MOLECULAR CHARACTERIZATIONS OF AQUATIC NITROGEN-CYCLING BACTERIAL COMMUNITIES

by

JAMES BYRON SULLIVAN

(Under the direction of Robert E. Hodson)

ABSTRACT

Investigations into the structure of aquatic bacterial communities involved in nitrogen cycling are necessary for the understanding and management of the systems in which they occur, as well as for their potential impact on global nitrogen dynamics. In aquatic systems, nitrogen moves from organism to organism via the dissolved nitrogen pool or by direct ingestion of biomass. At some point available nitrogen can be lost from a system. The loss of fixed nitrogen in aquatic systems occurs through physical removal or biogeochemical removal. It is the analysis of the bacterial communities responsible for the biogeochemical loss of fixed nitrogen that is the focus of this dissertation.

In this study, DNA-based molecular methods are employed to investigate the occurrence and distribution of nitrogen cycling bacterial genes that code for enzymes involved in nitrification and denitrification. Initially, the ammonia-oxidizing bacterial community associated with finfish aquaculture in southern China is examined using T-RFLP and principal component analysis and correlations are drawn using various dissolved nitrogen species. These anaylses indicate that the ammonia oxidizing bacterial community is altered in water samples taken above the sediment below the fish cages.

Next, the presence and phylogenetic distribution of nitrite reductase (*nirK*) gene sequences closely related to those recently found in marine ammonia-oxidizing isolates is examined in DNA extracted from an activated sludge wastewater treatment plant. Primers designed in this study from the ammonia oxidizer *nirK* sequences specifically amplify sequences that are very closely related to ones from which they were designed. These sequences are not closely related to known heterotrophic denitrifier nitrite reductase sequences and probably represent novel ammonia-oxidizer *nirK* genes.

Finally, current prokaryotic *in situ* PCR methodology is improved upon and applied to the study of the microscale distribution of bacteria containing heterotrophic denitrification genes in activated sludge flocs. Nitrite reductase and nitrous oxide genes are amplified and detected inside intact bacterial cells. Additionally, an initial attempt at performing a simultaneous taxonomic and functional *in situ* identification is reported.

INDEX WORDS: T-RFLP, Nitrification, Denitrification, NirK, NirS, NosZ, PI-PCR, Aquaculture, Activated sludge, Wastewater, Community analysis

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DEDICATION

This dissertation is dedicated to my beautiful, intelligent, hard-working, God-fearing wife, T., and my extraordinary daughter, Jessica. You have both been by my side as we strived to reach this goal. I have undoubtedly been blessed by our Lord, Jesus, to have been entrusted with, and supported by, the two of you. Thank you both from the bottom of my heart. I love you.

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CHAPTER 1

INTRODUCTION

Nitrogen availability is generally considered to be one of the major factors regulating primary production in coastal marine environments. Coastal regions often receive large anthropogenic inputs of nitrogen that can lead to eutrophication. The impact of these nitrogen additions has a profound effect in estuaries and coastal lagoons where water exchange is limited (12). Therefore, bacterially mediated nitrogen transformations are a key component of the biological health of these ecosystems.

Bacterial nitrogen fixation is the only biological source for "new" nitrogen in a system. Inert N_2 gas in the atmosphere is transformed into a form useful to organisms via this process. In well-mixed aquatic systems, the nitrogen in a system moves from organism to organism via the water or by direct ingestion of biomass and subsequent incorporation. At some point, however, available nitrogen can be lost from a system. The loss of fixed nitrogen in aquatic systems occurs through physical removal (burial, fish removal, etc.) or biogeochemical removal. It is the analysis of the bacterial communities responsible for the biogeochemical loss of "fixed" nitrogen that is the focus of this dissertation.

The conversion of ammonia to nitrite in the environment by chemolithotrophic ammonia-oxidizing bacteria is an essential step in the loss of fixed nitrogen from ecosystems (20). This transformation, when coupled with nitrite oxidation, completes the

nitrification process. The bacteria responsible for this process are taxonomically distinct. The characterized ammonia oxidizers are almost all members of the beta subclass of *Proteobacteria*. The few known exceptions are gamma *Proteobacteria*. Since the majority of ammonia-oxidizing bacterial lineages belong to a single phylogenetic group, an examination of 16S ribosomal RNA gene sequences belonging to this group can provide information about the structure of the ammonia-oxidizing bacteria in natural samples. Nitrification does not result in the loss of labile nitrogen sources directly. Rather, the end products, nitrite and nitrate, serve as terminal electron acceptors during bacterial anoxygenic respiration during which nitric oxide, nitrous oxide and dinitrogen are reduced to gaseous products which can be lost to the system. The utilization of oxidized nitrogen sources as terminal electron acceptors is termed denitrification. A key step in the process is the reduction of nitrite to nitric oxide (NO). Subsequent reductive steps form nitrous oxide (N_2O) and dinitrogen (N_2). It is at the point of transformation from nitrite to nitric oxide that the nitrogen is lost to the system, because no known organism can assimilate or reduce (except to dinitrogen) nitric oxide or nitrous oxide. Unlike the nitrifiers, bacteria responsible for denitrification are phylogenetically diverse (40) making it impossible to infer information about biogeochemical functionality from the phylogenetic study of these bacterial communities.

A comprehensive knowledge of the bacterial communities involved in nitrogen cycling is important for understanding aquatic ecosystem function. Many studies have sought to elucidate bulk denitrification and/or nitrification in nearshore aquatic systems (18, 31, 45), in lakes (36, 39), and in wetlands (9, 34). Through advances in molecular biology, it has become possible to characterize nitrogen-cycling bacterial communities in

much greater detail. Traditional methods for the study of nitrifying and denitrifying bacteria involve enrichment of samples and cultivation. The extremely slow growth rate of nitrifying bacteria make isolation-based methods very time consuming (33). Also, the selective cultivation of bacteria, by definition, changes the community structure, making conclusions drawn from laboratory investigations questionable when applied to natural systems. Enrichment can select for portions of the community involved in a process, while not enriching for all active species. Therefore, studies that allow for an investigation of an *in situ* community give a more accurate representation of the active species. Immunoassay detection on the basis of serological distinction has been shown to be useful for the identification and the enumeration of specific strains of nitrifying and denitrifying bacteria (41, 42). However, these methods still require the cultivation of target strains in order to develop the antibodies. Another major problem with immunological techniques is that they are species, or even strain, specific. This makes a comprehensive study of all, or even many, of the bacteria involved in a certain process impossible.

DNA-based methodologies allow for the study of nitrogen cycling bacterial communities without the need for cultivation. The polymerase chain reaction (PCR) enables the selective amplification of specific sequences of DNA from an environmental sample (32). Although PCR does not allow for an absolute measure of species abundance due to nonrandom amplification, or PCR bias, it does allow for an excellent estimate of overall diversity and structure of bacterial communities (26). Following the extraction of the total DNA from a sample, a portion of the target gene can be amplified from all target sequence-containing bacterial DNA present. It is possible to target

taxonomic (1, 13, 35) or functional DNA sequences for study (5, 42). All eubacteria possess DNA sequences that code for the 16S subunit of ribosomal RNA. The 16S rRNA gene contains regions of sequence that are highly conserved among all known species of bacteria while being a manageable length of approximately 1600 bases, making it an ideal target for phylogenetic identification. There are also regions of variation in the DNA sequence. These variations can be used for discerning the phylogenetic relationships among different species of bacteria. The greater the variation between 16S rDNA sequences, the more distantly related the bacteria are considered to be (43). Homologous functional genes are also investigated in a similar fashion.

PCR amplification and subsequent cloning and sequencing analysis can give excellent information about which bacteria are a part of the community of interest. However, it cannot show the overall bacterial sequence diversity of the sample since it is not possible to sequence every amplicon present. Other PCR-based techniques have been developed which allow for an estimate of overall diversity. Denaturing Gradient Gel Electrophoresis (DGGE) analysis separates amplicons of similar length by differences in sequence using an increasing linear gradient of denaturing agents such as urea or formamide (24). This method has been used to investigate coral-associated bacteria (30) and Arctic Ocean nitrifier communities (2, 10).

Terminal Restriction Fragment Length Polymorphism (T-RFLP) allows for an estimation of the minimum number of bacterial taxa present in a sample as well as an estimate of the relative abundance of the various species or groups of species present (21). T-RFLP has been used to investigate functional gene diversity of denitrifying (6) and ammonia oxidizing (16) bacterial DNA sequences occurring in environmental

samples. Prokaryotic *in situ* PCR (PI-PCR) is a technique that allows single cells within complex communities to be identified and characterized genetically (14). This method was adapted from usage in eukaryotic tissue research (19). PI-PCR has been used to identify naphthalene and toluene-degrading bacteria in seawater samples by detecting the functional genes associated with these processes (8, 14). Successful detection of E. coli cells using 16S rDNA-targeted primers has also been achieved (37).

OVERVIEW

1. The characterization of bacterial communities involved in nitrogen transformations in multiple use marine systems is important for the proper understanding and management of those systems. In the case of marine fish culture in coastal waters, it would be helpful to understand the bacterial community involved in nitrification, as this process could ameliorate the build-up of ammonia excreted as a fish waste product. The sediment below finfish aquaculture cages may have decreased rates of nitrification relative to pristine sediment due to factors associated with the greatly increased bacterial remineralization of uneaten food and fish excretia (22). Decreased sediment pore water oxygen levels due to bacterial respiration have been linked to decreased nitrification in aquaculture ponds (11). It has further been shown that the presence of sulfide as HS^-, a product of anaerobic sulfate reduction, decreases or completely inhibits rates of nitrification (17). Excessive ammonia or nitrite levels may also inhibit nitrification (3).

With regard to the effects of fish cage culture on ammonia oxidizing bacterial community structure, it has been shown that, in addition to decreased sediment nitrification rates beneath fish cage culture, the benthic community responsible for this

process is drastically altered as well. McCaig et. al. reported differences in both the species present and their relative abundances (22). However, little is known about the species composition and distribution of fish cage-associated water column nitrifying bacterial communities. It is one of the goals of this work to make an initial characterization of the ammonia-oxidizing bacterial (AOB) community that is associated with finfish aquaculture in Xincun Bay, Hainan, Peoples Republic of China and compare it to nearby water column samples from areas not directly associated with cage fish culture. The methods employed included PCR amplification, cloning and sequencing and T-RFLP analysis. Water samples will be compared as the whole-water and smaller than 5 μm fraction. Principal component analysis and regression analysis will be used to investigate correlation among the samples and between the samples and environmental parameters.

2. Since nitrifying bacteria form nitrite and denitrifying bacteria reduce nitrite (among other nitrogen species), the formation of denitrification intermediates in pure cultures of ammonia-oxidizers was a surprising phenomenon. The reduction of nitrite to nitric oxide and nitrous oxide by ammonia-oxidizing bacteria, a process termed nitrifier denitrification, was first described in *Nitrosomonas europaea* (15). Initially, it was thought that the gases produced might have been the products of the breakdown of unknown intermediates of the nitrification process. Later, the mechanism for the production of nitric oxide and nitrous oxide from nitrite was described with the use of ^{15}N tracers (27). Laboratory experiments and biochemical characterizations of enzyme properties such as rate enhancement at low oxygen tensions and reaction rate profiles

further demonstrated the existence of an enzyme that was very similar to the nitrite reductase possessed by heterotrophic denitrifying bacteria (4, 23, 29).

Recently, it has been shown that other ammonia-oxidizing bacteria also possess the genes coding for a nitrite reductase enzyme with very high DNA sequence and amino acid homology to the previously characterized copper-containing nitrite reductase (NirK) sequences (7). These bacterial isolates have been reported to express a functional form of NirK, one of the two forms of the enzyme (25). This discovery is interesting both from the standpoint of basic bacterial physiology and for understanding the sources of global production of nitric oxide and nitrous oxide (44). Unlike the pathway in most heterotrophic denitrifiers, dinitrogen (N_2) does not appear to be the ultimate end product of nitrite reduction in chemolithotrophic ammonia-oxidizers, nitric oxide and nitrous oxide are released instead. Nitric and nitrous oxide are important due to the role they play in contributing to global warming and stratospheric ozone destruction (38).

Although the ammonia-oxidizers that previously tested positive for nitrite reductase were all isolated from saltwater, other ammonia-oxidizers may also have this capability. One readily accessible (and, in the developed world, ubiquitous) type of environment with known nitrifier populations, high ammonia concentrations and limited oxygen concentrations is the wastewater treatment facility (28). In this investigation we demonstrate the presence of bacterial DNA sequences in a Georgia activated sludge municipal wastewater treatment facility that are highly homologous to the nitrite reductase sequences recently found to exist in marine ammonia-oxidizing bacteria. We have done this by designing PCR primers specific for the NirK gene in the recently characterized ammonia oxidizing 'denitrifiers' and using them to amplify purified DNA

from a local wastewater treatment plant. Phylogenetic analysis groups these DNA sequences very closely with the nitrite reductase from the isolates.

3. As mentioned previously, the development of Prokaryotic *In Situ* PCR (PI-PCR) carries a great deal of promise for the single cell scale investigations of bacterial communities. Nevertheless, as is true of most new techniques, there can be difficulties in applying this technique due to methodological problems, namely false positive signals. The third portion of this work builds on each of the previous two chapters by focussing on the bacterial communities involved in nitrogen cycling - in this case, denitrifiers. This chapter also addresses the difficulties in PI-PCR by "going back to the drawing board" and examining every step of the process, until finally arriving at an improvement upon the existing methodologies, specifically in the detection step of the protocol.

The bacteria that are the subject of this investigation include well-characterized strains like, *Pseudomonas stutzeri, Pseudomonas aureofaciens*, and *Paracoccus denitrificans.* Also investigated are several coastal isolates, some of which test positive for denitrification genes. Lastly, the new techniques are applied to activated sludge flocs from a wastewater treatment plant. The targets of the PI-PCR include nitrite reductase in heterotrophic bacteria, nitrous oxide reductase in heterotrophic bacteria, and the ammonia-oxidizer nitrite reductase described in the third chapter of this work.

CHAPTER 2

T-RFLP ANALYSIS OF THE AMMONIA-OXIDIZING BACTERIAL COMMUNITY ASSOCIATED WITH FINFISH AQUACULTURE IN A SOUTH CHINA LAGOON[1]

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[1]Sullivan, J.B. and R.E. Hodson. To be submitted to *Applied and Environmental Microbiology.*

Introduction

The characterization of bacterial communities involved in nitrogen transformations in multiple use marine systems is important for the proper understanding and management of those systems. In the case of marine fish culture in coastal waters, it would be helpful to understand the bacterial community involved in nitrification, as this process could ameliorate the build-up of ammonia excreted as a fish waste product. The sediment below finfish aquaculture cages may have decreased rates of nitrification relative to pristine sediment due to factors associated with the greatly increased bacterial remineralization of uneaten food and fish excretia (18). Decreased sediment pore water oxygen levels due to bacterial respiration have been linked to decreased nitrification in aquaculture ponds (11). It has further been shown that the presence of sulfide as HS^-, a product of anaerobic sulfate reduction, decreases or completely inhibits nitrification (14). Excessive ammonia or nitrite levels may also inhibit nitrification (5).

Nitrifying bacteria obtain the energy necessary for carbon dioxide fixation from the nitrogen oxidations they perform (see reviews in (6, 12)). Nitrification is a two-step process. In the first step, ammonia-oxidizing bacteria oxidize ammonia to hydroxylamine and then nitrite. Next, nitrite oxidizers produce nitrate from nitrite. Ammonia oxidation is performed by two select groups of chemolithotrophs. The largest group of these obligate aerobes is a subset of the Beta *Proteobacteria*; the other is limited to a few species within the Gamma *Proteobacteria*.

Ammonia oxidizers historically have been difficult to culture due to slow growth rates, perhaps resulting from the presence of only one copy of the *rrn* operon per

ammonia oxidizer genome (1). The *rrn* operon codes for the ribosomal RNA subunits 16S, 23S and 5S. The presence of multiple copies in a genome may lead to high ribosome numbers in bacteria and allows a cell to grow rapidly when conditions are optimal (9, 21).

Not only are nitrifying bacteria slow growing, but those cultured from a sample are not necessarily representative of the nitrifier community from whence they came. Investigations that have used both culturing and culture-independent techniques have produced discordant results (6, 23). For instance, Stephen et. al. (23) reported that *Nitrosomonas* spp. were selected in culture experiments while *Nitrosospira* spp. dominated culture-independent examinations from the same marine sediment samples. It has not been unequivocally determined which of the suites of techniques is more accurate, but diversity has been shown to be higher in samples examined using culture-independent methods (6). Also, despite the bias introduced in PCR amplification of environmental DNA samples, it is likely that the final ratio of DNA sequences following PCR amplification is similar to their ratio in the original sample (20). This suggests that culture-independent methods may provide more accurate information regarding ammonia oxidizing bacterial communities structure.

With regard to the effects of fish cage culture on ammonia oxidizing bacterial community structure, it has been shown that, in addition to decreased sediment nitrification rates beneath fish cage culture, the benthic community responsible for this process is drastically altered as well. McCaig et. al. reported differences in both the species present and their relative abundances (18). A novel *Nitrosomonas* clone sequence was shown to be most abundant directly beneath the fish cage, decreasing to

below Southern hybridization detection limits within 40 m. Conversely, certain *Nitrosospira* sp. and *Nitrosomonas* species were shown to be least abundant directly beneath the cages while increasing in abundance with distance from the cages. However, little is known about the species composition and distribution of fish cage-associated water column nitrifying bacterial communities. It is the goal of this work to make an initial characterization of the ammonia-oxidizing bacterial (AOB) community that is associated with finfish aquaculture in Xincun Bay, Hainan, Peoples Republic of China and compare it to nearby water column samples from areas not directly associated with cage fish culture.

Terminal restriction fragment length polymorphism (T-RFLP) analysis, a rapid, culture-independent method for characterization of the diversity and relative abundance of specific bacterial genes, was selected as our method (3). T-RFLP allows for the determination of the minimum number of bacterial taxa present in the sample as well as an estimate of the relative abundance of these taxa. This method begins with PCR amplification of isolated environmental DNA with primers specific to the gene of choice. One of the two PCR primers is fluorescently labeled. After isolation of the labeled amplicons, samples are treated with restriction enzymes. The result is that each copy of each of the bacterial 16S DNA sequences found in the original PCR mixture yields only one fluorescently labeled fragment. The length of the fragment corresponds to the position from the labeled end of the amplicon to the nearest restriction enzyme cut site, ant therefore can be taxonomically informative.

In order to characterize only the terminally labeled DNA pieces, the sample is examined using capillary electrophoresis and a laser. The laser causes the fluorescently

labeled pieces of DNA to be recognized, while unlabeled fragments are not. To measure the length of the terminal restriction fragments, a DNA size standard labeled with a different fluorophore is run with the sample. The resultant data is given in both tabular and graphical formats. The graphic format is an electropherogram.

Each electropherogram peak represents a bacterial taxon that has an initial cut site the same distance from the labeled terminus. A single peak can be comprised of sequences unique to a single species or to a group of species that share sequence length homology at that particular cut site. Therefore, the number of peaks indicates the minimum number of bacterial species present and thus the minimum level of diversity of the specific genes being studied from the bacterial community in that sample. The peak height indicates the relative quantity of DNA pieces of that length in the sample. Comparison of results from different environmental samples allows for identification of those peaks that do not occur in other samples and, thus, are indicative of different bacterial communities. Also, species whose relative abundance varies from sample to sample are identifiable and quantifiable, as measured by relative peak height expressed as percentage of total height of all peaks. It should be noted that PCR has been shown to non-randomly amplify DNA from environmental samples, thus skewing the results regarding relative abundances of the bacteria present (20). Therefore, it is necessary to treat all samples identically in order to minimize PCR bias.

When coupled with cloning and sequencing of the gene used for fragment length comparison, T-RFLP analysis can be a powerful tool for examining environmental bacterial communities (22). T-RFLP analysis has been used to describe the diversity and relative abundance for functional genes from denitrifying (7) and nitrifying (13) bacterial

communities. Since, as mentioned above, the majority of ammonia-oxidizing bacterial lineages belong to a single phylogenetic group, an examination of 16S ribosomal RNA gene sequences belonging to this group can provide information about the structure of the ammonia-oxidizing bacteria in natural samples. PCR-based 16S rDNA assays specific to the autotrophic ammonia oxidizers of the beta-subclass of *Proteobacteria* have been developed previously (27, 28) and widely applied (4, 16, 17, 19, 29).

Xincun Bay is located on the southeast coast in Lingshui County, Hainan Island, P.R. China (Figure 2.1). The bay has an elliptical basin 6 km long and 4 km wide, covering an area of 22 km^2. The maximum depth is 10.6 m, and the bay is connected to the South China Sea by a single tidal inlet 120 m wide. The tidal current in the channel can exceed 1.0 m/s, while in the middle of the bay currents rarely exceed 0.1 m/s. The town of Xincun has 15,000 residents and includes 3000-5000 people who live on floating houses with associated fish pens (Figure 2.2). The fish houses begin inside the mouth of the bay and border the channel across from the town for approximately 1.5 kilometers. Each house may have 4-8 active pens. A pen contains up to 30 large fish in a 4x8-foot area. Several species are grown in the pen from fingerlings, including cobia, *Rachycentron canadum* (L.), local grouper and pompano. The fish are collected from the local reefs by the local offshore net fishery. The fish that are ground-up to serve as food for the fish in the cages are caught in the same manner.

Figure 2.1. Map of China highlighting the location of the province of Hainan.

Hainan island is located in the South China Sea due east of Vietnam and separated from Vietnam by the Gulf of Tonkin. Hainan is the southernmost point of the People's Republic of China.

Figure 2.1. Map of China.

Figure 2.2. Xincun township and Xincun bay, Lingshui County, Hainan, PRC.

A composite photograph (Figure 2.2a) of Xincun township (far side of the water) and Xincun Bay as taken from a skylift that runs from the town to Monkey Island, a wildlife tourist attraction. The mouth of the bay is at the left of the photograph to the West. There is a sewage outfall for the town along the far shore of the channel, and a port servicing the offshore fishing industry. The sewage is treated by chlorination. The bay extends eastward beyond the right of the photograph. In the water are several hundred floating houses where the fish cage aquaculture takes place (Figure 2.2b). There is no sewage treatment for these homes.

Figure 2.2a. Xincun township and Bay, Lingshui County, Hainan, PRC.

Figure 2.2b. Aerial view of the finfish aquaculture in Xincun Bay.

Materials and methods

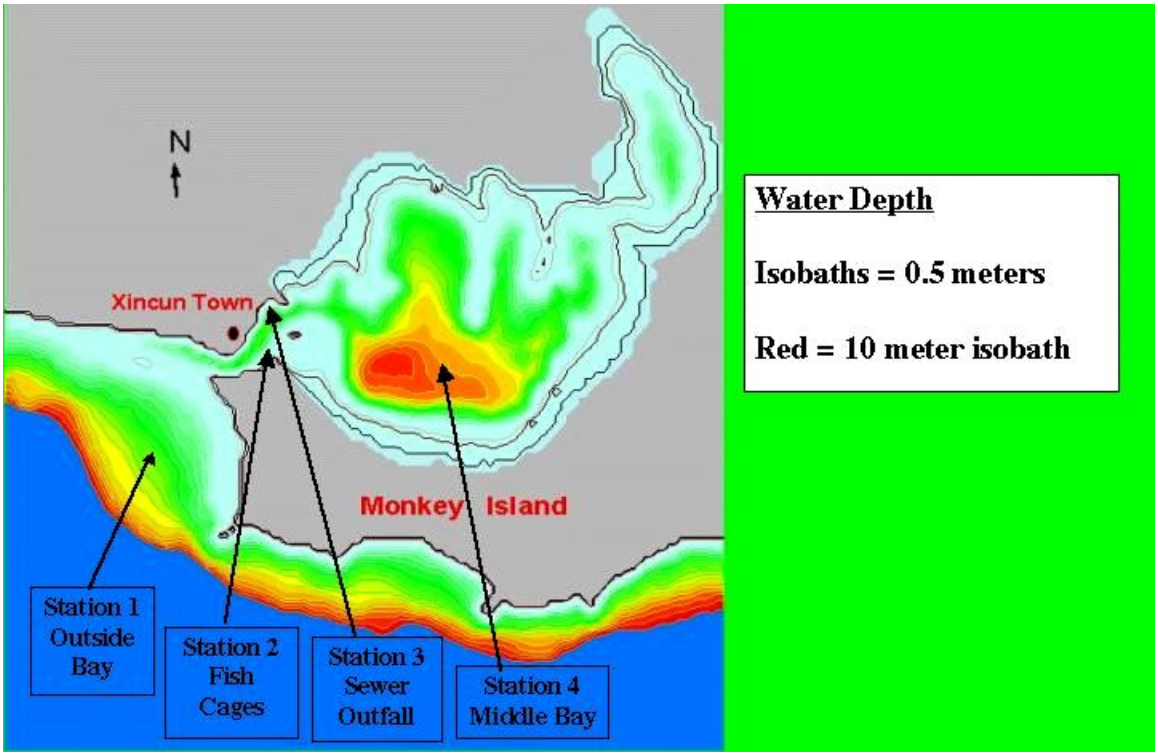
Sample Collection. On August 7, 2000, duplicate water samples were collected at four stations (Figure 2.3) from 0.5 m below the surface and just above the sediment (at station 1 samples were collected at 4 m depth, which is the depth at which oceanic salinity [~34 ppt] was first encountered) using a 5 L Niskin bottle. Water samples were transferred to 500 ml Nalgene bottles and immediately placed on ice until collection of bacteria by filtration (within 24 hours). Samples were filtered following the protocol of Somerville, et. al. (24). Briefly, two 150 to 200 ml water samples from each depth at each site were filtered through a 0.22 μm pore-size Sterivex SVG filter cartridge. One of the two samples was prefiltered with a 5.0 μm syringe filter before collection (all even numbered samples are filtered). 2.0 ml of lysis buffer consisting of 0.75 M sucrose, 40 mM EDTA, and 50 mM Tris (pH 8.0) was placed in each filter for storage at -20°C until processing. Filters were place on ice immediately until frozen (within 24 hours).

Concurrent to sample collection for bacterial community analysis, depth integrated water samples were taken for chemical analyses. Additionally, several environmental parameters were measured *in situ* at the time of sampling. Temperature, salinity, dissolved oxygen (DO), DO%, and pH measurements were collected. During sample collection at the station 2, the fish cages, a swimmer collected a sediment grab sample from the top 3-5 cm of the sediment surface in a 500 ml Nalgene bottle. After visual inspection, but before chemical analysis could be performed, the sample was spoiled and was discarded.

Figure 2.3. Map of sampling sites in Xincun bay.

Beginning at 11:00 AM on August 7, 2000, samples were collected from 0.5 m and at 4.0 m or just above the bottom from a small outboard rigged boat. Water samples for nutrient analysis were taken concurrently with samples for bacterial community analysis. Sampling took approximately 3 hours, with sample bottles being placed in crushed ice immediately after collection. Samples were taken in duplicate Niskin bottle casts.

Figure 2.3. Map of sampling sites in Xincun bay.



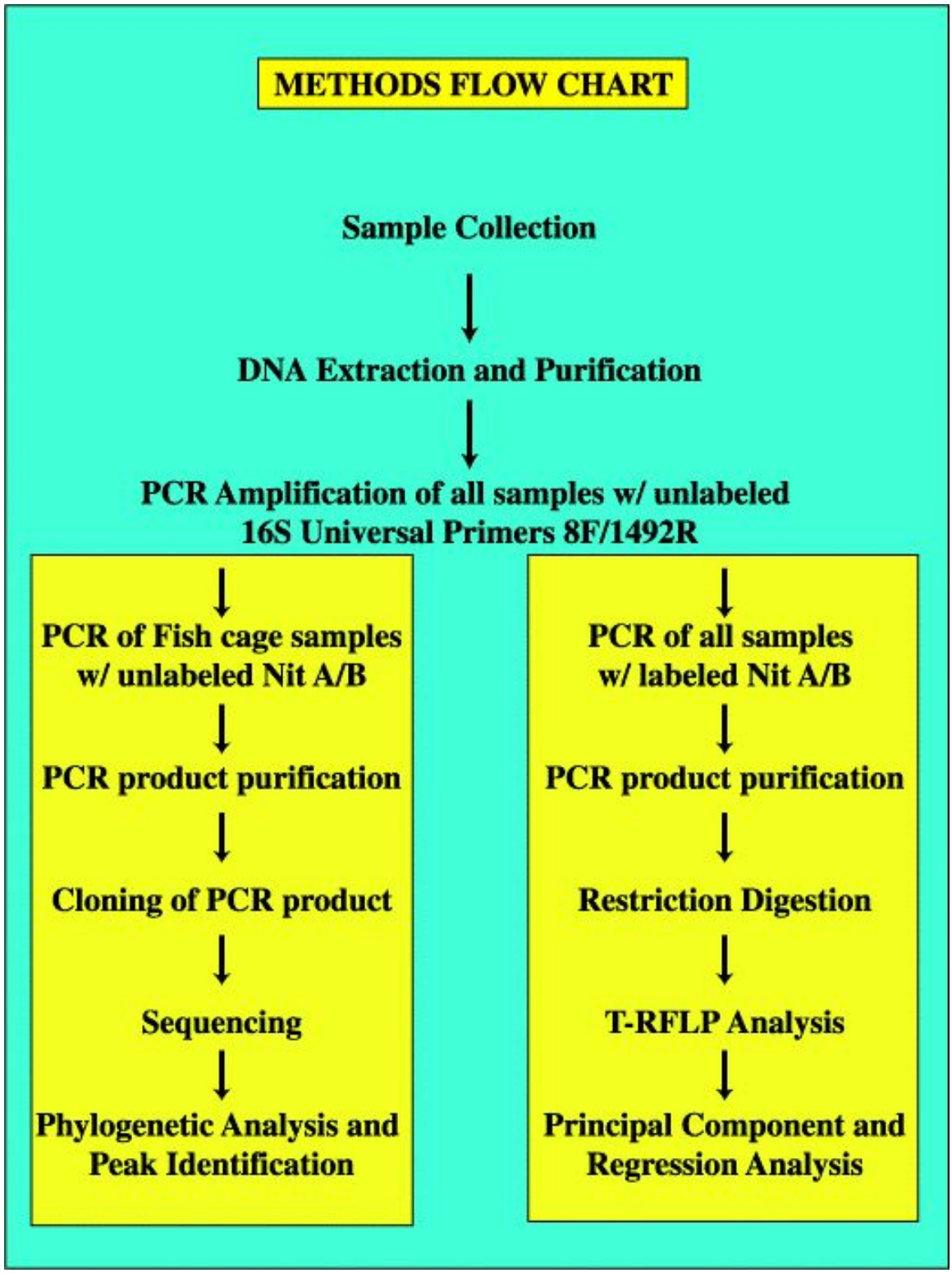
Methods overview. A flow chart was constructed to provide a summary of the methods employed in this investigation (Figure 2.4).

DNA Extraction. DNA was extracted from bacteria on the filters using the Ultra Clean Soil DNA Isolation Kit (MoBio) with a procedure modified for use with Sterivex filters (26). Briefly, the filter and lysis buffer for each sample were placed in a 50 ml plastic centrifuge tube with the first 3 reagents (bead solution, IRS and reagent S1) from the kit and the tube was vortexed at maximum speed for 20 minutes. The manufacturer's protocol was then followed on a 450 μl sample of the resulting slurry. The DNA samples were then used as templates in nested PCR reactions for T-RFLP analysis and cloning and sequencing reactions.

PCR Primers. The NitA/NitB primer set was developed by Voytek and Ward to specifically amplify 16S rDNA of ammonia-oxidizers from the beta *Proteobacteria* (28). It was the primer set we selected for T-RFLP and cloning and sequencing analysis. The sequences are: NitA, 5'CTTAAGTGGGGAATAACGCATCG3', and NitB, 5'TTACGTGTGAAGCCCTACCCA3'. The Nit A sequence is equivalent to the sequence beginning at position 137 of the 16S rDNA molecule in β-proteobacterial ammonia oxidizers and Nit B is homologous to the sequence ending at position 1,234. PCR product size is ~1097 base pairs.

Figure 2.4. Methods flow chart for Nit A/ Nit B PCR amplification and T-RFLP and clone sequence analysis of 16S rDNA from the bacterial community from Xincun bay.

Figure 2.4. Methods overview for T-RFLP and clone sequence analysis in Xincun bay.



Nested *in vitro* PCR amplification and product purification. PCR products from the NitA/NitB primer set were retrieved from several of the sixteen samples by direct amplification (sewage outfall whole-water and fish cage surface whole-water samples). It was necessary to perform a nested PCR in order to amplify NitA/NitB PCR products for the remainder of the samples. Nested PCR is an initial amplification of DNA with a eubacterial universal primer set that produces a product that includes the binding sites for NitA and NitB, followed by the NitA/B amplification using the product of the first reaction as the template for the second. Voytek and Ward also found it necessary to use nested amplification with this primer set (28). T-RFLP profiles run for direct versus nested amplification for the three samples that gave PCR product with NitA/NitB directly showed no significant difference (data not shown), so, for uniformity, all samples were processed following nested amplification.

The initial PCR protocol consisted of 1 μl of the purified DNA (~ 50 ng) from each sample as template, 0.2 μM of each primer, 0.25 mM dNTPs, 1.0 μl of Expand High Fidelity DNA polymerase mix (Roche Biochemicals) and 1X manufacturer-supplied buffer in a final reaction volume of 25 μl. The primer set used in the initial amplification was the universal 16S rDNA 8F/1492R set. The thermal protocol was: initial denaturation at 94°C for 4 minutes, followed by 30 cycles with denaturation at 94°C for 30 seconds, an annealing temperature of 60°C for 45 seconds and an elongation step of 72°C for 1 minute. A final elongation of 7 minutes at 72°C completed the amplification reaction.

The nested PCR protocol was identical with three exceptions. First, 1 μl of the first PCR reaction was used as the template for the second reaction. Second, the

NitA/NitB primer set was used. Lastly, for cloning and sequencing of the fish cage samples (X5-X8), an unlabeled primer set was used in the nested amplification. For T-RFLP analysis of all samples (X1-X16), Nit A was labeled with 6-FAM (5-[6]-carboxy-fluorscein) on the 5' end.

Cloning and Sequencing. For cloning of PCR products from the fish cage samples (X5, X6, X7 and X8), the unlabeled Nit A/B amplicon was purified from a 1.0% agarose gel using the Ultra Clean PCR CleanUp DNA Purification Kit (MoBio) following manufacturer's instructions. The purified PCR product was cloned using the Promega pGEMT-easy Kit. Transformants were grown overnight on LB plates with ampicillin (100 μg/ml), IPTG (isopropylthiogalactopyranoside [0.5 mM]) and X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside [80 μg/ml]) at 37°C. Plasmid DNA was extracted from a total of 15 white colonies for each of the four samples (60 total) using the QiaPrep Spin Miniprep Kit, (Qiagen, Inc.). One μl of purified plasmid DNA (~150 ng) was used in a 10 μl BigDye Terminator (Applied Biosystems, Inc.) cycle-sequencing reaction with the T7 primer. Each sample was also amplified separately for sequencing with the Sp6 primer in order to sequence the entire 1100 base pair amplicon. After removal of unincorporated dideoxy terminators by Sephadex G-50 purification, the PCR product was dried in a Speed Vac and resuspended in 10 μl of deionized water and sequenced on an ABI 377 Genetic Analyzer.

Phylogenetic analysis. Eighteen of the clone sequences were not used because they were unreadable (n=14) or contained chimeras (n=4). Sequence data for the 42 clear 16S

rDNA sequences retrieved from the 60 clones were reviewed using the Sequencher (Gene Codes Corporation, Ann Arbor, MI) program in order to ensure that the ABI software analysis program identified all bases correctly. Subsequently, sequences were exported in GCG (The Wisconsin Program Package for Biological Sequence Analysis, Genetics Computer Group, Oxford Molecular) sequence format, imported to RCR (Research Computing Resource, Biological Sciences Software, Databases and Services, University of Georgia, Athens, GA) and included in a multiple alignment using the PILEUP command. The resultant multiple sequence alignment was imported to SeqPup (Indiana University) for final alignment and length normalization. The finalized sequences were 1068 bp in length. Sequences that were less than 0.5% different were assumed to be identical and a representative sequence was used. The aligned sequences were each bootstrapped 100 times using the SEQBOOT program of the phylogenetic inference package PHYLIP 3.572 (10). Neighbor-joining trees were produced for each pseudoreplicate analysis. Next, the SEQBOOT output file was input for distance matrix analysis using DNADIST in PHYLIP. The Kimura model was used to calculate nucleic acid sequence distance (15). The CONSENSE program produced a majority-rule consensus tree for the sequences, and a phylogenetic tree was drawn using the DRAWGRAM program from PHYLIP.

Restriction Digestion and T-RFLP analysis. For T-RFLP analysis, the labeled Nit A/B amplicon for each sample (n=16) was cut out from a 1.0% agarose gel and purified using the UltraClean PCR CleanUp DNA Purification Kit (MoBio) following manufacturer's instructions. Approximately 150 ng of purified product was subjected to a restriction

digestion with *Cfo*I restriction enzyme. The protocol was: 1 μl of manufacturer-supplied buffer, 1 μl of enzyme, FAM-labeled Nit A/B PCR product in a final volume of 10 μl at 37°C for 3 h. A 4 μl subsample was removed and dried in a speed vac for 30 minutes and resuspended in 12.5 μl deionized formamide with 0.5 μl of GeneScan 500 ROX size standard (Perkin Elmer). Samples were then denatured at 95°C for 5 minutes, iced for 3 minutes and loaded on an ABI Prism 310 genetic analyzer. Analysis conditions were: 10 second injection time, 15kV voltage and 60°C run temperature in a 47 cm capillary with POP-4 polymer for 33 minutes (Perkin Elmer).

T-RFLP Data Analysis. Peak height was selected as the basis for comparison of the relative abundance of peaks. In order to process the data, a minimum peak height and peak height percentage was operationally determined. In this work, we examined peaks that were a minimum of 100 relative fluorescent units in height and represented a minimum of 2 percent of total peak height for that sample. Prior to analysis, a Microsoft Excel visual basic program was used to prepare the data (26). Peaks less that 1.5 bases apart were binned together. Subsequently, due to the potential for small run time differences among samples, peaks that were less than one base pair apart were considered the same and pooled.

Principal component analysis (PCA) was performed on the tabular T-RFLP data with Unscrambler 6.11 software (Camo). Peaks that occurred in 5 or more samples were included in the PCA of all stations. For the PCA of the whole-water (ww) or <5 μm (filtered) samples alone, peaks that occurred 3 or more times within those samples were included for analysis.

Regression analysis was performed to determine the total proportion of variance of the height of a given peak explained by the concentration of various dissolved nitrogen compounds. Peaks were examined that occurred at least 3 times in either the whole water samples or the filtered samples and that accounted for 2% or more of the total peak height for that sample. The independent variables considered were the concentration of nitrate, nitrite, ammonium, and total dissolved inorganic nitrogen (the sum of the three previous nitrogen species). Regression analysis was performed with Microsoft Excel.

R-squared values are reported for all peaks that were examined. R-squared values for which the correlation coefficient (R) is significant are discussed. Significance is defined here as a p-value of $p<0.10$.

Results and discussion

Overview. It appears that the ammonia-oxidizing bacterial community associated with finfish aquaculture in Xincun Bay differs significantly between surface and bottom samples. This was determined by the identification of peaks that include known ammonia oxidizers, the examination of the peaks that co-occur with them, correlations shown between the co-occurring peaks and dissolved inorganic nitrogen data and examination of total percent peak height for all known or inferred peaks that include ammonia-oxidizing bacteria for each sample.

Cloning and sequencing and phylogenetic analysis. The clone libraries from the fish cage samples, X5 – X8, yielded 42 clear sequences. Of those, 25 were unique, both in this investigation and as compared to 16S rDNA sequences in GenBank. Ten of these sequences clustered within the known ammonia-oxidizing beta *Proteobacteria*. A phylogenetic tree (Figure 2.5) was constructed using the 1068 base-pair clone sequence and equivalent-length sequences from known ammonia-oxidizer species as determined by BLAST analysis (2). Examination of the phylogenetic tree revealed the phylogeny of the NitA/B clones.

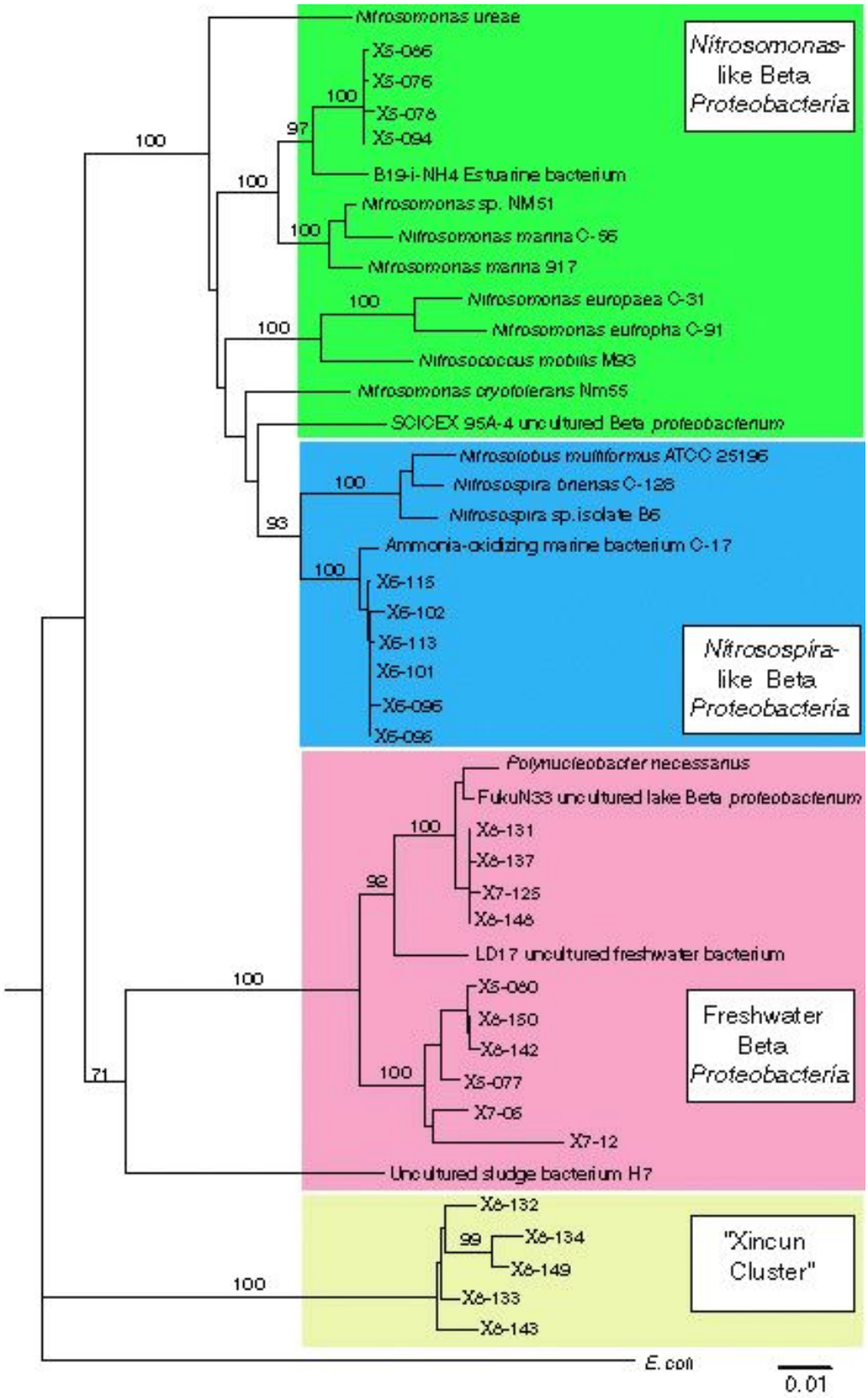
Sample X5, the fish cage surface whole-water sample, yielded 6 unique sequences, including 4 that are closely related to one another and to several *Nitrosomonas* species (most closely with *Nitrosomonas marina*). The two other unique X5 sequences (X5-077, X5-080) are most closely related to a non-ammonia oxidizing beta *Proteobacteria, Polynucleobacter necessarius*, a freshwater obligate ciliate symbiont (25). Clone sequences closely related to *P. necessarius* have been found in the free-living bacteria-sized particles in water samples from the Columbia River and its estuary (8).

Of the fourteen clone sequences from the X6 sample, the surface, <5 μm sample, 6 are unique. The unique clone sequences from this sample are closely related to one another and are most closely related to the 16S rDNA sequence from *Nitrosospira briensis.* It was shown previously in a Mediterranean Sea study that *Nitrosospira*-like AOB sequences are associated with free-living marine bacteria and *Nitrosomonas*-like sequences are associated with particle-associated communities (19). No non-AOB sequences were found in the clone library made from this sample.

Figure 2.5. Nit A/B phylogenetic tree from Xincun bay clone library.

Clone Nit A/B 16S rDNA sequences were placed in a phylogenetic tree with several known isolate and clone sequences from GenBank. The sequences formed three clusters. One cluster is comprised of known ammonia-oxidizing beta *Proteobacteria*. This cluster subdivides into *Nitrosomonas* species and *Nitrosomonas*-like sequences and *Nitrosospira* species and *Nitrosospira*-like sequences. The second cluster contains non-ammonia-oxidizing beta *Proteobacteria.* The third cluster is formed only by Xincun sample X8 clone sequences; the closest relative is an *Azoarcus* sp. that shares 88% nucleotide sequence identity. *E. coli* is the outgroup. Bootstrap values in excess of 70 are included in the tree.

Figure 2.5. Nit A/B phylogenetic tree from Xincun bay clone library.



In the clone libraries from the two bottom samples, X7 (ww) and X8 (<5 μm), no AOB-like sequences were found. Instead, the 3 X7 clones clustered with the freshwater beta *Proteobacteria*. Five of the X8 clones were also closely related to the *Polynucleobacter necessarius* cluster, but the remaining 5 were not. The latter five sequences form a separate cluster apart from both the AOB and the non-AOB clusters. The closest relative to these sequences is an *Azoarcus* sp. that shares 88% sequence identity with this cluster. The cluster is labeled "Xincun cluster" in Figure 2.5. Efforts are ongoing to isolate and identify the bacteria that form this group.

It is clear that, in this experiment, the NitA/B primer set was not specific for the 16S rDNA of ammonia-oxidizing bacteria belonging to the beta subclass of the *Proteobacteria*. In contrast, Voytek and Ward (28) applied this primer set to bacterioplankton samples collected from Lake Bonney, Antarctica and the Southern California Bight and found only ammonia oxidizer bacterial sequences. Another investigation using this primer set that found only ammonia-oxidizer-like 16s rDNA sequences was an extensive study of ammonia-oxidizers in Arctic waters (4).

There are several differences between the study sites in these investigations and the one being discussed here. As previously mentioned, there is aquaculture, a port, sewage, and tourism in and around Xincun bay. Perhaps one or more of these factors somehow affected the community structure in such a way as to cause beta Proteobacteria that are closely related to AOB to exist in the bay in high enough numbers to amplify without a 100% primer match. Additionally, all three of the sites were oligotrophic in comparison to the highly impacted water of Xincun Bay. Also, and perhaps most importantly, there is a constant, albeit small, inflow of freshwater to the bay, while the

other sites were did not have this allochthonous input. With the exception of the Xincun cluster of sequences, which appears to be distantly related subset of beta *Proteobacteria*, all of the non-AOB sequences amplified with the NitA/ NitB primer set were closely related to freshwater beta *Proteobacteria*. Hence, it seems likely that the primer set is specific for beta Proteobacteria and perhaps in oceanic environments, the non-AOB beta *Proteobacteria* are too distantly related to the ammonia-oxidizers to be amplified with this primer set.

T-RFLP Data and peak identification. Each of the sixteen water column samples was subjected to T-RFLP analysis. After normalization such that the peak with the greatest height is equal to 1000 relative fluorescence units, the electropherograms are shown in Figure 2.6 and 2.7. In Figure 2.6, the electropherograms are grouped by station and divided into whole water (A) and filtered water (B) samples. The surface sample in each is shown in blue and the bottom sample in red. Looking at figure 2.6, one can see that the diversity of the bacterial NitA/B positive community is higher for the whole water samples. Additionally, it appears that the diversity is highest for the fish cage whole water samples (Figure 2.6A-2).

In Figure 2.7, the sixteen electropherograms are grouped by sample and divided into surface (A) and bottom (B) samples. The difference in diversity is less obvious when comparing surface and bottom samples. However, it appears that the fish cage bottom samples (Figure 2.7B-2) have the highest diversity and/or the relative abundance of several peaks is very high relative to the other panels, which have at most three peaks that are tall relative to the others in the same panel. Further, it appears that the bottom,

whole water sample, X7 (in blue in Figure 2.7B-2) is the most diverse sample. However, a visual inspection offers no indication of which bacterial taxa are present in the bacterial community being investigated.

A key component of understanding diversity is knowledge about the members of the community of interest. From the discussion above regarding the specificity of the PCR primers, it is obvious that the T-RFLP peaks represent more than beta AOB. Therefore, it is necessary to identify which peaks may or may not represent AOB. There are several lines of evidence available to assist in the identification of peak constituents. The best method available for peak identification is to utilize the clone library data. Computer-generated (or virtual) digestions were performed on the clone and selected isolate sequences using the *Cfo*I restriction enzyme, which cuts double stranded DNA at 'GCGC' sequences sites after the second 'G', to determine expected terminal restriction fragment (TRF) sizes. *Nitrosomonas ureae* and the four unique X5 (fish cage, surface, whole- water sample) AOB clone sequences were found to produce TRFs of 242 – 244 bases. No other clone or isolate sequence examined produced a TRF of this size. Interestingly, sample X1 (the surface, whole water, offshore sample) was the only sample that had a peak of more than 2% total height at this position. However, the clone sequences were derived from sample X5, so it is clear that the bacteria containing these sequences were present in the surface water at the fish cages as well. Considering the absence of a peak of more than 2% total height in the X5 sample at position 242, the *Nitrosomonas*-like bacteria in X5 (the surface, whole-water, fish cage sample) are assumed to comprise a much smaller percentage of the total Nit A/B-positive bacterial

Figure 2.6. Xincun samples X1 – X16 T-RFLP electropherograms grouped by station and divided into whole water and <5 μm size fraction.

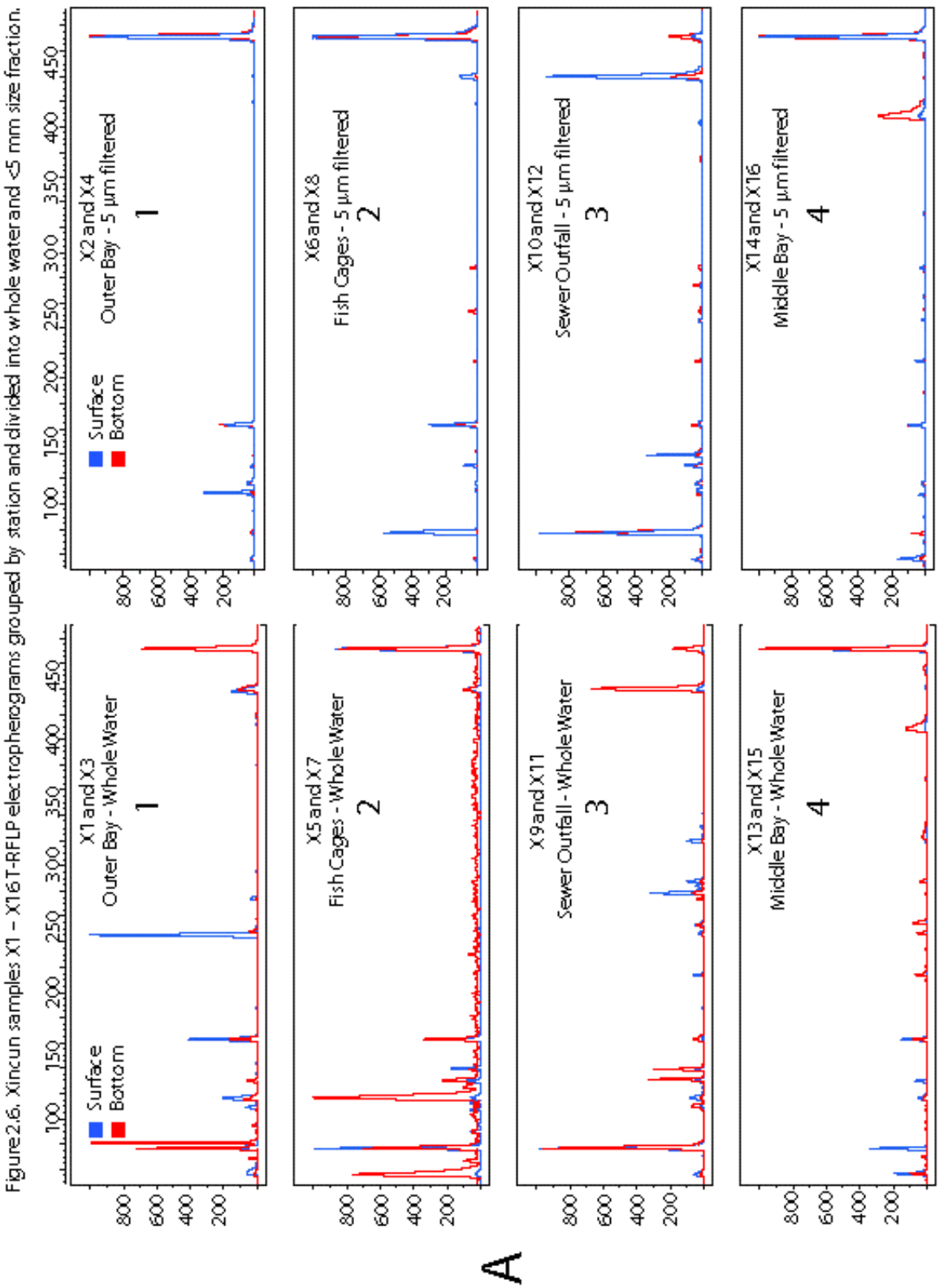


Figure 2.6. Xincun samples X1 – X16 T-RFLP electropherograms grouped by station and divided into whole water and <5 mm size fraction.

Figure 2.7. Xincun samples X1 – X16 T-RFLP electropherograms grouped by station and divided into surface and bottom samples.

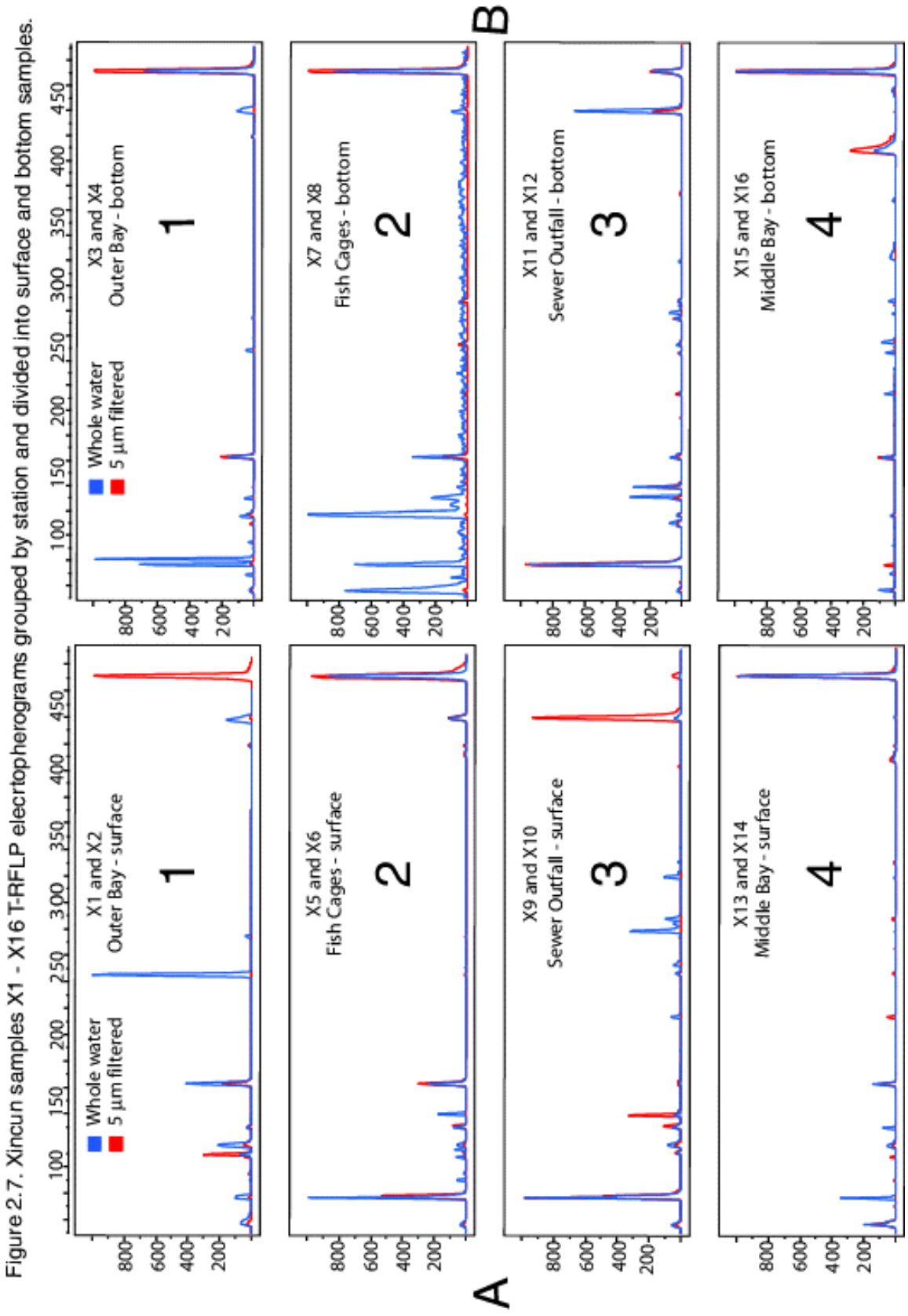


Figure 2.7. Xincun samples X1 - X16 T-RFLP elecrtopherograms grouped by station and divided into surface and bottom samples.

community than they do in sample X1. Regardless, peak 242 has been identified as representing a portion of the AOB community.

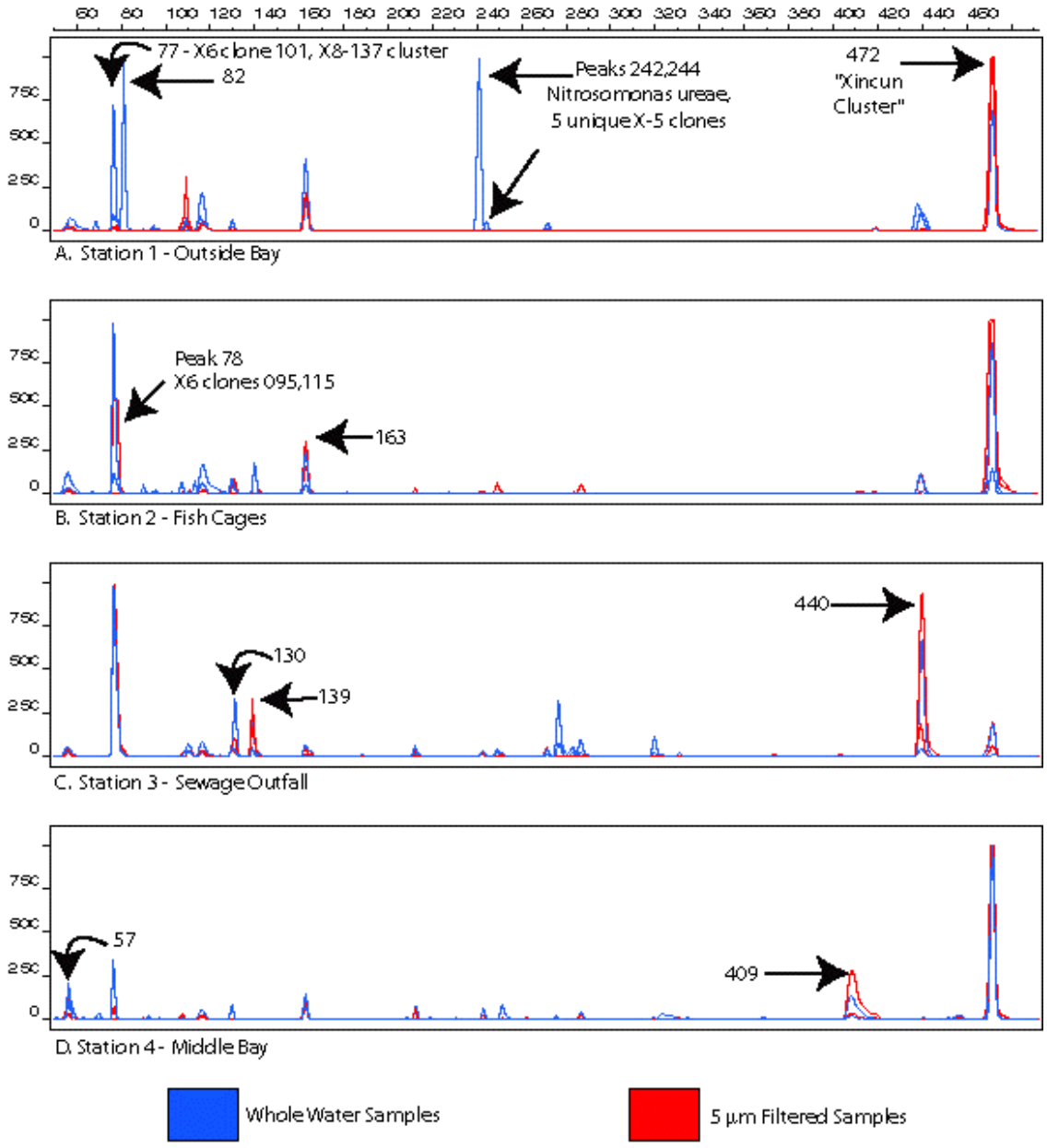
Virtual digestion of the X6 AOB sequences revealed expected TRFs of 81-84 bases. However, this was also true of the Nit A/B clone sequences associated with the *P. necessarius* cluster, as well as *Nitrosomonas europaea* C-31, *Nitrosomonas marina* 917 and *Nitrosospira briensis*. In order to determine exactly which, if any, peak represented the clone sequences, clones X5-094, X6-095, -101, -115 and X8-137 were analyzed separately as T-RFLP samples. The cloned DNA was amplified with labeled NitA and unlabeled NitB, a restriction digestion was performed on the PCR products and they were run on an ABI 310 Genetic Analyzer. Clone sequences X6-101 and X8-137, whose expected TRFs were 81 bases in length, produced peaks at position 77. Also, X6-095 and X6-115 yielded peaks of 78 bases, not the expected 82. However, clone X5-094, one of the *Nitrosomonas*-like sequences, yielded a peak at the expected position, 242 bases. While some variation in exact peak location is not uncommon, the reasons explaining the 4 base shift for some sequences and not others are not clear.

With the virtual and actual restriction digestion information from the clone library, certain peaks were identified. Peak 77 is assumed to represent an unresolved mixture of *Nitrosospira*-like bacteria and freshwater beta *Proteobacteria*. Peak 78 is assumed to represent *Nitrosospira*-like bacteria, although other unidentified bacteria may also be represented. Similarly, peak 242 represents the *Nitrosomonas*-like AOB. Lastly, the clone sequences that comprise a portion of the Xincun cluster (X8-132, X8-133, and X8-143) were determined to produce TRFs of 472 bp and are assumed to comprise all or part of the community represented by the peak at that position. Figure 2.8 shows all

Figure 2.8. Xincun samples X1 – X16 T-RFLP electropherograms grouped by station.

Whole-water samples are shown in blue and filtered samples are red. Arrows indicate peaks that have been identified or are discussed later in this work. All T-RFLP electropherograms have been normalized with the tallest peak larger than 50 base pairs in length equal to 1000 relative fluorescent units. Samples are grouped by station.

Figure 2.8. Xincun sample X1-X16 T-RFLP electropherograms.



electropherograms grouped by site. Peaks that were identified by the clone library are indicated.

Statistical Correlation Analyses. Tabular data (Table 2.1) from the T-RFLP analyses were used for statistical analysis. Principal component analysis (PCA) comparing the samples was performed using peaks that each represented a minimum of 2% of the total height of all peaks for any one sample and that occurred in 5 or more samples (Figure 2.9). The scatterplot represents the similarity or dissimilarity of the samples by comparing the T-RFLP tabular percent peak height data. Each of the samples from stations 1, 3 and 4 form clusters. Samples from station 2, the location of the fish cages, do not group together. Instead, the surface water samples group closely with the sewer outfall samples, indicating that the portions of the bacterial community that amplify with the NitA/NitB primer set are similar. These six samples (X5 – X6, and X9 – X12) are different from station 1 and station 4 samples and the fish cage bottom samples (X7 and X8) as determined by principal component 1, which accounts for 37% of the difference among the samples. Further, the groupings seem to coincide with the station and not whole water versus filtered water or surface versus bottom, with the exception of the fish cage site, station 2, for which the surface samples group together, while the bottom samples do not. When discussing the visual peak data, it was noted that X7 (the fish cage, bottom, whole water sample) seemed to have a more diverse, or at least, a more evenly balanced community structure. Similarly, in Figure 2.9, sample X7 is different from all 15 of the other stations. Also, sample X8, does not cluster with either the other fish cage samples or X7, but rather with the middle bay samples X14 (surface, filtered)

Table 2.1. Nit A/B peak heights shown as percent of total peak height.

Peak size (in base pairs) is shown across the first row. Sample number is shown in the first column. The numbers in the chart are percentages of total peak height represented by each peak in a given sample. Peaks that occurred as more than 2% of total peak height and were in at least four samples are included in the table. Peaks that appear only once in the 16 samples, but are discussed in the text are included. The samples each station (outside bay, fish cages, sewer outfall, mid-bay) are highlighted as a group.

Table 2.1. Nit A/B peak heights shown as percent of total peak height.

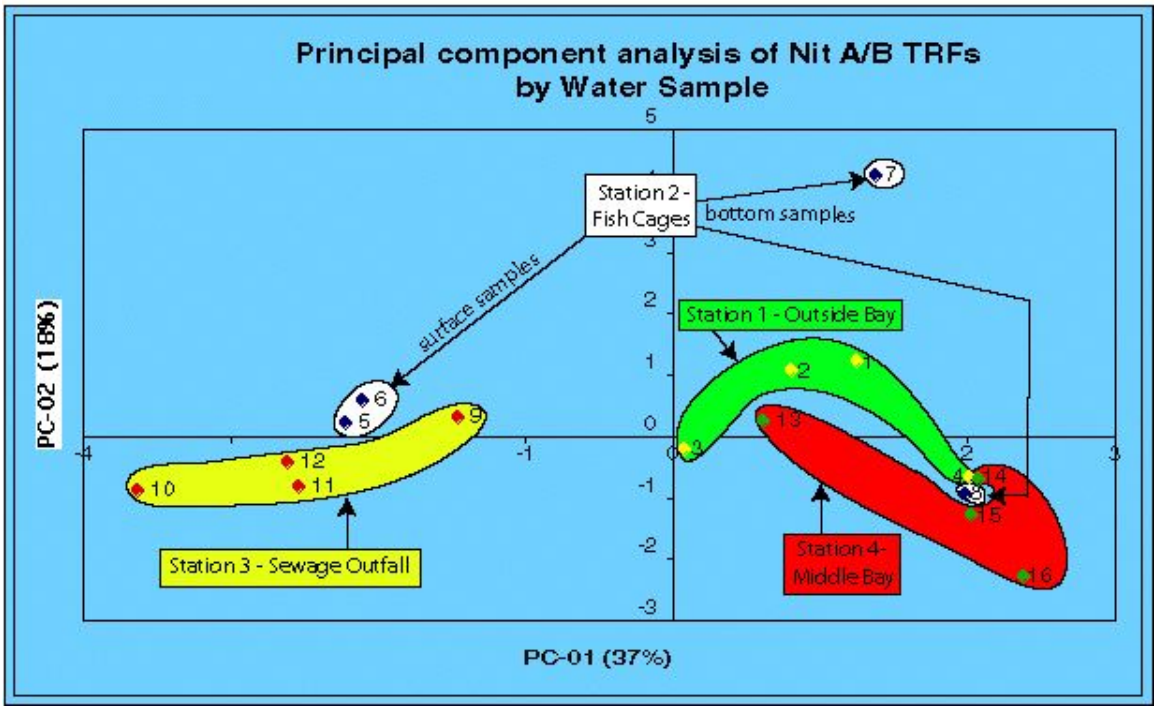
Peak size *sample	57	59	77	78	82	110	117	130	139	163	213	242	287	409	440	472
X1 – S, W	3	5	5	5		3	9			18		44				
X2 – S, F	4	3	3			38	6	3		22					2	
X3 – B, W			25		25		3	2		6					4	23
X4 – B, F				3		3	3			16						76
X5 – S, W			47	12			3	4	9	11					5	
X6 – S, F			36	33				6		19					7	
X7 – B, W	31		17	10			24			8						
X8 –B, F	3									12	2		4			74
X9 – S, W	2		41	18			3	2	2		3		4		2	
X10 – S, F	2		33	16				4	11						31	2
X11 – B, W			30	15		2		2	9	2					20	6
X12 – B, F			40	29				2	9	2					8	8
X13 – S, W	11	4	18				3	4		8						52
X14 – S, F	11	3								7	4		2	3		67
X15 – B, W	6									5	4		3	8		57
X16 – B, F	2		5							7				18		63
Occurrence	10	4	12	9	1	4	8	9	5	14	4	1	4	3	8	10

*Sample Origin: X1–X4 - Outer Bay; X5-X8 – Fish Cages; X9-X12 – Sewer outfall; X13-X16 – Middle Bay
S – Surface water sample; B – Bottom water sample. W – Whole water sample; F – 5 μm prefiltered sample.

Figure 2.9. Principle Component Analysis of T-RFLP data from all 16 samples from 4 stations.

Analyses were performed using only peaks that occurred in 5 or more samples. Samples from the same station are highlighted for clarity.

Figure 2.9. Principle Component Analysis of T-RFLP data from all 16 samples from 4 stations.



and X15 (bottom, whole water) and the outer bay, deep, filtered sample. This seemingly incongruous mix of samples that is so strongly correlated has one thing in common – all of the samples have 57% to 76% of their total peak height in peak 472, the non-AOB 'Xincun cluster.'

In order to determine which peaks co-occur, PCA was also performed to compare the peaks that occur 3 or more times within each of the 8 whole-water (odd numbered samples) or filtered samples (Figure 2.10). In the whole-water sample, peaks 77, 78, 130, 139 and 440 group together (Figure 2.10a). Peak 472 is the least similar to this group as determined by principal component 1 (PC-01). Examination of the peak occurrences in the filtered samples is shown in Figure 2.10b. Once again peaks 77, 78 and 440 group together and peaks 163 and 472 are the most different from that group. Although the grouping of peaks does not mean that the bacterial species that comprise them are the identical, co-occurrence does indicate a correlation between peaks. Therefore, since peak 472 is known to contain non-AOB, and peaks 77 and 78 do represent some portion of the AOB and primers supposedly specific to AOB were used in this analysis, it is possible that peaks 130, 139, and 440 are also AOB-representative peaks. Also, since peak 163 never groups with the AOB peaks, it may not represent beta AOB. However, there remains one piece of evidence to examine before final conclusions can be drawn – comparison of percent peak height (or abundance) to dissolved inorganic nitrogen data.

Comparison with environmental data. At the time of sample collection for the bottom samples at the fish cages, X7 and X8, a swimmer collected a sediment sample from the top 5-7 cm of sediment. The sediment was black and smelled strongly of sulfide. No

Figure 2.10. PCA analysis of T-RFLP peak occurrence.

In figure 2.10a, peaks that occur at least 3 times in the whole-water samples are shown. Peaks that appear to group together have been highlighted. Each data point is labeled with the corresponding peak that it represents.

In figure 2.10b, the peaks that occur at least 3 times in the filtered samples are shown. The peaks that appear to group together have been highlighted. Each data point is labeled with the corresponding peak that it represents.

Figure 2.10a. PCA analysis of peak occurrence in whole-water samples.

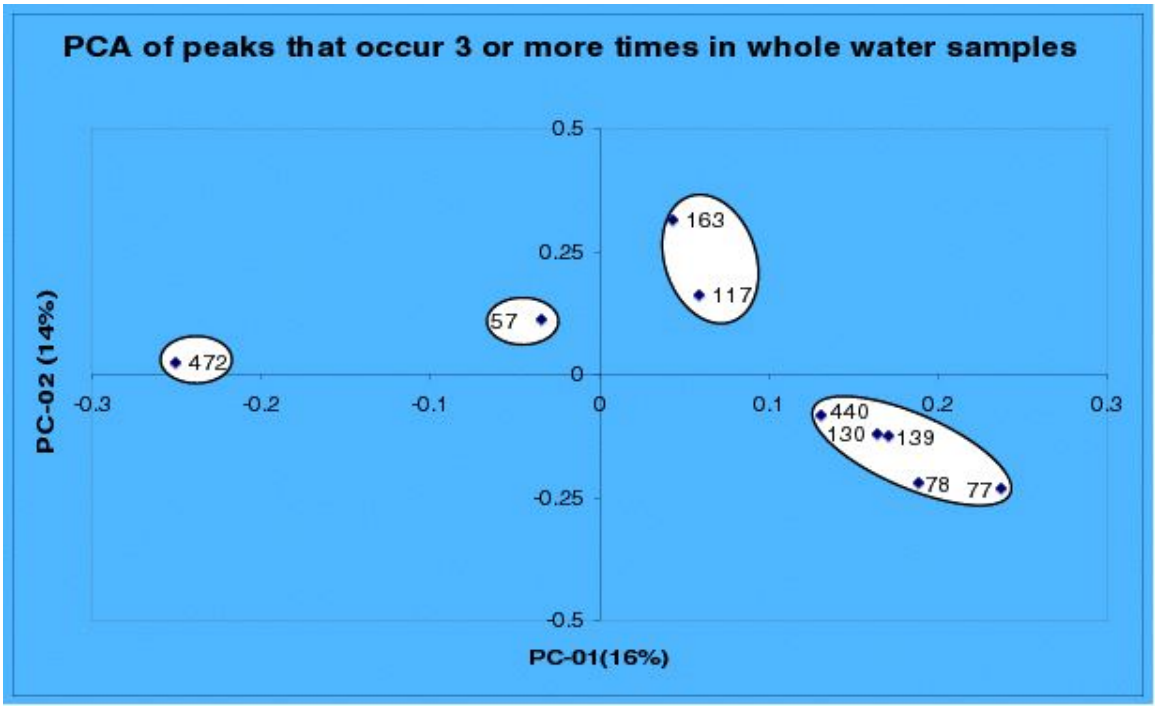
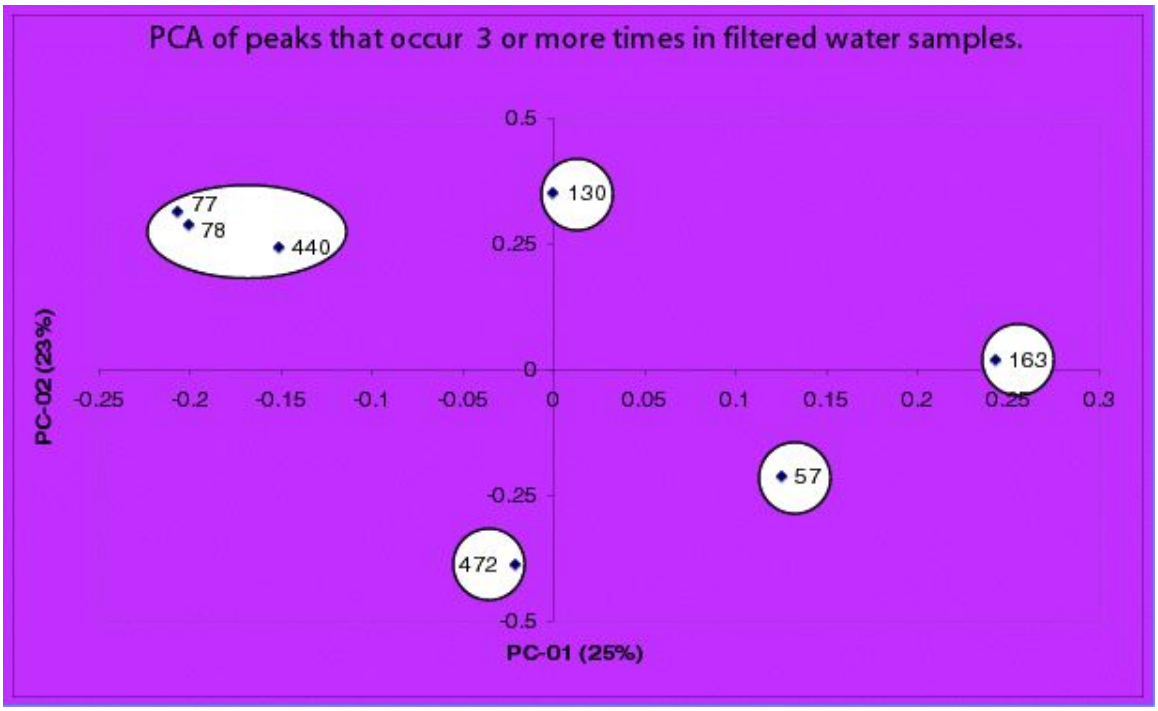


Figure 2.10b. PCA of peaks that occur in at least 3 filtered samples.



analysis for sulfide was performed, due to subsequent sample contamination. If the ammonia oxidizing bacterial community is indeed disrupted above the sediments at the fish cages, perhaps it is due, in part, to the inhibitory effects of sulfide.

The environmental parameter data (Table 2.2) were assayed and communicated by Chen Chunhua of the Hainan Marine Development, Planning and Design Institute, Haikou, Hainan, P.R.C. The percent peak height data for the peaks included in the PCA analyses were compared directly to the environmental data collected concurrent to sampling. Regression analysis revealed that as much as 95% of total proportion of variance in the abundance (percent heights) for several peaks can be explained by dissolved inorganic nitrogen concentrations in a positive correlation (Peak 78 - Figure 2.11). In whole water samples compared to total dissolved inorganic nitrogen (TDIN), peak 77 has an R-squared value of 0.53 ($R=0.73$, $n=8$, $p<0.05$) and peak 78 an R-squared value of 0.95 ($R=0.98$, $n=8$, $p<0.01$). For filtered samples compared with TDIN, positive correlations with an R-squared of at least 0.54 were found for peak 77 ($R=0.81$, $n=8$, $p<0.02$) and peak 78 ($R=0.73$, $n=8$, $p<0.05$). Peaks 77 and 78 were the only two peaks known to contain AOB (*Nitrosospira*-like clone sequences) that occurred frequently enough to perform regression analysis (peak 242 [n=1] was identified as representing *Nitrosomonas*-like bacteria). Interestingly, the only peak for which only non-AOB were identified, peak 472 (Xincun cluster), showed strong negative correlations with TDIN in whole water ($R = 0.75$, $n=8$, $p<0.02$) samples.

The other peaks that were examined in using PCA (Figure 2.10a and 2.10b) were examined using regression analysis (Figure 2.11). Peaks 57 and 130 showed no significant correlations with any of the dissolved nitrogen concentrations. Peak 163 had

Table 2.2. Environmental Parameters.

Environmental parameters that were measured or for which samples were taken at the time of water sampling for this study. Dr. Chen Chunhua, Hainan Marine Planning and Design Institute, Haikou, Hainan, P.R.C performed nitrogen species analyses.

Table 2.2. Environmental Parameters.

Sample#	Depth (m)	°C	pH	DO (mg/L)	DO%	Salinity (ppt)	Nitrate (µg/l)	Nitrite (µg/l)	Ammonia (µg/l)	Total DIN
1,2	0.5	27.63	8.1	5.04	77.3	31.53	0.83	0.1	2.27	3.2
3,4	4.0	26.61	8.14	6.01	92	33.54	0.83	0.1	2.27	3.2
5,6	0.5	29.98	8.16	4.93	77.6	29	1.69	0.27	9.03	10.99
7,8	3.0	29.13	8.1	4.19	65.5	30.28	1.69	0.27	9.03	10.99
9,10	0.5	29.88	8.09	5.05	79	27.98	5.97	0.98	8.26	15.21
11,12	3.0	28.45	8.05	4.07	62.7	29.74	5.97	0.98	8.26	15.21
13,14	0.5	30.54	8.21	6.2	98.5	28.51	0.7	0.1	0.09	0.89
15,16	4.0	29.47	8.09	5.25	83	31.24	0.7	0.1	0.09	0.89

Figure 2.11. - R-squared values for regression analyses comparing peaks used in PCA to various dissolved nitrogen species and total dissolved inorganic nitrogen.

R-squared values for all of the peaks used in PCA that occurred at least 4 times. R-squared values were calculated for each peak for regressions comparing peak height to nitrate, nitrite, ammonium and total dissolved inorganic nitrogen. A negative value is used to indicate a negative correlation. Values in bold indicate a p-value of $p<0.10$ for the correlation coefficient (R value) for that analysis. For each analysis, n=8. This is accomplished by using a value of zero for any peak height that is not equal to at least 2% of the total peak height for that sample.

Figure 2.11.

ww Samples

Peak size (n)	Nitrate	Nitrite	Ammonium	Total DIN
57 (8)	-0.06	-0.05	0.24	0.00
77 (8)	0.33	0.33	**0.52**	**0.53**
78 (8)	**0.75**	**0.75**	**0.83**	**0.95**
130 (8)	0.02	0.02	0.01	0.02
163 (8)	**-0.44**	**-0.45**	-0.06	-0.20
440 (8)	**0.43**	**0.43**	0.19	0.33
472 (8)	-0.23	-0.22	**-0.67**	**-0.56**

<5um samples

Peak size (n)	Nitrate	Nitrite	Ammonium	Total DIN
57 (8)	-0.13	-0.12	-0.30	-0.27
77 (8)	**0.65**	**0.65**	**0.48**	**0.65**
78 (8)	**0.39**	**0.40**	**0.49**	**0.54**
130 (8)	0.14	0.14	0.29	0.27
163 (8)	**-0.48**	**-0.49**	-0.02	-0.16
440 (8)	**0.61**	**0.61**	0.25	**0.45**
472 (8)	-0.31	-0.31	-0.22	-0.31

significant negative correlations with nitrate and nitrite for whole water ($R=0.66$ and 0.67 respectively, $p<0.10$) and filtered samples, ($R=0.69$ and 0.70 respectively, $p<0.10$). Peak 440 showed significant positive correlations for whole water samples with nitrate ($R=0.66$, $n=8$, $p<0.1$) and nitrite ($R=0.65$, $n=8$, $p<0.1$). Moreover, peak 440 in filtered samples had strong positive correlations for nitrate ($R=0.78$, $n=8$, $p<0.05$), nitrite ($R=0.78$, $n=8$, $p<0.05$) and TDIN ($R=0.67$, $n=8$, $p<0.1$).

Before attempting to correlate environmental data to peak height, it must be mentioned that the conclusions drawn from such analyses have not been supported experimentally. Such conclusions are speculative and may merely serve to restate the similarities shown by PCA. Nevertheless, it is possible that such correlations may prove to be accurate to some degree and thus may shed some light on the bacteria that are represented by unidentified peaks.

The hypothesis stated after PCA that peaks 130, 139, and 440 may be AOB and that peak 163 represents non-AOB can now be revisited. Since peak 139 could not be subjected to regression analysis, it cannot be further examined. PCA is the only indicator that peak 139 may contain AOB, but the correlation could be due to other peaks (77, 78) making up a large percentage of total height and lead to false conclusions. So we will not speculate as to the phylogenetic affiliation of this peak.

Peak 130 occurred frequently enough to perform regression analysis. However, the resultant correlation coefficients did not prove to be statistically significant. Therefore, we feel that, despite its clustering with the known AOB peaks 77 and 78 by PCA, peak 130 might not represent AOB.

Peak 163 comprises at least 2% of total peak height in 14 of the 16 samples studied. In every case but one (sample X13 is 8% and X14 is 7%), the percent height accounted for by this peak is higher for the filtered sample than for its whole-water counterpart. This is an indication that peak 163 represents free-living bacteria. PCA clearly separates peak 163 from the known ammonia-oxidizer peaks and regression analysis showed significant negative correlation between abundance, as defined by percent total peak height, and dissolved nitrate and nitrite concentrations. As a result, it is our conclusion that peak 163 probably does not represent AOB.

Conversely, PCA indicates that peak 440 is very similar to peaks 77 and 78 in both whole water and filtered analyses. Also, regression analysis revealed a positive correlation between the concentration of nitrate and nitrite and peak 440 percent of total peak height for whole water and filtered water as well as a positive correlation between peak 440 in the filtered samples and TDIN. Thus, it is our opinion that peak 440 may represent a subset of AOB in Xincun bay.

Summary. As stated above, it appears that the ammonia-oxidizing bacterial community associated with finfish aquaculture differs between surface and bottom samples. The visual inspection of T-RFLP electropherograms (Figure 2.7) indicated that X7, the fish cage bottom, whole-water sample was more diverse and/or balanced than the other samples. PCA analysis clearly indicated that X7 and X8 (the bottom filtered sample) differ from the surface fish cage samples, X5 and X6. In fact, X7 was different from all other samples and X8 clustered very closely with samples from both the middle and outer bay. Further, X8 was dominated by peak 472, which accounted for 74% of the total peak

height for that sample. Conversely, peak 472 accounted for less than 2% in X5, X6 and X7.

The total peak height accounted for by peaks 77 and 78 (known to include AOB) in samples X5 and X6, the surface fish cage samples, is 59% and 69%, respectively. In contrast, these same two peaks account for 27% of the total for sample X7 and less than 4% for X8. If peak 440 is included, then the percentages for each sample are X5 – 64%, X6 – 76%, X7 – 27%, X8 – less than 6%.

The differences in AOB community structure found in the surface water versus bottom water (3.5m depth) is in agreement with the findings of McCaig, et.al., who found the nitrification potentials and the bacterial communities associated with this process in sediments beneath aquaculture fish cages to be significantly different from sediment samples taken nearby (18).

In order to make a more thorough identification of the ammonia oxidizer bacterial community in these samples a more targeted approach is now in order. A restriction digest of unlabeled Nit A/B amplicons run on an agarose gel should allow isolation of bands that represent specific TRFs, for example, the 77, 78, 130, 139, 163, and 440 base pair bands. Cloning and sequencing of those bands would provide information to support or reject our hypothesis that these peaks do or do not represent AOB. Also, repeating the T-RFLP analysis with different restriction enzymes would assist in delineating the species represented by each peak especially peaks 77 and 78.

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CHAPTER 3

MOLECULAR CHARACTERIZATION OF NITRITE REDUCTASE GENES OF PUTATIVE AMMONIA-OXIDIZERS FROM A MUNICIPAL WASTEWATER TREATMENT PLANT[1]

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[1]Sullivan, J.B., P. Principi and R.E. Hodson. To be submitted to *Applied and Environmental Microbiology*.

Introduction

Excess or insufficient biologically available nitrogen is of primary importance in studies of aquatic system health. Of the primary biogeochemical cycles (carbon, nitrogen, sulfur, phosphorus, and oxygen), perhaps the least clearly understood is the nitrogen cycle. However, with its linkages to global carbon cycling (as an essential macronutrient for all life, primary production limitations in open ocean systems, fertilization applications in agriculture, coastal eutrophication events, and wastewater treatment practices), understanding the various biological interactions of the nitrogen cycle is paramount (11, 16). Therefore, a study into the presence of DNA sequences recently characterized in ammonia-oxidizing marine isolates that code for an important denitrification enzyme was undertaken.

Two of the component processes of the nitrogen cycle are nitrification and denitrification. Nitrification is a two step process. In the first step, ammonia-oxidizing bacteria oxidize ammonia to hydroxylamine and then nitrite. Next, nitrite oxidizers produce nitrate from nitrite. Ammonia oxidation is performed by two select groups of chemolithoautotrophs. The largest group of these obligate aerobes is a subset of the Beta Proteobacteria; the other is limited to a few species within the Gamma Proteobacteria. Nitrite oxidation is phylogenetically limited to a group within the Gamma Proteobacteria. Nitrifying bacteria obtain the energy necessary for carbon dioxide fixation from the nitrogen oxidations they perform. Denitrification is an alternate respiratory pathway that is carried out by a wide variety of facultative anaerobes. These bacteria possess the

ability to respire using oxidized nitrogen species in place of oxygen as terminal electron acceptors for metabolism of exogenous carbon compounds (18).

Denitrifying bacteria possess a series of enzymes, nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase, that allow them to respire anaerobically using nitrate, nitrite, nitric oxide, and nitrous oxide as terminal electron acceptors (22). Nitrite reductase is considered especially important to global nitrogen cycling because it produces nitric oxide, the first gaseous product in the pathway. This is the point at which biologically available, or fixed, nitrogen is generally considered to be lost from a system (20). There are two well-characterized forms of dissimilatory nitrite reductase, one iron-containing with a cytochrome cd1-heme structure and the other containing two copper molecules at its active site. The gene that codes for the iron-containing moiety is nirS, while the copper enzyme is coded for by nirK. Though having identical function in denitrifying bacteria, these two enzymes have no more similarity than do other non-homologous proteins and are never present in a single organism (22).

Since nitrifying bacteria form nitrite and denitrifying bacteria reduce nitrite (among other nitrogen species), the formation of denitrification intermediates in pure cultures of ammonia-oxidizers was a surprising phenomenon. The reduction of nitrite to nitric oxide and nitrous oxide by ammonia-oxidizing bacteria, a process termed nitrifier denitrification, was first described in *Nitrosomonas europaea* (6). Initially, it was thought that the gases produced might have been the products of the breakdown of unknown intermediates of the nitrification process. Later, the mechanism for the production of nitric oxide and nitrous oxide from nitrite was described with the use of ^{15}N tracers (12). Laboratory experiments and biochemical characterizations of enzyme

properties such as rate enhancement at low oxygen tensions and reaction rate profiles further demonstrated the existence of an enzyme that was very similar to the nitrite reductase possessed by heterotrophic denitrifying bacteria (2, 10, 15).

Recently, it has been shown that other ammonia-oxidizing bacteria also possess the genes coding for a nitrite reductase enzyme with very high DNA sequence and amino acid homology to the previously characterized copper-containing nitrite reductase (NirK) sequences (4). These bacterial isolates have been reported to express a functional form of NirK (21). This discovery is interesting both from the standpoint of basic bacterial physiology and for understanding the sources of global production of nitric oxide and nitrous oxide (19). Unlike the pathway in most heterotrophic denitrifiers, dinitrogen (N_2) does not appear to be the ultimate end product of nitrite reduction in chemolithotrophic ammonia-oxidizers. Nitric and nitrous oxide are important due to the role they play in contributing to "the greenhouse effect," global warming, and stratospheric ozone destruction (17).

Although the ammonia-oxidizers that previously tested positive for nitrite reductase were all isolated from saltwater, other ammonia-oxidizers may also have this capability. One readily accessible (and, in the developed world, ubiquitous) type of environment with known nitrifier populations, high ammonia concentrations and limited oxygen concentrations is the wastewater treatment facility (13). In this investigation we demonstrate the presence of bacterial DNA sequences in a Georgia activated sludge municipal wastewater treatment facility that are highly homologous to the nitrite reductase sequences recently found to exist in marine ammonia-oxidizing bacteria.

Material and Methods

Overview. DNA purified from activated sludge was probed with primers designed to be specific for a cluster of marine ammonia oxidizer NirK sequences. Following PCR amplification, the product was cloned and sequenced yielding 6 unique sequences. A phylogenetic tree was constructed to determine the relatedness of the unique sequences to those from which the primers were designed.

Bacterial strains. A pure culture of *Nitrosomonas marina* previously shown to contain the *nirK* gene, was obtained from Dr. Bess Ward (Department of Geosciences, Princeton University) and grown in 50% *Nitrosococcus oceanus* medium (10.0 mM $(NH_4)_2SO_4$, 0.14 mM $CaCl_2$, 1.45 mM $MgSO_4$, SL-8 micronutrient mix, 0.09 mM K_2HPO_4, Phenol red for pH detection, and 1M Na_2CO_3 dropwise until pH=7.75) and 50% 0.2 μm-filtered seawater. The culture was grown at 25°C in the dark while being shaken at 50 rpm.

Activated Sludge Samples. Four aliquots of 50ml each of activated sludge were collected from the primary aeration tank at a good mixing point from the Middle Oconee Water Pollution Control Plant (Athens-Clarke County, Georgia), during December 2001. Samples were stored for several days at 4°C immediately after collection until analyses were performed.

DNA extraction. For DNA from activated sludge, four 1 ml aliquots of sludge were centrifuged at 13,000 rpm for 5 minutes to harvest sludge flocs, and washed twice with Tris-EDTA buffer (pH 8). Total genomic DNA was extracted following the protocol

described in Short Protocols in Molecular Biology [#414]. Briefly, pellets were resuspended in Tris-EDTA buffer (pH 7.6) and lysed with 10% SDS and Proteinase K at 65°C. Cellular debris was further broken down for removal by addition of 5 M NaCl and CTAB. DNA was isolated by repeated phenol/chloroform/isoamyl alcohol and phenol/chloroform treatments. Purified DNA was precipitated with chilled ethanol at 4°C overnight, centrifuged at 15,000 rpm for 15 minutes, washed with 70% ethanol, and resuspended in Biograde dH2O. For primer testing, *N. marina* DNA was obtained by centrifuging 10 ml of cultured bacteria in growth medium as described above for 7 minutes at 13,000 rpm in 2 ml aliquots. Following centrifugation, the cell pellets were resuspended in 100 μl and combined in one tube. After pelleting again, the cells were resuspended in 100 μl deionized/distilledH_2O and the tubes were placed in boiling water for 10 minutes. One μl aliquots were used as template in 25 μl PCR reactions.

***NirK* primer design and verification.** In Casciotti and Ward (AEM 2001), five of the six NirK sequences described from marine ammonia oxidizer isolates formed a cluster that was distinct from the NirK genes of denitrifying bacteria. We designed several oligonucleotide PCR primers to specifically target sequences within this cluster. Multiple sequence alignment was performed using the nucleotide sequences for the NirK gene from each of the six NirK-containing ammonia oxidizers: *Nitrosomonas marina*, marine isolates NO3W, URW, C-45, C-113a, and TA-921i-NH4 as obtained from GenBank. Also included in the multiple sequence alignment were several typical heterotrophic denitrifier NirK sequences (*Rhizobium melitoti*, *Hyphomicrobium zavarzinii*, *Ochrobactrum anthropi*, *Bradyrhizobium japonicum* and *Rhodobacter sphaeroides*).

Primers AOBNirK546F [5’CGGCCTGAAAGATGCWGCA3’], AOBNirk571F [5'CCATATCRMTATGACCGYGC3'], AOBNirK873r [5'TGARCCR-CCTACCCAATACA3'], AOBNirK896r [5'TKGTCAGCGGYGTATCA3'], AOBNirK947R [5’TCATAMGCWGCAGCRACT3’] are numbered according to the NirK sequence of *Pseudomonas aureofaciens* (GenBank accession number Z21945). Primers were designed at regions that showed high sequence homology for the ammonia-oxidizing nitrite reductase (AOBNirK) cluster and low homology for the other known NirK sequences (including the NirK sequence for ammonia-oxidizer TA-921I-NH4, which clustered with classical heterotrophic denitrifiers). BLAST analysis verified the specificity of the primers. BLAST (Basic local alignment search tool) is available through the National Center for Biotechnology Information, National Institutes of Health and compares an input nucleotide sequence to an existing database of DNA sequences and returns the closest matches (1).

PCR conditions. The optimized PCR protocol consisted of 1 μl of a ten-fold dilution of the extracted activated sludge DNA as template; 0.5 μM of each primer, 0.25 mM dNTPs, 1.0 μl of Expand High Fidelity DNA polymerase mix (Roche Biochemicals) and 1X manufacturer-supplied buffer in a final reaction volume of 25 μl. The thermal protocol was: initial denaturation at 94°C for 4 minutes, followed by 35 cycles with denaturation at 94°C for 30 seconds, an annealing temperature of 58°C for primer set AOBNirk571F - AOBNirK873R for 45 seconds and an elongation step of 72°C for 1 minute. A final elongation of 7 minutes at 72°C completed the amplification reaction.

Sequencing. For cloned PCR products, the amplicon was purified from a 1.0% agarose gel using the UltraClean PCR CleanUp DNA Purification Kit (MoBio) following manufacturer's instructions. The purified PCR product was cloned using the Promega pGEMT-easy Kit. Transformants were grown overnight on LB plates with ampicillin (100 μg/ml), 0.5 mM IPTG (isopropylthiogalactopyranoside) and 80 μg/ml X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) at 37°C. Plasmid DNA was extracted from a total of 15 white and pale blue clones using the QiaPrep Spin Miniprep Kit, (Qiagen, Inc.) and digested with EcoRI restriction enzyme. The plasmid inserts that were the expected size were selected for sequencing. 1ul of purified plasmid DNA (~150 ng) was used in a 10 μl BigDye Terminator (Applied Biosystems, Inc.) cycle-sequencing reaction with the T7 primer. After removal of unincorporated dideoxy terminators by Sephadex G-50 purification, the labelled PCR product was dried in a Speed Vac and resuspended in 15 μl of HiDi formamide (ABI) and sequenced on an ABI 310 Genetic Analyzer. For direct sequencing of PCR products, the amplicon was purified directly from the PCR reaction mix using the Wizard PCR Product Purification Kit (Promega Corp). After purification, 1μl of product was used as a template in a cycle-sequencing reaction as outlined above.

Phylogenetic analysis. Sequence data were reviewed using the Sequencher (Gene Codes Corporation, Ann Arbor, MI) program in order to ensure that the software analysis program identified all bases correctly. Subsequently, sequences were exported in GCG (The Wisconsin Program Package for Biological Sequence Analysis, Genetics Computer Group, Oxford Molecular) sequence format, imported to RCR (Research Computing

Resource, Biological Sciences Software, Databases and Services, University of Georgia, Athens, GA) and included in a multiple alignment using the PILEUP command. The resultant multiple sequence alignment was imported to SeqPup for final alignment and length normalization. The finalized sequences were 302 base pairs long. These aligned sequences were each bootstrapped 100 times using the SEQBOOT program of the phylogenetic inference package PHYLIP 3.572 (5). Neighbor-joining trees were produced for each pseudoreplicate analysis. Next, the SEQBOOT output file was input for distance matrix analysis using DNADIST in PHYLIP. The Kimura model was used to calculate nucleic acid sequence distance (8). The CONSENSE program produced the majority-rule consensus tree for the sequences, and the phylogenetic tree was drawn using the DRAWGRAM program from PHYLIP.

Results

Primer design and verification. Several PCR primers specific for the nitrite reductase gene (NirK) possessed by several marine ammonia-oxidizing bacteria were designed; two forward and three reverse primers (Figure 3.1). The primers ranged in length from 17 to 20 bases with a G+C content of 44-60% (Table 3.1). Primer annealing temperature ranged from 50°C for the least GC-rich sequence of AOBNirK 896R (the primer contains 2 degenerate bases – Table 3.1) to 63°C for the most GC-rich version of AOBNirK 571F.

During PCR testing with *N. marina* genomic DNA, all 6 possible primer sets yielded a single product of the expected size after PCR optimization. Following amplification with the primer set AOBNirK 571F/873R, the *Nitrosomonas marina* PCR

Figure 3.1. Ammonia-Oxidizer Nitrite Reductase Primer Design

Aligned NirK sequences used to develop AOBNirK primer set. The bottom five sequences are ammonia-oxidizing isolates that have been shown to possess NirK. The remaining six sequences include five heterotrophic denitrifiers and one, TA-921i-NH4, ammonia oxidizer that are NirK positive. Darker highlighting indicates identity with the primer developed at each site. Lighter highlighting indicates a base pair mismatch within the primer binding sequence of each organism shown. Primers names ending in “F” are forward primers. Primer names ending in “R” are reverse primers whose sequence is the reverse complement of the genomic sequence shown.

Figure 3.1. Ammonia-Oxidizer Nitrite Reductase Primer Design

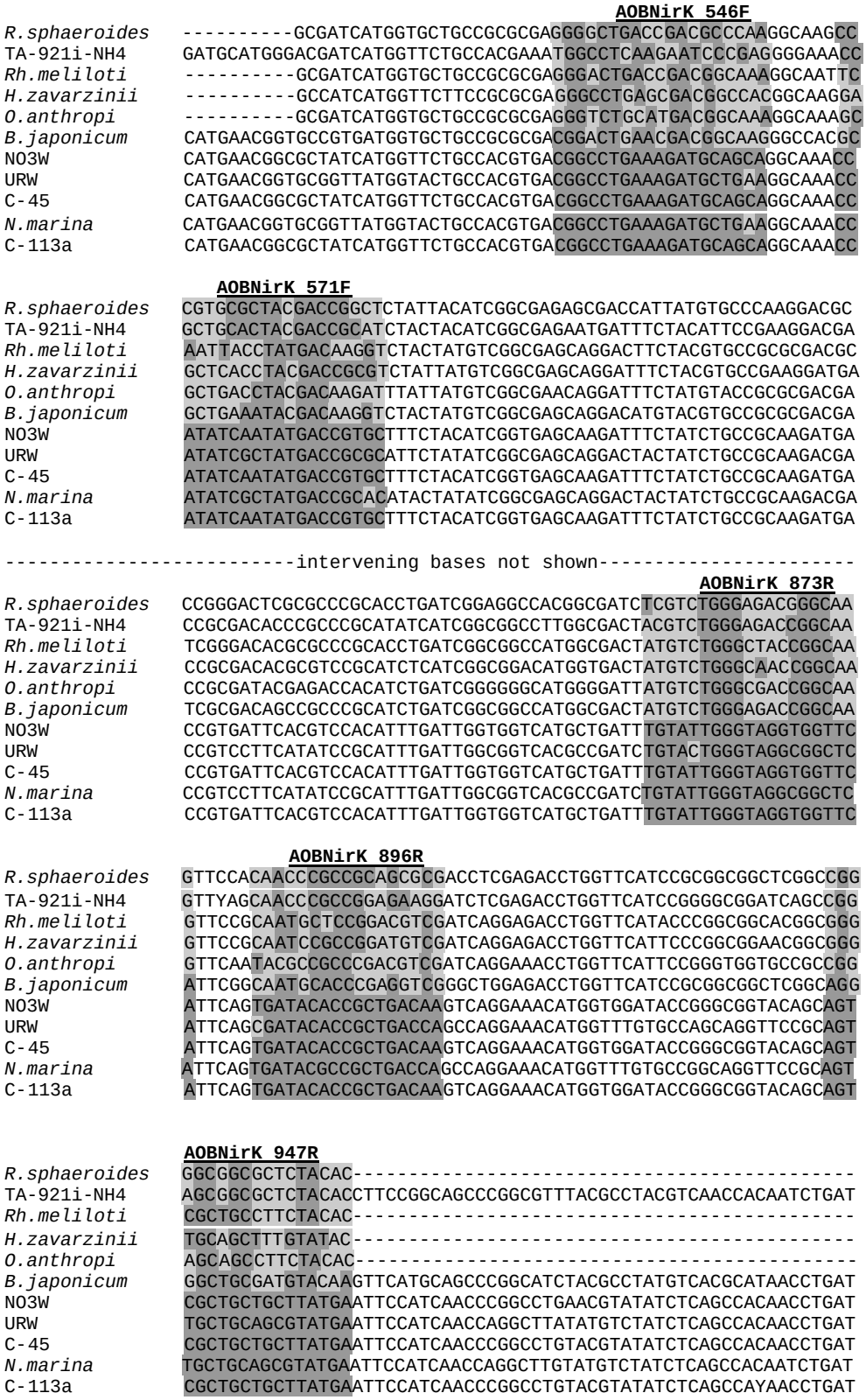


Table 3.1. PCR primers designed in this study.

A summary table of the primers designed in this study. Primer sequences ending in “F” are forward primers and require a reverse primer, “R”, for amplification. The primers were designed to target NirK sequences specific to those found recently that were specific to ammonia-oxidizing β-proteobacteria. The ammonia-oxidizing bacterial NirK (AOBNirK) sequences form a cluster separate from all known NirK sequences.

Table 3.1. PCR primers designed in this study.

Name	Numbering from NirK *P.aureofaciens* accession #Z21945	Annealing Temp (Tm)	Length (bases)
AOBNirK546F	5'CGGCCTGAAAGATGCWGCA	58	19
AOBNirK571F	5'CCATATCRMTATGACCGYGC	56-63	20
AOBNirK873R	5'TGARCCRCCTACCCAATACA	56-60	20
AOBNirK896R	5'TKGTCAGCGGYGTATCA	50-55	17
AOBNirK947R	5'TCATAMGCWGCAGCRACT	51-56	18

product was purified directly from the PCR reaction mix and sequenced. The nucleotide sequence obtained was 100% homologous over the 302 base pair length with the *N. marina* NirK sequence in GenBank.

When isolated DNA from activated sludge was used as a template with the AOBNirK 546F/947R primer set, a band of the expected size was among several bands visualized when viewed by gel electrophoresis. This band was excised, purified, cloned and sequenced. None of the sequences obtained were AOBNirK. Inexplicably, cloning and sequence analysis revealed that most of the amplicons contained AOBNirK546F as both the forward and the reverse primer. Therefore, AOBNirK 571F was employed as the forward primer. When sludge DNA was used as template and the AOBNirK 571F and AOBNirK 873R primer set was used, a single band of the expected size was the result and, when purified, was used for subsequent cloning and sequencing experiments.

Phylogenetic Analysis. Following sequencing, the 6 unique AOBNirK clone sequences obtained using the AOBNirK 571F/873R primer set were compared to one another and representative NirK-containing ammonia-oxidizing and heterotrophic denitrifying isolates. The clone DNA sequences were first run in the BLASTX program of NCBI. This program converts an input nucleotide sequence to amino acid sequence in each of the six possible reading frames and compares each to the existing database of amino acid sequences and returns the closest matches. All of the clone sequences returned the previously published ammonia-oxidizer sequences (4) as the most closely related sequences in the database. Subsequently, the nucleotide sequences were examined for deduced amino acid sequence. Comparison of the deduced amino acid sequences (Table

3.2) showed a 92.7% to 100% identity of the clones to one another. Similarly, the *N. marina*, NO3W, URW, C-45, C-113a cluster of NirK-containing AOB share an amino acid sequence identity of 90.0% - 99.4%. The amino acid sequence identity between the AOBNirK clones and the *N. marina* NirK cluster is 80.2% to 85.1%.

In contrast, the amino acid sequence identity between the clones and the denitrifier NirK sequences was no greater than 65.3%. Similarly, the *N. marina* cluster shared an amino acid identity of no more than 63.3% with the denitrifier NirK sequences. Due to the existence of 100% amino acid homology among four of the AOBNirK clones (AOBNirK 07, 16, 26 and 30), a comparison of nucleotide sequences among the clones was also conducted (Table 3.3). The AOBNirK clone nucleotide sequences range in homology from 84.2% to 99.3%.

Following amino acid and nucleotide sequence analysis, phylogenetic trees for each were constructed. Due to the amino acid identity of some of the isolates in the *N.marina* cluster to one another and the identity between the amino acid sequences of the clones, a nucleotide sequence tree was selected as the most informative (Figure 3.2).

Discussion

Phylogenetic analysis of the AOBNirK clone sequences revealed that they are closely related to one another and are highly homologous with known ammonia-oxidizer NirK sequences. The nucleotide sequences retrieved from the wastewater treatment plant activated sludge for AOBNirK clones 07 and 30 were 99.3% similar. With a length of

Table 3.2. NirK amino acid identities for activated sludge clones and selected isolates.

NirK amino acid sequences were compared between the bacterial NirK sequences used for phylogenetic comparisons. The length of the sequence is 100 residues. The outgroup was *Neisseria gonorrhoeae* Pan 1, an anaerobically induced outer membrane protein. It was used as the outgroup by Casciotti and Ward, AEM, 2001. The heterotrophic denitrifiers were *Rhizobium melitoti, Hyphomicrobium zavarzinii, Ochrobactrum anthropi, Bradyrhizobium japonicum* and *Rhodobacter sphaeroides.* The ammonia-oxidizing isolates whose NirK nucleotide sequences the AOBNirK primers target are referred to as AOB. Amino acid sequence identities are shown in the table.

Table 3.2. NirK Amino Acid Identities for Activated Sludge Clones and Selected Isolates.

Sequence	AOBNirK 16	AOBNirK 30	AOBNirK 06	AOBNirK 07	AOBNirK 11	AOBNirK 26
TA-921i-NH4	59.4	59.4	60.4	59.4	60.4	59.4
R. sphaeroides	65.4	65.4	64.4	65.3	64.4	65.3
B. japonicum	62.4	62.4	61.4	62.4	61.4	62.4
H. zavarzinii	60.4	60.4	59.4	60.4	60.4	60.4
N. marina	83.2	83.2	83.2	83.2	84.2	83.2
NO3W	82.2	82.2	80.2	82.2	83.2	82.2
URW	82.2	82.2	80.2	82.2	83.2	82.2
C-113a	84.2	84.2	84.2	84.2	85.1	84.2
AOBNirK16	-	100.0	92.7	100.0	99.0	100.0
AOBNirK 30		-	92.7	100.0	99.0	100.0
AOBNirK 06			-	92.7	93.1	92.1
AOBNirK 07				-	99.0	100.0
AOBNirK 11					-	99.0
AOBNirK 26						-

Table 3.3. NirK Nucleotide Identities for Activated Sludge Clones.

Due to the fact that several of the AOBNirK clones showed 100% amino acid sequence identity, the nucleotide identities between the clone sequences was examined. The length of the nucleotide sequences used for comparison is 302 bases. Therefore, a single base pair mismatch represents a difference of 0.3% in identity.

Table 3.3. NirK Nucleotide Identities for Activated Sludge Clones

Sequence	AOBNirK 16	AOBNirK 30	AOBNirK 06	AOBNirK 07	AOBNirK 11	AOBNirK 26
AOBNirK16	-					
AOBNirK 30	97.0	-				
AOBNirK 06	84.2	84.2	-			
AOBNirK 07	97.7	99.3	84.2	-		
AOBNirK 11	93.1	94.7	85.1	94.7	-	
AOBNirK 26	96.1	96.0	85.5	96.7	94.7	-

Table 3.4. Amino acid identity summary

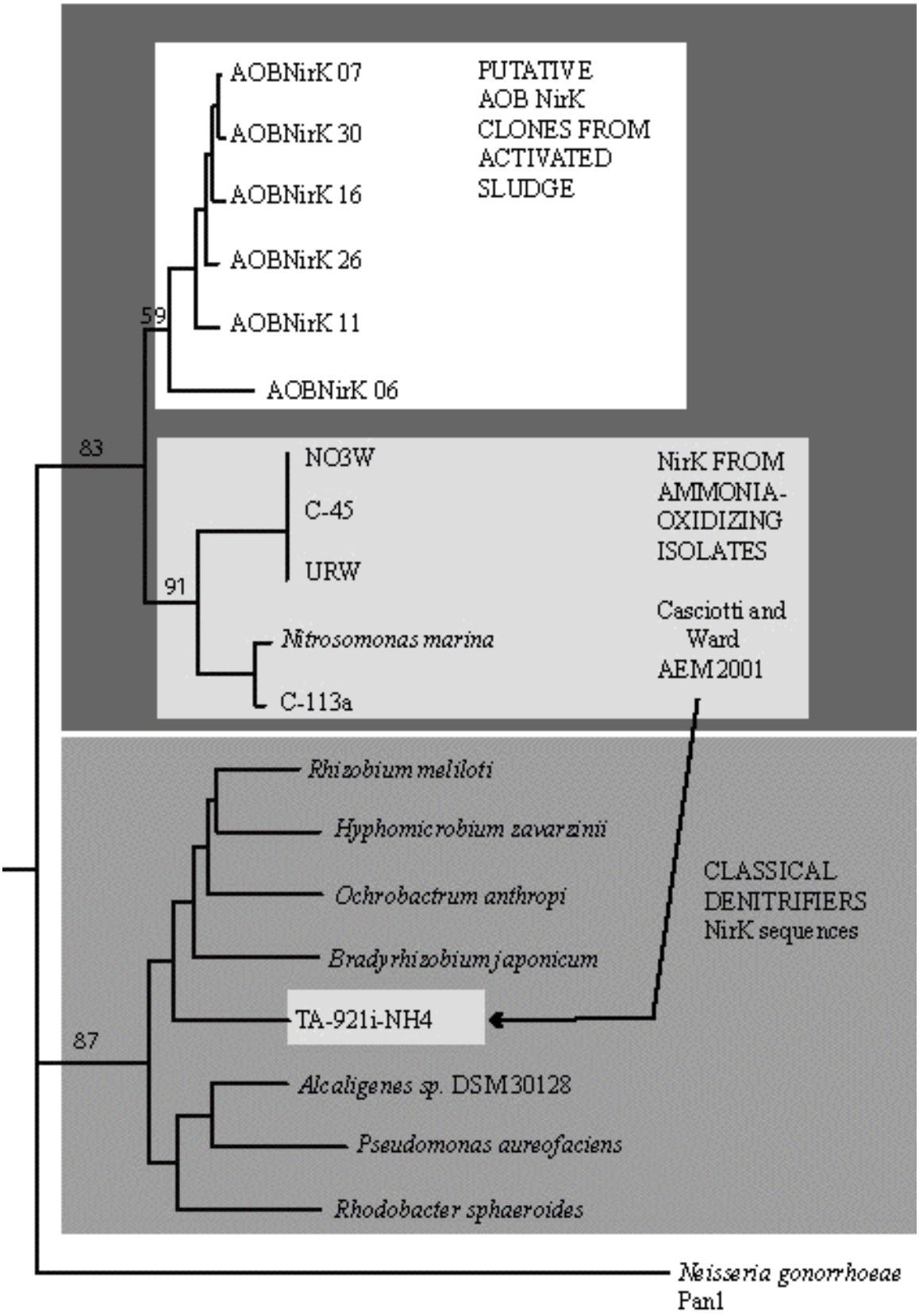
NirK amino acid sequences were compared between the bacterial NirK sequences used for phylogenetic comparisons. The outgroup was *Neisseria gonorrhoeae* Pan 1, an anaerobically induced outer membrane protein. It is used as the outgroup by Casciotti and Ward, AEM, 2001. The heterotrophic denitrifiers were *Rhizobium melitoti*, *Hyphomicrobium zavarzinii*, *Ochrobactrum anthropi*, *Bradyrhizobium japonicum* and *Rhodobacter sphaeroides.* The ammonia-oxidizing isolates whose NirK nucleotide sequences the AOBNirK primers target are referred to as AOB. Amino acid sequence identity ranges between each group are shown in the table. The darker shaded cell on the table indicates the amino acid identity range between the AOB and the AOBNirK clones.

Table 3.4. Amino acid identity summary

	Denitrifiers	AOB isolates	AOBNirK Clones
outgroup	30.5 - 38.9	38.6 - 40.5	42.9 - 46.7
Denitrifiers	63.4 - 71.5	55.6- 63.3	59.4 - 65.3
AOB isolates		90.0 - 99.4	80.2 - 85.1
AOBNirK Clones			92.7 - 100.0

Figure 3.2. Neighbor-joining tree of putative AOBNirK nucleotide sequences. Bootstrap values are shown for significant nodes and are out of 100 replicates.

Figure 3-2. Phylogenetic tree of putative AOBNirK nucleotide sequences.



more than 300 base pairs, this constitutes only 2 base mismatches, which could be explained by cloning or sequencing error. All other clones have nucleotide identities of no more than 97.7%, which is distant enough to be considered a unique sequence. For example, clone AOBNirK 11 and AOBNirK 16 are 99.0% similar at the amino acid sequence level, or 1 mismatch over the entire length sequenced. At the nucleotide sequence level, however, the two are 93.1% similar. Over a length 300 bases, this amounts to approximately 20 mismatches. Clearly, the two clone sequences are not identical, but closely related.

When considering the NirK sequences retrieved from the ammonia-oxidizing bacterial isolates of Casciotti and Ward (AEM, 2001), it is interesting to note that the amino acid sequence from *Nitrosomonas marina* over the 100 residues is only 90.0% similar to NO3W and C-45. Similarly, the amino acid sequence identity for AOBNirK 11 and *N. marina* is 84.2%. Also, the C-113a and AOBNirK 11 sequences share 85.2% identity. So, even though the ammonia-oxidizer isolate NirK sequences are more similar to one another than to the AOBNirK clone sequences, the two groups are clearly closely related.

For nitrite reductase enzymes, amino acid similarities of >50% indicate that the enzymes are homologs (23). In this investigation, the partial NirK amino acid sequences for the known denitrifiers are between 55.6% and 65.3% homologous to those for the *N. marina* ammonia-oxidizing isolates and cloned sequences. Further, the *N. marina*-cluster isolates have been shown to have a functional nitrite reductase (21). Therefore, a sequence with an amino acid homology of >80% to one of these known functional

enzymes - as are all of the AOBNirK clones sequences - would almost assuredly code for a copper-containing nitrite reductase.

Although it may be reasonable to accept that the cloned DNA sequences obtained from the activated sludge code for nitrite reductase enzymes, perhaps even functional enzymes, the larger question involves the taxonomy of the bacteria that contain these genes. Although the amino acid sequences are highly homologous, establishing that the source of these sequences are ammonia-oxidizing bacteria and not some previously unknown heterotrophic denitrifier is not possible at this time. However, there is some evidence to support the assertion that these sequences come from ammonia-oxidizers.

Figure 3.3 is taken directly from Casciotti and Ward (AEM, 2001). The figure consists of three phylogenetic trees that, taken together, demonstrate a trait of ammonia-oxidizing bacteria – the lack of lateral gene transfer. That is to say, ammonia-oxidizers have not been conclusively shown to incorporate exogenous DNA into their genomes (7), perhaps due to the fact that they are obligate chemolithoautotrophs. Unlike denitrifying bacteria, which can be distantly related taxonomically, yet have highly homologous functional genes, presumably through lateral transfer, ammonia-oxidizing bacterial functional relatedness appears to mirror taxonomy.

The three trees in Figure 3.3 are labelled: A, for the NirK tree, B, for the amoA tree (amoA is the gene that codes for the functional subunit of the ammonium monooxygenase enzyme), and C, for the 16S rRNA taxonomic tree. In tree C of Figure 3.3, the 16S tree for several well-characterized ammonia-oxidizers as well as the isolates are shown. Notice that the isolate TA-921i-NH4 16S rRNA gene sequence falls outside the *N. marina* cluster. The same pattern is repeated in each of the three trees. In fact, the

trees have identical topology. This topological identity is in agreement with the assertion that ammonia-oxidizing bacteria do not add genes through lateral transfer (3, 9). Comparing this observation with the fact that the cluster of AOB isolates shares a common ancestral node with the AOBNirK sequences in the phylogenetic tree (Figure 3.2), one can clearly see that the cloned DNA sequences are a sister cluster of the *N. marina* NirK cluster shown in Figure 3.3A. Additionally, the amino acid identities between the cloned sequences and the *N. marina*-cluster isolates range from 80.2% to 85.2% while the *N. marina* cluster sequences are 90% - 99.4% identical. It must be noted that the NirK sequence for isolate TA-921i-NH4 obviously falls within the known denitrifier cluster and raises questions about the validity of this argument.

The environment in which these organisms originated may partly explain the reason that the clone sequences form a distinct cluster, albeit closely related, from the ammonia-oxidizer isolates. The salinity in the wastewater treatment plant is 0-2 parts per thousand (ppt) and the isolates are all marine strains. Therefore, it makes sense that in such a vastly different environment the ammonia-oxidizing bacteria present would differ in 16S rRNA gene sequence (13). The NirK sequences might be expected to be closely related, but not identical. Similarly, it is interesting to note that all of the bacteria in the *N. marina* cluster are oceanic (4) and the TA-921i-NH4 isolate is from Chesapeake Bay. Although the salinities may have been the same, certainly the environments differ.

If it is shown that the ammonia-oxidizing bacterial communities of municipal wastewater treatment plants contain functional nitrite reductase enzymes, it would be a very important finding with regard to global nitrogen trace gas emissions. Nitrifier

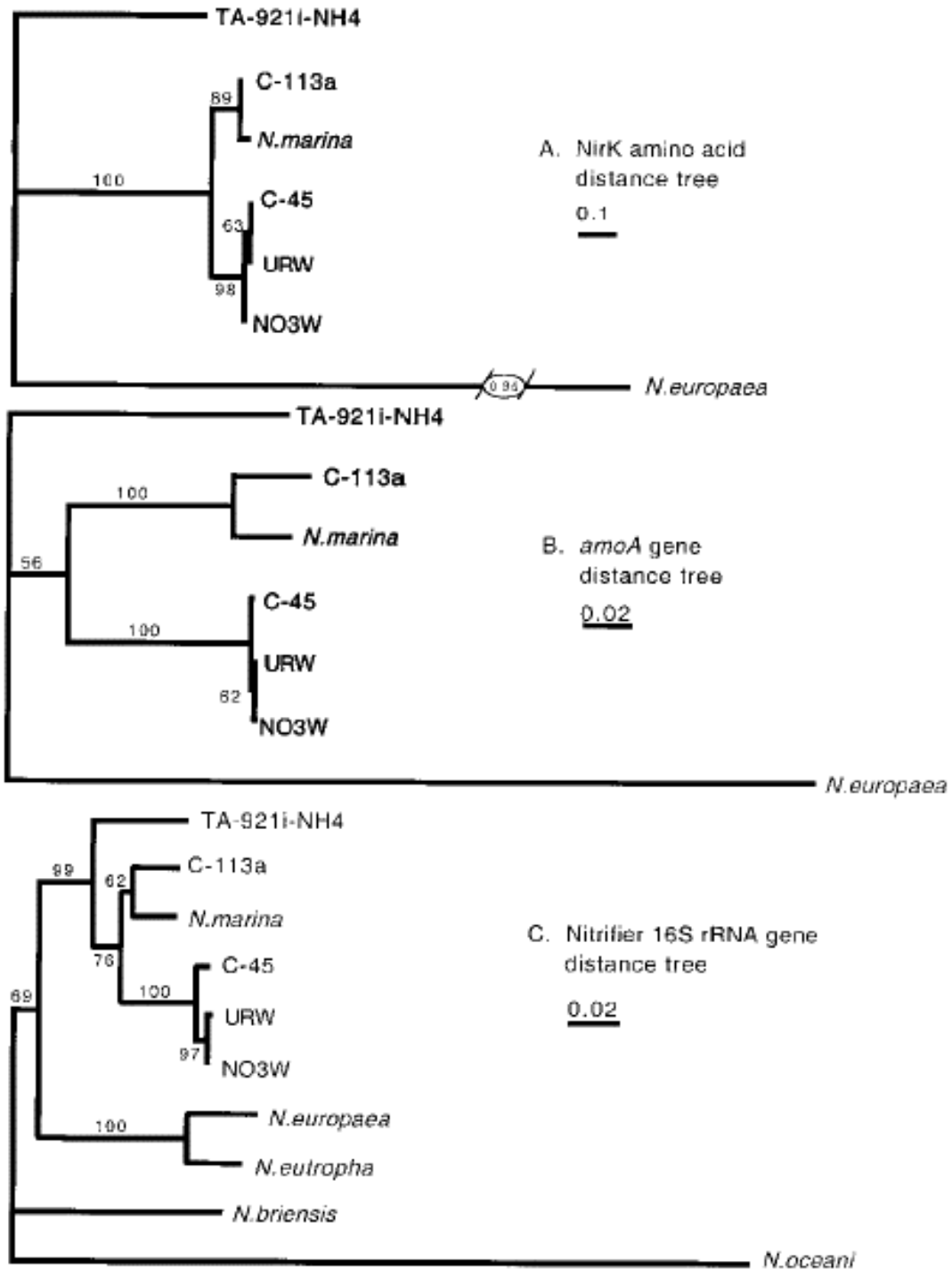
Figure 3.3. From Casciotti and Ward, AEM 2001.

Panel A – Nir K amino acid distance tree.

Panel B – Amo A nucleotide distance tree.

Panel C – Nitrifier 16S rDNA nucleotide distance tree.

Figure 3.3. From Casciotti and Ward 2001



populations in wastewater treatment plants are stable. Additionally, there are a large and growing number of wastewater treatment plants in the developed world. Therefore, the expression of a nitrite reductase gene in ammonia-oxidizing bacteria could contribute significantly to NO and N_2O production. This is especially true since the pathway in nitrifiers seems to lack a nitrous oxide reductase and, hence, the end product of all nitrite reduction in these bacteria is nitric oxide or nitrous oxide.

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CHAPTER 4

THE DEVELOPMENT OF AN IMPROVED PROKARYOTIC *INSITU* PCR PROTOCOL AND ITS APPLICATION IN THE DETECTION OF DENITRIFICATION GENES IN BACTERIA [1]

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[1]Sullivan, J.B. and R.E. Hodson. To be submitted to *Applied and Environmental Microbiology*.

Introduction

A wealth of field data suggests that nitrogen is the principal limiting nutrient for primary production in many marine ecosystems (10, 15). As such, ecosystems evolve complex interactions to retain bioavailable nitrogen and limit its loss from the system (14). Conversely, excessive input of allochthonous fixed nitrogen also can cause problems including, at its extreme, riverine and coastal eutrophication (20, 21, 24). Eutrophication in turn can result in hypoxic or anoxic events that adversely affect essential habitat for fish. The wastewater treatment industry exists, in part, to ameliorate the problem of delivering excess nitrogen to aquatic systems. Given the human and environmental impacts that are associated with aquatic nitrogen, it is imperative to understand the organisms that influence its biological availability.

Reduction of oxidized forms of nitrogen within the global nitrogen cycle is caused, biologically, only by microbial denitrification. Denitrification is an alternate respiratory pathway possessed by a wide variety of facultative anaerobes. Heterotrophic bacteria that possess this capability are able to use oxidized nitrogen species in place of molecular oxygen as terminal electron acceptors in the respiration of exogenous carbon compounds (26). Surprisingly, it has also been shown that some strains of marine chemolithotrophic ammonia oxidizing bacteria are capable of conducting dissimilatory reduction of fixed nitrogen (4). Recently, DNA sequences coding for enzymes in the denitrification pathway that are highly similar to those in the ammonia oxidizers were found to exist in a freshwater municipal wastewater treatment plant (see Chapter 3).

The enzymes involved in the process of denitrification include nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase. These enzymes enable denitrifiers to respire anaerobically using nitrate, nitrite, nitric oxide, and nitrous oxide, respectively, as terminal electron acceptors. Nitrite reductase is especially important in global nitrogen cycling in that it converts nitrite to nitric oxide, the first of the gaseous

products in the pathway. This is the point at which biologically available, or fixed, nitrogen is generally considered to be lost from a system (28).

There are two well-characterized forms of respiratory nitrite reductase, one containing iron in a cytochrome cd1-heme structure and the other containing two copper molecules at the enzyme's active site. The gene that codes for the iron-containing moiety is nirS, whereas the copper enzyme is coded for by nirK. The two enzymes have identical function, both reduce nitrite to nitric oxide. However, these two gene products have no more similarity to one another than do other non-homologous proteins and have never been found to both be present within a single species (29). Due to the large body of work that had previously been conducted on this gene, NirS, the structural gene for the cd1-heme-type nitrite reductase, was selected for the present study of the distribution of specific enzymes in bacterial communities (7, 12, 18, 19, 27, 30).

When asking questions about the structure and function of bacterial communities, microbial ecologists encounter difficulties due to the limitations of the available methods. The development of prokaryotic *in situ* PCR, or PI-PCR, which allows for the amplification of single- or low-copy number genes inside individual bacterial cells, promises to be a potentially powerful tool for describing bacterial communities at the single cell scale (11). Difficulties remain in interpretation of results generated by this approach, but the list of genes and gene expression products (mRNA) that can be detected *in situ* using this approach is growing. In the past several years, gene occurrence and expression has been investigated using various protocols for PI-PCR (6, 11, 16, 22). The diagram of the modified PI-PCR protocol described below is useful for understanding this approach, as only the detection step varies from other protocols (Figure 4.1).

Before discussing the state of current PI-PCR protocols, it is important to note that in utilizing each of the methods discussed below the investigators were able to overcome methodological difficulties for the specific gene in that study. Diligent experimental optimization notwithstanding, interpretational difficulties in data interpretation remain. It is

the intent of this investigation to address the potential pitfalls in the extant methods by first reviewing the theory behind each and experimentally addressing perceived weaknesses.

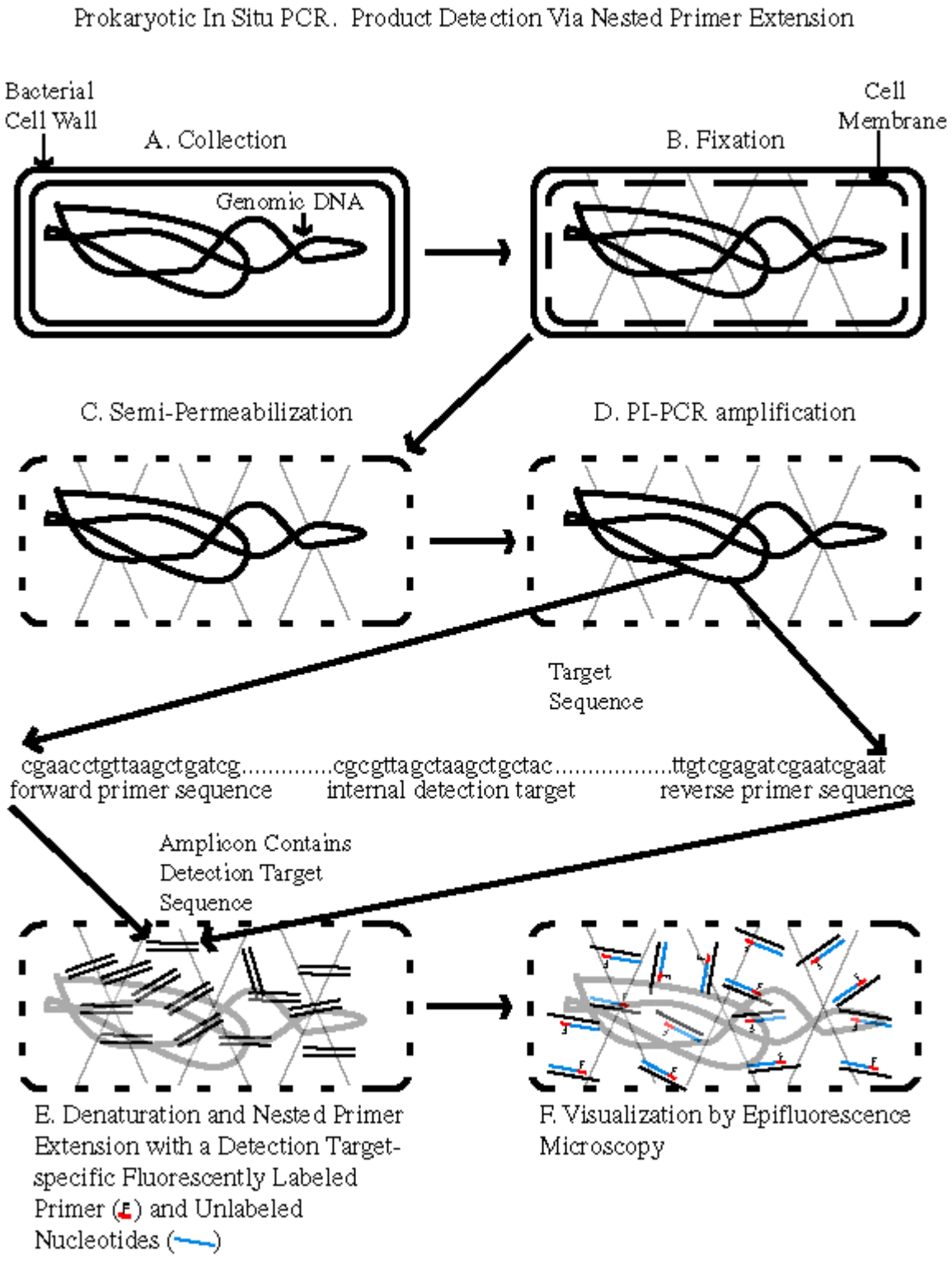
Some PI-PCR methods employ direct detection of the PCR products. These protocols call for the use of fluorescently-labeled or digoxygenin-labeled nucleotides in the elongation stage of the PCR reaction or for the incorporation of labeled primers in the amplicon. Both types of methods may encounter the problem of nonspecifically primed PCR reactions, which produce spurious PCR products. During the inclusion of the fluorescent reporter molecules, unintended products are labeled and visually (microscopically) appear identical to the properly primed and amplified target amplicons when viewed under epifluorescence microscopy. Incorporation of digoxygenin-labeled nucleotides or primers can present similar difficulties in interpreting results for similar reasons.

Indirect PI-PCR methods also have been developed in which sequences are amplified and a second step is added to detect target amplicons within bacterial cells. Potential problems with this approach are different, but no less significant. Post-amplification annealing of a labeled probe to a previously unlabelled amplicon via *in situ* hybridization is one method of indirect detection (22). Unlike FISH (fluorescent *in situ* hybridization), which uses single-stranded ribosomal RNA as a target site for a fluorescently labeled probe, the bacteria subjected to PI-PCR must undergo a pre-hybridization denaturation step to make the target site accessible to the probe since PCR products are double-stranded. After denaturation, all of the DNA and RNA in the cell is single-stranded and capable of binding as the double stranded DNA “seeks” to re-anneal (23). This fact coupled with the length of time that the labeled probe is in contact with the cells (typically from 2-16 hours) can lead to false positive results due to non-specific hybridization of the probe. Raising the wash temperature and/or time and lowering wash salt concentration can increase the level of stringency and remove improperly bound probe.

Figure 4.1. Conceptual diagram of PI-PCR with nested primer extension for detection.

A. Sample collection. Typically, water samples are collected and stored on ice until fixation, optimally within a few hours.
B. Fixation. The addition of paraformaldehyde in phosphate buffered saline to a final concentration of 4% causes the cell membrane to be compromised, but also serves to strengthen the cell wall by crosslinking embedded proteins. Fixation is time-limited as excessive crosslinking can make semipermeablization difficult.
C. Semipermeablization. Lysozyme weakens the structure of the peptidoglycan layer in the cell wall. Optimally, the openings are large enough for the DNA polymerase to enter, but do not cause total cellular lysis.
D. PI-PCR. The target sequence is amplified using standard PCR reagents.
E. Denaturation and Nested Primer Extension. Target sequence-containing amplicons are denatured and labeled primers specific to the target site are bound and extended using DNA polymerase and unlabeled nucleotides.
F. Visualization. Counterstaining with DAPI or another DNA specific dye allows for visualization using an Epifluorescent microscope equipped with the proper filters for excitation and viewing of dyes and fluorophores.

Figure 4.1.


Prokaryotic In Situ PCR. Product Detection Via Nested Primer Extension
Bacterial Cell Wall
A. Collection
Genomic DNA
B. Fixation
Cell Membrane
C. Semi-Permeabilization
D. PI-PCR amplification
Target Sequence
cgaacctgttaagctgatcg.....cgcgttagctaagctgctac.....ttgtcgagatcgaatcgaat
forward primer sequence
internal detection target
reverse primer sequence
Amplicon Contains Detection Target Sequence
E. Denaturation and Nested Primer Extension with a Detection Target-specific Fluorescently Labeled Primer (F) and Unlabeled Nucleotides (—)
F. Visualization by Epifluorescence Microscopy

It is important to remember, however, that the higher the wash stringency, the greater the chance of losing correctly labeled positive signal.

Another indirect method of detection utilizes labeled nucleotides in an arithmetic amplification with a single primer that is internal to the original amplicon (10). As discussed above, the incorporation of labeled nucleotides can lead to false positives. If the first round of unlabeled PI-PCR failed in a cell, then the false priming during the detection step would most likely occur on genomic DNA or large (several Kb) genomic fragments. The product of incorrect primer extension could be hundreds of times longer than the target amplicons, each incorporating hundreds of labels. For example, a PCR mix that used 35% labeled dUTP, 65% unlabeled dTTP and equal concentrations dATP, dCTP and dGTP would place a label on average once every 12 bases (25% chance of dTTP X 35% chance of labeled dUTP = 0.0875 = 1/12). Over the length of a 6000 Kb fragement, roughly 500 labels would be incorporated. Since DNA polymerase can produce DNA at 1,000 bases per second, it is possible to have large spurious products.

The modified PI-PCR method described herein attempts to address and eliminate the difficulties in interpretation of results of previous PI-PCR methods. In order to minimize the occurrence of false positive signals, several factors were considered. First, it was reasoned that an indirect method of detection should result in a lower incidence of false positive results. The logic behind this is as follows: by amplifying the target DNA sequence with unlabeled nucleotides and primers one does not generate direct false positives (Figure 4.2). Following an adequately controlled detection step of PI-PCR, a bacterial cell containing an unlabeled non-target amplicon is indistinguishable from a cell with no amplicon. The probability of an internal, labeled probe matching the DNA sequence of a spurious PCR product closely enough to remain bound after washing is very low. That is to say, it is possible that there exists a portion of DNA sequence in the genome of a non-target organism that matches the probe sequence moderately well (Figure 4.2 panel B). However, the non-target genomic DNA would also need to contain a close

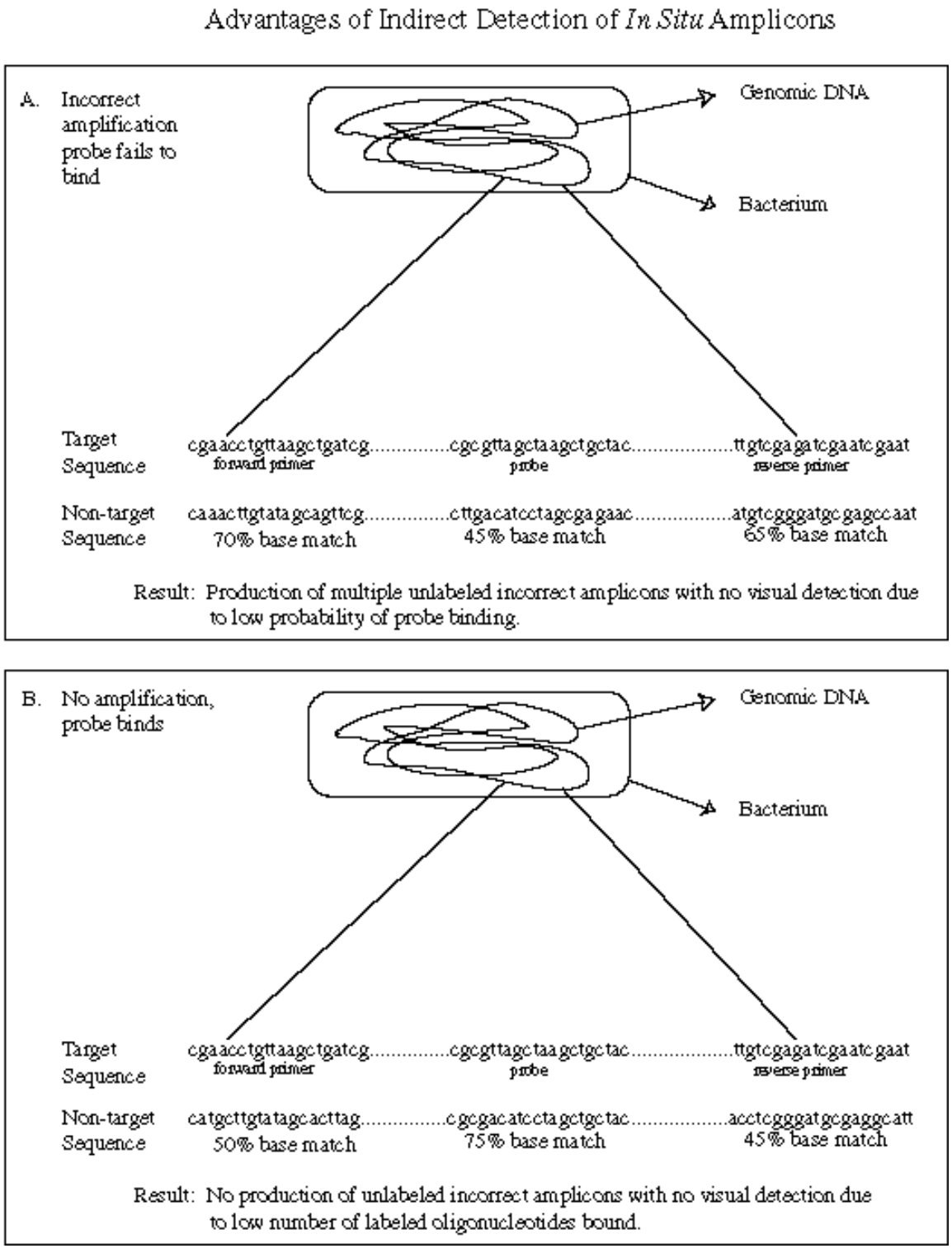
Figure 4.2. Graphical explanation of potential outcomes from the incorrect binding of primers or probes as applied in PI-PCR with indirect detection of *in situ* amplicon.

Panel A – If spurious PCR products result from PI-PCR, they will not be detected if the non-target amplicon does not contain a sequence that shares moderately high sequence homology with the labeled detection probe/primer. In direct detection protocols using labeled primers or nucleotides, the incorrect amplicon will appear identical to the correct signals and a false positive will result.

Panel B. There are no incorrect PCR products formed during the amplification step of indirect PI-PCR. However, there is a non-target sequence with high sequence similarity to the labeled detection probe/primer in the bacterial genome. The incorrect annealing and elongation of the detector molecule will not result in a positive signal due to the low copy number of the incorrect target. It should be noted that this is scenario would not produce a false positive in direct detection protocols either.

A third scenario is possible in which a spurious PCR product contains a sequence of high homology to the detection molecule. The result will be a false positive, even with indirect detection. This scenario is considered to be much less likely than events depicted in Panel A or Panel B.

Figure 4.2.


Advantages of Indirect Detection of *In Situ* Amplicons
A. Incorrect amplification probe fails to bind
Genomic DNA
Bacterium
Target Sequence
cgaacctgttaagctgatcg.....cgcgttagctaagctgctac.....ttgtcgagatcgaatcgaat
forward primer
probe
reverse primer
Non-target Sequence
caaacttgtatagcagttcg.....cttgacatcctagcgagaac.....atgtcgggatgcgagccaat
70% base match
45% base match
65% base match
Result: Production of multiple unlabeled incorrect amplicons with no visual detection due to low probability of probe binding.
B. No amplification, probe binds
Genomic DNA
Bacterium
Target Sequence
cgaacctgttaagctgatcg.....cgcgttagctaagctgctac.....ttgtcgagatcgaatcgaat
forward primer
probe
reverse primer
Non-target Sequence
catgcttgtatagcacttag.....cgcgacatcctagctgctac.....acctcgggatgcgaggcatt
50% base match
75% base match
45% base match
Result: No production of unlabeled incorrect amplicons with no visual detection due to low number of labeled oligonucleotides bound.

match with *each* of the two primers used in the initial amplification reaction. Moreover, the primer-matching DNA regions would have to occur in the correct orientation on either side of the probe-matching sequence in order to amplify the non-target sequence (Figure 4.2 panel A). Second, the time that a hybridization probe is in contact with a cell should be minimized so as to lessen the chance of non-specifically binding to DNA, cellular proteins, etc. Third, being able to dramatically increase the stringency of the post-hybridization wash procedure would further reduce false positive signals. This must be done is such a way so that true positive signals are not lost.

In this report we apply a nested primer extension detection protocol. Briefly, a low cycle number (1 or 2 cycles) primer annealing and extension step using a labeled probe/PCR primer and unlabeled nucleotides is performed. This step follows non-labeled PI- PCR. Nested primer extension addresses each of the experimental difficulties discussed above.

First, as discussed above, the indirect method avoids incorporation of labels in incorrect PI-PCR products. Spurious amplification produces unlabeled, and hence undetected, amplicons. Barring the unlikely event of non-targeted sequence containing an internal sequence with high identity to the detection primer, spurious products will remain undetected.

Second, the low cycle number of primer extension greatly decreases the time that the labeled probe is in contact with the cell. In an *in vitro* PCR reaction, one assumes that the primers in the reaction mix will bind to the correct target DNA sequence in a matter of seconds, not hours as in a hybridization reaction. The positive results achieved routinely using *in vitro* PCR attest to the veracity of this assumption. Using nested primer extension for detection in PI-PCR is based on this same assumption. One or two cycles of PI-PCR is run using the cells previously subjected to 20-25 cycles of unlabelled PI-PCR as the template. The reaction mix for the nested primer extension contains only one primer that is

labeled at the 5' end, so as not to interfere with the PCR reaction. The total time that the labeled probe/primer is in contact with the cells is usually less than 20 minutes.

An additional advantage of primer extension detection is that it serves to anchor the probe to the correct target so that the stringency of the wash can be dramatically increased if necessary. Since the reaction mix includes DNA polymerase and nucleotides as well as a single labeled primer, the primer binds to the target site and is extended to the terminus of the PCR product on which it is bound. This extension of the primer serves to greatly increase the dissociation temperature of the labeled product. For instance, an oligonucleotide of 20 bases in length with a 50% G+C content has a Tm or melting temperature of ~50°C whereas an extended primer of only 150 bases has a Tm approaching 90°C. The primers used for extension in this study result in products ranging from 140 bases to over 650 bases and the primer extension products should have correspondingly higher Tm values. Although this step would also anchor any incorrectly bound probe, the incidence of such binding should be extremely low in comparison with the number of correctly bound labels in a cell containing the correct PCR product. This is especially true since the use of only one primer and very low cycle number eliminates the chance for an exponential increase in false signal.

We applied nested primer extension to laboratory cultures of known denitrifying bacterial species and to samples of denitrifying activated sludge flocs from a municipal wastewater treatment plant in Athens, Georgia, USA. The bacterial genes coding for the structural portion of the iron-containing nitrite reductase (*nirS*) and for nitrous oxide reductase (*nosZ*) in heterotrophic bacteria were detected using this method, as were the genes coding for the copper containing nitrite reductase (*nirK*) in putative ammonia oxidizing chemolithotrophic bacteria.

Finally, in order to begin to address the question of taxonomy and function in a single bacterial cell, initial attempts have been made at dual labeling specific bacteria.

Utilizing the nested primer extension concept, PI-PCR targeting the nitrous oxide reductase gene was followed dual primer extension step. One probe/primer targeted the *nosZ* amplicon in a nested extension while the other targeted 23S rRNA directly and was specific for *Pseudomonas sp*. *Pseudomonas sp*. were targeted because many of the positive control species in our laboratory experiment were of this genus.

Materials and Methods

Bacterial Cultures. Known denitrifying bacterial species *Pseudomonas stutzeri* (ATCC 14405), *Pseudomonas aureofaciens* (ATCC 13985) and *Paracoccus denitrificans* (ATCC 19367) were obtained from the American Type Culture Collection (Manassas, VA). *Pseudomonas stutzeri* and *Paracoccus denitrificans* were grown in Marine Broth 2216 (Difco) and *Pseudomonas aureofaciens* was grown in Nutrient Broth (Difco) for use in the experiments. Isolates GAI-037 (9), EE36, Y4I, ZAH511, O16, O21, E37 and *Sulfitobacter pontiacus* were supplied by Dr. Mary Ann Moran, University of Georgia, and were also grown in Marine Broth. All cell cultures were grown at 25°C and shaken at a rate of 100 rpm.

Cell Fixation. Cultures were resuspended per ATCC instructions in the appropriate media and grown overnight in test tubes as described above. The next day, a 0.1 ml subsample was spread on a plate of the same media with 15 g/L agar added. Following 12 hours of growth, single colonies were used to inoculate 10 ml of autoclaved media in test tubes. Cells were grown overnight as above. One ml each culture was placed into each of four 1.5 ml microcentrifuge tubes. The tubes were centrifuged at 10,000 x*g* for 5 minutes. The supernatant was removed and two microcentrifuge tubes per culture were set aside for DNA extraction. Additionally, four 1.0 ml samples of activated sludge wastewater

collected on December 9, 2001 from Wastewater Treatment Plant #2, Athens, GA were prepared in the same manner. Cell pellets in the tubes for fixation were rinsed by resuspension in Phosphate Buffered Saline (PBS – 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2PO_4, and 0.24 g of KH_2PO_4 per liter) and centrifuged under the same conditions in order to remove remaining media/wastewater. Following removal of the PBS supernatant, the cells were fixed by resuspension in 4% paraformaldehyde in PBS and placed on ice for 2 hours. After fixation, cells were again pelleted at 10,000 x*g*. The paraformaldehyde was removed. Cell pellets were rinsed twice in PBS following the same steps as above and were stored at -20°C in a 50/50 mix of PBS and ethanol.

DNA extraction. The cell pellets from the two microcentrifuge tubes per culture/isolate/sample prepared as described above were washed twice with Tris-EDTA buffer (pH 8) by repeated resuspension and centrifugation. Total genomic DNA was extracted following the protocol described in Short Protocols for Molecular Biology (3). Briefly, pellets were resuspended in a Tris-EDTA buffer and lysed with 10% SDS and ProteinaseK while heating. Cellular debris was further broken down for removal by treatment with high NaCl concentrations and CTAB. DNA was isolated by repeated phenol/chloroform/isoamyl alcohol and phenol/chloroform treatments. Purified DNA was precipitated with chilled ethanol at 4°C overnight, centrifuged at 15,000 rpm for 15 minutes, washed with 70% ethanol, and resuspended in biograde dH_2O. The purified DNA was stored at -20°C.

PCR primers and probes. See Table 4.1 for a list of primers and probes used in this study.

NirS cd-1 heme nitrite reductase. PCR primers were developed from existing NirS sequences in GenBank by aligning the available NirS DNA sequences. The species used

Table 4.1. PCR primers/probes that were used in this study. The source of the oligonucleotide is noted beneath the table. All primers/probes are designated ‘F’ for a forward primer or ‘R’ for a reverse primer. Except for the *Pseudomonas* group specific primers, all numbering begins at the first base in the coding sequences of the gene in a model organism that was included in the primer design.

Table 4.1. Primers and Probes

Target/ Primer Name	Sequence	Tm(°C)
NirS[a]		
NirS1045aF[1]	5'CCTA(CT)TGGCCGCC(AG)CA(AG)T(AT)C	60
NirS1137F[1]	CY3*5'TACCACCCCGAGCCGCGCGT	70
NirS1795R[1]	5'CCTTGTTGTACTCGGCCTGC	64
Putative AOBNirK[b]		
AOBNirK571F[2]	5'CCATATC(AG)(AC)TATGACCG(CT)GC	60
AOBNirK873R[2]	FITC*5'TGA(AG)CC(AG)CCTACCCAATACA	58
AOBNirK947R[2]	5'TCATA(AC)GC(AT)GCAGC(AG)ACT	54
NosZ[c]		
NosZ1527F[3]	5'CGCTGTTC(ACT)TCGACAC(CT)CA	63
NosZ1647R[1]	TR*5'CACTACCAGCCGTGTCAC	60
NosZ1773R[3]	5'AT(AG)TCGATCA(AG)CTG(CGT)TCGTT	59
Pseudomonas 23S[d]		
Ps23S1F[1]	5'AGGCGATGGGAAACAGGTT	65
Ps23S55F[4]	FITC*5'GCTGGCCTAGCCTTCTCCGT	60
Ps23S196R[1]	5'GAACCAAGGCATCAACCACT	64

[a] - Numbering from *P. stutzeri* – GenBank accession #X56813
[b] - Numbering from NirK *P.aureofaciens* – GenBank accession #Z21945
[c] - Numbering from *P. stutzeri* ZoBell
[d] - Numbering based on expected size of amplicon
[1] – Primers developed in this study
[2] – Primers developed in another study, see Chapter 3 "Molecular Characterization of Putative Ammonia-Oxidizer Nitrite Reductase genes from a Municipal Wastewater Treatment Plant"
[3] – Primers developed by Scala and Kerkhof (17).
[4] – Probe Ps23S55F sequence was taken from Amann et. al. (1).

were *Pseudomonas stutzeri* (GenBank accession # M80653), *Pseudomonas stutzeri* (X56813), *Paracoccus denitrificans* (U05002), *Paracoccus denitrificans* (U75413), *Pseudomonas aureginosa* (X16452) and *Alcaligenes eutrophus* (X91394). Regions of DNA sequence that were conserved among the NirS were selected as potential primer sites. Subsequent BLAST analysis of the selected sequences against known bacterial DNA databases indicated that only NirS sequences matched significantly (>85%). Moreover, the closest non-NirS match of a single primer showed a sequence similarity of less than 70% and all potential complementary primers shared sequence identity with the same non-NirS sequence at no more than 50%. BLAST analysis is available through the National Center for Biotechnology Information, National Institutes of Health and compares an input nucleotide sequence to an existing database of DNA sequences and returns the closest matches.

NosZ nitrous oxide reductase. Primers 1527F and 1773R were taken directly from the work of Scala and Kerkhof (17). Primer 1647R was developed in this study to use as a labeled detection molecule by the same alignment method as described above using available NosZ sequences.

AOBNirK copper nitrite reductase. Primers were developed for use in a previous study. See Chapter 3, "Molecular Characterization of Putative Ammonia-Oxidizer Nitrite Reductase genes from a Municipal Wastewater Treatment Plant."

Pseudomonas group specific primers/probe. Probe sequence was taken directly from Amann et. al. (1). Primers were designed by aligning *Pseudomonas chlororaphis, P. aureofaciens, P. putida, P. fluorescens, P. cichorii, P. stutzeri,* and *P. pseudoalcaligines* 23S rDNA sequences and selecting appropriate regions of similarity

flanking the probe sequence that returned no significant non-pseudomonad BLAST matches.

PI-PCR Methodological Controls. *In vitro* PCR and agarose gel electrophoresis for all lab cultures and DNA extracted from environmental samples for each primer set used were performed. Lab culture PCR products for the following species and genes were sequenced to ensure the correct product was amplified: *Pseudomonas stutzeri* – NirS and *Nitrosomonas europaea* – AOBNirK.

For AOBNirK, since *in vitro* PCR using the environmental DNA produced multiple bands, DNA from all bands was cut out of an agarose gel and tested for reactivity with the fluoroscein-labeled detector molecule through dot blot hybridization. Only the correctly sized band exhibited visually identifiable hybridization with the probe as detected by the use of anti-fluoroscein conjugation and colorimetric detection. Further, the environmental DNA was also cloned and sequenced (see Ch. 3 "Molecular Characterization of Putative Ammonia-Oxidizer Nitrite Reductase genes from a Municipal Wastewater Treatment Plant") to determine whether the correct-sized band contained the target sequence alone.

DNase and RNase treatment was performed in order to use *P. stutzeri* as a negative control during method development. Briefly, RNase-free DNase was applied overnight at 25°C to degrade cellular DNA. After rinsing to remove the DNase mix, the cells were subjected to an RNase treatment of 1 hour at 37°C. Heat inactivation of the RNase and rinsing prepared the cells for use in PI-PCR.

Finally, DNA extraction from fixed cells after PI-PCR in suspension was performed. This was followed by visualization of the PCR product by agarose gel electrophoresis.

PI-PCR Experimental Controls. As a positive control samples of fixed, known positive cells were spotted onto a separate slide well on the same slide. They were then subjected to amplification and detection protocols using aliquots of the same master mixes as those used for the fixed cells in the experimental slide wells.

For negative controls, several different methods were employed. A known negative was amplified and detected using aliquots of the same master mixes as those used for the experimental subject. Also, the experimental subject bacterial mix was subjected to amplification using a reaction mix with no primer. This control addresses the issue of naturally occurring oligonucleotides acting as primers in the amplification reaction. Additionally, the experimental bacterial mix was subjected to amplification using reaction mix with no DNA polymerase. This control addresses the issue of detector molecule binding and visualization occurring with no amplification. Finally, the experimental bacterial mix was subjected to amplification using complete reaction mix. In the detection step of primer extension, however, no labeled primer was added to the reaction mix.

PI-PCR On Slides: Five μl of concentrated, fixed cells were spotted onto slides and oven-dried at 45°C for 3 minutes to adhere the cells to the slide. Semi-permeabilization of the cell membranes was achieved using 1.0 mg/ml lysozyme in PBS for 20 minutes at room temperature. Following the lysozyme treatment, the slides were rinsed with PBS. To encourage the cells to take up the reaction mix, sequential dehydration with 50%, 80% and 98% ethanol was performed. Beginning with the 50% dehydration step, the ethanol mixes were placed on each slide well for 1 minute. After allowing the last dehydration step to dry, a 25 μl Frame Seal Chamber (MJ Research, Inc.) was attached around each well. The frame seal chamber is a double-sided adhesive square piece of tape with the center removed so that it surrounds the well. 25 μl of the appropriate PCR reaction mix was

placed in the well and a plastic cover was carefully placed on top of the frame seal chamber and gently pressed onto the upper adhesive to seal the chamber for PI-PCR.

PI-PCR In Suspension: Twenty μl of concentrated, fixed cells (~10^9 cells per ml) were placed in a 600 μl microcentrifuge tube for each bacterial species tested. The cells were centrifuged for 3 minutes at 10,000 X g and the PBS/ethanol supernatant was removed. To rinse the ethanol from the fixed cells, the pelleted cells were resuspended in PBS and centrifuged again. Following removal of the supernatant, the pellet was resuspended in 50 μl of 1.0 mg/ml lysozyme solution for 20 minutes at room temperature followed by two PBS rinses as described above. The cell pellet was resuspended in PCR mix for amplification.

PCR mix and conditions: The optimized PCR cocktail for PI-PCR consisted of 1.0 μM of each unlabeled primer, 0.25 mM dNTPs, 1.0 μl of Expand High Fidelity DNA polymerase mix (Roche Biochemicals) and 1X manufacturer supplied buffer in a final reaction volume of 25 μl. The thermal protocol was: initial denaturation at 94°C for 5 minutes, followed by 30 cycles with denaturation at 94°C for 1 minute, an annealing temperature appropriate for each primer set for 1 minute and an elongation step of 72°C for 1.5 minutes. A final elongation of 7 minutes at 72°C completed the amplification reaction. For in *vitro* PCR, between 0.2 and 0.5 μM of each primer produced optimal results.

***In Situ* Amplification verification**: Following PI-PCR in suspension as described above, the cells were centrifuged for 5 minutes at 10,000 Xg and the PCR supernatant was removed and retained. The post PI-PCR cell pellet was resuspended in PBS, vortexed, centrifuged as before and the PBS was removed and retained. The DNA remaining inside

the cells was extracted as described above. Subsequently, the 5 μl each of PI-PCR supernatant and extracted DNA were run in a 1.5% agarose gel at 50 V in 1X TAE buffer.

Sequencing. For direct sequencing of pure culture PCR products, the amplicon was purified directly from the PCR reaction mix using the Wizard PCR Product Purification Kit (Promega Corp). After purification and elution in 30 μl of ddH_2O, 1.0 μl was used in a 10 μl BigDye Terminator (Applied Biosystems, Inc.) cycle-sequencing reaction with the appropriate primer forward primer. After removal of unincorporated dideoxy terminators by Sephadex G-50 purification, the labelled PCR product was dried in a Speed Vac and resuspended in 15 μl of HiDi formamide (ABI) and sequenced on an ABI 310 Genetic Analyzer. For the AOBNirK cloned PCR products, the wastewater community DNA was used as the template for *in vitro* PCR. The amplicon was excised and purified from a 1.0% agarose gel using the UltraClean PCR CleanUp DNA Purification Kit (MoBio) following manufacturer's instructions. The purified PCR product was cloned using the Promega pGEMT-easy Kit. Transformants were grown overnight on LB plates with ampicillin 100 μg/ml, IPTG (isopropylthiogalactopyranoside) 0.5 mM and X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) 80 ug/ml at 37°C. Plasmid DNA was extracted from a total of 15 white and pale blue clones using the QiaPrep Spin Miniprep Kit (Qiagen), and digested with EcoRI restriction enzyme in order to assure that all that plasmid inserts were the expected size.

Epifluorescent microscopic visualization: In order to visualize all bacterial cells regardless of positive PI-PCR signal, a double-stranded DNA dye was employed. Accordingly, each slide was counter-stained with DAPI (1 μg/ml for 5 minutes) after the PCR cocktail was removed by PBS rinse. Slides were examined under an epifluorescence

microscope (Olympus BX-40) with a 100X U Plan Oil objective lens (C. Squared Corp., Marietta, CA) under the appropriate light wavelengths to excite the fluorophores used: CY3, DAPI, Fluoroscein, or Texas Red. Images were acquired with a SenSys: 1400 CCD camera (Photometrics, Tuscon, AZ) and processed by Oncor Image Analysis software.

Results

NirS. *In vitro* PCR produced a single band of approximately 750 bp when using the primer set NirS 1045aF and NirS 1795R and *Pseudomonas stutzeri* DNA as the template. A no-DNA control and *Pseudomonas aureofaciens* produced no discernible bands (Figure 4.3). The band from *P. stutzeri* was excised and sequenced. The sequence showed 100% identity with the published *P. stutzeri* sequence, GenBank accession number X56813.

In order to verify that amplification of the target sequence occurred inside semi-permeablized, fixed cells, post-PI-PCR DNA extraction was performed (Figure 4.4). *Pseudomonas stutzeri, Pseudomonas aureofaciens* and a Roseobacter isolate, GAI-037 were all subjected to PI-PCR in suspension with the primers NirS 1045aF and NirS 1795R. A band of the correct size was detected in the supernatant from the PI-PCR reaction and in the extracted DNA from *P. stutzeri* only. There were no visible bands for either *P. aureofaciens* or GAI-037. An aliquot of phosphate buffered saline (PBS) that was used to rinse the cells after removal of the PI-PCR supernatant was also run and no visible PCR product was found. This step was done to ensure that no DNA was in the liquid matrix between cells in the pellet.

The initial PI-PCR studies on slides were run using *Pseudomonas stutzeri* as a positive. GAI-037, an aerobic coastal isolate that has been shown to be capable of substantial dimethyl sulfoniopropionate (DMSP) degradation was chosen as the negative (8). The selection of GAI-037 as the negative was made because it is distantly related phylogenetically to *P. stutzeri* and does not have the ability to denitrify. The cultured cells

Figure 4.3. *In vitro* PCR with the primer set NirS 1045aF/ NirS1795R.

PCR amplification was performed using purified DNA from cultures and the primer set NirS 1045aF and NirS 1795R.

Lane 1 - *P. stutzeri* - a known positive for the iron-containing nitrite reductase, NirS.

Lane 2 – Control – no DNA, ddH_2O used as template

Lane 3 – *P. aureofaciens* has the copper containing nitrite reductase, NirK and is a known negative for NirS.

Figure 4.3. Gel electrophoresis of *In vitro* PCR with NirS primer set.

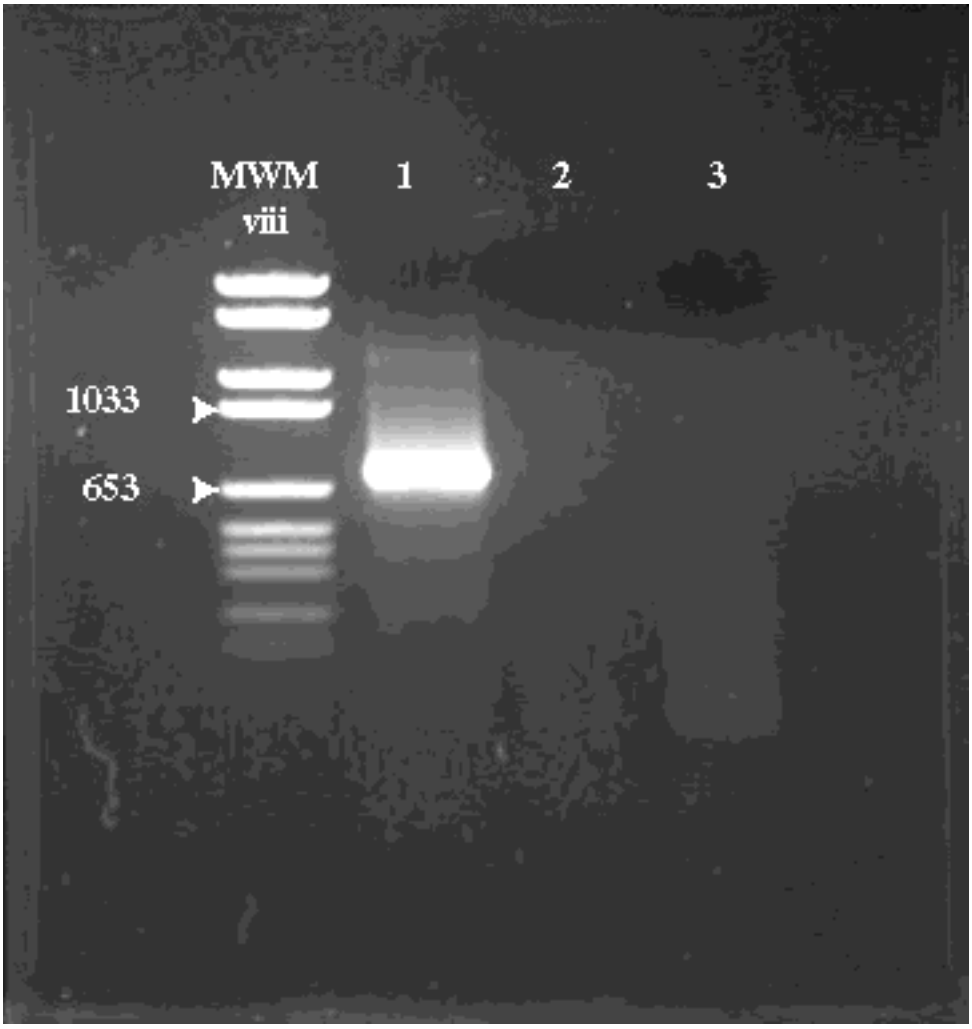
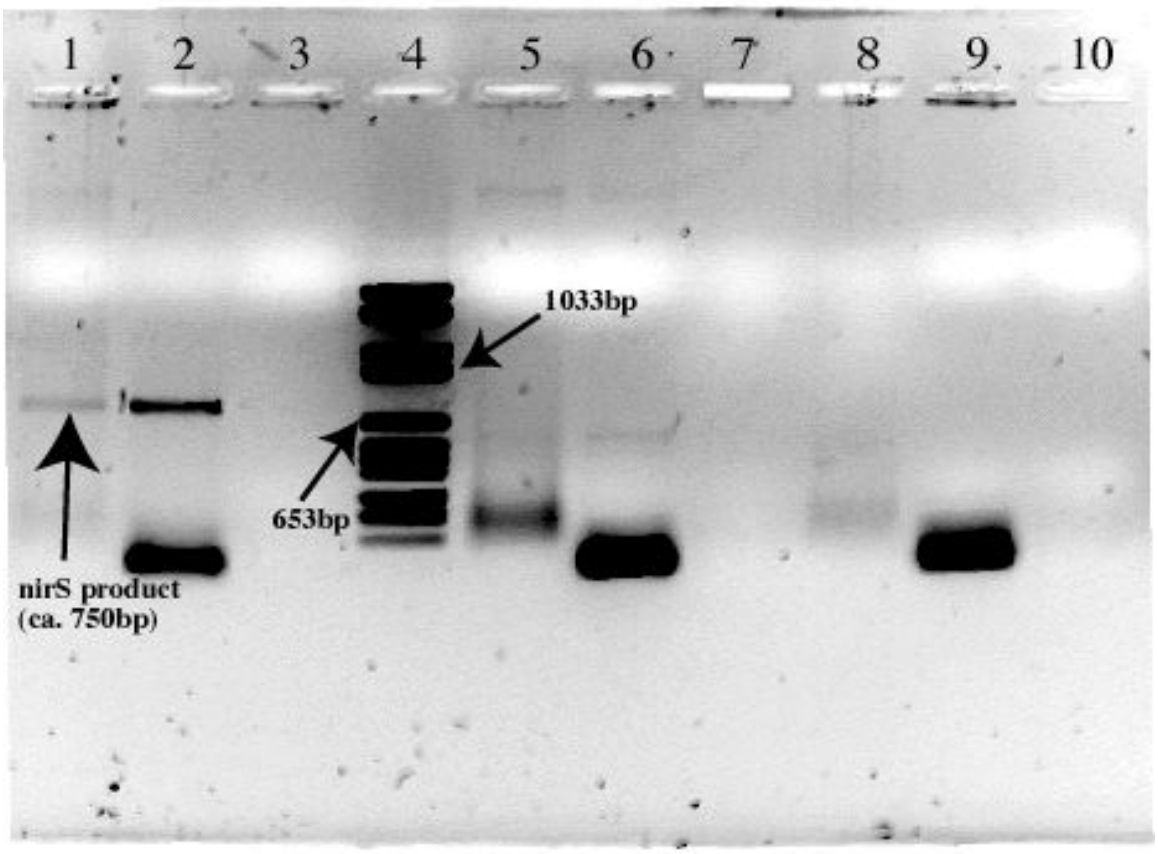


Figure 4.4. Gel electrophoresis following PI-PCR in suspension. Primers were NirS 1045aF and NirS 1795R.

Lane 1 – DNA extracted from *P. stutzeri* cells following PI-PCR in suspension and cell pellet wash. Lane 2 – PCR cocktail supernatant from *P. stutzeri* PI-PCR reaction in suspension. Lane 3 – PBS rinse of *P. stutzeri* cell pellet following removal of PCR cocktail supernatant. Lane 4 – Molecular Weight Marker VI (Roche Biochemicals). Lane 5 – DNA extracted from *P. aureofaciens* cells following PI-PCR in suspension and cell pellet wash. Lane 6 – PCR cocktail supernatant from P. *aureofaciens* PI-PCR reaction in suspension. Lane 7 – PBS rinse of *P. aureofaciens* cells following removal of PCR cocktail supernatant. Lane 8 – DNA extracted from GAI-037 cells following PI-PCR in suspension and cell pellet wash. Lane 9 – PCR cocktail supernatant from GAI-037 PI-PCR in suspension. Lane 10 – PBS rinse of GAI-037 cell pellet following removal of PCR cocktail supernatant.

Figure 4.4. Gel electrophoresis verification of *in situ* amplification of NirS



were fixed separately and mixed to yield equal cell densities prior to being spotted onto the slide and subjected to PI-PCR. Illumination under CY3-exciting light indicated which cells had incorporated detectable levels of labels. The result was a clearly positive signal for one group of cells (presumably, *P. stutzeri*) and a clearly negative for the other (Figure 4.5).

The next step in determining the robustness of the method was to perform PI-PCR on a mix of cells that contained known positives for NirS and closely related cells that were known negatives for the gene. Including negative cells that were known to contain the other form of nitrite reductase, NirK would be useful for discussing the specificity of the protocol. Also, selecting a species of bacteria with morphology distinguishable from *P. stutzeri* would be helpful in determining the veracity of the results. Given these requirements, *Pseudomonas aureofaciens* was selected as an appropriate negative control (Figure 4.6). *P. aureofaciens* cells during exponential growth are short thick rods and *P. stutzeri* cells after exponential growth and brief refrigeration are long rods or chains. The results show the *P. stutzeri* cells as positive (red) and the *P. aureofaciens* as negative.

NosZ. *In vitro* amplification using the NosZ 1527F and NosZ1773R primer set was performed for *Pseudomonas stutzeri, Pseudomonas aureofaciens, Pseudomonas denitrificans* and several coastal isolates (Figure 4.7). A clear band of the correct size was visualized for *P. stutzeri, P. denitrificans,* and isolates Y4I and Y3F. *P. aureofaciens* and ZAH511 produced multiple bands of incorrect size. The remaining isolates yielded negative results. For PI-PCR with the NosZ primer set, four distinct morphological types of bacteria were selected from those tested using *in vitro* PCR. The positive was *P. stutzeri* with a chained rod morphotype. Two known negatives were selected: barbell-shaped O16 and the large, rod Roseobacter, EE36. The short, fat rods of ZAH511 were included in order to determine if, after producing multiple incorrect-sized bands during *in vitro* PCR, a false positive would result following nested primer extension (Figure 4.8).

Figure 4.5. Nitrite Reductase PI-PCR

Panel A – DAPI-stained *Pseudomonas stutzeri* and coastal isolate GAI-37 cells following PI-PCR and nested primer extension for NirS using a CY3-labeled probe. Both species are visible under UV excitation due to DAPI fluorescence.

Panel B – The same microscope field as in Panel A. Visualization of cells that test positive for the NirS gene. Under the appropriate higher wavelength excitation, only the NirS-positive cells (*Pseudomonas stutzeri*, the larger rods) are visible due to CY3 fluorescence. GAI-37 does not denitrify. The primer set used was NirS 1045aF and NirS 1795R. Detection of specific amplification product was accomplished by annealing and extending with unlabelled nucleotides and DNA polymerase a CY3-labeled oligonucleotide, NirS1137P, to the PI-PCR product. A single cycle primer extension was used for detection.

Panel C – A composite image of A and B mimicking what the observer sees microscopically by alternating between DAPI-specific and CY3-specific illumination.

Figure 4.5.

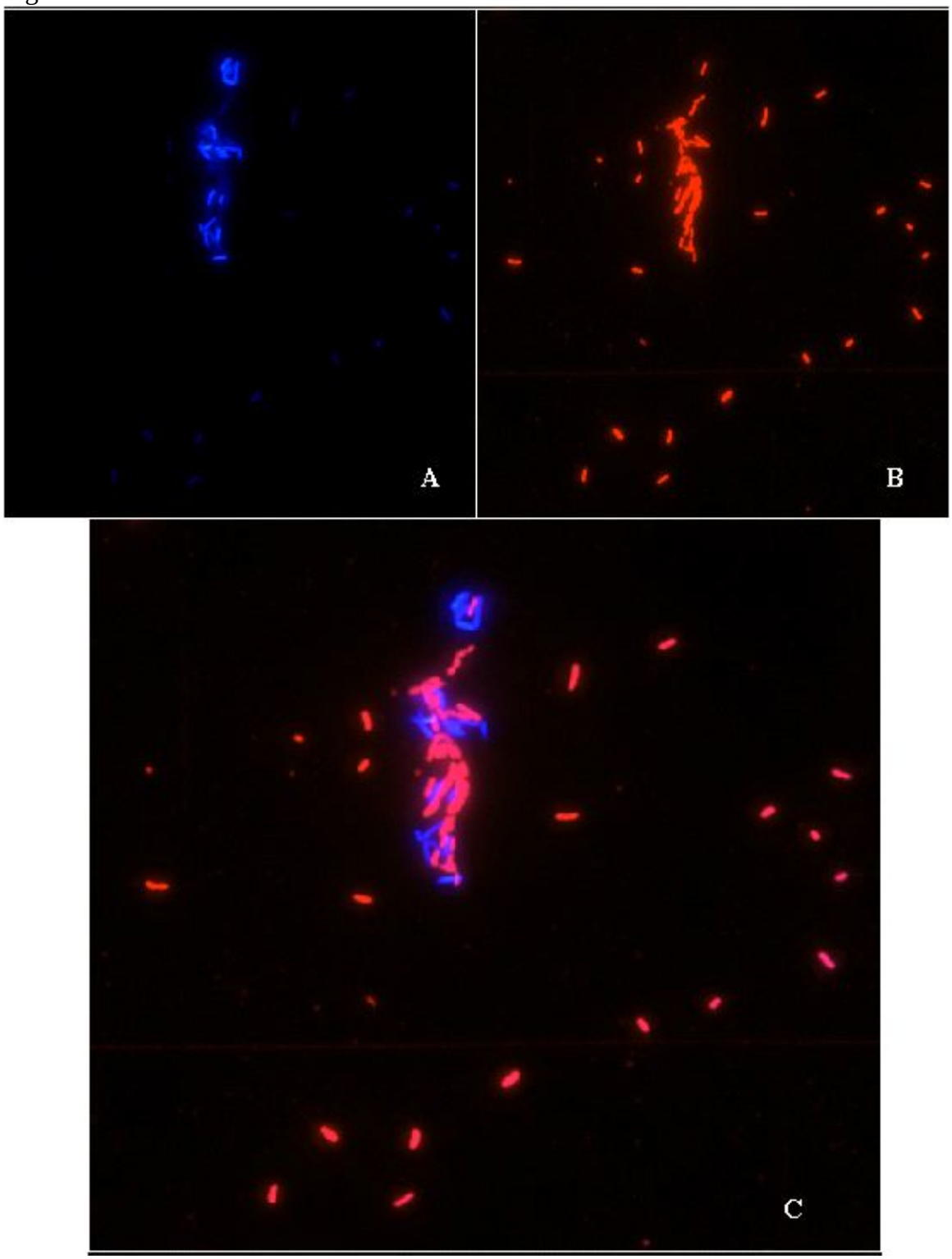


Figure 4.6. NirS Nitrite Reductase PI-PCR.

Panel A – DAPI-stained *Pseudomonas stutzeri* and *Pseudomonas aureofaciens* cells following PI-PCR affixed to a slide and cells were viewed by epifluorescence microscopy under UV excitation so that cells of both species are visible. *P. stutzeri* cells were harvested after they had passed the exponential growth phase and were stored at 4°C for several hours after growth which caused the variation in length. *P. aureofaciens* cells were harvested during exponential growth and the cells were of uniform size and shape. The primer set used was NirS 1045aF and NirS 1795R.

Panel B – The same microscope field as in Panel A viewed under higher wavelength excitation resulting in red fluorescence of cells that test positive for the NirS gene. Detection of specific amplification product was accomplished by annealing and extending a CY3-labeled oligonucleotide, NirS 1137P, to the PI-PCR product. A single cycle primer extension was used for detection.

Panel C – A composite image of A and B.

Figure 4.6.

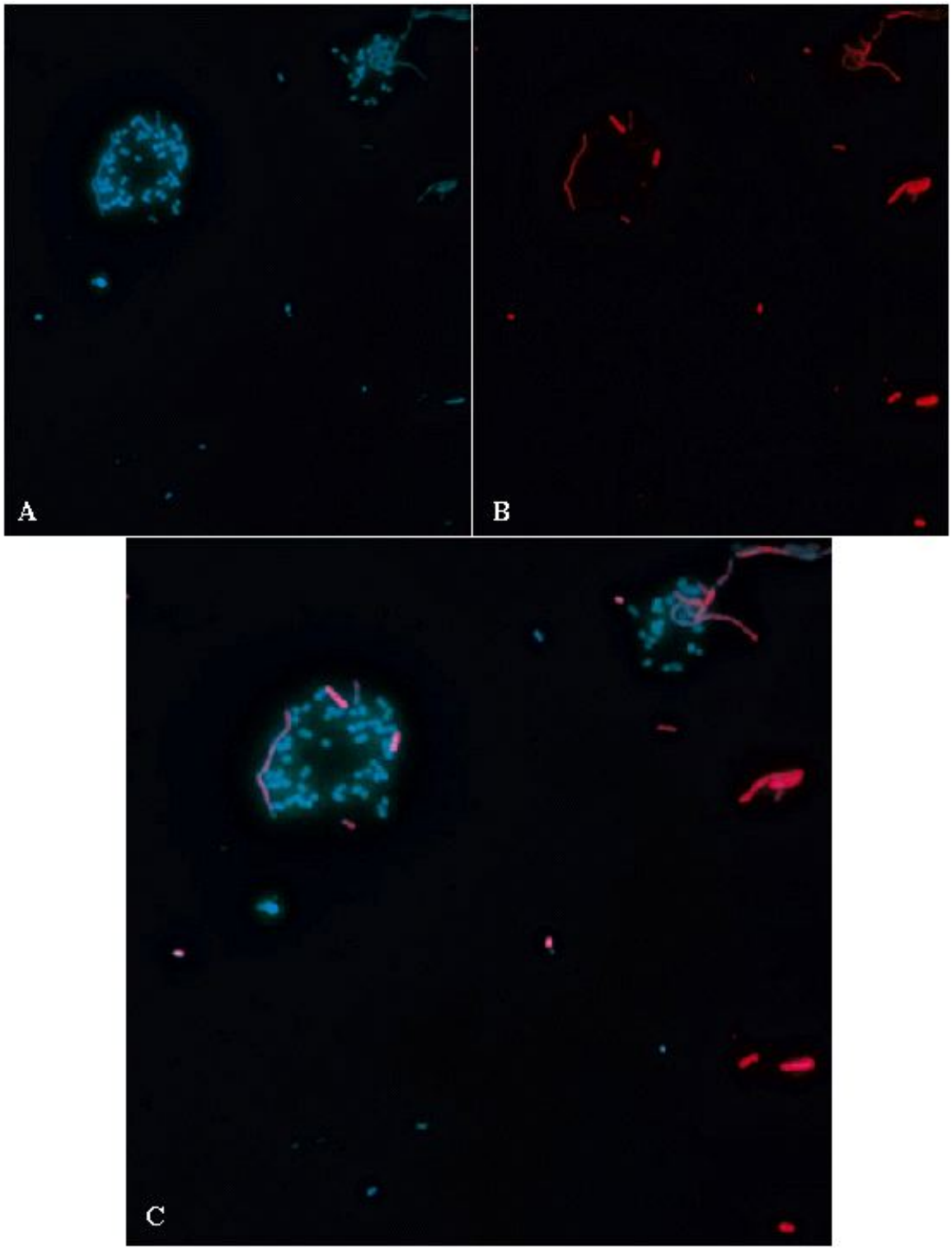


Figure 4.7. Gel electrophoresis with NosZ 1527/1773R.

In vitro PCR amplification of purified DNA from two Pseudomonas species and several environmental isolates targeting NosZ and using the NosZ 1527F and NosZ 1773R as the primer set. The annealing temperature for the thermal protocol was 61°C. The name of the DNA template source for the PCR product in each lane is listed below:
Lane 1 – *Pseudomonas stutzeri*. Lane 2 – *Pseudomonas aureofaciens*. Lane 3 – *Pseudomonas denitrificans*. Lane 4 – Y4I. Lane 5 – O16. Lane 6 – O21. Lane 7 – Molecular Weight Marker VIII, Roche Biochemicals. Lane 8 – ZAH511. Lane 9 – E37. Lane 10 – Sulfitobacter pontiacus. Lane 11 – Y3F. Lane 12 – EE36.

Figure 4.7. gel electrophoresis with NosZ

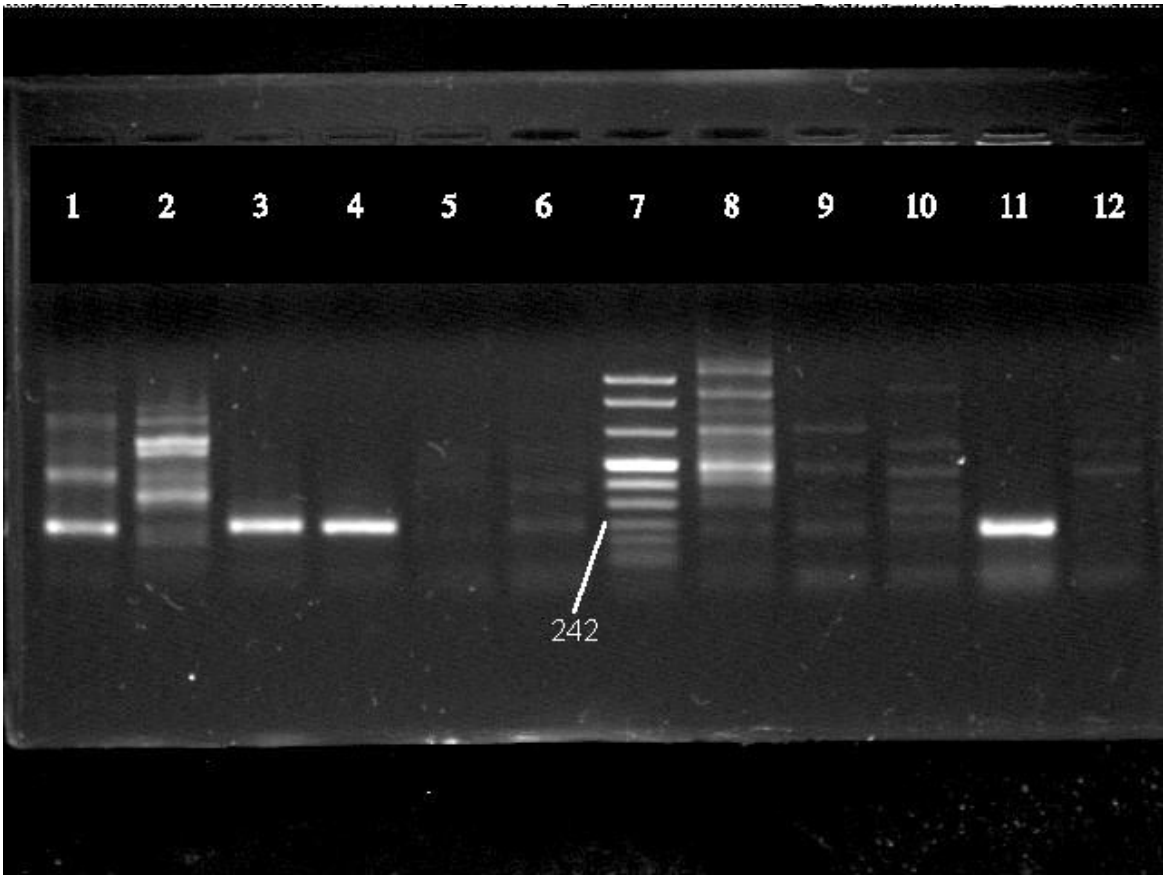


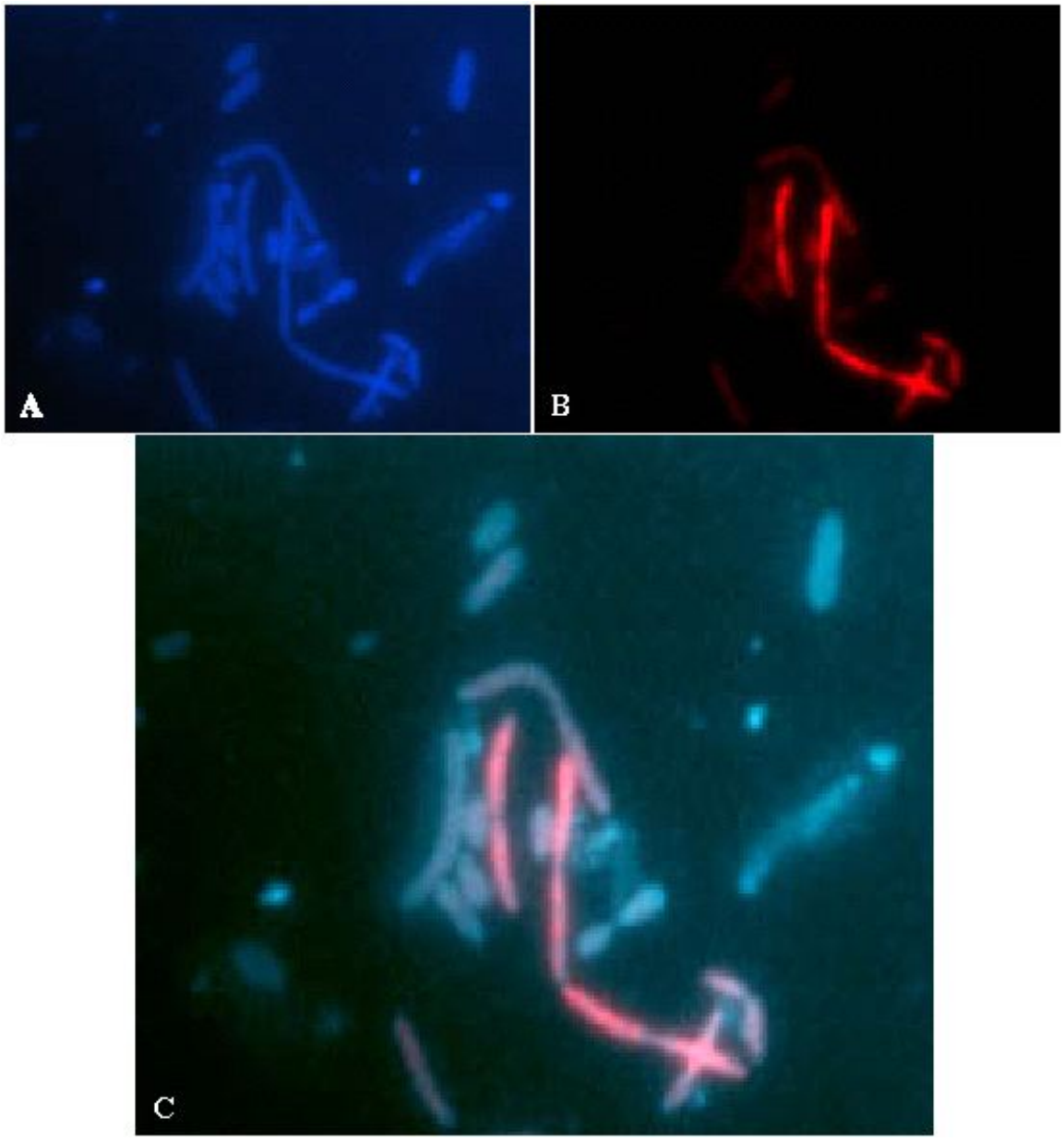
Figure 4.8. Discrimination between NosZ-positive and NosZ-negative bacteria using PI-PCR. Epifluorescence micrograph of DAPI-stained and CY3-stained cells.

Panel A –*Pseudomonas stutzeri* cells (chained rods), and three coastal isolates – O16, (barbell), ZAH 511 (short, fat rods) and EE36 (large rods) following PI-PCR on a slide. All cells were counter-stained with DAPI and viewed under UV epifluorescent illumination. *In vitro* PCR (Figure 4.6) experiments had previously indicated that the coastal isolates did not contain the NosZ gene. The primer set used was NosZ 1527F and NosZ 1773R (17).

Panel B – Visualization of cells that test positive for the NosZ gene by epifluorescent illumination at ~650 nm excitation. Detection of specific amplicon was accomplished by annealing and extending a Texas Red-labeled oligonucleotide, NosZ 1647R, to the PI-PCR product. A single cycle primer extension was used for detection.

Panel C – A composite image of A and B showing effective microscopic discrimination among NirS-positive (red, long rods) and the negative (blue) cells.

Figure 4.8.



NosZ Functional and 23S Taxonomic Dual labeling. After the development of a pair of primers that flank the published 23S *Pseudomonas*-group specific probe, *in vitro* PCR was performed on several cultures and isolates (Figure 4.9). As expected, *P. stutzeri*, P. *aureofaciens*, and *P. denitrificans* tested positive for *Pseudomonas* 23S. Isolate ZAH511 also tested positive. Y4I, O16, O21, E37, *Sulfitobacter pontiacus*, Y3F, and EE36 produced no distinct PCR products.

The results from this *in vitro* PCR experiment and from the previous NosZ experiments were used to select species for a dual functional (NosZ) and taxonomic (*Pseudomonas*-group) PI-PCR experiment (Figure 4.10). First, the NosZ PI-PCR, as described above, was performed. For dual labeling detection, a multiplex nested primer extension using the Texas Red-labeled NosZ1647R and the fluoroscein-labeled rRNA-targeted Ps23Sprobe. In order to verify that the NosZ1647R primer and the Ps23Sprobe did not produce a PCR product using DNA from the cultures and isolates used in this experiment, an *in vitro* PCR using the two primers as a set was performed. No PCR products were seen when viewed via agarose gel electrophoresis (data not shown).

In Figure 4.10-D, the cells in red are those positive for NosZ and negative for *Pseudomonas* 23S (Y4I – medium rods). The cells in blue are negative for both genes (*Sulfitobacter pontiacus* – fat rods). Those cells positive for the 23S gene and not the NosZ gene appear green and are identified as ZAH511 (short rods). The yellow-appearing cells are positive for both NosZ and 23S and are reasonably assured to be *P. denitrificans* cells (short chain or barbell).

Activated Sludge from a Municipal Wastewater Treatment Facility. The next step in the development of the method was to apply these techniques in a non-laboratory setting that would likely contain bacteria capable of denitrification. Accordingly, water samples from an activated sludge waste water treatment facility were collected and examined for the presence or absence of bacteria capable of nitrite reduction and nitrous

Figure 4.9. Gel electrophoresis of cultures and isolates following amplification with *Pseudomonas* 23S rDNA primer set 1F and 196R, which flank the probe-binding site.

Lane 23 – *P. stutzeri.* Lane 24 – *P. aureofaciens*. Lane 25 – *P. denitrificans*. Lane 26 – Isolate Y4I. Lane 27 – O16. Lane 28 – O21. Lane 29 – ZAH511. Lane 30 - E 37. Lane 31 – *Sulfitobacter pontiacus*. Lane 32 – Y3F. Lane 33 – EE36.

viii - Molecular weight Marker VIII (Roche Biochemicals).

Figure 4.9. gel electrophoresis with *Pseudomonas* 23S primers.

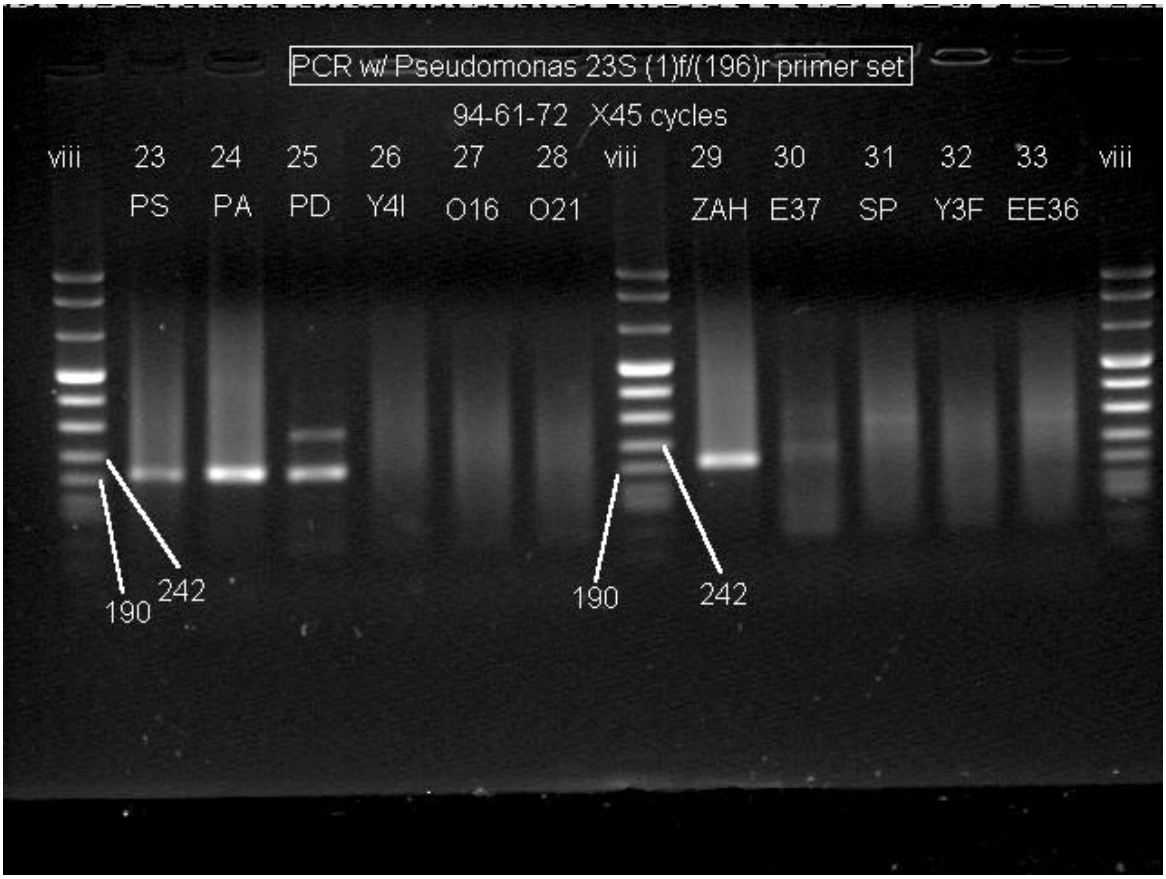


Figure 4.10. Dual functional/taxonomic labeling.

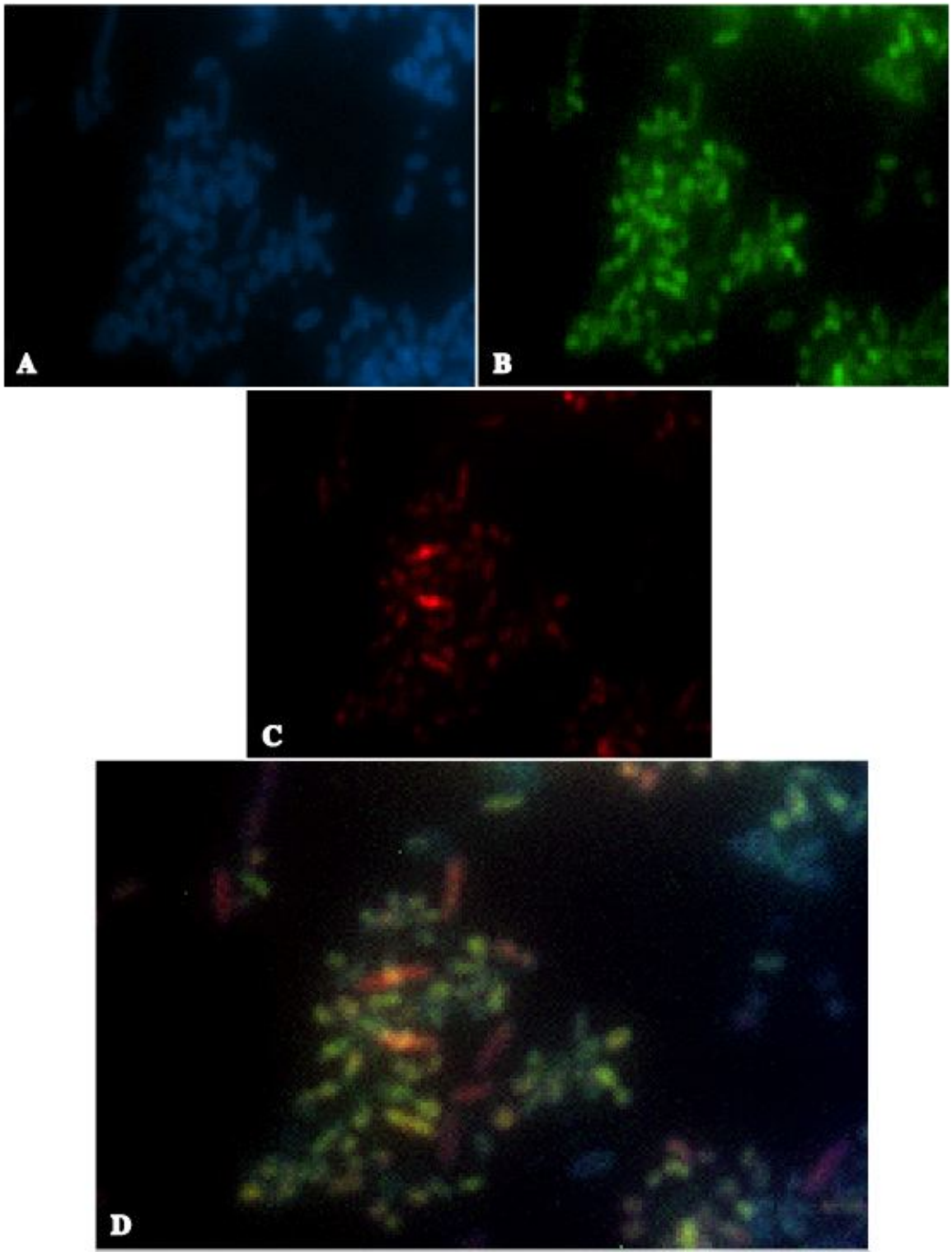
Panel A – All cells are visible under UV excitation. The DAPI-stained cell mix contains cells tested through *in vitro* PCR to be 1) positive for NosZ and positive for *Pseudomonas* 23S (*Pseudomonas denitrificans* - rods) 2) positive for NosZ and negative for *Pseudomonas* 23S (Y4I) 3) negative for NosZ and positive for *Pseudomonas* 23S (ZAH 511) and 4) negative for both (*Sulfitobacter pontiacus*).

Panel B – Under fluoroscein-exciting illumination, the cells that contain the fluoroscein-labeled *Pseudomonas* 23S primer are visualized. Detection was accomplished using a single cycle of labeled-primer annealing and extension using unlabeled nucleotides.

Panel C – Visualization of cells that test positive for the NosZ gene by excitation with ~650nm light. Detection of NosZ-specific amplification product was accomplished by annealing and extending a Texas Red-labeled oligonucleotide, NosZ 1647F, to the PI-PCR product. A single cycle of labeled primer annealing and extension with unlabeled dNTP's was used following PI-PCR. The single cycle used for detection of NosZ was conducted concurrently with a nested taxonomic primer/probe extension in a multiplex reaction.

Panel D – A composite of A, B, and C.

Figure 4.10.



oxide reduction. *In vitro* PCR indicated that the DNA purified from the activated sludge and water from the wastewater treatment plant contained bacterial cells that were positive for the NirS and NosZ genes (Figure 4.11).

NirS. In Figure 4.12, NirS PI-PCR using the NirS 1045aF and NirS 1795R primer set has been performed. Nested primer extension using the CY3-labeled NirS1137f oligonucleotide was used for detection. Shown is an activated sludge floc. The 3-dimensional structure is the result of the filamentous bacterial "skeleton" that is characteristic of a floc. In Panel A, the DAPI counter-stain showing all DNA-containing cells appears green in order to enhance clarity. Those cells that fluoresce under CY3-exciting illumination test positive for the NirS gene (Panel B). The variation in fluorescence intensity is attributed to the 3-dimensional nature of the floc.

NosZ. In Figure 4.13, NosZ PI-PCR has been performed on an activated sludge floc. The primer set NosZ 1527F and NosZ1773R were used for amplification and the Texas Red-labeled primer/probe NosZ 1647R was used for nested primer extension. The cells that fluoresce red are considered to be positive for NosZ.

AOBNirK. As reported in Chapter 3, putative ammonia-oxidizer nitrite reduction genes have been characterized from purified DNA from the same wastewater treatment plant sample as was used in the preceding PI-PCR experiment. Figure 4.14 shows the result of PI-PCR on an activated sludge floc using AOBNirK571F and AOBNirK896R. Nested primer extension using fluoroscein-labeled AOBNirK 873R allowed detection of positive cells. Fluoroscein-exciting illumination reveals positive cells as bright green (Panel B).

Figure 4.11. Gel electrophoresis of PCR using purified DNA from a municipal wastewater facility and primer sets NirS 1045aF/NirS 1795R and NosZ 1527F/NosZ1773R.
Four different dilutions of purified DNA from a municipal wastewater treatment plant were tested for the presence of the denitrification genes, NirS and NosZ.

1/1 - 1μl of purified DNA in a 25μl PCR reaction

1/10 - 0.1μl of purified DNA in a 25μl PCR reaction

1/100 – 0.01μl of purified DNA in a 25μl PCR reaction

1/1000 – 0.001μl of purified DNA in a 25μl PCR reaction

Figure 4.11. Gel electrophoresis of wastewater PCR with NirS and NosZ primers.

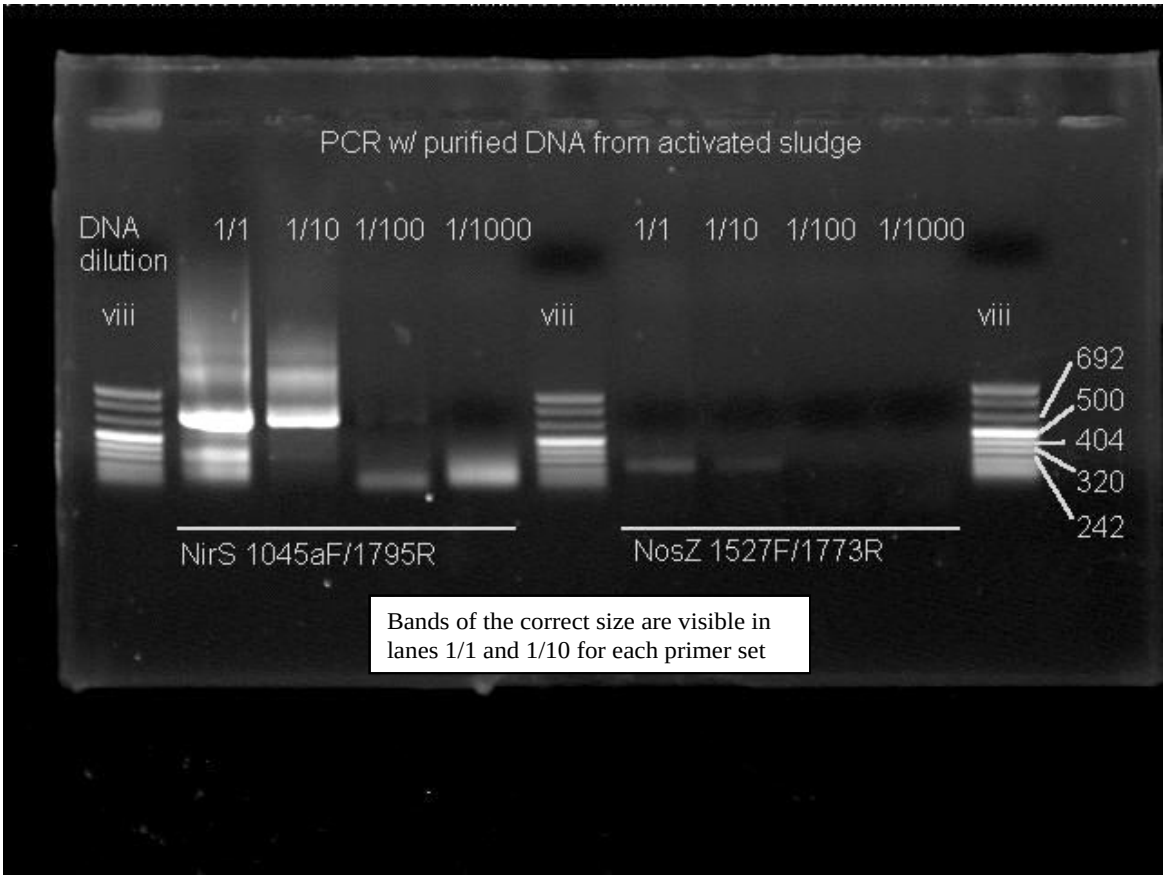


Figure 4.12. NirS PI-PCR demonstrated in activated sludge from a municipal freshwater wastewater treatment.

Panel A – DAPI-stained activated sludge floc following PI-PCR affixed to a slide and viewed by epifluorescence microscopy under UV excitation so that all cells are visible. Showed in green pseudocolor to enhance clarity.

Panel B – The same microscope field as in Panel A viewed under ~650nm excitation resulting in red fluorescence of cells that test positive for the NirS gene due to incorporation of CY3-labeled NirS1137F primer. PI-PCR was performed with primer set NirS1045aF and NirS1795R followed by nested primer extension detection.

Panel C – A composite combining Panel A in blue and green pseudocolor with Panel B in red.

Figure 4.12.

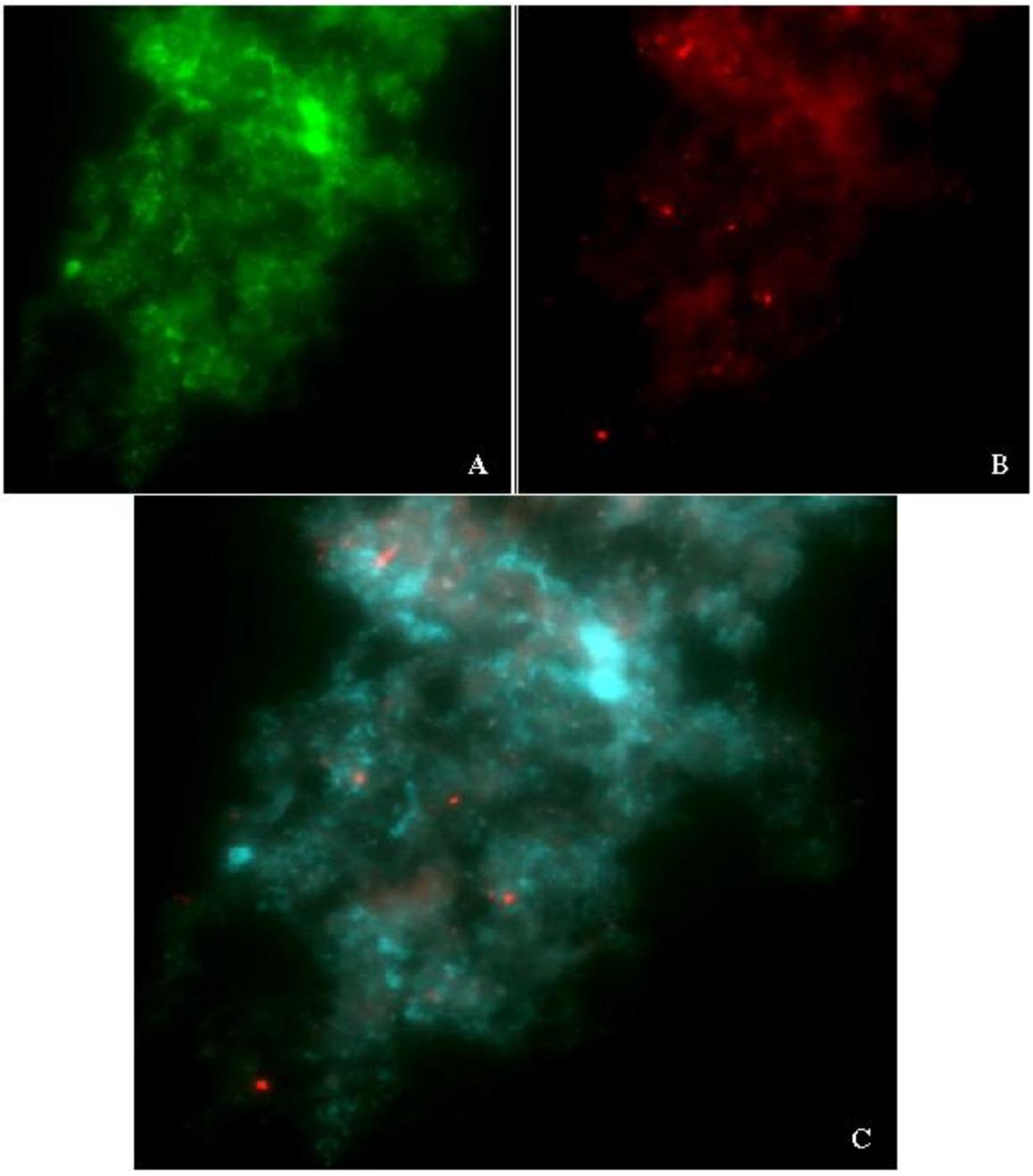


Figure 4.13. NosZ PI-PCR on activated sludge from a municipal freshwater wastewater treatment.

Panel A – DAPI-stained activated sludge floc following PI-PCR affixed to a slide and viewed by epifluorescence microscopy under UV excitation so that all cells are visible. Shown in green pseudocolor to enhance clarity.

Panel B – The same microscope field as in Panel A viewed under ~650nm excitation resulting in red fluorescence of cells that test positive for the NosZ gene due to incorporation of Texas Red-labeled NosZ 1647R primer. PI-PCR was performed with primer set NosZ1527F and NosZ1773R followed by nested primer extension detection. detected by nested primer extension with Texas Red-labeled NosZ 1647R.

Panel C – A composite combining Panel A in blue and green pseudocolor with Panel B in red.

Figure 4.13. NosZ PI-PCR on activated sludge.

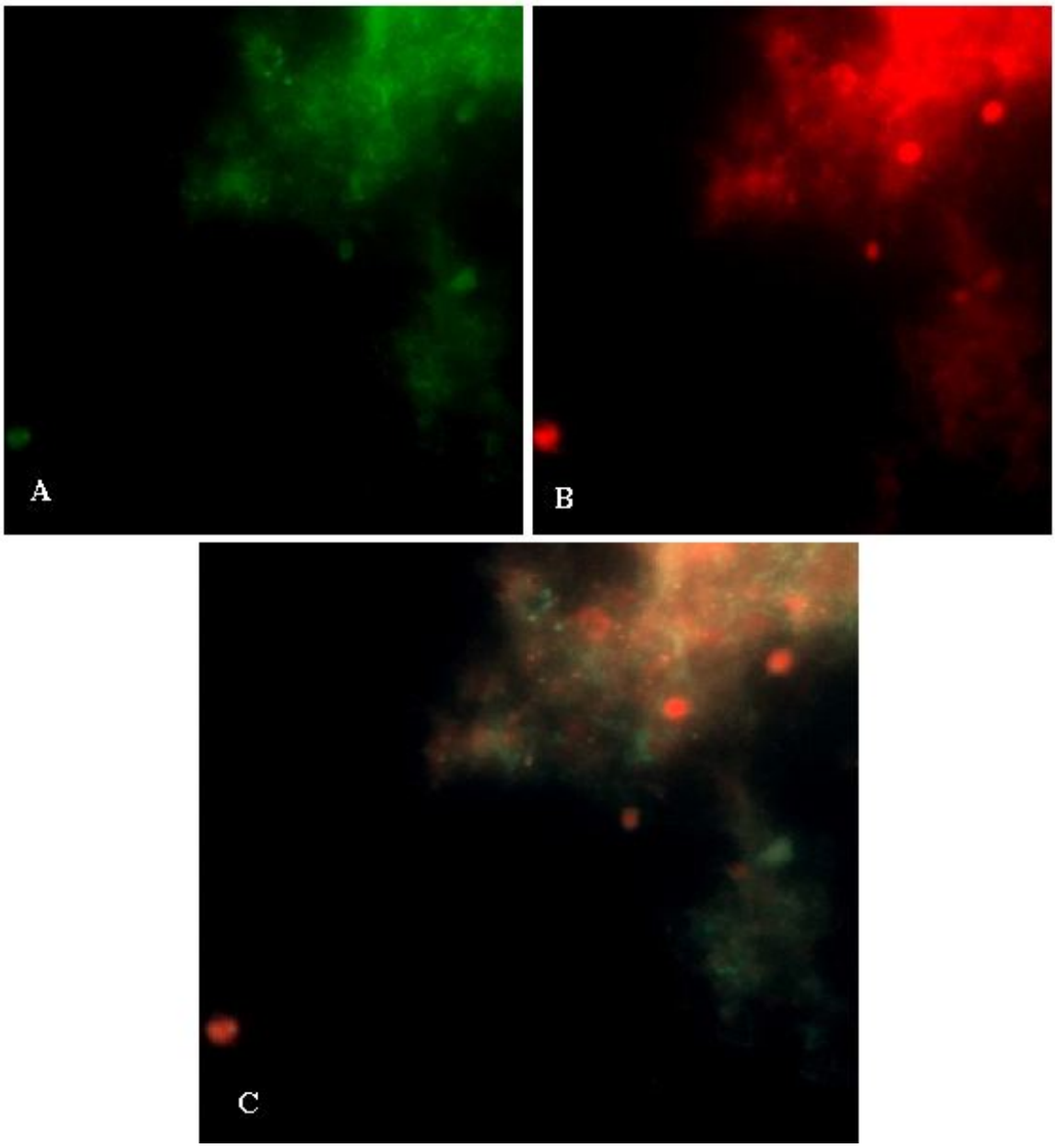


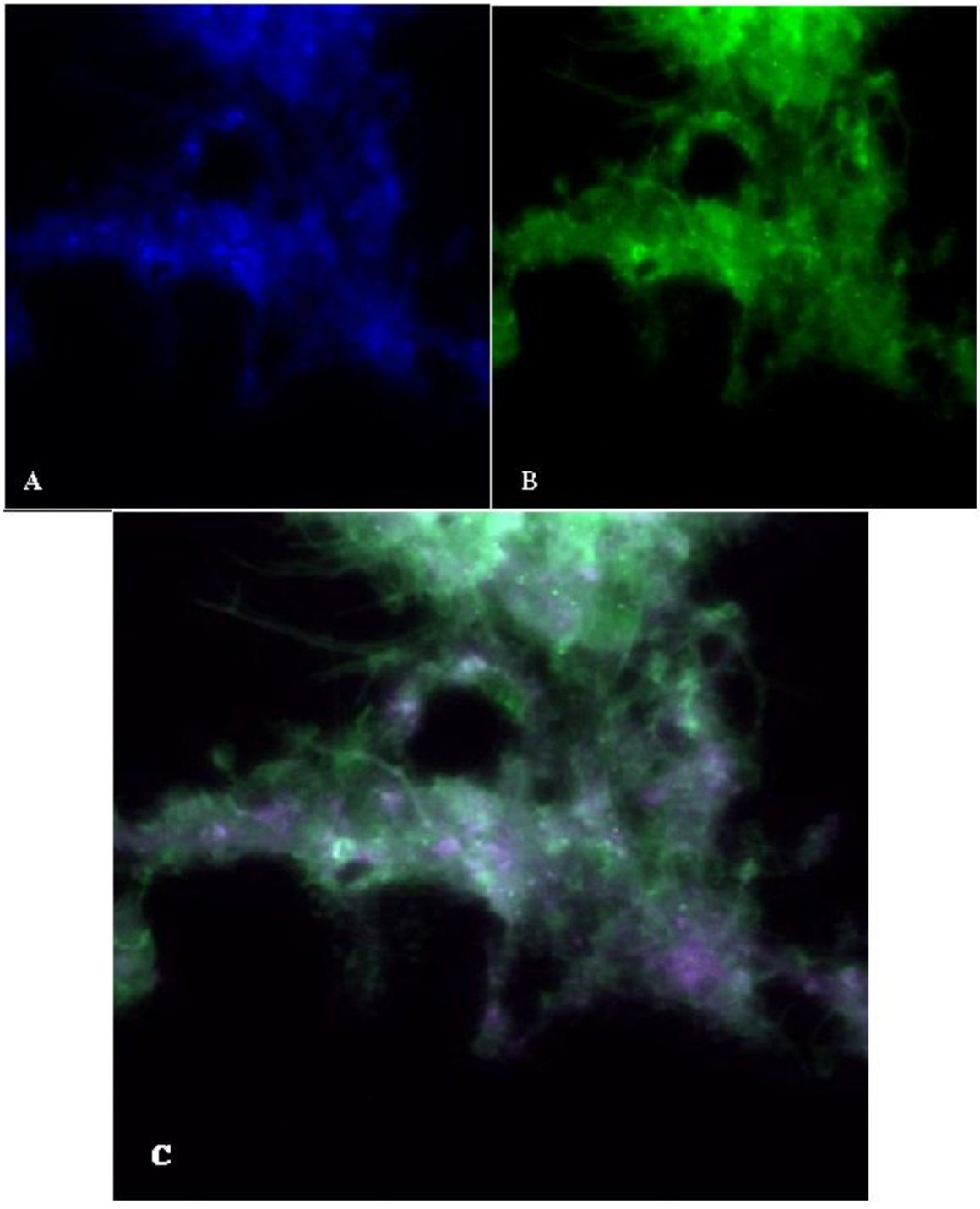
Figure 4.14. AOBNirK PI-PCR demonstrated on an activated sludge floc from a freshwater municipal wastewater treatment.

Panel A – DAPI-stained activated sludge floc following PI-PCR affixed to a slide and viewed by epifluorescence microscopy under UV excitation so that all cells are visible.

Panel B – The same microscope field as in Panel A viewed under ~488nm excitation resulting in green fluorescence of cells that test positive for the AOBNirK gene due to incorporation of fluoroscein-labeled AOBNirK873R primer. PI-PCR was performed with primer set AOBNirK571F and AOBNirK896R followed by nested primer extension detection.

Panel C – A composite combining Panel A in blue and red pseudocolor with Panel B in green.

Figure 4.14.



Discussion

Determining the microscale distribution of bacteria capable of denitrification was not possible until Prokaryotic *in situ* PCR was developed (10). Although *in situ* hybridization techniques, such as FISH, greatly improved our ability to study the microscale distribution of bacterial taxa, they lack the sensitivity to detect low- or single-copy genes. For this reason, and the fact that denitrifiers are phylogenetically diverse, a new approach was needed to study the distribution of bacteria capable of this process. However, the existing methodologies in *in situ* hybridization and PI-PCR produced equivocal results, and it became apparent that a new method was needed. In this study, each step of the current *in situ* gene amplification protocol was revisited in order to determine the strengths and weaknesses and to address the weaknesses.

In order to meet these criteria, several key elements were considered. First, the primer set specificity was ascertained and controlled. Second, the occurrence of *in situ* amplification was verified. Third, the potential pitfalls of the various detection methods were addressed. Such rigor was important not only for the development of the method, but for its application and interpretation of results as well.

NirS. Sequencing of the NirS PCR product from our control strain *Pseudomonas stutzeri* addressed several potential methodological difficulties (Figure 4.3). It confirmed that the culture of *P. stutzeri* was, in fact, *P. stutzeri* and that it was uncontaminated with any other NirS-containing bacteria. In addition, sequencing demonstrated that the primer set was specific to the desired target sequence. By extracting the DNA from *P. stutzeri* cells after they had been subjected to PI-PCR, it was shown that *in situ* amplification had in fact occurred (Figure 4.4). Also, the direct sequencing of the extracted PI-PCR product verified that the target sequence alone had been amplified. If there had been multiple

amplicons of the same size, no intelligible sequence would have been found by direct sequencing (as opposed to cloning and sequencing).

In the detection step of the protocol, the decision to utilize a low cycle number and nested primer extension followed numerous attempts to use existing fluorescent *in situ* hybridization (FISH) methods with positive and negative control strains. The results obtained with these methods were not robust or gave false positives and negatives despite the fact that many different parameters were varied (hybridization temperature, concentrations of buffers and probe, formamide use and percentage, washing times and buffer concentrations, etc). We also found that using more than two cycles for nested primer extension produced significantly increased background fluorescence compared to using a single cycle.

In discussing the micrographic figures, it is important to note two things. First, that all laboratory cultures and isolate strains were grown and fixed separately and were mixed together immediately prior to being spotted on the slide. Therefore, no spatial inferences can be drawn from the laboratory samples. This is, of course, not true for the wastewater sludge floc figures. Secondly, as with all visual technologies for the study of *in situ* bacterial communities, there exists a degree of interpretation of results that may vary among researchers. Therefore, it is important for the researcher to be consistent in his/her judgement in deciding whether a particular cell is positive or negative.

With those caveats in mind, we can discuss the PI-PCR figures. In Figure 4.5, the obvious difference in fluorescence between the cells positive (*P. stutzeri*) and negative (GAI-037) for NirS, is based solely on detection of the single gene. The next step in progressively testing for the robustness of our methodology included using an organism more closely related to the positive control. In Figure 4.6, *P. aureofaciens* cells (blue) that possess the NirK form of nitrite reductase were used as the negative control. *P. stutzeri* cells yielded bright CY3 fluorescence demonstrating the amplification of NirS within the cells. Conversely, *P. aureofaciens* cells are visible only under DAPI-counterstain-specific

UV epifluorescence illumination. This experiment verifies that the NirS primer set does not amplify NirK *in situ* and suggests that neither phylogenetic relatedness to the positive strain nor the presence of another form of nitrite reductase confounded our method.

Part of the great promise, to date, of the emerging methodologies for *in situ* gene amplification in bacteria has been its adaptability to an increasing number of genes and host organisms. After successful application of PI-PCR with nested primer extension detection to the important denitrifying gene NirS, it was our hope and expectation that our methods would be readily adaptable to other genes involved in denitrification.

NosZ. We wanted to determine whether or not the method is robust overall and not successful only with a particular primer set we optimized for NirS. Therefore, a different denitrification gene, NosZ, was examined using the same methodologies. Initially, our *in vitro* PCR experiments had confirmed the specificity of the published NosZ primers (Figure 4.7). By utilizing a variety of available isolates, we could select a number of morphologically distinct species for *in situ* investigation and further verify the strength of the method (Figure 4.8). The only strain that tested positive *in vitro* for NosZ was the known positive, *P. stutzeri.* One of the isolates, ZAH511, yielded multiple bands of incorrect size during the *in vitro* NosZ studies. The fact that, ZAH511 tested negative for NosZ during PI-PCR strongly supported our hypothesis that spurious, unlabeled PCR products are not detected by this method.

NosZ functional /*Pseudomonas* 23S taxonomic investigation. As stated above, one of the longstanding goals of developing PI-PCR was to adapt the methodologies for dual functional gene/taxonomic determinations in specific individual cells in natural aquatic samples, soils and mineral deposits. While we are not yet routinely capable of such dual, *in situ* detection, the preliminary advances made in this study bring us much closer to that goal.

The development of primers specific for Pseudomonads was useful for the selection of appropriate positive and negative controls from the available isolates for a dual functional/taxonomic approach (Figure 4.9).

By comparing results of detection of NosZ *in vitro* with 23S *in vitro* PCR amplification results, one bacterial species representing a positive control for each of the possible combinations of NosZ and/or *Pseudomonas* 23S was identified and selected. One can clearly see that each of the combinations is represented in Figure 4.10. With the blue cells representing only DAPI-staining, the green cells only those that contain sufficient quantities of bound 23S rRNA probe and DAPI, the red cells only NosZ primer and DAPI, and the yellow cells both 23S and NosZ, the potential for application is enormous. This is an area for additional research, but these results demonstrate, for the first time, that *in situ* amplification and nested primer extension of single gene sequences and identification of bacterial taxonomic sequences can be used to visually explore a single cell for its genetic capability and its phylogenetic position.

Additionally, while it did not prove necessary in this experiment, the need to amplify *in situ* the 23S ribosomal DNA in order to facilitate detection may be necessary when this method is applied to environmental samples. Low cellular rRNA levels may be encountered in quiescent, starved or otherwise stressed cells or possibly in healthy, active cells that are small in size (2).

Activated Sludge from a Municipal Wastewater Treatment Facility. The application of the PI-PCR primer extension method to genetic analysis of an environmental sample yielded interesting results. *In vitro* PCR had revealed the presence of NirS and NosZ genes in the bulk extracted DNA from the wastewater treatment plant sample (Figure 4.11). Application of the PI-PCR methodology targeting NirS (Figure 4.12) or NosZ (Figure 4.13) showed both NirS- and NosZ-positive cells distributed throughout the activated sludge floc.

When comparing numbers of cells identified as possessing NirS relative to cells with NosZ (Figures 4.12 and 4.13), it appears that the NosZ-positive cells are more numerous. Although it is important to point out that no quantitative studies have yet been undertaken with these primer sets and this methodology, it is not surprising to find higher apparent numbers of NosZ positive cells. To date, there is only one known form of nitrous oxide reductase and one known form of its structural gene, NosZ. Even though several species of bacteria capable of denitrification have been shown to lack a nitrous oxide reductase gene, all the species that express this gene should be expressing NosZ. Conversely, since there are two forms of nitrite reductase, only a subset of the bacteria capable of nitrite reduction would test positive for NirS.

Finally, another experiment was performed to expand our work to encompass studying the occurrence of nitrite reductase in chemolithoautotrophs. As stated above, the *in vitro* PCR results established the presence of organisms possessing the heterotrophic NirS and NosZ dissimilatory nitrogen cycling genes in our municipal sewage sludge samples. Using similar *in vitro* methods we have also detected the presence of DNA sequences coding for NirK typical of chemolithotrophic ammonia oxidizers (AOB) in the same sludge (see Chapter 3, “Molecular Characterization Of Putative Ammonia-Oxidizer Nitrite Reductase Genes From A Municipal Wastewater Treatment Plant”). AOB are widely distributed in sewage sludge (13, 25). Hence, we decided to attempt to utilize our new PI-PCR primer extension methodologies to see if we could detect the AOBNirK in individual bacteria in the sewage sludge. Figure 4.14 clearly demonstrates their presence and our ability to distinguish them from other bacteria.

These findings all suggest that dissimilatory nitrite reductase enzymes and their associated hosts could be further studied for their microscale layering in flocs and denser particles, or even discriminated by flow cytometry (5) and sorted for individual genetic or biochemical investigations. Oceanographic studies of the large scale depth and spatial distribution of nitrogen cycling bacteria should also be greatly facilitated by these

approaches; in turn, we anticipate these methods potentially leading to new insights into bacterially mediated N-cycling and changes in global elemental cycling due to altered biogeochemical processes.

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CHAPTER 5

CONCLUSIONS

The study of marine microbial ecology has changed dramatically several times over the past several decades. Pioneers like Claude ZoBell made major discoveries with the cultivation and study of marine bacterial species. Most probable number studies yielded new information regarding bacterial diversity and abundance. Detailed studies of the taxonomic and physiological characteristics of now well-known species, such as *Pseudomonas stutzeri*, yielded a great deal of insight into microbial ecology, including the mechanisms of marine nitrogen cycling. It was thought that cultivation of environmental species was the key to understanding bacterial community structure and function.

By the 1970's, however, studies by scientists such as John Hobbie, cast doubt on the veracity of this line of thinking. With the advent of methods for enumerating all bacteria in an aquatic sample, for example, acridine orange staining, it quickly became obvious that a very low percentage of existing aquatic bacteria were culturable. It followed logically that bacterial culturability might not mirror importance in terms of microbial function in marine samples. Thus, the study of isolates fell out of favor with marine microbial ecologists.

Studies of the rates of important processes overtook isolate work in terms of what was viewed as the most informative type of study of aquatic bacterial communities. It

was not necessary to know which bacteria were active to be able to measure environmentally important processes, like mineralization or organic transformations. Techniques using radiolabeled substrate were very popular and useful in describing the activity of bacterial communities.

With regard to aquatic nitrogen cycling, much was learned about nitrification and denitrification by the use of ^{15}N-labeled ammonium and nitrate as substrate for bacterial communities involved in these processes. The advent and application of inhibitors to specific steps in these processes allowed even more information to be discerned about the rates of nitrification (low level acetylene block and N-serv sensitive ammonia loss) and denitrification (acetylene block technique). Nevertheless, the actual mediators were still largely unknown, or at least, the diversity of the bacteria capable of these processes escaped discovery. The questions still remained – Who is doing what, when and where?

As reviewed earlier, the advent of culture-independent methods for describing the members of the bacterial communities allowed for a much more thorough look at bacterial community diversity and composition. This dissertation represents our attempt to adapt several of the recently developed DNA-based techniques to the study of the nitrogen cycle in aquatic systems. The techniques employed in our studies are partly existent and partly *de novo*. We have utilized Terminal Restriction Fragment Length Polymorphism analysis to examine the structure of a functional group of bacteria, namely ammonia-oxidizers. The success with which this method was applied in this case is tempered by the realization that the primer set selected proved to be less specific in the system in which we applied it than in the system for which it was developed or used subsequently by other investigators.

The development of a primer set that proved to be useful for describing DNA sequences very closely related to ammonia oxidizer nitrite reductase sequences illustrates the limits of our knowledge regarding the true complexity of nitrogen cycling bacterial communities. By describing a previously unknown group of putative nitrite reductase gene sequences, we have demonstrated the usefulness of basic applications of DNA-based molecular methods in the study of nitrogen cycling bacteria. It is possible that this discovery will lead to an increased understanding of global nitrous oxide production.

Finally, by improving the detection methodology for prokaryotic *in situ* PCR and applying it to several denitrification enzymes, we have helped to advance the state of knowledge regarding the understanding of aquatic nitrogen-cycling bacterial structure. In elucidating the spatial distribution of various denitrifying (and nitrifying) bacteria, we hope to have moved the state of the art forward, such that the next methodological improvements may make PI-PCR widely applicable to aquatic systems. Especially exciting is the potential for a simultaneous *in situ* functional and taxonomic approach.

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