DETERMINING THE INVASION POTENTIAL FOR THE HARMFUL BLUE-GREEN ALGA (CYANOBACTERIUM) *CYLINDROSPERMOPSIS RACIBORSKII* AT THE CURRITUCK BANKS NERRS SITE, CURRITUCK SOUND, NORTH CAROLINA

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ABSTRACT

ELIZABETH SAUNDERS CALANDRINO: Determining the Invasion Potential for the Harmful Blue-green Alga (Cyanobacterium) *Cylindrospermopsis raciborskii* at the Currituck Banks NERRS Site, Currituck Sound, North Carolina
(Under the direction of Dr. Hans Paerl)

Cylindrospermopsis raciborskii is an invasive, toxin-producing, filamentous-heterocystous, N_2-fixing cyanobacterium that has recently expanded its range to temperate waterways. Because it is tolerant of a range of environmental conditions, brackish systems like Currituck Sound in northeastern NC, may be susceptible to invasion. Two key research questions were addressed: 1) Is *C. raciborskii* currently present in Currituck Sound? and 2) What conditions would favor its growth and expansion? In 2007, microscopic analysis confirmed that *C. raciborskii* was present in Currituck Sound. Metagenomic analyses demonstrated that it has the genetic potential to produce the cyanotoxin cylindrospermopsin. In 2008, salinity in Currituck Sound had risen significantly and *C. raciborskii* was no longer present in Currituck Sound. The ability of *C. raciborskii* to grow in Currituck Sound water was assessed using a series of nutrient addition bioassay experiments and demonstrated that *C. raciborskii* can grow in this water and nitrogen additions increase its growth potential.

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I. EXTENDED ABSTRACT

Opportunistic invasive species can negatively impact an ecosystem in multiple ways, and estuaries that have been significantly altered by anthropogenic effects like nutrient enrichment may be particularly susceptible to invasion. *Cylindrospermopsis raciborskii* is an invasive, toxin-producing, filamentous-heterocystous, N_2-fixing cyanobacterium that has recently expanded its range to temperate waterways, including the St. Johns River, FL, and rivers and estuaries in NC. Because it is tolerant of a range of environmental conditions, including oligohaline waters experiencing nutrient enrichment, it opens up brackish systems like Currituck Sound in northeastern NC, to potential invasion. The goal of this research was to assess the susceptibility of Currituck Sound to the proliferation of *C. raciborskii*, utilizing the Currituck Banks NERRS site as the main study site, thus answering two main research questions: 1) Is *C. raciborskii* currently present in Currituck Sound and 2) What conditions would favor its growth and expansion? In 2007, the presence of *C. raciborskii* in Currituck Sound was established using microscopy, metagenomic and diagnostic photopigment analyses, which showed that it is not only present, but also has the genetic potential to produce the cyanotoxin cylindrospermopsin. This toxin has been linked to illness in humans and domesticated animals in other parts of the world, and therefore the presence of *C. raciborskii* with the genetic potential for producing toxin is of particular interest to water quality managers. In 2008, salinity in Currituck Sound had risen significantly, due to a severe drought in North Carolina, and *C. raciborskii* was no longer present in Currituck

Sound. The ability of *C. raciborskii* to grow in Currituck Sound water was assessed using a series of nutrient addition bioassay experiments, in which cultured *C. raciborskii* was be added to surface sound water, in a series of nutrient treatments, and incubated up to 8 days. Primary productivity, nitrogen fixation rates and chlorophyll *a* measurements, made every other day for the length of the experiment, were used to quantify cyanobacterial growth and demonstrated that *C. raciborskii* can survive in this water and that nitrogen additions increase its growth potential. Salinity is the main factor influencing *C. raciborskii* growth, with high salinity significantly limiting biomass accumulation and growth and production rates. Nitrogen additions can enable *C. raciborskii* to withstand high salinities and increases its competitive success within the existing phytoplankton community of Currituck Sound. Water quality managers of Currituck Sound are therefore encouraged to limit nutrient, particularly nitrogen enrichment of this area in order to prevent the expansion of *C. raciborskii* in this area, thereby protecting this unique low salinity estuarine and barrier island habitat for the native species, especially submerged aquatic vegetation, fisheries, migrating waterfowl, and humans that rely on this ecosystem.

II. RESEARCH MOTIVATION

Introduction:

Invasive species, i.e. species that have had a minor presence within an ecosystem, but because of some perturbation have increased their dominance and impact on the ecosystem (Colautti and MacIssac 2004), are among the most important challenges facing the protection, conservation and management of aquatic ecosystems. This is especially true in estuaries that are influenced by watershed land use changes, and associated anthropogenic stressors (nutrient, sediment and contaminant pollution, alteration of natural water flow), potentially causing ecosystem-level changes (Allen et al. 2006). Invasive species can negatively impact ecosystems in many ways, including out-competing more desirable native species, reducing biodiversity, altering physical habitats and food webs, and adversely affecting human use of local habitats (Allen et al. 2006).

One microorganism that is currently on the rise in temperate to tropical nutrient-enriched waters is the toxin-producing, filamentous-heterocystous, N_2-fixing cyanobacterium *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya et Subba Raju. *C. raciborskii* has two distinct morphological phenotypes in which the filament, typically between 2-3 μm wide and 10-120 μm long, is straight or coiled (Padisak 1997, **FIGURE 1**), and it is characterized by terminal heterocysts utilized by the filament for N_2-fixation (Seenayya and Raju 1972),

the enzymatic process by which "inert" atmospheric dinitrogen is converted into the biologically-available form of N, ammonium (Postgate 1998).

Cyanobacteria, which are among the planet's oldest known photosynthetic organisms (~2 bya; Schopf 2000), have demonstrated, over millions of years, a remarkable ability to adapt to environmental change, both natural and anthropogenic, which allows them to be highly successful invaders (Paerl and Fulton 2006, Paerl and Huisman 2009). Since its initial documentation in tropical and subtropical habitats (Seenayya and Raju 1972), *C. raciborskii* has significantly expanded its range over the last decade in diverse aquatic ecosystems, including lakes, reservoirs, rivers, and estuaries in Australia, Europe, South and North America (Branco and Senna 1994, Dokulil and Mayer 1996, Hawkins 1996, Chapman and Schelske 1997, Padisak 1997, Wood and Stirling 2003, Codd et al. 2005). *C. raciborskii* is a particularly-aggressive invader in Southeast US waters (Chapman and Schelske 1997, Dyble et al. 2002, Paerl and Fulton 2006).

Today, many aquatic environments are facing large-scale changes due to human-induced nutrient over-enrichment and global climate change, and researchers suggest that, over time, both of these changes may favor the growth and expansion of cyanobacteria (Paerl et al. 2001, Paerl and Husiman 2009). In particular, the combination of eutrophication and global warming may specifically increase the invasion potential for *C. raciborskii* in many areas, due to its efficient unique nutrient and light uptake and utilization strategies (Paerl and Huisman 2009).

C. raciborskii is of water quality and human health concern because it can be toxic. It produces the cyanotoxin tri-cyclic alkaloid cylindrospermopsin (CYN; Hawkins et al. 1985, Saker and Neilan 2001, **FIGURE 2**), which has been linked to illness in humans (Byth 1980,

Bourke et al. 1983, Hawkins et al 1985, Hayman 1992) and animals (Thomas et al. 1998, Saker et al. 1999). CYN adversely affects liver, lung and kidney function, and can even lead to death at high doses (Hawkins et al. 1985, Falconer and Humpage 2006). The most significant instance of *C. raciborskii* toxicity was a large-scale outbreak of Palm Island Mystery Disease (Byth 1980) in November 1979 in Palm Island, Australia. 138 children and 10 adults were sickened about 3 days after the main water supply, which was experiencing a persistent algal bloom, was treated with copper sulfate (Griffiths and Saker 2003). After many studies, it was determined that *C. raciborskii* was responsible for the bloom, and that the copper sulfate had caused cell lysis and the release of CYN into the water, directly leading to illnesses (Griffiths and Saker 2003). New evidence has also suggested that CYN may be bioaccumulated in invertebrates, including crayfish (Saker and Eaglesham 1999) and mussels (Saker et al. 2004), illustrating that there may be significant health concerns from human consumption of shellfish. Little is yet known about what triggers toxin-production in *C. raciborskii*, though there is some evidence of a negative correlation between ammonium concentration and toxin production (Saker and Neilan 2001, Griffiths and Saker 2003), and that environmental stress, particularly grazing pressure, may be responsible for increased toxicity (Fabbro et al. 2001). This limited knowledge makes it difficult to predict in which environments *C. raciborskii* would pose the most significant human-health threat, but it is clear that toxic blooms could have far reaching effects on the native aquatic flora, commercial and recreational fisheries, water use for bathing, and other recreational/tourist activities.

C. raciborskii has several physiological attributes that contribute to its ability to invade aquatic habitats. *C. raciborskii* dominance is favored by high temperatures and low

incident irradiation (optimum growth at 30°C and about 10% full light, or 120 μmol photons m^{-2} s^{-1}; Shafik et al. 2001), high pH, environmental consistency, long residence time, and a thermally stratified water column (Dokulil and Mayer 1996, McGregor and Fabbro 2000, Briand et al. 2002, Briand et al. 2004). Despite these optimum growing conditions, *C. raciborskii* tolerates a wide range of environmental conditions, and can form blooms under varying light, temperature and nutrient regimes (Isvánovics et al. 2000, Sprober et al. 2003, Briand et al. 2004). It can compete in nitrogen poor waters by fixing atmospheric nitrogen (N_2), while in nitrogen-enriched waters it effectively competes for combined N sources (Bouvy et al. 2000, Paerl and Fulton 2006). It also has a high affinity and storage capacity for phosphorous (Isvánovics et al. 2000). While its preferred habitat is fresh to slightly brackish waters, it tolerates elevated dissolved minerals (Briand et al. 2002), including low levels of salinity (4 g L^{-1}; Moisander et al. 2002).

Light utilization may play a key role in *C. raciborskii's* competitive success within the phytoplankton community. Whereas many other cyanobacterial species require high irradiance to reach their maximum growth potential and therefore form surface blooms, *C. raciborskii* is unique in that it forms subsurface blooms (Padisak 1997). Research has in fact shown that *C. raciborskii* reaches its maximum photosynthetic rate (P^b_{max}) at low light levels (30 to 400 μmol photons m^{-2} s^{-1}, Briand et al. 2004) enabling this species to bloom beneath other algal species or to form blooms in areas where water clarity is impaired. **FIGURE 3** demonstrates this fact with a Photosynthesis vs. Irradiance (PE) curve for a culture of *C. raciborskii*, Cyl L. Several PE experiments with Cyl L, as well as other *C. raciborskii* cultures were performed throughout the duration of this experiment using the photosynthetron method described by Lewis and Smith (1983). Photosynthetic parameters,

including P^b_{max}, α, or the light limited slop of the PE curve, and I_k, the irradiance level at which light saturated photosynthesis is reached, were determined from the figures by fitting the data with a curve using the hyperbolic tangent function proposed by Jassby and Platt (1976, **FIGURE 3**). In this case, a very typical example of the outcome of these experiments, the P^b_{max} for Cyl L is reached at about 200 μEinsteins m^{-2} s^{-1} after which saturation and photoinhition set in. This low P^b_{max} and high α make *C. raciborskii* an effective competitor in areas where light availability is limited either by existing algal populations or by high suspended sediment loads.

One other potential key to *C. raciborskii's* invasion success, particularly its expansion into northern waters, is its ability to produce akinetes (Briand 2004). These thick-walled, spore-like reproductive structures have long been recognized as playing a key role in cyanobacterial dominance (Rother and Fay 1977, Paerl 1988, Hansson 1996, Baker and Bellifemine 2004) and can ensure survival during adverse growth conditions by providing a resting stage for *C. raciborskii* (Moore et al. 2005). For instance, in northern water bodies, these akinetes can over-winter in the sediments and germinate only when temperatures reach 22-23°C (Padisak 1997, Moore et al. 2005). In addition, akinetes may be the vector for *C. raciborskii* invasions, as they may be transported to new aquatic habitats by migrating birds and fish (Padisak 1997). As global climate change continues, and periods of severe weather increase in frequency, duration, and degree, akinetes may provide a significant advantage for cyanobacteria and *C. raciborskii* to increase their presence and dominance in phytoplankton communities.

Many studies have demonstrated that *C. raciborskii* can be a highly successful competitor within the phytoplankton community and its invasion success often results in

communities shifting to *C. raciborskii* dominance from other, native cyanobacterial species including *Microcystis spp.* and other diazotrophic, or N_2-fixing, cyanobacteria like *Anabaena spp.* and *Aphanizomenon spp.* (Chapman and Schelske 1997, Saker and Griffiths 2001, Dobberfuhl 2003). In addition, as *C. raciborskii* dominance increases, species richness and diversity within phytoplankton communities decreases (Dobberfuhl 2003). One of the most documented cases of this is in St. Johns River, FL, where *C. raciborskii* went from a very minor to the dominant player in the phytoplankton community, dramatically out-competing the native *Anabaena*, following a steep increase in nitrogen and phosphorus enrichment of this waterway in the latter part of the 20th century (Chapman and Schelske 1997). The effect of *C. raciborskii* on phytoplankton community dynamics may have far-reaching consequences, particularly on food webs. Though some studies have found that blooms of *C. raciborskii* can be effectively controlled by grazing (Fabbro et al. 2001), other studies have shown that *C. raciborskii* is toxic or unpalatable to most common zooplankton grazers (Padisak 1997), and the presence of *C. raciborskii* can significantly change the composition of the zooplankton community (Leonard and Paerl 2005).

In North Carolina, *C. raciborskii* has already shown a widespread ability to proliferate. Routine monitoring by the North Carolina Department of Environment and Natural Resources (DENR) Division of Water Quality (DWQ) has indicated widespread presence of *C. raciborskii* in many of the State's waterways, including reservoirs (e.g., Falls of the Neuse reservoir) and rivers making up the headwaters of the Pamlico and Albemarle Sounds (E. Fensin, pers. comm.). Researchers suspect that other waterways, not currently monitored, could contain *C. raciborskii* or could be susceptible to future invasion in response to changing environmental conditions, especially anthropogenic nutrient enrichment.

One area that could be susceptible to invasion by *C. raciborskii* is the brackish Currituck Sound, in northeastern North Carolina (**FIGURE 4**). Currituck Sound receives discharge from the State's northernmost riverine tributaries (Chowan, Roanoake, Albemarle, and local systems) and drains into the Albemarle-Pamlico estuarine system, the largest estuary-lagoon system in the United States. Currituck Sound encompasses 39,600 ha with 1.6 m mean depth (Davis and Brinson 1983, Wicker and Endres 1995). It is a unique estuarine environment due to its low salinity, usually ranging from fresh (0 psu) to oligohaline (~3.5 psu; Caldwell 2001). This low salinity range, in particular, makes Currituck Sound a habitat in which *C. raciborskii* could thrive, given that it is a fresh water species and has been shown to invade fresh water habitats, like St. Johns River, in the past. Currituck Sound supports both commercial and recreational fisheries, numerous important plant species, including submerged aquatic vegetation (SAV), and is a critical habitat for migrating waterfowl. Currituck Banks is a National Estuarine Research Reserve System (NERRS) site located on a barrier spit separating Currituck Sound from the Atlantic Ocean. It supports a diverse array of waterfowl, fish and shellfish, including endangered or threatened species. The combined effect of low salinity and increasing nutrient enrichment associated with increasing coastal development in N.E. North Carolina may make Currituck Sound susceptible to invasion by *C. raciborskii*. In fact, Currituck Sound, in addition to having optimal salinity for growth of *C. raciborskii*, possesses the necessary light, temperature, and nutrient conditions to support the invasion or significant expansion of existing populations of this species. **Therefore, I hypothesize that *C. raciborskii* is present and that the environmental conditions of Currituck Sound are suitable for invasion.**

Research Questions and Goals:

Having made the hypothesis above, I determined to test this hypothesis by asking two main questions:

1. **Is *C. raciborskii* currently present in Currituck Sound?**

   Prior to my beginning this work, no one had looked for *C. raciborskii* in Currituck Sound, and very little information about the existing phytoplankton community was available (Caldwell 2001). In addition to determining the presence or absence of *C. raciborskii* in Currituck Sound, I will answer other basic questions including:

   a. What is the abundance of *C. raciborskii* in this system?
   b. Are *C. raciborskii's* main N_2-fixing cyanobacterial competitors, including *Anabaena spp*., *Aphanizomenon spp*, and *Anabaenopsis spp.* present or absent in Currituck Sound?
   c. Is *C. raciborskii* in Currituck Sound able to produce the CYN toxin? If so, what are the current levels of CYN toxin, as well as other cyanobacterial toxins saxotoxin and microcystin, in this system?
   d. What is the relatedness of the *C. raciborskii* strain in North Carolina to the strain extensively studied in the St. Johns River, FL?

   In order to answer these questions, I used pigment analysis, microscopy, extensive metagenomic work, and toxin analysis.

2. **What conditions would favor the invasion or expansion of this species?**

   Without knowing, at the start of this experiment, whether *C. raciborskii* was already present in Currituck Sound, I set out to investigate its ability to grow in Currituck Sound water. To do this, I conducted a series of nutrient addition bioassays designed to determine if *C. raciborskii* could grow in the water itself, i.e., if the environmental conditions, including salinity (*C. raciborskii* has been shown to tolerate salinity up to 4 g L^{-1}; Moisander et al. 2002), are adequate for *C. raciborskii* growth, whether *C. raciborskii* can adequately compete with the existing phytoplankton community, and under what nutrient conditions it grows best. This last question is aimed at identifying nutrient conditions and concentrations that will need to be avoided *in situ* to prevent problems with this species in Currituck Sound. In addition to the nutrient addition bioassays, the salinity threshold for *C. raciborskii* was examined using a growth chamber salinity experiment to further evaluate the susceptibility of Currituck Sound and other water bodies with similar salinity conditions to invasion or expansion of *C. raciborskii*, considering both current salinity conditions and projected conditions based on different climate change scenarios.

In addition to answering these two main research questions, this work had an additional research goal. A Graduate Research Fellowship (GRF) from NERRS primarily funded this research, and the Currituck Banks NERRS site served as the main sampling site for this study. Therefore, a main goal was to use the data obtained from this study to formulate long-term nutrient and water quality management strategies for Currituck Sound

that can be used by NERRS and North Carolina (DENR-DWQ) to minimize potential invasions of *C. raciborskii*. Results will be used to formulate a management plan to minimize the invasion and proliferation of this and possibly other harmful, toxic, N_2-fixing cyanobacterial species, with the goal of preserving water quality of the Sound for native species, especially SAV, fisheries, migrating waterfowl, and humans who rely on this ecosystem. When completed, this research will be useful in formulating similar strategies to protect other estuarine systems susceptible to *C. raciborskii* invasion, both in North Carolina and the nation.

III. MATERIALS AND METHODS:

Study Site:

The main study site was the North Carolina NERRS site of Currituck Banks (**FIGURE 5**). This 950-acre reserve is located just north of the town of Corolla, and about ten miles south of the Virginia border. It separates the Atlantic Ocean from the rest of Currituck Sound, and is characterized by its low salinity estuarine and barrier island habitat. Other sampling sites throughout the Currituck Sound and North Carolina were used to complement the work done at Currituck Banks, and to broaden application of research findings. All experimental work was conducted in an outdoor circulating research pond at the University of North Carolina – Chapel Hill's Institute of Marine Sciences (UNC-IMS), Morehead City, NC (**FIGURE 6**).

Determining *C. raciborskii's* presence in Currituck Banks:

Sample Collection: Surface water was collected from several locations within Currituck Banks and the surrounding Currituck Sound in summer 2007 and throughout eastern North Carolina in summer 2008 using pre-cleaned (stored in dilute HCl, followed by several rinses of deionized water and a sample rinse) polyethylene bottles. **FIGURE 7** and **TABLE 1** show

the locations of each sampling. Water was then transported back to IMS under approximate ambient temperature and light conditions for analysis.

Microscopy: At each location, sub-samples for species identification were collected in 80 ml glass vials and preserved immediately with 1% acid Lugol's solution. The samples were then allowed to settle and viewed at 400x (10x eyepiece and 40x objective) magnification under an inverted microscope (Leica M-20), and the presence and abundance of *C. raciborskii* was estimated by counting filaments and heterocysts.

Pigment Analysis: Diagnostic photopigment analysis was conducted on each water sample, using high performance liquid chromatography (HPLC) described by Pinckney et al. (2001). Water samples were filtered onto 4.7 cm diameter glass fiber filters (GF/F, 0.6-0.8 µm porosity), placed in 100% acetone, sonicated and extracted at -20°C overnight. 200 µl aliquots of filtered extracts were injected into the HPLC system, equipped with a column configuration designed to separate structurally different photopigments. The absorption spectra and chromatograms of each pigment were then analyzed to identify and quantify the pigments present (Pinckney et al. 2001). Although species-specific identification of *C. raciborskii* is not possible, the pigments zeaxanthin and myxoxanthophyll are diagnostic for cyanobacteria. High concentrations of myxoxanthophyll in particular indicate N_2-fixing cyanobacteria like *C. raciborskii* and its N_2-fixing cyanobacterial competitors, like *Anabaena spp*. and *Anabaenopsis spp*.

Metagenomic Analyses: Metagenomic techniques were used to confirm microscope analysis. Deoxyribonucleic acid (DNA) was extracted from Millipore, 0.7µm porosity glass fiber filtered samples using an UltraClean soil DNA purification kit (MO BIO Laboratories Inc). We targeted the N_2-fixing gene *nif*H, utilizing primers designed by Dyble et al. (2002). *Nif*H encodes dinitrogenase reductase, which is an iron protein subunit of nitrogenase, the enzyme used in nitrogen fixation. It is highly conserved among N_2-fixers, but has enough variable regions that species distinction is possible (Zehr and Paerl 2008). DNA samples were amplified with the cyano-*nif*H primer using polymerase chain reaction (PCR). The PCR was prepared using PCR reagents (Fisher Biotec Index) and the following recipe: a 50 µl reaction volume containing 10 µl manufacturer's buffer, 5 µl $MgCl_2$, 1 µl dNTPs, 1 µl BSA, 1 µl of forward and reverse primer, 0.4 µl Taq, 28.6 µl sterile water, and 2 µl purified DNA. The PCR was done using a Techne TC-512 thermal cycler and the amplification parameters were 94°C for 5 minutes, followed by 30 cycles of 94°C for 10 seconds, 55°C for 20 seconds, and 72°C for 1 minute, followed by an extension at 72°C for 7 minutes. Results were visualized using gel electrophoresis and used to determine the presence of N_2-fixers. The more specific cylindro-*nif*H was then used to determine whether or not *C. raciborskii* is present in the sample, using the same amplification parameters as above.

In addition to determining the presence or absence of *C. raciborskii* at each site, the ability of the *C. raciborskii*, when present, to produce CYN toxin was evaluated using a suite of primers designed by Wilson et al. (2000) and Schembri et al. (2001) and a multiplex method described by Fergussen and Saint (2003). Three main genes in the pathway to create CYN were targeted, *cyl*, which, like the cylindro-*nif*H gene, is specific for *C. raciborskii* and *ps* and *pks*, both of which code for necessary proteins in CYN synthesis. If a sample was

positive for all three genes, it was determined to be positive for *C. raciborskii* capable of producing CYN. The PCR was prepared using PCR reagents (Fisher Biotec Index) and the following recipe: a 50 µl reaction volume containing 10 µl manufacturer's buffer, 5 µl $MgCl_2$, 1 µl dNTPs, 1 µl BSA, 1 µl of forward and reverse primer, 0.4 µl Taq, 28.6 µl sterile water, and 2 µl purified DNA. The PCR was done using a Techne TC-512 thermal cycler and the amplification parameters for this suite of primers were 94°C for 10 minutes, followed by 30 cycles of 94°C for 30 seconds, 45°C for 30 seconds, and 72°C for 1 minute, followed by an extension at 72°C for 7 minutes.

Phylogenetic Analysis: The PCR results using the cyano-nif*H* primers were further used for sequence analysis. The PCR product was run on a 1.5% agarose gel and the visible bands were cut out and extracted using a QIAquick gel extraction kit (Qiagen).

The purified DNA was then cloned, by first ligating the DNA and transforming cells by introducing the DNA to competent *E. coli* cells (Invitrogen TOPO TA 2.1 cloning kit). After transformation, the cells were grown on LB plates and individual colonies were selected for sequencing. Forward and reverse sequencing was performed by the Biotech Department at the University of Florida. The sequences were aligned and trimmed using the BioEdit program. Also, using BioEdit as an interface, a phylogenetic tree was generated with the Dayhoff PAM matrix and neighbor-joining algorithm using the ProtDist application of PHYLIP (Felsenstein 1989).

Toxin Analysis: For water samples collected in summer 2008, concentrations of CYN, in addition to other cyanobacterial toxins including microcystin and saxotoxin, were determined

using ELISA kits (Abraxis LLC). These kits utilize immunoassays to quantify the concentrations of these toxins using antibodies and color solutions, which generate an intensity of color based on the concentration of toxin. The kits were read using a Multiskan Spectrum Spectrophotometer (ThermoFisher Scientific) and the exact concentrations determined using a standard curve. The detection limit for the Abraxis Cylindrospermopsin ELISA is 0.040 ppb (g/L), for the Abraxis Microcystins-ADDA ELISA kit it is 0.10 ppb (g/L) and for the Abraxis Saxotoxin (PSP) ELISA it is 0.015 ng/mL.

Determining *C. raciborskii's* growth potential in Currituck Banks:

A series of nutrient addition bioassay experiments, similar to those performed by Moisander and Paerl (2000) was completed during a two-year period (2007-2008). Bioassays were conducted in June and September of each year to capture the maximum *in situ* growth and bloom periods of *C. raciborskii*.

Water Collection: In June 2007, surface water was collected from two representative locations, one within the Currituck Banks reserve, and one from the surrounding Currituck Sound (**FIGURE 7**). For the remaining three experiments, water from the site within the reserve was used. Water was filtered through a 53 μm Nitrex mesh onsite to remove any large grazers from water samples. At each sampling site, vertical temperature, salinity, dissolved oxygen and photosynthetically-active radiation (PAR) profiles were analyzed using a YSI 6600 sonde. The water was then transported from the reserve to IMS (see **FIGURE 6**) under approximate ambient light and temperature conditions.

Filter treatments: The water from each site was divided into two treatments. For the unfiltered treatment, 40 L of water was left completely un-manipulated. For the filtered treatment, 40 L of water from each site was filtered through pre-combusted Millipore GF/Fs.

Nutrient Treatments: Once the filtration was complete, the 40 L of both filter treatments were divided into the incubation vessels, with a water sample reserved for initial or T_0 measurements. 2.5 L of water was dispensed into each acid-washed, 3.8-L polyethylene Cubitainers (Hedwin Corporation; Moisander and Paerl 2000) to make up the nutrient treatments. Cubitainers are chemically inert, 85% transparent to PAR, and easily deployed in the outdoor ponds. Dissolved inorganic carbon (DIC) solution ($NaHCO_3$ stock solution, 2 mg C/L ending concentration) was added to each Cubitainer to ensure that growth and production in the vessel was never limited by DIC. In June 2007, there were 2 sites, each with a filtered and an unfiltered treatment, and each filter treatment was further divided into 4 nutrient treatments, done in quadruplicate: Control (**C**; no nutrients added), Nitrate (**N**; KNO_3 stock solution, 10 μM final concentration), Phosphate (**P**; KH_2PO_4 stock solution, 5 μM final concentration), and Nitrate and Phosphate (**N+P**; KNO_3 and KH_2PO_4 stocks added, 10 μM and 5 μM final concentrations respectively). For the September 2007 and both 2008 bioassays, there was only one sampling site, with a filtered and unfiltered treatment divided into 8 nutrient treatments, each done in quadruplicate: **C**, **N**, Ammonium (**A**; NH_4Cl stock solution, 10 μM final concentration), **P**, **N+P**, Ammonium and Phosphate (**A+P**, NH_4Cl and KH_2PO_4 stocks added, 10 μM and 5 μM final concentrations respectively), Nitrate and Ammonium (**N+A**; KNO_3 (5 μM) and NH_4Cl (5 μM) stocks added, 10 μM total nitrogen final

concentration), and Nitrate, Ammonium, and Phosphate (**N+A+P**; KNO_3 (5 μM), NH_4Cl (5 μM) and KH_2PO_4 stocks added).

Inoculation: After nutrient additions, an inoculum of a representative *C. raciborskii* strain was then added to each Cubitainer. The inoculum is *C. raciborskii* strain Cyl L (maintained at the UNC-IMS). This strain was originally isolated from Lake Griffin, Florida, and was purified by Dr. P. Moisander (pers. comm.). Inocula were grown in batch cultures in Z8 medium (Rippka 1988) in a growth chamber (23-28ºC, 15:9 L:D light cycle), and experiments were conducted 10 days into the inocula's growth cycle, just as it entered the exponential growth phase. 50 mL of Cyl L was added to each Cubitainer.

Controls: In addition to the estuarine water treatments, each bioassay also included a Media control, consisting of 4 Cubitainers containing 2.5 L Z8 media. These Cubitainers received DIC ($NaHCO_3$, 2 mg C/L or 0.17 mM final concentration), but no nutrients were added. In the 2008 bioassays, a second Media control was added, consisting of 4 Cubitainers containing 2.5 L Z8 media with additional nitrate added (10 μM KNO_3). Starting in September 2007, an additional control was added consisting of unfiltered water with no Cyl L added.

Incubation: Once the Cubitainers were set up, they were incubated in large corrals covered with a layer of neutral density screening under natural light and temperature conditions in a circulating seawater research pond located behind IMS. Every other day for 8 days (T_2, T_4, T_6, and T_8), the Cubitainers were sub-sampled at 8 a.m., with 300 to 500 mL removed, to

track the progress of the bioassay. These samples were analyzed for salinity, pH, chlorophyll *a*, diagnostic pigments, primary productivity, N_2-fixation (using acetylene reduction), DIC, CHN (carbon, hydrogen and nitrogen concentrations), nutrient (particularly NO_3^-, NH_4^+, and PO_4^{3-}) concentrations, and *C. raciborskii* abundance. Ambient light and temperature data were also recorded.

Laboratory Analyses: As indicators of cyanobacterial growth, primary productivity and N_2 fixation were assayed. Subsamples were incubated for 4 hours in a small corral covered with a layer of neutral density screening in the research pond. Primary productivity (PP) was measured using the $NaH^{14}CO_3$ incorporation method originally described by Parsons et al. (1984) and modified by Rudek et al. (1991). DIC was measured on acidified samples using a LI-COR CO_2 model 6252 infrared gas analyzer. N_2-fixation rates were estimated using the acetylene reduction (AR) assay as described by Burris (1972) and modified by Paerl (1998) with a 30 mL sample volume and 3.5 mL acetylene addition. As a measure of algal biomass and growth, chlorophyll *a* concentration (Chl *a*) was determined in parallel with activity measurements. The fluorometric technique detailed by Welschmeyer (1994) was used for determining Chl *a*, using a Turner TD-700 fluorometer. Growth rates were then calculated based on Chl *a* data using a best curve fit for exponential growth, using the following equation, described by Slater (1988):

$$x_t = x_0 e^{\mu t}$$

where x_t is the Chl *a* concentration (μg/L) at a specific time-point, x_0 is the initial Chl *a* concentration, t is the time-point of interest (d), and μ is the growth rate (d^{-1}). For all bioassays, growth rate was calculated considering that first 6 days of growth (t=6) because

cell die-off within the incubation vessels usually began after this point. Dissolved inorganic nutrients (NO_x, NH_4, PO_4, SiO_2) were measured using an autoanalyzer (Lachat Quick Chem. IV, Lachat Inc.). Diagnostic photopigment analysis was conducted using high performance liquid chromatography (HPLC) described by Pinckney et al. (2001). *C. raciborskii* abundance was determined from subsamples of water collected in 80 ml glass vials and preserved immediately with 1% acid Lugol's solution. The samples were then settled and viewed at 400x magnification under an inverted microscope (Leica M-20), and the abundance of *C. raciborskii* evaluated. The concentrations of carbon, hydrogen and nitrogen were assessed using a 2400 Series II CHN analyzer (Perkin Elmer). Salinity of the water was evaluated at the start of the experiment using the YSI 6600 sonde and modem, and was confirmed throughout the course of the experiment using a hand-held refractometer. pH was determined using a BASIC pH meter (Denver Instruments).

Statistical Analyses: All statistical analyses were performed using MATLAB (MathWorks). Differences in Chl *a* concentration, growth rate and productivity in different treatments between water from Currituck Sound and artificial medium across each treatment were analyzed using a repeated-measures analysis of variance (ANOVA) with water (Z8 medium or water from different sites) as the between-subject factor. P values less than 0.05 were used to indicate statistical significance.

Salinity Experiment: To investigate the salinity threshold of *C. raciborskii*, a growth chamber experiment was performed in the July 2008. 4 L of Z8 media was prepared, filtered through a 0.2 μm filter, and divided into 4 salinity treatments, 0 psu, 3 psu, 6 psu, and 9 psu.

Salinity was manipulated using NaCl. Each salinity treatment was further divided into two nutrient treatments, Control (no nutrients added) and Nitrate (NO_3^- added). For each treatment, quadruplicates of 250 ml of media were placed in glass Pyrex flasks with foam caps, inoculated with *C. raciborskii* (Cyl L strain) and were incubated in a growth chamber (23-28ºC, 15:9 L:D light cycle) for 14 days. Chlorophyll *a* concentration, used as a proxy for *C. raciborskii* biomass, was analyzed every other day throughout the course of the experiment.

IV. RESULTS AND DISCUSSIONS:

2007 vs. 2008:

As mentioned above, this project took place over two consecutive summers, 2007 and 2008. Due to hydrological conditions in North Carolina during this period, mainly a prolonged severe drought and the resulting salinity regime in Currituck Sound, these two years had markedly different results, both in terms of *C. raciborskii*'s presence in Currituck Sound and its growth potential once introduced. This difference makes it very difficult to draw comparison between these two summers, but instead offers the opportunity to examine this issue under these two very different set of conditions. For this reason, I will be presenting the results from each year of this study separately. For the first portion of the study, determining the presence of *C. raciborskii* in Currituck Sound, the 2007 results will encompass results from sampling sites 1-6 and 2008 will encompass 7-26 (**TABLE 1**). For the second portion of this study, determining *C. raciborskii's* growth potential in Currituck Sound, the 2007 results will encompass the June and September 2007 bioassays and the 2008 will encompass the June and September 2008 bioassays.

North Carolina Drought Conditions:

According to the North Carolina Division of Water Resources Drought Management Advisory Council, North Carolina began experiencing moderate drought conditions in late May 2007 (W. Yonts, pers. comm.). These conditions became severe in August, and were extreme or exceptional until March 2008. Despite periods of renewed precipitation throughout 2008, moderate drought conditions have persisted into early 2009, making this the worse drought in North Carolina on record (W. Yonts, pers. comm). For the purpose of this study, I visited the main study site at Currituck Banks 4 times to collect water samples, and the effect of the drought on the salinity regime in Currituck Sounds could be seen (**TABLE 2**). A lag effect in the salinity in Currituck Sound, produced by the severity of the drought from May 2007 until March 2008, caused the salinity at the study site to steadily increase throughout the course of the study. This, in turn, had large implications for the phytoplankton community composition, especially as the salinity in September 2008 was the highest on record for Currituck Sound, based on historical records (Caldwell 2001) and recent YSI deployments by NC-DENR (J. Fine pers. comm).

Determining *C. raciborskii's* presence in Currituck Banks:

***2007*:**

Pigment Analysis: In both June 2007 and September 2007, HPLC analysis of water samples both from within the Currituck Banks reserve (Sites 2, 3, and 6) and from Currituck Sound (Site 1) had high concentrations of myxoxanthophyll and zeaxanthin (ranging from about 3 to 10 μg/L of zeaxanthin and 6 to 20 μg/L of myxoxanthophyll). Though species-level distinction is not possible with this analysis, the combination of these two pigments generally indicates the presence of N_2-fixing cyanobacteria (Rowan 1989), like *C. raciborskii* and its direct competitors. The pigment analysis from the other sampling sites (Sites 4 and 5) did not indicate cyanobacterial presence (less than 1 μg/L for both myxoxanthophyll and zeaxanthin).

Microscopy: Microscopic analysis of these water samples confirmed the HPLC results. Both in June and September 2007, the phytoplankton community at the sampling sites with high myxoxanthophyll and zeaxanthin concentrations was very rich in N_2-fixing cyanobacteria. **FIGURES 8 and 9** shows examples of phytoplankton community for both Site 1 (outside the reserve, **FIGURE 8**) and Site 2 (inside the reserve, **FIGURE 9**). These samples confirm the presence of *C. raciborskii* in Currituck Sound (*C. raciborskii* concentrations ranged from 910 to 1500 cells/mL), in addition to several of its key competitors, including *Anabaena spp*., *Aphanizomenon spp*., and *Anabaenopsis spp*. These results confirm that Currituck Sound, at least in 2007, was a suitable habitat for *C. raciborskii*, having the necessary environmental conditions for *C. raciborskii* to survive.

Despite its presence in the phytoplankton community, *C. raciborskii* was by no means the dominant player, having concentrations of 910 to 1500 cells/mL, very low compared with *Anabaena spp*. This makes the current state of Currituck Sound similar to the situation in St. Johns River, FL a few decades ago, before the nitrogen and phosphorus over-enrichment of this system triggered a shift in dominance within the phytoplankton community from *Anabaena spp.* to *C. raciborskii* (Chapman and Schelske 1997). *C. raciborskii* in the St. Johns River today can in fact have concentrations up to ten times what was found to be in Currituck Sound in 2007. The cause of this shift is still largely unknown, but while superior nutrient uptake and utilization strategies may be responsible for this shift, advantageous light regimes may have also helped *C. raciborskii* out-compete *Anabaena spp.* in this system. *C. raciborskii*, as previously mentioned, reaches its P^b_{max} at low light levels, enabling this species to form blooms in areas of reduced water clarity. It is possible, therefore, that *Anabaena spp*. is being out-competed by *C. raciborskii* in the St. Johns River now because of the increased available nitrogen and phosphorus in the system, both of which also significantly decrease the water clarity, thereby allowing an opportunistic species like *C. raciborskii* to take advantage of changes to an environment.

The similarity between Currituck Sound now and the St. Johns River system of a few decades ago is interesting, and may serve as a cautionary tale for the water quality managers of Currituck Sound. Currently, the water quality of Currituck Sound is good, especially compared with other impaired estuaries like Chesapeake Bay. Currituck Sound not only currently supports thriving commercial and recreational fisheries but large populations of SAV, which are often seen as a barometer for estuarine health because of how sensitive SAV are to changes in water quality and clarity. Nutrient and suspended solid enrichment has

been minimal in this area (Caldwell 2001), and large stretches of the land surrounding Currituck Sound, especially around and north of the Currituck Banks reserve is currently undeveloped. The Outer Banks area of North Carolina, however, is expanding and developing, and therefore the anthropogenic effects, including increased runoff due to impervious surfaces, increased recreational use of waterways, and increased nutrient and sediment loads to Currituck Sound, on this area are increasing dramatically with every year (Frankenberg 1995). If nutrient inputs, both nitrogen and phosphorus, in this area were to increase significantly, conditions in Currituck Sound could come to resemble those in the St. Johns River now, mainly that water clarity would decrease. This could potentially pose two significant problems for the area, the first being that the populations of SAV, and the fish and migrating waterfowl that rely on them, would be in jeopardy, greatly impairing the ecosystem services that Currituck Sound currently provides. The second could be that these increased nutrients and decreased water clarity might allow *C. raciborskii* to expand its dominance within this phytoplankton community as it did in St. Johns River, and all the potential problems that might come with this expansion would follow, particularly if the *C. raciborskii* in Currituck Sound had the potential to be toxic, which will be covered in the next section. This eventuality may serve to motivate managers as one further reason to limit the human impact on this waterway.

Metagenomic Analysis: Metagenomic analysis was used to further confirm the results from the pigment and microscopic analysis. DNA samples were collected and processed from all water samples, and all results were visualized using gel electrophoresis. First we targeted cyano-*nif*H gene to determine the presence or absence of N_2-fixing cyanobacteria (Dyble et

al. 2002). As previously determined by the pigment and microscopic analysis, the gel electrophoresis, shown in **FIGURE 10**, confirmed the presence of N_2-fixing cyanobacteria at sites 1, 2, 3 and 6 with dark bands at 324 base pairs (bp). There were also faint bands for sites 4 and 5, though neither the pigment nor microscopic analysis showed N_2-fixing cyanobacteria at these sites. This demonstrates that metagenomic analysis is a much more sensitive tool for evaluating the presence of these organisms, but also that the most significant limitation of this tool is that it is not quantitative and can tell us nothing about the abundance of cyanobacterial N_2-fixers. Since they were not apparent in the microscopic analysis of these samples, we can assume they comprise a very small portion of the phytoplankton community at these sites, whereas they made up the majority of the community at sites 1, 2, 3 and 6.

Next, the more specific cylindro-*nif*H gene was targeted to determine whether or not *C. raciborskii* is present in each sample (Dyble et al. 2002). Again, microscopic analysis had shown *C. raciborskii* present in sites 1, 2, 3, and 6, but not in 4 and 5, but because identification of species is not always an exact science, the results from this PCR were important to determine *C. raciborskii*'s presence without a doubt. The results are shown in **FIGURE 11**, with bands at 224 bp clearly visible for sites 1, 2, and 6, a faint band for site 3, and no bands for sites 4 and 5. These results demonstrate without a doubt that *C. raciborskii* is a player in the phytoplankton community within, locally, Currituck Banks (sites 2, 3, and 6), and more broadly in at least some locations in Currituck Sound (site 1).

Next it was necessary to determine if the *C. raciborskii* strain present in Currituck Sound has the potential to produce the CYN toxin and therefore pose a significant human or animal health threat. Metagenomic analysis can identify the potential to produce toxin by

targeting three main genes in the pathway to create CYN (Wilson et al. 2000, Schembri et al. 2001, Fergussen and Saint 2003). These three genes, including *cyl*, which, like the cylindro-*nif*H gene, is specific for *C. raciborskii*, and *ps* and *pks*, both of which code for necessary proteins in CYN synthesis, when present in one sample, establish that that sample contains *C. raciborskii* capable of producing CYN (Fergussen and Saint 2003). The results from these three PCRs are shown in **FIGURE 12**, with bands for *cyl* at 308 bp, bands for *ps* at 597 bp and bands for *pks* at 422 bp. The results from this suite of PCRs show that the *C. raciborskii* at sites 1, 2, 3, and 6 all have the potential to produce the CYN toxin.

Metagenomic analysis, however, can only identify the potential to produce toxin, and does not necessarily indicate the cells in Currituck Sound are currently producing that toxin. In order to establish actual CYN production and quantify ambient toxin concentrations, toxin analysis is needed. Unfortunately, due to lack of equipment and supplies, this analysis was not performed on the samples from 2007, but was available and used on samples from 2008.

Phylogenetic Analysis: Sequences of nif*H* were analyzed from Currituck Sound (NC Sites 1, 2, 3, 5, and 6; **FIGURE 7** and **TABLE 1**), cultures of *C. raciborskii* isolated from Florida and maintained at IMS (Cyl D, Cyl F, and Cyl L), and randomly selected samples locations throughout St. Johns River, FL (SJR 1-4). GenBank BLAST searches showed that some of the *C. raciborskii* sequences from North Carolina matched 100% with sequences from previous *C. raciborskii* genetic studies utilizing samples from St. Johns River, FL (Dyble et al. 2002, Moisander et al. 2002). These results suggest that the *C. raciborskii* strains in Currituck Sound are very closely related to the Florida strains that have been extensively studied in the past. **FIGURE 13** shows a phylogenetic tree based upon nif*H* sequences of the

C. raciborskii strains, with a culture of *Anabaena aphanizomenoides*, maintained at IMS, as an out-group. The similarity between the Florida and the North Carolina strains is apparent. The main cluster contains many of the samples from North Carolina (NC 1A, 1B, 5 and 6), all of the *C. raciborskii* cultures, and two of the samples from Florida (SJR 2 and 3), all almost 100% similar to each other. Additionally, a smaller cluster shows a Florida sample (SJR 4) and two North Carolina samples (NC 2 and 3), again matching with 100% similarity. These results indicate that there is no significant difference between the strains of *C. raciborskii* in Currituck Sound and St. Johns River, FL, at least in terms of the nif*H* gene.

***Conclusions from 2007*:**

From the results of these analyses, I can conclude that *C. raciborskii* is present in the phytoplankton community in Currituck Banks and Currituck Sound. At the time that these samples were collected, the abundance of *C. raciborskii* in the samples ranged from 910 to1500 cells/mL, making it a minor player within a community constituted by many of *C. raciborskii*'s main competitors, including the N_2-fixing *Anabaena spp*., *Aphanizomenon spp*. and *Anabaenopsis spp*. In addition, these results show that the *C. raciborskii* strain in Currituck Sound has the genetic potential to produce CYN, and that the strains of *C. raciborskii* present in Currituck Sound are very closely related, it terms of the nif*H* gene, to those in St. Johns River, FL**.**

***2008*:**

Pigment Analysis: The pigment analysis from 2008 was markedly different from that of 2007. While some of the sites from the greater North Carolina area contained the pigments

myxoxanthophyll and zeaxanthin (sites 9 and 10**, TABLE 1**), the samples taken directly from Currituck Sound (sites 15, 16, 25, and 26) did not. This suggests that in 2008, N_2-fixing cyanobacteria did not make up a large portion of the phytoplankton community in Currituck Sound. This is, of course, in contrast to the 2007 findings, when N_2-fixing cyanobacteria were the key phytoplankton players. The pigment analysis from these samples, particularly the concentrations of fucoxanthin and lutein suggested a phytoplankton community similar to that of other estuaries in North Carolina, particularly the Neuse River (NRE), where the community is more influenced by coastal or oceanic species. The dramatic increase in the salinity of Currituck Sound within the period of this study (0 psu to 8.4 psu**, TABLE 2**) is probably responsible for this shift in pigments and therefore the phytoplankton community composition.

Microscopy: The microscopic analysis of the 2008 water samples again confirmed the results from the pigment analysis. In the samples from Currituck Sound, none of the players from 2007, including *C. raciborskii*, *Anabaena spp*., *Aphanizomenon spp*., and *Anabaenopsis spp*. were visible. In the place of these cyanobacterial species were primarily species of diatoms and dinoflagellates, including *Thalassiosira spp.* and *Prorocentrum spp*. In the greater North Carolina area, there were a few samples, including sites 9 and 10 that contained *Anabaena spp*. but no sample had *C. raciborskii.*

Metagenomic and Phylogenetic Analysis: Based on the pigment and microscopic analysis, it was unlikely that metagenomic analyses would indicate that any of these 2008 samples would be positive for *C. raciborskii*. The PCR assay for cyano-*nif*H gene to determine the

presence or absence of N_2-fixing cyanobacteria was negative for all but 3 sites in 2008 (site 9, 10, and 17), none of which was located in Currituck Sound. As such, phylogenetic analysis was not possible for these samples. The PCR assay for more specific cylindro-*nif*H gene was further used to determine that *C. raciborskii* was not present in any of the samples, which was again shown by negative results for the *C. raciborskii* toxin suite of *cyl*, *ps* and *pks*. The metagenomic analyses confirm that the phytoplankton community in Currituck Sound in 2008 was drastically different from that in 2007.

Toxin Analysis: While unavailable to us in 2007, in 2008 we had the ability to test all water samples for concentrations of cylindrospermopsin, microcystin, and saxotoxin using ELISA kits. All samples collected (sites 7-26) were assayed for all three toxins, and while a few sites had detectable levels of microcystin (site 8) and saxotoxin (site 12), these levels were very low and within water quality standards (1 μg/L concentration in water for microcystin, 40 to 80 μg per 100 μg of fish flesh for saxotoxin). Not surprisingly, cylindrospermopsin was undetectable at all sites, considering that *C. raciborskii* was not present in 2008. Toxin analysis of Currituck Sound in particular should be completed at some point in the future when *C. raciborskii* is present within the phytoplankton community in order to determine whether the cells, which have the genetic potential to produce toxin, are producing in this environment (See Future Work).

***Conclusions from 2008*:**

From the results of these analyses, I can conclude that *C. raciborskii* was not present in the phytoplankton community in Currituck Banks and Currituck Sound in 2008. The

difference between the results of 2007 and 2008 were in very stark contrast. While in 2007, *C. raciborskii* was a minor player (910 to 1500 cells/mL) within the phytoplankton community very rich in N_2-fixing filamentous cyanobacteria, pigment and microscopic analysis of the 2008 samples showed virtually no diazotrophic cyanobacteria. These results were affirmed by negative results in PCR assays for cyano-*nif*H, cylindro-*nif*H, *cyl*, *pks*, and *ps* and the cylindrospermopsin toxin analysis. From these results, I suspect that the severity of drought that North Carolina experienced throughout 2007 and 2008 produced a salinity regime that was inhospitable both to *C. raciborskii* and its competitors. This led to a phytoplankton community shift to species more tolerant of the elevated salinity. It was disappointing for this study to find that this shift had occurred, but since many of the species lost, including *C. raciborskii*, *Anabaena spp*. and other cyanobacterial species, have the ability to produce akinetes, and the ability of these akinetes to survive adverse conditions including significant salinity increases (Baker and Bellifemine 2000), I expect that these species will return to Currituck Sound when favorable conditions return. I will discuss this further in Overall Conclusions and Future Work.

Determining *C. raciborskii* growth potential in Currituck Banks:

Filtered vs. Unfiltered:

As mentioned above, I will be presenting the results of the 2007 bioassays separate from those in 2008 due to the drastic difference in the salinity conditions between these two years. In addition, I will also be separating the results from the filtered treatments and the unfiltered treatments. These two treatments were designed to answer very different research questions. The filtered treatments, in which *C. raciborskii* culture was added to Currituck Sound water that had been filtered through GF/F filters to remove all existing phytoplankton, were used to determine whether *C. raciborskii* can grow in Currituck Sound water when added in isolation, without the added pressure of competing for light, nutrients, and space with other species. As a result, this treatment will determine whether the environmental or chemical conditions of Currituck Sound water, in particular the salinity regime, are satisfactory for *C. raciborskii* growth and will also be used to speculate under what nutrient conditions *C. raciborskii* growth is favored. The unfiltered treatments, in which *C. raciborskii* added to unmanipulated Currituck Sound water, were used to determine whether *C. raciborskii* could effectively compete within the existing phytoplankton community of Currituck Sound, and what nutrient conditions would increase its competitiveness.

Bioassay Results from the Filtered Treatments:

***Summer 2007*:**

The first bioassay took place in late June of 2007. Water was collected from 2 sites, one inside the Currituck Banks reserve and one from Currituck Sound outside of the reserve.

The water from both sites was 0 psu at the time of collection (**TABLE 2**). For each of the two sites, filtered and unfiltered treatments were prepared. For the filtered treatment, a Z8 media control was used. Z8 media is designed specifically for culturing N2 fixing cyanobacterial and should provide these organisms with an ideal growth media, containing all necessary nutrients except nitrogen (therefore the filaments would need to fix atmospheric nitrogen in this media in order to grow). This control allowed us to establish whether *C. raciborskii* preferred to grow in the media or within the Currituck Sound water. My initial hypothesis here was that *C. raciborskii* would grow better in the media as compared with the water treatments. Four nutrient treatments were used in this first bioassay; Control (**C**), Nitrate (**N**), Phosphate (**P**) and Nitrate and Phosphate (**N+P**).

Chl a and AR: **FIGURE 14** shows the results of Chl *a*, used here as a proxy for *C. raciborskii* biomass, for Site 1 (outside the reserve) and Site 2 (inside the reserve). The first noticeable element of these figures is that contrary to my initial hypothesis, the added *C. raciborskii* thrived in the Currituck Sound water from both sites. Biomass increases were significantly lower in the media (**M**) treatments. **FIGURE 15** may demonstrate why this was the case. It shows the acetylene reduction (AR) rates for these samples, used as a proxy for nitrogen fixation (Burris 1972, Paerl 1998). As mentioned above, the Z8 media does not contain nitrogen, so nitrogen fixation is required to grow in this media. As such, fixation rates in **M** were high throughout the course of the bioassay. Because nitrogen fixation is an expensive process, the necessity of maintaining such high nitrogen fixation rates may have resulted in decreased growth and thereby decreased biomass and lower Chl *a* as compared with the control (**C**) treatments, Currituck Sound water with no nutrients added, as seen in

FIGURE 14. Because the Currituck Sound water contained nitrogen (0.0 μg N-NO_x/L for both sites, but 34.3 μg N-NH_4/L for Site 1 and 40.4 μg N-NH_4/L for Site 2), the *C. raciborskii* added could suspend nitrogen fixation and switch to uptaking ambient nitrogen and utilizing it for growth and building biomass. In fact, even in the later days of the bioassay (T_6, T_8), AR rates were low across all the nutrient treatments, suggesting that ambient nitrogen levels were sufficient to support *C. raciborskii* growth even in the **C** and **P** treatments, where no additional nitrogen was added. These results suggest that Currituck Sound water in 2007 had the necessary environmental factors, including salinity and nutrients, not only to sustain the growth of *C. raciborskii*, but also to provide *C. raciborskii* with a very favorable growth environment where large increases in biomass were possible.

Looking at **TABLE 3** and more closely at **FIGURE 14**, we can evaluate the differences between Site 1 (outside the reserve) and Site 2 (inside the reserve). Initially, prior to filtration and bioassay set-up, the Chl *a* concentrations for the two sites were very similar (**TABLE 3**; 17.2 μg/L for Site 1, 14.1 μg/L for Site 2), with a slightly higher concentration outside the reserve. During the course of the bioassay, there was no significant difference between the two sampling sites, at least for first time point (T_2) of the bioassay (**FIGURE 14**). In T_4 and T_6, Site 1 had a significant increase in Chl *a* concentration before a rapid die-off in T_8, whereas Site 2 seemed to repeat this pattern but with a slight lag behind Site 1, in T_6 and T_8. There was also no significant difference between Site 1 and Site 2 in terms of the AR rates (**FIGURE 15**).

Across the different nutrient treatments, it was clear that for both sites, Chl *a* concentration was limited by nitrogen. In both cases, across all time points, the treatments with nitrogen additions (**N** and **N+P**) had a significantly higher increase in Chl *a*

concentration. **TABLE 4** shows an example of this for both sites at T_4, with nitrogen additions statistically significant (P value 0.0071). This suggests that when *C. raciborskii* is alone, removed from competition with other phytoplankton species, that nitrogen enrichment would favor an increase in its biomass.

GR: Growth rates were calculated using the Chl *a* data, by fitting a curve for exponential growth (Slater 1988). The growth rates reported are for the first 6 days of the bioassay, with the T8 time-point eliminated due to the fact that by this point in the bioassay, cell die-off had begun within the incubation vessels. As expected, these results mimic those of Chl *a*. In June 2007, growth rates in the Currituck water treatments were significantly higher than the media treatment. In addition, growth was limited by nitrogen at both sites throughout the course of the bioassay. **TABLE 5** shows an example of this for both sites, with nitrogen additions statistically significant (P value 0.0012). There was no significant difference in GR between Sites 1 and 2. These results suggest, again, that when *C. raciborskii* is in isolation, nitrogen enrichment favors its growth.

PP: Primary productivity (PP) was measured using the $NaH^{14}CO_3$ incorporation method (Parsons et al. 1984, Rudek et al. 1991). **FIGURE 16** shows the results from the PP analysis. PP was very low in **M**, probably due to the fact that all energy was being utilized by the filaments for nitrogen fixation instead of production (**FIGURE 15**). Like Chl *a* and GR, primary production appeared to be limited by nitrogen. **TABLE 6** shows an example of this for both sites at T_4, with nitrogen additions again statistically significant (P value 0.0348). While there was no statistically significant difference between the two sampling sites in Chl

a, AR, or GR (P values 0.7662, 0.8224, and 0.8483), Site 1 did show higher production rates than Site 2. PP was in fact the only factor measured in which the two sites noticeably differed, though still not statistically significant (P value 0.1144), making it difficult to ascertain the reason. Despite the differences in the magnitude of the production rates, the pattern remained was the same, with PP greater in the treatments with added nitrogen. These results again seem to indicate that isolated *C. raciborskii* is limited in its biomass, its growth, and its production by nitrogen and that nitrogen enrichment would significantly favor its expansion in this area.

Fall 2007:

Based on the results from the first bioassay, in which for all of the parameters analyzed Site 1 and Site 2 did not differ significantly, the second bioassay utilized water from only one sampling site, within the Currituck Banks reserve. In order to make up for this, additional treatments were added. For the filtered treatments, Z8 media control was again used to establish whether *C. raciborskii* preferred to grow in the media or within the Currituck Sound water. The nutrient treatments were increased from four to eight, including **C**, **N**, Ammonium (**A**), **P**, **N+P**, Ammonium and Phosphate (**A+P**), Nitrate and Ammonium (**N+P**), and Nitrate, Ammonium, and Phosphate (**N+A+P**). For this bioassay, conducted in September 2007, the salinity of the Currituck Banks sampling site had increased from 0 psu to 4.7 psu (**TABLE 2**). While this salinity is significantly higher than that of the first bioassay, it is on the cusp of the salinity tolerance for *C. raciborskii* reported by Moisander (2002) and microscopic analysis of the existing phytoplankton community confirmed the

presence of *C. raciborskii* in Currituck Sound before the start of the bioassay, suggesting that this salinity may be tolerated by *C. raciborskii in situ*.

Chl a: **FIGURE 17** shows the results from the Chl *a* analysis for the filtered treatments of the Fall 2007 bioassay. An immediate difference from the previous bioassay is evident. Here, the added *C. raciborskii* grew better in the media than it did in the sound water. This is probably an affect of the high salinity of the water, which shocked the cultured *C. raciborskii*, preventing it from growing well in any of the treatments, especially when compared with the summer bioassay. Another potential contributing factor was poor weather conditions resulting in low light for the first two days of incubation, which made production rates lower and inhibited growth (**FIGURE 18**). Still, the sound water treatments, even with significant nitrogen additions, had lower biomass than the Z8 media which has no nitrogen, indicating that the water was inhospitable at this salinity level. The cultured *C. raciborskii* was able to bounce back and accumulate some biomass by the conclusion of the bioassay (T_8), particularly in the nitrogen treatments (**TABLE 7**). This indicates that no only is the *C. raciborskii* biomass limited by nitrogen (P value for nitrogen additions 0.0458), as shown by the first bioassay, but that nitrogen may in some way help *C. raciborskii* function in higher salinity environments. Within the nitrogen treatments, there didn't seem to be any statistical difference between nitrate and ammonium as the nitrogen source.

PP: Results were similar for primary production, shown in **FIGURE 18**. Initially, the media treatment seems to do better, though production rates were very low, probably reflecting the low light levels experienced during the first two time points. The Currituck Sound water

treatments had virtually no significant production until T_8, most likely due to a combination of low light and the shock of the high salinity. By T_8, the *C. raciborskii* in the water treatments did begin to recover, at least in terms of primary production. The production in the nitrogen treatments outpaced that in the media treatment (**TABLE 8**). Production did appear to be limited by nitrogen, with neither nitrate nor ammonium being clearly favored (P value for nitrate additions 0.0213, P value for ammonium additions 0.0066), and phosphorus additions also seem to increase production in T_8 (**TABLE 8**). These results combined with those from Chl *a* seem to indicate that isolated *C. raciborskii* is limited in its biomass and production by nitrogen, with phosphorus limitation of production also indicated, demonstrating that dual nutrient enrichment would significantly favor *C. raciborskii*'s expansion in this area. Nutrient enrichment, by increasing the bioavailability of nitrogen in particular, may further enable *C. raciborskii* to expand into areas that would have previously been protected by higher salinity regimes.

Summer and Fall 2008:

Based on the results from the first two bioassays, both bioassays in 2008 had the same procedure, using just one sampling site within the Currituck Banks reserve with the same nutrient treatments as described for Fall 2007. For the filtered treatments, Z8 media control was again used to establish whether *C. raciborskii* preferred to grow in the media or within the Currituck Sound water. Additionally, a second Z8 media control was introduced in which nitrate was added to the media. This control was used to determine if the necessity of fixing nitrogen when in the Z8 media had a significant effect on the ability of *C. raciborskii* to grow. In June 2008, the salinity of the Currituck Banks site had increased to 7.4 psu, and

increased further in September 2008 to 8.4 psu (**TABLE 2**). This is significant because these salinities are well beyond the excepted salinity tolerance of *C. raciborskii*. In addition, these bioassays also differed from the 2007 bioassays in that *C. raciborskii* was not present in the existing phytoplankton community before beginning this bioassay, suggesting that the salinity had become prohibitive to *C. raciborskii* growth *in situ*.

Chl a and GR: The added *C. raciborskii* was shocked when added into these very high salinity environments. **FIGURE 19** shows the Chl *a* concentrations for June 2008. Like the results from Fall 2007, here *C. raciborskii* biomass was lower in the Currituck Sound water treatments than the media treatment earlier in the bioassay, but it did appear that by T_8 the added *C. raciborskii* had recovered. **TABLE 9A** shows the Chl *a* concentrations for T_8. The results indicate nitrogen limitation, though this was not statistically significant (P value 0.1705) and further show that by the conclusion of this bioassay, the *C. raciborskii* biomass in the nitrogen treatments had surpassed that in the media treatments.

The growth rate analysis further suggests nitrogen limitation. **TABLE 10A** shows the GR results, and displays that with the exception of the **N+A+P** treatment, the nitrogen treatments had higher growth rates than both the media treatments and the treatments with no nitrogen added. The results of these two analyses may indicate that *C. raciborskii* may have a higher salinity tolerance that previously reported or that high nitrogen bioavailability may increase *C. raciborskii* ability to withstand high salinity conditions. I will discuss this further in Overall Conclusions and Future Work.

FIGURE 20 shows the Chl *a* results for September 2008. Here, *C. raciborskii* biomass was highest in the media treatments throughout the course of the bioassay. In the Currituck Sound water treatments, Chl *a* was very low throughout, not even reaching 20 μg/L in any of the treatments by T_8, as shown in **TABLE 9B**. There was no significant evidence that the *C. raciborskii* had recovered from salinity shock, though the nitrogen treatments did have higher biomass than the treatments without added nitrogen. This was particularly true for ammonium (P value for ammonium additions 0.0058).

Growth rates in September 2008, particularly in the N treatments, were also very low as compared with the 3 other bioassays (**FIGURE 21)**. **TABLE 10B** shows the GR results, and demonstrates that for the most part, there was no real nutrient limitation of growth. The **A+P**, **N+A**, and **N+A+P** treatments were slightly higher than the other treatments, but no additions were statistically significant. In general GR was very low in this bioassay, and seem to indicate the struggle *C. raciborskii* had in this 8.4 psu salinity water.

PP: In both June 2008 and September 2008, primary production rates were very low throughout the course of the bioassays, as shown in **FIGURE 22** and **FIGURE 23**. For both of these bioassays, several days of the bioassay incubation were cloudy, so low light may have been responsible for the low productivity. This is further evidenced by the low production in the media treatments in both bioassays. The added *C. raciborskii* seems to have had difficulty ramping up production and growth even in the media, especially in June 2008 (**FIGURE 22**). Beyond that, the low production rates in the rest of the treatments suggest that the *C. raciborskii* was, again, inhibited at the high salinity treatments.

In June 2008, the *C. raciborskii* in some treatments was able to outpace the media treatments by T_6, but in September 2008, media treatments did better than the Currituck water treatments throughout the course of the bioassay (**FIGURE 23**). This is consistent with the results from the Chl *a* analysis which showed highest biomass in the media treatments. Across all bioassays, a pattern has emerged. *C. raciborskii* growth is slower in high salinity water, and added nitrogen does make a difference in enabling *C. raciborskii* to withstand higher salinities (**FIGURE 21**).

TABLE 11A shows the production rates for T_6 of the June 2008 bioassay. Here, we see an additional indication that ammonium may be the favored nitrogen source for *C. raciborskii*. Ammonium additions triggered a stronger response than nitrate additions in increased production, shown by the higher production rates in the **A**, **A+P**, **N+A**, and **N+A+P** treatments (P value 0.0437). September 2008 had a similar result, as seen in **TABLE 11B**, with the highest production rates in the same 4 treatments, all of which featured an ammonium addition (P value 0.0062). In the 2007 bioassays, nitrogen limitation of primary production was clear, but no favored nitrogen source was indicated by the data. In the 2008 bioassays, *C. raciborskii* production appears to be favored not by nitrogen additions, but specifically by ammonium additions. This may be due to the fact that the utilization of nitrate as a nitrogen source is more energetically costly to the cell than ammonium, as nitrate first needs to be reduced to ammonium before it can be used. This reduction is a two part process in which the nitrate is first reduced to nitrite, catalyzed by the enzyme ferredoxin-nitrate reductase, and then to ammonium, catalyzed by ferredoxin-nitrite reductase, requiring a substantial amount of energy (Syrett 1981, Herrero et al. 2001). Ammonium has long been recognized as the preferred nitrogen source for most cyanobacteria and other phytoplankton,

and the presence of ammonium has been shown to inhibit the uptake of nitrate in cyanobacterial species like *Anabaena* and *Nostoc* (Flores et al. 1980). These energy costs may have proved prohibitive when *C. raciborskii* was already stressed by high salinities, as experienced in the 2008 bioassays, thus causing low production rates except in treatments where ample ammonium was added. This indicates that high ammonium additions may be necessary for *C. raciborskii* to subsist in habitats with salinities ranging from 5 to 9 psu, and that limiting nitrogen, specifically ammonium inputs, may protect these areas for *C. raciborskii* invasion.

***Conclusions from the Filtered Treatments*:**

The filtered treatments were designed to determine whether the environmental conditions of Currituck Sound water, in particular the salinity regime, were satisfactory for *C. raciborskii* growth when the added pressures of grazing and competing with other species for light, nutrients, and space were removed. They were also designed to determine under what nutrient conditions *C. raciborskii* growth is favored. When looking at the results across all four bioassay, some interesting patterns emerge. First and foremost is that salinity plays a large role in determining whether *C. raciborskii* can grow in Currituck Sound. When the salinity was near 0 psu, as in the June 2007 bioassay, Currituck Sound was a very good habitat for *C. raciborskii*, providing an even better medium for growth than Z8 media, probably because of the available nitrogen. As salinity increased from 0 psu to 4.7 psu (September 2007), 7.4 psu (June 2008), and 8.4 psu (September 2008), *C. raciborskii's* growth potential in Currituck Sound was reduced, resulting in lower biomass, growth rates, and production rates in the Currituck Sound water treatments as compared to the Z8 media

treatments. These results suggest that during periods when salinity is high, like periods of drought such as was seen during the course of this study, Currituck Sound may be protected from the invasion or increased expansion of *C. raciborskii*, simply because the high salinity is prohibitive to its growth. This finding has interesting implications for the area based on the time in which we now live. Climate change is occurring, and several different climate change scenarios have been suggested for eastern North Carolina which may influence the salinity regime in Currituck Sound. These scenarios and their implications will be discussed in Overall Conclusions.

Across all bioassays, a second pattern related to salinity also emerged. *C. raciborskii* growth, as mentioned above, was slower in high salinity water, and in these cases, added nitrogen did make a difference in enabling *C. raciborskii* to withstand higher salinities. Even in 0 psu water, nitrogen limited biomass accumulation, growth, and production of *C. raciborskii*. As salinity increased, added nitrogen greatly increased *C. raciborskii's* ability to recover from salinity stress and achieve high Chl *a* concentrations and primary production rates. Nitrogen additions allowed *C. raciborskii* to grow at salinities higher the salinity tolerance for *C. raciborskii* reported in the literature (Moisander 2002). At the highest salinities of this study, nitrogen limitation became ammonium limitation, further suggesting that withstanding high salinities had a significant energetic cost for *C. raciborskii*, eventually requiring that it stop reducing nitrate and switch to solely utilizing ammonium as its nitrogen source. This has significant implications for Currituck Sound. Although the area is currently close to pristine, especially compared with other water bodies in North Carolina, the development in the area surrounding it is increasing every day, increasing the potential for human-induced nutrient enrichment. If nitrogen enrichment was to significantly increase in

the coming years, these results of the filtered treatments suggest that *C. raciborskii* may become a more significant problem for the area and may allow *C. raciborskii* to persist even in periods of increased salinity. These results further suggest that limiting nitrogen inputs may be the most successful tool for controlling *C. raciborskii* growth in Currituck Sound.

***Bioassay Results from the Unfiltered Treatments*:**

The unfiltered treatments of the bioassays, in which *C. raciborskii* added to unmanipulated Currituck Sound water, were used to determine whether *C. raciborskii* could effectively compete within the existing phytoplankton community of Currituck Sound, and what nutrient conditions would increase its competitiveness. Since *C. raciborskii* was added to the existing phytoplankton community, many of the analyses used in for filtered treatments, like Chl *a*, PP, GR and AR, were not as useful in determining *C. raciborskii's* growth potential. This was due to the fact that it was impossible to tease apart the effect of different nutrients on *C. raciborskii* from the effect these nutrients had on the phytoplankton community as a whole.

As an example of this, **FIGURE 24** shows the Chl *a* results for the unfiltered treatments of the June 2007 bioassay, in which the existing phytoplankton community was dominated by N_2-fixing cyanobacteria including *C. raciborskii* (**FIGURE 8**). **FIGURE 24** shows that, for both sampling sites, the phytoplankton community was initially nitrogen limited, with the T_2 and T_4 treatments with nitrogen additions having higher Chl *a* concentrations than the other treatments (P value 0.002). The Chl *a* concentrations at T_4 are shown in **TABLE 12**. Between T_4 and T_6, there was a switch, and the phytoplankton community became phosphorus limited. This is due to the fact that, by that time, biomass

and growth had increased so much that nutrient stores had been depleted, and while nitrogen fixation could provide *C. raciborskii* and the other N_2-fixing cyanobacteria within the phytoplankton community with nitrogen, there was no other source of phosphorus. **FIGURE 25** and **TABLE 13** show similar results for primary production (P value 0.0031). While these results show the effect of different nutrients on the phytoplankton community as a whole, they are unable to distinguish between phytoplankton species and therefore cannot provide any insight into the effect of these nutrients on *C. raciborskii*.

C. raciborskii filament counts:

There is one analysis that can provide insight into whether *C. raciborskii* can effectively compete within the Currituck Sound phytoplankton community and what nutrient conditions increase its competitiveness. *C. raciborskii* filament counts were done before the beginning of the bioassay (T_0) and at the end of the bioassay (T_8) for all the treatments. In addition, for each sample, qualitative assessment was made about the other organisms present within the phytoplankton community and their general abundance as compared with that of *C. raciborskii*. The results from each bioassay will be discussed below.

Summer 2007: The T_0 samples from the June 2007 bioassay were rich in N_2-fixing cyanobacteria. For both sites, *Anabaena* spp. and *Anabaenopsis* spp. appeared to be the dominant players, but also present were *C. raciborskii*, *Aphanizomenon* spp., *Nostoc* spp. as well as other phytoplankton like *Microcystis* spp. and numerous species of filamentous green algae. **FIGURE 26** shows the results of the *C. raciborskii* counts. All filaments counted had at least one terminal heterocyst. For both sites, the pattern is the same. Compared with the

T_0 samples, the **N** treatment had a far higher abundance of *C. raciborskii* than any of the other treatments. In fact, for both sites, the **C** treatments were dominated by *Anabaena* spp. and *Aphanizomenon* spp., with fewer *C. raciborskii* filaments than seen in T_0. The **N** treatment favored *C. raciborskii*, as this was the only treatment in which the *C. raciborskii* abundance seemed to surpass that of *Anabaena* spp. The **P** treatment was very interesting, as it clearly favored *Anabaena* spp. to the point that very few *C. raciborskii* were visible. The **N+P** treatment was the most mixed, with several different species, including the filamentous green algae, being favored by the dual nutrient additions. The relative abundances of *C. raciborskii*, *Anabaena* spp., *Aphanizomenon* spp. and *Anabaenopsis* spp. were all very similar in this treatment. The only clear difference between Site 1 and Site 2 was that Site 2 was less dominated by *Anabaena* spp., contained more diatoms, and that the initial abundance of *C. raciborskii* was higher, but the general pattern was the same. **TABLE 14** shows the results of the *C. raciborskii* filament counts for both sites. Here it is again clear that nitrate addition alone favored *C. raciborskii* while coupling nitrate and phosphate favored many species, resulting in more competition for *C. raciborskii*. No nutrient additions favored the more dominant N_2-fixing cyanobacterial species, like *Anabaena* spp., suggesting that these species may be more efficient than *C. raciborskii* in either nutrient uptake or nitrogen fixation. The fact that phosphate additions also favored *Anabaena* spp. further supports this point.

Fall 2007: **FIGURE 27** shows the results for the September 2007 bioassay. The T_0 sample from this bioassay showed higher abundance of *C. raciborskii*, *Anabaena* spp. and other N_2-fixing cyanobacterial species as compared with the June 2007 bioassay, probably since this

bioassay was completed at the end of the optimal growth period for these species, while the June 2007 bioassay was right at the beginning, before the most significant growth had taken place. *C. raciborskii* seemed to play a more dominate role in the phytoplankton community at this time, though there was also a lot of filamentous green algae and diatoms competing for dominance. The T_8 samples showed results similar to those from the June 2007 bioassay, but with more treatments. In the **CC** treatment, a control in which no *C. raciborskii* was added, the phytoplankton community was very similar to T_0, with some die-off that is expected due to the absence of nutrient additions. The **C** treatment had higher abundances of both *C. raciborskii* and *Anabaena* spp. as compared with T_0. Again, the **N** treatment clearly favored *C. raciborskii*, as did the **A** treatment, though this treatment also seemed to favor *Anabaena* spp. and *Anabaenopsis* spp. The **P** treatment again favored *Anabaena* spp. and it dominated the community in this treatment. The **N+P** and **A+P** treatments were very mixed, with fairly equal distributions of *C. raciborskii*, *Anabaena* spp., *Anabaenopsis* spp., filamentous green algae, and diatoms. The **N+A** treatment had very interesting results, favoring *C. raciborskii* and filamentous green algae, with the abundance of *Anabaena* spp. decreasing sharply in this treatment. In the **N+A+P** treatment, the abundance of all the N_2-fixers was decreased with the phytoplankton community being dominated by the green algae and the diatoms. **TABLE 15** shows the *C. raciborskii* abundances for each treatment, again demonstrating that the treatments in which *C. raciborskii* was most competitive were the **N** and the **N+A** treatments, while phosphate or dual nutrient additions favored other species that then outcompeted *C. raciborskii*.

Summer 2008: The salinity difference between 2007 and 2008 were visible in the *C. raciborskii* filament counts and the phytoplankton community composition. The results from the June 2008 bioassay are shown in **FIGURE 28**. As seen with the microscopic and metagenomic analyses from this bioassay, there was no *C. raciborskii* in the T_0 sample (**TABLE 16**). The phytoplankton community was dominated by filamentous green algae, diatoms and dinoflagellates. There was the occasional N_2-fixer, primarily *Anabaena* spp., but the phytoplankton community was very different from that seen in 2007. The **CC** treatment again had no *C. raciborskii*, but did demonstrate some growth of the other N_2-fixers, with more *Anabaena* spp. than was seen in T_0. The **C** treatment had even more N_2-fixers, all with prominent heterocysts, including *Aphanizomenon* spp. There was some *C. raciborskii*, but not much, indicating that the added *C. raciborskii* was struggling to exist in the high salinity water without added nutrients. The **N** treatment was very dense with phytoplankton, and had a higher abundance of *C. raciborskii* compared with other N_2-fixers. The abundance of diatoms and green algae was also higher in this treatment than in the previous treatments, with these being the dominant players of the phytoplankton community. The **A** treatment was very similar to the **N** treatment, with high phytoplankton biomass of mostly green algae, diatoms, and some N_2-fixers, including *C. raciborskii*, *Anabaena* spp., *Anabaenopsis* spp., and *Aphanizomenon* spp. The **P** treatment had mostly N_2-fixers, primarily *Anabaena* spp. and *Aphanizomenon* spp., with very little *C. raciborskii*. The **N+P** and **A+P** treatments very both very mixed, having good distributions of N_2-fixers, green algae, diatoms, and dinoflagellates. The **N+A** treatment favored *C. raciborskii* the most, with noticeably higher abundance of *C. raciborskii* in this treatment as compared with all others. The **N+A+P** treatment had a decreased abundance of N_2-fixers as the other

phytoplankton players outcompeted them, with the green algae becoming the dominant player in this treatment. These results, shown quantitatively in **TABLE 16**, show the same pattern as seen in 2007, with *C. raciborskii* being most competitive in the **N+A** treatment, while phosphate or dual nutrient additions favored other species that then outcompeted *C. raciborskii*, like green algae and diatoms. There is a clear difference in the magnitude of the *C. raciborskii* abundances between the two years of the study, showing again the difficulty *C. raciborskii* had with remaining competitive in the higher salinity water.

Fall 2008: **FIGURE 29** shows the results from the September 2008 bioassay. Again there was no *C. raciborskii* in the T_0 sample (**TABLE 17**). There were a small number of N_2-fixers, including one *Anabaena* spp. filament and a few filaments of *Aphanizomenon* spp., but the community mainly consisted of diatoms and filamentous green algae. Remarkably, the pattern of *C. raciborskii* abundance across the remaining treatments was very similar to that seen in the previous bioassay. The **CC** treatment again had no *C. raciborskii*, since the T_0 sample had none, and none was added. **C** treatment had some *C. raciborskii*, but the abundance was very limited, even as compared with the abundance of other N_2-fixers, like *Aphanizomenon spp*, which was more abundant here than in the T_0 sample. This suggests that the added *C. raciborskii* had significant difficulty growing and reproducing in the high salinity water. The **N** treatment favored *C. raciborskii* growth, indicated by a higher abundance of *C. raciborskii* compared with other N_2-fixers. Despite this, diatoms and green algae remained the dominant players of the phytoplankton community in this treatment. Similarly, the **A** treatment had high phytoplankton biomass of mostly green algae, diatoms, and some N_2-fixers, including *C. raciborskii* and *Aphanizomenon* spp. The **P** treatment most

significantly favored the N_2-fixers, with *Anabaena* spp. and *Aphanizomenon* spp. dominating in this treatment and effectively outcompeting *C. raciborskii*. The abundance of *C. raciborskii* was the lowest in this treatments, indicating significant competitive stress. The **N+P** and **A+P** treatments very both very mixed, with equal distributions of N_2-fixers, green algae, diatoms, and dinoflagellates, all competing for dominance. As seen in June 2008, the **N+A** treatment was the treatment that most favored *C. raciborskii*. This suggests that *C. raciborskii* may be more efficient that the other N_2-fixing cyanobacterial species at uptaking nitrogen when it is bioavailable. It is also possible that *C. raciborskii* may be able to more efficiently switch between fixing nitrogen when this nutrient is absent and uptaking it when it becomes bioavailable, as has been indicated in other studies (Bouvy et al. 2000, Burford et al. 2006, Paerl and Fulton 2006). The **N+A+P** treatment significantly favored the non-N_2-fixing phytoplankton, especially the fast-growing green algae. With ample available nutrients, green algae were able to effectively out-compete the N_2-fixers, including *C. raciborskii* for nutrients. Again the results from this bioassay, shown quantitatively in **TABLE 17**, show the same pattern as seen in all other bioassays, with *C. raciborskii* being most competitive in the **N+A** treatment. Again, it appeared that phosphate or dual nutrient additions favored other species that then out-competed *C. raciborskii,* like green algae and diatoms. The Fall 2008 *C. raciborskii* abundance results also reflect the conclusion that *C. raciborskii* had significant difficulty remaining competitive in the higher salinity water. This is especially evident when comparing the *C. raciborskii* filament counts for all four bioassays, as seen in **FIGURE 30**. The difference in the magnitude of the abundances from 2007 and 2008 demonstrates the effect of the salinity change on *C. raciborskii's* ability to grow and compete in Currituck Sound water.

***Conclusions from the Unfiltered Treatments*:**

The unfiltered treatments of the bioassays were used to determine whether *C. raciborskii* could effectively compete within the existing phytoplankton community of Currituck Sound, and what nutrient conditions would increase its competitiveness. *C. raciborskii* abundance was the main tool used to determine this, in that the relative abundance of *C. raciborskii* as compared with other species within the phytoplankton community in the different treatments demonstrated its competiveness under different nutrient conditions. The results were very similar across all four bioassays. *C. raciborskii's* abundance was highest in the treatments with nitrogen, either nitrate or ammonium, additions. It was within these treatments that *C. raciborskii* was most able to compete with other N_2-fixers and other phytoplankton. In cases where no nitrogen was added or when phosphate alone was added, it appeared that the dominant N_2-fixer in Currituck Sound, *Anabaena* spp., continued to out-compete *C. raciborskii* and maintain its dominance within the phytoplankton community. In cases where both nitrogen and phosphorus were added, other, non-N_2-fixing phytoplankton, like green algae, were most competitive, probably because they are fast growing, especially when nutrient limitation is removed. These results suggest that nitrogen inputs would be most beneficial to *C. raciborskii* growth, in part because it would make it more competitive within the phytoplankton community. These results also show that *C. raciborskii* abundance is clearly influenced by salinity but that nitrogen availability significantly increases its ability to survive and maintain competitiveness in these conditions. The effect of salinity on *C. raciborskii* will be further discussed below with the results of the salinity experiment.

Salinity Experiment:

The salinity experiment was to designed to provide more insight into *C. raciborskii's* salinity tolerance and to examine the effect that added nitrogen had on this tolerance. The experiment was performed using Z8 media that was adjusted to desired salinity using NaCl. Four different salinity treatments and two nutrient treatments were used: 0 psu, 3 psu, 6 psu, and 9 psu, each with control and nitrate added. *C. raciborskii* biomass throughout the course of the experiment was tracked, using Chl *a* concentration as a proxy for biomass. The results are shown in **FIGURE 31**. The results are what I would have expected, with the *C. raciborskii* biomass highest in the 0 psu Z8 media, followed by 3 psu, 6 psu, and finally 9 psu. In each salinity, the addition of nitrate lead to higher biomass. The results from the final sub-sampling point, T_{10}, are shown in **TABLE 18**. It appears that the salinity tolerance of *C. raciborskii*, according to these results, is between 3 and 6 psu, with very little growth beyond that salinity. These results are very consistent with the salinity tolerance of *C. raciborskii* reported in the literature (Moisander 2002), although my bioassay results indicated that *C. raciborskii*, when given enough nutrients, was able to survive and grow in salinities slightly higher than 6 psu in Currituck Sound water. This may be due to the fact that Currituck Sound water may contain higher concentrations of organic matter, essential nutrients and trace metals than are available in Z8 media, making it easier for *C. raciborskii* to survive in this water than the media when the salinity is the same. Organic matter in particular has been shown to favor cyanobacterial growth (Stewart 1974), and is completely absent from Z8 media. This hypothesis could be tested by performing a salinity experiment using 0 psu Currituck Sound water as the medium and adjusting the salinity of it, but this was

not possible during this study as Currituck Sound was only 0 psu at the beginning of the study. This will be discussed further in Future Work. Regardless, this experiment does show that higher salinity can greatly decrease *C. raciborskii's* growth potential, and that nitrogen inputs may increase its ability to withstand these conditions. This is additional evidence that nitrogen inputs need to be limited to prohibit *C. raciborskii's* expansion in Currituck Sound.

V. OVERALL CONCLUSIONS

The purpose of this study was to answer two main research questions.

1. **Is *C. raciborskii* currently present in Currituck Sound?**

   Before beginning this work, *C. raciborskii* had not been identified in Currituck Sound, NC, due mostly to the fact that very little information about the existing phytoplankton community was known (Caldwell 2001). In the course of this study, I identified *C. raciborskii* in Currituck Sound in both June and September of 2007. The abundance of *C. raciborskii* at that time ranged from 910 to1500 cells/mL, making it a minor player within a phytoplankton community very rich in N_2-fixing filamentous cyanobacteria, like *Anabaena* spp., *Anabaenopsis* spp. and *Aphanizomenon* spp. Although toxin analysis was not available to me in 2007 and therefore I was unable to quantify the concentration of toxin in Currituck Sound, I was able to confirm, using the toxin primers *cyl*, *pks*, and *ps*, that the *C. raciborskii* in Currituck Sound was able to produce the cylindrospermopsin toxin at that time. Additionally, phylogenetic analysis of the *C. raciborskii* strain in Currituck Sound showed that this strain was very closely related to the strain previously isolated and studied from St. Johns River, FL.

In 2008, the results were very different. In this year, pigment and microscopic analysis of Currituck Sound water samples showed that *C. raciborskii* was not a member of the phytoplankton community at that time, and these results were affirmed by negative results in PCR assays for cyano-*nif*H, cylindro-*nif*H, *cyl*, *pks*, and *ps* and the cylindrospermopsin toxin analysis. Between September 2007 and June 2008, the entire phytoplankton community had shifted away from N_2-fixing filamentous cyanobacteria to species more tolerant of the elevated salinity like green algae, diatoms and dinoflagellates. I concluded that the severity of drought that North Carolina experienced throughout 2007 and 2008 resulted in high salinities (7.4 psu in June 2008, 8.4 psu in September 2008) that were inhospitable both to *C. raciborskii* and its competitors, and proved prohibitive to their growth.

2. What conditions would favor the invasion or expansion of this species?

To answer this question, I conducted a series of nutrient addition bioassays designed to determine if the environmental or chemical conditions of Currituck Sound, including salinity, were adequate for *C. raciborskii* growth, whether *C. raciborskii* can adequately compete with the existing phytoplankton community, and under what nutrient conditions it grows best, though it did not determine whether the physical or hydrological conditions or the grazing pressures of Currituck Sound were suitable for *C. raciborskii* growth. The bioassay results confirmed a major result from above, namely that salinity the plays a large role in determining whether *C. raciborskii* can grow in Currituck Sound. In 2007, when

C. raciborskii was a player within the phytoplankton community in Currituck Sound, water from the sound was also a very good habitat for the cultured *C. raciborskii* added for the bioassays. In fact, in June 2007 when the salinity of Currituck Sound was 0 psu, the water provided the *C. raciborskii* with an even better medium for growth than the nitrogen-deficient Z8 media. As salinity increased from 0 psu to 4.7 psu (September 2007), 7.4 psu (June 2008), and 8.4 psu (September 2008), *C. raciborskii's* growth potential in Currituck Sound was further and further reduced, resulting in lower biomass, growth rates, and production rates in the Currituck Sound water treatments as compared to the Z8 media treatments. These results show that salinity is a major determinant of *C. raciborskii*'s invasion potential in any waterway and that the fresher the water, the more likely it is that *C. raciborskii* will be able to invade or increase its dominance within an aquatic habitat.

C. raciborskii abundance was used to determine whether it can adequately compete with the existing phytoplankton community of Currituck Sound. Based on the initial abundances of *C. raciborskii* in Currituck Sound (910 cells/mL in June 2007, 1500 cells/mL in September 2007), *C. raciborskii* was able to compete within the phytoplankton community, but only to the extent of maintaining a minor role as compared to the more significant players like *Anabaena* spp. The nutrient addition bioassays demonstrated *C. raciborskii's* competiveness under different nutrient conditions, showing what conditions would make *C. raciborskii* most competitive within the phytoplankton community. All four bioassays

showed that *C. raciborskii's* abundance was highest in the treatments where competition for nitrogen was eliminated by nitrogen, either nitrate or ammonium, additions, and that it was within these treatments that *C. raciborskii* was able to grow with other N_2-fixers and other phytoplankton. These results suggest that *C. raciborskii* is most competitive within the phytoplankton community when concentrations of nitrogen are high, and that increased nitrogen availability through nitrogen inputs would be most beneficial to its growth.

The bioassay results also suggest that increased nitrogen availability may significantly increase *C. raciborskii's* ability to survive in high salinity conditions, though the direct test of this had ambiguous results. Even in June 2007 in fresh water, nitrogen limited the biomass accumulation, growth, and production of *C. raciborskii*. As salinity increased over the three remaining bioassays, added nitrogen greatly increased *C. raciborskii's* ability to recover from salinity stress and achieve high Chl *a* concentrations and primary production rates. Nitrogen additions also allowed *C. raciborskii* to grow at salinities higher the salinity tolerance for *C. raciborskii* reported in the literature (Moisander 2002). At the highest salinities of this study, *C. raciborskii* growth was limited not by nitrogen, but by ammonium, further suggesting that withstanding high salinities had a significant energetic cost for *C. raciborskii*. These costs became so great in 7.4 psu and 8.4 psu salinity water that *C. raciborskii* could no longer expend energy to reduce nitrate and was forced to switch to solely utilize ammonium as its nitrogen source. The salinity experiment further investigated the effect that added

nitrogen had on *C. raciborskii's* salinity tolerance. This experiment showed that, as suggested by the bioassay results, higher salinity can greatly decrease *C. raciborskii's* growth potential, but that nitrogen inputs may have an effect, though perhaps minimal, on its ability to withstand these conditions. These results, along with the other results discussed above, suggest that conditions of low salinity and increased nitrogen availability would favor the invasion or expansion of this species, without considering other complicating factors like grazing pressure and *C. raciborskii* growth regulation by physical processes.

These results have significant implications for the control of *C. raciborskii* in Currituck Sound. First, salinity appears to be a main factor in determining the habitats in which *C. raciborskii* can successfully grow. The results from this study suggest that during periods when Currituck Sound experiences high salinity, like the period of severe drought seen in North Carolina in 2007 and 2008, Currituck Sound may be protected from the invasion or increased expansion of *C. raciborskii*, simply because the high salinity is prohibitive to its growth. This is particularly important in light of the climate change scenarios predicted for North Carolina in the coming decades, and what they might mean for the salinity regime in Currituck Sound.

The UNC Climate Change Committee Report predicts three main changes to North Carolina's climate due to global warming: higher temperatures resulting in hotter summers and warmer winters, more extreme events, including heat waves, droughts, and severe storms, and sea level rise, which could result in the loss of coastal land, including large portions of the Outer Banks (Bland and Salvesen 2009). All of these factors have the

potential to effect *C. raciborskii* growth in North Carolina. Initially, rising temperatures may favor its growth in Currituck Sound and other water bodies in North Carolina, as *C. raciborskii* has been shown to exhibit its optimal growth rate at 30°C (Shafik et al. 2001), allowing it and other cyanobacterial species to outcompete other phytoplankton, especially eukaryotic species at this high temperature (Paerl and Huisman 2008, Paerl and Huisman 2009). However, these increased temperatures may also result in more extreme events. The increased frequency of both droughts and hurricanes may effectively reverse any increases in *C. raciborskii*'s growth potential brought about by higher temperatures by influencing the salinity regime of Currituck Sound. As seen in this study, drought can have a significant impact on the salinity in Currituck Sound, as it increased from 0 psu in June 2007 to 8.4 psu in September 2008 due to the severe drought in North Carolina during this time. These were salinity conditions that had rarely been seen in Currituck Sound before this study, but with an increased frequency and intensity of droughts due to global climate change, these conditions may become the norm in Currituck Sound, effectively prohibiting *C. raciborskii* growth.

The increased frequency and severity of hurricanes in North Carolina may have one of two effects on Currituck Sound salinity. First, these storms may result in high amounts of precipitation and coastal flooding that may make Currituck Sound a fresher, lower salinity environment. This would make Currituck Sound a more hospitable environment for *C. raciborskii*, especially if these events also delivered significant amounts of nitrogen along with the rain to the Sound. Hurricanes and intense storms, however, may also result in events of ocean wash-over, in which oceanic water may be introduced to the Sound during a storm, or, more significantly, the creation of new inlets along the Outer Banks. Currently, Currituck Sound maintains its low salinity due to the increased influence of rivers and the

decreased influence of the ocean in this estuary. Currituck Sound is protected from oceanic influence by the Outer Banks, and this protection would be greatly decreased if more inlets in the Outer Banks were created, as new inlets may flood both the Albemarle-Pamlico estuarine system and Currituck Sound with ocean water. In addition to the creation of new inlets from storm events, raising sea level may also result in the breach of Currituck Sound by oceanic water. As sea level rises, low lying coastal areas in North Carolina, including large portions of the Outer Banks may be lost, potentially creating new inlets and exposing Currituck Sound to oceanic water. In both cases, the influx of oceanic water into Albemarle-Pamlico or Currituck Sound would significantly increase the salinity in these estuaries. Higher salinity would, again, protect Currituck Sound from the expansion of *C. raciborskii* in this area.

While these climate changes scenarios suggest that *C. raciborskii* may not pose a significant threat to Currituck Sound in the future if high salinity conditions become prevalent, it is important to remember that when faced with adverse conditions, *C. raciborskii* and other species of cyanobacteria are able to produce akinetes. These spore-like reproductive structures could ensure survival of *C. raciborskii* during inhospitable conditions, and allow them to return to the phytoplankton community when favorable conditions return (Moore et al. 2005). Although the salinity tolerance of *C. raciborskii* akinetes is not known, studies have shown that akinetes of similar species have been able to withstand high salinities (up to 10,000 $\mu S\ cm^{-1}$ for *Anabaena circinalis*; Baker and Bellifemine 2000). This suggests that *C. raciborskii* and its N_2-fixing competitors may be able to form akinetes when salinity conditions are high, following a drought or an event of ocean wash-over, and that these akinetes may germinate when favorable conditions return. Therefore, the ability to produce akinetes may provide a significant advantage for *C.*

raciborskii and other diazotrophic cyanobacteria, allowing them to maintain a presence within the Currituck Sound phytoplankton community even when salinity conditions are not optimal. It is also important to remember that cyanobacterial species, as some of the planet's oldest known photosynthetic organisms (Schopf 2000), have persisted through millions of years of environmental change, both natural and anthropogenic, displaying a remarkable ability for adaptation (Paerl and Huisman 2009). As global climate change intensifies, this ability to adapt may help cyanobacteria, including *C. raciborskii*, survive the challenges of climate change. It is important, therefore, to consider other strategies for minimizing the invasion and proliferation of *C. raciborskii* in Currituck Sound.

Another key result from this study was that nitrogen availability is another main factor in determining how successful *C. raciborskii* is in a particular habitat. The bioassay results show that *C. raciborskii* growth is positively influenced by nitrogen additions and that access to bioavailable nitrogen may even allow *C. raciborskii* to persist in high salinity environments where its growth would otherwise be stunted. This suggests that nitrogen input limitation may be necessary to safeguard Currituck Sound and other water bodies from *C. raciborskii*.

This fact is slightly counter-intuitive, given that many studies have shown that N2-fixing cyanobacteria, since they can create their own nitrogen, are more problematic in N-deficient waterways where the ratio of total nitrogen to phosphorus (N:P) is less than 20 (Smith 1983, Smith 1990). In these cases, it has been suggested that either phosphorus or dual nutrient input limitation is needed to control diazotrophic cyanobacterial blooms (Paerl 1999). The results of this study, however, do not support these conclusions. In the filtered treatments of the bioassays, *C. raciborskii's* growth was almost always limited by nitrogen,

with phosphorus additions making little difference in Chl *a* concentration or primary production rates when *C. raciborskii* was by itself. This could be due to the fact that *C. raciborskii* is very good at taking up phosphorus at very low concentrations and storing it, which would minimize short-term phosphorus limitation for this species, making nitrogen the element limiting growth. In most cases, the nitrogen source, either nitrate or ammonium, did not appear to matter, although ammonium emerged as the preferred source when salinity stress was at its greatest in the final bioassay.

More significantly, when competing with other phytoplankton for resources in the unfiltered treatments, *C. raciborskii* abundance was highest in the treatments with nitrogen, either as nitrate or ammonium, additions. It was within these treatments, where competition for nitrogen was eliminated, that *C. raciborskii* was most able to grow as well as the other phytoplankton, including other diazotrophic cyanobacteria. When no nutrients were added or when phosphate alone was added, other cyanobacterial species, like *Anabaena* spp., were able to out-compete *C. raciborskii* within the phytoplankton community. In cases where both nitrogen and phosphorus were added, other, non-diazotrophic phytoplankton, like green algae, were most competitive, easily out-competing *C. raciborskii*. Complicating things further is the effect that added nitrogen may have on *C. raciborskii*'s salinity tolerance. The bioassay results combined with the results from the salinity experiment indicate that *C. raciborskii* abundance is influenced by salinity and that nitrogen availability may or may not increase its ability to survive and maintain competitiveness in these conditions. These results suggest that when simply considering what nutrient conditions would promote *C. raciborskii* growth within Currituck Sound, nitrogen inputs would be most beneficial. This is due to the three main factors discussed above: that nitrogen limits *C. raciborskii* growth, that nitrogen

makes *C. raciborskii* more competitive within the phytoplankton community, and that nitrogen availability may or may not help *C. raciborskii* survive and recover from salinity stress.

Another main goal of this study was to formulate long-term nutrient and water quality management strategies for Currituck Sound that can be used by NERRS and NC DENR-DWQ to minimize potential invasion and proliferation of *C. raciborskii*. As *C. raciborskii* is already a member of the phytoplankton community in Currituck Sound under certain conditions, these strategies should be aimed at limiting the expansion of this species by maintaining conditions that keep *C. raciborskii* as a minor player and prevent it from increasing its dominance. As the results from this study have shown, limiting nutrient, particularly nitrogen, inputs should be a central strategy employed by managers in this area.

In many ways, the current state of the phytoplankton community in Currituck Sound is similar to that of the St. Johns River, FL a few decades ago, before the anthropogenic nutrient enrichment of this system triggered a shift in dominance within the phytoplankton community from *Anabaena* spp. to *C. raciborskii* (Chapman and Schelske 1997). *C. raciborskii* is an opportunistic species, and demonstrated in this case a remarkable ability to take advantage of human-induced environmental change to increase its dominance within the phytoplankton community. The case of *C. raciborskii* in St. Johns River should serve as a cautionary tale for the water quality managers of Currituck Sound, especially since, as shown by the phylogenetic analysis is this study, the strains of *C. raciborskii* from both systems are so closely related. While the water quality of Currituck Sound is currently very good and nutrient enrichment has been minimal in this area, land development of the Outer Banks is continuously increasing, and potential for nutrient enrichment of Currituck Sound is growing.

The results of this bioassay suggest that limiting nitrogen inputs is necessary to ensure that *C. raciborskii* does not increase its dominance within the phytoplankton community of Currituck Sound, as it did in the St. Johns River following significant nutrient, particularly nitrate enrichment. Keeping nitrogen concentrations low would also protect against *C. raciborskii* survival when salinity increases in Currituck Sound would normally prevent its growth. As *C. raciborskii* in Currituck Sound has the potential to be toxic, limiting its expansion is important to protect all those, both animals and humans alike, who live in or around Currituck Sound. Additionally, limiting nutrient enrichment has an added benefit of suppressing in only *C. raciborskii* growth, but phytoplankton growth in general, which is necessary to maintain high water clarity and the SAV populations that make Currituck Sound important as an ecosystem for the fish and waterfowl species that rely on them. If the water quality managers of Currituck Sound and other water bodies throughout the country with similar salinity conditions limit or significantly reduce nitrogen concentrations in their system, *C. raciborskii* populations should be kept low enough to prevent severe problems with this organism.

VI. FUTURE WORK:

As is the case with many studies, the experimental results presented here open up avenues for future work. There were, in fact, several experiments that were planned for the 2008 field season that were not feasible because of the high salinity of Currituck Sound during that time, and the fact that *C. raciborskii* was no longer present in that estuary. These experiments would have helped refine the salinity tolerance of *C. raciborskii* and determine how great the threat of *C. raciborskii* is in and around the Currituck Banks reserve.

The first experiment planned was a transect of Currituck Sound along its north-south axis. This experiment was designed to determine how widespread *C. raciborskii* was within Currituck Sound, with the transition from the fresher north to more brackish south presumably showing the threshold salinity level, above which *C. raciborskii* could no longer be a member of the phytoplankton community Toxin analysis along this transect would have helped identify areas most at risk for toxin-related health problems. This experiment was designed in 2007 under the assumption that *C. raciborskii* would be present at some locations within Currituck Sound, and was abandoned when this turned out to be false. I believe the information gained from such an experiment would still be very relevant in terms of addressing *C. raciborskii*'s growth potential in Currituck Sound.

In addition to addressing the extent of *C. raciborskii*'s presence in Currituck Sound, this transect could be used to determine whether or not the filaments are actively growing in a particular location, or if they are, in fact, growing further up in the rivers that feed

Currituck Sound and being pushed into the Sound by the currents or wind. If it were the case that the cells seen in Currituck Sound were effectively dead, having no potential for production or growth in Currituck Sound, then *C. raciborskii* would ultimately present no real threat to Currituck Sound after all. Although the bioassay results do indicate that *C. raciborskii* growth is possible in Currituck Sound, there are two analytical tools that could be used to answer this question.

The first tool is autoradiography, which can be used to determine whether a cell is metabolically active (Paerl 1982). The process is as follows: radioactive material, like ^{55}Fe, is added to a water sample containing *C. raciborskii*, it is incubated, filtered, the filter is fixed to a slide, and the slide is dipped in emulsion. The slide is then developed like a photograph, and the radioactive atoms that were added will have emitted some beta particles, leaving behind tiny black dots that are visible microscopically (Paerl 1982). The uptake of these now visible radioactive iron atoms by the vegetative portion of the cell and by the heterocysts can demonstrate that the cell is actively taking up nitrogen, and you can inferred that photosynthesis or nitrogen fixation is occurring, as both of these processed utilize iron (Paerl 1982). ^{14}C could also be used to more directly demonstrate active photosynthesis. Tetrazolium salt reduction is another tool that can be used similarly to demonstrate the metabolic activity of a cell (Paerl and Bland 1982). In the current work, tetrazolium salt solutions could be added to a sample containing *C. raciborskii*, it is incubated, formalin is added to stop the reaction, and the cells are then visualized under the microscope. The reduction of the tetrazolium salts will cause metabolically active cells to darken, thereby distinguishing live cells actively undergoing carbon and nitrogen fixation from cells that are metabolically inactive and essentially dead. The use of these two tools on water samples

from Currituck Sound could show whether or not the *C. raciborskii* cells observed there are actually able to grow in that environment.

This study would have also benefited from more extensive salinity tolerance testing of *C. raciborskii*. As mentioned previously, a salinity experiment utilizing fresh (0 psu) Currituck Sound water as its base would be the ideal experiment for comparison with the results of the media salinity experiment performed in July 2008 (**TABLE 18** and **FIGURE 31**). Salinity could be introduced as an additional variable in a nutrient addition bioassay, where instead of utilizing different sample sites, different salinities could be used to show the differing effects of nutrients on *C. raciborskii* from location exposed to different salinity regimes. An experiment like this could provide useful insights not only into the salinity tolerance of *C. raciborskii*, but also on how nutrient availability may influence this tolerance.

There were also some limitations and pitfalls to this work that could be circumvented in future works. For instance, the bioassay experiments of this study relied on the addition of cultured *C. raciborskii*. The culture used was isolated from a Florida lake and has been in culture at least 5 years at IMS (Cyl L). Although the phylogenetic analysis of the nif*H* gene of *C. raciborskii* strains found in Currituck Sound revealed that they were very similar to the culture strain used in these experiments (**FIGURE 13**), it is not known whether these strains differed significantly in other genes influencing other aspects of growth, including salinity tolerance or nutrient utilization. For this reason, it would be interesting to repeat these experiments utilizing a culture of *C. raciborskii* isolated from Currituck Sound. Based on the sampling for this experiment, we now have access to mixed cultures of the N_2-fixing cyanobacteria of Currituck Sound, but the isolation of *C. raciborskii* from this mix will take more time. Additionally, there is little research available clarifying the effects keeping cells

in culture over many years may have on their ability to adapt to environmental change, like changes in nutrient, light or salinity regimes. For instance, some diagnostic pigment analysis (by HPLC) of *C. raciborskii* strains in culture suggest that when kept in growth chambers for many years, cells adapt by limiting the production of pigments associated with light capture at low light levels and increase production of photo-protective pigments that protect the cell's photosynthetic architecture in high light conditions. These changes may result in cultured *C. raciborskii* behaving very differently in experiments as compared to *C. raciborskii* growing *in situ*. These differences need to be accounted for and qualified.

Finally, nutrient addition bioassays were one of the main experimental tools utilized in this study and although bioassays have been used successfully for many years to determine nutrient limitation of primary production and phytoplankton growth (D'Elia et al. 1986, Howarth 1988, Kareiva 1994), other studies have questioned their effectiveness and suggested that their results may be misleading (Healey and Hendzel 1980, Elser and Kimmel 1985, Elser et al. 1990). Large-scale microcosm experiments and field studies of *C. raciborskii* in its native habitat (Carpenter 1996) may alleviate some of the limitations of traditional bioassay experiments and would help affirm these results.

APPENDIX A: Abbreviations

A	ammonium treatment
ANOVA	analysis of variance
AR	acetylene reduction
Bp	base pairs
C	control treatment
C. raciborskii	*Cylindrospermopsis raciborskii*
Chl *a*	chlorophyll *a*
CYN	cylindrospermopsin
DIC	dissolved inorganic carbon
DNA	Deoxyribonucleic acid
HPLC	high performance liquid chromatography
M	media treatment
N	nitrate treatment
NC	negative control
N:P	ratio of total nitrogen to phosphorus by weight
DENR	Department of the Environment and Natural Resources
DWQ	Division of Water Quality
GF/F	glass fiber filters, 0.6-0.8 µm porosity
GRF	Graduate Research Fellowship
NERRS	National Estuarine Research Reserve
NRE	Neuse River Estuary

P	phosphate treatment
PCR	polymerase chain reaction
PP	primary productivity
SAV	submerged aquatic vegetation
SJR	St. Johns River, FL
UNC-IMS	University of North Carolina at Chapel Hill's Institute of Marine Science

APPENDIX B: Figures

Figure 1: *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya et Subba Raju shown in both its straight and wavy morphologies. Also evident are the terminal heterocysts used by the filament for N_2 fixation. Photograph by Pia Moisander.

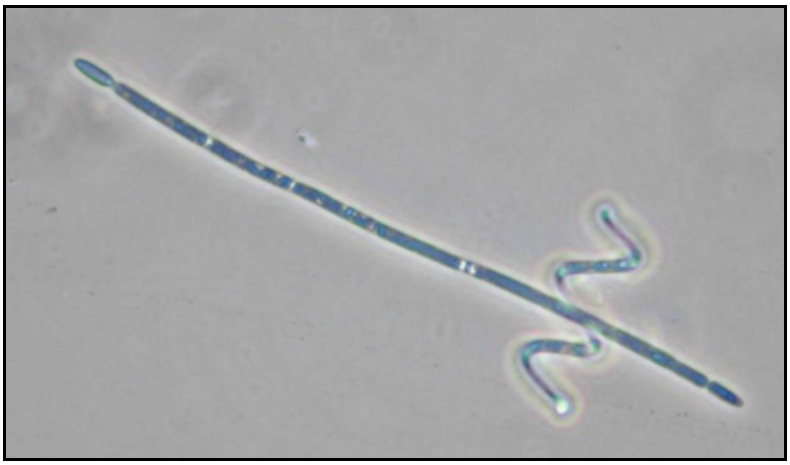
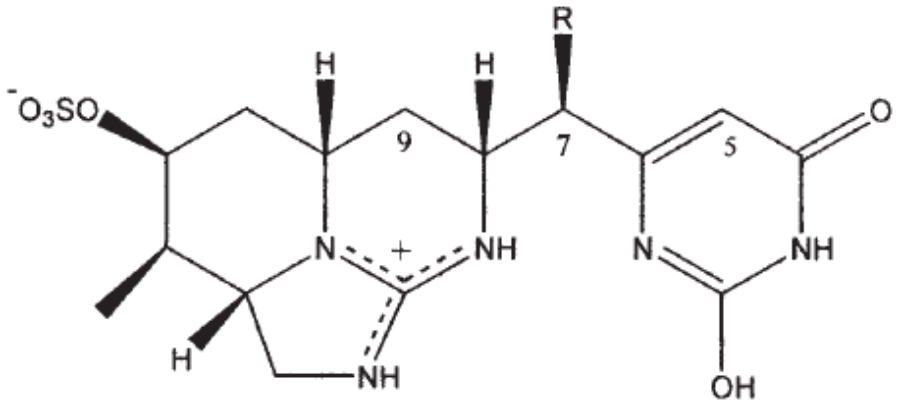


Figure 2: The structure of the cylindrospermopsin (CYN) toxin molecule. Figure reproduced from Wood and Stirling (2003).



	R	
cylindrospermopsin(CYN)	OH	
7-epicylindrospermopsin	OH	epimer at C7
deoxycylindrospermopsin (do-CYN)	H	

Figure 3: A Photosynthesis vs. Irradiance (PE) curve for a culture of *C. raciborskii*, Cyl L, (isolated by P. Moisander and maintained at UNC-IMS for at least 5 years). The data are fitted with a curve using the hyperbolic tangent function proposed by Jassby and Platt (1976) and shows that the P^{b}_{max}, or maximum photosynthetic rate normalized for Chl *a* concentration (designated PB on figure), for Cyl L is reached at a low irradiance of about 200 μEinsteins m^{-2} s^{-1}.

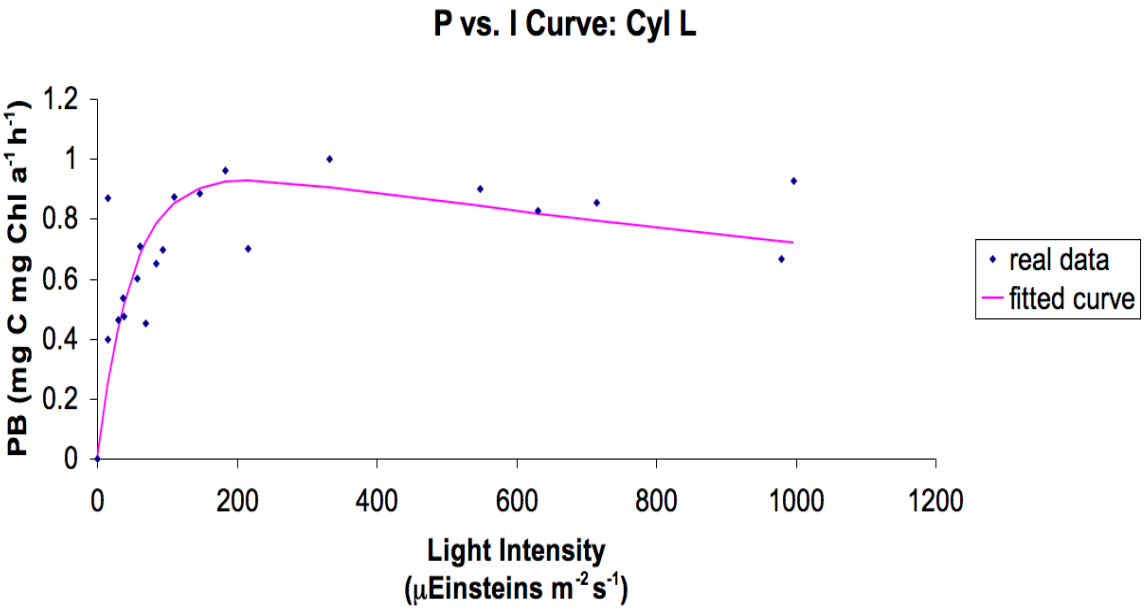


Figure 4: A) A satellite (Google Earth) image of eastern North Carolina, showing the key locations for reference of Chapel Hill, NC, Morehead City, NC, and Virginia Beach, VA. The red box denotes the location of Currituck Sound, north of the Albemarle-Pamlico estuarine system.

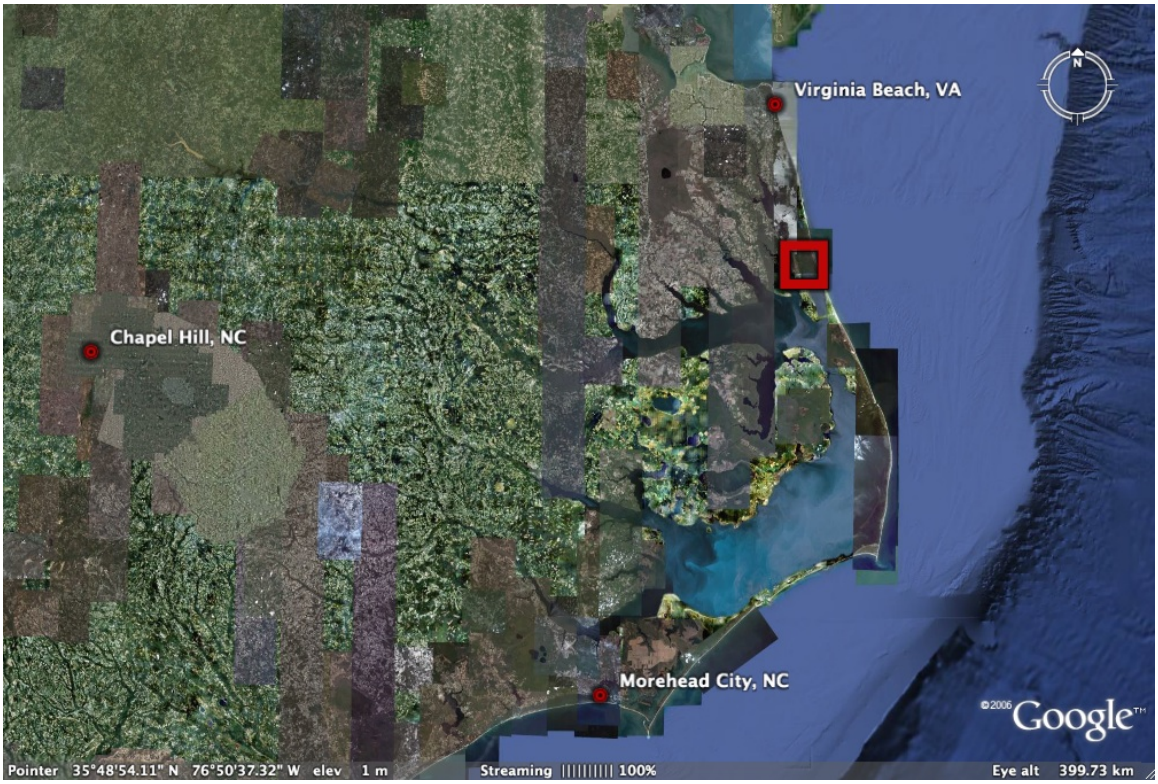


Figure 4 (continued): **B)** A zoomed-in view (from the red box of **FIGURE 4A**) of Currituck Sound, with Corolla, NC, one of the northern-most towns of North Carolina's outer banks as a reference point.

Figure 5: The Currituck Banks reserve that served at the main study site for this project. The reserve encompasses 950 acres and is located between the town of Corolla, NC and the Virginia border (map reproduced courtesy of NC NERRS).

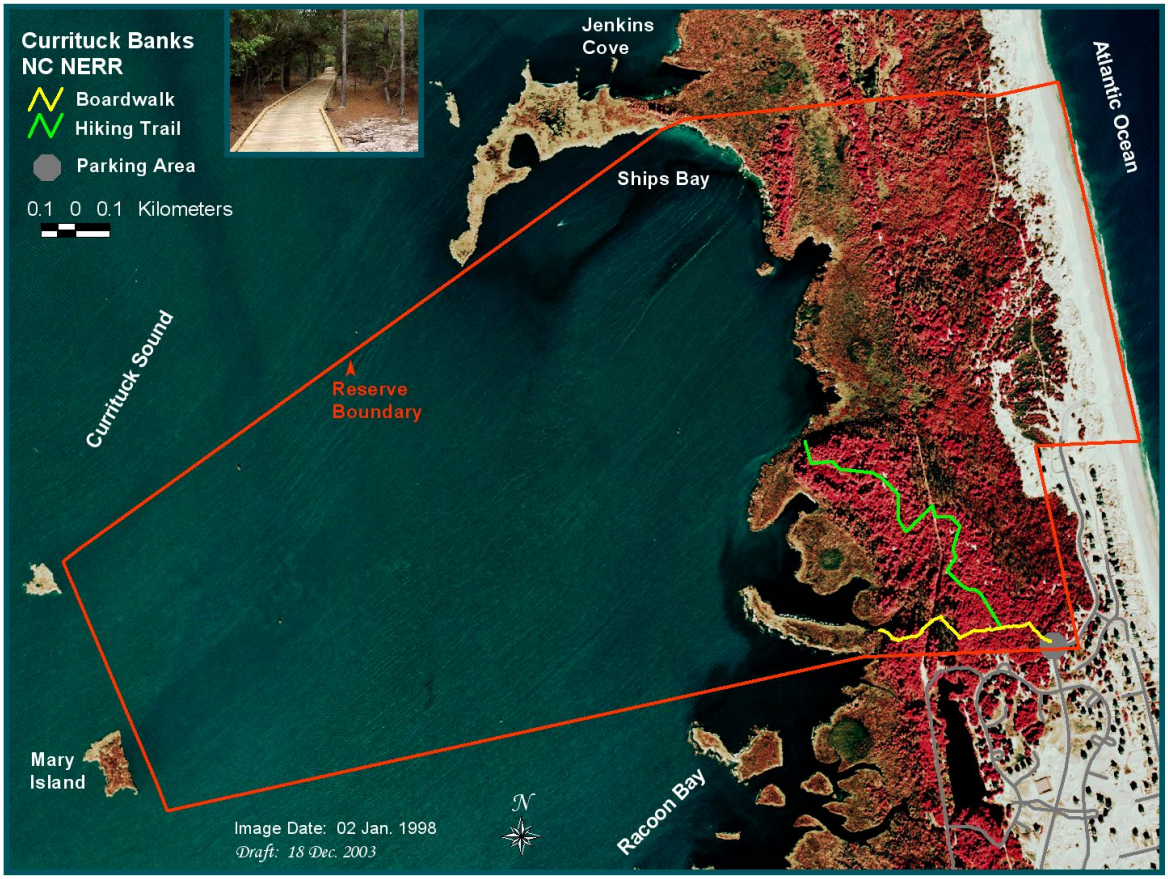


Figure 6: Map of eastern North Carolina showing the relationship between the bioassay collection site in Currituck Sound, located near Corolla, NC, and location where the bioassays were conducted, the University of North Carolina – Chapel Hill's Institute of Marine Sciences (UNC-IMS), in Morehead City, NC.

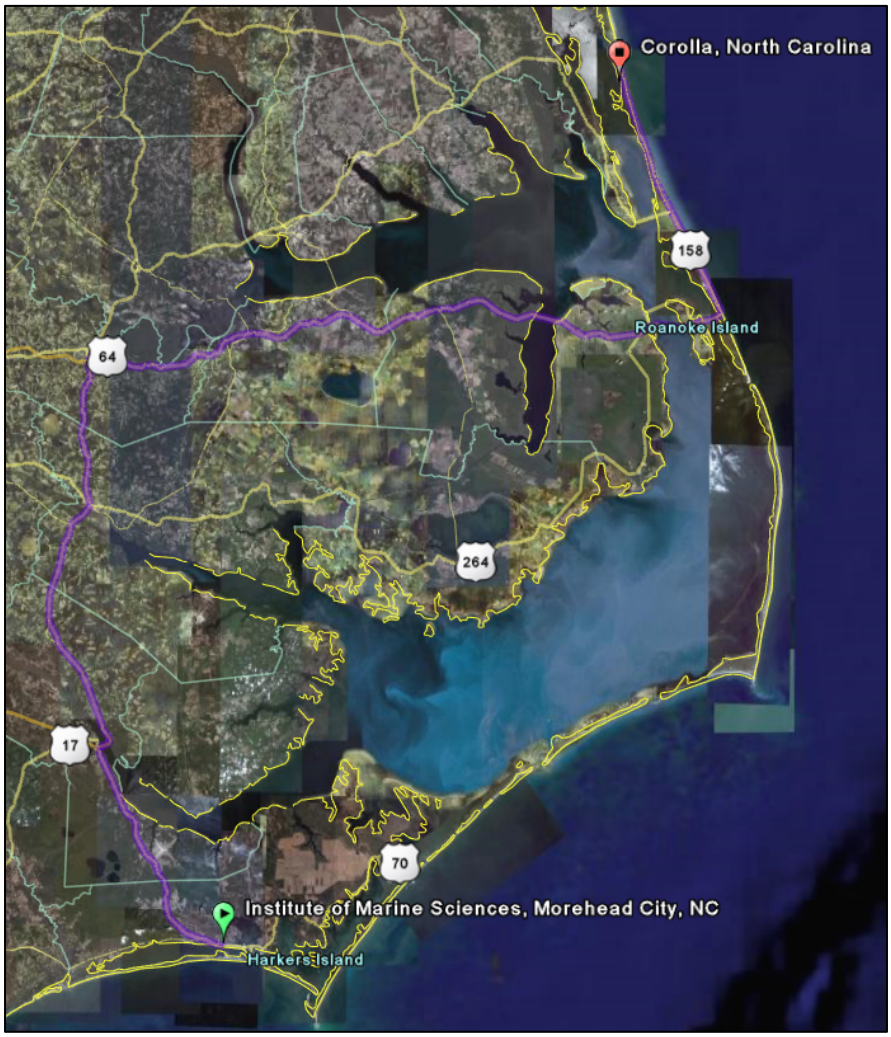


Figure 7: A) A satellite image (Google Earth) showing all the water collection sites throughout North Carolina used for this project. These numbered sites correspond to those listed in **TABLE 1**. The sites within the red box are shown in the zoomed-in image in **FIGURE 7B**.

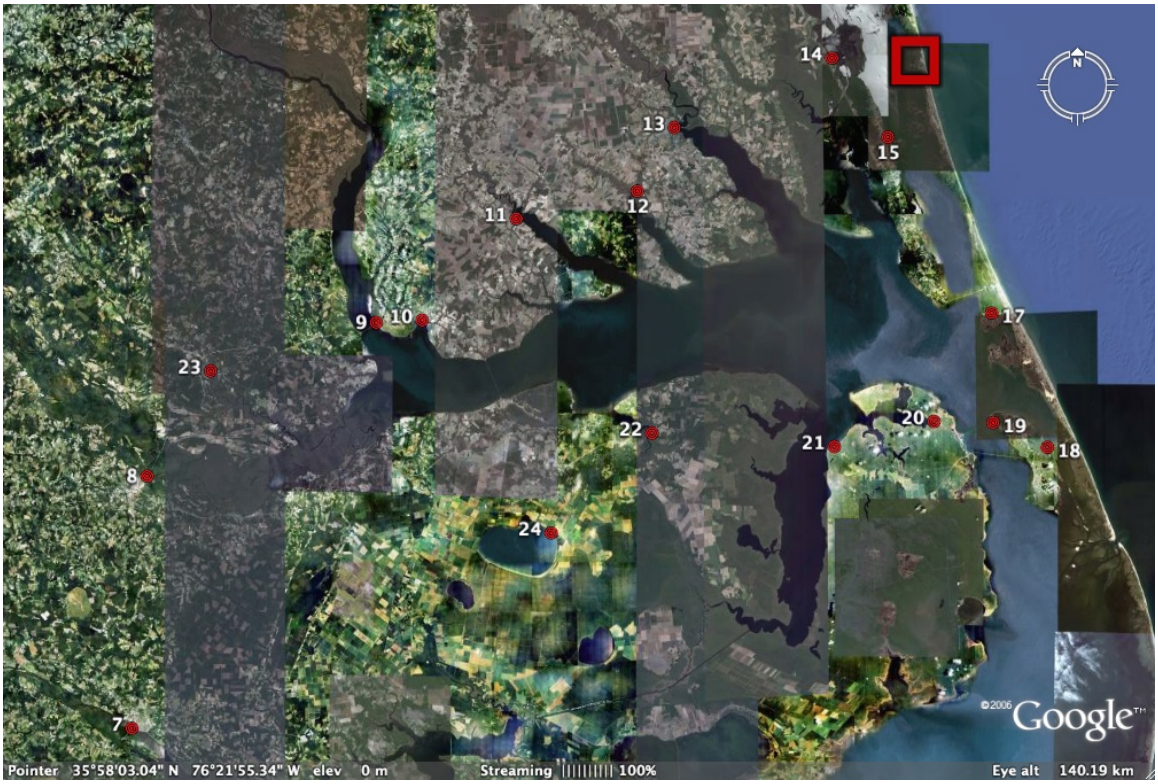


Figure 7 (continued): **B)** The water collection sites within Currituck Sound throughout the course of this study. The numbered sites correspond to those listed in **TABLE 1**.

Figure 8: Examples of the phytoplankton community from collection site 1, in Currituck Sound, NC, but outside the NERRS Currituck Banks reserve: **A**) *Anabaenopsis* spp. **B**) *Anabaena* spp. and *C. raciborskii* **C**) *C. raciborskii* (wavy morphology) **D**) *C. raciborskii* (straight morphology). Photographs by Melissa Leonard.

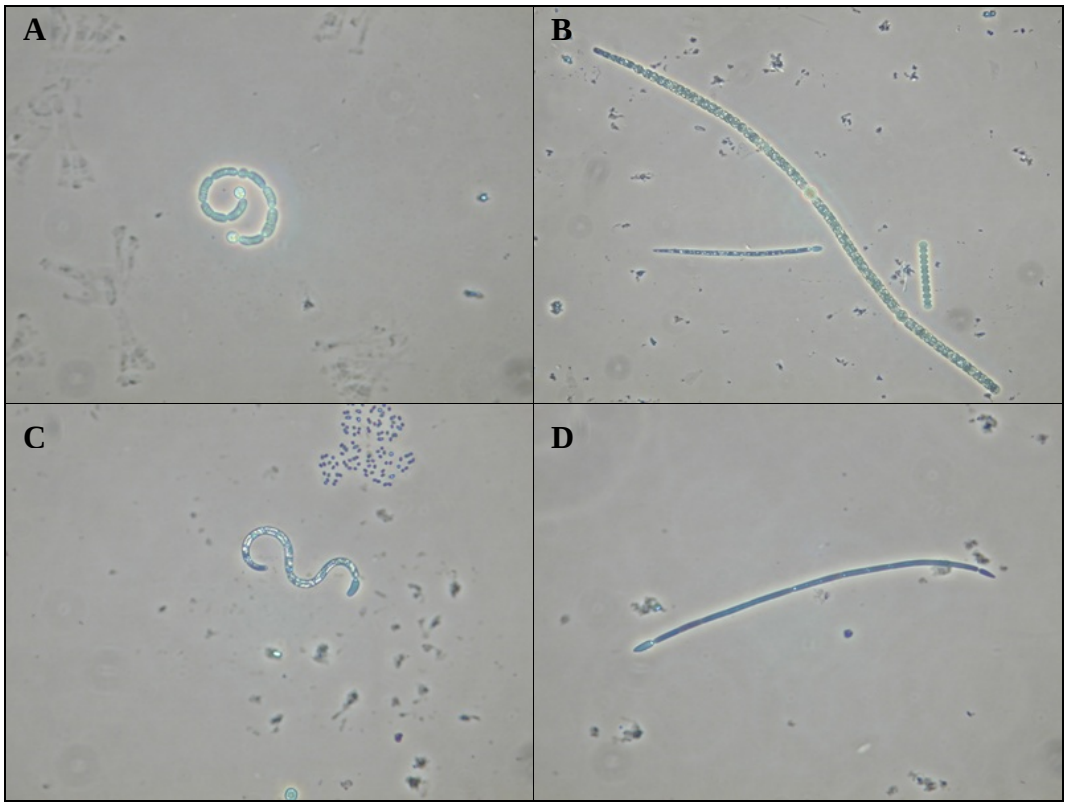


Figure 9: Examples of the phytoplankton community from collection site 2, within NERRS Currituck Banks reserve: **A**) *Anabaenopsis* spp. **B**) *Anabaena* spp. **C**) *C. raciborskii* (wavy morphology) **D**) *C. raciborskii* (straight morphology). Photographs by Melissa Leonard.

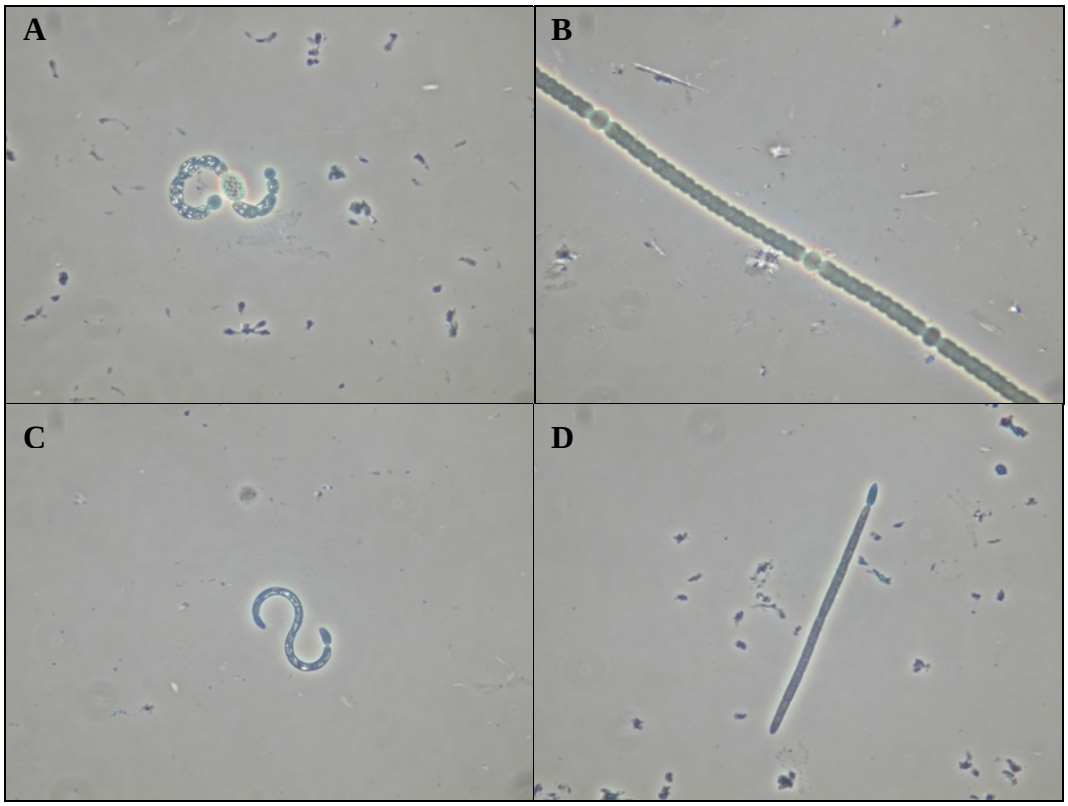


Figure 10: The results from the PCR assay of the cyano-*nif*H gene, used to determine the presence or absence of N_2-fixing cyanobacteria. The dark bands at 324 base pairs (bp) for collection sites 1, 2, 3 and 6 confirmed the presence of N_2-fixing cyanobacteria at these sites, as previously shown by the microscopic analysis. There were also faint bands for sites 4 and 5 despite the absence of visible N_2-fixing cyanobacteria in these samples.

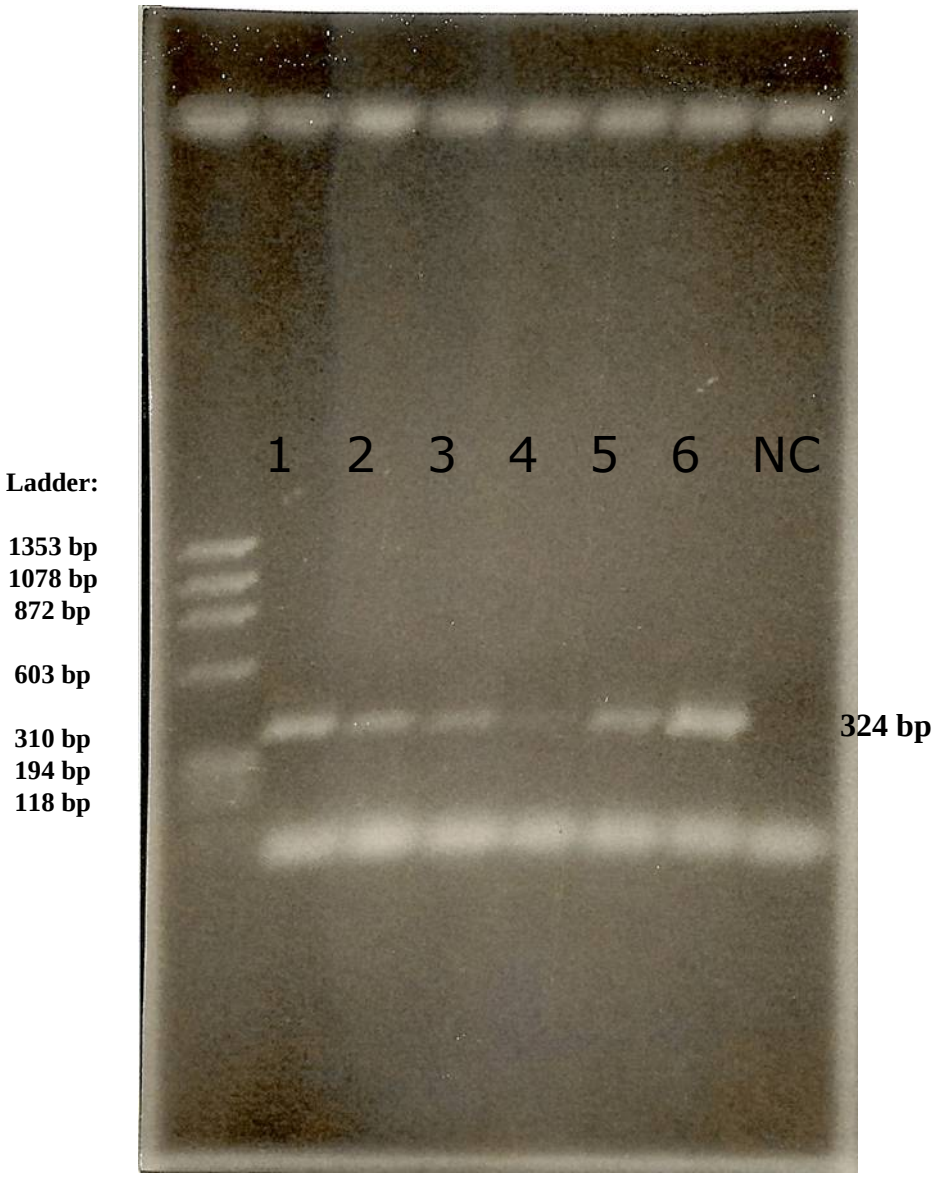


Figure 11: The results from the PCR assay of the more specific cylindro-*nif*H gene, used to determine the presence or absence of *C. raciborskii*. The bands at 224 bp are clearly visible for sites 1, 2, and 6, a faintly visible for site 3, and absent for sites 4 and 5 confirmed the results from the microscopic analysis.

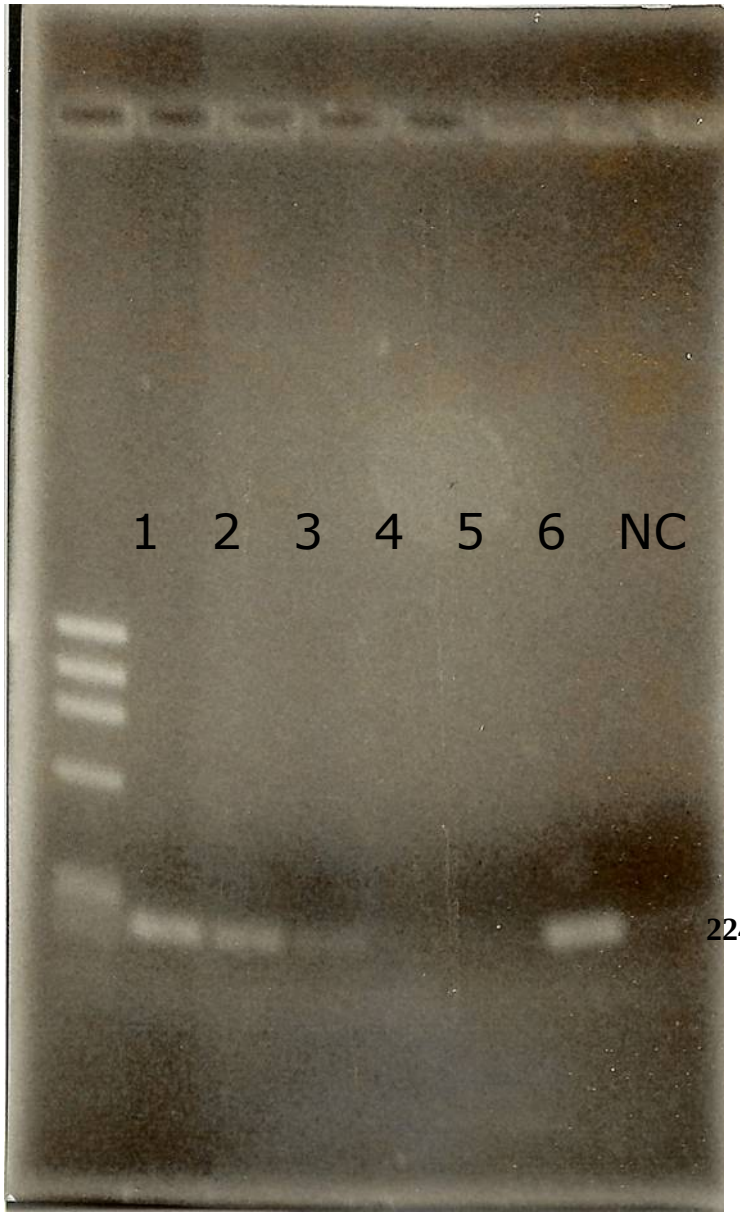


Figure 12: The results of the PCR assays targeting three main genes in the pathway to create CYN. These three genes, when present in one sample, establish that that sample contains *C. raciborskii* capable of producing CYN. In this case, sites 1, 2, 3, and 6 are positive for all three genes, establishing that the *C. raciborskii* at these sites are capable of producing CYN.

A) The results from the PCR assay of *cyl*, which, like the cylindro-*nif*H gene, is specific for *C. raciborskii*. Sites 1, 2, 3, and 6 have bands at 308 bp.

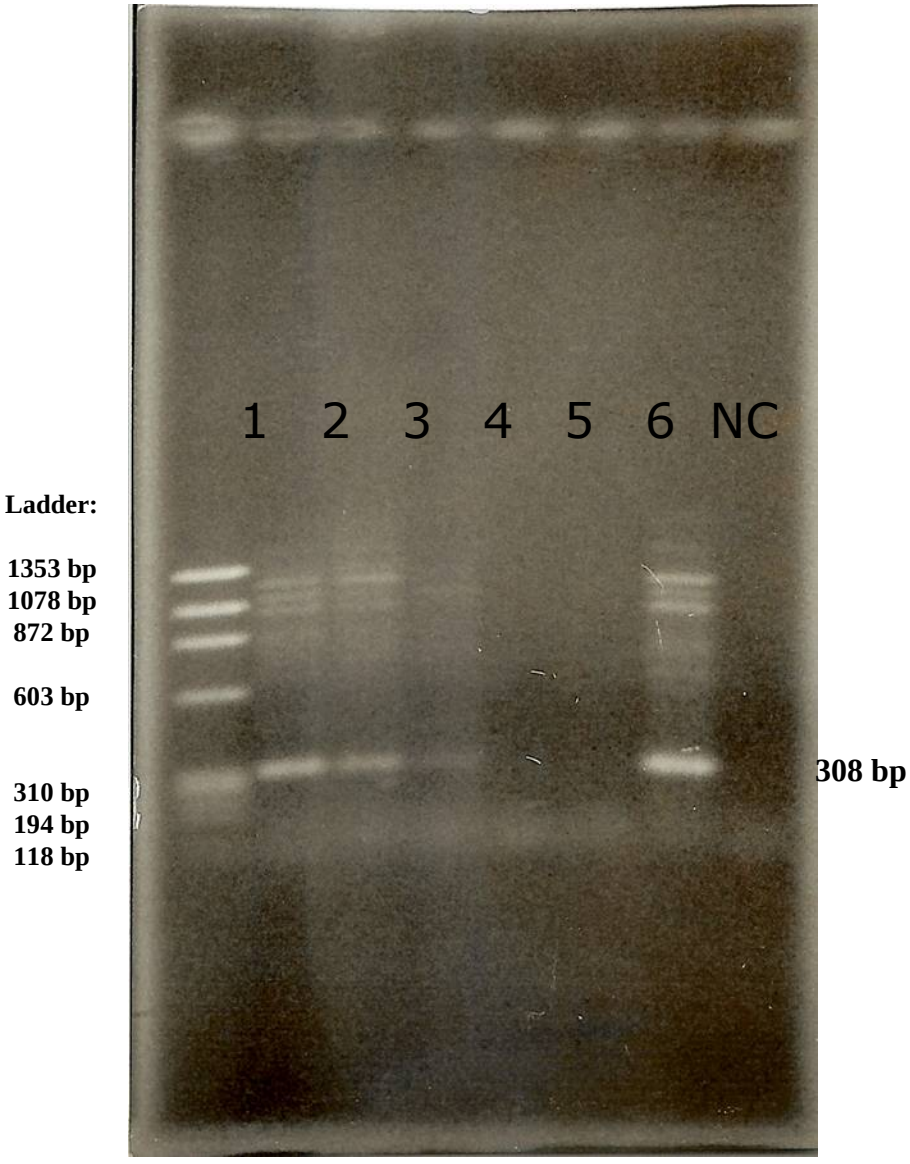


Figure 12 (continued): **B)** The results from the PCR assay of *pks*, which codes for a necessary protein in CYN synthesis. Sites 1, 2, 3, and 6 have bands at 597 bp.

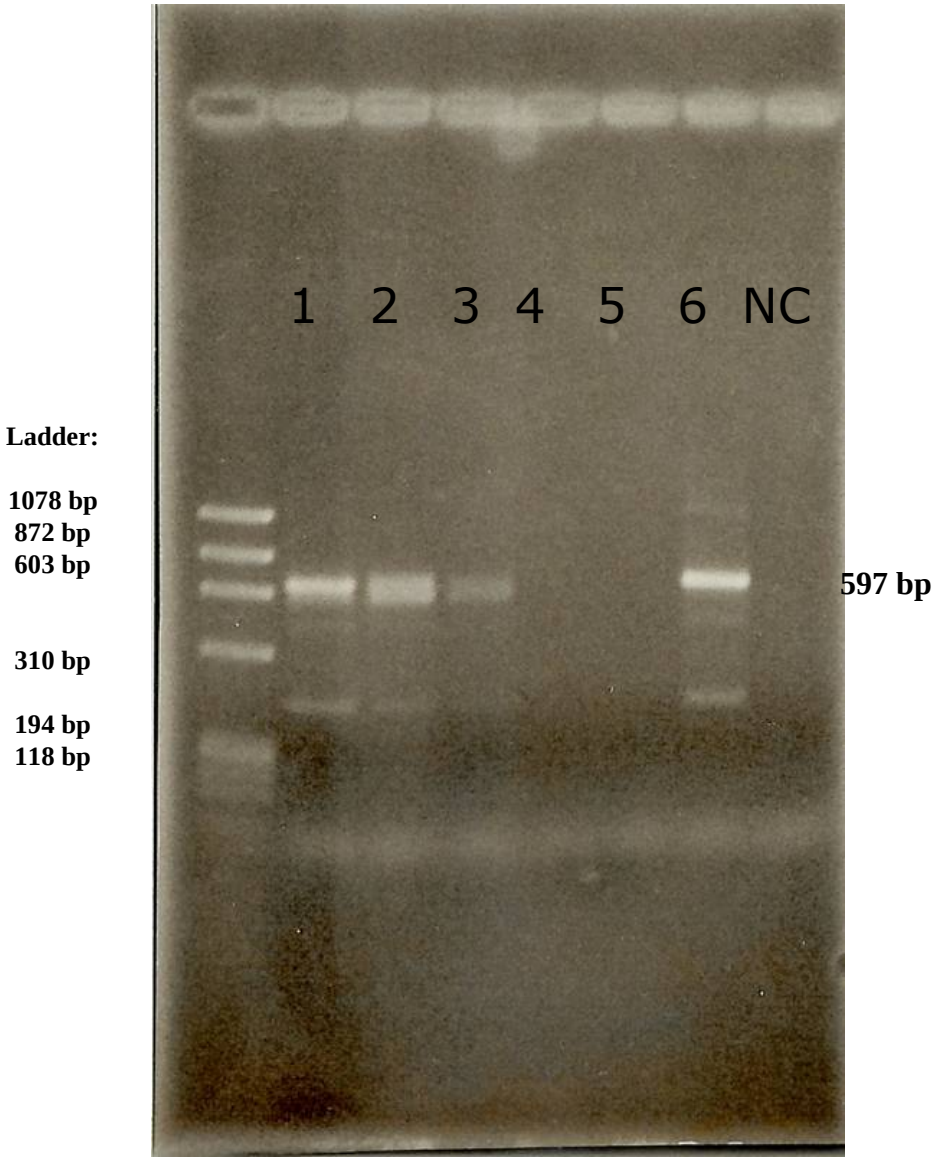


Figure 12 (continued): **C)** The results from the PCR assay of *ps*, which codes for a necessary protein in CYN synthesis. Sites 1, 2, 3, and 6 have bands at 422 bp.

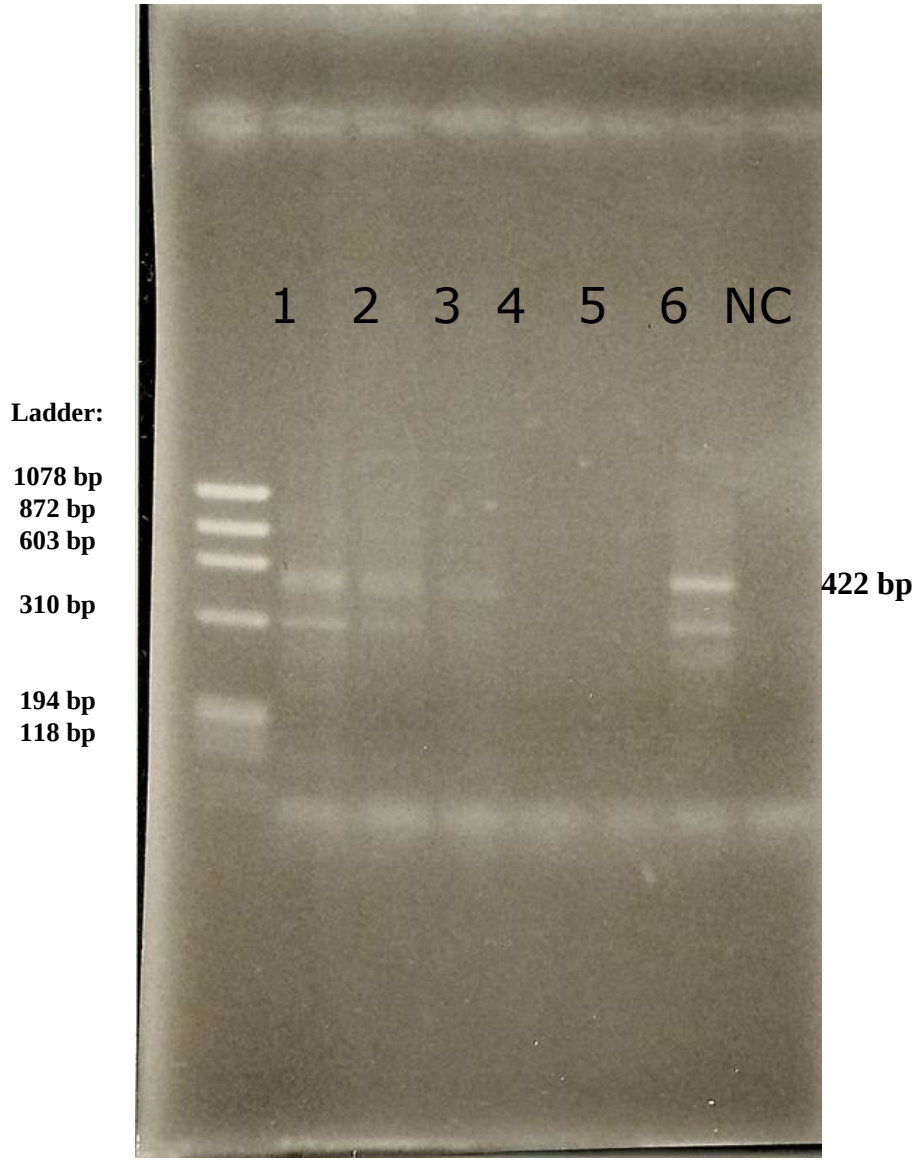


Figure 13: A phylogenetic tree based upon nif*H* sequences of the *C. raciborskii* strains. These samples include Currituck Sound samples NC 1, 2, 3, 5, and 6 (corresponding to sites in **FIGURE 7** and **TABLE 1**), cultures of *C. raciborskii* isolated from Florida (Cyl D, Cyl F, and Cyl L), and randomly selected samples locations throughout St. Johns River, FL (SJR 1-4). A culture of *Anabaena aphanizomenoides* was used as an out-group. The legend indicates the length of 0.1 substitutions per site. The similarity between the Florida and the North Carolina strains is apparent.

Figure 14: Chl *a* concentrations, used as a proxy for biomass, for the filtered treatments of the June 2007 bioassay. Site 1 is located outside the Currituck Banks reserve and Site 2 is within the reserve (**FIGURE 7B**). The index shows the different time points (T_0, T_2, T_4, T_6, and T_8) of subsampling during the bioassay. The nutrient treatments are Z8 media (**M**), control (**C**), nitrate added (**N**), phosphate added (**P**), and nitrate and phosphate added (**N+P**). Nitrogen limitation of biomass is indicated by increased Chl *a* concentration in the treatments with nitrogen added.

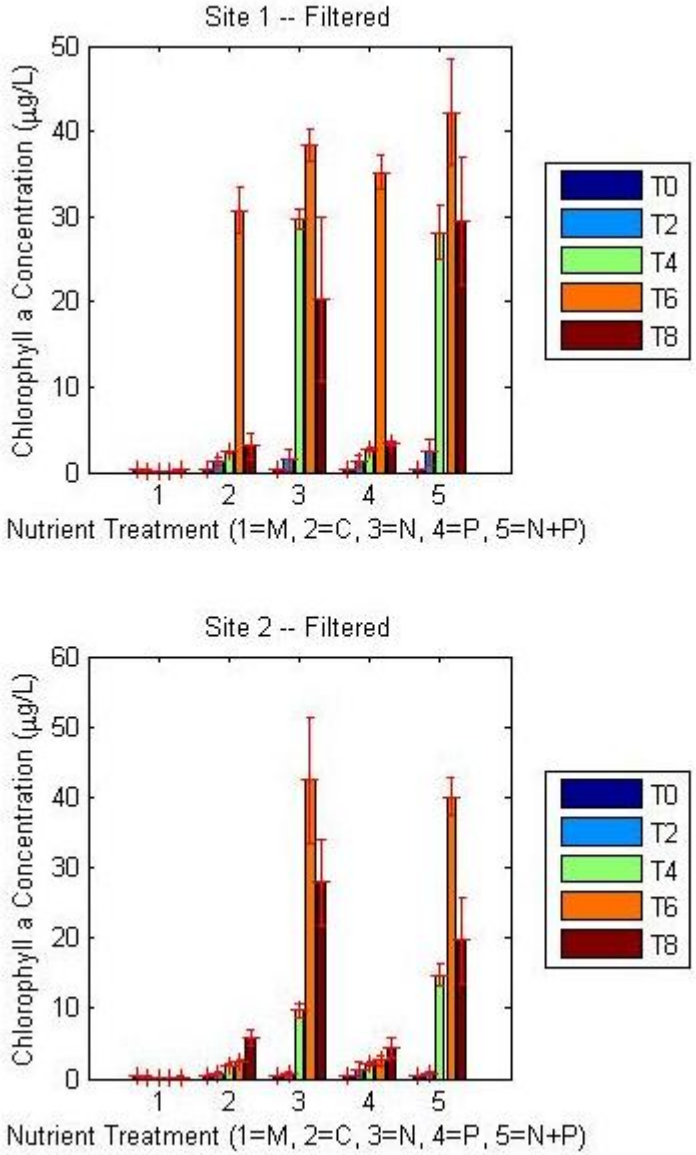


Figure 15: Acetylene reduction rates, used as a proxy for nitrogen fixation, for the filtered treatments of the June 2007 bioassay. The highest acetylene reduction rates occurred in the Z8 media, which has no bioavailable nitrogen, while the Currituck Sound water treatments all had low fixation throughout the bioassay.

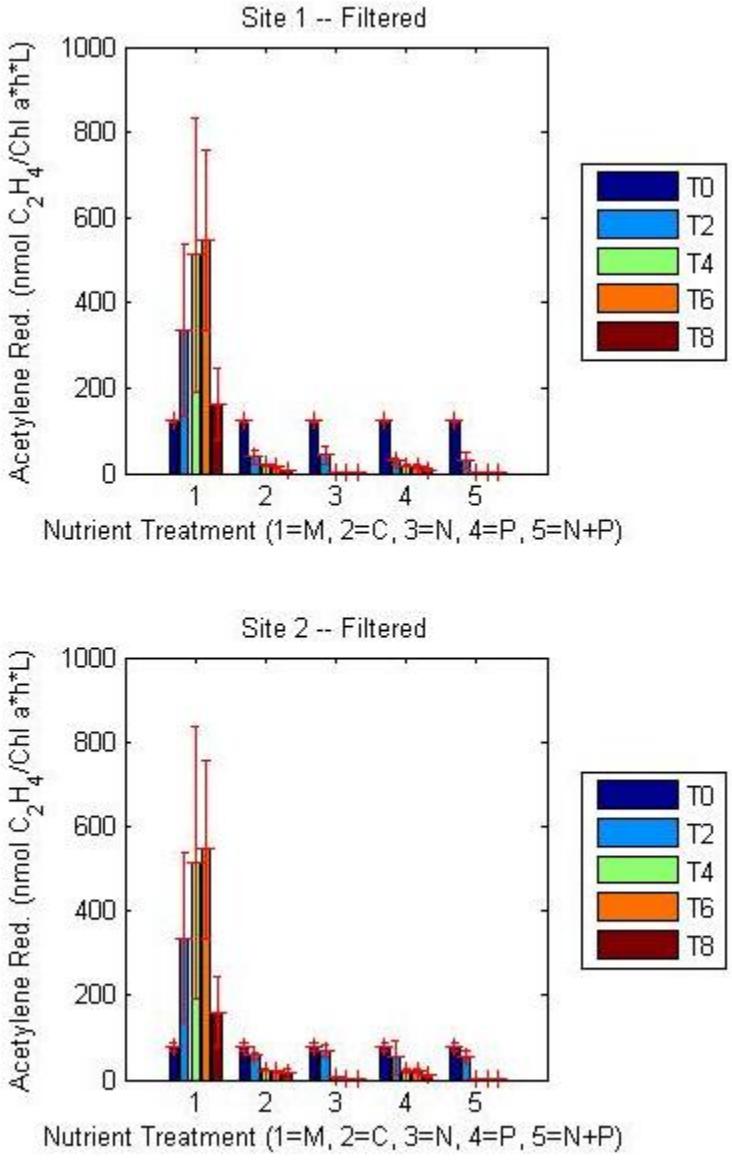


Figure 16: Primary production for the filtered treatments of the June 2007 bioassay. Nitrogen limitation of production is indicated by increased primary production rates in the treatments with nitrogen added.

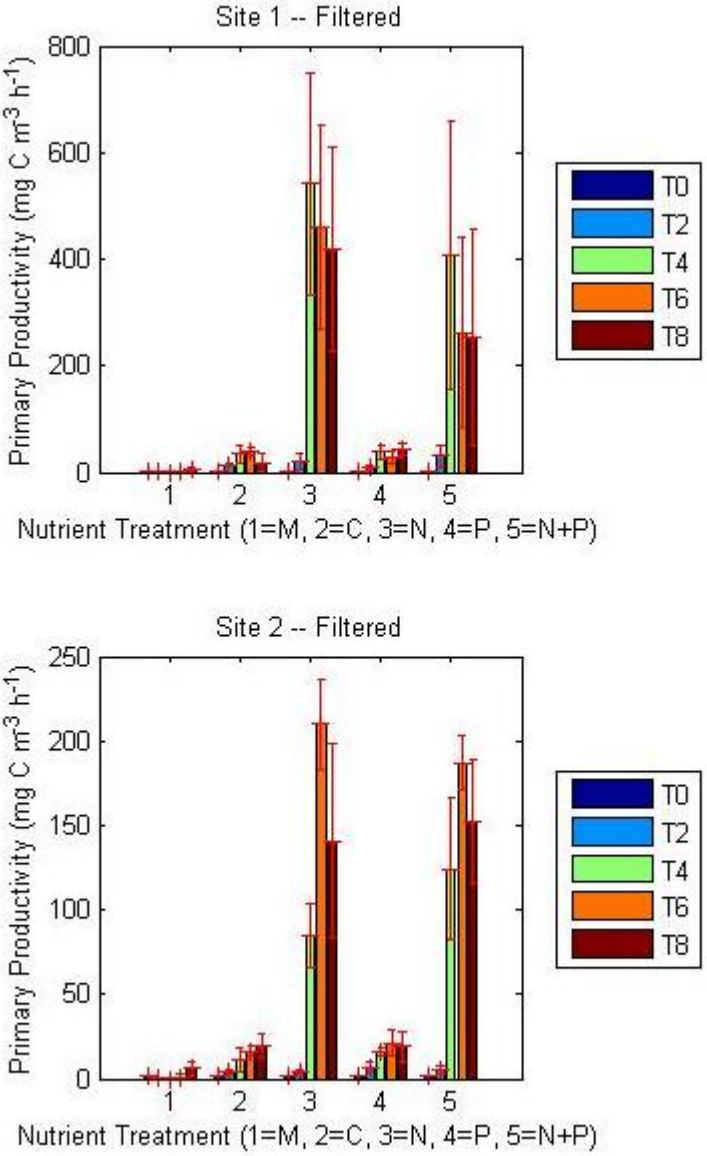


Figure 17: Chl *a* concentrations, used as a proxy for *C. raciborskii* biomass, for the filtered treatment of the September 2007 bioassay. Only one sampling site was used, located within the reserve (**Site 6, FIGURE 7B**). The index shows the different time points (T_0, T_2, T_4, T_6, and T_8) of subsampling during the bioassay. The nutrient treatments are **M**, **C**, **N**, ammonium added (**A**), **P**, **N+P**, ammonium and phosphate added (**A+P**), nitrate and ammonium added (**N+A**), and nitrate, ammonium, and phosphate added (**N+A+P**). Unlike the June 2007 bioassay, here the added *C. raciborskii* added did better in **M** than the Currituck Sound water treatments. Nitrogen limitation of biomass is indicated by increased Chl *a* concentration in the treatments with nitrogen added.

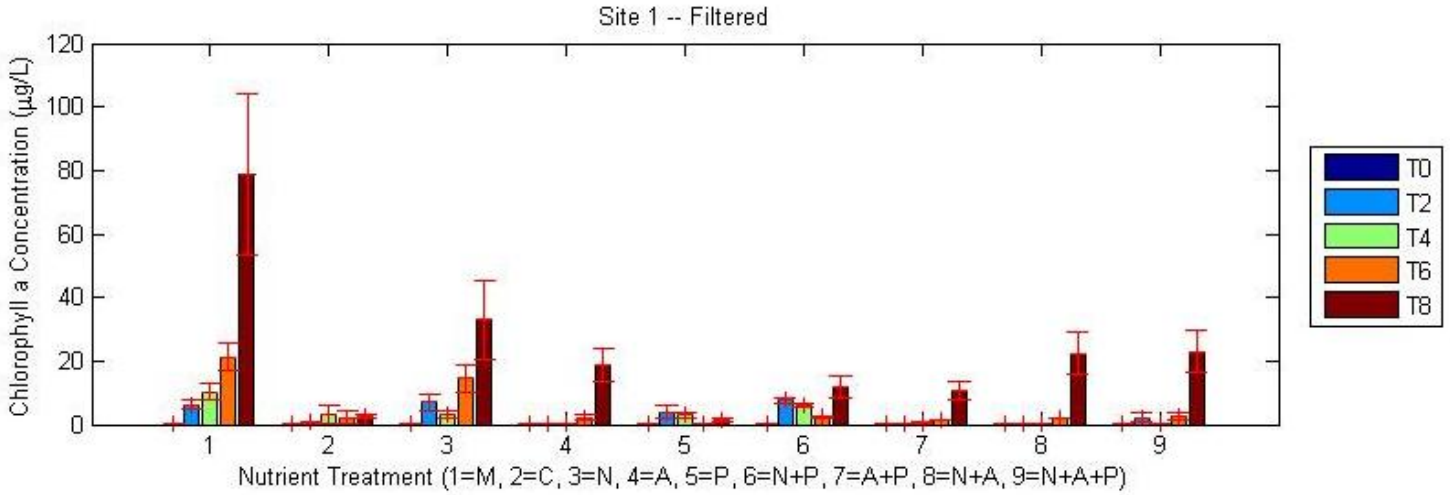


Figure 18: Primary production rates for the filtered treatment of the September 2007 bioassay. Here the added *C. raciborskii* added initially did better in **M** than the Currituck Sound water treatments, but recovered in these treatments toward the end of the experiment, especially in the treatments with nitrogen added, indicating nitrogen limitation.

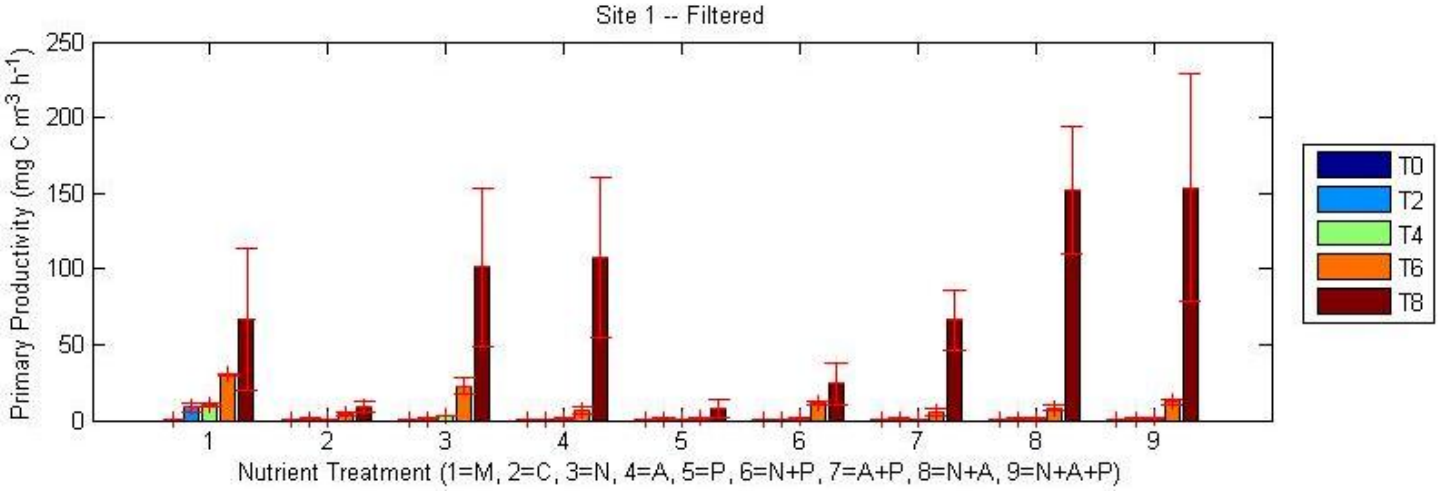


Figure 19: Chl *a* concentrations, used as a proxy for *C. raciborskii* biomass, for the filtered treatment of the June 2008 bioassay. Only one sampling site was used, located within the reserve (**Site 25, FIGURE 7B**). The index shows the different time points (T_0, T_2, T_4, T_6, and T_8) of subsampling during the bioassay. The nutrient treatments are **M**, Z8 media with added nitrate (**MN**), **C**, **N**, **A**, **P**, **N+P**, **A+P**, **N+A**, and **N+A+P**. Initially, the added *C. raciborskii* added did better in the media treatments than the Currituck Sound water treatments, but recovered in these treatments by the end of the experiment, especially in the treatments with nitrogen added, indicating nitrogen limitation.

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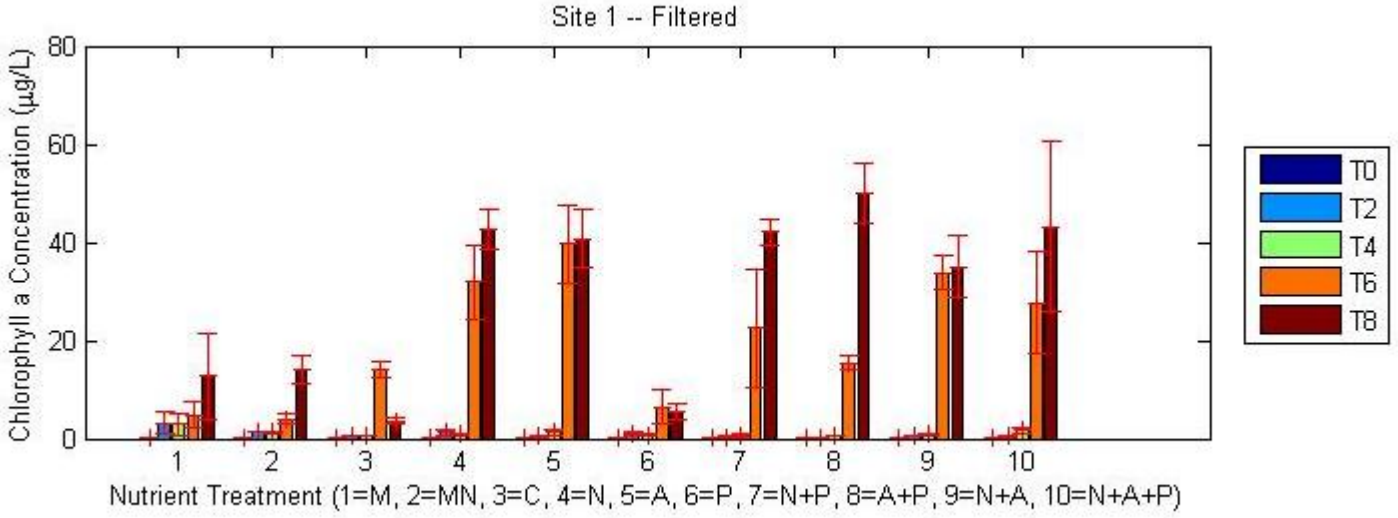


Figure 20: Chl *a* concentrations, used as a proxy for *C. raciborskii* biomass, for the filtered treatment of the September 2008 bioassay. Only one sampling site was used, located within the reserve (**Site 26, FIGURE 7B**). The index shows the different time points (T_0, T_2, T_4, T_6, and T_8) of subsampling during the bioassay. The nutrient treatments are **M**, **MN**, **C**, **N**, **A**, **P**, **N+P**, **A+P**, **N+A**, and **N+A+P**. The added *C. raciborskii* added did better in the media treatments than the Currituck Sound water treatments throughout the bioassay, though the nitrogen treatments did have higher biomass than the treatments without added nitrogen. Overall, biomass was very low throughout, suggesting salinity stress.

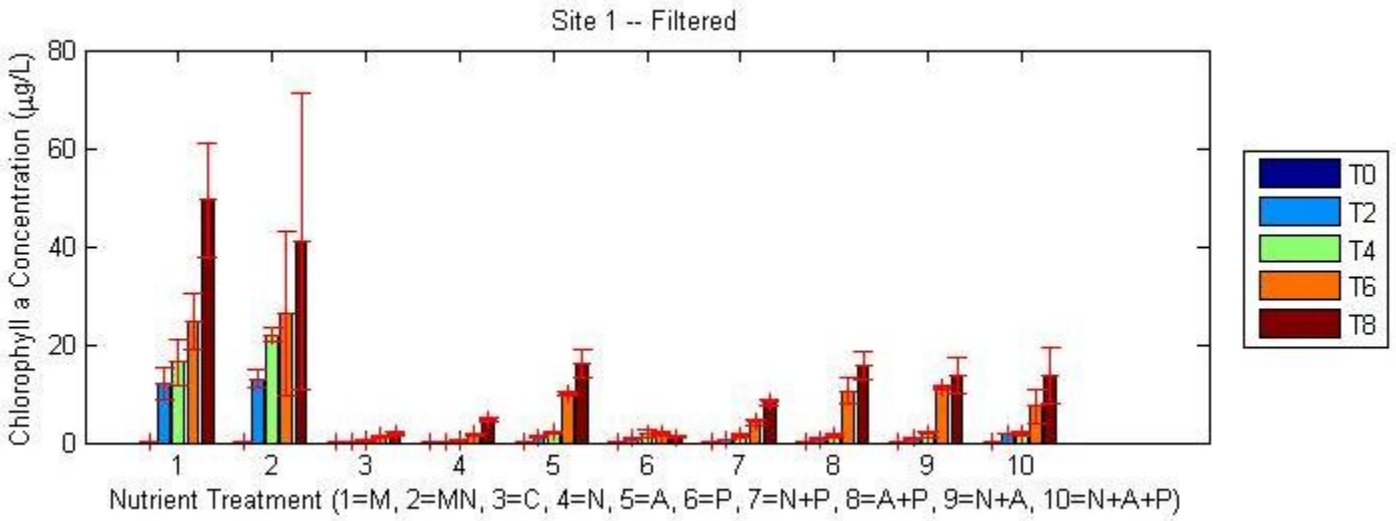


Figure 21: Growth rates for the filtered treatments of all 4 bioassays, showing that growth was lowest in Fall 2008, when salinity stress was at its greatest (8.4 psu, **TABLE 2**).

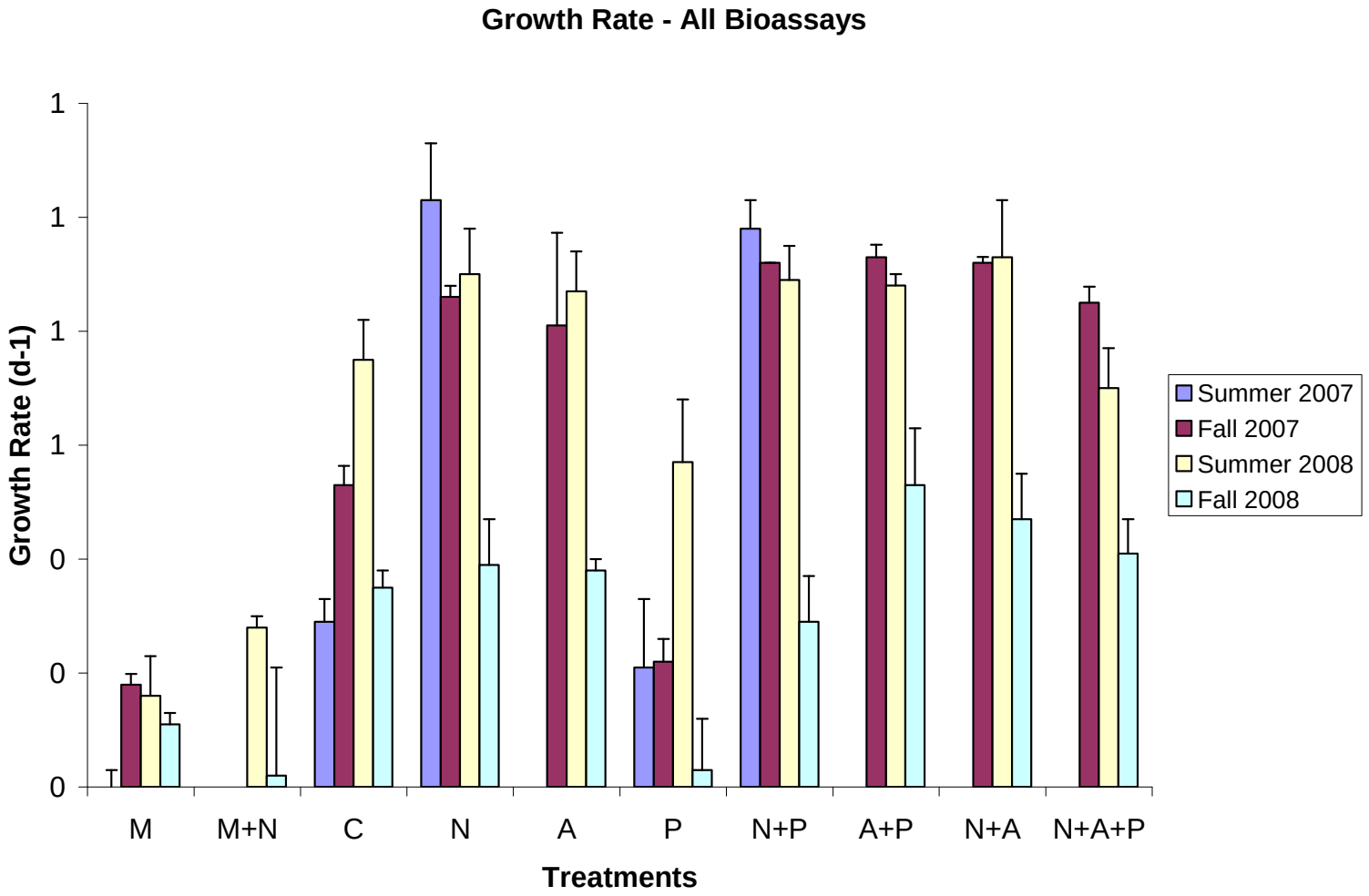


Figure 22: Primary production rates for the filtered treatment of the June 2008 bioassay. Production rates were low throughout the bioassay across all treatments. The results suggest that production in this bioassay may have been limited not by nitrogen but by ammonium, as ammonium additions triggered a stronger response than nitrate additions in increased production. This is shown by the higher production rates in the **A**, **A+P**, **N+A**, and **N+A+P** treatments.

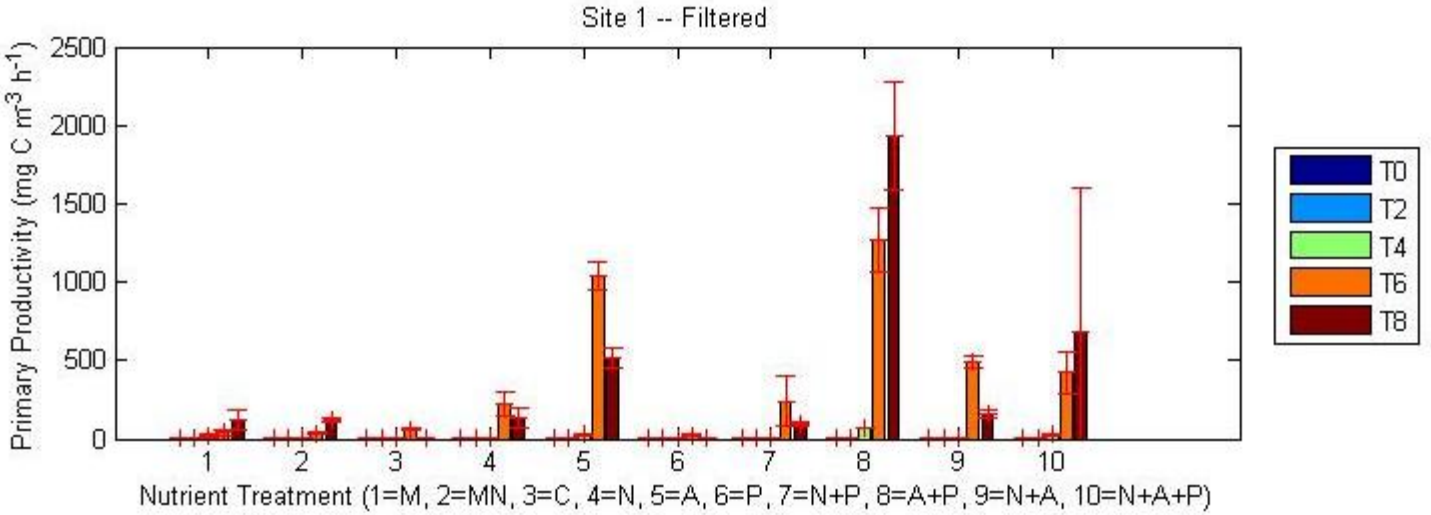


Figure 23: Primary production rates for the filtered treatment of the September 2008 bioassay. Production rates were low throughout the bioassay across all treatments, with the media treatments doing better than the Currituck Sound water treatments, suggesting salinity stress. Like the June 2008 production results, these results suggest that production in Currituck Sound may have been limited not by nitrogen but by ammonium, as ammonium additions triggered a stronger response than nitrate additions in increased production.

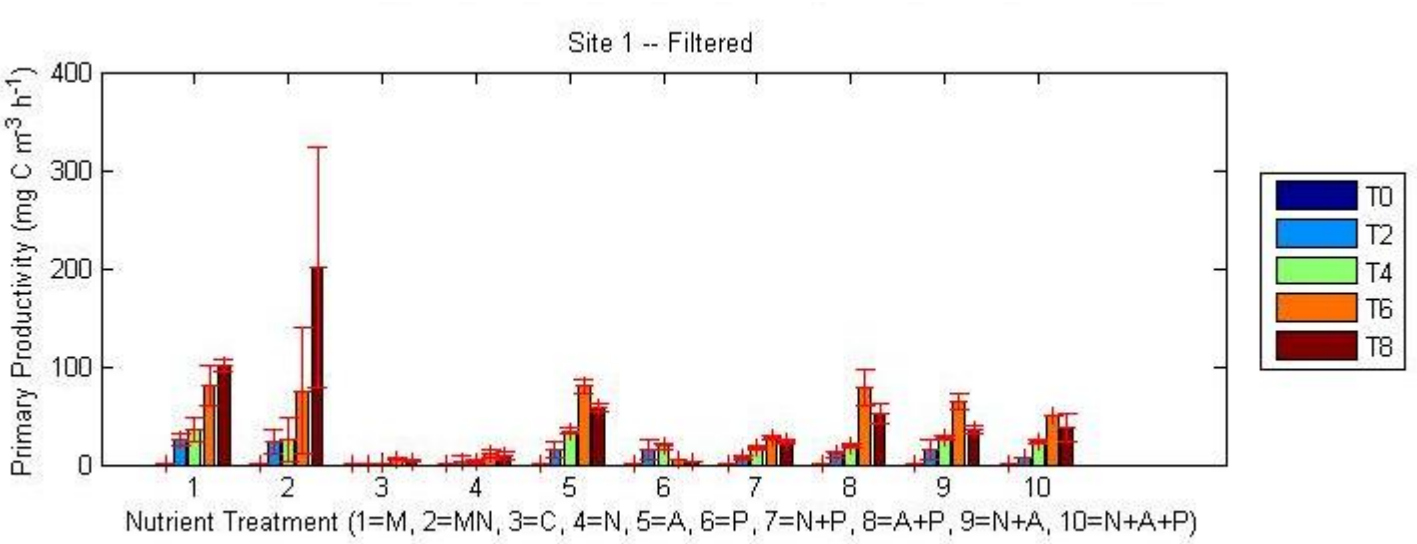
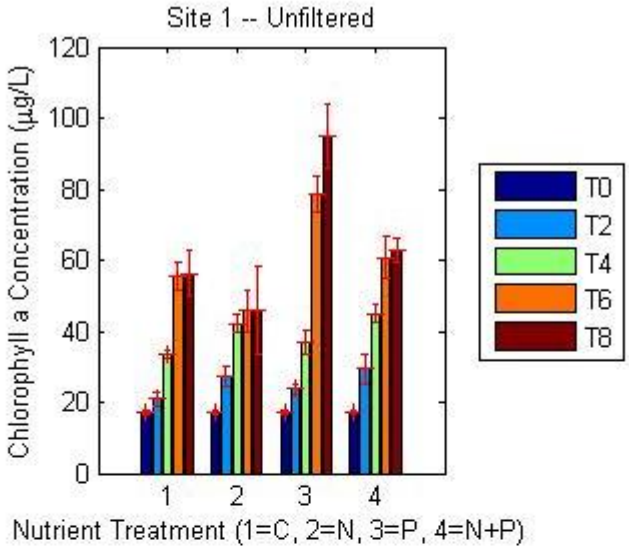


Figure 24: Chl *a* concentrations, used as a proxy for biomass, for the unfiltered treatments of the June 2007 bioassay. Site 1 is located outside the Currituck Banks reserve and Site 2 is within the reserve (**FIGURE 7B**). The index shows the different time points (T_0, T_2, T_4, T_6, and T_8) of subsampling during the bioassay. The nutrient treatments are control (**C**), nitrate added (**N**), phosphate added (**P**), and nitrate and phosphate added (**N+P**). Biomass is initially limited by nitrogen, but becomes phosphorus limited by the end of the bioassay.



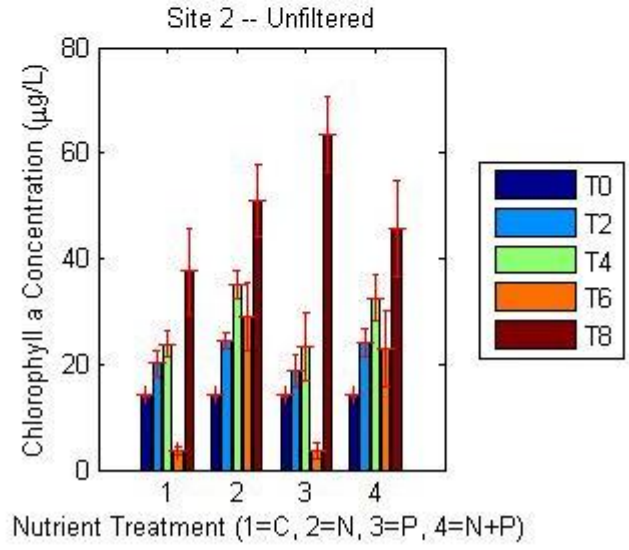


Figure 25: Primary production for the unfiltered treatments of the June 2007 bioassay. Nitrogen limitation of production in the first two subsampling points is indicated by increased primary production rates in the treatments with nitrogen added, after which production becomes phosphorus limited.

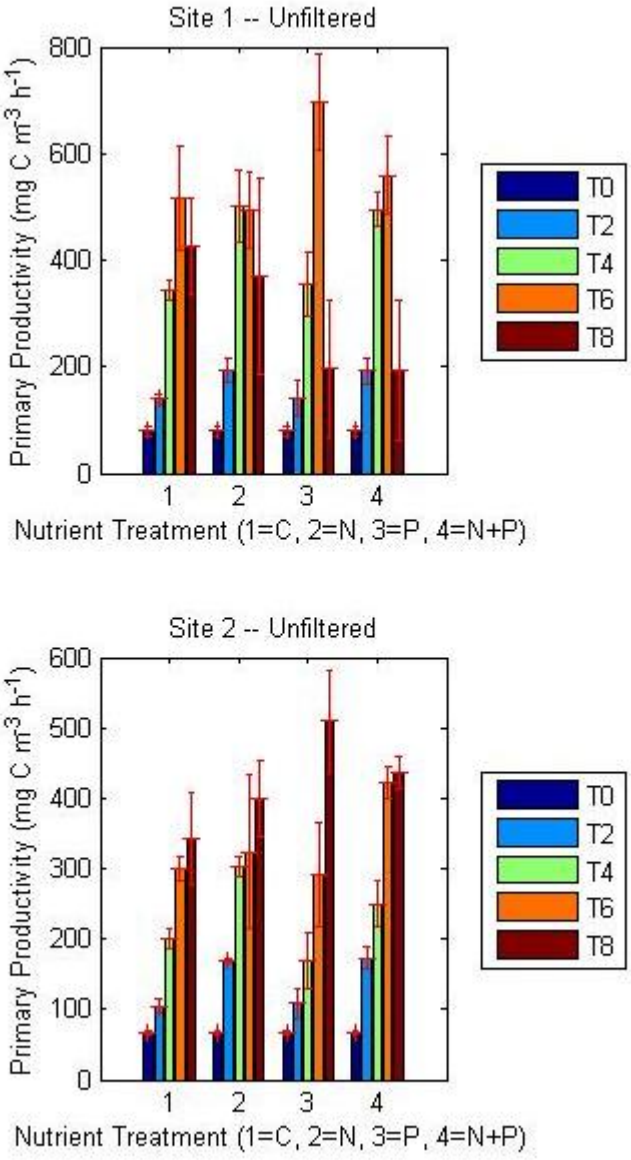


Figure 26: *C. raciborskii* filament counts for T_8 for the unfiltered treatments of the June 2007 bioassay. *C. raciborskii* was the most competitive in the nitrate added treatments, shown by the high *C. raciborskii* abundance.

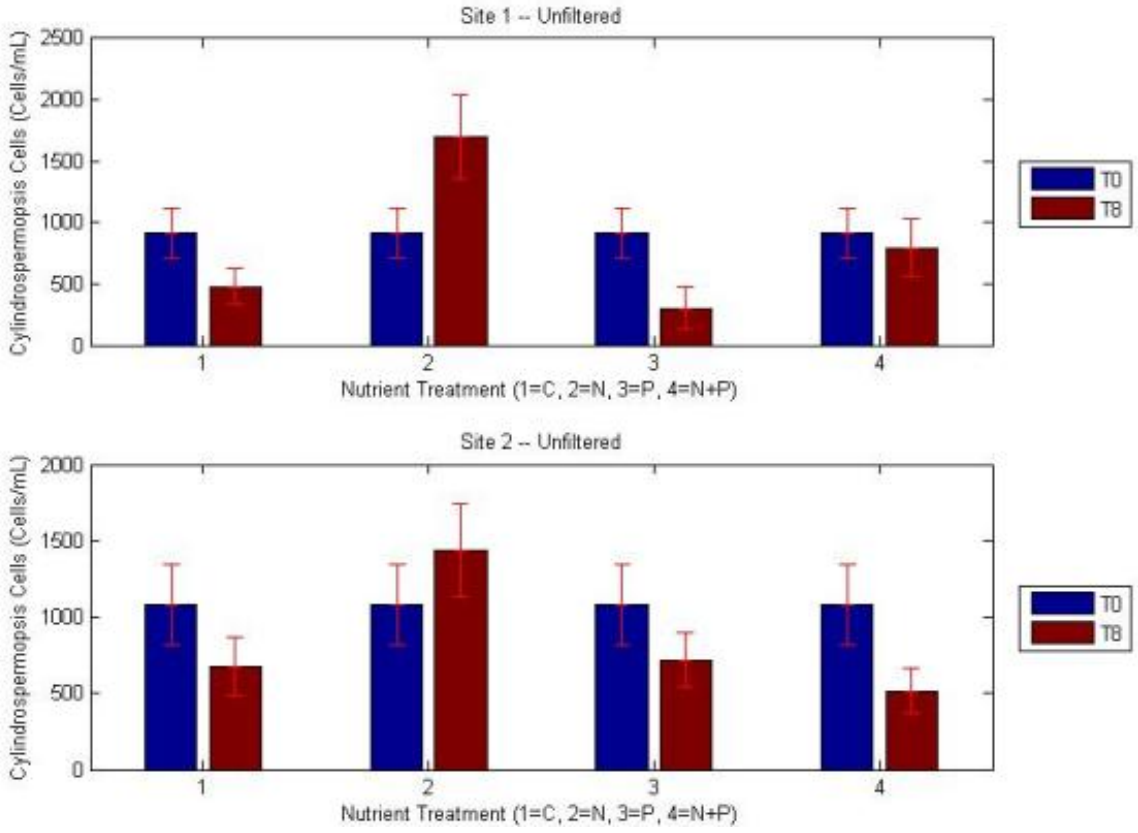


Figure 27: *C. raciborskii* filament counts for T_8 for the unfiltered treatments of the September 2007 bioassay. *C. raciborskii* was the most competitive in the nitrogen added treatments, shown by the high *C. raciborskii* abundances in these treatments.

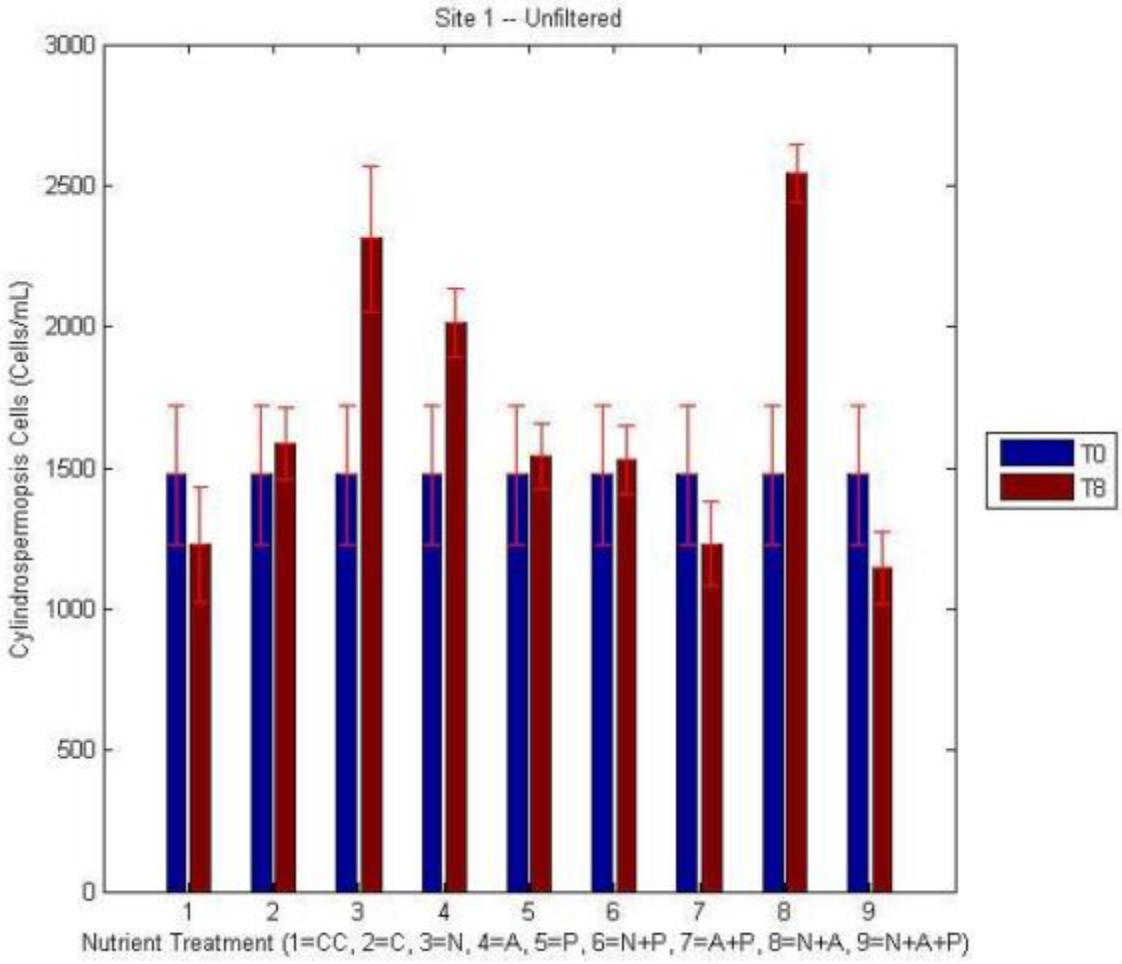


Figure 28: *C. raciborskii* filament counts for T_8 for the unfiltered treatments of the June 2008 bioassay. *C. raciborskii* was the most competitive in the nitrogen added treatments, shown by the high *C. raciborskii* abundances in these treatments.

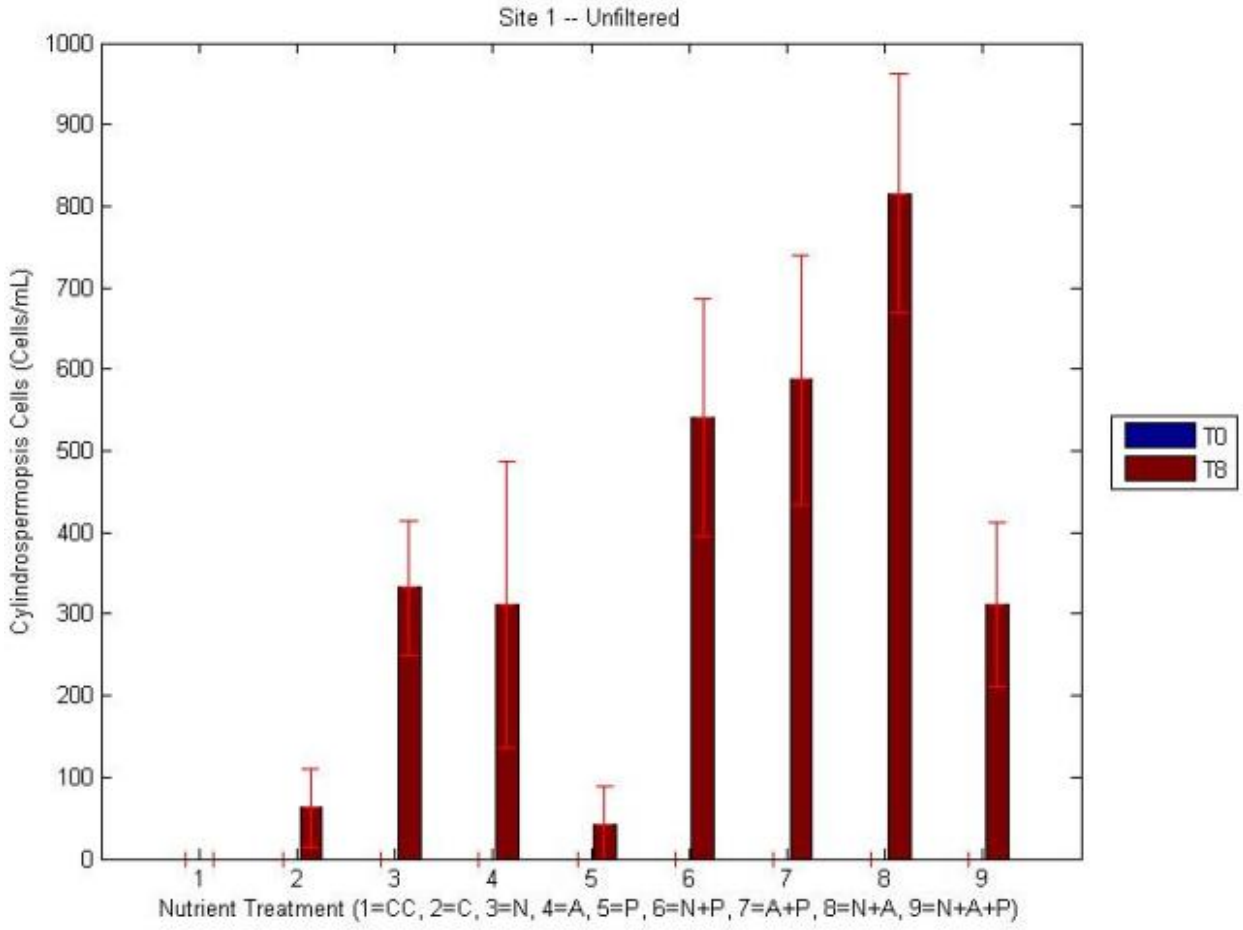


Figure 29: *C. raciborskii* filament counts for T_8 for the unfiltered treatments of the September 2008 bioassay. *C. raciborskii* was the most competitive in the nitrogen added treatments, shown by the high *C. raciborskii* abundances in these treatments.

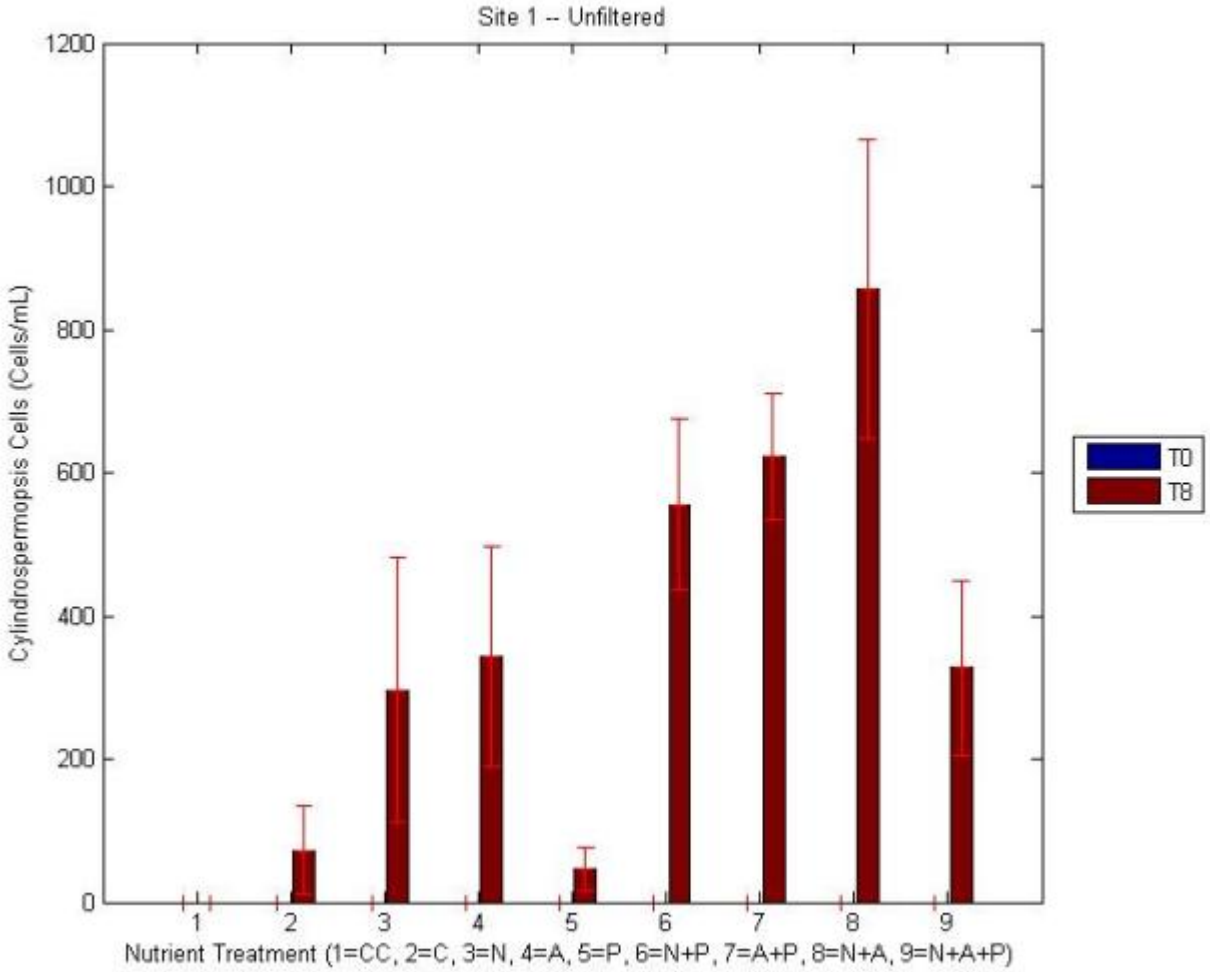


Figure 30: *C. raciborskii* filament counts for T_8 for the unfiltered treatments of all 4 bioassays. The comparison between the *C. raciborskii* abundances in the 2007 bioassays and the 2008 show the effect of increased salinity on *C. raciborskii*'s ability to survive and grow.

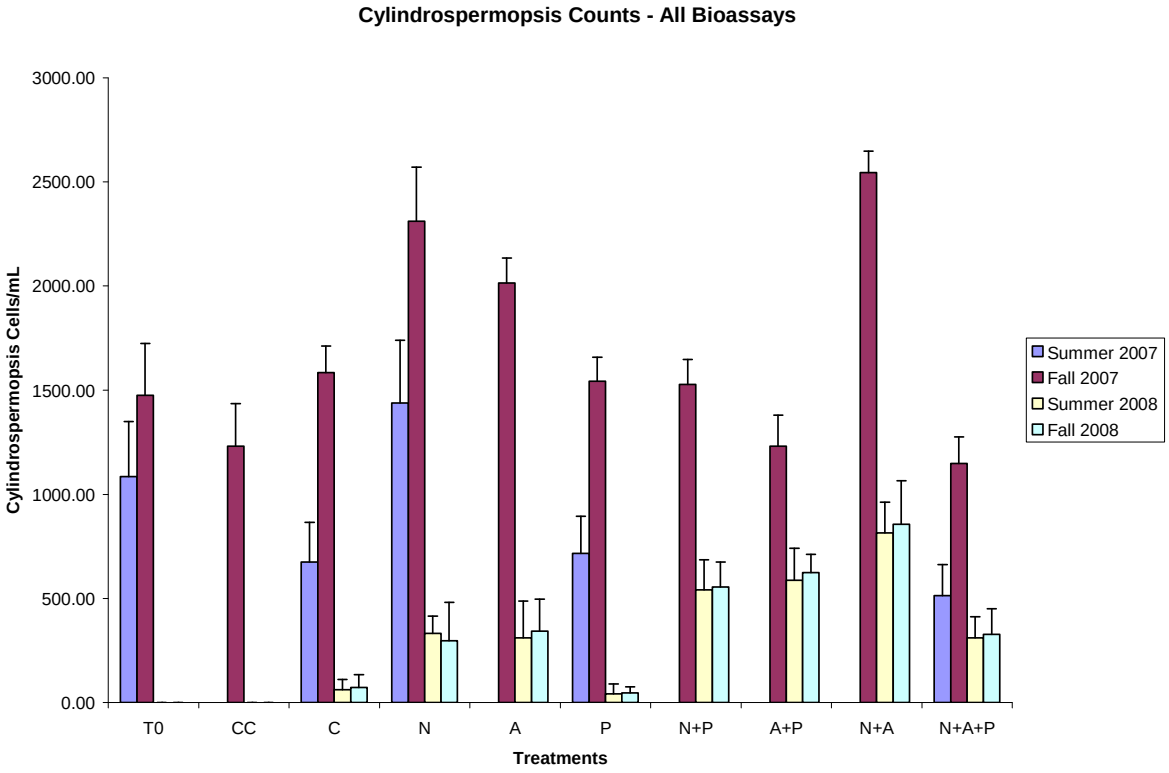
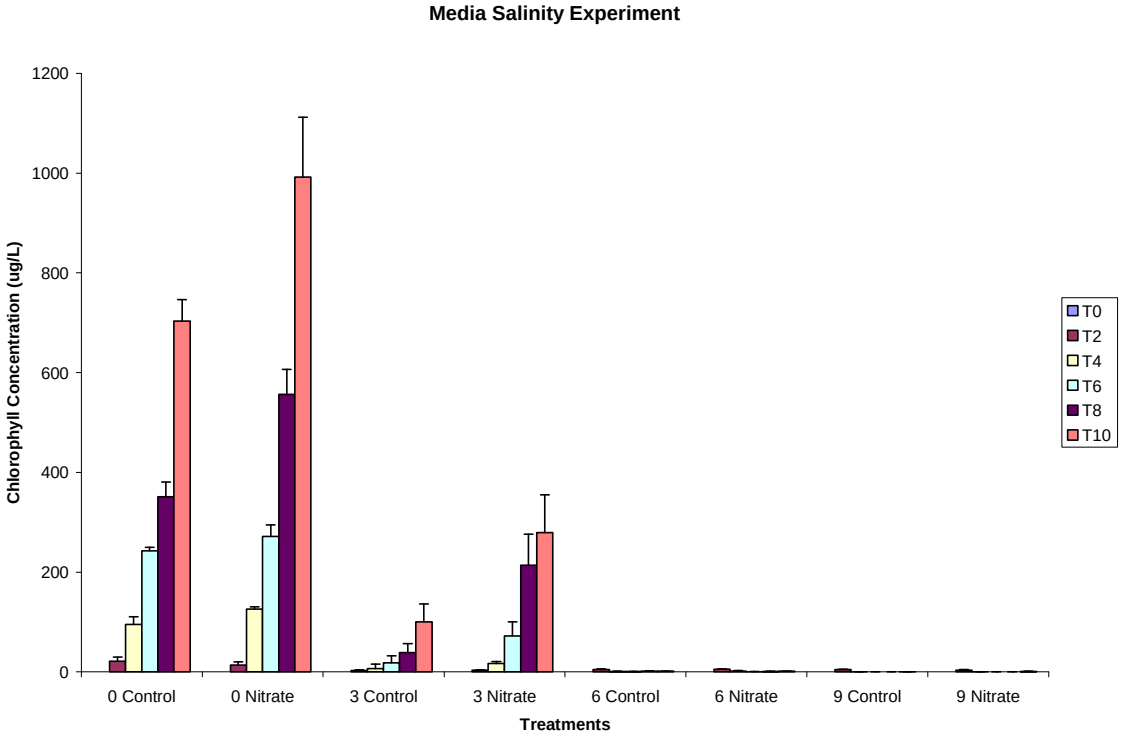


Figure 31: Chl *a* concentrations for T_{10} of the salinity experiment. The results indicate that *C. raciborskii* biomass is significantly limited by salinity and that nitrogen increases its ability to recover from salinity stress.



APPENDIX C: Tables

Table 1: A summary of the water collection sites throughout North Carolina utilized in this study to determine how widespread *C. raciborskii* currently is in the state. The site numbers correspond to the map in **FIGURE 7**.

Site #	Site Location	Latitude/Longitude	Date Collected	Salinity
1	Currituck Sound, outside reserve	N 36.37283° W 75.83529°	6/22/07	0 psu
2	Currituck Banks, at duck blind	N 36.39212° W 75.85087°	6/22/07	0 psu
3	Currituck Banks, front of marsh	N 36.39545° W 75.84138°	6/22/07	0 psu
4	Currituck Banks, behind marsh	N 36.39010° W 75.83477°	6/22/07	0 psu
5	Currituck Banks, front of forest	N 36.39473° W 75.83854°	6/22/07	0 psu
6	Currituck Banks, at duck blind	N 36.39212° W 75.85087°	9/14/07	4.7 psu
7	Pamlico River, at Washington, NC	N 35.54095° W 77.06342°	5/31/08	0 psu
8	Roanoke River, at Williamston, NC	N 35.85981° W 77.04091°	5/31/08	0 psu
9	Chowan River, at Edenhouse Bridge	N 36.05458° W 76.68387°	5/31/08	0 psu
10	Edenton Bay, at Edenton, NC	N 36.05755° W 76.61228°	5/31/08	0 psu
11	Perquimans River, at Hertford, NC	N 36.18554° W 76.46536°	5/31/08	4 psu
12	Little River, at Nixontown, NC	N 36.22033° W 76.27652°	5/31/08	4 psu
13	Pasquotank River, at Elizabeth City, NC	N 36.30029° W 76.21805°	5/31/08	4 psu
14	Conjock Bay, at Barco, NC	N 36.38747° W 75.97060°	5/31/08	5 psu
15	Currituck Sound, at Poplar Branch, NC	N 36.28722° W 75.88365°	5/31/08	6 psu
16	Currituck Banks, behind marsh	N 36.39002° W 75.83647°	5/31/08	4 psu
17	Kitty Hawk Bay, at Kitty Hawk, NC	N 36.06453° W 75.72331°	5/31/08	9 psu
18	Roanoke Sound, at Roanoke Island	N 35.89433° W 75.63760°	5/31/08	15 psu
19	Croatan Sound, at Roanoke Island	N 35.92626° W 75.72263°	5/31/08	13 psu

20	East Lake, at Marshes, NC	N 35.92826° W 75.81487°	5/31/08	1 psu
21	Alligator River, at East Lake Landing	N 35.89732° W 75.97051°	5/31/08	12 psu
22	Scuppernong River, at Columbia, NC	N 35.91528° W 76.25456°	5/31/08	3 psu
23	Cashie River, at Windsor, NC	N 35.99297° W 76.94241°	6/1/08	0 psu
24	Lake Phelps, at Pettigrew State Park	N 35.78972° W 76.41141°	5/31/08	0 psu
25	Currituck Banks, at duck blind	N 36.38718° W 75.84788°	6/20/08	7.4 psu
26	Currituck Banks, at duck blind	N 36.38699° W 75.84828°	9/12/08	8.4 psu

Table 2: The changes in the salinity regime at the main Currituck Banks sampling site, utilized in all 4 bioassays, throughout the course of the study period.

Date Sampled	Experiment	Salinity
6/22/07	Bioassay 1	0.0 psu
9/14/07	Bioassay 2	4.7 psu
6/20/08	Bioassay 3	7.4 psu
9/12/08	Bioassay 4	8.4 psu

Table 3: The initial chlorophyll *a* concentrations, before filtering or any *C. raciborskii* additions, of the sites used in the bioassays.

Date Sampled	Site	Experiment	Chlorophyll *a* Concentration (µg/L)
6/22/07	1	Bioassay 1	17.8
6/22/07	2	Bioassay 1	14.5
9/14/07	2	Bioassay 2	43.5
6/20/08	2	Bioassay 3	17.0
9/12/08	2	Bioassay 4	22.3

Table 4: Chlorophyll *a* concentrations for T_4 for the filtered treatments for both sampling sites of Bioassay 1, performed in June 2007. The results indicate that *C. raciborskii* biomass, as indicated by Chl *a*, is limited by nitrogen at both sites. The P value for nitrogen additions across both sites was 0.0071.

A) Site 1

Treatment	**Chlorophyll *a* Concentration (µg/L)**	**SD**
Media	0.12	0.07
Control	2.46	0.12
Nitrate	**29.7**	**1.2**
Phosphate	2.67	0.25
Nitrate + Phosphate	**28.1**	**3.2**

B) Site 2

Treatment	**Chlorophyll *a* Concentration (µg/L)**	**SD**
Media	0.12	0.07
Control	1.97	0.14
Nitrate	**9.69**	**1.00**
Phosphate	2.24	0.30
Nitrate + Phosphate	**14.8**	**1.60**

Table 5: Growth rates, calculated using the formula suggested by Slater 1988, for the filtered treatments for both sampling sites of Bioassay 1, performed in June 2007. The results indicate that *C. raciborskii* growth is limited by nitrogen at both sites. The P value for nitrogen additions across both sites was 0.0012.

A) Site 1

Treatment	Growth Rate (d^{-1})	SD
Media	-0.13	0.16
Control	0.25	0.06
Nitrate	**0.79**	**0.21**
Phosphate	0.26	0.16
Nitrate + Phosphate	**0.61**	**0.26**

B) Site 2

Treatment	Growth Rate (d^{-1})	SD
Media	-0.13	0.16
Control	0.29	0.04
Nitrate	**1.03**	**0.10**
Phosphate	0.21	0.12
Nitrate + Phosphate	**0.98**	**0.05**

Table 6: Primary production rates for T_4 for the filtered treatments for both sampling sites of Bioassay 1, performed in June 2007. The results indicate that *C. raciborskii* production is limited by nitrogen at both sites. The P value for nitrogen additions across both sites was 0.0348.

A) Site 1

Treatment	^{12}C Assimilated (mg C m^{-3} h^{-1})	SD
Media	0.02	0.18
Control	34.3	16.7
Nitrate	**542**	**209**
Phosphate	38.9	12.7
Nitrate + Phosphate	**406.4**	**251**

B) Site 2

Treatment	^{12}C Assimilated (mg C m^{-3} h^{-1})	SD
Media	0.02	0.18
Control	11.4	7.09
Nitrate	**84.9**	**19.4**
Phosphate	16.0	2.40
Nitrate + Phosphate	**124**	**42.4**

Table 7: Chlorophyll *a* concentrations for T_8 for the filtered treatment of Bioassay 2, performed in September 2007. The results indicate that *C. raciborskii* had a higher biomass in the Z8 media, and that in the Currituck Sound water, *C. raciborskii* biomass, as indicated by Chl *a*, is limited by nitrogen (The P value for nitrogen additions was 0.0458).

Treatment	Chlorophyll *a* Concentration (µg/L)	SD
Media	78.7	25.5
Control	2.35	0.54
Nitrate	**33.0**	**12.3**
Ammonium	**18.7**	**5.18**
Phosphate	1.48	0.43
Nitrate + Phosphate	**11.9**	**3.25**
Ammonium + Phosphate	**10.8**	**2.79**
Nitrate + Ammonium	**22.3**	**6.59**
Nitrate + Ammonium + Phosphate	**22.8**	**6.67**

Table 8: Primary production rates for T_8 for the filtered treatment of Bioassay 2, performed in September 2007. *C. raciborskii* production was limited by nitrogen and, to a certain extent, by phosphorus as well. Though production rates were lower in the Currituck Sound water treatments that the media treatment earlier in the bioassay (**FIGURE 18**), by T_8 production is highest in the nitrogen treatments (P value for nitrate additions was 0.0213, P value for ammonium additions was 0.0066).

Treatment	^{12}C Assimilated (mg C m^{-3} h^{-1})	SD
Media	66.8	47.3
Control	8.45	3.62
Nitrate	**101**	**52.1**
Ammonium	**108**	**53.0**
Phosphate	7.31	5.94
Nitrate + Phosphate	**24.4**	**13.9**
Ammonium + Phosphate	**66.4**	**19.6**
Nitrate + Ammonium	**152**	**42.1**
Nitrate + Ammonium + Phosphate	**154**	**75.0**

Table 9: Chlorophyll *a* concentrations for T_8 for the filtered treatment of Bioassay 3 (A) and 4 (B), performed in June and September 2008.

A) In June 2008, though *C. raciborskii* biomass was lower in the Currituck Sound water treatments than the media treatment earlier in the bioassay (**FIGURE 19**), by T_8 the added *C. raciborskii* had recovered and Chl *a* concentration was highest in the nitrogen treatments, but this was not statistically significant.

Treatment	**Chlorophyll *a* Concentration (µg/L)**	**SD**
Media	12.7	8.75
Media + Nitrate	14.1	2.95
Control	3.64	0.74
Nitrate	42.7	3.96
Ammonium	40.8	6.06
Phosphate	5.36	1.68
Nitrate + Phosphate	42.2	2.60
Ammonium + Phosphate	49.9	6.10
Nitrate + Ammonium	35.0	6.37
Nitrate + Ammonium + Phosphate	43.2	17.3

Table 9 (continued): **B**) In September 2008, *C. raciborskii* biomass was highest in the media treatments throughout the course of the bioassay (**FIGURE 20**). In the Currituck Sound water treatments, Chl *a* was very low throughout, not even reaching 20 µg/L in any of the treatments by T_8. The ammonium treatments did have higher biomass than the treatments without added ammonium (P value 0.0058).

Treatment	Chlorophyll *a* Concentration (µg/L)	SD
Media	49.5	11.8
Media + Nitrate	40.9	30.2
Control	1.83	0.38
Nitrate	4.85	0.41
Ammonium	**16.1**	**2.74**
Phosphate	1.22	0.27
Nitrate + Phosphate	8.19	0.73
Ammonium + Phosphate	**15.8**	**2.79**
Nitrate + Ammonium	**13.6**	**3.55**
Nitrate + Ammonium + Phosphate	**13.6**	**5.76**

Table 10: Growth rates for the filtered treatment of Bioassay 3 (A) and 4 (B), performed in June and September 2008.

A) In June 2008, growth rates in general were very low. These results show that the nitrogen treatments, with the exception of the **N+A+P** treatment, appeared to have higher growth rates than both the media treatments and the treatments with no nitrogen added, but this was not statistically significant.

Treatment	**Growth Rate (d^{-1})**	**SD**
Media	0.16	0.07
Media + Nitrate	0.28	0.02
Control	0.75	0.07
Nitrate	0.90	0.08
Ammonium	0.87	0.07
Phosphate	0.57	0.11
Nitrate + Phosphate	0.89	0.06
Ammonium + Phosphate	0.88	0.02
Nitrate + Ammonium	0.93	0.10
Nitrate + Ammonium + Phosphate	0.70	0.07

Table 10 (continued): **B**) In September 2008, growth rates were the lowest of all 4 bioassay, indicating significant salinity stress in water, which was 8.4 psu**.** No real nutrient limitation was indicated by these results. Three treatments, **A+P**, **N+A**, and **N+A+P,** were slightly higher than the other treatments, but this was not statistically significant. In general, GR was very low.

Treatment	**Growth Rate (d^{-1})**	**SD**
Media	0.11	0.02
Media + Nitrate	0.02	0.19
Control	0.35	0.03
Nitrate	0.39	0.08
Ammonium	0.38	0.02
Phosphate	0.03	0.09
Nitrate + Phosphate	0.29	0.08
Ammonium + Phosphate	0.53	0.10
Nitrate + Ammonium	0.47	0.08
Nitrate + Ammonium + Phosphate	0.41	0.06

Table 11: Primary production rates for T_6 for the filtered treatment of Bioassay 3 (A) and 4 (B), performed in June and September 2008.

A) In June 2008, there was a response in primary production rates to ammonium additions (P value 0.0437), suggesting that nitrogen is limiting, but that the energetic cost of reducing nitrate to ammonium may be too great for *C. raciborskii* when already stressed by high salinity (7.4 psu).

Treatment	**^{12}C Assimilated (mg C m^{-3} h^{-1})**	**SD**
Media	39.5	20.0
Media + Nitrate	37.4	7.13
Control	60.7	6.27
Nitrate	225	77.9
Ammonium	**1040**	**92.4**
Phosphate	23.6	3.19
Nitrate + Phosphate	242	159
Ammonium + Phosphate	**1270**	**210**
Nitrate + Ammonium	**490**	**35.0**
Nitrate + Ammonium + Phosphate	**424**	**131**

Table 11 (continued): **B**) In September 2008, primary production rates were very low throughout the course of the bioassay. As in June 2008, there was a response in primary production rates to ammonium additions (P value 0.0062), again suggesting that nitrogen is limiting, but when already faced with high salinity conditions (8.4 psu), the energetic cost of reducing nitrate would be too great, resulting in a strong preference for ammonium.

Treatment	**^{12}C Assimilated (mg C m^{-3} h^{-1})**	**SD**
Media	80.6	20.5
Media + Nitrate	75.3	64.0
Control	6.09	0.98
Nitrate	10.5	4.25
Ammonium	**79.7**	**7.48**
Phosphate	5.20	0.76
Nitrate + Phosphate	28.1	2.45
Ammonium + Phosphate	**78.7**	**18.4**
Nitrate + Ammonium	**64.4**	**8.11**
Nitrate + Ammonium + Phosphate	**50.0**	**0.37**

Table 12: Chlorophyll *a* concentrations for T_4 for the unfiltered treatments for both sampling sites of Bioassay 1, performed in June 2007. The results indicate that *C. raciborskii* biomass, as indicated by Chl *a*, was limited by nitrogen at both sites at this time point, although phosphorus becomes limiting later in the bioassay. The P value for nitrogen additions across both sites was 0.002.

A) Site 1

Treatment	**Chlorophyll *a* Concentration (µg/L)**	**SD**
Control	33.7	1.23
Nitrate	**42.2**	**2.63**
Phosphate	37.0	3.49
Nitrate + Phosphate	**44.8**	**2.58**

B) Site 2

Treatment	**Chlorophyll *a* Concentration (µg/L)**	**SD**
Control	23.7	2.48
Nitrate	**35.1**	**2.52**
Phosphate	23.2	6.49
Nitrate + Phosphate	**32.5**	**4.34**

Table 13: Primary production rates for T_4 for the unfiltered treatments for both sampling sites of Bioassay 1, performed in June 2007. The results indicate that *C. raciborskii* production was limited by nitrogen at both sites at this time point, although phosphorus becomes limiting later in the bioassay. The P value for nitrogen additions across both sites was 0.0031.

A) Site 1

Treatment	**^{12}C Assimilated (mg C m^{-3} h^{-1})**	**SD**
Control	343	19.4
Nitrate	**502**	**67.0**
Phosphate	354	59.3
Nitrate + Phosphate	**495**	**31.4**

B) Site 2

Treatment	**^{12}C Assimilated (mg C m^{-3} h^{-1})**	**SD**
Control	201	13.5
Nitrate	**303**	**13.7**
Phosphate	168	40.3
Nitrate + Phosphate	**250**	**32.3**

Table 14: *C. raciborskii* counts for T_8 for the unfiltered treatments for both sampling sites of Bioassay 1, performed in June 2007. The results indicate that *C. raciborskii* abundance is highest in the nitrate treatment, making it most competitive in this treatment.

A) Site 1

Treatment	***C. raciborskii* Concentration (filaments/mL)**	**SD**
T_0	910	200
T_8 Control	470	150
T_8 Nitrate	**1700**	**340**
T_8 Phosphate	301	170
T_8 Nitrate + Phosphate	790	240

B) Site 2

Treatment	***C. raciborskii* Concentration (filaments/mL)**	**SD**
T_0	1090	260
T_8 Control	680	190
T_8 Nitrate	**1400**	**301**
T_8 Phosphate	720	180
T_8 Nitrate + Phosphate	510	150

Table 15: *C. raciborskii* counts for T_8 for the unfiltered treatments for Bioassay 2, performed in September 2007. The results indicate that *C. raciborskii* abundance is highest in the nitrogen added treatments. No nutrient additions and phosphorus additions favored other members of the phytoplankton community.

Treatment	***C. raciborskii* Concentration (filaments/mL)**	**SD**
T_0	1500	250
T_8 Cyl Control	1200	204
T_8 Control	1600	130
T_8 Nitrate	**2300**	**260**
T_8 Ammonium	**2000**	**120**
T_8 Phosphate	1500	120
T_8 Nitrate + Phosphate	1500	120
T_8 Ammonium + Phosphate	1200	150
T_8 Nitrate + Ammonium	**2500**	**100**
T_8 Nitrate + Ammonium + Phosphate	1100	120

Table 16: *C. raciborskii* counts for T_8 for the unfiltered treatments for Bioassay 3, performed in June 2008. The results indicate that *C. raciborskii* abundance is highest in the nitrogen added treatments. No nutrient additions and phosphorus additions favored other members of the phytoplankton community.

Treatment	***C. raciborskii* Concentration (filaments/mL)**	**SD**
T_0	0.0	0.0
T_8 Cyl Control	0.0	0.0
T_8 Control	62	48
T_8 Nitrate	330	82
T_8 Ammonium	310	180
T_8 Phosphate	42	48
T_8 Nitrate + Phosphate	540	150
T_8 Ammonium + Phosphate	590	150
T_8 Nitrate + Ammonium	**820**	**150**
T_8 Nitrate + Ammonium + Phosphate	310	100

Table 17: *C. raciborskii* counts for T_8 for the unfiltered treatments for Bioassay 4, performed in September 2008. The results indicate that *C. raciborskii* abundance is highest in the nitrogen added treatments. No nutrient additions and phosphorus additions favored other members of the phytoplankton community.

Treatment	***C. raciborskii* Concentration (filaments/mL)**	**SD**
T_0	0.0	0.0
T_8 Cyl Control	0.0	0.0
T_8 Control	73	61
T_8 Nitrate	300	190
T_8 Ammonium	340	150
T_8 Phosphate	47	29
T_8 Nitrate + Phosphate	560	120
T_8 Ammonium + Phosphate	620	88
T_8 Nitrate + Ammonium	**860**	**210**
T_8 Nitrate + Ammonium + Phosphate	330	120

Table 18: Chl *a* concentration for T_{10} of the salinity experiment, performed in July 2008. The results indicate that *C. raciborskii* biomass is significantly limited by salinity and that nitrogen increases its ability to recover from salinity stress.

Treatment	Chl *a* Concentration (µg/L)	SD
0 psu Control	703.7	43.1
0 psu + Nitrate	992	120.0
3 psu Control	100.0	36.2
3 psu + Nitrate	279	75.4
6 psu Control	1.02	0.55
6 psu +Nitrate	1.13	0.65
9 psu Control	0.35	0.14
9 psu + Nitrate	0.61	0.88

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