

ABSTRACT

Title of Thesis: NITROGEN FIXATION IN BENTHIC
MICROALGAL MATS: AN IMPORTANT,
INTERNAL SOURCE OF “NEW” NITROGEN TO
BENTHIC COMMUNITIES IN FLORIDA BAY

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Science

The introduction of “new” nitrogen via nitrogen fixation may have a significant effect on nitrogen availability in estuaries and other partially enclosed aquatic systems. This thesis examines rates of nitrogen fixation associated with benthic microalgal communities in Florida Bay to determine the relative importance of this source as compared to external loads of nitrogen to the system.

Nitrogen fixation was measured using a number of experimental techniques to calibrate the use of the acetylene reduction assay and to determine the suitability of the N₂:Ar comparison technique for measuring rates of nitrogen fixation. Nitrogen fixation was found to be mediated by benthic microalgae at the surface of the sediments in Florida Bay, and higher rates of fixation were measured in areas dominated by benthic microalgal mats. Nitrogen fixation was highest in the relatively phosphorus-rich western basins and was stimulated by experimental phosphorus additions, suggesting that nitrogen fixation is partially phosphorus-limited.

NITROGEN FIXATION IN BENTHIC MICROALGAL MATS: AN IMPORTANT,
INTERNAL SOURCE OF “NEW” NITROGEN TO BENTHIC COMMUNITIES IN
FLORIDA BAY

by

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TABLE OF CONTENTS

Acknowledgements.....	ii
Table of Contents.....	iii
List of Tables.....	iv
List of Figures.....	v
Chapter 1: Building a case for benthic nitrogen fixation as a significant nitrogen source.....	1
Background and Description of Study Site.....	2
Chapter 2: An analysis of methods.....	6
Introduction.....	6
Materials and methods.....	13
Study site.....	13
Sampling.....	15
Whole cores and slurries.....	17
Acetylene reduction assay (ARA).....	19
¹⁵ N amendment.....	21
N ₂ :Ar comparison.....	22
Results.....	24
Discussion.....	30
Conclusions.....	46
Chapter 3: Magnitudes, patterns and factors of limitation.....	48
Introduction.....	48
Methods.....	52
Study site.....	52
Sampling.....	56
Acetylene reduction assay.....	57
Inhibitors.....	59
Nutrient amendments.....	61
Sediment chlorophyll measurements.....	61
Other measurements.....	62
Results.....	63
Description of nitrogen fixation: magnitudes, annual and spatial patterns.....	63
Factors controlling nitrogen fixation.....	66
Discussion.....	75
Conclusions.....	95
Bibliography.....	97

LIST OF TABLES

Table 1: Review of nitrogen fixation rates measured by the acetylene reduction assay and ¹⁵ N amendment techniques that have been reported in the scientific literature.....	10
Table 2: Identification and description of study sites.....	16
Table 3: Experimentally-determined ratios of nitrogen fixation measured by the ¹⁵ N amendment and acetylene reduction assay techniques.....	31
Table 4: Summary of average nitrogen fixation rates by basin and by season.....	68
Table 5: Comparison of nitrogen fixation rates measures in this study to rates reported in the scientific literature.....	89
Table 6: Comparison of internal inputs of nitrogen calculated by this study to reported external inputs of nitrogen.....	94

LIST OF FIGURES

Figure 1: Comprehensive Everglades Restoration Plan.....	4
Figure 2: Map of Florida Bay including location of the study sites.....	14
Figure 3: Diagram of whole core assays.....	18
Figure 4: Characteristic regression curve used to determine nitrogen fixation rate..	26
Figure 5: Comparison of nitrogen fixation rates measured in whole cores and slurries.....	27
Figure 6: Comparison of nitrogen fixation rates measured in seagrass-dominated and BMA-dominated areas.....	28
Figure 7: Nitrogen fixation in the dark as compared to rates measured in the photoperiod.....	32
Figure 8: Variation in the rate of nitrogen fixation over a multiple day incubation..	33
Figure 9: Oxic/anoxic effects on nitrogen fixation.....	34
Figure 10: ¹⁵ N enrichment as a means of determining nitrogen fixation.....	35
Figure 11: Comparison of nitrogen fixation rates measured by the acetylene reduction assay versus ¹⁵ N enrichment.....	36
Figure 12: Comparison of nitrogen fixation rates measured by the acetylene reduction assay versus the N ₂ :Ar comparison technique.....	38
Figure 13: Map of Florida Bay including location of the study sites.....	54
Figure 14: Map of Taylor Slough.....	55
Figure 15: Diagram of whole core assays.....	60
Figure 16: Photograph of the benthic microalgal assemblage under microscopy....	65

Figure 17: Histogram displaying the frequency of measured rates of nitrogen Fixation.....	67
Figure 18: Annual profile of nitrogen fixation rates at site 5.....	69
Figure 19: Comparison of nitrogen fixation rates along an east-west transect.....	70
Figure 20: Comparison of water column ammonium concentrations along an east- west transect.....	71
Figure 21: Comparison of pore water ammonium concentrations along an east-west transect.....	72
Figure 22: Linear regression relating rates of nitrogen fixation and measurements of sediment chlorophyll.....	74
Figure 23: Effects of specific inhibitors on nitrogen fixation rates.....	76
Figure 24: Effect of sodium molybdate.....	77
Figure 25: Effect of ammonium amendments on nitrogen fixation rates.....	78
Figure 26: Effect of phosphorus amendments on nitrogen fixation rates.....	79
Figure 27: Effects of nutrient amendments on rates of nitrogen fixation.....	81

Nitrogen fixation in benthic microalgal mats: an important, internal source of “new” nitrogen to benthic communities in Florida Bay

Chapter 1 – Background and Introduction: Building a case for benthic nitrogen fixation as a significant nitrogen source

Benthic nitrogen fixation has been identified as a significant internal source of “new” nitrogen (*sensu* Dugdale and Goering 1967) in numerous subtropical and tropical estuaries worldwide (Patriquin and Knowles 1972, Capone et al. 1979, Charpy-Roubaud et al. 2001). Nitrogen availability in these estuarine systems can be described as a function of numerous internal and external processes that increase and consume available concentrations of nitrogen. Introductions of “new” nitrogen through processes such as nitrogen fixation have been shown to increase rates of primary production (*sensu* Dugdale and Goering 1967) and have been identified as major nitrogen sources to satisfy, in part, the nitrogen demand within estuarine systems (Charpy-Roubaud et al. 2001). This thesis examines rates and patterns of nitrogen fixation in Florida Bay and attempts to determine the relative significance of this nitrogen source to overall nitrogen availability.

Nutrient availability in Florida Bay appears to be heavily influenced by internal processes, as compared to other subtropical estuarine systems. Florida Bay is a very shallow system (~ 1 m on average) and is sectioned by numerous, natural carbonate mud-bank formations that partially impede the movement of water (Holmquist et al. 1989). These mud banks subdivide the bay into a series of partially

isolated basins that often display differences in salinities and other parameters from adjacent basins. The observation of these differences between waters located so close to each other suggests that there may also be distinct differences in concentrations of nitrogen, phosphorus and other nutrients that result from internal processes that govern the generation and consumption of these nutrients.

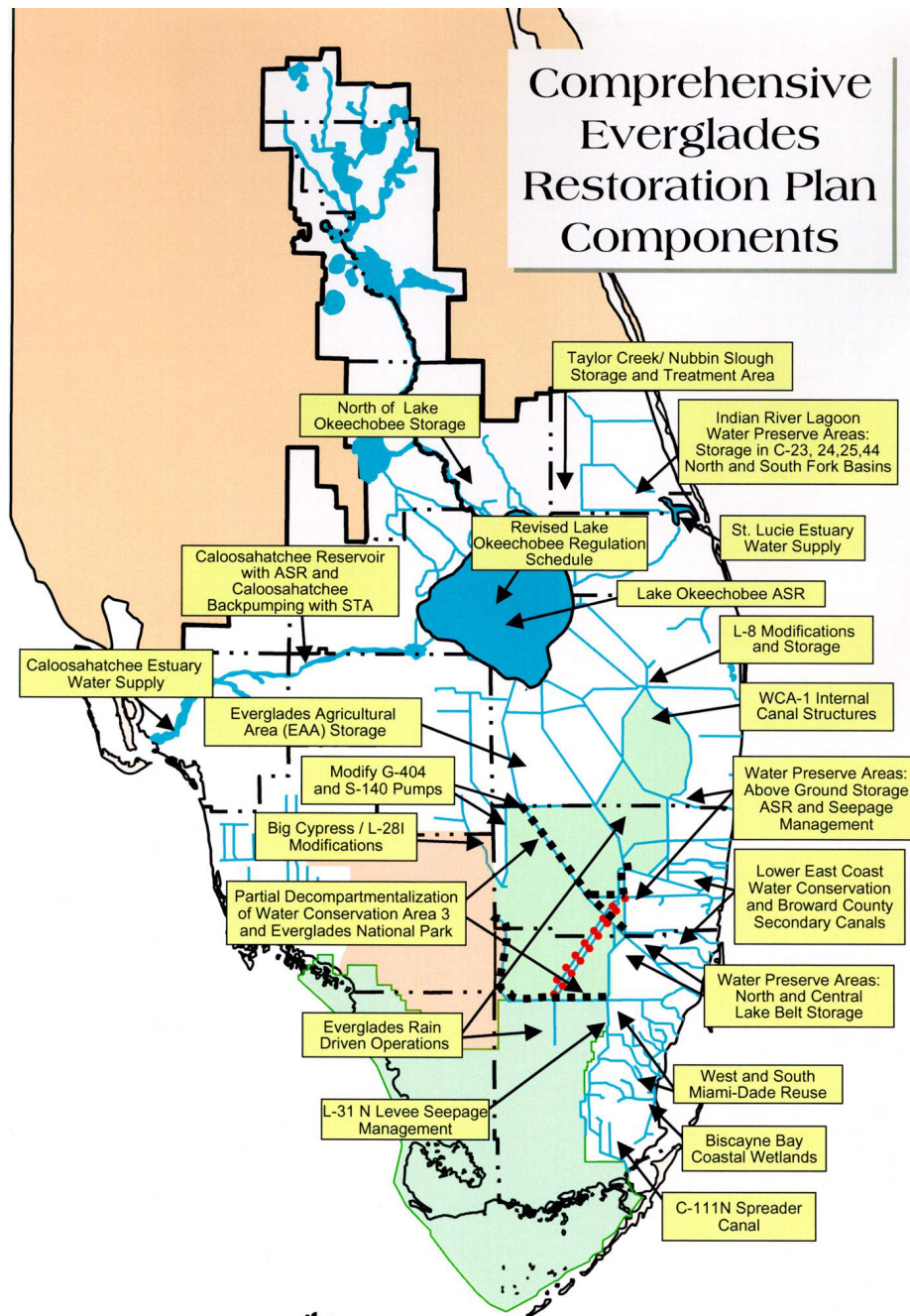
Background and Description of the Study Site

Large-scale modifications to the flow of water through the Kissimmee-Okeechobee-Everglades system over the past century have combined with the natural geography of Florida Bay to further isolate the system from potential nutrient sources both upstream and downstream of the bay. Under natural conditions, freshwater moved south from the Florida peninsula as sheetflow through the Taylor Slough system into northeastern reaches of Florida Bay. This flow has, however, been interrupted by the construction of numerous canals and flow control structures that have resulted in the diversion of large volumes of freshwater to agricultural areas, municipalities and to sea. Freshwater flow rates into Florida Bay from 1940-1986 decreased 59% from the levels measured in 1881-1939 (Smith et al. 1989). This reduction in the amount of freshwater flowing into Florida Bay lessens the effect of external loading from upstream systems as a source of nutrients to the waters of Florida Bay. Furthermore, natural impediments to water flow in the form of the carbonate mud-banks, diminish the effects of these upstream sources on waters in the central and western parts of the bay even more.

Concurrent with these changes in water flow has been the increasing incidence and duration of hypersalinity events in Florida Bay (greater than 50 psu) and seagrass die-back (Zieman et al. 1999). These events have spawned great interest in both the research and management communities in understanding the current state of Florida Bay and what strategies can be taken (if there are any) to restore Florida Bay to the “healthy” state that was observed only decades ago.

The Federal government, in cooperation with the State of Florida and local entities, has recently initiated the Comprehensive Everglades Restoration Plan (CERP) (Figure 1). CERP is a multi-year, large-scale project that will restore water flow through the Florida Everglades and eventually into Florida Bay in order to return the overall system to a more natural state. However, one of the consequences of returning water flows to historical (or nearer to historical) conditions may be an influx of nutrients from areas that support significant agriculture at the northern margin of the present-day Everglades. In order to determine the possible effect of this influx, it is necessary to understand the present state of nutrient availability within Florida Bay.

Previous investigations into nitrogen cycling within Florida Bay have observed a high influx of nitrogen via advection of waters from the Gulf of Mexico across the western reaches of Florida Bay (Rudnick et al. 1999). Inputs of nitrogen from the Florida peninsula, the islands of the Florida Keys, advection from the Florida Strait and via precipitation were orders of magnitude lower. However, the effect that inputs from the Gulf of Mexico may have on overall nitrogen availability in Florida Bay is very much uncertain since much of the water that enters western



Courtesy U.S. Army Corps of Engineers, CERP

Figure 1. The Comprehensive Everglades Restoration Plan (CERP) is a multi-year, multi-million dollar federal-state partnership to restore water flow through the Kissimmee-Okeechobee-Everglades system. The project includes more than 40 restoration projects and is designed to bring about environmental improvements in the Florida Everglades and Florida Bay by 2010.

Florida Bay quickly moves to the Atlantic Ocean via a gap in the Florida Keys (Rudnick et al. 1999). Therefore, internal processes, such as nitrogen fixation, are hypothesized to have a significant role in determining overall nitrogen availability in the system.

Placing the Focus on Internal Processes

Preliminary investigations into internal cycling processes in Florida Bay have observed very high rates of denitrification throughout the bay and have suggested that rates of nitrogen fixation may also be extremely high (Cornwell et al. in prep). These measurements were made by employing the $N_2:Ar$ comparison technique (Kana et al. 1994, 1998) which measures nitrogen fixation indirectly by analyzing the disappearance of dinitrogen from the water column, presumably due to nitrogen fixation that occurs in the sediments.

This thesis seeks to directly measure rates of benthic nitrogen fixation in Florida Bay, to determine the significance of this source to overall nitrogen loading in the system, and to verify the accuracy of measurements of nitrogen fixation rates as determined by the $N_2:Ar$ comparison technique. In addition, investigations were made to describe seasonal patterns of nitrogen fixation as well as additional factors that exert control over benthic nitrogen fixation in Florida Bay.

Nitrogen fixation in benthic microalgal mats: an important, internal source of bioavailable nitrogen to benthic communities in Florida Bay

Chapter 2 – A methods comparison

Introduction

Nitrogen is often identified as the most limiting nutrient required for aquatic vegetative growth and production in estuarine and marine systems (Ryther and Dunstan 1971, Howarth et al. 1988a). The size of the available nitrogen pool within these systems is controlled by nitrogen loading from external sources and the internal generation of nitrogen by internal processes (Kemp and Cornwell 2002). Fixation of atmospheric dinitrogen (N_2) by microbial organisms is one internal process that may significantly increase the availability of this necessary resource to biota. Nitrogen fixation has been recognized as a significant source of “new” nitrogen (*sensu* Dugdale and Goering 1967) that can support high rates of production in numerous estuarine and marine systems worldwide (Capone 1988).

In shallow, subtropical systems such as Florida Bay, sufficient light penetrates to the sediments year-round to support large communities of seagrass and benthic microalgae (sometimes referred to as microphytobenthos). Oligotrophic conditions in the water column do not support abundant phytoplankton populations, resulting in low turbidity and little attenuation of light prior to reaching the sediment surface. Rates of primary production in Florida Bay are dominated by benthic vegetation as

opposed to organisms within the water column (Forqurean et al. 1992). The predominance of benthic productivity over productivity within the water column leads to an increased significance in nutrient cycling processes at the sediment-water interface (Kemp and Cornwell 2002). In systems dominated by benthic vegetation such as Florida Bay, sediment pore waters have been identified as the dominant source of nutrients (Short 1987). Thus, processes that increase the availability of necessary nutrients near and within the sediments likely have an important role in sustaining growth of seagrasses and other aquatic vegetation. Nitrogen fixation within the benthos has been identified as a major internal source of “new” nitrogen in other systems (Smith 1984, Charpy-Roubaud et al. 2001) and may be responsible, in part, for supporting the abundant benthic vegetation observed in Florida Bay.

Nitrogen fixation is a common feature in marine and estuarine systems worldwide and has been partially credited with supporting high rates of vegetative growth and production in oligotrophic estuarine and marine systems in the tropics and subtropics (Capone 1988, Howarth et al. 1988a, Paerl et al. 1994). The process of nitrogen fixation involves the reduction of triple-bonded atmospheric dinitrogen to a simple amine form (NH_x) which can then be incorporated into cellular material (Postgate 1982, Capone 1988). This chemical reaction is mediated by the enzyme, nitrogenase, and is an energy-consuming process. The energy required to run the reaction is derived from the respiration of photosynthetically or chemosynthetically-produced organic matter (Capone 1988). Nitrogen fixation is a process that is carried out only by prokaryotic organisms including numerous bacterial classes and photoautotrophic cyanobacteria (Postgate 1982, Capone 1988).

Rates of benthic nitrogen fixation have been assessed using numerous techniques in marine and estuarine systems worldwide over the past half-century. However, the methods for determining nitrogen fixation rates are fraught with methodological drawbacks and assumptions that make the assessment of nitrogen fixation under natural conditions a difficult endeavor (Seitzinger and Garber 1988). The two most prevalent techniques found within the literature are ^{15}N addition, a technique that measures the production of reduced nitrogen ($^{15}\text{NH}_x$), and the acetylene reduction assay (ARA), an approach that measures the activity of the nitrogenase enzyme.

Much of the literature describing nitrogen fixation is based on rates calculated using the acetylene reduction assay (ARA) as first described by Stewart et al. (1967) and Hardy et al. (1968). ARA takes advantage of the non-specificity of the nitrogen fixing enzyme, nitrogenase, to triple-bonded molecules. The reduction of the triple-bonded acetylene (C_2H_2) molecule to ethylene (C_2H_4) is measured as a proxy indicator for the rate of dinitrogen reduction to ammonia (Capone 1988). The technique assumes a 3:1 ratio between the total moles of acetylene reduced to moles of N_2 reduced based on the number of electrons transferred in each reaction.

However, the theoretical 3:1 ratio has been examined in a number of studies (Patriquin and Knowles 1972, Carpenter et al 1978, Seitzinger and Garber 1987, O'Donohue et al 1991, and Charpy-Roubaud et al. 2001) using concurrent ^{15}N addition experiments and experimentally-determined ratios have been found to vary somewhat from the theoretical value. These observed variations range from slight to significant differences from the theoretical ratio and have led to a call by some

authors for calibration of the ARA with simultaneous ^{15}N amendment assays in all studies (Table 1).

^{15}N amendment assays have the advantage of directly measuring the process of nitrogen fixation as opposed to a measurement of nitrogenase activity, ARA. However, ^{15}N amendments are much more expensive to analyze than ARA and require the “sacrifice” of cores at prescribed time points in order to assess the relative ^{15}N enrichment over time. In contrast, ARA assays can be repeatedly sampled by removing small volumes of the gas headspace without adversely affecting the core or living biomass and are analyzed rapidly and cost-effectively by gas chromatography. The approach of calculating fixation rates within single cores rather than across a series of cores, as is the case in ^{15}N amendment, may result in more accurate measurements of nitrogen fixation over time. Single cores may also be useful in the identification and description of the effects of small variations observed between individual cores (e.g. BMA biomass, sediment column height etc.) on rates of nitrogen fixation.

Over the last decade, rates of benthic nitrogen fixation have also been measured by membrane-inlet mass spectrometry (MIMS) as described in Kana et al. (1994). This method of $\text{N}_2:\text{Ar}$ comparison has been used to describe patterns of nitrogen and oxygen cycling between the sediments and water columns in numerous estuarine systems (Kana et al. 1998, Sundback et al. 2000, Newell et al. 2002, Bernot et al. 2003). $\text{N}_2:\text{Ar}$ ratios are a measure of the net appearance/disappearance of N_2 relative to argon concentrations from the water phase as a function of denitrification and nitrogen fixation. Denitrification results in a net increase in the appearance of N_2

System	Reported C ₂ H ₂ : N ₂ value	Source
Benthic Microalgal Mats		
Salt marsh	3.6	Patriquin et al. (1978)
Intertidal mud flats	4.7-6.9	Potts et al. (1978)
Benthic Microalgae	1.8-4.8	Charpy-Roubaud et al. (2001)
	1.2-5.9	This Study
Seagrass Rhizospheres		
<i>Zostera marina</i>	0.5-6.2	Patriquin and Knowles (1972)
	1.7-4.1	Capone and Budin (1982), Capone (1983)
<i>Zostera capricorni</i>	3.1	O'Donohue et al. (1991)
<i>Syringodium filiforme</i>	0.8-15.5	Patriquin and Knowles (1972)
<i>Thalassia testudinum</i>	2.0-3.3	Patriquin and Knowles (1972)
Subtidal sediments		
Anaerobic	12-94	Seitzinger et al. (1987)

adapted from Seitzinger et al. (1987)

Table 1. A table listing experimentally-determined ratios of nitrogen fixation as determined by use of the acetylene reduction assay and ¹⁵N amendment techniques. Nitrogen fixation rates were determined using both methods contemporaneously and results were compared to calculate a ratio describing the relationship between nitrogenase activity (as measured by ARA) and direct measurements of nitrogen fixation (as measured by ¹⁵N amendment). ARA assumes a theoretical ratio of 3:1 to relate the reduction of acetylene to the reduction of dinitrogen. In some cases, experimentally-calculated ratios have been found to vary greatly from this ratio; however, the majority of calculations have been found in the vicinity of the theoretical 3:1 ratio.

while nitrogen fixation causes a net decrease of water phase dinitrogen. The final figure calculated by N₂:Ar comparison is a net estimate and gives a comparative magnitude of nitrogen fixation in terms of denitrification rather than a true rate of nitrogen fixation. However, periods and magnitudes of net nitrogen inputs (via fixation as compared to denitrification) can be calculated using N₂:Ar comparison and, thus, it can be a useful instrument when investigating nitrogen fixation.

Assessments of nitrogen fixation rates in benthic regimes have also differed in terms of the treatment of the sediments during the duration of the experiment. Slurries were formed for ARA analysis by mixing a sediment column of known depth and a volume of overlying water. The homogenization of the sediments allows for complete introduction of the amended gases in the headspace to all parts of the sediments. However, slurry formation also results in the destruction of vertical structure within the sediment column as well as possible burial of photoautotrophic organisms that may be diazotrophic. Treatment of the sediment in the form of a whole core allows for the structural integrity and vertical zonation within the sediment column to remain intact. The use of whole cores also has the advantage of being directly comparable to whole cores analyzed by the N₂:Ar comparison techniques.

However, analysis of nitrogen fixation via ARA in intact cores may underestimate total values of nitrogen fixation. Incomplete diffusion into and out of the sediments may preclude the penetration of sample gases to nitrogen fixing zones at depth (Patriquin and Denike 1978) or may lead to the incomplete recovery of signal of reduced compounds from the sediments (Flett et al. 1975). Conversely, slurry

formation likely results in the release of organic substrates and other nutrients from deep in the sediment column to surficial nitrogen fixing organisms and, thus, may represent more of a potential rate of nitrogen fixation than that seen under natural conditions (Welsh et al. 1996a; 1996b).

This study focused on three objectives: 1) to calibrate the use of the acetylene reduction assay by simultaneously measuring nitrogen fixation via ^{15}N amendment; 2) to determine the accuracy of nitrogen fixation measured via the acetylene reduction assay in whole cores; and 3) to compare measurements of nitrogen fixation as determined by the $\text{N}_2:\text{Ar}$ comparison technique with simultaneous measurements using other analyses.

In this study, several techniques and treatments; acetylene reduction assays in intact cores and sediment slurries, ^{15}N amendment to whole cores and the $\text{N}_2:\text{Ar}$ comparison method; were used concurrently to determine rates of benthic nitrogen fixation in Florida Bay from August 2002-August 2003. To account for potential sources of error associated with each procedure, intact cores and slurries were assayed simultaneously in order to accurately portray rates of BMA-mediated nitrogen fixation in Florida Bay. Incubations were done over numerous natural diel cycles to ensure sufficient diffusion of acetylene and ^{15}N .

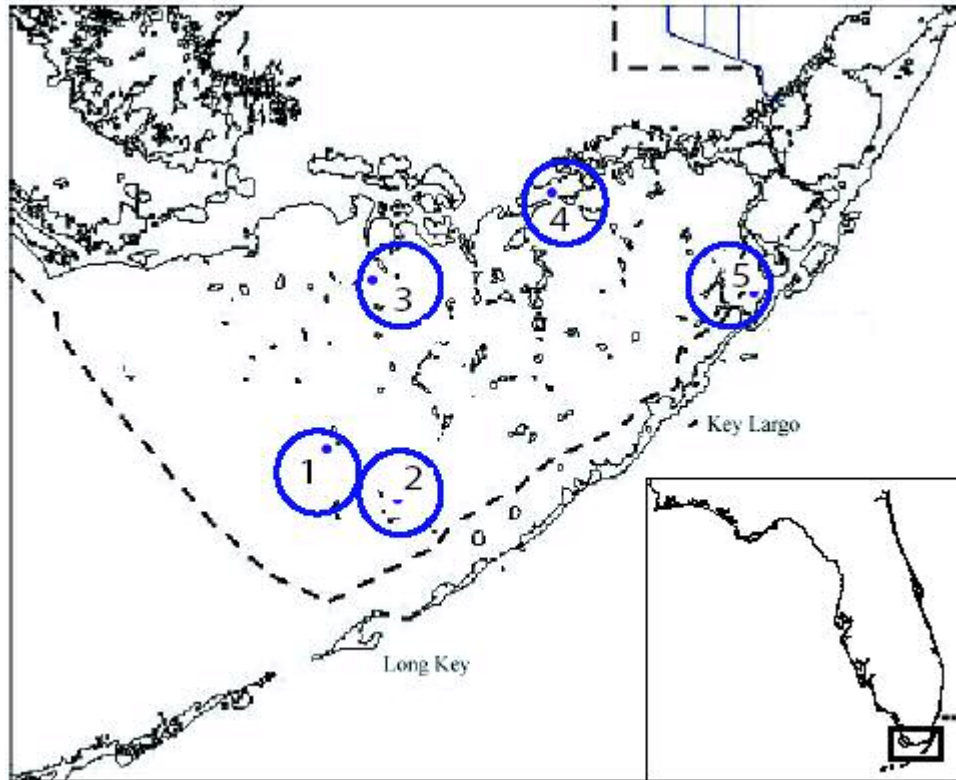
Materials and Methods

Study site

The study was set in Florida Bay (Fig. 2), a subtropical estuary situated between the Florida peninsula and the nearly-solid line of islands that make up the Florida Keys. Florida Bay is a shallow body of water (< 3 m) partitioned by numerous natural carbonate mud bank formations that divide the bay into a series of smaller basins. Adjacent basins often show distinct differences in salinity and nutrient profiles as a result of diminished water exchange over the banks and, thus, were treated and sampled separately to determine overall patterns of benthic nitrogen fixation throughout the bay.

Intact sediment cores and bulk sediment samples were collected in 5 basins located throughout Florida Bay (Fig. 2). These sites were numbered along a transect from the mouth to the head of the bay (roughly a southwest to northeast direction) (Table 2). These sites were selected to give the greatest spatial coverage over discrete zones identified by previous studies (Turney and Perkins 1972, Wanless and Tagett 1989, Zieman et al. 1989, and Boyer et al. 1997). These locations were also sampled in previous projects to describe patterns of nitrogen cycling (Cornwell unpublished data).

All sites were shallow in depth (maximum depth ~ 2 m) and were generally covered with well-developed seagrass beds dominated by *Thalassia testudinum*. Interspersed within seagrass meadows were smaller patches completely devoid of macrophytes. These patches, termed “BMA-dominated” in this paper, are marked by



Modified from Forqurean and Robblee (1999)
Inset graphic courtesy of the Integration and Application Network's,
University of Maryland Center for Environmental Science, symbol library

Figure 2 – Map of Florida Bay showing the five sample areas: Rabbit Key Basin (Site 1), Barnes Key (Site 2), Rankin Key (Site 3), Little Madeira Bay (Site 4), and Sunset Cove (Site 5). The sampling areas were located so as to measure rates of nitrogen fixation in a number of discrete zones described in previous studies (Zieman et al. 1989).

the presence of a well-developed mat consisting of numerous classes of benthic microalgae and associated microorganisms, hereafter termed “BMA.” BMA communities were found throughout the bay in both seagrass-dominated and BMA-dominated areas at the sediment-water interface. The five sites differed in relative coverage by seagrass and BMA with two basins, Sites 1 and 4 being completely dominated by seagrasses and devoid of any BMA-dominated zones (Table 2).

Sampling

All samples were taken via SCUBA and transported to the laboratory for analysis. Experiments with sediment samples were initiated no more than 12 h after collection. Samples were collected as intact cores in clear acrylic plastic tubes (15 cm height, 3.75 cm inner diameter or 30 cm height, 6.25 cm inner diameter) or cutoff 60 mL plastic syringes (5 cm height, 1.6 cm inner diameter) for slurring with water collected at from each site. Replicate cores and sediment samples for slurries were collected (N = 2-5, nearly all experiments consisted of at least triplicate samples) to account for natural variability.

Site	Basin	Location	Vegetation Type
1	Rabbit Key Basin	24° 58.355' N, 80° 50.736' W	100% seagrass-dominated 0% BMA-dominated
2	Barnes Key Basin	24° 58.368' N, 80° 47.234' W	75% seagrass-dominated 25% BMA-dominated
3	Rankin Key	25° 07.410' N, 80° 47.524' W	50% seagrass-dominated 50% BMA-dominated
4	Little Madeira Bay	25° 11.447' N, 80° 38.169' W	100% seagrass-dominated 0% BMA-dominated
5	Sunset Cove	25° 05.737' N, 80° 27.476' W	50% seagrass-dominated 50% BMA-dominated

Table 2: Five sites were sampled throughout Florida Bay. The sites were numbered as above. All sites contained areas that were dominated by seagrasses (seagrass-dominated) and three of the five sites contained significant areas that were dominated by benthic microalgae (BMA-dominated) and marked by the complete absence of seagrasses. The spatial extent of BMA-dominated areas varied between sampling periods; however BMA-dominated zones were consistently present. Site 4 was observed to alternate between seagrass-dominated and BMA-dominated states; however the site was completely seagrass-dominated during the only season that nitrogen fixation was measured.

Whole cores and slurries

Intact cores consisted of ~ 7.5 cm height of sediment overlain by a short water phase and 29 mL gas headspace (Fig. 3). Cores were sealed with a gas-tight clear acrylic lid and bottom stopper. Suspended from the lid was a short, magnetic stir bar to gently stir the water phase without physically disturbing the surficial BMA layer. A rubber septum was placed into the plastic lid as an injection port through which the gas head space could be amended and sampled.

Sediment slurries were made in glass serum vials (72 and 145 mL total volume) by adding approximately 10 mL of site water to a 5 cm high sediment column (~ 40 mL of wet sediment). The sediment/water mixture was sealed in a gas-tight serum vial with a rubber septum held in place by a crimped aluminum seal (22 mL headspace) or Erlenmeyer flask capped with a gas-tight rubber stopper (90 mL headspace).

Both cores and slurries were incubated in a water bath held inside clear, acrylic manifolds that allowed light to reach sediment surfaces. The experimental setup was located under full ambient sunlight and the water bath was intermittently replaced to maintain a natural temperature range (25-32 °C) throughout the course of the experiment. Experiments were conducted over one or multiple natural diel cycles and manifolds were completely covered during overnight periods to negate any possible stimulatory effects from artificial lights located near the experimental setup. Samples were then incubated according to one of three treatments to obtain rates of nitrogen fixation: the acetylene reduction assay, ¹⁵N amendment or N₂:Ar comparison techniques.

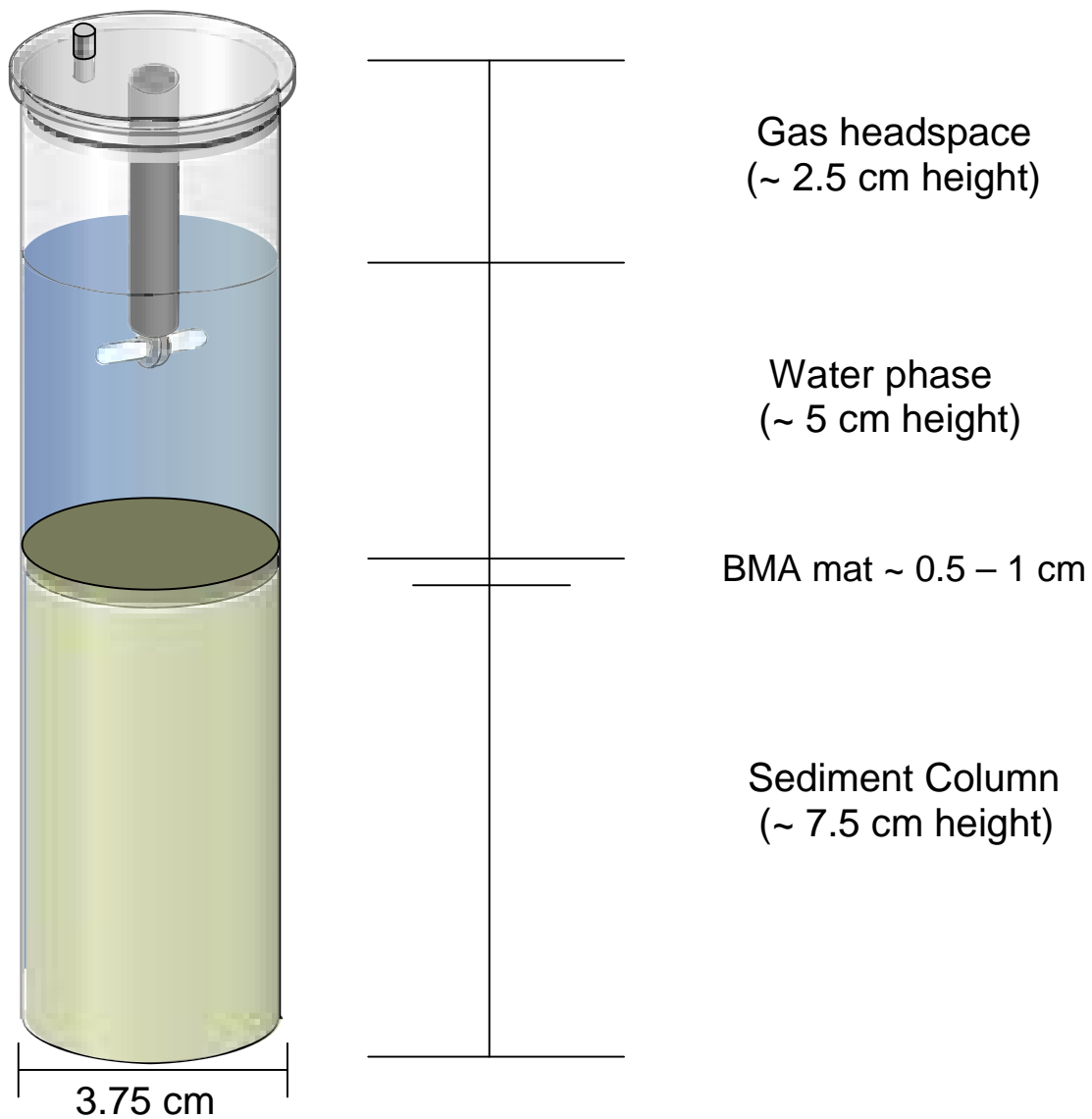


Image courtesy of the Integration and Application Network,
University of Maryland Center for Environmental Science

Figure 3 – Diagram of whole cores showing the air headspace, water phase, and sediment column. The benthic microalgal mat was found at the surface of the sediment column and was generally 0.5 – 1 cm thick. A magnetic stir bar created flow within the water phase to bring about a more complete diffusion in the water phase and into and out of the sediment column. Gas samples were obtained through a rubber septum set into the top of the core.

Acetylene Reduction Assay (ARA)

Intact sediment cores and sediment slurries were analyzed using the acetylene reduction assay (Stewart et al. 1967, Hardy et al. 1968 as modified by Bebout et al. 1987). Acetylene was generated by reacting calcium carbide (CaC_2) with deionized water. Assays were initiated by adding a volume of acetylene equal to ten percent of the total headspace to cores and slurries following the removal of an equal volume of air from the headspace. Assays were initiated during the dark period and allowed to incubate approximately 6-7 hours before the first sample was taken. The first sample was always taken just prior to the beginning of the photoperiod (sunrise) or shortly thereafter. The headspace of each core/slurry was sampled by removing a 0.5 mL air volume via a Hamilton gas-tight syringe approximately 7-8 times per 24-hour cycle.

Air samples were injected into pre-evacuated Exetainer (Labco Ltd. UK) vials (2 mL volume) and diluted with air to a final volume of 2 mL. Samples were diluted to minimize the amount of gas that was removed from the headspace from each experiment during each sampling period. Dilution effects were tested using a known concentration of ethylene gas and diluted samples were consistently found to have one quarter of the signal strength from undiluted samples. Blanks using air were also analyzed and no ethylene signal was observed, suggesting that the dilution had no effect on the ethylene concentration that was measured.

Gas samples were transported to Horn Point Laboratory, Cambridge, MD, USA for analysis by gas chromatography. A Shimadzu GC-8A instrument (flame ionization detector and HayeSep A column) was used to measure ethylene levels as calibrated by the use of a known ethylene standard. Gas analyses were done within

one month of sampling. Separate experiments showed that no appreciable ethylene signal was lost from the Exetainer vials during this time (data not shown). Ultra-pure carrier (UPC) grade Helium was used as the carrier gas.

Concentrations of dissolved ethylene in liquid phase were determined based on recorded water temperatures and ethylene solubility coefficients in water (Larkum et al. 1988), in addition to concentrations of ethylene in the headspace to obtain total ethylene measurements for each core/slurry. Total ethylene values were converted to total concentrations of fixed nitrogen by applying the theoretical 3:1 ratio (moles acetylene reduced : moles dinitrogen fixed) assumed by the acetylene reduction assay. Total micromoles of nitrogen were calculated on an areal basis (using surface area enclosed by the acrylic core) and plotted against incubation time. The linear regression was then reported as the rate of nitrogen fixation for each core or slurry.

A series of slurries were treated anaerobically to investigate rates of nitrogen fixation beyond the oxygen penetration depth within the sediment column. Anaerobic headspaces were created by placing slurry mixtures in a sealed glove bag and removing air by vacuum. Slurries were then amended with nitrogen (N_2) gas and subsequently evacuated before again amending the slurries with N_2 gas in order to establish a completely oxygen-free head space. Anaerobic slurries were then treated with acetylene and monitored for ethylene production as mentioned above.

Blank cores were employed to account for the possible production of ethylene that could not be attributed to nitrogenase activity. Blanks consisted of water phase with and without additions of acetylene and sediment cores/slurries with and without acetylene amendments. No production of ethylene was observed under any of the

blank treatments leading to the conclusion that all ethylene produced in cores and slurries was the result of the reduction of acetylene by nitrogenase.

¹⁵N Amendment

A series of whole cores (15 cm height, 3.75 cm inner diameter) was amended with volumes of 98 % + ¹⁵N₂ gas (Cambridge Isotope Laboratories, Inc.) to directly measure rates of nitrogen fixation in relation to simultaneous ARA experiments. To initiate amendment assays, approximately half of the headspace (15 mL) was removed by syringe through a septum set into a gas tight lid and replaced with an equal volume of ¹⁵N₂ gas. Cores were incubated as described above (ARA section) in a water bath under natural light conditions with continuous water movement created within each core by a rotating magnetic stir bar.

Duplicate cores were sacrificed at set time points to investigate nitrogen fixation over time. The top 0.5 cm of the sediment column (composed primarily of the BMA assemblage) were removed manually and quickly frozen. The frozen samples were then thawed to room temperature, dried, and ground manually before being sent for ¹⁵N/¹⁴N fractionation analysis by mass spectroscopy (Stable Isotope Facility, University of California at Davis). Total moles of ¹⁵N were calculated from total masses of nitrogen and $\delta^{15}\text{N}$ values observed in each sample. The relative enrichment of BMA and associated sediment material over natural occurrences of ¹⁵N (as measured in separate cores sampled prior to ¹⁵N incubation) was wholly attributed to nitrogen fixation and was plotted in relation to incubation time.

N₂:Ar Comparison

A series of whole cores (30 cm height, 6.25 cm inner diameter) were sampled at the same sites as described above to estimate the magnitude of nitrogen fixation as determined by the relative ratio of N₂:Ar gas dissolved in the liquid phase of the experiment (N₂:Ar comparison) (Kana et al. 1994, 1998). Cores consisted of an intact sediment column and BMA mats overlain by a volume of water (no air headspace). After sampling, cores were placed in a water bath consisting of surface seawater from the appropriate site and bubbled overnight to encourage full oxygenation of the water phase. In addition, bubbling resulted in the exchange of water within cores with surface water added to the incubator. This action decreased any effect of nutrients/materials that were released from the pore waters during the process of coring on N₂:Ar measurements. Cores were covered overnight and allowed to equilibrate before sampling commenced the next morning.

After bubbling overnight, cores were carefully sealed with acrylic plastic lids to exclude any air or bubbles from the core. Once sealed, water phases within cores were mixed continually by a magnetic stir-bar that was rotated by a motor-driven turntable.

Water phase samples were collected by displacing a small volume of water within the core with site water. The displaced volume was placed into an 8 mL glass vial, immediately fixed with a volume of mercury chloride (HgCl₂), sealed with a glass stopper and stored in a water bath until analysis. Water samples were analyzed within 2 weeks of collection. Cores were first sampled in the dark (opaque cover wrapped around and over the incubator) for a series of time points and then placed

under natural light conditions (cover off) for a series of “light” measurements. Total incubation time averaged 6-7 hours and was completed in one day.

Samples were analyzed for N₂ appearance/disappearance from the water phase over time within and between light and dark treatments. N₂ concentrations were measured in relation to argon (Ar) concentrations via membrane-inlet mass spectroscopy (Kana et al. 1994, 1998) to describe the production or consumption of dinitrogen in the assays. Due to the absence of any air headspace, all processes relating to the consumption and release of N₂ in these cores was attributed to processes within the sediment and water phases. Apparent decreases in N₂ concentrations within the water phase were recognized as uptake by the sediments (nitrogen fixation) and increased water phase concentrations were used to signify denitrification. In separate experiments, denitrification was inhibited by the addition of methylfluoride (CH₃F), a specific inhibitor of nitrification (Miller et al. 1993, Caffrey and Miller 1995) and thus denitrification, in order to accurately measure nitrogen fixation as measured by N₂:Ar comparison without the lessening effect of denitrification.

The total release/consumption of N₂ as measured by N₂:Ar comparison is a net figure (denitrification – nitrogen fixation) and cannot be compared directly to measurements of nitrogen fixation as described above. However, the direction and magnitude of N₂ appearance/disappearance as measured by the N₂:Ar comparison method was compared qualitatively to other methods of determining rates of nitrogen fixation in this study.

Results

Acetylene reduction was observed in all intact core and sediment slurry assays indicating that nitrogen was being actively fixed in all basins that were investigated throughout the year. Nitrogen fixation (as calculated from ethylene production) was generally linear over time (Fig. 4) and did not show a significant lag period before the initiation of ethylene production. Blanks consisting of acetylene-amended water phases and non-amended sediments and water phases showed no evolution of ethylene resulting in the conclusion that all observed ethylene had been generated via nitrogenase activity.

Measured rates of nitrogen fixation ranged from 1-11 $\mu\text{mol N fixed m}^{-2} \text{ h}^{-1}$ in slurry assays and from 0.5-14 $\mu\text{mol N fixed m}^{-2} \text{ h}^{-1}$ in whole core assays (Fig. 5). Sediments dominated by benthic microalgal mats (BMA-dominated) showed much higher rates of nitrogen fixation than assays taken from within seagrass beds (seagrass-dominated) when treated as whole cores (Fig. 6). Nitrogen fixation rates calculated in slurry assays showed much more similarity between seagrass-dominated and BMA-dominated assays. Assays of nitrogen fixation at depth (depth fractions of the sediment column treated as slurries) revealed that rates of nitrogen fixation were highest within the top most centimeter of the sediment column suggesting that the highest rates of nitrogen fixation were occurring within the benthic microalgal community. Highest rates of nitrogen fixation were observed during the photoperiod (Fig. 7) in both whole cores and slurries while slightly lower rates were found during the overnight hours for all seasons except late summer (August) when “dark” fixation

appeared to be more of an important component of overall BMA-mediated fixation (Fig. 8).

Sediment slurries showed similar to very high rates of nitrogen fixation as compared to rates derived from contemporaneous whole-core assays (Fig. 5). The observed differences between the two techniques varied in magnitude by season and sample site. The largest discrepancies between rates of nitrogen fixation as measured by the two techniques occurred in the summer (June). Rates calculated from both techniques in the winter (January) and early spring (March), however, showed more similar magnitudes and in one case were opposite the observed summer trend as rates measured by whole cores at Site 3 were higher than those calculated from slurries. Slurries incubated under oxic conditions fixed nitrogen at similar to slightly higher rates than anoxic slurries (Fig. 9).

Profiles of benthic microalgal-mediated nitrogen fixation determined by ^{15}N amendment showed a linear trend over the first light period (Fig. 10). This initial trend was followed by a stochastic array of high and low measurements of ^{15}N levels within the surficial BMA mat. The magnitude and rate of nitrogen fixation calculated from the linear trend observed during the first diel period in ^{15}N -amended cores agreed well with nitrogen fixation rates calculated from acetylene-amended whole cores (Fig. 11). Measurements of nitrogen fixation from ^{15}N amended cores were compared to measurements from ARA assayed cores, resulting in an average ARA: ^{15}N amendment of 3.40 (Table 3).

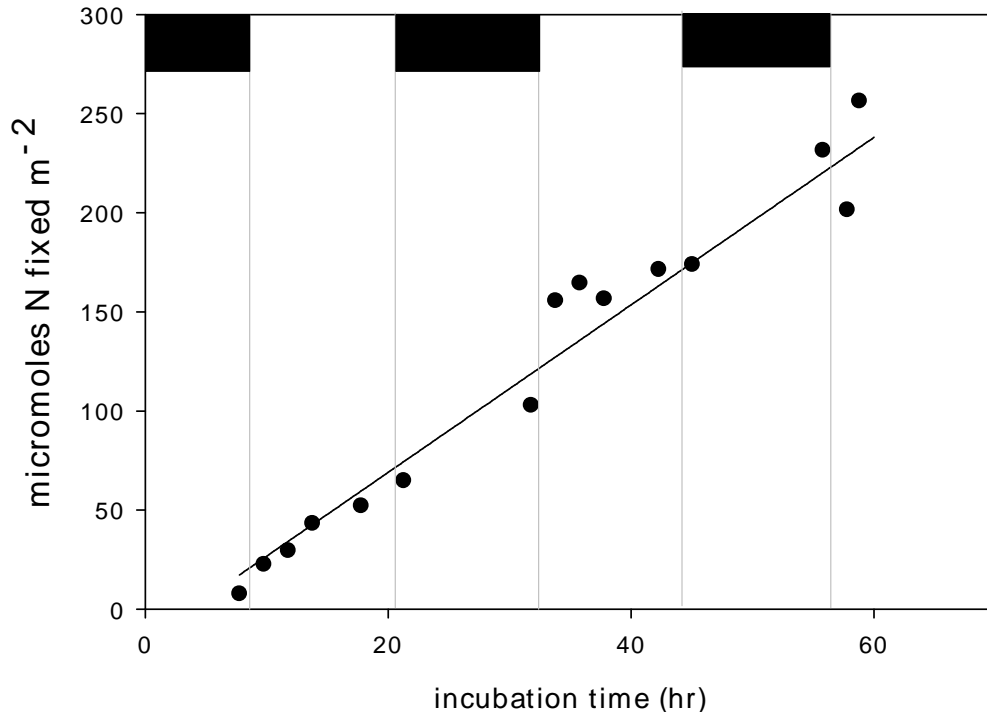


Figure 4 – Example of a linear regression curve ($y = 4.47x - 20.8$; $r^2 = 0.96$) used to determine nitrogen fixation rates over in whole cores and slurries over time. Measurements over numerous time points were plotted at their time of sampling to generate a linear relationship. The slope of the regression was reported as the rate of nitrogen fixation. Incubations were carried over multiple diel periods and the dark (night) periods are designated by the black boxes at the top of the panel.

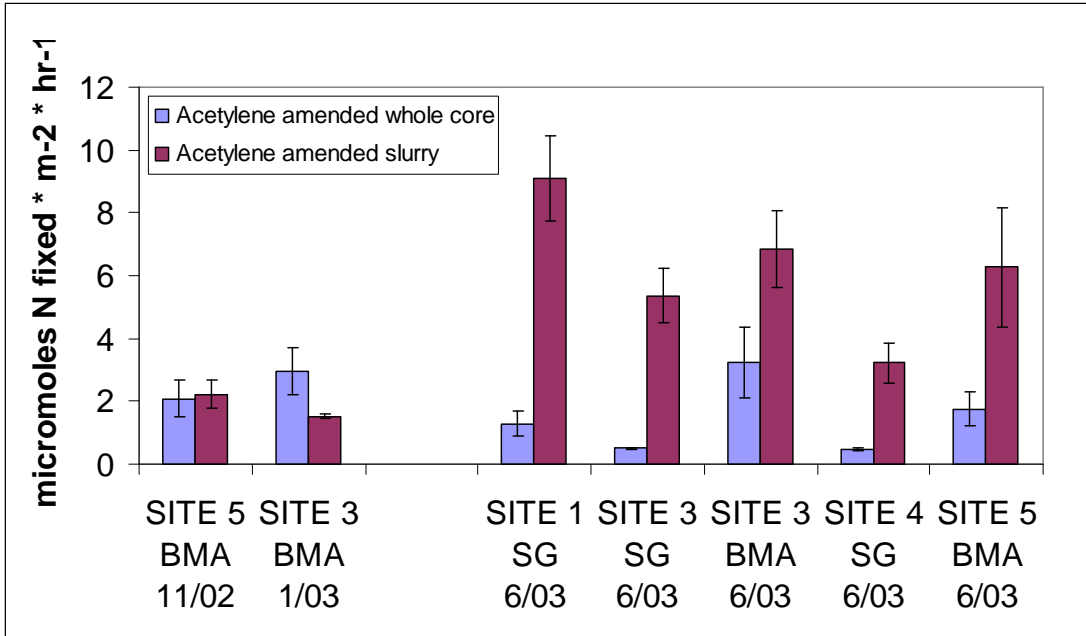


Figure 5 – Nitrogen fixation rates were measured in whole core assays and in sediment slurry assays. Sediments from BMA-dominated (BMA) and seagrass-dominated (SG) sediments were analyzed to determine rates of nitrogen fixation in slurry and whole core assays. In general, rates measured in slurry assays were equal or greater than rates measured in whole cores. Nitrogen fixation rates measured in slurries may reflect the effects of mixing the sediment column and increasing the availability of organic matter and nutrients to benthic microalgae at the sediment-water interface.

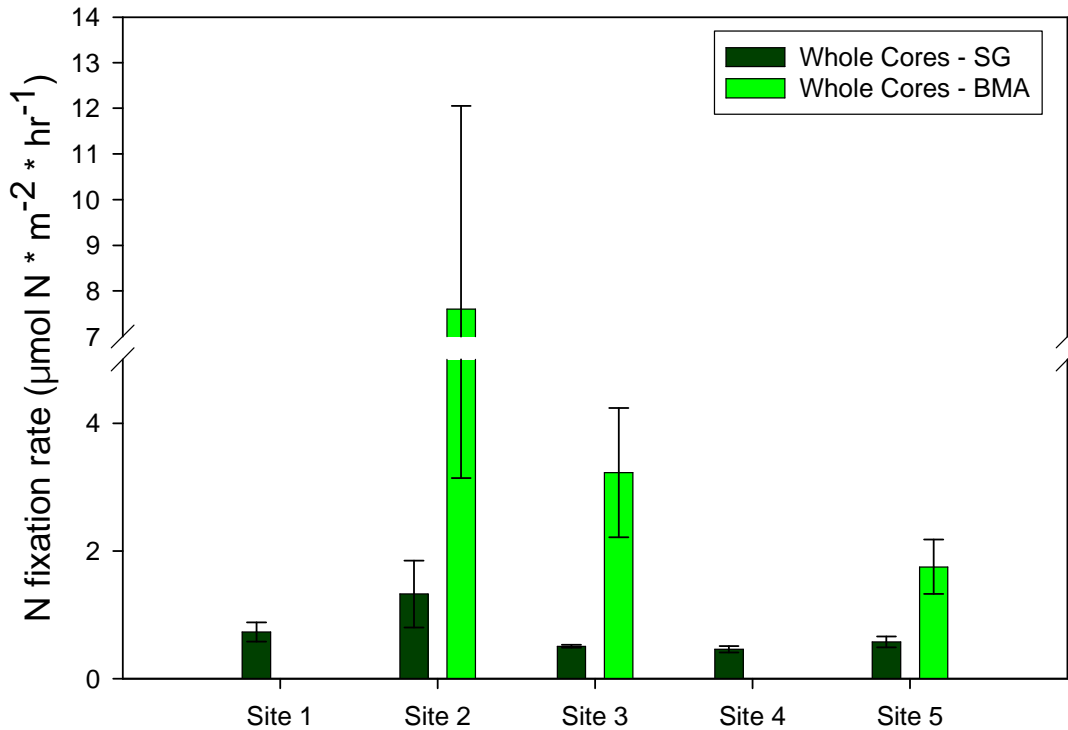


Figure 6 – Nitrogen fixation rates were measured in whole core assays obtained from seagrass-dominated regions (whole cores - SG) and areas dominated by benthic microalgae (whole cores - BMA) in five basins of Florida Bay in June 2003. Nitrogen fixation rates were consistently higher in areas dominated by benthic microalgae. An apparent trend of decreasing nitrogen fixation rates along a transect from the mouth of the bay (Site 1) to the head of the bay (Sites 4 and 5) was also observed. The sediments at two sites (Sites 1 and 4) were completely colonized by stands of seagrass; therefore no nitrogen fixation rates in BMA-dominated sediments were measured in these sites.

Nitrogen fixation rates calculated by acetylene reduction ranged from 0.4-1.9 times the rate as reported from ^{15}N -amended cores with nitrogen fixation rates as determined by ARA being an average of 1.3 times higher than rates as measured by ^{15}N amendment. This relative agreement between rates measured simultaneously by two separate techniques suggests that the 3:1 ratio (moles acetylene reduced to moles dinitrogen fixed) assumed by the acetylene reduction method is appropriate for benthic microalgal-mediated nitrogen fixation in Florida Bay.

Whole cores assayed by $\text{N}_2:\text{Ar}$ comparison, showed a general pattern of net denitrification when incubated in the dark and net nitrogen fixation when exposed to ambient light conditions. Variations in this pattern were observed, as denitrification was at times the dominant process during the photoperiod in certain basins. Variations in the magnitude of the nitrogen flux were also observed between basins and seasons and, in each case, were found to be much larger than measured rates of nitrogen fixation as determined by acetylene reduction and ^{15}N amendment. In June 2003, measurements of net nitrogen fixation by $\text{N}_2:\text{Ar}$ comparison (determined by the disappearance of N_2 from the water phase when exposed to light) overestimated measured rates of nitrogen fixation by a factor of 3-100 times (Fig. 12). This estimate of nitrogen fixation via $\text{N}_2:\text{Ar}$ comparison is a net figure that subtracts the magnitude of denitrification and, therefore, intimates a much higher apparent rate of fixation. Cores treated with methylfluoride, an inhibitor of nitrification, also showed very high rates of apparent nitrogen fixation ($120\text{-}544 \mu\text{mol N fixed m}^{-2} \text{ h}^{-1}$) in the light as calculated by the $\text{N}_2:\text{Ar}$ comparison method (data not shown). However, this high estimate of nitrogen fixation contradicts the relatively low rates ($1\text{-}10 \mu\text{mol N fixed}$

$\text{m}^{-2} \text{h}^{-1}$) found by acetylene reduction and ^{15}N amendment and may be an artifact of the $\text{N}_2:\text{Ar}$ comparison method rather than a true assessment of benthic nitrogen fixation.

Discussion

Nitrogen fixation was measured simultaneously using multiple methods in order to more accurately determine rates of benthic microalgal-mediated nitrogen fixation within Florida Bay. Rates calculated by the acetylene reduction assay (ARA) and ^{15}N amendment showed similar estimates of nitrogen fixation. Results obtained by $\text{N}_2:\text{Ar}$ comparison, however, consistently suggested rates of nitrogen fixation that were at least an order of magnitude higher than those found using other methods.

Previous studies have described rates of nitrogen fixation using a variety of techniques. The accuracy and validity of some of these methods have come into question, however, due to relationships and conditions assumed by each procedure. The accuracy of ARA, in particular, as a method of determining rates of nitrogen fixation has been a source of discussion within the scientific literature (Seitzinger and Garber 1987, Capone 1988, O'Donohue et al. 1991). Acetylene reduction has been the preferred method for measuring rates of nitrogen fixation in many studies due to the ability to calculate nitrogen fixation in real time and the relative low cost of analysis associated with the technique. However, acetylene reduction is an indirect measurement of nitrogen fixation because it describes the reduction of a similar triple-bonded substrate and assumes certain conditions to relate the magnitude and rate of reduced acetylene to nitrogen fixation.

Site	Date	ARA: ¹⁵ N amendment
Site 3	January 2003	1.20
		2.13
		3.74
Site 5	November 2002	2.34
		5.17
		5.82
Average		3.40

Table 3. Experimentally determined ratios of nitrogen fixation rates as measured by the Acetylene Reduction Assay (ARA) and ¹⁵N amendment. Nitrogen fixation was determined via the ARA technique in three replicate cores for each site. Rates of fixation were compared to measurements of nitrogen fixation as determined by the linear regression of ¹⁵N enrichment in the BMA assemblage over time. Individual comparisons varied from the theoretical 3:1 ratio (moles acetylene : moles nitrogen reduced by the nitrogenase enzyme per unit energy) assumed by the ARA method; however, the average value compared favorably to the expected ratio.

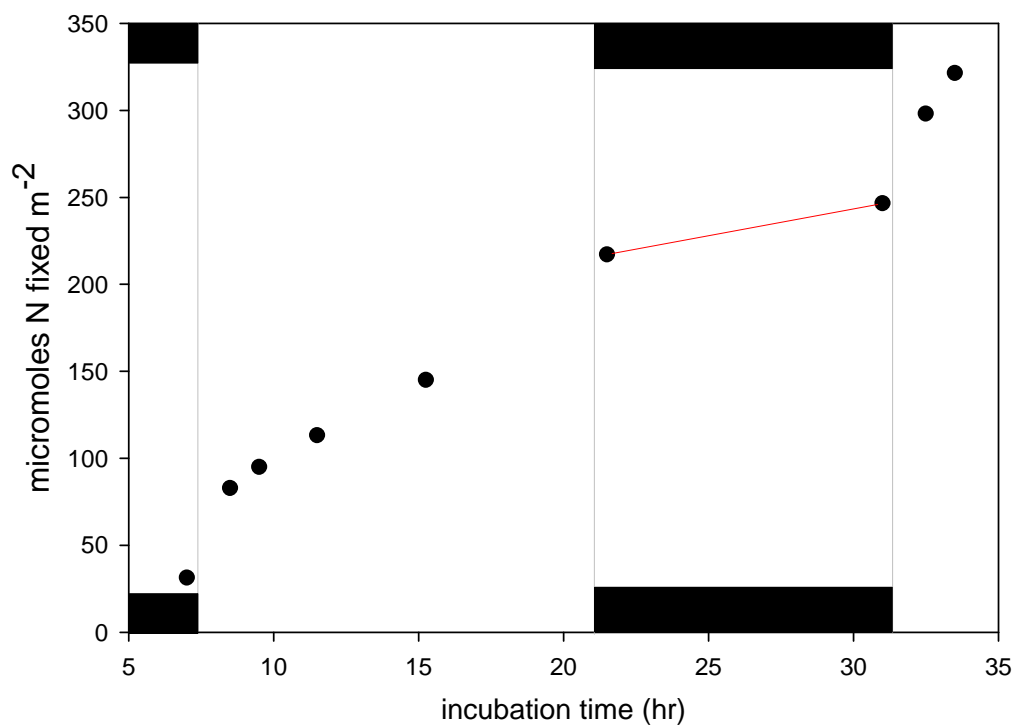


Figure 7 – A representative plot of nitrogen fixation over 1+ diel periods, showing a significant slowdown in the rate of fixation during the overnight period (designated by dark boxes at the top of the panel). Regression line shows the rate of fixation overnight as compared to points measured during the first and second light periods.

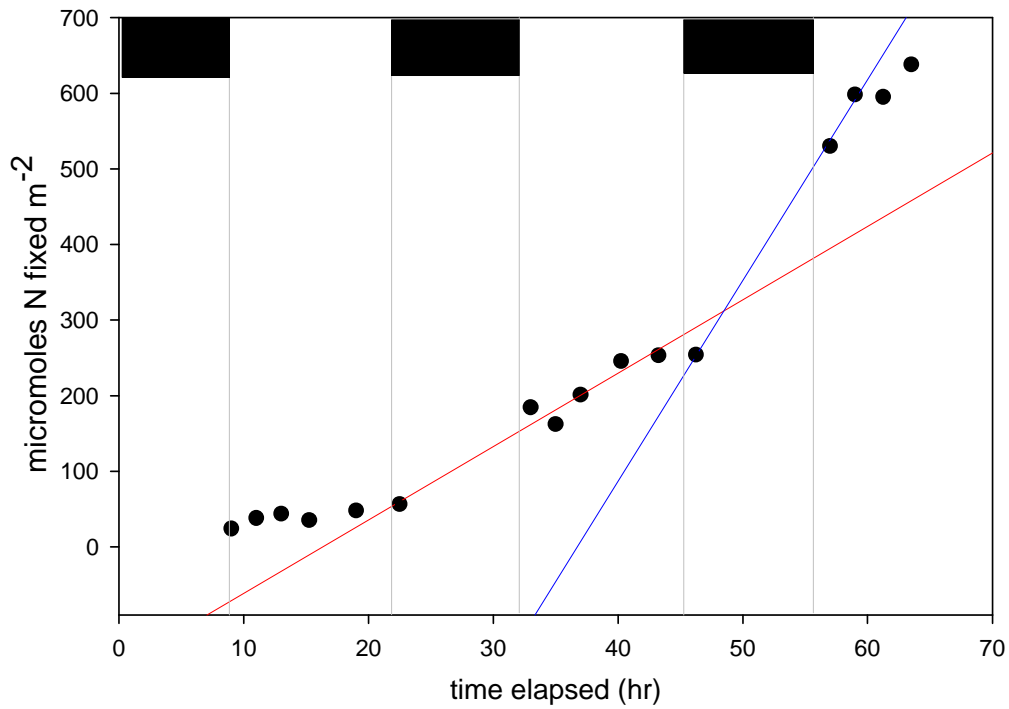


Figure 8 – A representative plot of nitrogen fixation over multiple diel periods displaying variable rates of fixation for each light period. Rates of nitrogen fixation were reported as the slope of the linear regression; however these slopes occasionally included noticeable deviations from the linear relationship. In this graph, nitrogen fixation during the overnight hours appears to have a significant effect on the overall relationship.

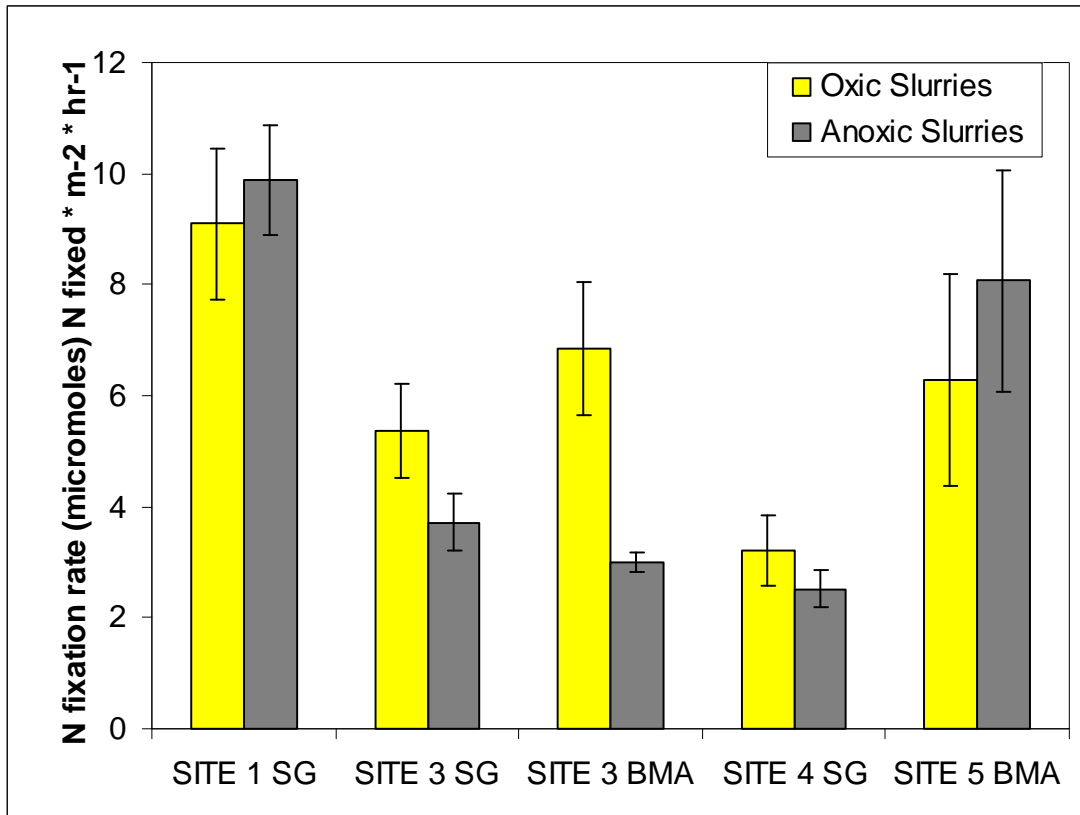


Figure 9 – Comparison of nitrogen fixation rates measured in both oxic and anoxic slurry assays using sediments obtained from several basins in Florida Bay. Nitrogen fixation was generally observed to occur at a similar rank when measured under oxic and anoxic conditions. In a few cases, anoxic conditions appeared to inhibit nitrogen fixation as compared to slurries incubated under oxic conditions.

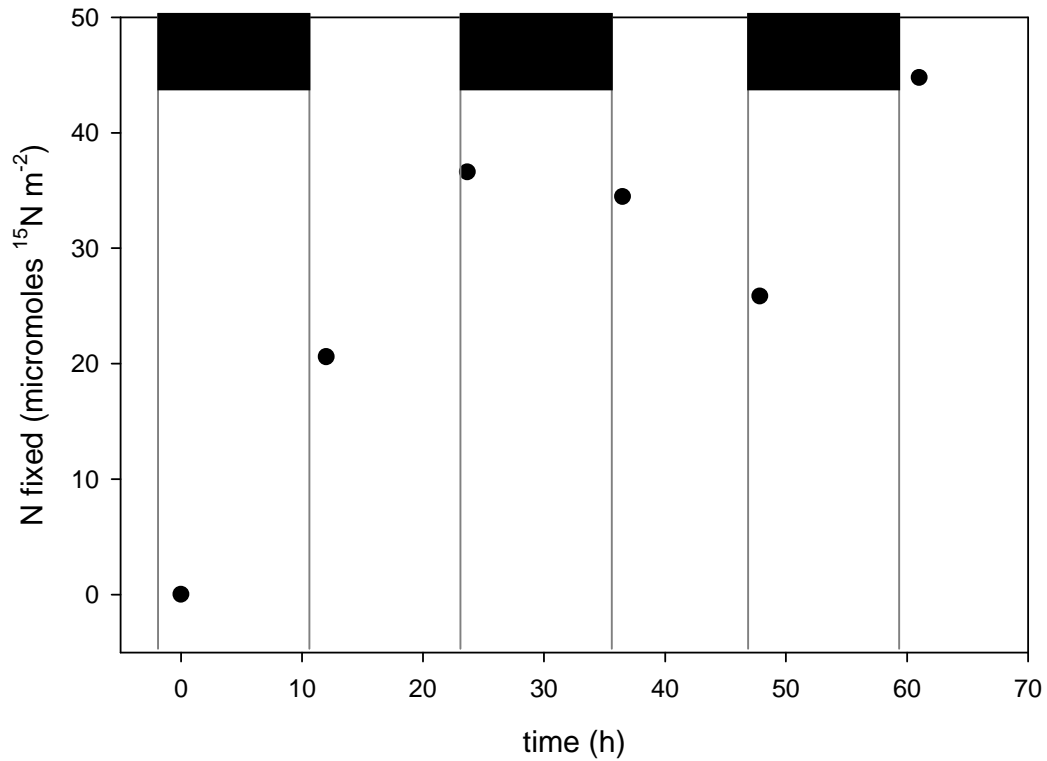


Figure 10 – A plot displaying ^{15}N enrichment (nitrogen fixation) in the benthic microalgal assemblage in whole core assays over time. ^{15}N enrichment progressed in a nearly linear fashion for the first complete diel cycle after which this trend appears to dissipate. The breakdown in the linear trend likely reflects regeneration and cycling mechanisms that removed the newly-fixed nitrogen from the BMA assemblage.

Figure 11 – Contemporaneous investigations into nitrogen fixation rates using the acetylene reduction assay (solid circle, square and inverted triangle) and the ^{15}N enrichment technique (dotted triangle) over multiple diel periods using whole cores obtained from site 5 (top) and site 3 (bottom) in January 2003. Regression lines accompany measurements of nitrogen fixation by acetylene reduction.

The equations for the regression lines at site 5 are as follows (from top to bottom):

$$y = 1.66x + 3.02; r^2 = 0.99$$

$$y = 1.14x + 5.01; r^2 = 0.98$$

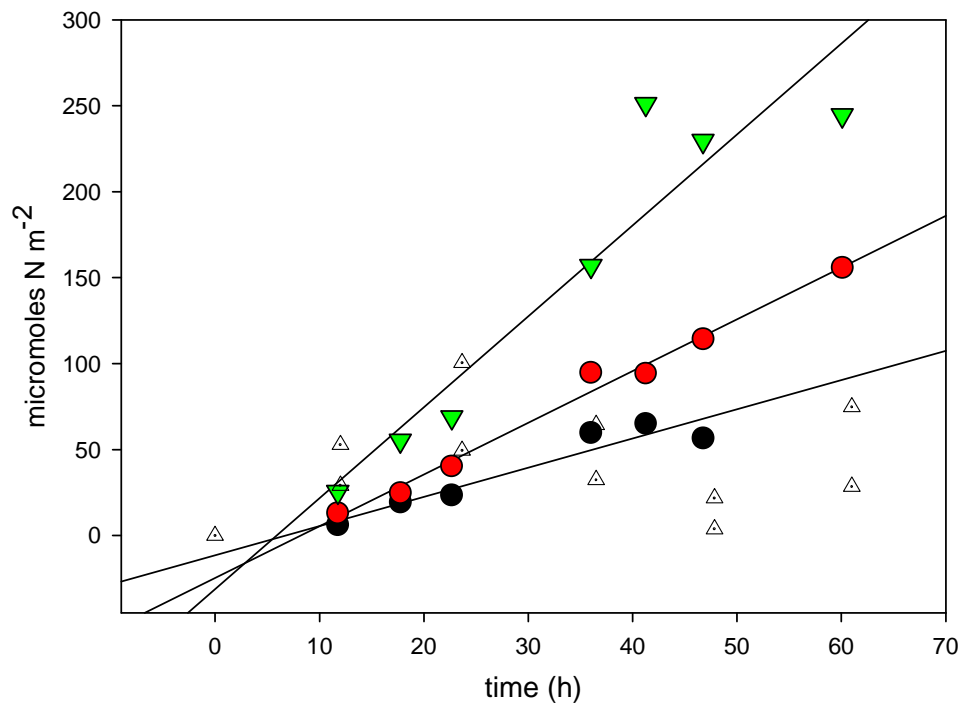
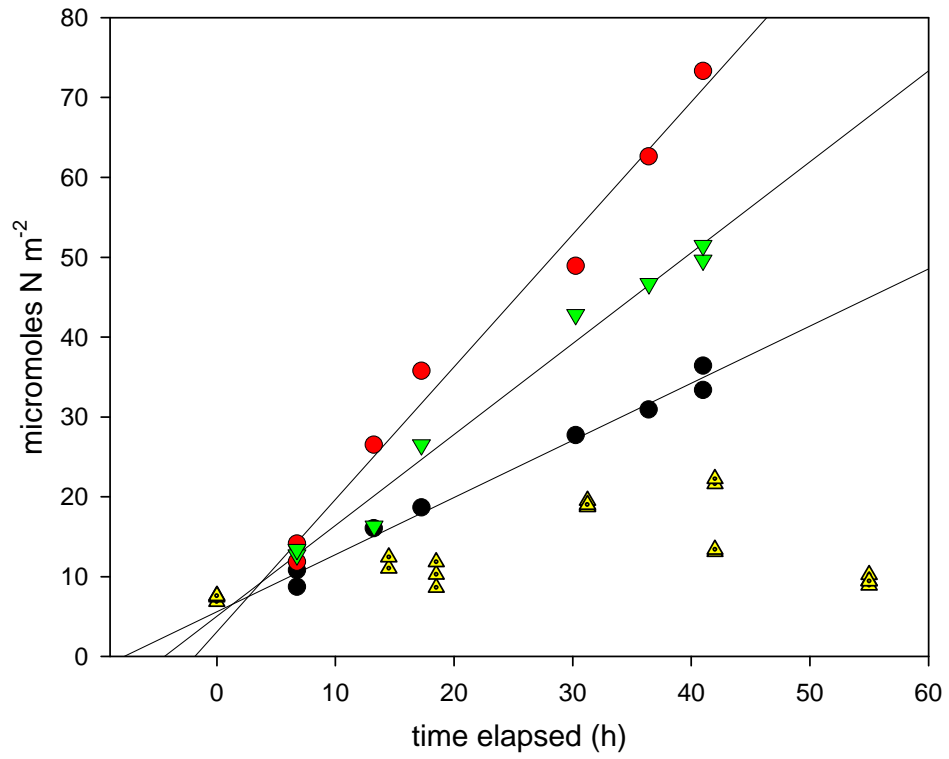
$$y = 0.72x + 5.59; r^2 = 0.99$$

The equations for the regression lines at site 3 are as follows (from top to bottom):

$$y = 5.29x - 31.19; r^2 = 0.88$$

$$y = 3.01x - 24.69; r^2 = 0.99$$

$$y = 1.70x - 11.38; r^2 = 0.91$$



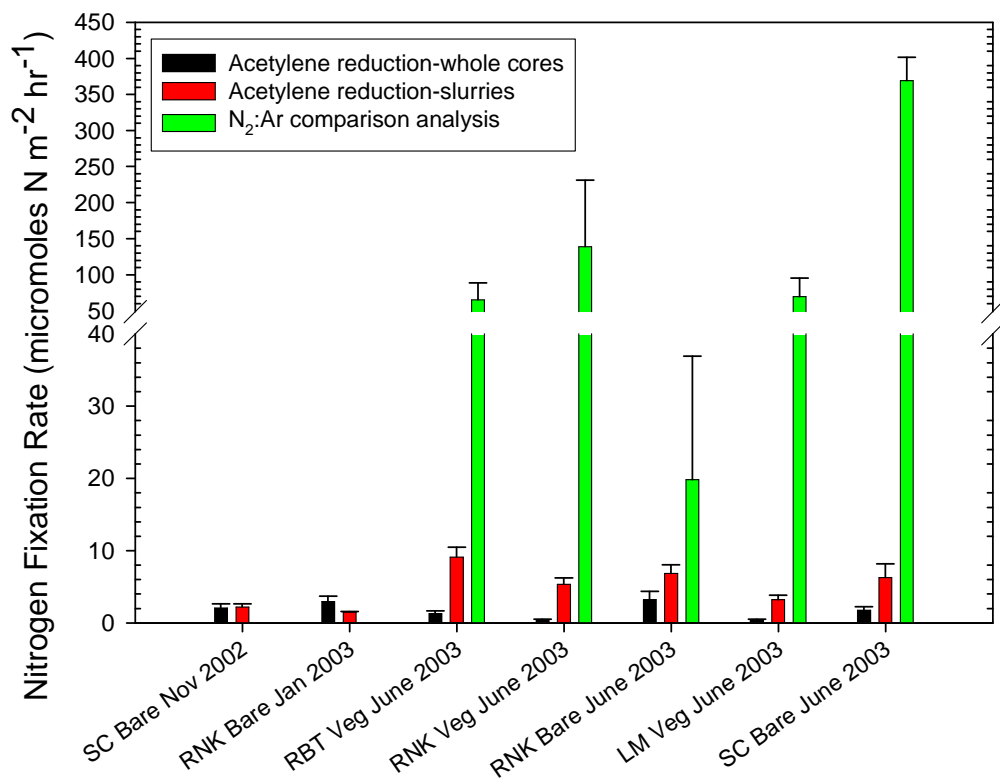


Figure 12 – Comparison of measured nitrogen fixation rates from whole cores and slurries analyzed contemporaneously via acetylene reduction assay (ARA) and the N₂:Ar comparison technique. In all cases rates of nitrogen fixation as measured by N₂:Ar comparison were orders of magnitude higher than those measured by ARA.

One of these assumptions is that acetylene and its reduced form, ethylene, diffuse freely and rapidly between gas and aqueous phases. In this study acetylene reduction was observed in all cores and slurries throughout incubation periods lasting up to 60 hours. No significant lag period was observed in ethylene production in slurry incubations suggesting that acetylene supply to the fixing organisms within the benthic microalgal community was sufficient and diffusion of ethylene to the gas phase was adequate to observe ethylene production. Rates of nitrogen fixation in whole core assays were generally very low during the first 8-10 hours of incubation. This period, however, was always during the overnight hours when apparent nitrogen fixation rates were generally lower than during the photoperiod (Nagel Chapter 3). In a few cases, nighttime nitrogen fixation was very high during this initial period and showed no evidence of a lag as would result from insufficient diffusion into and out of the sediments. Observations of high nitrogen fixation during the overnight hours may be representative of a change in species composition within the BMA assemblage between seasons.

Certain cyanobacteria species (especially *Lyngbya spp.*) have been shown to primarily fix nitrogen during dark periods (Bebout et al. 1993; Omoregie et al. 2004) and this could have been the source of the observed increase in overnight nitrogen fixation. *Lyngbya* was observed in the BMA assemblage during the summer sampling period; however no attempts were made to quantify the population size in this or other seasons.

Slurries were vigorously shaken following the addition of acetylene to each individual assay as well as immediately prior to each sampling time. This action

likely increased diffusive transport and resulted in equilibrium of ethylene between aqueous and gas phases within slurry assays (Pinckney et al. 1995, Welsh et al. 1996b). Whole cores, on the other hand, were shaken very gently prior to sampling to equilibrate gas and aqueous phases without disturbing the structural integrity of the sediments and benthic microalgal mats. Therefore, it is likely that there could have been incomplete diffusion of ethylene into the gas phase (headspace) in whole core assays as compared to well-mixed slurries.

However, the effect of partial or incomplete diffusion within whole cores assays is diminished due to the suggestion that most nitrogen is fixed within the BMA community at the sediment-water interface. Vertical surveys of nitrogen fixation within the sediment column suggest that nitrogen fixation primarily occurred within the topmost 1 cm of the sediment column. This depth was consistently colonized by abundant populations of benthic microalgal communities. While slurry formation addressed the uncertainty related to the diffusion of acetylene to all parts of the assay, it also likely resulted in a disruption of the BMA community structure and the burying of BMA community components. Measurements of nitrogen fixation under normal oxic conditions were observed to be similar to higher than rates of nitrogen fixation measured under anoxic conditions, further supporting the role of the BMA community at the sediment-water interface as the dominant source of nitrogen fixation. Therefore, although the degree to which acetylene diffused into the sediment column was not directly investigated, it does not reason that this unknown had a great effect at the surface of the sediments where the BMA community was found.

Rates of nitrogen fixation in slurry assays were generally higher than rates calculated from intact cores. This was especially true in the late summer (August) when rates derived from slurries were observed at 3 to 5 times higher than rates found in whole cores. While the disparity between rates derived from whole cores and slurries may be suggestive of lower diffusion of ethylene to the gas headspace in whole core assays, it may also be explained as a result of the homogenization of the sediment column in slurry assays (Welsh et al. 1996a, b). Slurry formation disrupts the vertical zonation of the sediments and results in the introduction of nutrients (DOC, ammonia and phosphorus) from senescing organic matter deep within the sediment column into contact with nitrogen fixing organisms in the surficial BMA assemblage. Therefore, rates of nitrogen fixation calculated within slurry assays may reflect the potential rate of nitrogen fixation at each sample site while whole cores provide a more accurate description of in situ rates of nitrogen fixation as proposed by Welsh et al. (1996a, b).

This hypothesis is supported by observations of nitrogen fixation in ^{15}N -amended cores. Nitrogen fixation was assayed simultaneously in intact cores by ARA and ^{15}N amendments in two basins in the fall and winter. Nitrogen fixation rates were calculated from rates of ethylene production in ARA cores using the standard 3:1 ratio (moles acetylene reduced to moles dinitrogen fixed) and compared to estimates of nitrogen fixation in ^{15}N -amended cores. Rates of ethylene production in acetylene-amended cores ranged from 1.2-5.9 times higher than rates of nitrogen fixation measured in ^{15}N -amended cores (Table 3). The average of the experimentally found ratios was 3.4; very similar to the theoretical 3:1 ratio assumed

by ARA. It appears that acetylene reduction generates accurate assessments of benthic microalgal-mediated nitrogen fixation within Florida Bay. Likewise, the similarity in rates of nitrogen fixation between acetylene and ^{15}N -amended whole cores suggests that acetylene reduction in whole cores is an appropriate method for measuring in situ rates of nitrogen fixation.

Rates of nitrogen fixation in ^{15}N -amended cores were calculated using the initial linear trend in ^{15}N accumulation within the BMA assemblage over time. This regression remained linear and significant over the first diel cycle (24 hours) in all incubations before degenerating into a series of high and low points observed further within the incubation (see Fig 10). The breakdown in the linear relationship likely reflects the impacts of regenerative and cycling processes that removed newly-fixed nitrogen from the BMA assemblage. For this reason measurements of nitrogen fixation as assayed by ^{15}N addition incorporated samples taken only during the first 24-hour period.

Measurements taken from duplicate cores at each sampling point agreed well with each other suggesting that the mass of ^{15}N within the solid phase in the BMA assemblage was reduced at times during the incubation. Measurements of labeled nitrogen within the water phase and pore waters would have likely accounted for this missing mass of ^{15}N (Glibert and Bronk 1994), however these assays were not performed. The possibility of extracellular release of newly fixed ^{15}N into phases not examined by this study may be a source of uncertainty regarding the reported values of total nitrogen fixation in ^{15}N -amended cores within this study. However, the reported rate of nitrogen fixation was a product of the first diel cycle only when such

loss of newly-fixed ^{15}N appeared to be at a minimum (judging from the continued linearity of the trend during this period). Therefore, while measurements of labeled nitrogen would result in a more complete estimate of nitrogen fixation it is likely that the reported values from the first diel period represent a very good estimate of nitrogen fixation by the BMA assemblage. Likewise, the observed similarity between estimates of nitrogen fixation determined by ARA and ^{15}N amendment suggests that calculated rates of nitrogen fixation via acetylene reduction also are sufficient measurements of BMA-mediated fixation.

Rates of nitrogen fixation as derived from acetylene reduction and ^{15}N amendment were compared to estimates of nitrogen fixation from whole cores analyzed by $\text{N}_2:\text{Ar}$ comparison. $\text{N}_2:\text{Ar}$ comparison has generally been used to describe patterns and magnitudes of denitrification within the upper sediment column (Kana et al. 1994, 1998). Results derived from $\text{N}_2:\text{Ar}$ comparison reflect a net appearance or disappearance of dinitrogen within the water phase as a result of dinitrogen consumption and production within the sediments. Trends of increasing N_2 within the water phase over the incubation period are described as periods of net denitrification while patterns of decreasing N_2 concentrations within the water phase are attributed to net nitrogen fixation. Both net figures indiscriminately incorporate rates of denitrification and nitrogen fixation within the final estimate of the N_2 flux and do not give finite estimates of either process. For this reason, $\text{N}_2:\text{Ar}$ comparison estimates of nitrogen fixation are not directly comparable to estimates of nitrogen fixation as derived from ARA and ^{15}N analysis.

However, periods when nitrogen fixation is the dominant process may result in quantitative estimates of nitrogen fixation (albeit diminished by some rate of contemporaneous denitrification) that can be compared to estimates of fixation derived from other methods. Rates of dinitrogen flux generally showed a net disappearance of N_2 from the water phase during the photoperiod, suggesting high rates of nitrogen fixation during the day. Therefore, the linear regression of N_2 disappearance over time was used to determine rates of nitrogen fixation within $N_2:Ar$ comparison cores with the caveat that these rates carried a degree of uncertainty due to the presence of simultaneous denitrification that likely resulted in a lower overall estimate of total nitrogen fixation.

Cores analyzed by $N_2:Ar$ comparison suggest rates of nitrogen fixation that are more than one order of magnitude higher than those calculated from simultaneous incubations of acetylene and ^{15}N -treated cores and slurries. Estimates of nitrogen fixation as analyzed by $N_2:Ar$ comparison also incorporate some component of denitrification suggesting that the disparity between actual rates of nitrogen fixation as determined by $N_2:Ar$ comparison and those calculated from ARA and ^{15}N amendment is even larger. This is supported by results from cores where nitrification and, thus, coupled denitrification were inhibited by methylfluoride amendments. Nitrogen fixation rates as calculated by $N_2:Ar$ comparison in CH_3F -amended cores were 10-50 times higher than high-end estimates of nitrogen fixation from acetylene and ^{15}N -amended cores. Additionally, no clear pattern or correlation was observed between nitrogen fixation rates derived from $N_2:Ar$ comparison treated cores and

other methods suggesting that changes in the N₂:Ar ratio in the water phase offer a poor estimation of BMA-mediated nitrogen fixation.

The estimates reported by N₂:Ar comparison may be a function of a number of processes that combine to create such high, apparent rates of nitrogen fixation. Net nitrogen fixation was generally observed during the photoperiod and therefore, was concurrent with photosynthesis. The production of oxygen bubbles by photosynthetic processes is a major concern in cores analyzed via N₂:Ar comparison as aqueous N₂ is preferentially stripped from the water phase into the newly-formed bubbles (Cornwell pers. comm.). Argon is stripped from the water phase at much lower rates than nitrogen resulting in a skewed N₂:Ar ratio and a large effect on apparent N₂ concentrations in the water phase (An et al. 2001, Cornwell pers. comm.). High rates of oxygen production that result from high rates of photosynthesis within the benthic microalgal mat would likely result in much higher rates of apparent N₂ disappearance and, therefore, apparent nitrogen fixation within these cores. Such bubble formation was a regular occurrence in all incubated whole cores (personal observation) and, as such, N₂:Ar comparison cores were incubated in the light for a maximum of 3 hours in order to lessen the effect of photosynthetically-produced oxygen on the assay.

Nitrogen fixation rates derived from such short incubation periods may not compare well to rates determined over longer incubations and may result in some of the large differences observed between rates of nitrogen fixation as estimated by N₂:Ar comparison and estimates from other methods. In multiple-day ARA incubations, short-term variations in the rate of nitrogen fixation were observed with somewhat higher rates of fixation apparent in the first hours of the photoperiod

(Nagel Chapter 3). These short-term variations, however, were for the most part lost, as a linear daily rate was observed over the whole of the incubation. The rates described by N₂:Ar comparison may be similar to the short-term increases observed in the rate of nitrogen fixation as the whole of the incubation period in N₂:Ar comparison assays encompasses only this initial exposure to sunlight. If this were the case, short-term measurements of a slightly elevated rate of fixation would be much higher than rates calculated over a much longer incubation period (24-60 hours) as was the case in ARA and ¹⁵N-amended cores. However, even when compared to nitrogen fixation rates calculated over the first three hours of the photoperiod, rates described by N₂:Ar comparison are more than an order of magnitude higher than those calculated from other methods.

Conclusions

Estimates of nitrogen fixation were calculated using numerous methods in order to determine an appropriate procedure for accurately describing rates of benthic microalgal-mediated nitrogen fixation in Florida Bay. Cores and slurries were analyzed by the acetylene reduction assay (ARA) and ¹⁵N amendment and were found to be very similar in magnitude and pattern. Nitrogen fixation rates derived from slurry assays were slightly higher than those calculated from intact cores. Differences between slurries and whole cores were significant in the summer yet less than one order of magnitude in all cases and may be more indicative of procedural differences

rather than real differences in the level of nitrogen fixation. Good replication between ^{15}N -amended cores and ARA treated whole cores suggests that the theoretical 3:1 ratio (moles acetylene reduced : moles dinitrogen fixed) is reasonable for calculating benthic microalgal-mediated nitrogen fixation in Florida Bay. Also, treatment of the sediments as an intact core appears to be an appropriate method for determining *in situ* rates of nitrogen fixation. However, intact cores likely do not account for rates of nitrogen fixation that can be attributed to rhizosphere-associated bacteria. Further investigation into nitrogen fixation within the rhizosphere is necessary to determine the overall significance of nitrogen fixation as a source of nitrogen in Florida Bay.

Nitrogen fixation as assayed by $\text{N}_2:\text{Ar}$ comparison did not correlate well with rates of nitrogen fixation calculated from cores and slurries analyzed simultaneously by other methods. For this reason, descriptions of nitrogen fixation by $\text{N}_2:\text{Ar}$ comparison alone should be treated with caution when directly compared to published rates of nitrogen fixation as determined by acetylene reduction and ^{15}N amendment.

Nitrogen fixation in benthic microalgal mats: an important, internal source of “new” nitrogen to benthic communities in Florida Bay

Chapter 3 – Magnitudes, patterns, and factors of limitation

Introduction

The availability of fixed nitrogen is generally cited as one of the factors most responsible in limiting rates of primary production in marine and estuarine systems worldwide (Ryther and Dunstan 1971, Howarth et al. 1998a). The largest global pool of nitrogen, atmospheric dinitrogen (N_2), is largely unavailable to biological processes unless first reduced to a bioavailable form (NH_3) via nitrogen fixation. Fixation is wholly mediated by a diverse array of bacteria found in both the water column and sediments in aquatic systems. Nitrogen fixing bacteria include photoautotrophic, chemoautotrophic and heterotrophic types and are found both free-living and associated with higher organisms in aquatic environments (Capone 1998). Nitrogen fixed by diazotrophic organisms may be introduced to higher trophic levels via extracellular release or direct grazing and can lead to increased ambient water column and pore water nitrogen concentrations (Glibert and Bronk 1994, O’Neil et al. 1996, O’Neil 1999).

Benthic nitrogen fixation is a potentially important nutrient source that can support high rates of vegetative growth and production in oligotrophic tropical estuarine and marine systems (Capone 1988, Howarth et al. 1988a, Paerl et al. 1994). Tropical estuaries and coastal bays are often characterized by high rates of biological production despite low nutrient concentrations in the water column and sediment pore waters (O'Neil and Capone 1989). This apparent contradiction in high rates of uptake and low rates of supply necessitates other sources of bioavailable nitrogen to support primary production in these systems. Rates of benthic nitrogen fixation have been calculated in coral reef (Larkum et al. 1988, O'Neil and Capone 1989), seagrass bed (Patriquin and Knowles 1972, Capone et al. 1979, O'Donohue et al. 1992, Moriarty and O'Donohue 1993), and benthic microalgal (BMA) mat (Capone 1983, Charpy-Roubaud et al. 2001) communities worldwide. Nitrogen fixation has been estimated to account for up to 50% of the total nitrogen requirements in seagrass beds and microbial mat systems (Patriquin and Knowles 1972, Capone et al. 1979, Charpy-Roubaud et al. 2001) and may be a poorly-understood nitrogen input term in many coastal systems worldwide.

Florida Bay is an oligotrophic estuary (Forqurean et al. 1992) that is dominated by seagrass communities that covered approximately 90-95% of the bay's sediments during recent surveys (Zieman et al. 1989, 1999). Over the past 20 years, numerous basins within Florida Bay have experienced seagrass die-back (Zieman et al. 1989, 1999) resulting in patches marked by the presence of well-defined mats consisting of benthic microalgae and associated microbial communities. Similar communities of benthic microalgae and cyanobacteria were termed

microphytobenthos by Macintyre et al. (1996) and Miller et al. (1996). Benthic microalgal (BMA) mats are a regular feature throughout the bay and are found where seagrass is present as well as in areas devoid of macrophytes (Brand 1999). BMA communities have been identified as sources of “new” nitrogen (Sensu, Dugdale and Goering 1967) via nitrogen fixation in shallow, estuarine and marine systems worldwide (Capone 1983, Paerl et al. 1991, 1996; Welsh et al. 1996a, b; Charpy-Roubaud et al. 2001). BMA are present in high abundances throughout Florida Bay and may represent a significant internal source of nitrogen to the benthic communities within the system that has not yet been accounted for in the literature.

Recent attempts to describe nitrogen cycling patterns in Florida Bay (Rudnick et al. 1999) have omitted values of nitrogen fixation and other potentially important internal processes. Internal nitrogen sources and sinks may be especially important in Florida Bay due to geological and anthropogenic factors that combine to isolate waters within Florida Bay from adjacent ecosystems and external nutrient loads. Florida Bay (Fig. 13) is a shallow (< 3 m) partially, enclosed estuary with one major source of freshwater inputs, Taylor Slough (Fig. 14). The bay is partitioned into discrete basins by natural carbonate mud banks that restrict the eastward advection of waters from the Gulf of Mexico into the bay as well as restricting exchange between individual basins (Holmquist et al. 1989, Forqurean and Robblee 1999). Freshwater inputs through Taylor Slough into the head of the bay have also been highly reduced by man-made water management structures that divert freshwater from the Everglades to sea (Forqurean and Robblee 1999). As a result, the impact of external sources of nitrogen on Florida Bay is likely diminished as compared to other

estuarine systems and may lead to an increased importance of internal nitrogen sources (recycling and nitrogen fixation) in supporting high rates of primary production.

Nitrogen fixation is often inhibited by high concentrations of labile nitrogen, generally in the form of ammonium (NH_4^+), due to the high metabolic costs associated with obtaining nitrogen via fixation (Postgate 1982, Capone 1998). Nitrogen fixation may also be limited by the availability of iron and molybdenum. Both of these micronutrients are essential components of the nitrogenase enzyme that reduces dinitrogen and have been identified as possible limiting factors on rates of nitrogen fixation (Capone 1988). Similarly, phosphorus availability may control rates of nitrogen fixation as high phosphorus concentrations may lead to increased nitrogen demand and create a more favorable environment for nitrogen-fixing organisms.

Florida Bay is considered to be a relatively nitrogen-replete system. Vegetative growth in Florida Bay has been shown to be stimulated by the additions of phosphorus and other micronutrients (Forqurean et al. 1992, Koch et al. 2001). However, previous investigations into internal nitrogen cycling processes have suggested very high rates of sediment-associated nitrogen fixation and denitrification in the system (Rudnick et al. 1999, Kemp and Cornwell 2001, Cornwell et al. in prep) suggesting that nitrogen availability is likely an important factor in supporting seagrass and BMA production in the system.

This project sought to quantify rates of benthic nitrogen fixation in Florida Bay and describe factors that may control the process *in situ*. Annual inputs of bioavailable nitrogen via fixation were estimated to assess the relative importance of

nitrogen fixation as compared to other nitrogen input terms as described in previously constructed system-wide budgets. Rates of nitrogen fixation were also measured in experimental manipulations designed to examine possible limitation by the availability of other nutrients and organic substrates.

Methods

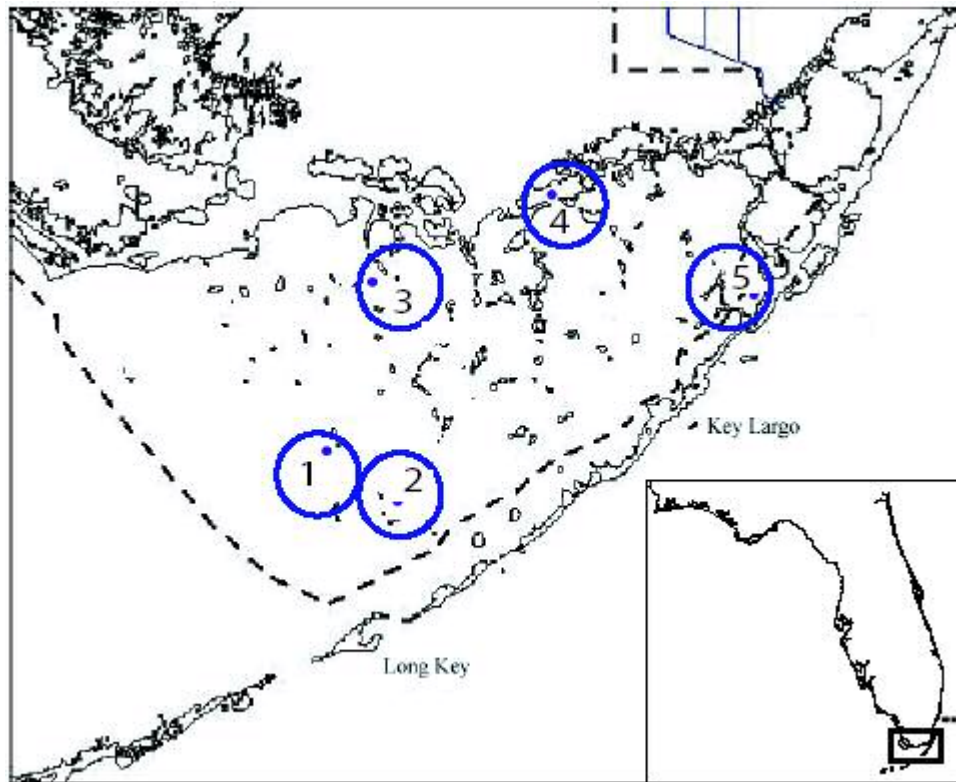
Study site

The study was set in Florida Bay, a subtropical estuary located between the Florida peninsula and the nearly-solid line of islands that make up the Florida Keys (Fig. 13). The system is a partially-enclosed, shallow (average depth ~ 1 m) coastal bay and is characterized by clear water columns and carbonate mud sediments. Extensive populations of benthic vegetation (seagrasses and benthic microalgae) are prevalent throughout the bay and dominate total primary production within the system (Zieman et al. 1989, Forqurean et al. 1992). Tidal influence is minimal as geological formations partially impede landward flow of Gulf of Mexico waters (Holmquist et al. 1989). Inputs of freshwater from terrestrial regions (Florida Everglades through Taylor Slough and C-111 Canal) are also small due to upstream water management structures that divert freshwater from moving into the head of the bay (Forqurean and Robblee 1999). In addition to relatively low rates of exchange with adjacent systems, interior Florida Bay is sectioned by numerous, natural carbonate mud bank formations that reduce water advection within the system (Holmquist et al. 1989, Forqurean and Robblee 1999). These banks divide the bay

into a series of smaller basins that often show distinct differences in salinity and nutrient profiles from adjacent basins. Thus, basins were treated and sampled separately to determine overall patterns of benthic nitrogen fixation throughout the bay.

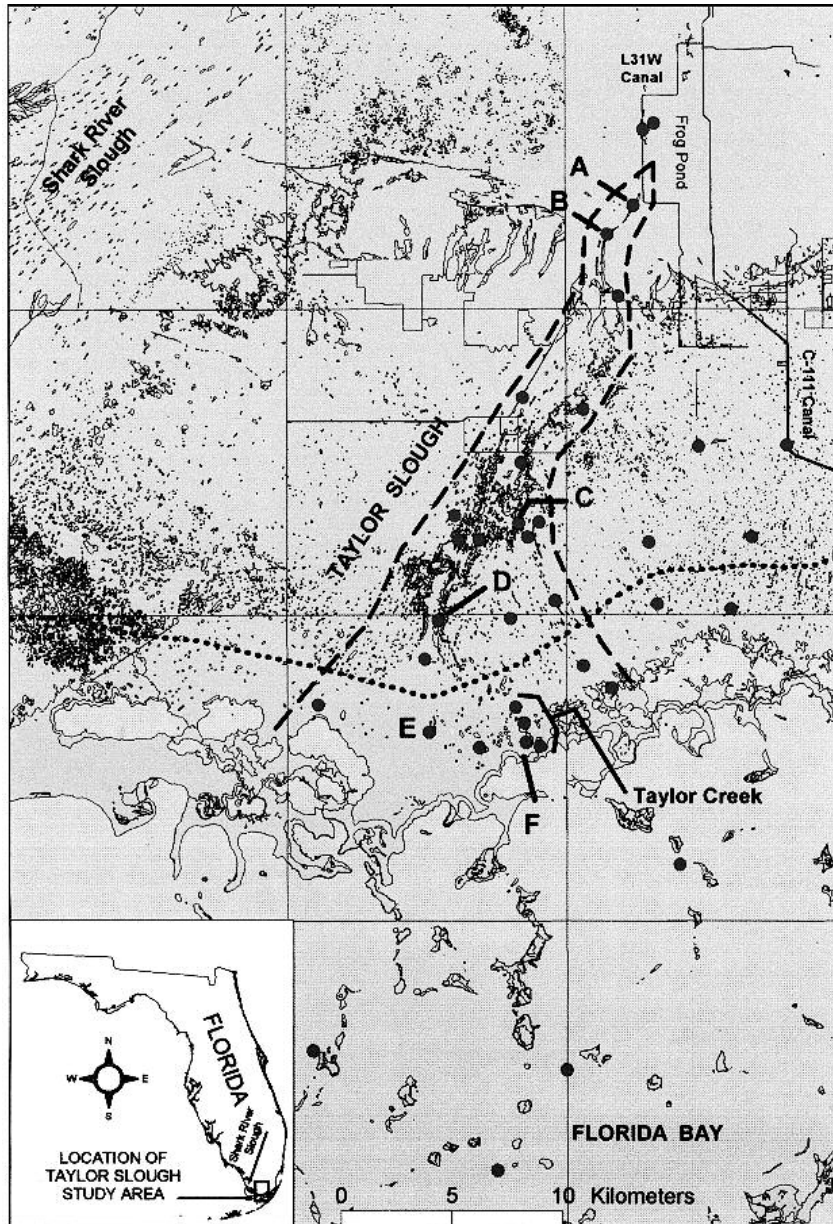
Intact sediment cores were collected in 5 basins located throughout Florida Bay (Table 2). These sites were selected to provide the greatest spatial coverage in discrete zones identified by previous studies (Turney and Perkins 1972, Wanless and Tagett 1989, Zieman et al. 1989, and Boyer et al. 1997) and to investigate nitrogen fixation along an east-west transect within Florida Bay.

Water column concentrations of nitrogen and phosphorus show strong gradients along an east-west transect within the bay (Forqurean et al. 1992). Eastern Florida Bay (characterized by site 4) is highly influenced by the input of nitrogen-replete freshwater from Taylor Slough (Fig. 14) into Little Madeira Bay and surroundings (Boyer et al. 1999). Freshwater in Taylor Slough flows through a dense patchwork of marshes in the southern Florida Everglades where phosphorus is readily removed, leaving waters with high N: P ratios (Rudnick et al. 1999). Western Florida Bay (characterized by sites 1 and 2) experiences much higher phosphorus concentrations due to advective exchange with relatively nutrient-rich Gulf of Mexico waters (Rudnick et al. 1999). Central Florida Bay (characterized by site 3) appears to be intermediate between the nutrient profiles seen in the eastern and western portions of the bay (Boyer et al. 1999).



Modified from Forqurean and Robblee (1999)
Inset graphic courtesy of the Integration and Application Network's,
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Fig.13 – Map of Florida Bay showing the five sample sites where nitrogen fixation was measured. The sampling areas were located to provide rates of nitrogen fixation in a number of discrete zones that have been described in previous studies (Zieman et al. 1989).



Modified from USGS – SOFIA project

http://sflwww.er.usgs.gov/publications/fs/taylor_slough/

Fig. 14 – Map of Taylor Slough in Florida Everglades National Park. The Slough drains a large portion of the Eastern Everglades including some areas that are highly developed and others that are the site of significant agricultural activities. The slough drains into Little Madeira Bay at the head of Florida Bay through a number of shallow creeks. Site 4 was situated in this basin at a short distance from the mouth of Taylor Creek, the largest of the five major creeks that drain into the bay. Taylor Creek is noted on the map.

An additional site (site 5) was investigated to ascertain possible impacts of anthropogenic nutrient inputs on rates of nitrogen fixation as compared to rates observed along the main east-west transect. The sampling station at site 5 was located near a heavily developed residential area on the island of Key Largo. As a result, nutrient profiles in Sunset Cove may be more affected by terrestrial inputs (runoff and sewage inputs) than the other 4 sites located further from land.

All sites were shallow in depth (maximum depth ~ 2 m) and were generally covered with well-developed seagrass beds dominated by *Thalassia testudinum*. Interspersed within seagrass meadows were smaller patches (1-15 m diameter) that were marked by the complete absence of macrophytes. These patches, termed “benthic microalgal-dominated” in this project, were marked by the presence of a well-developed mat consisting of numerous classes of benthic microalgae (BMA) and associated microorganisms. Benthic microalgal communities were found throughout the bay in both seagrass- dominated (seagrass present) and BMA-dominated (seagrass absent) areas at the sediment-water interface (Brand and Suzuki 1999).

Sampling

Whole cores (15 cm height, 3.75 cm inner diameter) and bulk sediment for slurry formation were sampled manually by SCUBA and transported to the laboratory for incubation. Experiments were initiated with sediment samples no more than 12 h after collection. Replicate samples (n = 3) were taken at each site. Samples were taken from both seagrass-dominated and BMA-dominated zones to ascertain the effect of dominant vegetation type on BMA-mediated nitrogen fixation. However,

due to the possibility of seagrass interactions affecting observed BMA-mediated nitrogen cycling processes, seagrass-dominated sediment cores were collected between plants so as to not include any aboveground macrophyte biomass.

Intact cores consisted of an approximately 7.5 cm height of sediment overlain by a short water phase and 29 mL gas headspace (Fig. 15). Cores were sealed with a gas-tight clear acrylic plastic lid and bottom stopper. A short magnetic stir bar was suspended from the lid of the acrylic core and gently stirred the water phase to increase diffusion without physically disturbing the surficial BMA layer. A rubber septum was placed into the plastic lid to provide an injection port through which the gas headspace could be amended and sampled.

Sediment slurries were created in glass serum vials (72 and 145 mL total volume) by adding approximately 10 mL of site water and a 5 cm height, 1.6 cm diameter sediment column (~ 40 mL of wet sediment) that was collected in a cut-off plastic syringe. The sediment/water mixture was sealed in a gas-tight serum vial with a rubber septum held in place by a crimped aluminum seal (22 mL headspace) or Erlenmeyer flask capped with a gas-tight rubber stopper (90 mL headspace).

Acetylene Reduction Assay

Intact sediment cores and slurried sediment mixtures were analyzed using the acetylene reduction assay (Stewart et al. 1967, Hardy et al. 1968 as modified by Bebout et al. 1987). Acetylene was generated by reacting calcium carbide (CaC_2) with deionized water. Assays were initiated by adding a volume of acetylene equal to 10% of the total headspace to cores and slurries after removal of the same volume of

air from the headspace. Cores were amended with acetylene during the dark period and allowed to incubate before the first sample was taken just prior to the beginning of the photoperiod (sunrise) or shortly thereafter. The headspace was sampled by removing a 0.5 mL air volume via a gas-tight syringe (Hamilton Co., USA) approximately 7-8 times per 24-hour cycle. Air samples were then injected into pre-evacuated Exetainer (Labco Ltd. UK) vials and diluted with air to a final volume of 2 mL. Samples were diluted so as to minimize the amount of gas that was removed from the headspace from each experiment during each sampling period. Dilution effects were tested using a known concentration of ethylene gas and diluted samples were consistently found to have one quarter of the signal strength from undiluted samples. Blanks using air were also analyzed and no ethylene signal was observed, suggesting that the dilution had no effect on the ethylene concentration that was measured.

Gas samples were then transported to Horn Point Laboratory, Cambridge, MD, USA for analysis by gas chromatography. A Shimadzu GC-8A instrument (flame ionization detector and HayeSep A column) was used to determine ethylene levels as calibrated by the use of a known ethylene gas mixture. Ultra-pure carrier (UPC) Helium was used as the carrier gas.

Volumes of ethylene in the water phase, as calculated from recorded water temperatures and ethylene solubility coefficients in sea water (Larkum et al. 1988), were added to measured volumes of ethylene in the headspace to obtain total ethylene volumes for each core/slurry. Due to the low solubility of ethylene in aqueous solution these volumes were quite low as compared to measurements of ethylene in

the headspace. However, estimates of ethylene in solution were added to gaseous-phase measurements in order to obtain a more complete measurement of total ethylene production. Total ethylene volumes were converted to total volumes of fixed nitrogen by applying the theoretical 3:1 ratio (moles acetylene reduced: moles dinitrogen fixed) assumed by the acetylene reduction assay. Total micromoles of nitrogen were calculated on an areal basis (using surface area of the BMA mat) and plotted against incubation time. The linear regression was then reported as the rate of nitrogen fixation for each core/slurry.

Blank cores were employed to account for the possible production of ethylene that could not be attributed to nitrogenase activity. Blanks consisted of water phases with and without injections of acetylene and sediment cores/slurries without acetylene amendments. No production of ethylene was observed under any of the blank treatments leading to the conclusion that all ethylene present in cores and slurries was the result of the reduction of acetylene by the nitrogenase enzyme.

Inhibitors

Whole cores were treated in several experiments with aqueous solutions of DCMU (3-(3,4-dichlorophenyl)-1,1-dimethyl urea) or sodium molybdate (Na_2MoO_4) to investigate the effects of inhibiting photosynthesis (Paerl et al. 1991) and sulfate-reduction (Oremland and Capone 1988, Welsh et al, 1996a,b), respectively, on rates of sediment nitrogen fixation. Inhibitor solutions were added to the water phase of individual whole cores to bring final concentrations to 20 μM DCMU and 10 mM molybdate (Pinckney and Paerl 1997, Welsh et al, 1996, Steppe and Paerl 2002).

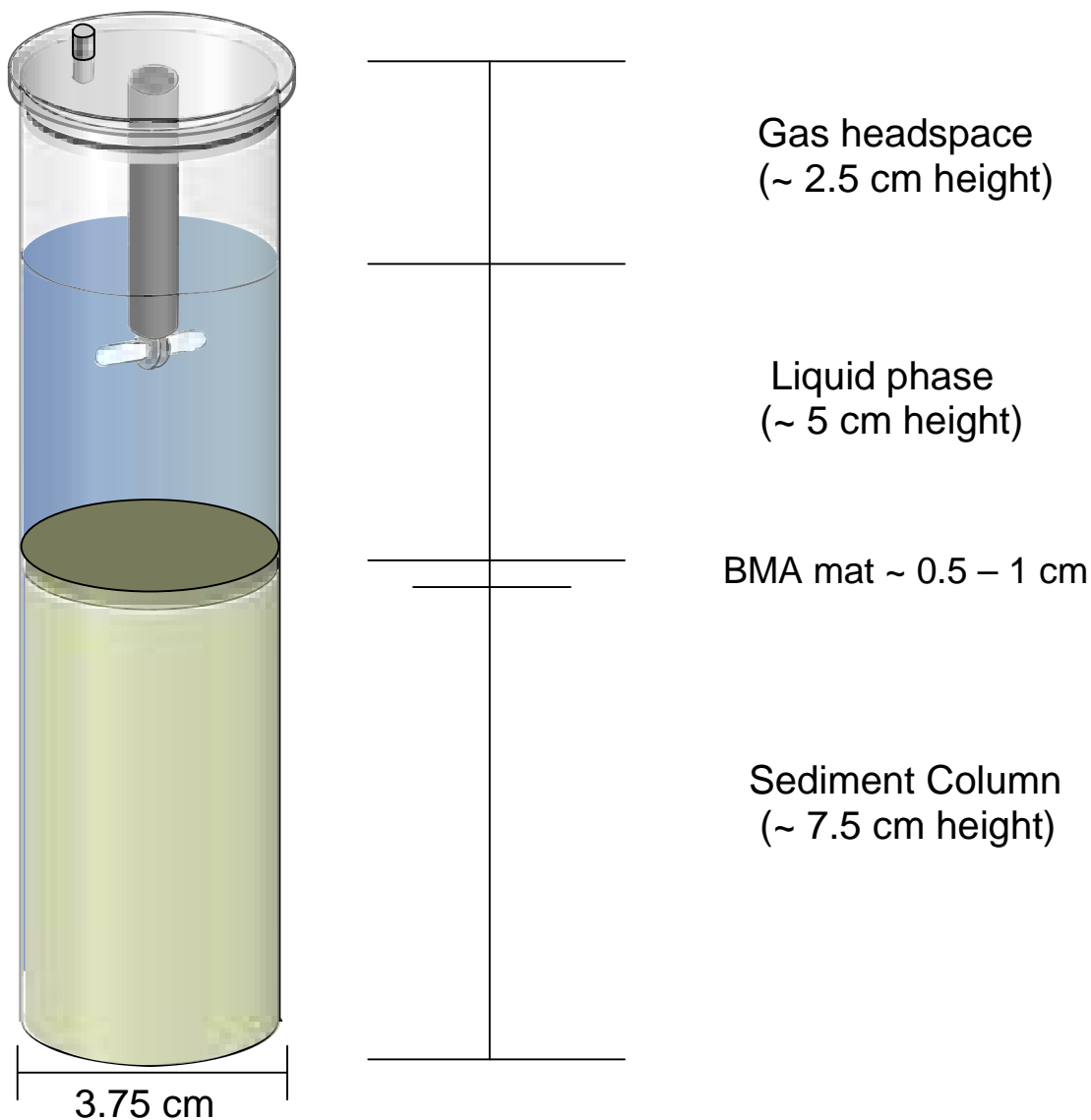


Image courtesy of the Integration and Application Network,
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Figure 15 – Diagram of whole cores showing the air headspace, liquid phase, and sediment column. The benthic microalgal mat was found at the surface of the sediment column and was generally 0.5 – 1 cm thick. A magnetic stir bar turned the liquid phase to create flow and more complete diffusion in the liquid phase and into and out of the sediment column. Gas samples were obtained through a rubber septum set into the top of the core.

Nutrient Amendments

Select cores were amended with solutions of inorganic nutrients to determine possible controls on nitrogen fixation. Triplicate cores were treated with additions of ammonium [NH_4Cl] (100 μM) to the liquid phase to investigate possible inhibition of nitrogen fixation under N-replete conditions. Inorganic phosphorus [NaH_2PO_4] (25 μM), iron [FeCl_3] (20 nM & 10 μM), EDTA [$\text{C}_{10}\text{H}_{14}\text{N}_2\text{Na}_2\text{O}_8$] (20 nM & 10 μM), and acetate [CH_3COONa] (100 μM) were added to the water phases within experimental cores to explore possible stimulation of nitrogen fixation under conditions of high micronutrient or high organic carbon availability. Combinations of the aforementioned nutrients (P + Fe 25 μM /20 nM; P + Fe + EDTA 25 μM /20 nM/20nM; and Fe + EDTA 20 nM/20 nM) were also added to assess possible co-stimulatory effects.

Nutrients were added to cores in aqueous solutions and placed directly into the water phase above the sediments and BMA layer. Nutrient solutions were added approximately 30-60 minutes before initiation of the experiment (injection of acetylene) to allow for more complete mixing and diffusion within the water phase. Nutrient amendment cores were incubated simultaneously with non-amended cores and nitrogen fixation rates were determined by ARA in both sets of cores.

Sediment Chlorophyll Measurements

Sediment chlorophyll- α (chl- α) was measured in each whole core assay using HPLC methods (Sun et al. 1991). Values of chl- α were used as proxy measurements of total BMA biomass in each core. A 1 cm depth column of sediment was removed

in a cut-off 10 mL plastic syringe (1.4 cm inner diameter) wrapped in aluminum foil and frozen immediately. Samples were thawed within 1-2 months for chl- α analysis. Sediment samples were amended with 9 mL of 100% acetone to create 10 mL total slurries with 90% acetone as the extracting solvent. Samples were vigorously shaken and placed in a freezer overnight to allow for the extraction of photopigments into the acetone solution. Samples were shaken again and then centrifuged for 10 min at 3000 rpm to separate the chlorophyll-containing acetone solvent from sediment and MPN solids. The liquid phase was then passed through a 25 μ m Teflon-cased filter (0.45 μ m pore size) and analyzed by high performance liquid chromatography (Van Heukelem et al. 1994). A subsample of extracts was analyzed for all photopigments in order to describe significant components of the BMA community.

Other Measurements

Ammonium concentrations in the water phase and sediment pore waters were analyzed colorimetrically (Parsons et al. 1984). Levels of incident light were measured using a Li-Cor data logger that was placed in the vicinity of the incubating samples. All statistical analyses were computed using SAS version 8.0 (SAS Institutes, Cary, NC).

Results

Magnitudes, Seasonal and Spatial Patterns of Nitrogen Fixation

Benthic microalgal communities consisted of a loosely-connected assemblage of living organisms and detrital material that covered the surface of the sediments in each of the five sampled basins. BMA mats in Florida Bay were dominated by benthic diatoms and filamentous cyanobacteria (Fig. 16). Purple sulfur bacteria were also found within the topmost centimeter suggesting that there were hypoxic, sulfide rich microzones present in parts of the mat. Visual observations of the BMA assemblage showed high concentrations of diatoms species with lesser numbers of cyanobacteria. These observations were corroborated by HPLC analyses of BMA mat samples; however no attempt was made to calculate the relative population size of benthic microalgal classes during each season or between each season.

Nitrogen fixation was investigated at depths up to 10 cm within the sediment column via the formation of sediment slurries. Slurries composed of surface sediments (with BMA mats) showed much higher rates of nitrogen fixation than those seen in slurries made with all other segments of the sediment column (results not shown) from both seagrass-dominated and BMA-dominated sites.

Rates of benthic microalgal-mediated nitrogen fixation ranged from 0.5-20 $\mu\text{mol N m}^{-2} \text{ h}^{-1}$ with the vast majority of readings ranging from 1-6 $\mu\text{mol N m}^{-2} \text{ h}^{-1}$ (Fig. 17), (Table 4). Nitrogen was fixed primarily during the photoperiod with lower rates of fixation observed during the overnight hours in all seasons except for late summer (August) when “dark” fixation became a more significant component of

overall fixation. Accelerations in the rate of nitrogen fixation were often seen shortly after sunrise and continued at a high rate for a number of hours. The impact of these short-term variations on the linear rate observed throughout the time course, however, was generally muted in multiple-day experiments. This suggests that longer incubations may give more accurate estimates of nitrogen fixation rates as opposed to short-term “snapshots” of rates that may or may not persist past a period of a few hours.

An annual profile at site 5 revealed significant seasonal differences in rates of nitrogen fixation. Nitrogen was fixed at significantly higher rates ($p < 0.05$) in August than any other season at that site (Fig. 18). Total BMA biomass as measured by sediment chlorophyll was also observed to be highest in August as compared to other seasons.

Significant ($p < 0.05$) variations in rates of nitrogen fixation were found between the 5 sampled basins within Florida Bay (Fig. 19). Magnitudes of nitrogen fixation decreased along a transect from sites 1 and 2 to sites 5 (from the mouth to the head of the bay; roughly from west to east). Ammonium (NH_4^+) concentrations in the top 1 cm of the sediment column, likewise, decreased along a west-east transect (Fig. 20) while water column ammonium concentrations showed the opposite trend; an increase from west to east (Fig. 21).



Figure 16 – Photograph of the benthic microalgal assemblage as observed under microscopy. BMA mats were dominated by benthic diatom species with sizeable populations of cyanobacteria also being present.

Factors Controlling Nitrogen Fixation

Higher rates of nitrogen fixation were observed in BMA-dominated cores than in those taken from seagrass-dominated sediments likely reflecting the effect of BMA biomass on magnitude of fixation. Benthic microalgae biomass (as determined by chlorophyll- α) was significantly higher in BMA-dominated sediments as compared to sediments covered by seagrass beds (seagrass-dominated). Rates of fixation were positively correlated with BMA biomass for sites 1, 2, 3, and 5 in January, June and August 2003 (Fig. 22). Rates of nitrogen fixation at site 4 were very low (0.46 ± 0.05 micromoles $\text{N m}^{-2} \text{ h}^{-1}$) in all incubations despite slight differences in chlorophyll and, therefore, were not included in the dataset.

Nitrogen fixation rates and total sediment chlorophyll were positively correlated for all sampling periods. However, the data appeared to fall into two distinct groupings with nitrogen fixation to BMA biomass ratios in June being more than three times greater than those observed during the rest of the year (in November, June and August) (Fig. 22). This observed shift in the relationship between BMA biomass and BMA-mediated nitrogen fixation may reflect a change in the composition of the microbial community toward the late spring or, perhaps, an increased nitrogen demand resulting from high rates of vegetative growth.

In select experiments, inhibitors were added to assess the extent of total BMA nitrogen fixation that could be attributed to photoautotrophic and sulfate-reducing communities within the BMA assemblage. Additions of DCMU, an inhibitor of photosystem II, and sodium molybdate, an inhibitor of sulfate reduction, to BMA-dominated cores each resulted in significant declines in total nitrogen fixation

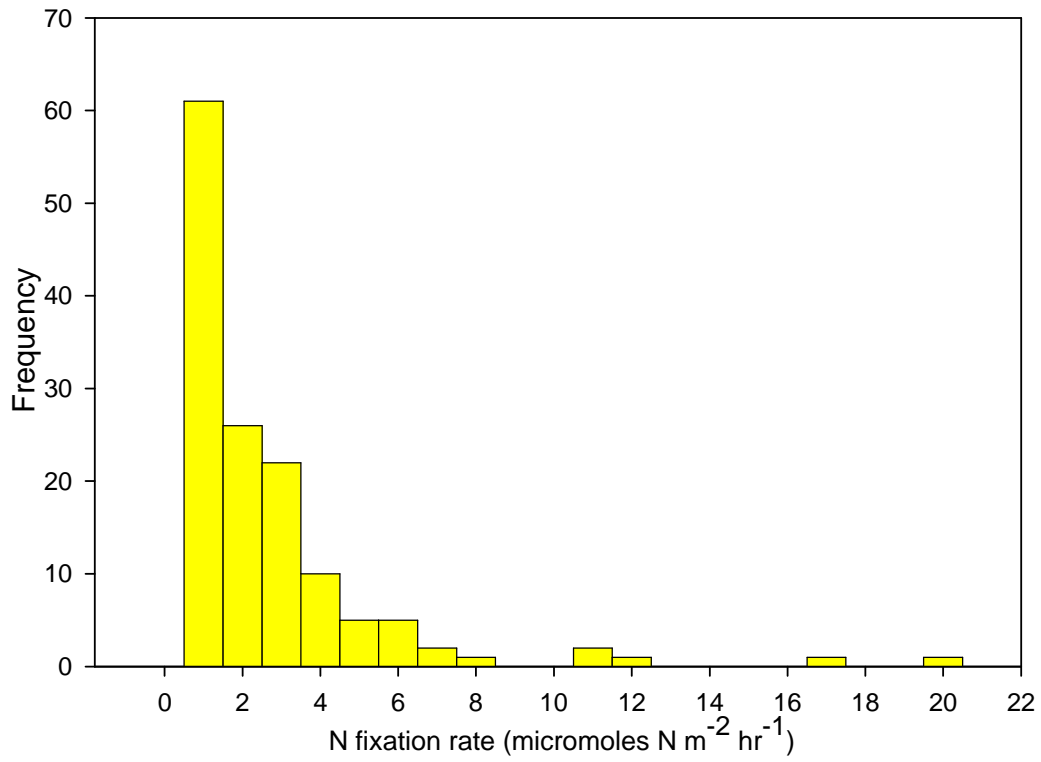


Figure 17 – Histogram displaying the frequency of measured rates of nitrogen fixation in whole core experiments (as rounded to the nearest whole number). The great majority of rates were measured between 1-5 micromoles N m⁻² hr⁻¹.

Basin	Season	N fix rate (micromoles N m ⁻² hr ⁻¹)
Site 1	June 2003	1.03 ± 0.24
	August 2003	0.99 ± 0.09
Site 2	June 2003	7.60 ± 2.23
	August 2003	2.60 ± 0.61
Site 3	January 2003	3.35 ± 0.85
	June 2003	3.87 ± 1.20
Site 4	June 2003	0.46 ± 0.05
Site 5	November 2002	0.80 ± 0.03
	January 2003	2.64 ± 0.73
	June 2003	1.75 ± 0.52
	August 2003	6.08 ± 2.48

Table 4. Rates of nitrogen fixation were measured in 5 sites located throughout Florida Bay. Rates of nitrogen fixation are reported for each season that a particular site was sampled. The rates reported in this table represent an average of replicate unamended whole cores plus or minus one standard error.

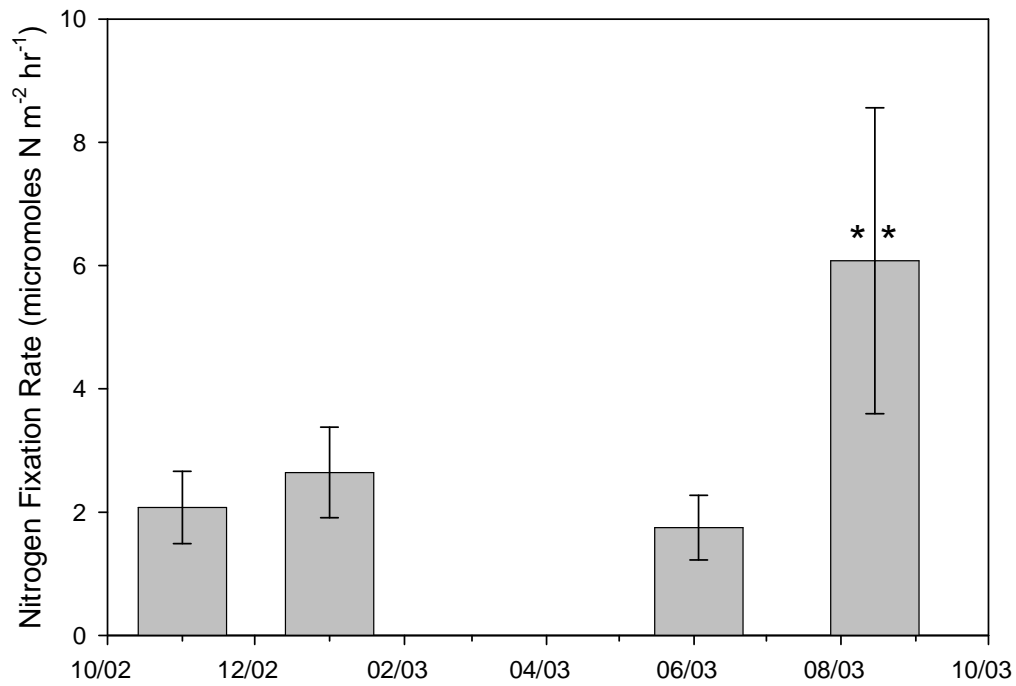


Figure 18 – An annual profile of nitrogen fixation rate as measured in whole cores taken from regions that were BMA-dominated at site 5. Rates of fixation were generally variable between seasons; however rates measured in August 2003 were significantly higher ($p < 0.05$) than rates measured in the same site during other seasons.

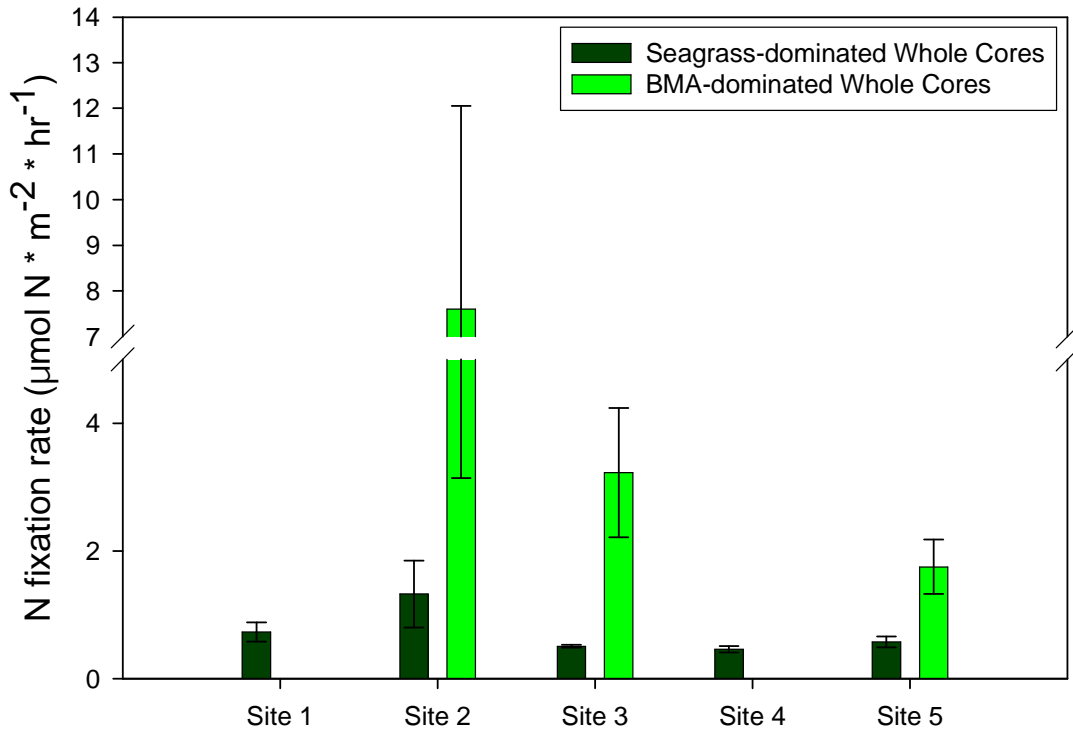


Figure 19 – Comparison of nitrogen fixation rates measured in whole cores extracted from seagrass-dominated areas (seagrass) and BMA-dominated areas (BMA) in five basins of Florida Bay in June 2003. Higher rates of nitrogen fixation were consistently observed in assays of BMA-dominated sediments as compared to seagrass-dominated sediments. A trend of decreasing rates of nitrogen fixation along a west to east transect was also observed. Sites are listed from west to east in this and the following figures.

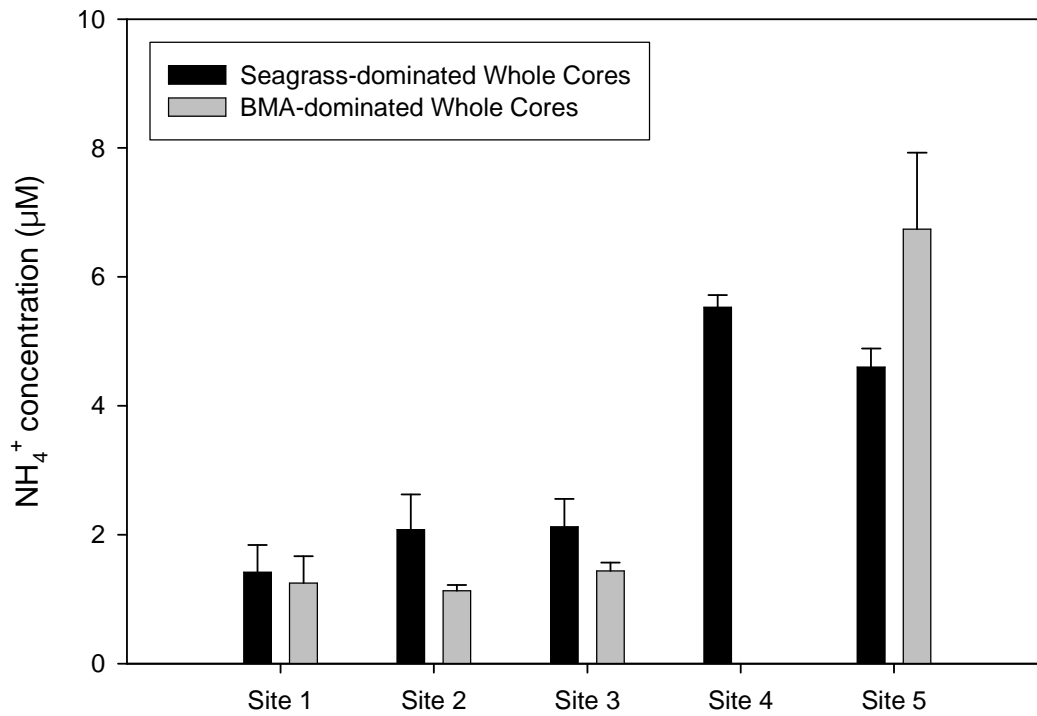


Figure 20: Ammonium concentrations were measured in the water column above seagrass-dominated areas (seagrass) and BMA-dominated areas (BMA) at 5 sites throughout Florida Bay in June 2003. Ammonium concentrations increased along a transect from west to east.

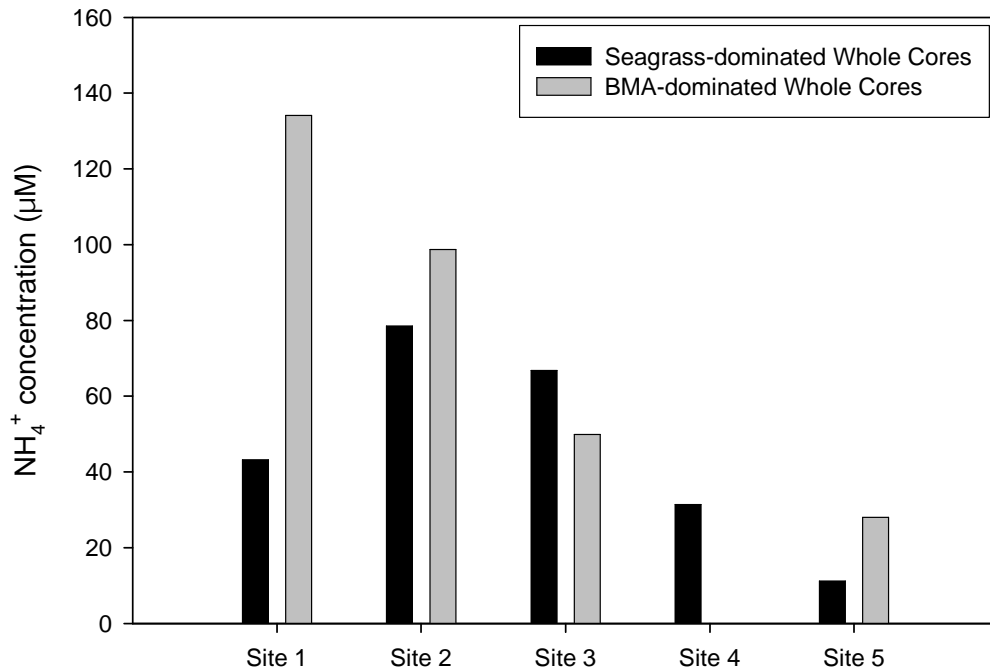


Figure 21. Ammonium concentrations were measured in the top 0.5 cm of the sediments at five sites within Florida Bay in June 2003 to determine the size of the ammonium pool within the BMA assemblage. BMA communities were consistently found within the top 1 cm of both seagrass and BMA-dominated sediments. Ammonium concentrations in the sediments decreased along a transect from west to east. This trend is negatively correlated with the trend observed within the water column.

as compared to non-amended BMA-dominated cores (Fig. 23). Total BMA biomass was also lower in DCMU- and molybdate-treated cores following a 36-hour incubation period. Total sediment chlorophyll- α and rates of nitrogen fixation were positively correlated by linear regression in seagrass-dominated, BMA-dominated, and DCMU-treated cores (Fig. 24). However, molybdate-amended cores displayed less than half the rate of nitrogen fixation expected by the linear regression based on measurements of total sediment chlorophyll- α .

Availability of ammonium and other nutrients were examined as a possible limiting control on BMA-mediated nitrogen fixation via nutrient amendment experiments. Aqueous solutions of 100 μ M ammonium (\sim 20-50 times greater than natural levels) were added to increase water phase NH_4^+ concentrations in intact core assays. Nitrogen amendments, however, did not elicit an inhibitory effect on the process of nitrogen fixation despite the high energy demands associated with fixation relative to ammonium uptake. Rates of nitrogen fixation in ammonium-amended cores were not significantly different than those calculated from cores incubated under natural conditions in all 5 basins (Fig. 25). Separate additions of iron and EDTA resulted in significant ($p < 0.05$) increases in rates of nitrogen fixation in only one basin (Sunset Cove) while acetate (DOC) and Fe/EDTA amendments did not result in increased rates of nitrogen fixation in any of the five basins (Fig. 26). Phosphorus amended cores, however, showed significantly higher nitrogen fixation rates (Fig. 27) than non-amended cores in the three western-most basins (sites 1, 2, and 3) suggesting phosphorus limiting conditions in these areas of Florida Bay.

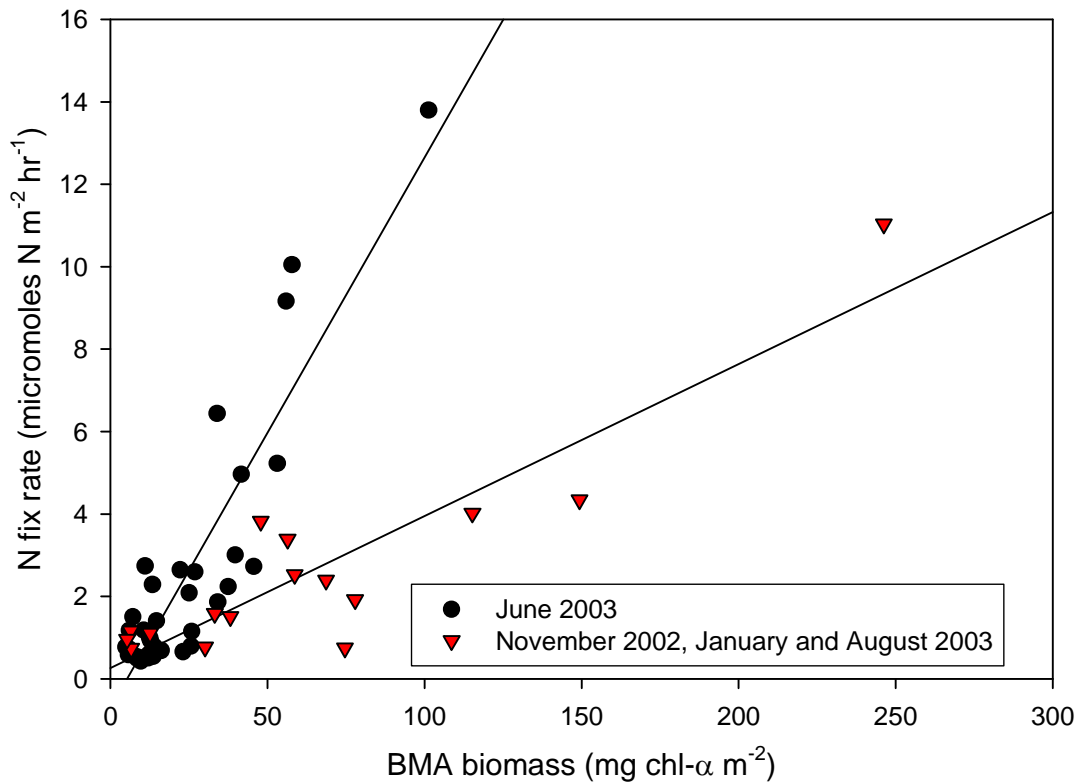


Figure 22 – Linear regression displaying a significant ($p < 0.05$), positive relationship between measured rates of nitrogen fixation and measurements of sediment chlorophyll- α (termed BMA biomass) for whole cores measures in basins of Florida Bay. Measurements made in June 2003 (filled circles) appear to have a slightly different relationship ($y = 0.13x - 0.71$; $r^2 = 0.81$) than the relationship observed (inverted triangles) during all other sampling periods ($y = 0.04x + 0.26$; $r^2 = 0.82$).

Discussion

Acetylene reduction was observed in every sample that was analyzed over a period from June 2002-August 2003, suggesting that nitrogen fixation is a common feature in the benthic environments of Florida Bay. Nitrogen fixation was surveyed within the sediment column up to a depth of 10 cm in order to determine the location of fixation on a vertical scale as well as the microbial communities that might be responsible for this process. The highest rates of nitrogen fixation were consistently observed within the 0-1 cm section. This fraction of the sediment column was characterized by the existence of a flocculent microbial mat consisting of benthic microalgae and associated microorganisms.

Estimates of benthic microalgal-mediated nitrogen fixation in Florida Bay were similar to results found in temperate and tropical coastal systems worldwide (Carpenter et al. 1978, Capone 1983, Stal et al. 1984, Paerl et al. 1996, Charpy-Roubaud et al. 2001). Higher rates of nitrogen fixation were observed in whole cores taken from BMA-dominated areas as opposed to those taken from sediments colonized by seagrasses. This is in contrast to the results of many studies that found much higher rates of nitrogen fixation in the rhizosphere of seagrass-dominated samples than those found in “uncolonized” sediments (Patriquin and Knowles 1972, O’Donohue et al. 1991, Moriarty and O’Donohue 1993, Welsh et al. 1996a, b, McGlathery et al. 1998). Preliminary attempts were made to measure rates of nitrogen fixation during this study using slurries composed of non-rinsed *Thalassia testudinum* roots and rhizomes as well as bulk sediment samples from the

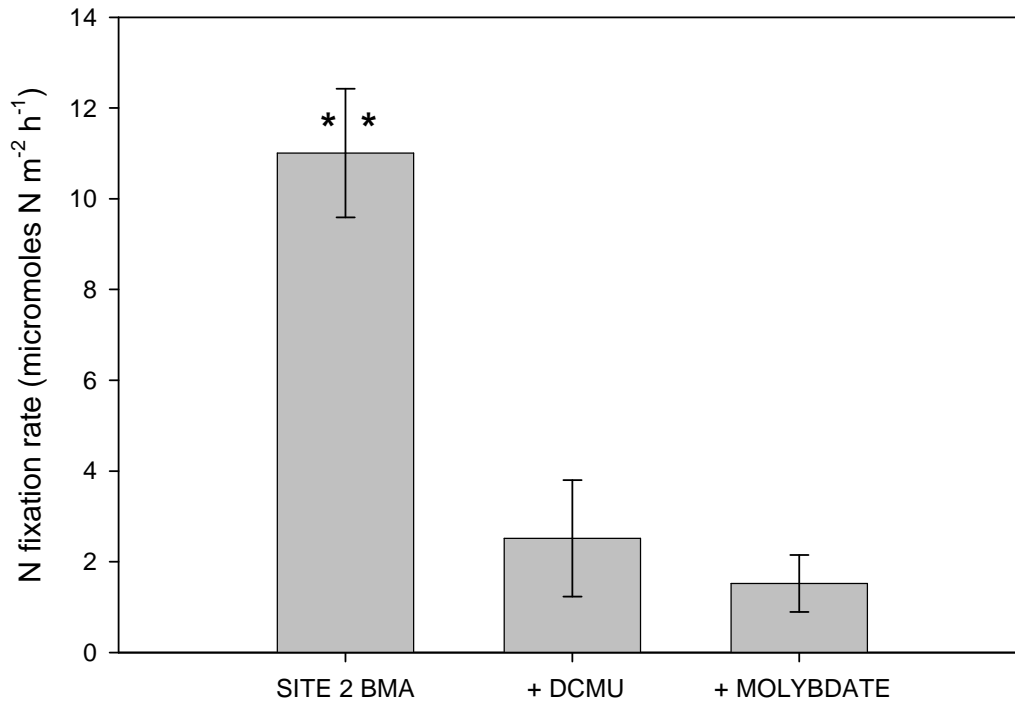


Figure 23 – Comparison of benthic nitrogen fixation rates measured in assays of unamended whole cores with whole cores amended with DCMU and sodium molybdate, inhibitors of photosynthesis II and sulfate reduction, respectively. Rates measured in unamended cores were significantly greater than rates measured in both DCMU and sodium molybdate-amended cores. All cores were sampled from a BMA-dominated region at Site 2 in June 2003.

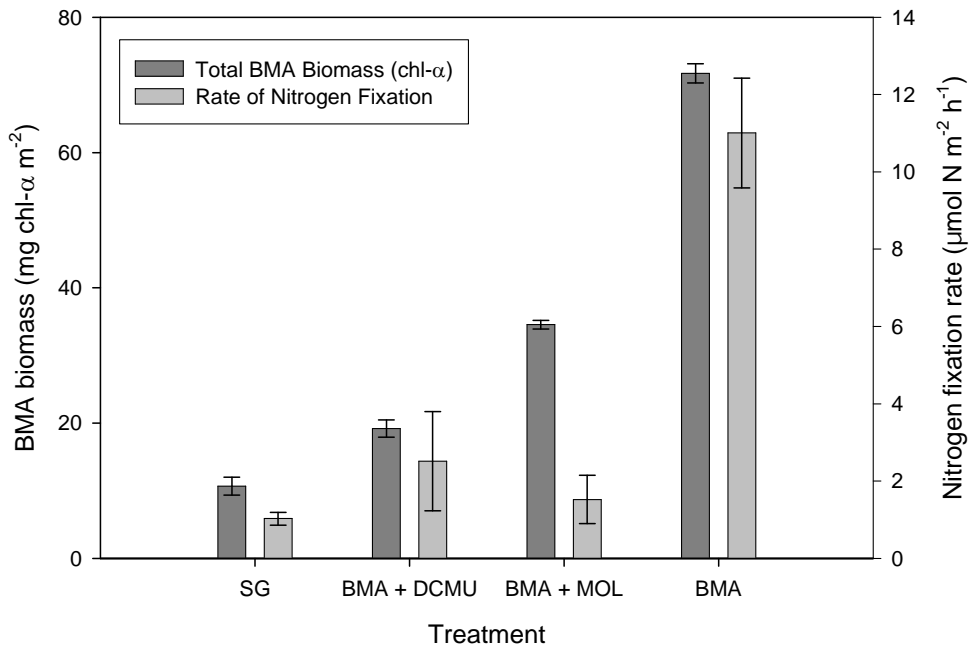
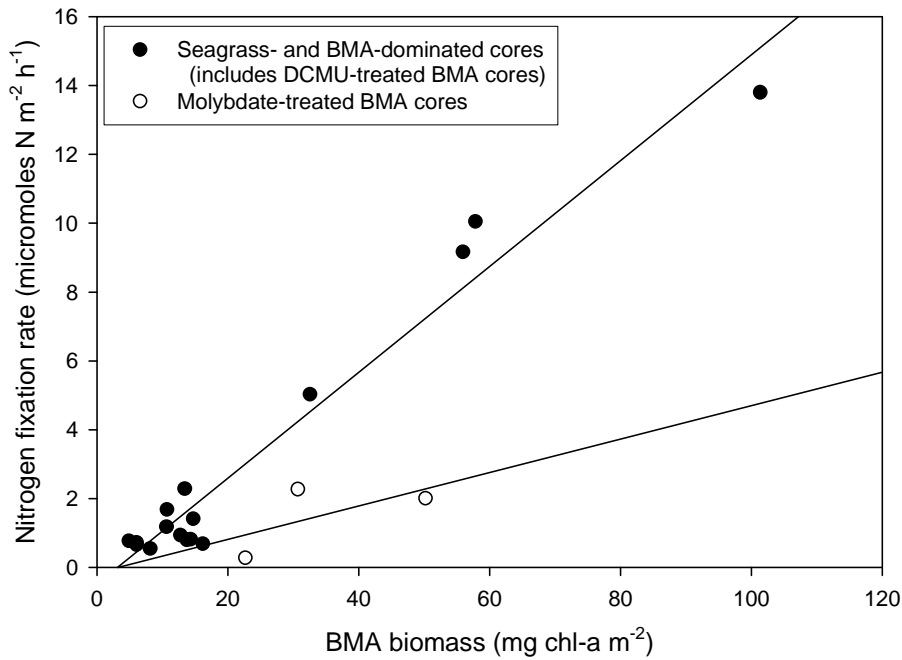


Figure 24 — Linear regression (top) and bar graph (bottom) showing a significant ($p < 0.05$), positive relationship ($y = 0.15x - 0.48$; $r^2 = 0.96$) between measurements of nitrogen fixation rates and measurements of sediment chlorophyll in whole core assays obtained from both seagrass-dominated (SG) and benthic microalgae-dominated (BMA) regions from numerous basins in Florida Bay. Cores treated with sodium molybdate (open circles), a specific inhibitor of sulfate reduction, fall outside of this relationship while cores treated with DCMU do not.

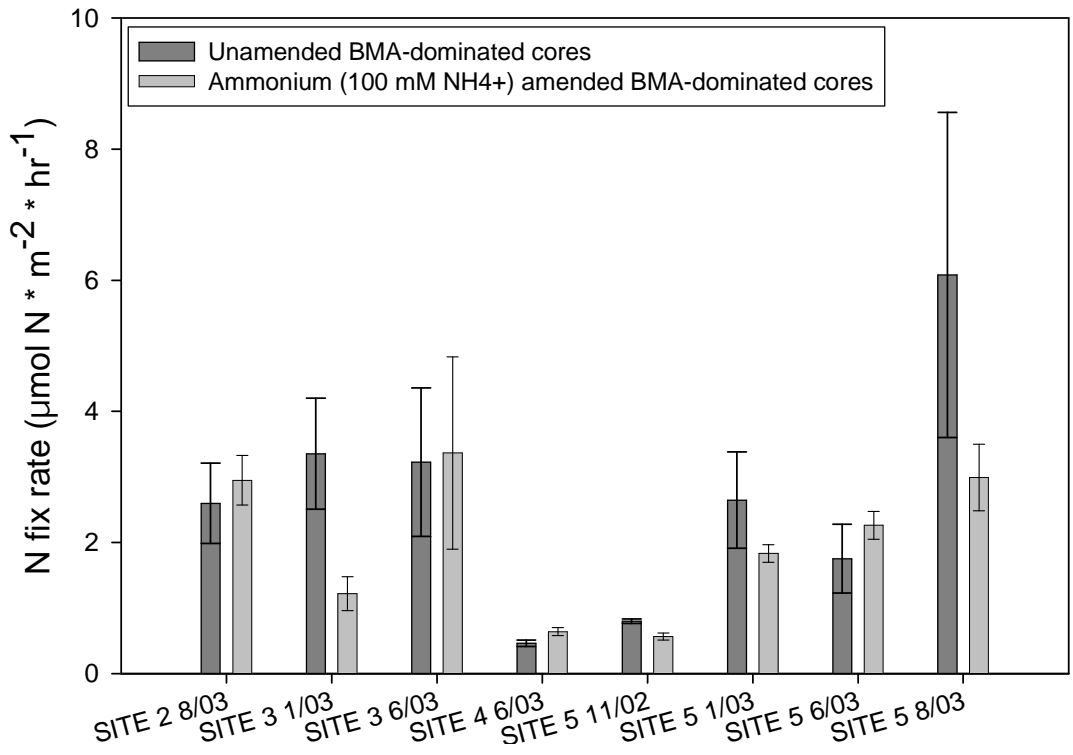


Figure 25 – Comparison of measured rates of nitrogen fixation in unamended BMA-dominated whole cores with rates measured in ammonium-amended BMA-dominated whole cores in numerous basins of Florida Bay over several seasons. Ammonium amendments did not result in significantly diminished rates of nitrogen fixation. This graph displays the average rate of nitrogen fixation measured in replicate cores with one standard error.

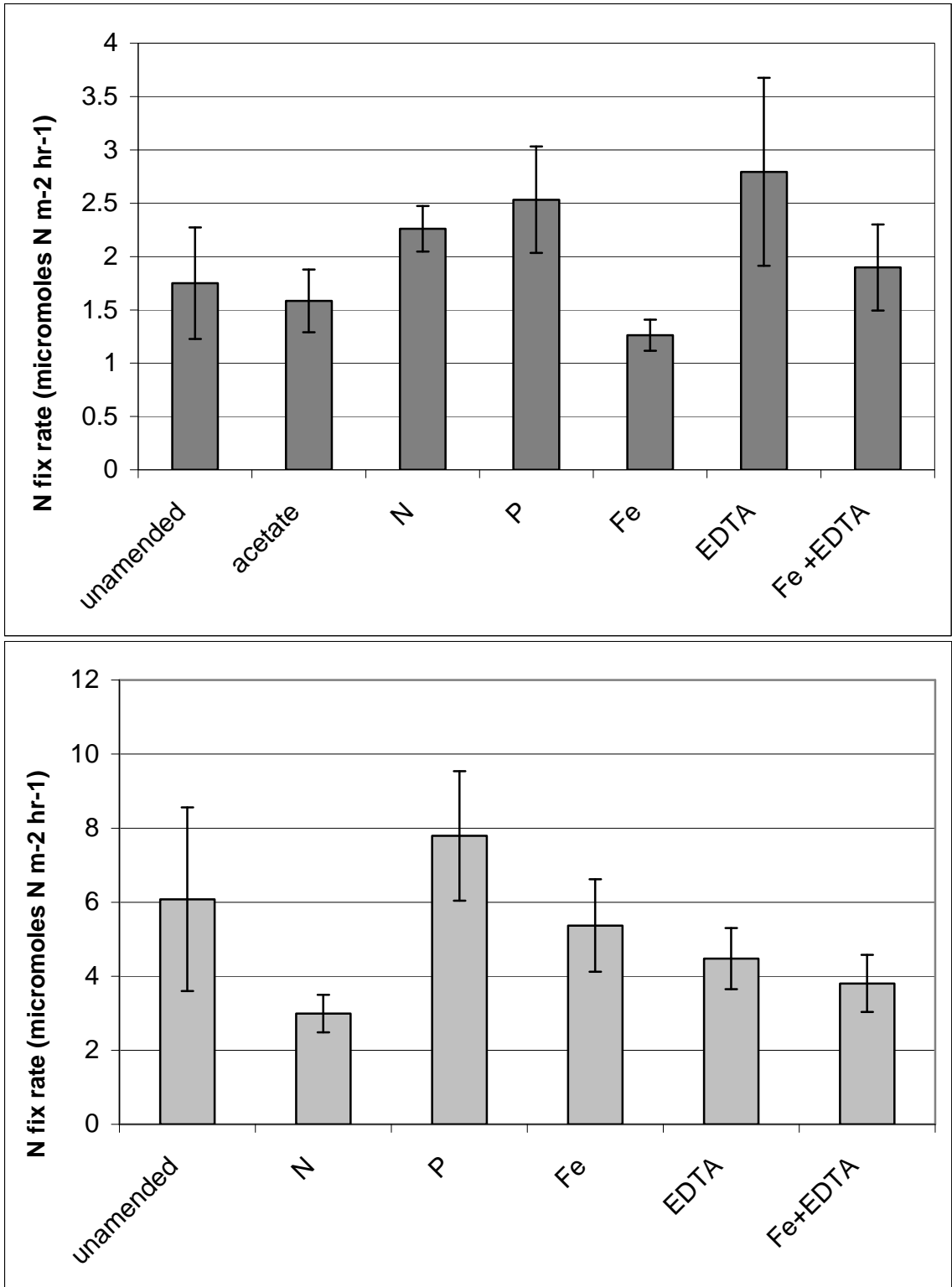


Figure 26 – Measurements of nitrogen fixation rates in unamended BMA-dominated whole cores with assays amended by the addition of ammonium (N), phosphorus (P), Iron (Fe), EDTA and Iron/EDTA (Fe + EDTA) at Site 5 in June 2003 (top) and August 2003 (bottom).

rhizosphere. Acetylene reduction was observed in these slurries suggesting that the rhizosphere likely is a significant source of nitrogen to the benthic communities of Florida Bay. These rates were, however, lower than those measured in slurries that incorporated samples of the BMA mat.

This observation of low rates of nitrogen fixation in the rhizosphere samples may have resulted from the separation of the bacterial community from seagrass roots and rhizomes. The sampling process excluded macrophyte biomass and physically removed heterotrophic bacteria from plant material as well as from the source of labile organic carbon necessary to fuel nitrogen fixation (O'Donohue et al. 1991, Moriarty and O'Donohue 1993). To fully describe nitrogen fixation within the rhizosphere, it would have been necessary to collect and incubate sediment samples together with intact *Thalassia* plants. However, this was not possible with the incubation cores utilized in this study and therefore, all reported values of nitrogen fixation in this study refer to the process within the benthic microalgal community alone.

BMA-mediated nitrogen fixation likely accounts for nearly all of the nitrogen fixed in areas of Florida Bay marked by the presence of BMA-dominated patches. These sediments were devoid of seagrasses and would not likely be the site of high

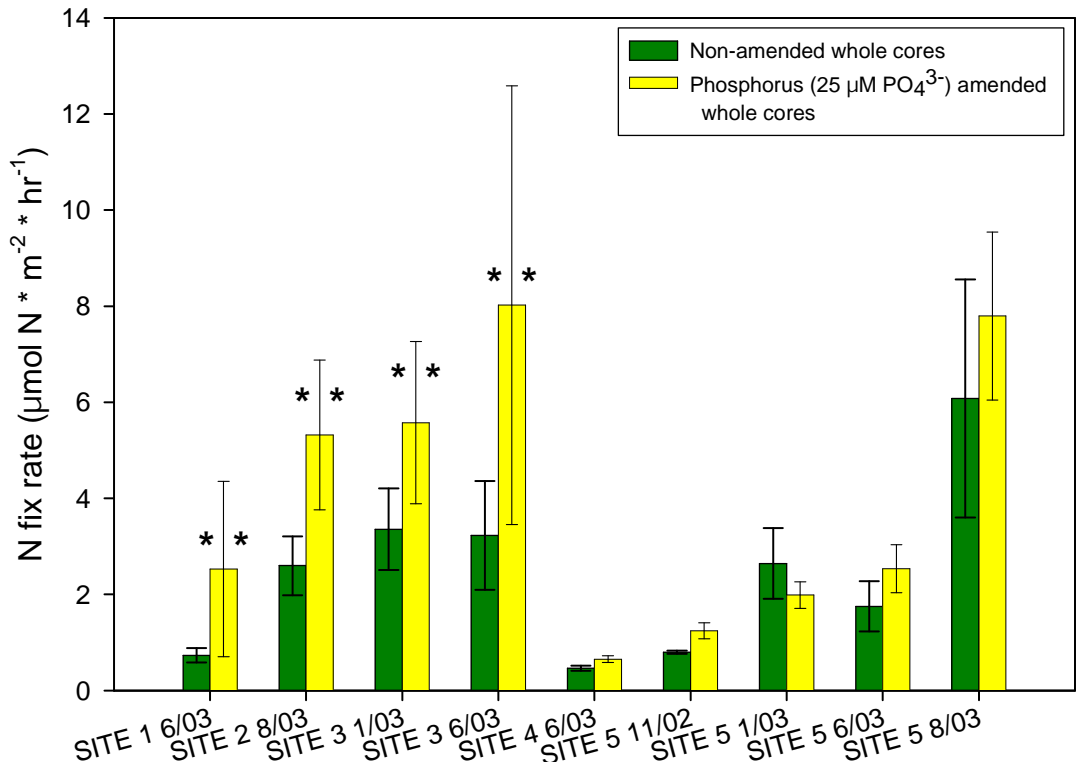


Figure 27 – Comparison of measured rates of nitrogen fixation in whole cores with rates measured from phosphorus-amended assays from numerous basins over several seasons. Phosphorus amendments were observed to significantly ($p < 0.05$) stimulate nitrogen fixation as compared to unamended assays in the western and central basins of Florida Bay (sites 1, 2, and 3) [signified by double asterisk]. The values shown in the figure represent the average of replicate cores with one standard error.

rhizosphere-associated nitrogen fixation due to the absence of living roots and rhizomes. While measurements of BMA-mediated nitrogen fixation likely underestimate, to some degree, total fixation in seagrass-dominated zones, measurements of fixation within the BMA community showed significant production of bioavailable nitrogen in samples taken from both seagrass-dominated areas (within seagrass beds) and BMA-dominated areas. Therefore, descriptions of the rates and magnitudes of nitrogen fixation by benthic microalgae are essential to better describe overall patterns of nitrogen availability within Florida Bay.

The ability to fix nitrogen (diazotrophy) has been observed in numerous classes of both autotrophic and heterotrophic bacteria commonly found in microbial mats and underlying marine sediments (Postgate 1982, Capone 1988). In each case the magnitude of nitrogen fixation appears to be closely coupled to the availability of photosynthetically-derived organic matter (Bebout et al. 1993, Paerl et al. 1996). Visual examination of the benthic microalgal mats in Florida Bay revealed abundant communities of filamentous cyanobacteria as well as numerous photosynthetic sulfur bacteria. Diazotrophy is common in many cyanobacteria and purple sulfur bacteria species (Capone 1988 and references within), and it is likely that these organisms composed a large fraction of the nitrogen fixing community within the benthic microalgal mats. Nitrogen fixation by heterotrophic, sulfate-reducing bacteria was also likely present within the sediments of Florida Bay based on the results of studies in similar systems (Postgate 1982, Bebout et al. 1993, Paerl et al. 1996).

Inhibitors of photosynthesis (photosystem II – DCMU) and sulfate reduction (molybdate) were employed to investigate the relative fraction of total nitrogen

fixation that could be attributed to photoautotrophic and sulfate-reducing bacteria, respectively. Additions of DCMU and molybdate both resulted in significantly decreased rates of nitrogen fixation and BMA abundance as compared to non-amended cores. Taken together the rates of nitrogen fixation from cores that were treated with molybdate or DCMU were less than half the rates found in untreated cores, suggesting that inhibition of both photosynthesis and sulfate reduction repress rates of BMA-mediated nitrogen fixation in Florida Bay. It appears that the addition of specific inhibitors may have had an effect on the interactions between the various components of the benthic microalgal assemblage and the overall magnitude of nitrogen fixation that occurs within the BMA community.

Samples taken from both seagrass-dominated and BMA-dominated sediments as well as samples treated with DCMU showed a very strong correlation between BMA biomass and nitrogen fixation rates. The linearity of the relationship suggests that nitrogen fixation is either 1) carried out overwhelmingly by oxygenic photoautotrophic cyanobacteria or 2) that nitrogen is fixed, in part, by associated heterotrophic or anoxygenic phototrophic organisms that are significantly limited by the availability of labile organic carbon derived from photosynthesis within the BMA community. The addition of DCMU seems to support both limiting possibilities as DCMU amendments resulted in highly reduced levels of BMA biomass and rates of nitrogen fixation as compared to non-amended cores.

However, molybdate-amended samples consistently fell outside of the linear relationship between fixation and BMA biomass with much lower rates of nitrogen fixation observed in relation to levels of BMA abundance found within each core.

Molybdate is a specific inhibitor of sulfate-reduction (Oremland and Capone 1989) which has no direct effect on photosynthesis and the production of organic carbon. Therefore, the observed discrepancy between rates of nitrogen fixation in molybdate-treated cores and non-amended cores must be attributed to sulfate reducing bacteria within the BMA mat and the sediments or some effect that sulfate reducing bacteria have on the BMA community's ability to fix nitrogen as a whole.

Declines in the rate of sulfate reduction may have affected nitrogen fixation in the BMA community in a number of ways. The inhibition of sulfate reduction may have had a direct effect on diazotrophic, sulfate-reducing bacteria by shutting down the respirative pathway by which energy is derived. Without available energy, nitrogen fixation, an energy-consuming process, is not likely to have occurred at rates similar to those in uninhibited communities. Inhibition of sulfate-reduction may also have brought about diminished sulfide levels within the microbial mat. Decreases in sulfide may have elicited lower rates of nitrogen fixation from sulfide-dependent photoautotrophs such as purple sulfur bacteria (Bebout et al. 1993, Steppe and Paerl 2002). Low production of sulfide may also have led to increased oxygen concentrations within the microbial mat and underlying sediments (Jorgensen et al. 1979, Stal et al. 1984). Increased oxygen availability may have resulted in an inhibitory effect on nitrogen fixation due to the sensitivity of the nitrogenase enzyme to the presence of oxygen (Capone 1988). However, this is not likely to be the factor most limiting nitrogen fixation in Florida Bay as low oxygen conditions in DCMU-amended cores displayed even lower rates of nitrogen fixation than those observed in molybdate-amended samples. Rates of sulfate reduction and sulfide availability were

not directly measured in this study, however, the inhibitor-treated cores suggest that sulfate reducing bacteria and/or purple sulfur bacteria are important nitrogen fixers in addition to benthic cyanobacteria within the BMA community of Florida Bay.

While the specifics of interactions between the photoautotrophic and anoxygenic autotrophic communities within the BMA assemblage remain unexplained, there appears to be significant interactions within the assemblage that affect rates of nitrogen fixation. For this reason, rates of nitrogen fixation are attributed to the community as a whole by this study. However, further study may further explain some of these relationships and may allow further insight into the role of the many components of the BMA assemblage in the fixation of nitrogen in Florida Bay.

Rates of benthic microalgal-mediated nitrogen fixation were measured between $0.3\text{-}20 \mu\text{mol N m}^{-2} \text{ h}^{-1}$ ($0.1\text{-}6.7 \text{ mg N m}^{-2} \text{ d}^{-1}$) in Florida Bay. These rates agree well with the range of estimates found in other temperate and tropical systems worldwide (Table 5). Estimates of nitrogen fixation in seagrass-dominated areas of Florida Bay are much lower than previously reported rates of nitrogen fixation in seagrass beds. However, this is likely due to the inclusion of rhizosphere-associated nitrogen fixation in other studies, while this projected reported rates of fixation only within the benthic microalgal community.

Rates of benthic microalgal-mediated nitrogen fixation were calculated over multiple days and reported on an hourly scale in conjunction with measurements of other nitrogen cycling processes in associated projects. However, estimates of nitrogen fixation in this study likely are more reflective of the average daily rate of

nitrogen fixation rather than ephemeral variations that were observed over shorter time scales. Nitrogen fixation was significantly linear over the entirety of the incubation with short periods of variability generally occurring in the early morning and overnight hours. Slight accelerations in the rate of nitrogen fixation were observed in the early morning hours before settling back into a linear trend throughout the rest of the photoperiod.

Stimulation of nitrogen fixation in the early morning hours has been observed in other microbial-mat systems and was attributed to the introduction of newly-produced labile organic matter at oxygen concentrations below the threshold that would inhibit nitrogenase activity (Stal et al. 1984). Bebout et al. (1993) speculated that anoxygenic photoautotrophs may be responsible for this increase in the rate of nitrogen fixation due to the high availability of sulfide, low concentrations of oxygen and presence of photoactive radiation. Whatever component or components of the BMA community are responsible for the observed change in the rate of nitrogen fixation, these effects are relatively small when considered over longer time scales. Additionally, scaling up short-term measurements to a daily or longer time scale may capture moments that are not necessarily indicative of overall fixation patterns. For this reason, estimates of nitrogen fixation on the daily time scale (one complete diel cycle or longer) likely provide more complete and accurate descriptions of in situ benthic microalgal-mediated nitrogen fixation.

Daily rates also incorporate patterns of nitrogen fixation in the dark (overnight hours), which were consistently lower than rates observed in the light. Such light-associated patterns of nitrogen fixation have been described by many studies and

further suggest the limitation of nitrogen fixation by the availability of organic carbon generated by photosynthesis (Carpenter et al. 1978, Stal et al. 1984, Bautista and Paerl 1985, Bebout et al. 1993, Charpy-Roubaud et al. 2001, Miyajima et al. 2001). No direct measurements of primary production were made in this study, however the presence of small bubbles, presumably oxygen generated by photosynthesis, was commonly observed at the surface of the microbial mat.

Rates of nitrogen fixation were investigated at five locations throughout Florida Bay and revealed differing patterns between the basins sampled in this project. Highest levels of nitrogen fixation were observed in the western-most basins with lower levels seen in the central and northeastern parts of the bay. This trend of decreasing nitrogen fixation along a roughly west to east transect was directly in contrast to concentrations of ammonium (NH_4^+) measured within the water column. High water column ammonium levels in the eastern and central areas of Florida Bay were likely the result of external loading of nitrogen via terrestrial runoff from the Everglades and Florida Keys (Rudnick et al. 1999). These high concentrations of relatively labile nitrogen may have created conditions in the eastern bay where nitrogen fixation would not have been favored as a mechanism for satisfying the nitrogen demands within the BMA community.

In the western-most reaches of Florida Bay, however, ammonium concentrations were lower as these basins were much further removed from terrestrial inputs of nitrogen. Additionally, phosphorus availability was higher at the western margin due to the advection of relatively phosphorus-rich waters into the bay from the Gulf of Mexico (Forqurean et al. 1992, Rudnick et al. 1999). Primary production

spurred by higher concentrations of available phosphorus likely resulted in a further drawdown of nitrogen from the water phase. Such conditions suggest that the western part of the bay is more nitrogen-limited than central and northeastern basins.

Measurements of nitrogen fixation by this study appear to support the idea of increasing nitrogen demand in the western bay as a clear gradient in the magnitude of nitrogen fixation was observed along an east-west transect with highest rates measured in the westernmost basins.

The trend of increasing rates of nitrogen fixation from east to west was also observed in measurements of pore water ammonium concentrations within the top half centimeter (0-0.5 cm fraction) of the sediment column. Pore water ammonium profiles from BMA-dominated and seagrass-dominated cores generally approximated the patterns of nitrogen fixation from similar samples. This correlation suggests that nitrogen fixation by the benthic microalgal community is the major source of ammonium present within the upper fraction of the sediment column in the vicinity of the microbial mat. High rates of nitrogen fixation coupled with relative isolation from terrestrial nitrogen loads suggest that benthic microalgal-mediated nitrogen fixation is an important nitrogen loading term for a large area of Florida Bay.

Nutrient amendments were added to samples to further examine the effects of nutrient availability within the water phase on rates of nitrogen fixation in the benthic microalgal community. Previous examinations of BMA-mediated nitrogen fixation under artificially high nitrogen conditions have reported both an inhibitory effect of ammonium amendments (Pinckney et al. 1995) and the absence of any such effect (O'Neil and Capone 1989).

Table 5. A survey of nitrogen fixation rates that have been reported in the literature.

Site	Nitrogen Fixation (mg N * m ⁻² * d ⁻¹)	Source
BMA-dominated Sediments		
Australia	0.20-1.41 0-4.33	O'Neil and Capone 1989 Capone et al. 1992
Bermuda	0.13-3.85	O'Neil and Capone 1989
Bassin d'Arcachon France	0.02-3.7	Welsh et al. 1996a
Card Sound (Florida)	0.37	Bunt et al. 1971
Florida Bay	0.1-6.7	This Study
Ishigaki Island Japan	1.38-2.28	Miyajima et al. 2001
Puerto Rico	0.71-5.17	O'Neil and Capone 1989
San Salvador	0.07-0.69	O'Neil and Capone 1989
Tikehau Lagoon French Polynesia	0.4-3.9	Charpy-Roubaud et al. 2001
Seagrass-dominated Sediments		
Australia	10-40 16-47 16-166	O'Donohue et al. 1991 Moriarty and O'Donohue 1993 Perry (1997)
Bahamas	14-41 6.0-9.0	Oremland et al. 1976 Capone et al. 1979
Barbados	27-140	Patriquin and Knowles 1972
Bassin d'Archachon France	0.1-7.3	Welsh et al. 1996
Florida	0.03 5.0-24 0.15-0.75	McRoy et al. 1973 Capone and Taylor 1980 Perry (1997)
Florida Bay	0.1-0.9	This Study
Ishigaki Island	2.4-4.4	Miyajima et al. 2001

Ammonium-amended cores showed very similar rates of nitrogen fixation to rates measured in non-amended cores. Water phase ammonium concentrations were augmented to approximately 100 μM , more than 20-50 times higher than natural levels. No measurements of ammonium in the pore water were made, yet it is likely that pore water concentrations rose appreciably in response to increased water phase values. However, no inhibition of nitrogen fixation was observed under these nitrogen-replete conditions despite the energy costs associated with the process. These results indicate that rates of nitrogen fixation in Florida Bay are relatively unaffected by changes in ammonium availability on the short term and suggest the presence of continued nitrogen demand in this system.

Phosphorus amendments, however, generally did result in increased rates of nitrogen fixation. Primary production in Florida Bay has been described as phosphorus-limited (Forqurean et al. 1992). Enhanced phosphorus availability likely created favorable conditions for the growth of diazotrophic cyanobacteria due to their ability to supply sufficient amounts of nitrogen to support increased rates of production (Paerl et al. 1994, Pinckney et al. 1995). Additions of iron (alone and in combination with EDTA) and dissolved organic carbon both failed to bring about consistent increases in nitrogen fixation over non-amended samples of the BMA assemblage (Figure 26). Thus, it appears that BMA-mediated nitrogen fixation is a major component in supporting the production and growth of cyanobacteria and is constrained by outside mechanisms (such as phosphorus availability) that limit these processes.

The results of this study indicate that the amount of nitrogen fixed by diazotrophic organisms in the benthic microalgal mats is a significant input term in basins located throughout Florida Bay. However, it is difficult to accurately estimate the total magnitude and impact of nitrogen fixation on scales large enough to compare inputs via fixation to previously reported values of external nitrogen loads to the system. Nitrogen fixation was observed at much higher rates in BMA-dominated patches as opposed to areas covered by seagrasses. Rates of nitrogen fixation also varied between basins with a strong gradient observed along an east to west tract. Therefore, a number of steps were taken to include larger-scale estimates of nitrogen fixation in order to construct an improved nitrogen budget for Florida Bay.

For this budget, the system was divided into five zones that included one of the five basins that were sampled in this study. The location and extent of these five zones were roughly based on previous divisions of Florida Bay on the basis of benthic plant communities as described by Zieman et al. (1989). Zones were further divided according to the relative area within each basin that was dominated by seagrass as compared to the area that was dominated by BMA to more accurately describe the total amount of BMA-mediated nitrogen fixation occurring within each part of the bay. Both the western bay (site 1) and eastern bay (site 4) were fully seagrass-dominated during the sampling period and were considered to be 100% seagrass-dominated. The north-central (site 3) and island-associated (site 5) portions of the bay were estimated on a 50% seagrass-dominated, 50% BMA-dominated basis and the south central (site 2) part of the bay was split 75% seagrass-dominated, 25% BMA-dominated. These estimates of percent vegetative cover were based primarily

on personal observations at sampling sites and adjacent basins throughout the sampling period. Measurements of nitrogen fixation from each of the five sampled basins were considered to be representative for the entire area of each zone, respectively. Total nitrogen fixation was then calculated as the sum of estimates of nitrogen fixation from each of the five basins.

Estimates of external inputs of total nitrogen (TN) to Florida Bay were reported by Rudnick et al. (1999) and have since been further refined by the same authors. These estimates suggest that nitrogen loading is dominated by the advection of nitrogen-rich waters into Florida Bay from the Gulf of Mexico at the western margin of the system. Smaller inputs of nitrogen are derived from terrestrial runoff from the Florida Everglades and Florida Keys as well as from the atmosphere through precipitation directly incident on the waters of the bay. Suggestions by Rudnick et al. (1999) that nitrogen fixation likely has a significant impact on nitrogen availability appear to be validated by the results of this study.

Using the budget with all of the assumptions mentioned above, benthic microalgal-mediated nitrogen fixation in Florida Bay was estimated at 382 metric tons N km⁻² yr⁻¹ (Table 6). This figure is of similar magnitude to external nitrogen loading resulting from terrestrial runoff from the Florida mainland and islands of the Florida Keys as well as from the atmosphere via precipitation. These input terms taken together are still far less than the estimated input of nitrogen into Florida Bay due to water advection from the Gulf of Mexico and, as such, it would appear that nitrogen availability within the system is highly dependent on this one source. However, the fate and availability of incoming nitrogen from the Gulf of Mexico is

very much in question as the presence of large, shallow mud banks likely prevents the introduction of nutrient-replete waters beyond the western-most fringes of the bay (Rudnick et al. 1999). Thus, upstream (terrestrial) and more localized inputs of nitrogen, like nitrogen fixation, are likely the most important sources of bioavailable nitrogen for much of Florida Bay.

The spatial extent that is impacted by both external and internal nitrogen sources is relatively unknown. Introduction of nitrogen from the Everglades and Florida Keys occurs on a fairly localized scale along the northern margin of the system, and patterns of water advection that may transport these pools of nitrogen between adjacent basins and beyond have not been well described. Similarly the impacts of external nitrogen inputs from the Gulf of Mexico beyond the western-most part of Florida Bay are not well known. This uncertainty surrounding the impacts of these external nitrogen sources may create a situation where nitrogen inputs via precipitation and nitrogen fixation comprise the largest input terms in interior parts of Florida Bay.

External Inputs	metric tons TN * yr ⁻¹	mg TN * m ⁻² * d ⁻¹
Everglades	250	0.31
Taylor Slough and C-111		
Atmosphere	710	0.88
Florida Keys Wastewater	170	0.21
Gulf of Mexico	13000	16.19
Advection across Boundary		
<hr/>		
Total Inputs (External Sources)	14000	17.43
from Rudnick et al. 1999		
<hr/>		
Internal Inputs	metric tons N * yr ⁻¹	mg N * m ⁻² * d ⁻¹
Western Bay (Site 1)	40	0.25
South Central Bay (Site 2)		
seagrass-dominated	54	0.45
BMA-dominated	102	2.55
North Central Bay (Site 3)		
seagrass-dominated	14	0.17
BMA-dominated	87	1.08
Eastern Bay (Site 4)	24	0.15
Island-Associated Bay (Site 5)		
Seagrass-dominated	15	0.19
BMA-dominated	47	0.59
<hr/>		
Total Inputs (Internal Sources)	383	0.47

Table 6. A comparison between external inputs of nitrogen as reported by Rudnick et al. (1999) and estimates of internal inputs based on measurements of nitrogen fixation in this study. Measured rates of nitrogen fixation were assumed to be representative of a section of Florida Bay. Estimates of relative seagrass-dominance vs. BMA-dominance were based on observations at each of the sample sites and were used to determine total nitrogen inputs to each area of the bay. Two sites (Site 1 and Site 5) were completely dominated by seagrass and, therefore, have no inputs of nitrogen from BMA-dominated zones. Estimated inputs of nitrogen compare to external inputs from the Everglades, Florida Keys, and atmospheric deposition. However, internal inputs of nitrogen via nitrogen fixation may be very significant to supporting productivity in Florida Bay as it is unknown to what extent external inputs of nitrogen are available to regions of the bay due to the structures that combine to isolate that bay from adjacent ecosystems.

Conclusions

Nitrogen fixation was a common occurrence in the benthic microalgal communities throughout Florida Bay. Periods of fixation were generally found during the photoperiod and were attributed to the availability of labile organic carbon produced via oxygenic photosynthesis. Significant correlations between nitrogen fixation and BMA biomass as measured by chlorophyll- α suggest that phototrophic cyanobacteria comprise a major fraction of the diazotrophic suite of organisms within the benthic microalgal mats. Sulfate-reducing bacteria and anoxygenic, sulfide-dependent phototrophs were also identified as likely nitrogen fixers.

A trend of increasing rates of nitrogen fixation was observed along an east to west transect which directly contrasted the pattern observed in water column ammonium concentrations. These patterns are likely indicative of increasing phosphorus concentrations in the western basins due to the influence of the Gulf of Mexico as well as diminished impacts of terrestrial nitrogen sources felt downstream as compared to basins located closer to the point of input.

Benthic microalgal nitrogen fixation was highest in “BMA-dominated” patches due to increased BMA abundance in the absence of competition and shading by seagrasses. BMA-mediated nitrogen fixation was stimulated by phosphorus suggesting BMA production was limited by phosphorus availability. Amendments of ammonium did not result in any consistent stimulation or inhibition of rates of nitrogen fixation indicating that this process is not affected by increased nitrogen loading, at least not on the short-term (multiple days). Similarly iron and dissolved

organic carbon (acetate) failed to bring about consistent changes in the rate of nitrogen fixation.

Benthic microalgal-mediated nitrogen fixation is a significant source of bioavailable nitrogen and may be one of the largest nitrogen inputs in interior and western basins of Florida Bay. As such, it is necessary to include rates of nitrogen fixation in future budgets and nitrogen cycling models for this system. Further work examining nitrogen fixation within the rhizosphere in seagrass-dominated areas of Florida Bay is suggested since this is likely a major source of nitrogen that is yet to be described.

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