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Determining the potential for the proliferation of the harmful cyanobacterium *Cylindrospermopsis raciborskii* in Currituck Sound, North Carolina

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ABSTRACT

Cylindrospermopsis raciborskii is a tropical invasive, toxin-producing, filamentous-heterocystous, N_2-fixing cyanobacterium that has recently expanded its range in temperate waterways. Because it is capable of exploiting low salinity (oligohaline) waters experiencing nutrient enrichment, it opens up brackish eutrophying systems to potential invasion. We examined the susceptibility of oligohaline (historically 0–3.5 salinity) Currituck Sound (CS), North Carolina to *C. raciborskii* proliferation during 2007–2008. This component of the Albemarle-Pamlico Sound estuarine system is experiencing incipient eutrophication. We addressed the following questions: (1) Is *C. raciborskii* currently present in CS, and (2) what conditions favor its growth and expansion? In 2007, *C. raciborskii* was confirmed by microscopy, diagnostic photopigment and molecular analyses, which further revealed its genetic potential to produce the cyanotoxin cylindrospermopsin. In 2008, CS salinity had risen due to a persistent drought, and *C. raciborskii* was no longer microscopically observed. The potential for *C. raciborskii* to grow in CS was assessed using *in situ* nutrient addition bioassays. Primary productivity, nitrogen fixation (nitrogenase activity) rates and chlorophyll *a* measurements demonstrated that *C. raciborskii* could grow in CS water, and dissolved inorganic nitrogen (nitrate and ammonium) additions increased its growth potential. Salinity was a key factor influencing *C. raciborskii* growth, with elevated salinity (8.4) significantly limiting biomass accumulation. Interestingly, nitrogen enrichment enabled *C. raciborskii* to better withstand elevated salinities and increased its competitive success in the CS phytoplankton community. Emphasis should be placed on controlling nutrient, particularly nitrogen, enrichment in order to prevent the expansion of *C. raciborskii* in this and other nutrient-sensitive oligohaline ecosystems.

1. Introduction

Invasive species, i.e., species that have had a minor presence within an ecosystem, but because of external perturbations have increased their dominance in and impact on the ecosystem (Colautti and MacIssac, 2004), are among the most important challenges facing protection, conservation and management of aquatic ecosystems. Ecosystem-level changes in estuaries include the increased influence by human urban, agricultural and industrial activities in watersheds, accompanied by nutrient and sediment enrichment, and alteration of natural water flow (Allen et al., 2006). Invasive species can negatively impact ecosystems in multiple ways, including out-competing native species, reducing biodiversity, altering biogeochemical cycling and food web dynamics, and adversely affecting human use of impacted habitats.

A phytoplankter exhibiting invasive behavior in temperate to tropical nutrient-enriched waters is the toxin-producing, filamentous-heterocystous, nitrogen (N_2)-fixing freshwater cyanobacterium *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya et Subba Raju. This species 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), and it is characterized by conical terminal heterocysts (Seenayya and Raju, 1972). *C. raciborskii* can potentially produce the tri-cyclic alkaloid cyanotoxin cylindrospermopsin (CYN; Hawkins et al., 1985; Saker and Neilan, 2001), which has been linked to illness in animals (Thomas et al., 1998; Saker et al., 1999), including humans (Byth, 1980; Bourke et al., 1983; Hawkins et al., 1985; Hayman, 1992).

Though prevalent in tropical and subtropical waters (Seenayya and Raju, 1972), *C. raciborskii* has significantly expanded its range over the past 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; Briand et al., 2004; Codd et al., 2005). It is a particularly aggressive invader in US Southeast, Midwest and Southwest lakes, reservoirs and rivers (Chapman and Schelske, 1997; Dyble et al., 2002; Paerl and Fulton, 2006).

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Cylindrospermopsis raciborskii has several physiological characteristics that contribute to its ability to invade non-native temperate environments. Its competitive abilities are favored by nutrient enrichment, relatively high temperatures and relatively low incident irradiation (optimal growth at 30 °C and at near 10% of surface irradiance, or 120 μmol photons m^{-2} s^{-1}; Shafik et al., 2001), high pH, poor flushing (long water residence time), and persistent vertical stratification (Dokulil and Mayer, 1996; McGregor and Fabbro, 2000; Briand et al., 2002, 2004). In addition, *C. raciborskii* tolerates a wide range of environmental conditions, as it can form blooms under varying light, temperature and nutrient regimes (Isvánovics et al., 2000; Sprober et al., 2003; Briand et al., 2004). It can at times thrive in nitrogen (N) deplete waters by fixing atmospheric nitrogen (N_2), while in N-enriched waters it effectively competes for combined N sources with eukaryotic phytoplankton species (Bouvy et al., 2000; Paerl and Fulton, 2006; Moisander et al., 2008; Piehler et al., 2009). It also has high uptake affinity and an excellent storage capacity for phosphorus (Isvánovics et al., 2000). While its preferred habitat is fresh to slightly brackish waters, it can tolerate elevated dissolved minerals (Briand et al., 2002), including low levels of salinity (up to ~4; Moisander et al., 2002). Whereas other cyanobacterial bloom species require high irradiance to reach their maximum growth potential and therefore form surface blooms, *C. raciborskii* is unique in that it generally forms subsurface blooms (Padisak, 1997), and its maximum photosynthetic rate (P^{b}_{max}) occurs at relatively low light levels (30–400 μmol photons m^{-2} s^{-1}, Briand et al., 2004). This enables it to bloom beneath other algal species, resist the effects of self-shading, and form blooms in waters where clarity is impaired.

In North Carolina, routine monitoring by the North Carolina Department of Environment and Natural Resources (DENR) Division of Water Quality (DWQ) indicates widespread presence of *C. raciborskii* throughout the State's waterways, including nutrient-enriched reservoirs (e.g., Falls of the Neuse reservoir) and riverine tributaries of the Pamlico and Albemarle Sounds (E. Fensin, NC-DENR, pers. comm.).

One large water body that could be susceptible to invasion by *C. raciborskii* is the brackish Currituck Sound (CS), in northeastern North Carolina. The CS receives discharge from the State's northernmost riverine tributaries (Chowan, Roanoke, Albemarle, and local systems) and drains into the Albemarle-Pamlico Sound Estuary, the largest lagoonal estuarine system in the United States (~79,000 ha). It is also connected to and exchanges water with the Chesapeake Bay via two canals. The CS encompasses 39,600 ha with 1.6 m mean depth (Davis and Brinson, 1983; Wicker and Endres, 1995; Fig. 1). It supports commercial and recreational fisheries and is a critical habitat for waterfowl. The CS is a unique estuarine environment due to its low salinity, which ranges from fresh (0) to oligohaline (~3.5; Caldwell, 2001). These low salinity conditions provide a habitat in which *C. raciborskii* could potentially thrive (Padisak, 1997; Chapman and Schelske, 1997). The combined effect of low salinity and increasing nutrient enrichment associated with development in Northeastern North Carolina and Southeastern Virginia coastal plain watersheds creates conditions amenable for invasion and proliferation by *C. raciborskii*. Also, CS exhibits highly variable light conditions, which favor algal species that reach maximum photosynthetic activity (P^{b}_{max}) at relatively low light levels, like *C. raciborskii* (Briand et al., 2004). In addition to exhibiting optimal salinity and light conditions for the growth of *C. raciborskii*, CS also possesses the requisite temperature and nutrient conditions to support the invasion and/or expansion of this species (Paerl and Huisman, 2009).

The purpose of this study was to determine whether *C. raciborskii* is currently present in CS, and to clarify its invasion potential for this large system. In order to answer the second question, we determined whether *C. raciborskii* could grow in CS water, i.e., if the environmental conditions, including salinity, are adequate for *C. raciborskii* growth and whether *C. raciborskii* can adequately compete with the existing phytoplankton community under nutrient-enriched conditions.

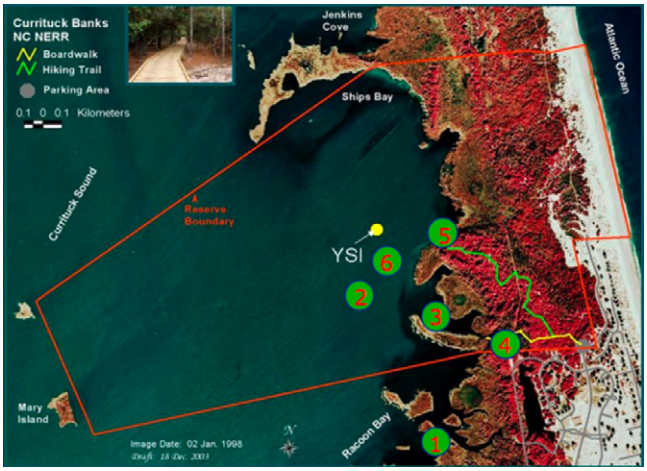

Fig. 1. A map, reproduced courtesy of NC NERRS, of the Currituck Banks reserve that served at the main study site for this project. The reserve encompasses 950 acres and is located between the town of Corolla, NC and the Virginia border. Sampling sites for 2007 and 2008 are shown.

2. Materials and methods

2.1. Study site

The study site was the Currituck Banks component of the North Carolina National Estuarine Research Reserve (CBNERR; www.nccoastalreserve.net; Fig. 1). This 950-acre reserve is located just north of town of Corolla, about 15 km south of the North Carolina-Virginia border. It separates the Atlantic Ocean from the rest of CS, and is characterized by its low salinity estuarine habitat. 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.

2.2. Determining *C. raciborskii*'s presence in CS

Surface water was collected from several locations within Currituck Banks and the surrounding CS in summer 2007 and 2008, using pre-cleaned (stored in dilute HCl, followed by several rinses of deionized water and a sample rinse) 25-L polyethylene bottles. Fig. 1 shows the sampling locations. Water was then transported back to IMS under ambient temperature and light conditions for analysis.

At each location, sub-samples for microscopic species identification were collected in 80 mL borosilicate vials and preserved immediately with 1% acid Lugol's solution. The samples were then allowed to settle and viewed at 400× (10× eyepiece and 40× objective) magnification under an inverted microscope (Leica M-20), and the presence and abundance of *C. raciborskii* was estimated by counting filaments (all filaments visualized were straight with one terminal heterocyst).

Molecular techniques were used to confirm microscopic analysis. Deoxyribonucleic acid (DNA) was extracted from Millipore, 0.7 μm 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 complex mediating N_2 fixation. *NifH* is highly conserved among N_2-fixers, but has enough variable regions such that species distinction is possible (Zehr and Paerl, 2008). DNA samples were amplified with the cylindro-*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 min, followed by 30 cycles of 94 °C for 10 s, 55 °C for 20 s, and 72 °C for 1 min, followed by an extension at 72 °C for 7 min (Dyble et al., 2002). Results were visualized using gel electrophoresis and used to determine the presence of *C. raciborskii*.

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 Fergusson and Saint (2003). Three 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 encode for necessary proteins in CYN synthesis. If a sample was positive for all three genes, it was determined to be positive for *C. raciborskii* production of 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 min, followed by 30 cycles of 94 °C for 30 s, 45 °C for 30 s, and 72 °C for 1 min, followed by an extension at 72 °C for 7 min (Fergusson and Saint, 2003).

2.3. Determining *C. raciborskii*'s growth potential in CS

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 assess *in situ* growth and bloom potentials of *C. raciborskii*. The results between the two years of the study differed significantly, and we focused on the results of the first and last bioassays, performed in June 2007 and September 2008 respectively, as these bioassays showed the greatest contrast in salinity conditions and cyanobacterial population composition.

In June 2007 and September 2008, surface water was collected from a representative location within the CBNERR (Fig. 1, sites 2 and 6 respectively), and filtered through a 53-μm porosity Nitex 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 under ambient light and temperature conditions.

The filtered water sample was then divided into two filtration treatments. The filtered treatments, in which cultured *C. raciborskii* was added to GF/F filtered CS water (to remove all naturally occurring phytoplankton), were used to determine whether the environmental conditions in the CS, specifically for the ambient salinity regime, were satisfactory for *C. raciborskii* growth and under what nutrient conditions *C. raciborskii* growth was favored. Unfiltered treatments, in which *C. raciborskii* was added to whole CS water, were used to determine whether *C. raciborskii* could effectively compete within the existing CS phytoplankton community, and which nutrient conditions would increase its competitiveness. 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 glass fiber filters (~0.7 μm porosity). Once the filtration was complete, 40 L of water from each filtration treatment was divided among 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 that are 85% transparent to PAR (Hedwin Corporation; Moisander and Paerl, 2000). Dissolved inorganic carbon (DIC) solution ($NaHCO_3$ stock solution, 2 mg C L^{-1} 1 final concentration) was added to each Cubitainer to ensure that growth and production in the vessel was not limited by DIC availability (Moisander and Paerl, 2000). In June 2007, each filtration treatment was divided into 4 nutrient treatments, each 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 2008 bioassay, 8 nutrient treatments were used, 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 and5 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). Following nutrient additions, an inoculum of a representative *C. raciborskii* strain was added to each Cubitainer. The inoculum was *C. raciborskii* strain Cyl L (maintained at the UNC-IMS). This strain was originally isolated from Lake Griffin, Florida, and purified by Dr. P. Moisander (pers. comm.). Inocula were grown in batch cultures in N-free Z8 medium (Rippka 1988) in a growth chamber (23–28 °C, 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.

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^{-1} or 0.17 mM final concentration), but no nutrients were added. Z8 media is designed specifically for culturing N_2-fixing cyanobacteria and should provide these organisms with an optimal 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* grew better in the media or within the CS water, in order to establish its preferred growth medium. In the 2008 bioassay, a second media control was added, consisting of 4 Cubitainers containing 2.5 L Z8 media with additional nitrate added (10 μM KNO_3) as well as an additional control consisting of unfiltered water with no Cyl L added.

Cubitainers were incubated in floating corrals covered with a layer of neutral density screening under natural light and temperature conditions in a circulating seawater pond located behind the UNC-IMS. This setup mimicked *in situ* conditions of CS. 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 300–500 mL removed, to track the progress of the bioassay. These samples were analyzed for salinity, pH, chlorophyll *a*, diagnostic photopigments (chlorophylls and carotenoids), primary productivity, N_2-fixation (nitrogenase activity, using the acetylene reduction technique), DIC, carbon, hydrogen and nitrogen (CHN) concentrations, nutrient (NO_3^-, NH_4^+, and PO_4^{3-}, SiO_2) concentrations, and *C. raciborskii* abundance (see methods below). Ambient light and temperature data were recorded.

As indicators of cyanobacterial growth, primary productivity and N_2 fixation were assayed (c.f. Paerl et al., 2005). Subsamples were incubated for 4 h in a small corral covered with a layer of neutral density screening in the UNC-IMS pond. Primary productivity (PP) was measured using the $NaH^{14}CO_3$ incorporation method 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 (nitrogenase activity) rates were estimated using the acetylene reduction (AR) assay as described by Burris (1972) and modified by Paerl (1998), using 25 mL AR vials, 15 mL sample volume and 2.5 mL acetylene addition. Ethylene production (from acetylene) mediated by nitrogenase was measured by flame ionization gas chromatography, using a Shimadzu GC9 gas chromatograph. As a measure of algal biomass and growth, chlorophyll *a* concentration (Chl *a*) was determined in parallel with activity measurements. The fluorometric technique of Welschmeyer (1994) was used for determining Chl *a*, using a Turner TD-700 fluorometer. Growth rates (GR) were then calculated based on Chl *a* data using a best curve fit for exponential growth, described by Slater (1988). Dissolved inorganic nutrients (NO_3^-, NH_4^+, and PO_4^{3-}, 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). Particulate carbon, hydrogen and nitrogen were assessed using a 2400 Series II CHN analyzer (Perkin Elmer). Salinity of the water was determined at the start of the experiment using a YSI 6600 sonde, and was reassessed throughout the course of the experiment using a hand-held refractometer. pH was determined using a BASIC pH meter (Denver Instruments).

All statistical analyses were performed using MATLAB (MathWorks). Differences in Chl *a* concentration, growth rate and productivity in different treatments between water from CS 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.

3. Results and discussion

3.1. Determining C. raciborskii's presence in CS

Microscopic and molecular analysis of water samples taken from CS, specifically the Currituck Banks component of the North Carolina National Estuarine Research Reserve (CBNERR, Fig. 1) in 2007 showed a phytoplankton community containing diverse N_2-fixing cyanobacterial species, including *C. raciborskii* (Fig. 2) (*C. raciborskii* concentrations ranged from 910 to 1500 cells mL^{-1}), *Anabaena* spp., *Aphanizomenon* spp., and *Anabaenopsis* spp. DNA samples were also collected and processed from all water samples, and amplification of the CYL toxin genes, including *cyl* (308 bp), *ps* (597 bp) and *pks* (422 bp)indicated that *C. raciborskii* populations at sites 1, 2, and 3 had the genetic potential to produce the CYN toxin (Fig. 3).

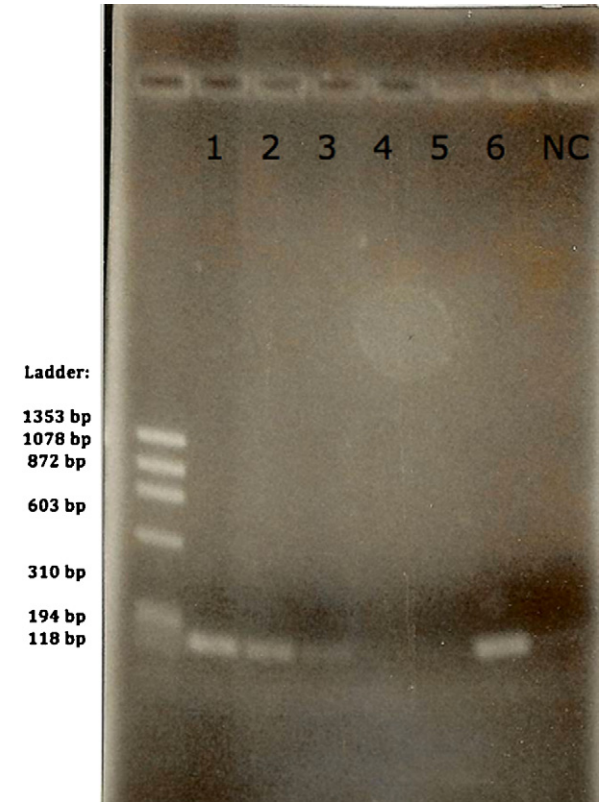

Fig. 2. The results from the PCR assay of the more specific cylindro-*nif*H gene, used to determine the presence or absence of *C. raciborskii*.

In 2008, microscopic results from sites 2, 3, and 6 (Fig. 1) were markedly different from 2007. The phytoplankton community had shifted, containing none of the cyanobacterial bloom species observed in 2007, including *C. raciborskii*. Instead, diatoms and dinoflagellates prevailed, including *Thalassiosira* spp. and

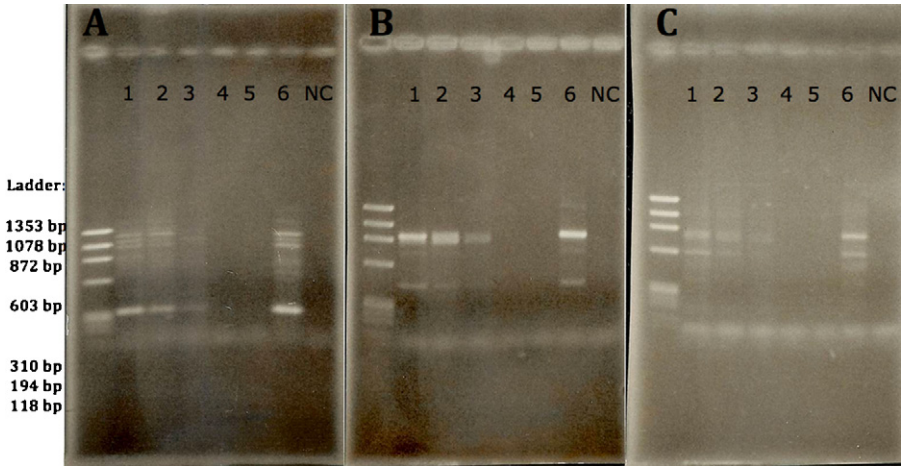

Fig. 3. The results of the PCR assays targeting three main genes in the pathway to create CYN. (A) The results from the PCR assay of *C. raciborskii*-specific *cyl*. (B) The results from the PCR assay of *pks*, which codes for a necessary protein in CYN synthesis. (C) The results from the PCR assay of *ps*, which codes for a necessary protein in CYN synthesis.

Table 1
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	Salinity
6/22/2007	0.0
9/14/2007	4.7
6/20/2008	7.4
9/12/2008	8.4

Prorocentrum spp. respectively. Assays of the cylindro-*nif*H gene and the *C. raciborskii* toxin suite of *cyl*, *ps* and *pks* were further used to determine that *C. raciborskii* was not present in any of the samples from CS in 2008.

This shift may have been due to extreme drought conditions in North Carolina, which started in 2007 and persisted through 2008. According to the drought classifications of 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 remained extreme or exceptional until March 2008, and despite some periods of precipitation in 2008, moderate drought conditions persisted into early 2009, making this the worst drought in North Carolina since record keeping began in 1895 (W. Yonts, pers. comm). During this study, water samples were collected at CBNERR at 4 different times, and the effect of the drought on the salinity regime was evident (Table 1). This strongly affected phytoplankton community composition, especially during September 2008, when salinity was the highest on record for CS (Caldwell, 2001; NC-DENR).

The severity of drought that North Carolina experienced throughout 2007 and 2008 produced salinity increases in CS that were inhospitable for both *C. raciborskii* and its N_2-fixing competitors. This caused the phytoplankton community to shift from largely freshwater cyanobacterial species to eukaryotic species more tolerant to the elevated salinity. Cyanobacterial species, including *C. raciborskii*, may have remained within the CS, either by forming akinetes or by taking refuge in the fresher feeder creeks to CS, although this was not investigated in this study. We expect that this situation will reverse when more favorable conditions (i.e., reduced salinity) prevail.

3.2. Determining *C. raciborskii* growth potential in CS

The first of a series of growth potential bioassays was conducted in late June 2007, when the CS water was essentially fresh (~0) at the time of collection (Table 1). Contrary to our initial hypothesis, *C. raciborskii* thrived in the CS water, indicated by elevated Chl *a* concentrations (used as a proxy for biomass) in CS water treatments as opposed to the media treatment (M, Fig. 4a). Acetylene reduction (AR) rates (nitrogenase activity), which were used as a proxy for nitrogen fixation (Burris, 1972; Paerl, 1998) were high in M throughout the course of the bioassay (Fig. 4b), indicating that the need for maintaining high nitrogen fixation rates in the nitrogen-free Z8 media resulted in decreased growth and thereby decreased biomass and lower Chl *a* as compared with the control (C) treatment. The CS water contained combined nitrogen (undetectable nitrate but a mean of 40.4 μg $N–NH_4$ L^{-1}), and the added *C. raciborskii* utilized ambient combined nitrogen for growth and biomass formation (Fig. 4a). These results suggest that CS water in 2007 possessed the necessary environmental conditions, including salinity and nutrients, to sustain *C. raciborskii* growth and support bloom development.

For both sites, the treatments containing nitrogen (N) additions (N and N + P) yielded significantly higher biomass (as Chl *a* concentration, Fig. 4a) than the treatments without N added (Table 2, P value 0.0071). This suggests that when *C. raciborskii* is removed

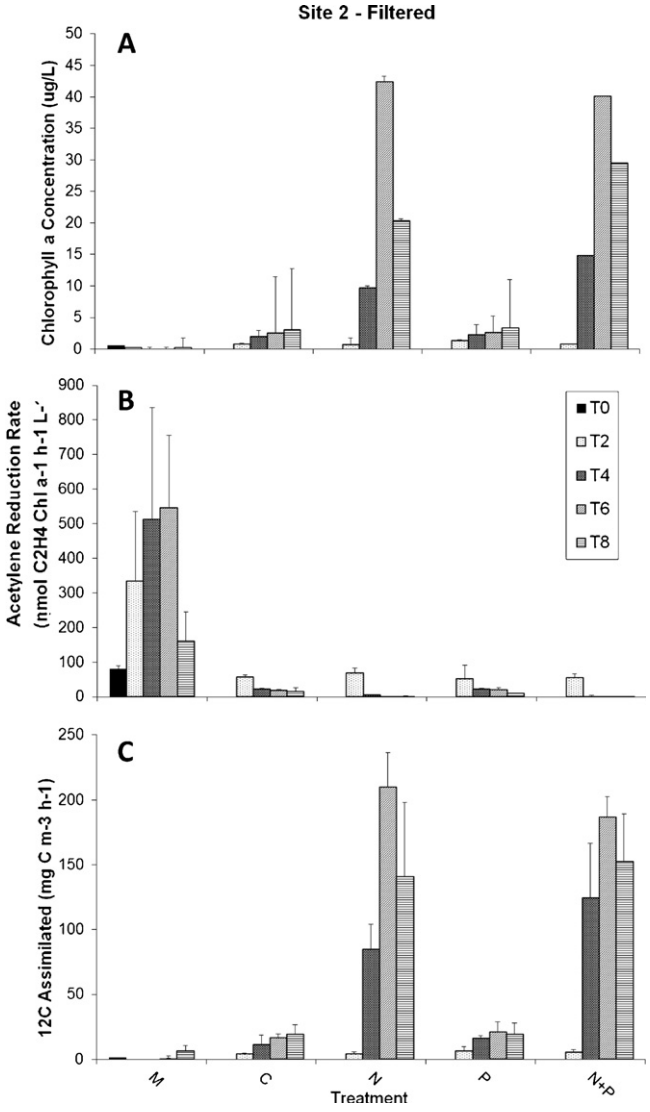

Fig. 4. Results for the filtered treatments of the June 2007 bioassay. (a) Chl *a* concentrations, used as a proxy for biomass. (b) Acetylene reduction rates, used as a proxy for nitrogen fixation. (c) Primary production.

from other, potentially competing phytoplankton species, N enrichment favors an increase in its biomass. Nitrogen additions also stimulated growth rates (Table 2, *P*-value 0.0012). Like Chl *a* and GR, primary production appeared controlled by nitrogen availability (Fig. 4c). For instance, at T_4, nitrate additions lead to statistically significant ($P < 0.0348$) stimulation of primary production (Table 2).

In September 2008, salinity of the Currituck Banks site had increased to 8.4 (Table 1). This increase was well beyond the previously determined salinity tolerance of *C. raciborskii* (Moisander et al., 2002). Furthermore, *C. raciborskii* was not present in the natural phytoplankton community before initiating this bioassay, suggesting that the elevated salinity was prohibitive for *C. raciborskii* growth *in situ*.

For this bioassay, a second Z8 media treatment was introduced with added nitrate to determine if *C. raciborskii* would grow better in the media if the necessity to fix atmospheric nitrogen was removed by introducing a source of bioavailable nitrogen. *C. raciborskii* biomass (Chl *a*, Fig. 5a) was greatest in the media treatments throughout the course of the bioassay. In the CS water treatments, Chl *a* was very low throughout the experiment, remaining below 20 μg L^{-1}in all of the treatments (Table 3). Both

Table 2
Chlorophyll a concentrations, growth rates, and primary production rates for T_4 for the filtered treatments in June 2007. The results indicate that *C. raciborskii* biomass, as indicated by Chl *a*, is limited by nitrogen, with a *P*-value of 0.0071. *C. raciborskii* growth was also limited by nitrogen, with a P value of 0.0012. Finally, *C. raciborskii* production is limited by nitrogen with a *P*-value of 0.0348.

Treatment	Chlorophyll *a* concentration		Growth rate		Primary productivity	
	($\mu g L^{-1}$)	SD	(d^{-1})	SD	($mg C m^{-3} h^{-1}$)	SD
Media	0.12	0.07	−0.23	0.59	0.02	0.18
Control	1.97	0.14	0.46	0.05	11.41	7.09
Nitrate	9.69	1.00	1.33	0.14	84.85	19.35
Phosphate	2.24	0.30	0.36	0.32	15.95	2.40
N + P	14.77	1.60	1.45	0.10	124.28	42.38

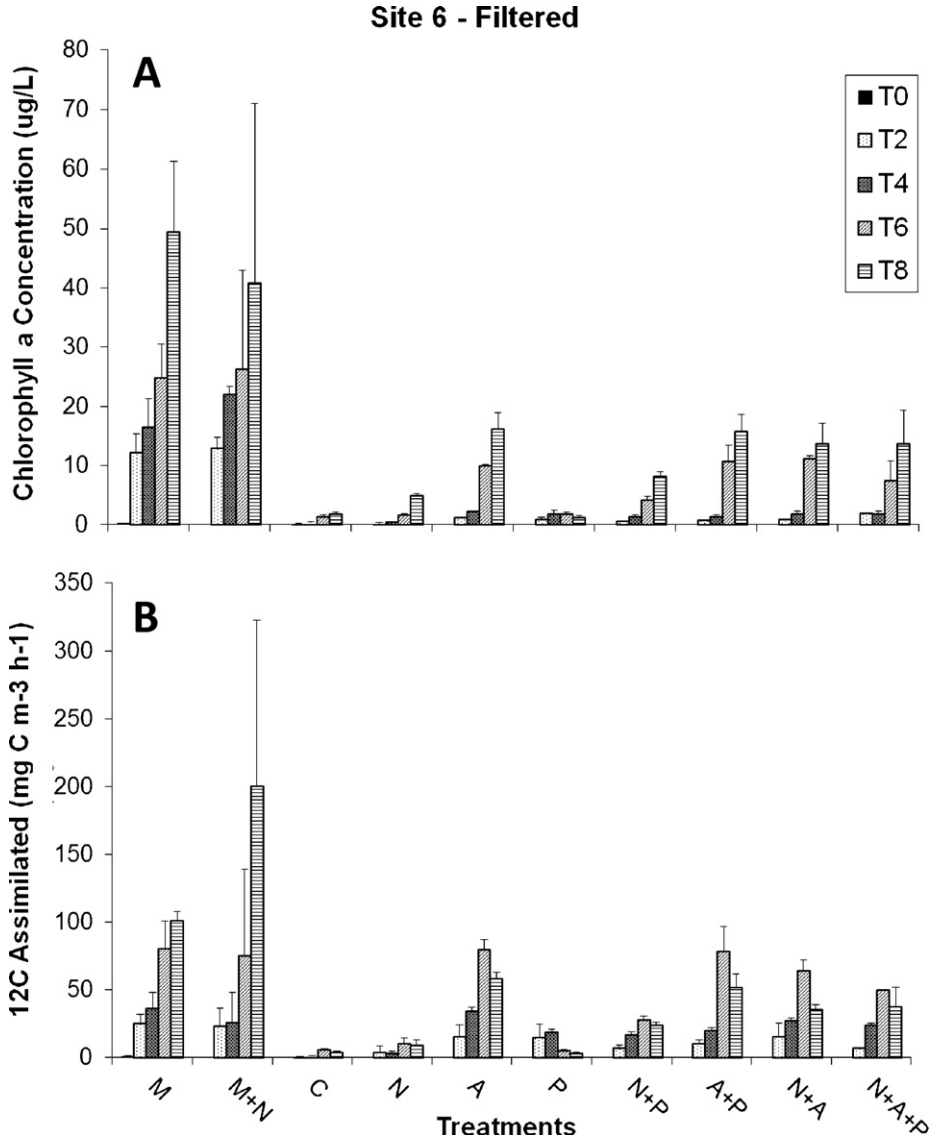

Fig. 5. Results for the filtered treatments of the September 2008 bioassay. (a) Chl *a* concentrations, used as a proxy for *C. raciborskii* biomass. (b) Primary production rates.

results suggest that salinity above 8 had a detrimental effect on *C. raciborskii* growth. Despite these negative effects of the elevated salinity, N treatments, particularly ammonium (*P*-value for ammonium additions is <0.006), had higher *C. raciborskii* biomass than the treatments receiving no nitrogen additions.

C. raciborskii primary production rates were also quite low throughout the course of the bioassay (Fig. 5b), further suggesting that *C. raciborskii* growth was greatly reduced under elevated salinity conditions. Under these conditions, ammonium–N additions triggered a stronger positive growth response than nitrate–N additions, as shown by the higher production rates in the A, A + P, N + A, and N + A + P treatments (Table 3, $P < 0.0062$). Overall, *C. raciborskii* production, appears to be favored by ammonium additions. Ammonium has long been recognized as the preferred nitrogen source for many cyanobacterial genera (Syrett, 1981) and other phytoplankton, and in the presence of high levels of

Table 3
In September 2008, the filtered ammonium treatments had higher biomass than the treatments without added ammonium (*P*-value 0.0058). There was also a response in primary production rates to ammonium additions (*P*-value 0.0062).

Treatment	Chlorophyll *a* concentration		Primary productivity	
	($\mu g L^{-1}$)	SD	($mg C m^{-3} h^{-1}$)	SD
Media	49.45	11.75	80.61	20.45
M + nitrate	40.87	30.18	75.25	63.98
Control	1.83	0.38	6.09	0.98
Nitrate	4.85	0.41	10.45	4.25
Ammonium	16.12	2.74	79.67	7.48
Phosphate	1.22	0.27	5.20	0.76
N + P	8.19	0.73	28.09	2.45
A + P	15.78	2.79	78.65	18.35
N + A	13.62	3.55	64.43	8.11
N + A + P	13.59	5.76	49.95	0.37

ammonium, nitrate uptake may be strongly suppressed (Flores et al., 1980). These energy costs, in addition to those associated with nitrogen fixation, may have proved prohibitive when *C. raciborskii* was already stressed by high salinities, as experienced in the 2008 bioassay. This would help explain the observed low primary production rates except in treatments receiving ammonium enrichment. This indicates that adequate ammonium supplies may be necessary for *C. raciborskii* to subsist in oligo- to mesohaline habitats with salinities ranging from 5 to 9. Therefore, controlling N inputs, especially ammonium, may help protect these areas from *C. raciborskii* expansion. This finding is relevant in the face of increasing ammonium inputs from agricultural development (expansion of confined animal operations, increased use of urea which hydrolyzes to ammonium) and ammonia-based fertilizers (Galloway and Cowling, 2002).

Microscopic observations provide insight into whether *C. raciborskii* could effectively compete within the CS phytoplankton community and what nutrient conditions increase its competitiveness in the unfiltered bioassay treatments. In June 2007, microscopic observations showed that at T_0 samples were abundant in N_2-fixing cyanobacteria. *Anabaena* spp. and *Anabaenopsis* spp. were the dominant species, but also present were *C. raciborskii*, *Aphanizomenon* spp., *Nostoc* spp. as well as non-N_2 fixing cyanobacteria (*Microcystis* spp.) and numerous species of filamentous green algae. In September 2008, no *C. raciborskii* filaments were observed at T_0, although there were a small number of other N_2-fixing species, including a few heterocyst-bearing *Anabaena* spp. and *Aphanizomenon* spp. filaments. The phytoplankton community at this time mainly consisted of diatoms and filamentous cihlorophytes.

In both bioassays, when compared with the T_0 samples, the nitrate–N treatment led to maximal abundance of *C. raciborskii* (Fig. 6). Qualitative assessment of the phytoplankton community showed that the other nutrient treatments favored additional phytoplankton groups, which may have outcompeted *C. raciborskii*. For instance, the P treatment favored other N_2-fixing genera, with *Anabaena* spp. and *Aphanizomenon* spp. dominating. Treatments containing both N and P resulted in a mixed phytoplankton community, with diatoms, dinoflagellates, green algae and cyanobacteria all in similar abundances. The fact that *C. raciborskii* was favored by N treatments alone suggests that it may be more efficient than the other diazotrophic cyanobacterial species at utilizing combined nitrogen when it is available. This finding supports earlier studies, indicating that *C. raciborskii* is able to effectively compete for combined N when it is present, before having to switch to N_2 fixation (Bouvy et al., 2000; Burford et al., 2006; Paerl and Fulton, 2006). The difference in the magnitude of its abundances between 2007 and 2008 demonstrate the effect of salinity on *C. raciborskii*'s ability to grow and compete in CS water, with severe salinity stress occurring in 2008.

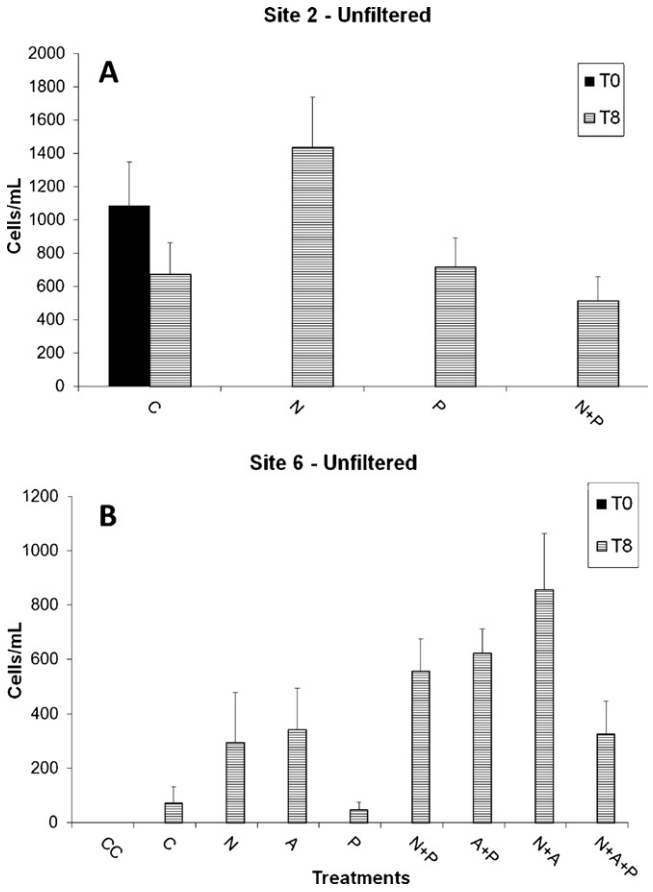

Fig. 6. *C. raciborskii* filament counts for T_8 for the unfiltered treatment in June 2007 (a) and September 2008 (b).

4. Conclusions

During the course of this study, *C. raciborskii* with the genetic capability to produce CYN was identified in CS as a minor component of a phytoplankton community rich in N_2-fixing filamentous cyanobacteria when salinities were near 0 in 2007. Increased salinity in 2008, resulting from a prolonged record drought, proved inhospitable to *C. raciborskii* and its N_2-fixing competitors. Bioassay results confirmed that salinity levels strongly control *C. raciborskii* growth and abundance in CS. However, we observed that salinity tolerance of *C. raciborskii* (7–8) was higher than previously reported (Moisander et al., 2002). Specifically, we found that specific N additions (ammonium–N) enhanced the competitive ability and salinity tolerance of *C. raciborskii*, indicating that increased anthropogenic N loading may favor the invasion potential for *C. raciborskii* into estuarine water bodies. These findings have implications for *C. raciborskii* management, both in CS and in other brackish water bodies worldwide potentially facing expansion of this species. As precipitation regimes and freshwater discharge are altered due to regional and global climate change, salinity regimes throughout estuarine systems are likely to change, and in some cases make systems previously protected by high salinities vulnerable to *C. raciborskii* invasion. Clearly, there is a need to consider salinity (and changes therein) as a major determinant in the survival of this potentially harmful species when formulating management strategies. Our results provide another reason for

controlling N in addition to P inputs to the freshwater-marine continuum that constitutes estuarine ecosystems worldwide (Paerl, 2009).

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