NITRATE UPTAKE BY HETEROTROPHIC BACTERIA AND THE DIVERSITY OF BACTERIAL NITRATE ASSIMILATION GENES IN MARINE SYSTEMS

by

ANDREW ALLEN

(Under the direction of Dr. Peter Verity and Dr. Marc Frischer)

ABSTRACT

This study focused on the development of molecular methods to assay the diversity and abundance of groups of heterotrophic bacteria that are capable of aerobic NO₃⁻ assimilation. The variability of the abundance and diversity of populations of heterotrophic bacteria capable of NO₃⁻ utilization was studied in relation to patterns of bacterial NO₃⁻ uptake as indicated by ¹⁵N uptake experiments. A PCR primer set that could be used to selectively amplify a fragment of the nasA gene (assimilatory nitrate reductase) from heterotrophic bacteria was designed. Results suggest that nine groups of heterotrophic bacterial nasA genes are common and widely distributed in oceanic environments. ¹⁵N tracer experiments conducted in the Barents Sea and in the South Atlantic Bight indicate that bacteria assimilate, on average, between 15 and 40 % of the available NO₃⁻. These results suggest that bacteria play a larger role in NO₃⁻ utilization than previously hypothesized and that bacterial uptake of NO₃⁻ should not be ignored in estimates of new production. In the Barents Sea Marinobacter sp. nasA gene abundance, measured via a SYBR Green real-time PCR assay, was positively correlated with NO₃⁻, showing a two-fold increase in concentration relative to total bacteria at 80 m compared to 5 m. Compared to other variables tested, NO₃⁻ is the best predictor, by a factor of 10, of the variability associated with nasA community structure (assayed via T-RFLP) across the different water masses sampled in the Barents Sea. Studies conducted in the Skidaway River estuary in the South Atlantic Bight indicated a strong correlation, across seasons, between Marinobacter sp. nasA gene abundance and the magnitude of bacterial NO₃⁻ uptake. Of the different variables assayed, NO₃⁻ uptake rate was the best predictor, by a factor of 15, of the variability associated with nasA community structure. The finding that NO₃⁻ availability and patterns of NO₃⁻ utilization are positively correlated with nasA community structure variability and the abundance of particular groups of nasA genes, indicates that patterns of NO₃⁻ supply, in the marine environment, are sufficiently important to be a factor in regulating bacterial communities.

INDEX WORDS: Nitrate uptake, ¹⁵N, PCR, Q-PCR, T-RFLP, nasA, Heterotrophic bacteria, Marine bacteria, Nitrogen cycle, estuary
NITRATE UPTAKE BY HETEROTROPHIC BACTERIA AND THE DIVERSITY OF BACTERIAL NITRATE ASSIMILATION GENES IN MARINE SYSTEMS

by

ANDREW E. ALLEN

B.A., VASSAR COLLEGE, 1996

A dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2002
NITRATE UPTAKE BY HETEROOTROPHIC BACTERIA AND DIVERSITY OF BACTERIAL NITRATE ASSIMILATION GENES IN MARINE SYSTEMS

by

ANDREW E. ALLEN

Approved:

Major Professor: Peter G. Verity
Committee: Marc E. Frischer
James T. Hollibaugh
Samantha B. Joye
Marry Ann Moran
Deborah A. Bronk

Electronic Version Approved:

Gordhan L. Patel
Dean of the Graduate School
The University of Georgia
May 2002
DEDICATION

To Grand Dot and Aunt Jane - I know you are somewhere having a “Big Time”.

Also to my parents, who have dedicated their lives to making a difference through excellence in teaching. You continue to be the best teachers I have ever known. Thank you for always emphasizing the importance of education and the value of intellectual curiosity.
ACKNOWLEDGEMENTS

There have been far too many people to name here who have helped me during my graduate studies and to get there, but I would like mention several of the most important ones.

First, to Dr. Marc Frischer and Dr. Peter Verity who have gave me a wonderful opportunity. Thank you for introducing me to marine microbial ecology; I am honored to have been your student. I have learned distinct but very complimentary lessons from both of you. Thank you for your professional and financial support and, as importantly, thank you for your friendship.

I would like to acknowledge the huge role Dr. Melissa Booth has played in this work and in my development and as a scientist. I will always remember the pain, joy and sense of potential discovery from collecting 50 L of water with 5 L Niskin bottles from 100 m beneath the Gulf Stream in the middle of the night aboard the R/V Bluefin. With a little help from Jack, Jill, Bonnie, Clyde, Fred, and Ginger we managed to make it work. From the early days of “Melissa’s Ocean Spreader Protector”, to filtering 40 L of water by hand through multiple 250 ml funnels, to the hand over hand Niskin bottle collection of water at Priest Landing to trying to filtering 40 L of water in serial the night before a cruise and determining the filters have no pores, you have been tremendously supportive and helpful to me in all of the crazy things I have attempted. Also, several of the most important parts of this work would not have been possible without your expertise, guidance, and tutelage. But, most importantly, thank you for your friendship. There have been several times when your friendship and personal concern for me have made the difference between misery and happiness.
Also special thanks to Dr. Deborah Bronk. I feel like this work is in large part the result of questions you have uncovered during your career. From day 1, when you began to teach me nutrient chemistry, you have been extremely helpful and supportive with everything I have needed. This work would not have been possible without your expertise and experience with regard to nitrogen cycling studies. Your insistence that I meet the pre-requisites of using a notebook with carbon copy pages and using endnote in order for you to be on my committee has served me well. Also, Marta Sanderson has played an enormous role in the data generated from these studies. Your careful preparation and prompt turn around of hundreds of samples has truly made this a collaborative effort and a success. I always feel productive when I put samples in the mail for you, because it is one of the few things that I do that I know will lead to quality data. Thank you.

Also, thanks to my committee, all of whom, have been very helpful and cooperative considering my off campus status. The fact that I have spent very little time is Athens has not been the most convenient arrangement at times, but all of you have helped make it easier than it otherwise could have been.

Special thanks also to Heidi Hendrickson. You were a very fast learner and I always enjoyed your positive attitude; you were always a pleasure to work with. Thank you to Jean Danforth, who is the one who has had to really bear the brunt of my steep learning curve on a day to day basis. Hope Howard-Jones and Victoria Ballard have been very helpful with microscopy related tasks and bacterial production measurements and also a pleasure to work along side; I wish you both the best of luck. Also, thanks to Lori Cowden who in my early days at Skidaway was extremely helpful in terms of solving all
kinds of daily problems. Charles Robertson has been very helpful by providing cruise supplies and equipment time and time again and also has been helpful in terms of general discussion regarding the role of bacteria in nitrogen assimilation.

I would like to thank the entire Skidaway Institute of Oceanography staff. The shop has been very supportive; Harry Carpenter, Raymond Thomas, Jimmy Williams have all designed and constructed useful sea-worthy equipment. John Baggett and Braxton Tesh have been helpful in several unfortunate late night and weekend emergency situations. Thanks to the crew of the R/V Bluefin: Jay Fripp, Raymond Sweatte, Mike Richter, and Raymond Thomas. Anna Boyette and Suzanne McInstosh have been very helpful in preparing figures and posters. Dee Peterson has been helpful in helping to prepare documents and figures numerous times. I would like to thank everyone in the business office. Bill McChesney, Beth Christiansen, Christel Morrison, Carol Finch and Shelia Wentz have been very helpful many times and especially helpful in many “last minute” situations. Also, thanks to Norman Thomas, Michael Sterling, William Scott, and David Walker who many times have provided much needed conversation about sports and current events. Liz Cooksey has also been helpful numerous times.

One final word on Skidaway: for nearly the past four years Skidaway has not only been the place where I have conducted my research, it has been my home. I am grateful for the experience of having lived in such a beautiful place; I will never tire of remembering the glassy and smooth and often rough and wild look of the river and the evening sun falling into the marsh. Thanks to the Skidaway Institute of Oceanography for providing housing to facilitate my research.
Special thanks to Dr. G.-A. Paffenhöfer for twice providing very valuable ship time aboard the R/V *Hatteras*. Dr. Paul Wassman also provided, what has proved to be, extremely valuable ship time in the Barents Sea aboard the R/V *Jan Mayen*. Thanks to the crews of the R/V *Hatteras* and the R/V *Jan Mayen*.

I also want to acknowledge the deep support I have gotten from many very close friends during graduate school. Perhaps as much as anything else, my graduate school experience has taught me the true value of meaningful friendships. Somehow all of the long isolated hours and late nights seem worth it when, at the end of the day, you can relax in the company of friends you feel truly at home around. Also, many of you are directly responsible for interest in Ecology, Oceanography, and wilderness and without your influence I would not be the scientist I am today. The following people have all been a very positive influence and helped me to remain calm and focused during this process and the concern for me that each of you has expressed is not forgotten: Ben Webber, Stuart and Alison Barkoff, Erica Field, Kristen Field, Melissa Booth, Matt Ferner, Jack White, David Govus, David Harrison, Pam Benvenue, Peter Koch, Terry Weber, Misha Hauer, Laura Burkle (expert plate poorer), Florion Rambow, Jamie March, Jon Benstead, All and Betts Field, Tom and Jonti Rodi, Arlene Alvarado.

Other people I would like to thank for help in getting here are Karen and Jim Porter, Patty Gowatty, Richard Hemmes, Roger Gay, and Beverley Herbert.

Nothing that I have accomplished would be possible without the deep love and support I have received from my family over the years. First, Uncle Ray and Aunt Diane - you have given me so much love throughout my entire life. I truly believe that my interest in Ecology and nature comes in many ways from the long trips you took me on to
the most beautiful parts of North America many times when I was young. I deeply appreciate all that you have done for me and the love and affection you continue to show me. I love you very much.

Mom and Dad, it is truly impossible for me to express in words the deep level of gratitude I feel for everything you have done for me. You are my heroes. You have always taught me to trust myself and to have confidence in myself and I cannot think of a more important gift that a parent can give. I love you very much and thank you for taking care of my precious girl.

Erica, I am not sure what to say, but I know that if I do not say anything I will feel like something very important and huge is missing. Over the past seven years, I have been closer to you than I ever have to anyone and we have shared so much. Without your faith in me, and the love you have shown me, I am sure I would not be where I am today.

From the San Juan Mountains to Grizzly Lake, from the oasis in the Valley of the Condors high in the Andes Mountains to Grandfather Mountain, North Carolina, from the canyons of North Texas to the marshes of Northern Germany, from the Outer Banks to St. Simons to Bald Head Island, from Crested Butte to Key West to Atlanta to Chapel Hill, from Tybee Island to the giant sand dunes of coastal Oregon to the Nazca lines and beaches on the Paracas peninsula, from the green fields and cows of Ellijay, Georgia to Duke forest, from Moab, Utah to Davis, California to Eugene, Oregon, from Sante Fe to Ariquillpa, from Iquitos and the Peruvian Rain Forest to the Olympic Peninsula, from Lima to Oslo, from New Orleans to Mannheim, from Cold Spring to Rhinebeck to New Paltz, from Arcata, California to New York City, from Athens, Georgia to Lilihammer to Poughkeepsie, from the Gardens of Tivoli to Cusco, from the glaciers and fjords of
Norway to the Catskills Mountains, from the Blue ridge Parkway to Janesville, Wisconsin, from Skidaway Island to Milledge Terrace to Maple street and so many places in between - I love you and I will never forget all that we have shared with each other during this time. Thank you for everything, I have missed you more than you know.
# TABLE OF CONTENTS

ACKNOWLEDGMENTS................................................................................................................................. v

CHAPTER

1 INTRODUCTION AND LITERATURE REVIEW................................................................. 1

   Evidence for Bacterial Assimilation of NO₃⁻ .......................................................................... 1

   The Significance of Bacterial Nitrate Assimilation in Marine Systems ........... 2

   Nitrate Assimilation by Heterotrophic Marine Bacteria: A Molecular
   Approach .................................................................................................................................................. 7

   Summary of Chapters ...................................................................................................................... 9

2 DIVERSITY AND DETECTION OF NITRATE ASSIMILATION GENES IN
   MARINE BACTERIA ................................................................................................................... 21

3 IMPORTANCE OF HETEROTROPHIC BACTERIAL ASSIMILATION OF
   AMMONIUM AND NITRATE IN THE BARENTS SEA DURING SUMMER......................................................... 47

4 TRFLP AND REAL-TIME PCR ANALYSIS OF BACTERIAL NITRATE
   ASSIMILATION GENES IN THE BARENTS SEA DURING SUMMER ......................................................... 91

5 SEASONAL VARIABILITY OF BACTERIAL NITRATE ASSIMILATION
   AND NITRATE ASSIMILATION GENES (nasA) IN A SUBTROPICAL
   ESTUARY .............................................................................................................................................. 137

6 HETEROTROPHIC BACTERIAL NITRATE ASSIMILATION:
   CONCLUDING REMARKS ............................................................................................................... 180

APPENDIX A: ACCESSION NUMBERS ................................................................................................. 192
CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

Evidence for Bacterial Assimilation of Nitrate

The primary role of heterotrophic bacteria in marine systems is classically considered to be the decomposition and mineralization of dissolved and particulate organic matter (Pomeroy 1974). Because heterotrophic bacteria represent a sink for nitrate (NO$_3^-$) that does not involve the incorporation of inorganic carbon, significant heterotrophic bacterial utilization of NO$_3^-$ would likely have profound effects on the fluxes of nitrogen and carbon in the water column. Despite the fact that bacterial assimilation of NO$_3^-$ has received very little attention in marine nitrogen (N) cycling studies, the ability of heterotrophic bacteria to assimilate NO$_3^-$ has been known for some time (Nicholas 1963, Painter 1970). There is convincing data that suggests that soil microbial communities often assimilate almost all of the NO$_3^-$ when bacterial turnover is high (Stark & Hart 1997). Although its ecological relevance is unclear, NO$_3^-$ assimilation by heterotrophic bacteria in marine systems has been documented in several systems (Table 1.1).

Studies, in which bacteria were grown on a rich carbon source, such as pulp mill effluent or glucose, total NO$_3^-$ disappearance was attributed to bacteria (Parker et al. 1975, Parsons et al. 1980). Kirchman et al. (1994) measured a bacterial contribution of 4 to 14 % of total NO$_3^-$ uptake in the North Atlantic during a spring bloom. Kirchman and Wheeler (1988) reported that heterotrophic bacteria contributed 5 to 60 % (average of 32%) to total NO$_3^-$ uptake in the sub-Arctic Pacific, where nitrate concentrations are
relatively high (6 to 20 µM). In the same study, it was reported that the percentage NO₃⁻ uptake was higher than the percentage ammonium (NH₄⁺) uptake in 17 out of 20 samples. A similar trend was observed in the Barents Sea where bacteria accounted for 16 – 40 % of the total NO₃⁻ uptake and 12 – 40 % of the total NH₄⁺ uptake, and in 12 out of 15 samples the percentage NO₃⁻ uptake was higher than the percentage NH₄⁺ uptake (Allen et al. in press). At the very least, this comparison suggests that heterotrophic bacterial assimilation of NO₃⁻ and NH₄⁺ can be equally important in some environments.

A review of the literature generally supports theoretical predictions based on the energetics of bacterial growth and the oxidation state of various N compounds. Significant assimilation of NO₃⁻ is expected and usually occurs in environments with large inputs of organic matter with a high C:N ratio, high bacterial turnover rates, and high concentrations of NO₃⁻ relative to NH₄⁺ and amino acids. It is not surprising, therefore, that some of the highest levels of bacterial NO₃⁻ assimilation documented have occurred in heterotrophic, dissolved organic carbon (DOC) and NO₃⁻ replete environments such as the Thames estuary (Middleburg & Nieuwenhuize 2000), the Hudson River (Caraco et al. 1998), and in marine sediments (Blackburn et al. 1996).

The Significance of Bacterial Nitrate Assimilation in Marine Systems

Microbial biomass and detrital organic matter can serve as a source of food and organic N to grazers in marine systems (Findlay & Tenore 1982, Tenore 1988). The origin of this organic N can be a result of autotrophic assimilation of dissolved inorganic N (DIN) or heterotrophic bacterial assimilation of DIN. Therefore, NO₃⁻ uptake by heterotrophic bacteria is important in the context of marine biogeochemical N budgets
because it represents a mechanism by which heterotrophic microbes contribute to the production of organic N. Nevertheless, it is generally assumed that autotrophic assimilation of DIN is the primary source of organic N to a system, and most estuarine and open ocean models of N cycling ignore bacterially assimilated DIN (Fasham et al., 1990, Boynton et al. 1995, Haupt et al. 1999, Olivieri & Chavez 2000, Dadou et al. 2001). In empirical and modeling studies of the impact of bacterially assimilated DIN on Hudson River ecosystem carbon (C) and N budgets, bacterially assimilated DIN was responsible for enriching the N content of the dissolved organic N (DON) and particulate organic (PON) pool by 10% and 50% respectively (Caraco et al. 1998).

Heterotrophic bacterial assimilation of NO$_3^-$ is important in marine biogeochemical C budgets as well because of its potential impact on estimates of new production and on the relationship between new production and C export out of the euphotic zone (Legendre & Grosselin 1989, Kirchman 2000). Traditional views of the marine N cycle hold that the downward flux of particulate N is equal to autotrophic or new production. Therefore, sinks for dissolved N that do not incorporate inorganic C, such as heterotrophic bacterial uptake of NO$_3^-$, represent mechanisms, which potentially reduce oceanic sequestration of CO$_2$ via the “biological pump”. The important point is that DIN that supports heterotrophic production contributes to the oxidation of organic C, as opposed to DIN that supports autotrophic production and contributes to the fixation of CO$_2$.

For three decades, oceanographers and biogeochemists have appreciated the importance of NO$_3^-$ uptake in marine ecosystems because it supports new production that over large geographic and temporal scales must equal export from the system, ignoring
N$_2$ fixation and other N inputs (Dugdale & Goering 1967, Eppley & Peterson 1979, Bronk et al. 1994). The ratio of NO$_3^-$ to total N uptake (the $f$-ratio) is often used as an index for characterizing different environments because it describes the relationship between primary production and the fraction of particulate and dissolved production exported from the system (Eppley & Peterson 1979). Some researchers have argued that the $f$-ratio should be corrected for bacterial NH$_4^+$ assimilation (Harrison et al. 1987, Legendre & Grosselin 1989), but the extent to which bacterial NO$_3^-$ uptake might affect estimates of the $f$-ratio has not been carefully explored.

It is critical to bear in mind that, regardless of heterotrophic bacterial assimilation of NO$_3^-$, uptake of NO$_3^-$ still has to be balanced by export, assuming steady state. The relationship between new production and N export, however, changes if bacteria assimilate NO$_3^-$ and the extent to which bacterial NO$_3^-$ uptake might cause systems to deviate from steady state has not been examined. Attempts to measure and equate $f$-ratio estimates with export flux sediment trap data often result in $f$-ratio estimates that are much larger than trap values (Murray et al. 1989), which, disregarding the possibility of sampling error, seems to indicate a state of imbalance with respect to steady state. In studies of NO$_3^-$ uptake in the Barents Sea, Allen et al. (in press) observed that in experiments where the ratio of total community NO$_3^-$ uptake to total DIN uptake was $>0.5$, bacteria accounted for approximately 40% of the NO$_3^-$ utilization. This implies that in regions where apparent high $f$-ratios are observed, bacteria may be partially responsible for new production measurements, which is not equivalent to autotrophic production. Most likely, these observations reflect high ambient NO$_3^-$ concentrations, which seem to increase levels of bacterial NO$_3^-$ assimilation (Vallino et al. 1996).
Because of small cell size, NO$_3^-$ uptake by heterotrophic bacteria would be likely to become uncoupled from particulate N export and require additional trophic transfers to be transformed into sinking material. If bacterially assimilated DIN is a major contributor to the DON pool in the open ocean, as it has been shown to be in estuaries (Caraco et al. 1998), then it is likely that the uncoupling between production and export that occurs as a result of bacterial NO$_3^-$ uptake is at least partially balanced, after a time-lag, by the downward advection of DON. Although the downward flux of DON has been shown to be an important part of the overall new production/export flux paradigm (Bronk et al. 1994), it is rarely considered and bacterial DIN assimilation could play an important role in DON production and advection dynamics.

Uptake of DIN by heterotrophic bacteria is also important to consider because of its potential impact on the utilization of DOC, the largest pool of reduced C in the ocean (Fuhrman 1992). For example, evidence suggests that DIN uptake determines the seasonal build-up in DOC concentrations in the North Atlantic (Carlson et al. 1994, Williams 1995). Bacterial assimilation of NO$_3^-$ could explain the negative correlation between the C:N ratio of dissolved organic matter (DOM) and NO$_3^-$ concentration across many of the world’s largest river systems (Caraco et al. 1998). This would imply, however, that bacterial production is DIN limited, which some studies have shown (Horriigan et al. 1988, Kuparinen & Heinanen 1993, Rivkin & Anderson 1997, Allen et al. accepted). Other studies, however, have demonstrated that bacterial production is not limited by DIN (Kirchman 1990).

Bacterioplankton are considered to be primarily limited by C (Pomeroy & Wiebe 1993, Kirchman et al. 1990) and the oxidation state of the available DOM (Vallino et al.
1996), and secondarily by nutrients such as N, phosphorous and iron (Kirchman et al. 2000, Cochlan 2001). The implies that, most of the time, marine bacteria are growing at relatively low growth efficiencies with slow doubling times and acting as a source of DIN (mineralizers of DON). However, immediately following an algal bloom in an upwelling environment or the input of an allochthonous source of DOM in an estuary, bacterioplankton populations likely undergo a brief transition to act as a strong sink for DIN, with the precise timing of this transition a function of the energy content of the DOM source. In a controlled experiment, for example, bacterial DIN demand and uptake exhibited contrasting patterns depending on the presence of different species of phytoplankton. Diatoms are thought to release organic material of a refractory and polymeric nature which leads to a delay in bacterial demand for DIN, and the coccolithophorid *Emiliania huxleyi* releases much more labile DOM which promotes a more immediate bacterial demand for DIN (Sanders & Purdie 1998). This scenario is consistent with observations that rapidly growing bacteria are inefficient remineralizers of organic N (Goldman et al. 1987).

Generally, there is very little known regarding which groups of heterotrophic bacteria are capable of NO₃⁻ assimilation. It has been shown that not all marine bacteria are metabolically capable of aerobic NO₃⁻ assimilation (Allen et al. 2001, Richardson et al. 2001). Therefore, it would appear that discrete and identifiable groups of marine bacteria are responsible for what is an important process for food web dynamics and C and N cycling. A better understanding of the diversity and distribution of bacterial groups capable of NO₃⁻ assimilation will help in trying to understand the role of heterotrophic bacteria in N fluxes through the microbial food web. Despite long standing
debate regarding whether or not heterotrophic bacteria are sources of DIN as a result mineralization processes or sinks for DIN as a result of assimilation processes (Wheeler & Kirchman 1986), heterotrophic bacteria likely function dynamically and are often times a DIN sink and other times a DIN source. Since not all groups of marine bacteria are capable of NO$_3^-$ assimilation, the composition of the bacterial community could influence whether or not the microbial food web operates as a source or sink of DIN.

**Nitrate Assimilation by Heterotrophic Marine Bacteria: A Molecular Approach**

Although conventional tracer approaches to N assimilation in aquatic environments are useful and informative with regard to rate and flux measurements, molecular approaches offer the opportunity to assay the functional significance, diversity, and activity of particular organisms and groups of organisms. The ability to distinguish between prokaryotic autotrophs and heterotrophs with regard to NO$_3^-$ assimilation is particularly intriguing since autotrophic uptake leads to CO$_2$ reduction and incorporation while heterotrophic uptake leads to DOC oxidation and CO$_2$ production. Fortunately, among prokaryotes there is enough phylogenetic divergence between autotrophic and heterotrophic *nasA* (called *narB* in cyanobacteria) genes, to facilitate a molecular approach to distinguish between the distribution and activity of the two groups (see Ch. 2, Fig. 2.2). Knowledge of the genes encoding enzymes involved in marine heterotrophic bacterial NO$_3^-$ assimilation will help provide a picture of the diversity of bacteria responsible for NO$_3^-$ uptake and strengthen our understanding of the ecology of the N cycle. The molecular approach to the problem of nitrate assimilation seems especially
promising because of the relative ease with which molecular information can be collected simultaneous to rate measurements.

Probes for specific metabolic pathways can be particularly useful for detecting and characterizing which groups of microbes are important in key steps of biogeochemical processes. Molecular techniques have been employed to illuminate factors that control the rates of fluxes and transformations in nitrogen cycling processes (Ward 1996, Zehr & Hiorns 1998, Zehr & Voytek 1999, Zani et al. 2000, Zehr and Ward 2002). Molecular techniques have also been successfully used to characterize bacteria and the genes that are important in several aspects of the nitrogen cycle, including nitrification, denitrification, nitrogen fixation as well as heterotrophic bacterial nitrate assimilation (Zehr & McReynolds 1989, Kirshtein et al. 1991, Voytek & Ward 1995, Voytek et al. 1997, Zehr & Paerl 1997, Scala & Kerkhof 1998, Zehr et al. 1998, Scala & Kerkhof 1999, Voytek et al. 1999, Zani et al. 2000, Allen et al. 2001). The research presented here represents a first attempt to construct a DNA sequence database of heterotrophic bacterial nitrate assimilation genes in a variety of marine environments and to identify which groups of these genes appear to be the most common to particular environments in relation to NO₃⁻ availability and patterns of bacterial NO₃⁻ utilization.

Summary of Chapters

Chapter 2 takes advantage of the knowledge regarding the structural genes that comprise the bacterial nitrate assimilation operon. The aim of the studies presented in Chapter 2 was the design and optimization of a series of nested heterotrophic bacterium-specific nasA primers. The detection of nasA genes in a variety of marine environments is presented in Chapter 2 and provides a basis for the hypothesis that the potential for NO$_3^-$ utilization by heterotrophic bacteria is significant. Also, phylogenetic analysis of nasA genes cloned from several diverse samples reaffirmed the indication, based on a limited database, that there is a clear genetic distinction between nasA genes from heterotrophic bacteria and nasA genes from autotrophic cyanobacteria.

Chapter 3 focuses on rate and magnitude measurements of $^{15}$N DIN uptake that were made during a cruise transect across the Barents Sea into the marginal ice zone (MIZ). Results indicated that the percent bacterial DIN uptake of total DIN uptake increased significantly from 10% in open Atlantic waters to 40% in the MIZ. On average, at each of five 24 hour stations bacteria accounted for 16-40% of the total NO$_3^-$ uptake and 12-40% of the total NH$_4^+$ uptake. Results from this study also indicate that in experiments where the total NO$_3^-$ uptake to total DIN uptake ratio is $>0.5$, bacteria accounted for approximately 40% of the NO$_3^-$ utilization. This finding provides the basis for the hypothesis that in regions where apparent high f-ratios are observed, bacteria may be partially responsible for the new production measurements, but do not result in increased new production.

Chapter 4 is aimed at explaining the variability associated with the $^{15}$N measurements of bacterial DIN uptake made in Chapter 3 through molecular methods.
The more comprehensive and quantitatively informative molecular techniques, terminal restriction fragment length polymorphism (T-RFLP) and real-time PCR are employed to determine how similar \( nasA \) populations are to one another in surface (5 m) and deeper (80 m) waters across the sampling transect. The major hypothesis addressed is that differences in \( \text{NO}_3^- \) concentrations and \( \text{NO}_3^- \) uptake rates are reflected in the variability associated with \( nasA \) population community structure and abundance. Results indicate that \textit{Marinobacter} sp. \( nasA \) gene abundance is positively correlated with \( \text{NO}_3^- \), showing a two-fold increase in concentration relative to total bacteria at 80 m compared to 5 m. Also, analysis of T-RFLP data suggests that \( \text{NO}_3^- \) concentration, compared to the other variables assayed (bacterial productivity, bacterial biomass, chlorophyll a, \( \text{NH}_4^+ \) and \( \text{NO}_3^- \) concentration) is the best predictor, by a factor of 10, of the variability associated with \( nasA \) community structure.

The objectives of Chapter 5 were to utilize T-RFLP and SYBR Green real-time PCR techniques in conjunction with \(^{15}\text{N}\) tracer techniques to assess the seasonal variability of bacterial \( \text{NO}_3^- \) assimilation and bacterial nitrate assimilation genes (\( nasA \)) in the Skidaway River estuary. The hypothesis that, on a seasonal basis, populations of \( nasA \) containing bacteria are not randomly distributed among the total bacterioplankton community, and that the variability associated with the abundance and community structure of \( nasA \) communities is reflected in the magnitude of bacterial \(^{15}\text{NO}_3^-\) uptake is addressed. Compared to \( \text{NO}_3^- \) and \( \text{NH}_4^+ \) concentration and \( \text{NH}_4^+ \) uptake rate, \( \text{NO}_3^- \) uptake rate is the best predictor, by a factor of 15, of \( nasA \) community structure.

The findings of Chapters 4 and 5 that \( \text{NO}_3^- \) availability and patterns of \( \text{NO}_3^- \) utilization are positively correlated with \( nasA \) community structure variability and the
abundance of particular groups of \textit{nasA} genes, indicates that patterns of NO$_3^-$ supply, in the marine environment, are sufficiently important to be a factor in regulating bacterial communities. Considering the high energetic costs associated with NO$_3^-$ assimilation as well the fact that NO$_3^-$ is traditionally considered a resource that regulates autotrophic activity and production, the result that bacterioplankton community structure is positively correlated with NO$_3^-$ availability and NO$_3^-$ uptake is interesting. These exciting findings are only possible through a marriage of traditional chemical tracer techniques and modern molecular approaches targeted to the level of the functional gene. The most profound insights regarding the distribution and activity levels of heterotrophic bacterial nitrate assimilation genes are yet to come.
Literature Cited


41:1591-1609

Voytek MA, Priscu JC, Ward BB (1999) The distribution and relative abundance of
ammonium-oxidizing bacteria in lakes of the McMurdo Dry Valley, Antarctica.
Hydrobiologia 401:113-130

subclass of the class Proteobacteria in aquatic samples with the PCR. Appl. Environ.
Microbiol. 61:1444-1450

bacteria in Lake Bonney, Antarctica determined by immunofluorescence, PCR and in
situ hybridization. In: (eds) Ecosystem Dynamics in a Polar Dessert: the McMurdo
Dry Valleys, Antarctica. American Geophysical Union, , p 217-228

Ward BB (1996) Nitrification and denitrification: Probing the nitrogen cycle in aquatic

Wheeler PA, Kirchman DL (1986) Utililization of inorganic and organic nitrogen by
bacteria in marine systems. Limnol. Oceanogr. 31:998-1009

Williams PJ (1995) Evidence for the seasonal accumulation of carbon-rich dissolved
organic material, its scale in comparison with changes in particulate material and the
consequential effect on net C/N assimilation ratios. Marine Chemistry 51:17-29

microbial assemblages in Lake George, New York, detected by reverse transcriptase
PCR. Appl. Environ. Microbiol. 66:3119-3124
Zehr JP, Hiorns W (1998) Molecular approach to studies of the activities of marine 
Chapman and Hall, 

Zehr JP, McReynolds LA (1989) Use of degenerate oligonucleotides for amplification of 
the \textit{nifH} gene from the marine cyanobacterium \textit{Trichodesmium thiebaudi}. Appl. 
Environ. Microbiol. 55:2522-2526

Zehr JP, Mellon MT, Zani S (1998) New nitrogen-fixing microorganisms detected in 
oligotrophic oceans by amplification of nitrogenase (\textit{nifH}) genes. Appl. Environ. 
Microbiol. 64:3444-3450

and nitrogenase expression. In: Cooksey KE (eds) Molecular Approaches to the Study 
of the Ocean. Chapman and Hall, London, United Kingdom

Zehr JP, Voytek MA (1999) Molecular ecology of aquatic communities: reflections and 
future directions. Hydrobiologia 401:1-8

Zehr and Ward (2002) Nitrogen cycling in the ocean: New perspectives on processes and 
<table>
<thead>
<tr>
<th>Location</th>
<th>NH$_4^+$</th>
<th>NO$_3^-$</th>
<th>Comments</th>
<th>Ref.</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alberni Inlet</td>
<td>Na$^a$</td>
<td>Na</td>
<td>Bacterial NO$_3^-$ uptake suppressed production</td>
<td>Parker et al. (1975)</td>
<td>$^{14}$C production vs. NO$_3^-$ depletion</td>
</tr>
<tr>
<td>Saanich Inlet (glucose enrichment exp.)</td>
<td>Na</td>
<td>Na</td>
<td>Bacterial NO$_3^-$ uptake suppressed production</td>
<td>Parsons et al. (1980)</td>
<td>$^{14}$C production vs. NO$_3^-$ depletion</td>
</tr>
<tr>
<td>Georgia Coastal water</td>
<td>78</td>
<td>0</td>
<td>Bacteria could account for ~200 mg N m$^{-2}$ d$^{-1}$ on nitrate alone</td>
<td>Wheeler and Kirchman (1986)</td>
<td>Used $^{15}$N with prokaryotic inhibitors</td>
</tr>
<tr>
<td>Scripps Pier</td>
<td>Na</td>
<td>Na</td>
<td>Chlorophyll detected in &lt;1.0 size fraction</td>
<td>Horrigan et al. (1988)</td>
<td>2-stage continuous flow culture study</td>
</tr>
<tr>
<td>Georges Bank</td>
<td>38</td>
<td>27</td>
<td></td>
<td>Harrison and Wood (1988)</td>
<td>$^{15}$N size-fractionation and GFF filters</td>
</tr>
<tr>
<td>North Atlantic</td>
<td>Na</td>
<td>Na</td>
<td>DIN preferred to DON</td>
<td>Kirchman et al. (1991)</td>
<td>Batch incubations</td>
</tr>
<tr>
<td>Roosevelt Inlet</td>
<td>Na</td>
<td>Na</td>
<td></td>
<td>Kirchman et al. (1992)</td>
<td>Batch incubations</td>
</tr>
<tr>
<td>North Atlantic</td>
<td>22 - 36%</td>
<td>4 -14%</td>
<td>Spring bloom</td>
<td>Kirchman et al. (1994)</td>
<td>$^{15}$N size-fractionation and GFF filters</td>
</tr>
<tr>
<td>Barents Sea</td>
<td>53</td>
<td>48</td>
<td></td>
<td>Kristiansen et al. (1994)</td>
<td>$^{15}$N size-fractionation and GFF filters</td>
</tr>
<tr>
<td>Svalbard, Norway</td>
<td>Na</td>
<td>Na</td>
<td>Significant NO$_3^-$ uptake in sediments not attributable to denitrification</td>
<td>Blackburn et al. (1996)</td>
<td>Used $^{15}$N with prokaryotic inhibitors</td>
</tr>
<tr>
<td>Hudson River</td>
<td>Na</td>
<td>Na</td>
<td>Bacterially assimilated DIN 4-fold larger than phytoplankton</td>
<td>Caraco et al. (1998)</td>
<td>$^{15}$N studies</td>
</tr>
<tr>
<td>Subarctic Pacific</td>
<td>31</td>
<td>32</td>
<td></td>
<td>Kirchman and Wheeler (1998)</td>
<td>$^{15}$N size-fractionation and GFF filters</td>
</tr>
<tr>
<td>Gulf of Mexico</td>
<td>Na</td>
<td>Na</td>
<td>DIN preferred to DON</td>
<td>Jorgensen et al. (1999)</td>
<td>Batch incubations</td>
</tr>
<tr>
<td>Thames Estuary</td>
<td>82</td>
<td>66</td>
<td></td>
<td>Middelburg and Nieuwenhuize (2000)</td>
<td>Used $^{15}$N with prokaryotic inhibitors</td>
</tr>
<tr>
<td>Barents Sea</td>
<td>12-40</td>
<td>16-40</td>
<td></td>
<td>Allen et al. (accepted)</td>
<td>$^{15}$N size-fractionation and silver filters</td>
</tr>
</tbody>
</table>

Na$^a$, not applicable.
CHAPTER 2

DIVERSITY AND DETECTION OF NITRATE ASSIMILATION GENES IN MARINE BACTERIA

ABSTRACT

A PCR approach has been used to construct a database of nasA (called narB in cyanobacteria) genes and to detect the genetic potential for heterotrophic bacterial nitrate utilization in marine environments. A nasA-specific PCR primer set that could be used to selectively amplify the nasA gene from heterotrophic bacteria was designed. Using seawater DNA extracts from microbial communities in the South Atlantic Bight (SAB), the Barents Sea, and the North Pacific Gyre, nasA genes were PCR-amplified and sequenced. Results indicate that several groups of heterotrophic bacterial nasA genes are common and widely distributed in oceanic environments.
The importance of inorganic N (NH$_4^+$ or NO$_3^-$) for the nutrition and growth of marine phytoplankton has long been recognized (5, 7, 8) while the utilization of inorganic N by bacteria has historically received less attention (11, 13, 15, 17, 43). The primary role of heterotrophic bacteria is classically considered to be the decomposition and mineralization of dissolved and particulate organic nitrogen (DON and PON) (27). Bacterial NO$_3^-$ assimilation is not a pathway currently considered in pelagic carbon and nitrogen cycling models (1, 6, 10). A recent review of freshwater and marine studies, however, reports that bacteria may rely on both NH$_4^+$ and NO$_3^-$ for growth and biomass synthesis, and overall they may be significant consumers of inorganic N: mean values of 30% and 40% are reported for NH$_4^+$ and NO$_3^-$ respectively (14). Under certain conditions, such as high concentrations of DOC relative to DON, bacteria may be responsible for most, if not all, of the observed NO$_3^-$ uptake and disappearance (16, 24, 25). Significant heterotrophic bacterial utilization of DIN likely would have profound effects on the fluxes of N and C in the water column.

Bacterial nitrate utilization in aquatic communities, however, is difficult to study using conventional tracer approaches. Within the bacterial size class, autotrophic cyanobacteria (picoplankton) are often abundant (4, 41) and likely to complicate conclusions regarding the total flux of labeled nitrogen into the heterotrophic fraction of the bacterial community. Also, size fractionation does not allow for the examination of nitrate uptake by attached bacteria or large cells caught filters.

It is known that some, but not all, heterotrophic bacteria are capable of growth on NO$_3^-$ as a sole N source (28). In _K. pneumoniae_, the structural genes for nitrate assimilation form an operon, _nasFEDCBA_ (19-21). The NASC protein is thought to
mediate electron transfer from NADH to NASA, which contains the active site for nitrate reduction (19, 20). The NASA protein has also been purified and characterized from the phototrophic alpha proteobacterium, *Rhodobacter capsulatus* (2, 22). Examination of currently available prokaryotic genome sequences suggests the presence of *nasA* in a wide diversity of organisms, although these observations need verification (28).

Molecular techniques can be employed to illuminate factors, which control the rates of fluxes and transformations in nitrogen cycling processes (40, 46, 47, 51). Molecular approaches have been successfully used to detect and characterize bacteria and the genes important in several aspects of the nitrogen cycle including nitrification, denitrification, and nitrogen fixation (18, 29, 30, 37-39, 45, 48-50).

Here we report the design and optimization of a series of nested heterotrophic bacteria-specific *nasA* PCR primers. The detection of *nasA* genes in a variety of marine environments provides a basis for the hypothesis that the potential for NO$_3^-$ utilization by heterotrophic bacteria is significant. Phylogenetic analysis of *nasA* genes cloned from diverse samples indicates the presence of several distinct clades and suggests a clear genetic distinction between *nasA* genes from heterotrophic bacteria and autotrophic cyanobacteria.

Initially, a set of three nested universal degenerate *nasA* primers were designed based on five previously sequenced sequences from cyanobacteria and one sequence from a heterotrophic bacterium (3, 12, 20, 23, 34). The sequences from cyanobacterial strains were from *Oscillatoria chalybea*, *Anabaena* sp. strain PCC7120, *Synechocystis* sp. strain PCC6803, *Synechococcus* sp., and *Synechococcus* sp. strain 7942, and the sequence from a heterotrophic strain was from *Klebsiella oxytoca*. GenBank accession
numbers for these sequences are X89445, L49163, BAA17488, CAA52675, P39458, L06800, respectively. An alignment of the nasA inferred amino acid sequences indicated conserved regions suitable for targeting PCR oligonucleotide primers. These primers were used to amplify nasA sequences in other heterotrophic bacteria, and a group-specific degenerate primer was designed to specifically amplify the nasA gene from heterotrophic bacteria. All primers used in this study are listed in Table 2.1.

Surface water samples were collected during 2 cruises in the South Atlantic Bight (SAB) off the Georgia coast aboard the R/V Bluefin during October 1998 and R/V Cape Hatteras during March 1999 (31-33°N 78-81°W). SAB samples were also collected from docks located on the Skidaway (March 1999) and Wilmington Rivers (July 1998). Additional water samples used in this study were collected from 5m, 30m, and 80m in the Barents Sea (70-78°N 30°E) aboard the R/V Jan Mayen during July 1999, and from the surface of the North Pacific Gyre at Hawaii Ocean Time Series (HOTS) stations (22°45’N 158°W) during May 1997 and aboard the R/V Melville during June 1999. For DNA extraction, bacteria from 40 L of water were collected. To remove eukaryotic plankton, water was consecutively prefilted, under vacuum, through a 3-μm-pore-size polycarbonate cartridge filter (Gelman Sciences Inc., Ann Arbor, MI) and a 142 mm-diameter 0.8-μm-pore-size polycarbonate Supor filter (Gelman) with a custom manufactured acrylic filter holder. Bacterial cells in the filtrate were collected onto a 142 mm-diameter 0.2-μm-pore-size polycarbonate Supor filter (Gelman) and stored at -20°C aboard the ship and transferred to -80°C in the lab. DNA from the SAB samples collected in October 1998 was extracted according to Gonzalez et al.1996 (9), and all other samples were extracted using the UltraClean™ Soil DNA Kit Mega Prep (Mo Bio
Laboratories Inc., Solana Beach, CA). For the latter technique, frozen filters were crushed inside of Whirl-Pak® bags (Nasco, Fort Atkinson, WI) and put directly into the lysing matrix of step one of the soil DNA extraction procedure. Visualization of purified DNA by gel electrophoresis revealed the presence of high molecular weight DNA with little shearing and no RNA contamination. From 40 L of seawater, this method typically yielded an average of 100-110 µg of DNA. Assuming an average concentration of 10^6 cells/ml of bacteria in seawater and an average DNA content of 3 fg/cell (31), the approximate extraction efficiency of this method was between 80 and 90%.

PCR was performed in a nested format to improve specificity and sensitivity. The PCR products of the outermost degenerate nasA/narB universal primers (nas22, nas1933) were subsequently used as templates in PCR reactions with the heterotrophic-specific internal primer set (nas964, nasA1735). Amplification was accomplished using the Qiagen Taq PCR Master Mix System following the standard protocol recommended by the vendor (Qiagen, Valencia, CA) with 35 cycles (94°C, 5 seconds; 55.5°C, 10 seconds; 72°C, 1 minute) initiated after a hot start at 94°C for 5 minutes and followed by a 7 minute final extension step at 72°C. DNA template (10-100 ng of community DNA or 0.1-10 ng of genomic DNA from a pure culture) was added per 25-µl PCR reaction. First round reactions contained 1 µM (each) primer (nas22 and nas1933) and 3.5 mM MgCl2. Round two of nested PCR contained 1-2 µl of product from the first round, 2.5 mM MgCl2, 1 µM (each) primer (nas964 and nasA1735), and the extension time in each cycle is cut to 30 seconds. The nasA-specific primers yielded a PCR product that was 750 – 800 bp. 16S rRNA amplification of the nearly complete 16S rRNA gene was facilitated using eubacterial primers fd1 and rp2 (Table 2.1) (42) at 100nM each.
Thermal cycling was performed using a 2400 or 9700 Perkin-Elmer thermal cycle (Perkin-Elmer Corp., Norwalk, Conn.).

Although nasA PCR reactions could be optimized for specific community DNA samples by raising the annealing temperature to 57 - 60°C, the somewhat less stringent annealing temperature of 55°C was used in the initial construction of clone libraries to increase yield and the likelihood of amplification between most primer template combinations.

The desired sized PCR product was excised from the gel and purified using GenElute™ Agarose Spin Columns (Supelco, Bellefonte, PA). PCR products were ligated and cloned using either the TOPO™ TA Cloning® Kit (for pure cultures) or the Original TA Cloning® Kit (for community DNA samples). In both cases, the PCR product was ligated into a pCR 2.1 plasmid vector and cloned into TOP10 One Shot competent E. coli cells (Invitrogen, Carlsbad, CA). Plasmid DNA was extracted and purified using the Wizard® Plus Minipreps DNA purification system (Promega, Madison, WI).

Sequences were determined by automated sequencing at the Molecular Genetics Facility (University of Georgia) using an ABI automated sequencer (models 373 and 377). Sequencing reactions were facilitated using the ABI Big Dye prism dideoxy sequencing dye terminator kit following manufacturer protocols. Sequence analysis was accomplished using ABI software version 3.3 (ABI, Foster City, Calif.). Sequencing primers are listed in Table 2.1.

Bacteria were isolated from seawater samples collected from the SAB continental shelf during March and June 1999 (31-33°N 78-81°W). Bacteria were also
isolated from Barents Sea water samples collected during July 1999 (70-80°N 30°E). Bacteria were isolated on either organic nitrogen or nitrate as the sole nitrogen source. Selected colonies were axenically transferred to new plates twice to ensure that pure cultures were obtained. For long-term storage each isolate strain was maintained in a 15% glycerol freezer stock (v.v) at -80°C.

To screen isolated strains for the presence of nasA, PCR-amenable DNA was extracted from each of the isolates following the Bio 101 FastDNA® spin protocol using the Bio 101 Fast Prep Instrument (Bio 101, Vista, CA). In all PCR reactions, appropriate negative controls without DNA and positive controls were included.

To test isolates for the capability of growth on nitrate as the sole N source, two tubes containing 5 mls NFG media (33) were prepared. One of the tubes received an aseptic addition of a sterile NaNO₃ solution for a final concentration of 10 mM NO₃⁻. The second tube did not receive such an addition and served as a negative control. Each of the two NFG tubes were then inoculated with a 1:100 addition of stationary phase culture grown in peptone-and yeast-enriched ASW (26). After 72 hours, the optical density of the two tubes was compared to the optical density of a NFG tube that did not receive an inoculation. Additionally, several isolates were selected for batch culture growth assays. These experiments were conducted in axenic 100 ml cultures of NFG media containing NO₃⁻ at 80 μM or 10 mM as the sole N source. Doubling rates were determined by estimating cell density at a minimum of four time points during exponential growth phase growth. Cell densities were determined by direct epiflourescent microscopy after staining with DAPI (44).
Phylogenetic relationships based on \textit{nasA} gene sequences were determined. Nucleotide sequences were translated to approximately 264 unambiguous amino acid positions. All of the available \textit{narB/nasA} amino acid sequences were then aligned using the CLUSTAL W version 1.7 multiple sequence alignment algorithm (32). Phylogenetic trees were inferred and drawn using the TREECON software package version 1.3b (35, 36), using the Kimura two-parameter model for inferring evolutionary distance. Bootstrap estimates (100 replicates) of confidence intervals were also made using the algorithms available in the TREECON package.

For 16S rRNA analysis, 464 unambiguously alignable nucleotide positions were used. The nucleotide sequences were compared to 16S rRNA gene sequences available in GenBank using the “Blast” program to determine the degree of sequence similarity to known organisms. All \textit{nasA} and 16S rRNA sequences determined in this study have been submitted to GenBank. GenBank accession numbers are listed in Table 2.2.

\textbf{Results.} Using the universal \textit{nasA} nested primer set, a fragment of 1000 bp was amplified, cloned and sequenced from a group of phylogenetically diverse bacteria including (i) \textit{Clostridium oceanica}, (ii) \textit{Vibrio diazotrophicus}, (iii) \textit{Pseudomonas sp.}, (iv) \textit{Trichodesmium IMS}, (v) \textit{Fischerella sp.}, (vi) \textit{Plectonema boryanum}, and (vii) DNA extracted from the bacterial size fraction of seawater collected at the HOTS station near Hawaii. Also, we attempted to amplify the 1000 bp \textit{nasA} fragment from \textit{Bacillus sp.}, \textit{Micrococcus luteus}, \textit{Vibrio strain S-14}, and \textit{Pseudomonas stutzeri}. These templates, however, did not yield a PCR product and were concluded to be \textit{nasA} negative (Table 2.2).
Using the expanded database of *nasA* sequences, an additional reverse primer, at amino acid residue position 579, was targeted to the heterotrophic organisms. The heterotrophic-specific primer is *nasA*1735 (Table 2.1), and is approximately 200 bp downstream from the universal *nasA* reverse primer.

Using a collection of isolates obtained during a cruise in the Barents Sea, the relationship between the presence of the *nasA* gene and the phenotype (the ability to utilize NO$_3^-$ as a sole N source during aerobic growth) was explored. Out of the 30 isolates screened, 17 were able to grow using NITRATE as a sole N source. All of these strains were PCR positive for the *nasA* gene fragment. Thirteen of the isolates screened could not grow on NO$_3^-$ only and each of these strains did not contain a *nasA* gene fragment. Three isolates from South Atlantic Bight waters and three from the Skidaway River Estuary, Georgia were also screened. Of these six isolates, two were NO$_3^-$ growth positive and *nasA* PCR positive, two were NO$_3^-$ growth negative and *nasA* PCR negative, and two were NO$_3^-$ growth negative and *nasA* PCR positive. Therefore, of 36 isolates examined, 19 were PCR positive for *nasA* and demonstrated the ability to utilize NO$_3^-$ as a sole N source, 15 were PCR negative for *nasA* and did not exhibit the ability to utilize NO$_3^-$, and two displayed the somewhat contradictory result of being PCR positive for *nasA* while apparently not being able to grow on NO$_3^-$ as a sole N source (Table 2.3).

Results of the batch growth assays indicated some degree of variability between isolates in their affinity for NO$_3^-$ (Table 2.3). Data is only reported for experiments conducted at 10 mM NO$_3^-$ . Experiments conducted at 80 µM generated similar doubling times for the different strains tested but resulted in lower final cell yields.
The presence of *nasA* was detected in all environments examined including the South Atlantic Bight, the North Pacific Gyre, a Norwegian Fjord, and the Barents Sea (Figure 2.1). The sensitivity of *nasA* detection by PCR was initially estimated by amending filtered seawater with 10⁵ and 10² cells/ml of *Klebsiella pneumoniae* and detecting *nasA* in those samples (Figure 2.1, only 10⁵ shown). This approach did not establish a minimum level of detection, but demonstrated that a concentration of at least 10² cells/ml positive for the *nasA* gene could be detected. Since a strong signal was detected in a wide range of marine samples, heterotrophic bacteria with the capacity for NO₃⁻ assimilation appear to be very common and well distributed.

In general, *nasA* genes from uncultured organisms do not form separate clades from cultured bacteria. Also, *nasA* genes in taxonomically related bacteria are not necessarily similar, except in the case of *Vibrio* representatives. *Vibrio diazotrophicus*, and two *Vibrio* isolates form a separate clade which includes seven clones from samples collected in the Skidaway River and the South Atlantic Bight (SAB) – mid-shelf (35 miles offshore). *Klebsiella pneumoniae* ATCC 13883 and a *Pseudoalteromonas* isolate typify another clade, which includes three clones from SAB mid-shelf samples. A third discrete cluster includes *Clostridium oceanica*, *Klebsiella oxytoca*, *Pseudomonas sp.*, and a *Marinobacter sp.* isolate. Also in this cluster are clones from the mid-shelf (25 and 35 miles offshore), the Skidaway River, and the adjacent Wilmington River (Figure 2.2).

Among the nitrate-assimilating (*nasA* positive) and non nitrate-assimilating (*nasA* negative) isolates for which the 16S rRNA gene has been sequenced, there are more different types of taxa associated with the *nasA* negative strains. For example, *nasA* negative isolates include organisms such as *Micrococcus sp.* (Gram positive phylum,
high G + C subdivision), *Erythrobacter sp.* (Alpha proteobacteria), *Aerococcus sp.* (Gram positive phylum, low G + C subdivision), *Sagittula stellata* E37 (Alpha proteobacteria), and *Pseudoalteromonas sp.* (Gamma proteobacteria) (Table 2.2). By contrast, the majority of the *nasA* positive strains for which 16S rRNA has been sequenced are Gamma proteobacteria. In particular, members of the *Pseudoalteromonas sp.* and *Vibrio sp.* account for 5 out of 7 of the *nasA* positive strains that were isolated.

**Conclusions.** The correlation between the presence of *nasA* and nitrate utilization assays on individual isolates (34 out of 36 isolates tested) supports the hypothesis that the *nasA*-specific primer sets developed in this study provide a reliable assay for a functional assimilatory nitrate reductase genes. Sequences derived from isolates that were unable to utilize NO$_3^-$ in culture (Table 2.3) are more related to dehydrogenases and other gene families and can be distinguished phylogenetically from functional assimilatory nitrate reductases (Figure 2.2). This illustrates the fact that, although degenerate primers are powerful in their ability to retrieve gene fragments from a wide diversity of organisms, it is important to sequence and phylogenetically analyze PCR products from as many different types of organisms as possible in order to identify potential non-specific PCR products.

We have demonstrated that genetic probes can be constructed to recognize the functional assimilatory nitrate reductase genes of specific groups of bacteria. Heterotrophic *nasA* genes have been detected in every marine sample tested thus far indicating that bacteria capable of assimilating nitrate are ubiquitous in diverse ocean margins and open waters. These observations suggest that heterotrophic bacteria are potentially important consumers of NO$_3^-$ in marine environments.
ACKNOWLEDGMENTS

For providing ship time, we thank G. P. Paffenhoffer, D. Bronk, J. Bower, and P. Wassman. We thank the crews of the R/V Bluefin, the R/V Hatteras and the R/V Jan Mayen. We also thank M. A. Moran for donating bacterial strains. Thanks to H. Howard-Jones for microscopy help, and we thank S. McIntosh and A. Boyette for preparing figures, and we thank Dee Peterson for preparing this manuscript.

This research was supported by the U.S. Department of Energy, DOE grants DE-FG02-88ER62531 and DE-FG02-98ER62531.

REFERENCES


Figure legends

Figure 2.1. PCR amplification of the expected 750-800 bp nasA gene fragment from various marine samples. The second lane is a PCR product from a sample that was prepared by amending filtered seawater to a final concentration of 10^3 cells/ml of *Klebsiella pneumoniae*. The remaining nasA PCR products were amplified in samples collected from a Norwegian Fjord (lane 3), the Barents Sea (lanes 4-6), the Skidaway River (lanes 7,8), the South Atlantic Bight (lane 9,10), and the North Pacific Gyre (lane 11,12). The PCR products are shown in relation to a molecular weight size standard PCR marker (Promega, Madison, WI).

Figure 2.2. Inferred phylogenetic relationship between nasA and narB amino acid sequences from heterotrophic and cyanobacteria respectively. The scale bar indicates 0.05 fixed amino acid substitutions per site. Numbers refer to bootstrap values for each node. Bootstrap values below 70 (out of 100) are not shown. The amino acid sequence of Formate Dehydrogenase from *Methanobacterium thermoautotrophicum*, GenBank accession number U52681, a putative evolutionary ancestor of the nasA/narB, genes was used to root the tree.
Table 2.1. Oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Amino acid sequence</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>nas22</td>
<td>TGYCCNTAYTGYYGGNGT</td>
<td>CPYCG</td>
<td>nasA/narB PCR amplification</td>
</tr>
<tr>
<td>nas964</td>
<td>CARCCNAAYGCNATGGG</td>
<td>QPNAM</td>
<td>nasA/narB PCR amplification</td>
</tr>
<tr>
<td>nasA1735</td>
<td>ATNGTRTGCCAYTGRTC</td>
<td>DQWHT</td>
<td>nasA PCR amplification</td>
</tr>
<tr>
<td>nas1933</td>
<td>CARTGCATNGGNAYRAA</td>
<td>F/L V/I/M PMH</td>
<td>nasA PCR amplification</td>
</tr>
<tr>
<td>M13F</td>
<td>GTAAACGACACGGCACG</td>
<td></td>
<td>Forward sequencing primer  - All</td>
</tr>
<tr>
<td>522F</td>
<td>CAGCGCGGTAATAC</td>
<td></td>
<td>Forward sequencing primer  - 16S</td>
</tr>
<tr>
<td>1056F</td>
<td>TGGCTGTCGCAGCTCGTGT</td>
<td></td>
<td>Forward sequencing primer  - 16S</td>
</tr>
<tr>
<td>M13R</td>
<td>CAGGAACAGCTATGAC</td>
<td></td>
<td>Reverse sequencing primer  - All</td>
</tr>
<tr>
<td>1056R</td>
<td>ACACGAGCTGACAGCACAGCA</td>
<td></td>
<td>Reverse sequencing primer  - 16S</td>
</tr>
<tr>
<td>522R</td>
<td>GTATTACCGGGCCTG</td>
<td></td>
<td>Reverse sequencing primer  - 16S</td>
</tr>
</tbody>
</table>
Table 2.2. Strains used for this study and 16S rRNA accession numbers

<table>
<thead>
<tr>
<th>Strain</th>
<th>Closest Relative</th>
<th>nasA PCR (+)/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Known Cultures</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Fischerella</em> sp. A</td>
<td>na C</td>
<td>+</td>
</tr>
<tr>
<td><em>Plectonema boryanum</em> A</td>
<td>na</td>
<td>+</td>
</tr>
<tr>
<td><em>Trichodesmium</em> sp. strain IMS101 A</td>
<td>na</td>
<td>+</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> ATCC 13883 A</td>
<td>na</td>
<td>+</td>
</tr>
<tr>
<td><em>Clostridium</em> oceanica A</td>
<td>na</td>
<td>+</td>
</tr>
<tr>
<td><em>Pseudomonas</em> sp. A</td>
<td>na</td>
<td>+</td>
</tr>
<tr>
<td><em>Vibrio diazotrophicus</em> A</td>
<td>na</td>
<td>+</td>
</tr>
<tr>
<td><em>Bacillus</em> sp.</td>
<td>na</td>
<td>-</td>
</tr>
<tr>
<td><em>Micrococcus leutens</em></td>
<td>na</td>
<td>-</td>
</tr>
<tr>
<td><em>Vibrio</em> S-14</td>
<td>na</td>
<td>-</td>
</tr>
<tr>
<td><em>Pseudomonas</em> stutzeri</td>
<td>na</td>
<td>-</td>
</tr>
<tr>
<td><em>Sagittula</em> stellata E37</td>
<td>na</td>
<td>-</td>
</tr>
<tr>
<td>Strains isolated in this study D</td>
<td></td>
<td></td>
</tr>
<tr>
<td>South Atlantic Bight(A) B</td>
<td><em>Cytophaga</em> sp.</td>
<td>+</td>
</tr>
<tr>
<td>South Atlantic Bight(B)</td>
<td><em>Aerococcus</em> viridans</td>
<td>-</td>
</tr>
<tr>
<td>South Atlantic Bight(C) B</td>
<td><em>Alteromonas</em> sp. strain MS23</td>
<td>+</td>
</tr>
<tr>
<td>Barents Sea Isolate #4 B</td>
<td><em>Pseudoalteromonas</em> sp. strain ANG.ro2</td>
<td>+</td>
</tr>
<tr>
<td>Barents Sea Isolate #10 B</td>
<td><em>Marinobacter</em> sp. strain DS40M8</td>
<td>+</td>
</tr>
<tr>
<td>Barents Sea Isolate #23</td>
<td><em>Pseudoalteromonas</em> haloplanktis</td>
<td>-</td>
</tr>
<tr>
<td>Barents Sea Isolate #25 B</td>
<td><em>Pseudoalteromonas</em> citrea</td>
<td>+</td>
</tr>
<tr>
<td>Barents Sea Isolate #32</td>
<td><em>Erythrobacter</em> citreus</td>
<td>-</td>
</tr>
<tr>
<td>Skidaway River #1 B</td>
<td><em>Vibrio</em> carchariae</td>
<td>+</td>
</tr>
<tr>
<td>Skidaway River #2 B</td>
<td><em>Vibrio</em> furnissii (ATCC 35016 T)</td>
<td>+</td>
</tr>
<tr>
<td>Skidaway River #3</td>
<td><em>Micrococcus luteus</em> strain FO-084a</td>
<td>-</td>
</tr>
</tbody>
</table>

A Sequence amplified in this study by using oligonucleotide primers nas964 and nas1934.

B Sequence amplified in this study by using oligonucleotide primers nas964 and nas1735.

C Not applicable.

D 16S rRNA accession numbers for the eleven isolated strains shown here are AF300973-AF300983.
Table 2.3. Comparison of the growth capability of bacterial isolates on sole nitrate media and the detection of \textit{nasA} PCR gene product.

<table>
<thead>
<tr>
<th>Isolate Location</th>
<th>Growth on NO$_3^-$ as sole Nitrogen source</th>
<th>nasA PCR (+/-)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Barents Sea</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Barents Sea Isolate #4</td>
<td>Y$^a$</td>
<td>+</td>
</tr>
<tr>
<td>Barents Sea Isolate #10</td>
<td>Y$^a$</td>
<td>+</td>
</tr>
<tr>
<td>Barents Sea Isolate #23</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>Barents Sea Isolate #25</td>
<td>Y$^a$</td>
<td>+</td>
</tr>
<tr>
<td>Barents Sea Isolate #32</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>Barents Sea Isolate #1</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>Barents Sea Isolate #2</td>
<td>Y</td>
<td>+</td>
</tr>
<tr>
<td>Barents Sea Isolate #3</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>Barents Sea Isolate #5</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>Barents Sea Isolate #6</td>
<td>Y</td>
<td>+</td>
</tr>
<tr>
<td>Barents Sea Isolate #7</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>Barents Sea Isolate #8</td>
<td>Y</td>
<td>+</td>
</tr>
<tr>
<td>Barents Sea Isolate #9</td>
<td>Y</td>
<td>+</td>
</tr>
<tr>
<td>Barents Sea Isolate #12</td>
<td>Y</td>
<td>+</td>
</tr>
<tr>
<td>Barents Sea Isolate #13</td>
<td>Y</td>
<td>+</td>
</tr>
<tr>
<td>Barents Sea Isolate #14</td>
<td>Y</td>
<td>+</td>
</tr>
<tr>
<td>Barents Sea Isolate #15</td>
<td>Y</td>
<td>+</td>
</tr>
<tr>
<td>Barents Sea Isolate #16</td>
<td>Y</td>
<td>+</td>
</tr>
<tr>
<td>Barents Sea Isolate #18</td>
<td>Y</td>
<td>+</td>
</tr>
<tr>
<td>Barents Sea Isolate #19</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>Barents Sea Isolate #20</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>Barents Sea Isolate #21</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>Barents Sea Isolate #22</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>Barents Sea Isolate #24</td>
<td>Y</td>
<td>+</td>
</tr>
<tr>
<td>Barents Sea Isolate #26</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>Barents Sea Isolate #27</td>
<td>Y</td>
<td>+</td>
</tr>
<tr>
<td>Barents Sea Isolate #28</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>Barents Sea Isolate #29</td>
<td>Y</td>
<td>+</td>
</tr>
<tr>
<td>Barents Sea Isolate #30</td>
<td>Y</td>
<td>+</td>
</tr>
<tr>
<td>Barents Sea Isolate #31</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td><strong>South Atlantic Bight</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>South Atlantic Bight(A)</td>
<td>N</td>
<td>+</td>
</tr>
<tr>
<td>South Atlantic Bight(B)</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>South Atlantic Bight(C)</td>
<td>N</td>
<td>+</td>
</tr>
<tr>
<td>Skidaway River #1</td>
<td>Y</td>
<td>+</td>
</tr>
<tr>
<td>Skidaway River #2</td>
<td>Y</td>
<td>+</td>
</tr>
<tr>
<td>Skidaway River #3</td>
<td>N</td>
<td>-</td>
</tr>
</tbody>
</table>

$^a$The doubling time of Barents Sea isolates 4, 10, and 25 on 10mM NO$_3^-$ as the sole N source is 3.78, 5.48, and 5.16 hours, respectively.
Fig. 2.2

Heterotrophic Bacteria
Cyanobacteria

Methanobacterium thermoautotrophicum (Formate Dehydrogenase)
CHAPTER 3

IMPORTANCE OF HETEROTROPHIC BACTERIAL ASSIMILATION OF AMMONIUM AND NITRATE IN THE BARENTS SEA DURING SUMMER

Abstract

In a transect across the Barents Sea into the marginal ice zone (MIZ), five 24 hour experimental stations were visited, uptake rates of NH$_4^+$ and NO$_3^-$ by bacteria was measured at five stations along with their contribution to total dissolved inorganic nitrogen (DIN) assimilation. The percent bacterial DIN uptake of total DIN uptake increased substantially from 10% in open Atlantic waters to 40% in the MIZ. The percentage of DIN that accounted for total bacterial nitrogen production also increased from south to north across the transect. On average, at each of the five 24 hour stations, bacteria accounted for 16 - 40% of the total NO$_3^-$ uptake and 12 - 40% of the total NH$_4^+$ uptake. As a function of depth, bacteria accounted for 17, 23, and 26% of the total NH$_4^+$ assimilation and 17, 37, and 36% of the total NO$_3^-$ assimilation at 5m, 30m, and 80m, respectively. Bacteria accounted for a higher percentage of total NO$_3^-$ uptake compared to total NH$_4^+$ uptake in 12 out of 15 samples. Bacterial productivity explains a substantial amount of the variability associated with bacterial DIN uptake, but the relationship between bacterial production and bacterial DIN uptake is best explained when the data from the open Atlantic water stations are grouped separately from the MIZ stations. The percentage of DIN that accounts for bacterial N production is approximately four-fold higher in 24 hour MIZ stations compared with open Atlantic stations. This suggests that bacteria play a larger role in NO$_3^-$ utilization, particularly in the MIZ, than previously hypothesized and that bacterial uptake of NO$_3^-$ should not be ignored in estimates of new production. Understanding processes that affect autotrophic based new production, such as heterotrophic bacterial utilization of NO$_3^-$, in polar oceans...
is of particular significance because of the role these regions may play in sequestering CO₂.

1. Introduction

The primary role of heterotrophic bacteria is classically considered to be the decomposition and mineralization of dissolved and particulate organic nitrogen (DON and PON) (Pomeroy 1974). The role of heterotrophic bacteria in the consumption of a significant fraction of the total NO₃⁻ or NH₄⁺ flux in marine environments is seldom considered in pelagic carbon and nitrogen cycling models (Fasham et al. 1990; Bissett et al. 1999; Haupt et al. 1999).

Several studies have documented high rates of NO₃⁻ utilization by bacteria (Parker et al. 1975; Parsons et al. 1980; Horrigan et al. 1988; Kirchman et al. 1991), but only a few have attempted to measure bacterial inorganic nitrogen utilization directly by separating the bacterial size fraction after incubation in the presence ¹⁵N tracers (Harrison and Wood 1988; Probyn 1990; Kirchman 1994; Kirchman and Wheeler 1998). One such study in the Barents Sea estimated that 53 and 48% of NH₄⁺ and NO₃⁻, respectively, was attributable to organisms <0.8 µm (Kristiansen et al. 1994). A recent review of marine studies that have measured NH₄⁺ and NO₃⁻ uptake by heterotrophic bacteria reports that bacteria account for 42 and 16% of NH₄⁺ and NO₃⁻ uptake, respectively (Kirchman 2000).

Significant heterotrophic bacterial utilization of dissolved inorganic nitrogen (DIN) would have profound effects on the fluxes of N and C in the water column (Kirchman et al. 1992; Kirchman 1994). In particular, NO₃⁻ uptake by heterotrophic
bacteria is important because of its potential impact on estimates of new production and on the relationship between new production and carbon export out of the euphotic zone (Legendre and Grosselin 1989; Kirchman 2000). The magnitude of the vertical flux is thought to be determined by new production, and biogenic matter vertical flux dynamics in the Barents Sea has been investigated previously (Wassmann et al. 1990; Wassmann et al. 1993; Andreassen et al. 1996; Andreassen et al. 1999).

The traditional view of the marine nitrogen cycle holds that the downward flux of particulate nitrogen would have to equal $\text{NO}_3^-$ uptake at steady state regardless of which group of microbes is using the $\text{NO}_3^-$ (Bronk et al. 1994; Kirchman 2000). What is not appreciated or understood, however, is the extent to which bacteria might cause the system to deviate from steady state or to at least complicate this picture by substantial utilization of $\text{NO}_3^-$ in the euphotic zone. $\text{NO}_3^-$ uptake by bacteria could potentially uncouple new and export production by directing more carbon into the microbial loop where more trophic transfers are required to produce large amounts of sinking particles (Kirchman 2000).

Understanding the variability and magnitude of new production in the polar regions is of particular interest because of the role these regions may play in sequestering $\text{CO}_2$ (Sarmiento and Toggweiler 1984; Walsh 1989; Erickson et al. 1990). Studies in the Northern Bering Sea, the Chukchi Sea, and the Barents Sea have measured some of the highest primary production rates in the world’s oceans (Sambrotto et al. 1984; Olsson et al. 1999; Luchetta et al. 2000). Although there is considerable disagreement over the role of bacteria in these cold waters (Pomeroy and Deibel 1986; Thingstad and Martinussen 1991), Arctic bacterioplankton appear to be important consumers of dissolved organic
matter (Cota et al. 1990; Rivkin et al. 1996; Steward et al. 1996), but their contribution to total DIN utilization remains unclear.

Kirchman et al. (1989) found that about 50% of heterotrophic bacteria from subarctic waters will pass through GF/F filters (nominal pore size 0.7 μm). The purpose of this study was to more accurately estimate bacterial and eukaryotic uptake by using 0.8 μm and 0.2 μm silver membrane filters (see methods section). The rate and amount of DIN utilized by bacterioplankton was determined along a transect that began in the Southern Barents Sea, crossed the Polar Front, and ended in the MIZ dominated by Arctic water.

2. Materials and Methods

Experiments were conducted during a cruise (ALV-3) aboard the R/V Jan Mayen in July 1999. Five stations (Fig. 3.1) were sampled over a 24 hour period along a transect beginning in 80% ice cover on July 1 (78º 13.67′ N, 34º 23.02′ E) and ending in the southern Barents Sea on July 9 (73º 47.99′ N, 31º 44.10′ E). The stations are referred to as Stations I-V from south to north. Water samples were collected using 10 L Niskin bottles.

2.1 Uptake of Inorganic Nitrogen Experiments
At each station, $^{15}$N tracer techniques were used to estimate uptake rates of NO$_3^-$ and NH$_4^+$ into the $>$0.8 µm and $<$0.8 µm size-fractions at 5m, 30m and 80m. Nutrient concentrations from the literature were used to estimate $^{15}$N additions that would yield approximately 10% enrichment over ambient levels. For NO$_3^-$ and NH$_4^+$, the enrichments were 0.01, 0.1, and 0.6 µM and 0.01, 0.01, and 1 µM at 5m, 30m, and 80m respectively. For the most part, target enrichment levels were achieved to within 1.0 µM. At no time, however, did the $^{15}$N additions result in enrichments of over 10% of ambient NH$_4^+$ or NO$_3^-$ concentrations.

Incubations were done in 1 l polycarbonate bottles in on-deck flow-through incubators under simulated in situ light and temperature conditions. Whole water (unfractionated) and fractionated treatments for NO$_3^-$ and NH$_4^+$ uptake experiments were prepared from samples collected at each depth. The fractionated treatment consisted of water that was gently filtered (<5 in.Hg) through a 1.0 µm filter prior to incubation. Incubations lasted 3 hrs. in order to minimize the risk of substrate depletion.

The incubations were terminated by passing the whole-water samples through a 0.8 µm silver filter (Osmonics Inc., Minnetonka, MN) to collect the $^{15}$N labeled particulate nitrogen in the $>$0.8 µm size class. The filtrate from these samples was collected and subsequently passed through a 0.2 µm silver filter to measure the $^{15}$N labeled particulate nitrogen in the bacterial size class. In the case of the pre-incubation 1.0 µm filtered treatments, the incubation was terminated by passing the sample through a 0.2 µm silver filter. The purpose of measuring $^{15}$N uptake in filtered and whole water treatments was to compare bacterial uptake in the presence of grazing and nutrient regeneration (the whole water treatments) and in samples where these processes were not
occurring (the 1.0 µm fractionated treatments). Filters were immediately frozen at -20ºC onboard the ship. Upon returning to the lab, filters were dried at 50ºC, ampulated and analyzed by mass spectrometry using a Europa GEO 20/20 with a ANCA prep. unit. Uptake rates were calculated as described by Bronk (1998).

2.2 Incorporation of $^{3}$[H]-Leucine

Rates of incorporation were determined by modifications of the micro-centrifugation method for $^{3}$[H]-leucine uptake (Sherr et al. 1999, Smith and Azam 1992). Triplicate water samples were incubated with leucine (final concentration 20 nM) from one to three hours at in situ temperatures in a flow through incubator on board the ship. For each sample, 1.7 ml aliquots were pipetted into 2 ml micro-centrifuge tubes. A fourth aliquot in each set, a kill sample, was amended with 190 µl of 50% TCA as a control for abiotic uptake. After incubation, leucine incorporation was terminated by adding 190 µl of 50% TCA to each of the live replicates. All of the samples (kill and live) were processed on board the ship immediately, and stored in 1.5 ml scintillation cocktail (EcoScint, National Diagnostics) at 4ºC for the duration of the cruise. Upon returning to the laboratory, the micro-centrifuge tubes containing the samples were placed in 20 ml scintillation vials and activity was determined with a scintillation counter.

Killed controls were subtracted from the average of triplicate live DPM values after values for molar incorporation rates of leucine were determined. Incorporation rates of leucine were converted to bacterial production assuming 1.15 x 10$^{17}$ cells mol$^{-1}$, which is the mean of all open oceanic studies (Ducklow and Carlson 1992; Kirchman 1992). Bacterial carbon content for Barents Sea bacterioplankton was assumed to be 15 fg C
These two assumptions lead to a 1.725 kg C mol\(^{-1}\) conversion factor, which results from assuming no isotope dilution, 7.3% leucine in protein, and that protein is 61.8% of total cellular carbon (Simon and Azam 1989). Prior work in cold-water environments (Carlson et al. 1999; Fagerbakke et al. 1996) has determined that the more commonly used 3.1 kg C mol\(^{-1}\) conversion factor (Simon and Azam, 1989) may overestimate production in these waters. Our conversions of leucine incorporation rates lead to lower production rates and therefore are likely to provide conservative estimates of heterotrophic bacteria productivity. Biomass production was expressed in N units by assuming 0.25 N/C ratio by weight for Barents Sea bacterioplankton (Fagerbakke et al. 1996).

### 2.3 Seawater Culture Enrichment Experiments

Two batch experiments were conducted to determine whether DIN limited bacterioplankton production in two different water masses. Batch experiment (1) was initiated with seawater collected at a coastal fjord station and batch experiment (2) was prepared with seawater collected at 24 hour station IV in the MIZ. Seawater for batch experiments was collected from the surface with a bucket. Seawater cultures were prepared by inoculating 0.22 µm filter sterilized seawater with a 0.8 µm filtered inoculum at a dilution of 10%. Experiments were conducted in 2 l Teflon bottles. Each experiment consisted of eight bottles: two controls (no nutrient amendment), a 2 µM NH\(_4^+\) amendment, a 2 µM NO\(_3^-\) amendment, two 5 µM NH\(_4^+\) amendments, and two 5 µM NO\(_3^-\) amendments.
Bottles were incubated in the dark at in situ temperatures. At six time points over the course of ninety-six hours the bottles were sampled to determine bacterial abundance and nutrient concentrations. For nutrient samples, 50 mls were filtered through a precombusted GF/F filter and the sample collected in an acid washed bottle and stored at -20°C. For bacterial cell counts, 20 ml samples were stored in 25% glycerol (final concentration) at -20°C. Cell densities were determined directly by epifluorescent microscopy after staining with DAPI (Williams et al. 1998).

2.4 Nutrient Concentrations and statistical analysis

Concentrations of NO₃⁻, and NH₄⁺, were measured with a Flow Solutions IV segmented flow analyzer (OI Analytical, College Station, TX.) according to analytical chemistries provided by the manufacturer. The data were analyzed by analysis of variance and regression models with Systat (Wilkinson 1990).

3. Results

3.1 Nutrient Concentrations

The concentration of DIN at the five stations reflected the origin of the water mass and the extent of ice cover (Table 3.1). Stations I and II had low concentrations of NO₃⁻, suggesting significant utilization by the plankton community and post bloom conditions (Verity et al., this issue). Station III, in the MIZ near the Polar Front, had 10-20% ice cover and much reduced NO₃⁻ concentrations associated with a larger sized phytoplankton community dominated by chained diatoms and Phaeocystis pouchetti single cells (Verity et al., this issue). Stations IV and V, which had 40-50% and 70-80%
ice cover respectively, had the highest NO$_3^-$ concentrations. NO$_3^-$ concentrations in surface waters were approximately 5 µM and between 6 µM and 9 µM at 30m, suggesting early bloom to prebloom conditions. There was less variability associated with NH$_4^+$ concentrations, which were equal to or less than 1.0 µM at 5m and 30m at all stations. For an overview of the hydrography, suspended biomass and nutrients along the transect, see Reigstad et al. (this issue).

3.2 Uptake of DIN by heterotrophic bacteria

Total DIN uptake and DIN uptake by bacteria (<0.8 µm size-fraction) varied approximately two-fold (Fig. 3.2A - B). The rate of total DIN uptake decreased slightly across the transect from Atlantic water into the MIZ, and the rate of bacterial DIN uptake increased gradually across the transect.

To compare relationships between bacterial DIN uptake at the different stations, the 5, 30, and 80m data were averaged. The <0.8 µm size fraction accounted for an average of between 10 and 40% of total DIN uptake (Fig. 3.3A) at the five stations. A significantly higher percentage of total DIN uptake was attributed to bacteria at Stns. IV and V than Stns. I,II, or III (P<0.001) (Fig. 3.3A).

As a percentage of the total NO$_3^-$ or NH$_4^+$ uptake, averaged for each station, bacteria accounted for between 16 and 40 % and 10 and 40 % respectively (Fig. 3.3B). In general, between stations, there was less variability associated with the percentage of total NO$_3^-$ uptake by bacteria than there was for the percentage of total NH$_4^+$ uptake. The difference between the percentage of total NO$_3^-$ uptake by bacteria at Stns. IV and V
(40 and 36%, respectively) and Stn. I (17%) was significant (P<0.05). The difference between the percentage of total NH$_4^+$ uptake by bacteria at Stns. IV and V (41 and 34%, respectively) and Stns. I, II, and III (12, 14, and 10%, respectively) was highly significant (ANOVA, P<0.001).

When the percentage of total NO$_3^-$ and NH$_4^+$ uptake by bacteria is compared (both were measured in the same sample), NO$_3^-$ uptake by bacteria was much higher (nearly two-fold) than NH$_4^+$ uptake (Fig. 3.4). The percentage of total NO$_3^-$ uptake by bacteria was higher than the percentage of total NH$_4^+$ uptake by bacteria in 12 out of 15 samples (Fig. 3.4).

Bacteria accounted for between 22 and 36%, and 19 and 24% of the total NO$_3^-$ and NH$_4^+$ uptake, averaged for 5, 30, and 80m, respectively (Fig. 3.5A). Because these data represent the averages from all five stations, the standard deviations are quite large, however, there is a trend of increasing percentage of NO$_3^-$ uptake by bacteria at 30 and 80m compared to 5m. The difference between average percent NO$_3^-$ uptake by bacteria at 5 (22%) and 30m (37%) was significant (P<0.05).

3.2.1 DIN Uptake in fractionated (pre-filtered) vs. unfractionated treatments

The ratio of NO$_3^-$ or NH$_4^+$ uptake in fractionated samples (where the >0.8 µm community was removed prior to incubation), compared to unfractionated samples were almost always less than one, indicating that bacterial NO$_3^-$ or NH$_4^+$ uptake was higher in the unfractionated treatments (Fig. 3.5B). The ratio of fractionated to unfractionated DIN uptake averaged for 5, 30, and 80m increased with depth. The
fractionated/unfractionated ratio for total DIN, NO₃⁻, and NH₄⁺ uptake was significantly higher at 80 compared to 5m (Fig. 3.5B) \((P<0.005)\). Therefore, the effect of fractionating prior to the incubation decreased with depth and the ratio of fractionated/unfractionated uptake approached one. The amount of total particulate nitrogen collected on the filter for the fractionated and unfractionated treatments, however, was not different, which implies that a significant amount of bacterial biomass was not caught on the filter during the fractionation prior to the incubation. The variability of particulate nitrogen collected on the filters was not significantly correlated with treatment, tracer \((¹⁵NO₃⁻\text{ or } ¹⁵NH₄⁺)\), depth, or station \((P>0.1)\).

### 3.3 Relationship between bacterial DIN uptake and biomass production

The ratio of DIN uptake to bacterial production (expressed in N units) was highly variable between Stns. I - II and Stns. III - V. DIN accounts for between 5 and 10\% of bacterial nitrogen production at Stns. I - II, and between 39 and 54\% of bacterial nitrogen production at Stns. IV - V (Fig 3.6A). The average percentage of bacterial nitrogen production which resulted from DIN uptake tended to increase with depth at most of the stations. Consequently, there was a large degree of variability associated with the average percentage of DIN that accounted for total bacterial N at each of the stations. Nevertheless, the percentage that DIN contributes to total bacterial N demand increased from south to north in the transect, and a significantly higher percentage of bacterial N resulted from DIN at Stns. IV - V compared to I - II (ANOVA, \(P<0.05\)).

Interestingly, the correlation between bacterial production and DIN uptake was significant only if data from Stns. I - II and Stns. III - V are considered separately (Fig. 3.6A).
3.6B). At Stns. I and II, where DIN accounts for between 5 and 10% of total bacterial N, the correlation between bacterial production and DIN uptake is high ($r^2=0.87; n=6; P<0.005$); the slope of the regression line is 0.07. At Stns. III - V, where DIN accounts for between 39 and 54% of estimated bacterial N demand, the correlation between bacterial production and DIN is also high ($r^2=0.80, n=9, P<0.001$); the slope of the regression line is 0.28. Although, the total number of samples is low for both of these relationships, these data suggest that bacterial production can account for much of the variability in bacterial DIN uptake and that approximately four times as much bacterial N resulted from DIN at stations III - V compared to Stns. I - II.

As a percentage of total bacterial DIN uptake, NO$_3^-$ and NH$_4^+$ varied in terms of their contributions to the total (Table 3.2). The percentage of bacterial NH$_4^+$ uptake of total bacterial DIN utilization ranged between 16 and 99%. On average, NH$_4^+$ accounted for 67% of bacterial DIN utilization and NO$_3^-$ was responsible for 33%. However, the relative contribution of bacterial NO$_3^-$ uptake to total bacterial DIN uptake increased, as averaged for each station, from 10% at station I to 57% at Stn. V (Table 3.2). Also, the contribution of bacterial NO$_3^-$ uptake to total bacterial DIN uptake increased, as averaged for each depth, from 15% at 5m to 50% and 32% at 30m and 80m, respectively (Fig. 3.7). The increase in NO$_3^-$ uptake as a contribution to total bacterial DIN uptake from 15% to 50% between 5m and 30m was significant (ANOVA, $P<0.05$).

3.3 Relationship between bacterial NO$_3^-$ uptake and new production

The contribution of NO$_3^-$ uptake to total DIN production provides a reasonable estimate of the $f$-ratio and the importance of new vs. regenerated production. The ratio of total
NO$_3^-$ uptake to total DIN uptake for each experiment is given in Table 3.3. In 11 out of 15 cases, the total NO$_3^-$ uptake to total DIN uptake ratio was equal to or below 0.28. At Stns. I - III, the total NO$_3^-$ uptake to total DIN uptake ratio was generally <0.1, which indicates that the relative importance of NO$_3^-$ based production was not very significant compared to regenerated production. NO$_3^-$ availability at Stns. I - III was also low and might partially explain this observation. At Stns. IV - V, however, NO$_3^-$ based production was much more important and the total NO$_3^-$ uptake to total DIN uptake ratio was often >0.5.

3.5 Seawater culture experiments

Ambient inorganic nitrogen concentrations in the controls and the treatments at the beginning of the experiments and the number of doublings of the bacterial community in each bottle are given in Table 3.4. In the control treatments for batch experiment 1, DIN concentrations were near the limit of detection throughout the 96 hour incubation, but in the DIN addition treatments and in batch experiment 2 nutrients concentrations became chaotic and randomly fluctuated after 48 hours. In batch experiment 1, the 5 µM NH$_4^+$ and NO$_3^-$ additions appear to have relieved DIN limitation and resulted in at least two doublings of the bacterial populations. In batch experiment 2, where the ambient NO$_3^-$ concentration was approximately 5 µM, the DIN additions did not have any effect relative to the controls.
4. Discussion

4.1 Bacterial NO$_3^-$ Uptake in Polar Waters

Although the bacterial size fraction (0.2 - 0.8 µm) could contain both small phytoplankton and heterotrophic bacteria, chlorophyll $a$ was not detected. Onboard microscopic examination of representative samples did not indicate the presence of autotrophic cells in the <0.8 µm size-fraction. Previous findings also support the observation that photosynthetic picoplankton are scarce in the Barents Sea (Throndsen and Kristiansen 1991). Therefore, the < 0.8 µm fraction is dominated by heterotrophic bacteria.

High rates of NH$_4^+$ utilization by marine bacteria have been observed several times and it known that heterotrophic bacteria are capable of assimilating NO$_3^-$ (Kirchman 2000). The substantial levels of NO$_3^-$ uptake by bacteria observed during this study, however, are noteworthy in that they are comparable to only one other study (Kirchman and Wheeler 1998).

Small cells such as bacteria and picophytoplankton usually account for relatively more NH$_4^+$ uptake than NO$_3^-$ uptake (Lipschultz 1995). It is likely that the high levels of NO$_3^-$ utilization observed in the Sub-Arctic Pacific by Kirchman and Wheeler (1998), and in the MIZ (this study), result from the high ambient NO$_3^-$ concentrations (5-20 µM), where only low per cell nitrogen uptake rates are necessary to supply bacteria with substantial nitrogen. A modeling study suggested that, for a given DIN uptake rate, fewer resources have to be allocated to NO$_3^-$ uptake than to NH$_4^+$ uptake when the former is at a higher concentration (Vallino et al. 1996).
4.2 Bacterial Productivity and Inorganic Nitrogen Utilization

Correlations between rates of nitrogen uptake and bacterial production provide insights into the variability associated with bacterial DIN dependence. Across the transect from the Atlantic water into the MIZ, bacteria utilization of DIN increased significantly. The relationship between bacterial production and DIN uptake at open Atlantic Stns. I - II compared to MIZ Stns. III - V indicates that at the latter stations bacterial nitrogen production was supported by four times as much DIN. According to nutrient data and plankton composition data (Ratkova et al., this issue; Reigstad et al.) Stns. I and II, showed signs of nitrogen limitation and were in postbloom phases and Stn. III was probably in declining bloom phase. Stns. IV and V, however, were not nutrient depleted. Although station III was nutrient depleted, the microbial food web was very diverse and Phaeocystis single cells were abundant. As a result there were likely very high levels of NH$_4^+$ regeneration, which contributed to a large spike in bacterial DIN uptake. Interestingly, however, NO$_3^-$ accounted for 56% of bacterial DIN utilization in the experiment at 30m, but only 17 and 18% at 5 and 80m respectively.

Although bacterioplankton populations at Stns. I - II and at Stns. III - V exhibited different levels of bacterial DIN dependence, bacterial productivity accounts for much of the variability observed in each case. The bioassay batch experiments demonstrated similarly DIN limited bacterial production in Norwegian Coastal Current waters (which are similar to open Atlantic waters), but not limiting bacterial production in the MIZ. Not surprisingly, $^{15}$N data indicated that bacterial DIN utilization is much higher in the MIZ where DIN was not limiting, compared to N limiting open Atlantic waters. It is possible that dissolved free amino acid (DFAA) production accounted for much of the bacterial
nitrogen production at Stns. I and II or that the bacteria were limited by carbon availability. Because bacterial production at Stns. I - II is higher on average compared to Stns. III - V, the former hypothesis is plausible.

It has been suggested that bacterial affinity for inorganic nutrients, particularly NO$_3^-$, is reduced at low temperatures (Reay et al. 1990). Therefore it was surprising that such high levels of bacterial NO$_3^-$ utilization were detected in the northern portion of the transect considering that there was a 9°C drop in surface water temperature across the five stations. The decrease in temperature did not appear to have any discernable effect on bacterial DIN utilization, although it may have influenced phytoplankton production and microzooplankton grazing (Verity et al., this issue).

Bacterial activity, as measured by the modified vital stain and probe technique (mVSP), indicates that the percentage of physiologically active bacterial cells at Stns. I, II and IV is on average 75>50%, compared to 25<50% for Stns. I and III (Howard-Jones et al., this issue). Higher rates of bacterial DIN uptake and higher percentages of total bacterial NH$_4^+$, NO$_3^-$, and DIN uptake were observed at Stns. IV, and V. Therefore, it appears that physiologically active cells, that are not DOC limited or inhibited by high concentrations of DFAA, will readily assimilate NH$_4^+$ and NO$_3^-$ simultaneously with a preference for NH$_4^+$ that can be offset by high NO$_3^-$ concentrations.

The observed DIN utilization by bacteria in the fractionated compared to unfractionated treatments also provides insight into some of the factors that are likely to control bacteria DIN uptake. It has previously been demonstrated that bacteria are likely to exhibit a higher growth efficiency and assimilate more DIN in the presence of remineralization processes such as grazing compared to situations where nitrogen
remineralization is absent (Hopkinson et al. 1989). Results from this study indicate a similar result for the case of Barents Sea bacterioplankton. Bacteria in the fractionated treatments, on average, took up less NH$_4^+$ and NO$_3^-$ compared to bacteria in the unfractionated treatment. The difference between DIN uptake in the different treatments decreases with depth, where it is expected that bacterial microzooplankton grazing processes are not as important. These results indicate that the removal of grazing processes reduced bacterial dependence on NO$_3^-$ as well as NH$_4^+$. A potential artifact of these experiments is that, during prefractionation, DON could be released into the fractionated samples and as a result bacterial DIN affinity is decreased. The removal of phytoplankton and production of carbon substrates could limit bacterial production as well, however the incubations were probably not long enough for this to occur. Nevertheless, the striking relationship between the ratio of bacterial DIN uptake in the fractionated compared to unfractionated treatments with depth strongly suggests that the removal of grazing processes in surface waters decreases bacterial activity, growth as well as DIN uptake.

4.3 Autotrophic Production and New Production Estimates

A previous study reported that new production as a percent of total production ($f$-ratio) was hyperbolically related to NO$_3^-$ concentration in the Barents Sea (Kristiansen et al. 1994). Results presented in this study suggest that high levels of bacterial NO$_3^-$ assimilation can inflate estimates of new production, and that high $f$-ratios should be interpreted cautiously if bacterial NO$_3^-$ utilization is not accounted for, especially if NO$_3^-$ concentrations are relatively high. One result from this study is that in experiments
where the total NO$_3^-$ uptake to total DIN uptake ratio is $>0.5$, bacteria accounted for approximately 40% of the NO$_3^-$ utilization. This implies that in regions where apparent high f-ratios are observed, bacteria may be partially responsible for the new production measurements, but do not result in increased new autotrophic production.

It has been demonstrated previously and in the present study using $^{15}$N techniques that bacteria can account for a substantial portion of the NO$_3^-$ uptake in the euphotic zone. Recently it has also been shown with molecular techniques that bacteria that are metabolically capable of assimilating NO$_3^-$ are common and widely distributed in the world’s oceans (Allen et al., submitted). Because bacteria can be responsible for close to 40% of the observed NO$_3^-$ uptake, their contribution can not be ignored in estimates of new production. Since Eppley and Peterson (1979), it has been assumed that the downward flux of nitrogen has to equal NO$_3^-$ uptake at steady state regardless of which group of microbes is using the NO$_3^-$ and therefore bacterial uptake of NO$_3^-$ is thought to have a negligible effect on f-ratios (Kirchman et al. 1992). However, substantial NO$_3^-$ utilization by bacteria is one mechanism which might cause the system to deviate from steady state because bacterial carbon production, which results from NO$_3^-$ utilization, represents POC that is more likely to remain in the water column and not to sink. Therefore, additional trophic transfers are required for the small bacterial particles to be transformed into sinking material. Also, it is clear that substantial bacterial NO$_3^-$ utilization can lead to the observation of high f-ratios, which should be interpreted cautiously.
Acknowledgements

The authors would like to thank C. Wexels-Riser, and M. Reigstad for their efforts in preparation for the cruise. We would also like to thank Paul Wassmann for the invitation to participate in the research expedition and for being such an accommodating host. Thanks especially to S. Øygarden for finding all of the supplies we requested, some on very short notice. Also, we acknowledge the captain and crew of the R/V Jan Mayen for logistic support and excellent meals. This research was supported by NSF grants OCE-95-21086 and 99-82133 and DOE grant FG-02-98ER62531. Thanks to D. Peterson and A. Boyette for help in preparing the manuscript and arranging the figures.
References


production and metabolic activities during austral autumn. Deep-Sea Research I 37, 1145-1167.


assemblages of marine bacteria in seawater culture. Mar. Ecol. Prog. Ser. 50, 147-
150.

Howard-Jones M.H., Ballard V.D., Allen A.E., Frischer M.E., Verity P.G. Distribution of
bacterial activity in the marginal ice zone of the central Barents Sea in summer. J.
Mar. Syst. (this issue).

Kirchman D.L. (1992). Incorporation of thymadine and leucine in the subarctic Pacific:


New York: John Wiley & Sons.

ammonium utilization and regeneration by heterotrophic bacteria in the subarctic


dissolved organic carbon during a spring phytoplankton bloom. Nature 352, 612-
614.


Reigstad M., Riser C.W., Øygarden S., Wassmann P., Rey F. Seasonal variation in hydrography, nutrients and suspended biomass in the marginal ice zone and the central Barents Sea. (this issue).

Ratkova T., Wassmann P., Reigstad M. Seasonal variation in phytoplankton species and abundance in the marginal ice zone and the central Barents Sea. (this issue).


Table 3.1. Concentrations of NH$_4^+$ and NO$_3^-$ (µM) at 5, 30, 80m (depths where $^{15}$N experiments were conducted) at the five stations. Ice cover extent is given in parentheses below the stations.

<table>
<thead>
<tr>
<th>Depth(m)</th>
<th>nr</th>
<th>[NH$_4^+$]</th>
<th>[NO$_3^-$]</th>
<th>[NH$_4^+$]</th>
<th>[NO$_3^-$]</th>
<th>[NH$_4^+$]</th>
<th>[NO$_3^-$]</th>
<th>[NH$_4^+$]</th>
<th>[NO$_3^-$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td></td>
<td>0.95</td>
<td>0.03</td>
<td>0.69</td>
<td>0.75</td>
<td>0.93</td>
<td>0.06</td>
<td>0.19</td>
<td>4.83</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>0.56</td>
<td>0.44</td>
<td>1.05</td>
<td>1.69</td>
<td>0.59</td>
<td>3.90</td>
<td>0.33</td>
<td>9.01</td>
</tr>
<tr>
<td>80</td>
<td></td>
<td>2.46</td>
<td>9.30</td>
<td>2.60</td>
<td>6.65</td>
<td>3.80</td>
<td>7.67</td>
<td>3.85</td>
<td>8.52</td>
</tr>
</tbody>
</table>

I (open water) II (open water) III (10-20%) IV (40-50%) V (70-80%)
Table 3.2. Percentage of bacterial NH$_4^+$ or NO$_3^-$ uptake to total bacterial DIN uptake at 5, 30, and 80m at the five stations.

<table>
<thead>
<tr>
<th>Depth(m)</th>
<th>NH$_4^+$</th>
<th>NO$_3^-$</th>
<th>NH$_4^+$</th>
<th>NO$_3^-$</th>
<th>NH$_4^+$</th>
<th>NO$_3^-$</th>
<th>NH$_4^+$</th>
<th>NO$_3^-$</th>
<th>NH$_4^+$</th>
<th>NO$_3^-$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>99.5</td>
<td>0.5</td>
<td>90.1</td>
<td>9.9</td>
<td>82.7</td>
<td>17.3</td>
<td>70.6</td>
<td>29.4</td>
<td>78.9</td>
<td>21.1</td>
</tr>
<tr>
<td>30</td>
<td>87.0</td>
<td>13.0</td>
<td>81.5</td>
<td>18.5</td>
<td>43.7</td>
<td>56.3</td>
<td>16.6</td>
<td>83.4</td>
<td>18.6</td>
<td>81.4</td>
</tr>
<tr>
<td>80</td>
<td>81.1</td>
<td>18.9</td>
<td>94.2</td>
<td>5.8</td>
<td>82.0</td>
<td>18.0</td>
<td>51.0</td>
<td>49.0</td>
<td>31.2</td>
<td>68.8</td>
</tr>
</tbody>
</table>
Table 3.3.
The percentage of total NO$_3^-$ uptake to total DIN uptake (both rates measured by combining the >0.8 $\mu$m fraction and the <0.8 $\mu$m fraction) for each experiment, and the % of bacterial NO$_3^-$ uptake (<0.8 $\mu$m fraction only) to total NO$_3^-$ uptake

<table>
<thead>
<tr>
<th>Depth(m)</th>
<th>$f$-ratio*</th>
<th>%Total NO$_3^-$ Uptake</th>
<th>Bacterial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stn. I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2.4</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>10.3</td>
<td>19.2</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>12.7</td>
<td>31.5</td>
<td></td>
</tr>
<tr>
<td>Stn. II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>7.1</td>
<td>25.8</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>7.2</td>
<td>46.9</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>3.6</td>
<td>26.1</td>
<td></td>
</tr>
<tr>
<td>Stn. III</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>6.5</td>
<td>17.5</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>28.9</td>
<td>36.5</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>7.4</td>
<td>40.6</td>
<td></td>
</tr>
<tr>
<td>Stn. IV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>32.2</td>
<td>39.1</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>83.1</td>
<td>37.5</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>21.5</td>
<td>40.2</td>
<td></td>
</tr>
<tr>
<td>Stn. V</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>22.3</td>
<td>27.2</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>75.2</td>
<td>45.7</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>66.9</td>
<td>44.4</td>
<td></td>
</tr>
</tbody>
</table>

* $f$-ratio is calculated as the ratio of total NO$_3^-$ uptake/total DIN uptake. $f$-ratios are expressed as percentages.
Table 3.4. Results of batch experiment (1) and batch experiment (2). For each treatment, the number of doublings of the bacterial community and the initial \( \text{NH}_4^+ \) and \( \text{NO}_3^- \) concentration in each of the bottles is reported. Batch experiment (1) was initiated in North Atlantic waters at a coastal fjord station (not one of the 24 hour stations). Batch experiment (2) was initiated at 24 hour Stn. IV in the MIZ.

<table>
<thead>
<tr>
<th>Batch Experiment 1</th>
<th>Number of Doublings</th>
<th>( \text{NH}_4^+ ) ( \mu \text{M} )</th>
<th>( \text{NO}_3^- ) ( \mu \text{M} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (A)</td>
<td>0</td>
<td>0.11</td>
<td>0.81</td>
</tr>
<tr>
<td>Control (B)</td>
<td>0</td>
<td>0.12</td>
<td>0.79</td>
</tr>
<tr>
<td>(5 ( \mu \text{M} )) ( \text{NH}_4^+ ) (A)</td>
<td>2.39</td>
<td>5.50</td>
<td>0.76</td>
</tr>
<tr>
<td>(5 ( \mu \text{M} )) ( \text{NH}_4^+ ) (B)</td>
<td>1.95</td>
<td>5.61</td>
<td>0.70</td>
</tr>
<tr>
<td>(5 ( \mu \text{M} )) ( \text{NO}_3^- ) (A)</td>
<td>1.62</td>
<td>0.10</td>
<td>5.62</td>
</tr>
<tr>
<td>(5 ( \mu \text{M} )) ( \text{NO}_3^- ) (B)</td>
<td>2.95</td>
<td>0.10</td>
<td>5.75</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Batch Experiment 2</th>
<th>Number of Doublings</th>
<th>( \text{NH}_4^+ ) ( \mu \text{M} )</th>
<th>( \text{NO}_3^- ) ( \mu \text{M} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (A)</td>
<td>2.68</td>
<td>0.51</td>
<td>4.91</td>
</tr>
<tr>
<td>Control (B)</td>
<td>2.24</td>
<td>0.46</td>
<td>4.99</td>
</tr>
<tr>
<td>(5 ( \mu \text{M} )) ( \text{NH}_4^+ ) (A)</td>
<td>2.16</td>
<td>5.61</td>
<td>4.79</td>
</tr>
<tr>
<td>(5 ( \mu \text{M} )) ( \text{NH}_4^+ ) (B)</td>
<td>1.83</td>
<td>5.77</td>
<td>4.81</td>
</tr>
<tr>
<td>(5 ( \mu \text{M} )) ( \text{NO}_3^- ) (A)</td>
<td>3.53</td>
<td>0.49</td>
<td>10.12</td>
</tr>
<tr>
<td>(5 ( \mu \text{M} )) ( \text{NO}_3^- ) (B)</td>
<td>2.44</td>
<td>0.55</td>
<td>10.01</td>
</tr>
</tbody>
</table>
**Figure Legends**

Figure 3.1. A map of the cruise track of the R/V Jan Mayen and locations of the five 24 hour experimental stations (I-V) in July 1999.

Figure 3.2. Uptake of dissolved inorganic nitrogen (DIN) at 5m and 30m at five stations during July 1999. (A) Total DIN by both size fractions (>0.8 µm size fraction + <0.8 µm size fraction). (B) Total DIN uptake by bacteria (<0.8 µm size fraction only).

Figure 3.3. Average percentage of total DIN uptake by bacteria (<0.8 µm size fraction) averaged between 5, 30, and 80m at five stations I-V. (A) Percentage of total DIN uptake attributable to bacteria at stations I-V. (B) Percentage of total NO₃⁻ or NH₄⁺ uptake that is bacterial at stations I-V.

Figure 3.4. Relative uptake of NO₃⁻ or NH₄⁺ by heterotrophic bacteria (<0.8 µm size fraction) for each sample where both uptake rates were measured simultaneously.

Figure 3.5. Percentage of total DIN, NO₃⁻, and NH₄⁺ uptake by bacteria in unfractionated and fractionated treatments (<0.8 µm size fraction) averaged between each station for 5, 30, and 80m. (A). Average percentage of total DIN, NO₃⁻, and NH₄⁺ uptake in unfractionated treatments at 5, 30, and 80m. (B). Average percentage of total DIN, NO₃⁻, and NH₄⁺ uptake in fractionated treatments at 5, 30, and 80m.

Figure 3.6. Relationship between bacterial production and bacterial DIN uptake. (A). Average percentage of bacterial production (converted to N units) accounted for by bacterial (<0.8 µm size fraction) DIN utilization. (B) Uptake of DIN by bacteria vs. bacterial production. Stations I and II (○), stations III, IV, and V (●).
Figure 3.7. Percentage of bacterial DIN uptake that is NO$_3^-$ at 5, 30 and 80m averaged over all five stations.
Fig. 3.1
Fig. 3.2A
Fig. 3.2B
Fig. 3.3A
Fig. 3.3B
Fig. 3.4
Fig. 3.5A
Fig. 3.5B
Fig. 3.6A
Fig. 3.6B
Fig. 3.7
CHAPTER 4

TRFLP AND REAL-TIME PCR ANALYSIS OF BACTERIAL NITRATE ASSIMILATION GENES IN THE BARENTS SEA DURING SUMMER

ABSTRACT

Bacterial assimilatory nitrate reductase (nasA) was assayed for diversity and changes in community structure (T-RFLP) in response to changes associated with different water masses sampled at 5 m and 80 m across a transect that began in Norwegian coastal waters and passed through the North Atlantic, across the Polar Front and into the marginal ice zone (MIZ). A SYBR Green real-time PCR assay was developed for a particular group of nasA genes characteristic of Marinobacter sp. Marinobacter sp. nasA gene abundance was positively correlated with NO$_3^-$, exhibiting a two-fold increase in abundance relative to total bacteria at 80 m compared to 5 m. In an attempt to explain nasA population variability and distribution as a function of relevant environmental variables, a partial least squares regression model was constructed. Bacterial productivity, bacterial biomass, chlorophyll, NH$_4^+$, and NO$_3^-$ were modeled as independent variables to determine how well each variable predicted the variation in nasA community structure. NO$_3^-$ concentration was the best predictor, by a factor of 10, of the variability associated with nasA community structure. In a previous study of $^{15}$NO$_3^-$ uptake across the same transect, bacteria were relatively more important in terms of total community uptake in the MIZ where NO$_3^-$ levels were high compared to the North Atlantic where DIN concentrations were lower. Results presented here, which indicate nasA communities were more diverse and that a specific nasA group was more abundant in waters where NO$_3^-$ levels were relatively high, agree well with the findings from the $^{15}$N experiments that suggested that the bacteria were relatively more important to overall community NO$_3^-$ assimilation in MIZ waters compared to North Atlantic waters.
INTRODUCTION

Significant heterotrophic bacterial utilization of dissolved inorganic nitrogen (DIN) would have profound effects on the fluxes of N and C in the water column (Kirchman et al. 1992, Kirchman 1994). NO$_3^-$ uptake by heterotrophic bacteria is especially important because of its potential impact on estimates of new production and on the relationship between new production and carbon export out of the euphotic zone (Legendre & Grosselin 1989, Kirchman 2000).

Based on $^{15}$N measurements of bacterial DIN assimilation across a transect in the Barents Sea from North Atlantic waters into the marginal ice zone (MIZ), bacteria accounted for 16-40% of the total NO$_3^-$ uptake. In 12 out of 15 experiments bacteria accounted for a higher percentage of total NO$_3^-$ uptake compared to total NH$_4^+$ uptake (Allen et al., in press). Several other studies have also documented high rates of NO$_3^-$ utilization by bacteria (Parker et al. 1975, Parsons et al. 1980, Horrigan et al. 1988, Kirchman et al. 1991, Caraco et al. 1998, Kirchman & Wheeler 1998, Kirchman 2000, Middelburg & Nieuwenhuize 2000). Despite the importance of bacterial assimilation of NO$_3^-$ to the introduction of organic N into the microbial food web and to geochemical mass balances, it is a process that has received very little attention in models of pelagic C and N fluxes (Fasham et al. 1990, Boynton et al. 1995, Haupt et al. 1999, Olivieri & Chavez 2000, Dadou et al. 2001).

Understanding the variability and magnitude of new production in the polar region is particularly significant because of the important role these regions play in sequestering CO$_2$ (Sarmiento & Toggweiler 1984, Walsh 1989, Erickson et al. 1990). Several studies have reported extremely high rates of primary production in the North Bering Sea, the
Chukchi Sea, and the Barents Sea (Sambrotto et al. 1984, Olsson et al. 1999, Luchetta et al. 2000). Arctic bacterioplankton appear to important consumers of dissolved organic matter and DIN (Cota et al., 1990, Rivkin et al., 1996, Steward et al., 1996, Howard-Jones et al. in press, Allen et al., in press). It is likely that microbial populations alternate between functioning as a source of DIN, which would act to enhance primary production, and as a sink for DIN, which would potentially reduce the magnitude of new production and result in an accumulation DON in the microbial food web (Caraco et al. 1998, Sanders & Purdie 1998). Molecular approaches can be useful for detecting and characterizing particular groups of bacteria involved in this important biogeochemical process (Zehr & Voytek 1999, Zehr & Ward 2002).

Previously, molecular techniques have been successfully used to characterize bacteria and the genes that are important in several aspects of the nitrogen cycle, including nitrification, denitrification, nitrogen fixation as well as heterotrophic bacterial nitrate assimilation (Zehr & McReynolds 1989, Kirshtein et al. 1991, Voytek & Ward 1995, Voytek et al. 1997, Zehr & Paerl 1997, Scala & Kerkhof 1998, Zehr et al. 1998, Scala & Kerkhof 1999, Voytek et al. 1999, Zani et al. 2000, Allen et al. 2001, Zehr and Ward 2002). Recently, molecular techniques, which are more comprehensive and quantitatively informative, such as terminal restriction fragment length polymorphism (T-RFLP) and real-time PCR have been used to evaluate the spatial heterogeneity of nitrous oxide reductase (\(\text{nosZ}\)) genes and to measure the abundance of \textit{Pseudomonas stutzeri} dissimilatory nitrite reductase (\(\text{nirS}\)) genes in sediments (Scala & Kerkhoff 2000, Gruntzig et al. 2001). T-RLFP analysis is a powerful technique that has been used to assay and compare microbial community structure and diversity in the lab and in the field.

In this study we apply T-RFLP and real-time PCR techniques to samples that were collected from the same water samples that were used to determine the rate and amount of DIN utilized by bacterioplankton in a previous study (Allen, et al. in press). The purpose of this study was to compare \( \text{nasA} \) populations in surface waters (5 m) and deeper waters (80 m) across a sampling transect that began in the Southern Barents Sea, crossed the Polar Front, and ended in the MIZ dominated by Arctic water. We address the hypothesis that differences in \( \text{NO}_3^- \) concentrations and \( \text{NO}_3^- \) uptake rates are reflected in the variability associated with \( \text{nasA} \) population community structure and abundance.

**MATERIAL AND METHODS**

Experiments were conducted during a cruise (ALV-3) aboard the R/V Jan Mayen in June 1999. Five stations (Figure 4.1) were sampled along a nineteen station transect beginning in complete ice cover on July 1 (78° 13.67′ N, 34° 23.02′ E) and ending near coastal waters on July 9 (73° 47.99′ N, 31° 44.10′ E). Water samples were collected from 10 L Niskin bottles. See Allen et al. (2001) for a detailed description of the sampling and collection method.

**DNA collection, PCR amplification and cloning.** DNA samples were collected from 5 m and 80 m at each of the five stations for a total of 10 samples. DNA samples were collected from 40 liters of water, DNA was extracted using the Ultra Clean Mega Prep soil DNA kit (Mo Bio Laborites, Inc., Solana Beach, Calif.), and nested PCR with
universal *nasA* primers was performed to detect the presence or absence of *nasA* as described by Allen et al. (2001).

PCR products were agarose gel purified and clone libraries were constructed from the 5 m Stn. III, 80 m Stn. III, 80 m Stn. IV, and 80 m Stn. V samples as described by Allen (2001). 35 clones from each 5 m Stn. III, and 80 m Stn. IV, and 10 clones each from 80 m Stn. III and 80 m Stn. V were screened and sequenced. *NasA* amino acid sequences were aligned using the CLUSTAL W (version 1.7) multiple-sequence-alignment algorithm (Thompson et al. 1994). Phylogenetic trees were inferred and drawn by using the TREECON software package (version 1.3b) (Van de Peer and De Wachter, 1997) and the Kimura two-parameter model for inferring evolutionary distances. Bootstrap estimates (100 replicates) of confidence intervals were also made by using the algorithms available in the TREECON package.

**SYBR Green Real-time PCR.** A primer pair suitable for use in a SYBR Green real-time PCR assay that is specific for a clade of bacterial *nasA* genes typified by two *Marinobacter* sp. isolates was designed. In order to estimate the contribution of *Marinobacter* like *nasA* genes as a percentage of the total bacteria between samples, an additional set of universal eubacterial 16S rRNA primers was designed to control for bacterioplankton abundance in the water column. All primer sequences used in this study are presented in Table 4.1. Expressing *Marinobacter* like *nasA* gene abundance as a percentage of total bacteria also controlled for variability in DNA extraction. *Marinobacter* sp. *nasA* primer design was facilitated by using the software package Primer Premier (version 5.0, Premier Biosoft International, Palo Alto, CA). Criteria for optimal primer pairs suitable for the SYBR Green PCR assay include a GC content of 40-
60%, a length of at least 18 bp, and a resulting PCR product of less than 200 bp with one melting domain.

SYBR Green is a minor-groove DNA binding dye with a high affinity for dsDNA and exhibits elevated levels of fluorescence upon binding to double stranded dsDNA (Witter et al. 1997). The SYBR Green dye is excited at a 485-nm wavelength, and the emission is measures at a 520-nm wavelength. In the Bio-Rad iCycler IQ Real-Time Detection System, the fluorescence of the SYBR Green dye is monitored at the end of PCR cycle, therefore allowing the detection of the product during the linear range of amplification. The specificity of the amplified product is monitored by its melting curve. Because the melting curve of a product is dependent on its GC content, length, and sequence, specific amplification can be distinguished from nonspecific amplification by examine the melting curve (Ririe et al. 1997).

SYBR Green PCR amplifications were performed using a Bio-Rad iCyclerIQ Real-Time PCR Detection System. The reactions were carried out in a 96-well plate in a 25-µl reaction volume containing 12.5 µl of 2x QuantiTech SYBR Green Master Mix (Qiagen, Valencia, Calif.), 0.2 µM concentration of each forward and reverse primer, and approximately 100 ng of DNA. The thermal profile for SYBR Green PCRs was 95°C for 10 min, followed by 25 cycles for 16S rRNA PCR and 30 cycles for Marinobacter nasA PCR of 95°C for 15s, 58°C for 10s, and 72°C for 1 min. This was followed immediately by a melt curve thermal profile, which was 65°C for 1 min, followed by 125 cycles of 0.2°C increments each for 10s. In each 96-well plate, a dilution series of the plasmid standard for the respective target gene was run along with the unknown samples. Each
sample was run in replicates of three and all reactions were repeated at least three times independently to ensure the reproducibility of the results.


**T-RFLP analysis (PCR amplification, digestion, and capillary electrophoresis).** The upstream primer, *nas*964 (Allen et al. 2001), was labeled at the 5’ end with a phosphoramidite linked D4 dye (Beckman Coulter, Fullerton, Calif.). Primers were synthesized by Invitrogen (Carlsbad, Calif.). A nested 25 µl PCR reaction was performed as described above with 50 ng of template DNA added to three first round reactions; each first round reaction was then used as a template in four second reactions for a total of PCR reactions for each DNA sample. First and second round reactions contained 500 pmol of primer and ran for 35 and 30 cycles respectively.

After amplification, each 25 µl PCR reaction was loaded into a separate well on a 0.8% agarose gel containing 1x GelStar nucleic acid stain (BMA, Rockland, ME), and separated by gel electrophoresis at 60V for 3 - 5 hours in 1x TAE buffer at room temperature. The approximately 800 bp *nasA* PCR products were then visualized on a Dark Reader (Clare Chemical Research, Denver, CO), which excites fluorophores between 420 and 500 nm and prevents oxidation of the phosphoramidite linked dye,
which occurs under standard agarose gel UV visualization. *nasA* PCR products were then excised from the gel and eluted using Quantum Prep DNA Gel Extraction Spin Columns (Bio-Rad, Hercules, Calif.). The eluted PCR products from each DNA were then pooled and precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes of 95% ethanol, followed by spinning at 14,000 rpm for 15 min. The DNA pellet was washed twice with 70% ethanol, dried, and resuspended in 40 µl of sterile water. The DNA concentration of the purified and pooled PCR products was then estimated by fluorometry with a Turner Design TD-700 fluorometer (Turner Designs, Sunnyvale, Calif.) using a PicoGreen dsDNA Quantitation Kit (Molecular Probes, Eugene, OR). 50 ng of purified PCR product was then digested separately in triplicate with 10 units of the restriction enzymes *Dde I*, *Mbo I*, and *Rsa I* (New England Biolabs). Digests were incubated at 37°C for 5 hours in buffer provided by the manufacturer.

The digested DNA was precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes of 95% ethanol, followed by spinning at 14,000 rpm for 15 min. The DNA pellet was washed twice with 70% ethanol, dried, and resuspended in 40 µl of deionized formamide and 0.5 µl of CEQ DNA Size Standard – 600 (Beckman Coulter, Fullerton, Calif.). T-RFLP digests were then separated via capillary electrophoresis on a Beckman CEQ 2000XL DNA Analysis System with a CEQ capillary array (33 centimeter long, 75 micrometer internal diameter) and CEQ linear polyacrylamide denaturing gel (LPA-1). After denaturing for 120 sec. at 90°C, samples were injected into the capillary for 30 sec. at 2.0kV and then separated for 70 min. at 4.8kV. The fluorescent dye at the end of the digested fragment was detected by a photo multiplier and analyzed using the Beckman
2000XL Fragment Analysis software package version 4.3.9, which displays the various T-RFLPs as a series of peaks.

Peak height was used a proxy for the relative abundance of taxa represented by restriction fragments. Integrated peak area was not used due to overlap between some peaks. Peak height was normalized against the sum of peak heights in a profile, and only peaks of at least 3000 relative fluorescent units were considered for analysis. Fragments less then 2 bases apart from a larger peak were discarded to account for signal noise in electropherograms. To account for small variations in run time between samples, peaks from different profiles with less than one base difference were considered the same. Also peaks that did not occur in at least two profiles from different samples were considered artifacts and removed.

**Multivariate Statistics.** Partial least squares regression and principal component analysis was performed using the Unscrambler 7.6 software package (CAMO Inc., Corvallis, OR.). Before the analysis, all the variables were autoscaled: the geometric means were centered to zero and all data were normalized for standard deviation. Full cross validation was used in the modeling procedure, one sample being omitted at a time. In partial least squares regression, the regression coefficient for each independent or environmental variable expresses the link between variation in that variable and the variation associated with a particular response variable. The independent variables used for analysis were chlorophyll a, bacterial biomass (estimated based on cell abundance), bacterial productivity (estimated via leucine incorporation), % activity (VSP) (Howard-Jones et al. 2000), NO₃⁻ and NH₄⁺ concentration. Data were provided by Howard-Jones et al. (in press) and Allen et al. (in press). These data were provided to the model for
each sample and the populations of T-RFLP fragments from all three of the digests for each sample were modeled as dependent variables.

RESULTS

*nasA* Sequence Diversity in the Barents Sea

The samples from which clone libraries were constructed were 5 m Stn. III, 80 m Stn. III, 80 m Stn. IV, and 80 m Stn. V. Altogether, 55 clones were analyzed and sequenced from the four libraries. Interestingly, the majority of *nasA* clones retrieved from each of these libraries grouped with the same clade, which is composed entirely of Barents Sea clones with the exception of 1 Sargasso Sea clone (Fig. 4.2). Of the nine major *nasA* clades described to date (Allen et al. 2001 and Fig 4.2), four do not have cultured representatives and two of those consist only of Barents Sea clones. One very deeply rooted clade was comprised only of two Barents Sea clones. The *nasA* containing isolates obtained from Barents Sea waters were more diverse than the Barents Sea clone libraries. Two *Pseudoalteromonas* sp. isolates retrieved from the Barents Sea clustered with clones retrieved from HOTS, BATS and the South Atlantic Bight (SAB) mid-shelf as well as with three isolates collected from the Sargasso Sea. *Marinobacter* sp., and *Marinomonas* sp. isolates that were collected from the Barents Sea typify major clades of *nasA* clones, and the *nasA* sequence from a *Psychrobacter* sp. isolate was not closely related to any of the other sequences.

**SYBR Green PCR Assay**

Because the DNA–binding dye SYBR green I binds all double-stranded DNA molecules, real-time PCR amplification that is monitored with SYBR Green I must be
optimized so that primer-dimer formation and non-specific amplification do not occur.

To evaluate whether or not primer-dimers and single PCR amplicons are generated, melt curve analysis can be used to identify the melting domains of double stranded DNA products that are generated during PCR amplification.

To evaluate the specificity of the reactions, PCR products were analyzed with melt curve analysis and inspected on agarose gels (Fig. 4.3). For both primer sets the generated PCR amplicon clearly dissociates as a solitary peak indicating that the PCR reaction was specific for the intended target. Also, melt curve analysis and examination of PCR reactions on agarose gels did not reveal any primer-dimer formation, which suggests that the increase in SYBR green fluorescence during PCR amplification is attributable to an increase of the intended target.

To evaluate the specificity of the Marinobacter sp. group nasA primers, DNA from the station IV 80 m was amplified and a clone library was constructed from the amplified material. 10 clones were sequenced. All of the sequences were identical, indicating that the primers are specific for the Marinobacter sp. nasA group. An alignment of the retrieved sequences with other types of typical nasA genes is shown in Fig. 4.4. Although the PCR amplicon is short (115 bp), there are motifs that are common only to Marinobacter sp. sequences, again indicating that PCR amplicon was unambiguously a Marinobacter sp. nasA PCR product.

In order to quantify unknowns using SYBR Green real-time PCR, it is critical that all of the PCR reactions exhibit a constant rate of doubling. In the standard curve, the threshold cycle (the point at which the fluorescence rises above the background) for each of the standards was plotted against log of the concentration of starting template copies
for that standard. The resulting slope of this line was informative with regard to how consistent the rate of doubling was between reactions, i.e. the efficiency. An efficiency of 100% corresponds to a slope of −3.332 in the regression equation describing the standard curve and indicates that all of the standards were amplifying at the exact same rate. The SYBR green PCR assays optimized in this study consistently produced linear standard curves with slopes that indicate that all of the reactions were amplifying at approximately the same rate (Fig 4.5).

The results for the real-time PCR assays are presented in table 4.2. Interestingly, *Marinobacter* sp. *nasA* gene copy number/picogram of DNA was approximately an order of magnitude higher for all of the 80 m samples compared to each of the 5 m samples. Gene copy concentrations in DNA extracts were used to estimate the abundance of gene copies in the water column by multiplying the number of gene copies/µl of DNA by the original volume (µls) that the DNA was resuspended and dividing by the volume of water that was filtered when the sample was collected. In order to estimate the fraction of total cells that contain *Marinobacter* sp. like *nasA* genes, the number of 16S rRNA genes/L of sea water was divided by 4, assuming an average of 4 16S rRNA gene copies / cell (Klappenbach et al., 2001), and *Marinobacter* sp. *nasA* gene concentration was divided by this number. Assuming there is 1 copy of *nasA* per cell (assumption based on 5 Genbank genomes), real-time PCR results indicated, on average for the entire transect, that there were approximately 4.0e2 *Marinobacter* like *nasA* containing cells/L, and 10⁷ total cells/L.

According to cell counts performed on samples collected during the same transect, these estimates of total cells underestimated the actual number by about a factor
of 10, and reflect cells losses that inevitably occur as a result of filtration and DNA extraction. Nevertheless, these estimates are valuable because they facilitate a reliable comparison by correcting for variation in DNA due to experimental handling and differences in cell abundance between samples. Fig. 4.6, shows that there is an average of a two fold difference in *Marinobacter nasA* genes relative to total cell abundance at 80m compared to 5m. Across all stations this result was significant (ANOVA, *P*<0.01). Also, NO$_3^-$ concentrations were substantially higher for all of the 80 m samples compared to the 5 m samples (Table 4.3).

**TRFLP Analysis of nasA genes (restriction enzyme selection)**

A phylogenetic tree of the most frequently recovered sequences from each clade was constructed to determine whether or not the major clusters remained coherent (Fig. 4.7). Although the deeper branching relationships between the major clades that are observed when a larger number of sequences are present in the alignment are not the same, all of the representative sequences cluster within the same major groups that they are affiliated with in the larger tree (Figure 4.2). This result indicated that these sequences were valid representatives for their respective groups and that they would be suitable for predicted T-RFLP analysis to determine which restriction enzymes are best for detecting accurate phylogenetic relationships among populations of *nasA* genes.

The same subset of sequences was then used in conjunction with various groups of restriction enzymes to generate predicted T-RFLP fragments and these fragments were used to construct UPGMA (unweighted pair group method using arithmetic averages) clustering dendrograms to determine whether or not the basic underlying relationships among the groups of sequences was preserved. UPGMA clustering analysis on the most
frequently observed clones from each of the major *nasA* clades with the restriction enzymes *Dde* I, *Mbo* I, and *Rsa* I maintained the major overall groupings of *nasA* sequences (Fig 4.8). No pair of *nasA* sequences in our database that phylogenetically group into different clades yields the same combination of putative terminal restriction fragments with this combination of enzymes.

**TRFLP Analysis of *nasA* genes in Barents Sea Samples**

The largest peaks in all of the samples corresponded to the putative terminal restriction fragments associated with major group of Barents Sea clones. Interestingly, there was not an isolate that is affiliated with this group (Fig. 4.9, Fig. 4.2). 95 % of the clones recovered from Barents Sea samples belonged to this group. Two of most commonly identified groups by T-RFLP were the unknown Barents Sea group and clones related to *Marinobacter* sp. and both groups have representatives that yield different putative terminal restriction fragments that could be identified in electropherograms (Fig. 4.9). Some of the other common terminal restriction fragments observed in this study have corresponding putative terminal restriction fragments the same as clones in the *Alteromonas* and *Marinobacter* group. In general, for the samples collected at the MIZ stations (III, IV, V) there were more terminal restriction fragments compared to the North Atlantic stations (I and II). As an average between 5m and 80m there were 23, 13, 43, 30, and 36 terminal restriction fragments at stations I – V respectively.

To discern general patterns of *nasA* population distribution between the different water masses, a simple dendrogram was constructed based on the comparison of T-RFLP patterns from the different samples. The T-RFLP patterns from the different water masses sampled show two distinct clusters. Samples from open Atlantic Stns. I – II
cluster together and samples from the MIZ stations III – V also form a distinct cluster (Fig. 4.10). Principal component analysis (PCA) was performed using normalized heights of terminal restriction fragment peaks as input variables. The first two principal components, PC1 and PC2, explained 34% and 17%, respectively, of the total variation in peak height and presence or absence between the different samples (Fig. 4.11). PCA revealed clear separation between the \( nasA \) populations sampled from the open ocean samples collected during the southern leg of the transect compared to the samples collected from northern stations dominated by ice cover.

To test if the variance in peak height, presence and absence (Y variables) \( (nasA \) gene populations) was related to bacterial production, chlorophyll, bacterial biomass, cell activity (measured via fluorescent in situ hybridization), or \( NO_3^- \) or \( NH_4^+ \) concentrations (X variables), a partial least squares regression model was constructed (Fig. 4.12). \( NH_4^+ \), % activity, \( NO_3^- \) were each positively correlated with the observed variability in the relative abundance and presence and absence of the \( nasA \) terminal restriction fragments. Biomass, bacterial productivity, and chlorophyll are each negatively correlation with the variability in the population of \( nasA \) terminal restriction fragments. Although, \( NH_4^+ \), and % activity were each positively correlated with the variability associated with \( nasA \) populations, \( NO_3^- \) concentration was the most important variable in explaining the variation in \( nasA \) terminal restriction fragments.

Regression coefficients for each dependent variable can be useful in determining how strong the link is between that variable and a given response variable. In this case, the regression coefficient for every possible combination of independent variable and terminal restriction fragment was analyzed to determine how strongly an independent
variable of interest was linked to the variation of a given terminal restriction fragment across all of the samples. Fig. 4.13 shows the median value of all of the regression coefficients (n = 46) for the different dependent variables used in the model. Although the high degree of noise associated the data set makes it difficult to draw conclusions regarding statistical significance, NO$_3^-$ was a better predictor of $nasA$ community structure than any of the other variables modeled variation by a factor 10. Clearly, $nasA$ populations are influenced by variability in NO$_3^-$ concentration.

**Discussion**

Analysis of clone libraries and T-RFLP assays yielded similar results regarding the most common group of $nasA$ genes in detected in Barents Sea DNA samples. Analysis of clone libraries produced a very low number of different sequences. 95% of the clones sequenced belonged to the same group of unknown Barents Sea clones. Although putative peaks for these sequences were the largest and most commonly observed peaks in the T-RFLP assays, T-RFLP did show some other major groups of $nasA$ containing organisms distributed throughout the samples.

Molecular techniques targeted to the $nasA$ gene were able to uncover two potentially important (one apparently major) populations of heterotrophic NO$_3^-$ assimilating bacteria based on frequency in sequence analysis of clone libraries and the presence and absence of T-RFLP peaks and peak height. Molecular approaches therefore, especially targeted to function genes, continue to be useful for detecting organisms that are difficult to culture. It is important to emphasize that in order to collect information regarding the types of organisms that are important for a particular process,
especially in the case of *nasA*, it is necessary to conduct studies at the level of the functional gene. For most of the major groups of cultured organisms that appear to be important in terms of NO$_3^-$ assimilation in the marine environment (i.e. *Vibrio* sp., *Marinobacter* sp., *Alteromonas* sp.), highly related strains that are *nasA* negative and do not display the NO$_3^-$ assimilation phenotype have also been cultured. Therefore, 16S rRNA based molecular assays designed to provide information about the activity and distribution of *Marinobacter* sp. would not necessarily reveal important populations of bacterioplankton that may be responsible for a substantial fraction of the total pelagic NO$_3^-$ uptake.

Real-time PCR and T-RFLP assays both suggest that the distribution and abundance of populations of *nasA* containing bacteria are influenced strongly by NO$_3^-$ availability. This is not necessarily an intuitive finding, because NO$_3^-$ is the most oxidized and least preferred nitrogen source. Thus, one might predict that the distribution and abundance of *nasA* containing bacteria is always primarily driven by the availability of other resources, such as carbon substrates, to the extent that there is not a discernable relationship between NO$_3^-$ resource availability and *nasA* abundance and community structure. This finding suggests that NO$_3^-$ availability is sufficiently strong enough of a resource to influence the structure of aerobic heterotrophic bacterioplankton communities.

The observation that all pelagic bacterioplankton are not capable of NO$_3^-$ assimilation coupled with the result that specific groups of *nasA* containing bacteria appear to respond to NO$_3^-$ resource availability implies that the ability to aerobically assimilate NO$_3^-$ for the purpose of biomass synthesis is likely a specialized niche. The
finding that \textit{nasA} population community structure is most strongly influenced by NO$_3^-$ availability helps to clarify the previous findings of Kirchman and Wheeler (1998) and Allen et al. (in press) that reported high levels of bacterial NO$_3^-$ utilization in the subarctic Pacific and Barents Sea MIZ, possibly resulting from high NO$_3^-$ concentrations. It is thought that in such environments, fewer resources have to be allocated to NO$_3^-$ uptake than to NH$_4^+$ uptake when the former is at much higher concentration (Vallino et al. 1996). In both studies NO$_3^-$ concentration was associated more closely with the measured variation in bacterial NO$_3^-$ uptake than any other variable, however there was significant variation in both studies that could not be accounted for by any measured variable. If \textit{nasA} population community structure is responsible for some of the observed variation, it makes sense that detectable patterns between NO$_3^-$ concentration and uptake would be difficult to uncover. Although the distribution of \textit{nasA} populations is clearly influenced by NO$_3^-$, the distribution of \textit{nasA} containing bacteria is also determined by many other abiotic and biotic factors. All of these variables interact to determine the distribution of \textit{nasA} populations, and the resulting bacterial community structure is likely to introduce a source of variability into measurements of NO$_3^-$ uptake.

Because the sampling transect in this study included several different water masses, it is not surprising that T-RFLP analysis revealed that the southern North Atlantic stations (I and II) and the MIZ stations (III, IV, and V) had distinct \textit{nasA} populations. It is especially interesting that these differences are also reflected in differences in the overall pattern of bacterial DIN utilization across the transect. The fact that the MIZ stations, on average, had more diverse \textit{nasA} communities compared to the North Atlantic stations has important implications for the interpretation of some of the data previously reported from
this transect regarding bacterial $^{15}$N DIN uptake and the percentage of metabolically active cells (VSP) (Allen et al. in press, Howard-Jones et al. in press). According to measurements of $^{15}$N uptake and bacterial productivity, DIN was a much more important N resource for bacteria in the MIZ stations compared to the North Atlantic stations. At stations III – V, it was determined that bacterial N production was supported by four times as much DIN compared to stations I – II (Allen, et al. in press).

Stns. I and II showed signs of nitrogen limitation and were in post bloom phases, Stn. III was in declining bloom phase and Stns. IV and V were not DIN limited. It was also determined that bacterioplankton in the open ocean Stns. I and II exhibited much higher rates of leucine incorporation compared to bacteria at the MIZ stations. However, estimates of cell activity (VSP) indicated that the fraction of the total cells that contained a sufficient amount of rRNA to be considered metabolically active was <20% at the southern North Atlantic stations and >50% at the MIZ stations (Howard-Jones, et al. in press).

Together these observations support a scenario that bacteria likely resorted to other N sources at the southern stations where DIN was limiting and competition for DIN substrates probably severe. This might also partially explain why estimates of bacterial productivity based on incorporation rates of leucine (a rich source of N) were relatively high at the stations (Howard-Jones, in press). Measurements of bacterial DIN uptake at North Atlantic stations (I and II) were not significantly lower than they were at the northern MIZ stations. However, as a fraction of total community uptake, the bacterial contribution (% of total uptake) was significantly higher at the MIZ stations. Results obtained in this study suggest that the total community of NO$_3^-$ assimilating bacteria was
responding to the higher concentrations of NO$_3^-$ at these stations, and therefore were likely to be having a much larger impact on total community uptake.

The strong negative correlation between nasA population variability and chlorophyll a suggests that heterotrophic and autotrophic uptake are de-coupled. Perhaps when DIN is scarce (North Atlantic stations) and bacteria resort to other N resources to support production, this alleviates some of the competition for DIN which is reflected in the relatively higher percentage of total uptake contributed by the larger size fraction (>0.8) at these stations. Contrastingly, when DIN is more abundant, competition for N resources is not as intense and bacterial populations (nasA communities) that can utilize NO$_3^-$ become a more important fraction of the total, which is reflected in N uptake measurements that reveal a much higher percentage of total uptake attributable to bacteria at the MIZ stations. DIN uptake is a substantial fraction of the total DIN uptake at the MIZ stations (>40 %), and these stations also have the highest fraction of metabolically active cells, which suggests that cell activity can in large part be sustained by DIN, and to a large extent by NO$_3^-$. 

nasA containing heterotrophic bacteria represent a biogeochemically important fraction of the total bacterioplankton because they represent a possible NO$_3^-$ sink which does not involve to incorporation of CO$_2$. This study reveals that nasA populations do in fact respond in terms of abundance and changes community structure to NO$_3^-$ resource availability. Previously, we might have hypothesized that most bacteria are capable of NO$_3^-$ uptake, but only occasionally exhibit the phenotype because of the energetic costs associated with NO$_3^-$ uptake. The results presented here suggest that specific groups of nasA positive bacteria are associated with high levels of NO$_3^-$ and that detectable changes
in *nasA* population community structure occur as a result of changes in patterns of NO$_3^-$ supply.

**Literature Cited**


<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>PCR product Size (bp)</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>932F</td>
<td>CGCACAAGCRGYGGAGYATGTG</td>
<td>131</td>
<td>16S rRNA forward</td>
</tr>
<tr>
<td>1062R</td>
<td>CACRRCAGGACTGACGA</td>
<td></td>
<td>16S rRNA reverse</td>
</tr>
<tr>
<td>Mar259F</td>
<td>GCGTTGTCCCACCGTGATTGT</td>
<td>115</td>
<td>Marino sp. nasA forward</td>
</tr>
<tr>
<td>Mar373R</td>
<td>ATTGGTGACGGTGCATCCT</td>
<td></td>
<td>Marino sp. nasA reverse</td>
</tr>
</tbody>
</table>
Table 4.2. Results of SYBR Green PCR assays and estimates of cell abundances (after filtration and DNA extraction).

<table>
<thead>
<tr>
<th>Date</th>
<th>Copies of <em>Marinobacter sp.</em> nasA genes / pg DNA</th>
<th>Copies of 16s rRNA genes / pg DNA</th>
<th>Estimated copies of <em>Marinobacter sp.</em> nasA genes / L sea water</th>
<th>Estimated copies of 16s rRNA genes / L sea water</th>
<th>Estimated Ratio of <em>Marinobacter sp.</em> NasA Containing Cells/Total Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AVG</td>
<td>SD</td>
<td>AVG</td>
<td>SD</td>
<td>AVG. Copies/L</td>
</tr>
<tr>
<td>5ml</td>
<td>0.76E3</td>
<td>0.26E2</td>
<td>1.04E8</td>
<td>0.71E7</td>
<td>3.26E2</td>
</tr>
<tr>
<td>80ml</td>
<td><strong>2.23E3</strong></td>
<td>3.14E2</td>
<td><strong>2.23E3</strong></td>
<td>3.14E2</td>
<td><strong>2.23E3</strong></td>
</tr>
<tr>
<td>5mlI</td>
<td>0.55E3</td>
<td>0.62E2</td>
<td>0.53E8</td>
<td>1.17E7</td>
<td>3.62E2</td>
</tr>
<tr>
<td>80mlI</td>
<td>1.76E3</td>
<td>2.62E2</td>
<td>0.87E8</td>
<td>1.15E7</td>
<td>3.73E2</td>
</tr>
<tr>
<td>5mlII</td>
<td><strong>1.93E3</strong></td>
<td>3.94E2</td>
<td><strong>0.93E8</strong></td>
<td><strong>0.16E7</strong></td>
<td><strong>4.83E2</strong></td>
</tr>
<tr>
<td>80mlII</td>
<td>12.6E3</td>
<td>11.8E2</td>
<td>1.4E8</td>
<td>1.91E7</td>
<td>7.58E2</td>
</tr>
<tr>
<td>5mlIV</td>
<td>0.45E3</td>
<td>1.37E2</td>
<td>1.43E8</td>
<td>1.31E7</td>
<td>1.11E2</td>
</tr>
<tr>
<td>80mlIV</td>
<td><strong>91.5E3</strong></td>
<td>92.5E2</td>
<td>2.98E8</td>
<td>2.6E7</td>
<td>12.8E2</td>
</tr>
<tr>
<td>5mV</td>
<td>0.54E3</td>
<td>2.30E2</td>
<td>1.98E8</td>
<td>2.53E7</td>
<td>1.02E2</td>
</tr>
<tr>
<td>80mV</td>
<td><strong>8.86E3</strong></td>
<td>5.96E2</td>
<td>1.84E8</td>
<td>1.6E7</td>
<td>16.5E2</td>
</tr>
</tbody>
</table>
4.3. Concentrations of $\text{NH}_4^+$ and $\text{NO}_3^-$ at 5m, 30m, 80m at the five 24 hour stations. DNA Samples were collected at 5 m and 80 m.

<table>
<thead>
<tr>
<th></th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\text{NH}_4^+$</td>
<td>$\text{NO}_3^-$</td>
<td>$\text{NH}_4^+$</td>
<td>$\text{NO}_3^-$</td>
<td>$\text{NH}_4^+$</td>
</tr>
<tr>
<td>5m</td>
<td>0.947</td>
<td>0.028</td>
<td>0.688</td>
<td>0.747</td>
<td>0.930</td>
</tr>
<tr>
<td>30m</td>
<td>0.56</td>
<td>0.438</td>
<td>1.05</td>
<td>1.686</td>
<td>0.590</td>
</tr>
<tr>
<td>80m</td>
<td>2.46</td>
<td>9.304</td>
<td>2.6</td>
<td>6.651</td>
<td>3.802</td>
</tr>
</tbody>
</table>
Figure Legends

Figure 4.1. A map of the cruise track of the R/V Jan Mayen in July 1999.

Figure 4.2. Inferred phylogenetic relationships of *nasA*- and *narB*- encoded amino acids sequences from heterotrophic bacteria and cyanobacteria, respectively. The numbers at the nodes are the bootstrap values greater than 50 (out of 100). The scale bar indicates 0.1 fixed amino acid substitutions per site. The amino acid sequence of formate dehydrogenase from *Methanobacterium thermoautotrophicum* (GenBank accession number U52681), a putative evolutionary ancestor of the proteins encoded by the *nasA* and *narB* genes, was used to root the tree. All of the *nasA* sequences from clones and isolates collected in the Barents Sea are indicated with arrows. All *nasA* GenBank accession numbers are given next to their designated sequence.

Figure 4.3. Dissociation curves (A and C) and agarose gels of PCR products amplified with mar259F and mar373R and 16S rRNA primers 932F and 1062R. DNA samples were collected from the Barents Sea. The dissociation curves each show one standard, an unknown, and a blank. The PCR assays were loaded into agarose gels (B and D) after PCR amplification and melt curve analysis. The samples correspond to (station and depth): lane 1) I 5 m, lane 2) I 80 m, lane 3) II 5 m, lane 4) II 80 m, lane 5) III 5 m, lane 6) III 80 m, lane 7) IV 5 m, lane 8) IV 80 m, lane 9) V 5 m, lane 10) V 80 m

Figure 4.4. Amino acid alignment of the amplified region between mar259F and mar373R of various *nasA* sequences which are commonly detected in clone libraries, a *Marinobacter* sp., several *Marinobacter* group clone sequences (shown in bold) and a clone which was retrieved from a Barents Sea sample using the PCR primers mar259F and mar373R.
Figure 4.5. Linear relationship between Threshold Cycle values and dilutions of plasmid DNA (●) standards, which contain copies of either a 16S rRNA gene fragment or a *Marinobacter nasA* gene fragment, and unknowns which were measured by the curve (○). The exact numbers of copies of the 16S rRNA plasmid added to each reaction were as follows: (1) 2 x 10⁷, (2) 2 x 10⁶, (3) 2 x 10⁵, and (4) 2 x 10⁴ copies/µl. For *Marinobacter* group *nasA* assays, the exact numbers of copies of the *nasA* plasmids added were as follows: (1) 2 x 10⁶, (2) 2 x 10⁵, (3) 2 x 10⁴, (4) 2 x 10³, (5) 2 x 10².

Figure 4.6. Ratio of *Marinobacter* sp. *nasA* genes to 16S rRNA genes at 5m and 80m at 5 transect stations.

Figure 4.7. Inferred phylogenetic relationships of *nasA* based on inferred encoded amino acid sequences from the most typical heterotrophic bacteria based on clone library analysis.

Figure 4.8. UPGMA dendrogram based on predicted T-RFLP patterns for the restriction enzymes *Dde* I, *Mbo* I, and *Rsa* I for each of the sequence. Below the group designation, the number of clones that have the same predicted T-RFLP pattern for a given group is given in relation to the total number of cloned recovered for that group.

Figure 4.9. Example of T-RFLP profile from the Stn. V, 5m sample. Putative peak identities are indicated.

Figure 4.10. UPGMA dendrogram based on comparison of T-RFLP patterns generated with the enzymes *Dde* I, *Mbo* I, and *Rsa* I. Sample are indicated with station designation and depth. The dashed line illustrates the difference distinction between the
Figure 4.11. Scores plot of the first two principal components (PC) from a principal components analysis of the T-RFLP profiles generated in this study. The different samples are represented by the circles.

Figure 4.12. PLS-model weight-vector plot for predictor (X) variables and the response variables for the first (LV1) and second (LV2) latent variables. Predictor variables are: bacterial productivity (leucine incorporations), bacterial biomass, chlorophyll, % activity (measured via VSP), and NO$_3^-$ and NH$_4^+$ concentrations. Each circle represents a unique T-RFLP fragment.

Figure 4.13. Box plot depicting the distribution of standardized regression coefficients for each dependent variable across the entire set of response variables (the entire set terminal restriction fragments); (n = 46). The median and 25% and 75% percentile values for the regression coefficients for each variable are depicted.
Fig. 4.3

A. *Marinobacter* sp. PCR product Dissociation Curve

B. *Marinobacter* sp. PCR Amplification From Barents Sea Samples

C. 16S rRNA PCR product Dissociation Curve

D. 16S rRNA PCR Amplification From Barents Sea Samples
Barents Sea Mar259 Clone
Marinobacter sp.
BATS (5m1)
SAB SkIO dock (sd36)
Alteromonas sp.
Barents Sea (III5m23)
Marinomonas sp.
Caulobacter vibrioides
Psychrobacter sp.
Vibrio furnissi
Synechococcus

Fig. 4.4
A.) *Marinobacter* sp. *nasA* Standard Curve and Barents Sea Unknowns Using SYBR Green PCR

\[ Y = -3.416(\log_{10}x) + 36.25 \]

\[ r^2 = 0.989 \]

B.) 16S rRNA Standard Curve and Barents Sea Unknowns Using SYBR Green PCR

\[ Y = -3.523(\log_{10}x) + 37.099 \]

\[ r^2 = 0.987 \]

Fig. 4.5.
Fig. 4.6.
Fig. 4.7
Fig. 4.8

similarity

- **Unknown SAB Group**
  - (4 out of 6 clones)

- **Marinomonas**
  - (6 out of 10 clones)

- **Marinobacter**
  - (40 out of 45 clones)

- **Alteromonas**
  - (7 out of 11 clones)

- **Barents Sea Group**
  - (45 out of 50 clones)

- **Vibrio**
  - (10 out of 14 clones)

- **Unknown Barents Sea Group**
  - (2 out of 2 clones)

- **αααα**
  - (3 out of 5 clones)
Fig. 4.9

5mV

*Rsa I* digest

Fluorescence (10^3)

25 30 35 40 45 50 55 60

110 bp. (Marinobacter sp.)

202 bp. (Barents Sea Group)

358 bp. Closer closely related to Marinobacter sp.

557 bp. Unidentified peak

658 bp. Barents Sea Group

5mV

25 30 35 40 45 50 55 60

175

150

125

75

25

110 bp. (Marinobacter sp.)

202 bp. (Barents Sea Group)

358 bp. Closer closely related to Marinobacter sp.

557 bp. Unidentified peak

658 bp. Barents Sea Group
Fig. 4.10
Fig. 4.11
Fig. 4.12

Biomass
Productivity
Chlorophyll
% Active Cells

LV1, x & y: 14% & 10%
LV1, x & y: 56% & 11%

NO₃⁻

NH₄⁺
Fig. 4.13

Chlorophyll Biomass Production % Activity NH₄⁺ NO₃⁻ Regression Coefficient
CHAPTER 5

SEASONAL VARIABILITY OF BACTERIAL NITRATE ASSIMILATION AND
NITRATE ASSIMILATION GENES (*nasA*) IN A SUBTROPICAL ESTUARY

---

1 Allen, A.E., Booth, M.G., Frischer, M.E., Bronk, D.A., Verity, P.G. To be submitted to *Marine Ecology Progress Series.*
ABSTRACT

The objectives of this study were to utilize T-RFLP and SYBR Green real-time PCR techniques in conjunction with $^{15}$N tracer techniques to assess the seasonal and spatial variability of bacterial NO$_3^-$ assimilation and bacterial nitrate assimilation genes (nasA). Here we evaluate the hypothesis that the fate of NO$_3^-$ (and consequently new production) depends on the presence and abundance of specific genes within complex microbial assemblages. Results indicated a significant level of correlation between *Marinobacter* sp. *nasA* gene abundance and the magnitude of bacterial NO$_3^-$ uptake during 2000-2001. Bacterial NO$_3^-$ uptake rates and *Marinobacter* sp. *nasA* gene abundance had an annual maximum in August and October and an annual minimum in March and April. In an attempt to explain *nasA* population variability as a function of NO$_3^-$ and NH$_4^+$ concentrations and uptake rates, a partial least squares model was constructed. NO$_3^-$ uptake rate was the best predictor, by a factor of 15, of *nasA* community structure. The association between variability in the magnitude of NO$_3^-$ uptake and the abundance of *Marinobacter* sp. *nasA* genes and overall *nasA* community structure implies that specific bacterial groups have devised physiological mechanisms to utilize NO$_3^-$ as an N source when conditions for heterotrophic NO$_3^-$ uptake are favorable.
INTRODUCTION

It is generally assumed that autotrophically assimilated dissolved inorganic nitrogen (DIN) is the primary source of organic N to marine systems (Mantoura & Ownes 1988, Boynton et al. 1995). The role of heterotrophic bacteria is classically considered to be the decomposition and mineralization of autotrophically produced organic N (Pomeroy 1974). However, depending on the C:N ratio and energy content of bacteria food substrates and DIN concentrations, heterotrophic bacteria have been shown to be both a source and a sink for DIN. Modeling exercises and field experiments have demonstrated that, under certain conditions, heterotrophic bacteria can be significant consumers of DIN (Parker et al. 1975, Parsons et al. 1980, Wheeler & Kirchman 1986, Kirchman et al. 1991, Vallino et al. 1996, Caraco et al. 1998, Sanders & Purdie 1998, Middelburg & Nieuwenhuize 2000, Allen et al. in press).

Despite the fact that many studies have documented high rates of bacterial DIN assimilation, most efforts to model C and N fluxes through pelagic ecosystems continue to neglect bacterial DIN assimilation (Fasham et al. 1990, Boynton et al. 1995, Haupt et al. 1999, Olivieri & Chavez 2000, Dadou et al. 2001). In one effort to measure and model the impact of bacterially assimilated DIN on Hudson River ecosystem C and N budgets it was determined that bacterially assimilated DIN was responsible for enriching the N content of the DON and PON pool by 10% and 50% respectively over the course of 1 month (Caraco et al. 1998). In estuaries, there have been conflicting reports concerning the significance of bacterial DIN assimilation. Hoch and Kirchman (1995), reported low levels of ammonium uptake by bacteria (5 to 10 % of total ammonium uptake), and suggested that this was typical in estuaries where there are often high concentrations of
dissolved free amino acids. In contrast, it was recently reported in the Thames estuary that bacteria are responsible for 82 and 66% of the ammonium and nitrate uptake, respectively (Middelburg & Nieuwenhuize 2000a), and the authors suggested that this most likely was not unusual in heterotrophic estuarine systems, where overall respiration exceeds primary production. Middelburg and Nieuwenhuize (2000a) based their estimates of bacterial $^{15}$N assimilation on comparisons between uptake assays conducted under ambient light and in the dark, with and without antibiotic additions (bacterial inhibitors). The authors reported that there was not a detectable light effect on nitrogen uptake in the Thames estuary in February 1999.

Open ocean environments, such as the Subarctic Pacific, the North Atlantic, and the Barents Sea, where heterotrophic bacterial utilization of DIN would likely have profound effects on the fluxes of N and C in the water column, vary considerably concerning the importance of heterotrophic bacterial assimilation of DIN. In the North Atlantic, heterotrophic bacteria were responsible for 23-39% and 4-14% of the total $\text{NH}_4^+$ and $\text{NO}_3^-$ uptake, respectively, and in the Subarctic Pacific heterotrophic bacteria were reported to assimilate 31 and 32% of the total $\text{NH}_4^+$ and $\text{NO}_3^-$ respectively (Kirchman 1994, Kirchman & Wheeler 1998). These studies were based on separation of the bacterial size fraction after incubation in the presence of $^{15}$N tracers. Bacteria were subsequently collected on GF/F filters, which, in subarctic waters, have been shown not to catch about 50% of heterotrophic bacteria (Kirchman et al. 1989). In the Barents Sea, using 0.8 $\mu$m and 0.2 $\mu$m silver membrane filters to separate bacterial and eukaryotic uptake, heterotrophic bacteria were reported to be responsible for between 15 and 40% of the total community $\text{NO}_3^-$ assimilation (Allen et al. in press).
It appears, therefore, that across aquatic and marine systems, heterotrophic bacteria are responsible for a highly variable, but often times substantial, contribution to total NH$_4^+$ and NO$_3^-$ assimilation. While differences in the magnitude of bacterial NO$_3^-$ assimilation are certainly a result of differences in variables such as DIN and DOC substrate concentrations, they are also probably due to differences in bacteria-phytoplankton community structure. It has been shown in a controlled experiment, for example, that bacterial DIN demand and uptake exhibits contrasting patterns depending in the presence of different species of phytoplankton. Diatoms are thought to release organic material of a refractory and polymeric nature which leads to a delay in bacterial demand for DIN and the cocolithophorid *Emiliania huxleyi* releases much more labile organic matter which promotes a more immediate bacterial demand for DIN (Sanders & Purdie 1998). Furthermore, it has also been shown that not all heterotrophic bacteria are capable of NO$_3^-$ assimilation (Allen et al. 2001). Therefore, it is likely that the community structure and metabolic diversity of the bacterioplankton exerts some influence on the magnitude of bacterial DIN assimilation.

Molecular techniques have been employed to illuminate factors that control the rates of fluxes and transformations in nitrogen cycling processes (Ward 1996, Zehr & Hiorns 1998, Zehr & Voytek 1999, Zani et al. 2000, Zehr & Ward 2002). Molecular techniques have been successfully used to characterize bacteria and the genes that are important in several aspects of the nitrogen cycle, including nitrification, denitrification, nitrogen fixation as well as heterotrophic bacterial nitrate assimilation (Zehr & McReynolds 1989, Kirshtein et al. 1991, Voytek & Ward 1995, Voytek et al. 1997, Zehr

Recently, more comprehensive and quantitatively informative, molecular techniques such as terminal restriction fragment length polymorphism (T-RFLP) and real-time PCR have been used to evaluate the spatial heterogeneity of nitrous oxide reductase (nosZ) genes and to measure the abundance of Pseudomonas stutzeri dissimilatory nitrite reductase (nirS) genes in sediments (Scala & Kerkhoff 2000, Gruntzig et al. 2001). T-RLFP analysis is a powerful technique that has been used to assay and compare microbial community structure and diversity in the lab and in the field (Bruce 1997, Liu et al. 1997, Horz et al. 2000, Dollopf et al. 2001, Moeseneder et al. 2001).

The objectives of the present study were to utilize T-RFLP and SYBR Green real-time PCR techniques in conjunction with $^{15}$N tracer techniques to assess the seasonal and spatial variability of bacterial NO$_3^-$ assimilation and bacterial nitrate assimilation genes (nasA), and to begin to evaluate the hypothesis that the fate of NO$_3^-$ (and consequently new production) depends on the presence and abundance of specific genes within complex microbial assemblages. We address the hypothesis that, on a seasonal basis, populations of nasA containing bacteria are not randomly distributed among the total bacterioplankton community, and that the variability associated with the abundance and community structure of nasA communities is reflected in the magnitude of bacterial $^{15}$NO$_3^-$ uptake.
MATERIAL AND METHODS

In the Skidaway River Estuary (31°N, 80°W), 15N experiments were conducted and 40 L water samples were filtered for DNA extraction in August and October 2000 and in January, March, April, May, June and July 2001. For the purpose of generating a more comprehensive nasA sequence database, additional water samples used to generate nasA clone libraries for this study were collected from the South Atlantic Bight (SAB) mid-shelf (31°31’N, 80°13’W) in October 2000 and from depths of 5 m and 800 m at the BATS station in the Sargasso Sea (32°10’N, 64°30’W) during July 2000 aboard the R/V Cape Hatteras.

Uptake of Inorganic Nitrogen. Chemical tracer techniques were used to estimate uptake rates of NO3− and NH4+ into the >0.8 µm and <0.8 µm size-fraction. Prior to the experiments in the estuary, an additional treatment of water pre-filtered through 3.0 µm-pore-size polycarbonate cartridge filters (Gelman Sciences, Inc., Ann Arbor, Mich.) was conducted to remove some of the large particles and suspended matter. Although bacterial activity and abundance might be correlated with these particles, our samples for nucleic acid extraction were pre-filtered through a 3.0 µm filter in order to filter a large volume of water and our goal was to conduct 15N experiments on the same size fraction that was used for nucleic acid extractions. Water samples (500 ml) were spiked with 0.1 µmol l−1 15NH4+ and 15NO3− (Cambridge Isotope Laboratories, Andover, Mass.). Samples were incubated in an on-deck incubator with running estuarine water. Incubations lasted ~2.5 h and were performed under ambient light in triplicate.

The incubations were terminated by passing the sample through a 0.8 µm silver filter (Osmonics Inc., Minnetonka, MN) to collect the 15N labeled particulate nitrogen in
the >0.8 \( \mu \)m size class. The filtrate from the samples was then collected and subsequently passed through a 0.2 \( \mu \)m silver filter to measure the \( ^{15} \)N labeled particulate nitrogen in the bacterial size class. Filters were immediately frozen at -20\( ^\circ \)C onboard the ship. Upon returning to the lab, filters were dried at 50\( ^\circ \)C, ampulated and analyzed by mass spectrometry using a Europa GEO 20/20 with a ANCA prep. unit. Uptake rates were calculated as described by Bronk (1998).

**DNA collection, PCR amplification and cloning.** DNA samples were extracted and purified from 40 liters of water. To remove large plankton cells, water was consecutively pre-filtered, under vacuum, through a 3-\( \mu \)m-pore-size polycarbonate cartridge filter (Gelman Sciences Inc., Ann Arbor, MI) and a 142 mm-diameter 0.8-\( \mu \)m-pore-size polycarbonate Supor filter (Gelman) with a custom manufactured acrylic filter holder. Bacterial cells in the filtrate were collected onto a 142 mm-diameter 0.2-\( \mu \)m-pore-size polycarbonate Supor filter (Gelman) and stored at -20\( ^\circ \)C aboard the ship and transferred to -80\( ^\circ \)C in the lab. DNA was extracted with an Ultra Clean Mega Prep soil DNA kit (Mo Bio Laboratories, Inc., Solana Beach, Calif.), and nested PCR with universal \( nasA \) primers was performed in order to detect the presence or absence of \( nasA \) as described by Allen et al. (2001).

From samples collected from the Skidaway River estuary and the SAB mid-shelf in October 2000, and from 5 m and 800 m at the BATS station in July 2000, PCR products were agarose gel purified and clone libraries were constructed as described by Allen et al. (2001). 35 clones from each of the 4 libraries were screened and sequenced. \( nasA \) amino acid sequences were aligned using the CLUSTAL W (version 1.7) multiple-sequence-alignment algorithm (Thompson et al. 1994). Phylogenetic trees were inferred.
and drawn by using the TREECON software package (version 1.3b) (Van de Peer and De Wachter, 1997) and the Kimura two-parameter model for inferring evolutionary distances. Bootstrap estimates (100 replicates) of confidence intervals were also made by using the algorithms available in the TREECON package.

**Sequencing.** Sequences were determined by automated sequencing with a Beckman CHQ 2000XL DNA Analysis System. Sequencing reactions were facilitated by using a CEQ DTCS dye terminator cycle sequencing quick start kit, following the protocol recommended by the manufacturer (Beckman Coulter, Fullerton, Calif.). Sequence analysis was accomplished by using Beckman CEQ 2000XL Sequence Analysis software, version 4.3.9. Sequencing was accomplished with standard M13F and M13R primers as described by Allen et al. (2001).

**SYBR Green Real-time PCR.** A primer pair suitable for use in a SYBR Green real-time PCR assay that is specific for a clade of bacterial *nasA* genes typified by two *Marinobacter* sp. isolates was designed. In order to estimate the contribution of *Marinobacter* like *nasA* genes as a percentage of the total bacteria between samples, an additional set of universal eubacterial 16S rRNA primers was designed to control for bacterioplankton abundance in the water column (Table 5.1). Expressing *Marinobacter* like *nasA* gene abundance as a percentage of total bacteria also controlled for variability in DNA extraction. *Marinobacter* sp. *nasA* primer design was facilitated by using the software package Primer Premier (version 5.0) (Premier Biosoft International, Palo Alto, CA). Criteria for optimal primer pairs suitable for the SYBR Green PCR assay include a GC content of 40-60%, a length of at least 18 bp, and a resulting PCR product of less than 200 bp with one melting domain.
SYBR Green is a minor-groove DNA binding dye with a high affinity for dsDNA and exhibits elevated levels of fluorescence upon binding to double stranded dsDNA (Witter et al. 1997). The SYBR Green dye is excited at a 485-nm wavelength, and the emission is measured at a 520-nm wavelength. In the Bio-Rad iCycler IQ Real-Time Detection System, the fluorescence of the SYBR Green dye is monitored during the extension step of the PCR cycles and the increase in fluorescence is used to quantify to concentration of starting template by estimating the rate of doubling during the linear range of amplification. The specificity of the amplified product is monitored by its melting curve. Because the melting curve of a product is dependent on its GC content, length, and sequence, specific amplification can be distinguished from nonspecific amplification by examining the melting curve (Rare et al. 1997). In this study a pair of SYBR Green real-time PCR primers which is specific for a clade of bacterial *nasA* genes typified by two *Marinobacter* sp. isolates genes was designed and used to quantify this group in relation to total bacteria, which was measured with a pair of SYBR Green real-time PCR primers that are specific for the 16S rRNA gene.

SYBR Green PCR amplifications were performed using a Bio-Rad iCyclerIQ Real-Time PCR Detection System (BioRad, Hercules, CA.). The reactions were carried out in a 96-well plate in a 25-µl reaction volume containing 12.5 µl of 2x QuantiTech SYBR Green Master Mix (Qiagen, Valencia, Calif.), 0.2 µM concentration of each forward and reverse primer, and approximately 100 ng of DNA. The thermal profile for SYBR Green PCRs was 95°C for 10 min, followed by 25 cycles for 16S rRNA PCR and 30 cycles for *Marinobacter nasA* PCR of 95°C for 15s, 58°C for 10s, and 72°C for 1 min. This was followed immediately by a melt curve thermal profile, which was 65°C for 1
min, followed by 125 cycles of 0.2°C increments each for 10s. In each 96-well plate, a
dilution series of the plasmid standard for the respective target gene was run along with the unknown samples. Each sample was run in replicates of three and all reaction were repeated at least three times independently to ensure the reproducibility of the results.

**T-RFLP analysis (PCR amplification, digestion, and capillary electrophoresis).** The upstream primer, *nas*964 (Allen et al. 2001), was labeled at the 5’ end with a phosphoramidite linked D4 dye (Beckman Coulter, Fullerton, Calif.). Primers were synthesized by Invitrogen (Carlsbad, Calif.). A nested 25 µl PCR reaction was performed as described above with 50 ng of template DNA added to three first round reactions; each first round reaction was then used as a template in four second reactions for a total of PCR reactions for each DNA sample. First and second round reactions contained 500 pmol of primer and ran for 35 and 30 cycles respectively.

After amplification, each 25 µl PCR reaction was loaded into a separate well on a
0.8% agarose gel containing 1x GelStar nucleic acid stain (BMA, Rockland, ME), and separated by gel electrophoresis at 60V for 3 - 5 hours in 1x TAE buffer at room temperature. The approximately 800 bp *nas*A PCR products were then visualized on a Dark Reader (Clare Chemical Research, Denver, CO), which excites fluorophores between 420 and 500 nm and prevents oxidation of the phosphoramidite linked dye, which occurs under standard agarose gel UV visualization. *nas*A PCR products were then excised from the gel and eluted using Quantum Prep DNA Gel Extraction Spin Columns (Bio-Rad, Hercules, Calif.). The eluted PCR products from each DNA were then pooled and precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes of 95% ethanol, followed by spinning at 14,000 rpm for 15 min. The DNA pellet was
washed twice with 70% ethanol, dried, and resuspended in 40 µl of sterile water. The DNA concentration of the purified and pooled PCR products was estimated by fluorometry with a Turner Design TD-700 fluorometer (Turner Designs, Sunnyvale, Calif.) after staining with the PicoGreen dsDNA Quantitation Kit (Molecular Probes, Eugene, OR). 50 ng of purified PCR product was then digested separately in triplicate with 10 units of the restriction enzymes Dde I, Mbo I, and Rsa I (New England Biolabs). Digests were incubated at 37°C for 5 hours in the appropriate digestion buffer provided by the manufacturer.

The digested DNA was precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes of 95% ethanol, followed by spinning at 14,000 rpm for 15 min. The DNA pellet was washed twice with 70% ethanol, dried, and resuspended in 40 µl of deionized formamide and 0.5 µl of CEQ DNA Size Standard – 600 (Beckman Coulter, Fullerton, Calif.). T-RFLP digests were then separated via capillary electrophoresis on a Beckman CEQ 2000XL DNA Analysis System with a CEQ capillary array (33 centimeter long, 75 micrometer internal diameter) and CEQ linear polyacrylamide denaturing gel (LPA-1). After denaturing for 120 sec. at 90°C, samples were injected into the capillary for 30 sec. at 2.0kV and then separated for 70 min. at 4.8kV. The fluorescent dye at the end of the digested fragment was detected by a photo multiplier and analyzed using the Beckman 2000XL Fragment Analysis software package version 4.3.9, which displays the various T-RFLPs as a series of peaks.

Peak height was used a proxy for the relative abundance of taxa represented by restriction fragments. Integrated peak area was not used due to overlap between some peaks. Peak height was normalized against the sum of peak heights in a profile, and only
peaks of at least 3000 relative fluorescent units were considered for analysis. Fragments less then 2 bases apart from a larger peak were discarded to account for signal noise in electropherograms. To account for small variations in run time between samples, peaks from different profiles with less than one base difference were considered the same. Also peaks that did not occur in at least two profiles from different samples were considered artifacts and removed.

**Multivariate Analysis.** Partial least squares regression and principal component analysis was performed using the Unscrambler 7.6 software package (CAMO Inc., Corvallis, OR.). Before the analysis, all the variables were autoscaled: the geometric means were centered to zero and all data were normalized for standard deviation. Full cross validation was used in the modeling procedure, one sample being omitted at a time. In partial least squares regression, the regression coefficient for each independent or environmental variable expresses the link between variation in that variable and the variation associated with a particular response variable. The independent variables used for analysis were NO$_3^-$ and NH$_4^+$ concentration, and NO$_3^-$ and NH$_4^+$ uptake rate. These data were provided to the model for each sample and the populations of T-RFLP fragments from all three of the digests for each sample were modeled as dependent variables.

**RESULTS**

**Analysis of nasA sequences from clones and isolates**

Thirty-five *nasA* clones from each of four clone libraries were analyzed and compared to previously sequenced *nasA* PCR products from clones and bacterial isolates (Fig. 5.1). *nasA* sequences clustered phylogenetically into eleven clades, if the Beta
Proteobacteria and Cyanobactreia are included. In general, SAB mid-shelf and especially Skidaway River estuary clone libraries contain \textit{nasA} sequences that belong to a larger number of different clades compared to Sargasso Sea or Barents Sea clone libraries, which almost exclusively contain clones that fall into the \textit{Marinobacter} sp. and Barents Sea groups, respectively. All nine of the major heterotrophic bacterial \textit{nasA} clades thus far identified have representatives from SAB mid-shelf and from the Skidaway River estuary. One deeply rooted clade, which does not currently contain a \textit{nasA} sequence from an identified organism, is comprised only of clones from the SAB mid-shelf and from the Skidaway River Estuary.

\textbf{SYBR Green Real-Time PCR}

Because the DNA–binding dye SYBR green I binds all double-stranded DNA molecules, real-time PCR amplification that is monitored with SYBR Green I must be optimized so that primer-dimer formation and non-specific amplification do not occur. To evaluate whether or not primer-dimers and single PCR amplicons are generated, melt curve analysis can be used to identify the melting domains of double stranded DNA products that are generated during PCR amplification.

To assess the specificity of the reactions, PCR products were evaluated with melt curve analysis and inspected on agarose gels (Fig. 5.2). For both primer sets the generated PCR amplicon clearly dissociates as a solitary peak indicating that the PCR reaction is specific for the intended target. Also, melt curve analysis and examination of PCR reactions on agarose gels did not reveal any primer-dimer formation, which suggests that the increase in SYBR green fluorescence during PCR amplification is attributable to an increase of the intended target.
In order to quantify unknowns using SYBR Green real-time PCR, it is critical that all of the PCR reactions exhibit a constant rate of doubling. In the standard curve, the threshold cycle (the point at which the fluorescence rises above the background) for each of the standards is plotted against log of the concentration of starting template copies for that standard. The resulting slope of this line is informative with regard to how consistent the rate of doubling is between reactions, i.e. the efficiency. An efficiency of 100 % corresponds to a slope of –3.332 in the regression equation describing the standard curve and indicates that all of the standards were amplifying at the exact same rate. The SYBR green PCR assays optimized in this study consistently produced linear standard curves with slopes that indicate that all of the reactions were amplifying at approximately the same rate (Fig 5.2).

The results of the real-time PCR assays are presented in Table 5.2. Multiplying the total number of copies in the DNA extract by the volume of water filtered generated estimates of cell concentrations in the water column. There is much more variability associated with the estimated number of Marinobacter nasA genes than there is with the 16S rRNA genes. Estimates of Marinobacter nasA vary by two orders of magnitude and estimates of 16S rRNA genes do not vary much at all. In order to estimate the fraction of total cells that contain Marinobacter sp. like nasA genes, the estimates of 16S rRNA genes/L of sea water were divided by 4, assuming an average of 4 16S rRNA gene copies/cell (Klappenbach et al., 2001), and Marinobacter sp. nasA gene concentration was divided by this number. Assuming there is 1 copy of nasA per cell (assumption based on 5 Genbank genomes), real-time PCR results indicate, on average for the year,
that there are approximately 4.0e4 *Marinobacter* like *nasA* containing cells/L, and 10^7 total cells/L.

According to cell counts performed on some of the samples, these estimates of cell abundance underestimate actual cell concentrations by approximately a factor of 10. Although these approximations of total cell concentration are underestimates, they are valuable because they provide a means of standardizing the samples to one another when attempting to compare *Marinobacter* sp. *nasA* abundances.

**15^N Uptake of DIN in the Skidaway River Estuary**

The < 0.8 µm size fraction appeared to take up a highly variable percentage of the NO_3^- and NH_4^+ in the estuary (Fig 5.4). Experiments were conducted with and without a 3.0 µm pre-filter. Whether or not the incubation is pre-filtered to remove large particles had a significant impact on measurements of percent uptake because of the amount of material that got caught on the 0.8 µm filter in samples that were not 3.0 µm pre-filtered. In the 3.0 µm pre-filter treatments, the 0.2 – 0.8 µm size fraction accounts for between 25 and 80 % of the total NH_4^+ and NO_3^- uptake, and in the whole water incubations that are not pre-filtered the 0.2 – 0.8 µm size fraction accounts for between 5 and 20 % of the total NH_4^+ and NO_3^- uptake. Interestingly, the uptake rate and total uptake by the < 0.8 µm size fraction size fraction is not significantly different in the treatments. Bacterial NO_3^- uptake rates vary considerably over the course of the year, ranging from an annual high of between 10 and 15 nmoles N l^-1 d^-1 in July, August and October to <1 nmoles N l^-1 d^-1 in March and April. NH_4^+ and NO_3^- concentrations follow a similar trend (Table 5.3), except in August and October when DIN concentrations are relatively low and bacterial DIN uptake remains high.
Relationship Between $^{15}$N Uptake of NO$_3^-$ and Marinobacter nasA abundance

NO$_3^-$ uptake by the < 0.8 µm size fraction and estimates of Marinobacter sp. nasA copy number, normalized to estimates of total cells, follow a seasonal trend of high uptake and gene abundance in the Summer and Fall and low uptake and gene abundance in the early Spring (Fig. 5.5). This relationship is significant (Fig 5.6) ($r^2 = 0.77$, $n = 8$, $P < 0.005$). As a percentage of total cells, Marinobacter sp. nasA containing cells are most abundant in October, June, July, August. Agreement between the molecular real-time PCR assay and $^{15}$N tracer assays is poorest in January when bacterial $^{15}$N uptake is 5 nmole N l$^{-1}$d$^{-1}$ and Marinobacter sp. nasA numbers are very low.

TRFLP Analysis of nasA genes

Over the course of the year T-RFLP patterns of nasA populations revealed several persistent terminal restriction fragments that were always detected. Mbo I digests had peaks of 84 and 192 bp in all of the samples with the exception of January (Fig 5.7.). The putative identities of these peaks are Marinobacter sp. and Marinomonas sp., respectively. The corresponding Dde I and Rsa I peaks for these groups also appear in all of the samples. In the April sample, the large 70 bp peak corresponds to clones in the Vibrio sp. group. This peak is also dominant in the March and July samples as are the putative Dde I and Rsa I peaks for the same clones in the Vibrio sp. group. One very persistent Mbo I peak, 146 bp, appears in every sample. This is the predicted terminal restriction fragment for the clone sd16, which is deeply rooted between the Vibrio sp. and Alpha groups (Fig. 5.1). The putative Dde I and Rsa I peaks for this clone also appear in all of the samples. All three of the enzymes combined yielded a total of 48 terminal restriction fragments for the entire data set. Each of the samples assayed yielded between
18 and 21 fragments for all of the enzymes, except for the August 2001 sample, which produced 31 terminal restriction fragments.

Principal components analysis was performed using normalized heights of terminal restriction fragment peaks as input variables. The first two principal components, PC1 and PC2, explained 30% and 23%, respectively, of total variation in peak height and presence and absence between the different samples (Fig. 5.8). PCA revealed that most of the samples, with the exception of the October 2000 and August 2001, are fairly similar to one another. This implies that, over the course of the year, *nasA* communities do not change substantially, and that most of the major groups are persistent through changes in season.

To test if the variance in peak height, presence and absence (*nasA* gene populations) is related to $^{15}\text{NO}_3^-$ and $^{15}\text{NH}_4^+$ uptake rates or $\text{NO}_3^-$ and $\text{NH}_4^+$ concentrations, a partial least squares regression model was constructed (Fig. 5.9). Of the four variables tested, $\text{NO}_3^-$ uptake rate was the best predictor of the variability associated with population of *nasA* terminal restriction fragments. This implies that variation in the magnitude of bacterial $\text{NO}_3^-$ uptake rates is reflected in changes in *nasA* community structure. $\text{NH}_4^+$ uptake and $\text{NH}_4^+$ concentration have a nearly neutral relationship to the population of response variables. Interestingly, $\text{NO}_3^-$ is not positively correlated with the overall variation in *nasA* communities, but several terminal restriction fragments are positively associated with $\text{NO}_3^-$. The fact that $\text{NO}_3^-$ concentration appears to be negatively correlated with the population *nasA* terminal restriction fragments is probably an artifact of the fact that in August, when $\text{NO}_3^-$ uptake rates are highest and more terminal restriction fragments are observed than in any other month, $\text{NO}_3^-$ concentrations
are very low (Fig. 5.6, Table 5.3). A comparison of the median of standardized regression coefficients for each independent variable shows that NO$_3^-$ uptake rate is a better predictor by a factor of 15 than any of the other variables. Also NO$_3^-$, which overall appears to have a neutral effect, has more positive regression coefficients that either NH$_4^+$ uptake or NH$_4^+$ concentration (Fig. 5.10).

**Discussion**

The overall richness of heterotrophic bacterial populations capable of NO$_3^-$ assimilation in marine systems remains unclear. We are still unable to assign any taxonomic designation to at least four major clades of *nasA* genes (Fig. 5.1). Despite the uncertainty associated with total taxonomic diversity of *nasA* populations, it does appear that the *Marinobacter* sp., *Marinomonas* sp., *Vibrio* sp., and *Alteromonas* sp. are each important contributors to the total *nasA* community. It is important to note, however, that organisms from each of these groups that are not capable of NO$_3^-$ assimilation have been recovered. Therefore, any *in situ* molecular approach designed to assay the distribution and abundance of populations of NO$_3^-$ assimilating bacteria must be targeted to the level of the functional gene.

The T-RFLP result that most of the major groups are persistent throughout most of the year is reflective of the fact that this estuary frequently receives episodic pulses of inorganic nitrogen (Verity, in press). Nutrient sources from inorganic- and organic-based fertilizers from surrounding urban and agricultural land use, groundwater-derived nutrients (Simmons, 1992), freshwater inputs from the Ogeechee River (Bronk, pers. comm.), and atmospheric inputs from rainfall have all been shown to be important in
terms of delivering fixed nitrogen to southeastern US estuaries (Paerl 1997, Seitzinger & Sanders, 1999). Therefore, it is plausible that populations of *nasA* containing heterotrophic bacteria, which are able to utilize NO₃⁻, are a stable component of the total bacterial assemblage. Based on our limited data, it also appears that there is a much larger number of *nasA* taxa in clone libraries generated from estuarine samples compared to clone libraries constructed from samples collected in non-estuarine environments. The larger number of different *nasA* taxa recovered from Skidaway River estuary samples reflects the relatively high level of NO₃⁻ resource availability in estuarine systems compared to other types of marine environments. Recent studies have shown the dominant role that heterotrophic bacteria can play in estuarine inorganic nitrogen assimilation (Middelburg and Nieuwenhuize, 2000a and b, Caraco, et al. 1998), and the results of this study reflect this by demonstrating that the major groups of *nasA* populations are present throughout the year.

It does appear, however, that the relative importance of bacterial NO₃⁻ utilization has a seasonal basis that is evident in measurements of the abundance *nasA* populations. An increase in the magnitude of bacterial inorganic nitrogen assimilation is often associated with a pulse of carbon rich organic material with a high C:N ratio, high bacterial turnover rates, and a dominance of heterotrophic processes (Vallino et al. 1996, Sanders & Purdies 1998, Middelburg & Nieuwenhuize 2000). In Chesapeake Bay, Bronk et al. (1998) observed that in the spring the Bay was reliant on allochthonous new nitrogen and was dominated by autotrophic processes but in the fall the Bay was dominated by heterotrophic processes and more reliant on autochthonous regenerated nitrogen. Therefore, it is not surprising that in the present study *nasA* populations
achieved their annual peak during late summer and fall when DIN demand and heterotrophic processes are likely to be most important. The good agreement between *Marinobacter* sp. *nasA* copy number, and $^{15}$N uptake is surprising considering genes from only one clade are being quantified. This implies that *Marinobacter* sp. organisms are responsible for much of the observed bacterial NO$_3^-$ uptake or alternatively that *Marinobacter* sp. populations are a reliable indicator of the overall importance of *nasA* populations. It is interesting to note that, according to patterns of T-RFLP peak height, *Marinobacter* sp. populations are a relatively minor contributor to the total *nasA* community, which supports the latter hypothesis.

The result that *nasA* population abundance, as a percentage of the total bacterioplankton, correlates positively with $^{15}$NO$_3^-$ uptake supports the hypothesis that *nasA* populations are not randomly distributed among the bacterioplankton and do not simply reflect the distribution and abundance of bacterial groups that contain *nasA* and are primarily driven by other processes and resources. The distribution and abundance of *nasA* populations is likely determined by the interaction of many biotic and abiotic variables, but the association between the magnitude of bacterial $^{15}$NO$_3^-$ uptake and the abundance of *Marinobacter* sp. *nasA* populations implies that there are specific bacterial groups that have devised physiological mechanisms to respond positively when conditions for heterotrophic NO$_3^-$ uptake are favorable.

The finding that NO$_3^-$ is a relatively weak predictor of *nasA* community structure conflicts with the findings of a previous study that *nasA* populations are primarily driven by patterns of NO$_3^-$ resource availability (Chapter 4). This can be explained by the fact that measurements of NO$_3^-$ concentrations actually reveal very little about NO$_3^-$ supply
rates when biological removal processes are important. Also, the observation that nasA abundance and uptake are coupled implies that, in this study, bacterial productivity is driving much of the uptake. We hypothesize that in situations where NO$_3^-$ concentrations are high but the magnitude of $^{15}$N uptake is relatively low (Allen et al. in press), the abundance, community structure, and, expression of nasA groups becomes uncoupled from uptake.

**Literature Cited**


Simmons GM Jr (1992) Importance of submarine groundwater discharge (SGWD) and seawater cycling material flux across sediment/water interfaces in marine environments. Maine Ecology Progress Series 84:173-184


TABLE 5.1. Oligonucleotide primers used for *Marinobacter*-like *nasA* and 16S rRNA genes

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>PCR product Size (bp)</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>932F</td>
<td>CGCACAAGCRGYYGGAGYATGTG</td>
<td>131</td>
<td>16S rRNA forward</td>
</tr>
<tr>
<td>1062R</td>
<td>CACRRCAAGTAGCTGACGA</td>
<td></td>
<td>16S rRNA reverse</td>
</tr>
<tr>
<td>Mar259F</td>
<td>GCGTTGTCCACCGTGATTGT</td>
<td>115</td>
<td><em>Marino. sp. nasA</em> forward</td>
</tr>
<tr>
<td>Mar73R</td>
<td>ATTGGTGACGTTGCCATCTTT</td>
<td></td>
<td><em>Marino. sp. nasA</em> reverse</td>
</tr>
</tbody>
</table>
Table 5.2. Results of SYBR Green Real-Time PCR assays and estimates of cell concentrations (after filtration and DNA extraction)

<table>
<thead>
<tr>
<th>Date</th>
<th>Copies of <em>Marinobacter</em> sp. <em>nasA</em> genes / pg DNA</th>
<th>Copies of 16s rRNA genes / pg DNA</th>
<th>Estimated copies of <em>Marinobacter</em> sp. <em>nasA</em> genes / L sea water</th>
<th>Estimated copies of 16s rRNA genes / L sea water</th>
<th>Estimated Ratio of <em>Marinobacter</em> sp. <em>nasA</em> Containing Cells/Total Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AVG</td>
<td>SD</td>
<td>AVG</td>
<td>SD</td>
<td>AVG</td>
</tr>
<tr>
<td>August 2000</td>
<td>1.54E5</td>
<td>3.26E4</td>
<td>0.8E8</td>
<td>0.32E7</td>
<td>8.28E4</td>
</tr>
<tr>
<td>October 2000</td>
<td>10.3E5</td>
<td>3.07E4</td>
<td>2.7E8</td>
<td>1.73E7</td>
<td>4.01E4</td>
</tr>
<tr>
<td>January 2001</td>
<td>0.22E5</td>
<td>0.44E4</td>
<td>1.91E8</td>
<td>3.29E7</td>
<td>0.38E4</td>
</tr>
<tr>
<td>March 2001</td>
<td>0.04E5</td>
<td>0.18E4</td>
<td>1.09E8</td>
<td>0.73E7</td>
<td>0.09E4</td>
</tr>
<tr>
<td>April 2001</td>
<td>0.29E5</td>
<td>0.17E4</td>
<td>1.7E8</td>
<td>0.62E7</td>
<td>0.71E4</td>
</tr>
<tr>
<td>May 2001</td>
<td>1.49E5</td>
<td>1.25E4</td>
<td>1.96E8</td>
<td>0.49E7</td>
<td>1.35E4</td>
</tr>
<tr>
<td>June 2001</td>
<td>1.6E5</td>
<td>1.08E4</td>
<td>0.69E8</td>
<td>1.02E7</td>
<td>3.56E4</td>
</tr>
<tr>
<td>July 2001</td>
<td>0.92E5</td>
<td>0.88E4</td>
<td>0.77E8</td>
<td>0.83E7</td>
<td>2.15E4</td>
</tr>
</tbody>
</table>
Table 5.3. Concentrations of NH$_4^+$ and NO$_3^-$ in the Skidaway River Estuary at the time (month/year) of $^{15}$N Experiments and DNA Collection

<table>
<thead>
<tr>
<th></th>
<th>8/00</th>
<th>10/00</th>
<th>1/01</th>
<th>3/01</th>
<th>4/01</th>
<th>5/01</th>
<th>6/01</th>
<th>7/01</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH$_4^+$ (µM)</td>
<td>0.1</td>
<td>0.3</td>
<td>0.6</td>
<td>0.98</td>
<td>1.2</td>
<td>1.2</td>
<td>1.5</td>
<td>2.2</td>
</tr>
<tr>
<td>NO$_3^-$ (µM)</td>
<td>0.35</td>
<td>2.0</td>
<td>1.2</td>
<td>2.6</td>
<td>0.52</td>
<td>2.0</td>
<td>7</td>
<td>6.5</td>
</tr>
</tbody>
</table>
Figure Legends

Figure 5.1. Inferred phylogenetic relationships of nasA- and narB- encoded amino acid sequences from heterotrophic bacteria and cyanobacteria, respectively. The numbers at the nodes are the bootstrap values greater than 50 (out of 100). The scale bar indicates 0.1 fixed amino acid substitutions per site. The amino acid sequence of formate dehydrogenase from Methanobacterium theromautrophicum (Genbank accession number U52681), a putative evolutionary ancestor of the proteins encoded nasA and narB genes, was used to root the tree. All of the nasA sequences from clones and isolates collected from the Skidaway River estuary and SAB waters are indicated in bold. All nasA GenBank accession numbers are given next to their designated sequence.

Figure 5.2. Dissociation curves (A and C) and agarose gels of PCR products amplified with mar259F and mar373R and 16S rRNA primers 932F and 1062R. DNA samples were collected from the Skidaway River Estuary. The dissociation curves each show one standard, an unknown, and blank. The PCR assays were loaded into agarose gels (B and D) after PCR amplification and melt curve analysis. The samples correspond to (month and year): lane 1) 8/00, 2) 10/00, 3) 1/01, 4) 3/01, 5) 4/01, 6) 5/01, 7) 6/01, and 8) 8/01.

Figure 5.3. Linear relationship between Threshold Cycle values and dilutions of plasmid DNA (●) standards, which contain copies of either a 16S rRNA gene fragment or a Marinobacter nasA gene fragment, and unknowns which were measured by the curve (○). The exact numbers of copies of the 16S rRNA plasmid added to each reaction were as follows: (1) 2 x 10^7, (2) 2 x 10^6, (3) 2 x 10^5, (4) 2 x 10^4 copies/µl. For Marinobacter group nasA assays, the exact numbers of copies of the nasA plasmids added were as follows: (1) 2 X 10^6, (2) 2 x 10^5, (3) 2 x 10^4, (4) 2 x 10^3, (5) 2 x 10^2
Figure 5.4. Average percentage of total NO$_3^-$ or NH$_4^+$ uptake by the < 0.8 µm size fraction. (A) Unfractionated, particle rich estuary water was incubated. (B) Samples were pre-filtered through a 3.0 µm cartridge filter prior to $^{15}$N incubation.

Figure 5.5. Seasonal measurements of $^{15}$NO$_3^-$ uptake by the < 0.8 µm size fraction and simultaneous measurements of *Marinobacter* group *nasA* gene abundance with SYBR Green real-time PCR assays.

Figure 5.6. Relationship between < 0.8 µm size fraction $^{15}$NO$_3^-$ assimilation and the ratio of *Marinobacter* group *nasA* genes to 16S rRNA genes.

Figure 5.7. Example of T-RFLP profiles from dock samples. Putative peak identity for *Marinobacter* sp. *nasA* sequences are indicated.

Figure 5.8. Scores plot of the first two principal components (PC) from a principal components analysis of the T-RFLP profiles generated in this study.

Figure 5.9. PLS-model weight-vector plot for predictor (X) variables and the response variables for the first (LV1) and second (LV2) latent variables. Predictor variables are: NO$_3^-$ concentration, NH$_4^+$ concentration, NO$_3^-$ uptake rate, and NH$_4^+$ rate.

Figure 5.10. Box plot depicting the distribution of standardized regression coefficients for each dependent variable across the complete data set of response variables (the entire set of restriction fragments); (n = 48). The median and 25% and 75% percentile values for the regression coefficients for each variable are depicted.
Fig. 5.2

A. *Marinobacter* sp. *nasA* PCR product Melt Curve

B. *Marinobacter* sp. *nasA* PCR Amplification from SAB Samples

C. 16S rRNA PCR product Melt Curve

D. 16S rRNA PCR Amplification from SAB samples
A.) Marinobacter sp. nasA Standard Curve and Skidaway River Estuary Unknowns Using SYBR Green PCR

\[ Y = -3.339 \log_{10}(x) + 36.336 \]
\[ r^2 = 0.993 \]

Fig. 5.3
Fig. 5.4
Fig. 5.5

NO$_3^-$ Uptake (n mole N l$^{-1}$ d$^{-1}$) (<0.8 um size-fraction)

Marinobacter sp. / 16s rRNA Genes

SYBR Green Real-Time PCR

August '00, October '00, January '01, March '01, April '01, May '01, June '01, July '01
Fig. 5.6.
Fig. 5.7

**Marinobacter sp. nasA**

---

**October 2000, Mbo I**

---

**January 2001, Mbo I**

---

**April 2001, Mbo I**

---

**June 2001, Mbo I**

---
Fig. 5.8
Fig. 5.9
Fig. 5.10
CHAPTER 6

HETEROTROPHIC MARINE BACTERIAL NITRATE ASSIMILATION:

SUMMARY OF CHAPTERS AND CONCLUDING REMARKS
Summary of Chapters and Concluding Remarks

An original overall goal this project was to develop molecular techniques to examine the production of bacterial biomass resulting from NO$_3^-$ utilization. Because chemical tracer techniques provide rates that represent the average uptake of a diverse community of autotrophs and heterotrophs, a molecular approach, which was capable of specifically identifying particular organisms and types of organisms, was developed. To date, 159 $nasA$ sequences from 10 different clone libraries have been generated. $nasA$ sequences cluster into eleven clades, if the Beta Proteobacteria and Cyanobacteria are included.

A subsequent goal of these investigations was to use T-RFLP and QPCR techniques to evaluate the hypothesis that the ability of heterotrophic bacterial communities to assimilate NO$_3^-$ is enhanced by the maintenance of diverse assemblages of bacteria capable of utilizing NO$_3^-$ . By simultaneously conducting molecular and chemical tracer assays, several interesting relationships between NO$_3^-$ availability, patterns of NO$_3^-$ assimilation by the bacteria size class and the abundance of diversity of specific groups of $nasA$ populations have emerged.

Chapter 2 takes advantage of the knowledge regarding the structural genes that comprise the bacterial nitrate assimilation operon. The aim of the studies presented in Chapter 2 was the design and optimization of a series of nested heterotrophic bacterium-specific $nasA$ primers. One major result from Chapter 2 is 100% correlation in marine isolates (n=36) between the presence of $nasA$, detected by PCR, and NO$_3^-$ utilization assays. This supported the hypothesis that the $nasA$-specific primer sets developed in Chapter 2 provide a reliable assay for functional assimilatory nitrate reductase genes.
The detection of \textit{nasA} genes in a variety of marine environments is also presented in Chapter 2 and provides a basis for the hypothesis that the potential for $\text{NO}_3^-$ utilization by heterotrophic bacteria is significant. Also, phylogenetic analysis of \textit{nasA} genes cloned from several diverse samples reaffirmed the indication, based on a limited database, that there is a clear genetic distinction between \textit{nasA} genes from heterotrophic bacteria and \textit{nasA} genes from autotrophic cyanobacteria.

Chapter 3 focuses on rate and magnitude measurements of $^{15}$N DIN uptake that were made during a cruise transect across the Barents Sea into the marginal ice zone (MIZ). Results indicated that the percent bacterial DIN uptake of total DIN uptake increased significantly from 10\% in open Atlantic waters to 40\% in the MIZ. On average, at each of five 24 hour stations bacteria accounted for 16-40\% of the total $\text{NO}_3^-$ uptake and 12-40\% of the total $\text{NH}_4^+$ uptake. Results from this study also indicate that in experiments where the total $\text{NO}_3^-$ uptake to total DIN uptake ratio is $>0.5$, bacteria accounted for approximately 40\% of the $\text{NO}_3^-$ utilization. This finding provides the basis for the hypothesis that in regions where apparent high $f$-ratios are observed, bacteria may be partially responsible for the new production measurements, but do not result in increased new production. Also, it is suggested that substantial $\text{NO}_3^-$ utilization by bacteria is one mechanism that might cause open ocean systems to deviate from the steady state that is assumed between $\text{NO}_3^-$ uptake and the downward flux of particulate nitrogen and DON. This results from the fact that bacterial biomass which results from $\text{NO}_3^-$ utilization represents organic material that is more likely to remain in the water column and not to sink. Therefore, additional trophic transfers are required for the small bacterial particles to be transformed into sinking material.
Chapter 4 is aimed at explaining the variability associated with the $^{15}$N measurements of bacterial DIN uptake made in Chapter 3 through molecular methods. The more comprehensive and quantitatively informative molecular techniques, terminal restriction fragment length polymorphism (T-RFLP) and real-time PCR are employed to determine how similar nasA populations are to one another in surface (5 m) and deeper (80 m) waters across the sampling transect. The major hypothesis addressed is that differences in NO$_3^-$ concentrations and NO$_3^-$ uptake rates are reflected in the variability associated with nasA population community structure and abundance. Results indicate that *Marinobacter* sp. nasA gene abundance is positively correlated with NO$_3^-$, showing a two-fold increase in concentration relative to total bacteria at 80 m compared to 5 m. Also, analysis of T-RFLP data suggests that NO$_3^-$ concentration, compared to the other variables assayed (bacterial productivity, bacterial biomass, chlorophyll a, NH$_4^+$ and NO$_3^-$ concentration) is the best predictor, by a factor of 10, of the variability associated with nasA community structure. The results presented in Chapter 4 that indicated that nasA communities contained a higher level of species richness and that a specific nasA group was more abundant in waters where NO$_3^-$ levels were relatively high agree well with the results of the $^{15}$N experiments presented in Chapter 3 that suggested that bacteria were relatively more important to overall community NO$_3^-$ assimilation in the MIZ where NO$_3^-$ levels were high compared to the North Atlantic where DIN concentrations were lower.

The objectives of Chapter 5 were to utilize T-RFLP and SYBR Green real-time PCR techniques in conjunction with $^{15}$N tracer techniques to assess the seasonal variability of bacterial NO$_3^-$ assimilation and bacterial nitrate assimilation genes (nasA) in
the Skidaway River estuary. The hypothesis that, on a seasonal basis, populations of *nasA* containing bacteria are not randomly distributed among the total bacterioplankton community, and that the variability associated with the abundance and community structure of *nasA* communities is reflected in the magnitude of bacterial $^{15}$NO$_3^-$ uptake is addressed. Compared to NO$_3^-$ and NH$_4^+$ concentration and NH$_4^+$ uptake rate, NO$_3^-$ uptake rate is the best predictor, by a factor of 15, of *nasA* community structure.

The findings of Chapters 4 and 5 that NO$_3^-$ availability and patterns of NO$_3^-$ utilization are positively correlated with *nasA* community structure variability and the abundance of particular groups of *nasA* genes, indicates that patterns of NO$_3^-$ supply, in the marine environment, are sufficiently important to be a factor in regulating bacterial communities. Considering the high energetic costs associated with NO$_3^-$ assimilation as well the fact that NO$_3^-$ is traditionally considered a resource that regulates autotrophic activity and production, the result that bacterioplankton community structure is positively correlated with NO$_3^-$ availability and NO$_3^-$ uptake is interesting. These results validate the approach of molecular microbial ecology at the level of the functional gene. If these studies were conducted with molecular tools at the level of the 16S gene we would not have any new information regarding NO$_3^-$ assimilation. In this study, several nearly identical isolates (99% 16S rRNA sequence similarity) that are similar enough to be the same species were found to contrast in their physiological ability to assimilate NO$_3^-$. 

Despite functional discrepancies within genera, we still wish to know which types of microbes (in terms of genera and life history strategies), in general, are responsible for important biogeochemical transformations. Based on this study, Gamma Proteobacteria appear to be the most common marine NO$_3^-$ assimilators. Gamma representatives typify
almost all of the major nasA clades. However, the probes, which have provided all of the nasA sequence information to date, were designed, based on *Klebsiella* – a Gamma Proteobacteria. This would seem to indicate our results could be biased towards Gamma Proteobacteria. However, DNA sequences from Alpha and Beta Proteobacteria representatives in GenBank would hybridize to the *nasA* primers designed in this study. Also, we do have some clones that cluster well with the Alpha sequences in GenBank; we just do not have nearly as many of them. The deeply rooted clade that contains the clones sd29, 3.1, 4m25, and 3m2 is very intriguing. Originally, I thought these were potentially the result of bad sequence data or that they might be non-functional genes, but all of these sequences were generated from separate clone libraries, which indicated that they might not be artifacts and T-RFLP peaks which they theoretically match are common.

In order to answer questions regarding which types of bacteria, as a function of genera and bacterial growth strategy, would be most likely to have *nasA* in their genome, it is important to continue efforts to isolate *nasA* positive bacteria and to sequence their 16S gene to attempt to assign taxonomic identity to some of the *nasA* clades that do not have cultured representatives. After a better representation of the different types of marine bacteria that are *nasA* positive are cultured, questions regarding what these different taxa have in common with regard to bacterial growth strategy can be addressed. However, it may not be possible to culture some of the more common *nasA* marine bacteria. Nevertheless, I think cultured representatives can potentially be informative regarding the general growth strategies of *nasA* positive bacteria. I hypothesize that *nasA* positive bacteria are also bacteria that oxidize to most reduced and energy rich carbon substrates.
In an interesting recent study, bacterial communities growing on high-molecular weight (HMW; >1000 Da) and low-molecular weight (LMW; <1000 Da) fractions of dissolved organic carbon were molecularly characterized (Covert & Moran, 2001). Not surprisingly the two communities were dominated by different phylotypes. Also, it was shown that bacterial utilization of the LMW weight fraction of DOC was higher, by 5 to 10 fold, than utilization of the HMW fraction or the whole DOC. It would be relatively easy to set up an experiment with HMW and LMW DOC fractions in aerated carboys or flasks. Isolates could be screened and sequenced for 16S and nasA genes or DNA and RNA could be extracted and molecularly assayed. I predict that the LMW DOC fraction, if also determined to be the more labile, would contain a higher percentage of nasA positive bacteria relative to total bacteria.

One of our primary goals has been to develop a comprehensively quantitative approach. This task has been very difficult due to the high level of nasA sequence diversity at the DNA level, which makes more comprehensive probe design difficult. For this reason, I attempted to design probes for a narrower group and this has been more successful than I originally thought it would be. I continue to get many clones from this clade, so I was not surprised that it amplified, but the level of the response the assay shows in relation to variables such as NO3⁻ and NO3⁻ uptake is intriguing.

In conjunction with T-RFLP, probes to specific clades could be very useful; accurately quantifying a group and knowing its relative abundance in relation to other groups can generate total abundance estimates. For example, Marinobacter sp. putative T-RFLP peaks are generally 5% of total peak height and Marinobacter sp. nasA gene abundance is generally about 3 x 10⁴/L, which indicates total nasA abundance is
approximately $6 \times 10^5$ L (plus losses that occur as a result of filtration, which from dock samples are likely to be substantial, and DNA extraction). We may then ask whether or not these estimates are reasonable based on what we know about the magnitude of NO$_3^-$ uptake by the bacterial size fraction. Based on dock experiments, bacteria, on average, account for about 7 nmole-N l$^{-1}$ d$^{-1}$, which is 0.5 ng-N l$^{-1}$ d$^{-1}$ or $5 \times 10^5$ fg-N l$^{-1}$ d$^{-1}$. If, on average, at the SkIO dock bacteria are doubling twice daily, they contain 5 fg of N/cell and NO$_3^-$ constitutes 10% of their N diet (Kirchman, 2001), it would require $5 \times 10^5$ cells/L to clear 7 nmole-N l$^{-1}$ d$^{-1}$. Therefore, our estimates of the total abundance of NO$_3^-$ assimilating bacteria are reasonable in the context of general physiological considerations.

The logical prediction that results from this is that between 0.1 and 10% of the total bacteria are responsible, on average, for between 10 and 40% of the total NO$_3^-$ uptake. Intuitively, 0.1 to 10% of the total bacteria does seem like a percentage capable of biogeochemical significance, but because bacteria are very specialized with regard to resource consumption perhaps it should not be surprising that what appear to be a minor percentage of total bacteria are capable of significantly impacting particular element cycles.

It has been estimated that the middle shelf region of the southern SAB receives $2.9 \times 10^4$ mtons of NO$_3$-N yr$^{-1}$ as a result of bottom intrusions (O’Malley, 1981) and estimated river and atmospheric inputs are $1.3 \times 10^4$ and $0.8 \times 10^4$ mtons N yr$^{-1}$ respectively (Haines, 1974). Also, over the past two decades, inputs of fixed nitrogen as a result of river, groundwater and atmospheric inputs have increased (Verity, in press).

The alternative ecological consequences of the fate of NO$_3^-$ in aquatic food webs as result
of partitioning between autotrophic and heterotrophic uptake are unknown but merit investigative modeling efforts.

In a very intriguing recent study that combined experimental data and modeling efforts, Caraco et al. (1998) suggested that ignoring bacterially assimilated DIN could lead to incorrectly interpreting food web dynamics. Specifically, they propose an ecosystem budget for the Hudson River estuary that suggests that net bacterial DIN assimilation is up to 13 g N m⁻² yr⁻¹, which is 4-fold larger than net N assimilation by phytoplankton. The major implications of this are the enrichment of detrital organic matter with N and the overall addition of organic N to the system.

Nevertheless models focused at determining the contribution of upwelling systems to the ocean’s carbon and nitrogen budgets do not consider bacterial NO₃⁻ uptake (Toggweiler & Carson, 1994). Hopefully the detection of specific bacterial populations that have been shown to be genetically and physiologically capable of NO₃⁻ uptake will encourage the consideration of NO₃⁻ uptake by bacteria in modeled marine C and N budgets. Attempts to model the impact of bacterial NO₃⁻ utilization should, however, make every effort to consider the effect and constraints of the energy content of carbon substrates on bacterial NO₃⁻ uptake (Vallino et al. 1996).

One of the major debates about molecular ecology is whether such methods can ever give information regarding the rate of activity of micro-organisms (Joint, 1995). Although expression assays can likely be used to infer levels of metabolic activity, I think there will always be a need for traditional chemical tracer based measurements of rates. The discovery of exactly where and how chemical tracer techniques and molecular assays do and do not overlap will the lead to new insights and hypotheses. At this point,
however, with regard to heterotrophic bacterial NO₃⁻ uptake, I think the major success of the molecular approach has been to open the bacterial “black box” and unambiguously identify some of the important groups of NO₃⁻ assimilating heterotrophic bacteria.
Literature Cited


# APPENDIX A

GenBank Accession Numbers

<table>
<thead>
<tr>
<th>Location sample for clone was collected</th>
<th>Abbreviation</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skidaway River estuary</td>
<td>sd21</td>
<td>AF503796</td>
</tr>
<tr>
<td>Skidaway River estuary</td>
<td>4h19</td>
<td>AF300585</td>
</tr>
<tr>
<td>Skidaway River estuary</td>
<td>sd2</td>
<td>AF593797</td>
</tr>
<tr>
<td>Skidaway River estuary</td>
<td>4h13</td>
<td>AF300584</td>
</tr>
<tr>
<td>Skidaway River estuary</td>
<td>sd16</td>
<td>AF503799</td>
</tr>
<tr>
<td>Skidaway River estuary</td>
<td>sd3.10</td>
<td>AF503801</td>
</tr>
<tr>
<td>Skidaway River estuary</td>
<td>sd11</td>
<td>AF503800</td>
</tr>
<tr>
<td>Skidaway River estuary</td>
<td>sd3.5</td>
<td>AF503802</td>
</tr>
<tr>
<td>Skidaway River estuary</td>
<td>sd13</td>
<td>AF503803</td>
</tr>
<tr>
<td>Skidaway River estuary</td>
<td>3h6</td>
<td>AF300579</td>
</tr>
<tr>
<td>Skidaway River estuary</td>
<td>yy5</td>
<td>AF300603</td>
</tr>
<tr>
<td>Skidaway River estuary</td>
<td>sd5</td>
<td>AF503804</td>
</tr>
<tr>
<td>Skidaway River estuary</td>
<td>sd23</td>
<td>AF503805</td>
</tr>
<tr>
<td>Skidaway River estuary</td>
<td>sd3.3</td>
<td>AF503806</td>
</tr>
<tr>
<td>Skidaway River estuary</td>
<td>sd36</td>
<td>AF503807</td>
</tr>
<tr>
<td>Skidaway River estuary</td>
<td>sd6</td>
<td>AF503808</td>
</tr>
<tr>
<td>Skidaway River estuary</td>
<td>sd3</td>
<td>AF503798</td>
</tr>
<tr>
<td>Skidaway River estuary</td>
<td>sd1.2</td>
<td>AF503809</td>
</tr>
<tr>
<td>Skidaway River estuary</td>
<td>sd29</td>
<td>AF503810</td>
</tr>
<tr>
<td>Skidaway River estuary</td>
<td>sd3.1</td>
<td>AF503811</td>
</tr>
<tr>
<td>Skidaway River estuary</td>
<td>3h22</td>
<td>AF300578</td>
</tr>
<tr>
<td>SAB (mid-shelf)</td>
<td>m230</td>
<td>AF503816</td>
</tr>
<tr>
<td>SAB (mid-shelf)</td>
<td>k7</td>
<td>AF300602</td>
</tr>
<tr>
<td>SAB (mid-shelf)</td>
<td>m215</td>
<td>AF503814</td>
</tr>
<tr>
<td>SAB (mid-shelf)</td>
<td>k8</td>
<td>AF300604</td>
</tr>
<tr>
<td>SAB (mid-shelf)</td>
<td>m21</td>
<td>AF503815</td>
</tr>
<tr>
<td>SAB (mid-shelf)</td>
<td>k9</td>
<td>AF300601</td>
</tr>
<tr>
<td>SAB (mid-shelf)</td>
<td>m220</td>
<td>AF503817</td>
</tr>
<tr>
<td>SAB (mid-shelf)</td>
<td>m214</td>
<td>AF503818</td>
</tr>
<tr>
<td>SAB (mid-shelf)</td>
<td>m216</td>
<td>AF503819</td>
</tr>
<tr>
<td>SAB (mid-shelf)</td>
<td>m221</td>
<td>AF503815</td>
</tr>
<tr>
<td>SAB (mid-shelf)</td>
<td>m222</td>
<td>AF503820</td>
</tr>
<tr>
<td>SAB (mid-shelf)</td>
<td>m210</td>
<td>AF503821</td>
</tr>
<tr>
<td>SAB (mid-shelf)</td>
<td>3m1</td>
<td>AF300580</td>
</tr>
<tr>
<td>SAB (mid-shelf)</td>
<td>3m3</td>
<td>AF300582</td>
</tr>
<tr>
<td>SAB (mid-shelf)</td>
<td>m234</td>
<td>AF503822</td>
</tr>
<tr>
<td>SAB (mid-shelf)</td>
<td>m225</td>
<td>AF503823</td>
</tr>
<tr>
<td>SAB (mid-shelf)</td>
<td>m2(5)</td>
<td>AF503823</td>
</tr>
<tr>
<td>SAB (mid-shelf)</td>
<td>3m2</td>
<td>AF300581</td>
</tr>
<tr>
<td>SAB (mid-shelf)</td>
<td>4m25</td>
<td>AF300587</td>
</tr>
<tr>
<td>SAB (mid-shelf)</td>
<td>4m12</td>
<td>AF300586</td>
</tr>
<tr>
<td>SAB (mid-shelf)</td>
<td>m1</td>
<td>AF300588</td>
</tr>
<tr>
<td>SAB (mid-shelf)</td>
<td>m18</td>
<td>AF300589</td>
</tr>
<tr>
<td>SAB (mid-shelf)</td>
<td>3m4</td>
<td>AF300583</td>
</tr>
<tr>
<td>Location sample for isolate was collected</td>
<td>Abbreviation</td>
<td>Closest relative</td>
</tr>
<tr>
<td>------------------------------------------</td>
<td>--------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>Skidaway River estuary</td>
<td>Sk. Dk. Islt. 1</td>
<td><em>Vibrio Campbellii</em></td>
</tr>
<tr>
<td>Skidaway River estuary</td>
<td>Sk. Dk. Islt. 2</td>
<td><em>Vibrio furnissii</em></td>
</tr>
<tr>
<td>Skidaway River estuary</td>
<td>Sk. Dk. Islt. 3</td>
<td><em>Micrococcus luteus</em></td>
</tr>
<tr>
<td>Barents Sea</td>
<td>Barents Islt. 8</td>
<td><em>Marinomonas protea</em></td>
</tr>
<tr>
<td>Barents Sea</td>
<td>Barents Islt. 16</td>
<td><em>Pseudoalteromonas sp. KMM 3548</em></td>
</tr>
<tr>
<td>Barents Sea</td>
<td>Barents Islt. 25</td>
<td><em>Pseudoalteromonas citrea</em></td>
</tr>
<tr>
<td>Barents Sea</td>
<td>Barents Islt. 19</td>
<td><em>Psychrobacter glacincola</em></td>
</tr>
<tr>
<td>Barents Sea</td>
<td>Barents Islt. 10</td>
<td><em>Marinobacter aquaeolei</em></td>
</tr>
<tr>
<td>Barents Sea</td>
<td>Barents Islt. 23</td>
<td><em>Pseudoalteromonas haloplanktis</em></td>
</tr>
<tr>
<td>Barents Sea</td>
<td>Barents Islt. 32</td>
<td><em>Erythrobacter citreus</em></td>
</tr>
<tr>
<td>Sargasso Sea</td>
<td>Sar450ao</td>
<td><em>Marinobacter sp. DS40M8</em></td>
</tr>
<tr>
<td>Sargasso Sea</td>
<td>Sar450co</td>
<td><em>Alteromonas macleodii</em></td>
</tr>
<tr>
<td>Sargasso Sea</td>
<td>Sar450ah</td>
<td><em>Alteromonas alvinellae</em></td>
</tr>
<tr>
<td>Sargasso Sea</td>
<td>Sar450bo</td>
<td><em>Alteromonas sp. T001</em></td>
</tr>
<tr>
<td>Location</td>
<td>Code</td>
<td>Species</td>
</tr>
<tr>
<td>----------</td>
<td>--------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>Belize</td>
<td>Cb2216s4</td>
<td><em>Vibrio harveyi</em></td>
</tr>
<tr>
<td>Belize</td>
<td>Cbno3r1</td>
<td><em>Halomonas meridiana</em></td>
</tr>
</tbody>
</table>