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Effects of modified nutrient concentrations and ratios on the structure and function of the native phytoplankton community in the Neuse River Estuary, North Carolina, USA

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Abstract

A variety of analyses were used to assess the structure (community composition) and function (assimilation number, nitrogen fixation) of phytoplankton in the Neuse River Estuary (NRE), NC under ambient and modified nutrient concentrations. Dilution bioassays were employed to reduce the concentration of nitrogen (N) or both N and phosphorus (P) and thus compare varied DIN:DIP ratios. Experimental manipulations created conditions that may result from mandated N load reductions to the estuary. We hypothesized that unilateral reduction of N loading to the NRE would increase the activity, abundance and diversity of N_2 fixing cyanobacteria. Changes in phytoplankton primary productivity, N_2 fixation (nitrogenase activity), genetic potential for N_2 fixation (presence of *nifH*), phytoplankton taxonomic composition (diagnostic photopigment concentration) and abundances of N_2 fixing cyanobacteria (microscopy) were determined. Decreasing ambient DIN:DIP ratios in NRE samples resulted in increased rates of N_2 fixation when seed populations were present and environmental conditions were amenable. Decreasing the DIN:DIP ratio did not lead to an increase in the abundance or diversity of N_2 fixing cyanobacteria. Because N_2 fixing cyanobacteria were only actively fixing nitrogen during periods of low riverine N discharge (summer and early autumn), lowering nutrient ratios may not have a major impact on the NRE. However, the maximum potential amount of N from N_2 fixation was calculated using rates from this study and was found to be approximately 3% of total riverine loading of N to the NRE. Because N_2 fixation occurs farther downstream and later in the year than riverine N loading to the NRE, there is potential for N_2 fixation to modify N dynamics. Analyses of the phytoplankton community as a whole in these relatively short term experiments indicated that reduced DIN:DIP may not have a major impact on their structure and function.

Introduction

Reducing the nitrogen (N) levels in N limited aquatic systems could have impacts beyond a simple reduction in phytoplankton productivity and biomass, including possible selection for species adapted to growth in waters with reduced nitrogen to phosphorus ratios (N:P) (Tilman et al., 1982; Smith, 1990). In particular, the phytoplankton community could potentially become dominated by N_2 fixing cyanobacteria that may circumvent N-limitation by drawing on an atmospheric source of N (Fogg, 1974; Paerl, 1990). Phytoplankton primary production in the lower NRE is controlled by N availability throughout much of the year (Paerl, 1987; Rudek et al., 1991; Boyer et al., 1994; Paerl et al., 1995). Riverine loading of dissolved inorganic nitrogen (DIN) is dominated by nitrate-nitrite (NO_x), which declines non-conservatively down-estuary under most flow conditions (Christian et al., 1991). The Neuse River Estuary (NRE) has experienced a deteri-

oration of water quality that has prompted a mandated nutrient management strategy intended to halt decline and provide eventual improvement in water quality (15A NCAC 2B.0232-.0240). Management plans for the NRE include a nitrogen loading 'cap' and an overall 30% reduction in N loading.

Cyanobacteria have exploited recent anthropogenic alterations of aquatic environments, most notably excessive nutrient enrichment (Fogg, 1969; Reynolds & Walsby, 1975; Paerl, 1988; Paerl & Tucker, 1995). Historically, cyanobacterial nuisance blooms have occurred in the oligohaline segments of North Carolina estuaries including the NRE. A problematic aspect of cyanobacterial opportunism is the development and proliferation of harmful blooms in nutrient enriched aquatic ecosystems (Fogg, 1969; Reynolds & Walsby, 1975; Horstmann, 1975; Paerl, 1988; Kahru et al., 1994; Sellner, 1997). Such blooms pose serious water quality, fisheries resource, aquaculture, and human health problems, including toxicity to higher order consumers, hypoxia and anoxia (Francis, 1878; Boyd et al., 1978; Codd et al., 1994; Carmichael, 1995; Paerl & Tucker, 1995). In addition, nutrient transformations and biogeochemical cycling can be altered if N_2 fixing cyanobacteria dominate the phytoplankton (Horne, 1977; Vincent, 1987; Howarth et al., 1988b).

Certain cyanobacterial genera are able to satisfy their N requirements by N_2 fixation. These genera are capable of growth in N-deficient waters, typified by many estuarine and coastal ecosystems. In these systems heterocystous cyanobacteria (e.g., *Anabaena, Aphanizomenon, Nodularia, Cylindrospermopsis*) and some non-heterocystous diazotrophic genera (e.g., *Lyngbya, Oscillatoria*) should be afforded a competitive advantage. Smith (1990) found N:P loading ratios may not determine the rates of N_2 fixation observed in estuaries, suggesting that total P loading may be more important, a claim that was disputed by Howarth and Marino (1990). Many estuarine and coastal waters have N:P ratios far below the stoichiometric thresholds of N limitation, thus they are largely predicted to be N limited (Nixon, 1986; D'Elia, 1987). However, these systems contain highly variable numbers of N_2 fixing cyanobacteria (Paerl, 1996; Paerl & Millie, 1996) and a high genetic potential for N_2 fixation (i.e. presence of *nifH* gene) (Paerl & Zehr, 2000). Abundance and activity of N_2 fixing cyanobacteria in response to reduced N:P ratios has been examined experimentally in lake phytoplankton communities (Seale et al., 1987; Levine & Schindler, 1992; 1999), but few data exist on the effects on the abundance and activity of estuarine N_2 fixers.

Here we used experimental manipulations to evaluate the effects of reducing the DIN:DIP ratio in the NRE on the abundance and activity of N_2 fixing cyanobacteria. Fourteen *in situ* dilution bioassays were conducted throughout 1997-1999 at a mesohaline site in the NRE. The concentration of N or both N and P was reduced, thereby altering the ratio of nutrient concentrations (Paerl & Bowles, 1987; Carrick et al., 1993; Dodds et al., 1993). Changes in primary productivity, N_2 fixation (nitrogenase activity), genetic potential for N_2 fixation (presence of *nifH*), phytoplankton taxonomic composition (diagnostic photopigment concentration) and abundances of N_2 fixing cyanobacteria (microscopy) were determined. Using these results we assessed the potential for increases in N_2 fixing cyanobacterial biomass and activity in response to nutrient load modifications.

Methods

Site description

The Neuse River watershed has an area of approximately 16 000 km^2 which discharges into the NRE and eventually Pamlico Sound (Figure 1). The river and estuary are shallow with a mean depth of 2.2 m. Salinity varies with changes in wind conditions and river discharge. Salinity stratification occurs in the river and estuary during periods of calm weather (low wind mixing) and moderate river discharge (Paerl et al., 1995; Robbins & Bales, 1995). Residence time in the estuary averages 51 d and can exceed 90 d during periods of reduced discharge (Christian et al., 1991; Robbins & Bales, 1995). Previous work has shown that stratification combined with long residence time promotes algal blooms (including cyanobacteria) (Paerl, 1987; Christian et al., 1986). The site for this study was mid-channel at channel marker 15 (Figure 1), at the upstream end of the widening of the estuary.

Experimental design

Water was collected in 20 l acid rinsed carboys. Samples were taken from just below the surface and transported in the dark to the UNC-CH Institute of Marine Sciences (IMS). The water samples were in transit for approximately 2 h. Nutrient dilution bioassays were used to reduce the concentrations of ambient dissolved

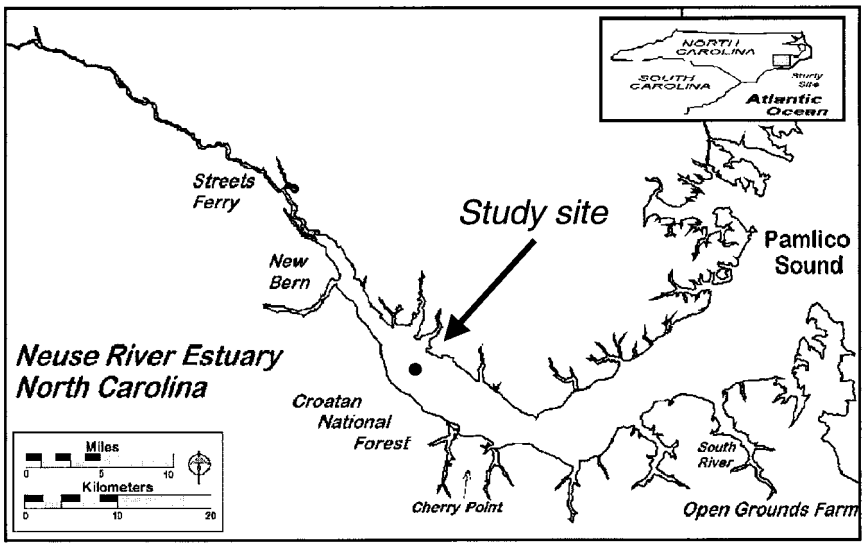

Figure 1. Map showing the study site's location within the Neuse River Estuary.

ions in NRE water samples. In this assay, concentrations of potential growth-limiting nutrients, chiefly N and P compounds, are reduced while maintaining non-growth-limiting ions at naturally occurring levels. Experiments were conducted 14 times in 1997–1999 (Table 1). Treatments for the all experiments (except the July 98) included: Control (unamended river water), 30% dilution (both N and P reduced, DIN:DIP same as ambient), and 30% dilution with P added back to the ambient concentration (N concentration alone reduced, DIN:DIP reduced compared to ambient). The July 98 dilution bioassay treatments included a series of dilutions from 20–50% and parallel dilutions with P added back to the ambient concentration. In each experiment dissolved inorganic carbon (DIC) was replenished to estimated ambient levels based on extensive historical data sets. Dilutions were made using a major ion solution, consisting of the major cations and anions of the Neuse River (Si, Ca, Mg, Na, K, Cl, and SO_4) (Paerl & Bowles 1987). Each treatment had four replicates and was incubated in 10 l Cubitainers (Hedwin Corp., Baltimore, MD) that are chemically inert and transmit 85% of incident photosynthetically active radiation. Cubitainers were placed outside IMS in holding ponds filled with water from Bogue Sound for temperature and irradiance control and incubated for 84 h. For additional details on the dilution bioassay procedure see Paerl & Bowles (1987). Pinckney et al. (1998) found 48 h a sufficient incubation period to detect effects of nutrient addition treatments in NRE phytoplankton communities (including cyanobacteria). We utilized an 84 h incubation based on additional previous work (Paerl & Bowles, 1987; Carrick et al., 1993; Dodds et al., 1993) that showed this time frame to be sufficient for observing effects of dilution treatments (which change nutrient concentrations less relative to ambient levels than do additions) while minimizing bottle effects. Cubitainers were inverted twice daily to minimize settling of biomass. Our design did not account for differential top-down controls in treatments. Because we had a relatively short incubation time and control cubitainers incorporated into all experiments, we believe that differential grazing effects would have been detected in these experiments.

Chlorophyll *a* concentration was measured using the non-acidification fluorometric method (Welschmeyer, 1994). Fifty ml water sub samples were filtered onto 25 mm Whatman GF/F glass microfiber filters (0.8 μ nominal pore size) and stored at -20 °C. Ten ml acetone (90% acetone: 10% DI) was added to filters in polypropylene centrifuge tubes. Filters in tubes were then sonicated on ice for 30 s and samples were extracted approximately 12 h at -20 °C.

Phytoplankton primary productivity was measured by addition of ^{14}C sodium bicarbonate ($\sim$6 kBq ml^{-1} final activity) to water samples. Samples were taken from each cubitainer and dispensed into 20 ml clear borosilicate vials for incubation. $NaH^{14}CO_3$ was added to the vials that were incubated under natural irradiance and temperature conditions in a rack just below the water surface. When neces-

Table 1. Physical and chemical data prior to the dilution bioassay. Absence or presence of nitrogen fixation and the genetic potential for nitrogen fixation are also indicated for each experiment

Date	temp (C)	DO (mg/L)	salinity (ppt)	NO_x (uM)	NH4 (uM)	PO4 (uM)	DIN:DIP	Nitrogenase Activty (Y/N)	*nif H* Present (Y/N)
7 Aug. 1997	26.69	8.0	0.3	0.4	0.9	1.7	0.7	Y	Y
14 Oct. 1997	24.09	8.9	9.0	0.7	4.0	0.8	6.0	N	*NA*
12 Jan. 1998	11.86	9.4	0.0	48.4	6.9	0.9	64.9	N	N
31 March 1998	18.57	7.9	0.0	31.6	2.8	1.0	34.5	N	Y
28 Apr. 1998	18.33	9.8	0.1	18.5	1.1	0.8	24.7	N	Y
27 May 1998	25.61	9.3	0.7	13.5	1.3	0.2	94.8	N	Y
22 June 1998	28.10	8.1	1.7	0.4	1.1	1.0	1.5	N	Y
21 July 1998	28.96	6.8	2.2	0.0	0.5	2.3	0.2	Y	Y
17 Aug. 1998	28.74	6.4	4.4	0.1	0.8	2.7	0.3	N	Y
9 Nov. 1998	12.72	10.6	6.4	0.8	0.7	0.6	2.7	N	Y
3 Feb. 1999	11.9	8.78	0.6	36.74	3.73	0.76	53.4	N	Y
21 June 1999	23.54	8.64	7.4	0	9.78	10.87	0.9	N	Y
19 July 1999	28.46	8.06	8.2	0	10.25	15.58	0.7	N	Y
13 Sept. 1999	24.64	5.52	1.3	197.92	95.04	63.49	4.6	N	Y

sary (> 800 μE m^{-2} s^{-1} irradiance), neutral density screens were used to reduce incident irradiance to prevent photoinhibition. Following 3 h incubations, samples were filtered onto 25 mm diameter Whatman GF/F filters. The filters were fumed immediately with concentrated HCl to remove unincorporated ^{14}C and then air-dried. Dry filters were placed in 7 ml plastic scintillation vials containing 5 ml Ecolume liquid scintillation cocktail (ICN, Inc.). Radioactivity was quantified using a Beckman model LS5000TD liquid scintillation counter. Counts per minute (CPM) were converted to disintegrations per minute (DPM) using quench curves derived from a calibrated ^{14}C-toluene standard. Dissolved inorganic carbon (DIC) in water samples was determined using an infrared gas analyzer (Li-Cor LI 6252) (Paerl, 1987). Dark vials were incubated for each treatment and their values were subtracted from the respective light values. Productivity rates were normalized to Chl *a* to remove variability due to unequal initial biomass in different treatments that resulted from the dilution treatments and presented as phytoplankton community assimilation number (C Chl a^{-1} h^{-1}).

N_2 fixation was estimated using the acetylene reduction assay (Stewart et al., 1967). 50 ml aliquots of sample water were added to 72 ml serum vials that were then capped with flanged red rubber stoppers. Acetylene was generated by placing CaC_2 in deionized water (DI) and was added to the headspace of the vial ($\sim$20% of the total volume). Acetylene blanks (DI + acetylene) and blanks with sample water only were run to test the purity of the acetylene generated and ethylene from sources other than acetylene reduction, respectively. Dark vials were also incubated for each of the treatments, and their rate of acetylene reduction was subtracted from light values to ensure the values presented were attributable to photosynthetic organisms. Serum vials were incubated in a running water bath for 4 h under appropriate natural light levels (modified using neutral density screens when necessary). Following incubation, 2 ml headspace subsamples were transferred to 2 ml evacuated autosampler vials for gas chromatographic analysis using a Shimadzu GC 9A gas chromatograph equipped with a flame ionization detector (FID). The GC was fitted with a 2 m stainless steel Poropak T column held at 80°C with high purity nitrogen as the carrier gas. Acetylene reduction rates were expressed in terms of ethylene generated per unit time and were normalized to Chl *a* concentration to remove variability due to unequal initial biomass in different treatments.

Phytoplankton community composition was determined using diagnostic photopigment analyses (Millie et al., 1993; Tester et al., 1995; Jeffery et al., 1997). Samples were collected from Cubitainers at specified time intervals, filtered onto 25mm Whatman GF/F filters, and frozen (-80 °C). Photopigments were extracted using a 90% aqueous acetone solvent

and sonication. High performance liquid chromatography (HPLC) was used to quantify the relative biomass of algal groups (cyanobacteria, diatoms, chlorophytes, cryptophytes and dinoflgellates) in the phytoplankton community based on biomarker photopigment (chemosystematic chlorophylls and carotenoids) concentrations. An in-line photodiode array spectrophotometer (PDAS, Shimadzu SPD M10av) provided identification of individual photopigments based on characteristic absorption spectra (380–700 nm) (Rowan 1989). Pigment concentrations from HPLC were analyzed using CHEMTAX to calculate the relative abundances of the major phytoplankton groups (Mackey et al., 1997). CHEMTAX is a matrix factorization program for calculating abundances of algal classes from concentrations of chemosystematic marker photopigments (chlorophylls and carotenoids) (Mackey et al., 1996; Mackey et al., 1997; Wright et al., 1996; Pinckney et al., 1998). The resulting class composition matrix was expressed as a relative value of the total chlorophyll *a*.

Sub-samples of water for each treatment were collected for microscopic counts. Lugol's solution was used to preserve 50 ml samples in polypropylene bottles. Filamentous cyanobacteria were counted under an inverted phase contrast microscope (Wild) using Utermöhl technique (Utermöhl, 1958) with PhycoTech counting chambers. N_2 fixing cyanobacteria were photographed using a Nikon Coolpix 950 digital camera and a Nikon Eclipse E800 microscope.

For molecular analysis of N_2 fixing cyanobacteria, sub-samples of water were filtered through 0.2 μm porosity Supor filters and stored at -80 °C. To extract DNA from the material on these filters, they were heated to 90 °C for a total of 5–6 hours. Three hours into the incubation the material was scraped off the filter and twice subjected to bead beating (150–200μm glass beads, 0.3 g) for 3 min each time. RNase was added near the end of the incubation (300 μg). Microscopic observation was used to verify that this process lysed all cells. The DNA was then purified first using DNAZol (Cincinnati, OH) and then the DNeasy Plant Kit (Qiagen, Valencia, OH) and eluted in water. The PCR amplification used degenerate oligonucleotide primers designed to pick up the *nifH* gene in all aerobic nitrogen fixers (Olson et al., 1998). The primer sequences used were:

forward primer : $5' -$ ATYGTCGGYTGYGAY

CCSAARGC $- 3'$,

reverse primer : $5' -$ ATGGTGTTGGCGGCR

TAVAKSGCCATCAT $- 3'$,

where Y is T or C; S is G or C; R is A or G; V is A, C, or G; and K is G or T.

The 334 bp fragment was amplified using 2 mM $MgCl_2$ in 10X buffer with 500 ng of each of the forward and reverse primers, dNTPs, and 2 U Taq polymerase in 50 μl volume for 30 cycles (1 min at 94 °C, 1 min at 53.5 °C, 1 min at 72 °C). The PCR product was visualized on a 1% SeaKem gel stained with ethidium bromide. The *nifH* gene fragment was ligated into a plasmid vector (pCR vector 2.1, Invitrogen) overnight at 14 °C and then transformed into *E. coli* INVαF′ ultracompetent cells (Invitrogen, Carlsbad, CA) grown on LB plates plus X-gal. The plasmids were isolated using the QIAprep miniprep kit (Qiagen). Clones with the correct size insert, confirmed by restriction cuts with *Eco*RI, were sequenced (700 ng plasmid with M13 forward primers). Sequences were analyzed using SeqLab and Phylip software (University of Wisconsin Genetic Computer Group) and compared against other nifH sequences in GenBank for identification.

This work coincided with the execution of a large-scale collaborative water quality modeling and monitoring project (ModMon: www.marine.unc.edu/neuse/modmon) in the NRE. Physical and chemical data were collected from the NRE bi-weekly along a mid-channel transect. Vertical profiles of temperature, salinity and dissolved oxygen were obtained using a Hydrolab probe coupled to a Surveyor 3 datalogger (Hydrolab Corporation, Austin TX). Both surface and bottom water samples were collected at each station for analysis of nutrient concentrations and photopigment concentrations (procedures described above). These data are included to provide environmental context for the bioassays described in this paper.

Community responses were analyzed using a one-way analysis of variance (ANOVA). Data were natural log transformed when necessary to satisfy the normality assumption. CHEMTAX percent composition data were arcsine square root transformed. Comparisons of means were made using a Bonferroni multiple comparisons of means (Moore & McCabe, 1993).

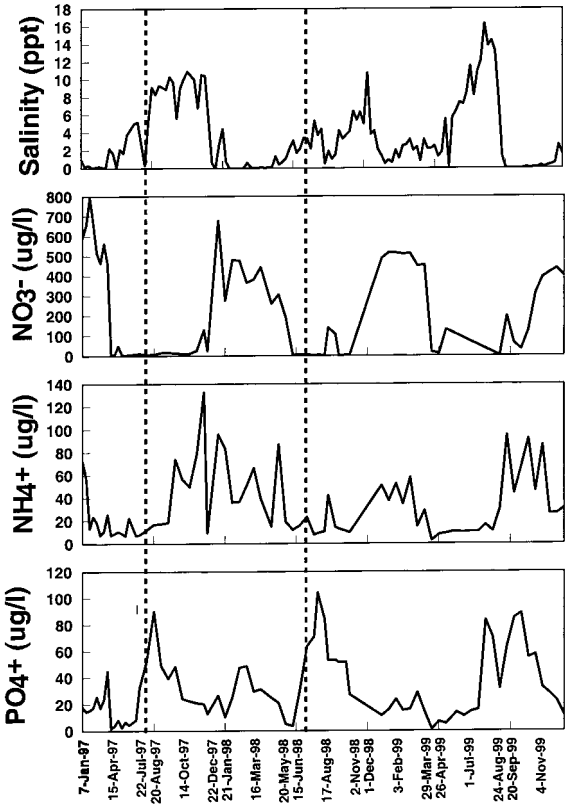

Figure 2. Nutrient and salinity monitoring data from surface waters at the site for the bioassays. Dashed lines indicate times when N_2 fixation was measured.

Results

N_2 fixation (nitrogenase activity) was detected twice during the two-year period of this study (Table 1). Figure 2 shows bi-weekly nutrient and salinity data for the duration of the study and indicates the two times when acetylene reduction was detected. Detection of acetylene reduction corresponded with the only times during the period of the study when DIN was low ($<20\ \mu g\ l^{-1}$), DIP was sufficient ($>20\ \mu g/\ l^{-1}$) and salinity was low (<4 ppt). Phytoplankton taxonomic composition in the estuary as determined by HPLC for each of the bioassays is presented in Figure 3. Cyanobacteria were dominant during periods of the summer through early fall of each year. Detailed data are presented below from four of the experiments; the two experiments in which N_2 fixation was detected (August 1997 and July 1998), an experiment in which N_2 fixation would have been expected to be present but was not (August 1998), and an experiment in which N_2 fixation would not have been predicted (January 1998).

In August 1997 both DIN:DIP and salinity were very low in the portion of the estuary from which water was sampled (Table 1). Following an 84 h incubation, phytoplankton assimilation number was not significantly reduced by the dilution of N and P (30% dilution) or by reduction of N alone (30% dilution +P) relative to the control (Figure 4A). Nitrogenase activity was detected in all treatments after 84 h incubations. Rates of acetylene reduction were significantly higher in the 30% dilution +P treatment compared to the control (Bonferroni, $p < 0.05$). Cyanobacteria were found to be the most abundant taxonomic group by photopigment analysis and their abundance did not change significantly with any treatment (Figure 5A). Microscopic analysis of phytoplankton community composition revealed filamentous heterocystous cyanobacteria to be dominant. Microscopic counts of the N_2 fixing cyanobacteria present identified *Anabaena (Aphanizomenon) aphanizomenoides* (FORTI) HORECKÁ et KOMÁREK to be present in all treatments in high densities (Figures 6A and 7A). Highest mean number of cells was observed in the 30% dilution +P treatment.

DIN:DIP was very high at the start of the January 1998 dilution bioassay, but salinity was very low (Table 1). Nitrogenase activity was not detected in any treatment (Figure 4B). Phytoplankton assimilation number was reduced in the 30% dilution +P treatment relative to the control. Cyanobacteria were less abundant than the previous experiment, comprising between 5–15% of total phytoplankton community composition (Figure 5B). Microscopic evaluation of the phytoplankton revealed that Chlorophytes and diatoms were dominant and that filamentous cyanobacteria were not present. There were no differences among treatments in percent cyanobacteria detected using HPLC (Figure 5B).

The July 1998 dilution bioassay water samples had both low salinity and a very low N:P ratio (Table 1). Assimilation number was not reduced by any dilution of either N alone or N or P (Figure 4C). There was, however, a significant increase in assimilation number in the 30% dilution +P and the 50% dilution +P treatments as compared to the control (Figure 4C)(Bonferroni, $p < 0.05$). Nitrogenase activity was detected in all treatments and was significantly higher than in the control in all levels of dilution except 20% (Figure 4C) (Bonferroni, $p < 0.05$). Nitrogenase activity in dilutions with P added was higher

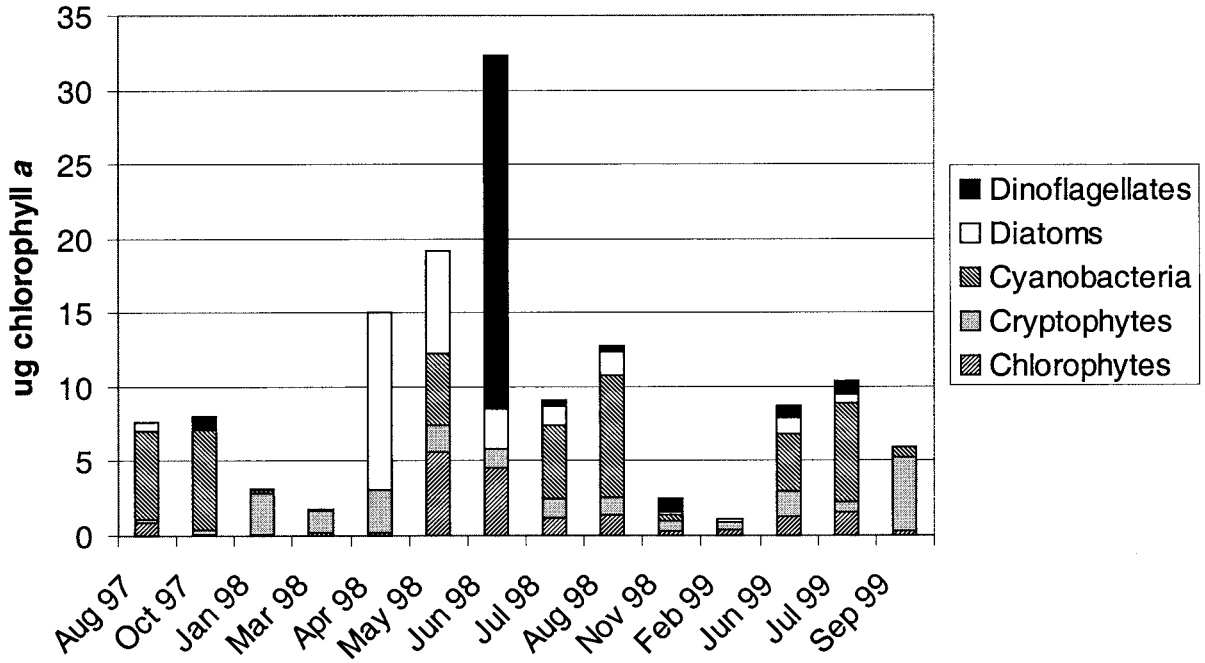

Figure 3. Phytoplankton taxonomic composition prior to each of the 14 bioassays conducted in this study. HPLC data were processed using the CHEMTAX matrix factorization program and show the contribution of each group to the total chlorophyll *a* pool.

than the parallel dilutions alone, with the exception of the 40% pair (Figure 4C). HPLC diagnostic photopigment analysis indicated cyanobacteria to be the most common taxonomic group (Figure 5C). There was no change in phytoplankton community composition in response to any of the treatments (Figure 5C). Microscopic analysis of the phytoplankton community showed filamentous cyanobacteria to be dominant. Counts of N_2 fixing cyanobacteria detected high numbers of *Anabaena compacta* (NYGAARD) HICKEL (Figures 7E and 7F) (Hickel, 1985), *Anabaenopsis* sp. (Figure 7B) and *Anabaena aphanizomenoides* (Figure 7A) in all treatments (Figure 6C). There was no apparent relationship between treatments and cell counts.

The August 1998 dilution bioassay samples had a very low N:P and slightly higher salinity than in other experiments (Table 1). Nitrogenase activity was not observed in any treatment. Assimilation number did not change in response to any treatment (Figure 4D). Photopigment analysis showed cyanobacteria to be the dominant taxa (Figure 5D). There were no significant changes in phytoplankton community composition in response to treatments (Figure 5D). *Anabaena aphanizomenoides* was present, but in much lower densities than in either August 1997 or July 1998 (Figure 6D). Small numbers of *Cylindrospermopsis raciborskii* (WOLOSZYNSKA) SEENAYA et SUBBA RAJU were observed in samples from both the July 1998 and August 1998 experiments (Figures 7C and 7D) (Chapman & Schelske, 1997).

The presence of N_2 fixers was also determined by detection of the *nifH* gene. For each of the 4 experiments, primers specific to *nifH* were used to amplify an approximately 340 nucleotide section of the *nifH* gene from the diazotrophs present. Using PCR *nifH* was detected in August 1997 (30%+P) (DNA from control and 30% dilution degraded), July 1998 (control, 20%, 20%+P, 50%, 50%+P), and August 1998 (ctrl, 30%+P). *nifH* was not amplified from any treatment in January 1998, indicating that the number of diazotrophs present was less than the lower PCR detection limit.

For control and dilution + P treatments in July 1998 and August 1998, the *nifH* PCR products were cloned and sequenced to determine the identity of the N_2 fixers present. Out of 40 clones sequenced, there were 5 *nifH* sequences identified in these samples. When compared to other *nifH* sequences in the GenBank database, four of these sequences clustered with the heterocystous cyanobacteria (Neuse #1,2,3,4) (Figure 8) and together comprised just over half

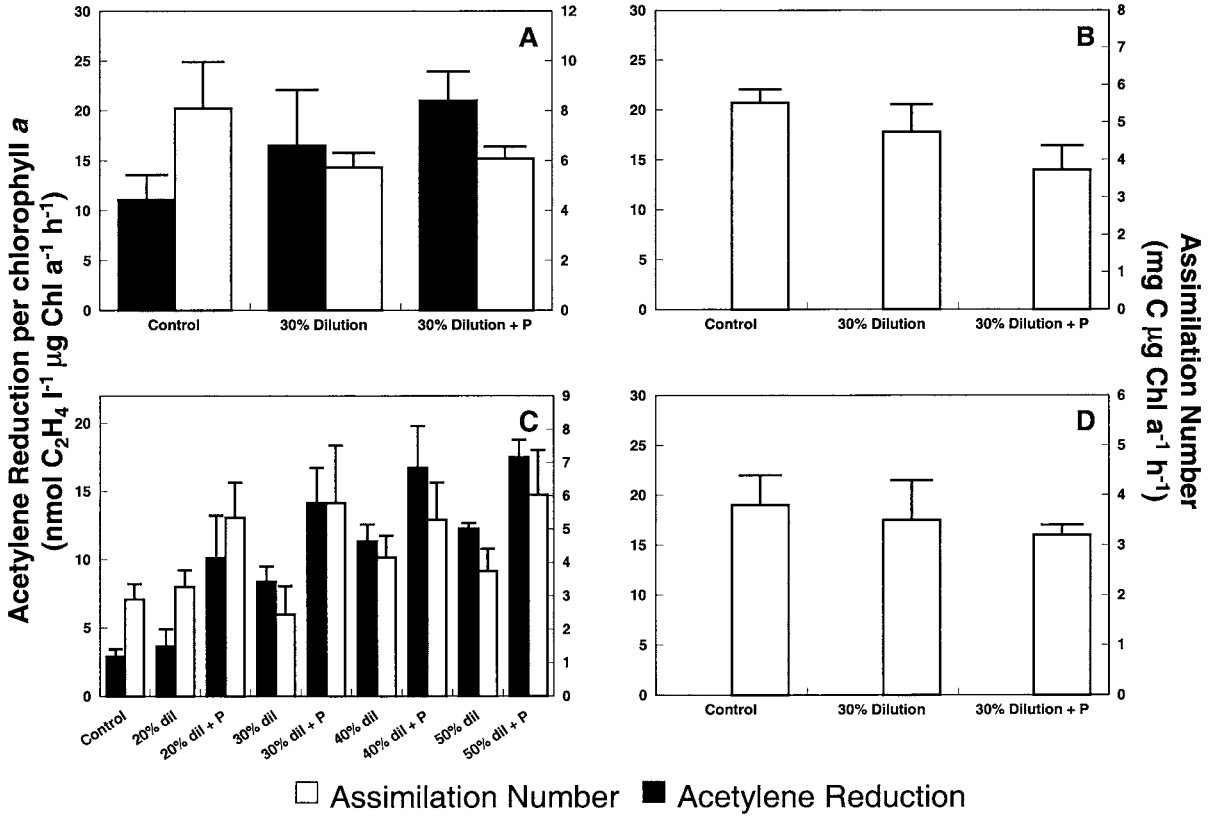

Figure 4. Acetylene reduction and assimilation number measurements from 84 h Cubitainer incubations with several treatments. Left side Y axes are acetylene reduction and right side Y axes are assimilation number in all four sub-figures. Experiments were conducted August 1997 (A), January 1998 (B), July 1998 (C), and August 1998 (D). Error bars are one standard deviation.

(52.5%) of the clones sequenced. The two sequences that were most prevalent in the clones sequenced (Neuse #1 and 2) were most similar to *Anabaena* spp. These two *nifH* sequences had an 86.7% similarity on the base pair level to each other and 97.3% similarity on the amino acid level. These two sequences were >99% similar to the sequences obtained from *Anabaena aphanizomenoides* and *Anabaenopsis* sp. isolated in culture from NRE samples. The other heterocystous cyanobacterial sequences (Neuse #3 and 4) were seen infrequently and only in samples from July 1998. These sequences could reflect the presence of *Anabaena compacta* seen in microscopic analysis or another filamentous heterocystous cyanobacteria indistinguishable by morphological characteristics. The fifth sequence (Neuse #5) fell outside of the distinct cluster of heterocystous cyanobacteria and was most similar to the filamentous nonheterocystous cyanobacteria *Lyngbya* spp. (Figure 8). Neuse #5 was ~83% similar to the heterocystous sequences on the nucleotide level, ~97% in amino acid sequence. This filamentous nonheterocystous *nifH* sequence comprised 47.5% of the clones sequenced. There was also a difference in the relative abundance of heterocystous and non-heterocystous sequences obtained for each time point. In July 1998, the heterocystous cyanobacteria (*Anabaena*-related) were more abundant (70% of clones) than the non-heterocystous filamentous cyanobacteria (*Lyngbya*-related) (30% of clones). However, in August 1998, 93% of clones sequenced were related to *Lyngbya* and only 7% showed a *nifH* sequence clustering with *Anabaena.*

Discussion

Changes in anthropogenic nutrient loading to aquatic ecosystems are likely to affect the structure and function of native phytoplankton communities (Fisher et al., 1992; Wallström et al., 1992; Reuter et al., 1993; Nixon, 1995). Some effects are easier to predict (e.g. higher rates of primary productivity following the

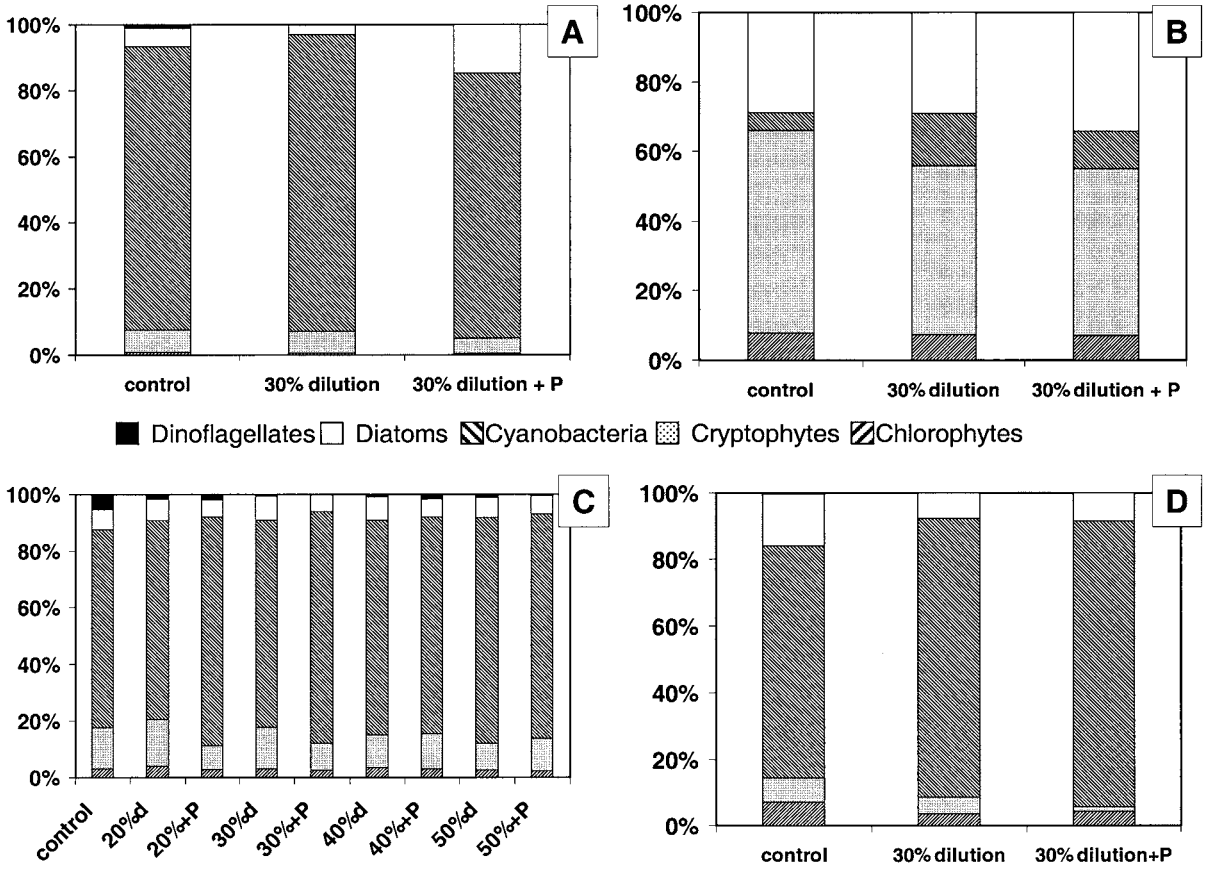

Figure 5. Phytoplankton taxonomic composition as measured using HPLC diagnostic photopigment concentrations and processed using the CHEMTAX matrix factorization program. Values are presented as mean percentage of total community chlorophyll *a*.

addition of a limiting nutrient), while others such as community composition shifts and changes in rates of specific processes (e.g. N_2 fixation) are more difficult to foresee. Management strategies designed to ameliorate previous anthropogenic water quality decline must consider unanticipated effects.

Low DIN:DIP has been suggested to select for N_2 fixers and N_2 fixation in both laboratory studies and field surveys (Niemi, 1979; Levine & Schindler, 1999). The presence of N_2 fixation solely during periods of low salinity could have been due to limited halotolerance (Apte et al., 1987), the presence of some other limiting factor in the less saline water, or the provision of seed populations of diazotrophs from upstream river water. Molecular data in this study confirmed the presence of N_2 fixers (through detection of *nifH*) over a wide range of salinities. Additionally, NRE N_2 fixing cyanobacteria identified in this study have been found to be halotolerant to levels significantly higher than those we report here (P. Moisander, unpublished). These results provide evidence that salinity alone did not control the presence of N_2 fixing cyanobacteria in this study.

Incremental increases in N_2 fixation were observed with larger decreases of DIN:DIP and with reduction of both DIN and DIP (same DIN:DIP). This result suggests a factor in addition to DIN:DIP contributed to the increases in N_2 fixation observed. Plausible explanations include changes in quality or quantity of incident light resulting from dilutions, dilution of some unknown inhibiting factor or container surface effects creating a competitive advantage for N_2 fixers. However, in 3 of 4 dilution pairs (e.g. 30% dilution and 30% dilution+P) N_2 fixation was significantly higher in the treatment with reduced DIN:DIP, indicating reduced DIN:DIP was largely responsible for the enhanced N_2 fixation. However, N_2 fixation was never initiated by reducing DIN:DIP (i.e. when it was not already occurring in the control). N_2 fixation was expected in August 1998 due to the very low DIN:DIP

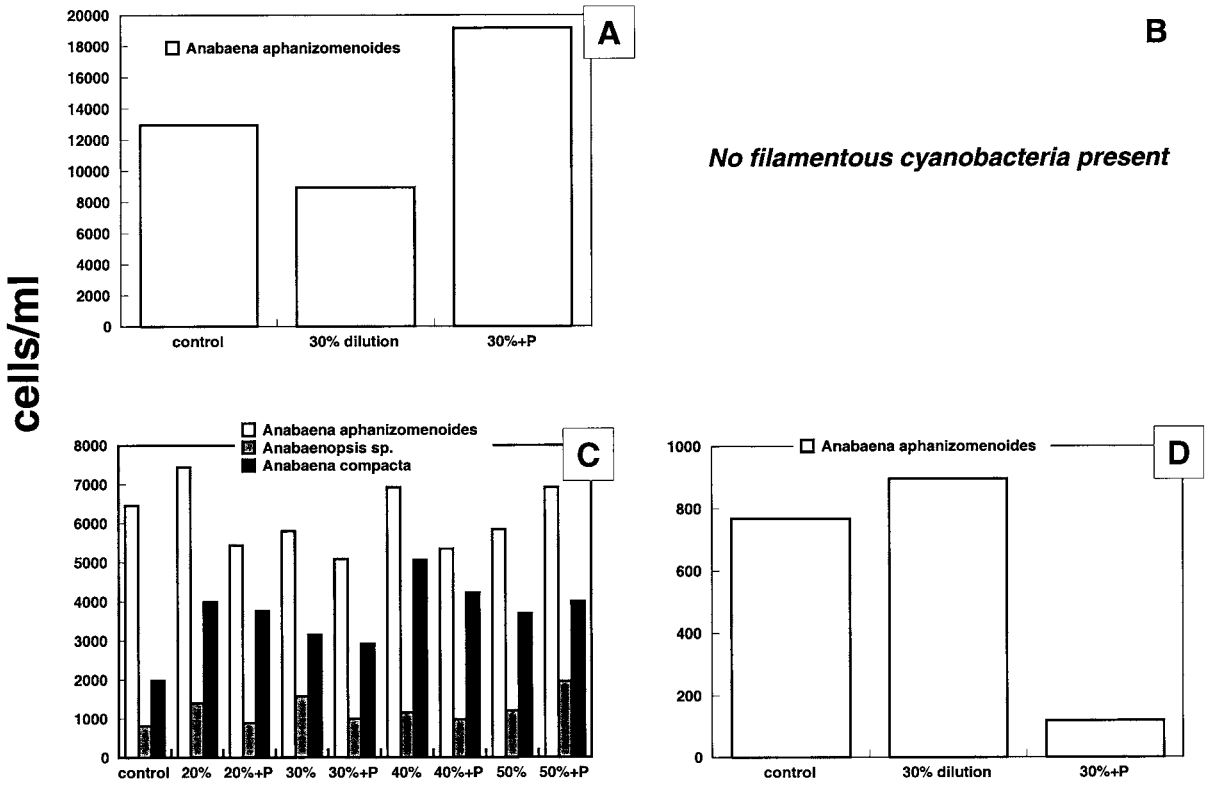

Figure 6. Microscopic counts of filamentous cyanobacteria from experiments in August 1997 (A), January 1998 (B), July 1998 (C), and August 1998 (D).

but it was not detected. Slightly higher salinity at this time either inhibited the activity of the freshwater N_2 fixers present or indicated insufficient transportation of upstream cyanobacteria to the estuary. In the January 1998 experiment the lack of N_2 fixation was not surprising due to the relatively high DIN:DIP. Reduction from the ambient DIN:DIP of more than 60 to a ratio around 40 should not have been sufficient to stimulate N_2 fixers in theory (Smith & Bennett, 1999), and it was not observed in our experiments. Additionally, the colder winter water temperatures decreased the likelihood of N_2 fixing cyanobacteria thriving under any nutrient regime (Paerl, 1990).

There are potential biogeochemical implications of increases in N_2 fixation rates caused by decreasing DIN:DIP. Management strategies such as the 30% reduction of N inputs adopted for the NRE call for the control of growth-limiting nutrients to decrease the negative effects of eutrophication. If N is reduced and the ambient DIN:DIP is also reduced, our results indicate that N_2 fixation will increase if a seed population is present. This increase in fixed N could circumvent a portion of the engineered reductions of N loading and biologically replace some N removed through management actions. The area in our estuarine study site where we believe freshwater N_2 fixing cyanobacteria would be present is approximately 7×10^7 m^2 and includes approximately 20% of the total NRE. Assuming that the N_2 fixers would actively fix at the highest rate we measured in control treatments (1.25×10^{-5} mol N m^{-3} h^{-1}), for 120 d y^{-1} (June, July, August and September), for 12 h d^{-1} and to a depth of 2 m in the water column, the total amount of N fixed in a year would be predicted to be approximately 35 metric tons. This calculation employed liberal values for each term and should be considered a maximum potential contribution of N from N_2 fixation to the NRE. The amount of N from N_2 fixation could be as much as 3% of the total riverine loading of N to the estuary (Stowe et al., 2001). Because N_2 fixation occurs farther downstream and later in the year than riverine N loading to the NRE, there is the potential for N_2 fixation to modify N dynamics in the NRE. This provides further evidence that N_2 fixation should be monitored during any management action designed to

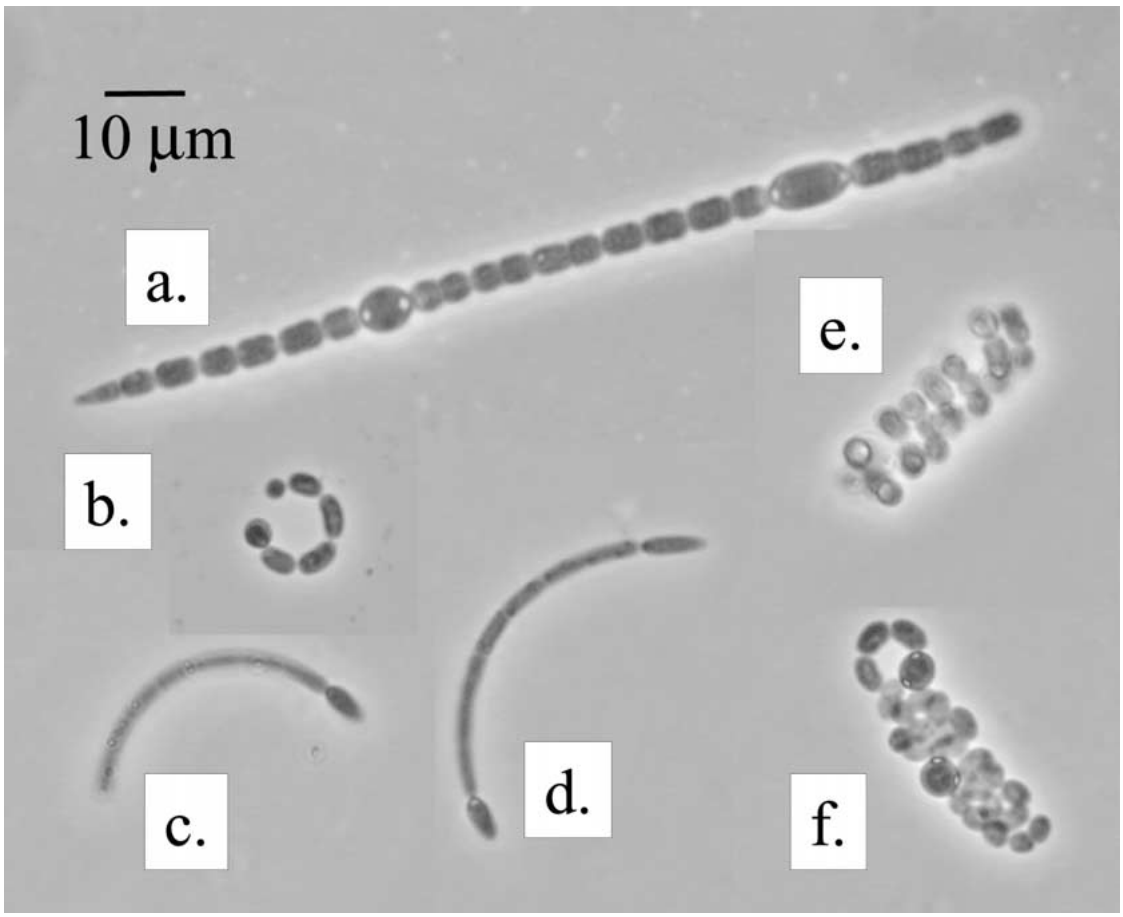

Figure 7. Photomicrographs of dominant heterocystous cyanobacteria from experiments in August 1997, July 1998, and August 1998. Organisms included: *Anabaena aphanizomenoides* (a), *Anabaenopsis* sp. (b), *Cylindrospermopsis raciborskii* (c, d), and *Anabaena compacta* (e, f).

reduce nutrient loading because it has the potential to be an appreciable source of N (Horne, 1977).

Since the goal of reducing nutrient loading to the NRE is to decrease eutrophication, the added carbon that may be fixed by the N_2 fixing cyanobacteria following reduction in DIN:DIP must be considered when assessing whether the management strategy will ultimately be effective. In experiments in which N_2 fixation occurred, assimilation number was never lowered by reduction of N. Ambient N was often nearly absent at these times, so it is possible that further reduction of N did not enhance the already severe N-limitation. More likely, the N gained from N_2 fixation allowed the phytoplankton community to be as productive in the N limited environment as in the controls. In July 1998, assimilation number was actually increased in two of the dilutions of N alone. Increased productivity by N_2 fixers in N limited (and P sufficient) environments has been observed by others (Niemi, 1979; Lukatelich & McComb, 1986). We found that the temporal distribution of actively fixing N_2 fixing cyanobacteria in the NRE are currently constrained enough that their activity following N reductions may not lead to problematic levels of phytoplankton carbon fixation. Other researchers have noted very similar temporal distributions of N_2 fixing cyanobacteria and also found our research site to be the most common location in the NRE for N_2 fixing cyanobacteria to occur (Mallin et al., 1991).

The structure of the phytoplankton community appeared to have been influenced, to some extent, by ambient DIN:DIP during this study. In the January 1998 experiment the phytoplankton community was relatively diverse as evidenced by both HPLC photopigment analysis and the microscopic counts. A similar diversity for the winter phytoplankton community was

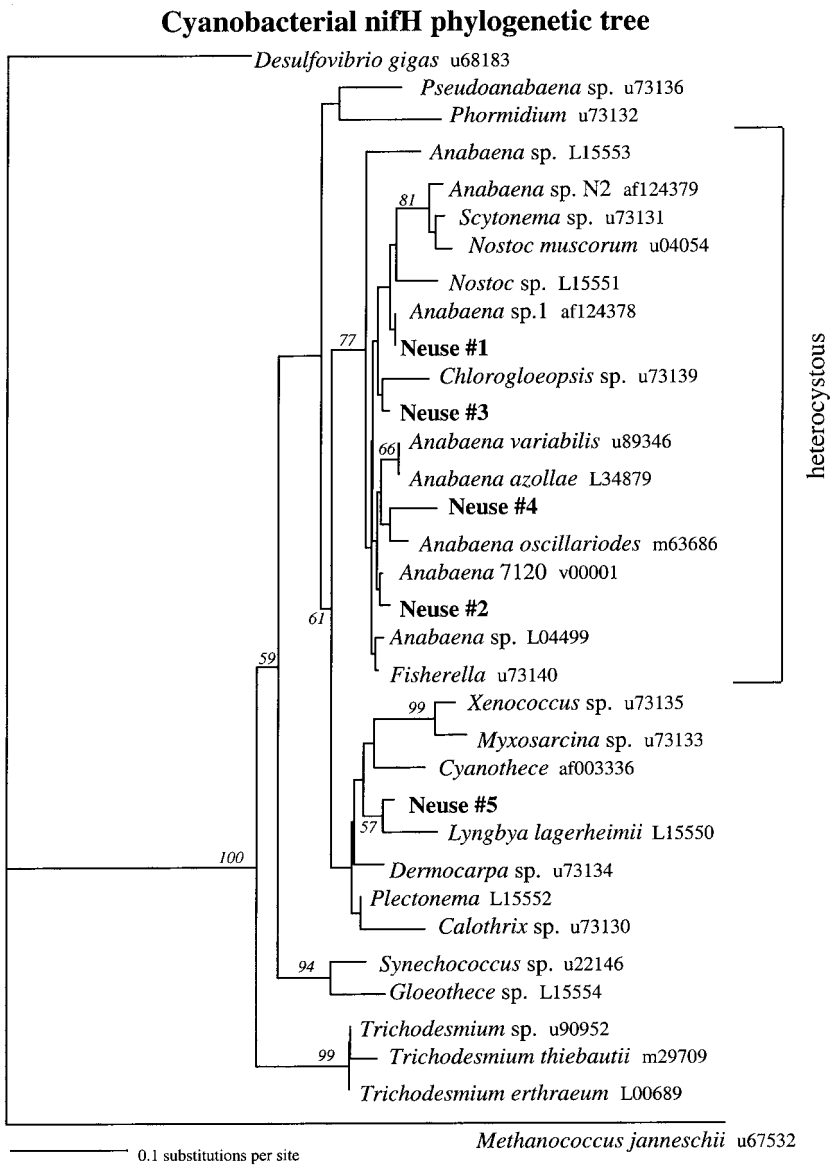

Figure 8. Phylogenetic analysis of cyanobacterial *nifH* amino acid sequences isolated from Neuse River Estuary dilution bioassays (Neuse #1–5) and from GenBank (accession numbers given). Bootstrap values of greater than 50% (generated by distance methods) are listed above each node. *Methanococcus janneschii* was used as the outgroup.

observed by Mallin et al. (1991). Both HPLC and microscopy detected some cyanobacteria in samples at this time point. However, these cyanobacteria were not likely N_2 fixers because *nifH* was not detected using molecular analyses. In August 1998, cyanobacteria were shown to be the dominant taxa by HPLC, but the microscopic analysis detected relatively few N_2 fixing cyanobacteria, supporting the lack of measurable N_2 fixation at that time. However, the *nifH* gene was detected. Presence of the genetic potential for N_2 fixation without measurable N_2 fixation can occur either because the environmental conditions do not favor N_2 fixation (Howarth, 1988a), or the organisms are not present in sufficient numbers to fix detectable amounts of nitrogen (Paerl, 1990). *nifH* clones from the August 1998 experiment were mostly the non-heterocystous *Lyngbya* related sequence as opposed to the heterocystous *Anabaena* related sequence found more often in the July 1998 experiment. If the N_2 fixers present during the August 1998 experiment were in fact *Lyngbya*

or some other closely related filamentous nonheterocystous cyanobacteria, it is possible that mixing in the estuary created conditions unfavorable for these nonheterocystous organisms to fix N_2, which is sensitive to O_2. It is also possible that N_2 fixation occurred at night and was not detected by daytime assays (Paerl, 1990)

When N_2 fixation was detected, ambient DIN:DIP and salinity were both very low. Phytoplankton community structure during these periods appeared to have been affected by the ambient nutrient conditions, with cyanobacteria the numerically dominant phytoplankton taxa. Though microscopy identified four different cyanobacterial N_2 fixers in the July 1998 experiment versus just two for the August 1997 experiment, the total cells per ml were similar for the two, possibly indicating a maximum biomass for N_2 fixers under these favorable conditions. The *nifH* sequences from the July 1998 experiment identified three distinct N_2 fixers present, but one of those, a *Lyngbya*-like filamentous non-heterocystous cyanobacterium, was not observed in microscopic counts. However, good correlation between the percentages of heterocystous *nifH* clones with the dominant cyanobacterial densities observed microscopically indicates that microscopic counts and *nifH* amplification are detecting the dominant organisms.

Experimental manipulations of nutrient concentrations did not have any measurable effect on the proportion of N_2 fixing cyanobacteria in phytoplankton community. Possible explanations for the lack of an observed treatment effect include extreme ambient DIN:DIP (both very high and very low) in this study, or the possibility that the incubation time was insufficient to allow reproduction of cyanobacteria. The latter is possible because the doubling time in nature for cyanobacteria may be several days (Reynolds & Walsby, 1975; Wallström et al., 1992), though optimal conditions would likely induce a detectable response. The more likely scenario was that the ambient conditions were already favorable at the beginning of the incubation, and a significant community of N_2 fixing cyanobacteria were already present in the river, and thus the controls, making further increases in the number of cyanobacteria less likely.

In this study we employed several methods to assess the biomass and activity of N_2 fixing cyanobacteria under ambient and modified nutrient concentrations. Decreasing ambient DIN:DIP ratio in NRE samples resulted in increased rates of N_2 fixation (provided that seed populations were available). We did not, however, detect an increase in the diversity or abundance of N_2 fixers resulting from experimental manipulations of DIN:DIP. Our results show that short term DIN:DIP reduction did not lead to changes in the native phytoplankton community composition. However, it is possible that longer-term reductions could lead to shifts in community structure.

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