UNC-WRRI-2002-339

BIOLOGICALLY MEDIATED NITROGEN DYNAMICS IN EUTROPHYING ESTUARIES. ASSESSING DENITRIFICATION, N2 FIXATION AND PRIMARY PRODUCTIVITY RESPONSES TO PROPOSED N LOADING REDUCTIONS IN THE NEUSE RIVER ESTUARY

by

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The research on which this report is based was financed in part by the Department of Interior, U.S. Geological Survey, through the North Carolina Water Resources Research Institute.

WRRI Project No. 70172

May 2002

One hundred thirty copies of this report were printed at a cost of $782.10 or $ 6.02 per copy.

ACKNOWLEDGMENTS

This project would not have been possible without the valuable technical and field support provided by the students and staff of the Paerl Laboratory. Particularly, we thank Lois Kelly, Pam Wyrick, Malia Go, Laura Hill, Kar Howe, Courtney Stephenson, Tom Nanni and Nathan Hall for their tremendous help. Finally, we thank Jay Pinckney for his guidance and expertise that significantly enhanced the quality of this work.

ABSTRACT

Experimental manipulations were used to predict the effects of reducing the ratio of dissolved inorganic nitrogen (DIN) to dissolved inorganic phosphorus (DIP) (DIN:DIP ratio) in the Neuse River Estuary (NRE) on the abundance and activity of N_2 fixing cyanobacteria. Changes in primary productivity, N_2 fixation (nitrogenase activity), genetic potential for N_2 fixation (presence of *nifH*), phytoplankton taxonomic composition (based on diagnostic photopigment concentrations) and numbers of N_2 fixing cyanobacteria (microscopy) were determined. Results from these experiments indicate that if reduction of N loading leads to a reduced ambient DIN:DIP ratio in the NRE, rates of N_2 fixation may be higher when diazotrophic cyanobacteria are present. We did not, however, detect an increase in either the diversity or abundance of N_2 fixers resulting from experimental manipulations of DIN: DIP ratio. A 30% reduction in nitrogen (N), phosphorus (P) or both N and P concentrations caused a reduction in phytoplankton assimilation number at the estuarine bioassay site. There was no reduction in assimilation number at the riverine site when assessing the data for the entire experimental period. When analyzing individual experiments, N dilution reduced assimilation number as often as dilution of both N and P concentration, while P reduction alone reduced assimilation number only once. Phytoplankton taxonomic composition as measured by HPLC diagnostic photopigment analysis was not altered by any dilution at any location in this work.

Spatial and temporal measurements of rates of denitrification, a potentially important sink for nitrogen in the NRE and similar systems, were assessed. Denitrification was measured using the acetylene block technique. Estimated *in situ* rates appeared to be regulated by nitrate-nitrite (NO_x) availability, both directly and through competition with benthic and pelagic primary producers. Rates were highest in the fall and winter, coincident with elevated NO_x levels. In addition, a positive correlation between estimated *in situ* denitrification rates and oxygen levels suggested an indirect regulation of denitrification via coupled nitrification. Potential rates of denitrification were elevated under conditions of anoxia during the summer and were apparently limited by supplies of labile carbon. A methods comparison (between the acetylene block and MIMS techniques) indicated that true rates of denitrification in the NRE might be closer to potential rates measured in the current study with an *estimated* 22 percent of DIN removed via denitrification. Estimations of the change in magnitude and distribution of microbially mediated denitrification following proposed reductions in N loading were also made. Based on the kinetics of nitrate utilization by denitrifiers, a 30% reduction in DIN loading to the NRE would not be expected to reduce the percentage of N removed via denitrification.

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SUMMARY AND CONCLUSIONS

Experimental manipulations were used to predict the effects of reducing the ratio of dissolved inorganic nitrogen (DIN) to dissolved inorganic phosphorus (DIP) (DIN:DIP ratio) in the Neuse River Estuary (NRE) on the structure and function of the native phytoplankton community. Dilution bioassays were conducted throughout 1997-1998 at a mesohaline site in the NRE. These manipulative experiments allowed reduction in concentration of one or more limiting nutrients by dilution and replacement of specific nutrient compounds to manipulate DIN:DIP ratio (Paerl and Bowles 1987, Carrick et al 1993, Dodds et al 1993). Changes in primary productivity, dinitrogen (N_2) fixation (nitrogenase activity), genetic potential for N_2 fixation (presence of *nifH*), phytoplankton taxonomic composition (diagnostic photopigment concentration) and numbers of N_2 fixing cyanobacteria (microscopy) were determined. Using these results we assessed the potential for N_2 fixing cyanobacteria to affect nutrient cycling and trophodynamics in the NRE both currently, and in response to nutrient load modifications. Additionally, spatial and temporal measurements of rates of denitrification, a potentially important fate of nitrogen (N) in the NRE and similar systems, were assessed. Estimations of the change in magnitude and distribution of microbially mediated denitrification following proposed reductions in N loading were also made. Because experiments were conducted over a relatively short-term (approximately 5 days) and were of moderate volume, they may not reflect changes that would occur on an ecosystem scale. However, these data are the first to provide any experimental prediction of potential responses of the NRE microbial community to nutrient management.

Several analyses were employed to assess the abundance and activity of N_2 fixing cyanobacteria under ambient and modified nutrient concentrations. Results from these experiments indicate that if a managed reduction of N loading leads to a reduced ambient DIN:DIP ratio in the NRE, rates of N_2 fixation may be higher when diazotrophic cyanobacteria are present. We did not, however, detect an increase in either the diversity or abundance of N_2 fixers resulting from experimental manipulations of DIN: DIP ratio. Our results indicated that there is the potential for significant N input through N_2 fixation in the NRE, however N_2 fixation was confined to a relatively narrow temporal window during this study.

A 30% reduction in concentration of N, P or both N and P caused a reduction in phytoplankton assimilation number at the estuarine bioassay site. There was no reduction in assimilation number at the riverine site when analyzing the data for entire experimental period. When analyzing individual experiments, decreasing N concentration reduced assimilation number as often as decreasing both N and P concentrations, while decreasing P concentration alone reduced assimilation number only once. It is difficult to identify a specific geographic region of the NRE where reducing levels of nutrients is likely to decrease phytoplankton productivity. However, it appeared that in areas further downstream, nutrient concentration reductions (most often N) are likely to be more effective. Phytoplankton taxonomic composition as measured by HPLC diagnostic photopigment analysis was not altered by any treatment at any location in this work.

Patterns have emerged that provide insight into regulatory factors and the seasonal dynamics of denitrification in this estuarine system. Denitrification measured as estimated *in situ* rates appeared to be regulated by NO_x availability, both directly and

through competition with benthic and pelagic primary producers. Rates were highest in the fall and winter, coincident with elevated NO_x levels. In addition, a positive correlation between estimated *in situ* denitrification and oxygen levels suggested an indirect regulation of denitrification via coupled nitrification. Potential rates of denitrification were elevated under conditions of anoxia during the summer and were apparently limited by supplies of labile carbon. A methods comparison (between the acetylene block and MIMS techniques) indicated that true rates of denitrification in the Neuse might be closer to potential rates measured in the current study with an *estimated* 22 percent of DIN removed via denitrification. Based on the kinetics of nitrate utilization by denitrifiers, a 30% reduction in DIN loading would not be expected to reduce the percentage of N removed via denitrification. These findings should be considered preliminary, pending direct assays of denitrification at fine temporal and spatial resolution. In addition, information on rates and dynamics of nitrification is needed to improve our understanding of N cycling in the NRE.

RECOMMENDATIONS

Results from this study provide information regarding the likelihood of success of North Carolina's nutrient management strategy for the NRE (15A NCAC 2B .0232-.0240) and experimentally based predictions about potential unanticipated collateral effects on the nutrient cycling and microbial community structure and function in the NRE. Our results indicate that a 30% reduction in N levels is likely to decrease phytoplankton productivity in the upper estuary. Reduction in levels of both N and P that may result from the implementation of some proposed Best Management Practices would likely lead to further reduction in phytoplankton productivity. Reduction of P levels is expected to be less effective at reducing phytoplankton productivity than reduction of nitrogen in the upper estuary. None of the nutrient reductions tested decreased phytoplankton productivity significantly at the riverine site. Should reduction of nitrogen levels in the NRE lead to decreased ratios of dissolved inorganic nitrogen to phosphorus, nitrogen-fixing cyanobacteria may be more active during periods in which they are present in the estuary. Our results indicate that there is the potential for disruption of N cycling in the NRE, however N_2 fixation was confined to a relatively narrow temporal window during this study. Denitrification is a significant sink for nitrogen in the NRE. Projections using current kinetic models indicate that a reduction in nitrogen levels in the NRE will not decrease the percentage of nitrogen removed via denitrification. As a whole, the results of this study support the likelihood of success of nitrogen management in the Neuse River watershed to control phytoplankton productivity. Additionally, we found little indication that unanticipated detrimental effects (e.g. a decrease in the importance of denitrification as a sink or an increase in the abundance and distribution on nitrogen-fixing cyanobacteria) of nitrogen management are likely to be major concerns. All experiments conducted were relatively short-term (ca 5 days) and moderate volume, so they may not reflect changes that would occur on an ecosystem scale. However, these data are the first to provide any experimental prediction of potential responses of the NRE microbial community to nutrient management.

INTRODUCTION

The NRE is experiencing deteriorating water quality that recently prompted a mandated nutrient management strategy intended to halt water quality decline and provide eventual improvement (15A NCAC 2B.0232-.0240). Phytoplankton primary production in the NRE is controlled by N availability throughout much of the year (Paerl 1987, Boyer et al. 1994, Paerl et al. 1995). Riverine loading of DIN is dominated by NO_x that declines non-conservatively down-estuary under most flow conditions (Christian et al. 1991). Management plans for the NRE include an N loading cap and an overall 30% reduction in N loading. However, management of N loading may result in shifts in the DIN:DIP ratio and may affect the importance of various sinks of N (e.g. denitrification) in the NRE.

Alterations in DIN:DIP ratio in the river water may have significant impacts on aquatic communities beyond a simple reduction in phytoplankton productivity and biomass, including shifts in species composition and possible selection for species adapted to growth in waters with reduced N:P (Tilman et al. 1982, Smith 1983). In particular, the phytoplankton community could conceivably become dominated by N_2 fixing cyanobacteria that may circumvent N-limitation imposed by the managed reduction by fixing atmospheric N_2 into ammonium (Fogg 1973, Paerl 1990). Evaluation of the impact of N-loading reductions on phytoplankton communities is necessary to assess whether additional controls may be required to effectively manage nuisance cyanobacterial growth in N-limited estuaries and to predict potential changes in nutrient cycling and trophic transfer. Because experiments were conducted over a relatively short-term (approximately 5 days) and were of moderate volume, they may not reflect changes that would occur on an ecosystem scale (Carpenter 1996).

Certain cyanobacterial genera are able to fulfill their N requirements by N_2 fixation. These species are capable of growth in N-deficient waters that typify many estuarine and coastal ecosystems. Heterocystous cyanobacteria (e.g., *Anabaena*, *Aphanizomenon*, *Nodularia*, *Cylindrospermopsis*) and some non-heterocystous diazotrophic genera (e.g., *Lyngbya*, *Oscillatoria*) should be afforded a competitive advantage in these N-deficient and P-replete waters. Smith (1983) showed a strong relationship between total N:P ratios and the prevalence of cyanobacterial bloom genera in a range of freshwater habitats. N:P ratios less than 29 (by weight) were conducive to the development and persistence of N_2 fixing genera. This stoichiometric predictor of cyanobacterial dominance has not been examined as thoroughly in estuarine systems. Many estuarine and coastal waters have N:P ratios far below 29 and thus they are largely N limited (Nixon 1986, D'Elia 1987), containing highly variable levels of N_2 fixing cyanobacteria (Paerl 1996, Paerl & Millie 1996). Enhanced abundance and activity of N_2 fixing cyanobacteria in response to reduced N:P has been verified experimentally with lake phytoplankton (Seale et al. 1987, Levine and Schindler 1992), but few data exist on effects on the abundance and activity of estuarine N_2 fixers.

Detection and identification of N_2 fixing cyanobacteria in these waters is critical to monitoring their presence and expansion. Direct microscopic counts provide data on cyanobacteria present often to the species level. However, accurate microscopic analyses are tedious and time consuming and sample preservation may cause damage to some cells. High performance liquid chromatography (HPLC) analysis of diagnostic photopigments can provide information about cyanobacterial abundances. This analysis offers an alternative to microscopic enumeration methods (Jeffrey et al. 1997), but does not provide information beyond the phytoplankton group level and therefore, may not distinguish diazotrophic from non-

diazotrophic cyanobacteria. Molecular approaches are able to circumvent many of these difficulties by targeting and identifying diazotrophic DNA sequences within an environmental sample, regardless of the abundance. DNA analysis provides a definitive identification that can be used to compare the degree of sequence similarity to other species. The *nifH* gene, which encodes the universally present Fe protein component of the nitrogenase enzyme, was used in these studies to characterize cyanobacterial communities. *NifH* is highly conserved among diazotrophs, yet maintains enough variation to be useful in differentiating cyanobacterial species (Zehr et al, 1997). Though new techniques are being developed, molecular approaches used in this study were not quantitative and were only able to discern the absence or presence of the genetic potential for N_2 fixation in these communities.

Denitrification is thought to be an important component of the N cycle of coastal and estuarine waters, potentially alleviating the impacts of excessive N loading (Kemp et al., 1990, Seitzinger 1988, Smith et al. 1985). Denitrification is the reduction of inorganic oxides (NO_3^- and NO_2^-) to gaseous endproducts (N_2O and N_2) carried out by a diverse group of facultative anaerobic heterotrophic bacteria (Knowles 1982). Few data are currently available on the potentially important loss of N to the atmosphere through denitrification in the Neuse River Estuary. It has been estimated that up to 50% of inorganic N entering coastal waters is denitrified (Seitzinger 1988, Yoon and Benner 1992, Nowicki 1994, Boynton et al, 1995). Constraints on the activity of microbial denitrifiers include substrate limitation (NO_3^- and organic carbon) as well as requisite anoxia for the induction of denitrifying enzymes (Knowles 1982). These requirements often determine specific locations and timing of denitrification in a given system.

Several researchers have described a seasonal pattern of peak denitrification in estuarine sediments in the spring that was attributed to an increase in NO_3^- supply from riverine sources coupled with warmer temperatures and increased organic carbon loading from phytodetritus (Jørgensen and Sørensen 1988, Jensen et al. 1988). A midsummer decline in denitrification noted in several estuaries has been explained by a repression of nitrification (oxidation of NH_4^+ to NO_3^-) by anoxia of the sediments (Seitzinger et al. 1984, Kemp et al. 1990). Nitrification is an important source of NO_3^- for denitrification in sediments where NO_3^- influx is low and NH_4^+ is the predominant form of available nitrogen. Because nitrification occurs only under aerobic conditions and denitrification is an obligate anaerobic process, peak rates of denitrification are predicted to occur at the interface of these two zones. Tracer studies with ^{15}N have provided direct evidence of this coupling in estuarine sediments (Nishio et al. 1983, Jenkins and Kemp 1984).

Few studies have examined spatial patterns of denitrification along a salinity gradient within an estuary. It is likely that limitations (organic matter, NO_3^- supplies and oxygen levels) on denitrification activity are seasonally operative in different regions of the estuary. For example, in the NO_3^--rich riverine sections of the NRE, increased organic carbon loading and anoxia of bottom waters following algal blooms and vertical stratification should stimulate denitrification. In contrast, anoxia could prevent nitrification of recycled NH_4^+ (and coupled denitrification) in the mesohaline reaches of the estuary where NO_3^- levels are typically low. Episodic hydrologic events (e.g. hurricanes and tropical storm-related discharge) are also likely to influence denitrification rates by altering levels of NO_3^-, organic carbon and oxygen (Joye and Paerl 1993). Extensive water column anoxia detected in the upper NRE during a 2-week period following Hurricane Fran in summer 1996, coupled with high flux of NH_4^+ from the sediment during this period (Paerl and Pinckney, UNC-IMS, unpublished data), indicates a possible de-

coupling of nitrification-denitrification. Clearly, both seasonal cycles and hydrologic events affect the dynamics of denitrification in estuarine sediments.

The quantification of denitrification is subject to debate since all methods suffer some limitations (Koike and Sørensen 1988, Knowles 1990, Seitzinger et al. 1993). Despite documented limitations of the acetylene inhibition technique (reviewed above), the relative simplicity and short incubation time required for this method makes it amenable to broad-scale field sampling. The kinetic approach, utilized here, provided both information on the dynamics of denitrifier activity and an estimate of *in situ* denitrification. Additionally, the kinetic approach allowed projection of the importance of denitrification at reduced N loading levels. The project was enhanced by additional denitrification measures with N_2/Ar ratio analyses using membrane inlet mass spectrometry (MIMS), a technique made possible by instrumentation suitable for measuring changes in N_2 relative to Ar, an inert reference gas (Kana et al. 1994).

This study used experimental manipulations to predict the effects of reducing the DIN:DIP ratio in the NRE on the structure and function of the native phytoplankton community. Dilution bioassays were conducted throughout 1997-1998 at a mesohaline site in the NRE. These manipulative experiments allowed reduction in concentration of one or more limiting nutrients by dilution and replacement of specific nutrient compounds to manipulate N:P ratio (Paerl and 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 numbers of N_2 fixing cyanobacteria (microscopy) were determined. Using these results we assessed the potential for N_2 fixing cyanobacteria to affect biogeochemistry and trophic cycling in the NRE both currently and in response to nutrient load modifications. Additionally, spatial and temporal measurements of rates of denitrification, a potentially important fate of nitrogen in the NRE and similar systems, were assessed. Estimations of the change in magnitude and distribution of microbially mediated denitrification following proposed reductions in N loading were also made.

MATERIALS AND METHODS

Site Description

The Neuse River watershed has an area of approximately 16,000 km^2 that discharges into the NRE and eventually, into Pamlico Sound (Fig. 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. Strong 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 52 d and can exceed 90 d during periods of reduced discharge (Christian et al. 1991, Robbins & Bales 1995). Denitrification measurements were made on a 5 site transect of the NRE that included Streets Ferry Bridge (SFB), channel Marker 38 (M38), Marker 22 (M22), Marker 15 (M15) and Marker 9 (M9) (Fig. 1). The sites for the dilution bioassays were mid-channel at M15 and at SFB (Fig. 1).

Dilution Bioassays

Water was collected in 20 L carboys rinsed once with 0.1N HCl and 3 times with site water. Samples were taken from just below the surface and transported in the dark to the UNC-CH Institute of Marine Sciences (IMS). Nutrient dilution bioassays were used to reduce the concentrations of ambient dissolved ions in Neuse River water samples. In this assay, concentrations of potential growth-limiting nutrients, chiefly N and P compounds, were reduced while maintaining non-growth-limiting ions at naturally-occurring levels. For details on the dilution bioassay procedure see Paerl and Bowles (1987). Treatments for the August 1997, January 1998 and August 1998 experiments included: Control (unamended river water), 30% dilution (both N and P concentrations reduced, DIN:DIP same as ambient), and 30% dilution with P added back to ambient concentration (N concentration reduced, reduced DIN:DIP compared to ambient). The July 1998 dilution bioassay treatments included a series of dilutions from 20-50% and parallel dilutions with P added back to ambient concentration. In the dilution bioassay designed to assess the effectiveness of nutrient reduction at controlling productivity, treatments included: Control (unamended river water), 30% dilution (both N and P concentrations reduced), 30% dilution with P added back to ambient concentration (N reduced), 30% dilution with N added back to ambient concentration (P reduced), 30% dilution with both N and P added back to ambient concentration (control for dilution of growth factors other than N and P). The two March 1999 dilution bioassays included dilution series similar to the August 1998 experiment described above. Finally, the April 1999 dilution bioassay included 30% dilution and 30% dilution with P added back to ambient levels at several sites through the estuary to assess the effectiveness of decreasing N concentration at reducing productivity over a broad spatial scale. In all experiments dissolved inorganic carbon (DIC) was replenished to estimated ambient levels based on parallel measurements and extensive historical data sets. Dilutions were made using a major ion solution, consisting of the major cations and anions of the NRE (Paerl and Bowles 1987). Each treatment had four replicates in 10 L Cubitainers made of chemically inert polyethylene that transmitted 85% of incident irradiance. Cubitainers were placed outside IMS in holding ponds filled with water from nearby Bogue Sound to mimic natural temperature and irradiance conditions and incubated for 96 h. Cubitainers were inverted twice daily to prevent settling of biomass. Subsamples were taken from each cubitainer and analyzed for phytoplankton biomass as chlorophyll *a* (Chl *a*) and primary productivity ($^{14}CO_2$ incorporation),

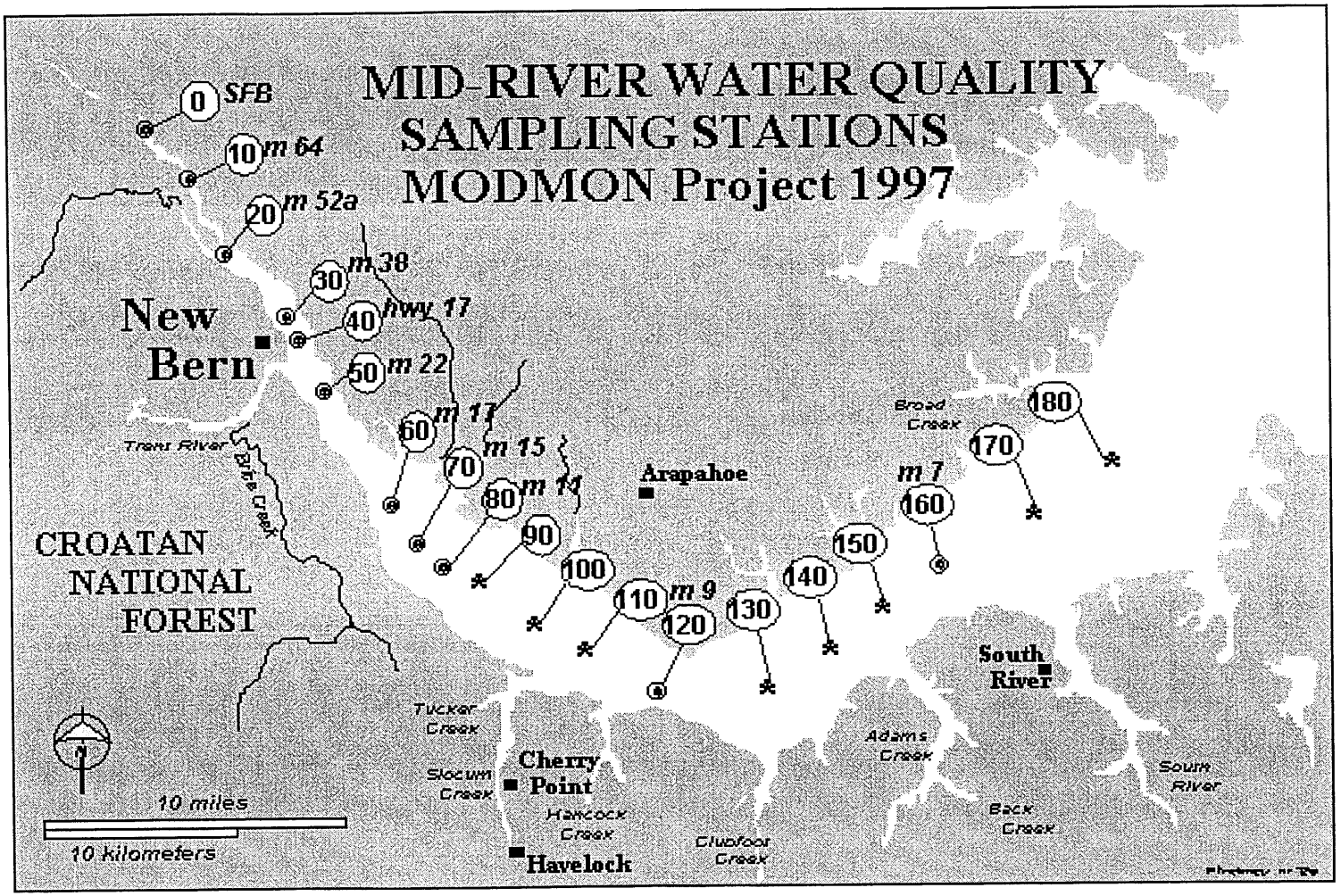

Figure 1: Map showing the study site's location within the Neuse River Estuary and the sampling stations for the assays

nitrogenase activity (acetylene reduction), phytoplankton community composition (HPLC diagnostic photopigment analysis, cell counts) and genetic potential for N_2 fixation (detection of *nifH* sequences).

Chlorophyll *a* concentration was measured using the non-acidification fluorometric method (Welschmeyer 1994). The fluorometer used was a Turner TD-700 that incorporates a set of very narrow bandpass excitation and emission filters that nearly eliminate spectral interference caused by the presence of pheophytin *a* and chlorophyll *b*. Fifty ml water subsamples were filtered onto 25 mm Whatman GF/F glass microfiber filters and stored at 0°C. 10 ml acetone (90% acetone: 10% DI) was added to filters in polypropylene centrifuge tubes. Filters were then sonicated on ice for 30 s and extracted for approximately 12 h at -20°C.

Phytoplankton primary productivity was measured using the ^{14}C method. Samples were taken from each cubitainer and dispensed into 20 ml clear borosilicate vials for incubation. $NaH^{14}CO_3$ was added to samples (~6 kBq ml^{-1} final activity) that were then incubated in a rack just below the water surface. Dark vials were also incubated for each treatment and their rates were subtracted from the light vial values. When necessary (ambient irradiance above 800 μE m^{-2} s^{-1}), neutral density screens were used to reduce incident irradiance in order to prevent photoinhibition. Following 3 h incubations, samples were filtered onto 25 mm 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 with 5 ml Ecolume 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 using a calibrated ^{14}C-toluene standard. Dissolved inorganic carbon (DIC) in water samples was determined using infrared gas analyzer (Li-Cor LI 6252) (Paerl 1987). Primary productivity rates were normalized to Chl *a* to remove variability due to unequal initial biomass in different treatments resulting from dilutions.

N_2 fixation (nitrogenase activity) was estimated using the acetylene reduction assay (Stewart et al. 1967). Fifty 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 adding CaC_2 to deionized water (DI) and was injected into the headspace of the vial (~20% of the total volume). Acetylene blanks (DI + acetylene) and sample water blanks (no acetylene added) were run to account for abiotic generation of ethylene and ethylene from sources other than acetylene reduction, respectively. Serum vials were incubated in a running water bath for 4 h under appropriate light levels (modified using neutral density screens when necessary). Dark vials were also incubated for each treatment and their rates were subtracted from the light vial values. Following the incubation, 2 ml headspace sub-samples were transferred to 2 ml evacuated autosampler vials for gas chromatographic analysis of ethylene 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. 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 Whatman GF/F filters (25 mm), 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 functional 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) 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. CHEMTAX software is a product of CSIRO Division of Oceanography (Hobart, Australia) and is available from D.J. Mackey (Denis.Mackey@marine.csiro.au) (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. 1997, Pinckney et al. 1998). The program uses a steepest descent algorithm to determine the best fit based on initial estimate of pigment ratios for algal classes. Input to the program includes raw matrices of photopigment concentrations from HPLC and an initial pigment ratio file. The data matrix is subjected to a factor minimization algorithm that calculates a best fit pigment ratio matrix and a final phytoplankton class composition matrix. The class composition matrix can be expressed as a relative or absolute value for specified photopigments. The absolute Chl *a* contribution of each class is a useful measure because it partitions the total Chl *a* into major phytoplankton groups.

Subsamples from each treatment were taken for microscopic cyanobacterial counts. Lugol's solution was used to preserve 50ml 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, water was filtered through Supor filters (pore size of 0.2 μm) and stored at -80°C. To extract DNA from the material on these filters, they were heated to 90°C for 5-6 hours. After 3 hours, the remaining material was scraped off the filter and twice subjected to bead beating (150-200 μm glass beads, 0.3g) for 3 min each time. RNase was added near the end of the incubation (300 μg). Microscopic observation ensured 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 polymerase chain reaction (PCR) amplification used degenerate oligonucleotide primers designed to pick up the *nifH* gene in all aerobic nitrogen fixers. The genes for nitrogenase are highly conserved, allowing primers to be constructed that will amplify *nifH* from a diverse set of organisms, but has enough variation to distinguish taxonomic groups and genera (Ben-Porath and Zehr, 1994). The primer sequences used were:

forward primer: 5' - ATYGTCGGYTGYGAYCCSAARGC - 3'
reverse primer: 5' - ATGGTGTTGGCGGCRTAVAKSGCCATCAT - 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 nucleotide fragment was amplified using 2mM $MgCl_2$ 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 minute at 94°C, 1 minute at 53.5°C, 1 minute 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 EcoRI, were sequenced (700 ng plasmid with M13 forward primers). Sequences were analyzed using SeqLab and Phylip software (University of Wisconsin Genetic Computer Group).

Community responses were analyzed using a one-way analysis of variance (ANOVA). Data were ln-transformed when necessary to satisfy the normality assumption. Pigment percent composition data were arcsine square root transformed. *Post-hoc* comparisons of means were made using a Bonferroni multiple comparison of means (Moore & McCabe 1993).

Denitrification

As mentioned previously, direct rate measurement of denitrification is currently hampered by methodological problems (Thompson et al. 1998) and by the large spatial and temporal variability of rates (Christensen et al. 1990, Folorunso and Rolston 1984, Parkin et al. 1987). Acetylene block of the reduction of N_2O to N_2 has been shown to be ineffective at low (<10 μM) NO_3^- concentrations (Kaspar 1982, Oremland et al. 1984, Slater and Capone 1989). Acetylene also inhibits nitrification (Hynes and Knowles 1984), potentially leading to underestimates of denitrification in systems where nitrification is a significant source of NO_3^- for (i.e. coupled to) denitrification. However, the simplicity of the acetylene inhibition technique allows for greater spatial replication relative to other techniques, and the short incubation time (minutes to hours) permits the measure of short-term changes in denitrification. The acetylene block technique has been adapted for use in estuarine sediments (Thompson et al. 1995, 1998) by measuring denitrification rates at incremental NO_3^- additions to determine saturation kinetics, providing both potential (V_{max}) and an estimate of *in situ* denitrification activity through interpolation of rate at ambient bottom water NO_x concentration.

Denitrification was assessed monthly at 5 sites on a transect in the NRE (June1997 through July 1999) using the acetylene block technique. Water depth at sediment collection sites was approximately 3 meters for all stations. Three replicate grab cores were collected with a Wildco Hand Corer. The cores were transported to the laboratory in a cooler, with incubations initiated within 2 to 4 hours following collection. Four sub cores (1 cm diameter X 2 cm deep) were taken from the surface of each larger core for a total of 12 cores. Determination of denitrification rates was made at incremental NO_3^- additions (final pore water concentrations of 10, 50, 100, 1000 μM NO_3). Line injections of acetylene-saturated solutions of the 4 concentrations of $NaNO_3$ were made at 1 cm intervals through silicon filled sideports at a volume equal to 10% porewater and overlying water and incubated for 1 hour. At the end of the incubation period, the cores were placed into 37 ml serum bottles containing 10 ml 1N KCl and immediately sealed. Background N_2O levels were determined seasonally by placing 3 replicate cores from each site in serum bottles containing KCl at the beginning of the incubation. Fifteen ml of headspace gas were removed and stored in previously sealed and evacuated 13 ml serum vials. Analysis of N_2O was performed within 1 week of incubation with a Shimadzu GC 14A gas chromatograph equipped with an electron capture detector. Denitrification rates were normalized to core weights and converted to an areal measure, accounting for sediment porosity differences. Estimated *in situ* rates were calculated by subtracting background levels of N_2O and entering measured concentrations of bottom water NO_3^- to equations derived from Lineweaver-Burke transformations of kinetic data:

$$1/V=[Ks/Vm]1/S+[1/Vm]$$

where V=denitrification rate, S=nitrate concentration, Vm=maximum or saturation denitrification rate and Ks=half saturation constant, assuming first order denitrification rate with respect to nitrate concentration. Potential rates are reported as the calculated Vm from this equation. Variability among replicates, expressed as r^2 of the regression for transformed data, was 0.632 (mean r^2 for all denitrification rates measured). The mean Ks for combined data was 198 ± 23.93 (standard error).

A comparison of two methods for measuring denitrification was conducted on five occasions between October 1999 and February 2000 at stations M9, M15 and M38. Membrane inlet mass spectrometry (MIMS) is a recently developed technique (Kana et al. 1994) for measuring ratios of N_2 , Ar and O_2 in water samples at relatively high precision (0.05%), providing direct rate measures during a relatively short incubation time (up to 12 hours). Sediment cores (10 cm x 35 cm) were collected with a custom built hand operated gravity coring device (designed by J. Fear) from sediments at water depths of 3 to 7 meters (downstream stations were at greater water depths). Upon return to the laboratory, cores were placed in baths of oxygenated water collected from each site and allowed to equilibrate overnight. At the start of the incubation, submerged cores were capped with gas-tight tops equipped with 2 sample ports and an attached stir bar. Cores were placed around a carousel housing 4 magnets that rotated stir bars at approximately 40 rpm. At each sampling point (3 h. interval), water was collected in 5 ml ground glass stoppered tubes in triplicate; sample water withdrawn from the sealed core was displaced by oxygenated water gravity fed to the inlet port. Overall sample water withdrawn was less than 10% of the total water volume overlying the core. N_2 , Ar and O_2 concentrations were analyzed with MIMS which utilizes a silicone membrane interface and components that provide temperature and flow control of water and trapping of interfering gases (Kana et al. 1994). N_2 flux was determined by comparison of N_2:Ar in core samples to standard air-equilibrated water (constant temperature and salinity) run in triplicate at each time point. Argon concentration was assumed to be at equilibrium with the water at the measured temperature and salinity. N_2 flux was calculated as the mean change in N_2 from 3 replicate cores over the 12 h incubation period. Incubations for the acetylene block technique were conducted as described above on subcores collected from replicate cores at each site.

Water samples (surface and bottom) collected bi-weekly were analyzed for NO_x and NH_4^+ and PO_4 concentrations, chlorophyll *a* and particulate carbon, hydrogen and nitrogen (CHN) content. Samples were filtered through pre-combusted glass fiber filters (pore size 0.45 mm) and analyzed with a Lachat QuickChem automated ion analyzer. Filters and surface sediments were analyzed for CHN with a Perkin Elmer 2400 Series II elemental Carbon Hydrogen Nitrogen analyzer.

Benthic biomass, measured as chlorophyll *a* content of the microalgal community, was determined monthly at each transect site. Two replicate cores (1.15 cm^2 x 0.5 cm deep) were obtained from the surface of a single grab core. Sediment samples were sonicated for 30 seconds over a bath of ice and chlorophyll *a* was extracted in a solvent solution of 45% methanol, 45% acetone, 10% deionized water for 24 hours in the freezer. Following extraction, the sediments were centrifuged (3000 rpm, 2 minutes) and filtered. Extracts were analyzed with a Turner 450 fluorometer standardized to dilutions of a known chlorophyll *a* concentration in the 45:45:10 solvent solution (Strickland and Parsons, 1972).

Sediment organic carbon (C) and nitrogen (N) were analyzed monthly in 2 replicate cores (1.15 cm^2 x 1 cm deep) collected from a single grab core at each site. Sediment cores were dried for 24 h at 70°C, ground with mortar and pestle, fumed for 48 h with 1N HCl to remove

inorganic C, and re-dried. Fumed sediment samples were analyzed for organic C and N content with a Perkin Elmer CHN analyzer (Model 2400 Series II) standardized with acetanilide. Replicate analyses were performed for each sediment core. Bottom water, collected bi-weekly from each station, was filtered through replicate muffled Whatman GF/F filters. Filters were dried for 24 h at 70°C, fumed for 6-8 h with 1N HCl to remove inorganic C, and re-dried. Filters were rolled, inserted into foil capsules and analyzed for CHN as described above for sediments.

NRE Monitoring Data

This work coincided with the a large-scale collaborative water quality modeling and monitoring project (ModMon: www.marine.unc.edu/neuse/modmon) in the Neuse River-Estuary. 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. Both surface and bottom water samples were collected at each station for analysis of nutrient concentrations and photopigment concentrations (procedures described above). These environmental data are used as context for the bioassays described in this report.

RESULTS AND DISCUSSION

Results will be discussed in sections defined by the particular hypothesis that was tested. The general sections are effects of nitrogen reduction on diazotrophic abundance and activity, effects of nutrient dilutions on phytoplankton primary productivity, and patterns and rates of denitrification.

Effects of N reductions on diazotrophs

Hypothesis *A 30% reduction in ambient NRE DIN concentrations will result in increased relative abundance and diversity of phytoplanktonic diazotrophic cyanobacteria.*

Analysis of the effects of N reduction on the relative importance of cyanobacteria to the overall phytoplankton community structure and the size and diversity of the diazotrophic portion of the phytoplankton community was assessed in four bioassays. In August 1997 both DIN:DIP ratio and salinity were very low at M15 (Table 1). Cyanobacteria were found to be the most abundant taxa by photopigment analysis though their abundance did not change significantly in response to any treatment (Fig 2A). Microscopic analysis of phytoplankton community composition revealed filamentous heterocystous cyanobacteria to be dominant. Microscopic counts of the N_2 fixing cyanobacteria present identified *Anabaena aphanizomenoides* to be present in all treatments in relatively high densities (Fig. 3A). Highest mean number of cells was observed in the 30% dilution +P treatment.

DIN:DIP ratio was considerably higher at the start of the January 1998 dilution bioassay and salinity was very low (Table 1). Cyanobacteria were far less abundant than in August 1997, comprising between 5-15% of total phytoplankton community composition (Fig. 2B). Microscopic evaluation of the phytoplankton observed chlorophytes and diatoms to be dominant. There were no differences detected in percent cyanobacteria among treatments using HPLC (Fig. 2B).

The July 1998, dilution bioassay water samples had both low salinity and a very low DIN:DIP ratio (Table 1). HPLC diagnostic photopigment analysis indicated cyanobacteria to be the most common taxa (Fig. 2C) and did not detect a change in phytoplankton community composition in response to any of the treatments (Fig. 2C). Microscopic analysis of the phytoplankton community showed filamentous cyanobacteria to be dominant. Counts of N_2 fixing cyanobacteria included high numbers of *Anabaena compacta, Anabaenopsis* sp. and *Anabaena aphanizomenoides* in all treatments (Fig. 3C). There was no apparent relationship between treatments and mean cell counts.

The August 1998 dilution bioassay samples had a very low DIN:DIP ratio and slightly higher salinity than the three previous experiments (Table 1). Photopigment analysis showed cyanobacteria to be the dominant taxa (Fig. 2D). There were no significant changes in phytoplankton community composition in response to treatments (Fig. 2D). Microscopic counts of N_2 fixing cyanobacteria showed *Anabaena aphanizomenoides* to be present, but at far lower densities than in either August 1997 or July 1998 (Fig. 3D).

Experimental manipulations of nutrient concentrations did not exert any measurable effect on the proportion of N_2 fixing cyanobacteria in phytoplankton community or on the overall diversity of N_2 fixers. Possible explanations for the lack of an observed treatment effect were the extremely high and low ambient DIN:DIP ratios during this study or an insufficient incubation

Date	station	temp (C)	DO (mg/L)	salinity (ppt)	NOx (ug/L)	NH4 (ug/L)	PO4 (ug/L)	chl a (ug/L)
8/7/97	m15 S	26.69	8.0	0.3	5.6	12.0	52.9	10.1
	SFB S	27.76	5.3	0.1	525.2	51.5	57.0	2.6
10/14/97	m15 S	24.09	8.9	9.0	9.3	56.1	24.0	4.5
	SFB S	23.24	6.0	0.1	807.9	49.6	52.7	1.0
1/12/98	m15 S	11.86	9.4	0.0	677.4	95.9	26.4	4.7
	SFB S	11.78	7.6	0.0	682.6	38.6	22.7	0.9
3/31/98	m15 S	18.57	7.9	0.0	442.8	39.4	31.0	1.1
	SFB S	18.72	6.9	0.0	369.8	24.1	26.6	1.7
4/28/98	m15 S	18.33	9.8	0.1	259.2	14.8	24.6	30.4
	SFB S	16.88	6.6	0.0	249.6	43.7	28.3	0.6
5/27/98	m15 S	25.61	9.3	0.7	189.4	18.7	4.9	12.2
	SFB S	25.55	6.1	0.0	987.1	90.5	43.7	0.5
6/22/98	m15 S	28.10	8.1	1.7	5.3	15.6	30.5	191.9
	SFB S	30.00	6.6	0.1	731.0	10.8	35.9	6.1
7/21/98	m15 S	28.96	6.8	2.2	0.0	7.6	70.9	14.2
8/17/98	m15 S	28.74	6.4	4.4	1.8	10.5	84.0	11.4
	SFB S	30.38	6.2	0.1	632.9	34.9	65.1	2.9
11/9/98	m15 S	12.72	10.6	6.4	11.44	10.4	18.1	11.1
	SFB S	12.16	8.9	0.0	820.29	54.9	29.1	0.4
2/3/99	m15 S	11.9	8.78	0.6	514.33	52.16	23.5	0.67
	SFB S	11.19	8.3	0.0	458.74	44.63	20.25	0.93
3/2/99	m15 S	10.04	10.36	2.6	511.86	57.7	16.04	3.20
3/29/99	SFB S	13.02	8.8	0.0	419.61	64.67	17.2	1.63
4/26/99	m9 S	18.89	8.14	7.6		12.76		8.41
	m15 S	19.97	9.76	2.5	9.31	7.8	6.77	7.60
	m38 S	20.65	8.17	0.1	485.04	128.47	44.89	2.86
	m52 S	21.51	6.51	0.1	575.51	168.08	46.55	0.72
	SFB S	21.92	6.79	0.1	626.3	92.29	35.73	0.41

Table 1: Physical data for dilution bioassays

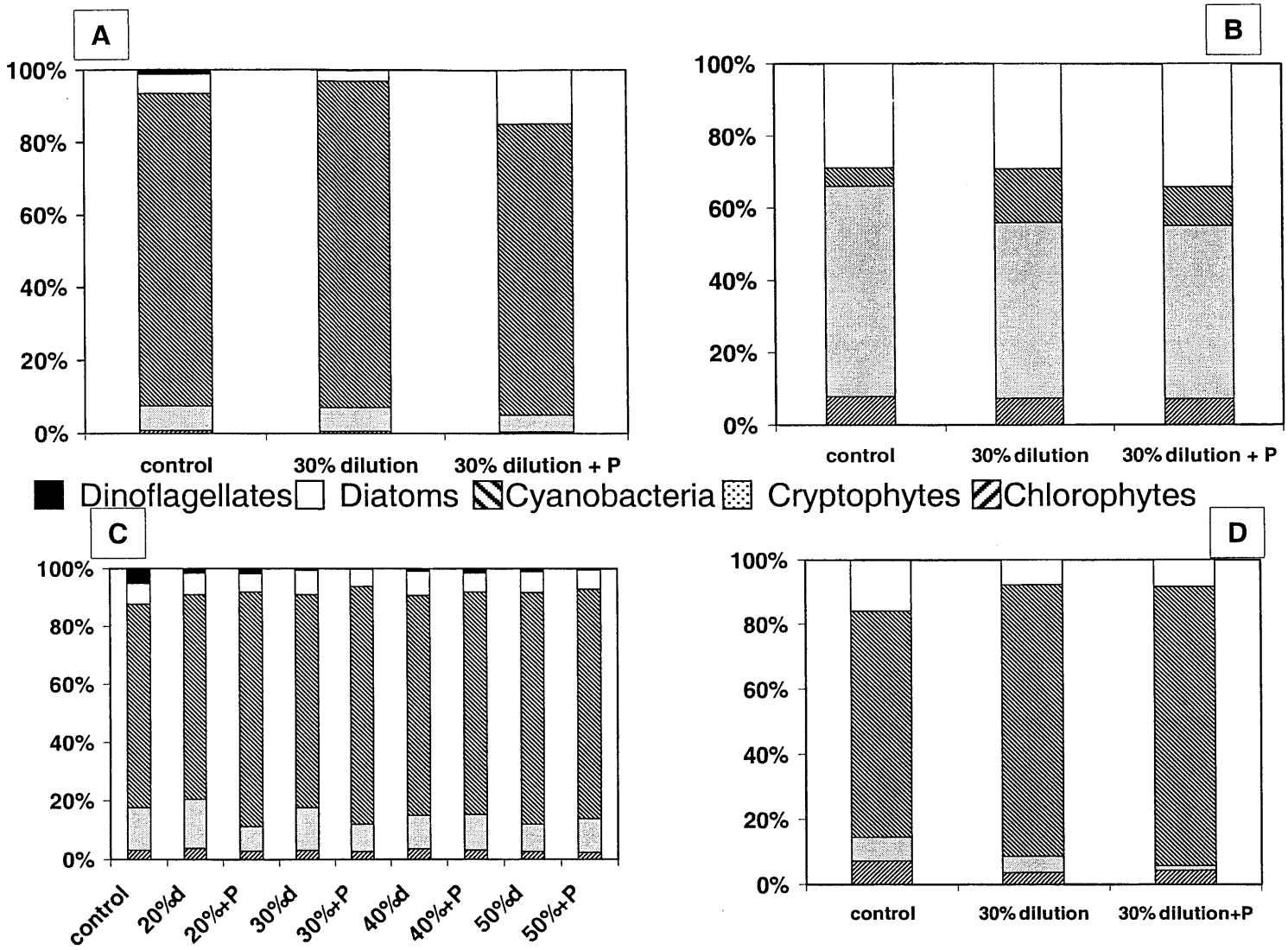

Figure 2: Phytoplankton taxonomic composition as measured using HPLC diagnostic photopigment concentrations processed using the CHEMTAX matrix factorization program. Values are presented as mean percentage of total community chlorophyll *a*.

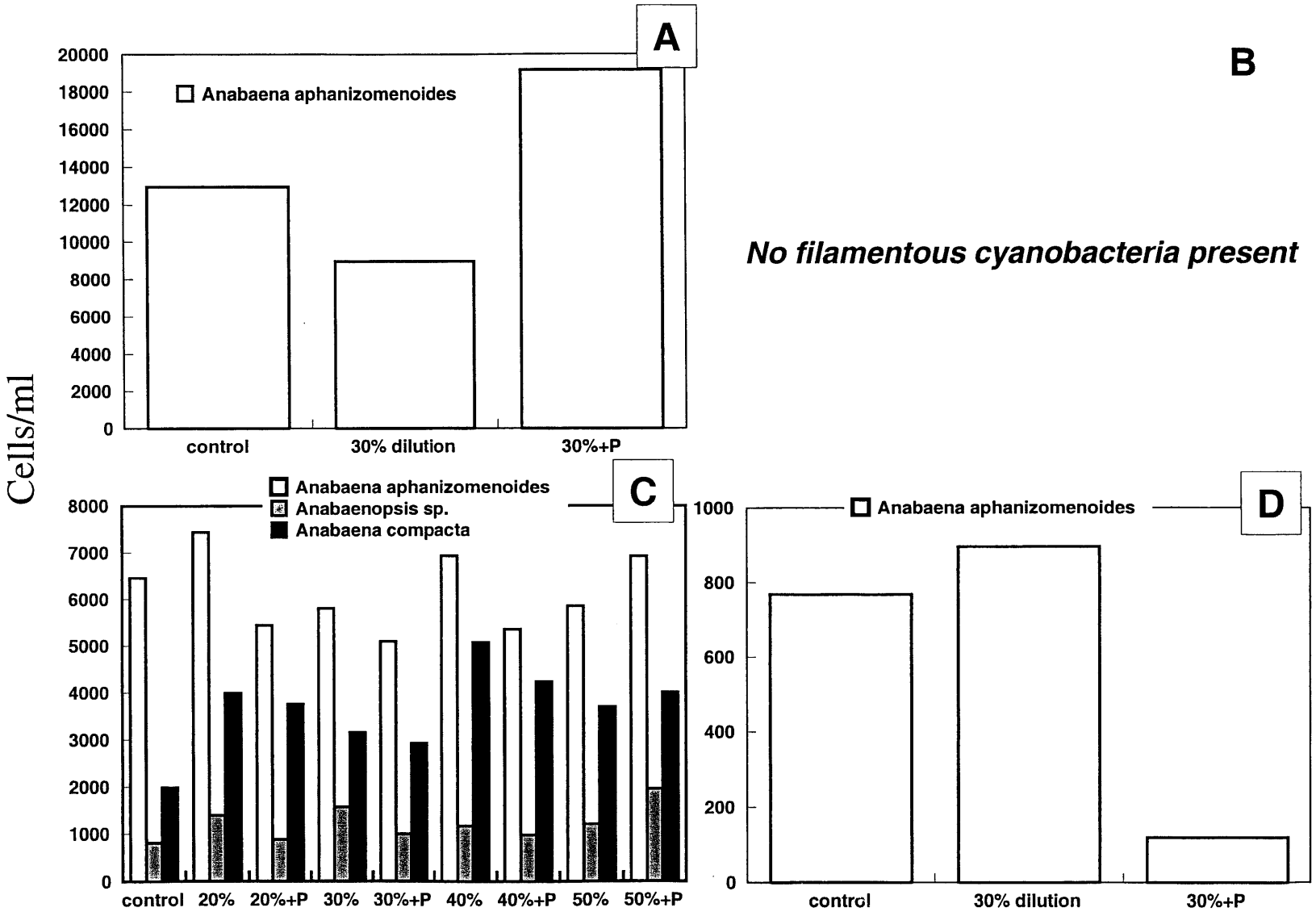

Figure 3: Microscopic counts of filamentous cyanobacteria from experiments in July 1997 (A), January 1998 (B), July 1998 (C), and August 1998 (D).

time for reproduction of cyanobacteria. The latter is possible because the doubling time for cyanobacteria may be as long as several days (Wallström et al. 1992), though optimal conditions would likely induce a detectable response. The more likely scenario was that in the dilution bioassays where N_2 fixers were dominant, the ambient conditions (low DIN:DIP ratio, low salinity) were favorable at the beginning of the incubations. Therefore, a significant community of N_2 fixing cyanobacteria were already present in the river, and thus the controls, making increases in their numbers less likely. Although varying DIN:DIP ratio did not induce a response in abundance, there did appear to be an effect on nitrogenase activity in these cyanobacteria.

Hypothesis *A 30% reduction in ambient NRE DIN concentrations will result in enhanced rates of in situ nitrogenase activity (N_2 fixation).*

Nitrogenase activity was detected in all treatments in the August 1997 and July 1998 dilution bioassays following a 3-day incubation. In August 1997, rates of nitrogenase activity were significantly higher in the 30% dilution +P treatment compared to the control (Fig 4A) (Bonferroni, $p<0.05$). In the July 1998 experiment, nitrogenase activity was significantly higher than the control in all dilutions with the exception of the 20% dilution (Fig. 4C) (Bonferroni, $p<0.05$). Nitrogenase activity in dilutions with P added back to ambient was higher than the parallel dilutions alone in all cases except the 40% dilutions (Fig. 4C) (Bonferroni, $p<0.05$).

Throughout this study ambient DIN:DIP ratio was generally either quite high (>30) or very low (<5) (Table 1). N_2 fixation occurred when both DIN:DIP ratio and salinity were low (<2ppt). Low DIN:DIP ratio has been found to favor N_2 fixers in both laboratory studies and field surveys (Niemi 1979, Smith and Bennett 1999). N_2 fixers may have been present during periods of low salinity because they often have limited halotolerance (Apte et al. 1987) and because the upstream river water was the likely origin of N_2 fixers in this study. Mallin and co-workers observed a similar pattern of cyanobacterial abundance in the NRE in their work (Mallin et al. 1991)

Reducing DIN:DIP ratio increased N_2 fixation rates relative to the control in all experiments in which N_2 fixation was observed. In July 1998 there were incremental increases in N_2 fixation observed with increasing reduction of DIN:DIP ratio. There was also an increase in N_2 fixation with reduction of both DIN and DIP concentration (same DIN:DIP ratio). This result suggests there was another factor beyond DIN:DIP ratio contributing to the observed increases in N_2 fixation observed. Two plausible explanations include changes in quality or quantity of incident light resulting from dilutions or container effects potentially 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 ratio, indicating reduced DIN:DIP was largely responsible for enhanced N_2 fixation. Decreasing DIN:DIP ration never caused N_2 fixation to occur when it was not present in the control. N_2 fixation was expected in August 1998 due to the very low DIN:DIP ratio, but it was not detected (Fig 4D). Slightly higher salinity at this time likely indicated either freshwater N_2 fixers were not competing effectively or were not being transported in sufficient concentrations from upstream. In the January 1998 experiment the lack of N_2 fixation (Fig 4B) was not surprising due to the relatively high DIN:DIP ratio. Reduction from the relatively high ambient DIN:DIP ratio may have been sufficient to stimulate N_2 fixers in theory (Smith and Bennett 1999), but it was not observed in our experiments. Additionally, the colder winter water temperatures

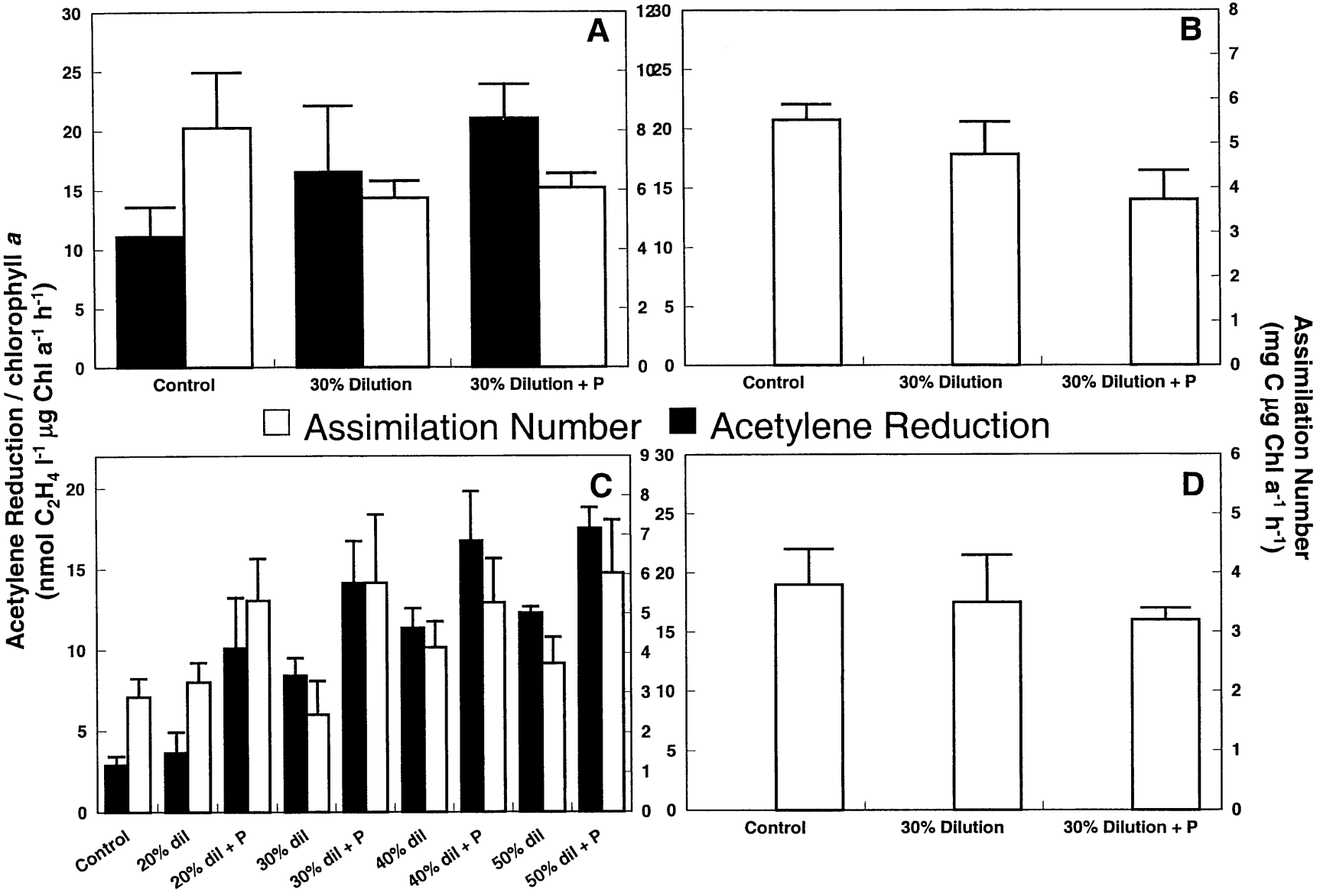

Figure 4: Acetylene reduction (left axis) and assimilation number (right axis) measurements from 3 day Cubitainer incubations with several treatments. Experiments were conducted August 1997 (A), January 1998 (B), July 1998 (C), and August 1998 (D). Error bars are one standard deviation.

decreased the likelihood of N_2 fixing cyanobacteria thriving under any nutrient regime (Paerl 1990).

Management strategies such as the N loading reductions adopted for the NRE may have biogechemical implications. 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 $7x10^7$ 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 ($1.25x10^{-5}$ 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. 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 nutrient management action because it has the potential to be an appreciable source of N (Horne 1977).

Hypothesis *A 30% reduction in ambient Neuse River-Estuary DIN concentrations will result in a higher diversity of diazotrophs (i.e. species possessing the nitrogenase gene)*

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 ctrl and 30% degraded), July 1998 (ctrl, 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 were less than the lower PCR detection limit. Detection of *nifH* confirmed cyanobacterial diazotrophs were present both at times when nitrogenase activity was measured (August 1997 and July 1998) and when it was not (August 1998).

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 distinct *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) (Fig. 5) and together comprised just over half (52.5%) of the clones sequenced. The two sequences that were most prevalent in the clones sequenced (Neuse #1 and 2, combined comprising 37.5%) were most similar to *Anabaena* spp. These two *nifH* sequences had an 86.7% similarity on the nucleotide 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* isolated in culture from Neuse River Estuary samples. The other heterocystous cyanobacterial sequences (Neuse #3 and 4) were seen infrequently and only in clones from July 1998. These sequences could reflect the presence of *Anabaena compacta* seen in microscopic analysis or another filamentous

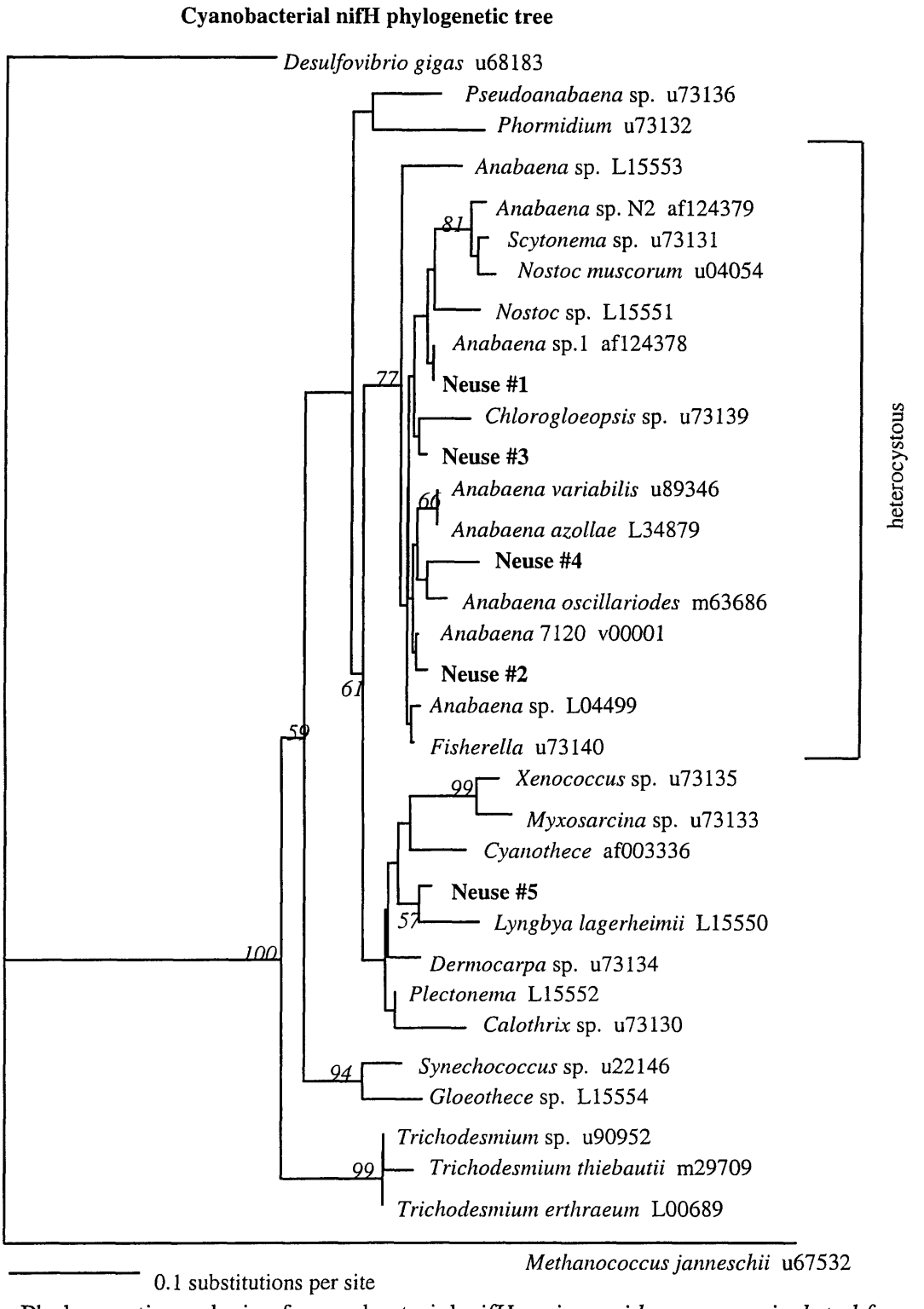

Figure 5: Phylogenetic analysis of cyanobacterial *nifH amino acid sequences isolated from Neuse River Estuary dilution bioassays (Neuse #1-5) and from GenBank (accession numbers given).* Bootstrap values of greater than 50% (generated by distance methods) are listed above each node. *Methanococcus janneschii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

heterocystous cyanobacteria indistinguishable by morphological characteristics. The fifth sequence (Neuse #5) fell outside of the distinct phylogenetic cluster of heterocystous cyanobacteria and was most similar to the filamentous nonheterocystous cyanobacteria *Lyngbya* spp. (Fig. 5). 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. When the *nifH* clones sequences were separated into time points, the relative percentage of *nifH* clones differed between July 1998 and August 1998. 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*.

The transition of the dominant cyanobacteria sequence from heterocystous to nonheterocystous cyanobacteria over a month period could suggest a seasonal succession, a spatial shift in the community, or a response to an increase in salinity. Problems inherent in N_2 fixation without the protection from oxygen provided by the heterocyst may contribute to the lack of nitrogenase activity measured in August 1998 despite the presence of N_2 fixers. The lack of quantitation in these methods makes it difficult to definitively correlate the percentage of clones of a particular sequence with the percentage of those organisms in the water sample. However, microscopic counts of heterocystous filamentous cyanobacteria confirming a decrease from July 1998 to August 1998 support the *nifH* data. Despite the temporal change in the cyanobacterial community, there does not appear to be a treatment effect. The same *nifH* sequences are present in both control and dilution + P (lower DIN:DIP ratio) for each experiment. That is, a reduction in N would not be expected to increase the diversity of diazotrophs in the Neuse River Estuary, though it may change the level of activity of those present. A logical next step would be to look at mRNA transcripts of *nifH* to monitor the expression of this gene instead of merely the genetic potential identified by its presence.

Summary of effects of N reductions on diazotrophs

In this study we employed several methods to assess the abundance and activity of N_2 fixing cyanobacteria under ambient and modified nutrient concentrations. Measuring the importance of N_2 fixing cyanobacteria to the structure and function of the NRE phytoplankton community required all of our analyses to be accurate in the varied environmental conditions that this study included. Results from these experiments indicate that if a managed reduction of N loading leads to a reduced ambient DIN:DIP ratio in the NRE, rates of N_2 fixation may be higher when diazotrophic cyanobacteria are present. We did not, however, detect an increase in the diversity or abundance of N_2 fixers resulting from experimental manipulations of DIN:DIP ratio. There is the potential for significant N input through N_2 fixation in the NRE, however N_2 fixation was confined to a relatively narrow temporal window during this study.

Assessment of reduction of phytoplankton productivity following nutrient reductions

Hypothesis *A 30% reduction in ambient Neuse River-Estuary DIN concentrations will result in a reduction in phytoplankton productivity.*

Dilution bioassays were conducted to assess the effectiveness of nutrient (N, P, N&P) concentration reductions at controlling phytoplankton productivity (assimilation number = C Chl a^{-1} h^{-1}). Regressions were run to assess the relationship of initial conditions and the effectiveness of treatments at reducing assimilation number. No significant correlations were found between percent reduction of assimilation number and any of the following, initial DIN concentration, initial DIP concentration, initial DIN:DIP ratio or initial chlorophyll a concentration. Pooled results from the study at Marker 15 revealed significant treatment (Fig. 6) and time of year effects (2-way ANOVA, $p<0.05$). Additionally, there was a significant interaction between treatment (type of nutrient reduction) and time of year ($p<0.05$). This interaction was expected because nutrient limitation of primary productivity has been found to be seasonally variable in both degree and nutrient type in the NRE (Paerl et al. 1995). A Bonferroni multiple comparisons of means test was applied to assess differences among the nutrient diluting treatments (Moore and McCabe 1993). Reduction in concentration of N, P or both N and P was found to significantly reduce assimilation number below the level of the control ($p<0.05$) (Fig. 6). Assimilation number in the dilution with both N and P added back to ambient concentrations was not different from the control. This result was encouraging in that it indicated that a dilution was not affecting assimilation number in ways other than reduction of N and P concentration. Pooled results for SFB showed no significant change in assimilation number resulting from any of the nutrient dilutions (Fig 7). Because this site is more upstream and the nutrient concentrations are almost always higher than levels seen downstream, the lack of reduction in assimilation number over the period of this study was anticipated.

Examination of results by individual experiment was also made. Nine experiments were conducted at two sites, for a total of 18 measurements. Out of the 18 possible occasions, N was found to limit productivity 3 times (SFB August 1998 and February 1999, M15 January 1998), P was found to limit one time (SFB January 1998) and N and P were determined to be co-limiting three times (SFB June 1998, M15 June 1998 and February 1999) (Figs 8 and 9). However, mean assimilation number was often reduced by nutrient dilutions and not determined to be significantly lower than the control. This was likely attributable to, in part, relatively small sample size (n=4) and relatively high variability of phytoplankton assimilation number in natural samples. Predictable and consistent seasonal patterns in nutrient limitation (e.g. strongly nutrient limited from late spring through fall) were not observed and SFB, the site generally exhibiting higher nutrient levels, was determined to be nutrient limited more often than M15. These results are difficult to explain and may be partly due to possible confounding factors resulting from the dilution bioassay (e.g. changes in quality and quantity of light) or from the differences in native phytoplankton communities.

Experiments were conducted in the spring of 1999 to evaluate the minimal dilution concentration at which reduction in primary productivity would occur. Results from M15 indicated that a 30% dilution reduced the mean assimilation number below the control following a 3-day incubation (Fig 10). In the March 1999 experiment, the reduction of both N and P concentrations was more effective at reducing assimilation number than was reduction of N concentration alone. This appeared to indicate a P limiting or co-limiting scenario. Significant

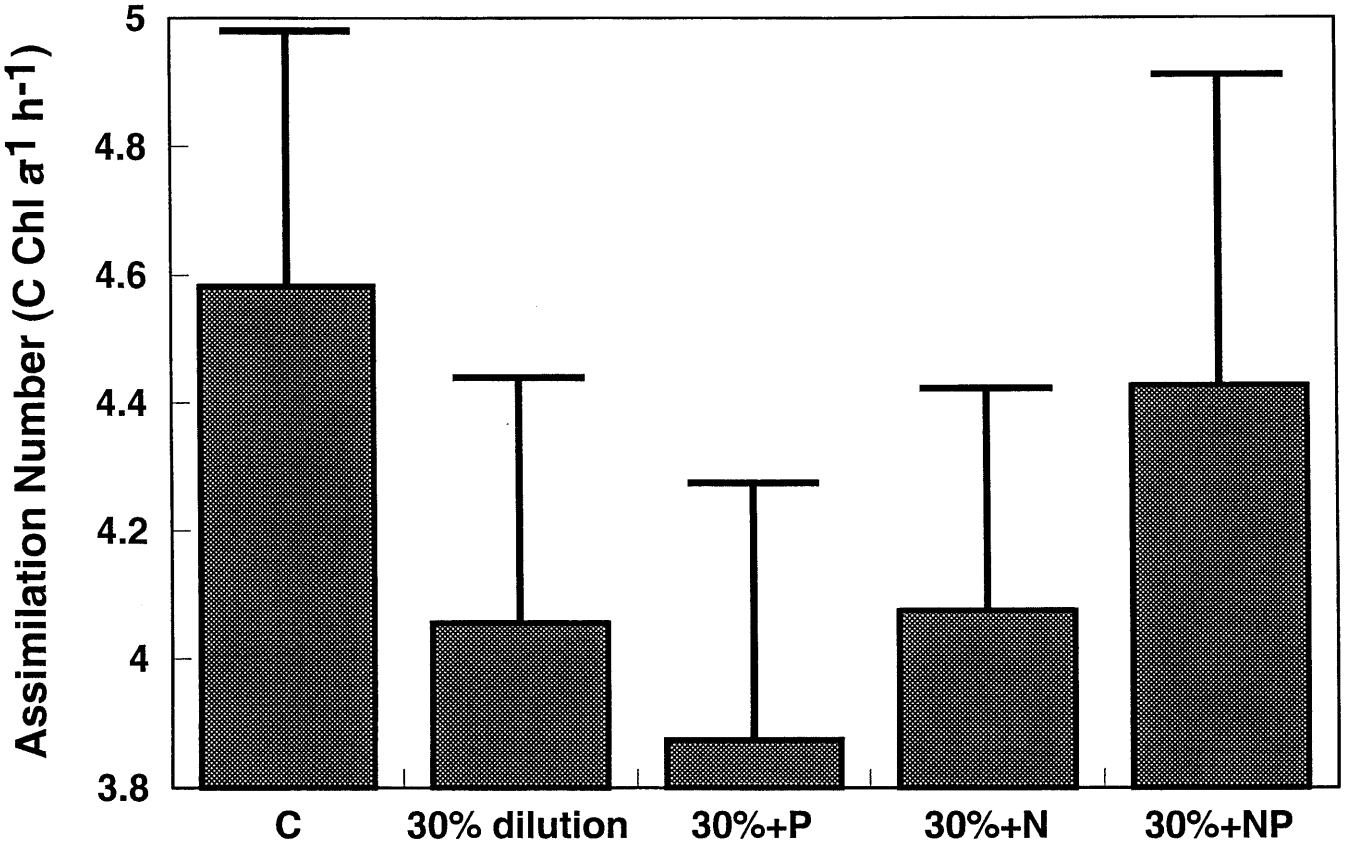

Figure 6: Phytoplankton assimilation number at Marker 15 following 3 day incubation with 5 different treatments. Treatments include control, 30% dilution (both N and P reduced), 30% dilution + P (N reduced), 30% dilution + N (P reduced), and 30% dilution + N + P. Values are means and error bars are one standard error.

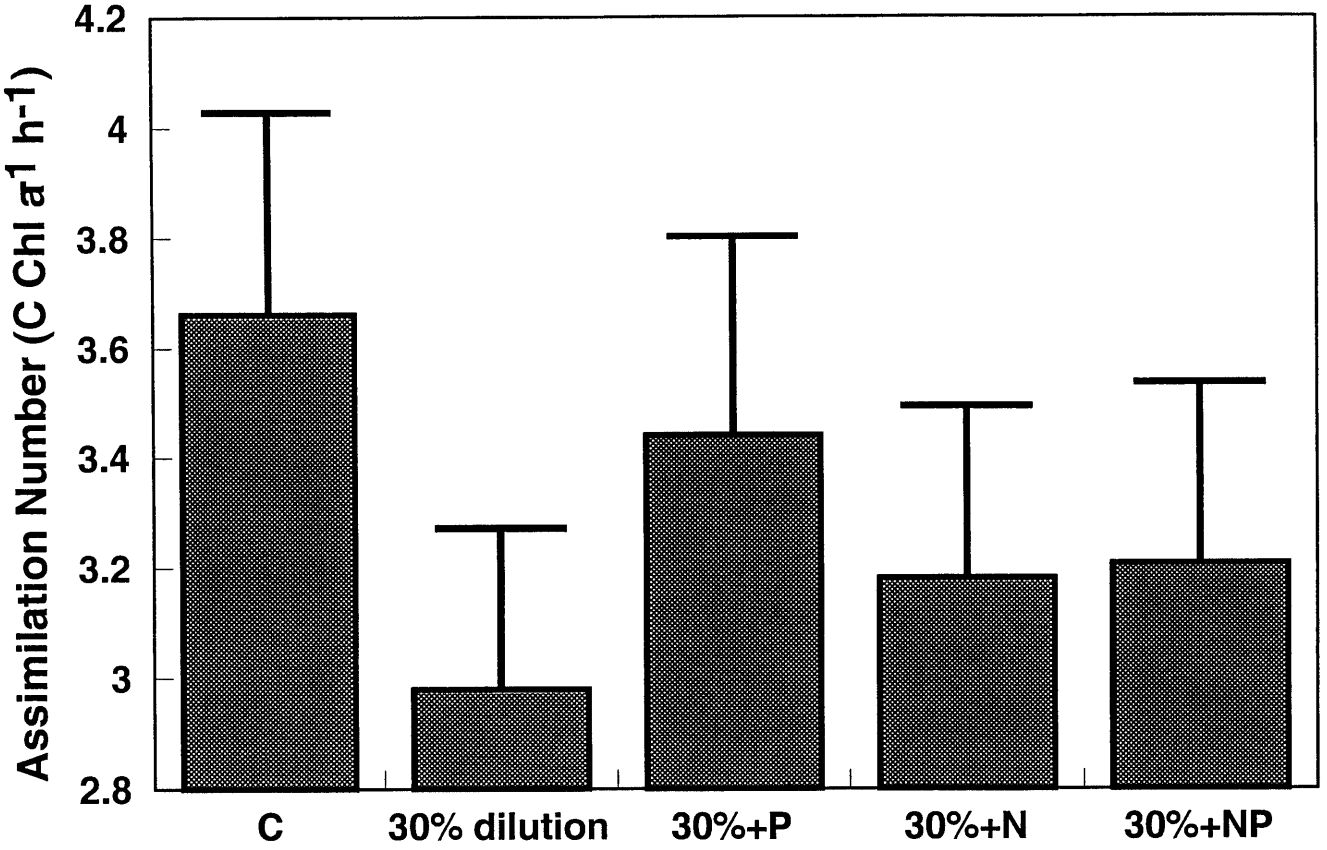

Figure 7: Phytoplankton assimilation number at Streets Ferry Bridge following 3 day incubation with 5 different treatments. Treatments include control, 30% dilution (both N and P reduced), 30% dilution + P (N reduced), 30% dilution + N (P reduced), and 30% dilution + N + P. Values are means and error bars are one standard error.

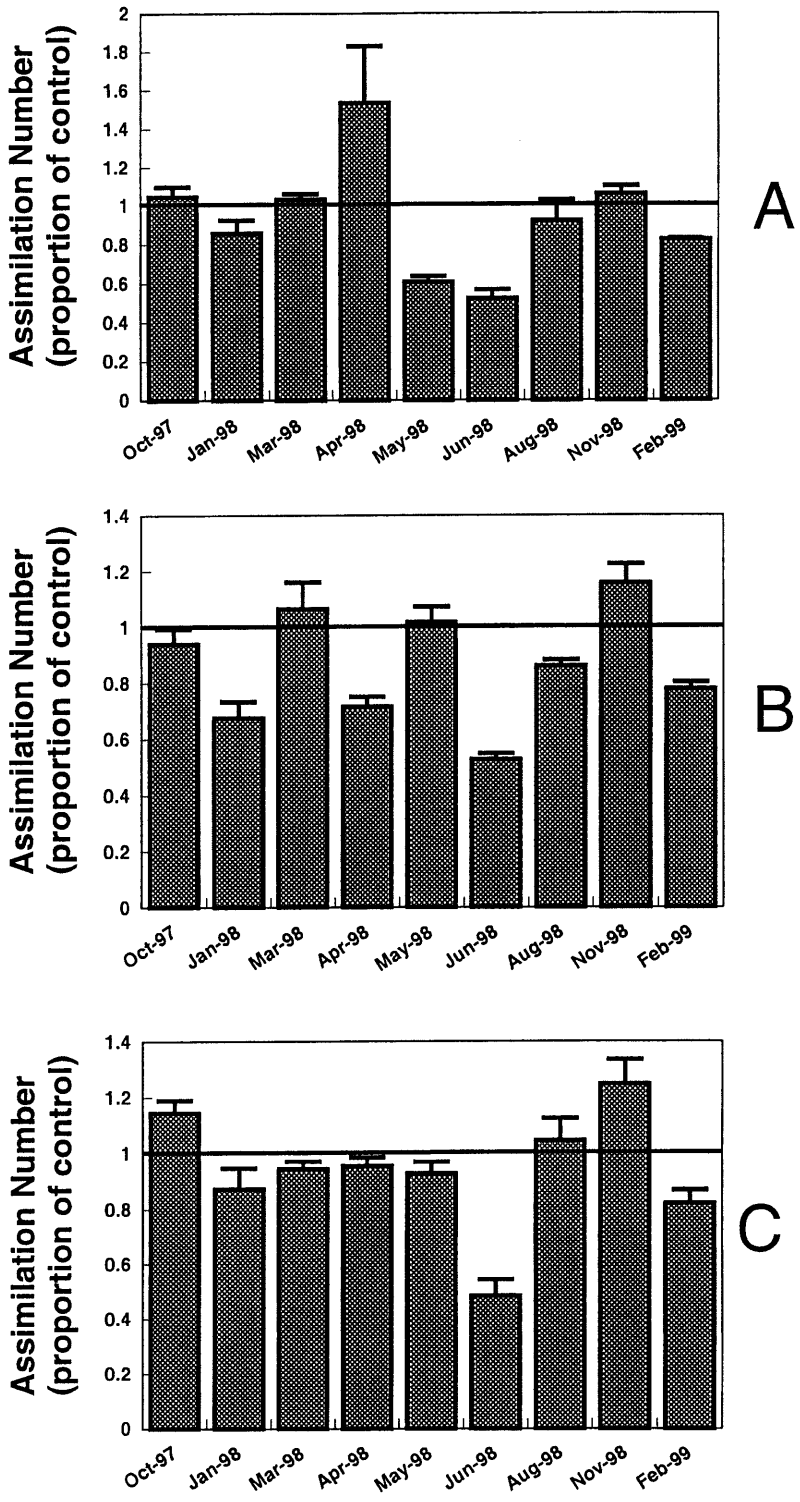

Figure 8: Phytoplankton assimilation number at Marker 15 following 3 day incubation with both N and P reduced (A), with N reduced (B) and with P reduced (C). Values are mean proportion of the control and error bars are one standard error.

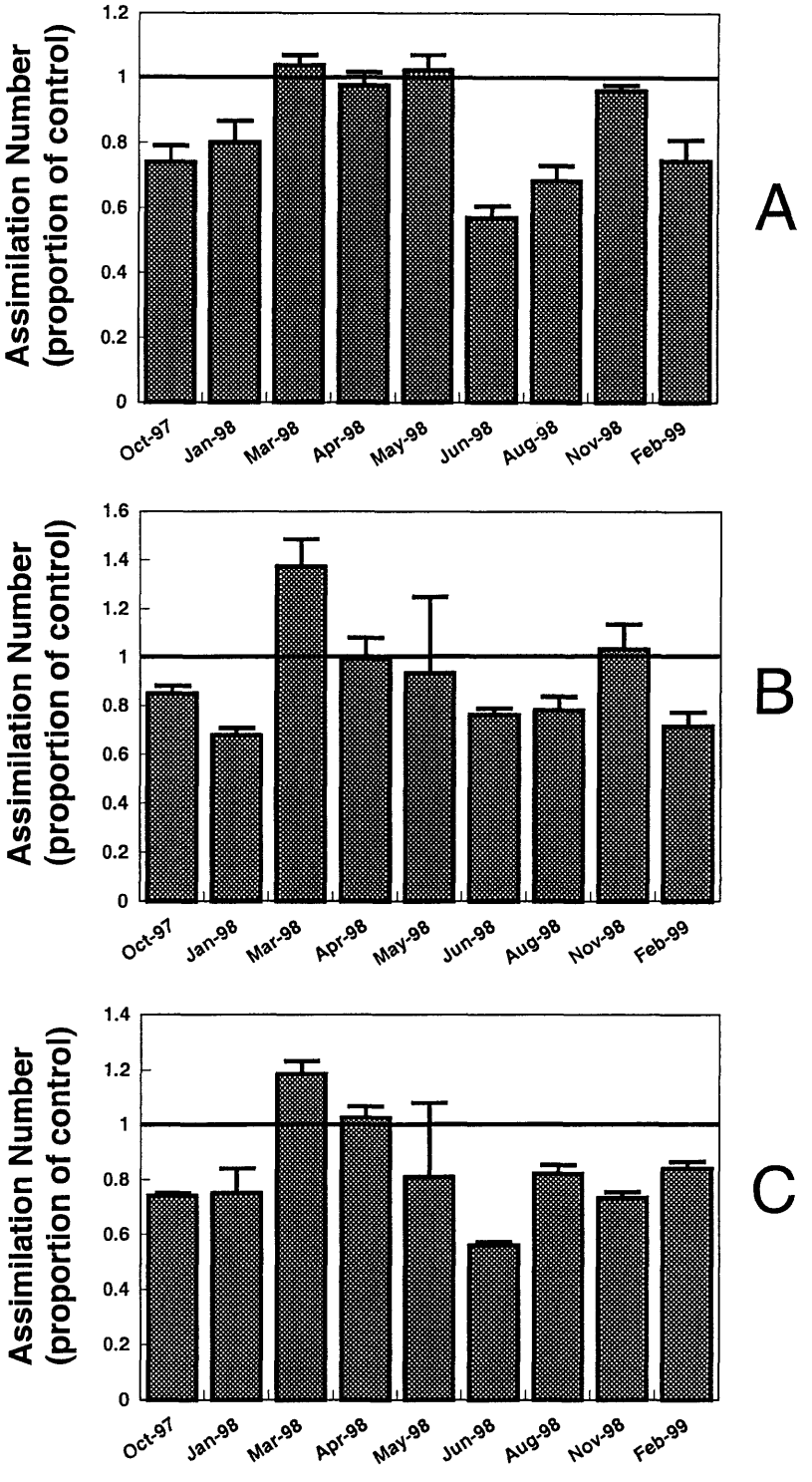

Figure 9: Phytoplankton assimilation number at Streets Ferry Bridge following 3 day incubation with both N and P reduced (A), with N reduced (B) and with P reduced (C). Values are mean proportion of the control and error bars are one standard error.

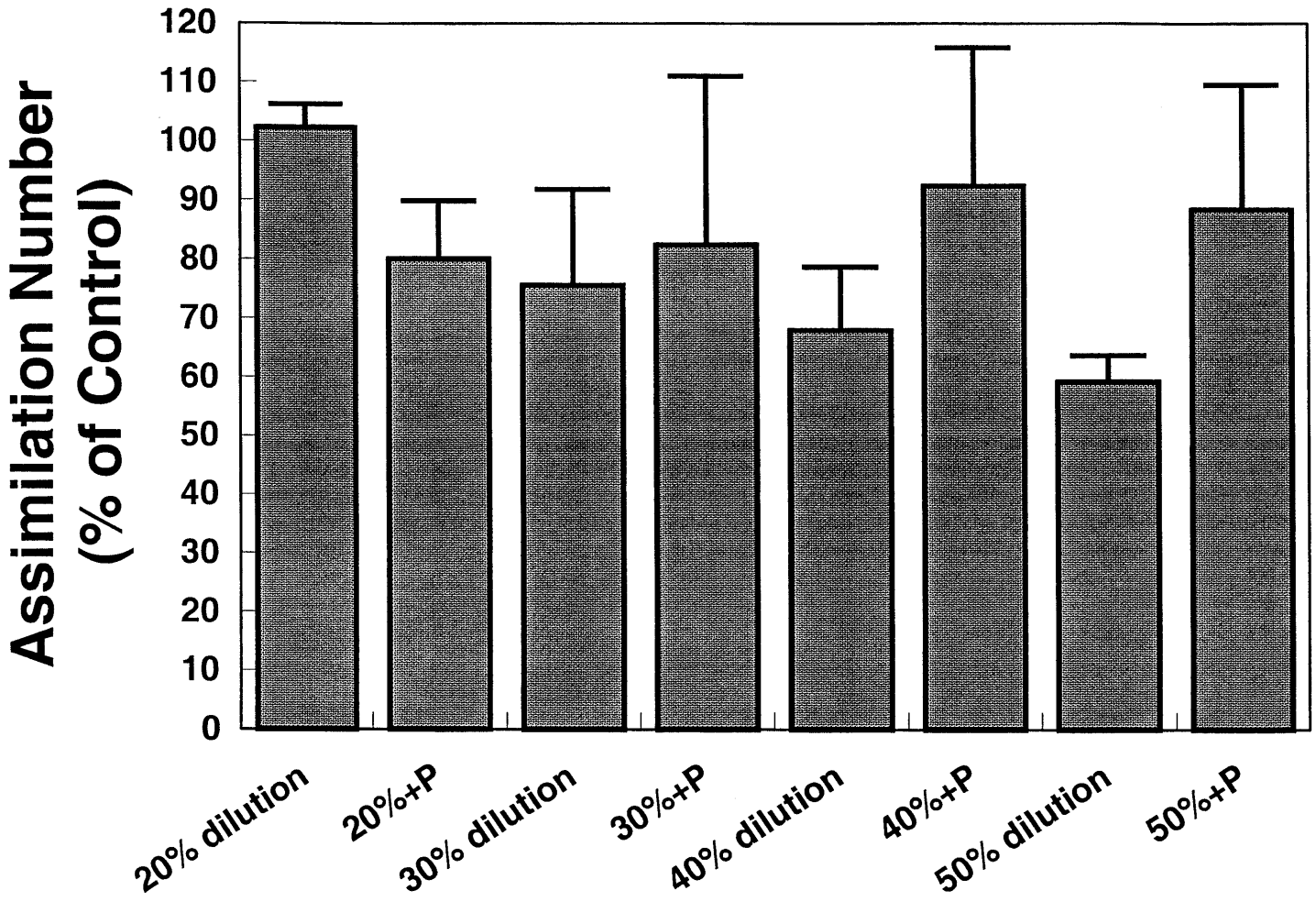

Figure 10: Phytoplankton assimilation number at Marker 15 following 3 day incubation with both N and P (20% dilution, 30% dilution, 40% dilution, 50% dilution) and N alone (20% dilution+P, 30% dilution+P, 40% dilution+P, 50% dilution+P) decreased by incremental amounts. Values are mean percent of control and error bars are one standard error

reductions in assimilation number occurred only with reduction of both N and P concentration by 50% (ANOVA, Bonferroni Multiple Comparison of Means, $p<0.05$). Ambient N concentration was very high at the start of this experiment and likely accounted for the lack of reduction in productivity. The parallel experiment at SFB yielded very different results. Ambient N was lower than that observed in the previous experiment and lower than generally seen at riverine stations during spring runoff periods (Table 1). Assimilation number was significantly reduced by decreases in N concentration ranging from 20% to 50% (Fig 11). An additional experiment was conducted in April to assess spatial variability in the effectiveness of a 30% reduction in N concentration. Results to this point had shown that reductions in N concentrations were generally more effective in reducing primary productivity at M15 than at SFB. Results from this experiment showed a trend towards reduction of assimilation number by reduced N concentrations at downstream sites (Fig. 12), but there were no significant reductions. Ambient N concentrations were elevated during this experiment at some sites and may have accounted for the lack of a significant reduction in assimilation number.

HPLC diagnostic photopigments were also analyzed for the dilution series at M15 and SFB and the dilution bioassay using sites throughout the river. The results from the CHEMTAX matrix factorization program revealed no shifts in community composition resulting from any of the dilutions at any sites (Figs 13,14,15) (ANOVA, $p<0.05$). The lack of a shift in phytoplankton taxa indicates the organisms were unaffected by the change in nutrient concentration in these experiments. The experimental period of 3 days should have been sufficient to include at least one generation time for most of the major phytoplankton groups. However, since the dilutions series at M15 and SFB were made during a period of relatively high ambient N concentration (Table 1), it is possible that the changes made were not enough to cause shifts in community composition over the period of the incubation. The results from the experiment throughout the river (Fig. 15) did provide a good illustration of the spatial diversity of the phytoplankton community in the NRE.

Summary of effects of nutrient dilution on phytoplankton assimilation number and community composition

A 30% decrease in N, P or both N and P concentrations caused a reduction in phytoplankton assimilation number at the estuarine bioassay site. There was no reduction in assimilation number at the riverine site when data for entire experimental period were analyzed. Individual experiment analysis showed N concentration decrease reduced assimilation number as often as reduction of both N and P concentration, while reduction of P concentration alone lowered assimilation number only once. It is difficult to identify a specific geographic region of the NRE where decreasing nutrient concentrations are likely to reduce phytoplankton productivity. However, these data do suggest that nutrient reductions (most often N) are likely to be more effective downstream in the NRE. Phytoplankton taxonomic composition was not altered by any treatment at any location in this work.

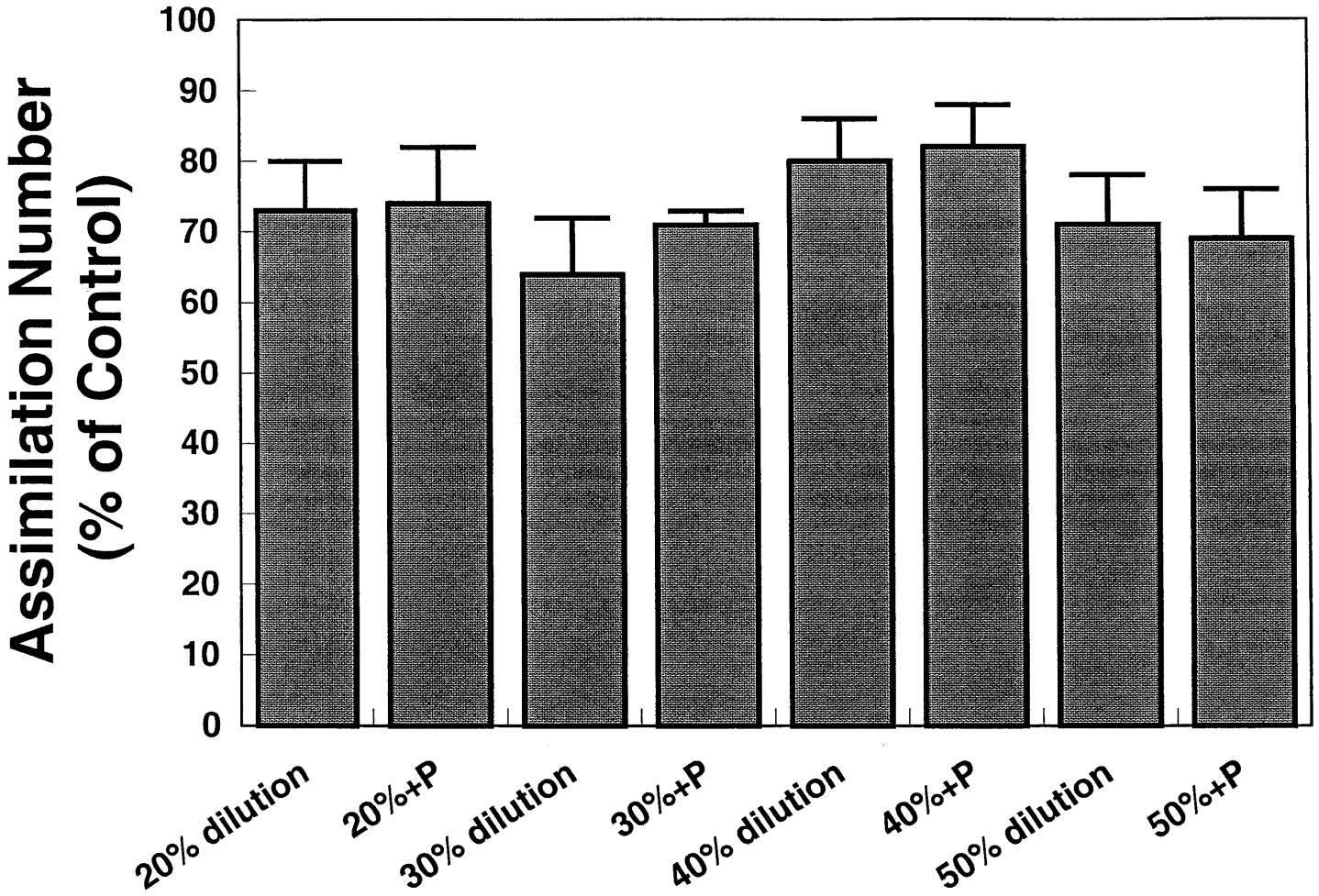

Figure 11: Phytoplankton assimilation number at Streets Ferry Bridge following 3 day incubation with both N and P (20% dilution, 30% dilution, 40% dilution, 50% dilution) and N alone (20% dilution+P, 30% dilution+P, 40% dilution+P, 50% dilution+P) decreased by incremental amounts. Values are mean percent of control and error bars are one standard error

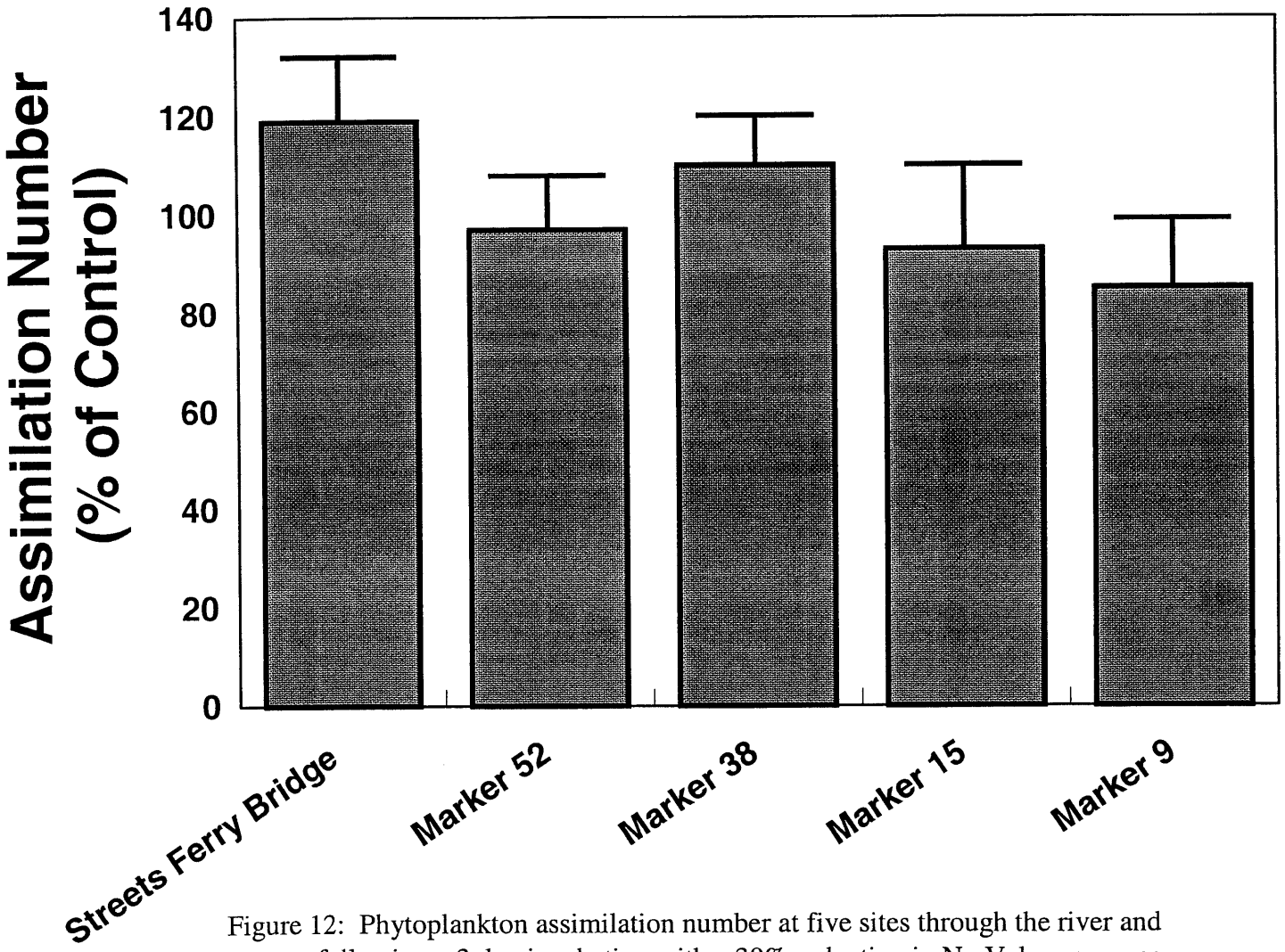

Figure 12: Phytoplankton assimilation number at five sites through the river and estuary following a 3 day incubation with a 30% reduction in N. Values are mean percent of control and error bars are one standard error.

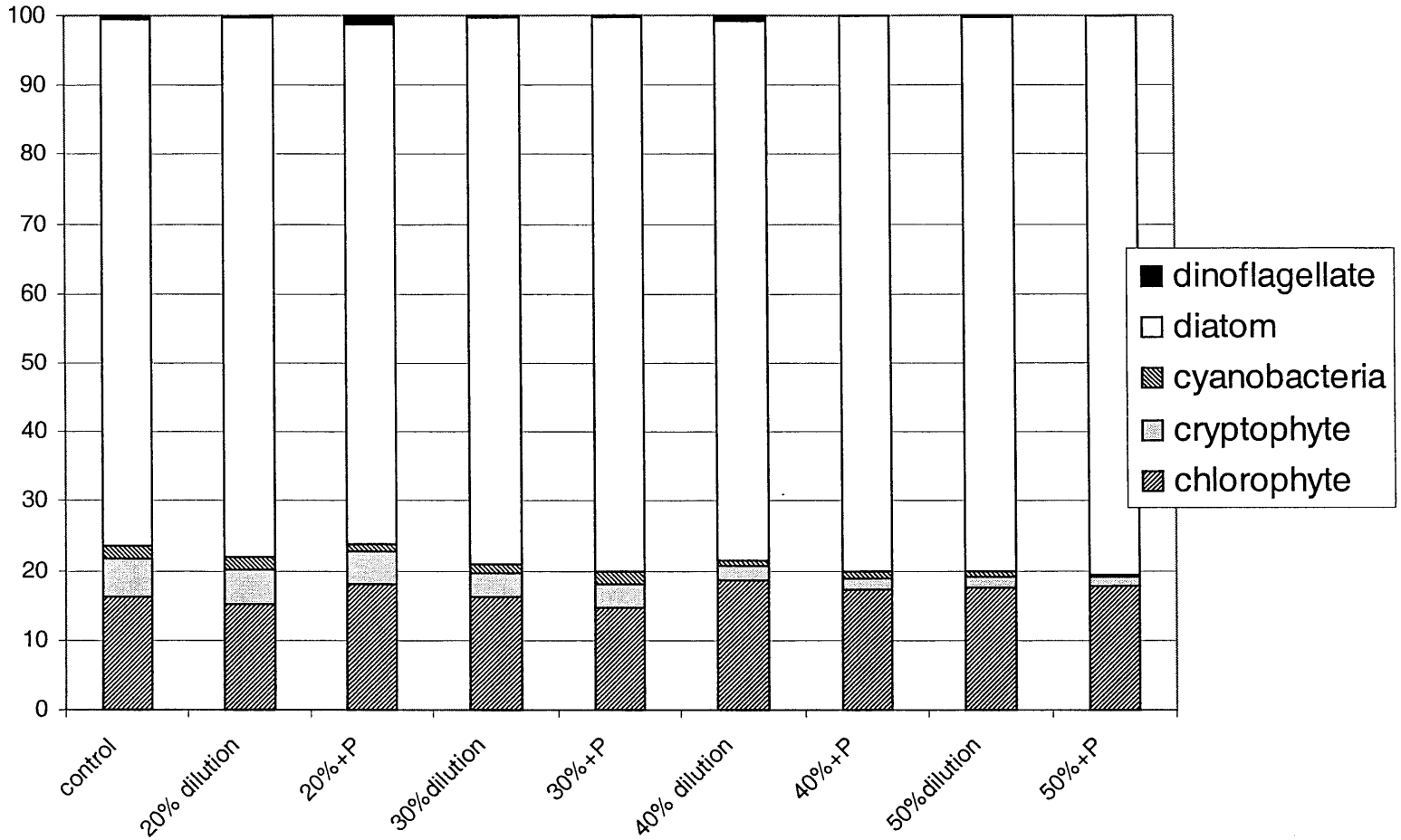

Figure 13: Phytoplankton taxonomic composition at Marker 15 following 3 day incubation with both N and P (20% dilution, 30% dilution, 40% dilution, 50% dilution) and N alone (20% dilution+P, 30% dilution+P, 40% dilution+P, 50% dilution+P) decreased by incremental amounts. Values are mean percent of total chlorophyll *a*.

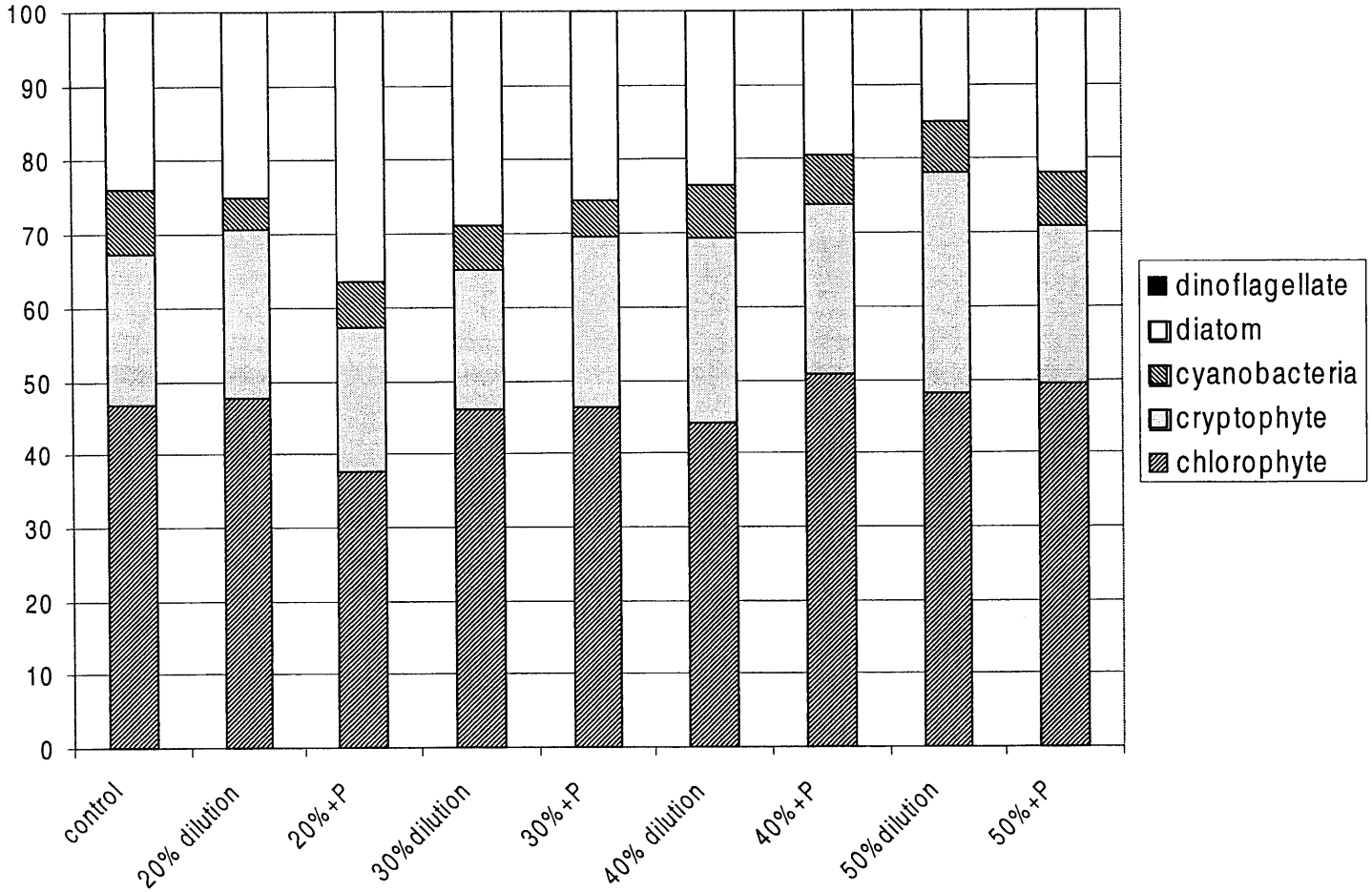

Figure 14: Phytoplankton taxonomic composition number at Streets Ferry Bridge following 3 day incubation with both N and P (20% dilution, 30% dilution, 40% dilution, 50% dilution) and N alone (20% dilution+P, 30% dilution+P, 40% dilution+P, 50% dilution+P) decreased by incremental amounts. Values are mean percent of total chlorophyll *a*.

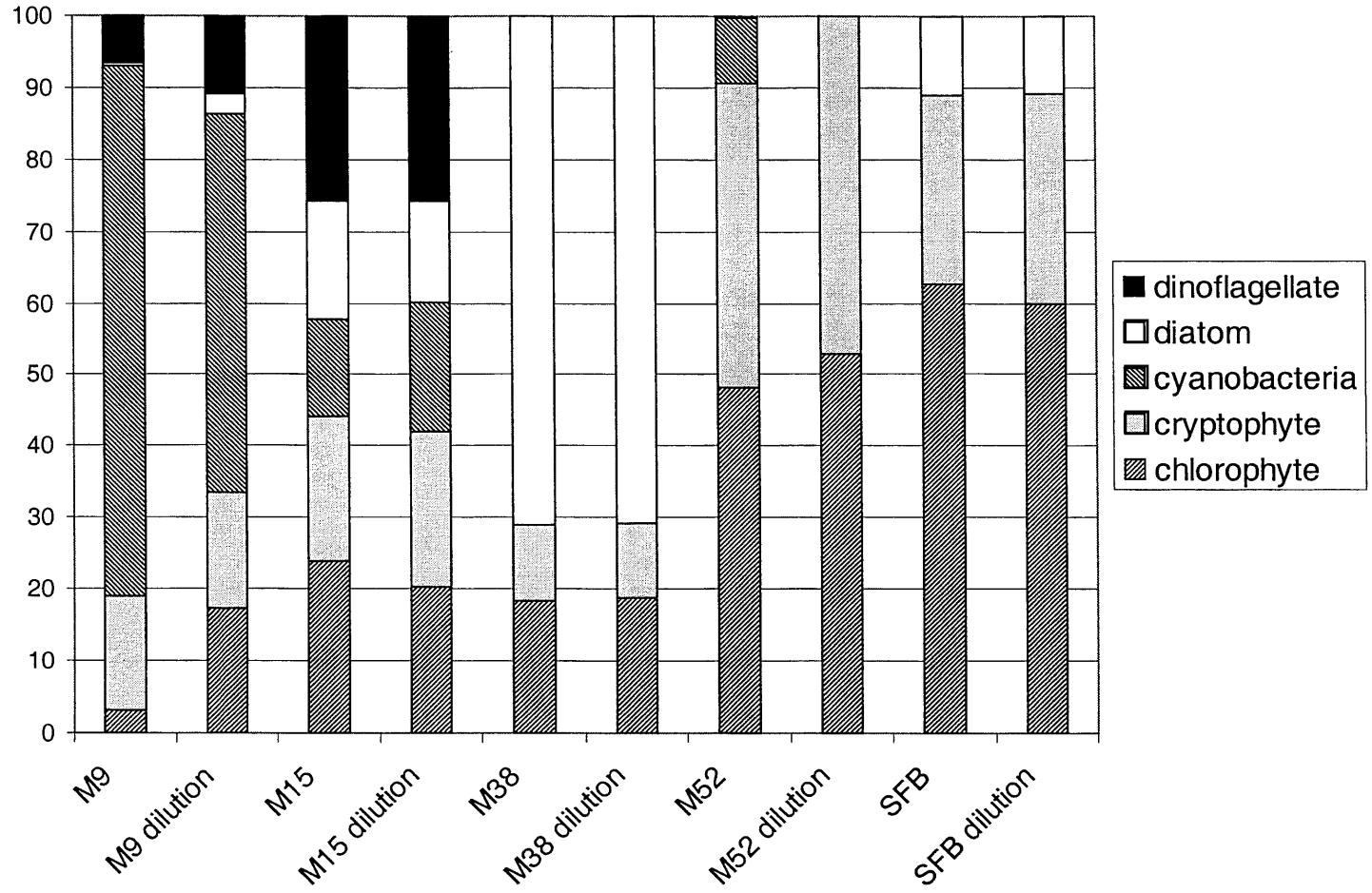

Figure 15: Phytoplankton taxonomic composition at five sites through the river and estuary following a 3 day incubation with a 30% reduction in N and in the control. Values are mean percent of total chlorophyll *a*.

Spatial-Temporal Patterns of Denitrification in the NRE

Hypothesis *Patterns of denitrification in the Neuse River are regulated by substrate availability, physical parameters, and interaction with associated microbially mediated N pathways over seasonal and annual time scales.*

Denitrification activity (estimated *in situ* rates) measured along the Neuse River transect showed a consistent spatial pattern of elevated rates upstream and decreasing downstream (Fig. 16). A comparison of means (Bonferroni test, $p<0.05$) showed significant differences in denitrification between each of the two upstream stations and all other stations, while denitrification among the 3 downstream stations was not significantly different from one another. Timing and location of elevated rates coincided with higher nitrate concentration of bottom water and freshwater input as indicated by low bottom salinity (Figs 17 B,C). Over the 3 year sampling period, highest denitrification rates occurred in the fall and winter (Fig.18). Elevated rates throughout the entire transect were evident in the winter, with seasonal peaks apparent in mid summer and mid-fall (97,98) rates. Lowest seasonal rates were observed in the spring months, usually coincident with lower nitrate levels in bottom water (Fig. 17). Recurrent spring phytoplankton blooms reached peak biomass and productivity in the middle and lower reaches of the transect (Pinckney et al. 1997) where NO_x levels and estimated *in situ* denitrification rates were low. Seasonal differences in estimated *in situ* denitrification rates were not statistically discernable by comparison of means (Bonferroni test, $p<0.05$) run by month or by season for combined data.

Statistical analyses were performed using bivariate correlations (Pearson correlation coefficients) to examine the relationship between denitrification rates and potential regulating factors (Table 2). Correlations were run on the complete data set, on separate data from each of 2 upstream stations, and from 3 downstream stations combined, the grouping suggested by the comparison of means discussed above. Additional correlations were performed on data from combined locations selected by season. Correlation coefficients for *in situ* denitrification with bottom [NO_x] (positive) and salinity (negative) were highest among all the variables considered, and were significant for most data groupings and for all seasons (Table 2). In addition, [NO_x] (positive) and salinity (negative) were related to estimated *in situ* rates of denitrification in stepwise linear regressions of log/log transformed data for combined data. These analyses support the hypothesis that denitrification activity is regulated by the direct input of riverine nitrate to the system. A direct proportionality between sediment denitrification rates and water column nitrate concentration in estuaries has been determined in recent studies (Cornwell et al.1999).

A secondary group of correlations suggested a competitive interaction between denitrifiers and primary producers for nitrate. Correlations were positive between estimated *in situ* denitrification and both water column and sediment carbon:nitrogen ratios (Table 2) for all locations/seasons except summer. Assuming higher C:N ratios are indicative of lower biomass (C:N was negatively correlated with chlorophyll *a* in both water column and sediments), it appears that denitrification activity was greater at times/locations of possibly reduced competition for nitrate. Likewise, estimated *in situ* denitrification was negatively correlated with chlorophyll *a* in the water column (downstream) and sediment (combined data) and for all seasons (combined locations) except summer. Tuominen et al. (1999) found reduced denitrification rates (measured with the isotope pairing method) in cores enriched with algae.

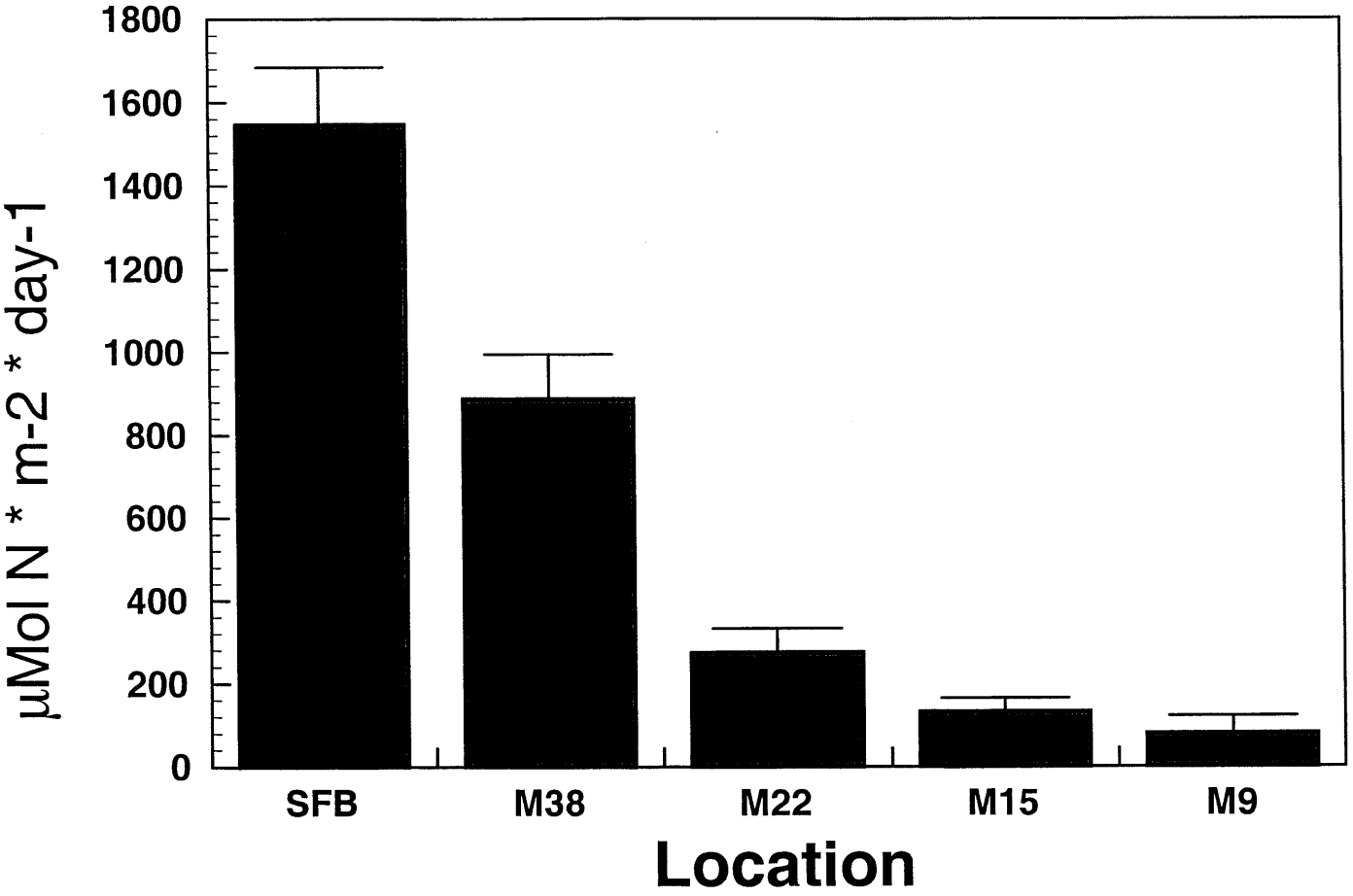

Figure 16: Estimated *in situ* denitrification (1996-1999) grouped by site. Error bars indicate standard error. SFB and M38 were significantly different from each other and from all other sites.

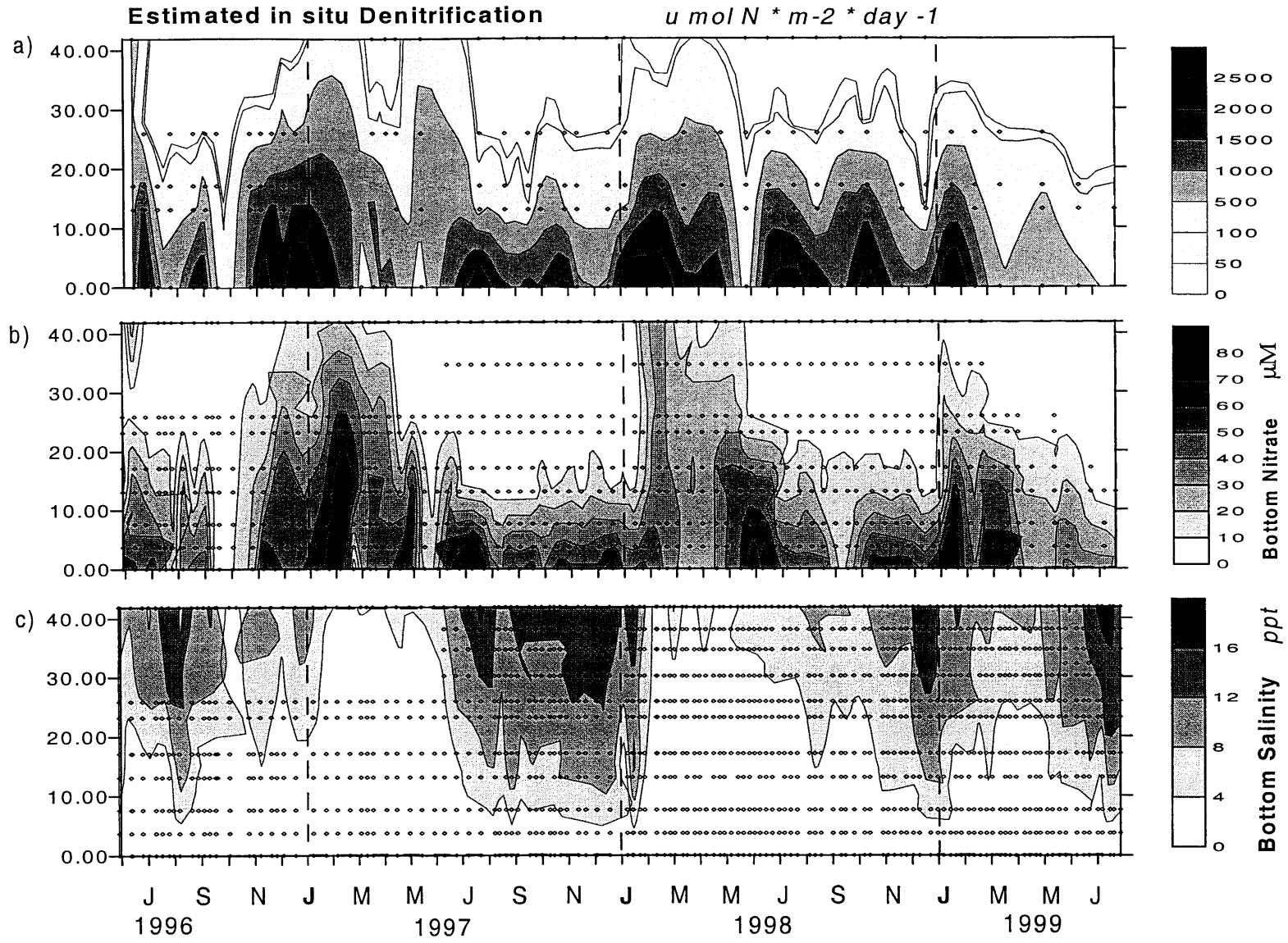

Figure 17 Multi-annual spatial plots of a) estimated in situ denitrification b)concentration of nitrate + nitrite in bottom water and c) bottom salinity

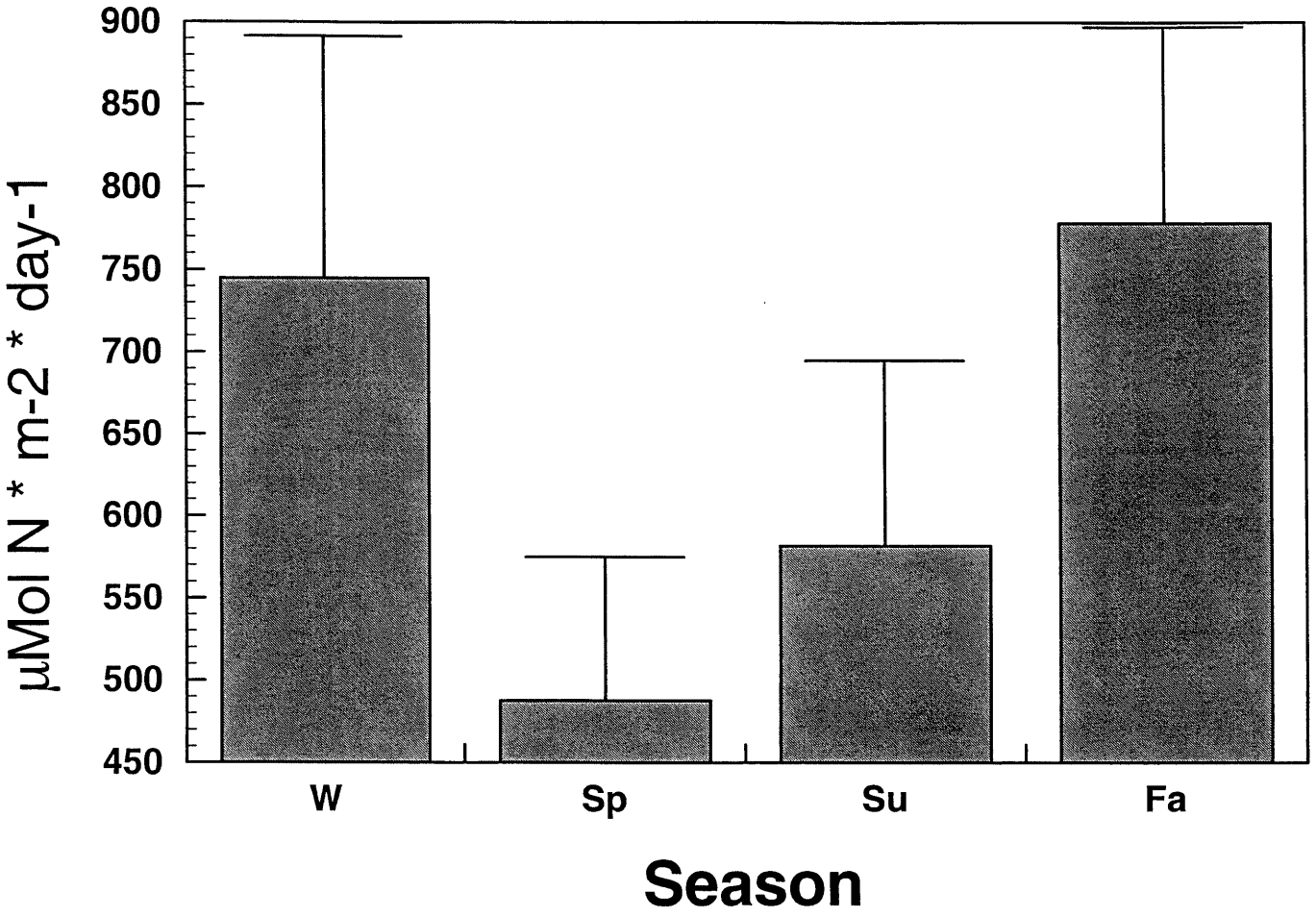

Figure 18: Estimated *in situ* denitrification (1996-1999) grouped by season. Error bars indicate standard error.

Sign	Variable	*All seasons combined*				*All stations combined*			
		ALL	*SFB*	*M38*	*Down*	*W*	*Sp*	*Su*	*Fall*
+	bottom NOx	.763	.431		.797	.595	.526	.911	.853
-	salinity	.595		.584	.503	.523	.462	.627	.747
+	C:N water column	.327		.353	.348	.600		.262	.430
+	C:N sediment	.338				.549		.414	.677
-	chlorophyll *a* water column	.363			.317	.415		.314	.462
-	chlorophyll *a* sediment	.398		.499				.328	.601
+	dissolved oxygen	.242		.348	.324			.564	
-	temperature				.339			.306	
-	pH					.416			.522

Table 2 Bivariate correlations (Pearson correlation coefficients) of measured variables with estimated *in situ* denitrification for indicated data groupings of location and season ($p<0.05$, blank cells were not significant).

Finally, estimated *in situ* denitrification rates were positively correlated with dissolved oxygen levels in bottom water (Table 2). This correlation reflects an indirect regulation of denitrification via control of nitrification, an obligate aerobic process. From figure 17 it is apparent that ambient NO_x levels were low at downstream locations except during winter periods of freshwater input. Denitrification in this portion of the estuary was likely dependent on nitrate derived from coupled nitrification, in turn controlled by dissolved oxygen levels. The correlation suggests such a relationship, particularly since the correlation for these variables grouped by season was only significant for the summer months when NO_x concentrations were negligible downstream (Table 2, Fig 17). Kemp et al. (1990) found both spring and fall peaks of sedimentary denitrification closely coupled to nitrification.

Potential denitrification rates provided less information about regulation since NO_3^- limitation was eliminated during the incubation. However, denitrification potentials provide information about secondary regulating factors, such as organic carbon supply and dissolved oxygen concentrations. These rates were useful as indicators of community response, given the appropriate conditions. Potential denitrification rates ranged to 7 times higher than estimated *in situ* rates (Figs. 17 and 19). Because denitrifiers are facultative anaerobes, i.e. organisms capable of switching from aerobic to anaerobic respiration, this adaptable microbial community exhibited a predictably wide spatial distribution. No significant differences were found among potential denitrification rates either spatially or temporally (by season or month) although mean rates were greatest at M22 and during the summer months (Figs. 20 and 21).

Potential denitrification was predicted to increase under conditions of anoxia. This was suggested at downstream stations where potential denitrification was negatively correlated with dissolved oxygen (Table 3). From figure 19 it appears that the timing of peak potential denitrification corresponded to periods of ammonium release in bottom water corresponding with anoxia. In addition, potential denitrification was correlated with bottom water increases of NH_4^+ and PO_4^- concentrations, both of which are known to flux out of sediments during periods of anoxia (Christian et al. 1991, Paerl at al. 1995, Paerl et al. 1998). Additional correlations suggested carbon limitation of denitrification, particularly at downstream stations. Negative correlations with C:N in the water column at M22, downstream stations, and for combined stations in the summer may indicate a positive relationship between potential denitrification and autotrophs that serve as a source of labile carbon for denitrifiers. Positive correlations between potential denitrification and chlorophyll *a* in the water column (M15) and in the sediment (combined data) support the hypothesis that carbon is limiting to denitrification. Caffrey et al. (1993) observed an increase in denitrification (under conditions of high water column NO_3^-) in coastal sediments amended with organic matter.

Stepwise multiple linear regressions were run on log/log transformed data with potential denitrification set as the dependent variable. Regressions included only salinity (negative) and temperature (positive) as significant for combined data (r^2 =0.097). For individual stations, the multiple regression equation included independent variables only at M15 where temperature was positively related (r^2 =0.135) and at M9 where NH_4^+ concentration was positively related, and dissolved oxygen and water column C:N ratios were negatively related (r^2 =0.752) to potential denitrification.

In addition to potential rates, the half saturation constant (K_s) was calculated from Lineweaver Burke transformations of the kinetic data as discussed in the methods. This constant is considered an affinity constant. That is, a lower K_s value indicates a greater affinity for NO_3^-, an adaptive advantage to low ambient NO_3^-, while higher K_s values are indicative of organisms

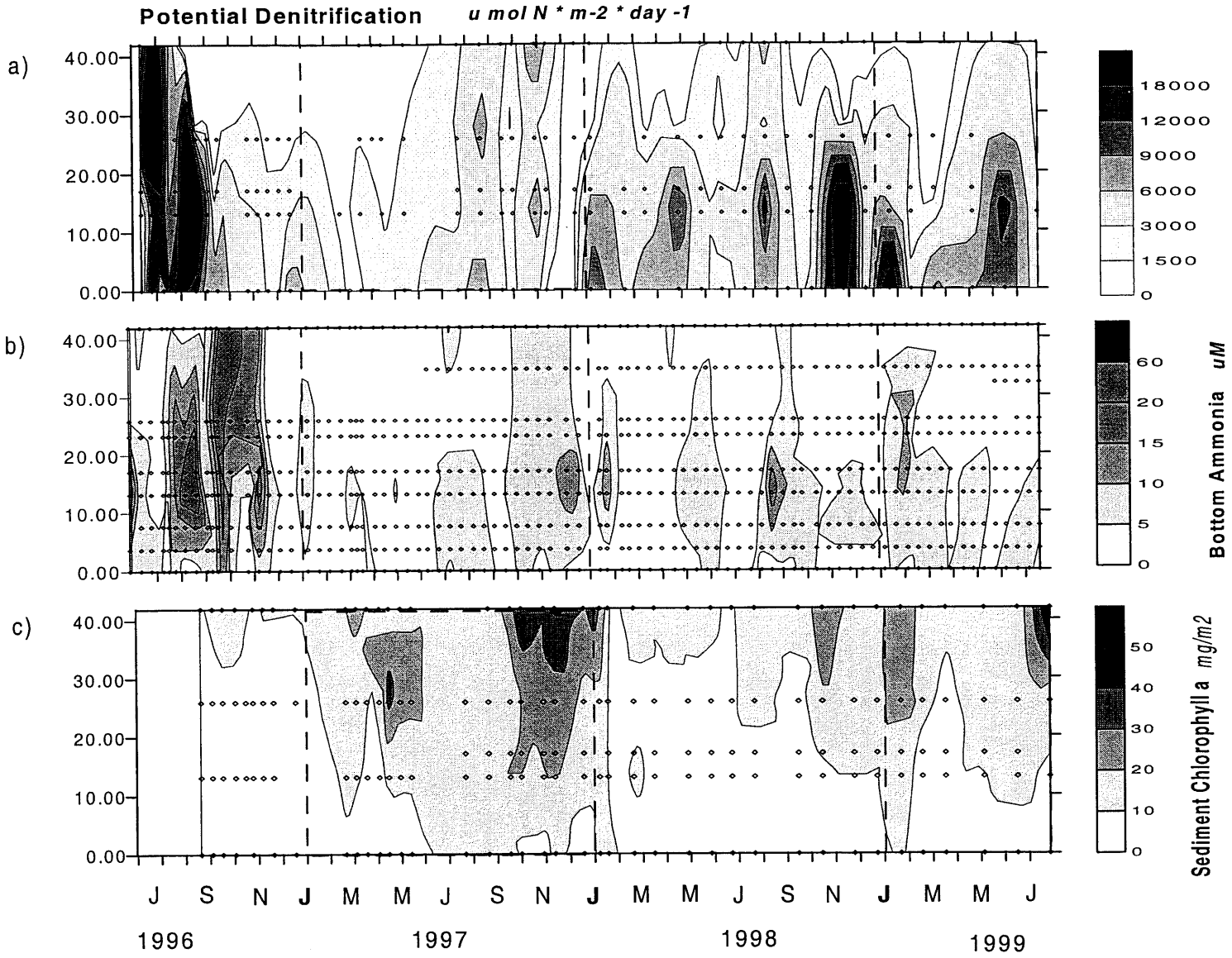

Figure 19 Multi-annual spatial plots of a) potential denitrification b) concentration of ammonia in bottom water and c) concentration of chlorophyll a in surface sediment

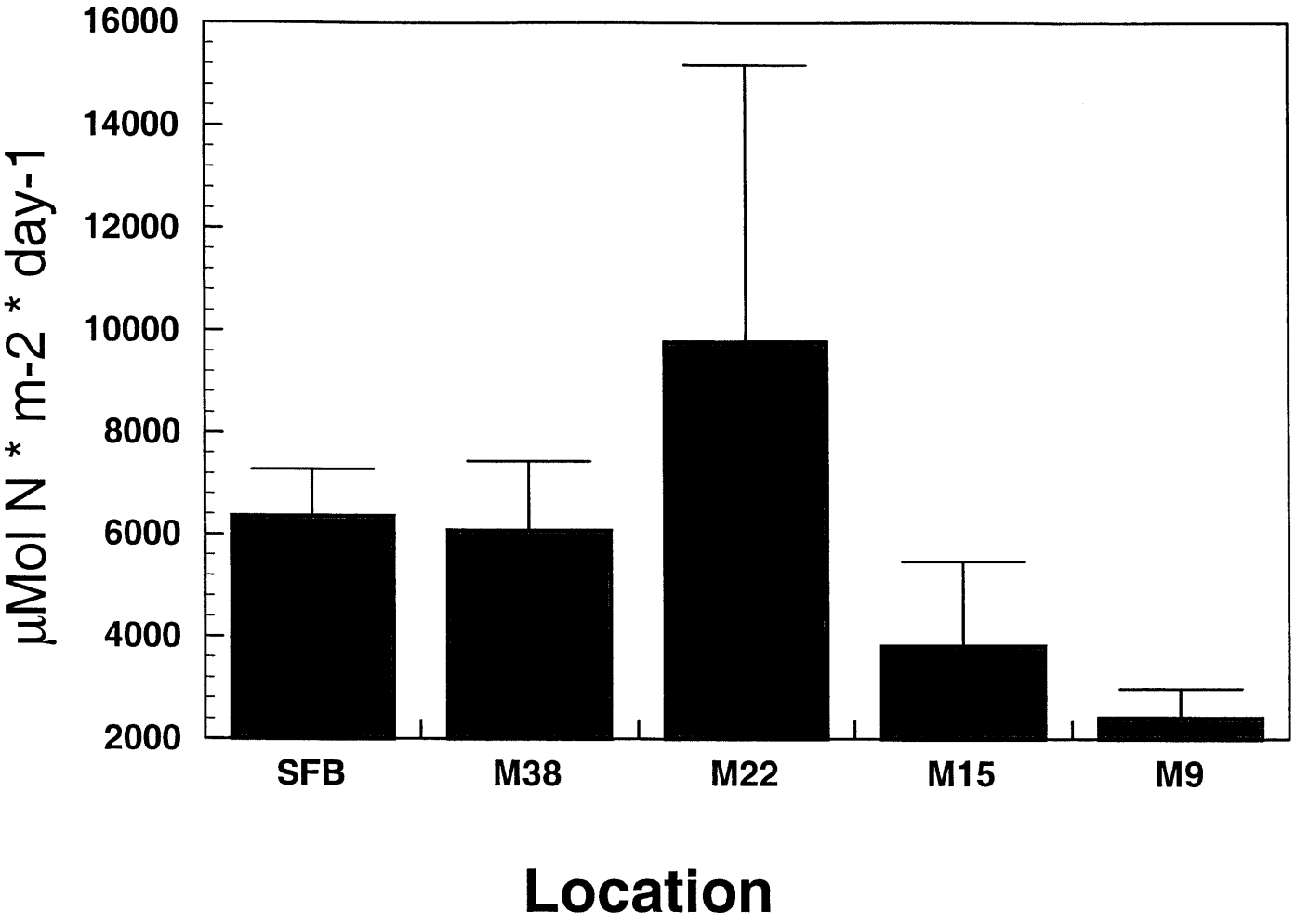

Figure 20: Potential denitrification (1996-1999) grouped by site. Error bars indicate standard error.

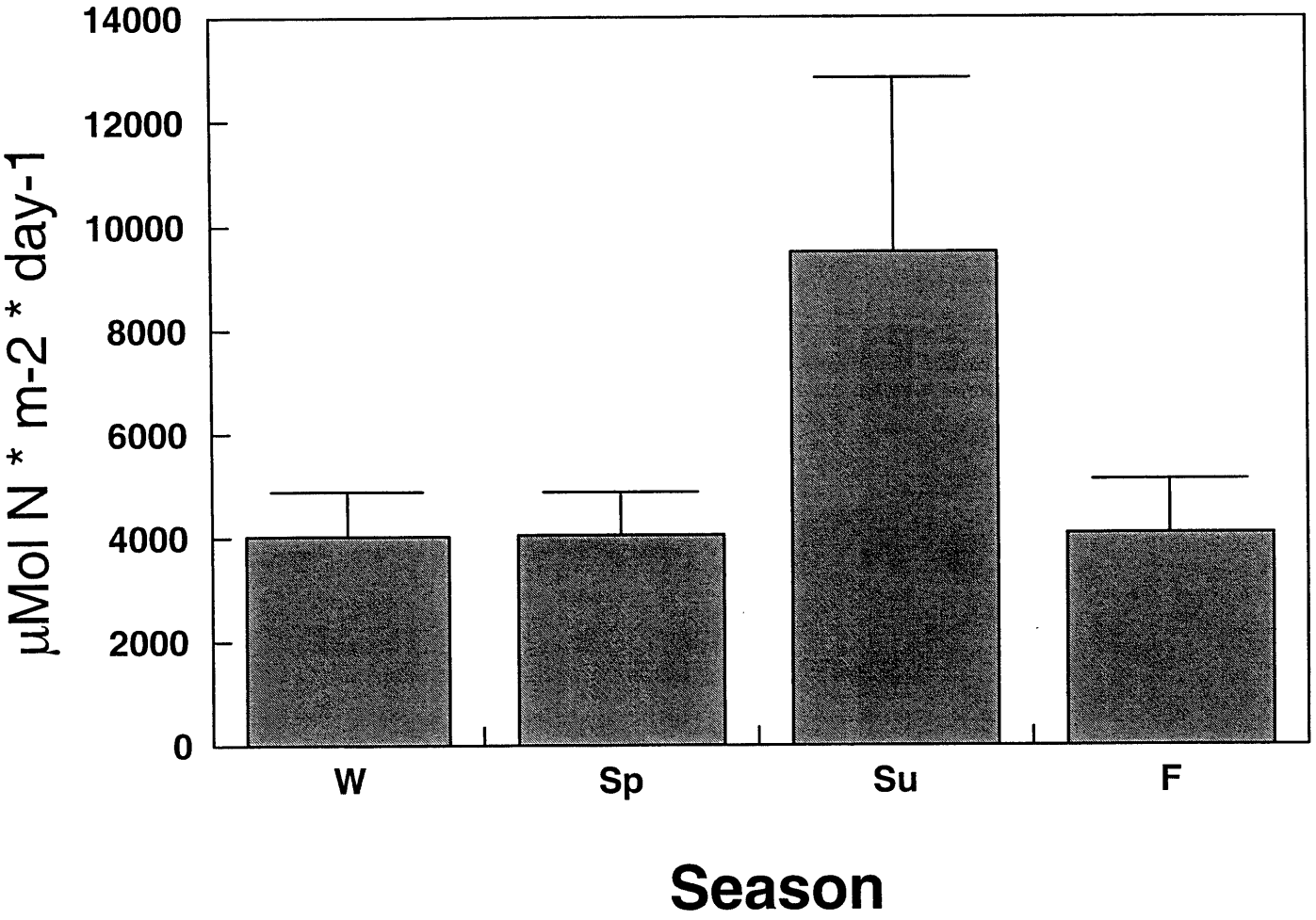

Figure 21: Potential denitrification (1996-1999) grouped by season. Error bars indicate standard error.

		All seasons				All locations
Sign	*Variable*	*ALL*	*M22*	*M15*	*Down*	*Summer*
-	salinity	.151				
-	Dissolved oxygen				.200	
+	Ammonia	.189	.427		.292	
+	phosphorous		.637		.419	
-	C:N water column		.484		.367	.341
+	C:N sediment				.238	.393
+	Chlorophyll *a* water column			.627		
+	Chlorophyll *a* sediments	.264				
+	temperature	.179				

Table 3 Bivariate correlations (Pearson correlation coefficients) of measured variables with potential denitrification by indicated data groupings ($p<0.05$, locations/seasons with no significant correlation were omitted from the table, blank cells were not significant).

that can take greater advantage of high levels of NO_3^-. Mean K_s for all denitrification rates measured was 198.46 (±23.93 standard error). This value falls in the range of K_s values reported in the literature for marine sediments (Billen 1978, Oren and Blackburn 1979, Nedwell 1982, and Esteves et al. 1986). Mean values for K_s were not significantly different among stations (Bonferroni), although the values were higher at M22 (325±69.5) relative to the other stations. Because NO_3^- levels were lower at M22 compared with upstream stations, the higher mean K_s at this station may be indicative of coupled nitrification-denitrification. Correlations were significant between K_s values and temperature (+0.176), salinity (+0.173) and dissolved oxygen (-0.186) for combined data. An expected correlation between K_s and bottom NO_3^- was not significant. This relationship could have been masked if nitrification was closely coupled to denitrification, (i.e. generated NO_3^- was quickly utilized and therefore not reflected in water concentrations).

Denitrification Methods Comparison

The comparison of the acetylene block (potential and *in situ* rates) with membrane inlet mass spectrometry (MIMS) is presented in figure 22. Potential rates determined with acetylene block were 1.6 times greater than MIMS rates which were in turn 7 times greater than estimated *in situ* rates for data from the three stations combined. Mean potential denitrification was not significantly different from MIMS (paired t test, $p<0.05$) for combined data. It is evident that the comparison between rates varied among stations, particularly at M38 where MIMS rates were significantly greater than potential denitrification rates. Sediments from M38 exhibited a lower sediment oxygen demand (J. Fear, UNC-IMS, unpublished data) relative to the other two stations. It is possible that the subcoring procedure performed for the acetylene block technique disrupted the anoxic zone that was not reestablished during the incubation in cores from this station. Because MIMS is a direct measure of N_2 flux from larger, undisrupted cores, data collected with this technique are preferable to acetylene block potential and estimated *in situ* numbers that are calculated from kinetic incubations amended with nitrate and inhibitor. The alteration of dissolved O_2 levels relative to *in situ* conditions remains problematic for MIMS incubations (incubations are started with aerated overlying water). This may result in an overestimate of measured rates with this technique in sediments where nitrification is closely coupled with denitrification, which could explain the similarity between MIMS and observed potential rates. Assuming MIMS as the standard, it is evident that estimated *in situ* rates grossly underestimated denitrification in the current study.

Estimated Mass Balance and Denitrification Rate Comparisons

Hypothesis *Denitrification in the NRE contributes to substantial removal of N input.*

Annual inputs of DIN to the Neuse transect were estimated from daily flow data at the Kinston gauging station (USGS) matched with interpolations of bi-weekly DIN concentrations in surface water at SFB for 1996-1999. Removal of nitrogen via denitrification was calculated by summation of rates from each station extrapolated to associated areal segments of the Neuse transect. Total N removal for the transect was then interpolated to daily values to match periodicity of N loading data. The proportion of N loading removed via denitrification (mean of daily comparisons) was 3.00% (estimated *in situ*) and 35.41% (potential rates). The large

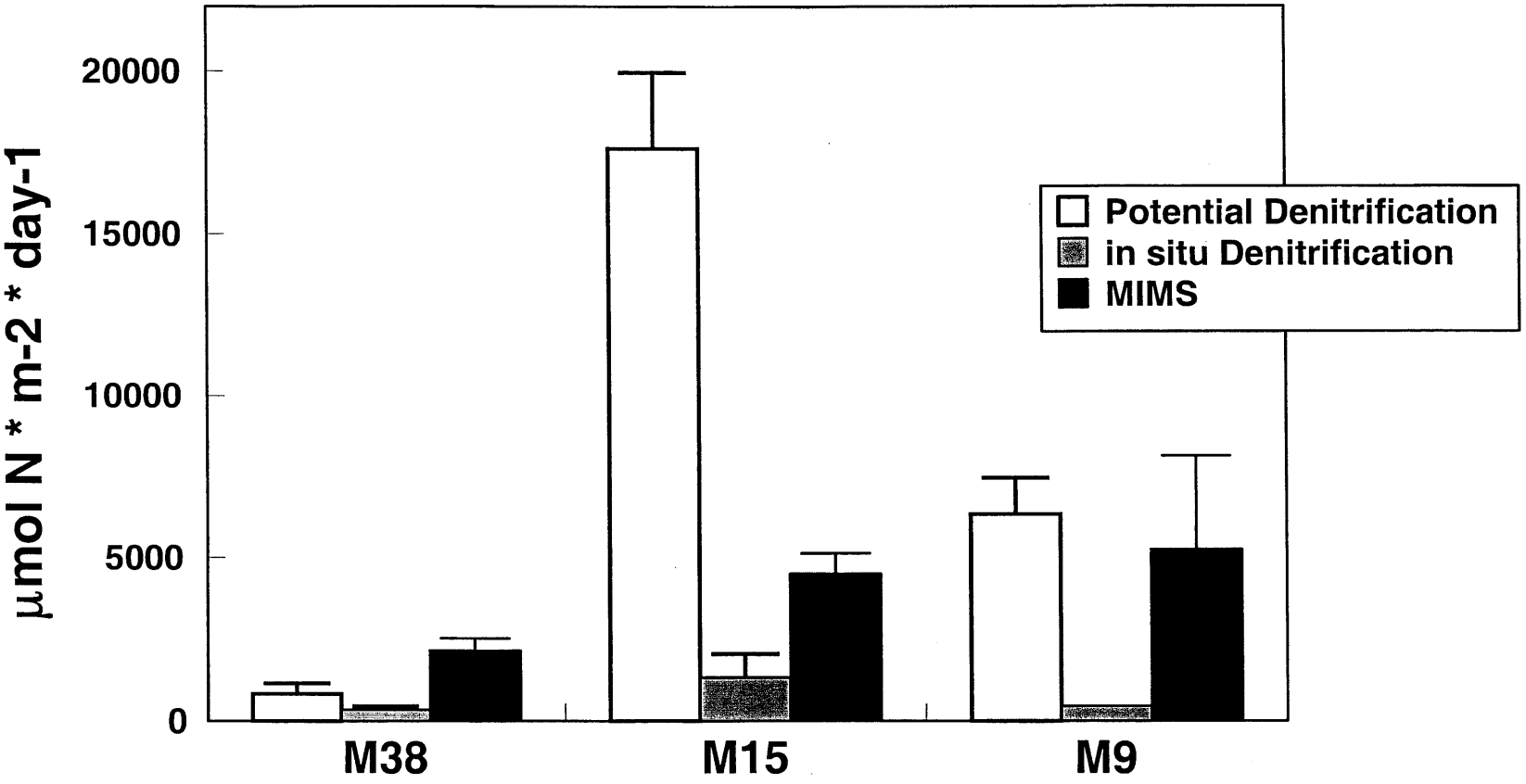

Figure 22: Comparison of denitrification rates determined with acetylene block (estimated in situ and potential rates) and MIMS techniques. Data for each site are pooled from repeated measures.

discrepancy between estimated *in situ* rates and potential rates is likely due, in part, to methodological problems that likely led to an underestimation of denitrification rates (discussed above). Assuming potential rates are 1.6 times greater than actual rates (from MIMS comparison), 22.2% of N loading was removed via denitrification in the Neuse transect. Previous estimates of N removal via denitrification in estuaries range up to 50% (Seitzinger 1988). The residence time of the water appears to be a controlling variable on the proportion of N loading that is denitrified (Nixon et al. 1996). From the loading/residence time relationship compiled by these researchers, the residence time of Neuse river water (>30 days, Pinckney et al. 1997) would lead to a predicted 20-25% denitrification removal.

Estimated *in situ* denitrification rates calculated at 30% reduction in NO_x levels were estimated to remove 3.19 % of DIN loading (assuming 30% reduction in DIN inputs). Based on this calculation, a reduction in N loading would not be predicted to lower denitrification rates in the Neuse. This prediction is obviously simplistic in that it does not take into account internal N cycling (such as nitrification), or the potential feedbacks that a reduction in N loading would entail. For example, if the N reduction resulted in lower carbon loading and higher oxygen levels in the sediment, nitrification (and coupled denitrification) would likely be enhanced.

A seasonal pattern of elevated % N removal during the summer months (when N loading was lower) was evident for potential denitrification (Fig 23). Nitrogen removal percentage based on estimated *in situ* rates showed a less distinct pattern of early summer and fall peaks. However, the interpretation of seasonal removal is limited because no adjustment was made to account for the time lag between loading upstream (SFB) and removal throughout the transect. Mean annual N loading was estimated as 1752 metric tons with estimated annual denitrification removal of 620 metric tons (potential) and 53 metric tons (estimated *in situ*). Mean (1996-1998) denitrification rates of 208 (estimated *in situ*) and 1358 μmol N m^{-2} day^{-1} (potential) fall in the range of rates measured in other estuaries using various techniques (for example Yoon and Benner 1992, Nowicki et al. 1997, Flemmer et al. 1998). Definitive conclusions about the quantitative significance of denitrification to N budgets in estuaries depend on more extensive use of recently improved techniques, including MIMS and additional experimental work to quantify controls on nitrification rates and coupling to denitrification. Data from this project suggest that denitrification is a significant sink for N in the Neuse River Estuary.

Summary of denitrification assessments

A complete understanding of denitrification continues to be hindered by methodological shortcomings. However, the patterns that have emerged provide insight into regulatory factors and on the seasonal dynamics of denitrification in this estuarine system. Denitrification measured as estimated *in situ* rates appeared to be regulated by NO_x availability, both directly and through competition with benthic and pelagic primary producers. Rates were highest in the fall and winter, coincident with elevated NO_x levels. In addition, a positive correlation between estimated *in situ* denitrification and oxygen levels suggested an indirect regulation of denitrification via coupled nitrification. Potential rates of denitrification were elevated under conditions of anoxia during the summer and were apparently limited by supplies of labile carbon. A methods comparison (between the acetylene block and MIMS techniques) indicated that true rates of denitrification in the Neuse may be closer to potential rates measured in the current study with an *estimated* 22 percent of DIN removed via denitrification. Based on the kinetics of nitrate utilization by denitrifiers, a 30% reduction in DIN loading would not be expected to reduce the percentage of N removed via denitrification. These findings should be considered preliminary,

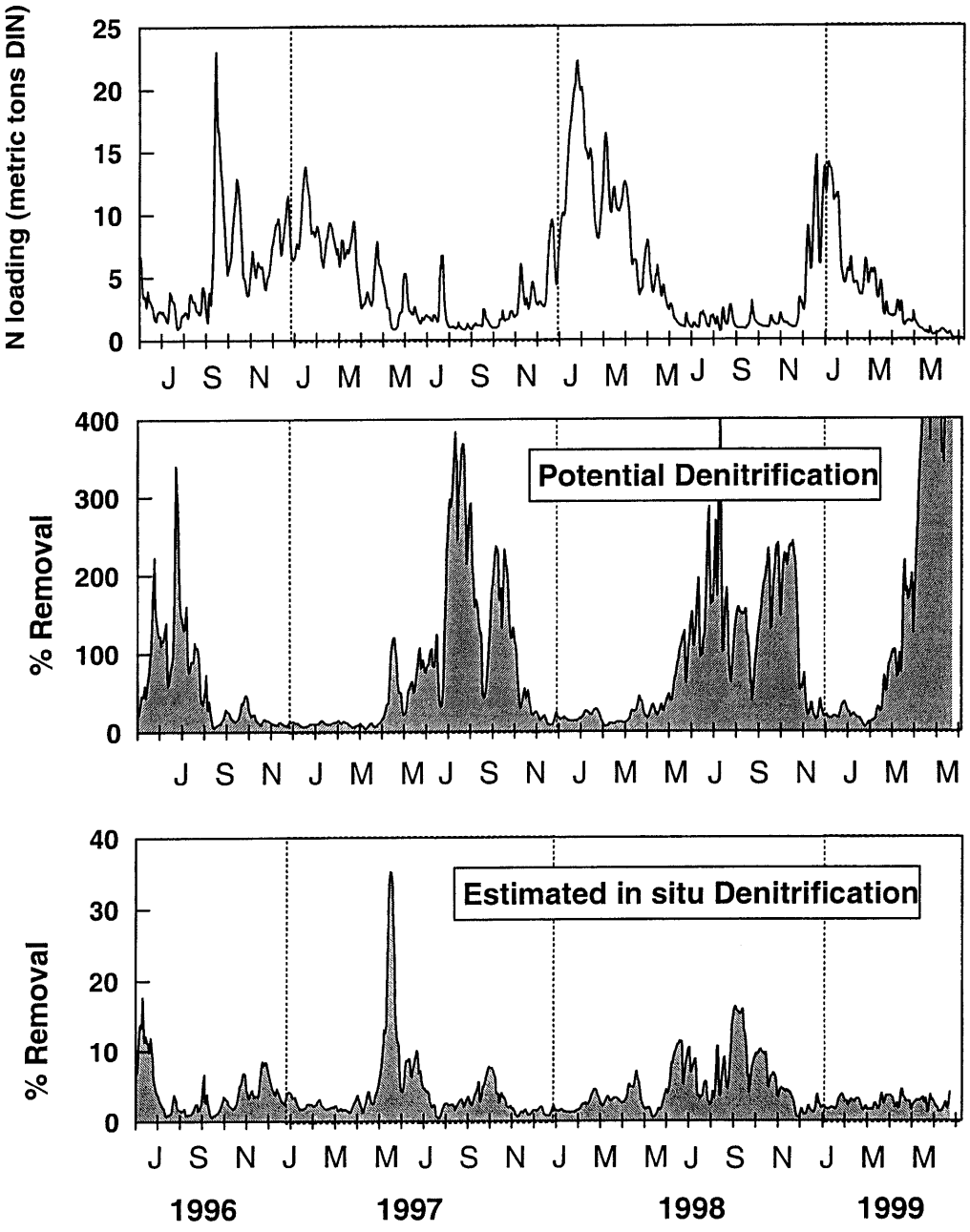

Figure 23: Calculated nitrogen loading and estimated percent removal via denitrification as potential and estimated *in situ* denitrification.

pending direct assays of denitrification at fine temporal and spatial resolution. In addition, information on rates and dynamics of nitrification is needed to improve our understanding of N cycling in the NRE.

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