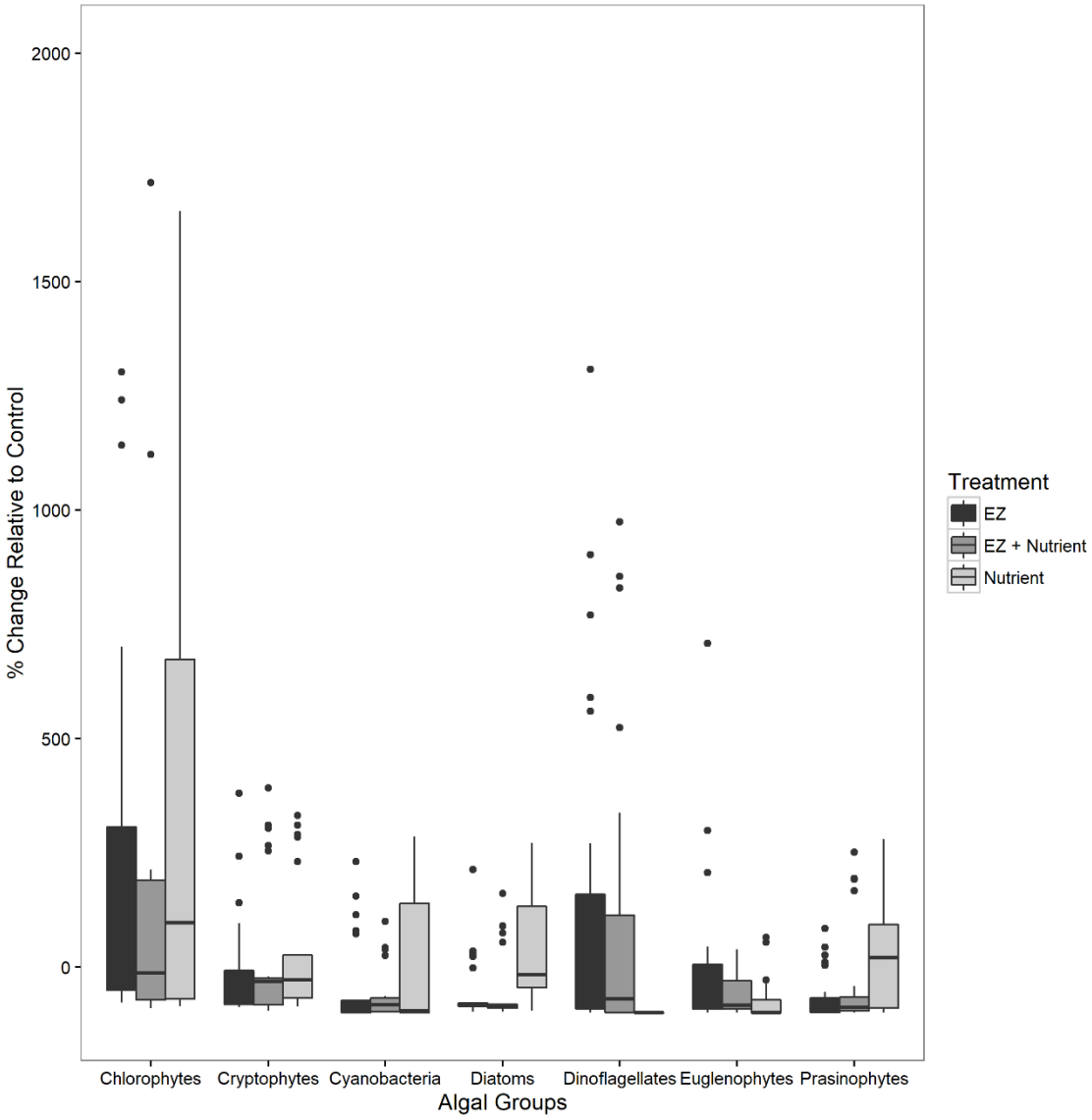

Figure 2.4: As in Fig. 2.3, for the low salinity site

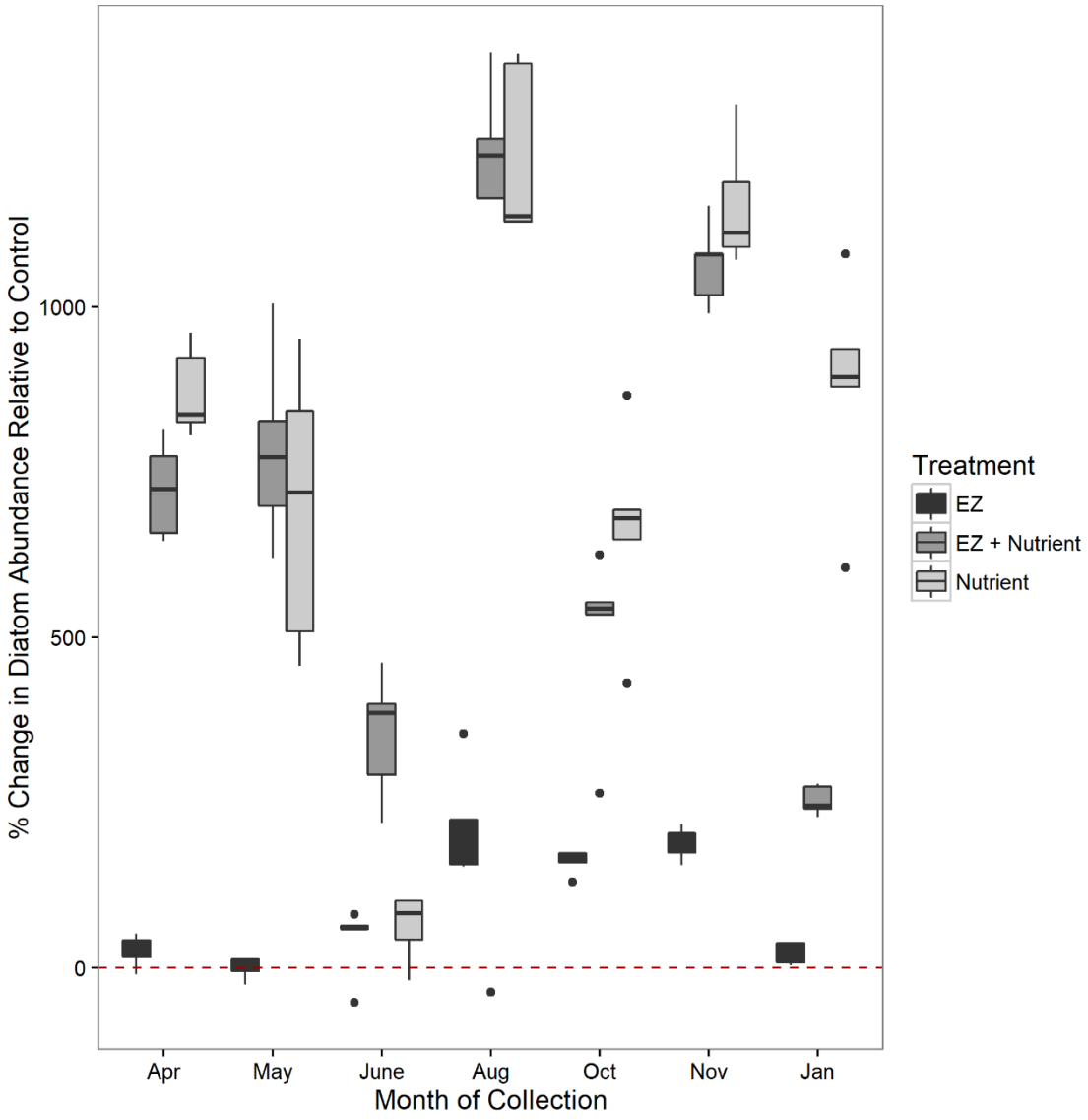

Figure 2.5: Percent change for diatoms in the 3 treatments relative to the control at the high salinity site across collection months. These values are derived from ChemTax analyses. The dashed line represents no change. Box plot parameters as in Fig. 2.3

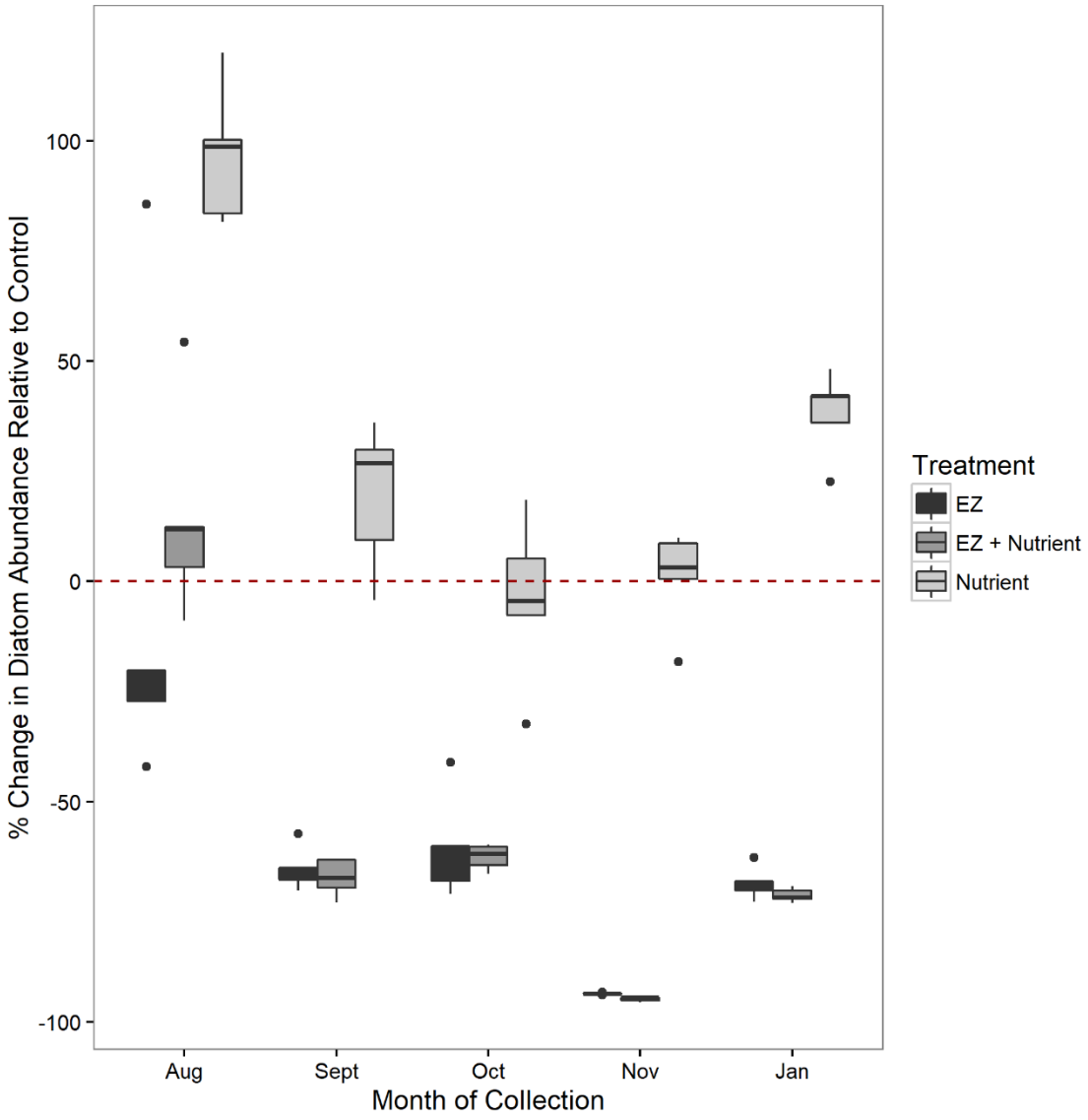

Figure 2.6: As in Fig. 2.5, for the low salinity site

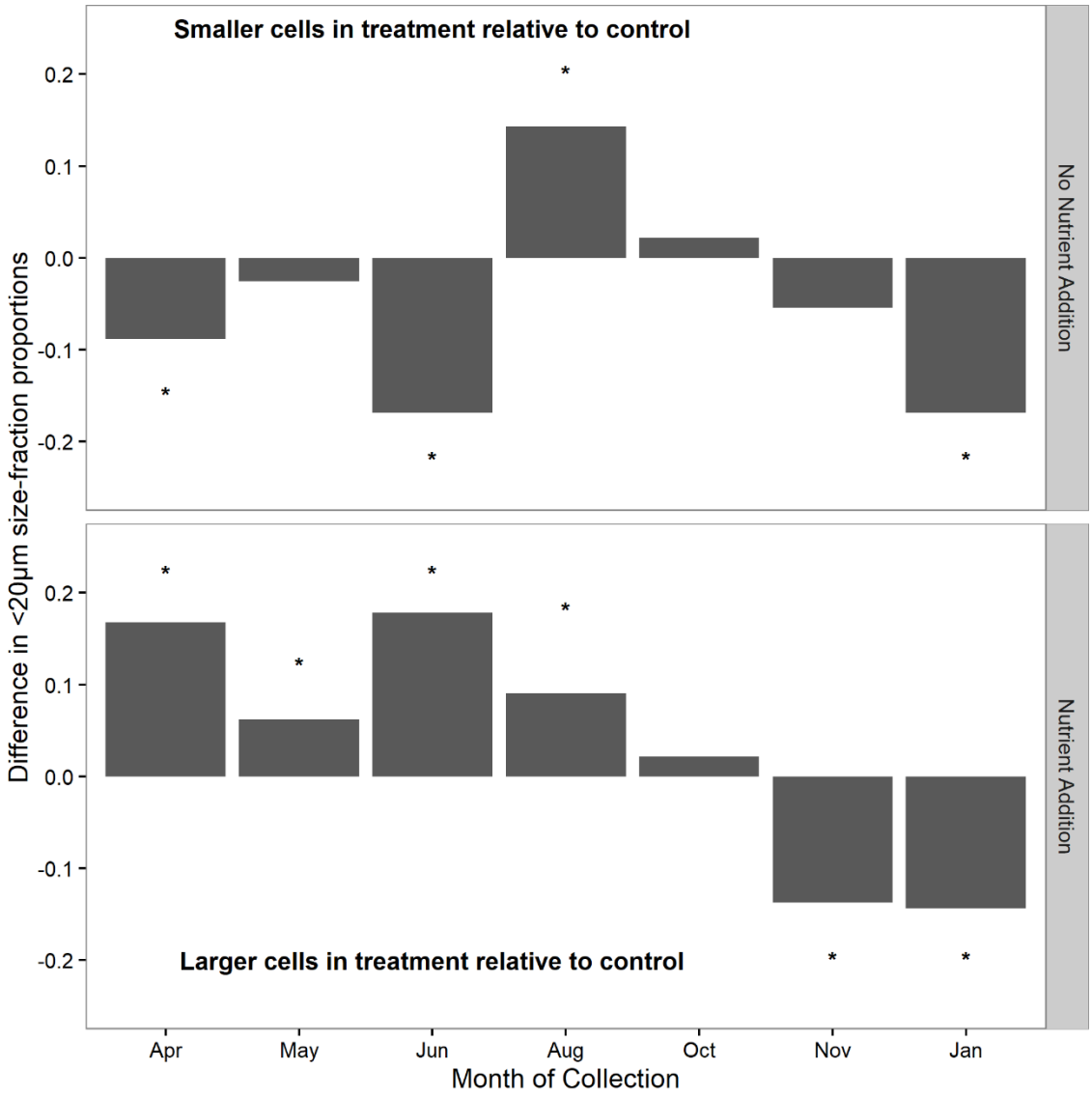

Figure 2.7: Differences in the <20 µm cell size-fraction proportions between the inhibitor treatments and the controls at the high salinity site. If the difference was <0, there was a decreased proportion of smaller cells in the treatment incubation relative to the control, indicating larger cells in that treatment sample. Asterisks (*) represent a single ANOVA with $p < 0.05$

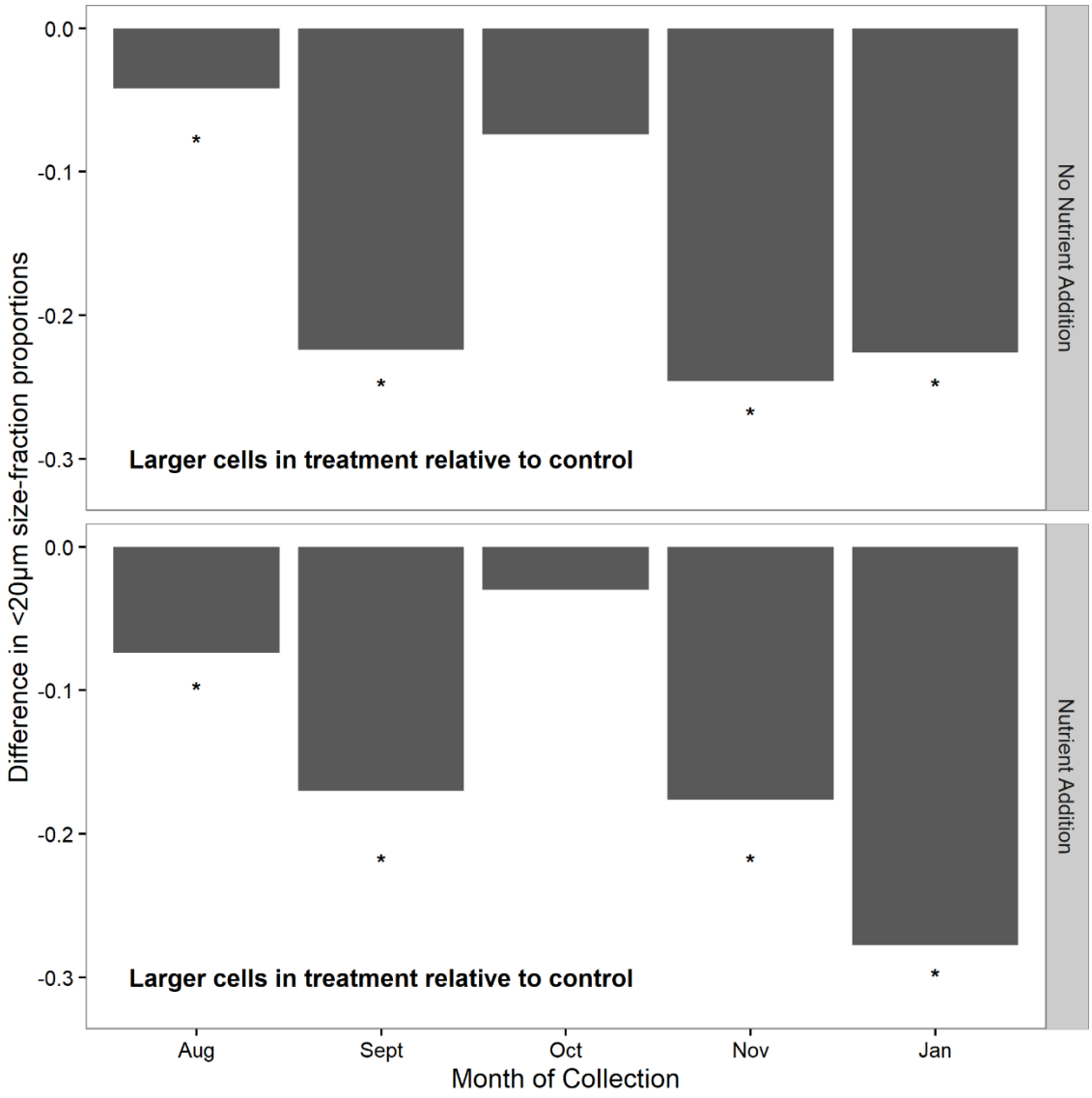

Figure 2.8: As in Fig. 2.7, for the low salinity site

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CHAPTER 3

EFFECTS OF CARBONIC ANHYDRASE INHIBITION ON BIOMASS AND PRIMARY PRODUCTION OF ESTUARINE BENTHIC MICROALGAL COMMUNITIES[1]

[1] Knotts, E. R., & Pinckney, J. L. (2019). Effects of carbonic anhydrase inhibition on biomass and primary production of estuarine benthic microalgal communities. Journal of Experimental Marine Biology and Ecology, 518, 151179. doi:10.1016/j.jembe.2019.151179
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3.1 ABSTRACT

Recent studies have focused on carbon concentrating mechanisms and the associated enzyme, carbonic anhydrase, to better understand the efficiency of CO_2uptake rates and carbon fixation in photoautotrophs. Some benthic microalgae (BMA) may be limited by inorganic carbon availability because high photosynthetic rates withdraw a large amount of CO_2 and HCO_3^- in the top layer of sediment. Investigating the mechanisms that affect carbon acquisition are necessary if we are to fully understand the functioning and structuring processes of these systems. From this, we can better predict the potential impacts of increasing atmospheric CO_2 concentrations on BMA communities. The purpose of this research was to examine a carbon concentrating mechanism used by BMA through their responses to induced carbon limitation. This approach was conducted through the removal of carbonic anhydrase (CA) activity using an inhibitor, ethoxyzolamide. Microcosm experiments were performed on intertidal muddy sediments from North Inlet Estuary, SC. Exposure to ethoxyzolamide resulted in a reduction of gross primary productivity (GPP) without a reduction in total BMA biomass. Furthermore, removed CA activity caused BMA cumulative GPP maxima to shift upward toward the surface in the sediment column. Active CA was necessary to maintain high GPP rates in these communities and allowed motile BMA to use a wider portion of the sediment column. Available HCO_3^- at lower depths could still be dehydrated into CO_2 by microalgae with CA. Changes in global atmospheric CO_2 concentrations leading to higher CO_2 availability at the atmosphere-sediment interface may alter the structure and function of these BMA systems, and the vertical distribution of GPP. These consequences may have important implications for the biogeochemical cycling occurring in estuaries.

3.2 INTRODUCTION

Marine photoautotrophic communities contain a range of taxa with different strategies and competitive abilities to acquire limiting resources. While most studies have focused on marine phytoplankton (Falkowski and Raven, 2007; Mercado et al., 2009), these competitive abilities are also expected to be employed in benthic microalgae and benthic diatom communities (hereafter referred to as 'BMA') (Raven et al., 2012). Within estuarine BMA communities, benthic diatoms, chlorophytes, and cyanobacteria are the main competitors that inhabit these sedimentary systems (Pinckney et al., 1994; Pinckney and Zingmark, 1993a; Underwood and Kromkamp, 1999). Benthic diatoms are usually the most abundant group and can be further split into two growth forms. Epipsammic benthic diatoms are generally attached to sediment grains and do not show migratory patterns, whereas epipelic benthic diatoms are highly-motile free-living forms that exhibit vertical migratory rhythms in the sediment column, and therefore may have a competitive advantage for resource (e.g. carbon, nutrients, light) acquisition (Barnett et al., 2015; Cartaxana et al. 2016; Consalvey et al., 2004; de Brouwer and Stal, 2001; Saburova and Polikarpov, 2003). In the North Inlet Estuary, both migrating and non-migrating constituents exist in the BMA community. Pinckney et al. (1994) found ca. 33% of the microalgal community were epipelic diatoms while the remaining 66% were epipsammic.

Regardless of whether the BMA are motile or attached, photosynthetic activity is restricted in estuarine muddy sediments to the top ca. 400 μm or less where 90% of the light is attenuated (Consalvey et al., 2004; Lassen et al., 1992). However, in that top layer, BMA vertical distributions may be affected by multiple factors due to the extreme

conditions these sedimentary environments experience. These conditions can involve sharp gradients in temperature and light (Consalvey et al., 2004; Pinckney et al., 1994; Pinckney and Zingmark, 1991), variable high pH levels (e.g. 7.3 to 11 due to photosynthetic CO_2 uptake and respiration) (de Jong et al., 1988; Ludden et al., 1985; Revsbech and Jørgensen, 1986) and limited carbon availability (Admiraal et al., 1982; da Silva et al., 2017; de Jong et al., 1988; Ludden et al., 1985).

There is high dissolved inorganic carbon (DIC) limitation in these benthic sediment systems (Ludden et al., 1985; Vieira et al., 2016). Current evidence suggests that there is a strong gradient of DIC availability with low concentrations at the surface and increasing concentrations with depth created by microbial respiration (Komada et al., 1998). This gradient was predicted because high light supply in the top photic layer would allow for high rates of photosynthesis, depleting the available DIC at the surface. Ludden et al. (1985) modeled the relationship between the diffusion of oxygen and inorganic carbon into the sediment and the carbon metabolism of BMA. The model predicted that the upper limitations in productivity and biomass were caused by oxygen accumulation and depletion of DIC. Higher CO_2 uptake rates are associated with higher production when microalgal films are visible at the sediment surface (Kristensen and Alongi, 2006). Those results together suggest that DIC is depleted in the top photic layer of the sediment by BMA photosynthetic activity. With higher DIC availability, higher production and biomass would be projected. Vieira et al. (2016) demonstrated that, when a surplus of DIC was supplied, photosynthetic capacity of the BMA significantly increased. Elevated DIC potentially could lead to higher biomass. Chen et al. (2019) confirmed that microphytobenthos affect the sediment to atmosphere CO_2 flux

offsetting the sediment carbon dioxide emission and turning the sediment into a carbon sink. That flux was significantly correlated to the Chlorophyll *a* concentration and diatom density. If we are to predict potential changes in community structure and production due to increasing concentrations of atmospheric CO_2, understanding DIC availability and the carbon acquisition strategies of BMA is crucial.

BMA are dependent on the supply of CO_2 for photosynthesis, growth, and division. In seawater, HCO_3^- is the predominant form of available DIC, but $CO_{2(aq)}$ is the necessary form used in the Calvin cycle by ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) (Lines and Beardall, 2018; Riebesell et al., 1993; Young et al., 2016). With a low diffusive coefficient (ca. $2.02 \pm 0.19 \times 10^{-9}$ m^{-2} s^{-1}) (Zeebe, 2011), $CO_{2(aq)}$ only accounts for ~1% of total inorganic carbon available in seawater. This limitation of CO_2 availability is even greater in muddy sediments, where high concentrations of CO_2-consuming photosynthetic cells occur. There is a thick diffusion boundary layer (ca. 1 mm) that induces low diffusion rates of $CO_2$from the bulk water medium to the cells (da Silva et al., 2017; Ludden et al., 1985; Raven et al., 2008; Raven et al., 2012). To overcome this limitation in carbon acquisition, marine photoautotrophs, including BMA, have evolved carbon concentrating mechanisms (CCMs) (Beardall et al., 1998; Raven et al., 2017). The biophysical mechanism of CCMs is based on the operation of membrane transporters with the enzyme carbonic anhydrase (CA). The process is used to increase concentrations of CO_2 internally at the site of carbon fixation (Burkhardt et al., 2001; Reinfelder, 2011; Zhang et al., 2014). Cyanobacteria has internal CA that is always localized to the carboxysome and other

eukaryotic phytoplankton have internal CA found in the chloroplast or thylakoid lumen (DiMario et al., 2018).

While there are some studies on the effects of environmental variability on CCMs in planktonic ecosystems, there are exceedingly few studies examining the mechanistic effect of CA in BMA communities although this carbon acquisition process is expected to be important. BMA communities provide an easily accessible and highly nutritious food source for microalgal grazers (Currin et al., 1995; Decho, 1990; Pinckney et al., 1994; Pinckney, 2018; Sullivan and Moncreiff, 1990). If different species-specific strategies for carbon acquisition are employed by different members of BMA communities, impacts to the effectiveness of strategies may affect their high productivity, biomass, and community structure, therefore, altering ecosystem function in intertidal muddy habitats of estuaries.

Studies investigating the interactions of climate change variables and autotrophic productivity are severely lacking in BMA communities that already experience high stress environments with drastically changing pH, water content, and carbon content in surrounding sediment on time scales of minutes to hours (da Silva et al., 2017; Johnson et al., 2013; Ludden et al., 1985). Given the experimental evidence for DIC limitation of photosynthesis in highly productive intertidal BMA communities (Cook and Roy, 2006; Ludden et al., 1985; Vieira et al., 2016), follow up with experiments investigating the role of CCMs and the enzymes involved (e.g. CA) in these communities are well-justified. For example, having efficient enzymes and CCMs may be more important to attached epipsammic benthic microalgae that are constrained to lower DIC conditions while epipelic microalgae might migrate lower in the sediment column to obtain sources

of higher DIC created by microbial respiration (da Silva et al., 2017). Several studies have demonstrated that increased CO_2 availability enhances BMA biomass and photosynthesis (Baragi and Anil, 2016; Cartaxana et al., 2015; Johnson et al., 2013), but a mechanistic demonstration of this result is lacking. In contrast, Hicks et al. (2011) reported no change in primary productivity with exposure to elevated CO_2 using BMA biomass as a proxy. One possible explanation for this result is that biomass is not the best indicator of whether elevated CO_2 is affecting photosynthesis and primary production.

If we are to accurately predict the trophodynamics of these estuarine systems, we need to recognize the mechanisms that potentially impact the functioning and structuring processes of the BMA community. The purpose of this study was to assess whether the contribution of CA-dependent CCMs in BMA communities influence biomass and gross primary production (GPP) in an estuarine, muddy environment. We also wanted to determine the regulatory effects of active CA on the vertical depth distribution of BMA in the upper millimeters of muddy sediment. Our primary hypothesis was that the estuarine BMA community's GPP and biomass would decrease without an active CA as having this enzyme should be crucial in a DIC limited environment. We also tested the hypothesis that the motile benthic diatoms would be more productive at lower depths of the sediment column if their CA enzyme was inhibited due to higher concentrations of DIC at depth. Given the well-documented role of BMA in the trophodynamics of estuaries, understanding the mechanisms of carbon acquisition in these systems is important in predicting how primary productivity and nutrient cycling might change in

response to increasing concentrations of atmospheric CO_2 (Hill and Hawkins, 1991; Johnson et al., 2013).

3.3 METHODS

3.3.1 STUDY SITE

Surficial muddy sediments for this study were taken from Oyster Landing (33° 21' 1" N, 79° 11' 24" W), located in the North Inlet Estuary near Georgetown, South Carolina, USA. This region is characterized by strong tidal exchange with the ocean, extensive stands of cord grass (*Sporobolus alternifolius*), and minimal anthropogenic impacts in a relatively undisturbed marsh system (Allen et al., 2014). Here, BMA is extremely abundant and widely distributed among five habitats – tall *Spartina* zones, short *Spartina* zones, shallow subtidal, intertidal mudflats, and intertidal sandflats – with highest biomass in intertidal mudflats and highest productivity in short *Spartina* zones (Pinckney and Zingmark, 1993a). In addition to productivity variability among habitats, short-term variability observed in primary productivity may be explained by vertical diatom migration occurring within the uppermost 5 mm of the sediment column (Jorgensen and Des Marais, 1986; Pinckney and Zingmark, 1991, Pinckney and Zingmark, 1993b; Pinckney and Zingmark, 1993c). During the periods of high primary productivity, most of the biomass, and thus productivity, is concentrated in the first hundreds of micrometers of sediment (de Brouwer and Stal, 2001; Pinckney et al., 1994; Pinckney, 1994). Finally, biomass for BMA follows a temporal trend generally increasing in late winter and early spring with lower levels throughout the rest of the year (Pinckney and Zingmark, 1993a; Pinckney and Zingmark, 1993c).

3.3.2 FIELD COLLECTIONS AND EXPERIMENTAL DESIGN

Twenty sediment cores (9.6 cm^2 × 6 cm) of unvegetated intertidal mud were collected within a square meter of each other at Oyster Landing, North Inlet in 2018 during low tide for a total of four replicated experiments. Specifically, the dates were February 27th, March 20th, April 10th, and April 25th with collections timed with low tides at 12:30 PM, 5:15 AM, 11:01 AM, and 11:44 AM, respectively. The cores were sealed and returned to the laboratory for incubations. Samples were acclimated for three days before the experiment was initiated to allow for resumption of normal vertical migration patterns (Hopkins, 1963; Pinckney et al., 1994). Cores were maintained in identical conditions that would be experienced during the experiment excluding the addition of an inhibitor (see below). The sediment at the collection site was composed of very fine grain sizes (62.5–125 μm) with ca. 36% silt/clay by weight (Pinckney et al., 2013).

Microcosm experiments were set up in two polyethylene trays (33 cm × 12 cm × 8 cm) which were connected to water reservoirs (10*L*). Diaphragm water pumps (Aqua Lifter®) controlled the water flow to simulate tidal periodicity at the collection site. Illumination was supplied using a 12 h light: 12 h dark photoperiod using fluorescent light (91 cm, 4 × 39 W Ocean Light T5 hood, 10,000 K 39 W –TRU fluorescent bulbs) with an in-situ irradiance of ca. 1000 μmol photons m^{-2} s^{-1} at the sediment surface when measured with a LI-COR LI-250 light meter.

There were two reservoirs, one was designated as the control and the other was designated the CA inhibited treatment. Our inhibitor was ethoxyzolamide (EZ, Sigma Aldrich, cat. # 333328-1G) – a commonly used inhibitor that penetrates the cell and

inhibits both external and internal CA in all evolutionarily distinct classes found in marine phytoplankton (Mercado et al., 1998; Mercado et al., 2009; Wu et al., 2015). Initial stock solutions of EZ were prepared in 0.05 M NaOH (Mercado et al., 1998). The CA inhibited treatment had a final EZ concentration of 100 μmol L^{-1}reached by adding an EZ stock solution accordingly in 8 L of sand-filtered seawater (~33 ppt) collected from the north inlet estuary.

3.3.3 PRODUCTIVITY AND BIOMASS MEASUREMENTS

Each bioassay tray contained 10 replicate sediment cores. During the incubation period, O_2 microprofiles of both assays were measured directly using a Clarke-style oxygen microelectrode (25 μm) connected to a picoammeter (Unisense) during simulated low tide. The light/dark shift method was used to determine gross primary productivity (GPP) across depth and treatment (Revsbech and Jørgensen, 1983). Microelectrodes were calibrated using a two-point procedure with 0% (0.1 M NaOH/ascorbate) and 100% (bubbling with air) saturation dissolved O_2concentrations as endpoints. All measurements were taken after O_2 profiles reached a steady-state as determined by the microelectrode profiles. The light/dark shift method required illuminating the sample at an irradiance of ca. 1200 μmol photons m^{-2} s^{-1} and then measuring the initial slope of oxygen decrease immediately (within 1 to 3 s) after darkening the sediment surface (Pinckney et al., 2003; Revsbech et al., 1986). The vertical position of the sensor was controlled at a 100 μm depth resolution using a motor-driven micromanipulator until there was no detectable difference in oxygen production. Five vertical profiles of production were obtained at random locations within each core to ensure microalgal patchiness did not confound the data (Pinckney et al., 2003). For each profile, every 100 μm depth interval

was integrated to obtain a depth integrated areal estimate of GPP. Five out of the ten cores were used during this analysis to estimate community GPP. The final GPP profile sample size was $n = 25$ per treatment for each replicate experiment.

Biomass (chlorophyll *a*, hereby referred to as "chl *a*") was measured at the conclusion of the three-day incubation period on all cores. Three subsamples were taken from a core ($n = 30$ per treatment) using a 11.1 mm diameter butyrate core tube and the upper 5 mm of the core was extruded, sectioned, and frozen in a 2.0 mL microfuge tubes. For analyses, sediment samples were lyophilized for 24 h at −50 °C, placed in 90% acetone, vortexed for 30s, and extracted at −20 °C for 24 h. Extracts were centrifuged at 13,400 RPM for 2 min to decrease turbidity following which extracts were diluted before analysis using a Trilogy fluorometer (Turner Designs). Raw fluorescent units were converted to chlorophyll concentrations using a standard curve. Sediments were retained, dried, and weighed for each sample to normalize chl *a* concentrations to sediment dry weight.

Productivity measurements were taken every day of the incubation creating a repeated measure, and biomass was measured on the final day of the incubation. To account for pseudo replication, this experimental design was repeated four separate times (see dates above).

3.3.4 DATA ANALYSIS

Analyses were conducted with the statistical program R, v.3.5.1 (RCoreTeam, 2012), *lme4* (Bates et al., 2015), and *lmerTest* (Kuznetsova et al., 2017) to perform a linear mixed effects analysis of the relationship between depth integrated areal estimate of GPP and exposure to the CA inhibitor, EZ. As fixed effects, we entered treatment type

and day of measurement with an interaction term. As random effects, we had replicate experiment number and the day of measurement. The random effect of replicate experiment number accounts for the variable differences between each experiment while the random effect of day accounts for the repeated measures. The initial model was Integrated GPP ~ Day * Treatment + (Day | Experiment number). *P*-values were obtained by likelihood ratio tests of the full model with the effect in question against the model without the effect in question. Using the values obtained by these likelihood ratio tests and confirming with the step function (*lmerTest*), our results show that the best fit model is Integrated GPP ~ Treatment + (Day | Experiment number).

To further investigate whether motile microalgae were moving deeper in the sediment or toward the sediment surface, linear interpolation was used between each two adjacent sampling depths starting at the surface. This method determined where 50% of the cumulative GPP was taking place. From this, inferences of BMA vertical location could be made. A non-parametric Friedman test was run to test the differences in the 50% cumulative GPP depth between the two treatments on the final day of incubation and replicate experiment number. All data was averaged to meet the assumption of an unreplicated complete block designs (i.e., there is exactly one observation for each combination of levels of treatment and experiment number). An ANOVA was used to analyze microalgae biomass differences between the two treatments at the end of the experiment. Chl *a* measures from experiment 1 were dropped because of measurement methodology differences. The data was square root transformed to meet the assumptions of normality and homogeneity of variance.

3.4 RESULTS

Using microcosm experiments, we found that there was a change in integrated GPP among our samples due to inhibition of CA activity. Model simplification using likelihood ratio tests indicated that integrated GPP was best explained using treatment as the explanatory factor while accounting for random effects of replicate experiment number and day of measurement. The full model removed both the interaction term between day and treatment and the fixed effect of day after it was determined these effects were non-significant ($X^2 = 5.36$, $p = .07$ and $X^2 = 4.31$, $p = .12$; respectively). The best-fit model established that there was a significant difference between the two treatments ($X^2 = 34.54$, $p < .001$, Fig. 3.1). Cores exposed to the CA inhibitor, EZ, had ca. 28% lower GPP compared to the cores with no addition (a difference of 0.11 μmol O_2 cm^{-2} h^{-1} ± 0.02 μmol O_2 cm^{-2} h^{-1}, SE) (Table 3.1). Across all the replicate experiments, the average GPP measures for the control and EZ treatments were 0.40 ± 0.02 and 0.29 ± 0.02, respectively (mean ± SE).

GPP on the third day of treatment was examined to investigate whether peaks in GPP were shifting in the sediment column (Fig. 3.2, Fig. 3.3). Generally, control treatments had higher GPP at lower depths and CA inhibitor treatments had their highest GPP rates occurring near the surface. The depth of 50% cumulative GPP for the inhibitor treatments occurred higher in the sediment column than in the control treatments. Fifty percent of the cumulative GPP in the EZ treatment was occurring at depths on average 25 μm or 54% higher in the sediment column than those in the control treatment ($\sigma\bar{x}$ = −29 μm depth and $\sigma\bar{x}$ = −54 μm depth; respectively, Fig. 3.4). A Friedman test showed there was also no significant difference between experiment number (Friedman chi-

squared = 2.4, df = 3, *p*-value = .4936). There was a statistically significant difference between the control and EZ addition (Friedman chi-squared = 4, df = 1, p-value = .0455).

Finally, biomass did not differ between the CA inhibitor treatments. Rather, there was a significant difference between our experimental replicates. The highest biomass was measured during the earliest experiment which was collected during early March and decreased with each replicate experiment to the end of April (Fig. 3.5). A two-way ANOVA analysis showed there was no statistically significant interaction between the effects of treatment and experiment on biomass ($F_{(2, 174)} = 1.16$, $p = .32$). There was also no significant difference between treatment type ($F_{(1, 174)} = 0.03$, $p = .86$). Tukey's HSD post hoc tests were carried out on experiment number. Experiment 2 was significantly different from experiment 3 ($p = .04$) and experiment 4 ($p < .001$), and experiment 3 significantly differed from experiment 4 ($p < .001$).

3.5 DISCUSSION

A major limiting factor for photosynthesis in seawater is the low CO_2concentrations leading to potential carbon limitation in photoautotrophs, both phytoplankton and BMA (Badger et al., 1998; Ludden et al., 1985; Riebesell et al., 1993). To counter-balance this sometimes-restricted carbon supply, CCMs evolved in conjunction with enzymes (i.e. CA) to saturate RuBisCO in the Calvin-Benson cycle through increased CO_2 concentrations and CO_2/O_2 ratios at the site of fixation in the carboxysome, chloroplast, or thylakoid lumen (DiMario et al., 2018). Yet, experiments of inorganic carbon enrichment have yielded variable results for photosynthesis, growth, and composition for BMA communities (Cartaxana et al., 2015; Hicks et al., 2011; Johnson et al., 2013; Torstensson et al., 2015). In this study, we investigated the

specific impact of inhibiting CA activity on integrated GPP, biomass, and depth profiles in natural estuarine BMA communities without carbon enrichment. We demonstrated that BMA communities depend on CA for greater integrated GPP, but inactivation of this enzyme did not necessarily affect total biomass. Investigations into relative productivity and cumulative production demonstrated that GPP within the sediment column shifts upward nearer to the sediment surface when CA is inhibited. Therefore, relative location of motile diatoms may be dependent on efficient CA activity for a broader distribution in the sediment column.

CCMs and the enzymes associated with it are important mechanisms to evaluate in marine photoautotrophs, both in water and sediment columns, because of their potential to influence carbon fixation and utilization, and therefore, productivity and biomass (Beardall and Giordano, 2002; Beardall and Raven, 2004; Knotts and Pinckney, 2018; Mercado et al., 2009; Reinfelder, 2011; Tortell, 2000). BMA are major contributors to ecosystem total primary production (Pinckney and Zingmark, 1993a), and thus, studying their carbon acquisition is essential. Relatively subtle environmental perturbations that either limit or supply carbon sources may alter the balance between autotrophy and heterotrophy, having profound effects on these estuarine ecosystems and the consumers that exploit them (Hicks et al., 2011; Montagna et al., 1995; Pinckney et al., 2003; Porubsky et al., 2008).

As studied here, GPP of the BMA in North Inlet estuarine muddy sediment was greatly influenced by CA activity to counterbalance DIC limitation in these high productivity environments. Previous investigations have recognized this occurrence in estuarine environments either by directly studying DIC limitation (Vieira et al., 2016) or

by elevating the DIC availability (Cartaxana et al., 2015; Cook and Roy, 2006). Using biomass as a proxy estimate, Hicks et al. (2011) found no significant increase in BMA productivity with elevated CO_2. Yet, when using the same proxy, Johnson et al. (2013), Cartaxana et al. (2015), and Vieira et al. (2016) all documented higher DIC led to increased primary productivity. Rather than manipulating CO_2 of DIC levels, our BMA communities experienced increased limitation to available DIC with the inhibition of CA. BMA could no longer actively accumulate available HCO_3^- without CA functioning to dehydrate bicarbonate to CO_2. Thus, inactive CA limited integrated GPP overall by 0.11 μmol O_2 cm^{-2} h^{-1} or ca. 28%. Our results suggest that, if exposed to higher DIC like Johnson et al. (2013) and Vieira et al. (2016), the BMA would show increased primary productivity. However, this present study only investigated CA inhibition effects over a short temporal scale of three days. Further studies should examine BMA production across a longer temporal scale since production is dependent on season (Pinckney and Zingmark, 1993a) and our measurements were taken during the months when BMA production was high. During low productivity months, CA may not be as critical for these systems.

BMA communities likely experience carbon limitation daily (Cook and Roy, 2006; Ludden et al., 1985; Vieira et al., 2016). Epipelic benthic diatoms may use their ability to migrate in the upper few mm of sediment to take advantage of areas with higher DIC or access to atmospheric CO_2. Multiple studies have demonstrated that DIC increased with depth (Burdige et al., 2010; Glud et al., 1998; Herczeg, 1988; Komada et al., 1998). While movement of epipelic diatoms has been linked to multiple factors including light, temperature, erosion, predation, and growth (Barnett et al.,

2015; Consalvey et al., 2004; Saburova and Polikarpov, 2003), motility may be another evolutionary advantage to overcome the DIC constraint. Thus, it may be expected that motile BMA would be found lower in the sediment column taking advantage of the available DIC. Alternatively, to overcome DIC limitation, epipelic movement may be toward the surface to make use of the atmosphere-sediment interface. Kristensen and Alongi (2006) determined that higher CO_2uptake rates occurred when microalgal films were visibly at the surface. Chen et al. (2019) further demonstrated that there were negative CO_2 fluxes with BMA at the surface and positive fluxes without visible BMA. Future studies should investigate whether epipelic diatoms are making use of the lower sediment profile or the surface to overcome DIC limitation. Our study only provides evidence that with active CA, productivity can be seen across a wider array of the sediment column. GPP for the BMA communities without active CA occurred nearer to the surface. This result was further supported when we investigated where 50% of the cumulative GPP was occurring. BMA communities that had CA inhibited were productive at a depth ~54% higher on average than the communities with active CA.

Microalgae in these muddy environments may be relying on atmospheric pCO_2because those concentrations are higher (350 µatm or 6.2×10^{16} mol C) than in porewater (5.77 µmol C cm^{-3}, Pinckney, unpublished data). The GPP in our sediment was measured in the top 500 µm of sediment. Therefore, we multiplied the porewater DIC (5.77 µmol C cm^{-3}) with the upper 0.05 cm to obtain the available DIC utilized by the BMA (ca. 0.29 µmol C cm^{-2}). The control treatments were utilizing 0.33 µmol C cm^{-2} h^{-1}. In this estuarine system, available DIC is limiting and can be depleted within an hour. This result is consistent with the study by Chen et al. (2019), where BMA are using

a significant amount of sediment CO_2 for photosynthesis in the sediments at the Jiulong River Estuary. However, movement of BMA to the surface to acquire CO_2 is not optimal since this location experiences high irradiance and UV exposure (Pinckney and Zingmark, 1993b; Pinckney, 1994). Thus, there is a trade-off between higher CO_2 availability but more light damage (Blanchard et al., 2004; Light and Beardall, 2001).

While a decrease in integrated GPP was measured in our CA inhibited treatments, our data analyses did not support the idea that the treatments would decrease BMA biomass as well. Biomass and production are commonly assumed to be related with chl *a* used as an indicator of production (Tremblay and Legendre, 1994). However, the presence of an organism doesn't necessarily mean it is contributing to production in a meaningful manner. This can be seen in recent studies that investigated the biomass of size-fractioned phytoplankton and their contributions to total productivity. Pommier et al. (2008) found North Atlantic phytoplankton >5 μm contributed 45% of total biomass but 79% of total productivity, and Hopcroft and Roff (1990) found microphytoplankton in Jamaica contributed 42% of biomass but 27% of total production. In our BMA system, the large benthic diatoms are probably contributing the most biomass and GPP, and smaller chlorophytes and cyanobacteria are contributing a smaller proportion of both biomass and GPP. We suspect that motile benthic diatoms are remaining present in the sediment column but contributing less total GPP because their main mechanism of carbon concentration has been removed. The chlorophytes and cyanobacteria were most likely removed from the community. Their lack of mobility and the removal of CA would have made access to $CO_{2(aq)}$ difficult conceivably leading to cell mortality. Perhaps the

removal of these smaller, non-motile BMA did not change the chl *a*content of the sediment. Alternatively, Gould and Gallagher (1990) measured in-situ growth rates of benthic diatoms to be 0.6–0.27 d^{-1}. Thus, it would likely take more than three days to see a change in biomass. This result may explain why, using biomass as a proxy estimate, Hicks et al. (2011) claimed that CO_2 elevation did not affect production. Future studies should examine this question in a longer time-scale experiment. Also, more in-depth investigations into community composition alterations are necessary to recognize whether biomass remained unchanged due to unaltered community composition or retained contributors that supply most of the primary production.

Finally, the biomass decrease across experiment number may be attributed to collection dates. Experiments 2 through 4 were conducted at the beginning of March through the end of April. BMA biomass at North Inlet has been shown to increase during late winter and early spring, followed by a decrease late spring (Gould and Gallagher, 1990; Montagna et al., 1983; Pinckney and Zingmark, 1993a). The measurements reported in this study agree with previous findings. The result that there was no interaction between experiment number and treatment on biomass may be indicative that, regardless of seasonal variation on community composition, the BMA biomass will respond the same way when CA is inhibited.

3.5.1 CONCLUSION

Results of our microcosm experiment illustrate the potential importance of a functioning CA-dependent CCM in BMA communities. Previous work shows that DIC limitation in these muddy estuarine sediments can influence community biomass and production (da Silva et al., 2017; Ludden et al., 1985). The incorporation of BMA

dynamics in our models and our study systems seems to be a logical first step to improve the understanding of ecosystem responses to global climate change and ocean acidification. Based on the results of this study, CA activity is critical to maintaining high GPP and making use of a wider portion of the sediment column, but not necessarily significant to sustaining biomass. Multiple factors may affect the ability of BMA to concentrate carbon or access DIC (e.g. burial, anoxia, or exposure to the atmosphere) (Consalvey et al., 2004; Saburova and Polikarpov, 2003). The consequences of altered DIC access could impact BMA biomass and productivity, and ultimately change our quantification of estuarine ecosystem trophodynamics. Attempts should be made to quantify the influence of CCMs on BMA production dynamics in addition to biomass measurements. Through the recognition of carbon acquisition strategies currently used by BMA, we can begin to characterize the important, yet not often considered, mechanisms that influence the community's functioning and structuring processes. This can further lead to accurately predicting changes in biogeochemical processes that are impacted by BMA communities that may occur in future climate scenarios.

3.6 ACKNOWLEDGEMENTS

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3.7 TABLES

Table 3.1: Average integrated GPP measurements (mean µmol O2 cm-2 h-1 ± standard error) in the control and inhibited treatment cores across day and replicate experiment (n = 5 cores).

	Day 1 ($\mu mol\ O_2\ cm^{-2}\ h^{-1}$)		Day 2 ($\mu mol\ O_2\ cm^{-2}\ h^{-1}$)		Day 3 ($\mu mol\ O_2\ cm^{-2}\ h^{-1}$)	
	Control	Inhibited	Control	Inhibited	Control	Inhibited
Experiment 1	0.25 (± 0.03)	0.16 (± 0.04)	0.36 (± 0.04)	0.27 (± 0.04)	0.42 (± 0.05)	0.24 (± 0.04)
Experiment 2	0.26 (± 0.02)	0.18 (± 0.01)	0.36 (± 0.07)	0.30 (± 0.04)	0.49 (± 0.03)	0.38 (± 0.06)
Experiment 3	0.43 (± 0.05)	0.32 (± 0.06)	0.50 (± 0.04)	0.40 (± 0.03)	0.52 (± 0.03)	0.34 (± 0.05)
Experiment 4	0.39 (± 0.02)	0.33 (± 0.05)	0.40 (± 0.03)	0.32 (± 0.03)	0.42 (± 0.08)	0.22 (± 0.04)

3.8 FIGURES

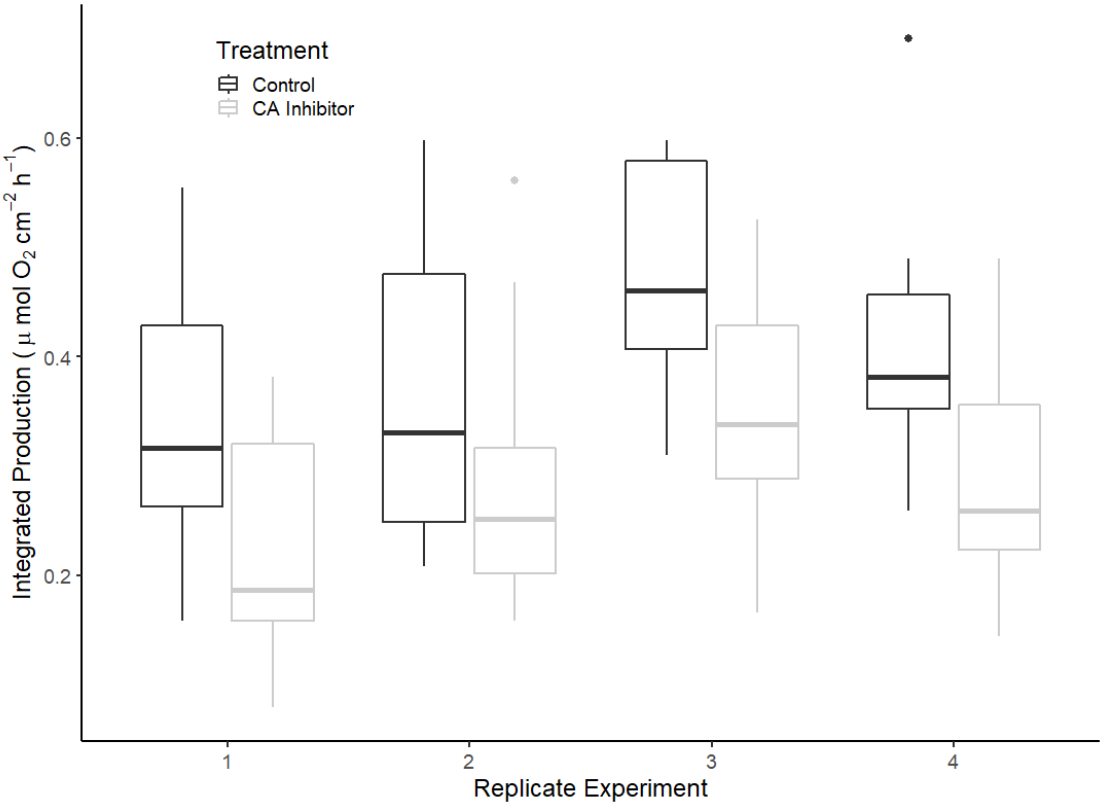

Figure 3.1: Boxplot of integrated GPP in the two treatments averaged across the three-day incubation (n = 5 cores per treatment per day). All four replicate experiments are included. There is a significant difference between the two treatments ($X2 = 34.538$, $p < 0.001$). The horizontal line is the median, with the box including the upper and lower quartiles of the data. The whiskers encompass 95% of the data.

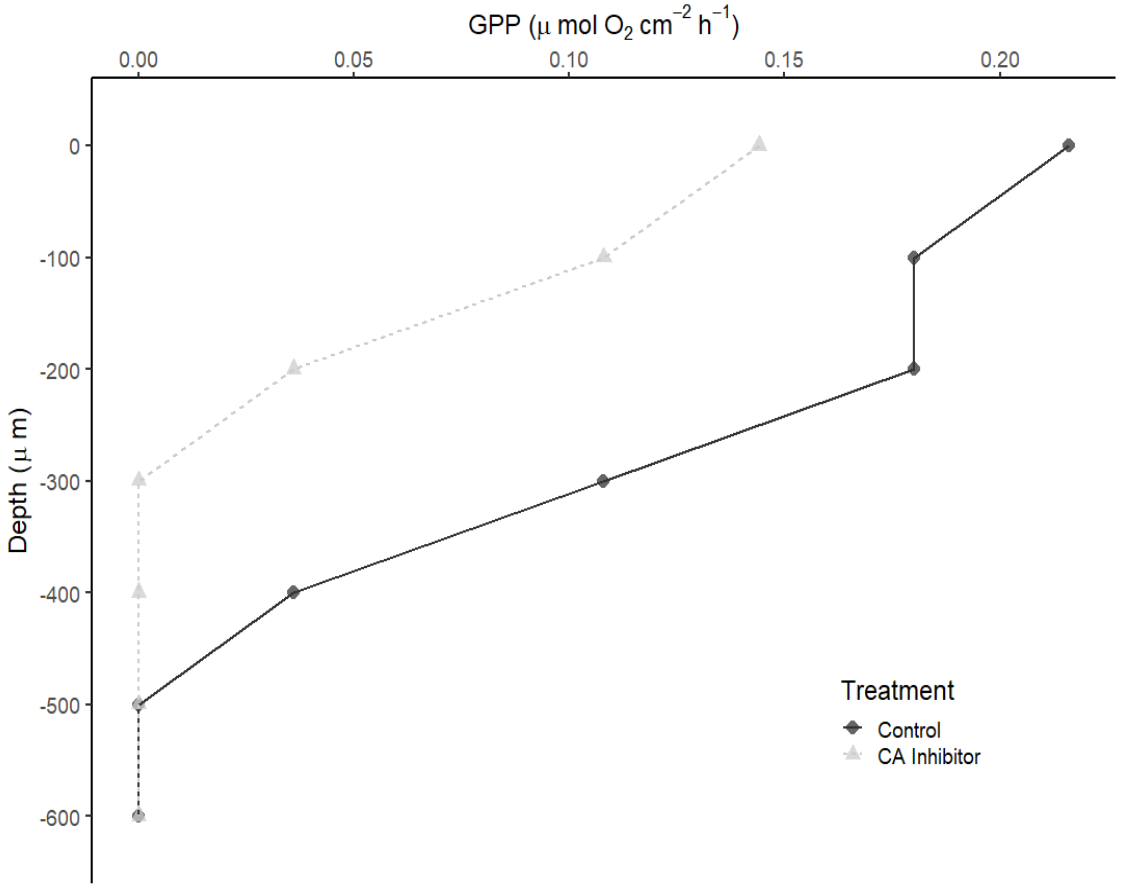

Figure 3.2: Representative GPP profile from a single core in experiment four on day three for the two treatments.

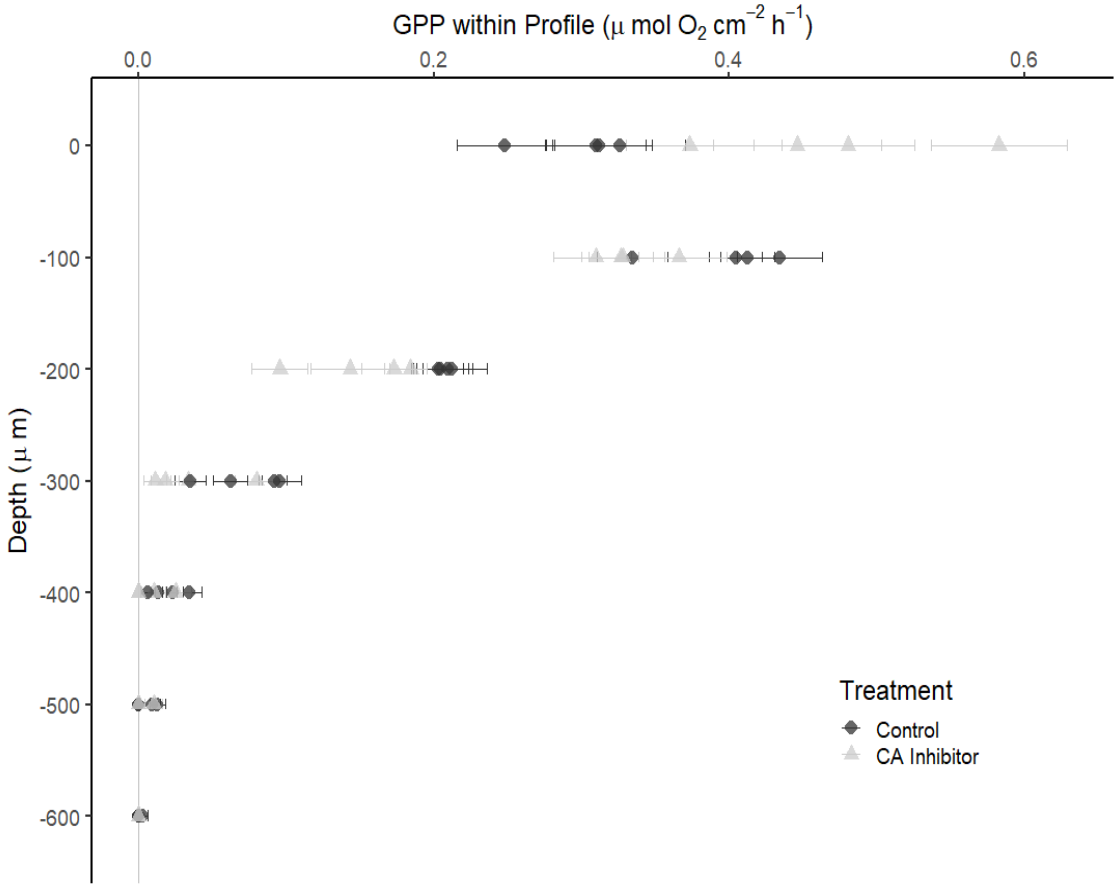

Figure 3.3: GPP (mean ± SE) across depth in the two treatments measured on day three (n = 25 per treatment for each replicate experiment). All four replicate experiments are included. Black circles are the control treatment. Grey triangles are the CA inhibitor treatment.

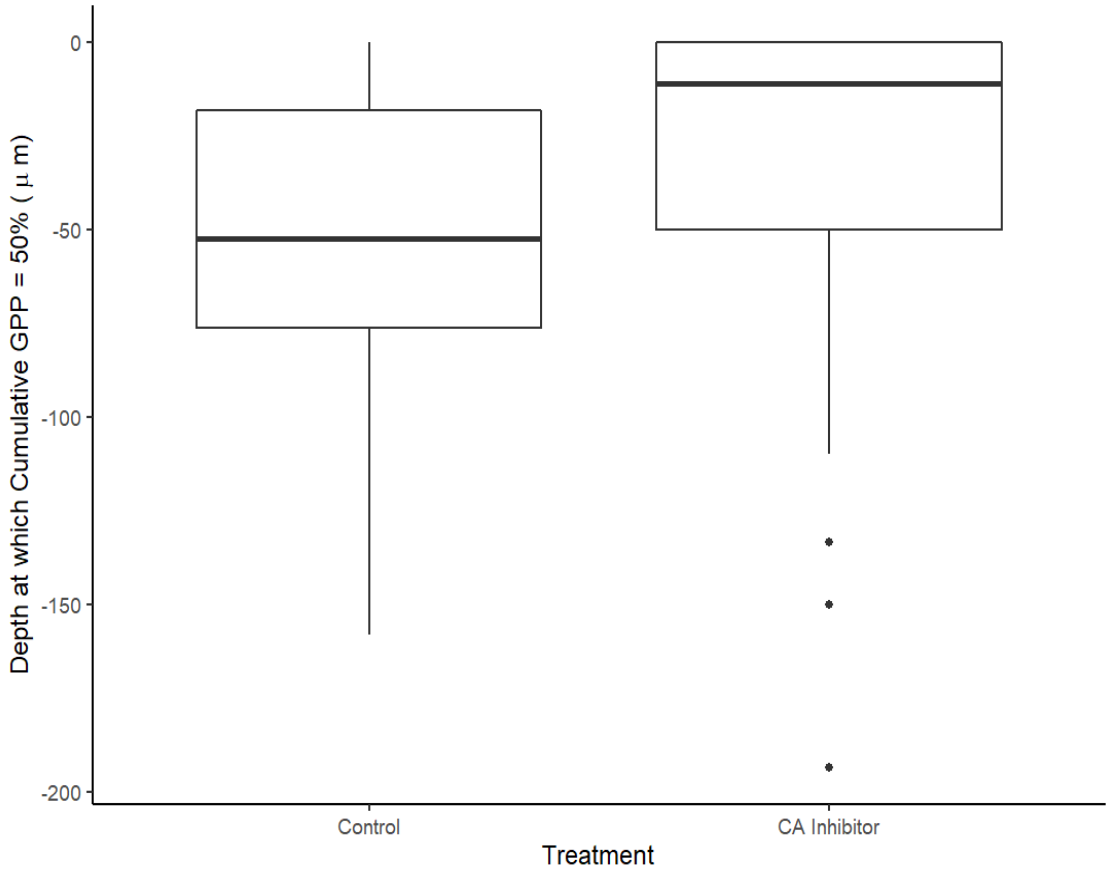

Figure 3.4: Depth at which cumulative GPP in a profile is equal to 50% in the two treatments on day three (n = 25 per treatment for each replicate experiment). All four replicate experiments are included. There is a significant difference between the two treatments (p = 0.0455). Individual data points indicate outliers.

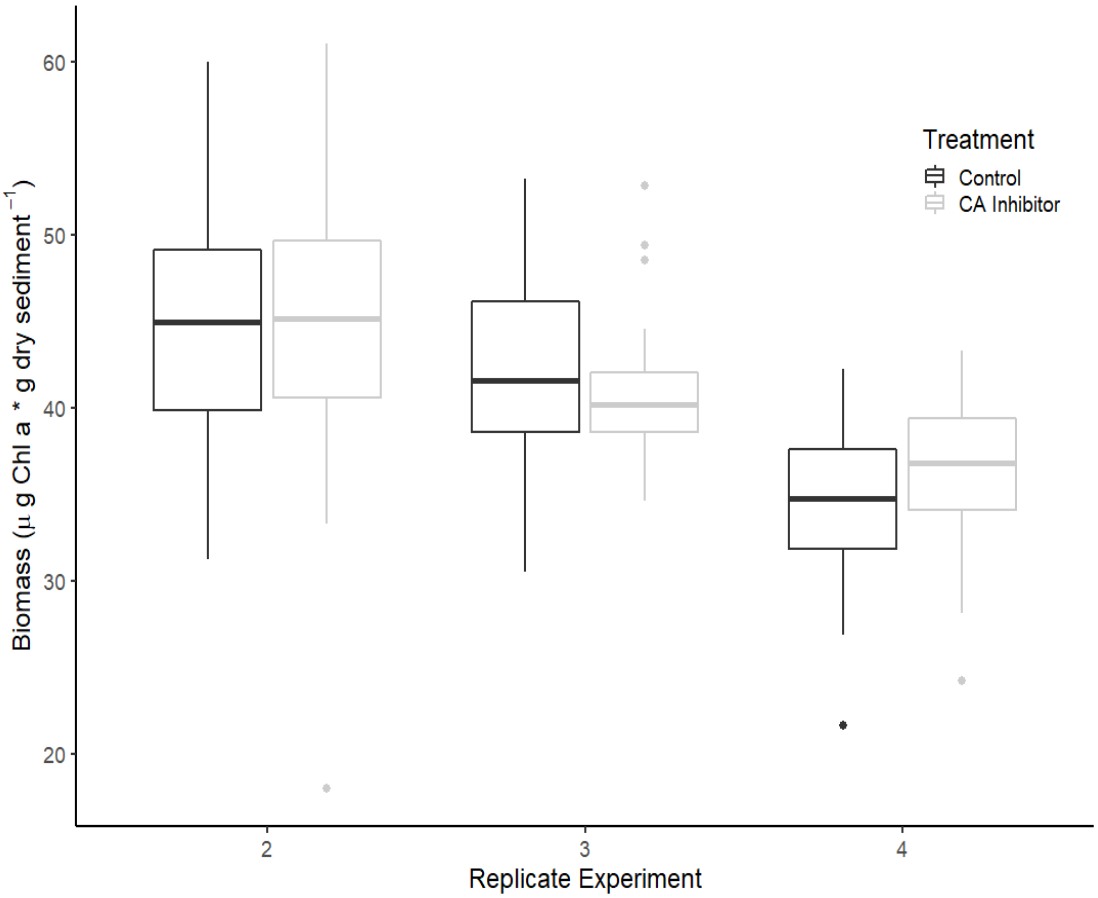

Figure 3.5: Average biomass measured on day three for each of the treatments across three experiments (n = 30 per treatment). Three replicate experiments are included with the first experiment dropped due to methodology differences. Experiment 2 significantly differed from experiment 3 ($p = 0.04$) and experiment 4 ($p < 0.001$), and experiment 3 significantly differed from experiment 4 ($p < 0.001$). Individual data points indicate outliers.

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CHAPTER 4

EFFECTS OF RISING CO_2 ON PHYTOPLANKTON CARBON CONCENTRATING MECHANISMS: A MINI-REVIEW ON RESOURCE CONSTRAINTS AND COMPETITION[1]

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4.1 SUMMARY

Biophysical carbon-concentrating mechanisms (CCMs) are found in marine phytoplankton to help overcome inorganic carbon limitations currently faced in surface seawater. However, rising atmospheric CO_2 is predicted to have consequences in seawater carbonate chemistry by increasing dissolved CO_2 concentrations. With these changes, it is hypothesized that marine phytoplankton communities will shift in dominance hierarchies and primary productivity, owing to the significant variability of carbon concentrating mechanisms (CCMs) among functional groups and species. Evaluations on how rising CO_2 will impact these mechanisms is still limited. This review aims to show (i) that resource limitation of important nutrients can have a variety of responses on CCM activity and/or growth rate depending on species, and (ii) CO_2-dependent shifts in species dominance hierarchies cannot be solely determined by monocultures that explore physiological responses of CCMs to CO_2 enrichment. Despite the difficulties involved in determining how CCM activity will alter communities in the future, methods combining pairwise comparisons, natural community investigations, and modeling may be useful when appropriate considerations are made regarding initial community composition and common environmental regimes.

4.2 INTRODUCTION

All photoautotrophs experience constraints on growth and reproduction due to limited resource availability (e.g. nitrogen, phosphorus, CO_2, silica, light) (see Liebig's Law of the minimum) (Liebig, 1840, Tilman, 1982). These limitations lead to competitive interactions between species that determine the composition and dynamics of

those photoautotroph communities. Fluctuating dynamics persist because different species possess unequal abilities to take up various resources (Petersen, 1975, Tilman *et al.*, 1982) and these variable resource acquisition strategies are associated with differential costs.

For phytoplankton, diversity in communities (termed "paradox of the plankton") (Hutchinson, 1961) is maintained through the different limitations of species based on a variety of abiotic (e.g. nutrients, carbon, light) and biotic (e.g. predators) factors (Tilman et al., 1982). Though phytoplankton competition is controlled through a variety of these conditions, Tortell (2000) made the assertion that taxonomic differences in carbon acquisition were exceptionally important when determining the ecological interactions of phytoplankton groups.

Among these photoautotrophs, carbon acquisition strategies are diverse. The first strategy involves catalytic kinetic properties of the enzyme, Ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO). In the first major stage of the Calvin cycle, the enzyme RuBisCO enables the reaction between CO_2 and ribulose bisphosphate (RuBP). RuBisCo plays a fundamental role in CO_2 assimilation because, as a main contributor of carbon fixation, this enzyme catalyzes the primary chemical reaction by which inorganic carbon is converted to organic molecules to be used as energy. However, imperfections in the low catalytic turnover rate and the affinity for oxygen have fostered evolutionary strategies to optimize the performance of RuBisCo which further diversified of the kinetic properties of this enzyme (Badger *et al.*, 1998, Young *et al.*, 2016). For example, Tortell (2000) showed a negative relationship between the capacity of cells to concentrate inorganic carbon and the CO_2 specificity factor of RuBisCo. He used the work of Badger

et al. (1998) on the enzyme's substrate specificity factors among major functional groups. Young et al. (2016) supported this concept with their demonstration of a negative relationship between the half-saturation coefficient for carbon and cellular RuBisCo content in different diatom strains.

These variations in kinetic properties of the first strategy led to the evolution of the second strategy which ensured that RuBisCo would be supplied with high levels of carbon. This tactic was to develop active uptake mechanisms that accumulated inorganic carbon significantly greater than concentrations in the bulk seawater environment. These carbon-concentrating mechanisms (CCMs) comprise of two types of biophysical mechanisms that raise the concentration of $CO_{2(aq)}$ at a cell's surface or internally at the site of fixation (Riebesell *et al.*, 1993, Rost *et al.*, 2003). The internal locations of fixation vary among marine phytoplankton and can be found in the carboxysome, chloroplasts, or thylakoid lumen (DiMario *et al.*, 2018). The first form restores the CO_2/HCO_3^- equilibrium at a cell's surface by carbonic anhydrase (CA)-catalyzed dehydration of HCO_3^- which allows passive diffusion through the membrane. The second form involves bicarbonate transporters carrying HCO_3^- across the membrane and then conversion of that ion to CO_2 by internal CA. Nearly all marine phytoplankton that have been studied have some form of a CCM, though efficiency varies interspecifically (across functional groups) (Mercado *et al.*, 2009, Reinfelder, 2011) and within functional groups and/or within genera (Martin & Tortell, 2008). Variation in the functional trait of dissolved inorganic carbon (DIC) uptake suggests that there is a capacity for future changes in phytoplankton communities (Griffiths *et al.*, 2017, Knotts & Pinckney, 2018).

As discussed in Tortell (2000) review of carbon acquisition strategies, competitive interactions among taxa are influenced by the differential resource efficiencies imposed by their mechanisms of inorganic carbon uptake and assimilation. With these different mechanisms for obtaining carbon, phytoplankton have become a topic of great importance as researchers attempt to determine how these photoautotrophs will respond individually or as a community to rising dissolved CO_2 (Raven, 1991, Raven, 2003).

The current review provides a more recent analysis of the resource constraints on carbon acquisition in phytoplankton and compares the shifts in species dominance hierarchies due to elevated $CO_{2(aq)}$. I also suggest potential studies that are critical in understanding the role CCMs have in driving community responses to rising CO_2. The purpose of this review is to summarize current information on phytoplankton responses to elevated CO_2 to provide insights into the potential major role of CCMs in structuring phytoplankton communities and the possible implications remodeled plankton communities may have on food web dynamics and biogeochemical cycling.

4.3 RESOURCE CONSTRAINTS ON CARBON ACQUISITION

Studies have investigated the nature of biophysical mechanisms and gene regulation involved in the repression of CCMs by high CO_2 concentrations (Badger et al., 1998). However, despite the concept that primary productivity is frequently limited by the availability of limiting nutrients (i.e. nitrogen, phosphorus, iron), few investigations have been directed toward understanding the effects of environmental factors (e.g. nutrient limitation) on CCM activity in microalgae (Beardall *et al.*, 2005). A table of

effects of limitation of growth by the availability of nitrogen (N), phosphorus (P), and iron (Fe) on CCMs can be found in Raven and Beardall (2014) and Raven *et al.* (2017), yet many of these studies do not address rising CO_2 in microalgae. Furthering our understanding of the ecological relevance of active DIC uptake requires evaluations of how nutrients may affect DIC acquisition in elevated CO_2 conditions (see Fig. 4.1) (Young & Beardall, 2005). Using natural assemblage samples taken from the Alborán Sea (southwest Mediterranean Sea), Sobrino *et al.* (2014) demonstrated that, in addition to elevated CO_2, nutrient concentrations functioned as important modulators regulating cell metabolism. To better understand how nutrient concentrations influence CCM activity, controlled experiments were used to examine reactions at a species level. This section focuses on those culture experiments that had both nutrient and elevated CO_2 treatments.

4.3.1 *NITROGEN*

Nitrogen is closely connected to carbon (C) energetically since N and C compete for the energy from photosynthesis (Beardall & Giordano, 2002, Raven *et al.*, 2008, Eberlein *et al.*, 2016, Pierangelini *et al.*, 2017). Eberlein et al. (2016) proposed that with CCM down-regulation under elevated pCO_2, more energy could be reallocated to other cellular processes such as the acquisition of limited resources. Two dinoflagellates from this study, *Scrippsoella trochoidea* and *Alexandrium fundyense*, had previously shown the ability to down-regulate their CCMs including the down-regulation of genes expressing CA (Eberlein *et al.*, 2014). *Thalassiosira pseudonana*, *Phaeodactylum tricornutum*, and *Thalassiosira weissflogii* also demonstrated CCM downregulation

through the decreased transcription of CA at high CO_2 in both N-replete and N-limited conditions (Hong *et al.*, 2017). Eberlein et al. (2016) suggested the increase in uptake and assimilation of N could be explained by the shift in energy from the down-regulated CCM to N uptake. In general, rising CO_2 improved the efficiency of N utilization by CCMs, provided that the nitrogen allocated to making the CCMs did not exceed the N saved through the CCM (Beardall *et al.*, 1998, Sciandra *et al.*, 2003).

To better assess the availability of inorganic nutrients and how they influence phytoplankton responses to elevated CO_2, multiple studies explored C/N elemental stoichiometry (Eberlein et al., 2016, Hong et al., 2017, Pierangelini et al., 2017). However, the few studies that looked at the combined effect of N limitation and elevated CO_2 on marine phytoplankton have shown a wide variety in elemental quotas depending on species and strains (Eberlein et al., 2016, Hong et al., 2017). For example, bloom-forming dinoflagellates (i.e. *S. trochoidea* and *A. fundyense*) showed increased particulate organic carbon (POC): particulate organic nitrogen (PON) ratios under N limitation and these ratios decreased with increasing CO_2 (Eberlein et al., 2016). Though these species showed changes in their cellular ratios, Pierangelini et al. (2017) showed that *Protoceratium reticulatum* did not change their internal C:N ratios. A difference in these studies was that N was never limiting in the study conducted by Pierangelini et al. (2017). According to Eberlein et al. (2016), C:N ratios are more sensitive to increased pCO_2 under N-limiting conditions than under -replete conditions. Hong et al. (2017) supported this concept using three diatom species which showed that in elevated CO_2 conditions, C:N ratios did not change when N was replete, whereas the ratios increased when N was limiting. This study went on to suggest that RuBisCo and other photosynthetic proteins

were down-regulated at high CO_2 which reduced the demand for cellular N to produce them. Therefore, there was a stronger decrease in the N quota compared to the C quota leading to the increased C:N ratios. Overall, N-limited diatoms could fix more C per unit of N in response to elevated CO_2 (Hong et al., 2017). Decreases in photosynthetic proteins does not necessarily imply the removal of CCMs. Young and Beardall (2005) showed maintaining CCM activity under N-limitation may be advantageous due to the improved N-use efficiency.

4.3.2 *PHOSPHORUS*

Similar to the data on nitrogen resource constraints, exposure to elevated CO_2 and phosphorus (P)-limited conditions shifted CCM activity to be either down-regulated as seen in *Cylindrospermopsis raciborskii* (Wu *et al.*, 2012) or maintained as seen in *Chlamydomonas acidophila* (Spijkerman *et al.*, 2014). In these studies, kinetic parameters (e.g. half-saturation coefficient of CO_2 [$K_{1/2}$], maximum rate of reaction [V_{max}]) are commonly used to study carbon assimilation and are calculated from the Michaelis-Menten equation. If the $K_{1/2}$ parameter increases, CCM activity is believed to be down-regulated by this measure of CO_2 affinity. Looking at P-replete conditions, Wu et al. (2012) reported significant increases in $K_{1/2}$ and V_{max} at both present and elevated CO_2 levels, though *C. raciborskii* had the highest parameters of photosynthesis when exposed to high CO_2. Similarly, specific growth rates increased after exposed to high CO_2 or P conditions which may be due to the lowering of energy requirements for the cyanobacterium's CCM or HCO_3^- transportation. In P-limited conditions *C. raciborskii* exhibited slight increases in $K_{1/2}$ and V_{max} at elevated CO_2 levels. Beardall et al. (2005)

saw similar results exhibiting downregulation of CCM activity under phosphorus limitation. Beardall et al. (2005) suggested that since CCMs are active energy-consuming processes requiring ATP, P deficiencies could have important impacts on CCM activity. Firstly, decreases in the available energy required for active transport and synthesis of proteins for transport could affect the capacity of cells to drive a CCM. Alternatively, P-limitation could decrease growth rates, which in turn decreases the demand for inorganic carbon supply and, therefore, decreases the need for C acquisition using CCM activity. P-limitation restrained *C. raciborskii*'s growth rate, photosynthetic capacity, and nutrient uptake rates sometimes regardless of CO_2 enrichment which supports the concept that P plays an important role in carbon fixation. The data from Kozlowska-Szerenos *et al.* (2004) was not reviewed here since the enrichment of CO_2 concentration was through the addition of DIC (i.e. bicarbonate) rather than CO_2. Contrasting the concept that P limitation downregulates CCM activity, *Chlamydomonas acidophila* demonstrated maximally expressed carbon concentrating factors (CCFs) under low CO_2 conditions along with P-limitation (Spijkerman et al., 2014). This study suggests that this species is already best suited for low CO_2 and P-limited conditions. However, Spijkerman et al. (2014) did propose that prolonged exposure to P-limited conditions might result in CCF regulation that a 10-day acclimation period was too short to capture. Regardless, affinity (V_{max}:$K_{0.5}$(CO2) for CO_2 uptake by O_2 evolution in *C. acidophila* did not differ between the phosphorus treatments at elevated CO_2 conditions. Overall, CCM characteristics were under regulation of CO_2 and not phosphorus condition.

4.3.3 *IRON*

Studies on the effects of iron deficiency on CCM expression and activity has remained limited since Raven and Beardall (2014) reviewed nutrient supply effects on CCMs. The one data set that has been previously discussed was a study done by Young and Beardall (2005). The data showed an increased inorganic C affinity for the chlorophyte, *Dunaliella*, exposed to iron limitation. This response is indicative of increased CCM activity. However, Young and Beardall (2005) did not investigate how elevated CO_2 conditions would interact with iron limitation. Although not looking at Fe limitation specifically, a freshwater alga, *Chlamydomonas acidophila*, was grown in variable P, Fe, and CO_2 conditions to study the effect of low P and high Fe concentrations on CO_2 acquisition (Spijkerman, 2011). This study demonstrated no differences in CCFs across the three iron concentrations used (0.2, 4, and 10 mM Fe) in both CO_2 conditions. Spijkerman (2011) concluded medium iron concentrations did not influence the expression of a CCM or the P_{max}. Perhaps iron limitation affects CCMs and any addition of iron reduces the nutrients impact on the mechanism's activity.

4.4 CO_2-DEPENDENT SHIFTS IN SPECIES DOMINANCE HIERARCHIES

Currently, marine phytoplankton exist in an environment characterized with high concentrations of HCO_3^- and low concentrations of $CO_{2(aq)}$. In these systems, dominant phytoplankton species differ in CO_2 requirements where some preferably use CO_2 as a carbon source while others use HCO_3^- (Elzenga *et al.*, 2000, Riebesell, 2004). However, regardless of uptake preferences, inorganic C must be in the form of $CO_{2(aq)}$ to be used by the RuBisCo enzyme. Tortell (2000) emphasized that it is critical to understand how

phytoplankton will respond to increasing atmospheric CO_2 over the next century. If environmental change allows for higher surface water CO_2, primary production may shift these microalgal communities (Beardall & Raven, 2004). Competition experiments demonstrating CO_2-dependent shifts in species dominance hierarchies are necessary both in monoculture and natural assemblages to examine the potential influence of C on species composition and succession.

4.4.1 *CULTURES*

While competition experiments are necessary to observe CO_2-dependent shifts in species hierarchies, many studies have focused on monocultures of multiple species to better understand the mechanisms determining efficiency and regulation of carbon acquisition. Some studies focused on single functional groups such as diatoms (Burkhardt *et al.*, 2001, Trimborn *et al.*, 2009, Yang & Gao, 2012) and raphidophytes (Fu *et al.*, 2008). Other studies tested marine microalgae across multiple functional groups such as diatoms, non-calcifying prymnesiophytes, and calcifying coccolithophores (Rost et al., 2003) or freshwater chlorophytes and cyanobacteria (Verschoor *et al.*, 2013, Ji *et al.*, 2017). While all the studies used multiple species, not all studies combined species together in a single apparatus to explore competition and therefore, are comparisons made based on physiological differences. However, Verschoor et al. (2013) and Ji et al. (2017) performed competition comparisons between freshwater chlorophytes (*Monoraphidium griffithii, Scenedesmus obliquus, Chlorella vulgaris*) and a cyanobacterium (*Synechocystis* sp. and *Microcystis aeruginosa*, respectively). Additionally, Low-DéCarie *et al.* (2011) used six species in the functional groups of

cyanobacteria, chlorophytes, and diatoms. Verschoor et al. (2013) first ran monoculture experiments and then, to observe species interactions, competition experiments where species were inoculated with a competitor. Ji et al. (2017) used their results from their monocultures to parameterize a resource competition model which predicted competitive interactions between the species. Next, competition experiments were carried out to test the model prediction. Using serial transfers, Low-DéCarie et al. (2011) investigated the competitive relationship between major taxa and how that relationship altered community composition (see later).

Like resource constraints, cultures exposed to high CO_2 demonstrated species-specific responses in their regulation of individual components of their CCM. One individual component of a CCM that is regularly observed is the CA activity, both external and internal. Burkhardt et al. (2001), Rost et al. (2003), and Trimborn et al. (2009) focused on these characteristics when evaluating the $K_{1/2}$ and V_{max} values for photosynthesis, CO_2 uptake, and HCO_3^- uptake. Burkhardt et al. (2001) restricted their focus on two widely studied diatoms, *Thalassiosira weissflogii* and *Phaeodactylum tricornutum*. While Rost et al. (2003) focused on different functional groups and Trimborn et al. (2009) examined only diatoms, both studied the bloom-forming species *Skeletonema costatum*. In all three studies, tables (Burkhardt et al., 2001, Rost et al., 2003, Trimborn et al., 2009) show the kinetic parameters that were obtained from a Michaelis-Menten fit for all the species across the pCO_2 treatment concentrations. For all diatoms, a similar trend of decreased CCM activity with elevated CO_2 levels were observed (Burkhardt et al., 2001, Trimborn et al., 2009). Only *Thalassiosira pseudonana*¸a non-blooming species, seemed not to regulate their CCM activity across

the tested CO_2 range (Trimborn et al., 2009). Rost et al. (2003) used previously reported K_M values for RuBisCo from Badger et al. (1998) and $K_{1/2}$ values obtained in this study to determine the ratio between K_M (CO_2) of RuBisCO and the apparent $K_{1/2}$ (CO_2) of O_2 evolution. This ratio was used to estimate the efficiency of the CCM across the functional groups of diatoms, non-calcifying prymnesiophytes, and calcifying coccolithophores. Rost et al. (2003) determined that, although *Emiliania huxleyi* had the highest $K_{1/2}$ values compared to the diatom *S. costatum* and both $K_{1/2}$ values increased with increasing CO_2, the diatom had higher regulation of the CCM. This study determined that the high saturation constant seen in *E. huxleyi* was most likely reflective of higher HCO_3^- uptake which may point to an involvement of the calcification process. These two studies concluded there would be shifts in the dominance between functional groups (Rost et al., 2003) and species within the diatom taxonomic group (Trimborn et al., 2009). Rost et al. (2003) expected the shifts would be away from coccolithophores toward diatom taxa. Trimborn et al. (2009) predicted large diatoms with lower CO_2 affinities ($K_{1/2}$), such as *S. costatum*, would benefit the most from elevated CO_2 conditions.

Above, I have focused on marine phytoplankton. The three studies reported below are based on freshwater species. The two studies that focused on competition experiments in freshwater algae and a cyanobacterium were Verschoor et al. (2013) and Ji et al. (2017). Both studies conducted experiments with low and elevated CO_2 levels. In the competition experiments with low pCO_2 treatments, the green alga *Scenedesmus obliquus* displaced the cyanobacterium *Synechocystis* sp. and *Microcystis aeruginosa*, respectively (Verschoor et al., 2013, Ji et al., 2017). When exposed to high pCO_2, both Verschoor et al. (2013) and Ji et al. (2017) observed coexistence between the cyanobacterium and

Scenedesmus. Verschoor et al. (2013) concluded that, under high CO_2, competition shifted the system from carbon limitation towards limitation by nitrogen and phosphorus which allowed the two species to coexist (see resource competition theory) (Tilman, 1982). Furthering the study, Ji et al. (2017) used two other green algae in their competition experiments. They concluded that *Monoraphidium griffithii* was the weakest competitor among the green algae in both CO_2 treatments. Also, at low CO_2 levels, *Chlorella vulgaris* could co-exist with the cyanobacterium *Microcystis aeruginosa* while at high CO_2 levels, *Chlorella* was outcompeted by *Microcystis*. There seems to be a pattern with elevated CO_2 and the coexistence of cyanobacterium and freshwater green algae which was predictive from monoculture work (Ji et al., 2017). Low-DéCarie et al. (2011) used linear modeling to predict community competition in elevated CO_2 conditions based on pure-culture, pairwise mixes, and full communities. With two species per functional group, Low-DéCarie et al. (2011) determined a strong predictive link between the pairwise mixtures and the competitive response in the whole community. In elevated CO_2 conditions chlorophytes had a strong positive response in competitive ability, diatoms had an intermediate response, and cyanobacteria had the weakest response. Further examination in whether this is the case for marine phytoplankton should be conducted.

4.4.2 *NATURAL ASSEMBLAGES*

Determining the CCM activities in a natural assemblage of marine phytoplankton has limitations since each species and/or functional group present has various physiological differences in their carbon acquisition capabilities. Most of the work

looking at elevated CO_2 levels on natural assemblages has focused on chlorophyll *a* (chl *a*) concentrations, growth rate, community shifts in major taxonomic groups, and particle size distribution (Tortell *et al.*, 2002, Kim *et al.*, 2006, Engel *et al.*, 2008, Tortell *et al.*, 2008, Feng *et al.*, 2009, Bach *et al.*, 2017, Grear *et al.*, 2017). Tortell (2000) urged future studies to examine the CO_2 dependent shifts in species dominance hierarchies to explain potential influences on inorganic C on species composition and succession. With the culture work examining CCM activity on a single species physiology, Tortell (2000) emphasized the importance of looking at the interactions among a community.

Chl *a* was generally seen to increase initially with the development of blooms and then peak when nutrients were depleted (Engel et al., 2008). There were no significant differences in total Chl *a* concentrations detected among pCO_2 treatments suggesting that observed increases in Chl *a* were not related to pCO_2 levels (Tortell et al., 2002, Engel et al., 2008, Bach et al., 2017, Grear et al., 2017). Similarly, Tortell et al. (2002) determined that there was no change in the growth rate and bulk productivity of the phytoplankton. Tortell et al. (2002) collected samples in September 2000 and used CO_2 concentrations of 150 and 750 ppm CO_2. When Tortell et al. (2008) conducted their experiment in the spring season (November-December) of 2005, a significant increase in primary productivity was reported between 100 and 380 ppm CO_2 but no further effects observed at 800 ppm CO_2. However, collections in the summer (January) of 2006 demonstrated a significant increase in bulk productivity across the CO_2 treatments. The growth rates followed a similar trend as productivity but were only significant between 380 ppm and 800 ppm CO_2. Season and treatment of ppm CO_2 may have influenced observed ^{14}C fixation. Regardless of whether biomass or productivity changes were noted across

elevated CO_2 conditions, Tortell et al. (2002) indicated that CO_2-dependent shifts in phytoplankton communities could occur in the absence of detectable differences in these factors.

The composition of a phytoplankton community is highly dynamic due to changing biotic and abiotic factors, but changes in CO_2 conditions may alter relative abundances of different functional groups and/or species. Additionally, phytoplankton cell size is often discussed in the context of responses to environmental change and therefore, was included in multiple studies investigating community-level shifts (Gao & Campbell, 2014). Many studies have indicated that diatoms increased in abundance when exposed to elevated CO_2 (Tortell et al., 2002, Bach et al., 2017) and larger chain forming diatoms were favored over pennate diatoms (i.e. *Skeletonema costatum* over *Nitzschia* spp., *Chaetoceros* spp. over *Pseudo-nitzschia*, *Chaetoceros lineola* over *Cylindrotheca closterium*) (Kim et al., 2006, Tortell et al., 2008, Feng *et al.*, 2010) yet there are contradictions. These range from diatoms being insensitive to the elevated CO_2 treatment and chlorophytes being the competitive functional group (Engel et al., 2008) to pennate diatom succession over centric diatoms (*Pseudo-nitzschia* and *Cylindrotheca closterium* dominated) (Feng et al., 2009). Bach et al. (2017) also demonstrated that cyanobacteria also grew in abundance under elevated CO_2 conditions, but went on to conclude that the group was most likely freed from grazing and competition pressures. This conclusion is complementary to the idea that increased CO_2 may affect bottom-up/top-down control mechanisms rather than direct effects of CO_2 on individual groups.

Diatoms may generally show an increase in abundance above other functional groups in elevated CO_2 conditions, but phytoplankton cell size varies among studies.

There seems to be support for all size classes (i.e. pico-, nano-, micro-plankton) to grow in abundance and maintain presence under elevated pCO_2 conditions. Both Bach et al. (2017) and Grear et al. (2017) showed picoeukaryotes (0.2 – 2 µm and 3-5 µm, respectively) with the strongest positive response in biomass and abundance to elevated CO_2. Grear et al. (2017) also reported that the abundance of nanoplankton (5 – 20 µm) had a negative response and the abundance of microplankton (>20 µm) showed no response to elevated CO_2. There was a general shift to smaller cells. This response was inconsistent with the evidence that < 4 µm cells had the worst response to elevated CO_2 (Engel et al. 2008) and that there was a general shift from small pennate diatoms to large centric diatoms (Tortell et al., 2008, Feng et al., 2010).

Overall, community level responses to rising pCO_2 vary across oceanic regions and bodies of water. The studies mentioned above spanned bays (Grear et al., 2017), fjords (Engel et al., 2008), seas (Tortell et al., 2008, Feng et al., 2010), and coasts (Kim et al., 2006, Bach et al., 2017). The initial ratio between major taxonomic classes can also be assumed to be different for many of these locations. The difference in initial community composition is critical to consider because, as concluded by Eggers *et al.* (2014), the initial community composition is a main driver behind community structure.

4.5 CONCLUSIONS AND FUTURE EFFORTS

According to Ji et al. (2017), simple dichotomies, such as, that eukaryotic phytoplankton are better competitors at high CO_2 levels and cyanobacteria at low CO_2 levels, do not capture the diversity of CCMs that are found among and within different

phytoplankton taxa. We need to further compare the performance of different CCMs across a wide range of different species interacting together.

There is immense complexity on how resource constraints (e.g. nitrogen, phosphorus, iron) and elevated CO_2 affect CCM activity among species both within and between functional groups. This also spans into how influential the initial community compositions are on competition under these conditions of resource limitation and elevated CO_2. A standardized method may be best to ascertain the mechanisms that remain constant across these environmental variations. Results may not be generalized to other coastal or oceanic ecosystems, but there may be similarities among systems with similar environmental regimes.

An example of a standardized method of approach could be based on the studies of Low-DéCarie et al. (2011) and Ji et al. (2017) which used culture experiments to create a model to test their results. Ji et al. (2017) tested their model's output in a controlled microcosm experiment looking at competitive interactions among functional groups. Both studies saw chlorophytes have a positive response to elevated CO_2 conditions and, in the treatment with a slight increase in CO_2 (Low-DéCarie et al., 2011), both studies saw cyanobacteria co-existing with the chlorophytes. While these were freshwater species, a pattern that pure-culture experimental responses could strongly predict competitive interactions is something to investigate in marine systems.

While culture experiments are needed to determine key physiological differences in CCMs among species to inform models, these studies lack the ecological adaptations and competition that occur in nature. Further long-term, community-level microcosm

studies are necessary to allow for evolutionary adaptation and its interaction with ecological responses.

4.6 FIGURES

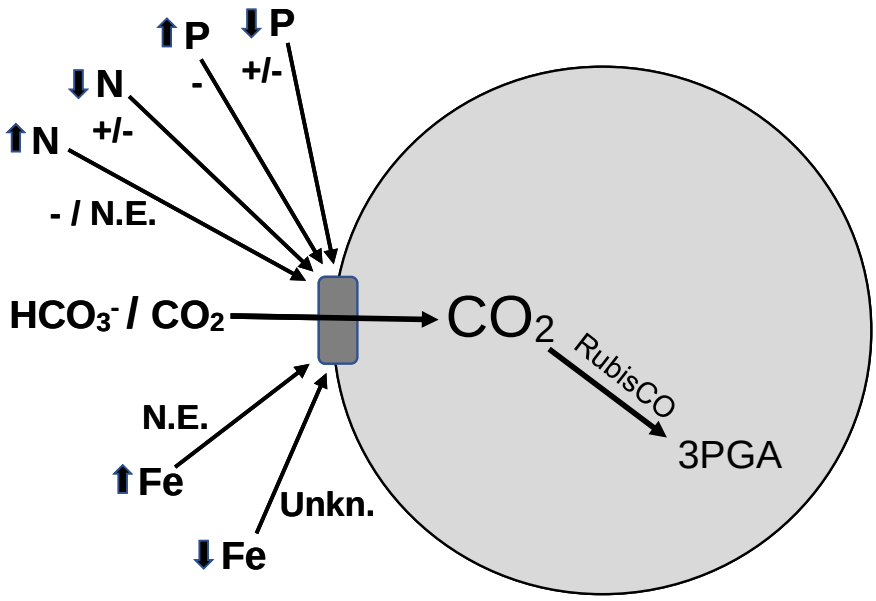

Figure 4.1: Summary of the interactions of elevated CO_2 and nutrient conditions on the expression of CCMs in marine phytoplankton. N = nitrogen, P = phosphorus, Fe = iron, N.E. = no effect, Unkn. = unknown effect, 3PGA = 3-Phosphoglyceric acid. Adapted from Raven and Beardall 2014.

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CHAPTER 5

CARBONIC ANHYDRASE INHIBITION EFFECTS ON GROWTH, GROSS PRIMARY PRODUCTION, AND PHOTOSYNTHETIC PERFORMANCE IN EXPERIMENTAL PHYTOPLANKTON COMMUNITIES[1]

[1]Knotts ER, Pinckney JL. Carbonic anhydrase inhibition effects on growth, gross primary production, and photosynthetic performance in experimental phytoplankton communities. Submitted to *Journal of Phycology*.

5.1 ABSTRACT

Differences in the ability to take up and utilize inorganic carbon exist between and within major phytoplankton taxa and suggests a capacity for future changes in phytoplankton communities in the form of hierarchies and production. Currently, most studies on how rising atmospheric CO_2 will impact phytoplankton has focused on monocultures and few investigations examine full community assemblages. Differences in acquisition strategies may cause predictable shifts in compositions of phytoplankton communities. We investigated whether induced carbon limitation shows predictable community shifts by inhibiting an enzyme used in carbon concentrating mechanisms (CCMs), carbonic anhydrase (CA). Using monocultures, pairwise mixtures, and community assemblages of four diatom genera, only *Cylindrotheca* experienced growth under CA inhibition while the other genera had strong population growth limitation and cell mortality. While the CA inhibited growth responses of the monocultures were good predictors of competition in pairwise mixtures ($R^2 = 0.95$), these mixtures were not good predictors of full assemblages when *Cylindrotheca* was removed from the analysis ($R^2 = 0.34$). Gross primary production (GPP) and relative electron transport rate (rETR) were analyzed to further investigate what advantages *Cylindrotheca* had in these communities. All genera experienced a decrease in GPP and rETR at high light levels (e.g. 1000 μmol photons m^{-2} s^{-1}) indicating that some other physiological trait gives *Cylindrotheca* a competitive benefit. Our study demonstrated that diatoms could not be clustered together by functional shape or taxonomic family to describe predictable responses to CA inhibition. Additional physiological mechanisms likely influence composition outcomes beyond CA activity in CCMs.

5.2 INTRODUCTION

Species composition of natural phytoplankton communities are created and maintained through the processes of resource competition in the absence of grazing. Under limiting resource competition theory, we should be able to predict the outcomes of interspecific competition by manipulating the resource requirements of the various competing species (Tilman, 1982). However, Hutchinson (1961) observed that phytoplankton communities support more species than would be predicted under the competitive exclusion principle (Hardin, 1960). Competition for essential resources has been proposed to contribute to the richness of the natural phytoplankton communities. Irwin and Finkel (2018) established that natural populations yielded realized niches that could be quite different from the fundamental niches as measured in the lab using monocultures. These realized niches allow the coexistence because each phytoplankton species has a different suite of favorable conditions that promote growth and reproduction. As a response, phytoplankton have developed different pathways to maximize growth and reproduction to increase their fitness. Recognizing the pathways that maximize successful nutrient uptake represents a means for understanding the coexistence within communities (Keeley, 1999).

In natural marine photoautotroph communities, competition for elements include, but are not limited to, various forms of nitrogen, phosphorus, iron, and carbon. While dissolved inorganic carbon (DIC) is not usually considered to be limiting in seawater with a concentration of ca. 2 mM, the aqueous form of CO_2 (hereafter referred to as $CO_{2(aq)}$) is typically less than 1% of that concentration (ca. 10 µM) in surface seawater. $CO_{2(aq)}$ is necessary in the first major stage of the Calvin cycle for photosynthesis where

the enzyme ribulose bisphosphate carboxylase (RubisCO) accelerates the chemical reaction by which inorganic carbon is fixed into organic material.

Phytoplankton species differ in $CO_{2(aq)}$ requirements and those taxonomic differences in carbon acquisition affinities are exceptionally important when determining the ecological interactions of phytoplankton groups (Mercado *et al.*, 2009). One of the strategies utilized for carbon acquisition are biophysical carbon-concentrating mechanisms (CCMs) (Riebesell *et al.*, 1993). One form involves bicarbonate transporters carrying HCO_3^- across the membrane and conversion of that ion to $CO_{2(aq)}$ by internal carbonic anhydrase (CA). The second form involves external cellular CA which restores the CO_2/HCO_3^- equilibrium at a cell's surface as $CO_{2(aq)}$ is taken up by the cell. The third form converts $CO_{2(aq)}$ back to HCO_3^- to slow $CO_{2(aq)}$ leakage from the cell and to maintain a large concentration of DIC near the site of carbon fixation. All these forms of a CCM help overcome inorganic carbon limitations for photosynthesis currently faced in surface seawater.

Variation in the functional trait of DIC uptake suggests that there is a capacity for alterations in phytoplankton community structure in response to increasing atmospheric CO_2 over the next century (Griffiths *et al.*, 2017). The resource requirements and competitive interactions of the dominant species under controlled carbon conditions are important, but it is also critical to understand how the effects of global scale changes in environmental conditions will impact species interactions in natural communities. Studies have used monocultures to better understand the mechanisms that determine efficiency and regulation of carbon acquisition (Martin & Tortell, 2008, Trimborn *et al.*, 2009, Spijkerman *et al.*, 2014, Wu *et al.*, 2015). While these monoculture experiments are

needed to identify key physiological differences in CCMs between species, these studies do not address how natural phytoplankton communities may shift under different seawater carbonate conditions. These studies lack critical information on the drivers behind community structure – ecological adaptations, initial community composition, and competition (Knotts, 2018 unpublished manuscript).

Reinfelder (2011) reviews CCMs in the three dominant groups of eukaryotic marine phytoplankton. Larger phytoplankton with efficient CCMs (e.g., diatoms) usually have the competitive advantage over smaller phytoplankton with less efficient CCMs (e.g., dinoflagellates, coccolithophores). Beardall and Raven (2004) suggested that environmental change allowing for higher surface water CO_2 might lead to greater primary production attributed to smaller, low-efficiency CCM species, and larger phytoplankton lose the competitive advantage of efficient CO_2 acquisition. However, these predictions have been countered by indications that down-regulation of CA activity in elevated CO_2 conditions may give an energy benefit to the larger algal species such as diatoms (Hopkinson *et al.*, 2011).

Diatoms increase in abundance when exposed to elevated CO_2 (Tortell *et al.*, 2002, Bach *et al.*, 2017) and larger chain forming diatoms are favored over pennate diatoms (Kim *et al.*, 2006, Feng *et al.*, 2009, Feng *et al.*, 2010), yet there are contradictions. Other studies have shown that diatoms are insensitive to elevated CO_2 concentrations, while chlorophytes are the competitively dominant functional group (Engel *et al.*, 2008) to pennate diatom outcompeting centric diatoms (Feng et al., 2009). Bach et al. (2017) also demonstrated that cyanobacteria grew in abundance under elevated CO_2 conditions, but went on to conclude that the group was most likely freed

from grazing and competition pressures. Overall, community level responses to rising pCO_2 varies across oceanic regions and bodies of water (Engel et al., 2008, Tortell *et al.*, 2008b, Feng et al., 2010, Bach et al., 2017, Grear *et al.*, 2017). The difference in initial community composition at these locations is critical to consider because, as concluded by Eggers *et al.* (2014), the initial community composition is a main driver behind community structure.

In fresh water, Low-DéCarie *et al.* (2011), Verschoor *et al.* (2013), Ji *et al.* (2017), and Pardew *et al.* (2018) reported chlorophytes as having a positive competitive response to elevated CO_2 conditions and, in the treatment with a slight increase in CO_2, these studies saw cyanobacteria co-existing with the chlorophytes. They concluded that pure-culture experimental responses could strongly predict competitive interactions and similar mechanisms should be examined in marine systems.

Natural phytoplankton communities in southeastern U.S. estuaries are mostly composed of diatoms with variable contributions of cryptophytes, cyanobacteria, chlorophytes, euglenophytes, dinoflagellates, and prasinophytes (Lewitus, 1998, Lawrenz *et al.*, 2013, Allen *et al.*, 2014). Knotts and Pinckney (2018) investigated whether functioning CCMs with CA provided a competitive advantage to some functional groups and contributed to structuring the phytoplankton community in their estuary. They established that diatoms persistently had a competitive advantage in abundance in these communities with or without active CA, but there were lower-level shifts in the dominant diatom genera when CA was inhibited. Centric diatoms (e.g. *Skeletonema, Guinardia,* and *Rhizosolenia*) dominated control samples suggesting that they had a competitive advantage through the use of an active CA enzyme. When CA was inhibited, pennate

diatoms (e.g. *Asterionellopsis*, *Thalassionema*, and *Cylindrotheca*) became more abundant relative to the centric diatoms. These results suggested that pennate genera were not dependent on CA activity to maintain a competitive advantage over other phytoplankton. Knotts and Pinckney (2018) hypothesized that the pennate genera listed above preferably uptake $CO_{2(aq)}$ and therefore, are not dependent on CA to rehydrate HCO_3^- to $CO_{2(aq)}$ near the site of carbon fixation (Mercado et al., 2009).

According to Ji et al. (2017), simple generic dichotomies in the outcomes of competition between major algal groups do not capture the diversity of CCMs that are found among and within different phytoplankton taxa. We need to further compare the performance of different CCMs across a wide range of different species interactions. Low-DéCarie et al. (2011) and Pardew et al. (2018) demonstrated that $CO_{2(aq)}$ uptake and utilization between major taxa could lead to predictable changes in competitive ability. Collectively, these studies showed that pure culture, pairwise mixtures, and full community experiments can be used to evaluate the growth rate changes attributable to changes in CO_2 concentration.

The purpose of our study was to similarly compare the predicated link between pure, pairwise, and full community cultures to changes in CA activity using growth rates and paired competitive abilities. Also, we measured oxygen production and photosynthetic performance changes due to CA inhibition to aid in identifying why certain genera can maintain a competitive advantage without active CA.

5.3 METHODS

5.3.1 *PHYTOPLANKTON CULTURES*

Phytoplankton cultures were collected and isolated from the North Inlet estuary near Georgetown, South Carolina, USA. We studied four genera from the marine diatom phytoplankton functional group - *Skeletonema* sp. (~6 μm by 4 μm), *Guinardia* sp. (~23 μm by 9 μm), *Cylindrotheca* sp. (28 μm by 5 μm), and *Asterionellopsis* sp. (12 μm by 10 μm). These four genera can be further divided into two groups that are distinguished by the shape of their frustule: centric and pennate. *Skeletonema* and *Guinardia* are classified as centric in the class Coscinodiscophyceae and clade Coscinodiscophytina characterized by their radially symmetric shape. *Cylindrotheca* and *Asterionellopsis* are classified as pennate diatoms in the class Bacillariophyceae and clade Bacillariophytina characterized by their bilateral symmetry.

These genera were selected based on their high prevalence in the North Inlet estuary and being clearly identifiable through morphological features visible under microscopy. Additionally, Knotts and Pinckney (2018) identified these genera as being highly responsive to carbonic anhydrase inhibition in a natural phytoplankton community.

5.3.2 *GROWTH CONDITIONS*

All phytoplankton genera were grown in f/2 + silicate medium as recommended by the National Center for Marine Algae and Microbiota (NCMA) at the Bigelow Laboratory for Ocean Sciences. Stock cultures were grown at 20 °C, under a 12:12 hr light:dark cycle at irradiance levels of 50 μmol photons • m-2 • s-1 as measured with a

Biospherical Instruments QS 2101 light meter (Biospherical Instruments, Inc., USA). Each of the phytoplankton genera were maintained in these environmental conditions in exponential growth for a period over two months before experimentation. Cultures were swirled daily by hand to ensure adequate mixing and prevent a settling bias, and were transferred to fresh culture medium every seven to ten days.

5.3.3 *CA INHIBITION*

Instead of increased CO_2 additions, we used a CA inhibitor to force the genera to experience the system as carbon limited. CCMs are an energy-consuming process that may experience down-regulation as a response rising CO_2 (Raven, 1991). A reduction in energetic cost of CCMs could yield energetic savings to be put toward growth and production (Hopkinson et al., 2011, Shi *et al.*, 2017). We assume that those genera who can competitively compete without CA will have a benefit in the future if its carbon concentrating mechanism (CCM) downregulates CA production. Therefore, our experiments have two treatments; a control treatment with cells in nutrient replete f/2 media and a CA inhibition treatment with a CA inhibitor, ethoxyzolamide (EZ, $C_9H_{10}N_2O_3S_2$, Sigma Aldrich, cat. no. 333328-1G). 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, Tortell *et al.*, 2000, Capasso & Supuran, 2015, Wu et al., 2015). A cell permeable inhibitor was used because different diatoms can have CA performing an essential role in CCMs in different cellular

locations such as in the cell walls and periplasmic spaces ((Hopkinson, 2014, Matsuda *et al.*, 2017, Young & Hopkinson, 2017).

5.3.4 *GROWTH RESPONSES AND COMPETITION EXPERIMENTS*

All experiments were conducted in 250 mL of medium in 500 mL Erlenmeyer flasks fitted with silicone stoppers attached to air pumps that gently bubbled 0.7 μm filtered air in the cultures throughout the entire experiment. The addition of bubbling air into the media was to maintain adequate mixing and to sustain CO_2 levels throughout the experiment. pH was monitored using an Orion pH meter model 250A. The average pH of the cultures through the experiment were 8.16 ± 0.03 (mean ± SE).

The pure cultures were initiated with a starting inoculation of 5×10^3 cells • mL-1, taken from each of the single genera stock cultures. Pairwise mixtures between centric and pennate diatoms were initiated with 2.5×10^3 cells • mL-1 for each of the two genera. The pairwise cultures were *Skeletonema* x *Cylindrotheca*, *Skeletonema* x *Asterionellopsis*, *Guinardia* x *Cylindrotheca*, and *Guinardia* x *Asterionellopsis*. Full communities of the four genera were initiated with 1.25×10^3 cells • mL-1 for each genus. All cultures remained in exponential growth throughout each of the 5- day experiments. Each culture type (i.e. pure, pairwise, full) and treatment (i.e. control and CA inhibition) had replication in quintuplicate for a total of 90 experimental cultures.

Culture cell densities were measured daily from the second day after inoculation until the final day of the experiment for the calculation of respective growth rates. Samples from each culture were immediately fixed with Lugol's solution and counted via microscopy with a 2.2 mL settling chamber and a light microscope at 100-400x

magnification, whereby the abundance of each genera comprising the cultures were counted using a total minimum count of 400 cells. However, in experimental CA inhibition cultures with high mortality, total counts were reduced to 100 cells.

5.3.5 *PRODUCTIVITY AND PHOTOSYNTHETIC PERFORMANCE MEASUREMENTS*

Light/dark dissolved oxygen (dO_2) productivity measurements were made separate from the growth and competition experiments. Culture genera were inoculated in separate 20 mL glass scintillation bottles at a density of 5×10^3 cell • mL-1 (7 replicates • genus-1 • treatment-1, n = 56). Glass coated mini magnetic stirrers were added and allowed to rotate at 600 rpm to prevent oxygen gradient development. These bottles were then sealed with parafilm, incubated for a fixed time interval (20 minutes), and then pierced with a Clark-type oxygen microelectrode needle (1.1 mm) connected to a picoammeter (Unisense). The size of the piercing was sufficiently small to ensure that gas–liquid exchange was minimized. The microelectrodes were calibrated using a two-point procedure with 0% (0.1 M NaOH/ascorbate) and 100% (bubbling with air) saturation dO_2 concentrations. Cultures were incubated at an illumination of 1000 μmol photons • m-2 • s-1 when measuring net primary production and in complete darkness when measuring respiration. There was no photoinhibition exhibited at this irradiance (see Fig. 5.8 control treatments).

All dO_2 measurements were started after the bottles were allowed to equilibrate for two minutes. The slope of increasing dO_2 in the light was net primary production (NPP) and the slope of decreasing dO_2 in the dark was respiration (R). These two slopes were then used to calculate gross primary production (GPP). Slopes were calculated from

a sampling interval that provided a regression line with a minimum r^2 value of 0.96 and 0.82 for NPP and respiration, respectively.

To assess the physiological responses of cultures to CA inhibition, 5 mL subculture of pure cultures with and without EZ were taken from each bottle during mid-exponential phase. Photosynthetic performance was estimated using pulse-amplitude-modulated chlorophyll *a* fluorescence (PAM) measurements using rapid light curves (RLCs) with a Walz Water-PAM, (Schreiber *et al.*, 1986). RLCs are different from traditional photosynthesis-irradiance (PI) curves because each step of illumination does not provide sufficient time for photosynthesis to reach steady state (Schreiber, 2004). These curves provide information on the present state of photosynthesis for photoautotrophs by measuring effective quantum yields and relative electron transport rates (rETR) (Ihnken *et al.*, 2011). The effective quantum yield of photosynthesis describes whether absorbed light photons are used to transport electrons through photosystem II and is calculated by the difference in maximal chlorophyll fluorescence and steady-state fluorescence, divided by the maximal chlorophyll fluorescence (Genty *et al.*, 1989). rETRs provide a relative rate of electrons passing through photosystem II and are calculated by multiplying the effective quantum yield by the photosynthetically active radiation (PAR). Finally, additional information on the efficiency of light capture can be obtained from the initial slope of the RLC in the form of α. (Schreiber, 2004).

Cells were dark-adapted for 20 minutes and then exposed to a saturation pulse at the end of nine different and increasing actinic photosynthetic active radiation (PAR) intensities, with a 30 s duration between each increment (Wu et al., 2015). The RLCs were fitted using the regression function introduced by Jassby and Platt (1976) that is

monotonically nondecreasing with no photoinhibition term. This function was selected because our data did not show photoinhibition at maximum irradiance exposure levels. From this function, the α and the maximum relative electron transport rate ($rETR_m$) were obtained and evaluated.

5.3.6 *DATA ANALYSIS*

5.3.6.1 GROWTH RESPONSES AND COMPETITION

Analyses were conducted with the statistical program R, v.3.5.1 (RCoreTeam, 2012). To measure the response of growth to the treatments, the *growthrates* package (Petzoldt, 2018) calculated the maximum growth rates from log-linear part of the growth curve for a series of experiments by using smoothing splines over the 5-day experimental period.

Competition coefficients were calculated using the methods of Pardew et al. (2018) and Low-DéCarie et al. (2011). Briefly, predicted competition coefficients were calculated from unialgal culture growth rates, while realized competition coefficients were calculated from changes in genera frequency.

The predicted competition coefficient of genus 1 (c_{1p}) was calculated as the difference of its growth rate (r_1) with the growth rate of a competing species (r_2) standardized by the growth rate of the entire competing community ($r_{community}$). The symbol "r" is the growth rate in doubling per day of either of the two species or the total population.

$$c_{1p} = \frac{r_1 - r_2}{r_{community}} \quad (1)$$

The realized competition coefficients (c_{1r}) were calculated from the change in the relative frequency (f) of each genus through time while also accounting for the growth of the entire community overall and g is the number of generations (doublings) of the total population. In the full community of four genera, f_2 was the frequency of all other species combined.

$$c_{1r} = \frac{1}{g_{community}} ln \left(\frac{\frac{f_{1\ final}}{f_{2\ final}}}{\frac{f_{1\ initial}}{f_{2\ initial}}} \right) \quad (2)$$

Competition coefficients were translated and transformed to fit ANOVA assumptions of normality and homogenous variance. This was accomplished with a $|\ln(x+1)|$ transformation.

A summary of the statistical analyses is given in Table 1. The responses of CA inhibition in unialgal growth rates were assessed using an analysis of variance (ANOVA) on the main effects and interactions of CA inhibition and genus. Functional shape of centric versus pennate was also investigated in place of the identifying genus. To assess the competitive response, individual three-way ANOVAs were conducted separately for each pairwise mixture of cultures using the realized competition coefficients as the response variable and the CA inhibition treatment, focal species, and competitor species as fixed factors. Full community competitions were assessed using a multivariate analysis of variance (MANOVA) when phytoplankton genera were separate and when grouped by functional shape to determine the effect of CA inhibition on the competition coefficient of each species in the mixture. Finally, to test whether the response of growth in pure culture or of competition in pairwise culture could predict the full community response, a least-squares linear regression model was fitted to relate the mean species response in

either pure culture or pairwise competition to the response of competitive ability in the full community.

5.3.6.2 PRODUCTION AND PHOTOSYNTHETIC PERFORMANCE

An ANOVA was used to analyze the differences of GPP between the treatment of CA inhibition and genus. The response variable was $\ln(x)$ transformed to satisfy the ANOVA assumptions of normality and homogenous variance.

RLCs were analyzed using the packages *lme4* (Bates et al., 2015) and *lmerTest* (Kuznetsova et al., 2017). A linear mixed effects analysis was performed on the relationship between the relative electron transport rate (rETR) and exposure to the CA inhibitor, EZ. As fixed effect, we entered treatment type and included the interaction term of genera and treatment. Genera were not compared because each genus and replicate experiment began with a different maximum photochemical efficiency of PS II (i.e. F_v/F_m ratio). As crossed random effects, we had replicate experiment number and PAR intensity. The random effects of replicate experiment number and PAR account for the variable differences between each replicate and each actinic light intensity shone on the phytoplankton. The initial model was rETR ~ Treatment + Treatment:Genus + (1 | Replicate Experiment) + (1 | Light Value). Using the step function (*lmerTest*), our results show that this was the best fit model.

Finally, initial slopes (α) of the rETR vs. irradiance curve of each genera between treatments were compared with t-tests in order to assess whether the RLCs on CA inhibited treatments experienced a change in photoacclimation status.

5.4 RESULTS

5.4.1 *GROWTH RESPONSES*

This experiment evaluated relative growth rate changes of four genera when exposed to a CA inhibitor in unialgal cultures, pairwise mixtures, and in a full community of all four diatom culture genera (Fig. 5.1). The growth rates were combined in the pairwise mixtures because there was no significant difference between each combination (two-tailed t-test, $p > 0.28$ per combination). In controlled unialgal and pairwise cultures, *Skeletonema* had the highest growth rates (mean = 0.96 d-1 and 0.95 d-1, Fig. 5.1D, H) followed by *Asterionellopsis* (mean = 0.79 d-1 and 0.89 d-1, Fig. 5.1A, E) and then *Cylindrotheca* (mean = 0.75 d-1 and 0.77 d-1, Fig. 5.1B, F). *Guinardia* had the slowest growth rate in all competition scenarios (mean = 0.61 d-1 and 0.68 d-1, Fig. 5.1C, G). Only in the full community scenario did *Asterionellopsis* have a higher growth rate than *Skeletonema* (mean = 0.93 d-1 and 0.90 d-1, Fig. 5.1 I, L). The difference in growth between the control and carbonic anhydrase inhibited treatments was significant across all genera ($F_{3,32} = 5.43$, $p = 0.004$, Table A.1). While there was an interaction between genus and treatment, treatment affected the growth rate in a negative manner across the four genera ($F_{1,32} = 115.45$, $p < 0.001$). This was corroborated when the functional shape of the respective algal species was investigated in place of genera. In this model, only the CA inhibition treatment explained the variance ($F_{1,36} = 75.72$, $p < 0.001$, Table A.2). All four genera saw a decrease in growth rate when carbonic anhydrase was inhibited across all cultures. Regardless of competitive environment, *Cylindrotheca* experienced a slower positive growth rate when exposed to the inhibitor. The three other genera had negative

growth rates representing strong growth limitation and cell mortality when active CA activity was inhibited.

5.4.2 *PAIRWISE COMPETITIONS*

The effect of CA inhibition upon the competitive ability of each genus within each of the pairwise mixtures was assessed using the centric diatoms, *Skeletonema* and *Guinardia*, as the focal genera and the pennate diatoms, *Asterionellopsis* and *Cylindrotheca*, as the competitor genera (Table A.3). The competitive ability of all the genera were impacted by the removal of CA; frequently in a negative manner ($F_{(1, 32)}$ = 10.82, p = 0.003). While the CA inhibition treatment affect the focal genera differently ($F_{(1, 32)}$ = 22.32, p < 0.001) and the competitor genera differently ($F_{(1, 32)}$ = 38.81, p < 0.001), there was no difference in how the focal and competitor species interacted together ($F_{(1, 32)}$ = 0.32, p = 0.58).

On average, genera with the fastest growth rates outcompeted the paired genus (Fig. 5.2). The pairing of *Skeletonema* and *Asterionellopsis* in both treatments (Fig. 5.2C, G) did not outcompete each other across the five-day experiment. In all other control experiments, the fastest growth rate gave that genus the competitive advantage in community frequency (Fig. 5.2A, B, D). The competitive advantage of a fast growth rate of these genera was not seen in the CA inhibitor treatments where there was strong growth limitation and cell mortality in *Skeletonema*, *Asterionellopsis*, and *Guinardia* (Fig. 5.2E-H). None of these genera had a competitive advantage and therefore, no distinct change in community frequency was observed. *Cylindrotheca* maintained a

competitive advantage with its positive growth and therefore outcompeted the paired genus (Fig. 5.2F, H).

5.4.3 *FULL COMMUNITY COMPETITIONS*

CA inhibition altered the abundances of the genera present relative to the control in the full assembled community of all four genera (Fig. 5.3). Consistent with the growth experiments and pairwise cultures, *Asterionellopsis* and *Skeletonema* had the greatest competitive advantage in the control treatment (Fig. 5.3A). Once CA was inhibited, all the genera that experienced high cell mortality and growth limitation demonstrated decreased competitive abilities, and decreased abundances in the community. *Cylindrotheca*, alternatively, benefited from the CA inhibition because the genus still maintained a positive growth rate that resulted in high abundances in the mixed community (Fig. 5.3B).

5.4.4 *PREDICTING COMMUNITY CHANGES*

For the treatments with CA inhibition, the growth rates in the unialgal cultures predicted the competitive abilities that we observed in the pairwise mixtures (linear regression, $R^2 = 0.95$, $p = 0.02$, Fig. 5.4). However, the shifts in the competitive abilities in the pairwise cultures did not successfully predict the changes in competition observed in the mixed community ($R^2 = 0.89$, $p = 0.06$, Fig. 5.5). Competition coefficients of *Cylindrotheca* were driving the entire relationship with its positive growth and low cell mortality. The high cell mortality experienced by the other community genera in the mixed culture resulted in equivalent coefficients (mean ± SE, -0.43 ± 0.04). The inability

to predict the competitive hierarchy of the full community was amplified when *Cylindrotheca*'s competition values were removed (R^2 = 0.34, p = 0.61). This may also explain why we were not able to detect an effect of CA inhibition on the competitive dynamics of different genera (i.e. competition coefficients) (MANOVA, $F_{2,37}$ = 0.056, p = 0.95). This was also the case when the competition coefficients were paired with functional shape (MANOVA, $F_{2,37}$ = 0.065, p = 0.94).

5.4.5 *PRODUCTION AND PHOTOSYNTHETIC PERFORMANCE*

We used GPP as a proxy when determining whether CA inhibition would affect primary production for the four genera (n = 7 measurements • genus-1 • treatment-1, Fig. 5.6). There was a significant difference among the genera ($F_{(3, 48)}$ = 11.91, p < 0.001). *Cylindrotheca* had the lowest production rates across treatments when normalized by Chl *a* content. Using a Tukey HSD test, the significant differences were found to be between *Cylindrotheca* and all the other genera, *Skeletonema*, *Asterionellopsis* and *Guinardia* (p = 0.012, p < 0.001, and p = 0.007, respectively), and between *Skeletonema* and *Asterionellopsis* (p = 0.042). There is also a significant difference between the CA inhibited and non-inhibited treatments ($F_{(1, 48)}$ = 12.81, p < 0.001). The unialgal cultures exposed to the CA inhibitor, EZ, had ca. 65% lower GPP compared to the cultures with no addition (a difference of 3.74 ± 1.04 μmol O_2 • Chl *a*-1 • h-1, mean ± SE). There was no significant interaction between the CA inhibition treatments and genera ($F_{(3, 48)}$ = 1.47, p = 0.23).

When investigating whether functional shape affected the GPP when CA was inhibited, no difference between the GPP of the two different functional shapes was

detected ($F_{(1, 52)} = 0.14$, $p = 0.715$) and there was no significant interaction between functional shape and CA inhibition treatment ($F_{(1, 52)} = 1.18$, $p = 0.28$, Fig. 5.7). As expected, there still was a significant difference in GPP between the two CA activity treatments in this model (($F_{(1, 52)} = 7.74$, $p = 0.008$).

A change in the curve shape for RLCs can be used to infer different photophysiological responses to increasing irradiance exposure. The best-fit model for rETR vs. irrandiance established that there was a significant difference between the two treatments ($X_2 = 97.70$, $p < 0.001$) and interaction between treatment and genus ($X_2 = 28.56$, $p < 0.001$). Using RLCs, a decrease in rETR due to CA inhibition was similarly exhibited by all four genera (Fig. 5.8). However, the genera responded differently to CA inhibition. The change in the shape of the RLCs was most apparent at the high irradiances and no photoinhibition was observed. While *Asterionellopsis* and *Cylindrotheca* both had a large separation in the CA inhibited RLCs relative to the control (Fig. 5.8A, B), *Skeletonema* and *Guinardia* had overlapping RLCs (Fig. 5.8C, D). This result seems to be associated with the amount of variation in rETR each genus exhibited at higher irradiances.

Of the four genera, the pennate diatoms *Asterionellopsis* and *Cylindrotheca* had $rETR_m$ values that were 35.4% higher than the centric diatoms when CA was active and 19% lower than centric diatoms when CA was inhibited. Overall, *Asterionellopsis*, *Cylindrotheca*, and *Skeletonema* exhibited a large decrease in their $rETR_m$ without active CA and *Guinardia* experienced only a slight decrease in its $rETR_m$ (control and CA inhibitor mean = 95.42 µmol electrons • m-2 • s-1 and 84.98 µmol electrons • m-2 • s-1, respectively).

To determine if each genus's photosynthetic efficiency was also negatively impacted by CA inhibition, the initial slope of the rETR vs. irradiance curve (α) was examined (Fig. 5.9). There was no significant difference in the treatment αs for all the genera indicating that the initial quantum efficiency remained equivalent (*Asterionellopsis* $t = 1.95$, $p = 0.09$; *Cylindrotheca* $t = 0.74$, $p = 0.48$; *Guinardia* $t = 2.16$, $p = 0.06$; *Skeletonema* $t = 1.13$, $p = 0.29$). CA inhibition did not alter photoacclimation status. The genera are experiencing similar rates of photosynthesis with active CA or CA inhibition under low amounts of irradiance.

5.5 DISCUSSION

Different physiological and environmental mechanisms control the growth rate and biomass of marine phytoplankton leading to complex community dynamics. Quantifying these parameters provides insights into how primary production in the ocean may respond to changing CO_2 concentrations. In surface seawater, where carbon limitation frequently occurs, CCMs evolved in conjunction with enzymes (i.e. CA) to supplement CO_2 supply to the Calvin-Benson cycle. In this study, we investigate the potential role of CA activity in CCMs in the control of growth rates, production, photosynthetic performance, and impacts on the competitive abilities of four common species of marine phytoplankton.

5.5.1 *GROWTH RESPONSE TO CA INHIBITION*

Previous studies have demonstrated that, in elevated CO_2 conditions, increased growth of phytoplankton usually occurs (Burkhardt *et al.*, 2001, Rost *et al.*, 2002, Low-

DéCarie et al., 2011, Trimborn *et al.*, 2013, Pardew et al., 2018). When CA activity was inhibited in our experiments, phytoplankton experienced decreased CO_2 availability, resulting in decreased growth. Knotts and Pinckney (2018) reported that, although diatoms maintained a majority in the population, there was a lower-level community shift in the relative abundances of functional cell shapes. This result suggests that centric diatoms had a competitive advantage with an active CA enzyme and pennate diatoms were not as dependent on CA to maintain a competitive abundance. Therefore, we predicted that centric diatoms would experience strong growth limitation and possible cell mortality compared to pennate diatoms under CA inhibition. The two centric and two pennate genera tested in this experiment experienced decreased growth with CA limitation. However, both centric and one pennate *Asterionellopsis*, experienced high growth limitation and cell mortality. CO_2 is a limiting resource in our experimental conditions but, without active CA, the rates of carbon fixation were insufficient to maintain positive growth rates for *Skeletonema*, *Guinardia*, and *Asterionellopsis*. Only *Cylindrotheca* maintained a slower but still positive growth rate. Competition in pairwise mixtures and full communities did not change the growth rate responses for these four genera.

Active CA, depending on cellular location, can help restore the CO_2/HCO_3^- equilibrium at a cell's surface as $CO_{2(aq)}$ is taken up, dehydrate HCO_3^- that was actively taken up to $CO_{2(aq)}$ internally, or rehydrate $CO_{2(aq)}$ back to HCO_3^- internally in an attempt to keep $CO_{2(aq)}$ from leaking out of the cell (Reinfelder, 2011). CCMs with CA are useful to overcome the slow dehydration kinetics of HCO_3^- and the electrochemical potential gradient that stops the passive transport of HCO_3^-. The noncatalyzed kinetic rate is ca.

0.037 s-1 (Johnson, 1982, Hopkinson, 2014) while the CA catalyzed rates speed up by a factor of 10^4 to 10^6 (Lindskog, 1997). The ability of *Cylindrotheca* to maintain positive growth suggests that this genus is not entirely dependent on CA-mediated uptake of HCO_3^- as a main form of inorganic carbon. However, our results suggest that *Skeletonema*, *Guinardia*, and *Asterionellopsis* are very dependent on CA activity to supplement photosynthesis and net primary production. Phytoplankton simultaneously take up CO_2 and HCO_3^- but there can be large species-specific differences in the preferred carbon source (Huertas & Lubian, 1998, Cassar *et al.*, 2004, Tortell *et al.*, 2008a, Neven *et al.*, 2011). While it is possible for species to switch preferred carbon sources in response to changing CO_2 conditions (Kranz *et al.*, 2015, Bercel & Kranz, 2019), some species maintain a preference irrespective of changing CO_2. For example, the diatom, *Fragilariopsis kerguelensis* continued to take up HCO_3^- regardless of changing P_{CO2} levels (Trimborn et al., 2013). Perhaps *Skeletonema*, *Guinardia*, and *Asterionellopsis* all overcome low CO_2 levels in the water column by taking up the more readily available but more energetically expensive carbon source of HCO_3^- (Trimborn et al., 2013, Bercel & Kranz, 2019). In our carbon limited conditions, these three genera may not have been able to switch to CO_2 and meet the metabolic costs on the slow internal dehydration kinetics of HCO_3^- without CA. In contrast, *Cylindrotheca* either already preferably took up CO_2 or was able to switch to that source once CA was inhibited.

All four genera in this study likely experienced CO_2 leakage from their cells since all phytoplankton are inherently permeable and cannot internally store CO_2 (Gutknecht *et al.*, 1977, Hopkinson et al., 2011). Dinoflagellates may leak ca. 50% of the inorganic

carbon taken up (Eberlein *et al.*, 2014). Of the four genera, it appears only *Cylindrotheca* was able to supply RubisCO at a rate fast enough to counter the diffusion out of the cell. As stated before, we assume *Cylindrotheca* is preferably taking up CO_2. One explanation for the faster carbon acquisition could be the cell shape or the lack of chain formation. Diffusion rates experienced by phytoplankton are influenced by their size, shape, and cellular chains (Crank, 1979). The shape, for instance, changes the surface area:volume ratio of an organism leading to altered diffusive transport per unit of surface area. *Cylindrotheca* was the only elongated, solitary cell and the other three genera were chain-formers. Pahlow *et al.* (1997) demonstrated that an elongated, solitary cell had a greater diffusive transport per cell compared to cells in a chain. This also meant that elongated, solitary cells could have a greater volume than a spherical cell for the same diffusive transport. They concluded that solitary cells with a prolate shape was always advantageous in terms of nutrient uptake compared to spherical cells. In our study, *Skeletonema* was the most spherically shaped and had the smallest radius (ca. 1.95 µm). A simple diffusion model indicates that *Skeletonema* would have the fastest diffusion rate of all the genera in our experimental communities. However, the larger volume of *Cylindrotheca* would give the benefit of more protein for production of RubisCO subunits in addition to its quick diffusion rate with its minor radius (ca. 2.53 µm).

5.5.2 *PREDICTING COMMUNITY CHANGES THROUGH COMPETITION*

In the absence of grazing, understanding how community compositions are maintained should be possible through the knowledge of each taxa's physiological mechanisms. Diatoms are very efficient at concentrating carbon using their CCMs, and

consequently have a competitive advantage in many marine systems (Reinfelder, 2011). However, understanding how CO_2 concentrations alter the growth of species alone and together becomes complicated when, in spite of similarities in carbon acquisition strategies, there are species-specific differences in carbon preference (Burkhardt et al., 2001).

Previous studies have investigated freshwater systems using this methodological approach to confirm the predictability of changes in community composition to a changing CO_2 environment. Low-DéCarie et al. (2011) and Ji et al. (2017) demonstrated that cyanobacteria grew faster and outcompeted chlorophytes under low CO_2 conditions, and those same cyanobacteria lost that competitive advantage in high CO_2 conditions. The outcomes in their pairwise competitions did predict the responses of the whole community. Using marine phytoplankton, Pardew et al. (2018) also was able to show a predictability in community composition under elevated CO_2 conditions. Yet, the competition between groups with similar capacities for carbon acquisition were less predictable than expected. This conclusion may explain why our community could not be predicted based on the pairwise competitions. *Skeletonema, Guinardia, and Asterionellopsis* all responded similarly when CA was inhibited. Further studies should investigate a larger group of diatoms and other taxa with known differences in carbon acquisition (Mercado & Gordillo, 2011). Another explanation for the unpredictability in our system could be that we did not use elevated CO_2 conditions. We approached our system with enhanced carbon limitation and assumed that genera expressing a competitive advantage without active CA would have a benefit in the future if CCMs downregulated CA production. Because these genera already live in a CO_2-limited

environment and CCMs were evolved to help cope with limited DIC availability in the water, further restrictions on their carbon acquisition strategies might not be desirable when trying to predict carbon competition. Conversely, Ji et al. (2017) did use low pCO_2 treatments (~100 ppm), saw growth in their cyanobacteria and green algae, and were able to demonstrate competitive interactions. Inhibited CA activity may make it too difficult to acquire and maintain carbon internally at the site of fixation.

5.5.3 *PRODUCTION AND PHOTOSYNTHETIC PERFORMANCE*

As studied here, GPP of the four genera was greatly influenced by CA inhibition and, in general, lowered the production ca. 65%. This result mirrors the result Knotts and Pinckney (2019) presented on benthic microalgae (BMA). Like phytoplankton, BMA communities experience carbon limitation and maintain their production with the use of CCMs and active CA. Similarly, zooxanthellae found in tropical cnidarians demonstrated a 56 – 85% reduction in photosynthetic activity when CA was inhibited (Weis *et al.*, 1989). When HCO_3^- is the dominant form for carbon uptake, this enzyme is critical for maintaining high production (Tortell & Morel, 2002, Martin & Tortell, 2006). Regardless of CA inhibition, *Cylindrotheca* had the lowest production rates. Perhaps *Cylindrotheca* can maintain positive growth when CA is inhibited because its slower rate of photosynthesis wouldn't require CO_2 as quickly as the other genera.

In elevated CO_2 conditions, CCMs may be downregulated (Raven *et al.*, 2011). Young *et al.* (2015) demonstrated this response by measuring increased CO_2 half saturation constants for cellular carbon fixation, decreased external CA activity (eCA), and increased biological fractionation of stable carbon isotopes. We predict that if we had

inhibited CA but elevated CO_2 conditions, our production would have been higher as well. Further studies should investigate how forced inhibited CA activity in high CO_2 conditions directly impacts production and growth.

In the case of photosynthetic performance measured with RLCs, increased rETR were obtained with increasing irradiance. At lower irradiances, the photochemical use of the excitation energy in the light (i.e. effective quantum yield) remained similar between the control and CA inhibition treatments with little variation. Higher irradiance levels resulted in a lower rETR for CA inhibited genera relative to controls. A reduction in rETR and $rETR_m$ at those irradiances were caused by lower effective quantum yields. While no downward trend was observed in the RLCs, the leveling-off of rETRs in the CA inhibition treatments at high irradiances indicates that photosynthetic light saturation had been reached. The quantum yield decreased as much as PAR increased causing the rETR to remain unchanged (Beer *et al.*, 2014).

While CA inhibition did lower the rETR significantly at high irradiances, at light limiting photosynthesis (100 μmol photons • m-2 • s-1) no significant differences were found for the efficiency of photon absorption, α, between the CA inhibited and control treatments. However, in general, α was lower in the CA inhibition treatments indicating lower light utilization efficiency. Wu et al. (2015), using the diatom *Thalassiosira pseudonana,* also saw a decline in rETR with the addition of a CA inhibitor regardless of CO_2 condition as well as no change in the α values at the end of their time-course experiments. Yet, pH conditions affected α values differently at early time points. They suggested that cells had to allocate extra energy for carbon transport or synthesis causing lower α values but the cells recovered by modulating their physiological processes by the

end of the time-course (Wu et al., 2015). Perhaps our cells would be more efficient at capturing photons if more time had been provided between the addition of our CA inhibitor and the measurement of photosynthetic parameters. Alternatively, a green alga *Chlamydomonas reinhardtii* showed no considerable differences in rETR when CA was inhibited, which suggests the enzyme is directly involved in the Calvin cycle and not the light-dependent reactions (Park *et al.*, 1999).

These trends of similar PSII activity in past studies and our own experiments at low levels of irradiance suggest that each genus is conducting similar rates of photosynthesis with or without active CA or CA. Yet, it is possible to have a high effective quantum yield with damaged Cavin cycle activity in weak light since there are not enough electrons to make dark enzymic steps of the Calvin cycle limiting. Next steps should test whether primary production shows no difference at lower irradiances given that our production measurements were conducted at high irradiances (ca. 1000 μmol photons • m-2 • s-1).

5.5.4 *CONCLUSION*

Our results do not provide a predictable pattern of changes in the phytoplankton community composition when CA is inhibited. In these already carbon-limited environments, most of our genera could not cope without the ability to dehydrate HCO_3^-. *Cylindrotheca* was the only genus capable of providing enough carbon to RubisCO during CA inhibition. Clearly, this genus has additional physiological mechanisms that we did not test that are accounting for its positive reaction. Although *Cylindrotheca*'s rETRs were similar to the other genera, the shape and chaining of this cell should be

considered along with its preferred carbon species. Perhaps, *Cylindrotheca* does not have an efficient CCM and does not strongly depend on CA for its carbon acquisition. The slower rates of production suggest that *Cylindrotheca* already does not acquire carbon as quickly as the other genera. Its solitary elongated shape may give it the competitive advantage of CO_2 diffusion through the cell wall rather than the more abundant HCO_3^-. Regardless, more investigations are necessary to determine what makes this genus the best competitor without CA activity and how it will fair in elevated CO_2 conditions. More attempts need to explore new physiological mechanisms and utilize additional taxa to advance our understanding of the dynamics of phytoplankton community assemblages. The ability to measure growth rates of phytoplankton exposed to a range of environmental conditions will help elucidate the relationships between carbon availability and phytoplankton composition.

5.6 ACKNOWLEDGEMENTS

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5.7 TABLES

Table 5.1: Summary of analyses for growth and competition: the effect of CA inhibition on growth rate in pure culture, competition between pairwise mixtures, and competition in the full community. The asterisks "*" represents an interaction between the parameters it is linking.

Analysis	**Algal Culture**	**Model: Response variable ~ Factors**
Growth Response	Unialgal	Growth rate ~ CA inhibition treatment * Genus
	Unialgal	Growth rate ~ CA inhibition treatment * Shape
Competition response	Pairwise mixture	Realized competition coefficient ~ CA inhibition treatment * Centric Genera * Pennate Genera
Competition response	Full community	Realized competition coefficient for four genera ~ CA inhibition treatment
	Full community	Realized competition coefficient for two functional shapes ~ CA inhibition treatment
Prediction of pairwise mixture response		Pairwise mixture response ~ Unialgal response
Prediction of community response (with and without *Cylindrotheca*)		Full community response ~ Pairwise mixture response

5.8 FIGURES

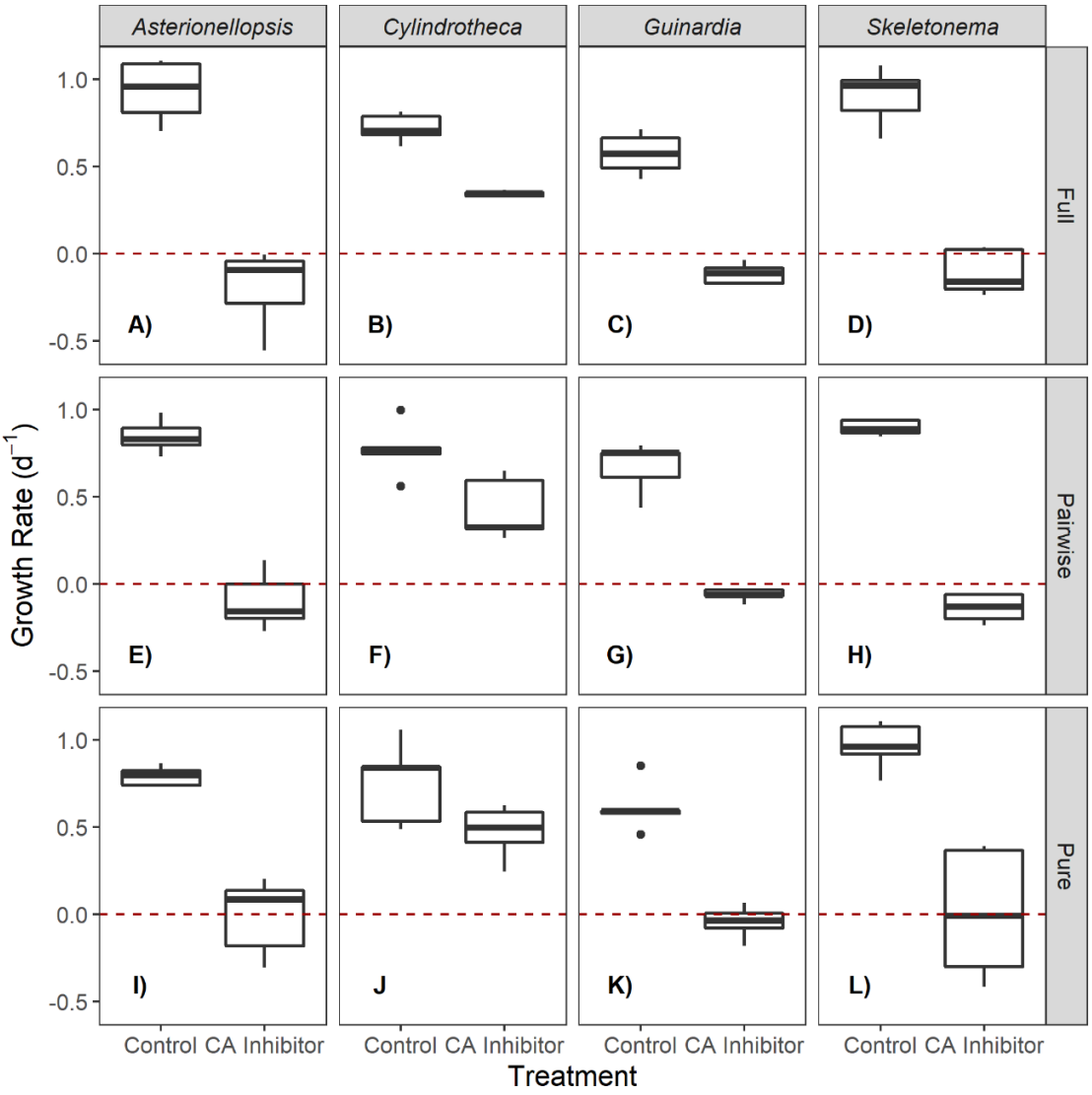

Figure 5.1: Boxplot of the cell-specific growth rate (d^{-1}) of each genus for each treatment seen across the competition types (A-D) pure unialgal, (E-H) pairwise mixture, and (I-L) full community. *Asterionellopsis* and *Cylindrotheca* are the pennate diatoms, and *Guinardia* and *Skeletonema* are the centric diatoms. The pairwise mixtures were always a centric and pennate diatom together in one flask. The full community mixture had all four genera present in one flask. 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. The dashed lines represent no growth. If the growth rate was < 0, there was strong growth limitation and cell mortality.

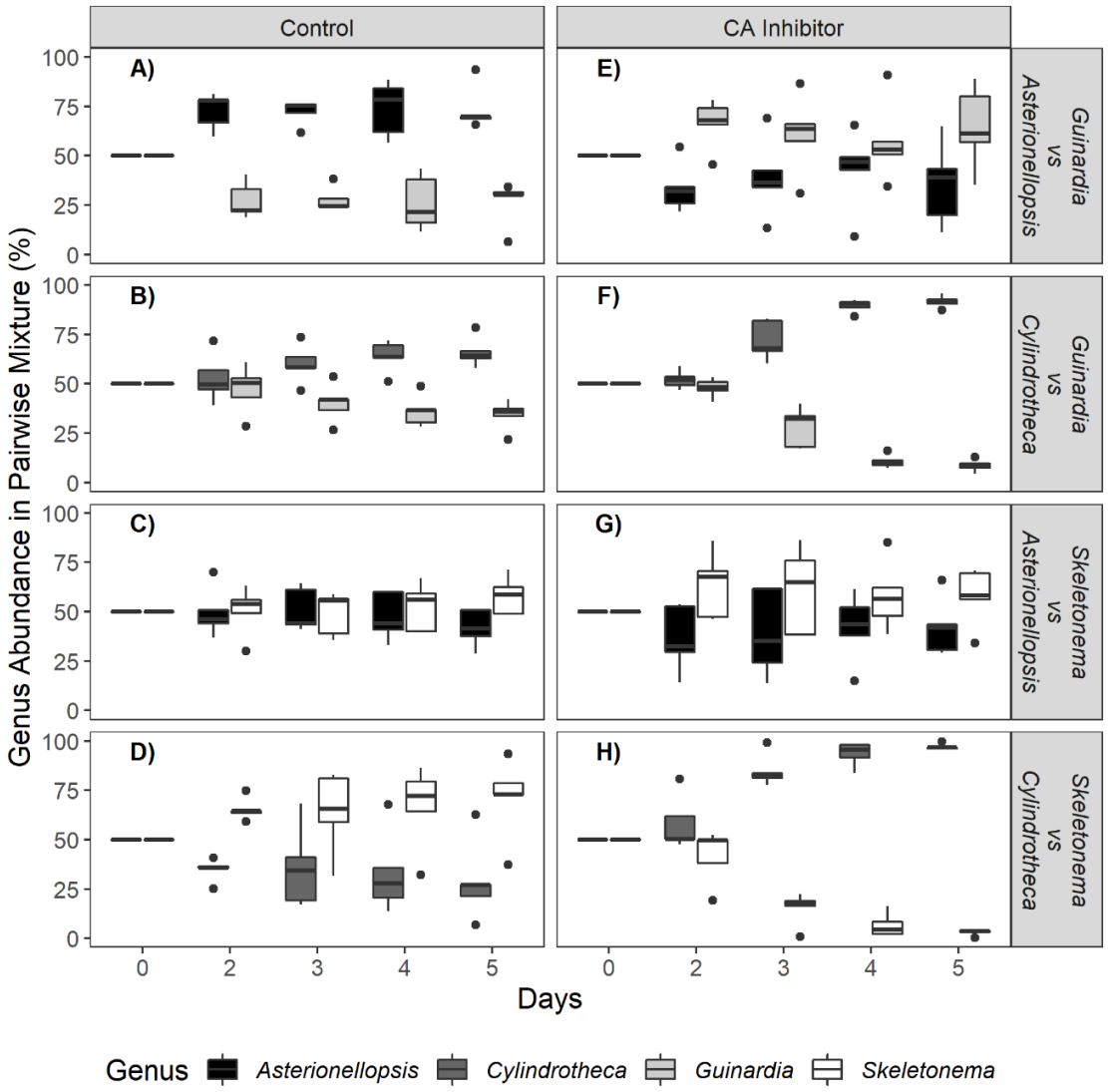

Figure 5.2: Pairwise community percentages for each genus across the five-day experiment for (A-D) the control treatment and (E-H) the CA inhibition treatment. All genera were initially inoculated at the same density for day zero (2.5 x 10^3 cells • mL-1).

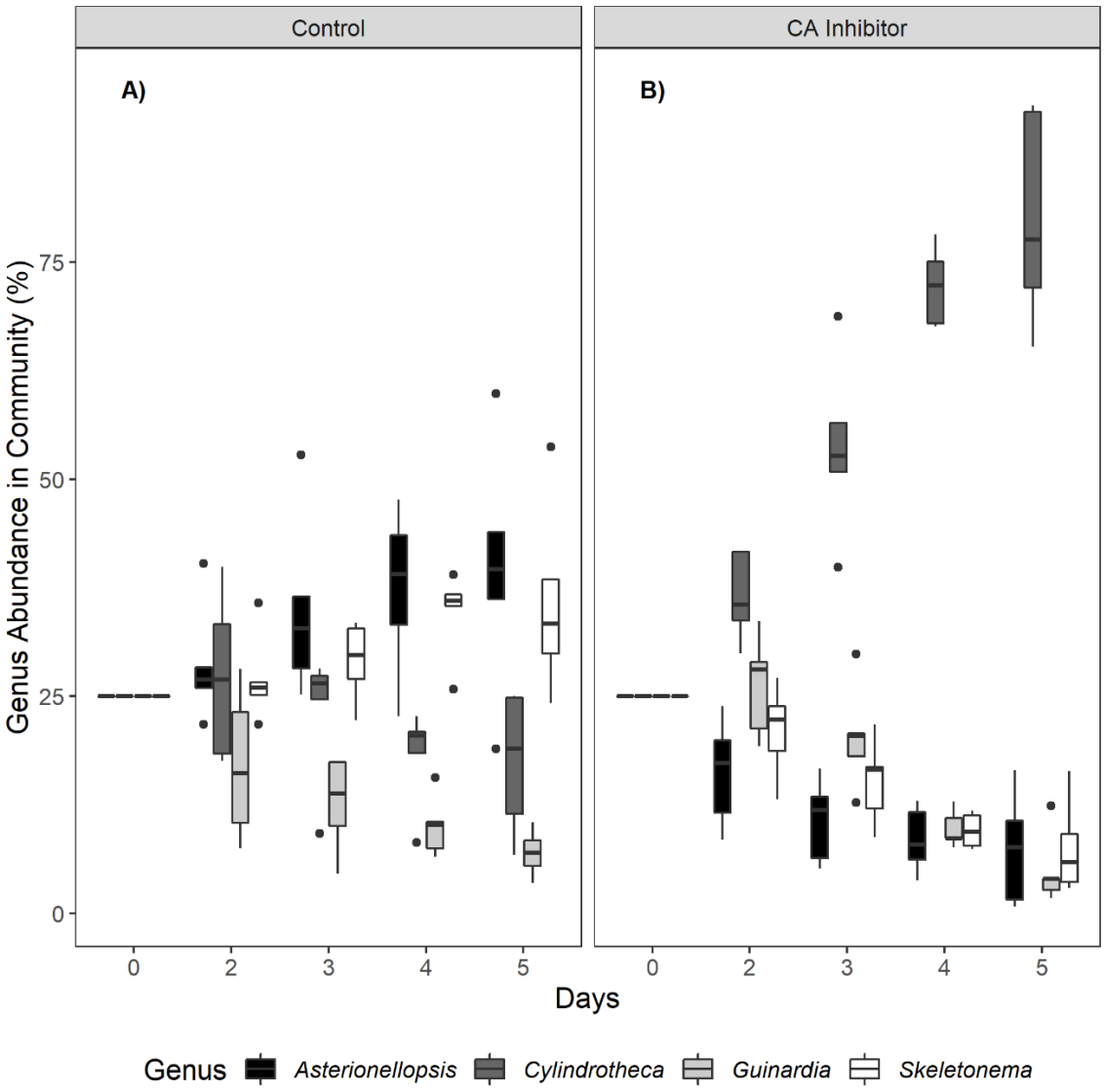

Figure 5.3: Full community percentages of each genus across the five-day experiment in A) the control treatment and B) CA inhibitor treatment. All genera were inoculated at the same density for day zero (1.25 x 10^3 cells • mL-1).

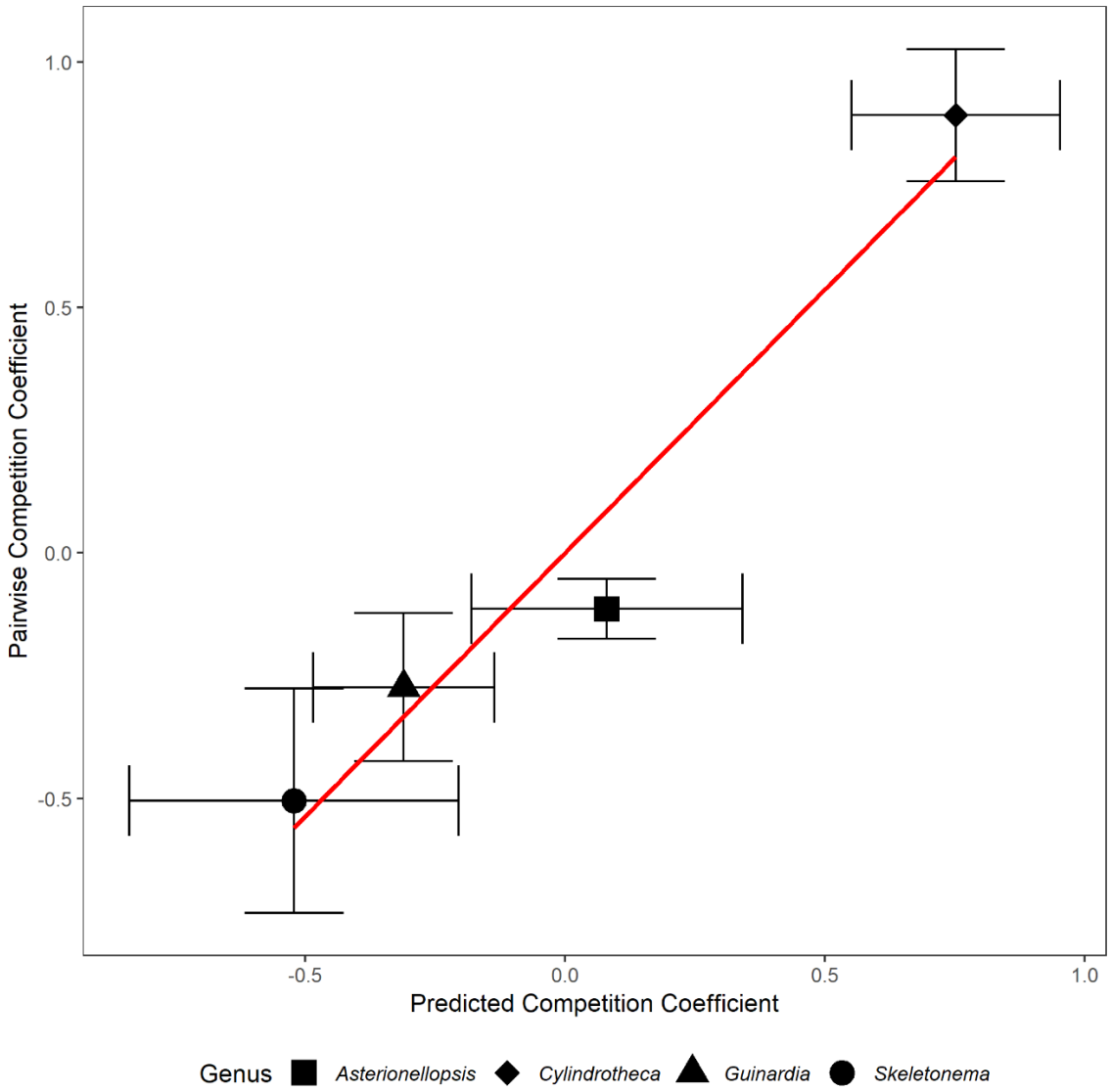

Figure 5.4: Relationship between the predicted competition coefficient and the pairwise competition coefficient of each of the genera in the CA Inhibitor treatment ($R^2 = 0.95$, $p = 0.02$). The predicted competition coefficients are calculated from the pure culture growth rates. The pairwise competition coefficients are calculated from the frequency changes that occurred in the experimental cultures (mean ± SE).

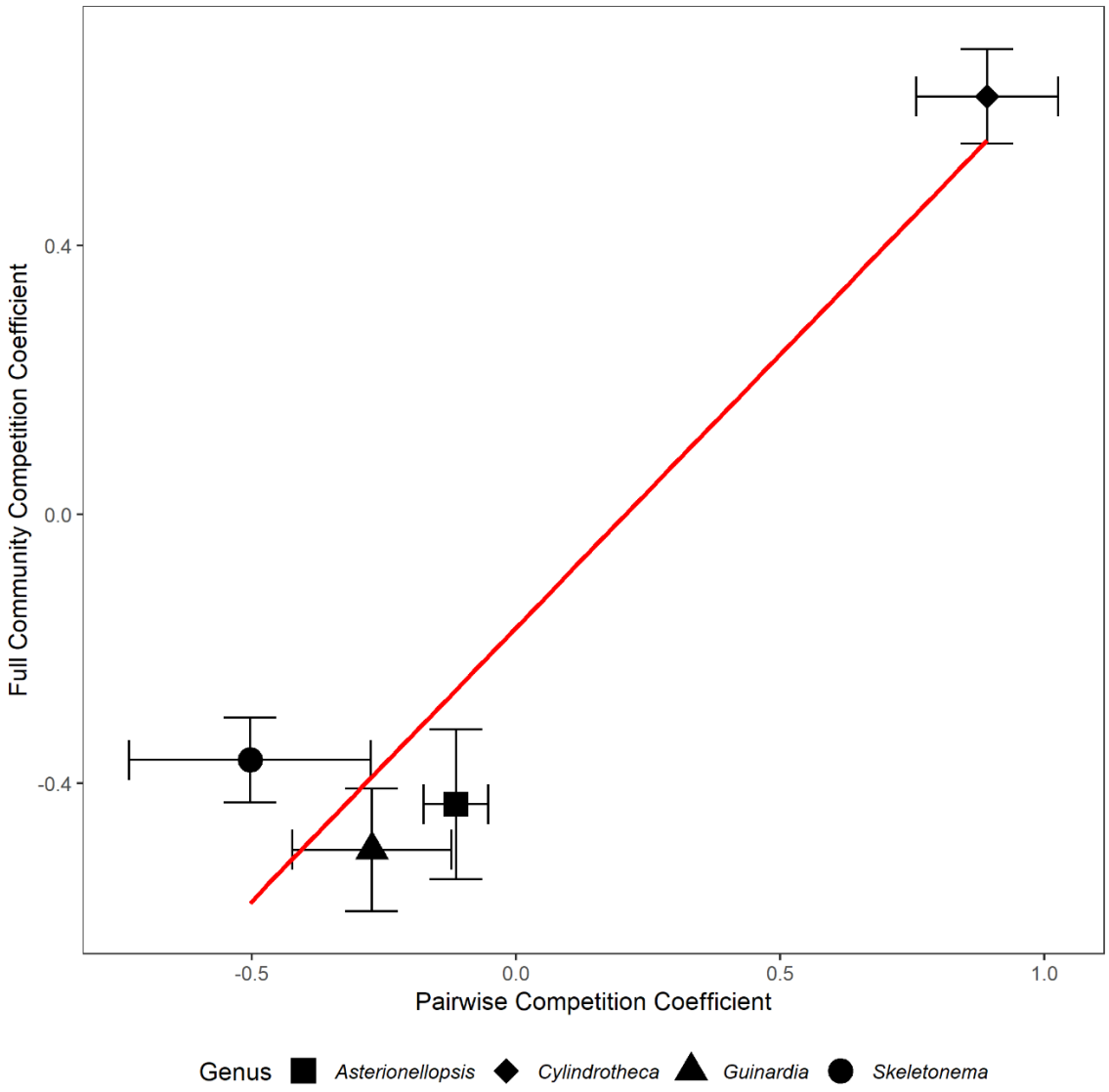

Figure 5.5: Relationship between the pairwise competition coefficient and the full community competition coefficient of each of the genera in the CA Inhibitor treatment ($R^2 = 0.89$, $p = 0.06$). The pairwise competition coefficients and full community competition coefficients are calculated from the frequency changes that occurred in the experimental cultures (mean ± SE). Removing values from competition with *Cylindrotheca* (the only genus for which the growth rate values are positive compared to the other genera) reduces the fit to $R^2 = 0.34$, $p = 0.61$.

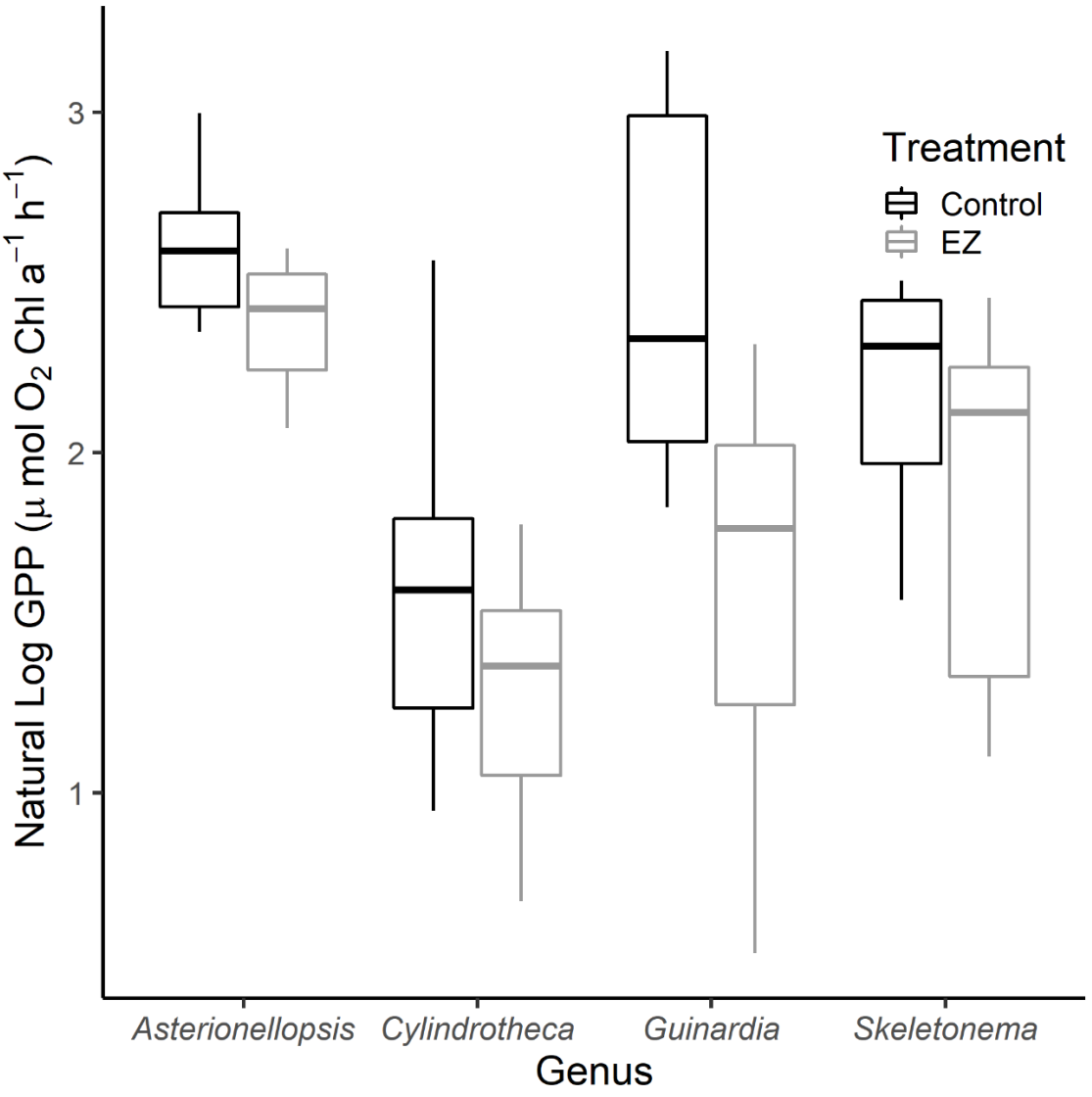

Figure 5.6: Boxplots of GPP for the four genera between the two treatments (n = 7 measurements • genus-1 • treatment-1) normalized to Chl *a*. There is a significant difference between *Cylindrotheca* and the three genera *Skeletonema*, *Asterionellopsis* and *Guinardia* ($p = 0.012$, $p < 0.001$, and $p = 0.007$, respectively) and between *Skeletonema* and *Asterionellopsis* ($p = 0.042$). There is also a significant difference between the two treatments ($p < 0.001$). There was no significant interaction.

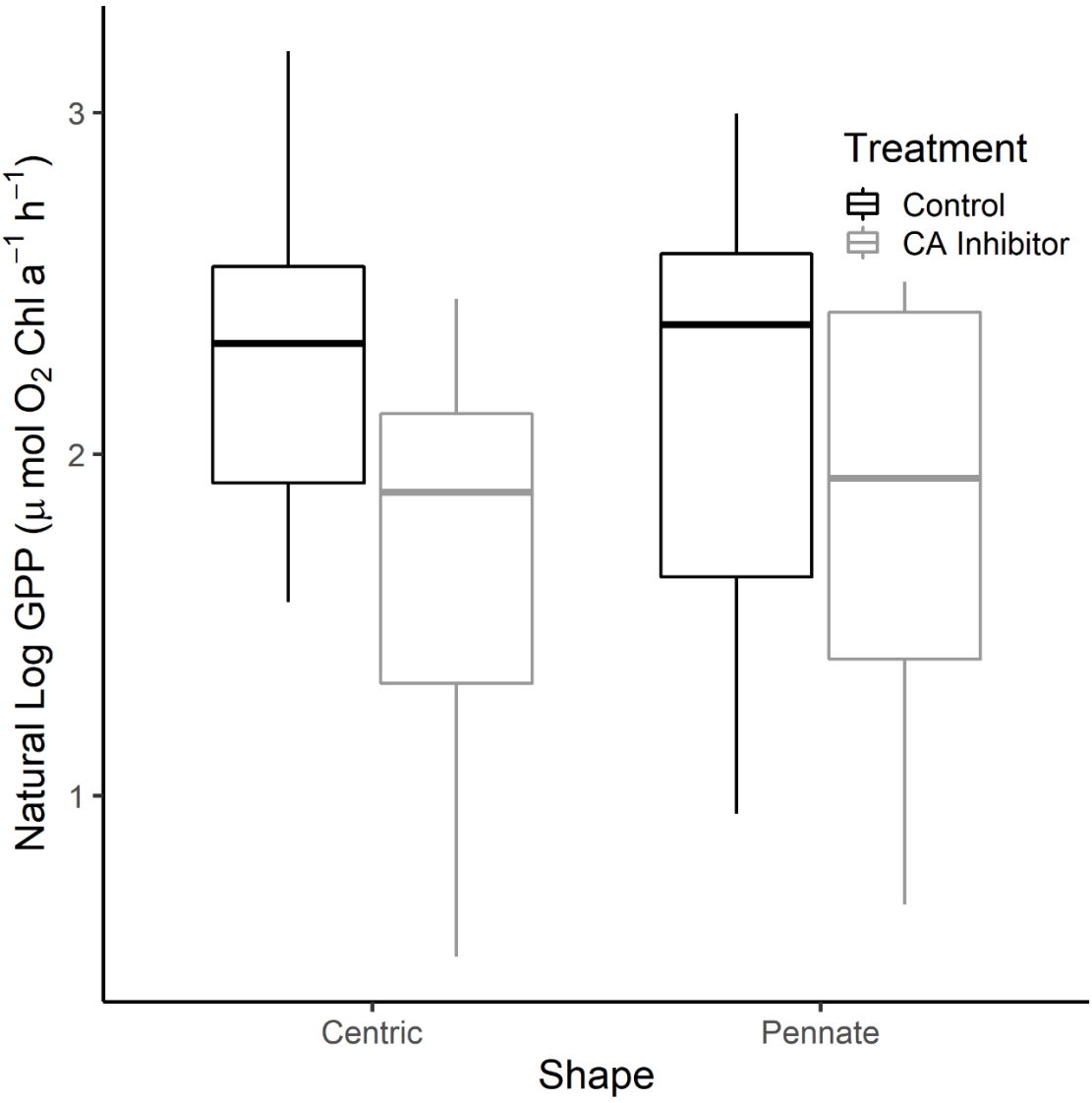

Figure 5.7: Boxplots of GPP for the two diatom shapes between the two treatments (n = 14 replicates • shape-1 • treatment-1). There is no difference between the shapes and no significant interaction. There is a significant difference between the two treatments (p = 0.002).

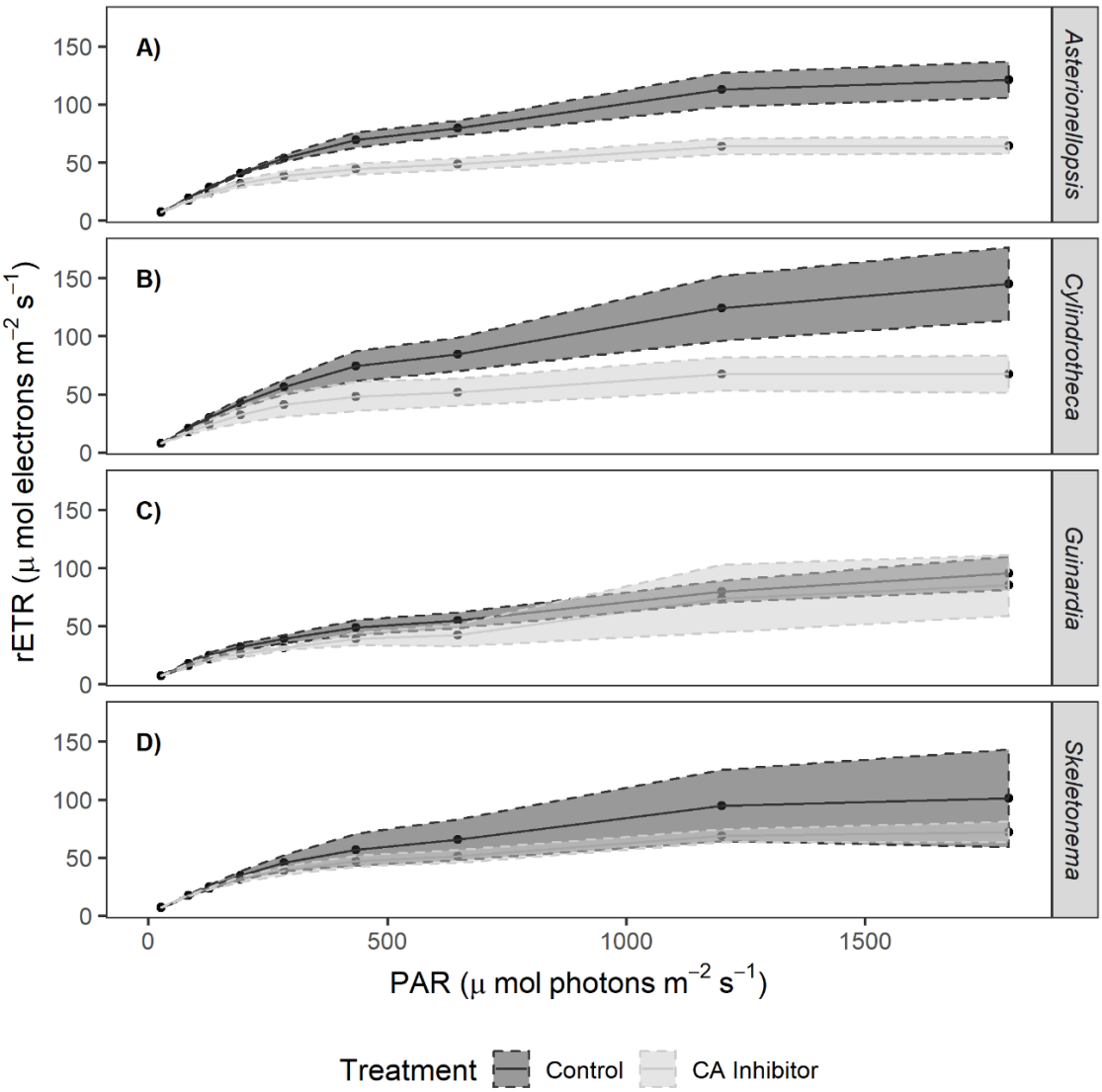

Figure 5.8: rETR between the two treatments (n = 5 replicates • genus-1 • treatment-1) across the actinic PAR intensities. A) *Asterionellopsis*, pennate diatom. B) *Cylindrotheca*, pennate diatom. C) *Guinardia*, centric diatom. D) *Skeletonema*, centric diatom. Shaded regions are 95% confidence intervals. There is a significant difference between the two treatments ($p < 0.001$) and a significant interaction between treatment and genus ($p < 0.001$).

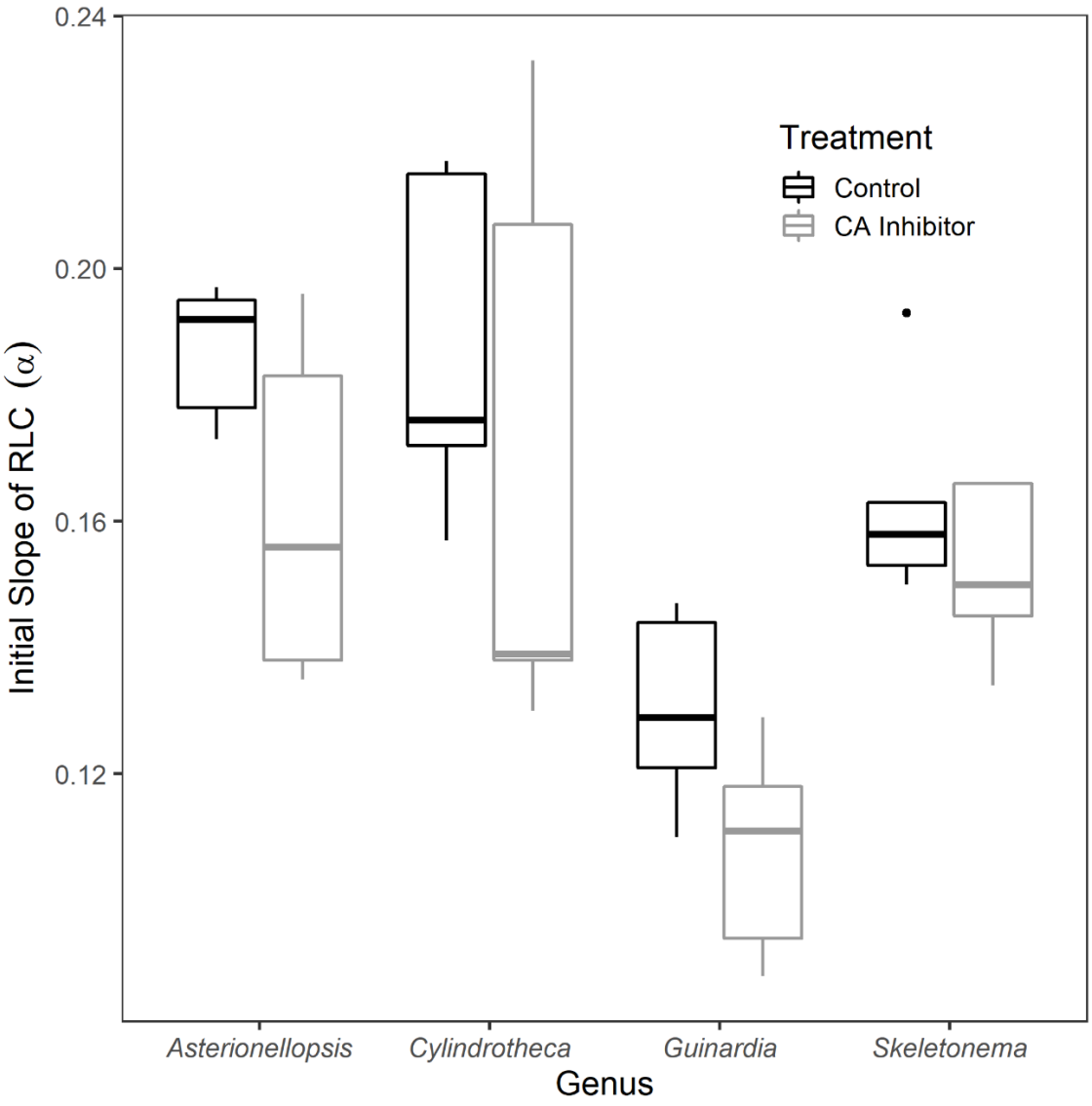

Figure 5.9: Boxplot of the initial slope of the RLC (α) for each genus between the two treatments (n = 5 replicates • genus-1 • treatment-1). Error bars are standard error. There is no significant difference between the treatments for each genus ($p > 0.05$).

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CHAPTER 6

GENERAL CONCLUSION

6.1 CONCLUSION

In this dissertation we examined the effects of carbonic anhydrase (CA) inhibition on the community structuring processes, both physical and physiological, on phytoplankton and benthic microalgae (BMA). We addressed our questions with two observational experiments, one review, and one manipulated experiment.

CHAPTER 2 described how different phytoplankton assemblages were dependent on CA. We provide evidence that CA inhibition altered the algal communities by mechanistically demonstrating that active CA helps structure phytoplankton community compositions in both high and low salinity sites. This was accomplished using two different salinity sites and examining changes in the distribution of taxonomic groups using pigment analyses and cell-size class distributions. CA provided a competitive advantage to phytoplankton that required efficient CCMs for carbon acquisition in our assemblages.

It has been suggested that at current low surface water CO_2 concentrations, larger phytoplankton with efficient CCMs (e.g. diatoms) have the competitive advantage over smaller phytoplankton with less efficient CCMs (e.g. dinoflagellates, coccolithophores) (Reinfelder 2011). Although diatoms were hypothesized to decrease in relative abundances without CA, these microalgae remained a high percentage of our estuarine

community as the dominant taxa. However, responses differed between salinity site. Diatoms experienced a decrease in abundances relative to the control when CA was inhibited at the low salinity site. This response is thought to be related to the differences in DIC availability. Carbon acquisition strategies may be more important in freshwater systems as these sites typically have low DIC availability. Instead of inhibition of abundances at the high salinity site, diatoms were mainly stimulated when CA was inhibited. Yet, there were lower-level shifts in the dominant genera. Centric diatoms (e.g. *Skeletonema*, *Guinardia*, and *Rhizosolenia*) dominated control samples suggesting that they had a competitive advantage with an active CA enzyme. Pennate diatoms (e.g. *Asterionellopsis*, *Thalassionema*, and *Cylindrotheca*) became more abundant when CA was inhibited indicating these genera may not depend on CA activity as a competitive CCM strategy. Although most studies have focused on diatoms because of high abundances and high efficiency CCMs, few of those investigations have studied pennate diatoms (Hobson et al. 2001 - *Cylindrotheca*; Martin & Tortell 2008 - *Asterionella*, *Pseudo-nitzschia*, *Cylindrotheca*). Pennate diatoms may preferably take up CO_2 over HCO_3^- and may not be dependent on CCMs using CA to acquire carbon (Mercado et al. 2009). Overall, this chapter explained coexistence and dominance among taxonomic algal groups present in a community by studying carbon acquisition strategies that used CA.

The role of CA on structuring processes in benthic microalgal (BMA) assemblages has been explored to a limited extent as research has mainly been focused on planktonic species. CHAPTER 3 expanded on the concept explored in CHAPTER 2 by investigating how CA activity affected BMA structuring processes. Specifically, we focused on their vertical sediment profiles, gross primary production (GPP), and biomass. Da Silva et al.

(2017) discussed evidence of carbon limitation in benthic diatom mats and BMA dominated by diatoms. Our estuarine sediments also experience carbon limitation and, therefore, CA activity is critical for carbon acquisition. With this enzyme, BMA can maintain higher GPP and use a wider portion of the sediment column. When CA was inhibited, production was limited to higher portions of the vertical profile and a greater portion of production occurring within the first 100 µm of sediment. We concluded that microalgae were making use of the atmosphere-sediment interface to overcome carbon acquisition limitations faced without CA. Kristensen & Alongi (2006) and Chen et. al. (2019) both demonstrated a similar phenomenon with higher CO_2 uptake rates occurring when microalgal films were visibly at the surface causing negative CO_2 fluxes. BMA might be experiencing a trade-off at the sediment surface where there is higher CO_2 availability but more light damage.

While carbon acquisition with CA in these systems is important, biomass was not affected by the inhibition of this enzyme. We can see immediate effects on inhibited this enzyme on production since, without it, the dehydration of HCO_3^- to CO_2 slows by a factor of 10^4 (Lindskog 1997, Reinfelder 2011). Typically, production and biomass are commonly assumed to be related, yet, the effect of slower production on biomass was not observed. We concluded that growth rates of benthic diatoms are typically ~0.6–0.27 d^{-1} and it would likely take more than our three-day incubation to see a change in biomass (Gould & Gallagher 1990).

With these results, we concluded that functioning CA-dependent CCMs are important in BMA communities. While a downregulation of CA is not likely to occur in these systems like predicted in phytoplankton communities (Young et al. 2015), understanding

carbon acquisition strategies is still essential. Consequences of altered DIC concentrations in BMA communities may change our quantification of estuarine ecosystem trophodynamics. Thus, this chapter demonstrates that CCMs using CA are essential in these communities and must be considered when investigating ecosystem responses to global climate change and ocean acidification.

CHAPTER 4 reviewed how taxonomic differences in carbon acquisition are exceptionally important when determining the ecological interactions of phytoplankton groups. With different strategies for obtaining inorganic carbon (Mercado et al. 2009), phytoplankton have become a topic of great importance as researchers attempt to determine how these photoautotrophs will respond individually or as a community to rising dissolved CO_2. Thus, this minireview provides a more recent analysis of the resource constraints on carbon acquisition in phytoplankton and compares the shifts in species dominance hierarchies due to elevated $CO_{2(aq)}$. Past studies are proposed as critical in understanding the role CCMs have in driving community responses to rising CO_2. Immense complexities in microalgal structuring processes can exist in response to elevated CO_2 since CCM activity can be altered by nutrient limitation, and grazing pressures and initial community assemblages will impact community outcomes.

CHAPTER 5 expanded on the concepts identified in CHAPTER 2 and reviewed in CHAPTER 4. This chapter uses a standard methodology that investigated competitive interactions by using monocultures and controlled competition comparisons in an attempt to determine if community responses could be predicted (Low-Décarie et al. 2011, Verschoor et al. 2013, Ji et al. 2017, Pardew et al. 2018). Our control monocultures show *Skeletonema* and *Asterionellopsis* having the fastest growth rate which gives them the

competitive advantage in both pairwise mixtures and full community assemblages. When CA is inhibited, *Cylindrotheca* is the only genus that maintains a slower but positive growth rate and competitively dominates all the mixed assemblages. To futher investigate what physiological mechanisms was giving this genus an advantage, we measured eletron transport rates (ETR) and oxygen production rates. All other genera had similar high production rates, yet *Cylindrotheca* had the lowest even in control treatments. We concluded that *Cylindrotheca* could provide enough carbon to RubisCO during CA inhibition because of its slower carbon turnover. Additionally, *Cylindrotheca* may be able to switch its preferred carbon sources in response to changing CO_2 availability (Kranz et al., 2015; Bercel & Kranz, 2019) or maintains a CO_2 preference irrespective of changing carbon availability. Thus, this chapter did not provide a predictable pattern of changes in the phytoplankton community composition when CA is inhibited. However, we did demonstrate that different mechanisms may influence diatom compositions and competition beyond CA activity in CCMs.

Collectively, this dissertation provides important insights into how diatoms maintain a dominant presence in our estuaries using carbon acquisition strategies. It explored the effects of CA inhibition on natural assemblages, cell size distributions, production, and biomass in different microalgal communities. This dissertation documented the alterations of the community composition and cell sizes without CA in two assemblages differing in salinity regime (CHAPTER 2). It further explored how BMA also responded to removed CA activity but investigated changes in production, biomass, and vertical profiles as a means to understand alterations in structuring processes (CHAPTER 3). It further reviewed the effects of resource limitation and carbon availability already

established in other studies (CHAPTER 4). Species specific requirements and preferences make establishing community compositions in future climate scenarios difficult. A standard methodology that controls for initial communities and allows for competition may provide an alternative technique to predicting how microalgal communities will respond to changing CO_2 conditions. Finally, this dissertation demonstrated that diatoms could not be grouped together by functional shape to describe responses to CA inhibition (CHAPTER 5). This finding furthers the argument that accounting for your starting community and allowing competition is critical in future studies. However, a larger diversity of species and physiological parameters are needed to capture a natural community picture.

Thus, this dissertation emphasizes the need to mechanistically explore and consider different carbon acquisition strategies, including CA activity and carbon species preference, when predicating and modeling current and future microalgal community processes. This is important because indirect effects of ocean acidification on productivity and composition of planktonic species may have long-term impacts on trophic interactions between planktonic food sources and higher-level coastal organisms.

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APPENDIX A

SUPPLEMENTAL TABLES FOR CHAPTER 5

A1.1 TABLES

Table A.1: A) ANOVA for the effect of CA inhibition on growth rate in pure culture. Genera are separated. B) Tukey test for significance among the multiple genera and interactions. Nonsignificant results were omitted. The letter "X" represents an interaction between the two terms it is between. Significance is indicated by * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$. Control represents the treatment with no addition of an inhibitor and EZ represents the CA inhibition treatment.

A) ANOVA						
	Df	**Sum Sq**	**Mean Sq**	**F value**	**p value**	**Sig**
Treatment	1	4.55	4.55	115.45	3.80E-12	***
Genera	3	0.58	0.20	4.93	6.30E-03	**
Treatment X Genera	3	0.63	0.21	5.34	4.28E-03	**
Residuals	32	1.26	0.04			

B) TUKEY HSD					
Genera	**diff**	**lwr**	**upr**	**p adj**	**Sig**
Guinardia-Cylindrotheca	-0.33	-0.57	-0.09	4.44E-03	**
Treatment X Genera	**diff**	**lwr**	**upr**	**p adj**	**Sig**
EZ X *Asterionellopsis* - Control X *Asterionellopsis*	-0.80	-1.21	-0.40	9.10E-06	***
EZ X *Guinardia* - Control X *Asterionellopsis*	-0.84	-1.24	-0.43	4.30E-06	***
EZ X *Skeletonema* - Control X *Asterionellopsis*	-0.78	-1.19	-0.38	1.40E-05	***
Control X *Cylindrotheca* - EZ X *Asterionellopsis*	0.76	0.36	1.17	2.18E-05	***
EZ X *Cylindrotheca* - EZ X *Asterionellopsis*	0.48	0.08	0.89	0.01	*
Control X *Guinardia* - EZ X *Asterionellopsis*	0.63	0.22	1.03	4.93E-04	***
Control X *Skeletonema* - EZ X *Asterionellopsis*	0.98	0.57	1.38	2.00E-07	***
EZ X *Guinardia* - Control X *Cylindrotheca*	-0.80	-1.20	-0.39	1.03E-05	***
EZ X *Skeletonema* - Control X *Cylindrotheca*	-0.74	-1.15	-0.34	3.36E-05	***
EZ X *Guinardia* - EZ X *Cylindrotheca*	-0.52	-0.92	-0.11	5.46E-03	**
Control X *Skeletonema* - EZ X *Cylindrotheca*	0.49	0.09	0.90	9.21E-03	**
EZ X *Skeletonema* - EZ X *Cylindrotheca*	-0.47	-0.87	-0.06	0.02	*
EZ X *Guinardia* - Control X *Guinardia*	-0.66	-1.07	-0.25	2.33E-04	***
EZ X *Skeletonema* - Control X *Guinardia*	-0.61	-1.01	-0.20	7.54E-04	***

Control X *Skeletonema* - EZ X *Guinardia*	1.01	0.60	1.42	1.00E-07	***
EZ X *Skeletonema* - Control X *Skeletonema*	-0.96	-1.36	-0.55	3.00E-07	***

Table A.2: ANOVA for the effect of CA inhibition on growth rate in pure culture. Functional shape (centric and pennate) are separated. The letter "X" represents an interaction between the two terms it is between. Significance is indicated by * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$

ANOVA						
	Df	**Sum Sq**	**Mean Sq**	**F value**	**p value**	**Sig**
Treatment	1	4.55	4.55	75.72	2.22E-10	***
Shape	1	0.13	0.13	2.21	0.15	
Treatment X Shape	1	0.18	0.18	2.98	0.09	
Residuals	36	2.17	0.06			

Table A.3: A) ANOVA for the effect of CA inhibition on realized competition coefficients between genera pairs. B) Tukey test for significance among the multiple genera and interactions. The focal genera were the centric shaped, *Skeletonema* and *Guinardia*. The competitor genera were pennate shaped, *Asterionellopsis* and *Cylindrotheca*. The letter "X" represents an interaction between the two terms it is between. Significance is indicated by * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$

A) ANOVA						
	Df	**Sum Sq**	**Mean Sq**	**F value**	**p value**	**Sig**
Treatment	1	1.21	1.21	10.82	2.45E-03	**
Focal	1	0.72	0.72	6.46	0.02	*
Competitor	1	1.21	1.21	10.79	2.48E-03	**
Treatment X Focal	1	2.49	2.49	22.32	4.42E-05	***
Treatment X Competitor	1	4.33	4.33	38.81	5.59E-07	***
Focal X Competitor	1	0.04	0.04	0.32	0.58	
Treatment X Focal X Competitor	1	0.12	0.12	1.05	0.31	
Residuals	32	3.57	0.11			

B) TUKEY HSD					
Treatment X Focal	**diff**	**lwr**	**upr**	**p adj**	**Sig**
Control X *Skeletonema* - Control X *Guinarda*	0.77	0.36	1.17	7.58E-05	***
Control X *Skeletonema* - EZ X *Guinarda*	0.62	0.21	1.02	1.35E-03	**
EZ X *Skeletonema* - Control X *Skeletonema*	-0.85	-1.25	-0.44	1.64E-05	***
Treatment X Competitor	**diff**	**lwr**	**upr**	**p adj**	**Sig**
EZ X *Cylindrotheca* - Control X *Asterionellopsis*	-0.69	-1.10	-0.29	3.08E-04	***
EZ X *Cylindrotheca* - EZ X *Asterionellopsis*	-1.01	-1.41	-0.60	8.00E-07	***
EZ X *Cylindrotheca* - Control X *Cylindrotheca*	-1.01	-1.41	-0.60	8.00E-07	***

APPENDIX B

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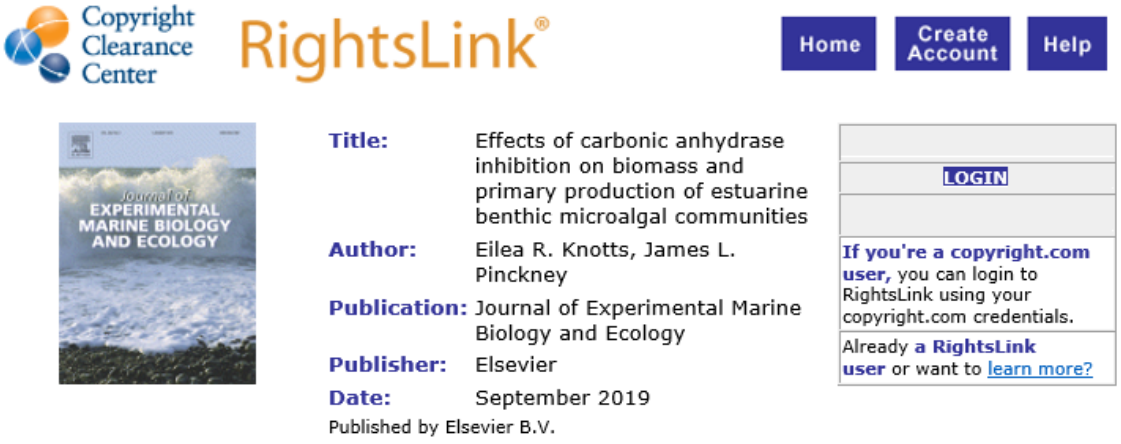

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