ISOLATION, CHARACTERIZATION AND DETECTION BY *IN SITU* GENE AMPLIFICATION OF MARINE DENITRIFYING BACTERIA

By

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ABSTRACT

Recent studies suggest that the oceanic nitrogen budget is unbalanced, primarily due to a high nitrogen removal in contrast to the fixation rate. This imbalance likely results from denitrification activity in continental shelf sediments. Denitrifying bacteria play a major role in marine sediment nitrogen balance. In order to assess the nitrogen balance characteristic, this study utilized slow-growth enrichment microcosms. These consisted of Puget Sound, WA seawater and sediment samples enriched with dimethyl sulfoxide (DMSO) as carbon source, and nitrate in order to stimulate denitrifying activity. Two sets of microcosms were prepared and incubated in the dark for 6 months at 25^{o}C and 4^{o}C. Of 82 strains isolated, 18 were positive for both nitrate reduction and gas production. Amplified ribosomal DNA restriction analysis (ARDRA) was performed to compare and establish similarities between the Puget Sound isolates and control cultures.

The novel *in situ* reverse transcription PCR (RT-PCR) method has been optimized to study the expression of the *nirS* gene in denitrifying bacteria. Pure cultures of reference denitrifying isolates from marine sediments were used to optimize the *in situ* RT-PCR protocol. We performed cell fixation after visible gas production was observed. *In situ* RT-PCR was performed after cell fixation and enzymatic permeabilization. The *nirS* 1F and *nirS* 6R primers were used for the amplification of cDNA and subsequently fluorescent *in situ* hybridization (FISH) was done to increase the detection specificity of the amplification product. Only active denitrifying cells were detected by this approach using fluorescence microscopy.

RESUMEN

Estudios recientes, sugieren que el presupuesto oceánico de nitrógeno no esta balanceado; debido a una alta remoción de nitrógeno en comparación con la tasa de fijación. Este imbalance puede ser el resultado de la denitrificación en sedimentos de los márgenes continentales. Las bacterias denitrificadoras juegan un papel importante en el balance de nitrógeno en sedimentos marinos. Para poder determinar las características del balance de nitrógeno, este estudio utilize microcosmos enriquecidos de crecimiento lento. Éstos, consistían de agua de mar de Puget Sound, WA y muestras de sedimento enriquecidos con sulfóxido de dimetilo (DMSO por sus siglas en inglés) como fuente de carbono, y nitrato para estimular la actividad de denitrificación. Se prepararon dos grupos de microcosmos y se incubaron en la oscuridad por 6 meses a 25°C y 4°C. De 82 cepas aisladas, 18 fueron positivas para la reducción de nitrato y producción de gas. Se realizó un análisis de restricción de la amplificación del ADN ribosomal (ARDRA por sus siglas en inglés) para comparar y establecer similitudes entre los cultivos aislados de Puget Sound y las cepas usadas como referencia.

El novedoso método de PCR y retrotranscripción *in situ* (*in situ* RT-PCR por sus siglas en inglés) se optimizó para estudiar la expresión del gen *nirS* en bacterias denitrificadoras. Cultivos puros de bacterias denitrificadoras aisladas de sedimentos marinos se usaron para optimizar el protocolo de RT-PCR *in situ.* Fijamos las células después de observar producción de gas. Se realizó RT-PCR *in situ* luego de fijar y permeabilizar enzimáticamente las células. Los “primers” NIRS 1F y NIRS 6R se usaron

para la amplificación del cDNA y luego se realizó hibridización fluorescente *in situ* (FISH por sus siglas en inglés) para aumentar la especificidad en la detección del producto de amplificación. Sólo, las células denitrificadoras activas fueron detectadas por este método utilizando microscopía de fluorescencia.

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I. INTRODUCTION

1.1 Introduction

Denitrification has been known for more than a century and is recognized as a key process in the nitrogen biogeochemical cycle (Ye et al. 1994). Through denitrification, nitrogenous oxides, principally nitrate (NO_3) and nitrite (NO_2), are reduced to dinitrogen gases (Groffman et al. 1988). Recently, denitrification importance has grown because of several reasons. Denitrification is a major source of nitric oxide (NO) and nitrous oxide N_2O. The accumulation of these gases contributes significantly to the ozone layer depletion and consequently to global warming. It is also an important process in waste treatment, by removing excess nitrate and stimulating carbon removal when aeration is difficult, for example, in using nitrate to stimulate pollutant bioremediation in aquifers (Ye et al., 1994). It is known that denitrification is also of agricultural importance since it is a major cause of soil fertility depletion (Groffman et al. 1992). All these reasons make clear the importance of gaining a better understanding of denitrification.

Although continental margin sediments constitute only about 10% of the total sediment surface area in the world's oceans, they are the dominant sites of nitrogen cycling. Thirty to fifty percent of marine primary productivity takes place in waters overlying continental margins (Romankevich, 1984; Walsh, 1991). Recent studies suggest that the oceanic nitrogen budget is unbalanced, primarily due to a higher nitrogen removal rate in contrast to the fixation rate (Ganeshram et al., 1995; Altabet et al., 1995;

Codispoti, 1995; Middelburg et al., 1996). This imbalance likely results from denitrification activity in continental shelf sediments (Christensen et al., 1987; Codispoti, 1989; Devol, 1991). However, prediction of denitrification is difficult with estimates of oceanic sedimentary denitrification varying by over more than an order of magnitude (Middelburg et al., 1996; Devol et al., 1997). Respiratory denitrification is widespread among prokaryotes, but the denitrifiers frequently found in soils and sediments are from more limited phylogenetic groups (Zumft 1992; Tiedje 1994). Although denitrification in marine environments has been studied intensively at the process level, little is known of the abundance, species composition, distribution, and functional differences of the denitrifying population in seawater and marine sediments (Ward et al., 1993, 1996). Such difficulties are compounded because there remains considerable uncertainty about mechanisms, processes and organisms that control the dynamics of denitrification in marine environment. Thus, understanding the diversity of microbial populations in marine environments, their responses to various changes of environmental factors such as O_2 and NO_3^-, and the impacts of the changes in microbial community structure and composition on the rates of denitrification and other processes in N cycling is critical. Hence, a key goal of this project is to link the N process information from the field with the microbial community structure and its activity.

Nitrite reductase (Nirs) is the key enzyme of denitrification in catalyzing the first committed step that leads to a gaseous intermediate (Zumft 1997). Two types of Nirs have been described: one contains a copper center (Cu-Nirs), and the other contains the hemes c and d_1 (cd_1-Nirs). The sequences of important functional regions such as the copper and heme binding domains appear to be more conserved. These make these

regions useful as priming sites for general PCR amplification primers for detecting denitrifying bacteria (Bracker and Witzel, 1997).

In situ hybridization (ISH) has been used in conjunction with epifluorescence microscopy to identify prokaryotic cells in various natural communities at phylogenetic levels ranging from species to kingdom (Amman et al., 1990, 1992, 1995; Hicks et al., 1992; Zarda et al., 1997; Chen et al., 1998). A major limitation of the ISH technique is that natural aquatic samples usually do not contain sufficient cellular rRNA to yield detectable fluorescent signal (Chen et al., 1998). Even with further modifications, it is doubtful that ISH can be a reliable method to detect low-copy or single-copy genes inside individual cells. Also, the applicability of ISH techniques is limited to taxonomic and/or phylogenetic identification of microbial communities, it cannot provide information on *in situ* genetic capabilities of individual cells within a microbial community (Chen et al., 1998)

The *In Situ* PCR (IS-PCR) and RT-PCR techniques are relatively new and still under development that were first initiated in 1990 (Bagasra, 1990; Hasse et al., 1990; Chen et al., 1998). For IS-PCR, amplification and detection of specific target nucleic acid sequences are carried out inside individual cells rather than on bulk extracted nucleic acid (Nuovo, 1994a), which are then visualized with a microscope. This new approach could greatly advance our understanding of specific genes and gene products at a single cell level. The detection and identification of bacterial species at the single-cell level through this novel approach can be a useful tool in enumerating bacterial populations, studying their microscale distribution and the interactions among different populations (Hodson et al., 1995; Chen et al., 1999).

Understanding the diversity of denitrifying populations in marine environments, and the impact of the changes in microbial community structure and composition on the rates of denitrification and nitrification and other processes in nitrogen cycling is critical to predict global nitrogen dynamics. Therefore, in situ amplification was targeted toward the *nir*S gene and the *nir*S mRNA of denitrifying bacteria, in order to have a better understanding of the abundance, distribution, activity, and diversity of denitrifying populations in marine sediments. Dominant denitrifying bacteria of marine environments are poorly understood. Thus isolating novel denitrifirers will also advance our understanding of the ecological, physiological and phylogenetic diversity of denitrifying bacteria in marine environments. Such knowledge will be important for a better understanding of the processes and mechanisms controlling N cycling (Tiedje, J.M., Personal communication).

1.2 Objectives

1. To isolate and characterize novel denitrifying bacteria from the Pacific North West marine sediment microcosm systems.

2. To develop an *in situ* PCR and RT-PCR protocol to study model marine denitrifiers based on the nitrite reductase gene.

3. To optimize the *in situ* PCR and RT-PCR detection methods for the dominant denitrifying populations in the marine sediments based on the *nirS* gene.

II. LITERATURE REVIEW

2.1 The Nitrogen Cycle (Denitrification)

Nitrogen is one of the chemical elements essential for life. All living cells require nitrogen since it is a major constituent of all amino acids, proteins, nucleic acids and many other important molecules. Molecular nitrogen (N_2) is abundant in the atmosphere representing approximately 79% of the atmospheric gases. Even though N_2 is very abundant it cannot be used by most organisms. In order to make it available to other organisms, N_2 must be fixed. Gaseous nitrogen fixation is a task that only a few specialized microorganisms have the ability to carry out. Thus microorganisms play a key role in the nitrogen cycle.

The nitrogen cycle represents the redox chemistry of the principal natural inorganic nitrogen species dinitrogen, ammonia, and nitrate. Nitrogen is introduced into the biosphere by biological and chemical fixation of dinitrogen (N_2) and removed from again by denitrification (Zumft, 1997). Between these two extremes, a host of reactions take place. The reactions include the oxidation of ammonia, produced by nitrogen fixation, to nitrite and nitrate (nitrification), the reduction of these oxidized nitrogen compounds to ammonia (dissimilatory nitrate reduction) and the incorporation of the various compounds in organic molecules (Gijs Kuenen and Robertson, 1988). A simplified version of the nitrogen cycle is shown on Figure 1 (Zumft, 1997).

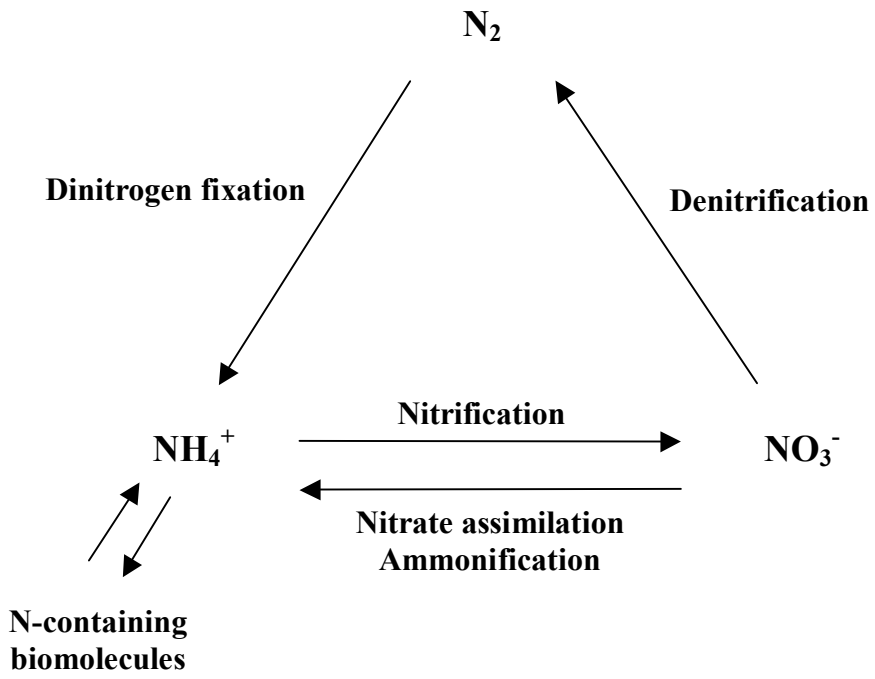

Figure 1**. Simplified Nitrogen biogeochemical cycle sustained by prokaryotes (Adapted from Zumft, 1997).**

The term denitrification was originally used to describe the loss of nitrate to gaseous products in decomposing organic matter. In early studies the intermediate gaseous species, nitric oxide (NO) and nitrous oxide (N_2O) were already observed. But their role was originally believed to be limited to supply oxygen for combustion (Zumft, 1992). Development of new concepts of energy metabolism led to the currently accepted view of anaerobic respiration. Denitrification is a bacterial respiratory process that occurs only in the absence of oxygen, or at particularly low oxygen concentrations (Rosswall, 1981; Lengler et al., 1999; Maier et al., 2000). It is an "anoxic" process that mainly occurs in facultative anaerobes. Thus, this respiratory pathway has to compete with oxygen, which usually suppresses the anaerobic pathways.

During denitrification the N oxyanions nitrate and nitrite and the gaseous N oxides NO and N_2O substitute dioxygen (O_2) as terminal electron acceptors for the generation of an electrochemical gradient across the cytoplasmic membrane. Denitrification can be considered as the assemblage of nitrate respiration, nitrite respiration combined with NO reduction, and N_2O respiration (Zumft, 1997).

A different enzyme catalyzes each step of the reduction reactions that take place in the denitrification process. All four of the enzymes involved in the denitrification have been identified as metaloproteins (Zumft, 1992; Lengler et al., 1999). Respiratory nitrate reduction is the first step, in which nitrate (NO_3) is reduced to nitrite (NO_2) by a nitrate reductase (Nar). Nitrite is then reduced by a nitrite reductase (Nir) into the gaseous product nitric oxide (NO). The NO is then further reduced by a nitric oxide reductase (Nor) to nitrous oxide (N_2O). Finally a nitrous oxide reductase (Nos) catalyzes N_2O reduction into dinitrogen gas (N_2).

The Nar and Nor enzymes, had been shown to be membrane integral proteins (Zumft, 1992, Ye, 1994). On the other hand Nir and Nos reductases have been shown to be periplasmic proteins (Figure 2).

2.2 Diversity of Denitrifying Bacteria

Denitrifiers are undoubtedly among the most successful physiological groups of microorganisms in nature (Tiedje et al., 1982). A recent survey of known denitrifying bacteria showed representatives totaling nearly 130 species within more than 50 genera. This number is more than double that given in the last survey (Jeter and Ingraham, 1981; Zumft, 1992). Denitrifiers are somewhat more frequent within the alpha and beta classes of the Proteobacteria, although there is no recognizable pattern of distribution. Denitrification is absent from enterobacteria although they respire nitrate to nitrite and direct the further reduction of nitrite to ammonification (Zumft 1992, 1997). Hypertermophily (Huber et al., 1992; Völkl et al., 1993; Zumft 1997) and alkaliphily (Berendes et al., 1996; Zumft 1997) are traits newly recognized within denitrifying prokaryotes.

The ubiquitous property of denitrifying bacteria has been widely shown (Gijs Kuenen and Robertson, 1988; Zumft, 1992, 1997). Denitrifying bacteria have been isolated from soil, fresh water, polluted habitats, the oceans, from plants, animals, man, and from exotic sources such as boiled ox blood, honey bee larvae, medicinal leeches and oil brine. Many are pathogenic for plants, animals, or humans, or have pathogenic potential under certain circumstances (Zumft, 1992). The denitrifying ability is found in

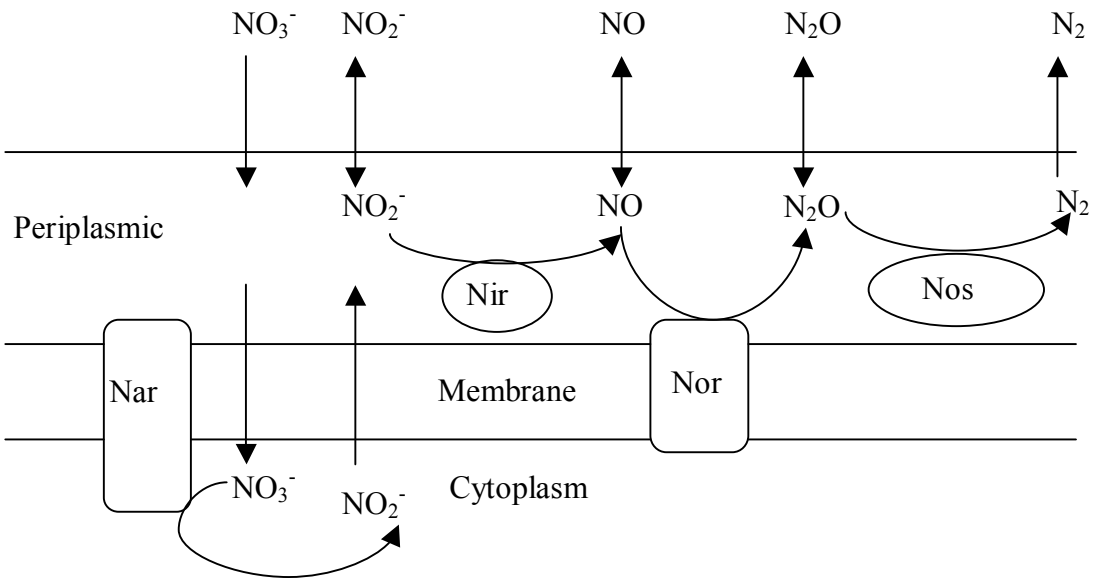

Figure 2. Denitrification pathway in gram-negative bacteria. Nar, nitrate reductase; Nir, nitrite reductase; Nor, nitric oxide reductase; Nos, nitrous oxide reductase (Adapted from Ye, Averill and Tiedje, 1994).

heterotrophic opportunists and chemoautotrophs, is widespread among *Bacteria* and *Archaea*, and has even been reported in Eukarya (Zehr, 2002; Zumft, 1997).

The overwhelming majority of denitrifying bacteria is mesophilic. Two exceptions are two psychrophilic isolates from Antarctica, *Azospirillum psychrophilum* and *Halomonas subglaciescola,* which grow at temperatures between –5 to 25°C. On the other hand there are moderately thermophilic *Bacillus stearothermophilus* and *Thermothrix thioparus* and hyperthermophilic archaeon *Pyrobaculum aerophilum* (Völkl et al., 1993; Zumft 1997). Since denitrification was discovered among archaebacteria, the largest group of denitrifying bacteria adapted to extreme environments is the extreme halophiles (Zumft, 1992). The archaeal nitrite reductase is active in the presence of 4M NaCl and almost inactive in its absence (Zumft, 1997).

The genes for denitrification encoding functions for nitrate respiration (*nar*), nitrite respiration (*nir*), NO respiration (*nor*), and N_2O respiration (*nos*) are assembled in clusters in various denitrifying strains as shown by various studies (Braun and Zumft, 1992; Jüngst et al., 1991; Arai et al., 1995; Berks et al., 1995; de Boer et al., 1996; Holloway et al., 1996; Zumft, 1997). For the reduction of nitrite, two entirely different enzymes in terms of structure and the prosthetic metal are found, although never within the same cell. About three-quarters of strains collected worldwide, with prevalence of pseudomonads among them, have the tetraheme protein cytochrome cd_1 as the respiratory nitrite reductase (NirS) which is coded by the *nirS* gene (Gamble et al., 1977; Zumft, 1997).

2.3 Available Molecular Techniques for Studying Denitrifying Populations

It is well known that we can culture only 0.1-5% of the microorganisms present in most natural samples. That is why knowing what microbial types make up a community in soil, water, the rumen, or other habitats have been a challenge for microbiologists (Tiedje, 1995; Tiedje and Zhou, 1996). In the past, researchers avoided attempting to describe the microbial composition of a habitat. Visual observation is often of little value when applied to the problem of describing microbial communities (Findlay, 1996). However, with the introduction of molecular biology tools into microbial ecology, along with advances in microscopy, automation, and computer databases, it has become feasible to overcome most of the previous obstacles (Tiedje, 1995).

There are many reasons that show how valuable it is to know the microbial composition of a habitat. Some strains produce novel and potentially important biotechnology products; other members play key roles in recycling nutrients and energy and sustain efficient agricultural, forestry of fishery ecosystems. Others detoxify hazardous wastes in our environment; and some members may provide new insight into how life is possible in unusual or extreme environments (Tiedje, 1995).

All of these reasons have made various culture independent methods useful tools for microbial populations' studies. Classical methods of isolation and identification as well as many modern molecular methods are highly selective because they fail to detect most organisms in a given environment. Only two accepted methods overcome the selectivity difficulty and provide a relatively unbiased view of the structure of complex microbial communities. These methods are analysis of microbial populations using rRNA or its corresponding DNA sequences and phospholipid fatty acid analysis.

Community analysis using rRNA (or rDNA) sequencing can detect and identify community members with a high degree of specificity (to species and even strains), as well as, detect and suggest phylogenetic affinities of as yet uncultured organisms. On the other hand these procedures are labor intensive and time has to be invested in the production, screening and sequencing of a recombinant library (a necessary step when characterizing a new environment). There are also unquantified potential selections introduced in various steps of the procedure. As such, it is not yet possible to relate the recovery of different gene sequences to the relative abundance of different organisms (Findlay, 1996). Other molecular biology approaches have been developed most of them depending on the recovery of DNA from the environmental sample. Two basic approaches can be used for the recovery of DNA from environmental samples such as soils and sediments. An indirect extraction where the microorganisms are first separated by repeated differential centrifugation from most of the soil material before lysis. The recovery efficiency of this method is usually less than 50% of the bacterial cells present (Holben et al., 1988; Tiedje and Zhou, 1996). An alternative direct extraction approach can be used where microorganisms are lysed in the soil and the free DNA is extracted and purified but it is usually contaminated with different substances which might interfere with subsequent applications (Ogram et al., 1987; Tiedje and Zhou, 1996).

Phylogenetic gene probes can also be developed based on 16S rRNA gene sequences (Amann, 1990; Tiedje and Zhou, 1996). Once phylogenetic relationships of the interesting organisms are known, PCR primers and oligonucleotide probes can be designed specific to these organisms. The probes developed can be used for monitoring organism groups of interest in the environment.

A number of recent studies have involved different molecular techniques to study bacterial communities and their effects on various environmental processes. *In situ hybridization* (ISH) has been one of the techniques that have proven to be a very important molecular tool that has significantly advanced studies in different areas of research such as gene structure and expression at individual cell level. It has provided powerful means to determine the level of gene expression in individual cells and tissues, thereby allowing the investigators to assess and compare varying levels of gene expression within a population (Bagasra et al., 1995). However, the usefulness of ISH is occasionally limited by low detection sensitivity, requiring at least 20 copies of the target of interest per cell. This is a serious limitation because it prevents the detection and quantitation of target nucleic acid sequences present at levels that cannot be clearly distinguished from background signal, (Haase, 1987; Bagasra et al., 1995; Van Elsas and Wolters, 1995).

In situ hybridization (ISH) using radioactively or fluorescently labeled oligonucleotide probes uniquely complemented to 16S rRNA sequences has been widely used in conjunction with epifluorescence microscopy to identify prokaryotic cells in various natural communities at phylogenetic levels ranging from species to kingdom (Amman et al., 1990, 1992, 1995; DeLong et al., 1989; Giovannoni et al., 1988; Hahn et al., 1992; Hicks et al., 1992; Manz et al., 1993; Ramsing et al., 1996; Tsien et al., 1990; Zarda et al., 1997; Chen et al., 1998). Most applications of monolabeled rRNA-targeted probes have been restricted to bacterial populations in relatively nutrient-rich environments, such as activated sludge (Harmsen et al., 1996; Weiss et al., 1996; Chen et al., 1998). A major limitation of the ISH technique is that natural aquatic samples usually

do not contain sufficient cellular rRNA to yield detectable fluorescent signal (Chen et al., 1998). Several modifications have been attempted to increase the signal strength and the applicability of ISH for the characterization of microbial community structure in oligotrophic environments. Some of these modifications are the use of multiple rRNA targeted fluorescent probes (Lee & Kemp, 1994; Chen et al., 1998), polyribonucleotide probes (Trebesius et al., 1994; Chen et al., 1998) or stronger fluorescent dyes (Alfreider et al., 1996; Chen et al., 1998). However, whole-cell hybridization techniques with rRNA-targeted probes can only be used for taxonomic or phylogenetic identification of microbial communities, and cannot provide information on *in situ* genetic capabilities of individual bacterial cells within a microbial community (Chen et al., 1998). Even with further modifications, it is doubtful that ISH can be a reliable method to detect low copy number or single copy genes in individual cells.

The Polymerase Chain Reaction (PCR) on the other hand, is an extremely sensitive technique with the potential to amplify rare or single copy gene sequences to levels easily detectable by gel electrophoresis and/or Southern blot hybridization. The development of this technique has been a major step forward in the study of microorganisms in the environment via their DNA and/or RNA (Erlich, 1989; Tsai and Olson, 1992). The 16S and 23S rRNA genes have been used for phylogenetic analysis of prokakaryotic and eukaryotic organisms. The amplified ribosomal gene (rDNA) is subjected to restriction endonuclease digestion; a process that has been termed ARDRA (Amplified Ribosomal DNA Restriction Analysis (Vaneechoutte et al., 1992)). The resulting restriction fragment pattern is then used as a fingerprint for the identification of bacterial genomes. This method is based on the principle that the restriction sites on the

RNA operon are conserved according to phylogenetic patterns. Although ARDRA has been used for the characterization of bacterial isolates, in theory this method has been used for analyzing mixed bacterial populations for a quick assessment of genotypic changes over time or between different locations reflecting different environmental conditions (Massol et al., 1995, 1997; Cho and Tiedje, 2000). However, since conventional PCR requires cell or tissue destruction to isolate nucleic acids, one cannot associate the amplification results to a specific cell, or measure the percentage of cells that contain the target sequence (Komminoth and Long, 1995).

2.4 *In Situ* Polymerase Chain Reaction (IS-PCR) and *In Situ* Reverse Transcription Polymerase Chain Reaction (IRT-PCR)

Several studies have described this new molecular technique that combines the high sensitivity of PCR with the specific cytological localization of sequences obtained with ISH (Komminoth and Long, 1995). The combination of these techniques have been termed "*in situ* PCR" (Hodson et al., 1995; Tani et al., 2002), "PCR *in situ*" (Bagasra et al., 1993), "PCR *in situ* hybridization" (Nuovo et al., 1994a), "in cell PCR" (Embleton et al., 1992) or "PCR-driven ISH" (Patterson et al., 1993). For the detection of RNA sequences, an intracellular reverse transcription (RT) step has been added to generate cDNA from RNA templates prior to *in situ* PCR (Komminoth & Long, 1995). This modification of *in situ* PCR has been termed "*in situ* RT-PCR" (Komminoth et al., 1994; Chen et al., 1998; Holmstrøm et al., 1999) or less precisely "RT *in situ* PCR" (Nuovo, 1994a) or "*in situ* cDNA PCR" (Chen and Fuggle, 1993). The use of PCR in conjunction with ISH (*in situ* PCR or ISPCR) has allowed specific amplification of previously

undetectable sequences (Haase et al., 1990; Nuovo et al., 1991; Nuovo, 1994b; Bagasra et al., 1992, 1993a; Embretson et al,. 1993).

In situ PCR is a unique modification of PCR in which amplification and detection of specific nucleic acid sequences (DNA and/or RNA) are carried out inside individual cells rather than on bulk extracted nucleic acids (Nuovo, 1994a). It has a great advantage to be used in characterizing the microscale genetic, physiological, ecological and phylogenetic properties of natural microbial communities. Since individual genes, rRNA and mRNA are all candidate targets for *in situ* PCR, the genetic capabilities, expression of those capabilities, ecological distribution, interaction and abundance, and phylogenetic information are all accessible on the individual cell level. *In situ* PCR and RT-PCR have been successfully used mostly in eukaryotic cells in biomedical applications (Haase et al., 1990; Bagasra et al., 1992, 1993; Long et al., 1993; Komminoth et al., 1994; Bagasra and Hansen, 1997). Recently, *in situ* PCR / RT-PCR have been successfully used for detecting prokaryotic cells (Hodson et al., 1995; Kurokawa et al., 1997; Chen et al., 1998, 1999; Holmstrøm et al., 1999). *In situ* PCR have thus been successfully used to specifically amplify and detect single copy nucleic acid sequences in single cells as well as low copy DNA sequences in tissue sections (Long and Komminoth, 1995). More recently, Hodson et al. (1995) have worked on the development of the IS-PCR method to visualize the microscale distribution of specific genes and gene products in individual bacterial cells in microbial communities. Also *in situ* RT-PCR has been used successfully to detect the presence and expression of the *nahA* gene and the *todC1* gene in *Pseudomonas putida* cells (Hodson et al., 1995; Chen et al., 1999). These studies

suggest that *in situ* PCR methods can provide microbial ecologists with new insight into genetic diversity and activities of microbes at the single-cell level (Chen et al., 1998).

Detection of intracellular PCR products has been achieved by two different approaches: indirectly by ISH (indirect *in situ* PCR) or through direct detection of labeled nucleotides which have been incorporated into PCR products during thermal cycling (direct *in situ* PCR) (Figure 3).

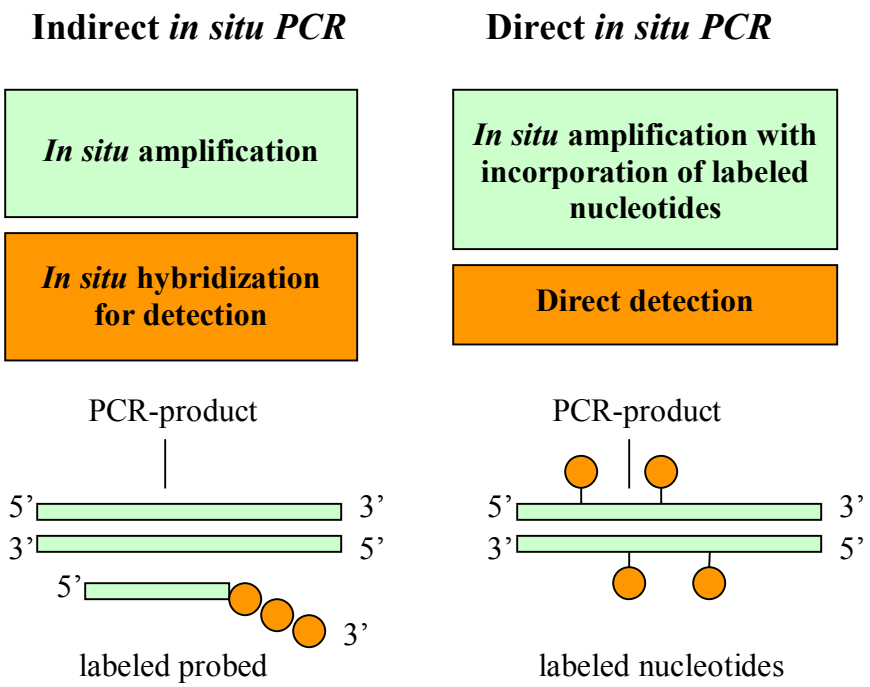

Figure 3. Direct and indirect *in situ* PCR detection methods (Komminoth and Long, 1995).

III. MATERIALS AND METHODS

3.1 Bacterial samples and growth conditions

Various control denitrifying bacterial strains including *P.stutzeri, P. aeruginosa, P. denitrificans, R. eutropha, A. brazilense,* which posses the *nir*S gene were used in this work. The *nir*K gene containing strain, *Pseudomonas sp.* G-179, was also used as a negative control. Other *nirS* positive denitrifying environmental isolates and clone cultures in *E. coli*, were also used throughout the optimization of *in situ* PCR and RT-PCR applications. The bacterial isolates and clones were obtained from Puget Sound, WA marine sediments (Figure 4) (Braker et al., 2000). Sediment and water samples were shipped on ice from Michigan State University. Clones were grown at 37°C on Luria-Bertani (LB) broth / agar broth (Difco, Detroit, MI) amended with ampicillin (Sigma, St. Louis, MO) at a final concentration of 100 μg/ml. They were incubated at 37°C with constant shaking at 150 rpm. For DNA extraction applications the bacterial cells were grown on LB. All denitrifying isolates and control strains were grown in nitrate broth/agar (pH 7.6) at 27°C for applications requiring denitrifying conditions and maintenance of the cultures. All of the microorganisms used in this study (Table 1) were provided by the Center for Microbial Ecology (CME) at Michigan State University, East Lansing, MI.

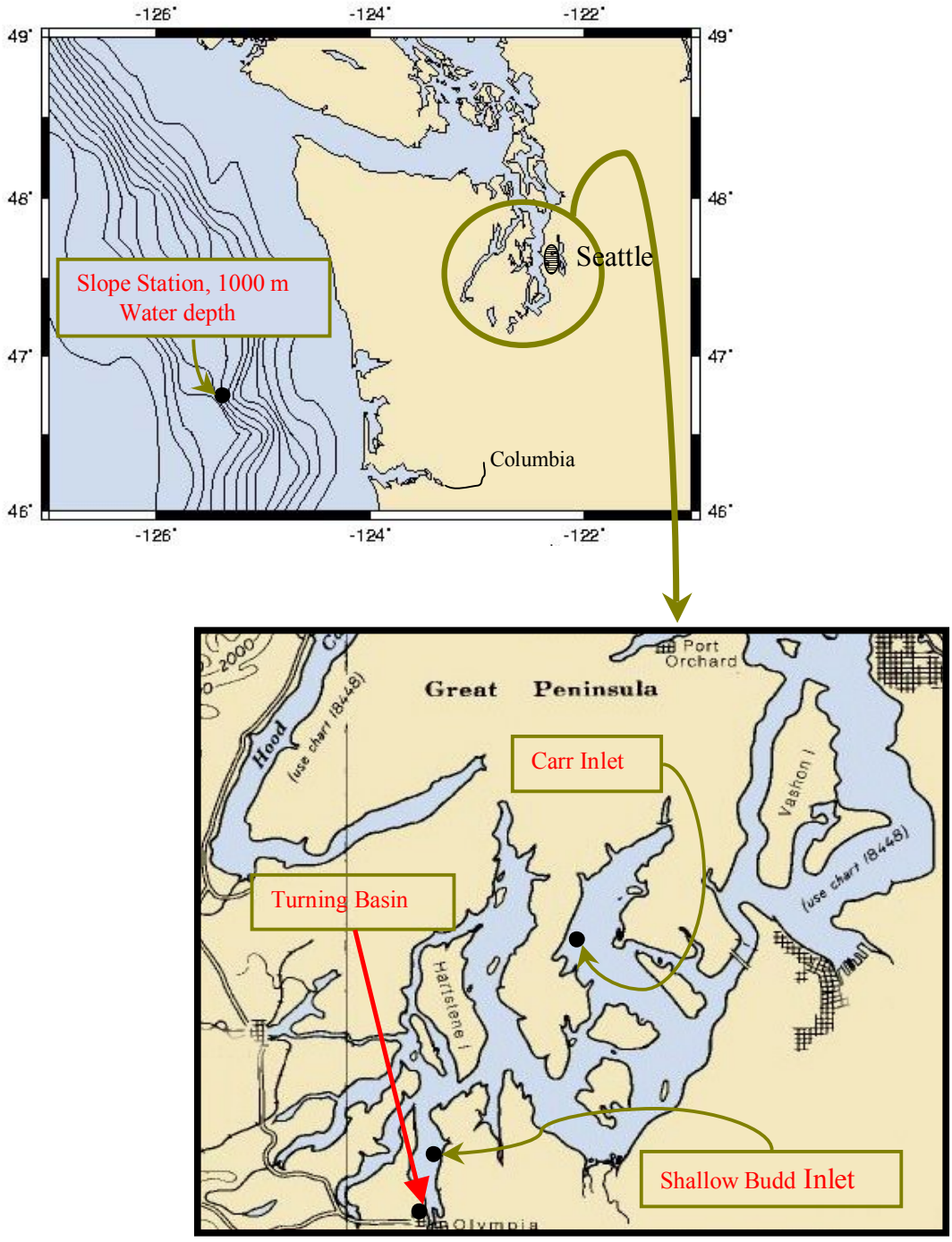

Figure 4. **Map of the Puget Sound, WA area. Marine sediment samples used in this research are from the Turning Basin area.**

Table 1. Bacterial strains used for *in situ* PCR and the RT-PCR protocol optimization.

Strains[a]	Source or Reference
E4-2 (99.5% *P. stutzeri*)[b]	Tiedje, 2000
Pseudomonas stutzeri JM-300[b]	Braker et al., 1998
D9-1 (100% *P. stutzeri*)[b]	Tiedje, 2000
F9-2 (99.4% *P. stutzeri*)[b]	Tiedje, 2000
A3-5 (99.3% *P. stutzeri*)[b]	Tiedje, 2000
(*E. coli* clone) pA17[b]	Tiedje, 2000
(*E. coli* clone) pA33[b]	Tiedje, 2000
(*E. coli* clone) pA63[b]	Tiedje, 2000
(*E. coli* clone) pA5[b]	Tiedje, 2000
(*E. coli* clone) pB6[b]	Tiedje, 2000
Pseudomonas stutzeri (ATCC 14405)[c]	Braker et al., 1998
Pseudomonas aeruginosa (DSM 6195)[c]	Braker et al., 1998
Paracoccus denitrificans Pd1222 (ATCC 19367)[c]	Braker et al., 1998
Azospirillum brasilense Sp7 (DSM 1690)[c]	Braker et al., 1998
Ralstonia eutropha[c]	Braker et al., 1998
Pseudomonas sp. Strain G-179 (M97294)[c]	Braker et al., 1998

[a] All bacterial strains possessed the *nirS* gene except *Pseudomonas sp.* Strain G-179 which has the *nirK* gene.
[b] Isolates and *E. coli* clones (pA17, pA33, pA63, pA5 and pB6) used for IS-PCR protocol optimization
[c] Bacterial strains used for the *in situ* RT-PCR protocol optimization

3.2 Extraction of genomic and plasmid DNA

A single colony of each bacterial sample (clones and isolates) was taken from 24 hour cultures and inoculated into 5-10 ml of LB broth. Ampicillin (100 μg/ml) was included in the media used to grow the *E. coli nirS* clones. The cultures were incubated at 27°C with constant shaking at 150 rpm for 24 hours. Cell harvesting was done by centrifugation at 14,000 rpm for 2 minutes in an Eppendorff microcentrifuge (Model 5415C). One hundred microliters of TE-sucrose (10 mM Tris, 1 mM EDTA pH 8.0, 25% sucrose) were added and the pellet was completely resuspended by vortexing. Then 40 μl of lysozyme ([5 mg/ml] (Sigma, St. Louis, MO) of 0.25 M Tris, pH 8.0), and 40 μl of 0.25 M EDTA were added to each sample. Mixing was done by vortexing followed by an incubation of 10 minutes at 37ºC. Subsequently 175 μl of sterile water, 50 μl of 10% SDS and 5 μl of ribonuclease A [10 mg/ml] (Sigma, St. Louis, MO) were added and vigorously by vortex. The tubes were incubated for 30 to 60 minutes at 37ºC. Ten microliters of Proteinase K [10 mg/ml] were added and mixed well (vortex) followed by an incubation at 37ºC for 30-60 minutes. We proceeded by adding 85 μl of TE buffer (10mM Tris, 1 mM EDTA pH 8.0 without sucrose), and 120 μl of 5M NaCl. The samples were mixed well (vortex) and incubated for 20 minutes at 65ºC. This step was followed by the addition of 440 μl of 8M potassium acetate and mixed by inverting the tube gently several times. The tubes were incubated on ice for 15 minutes and centrifuged at 14,000 rpm for 10 minutes. After centrifugation the clear liquid (bottom) was transferred to a new microcentrifuge tube avoiding the white material near the surface. The liquid was centrifuged again for 10 minutes at 14,000 rpm and the clear

liquid was transferred to a new tube avoiding again the white material near the surface. This last step was repeated twice. After this the tubes were filled with 95% ethanol and left for DNA precipitation overnight at room temperature. The next day the samples were spun at 14,000 rpm for 15 minutes and then proceeded to decant the supernatant very carefully with a smooth single movement. The DNA pellet was dried using a DNA Speed Vac device (DNA Speed Vac, DNA 110 Savant Instruments, Farmingdale, NY) device and finally the pellet was resuspended in approximately 50-500 µl of TE and stored at -20ºC. Genomic DNA and plasmid DNA was also extracted using the DNeasy™ Tissue Kit and the QIAprep® Miniprep extraction kits (QIAGEN Inc, Valencia, CA).

3.3 Bacterial cell fixation

Bacterial samples were grown as described before in 25 ml of the appropriate media. Cells were harvested by centrifugation at 5,000 rpm for 10 minutes and washed twice with 10 ml of phosphate-buffered saline (PBS: 120mM NaCl and 2.7 mM KCl in 10 mM phosphate buffer [pH 7.6]) (Hodson et al., 1995). Between washes cells were centrifuged at 5000 rpm for 5 min. Cells were resuspended in 10ml of freshly prepared 4% paraformaldehyde in PBS and incubated for 2-3 hours at on ice. The paraformaldehyde was prepared fresh by dissolving 4g of paraformaldehyde in 50 ml of 1X PBS pre-heated to 60ºC. While stirring two drops of sodium hydroxide 2M were added to facilitate the paraformaldehyde going into solution. The pH was adjusted to 7.6 and the volume was brought to 100 ml with 1X PBS prior to filter sterilizing the solution. After paraformaldehyde treatment cells were washed twice with 1X PBS and then

resuspended gently in 5 ml of 50% ethanol in 1X PBS. Fixed cells were stored in 300 µl aliquots at -20°C.

Bacterial cultures used for *in situ* RT-PCR were grown in nitrate broth at 27°C with constant shaking at 150 rpm until gas production was visible. Cells were pelleted by centrifugation at 7000 rpm for 10 min at 4°C. The pellet was resuspended in 2 ml of RNase free 1X PBS and 4ml of RNAprotect™ Bacteria Reagent (QIAGEN, Valencia, CA). The cells were mixed gently and incubated for 5 min at room temperature. The rest of the protocol for cell fixation is the same described earlier. As described by the QIAGEN® OneStep RT-PCR Kit Handbook, all solutions were treated to eliminate RNase, with 0.1% (v/v) DEPC. Solutions were incubated overnight at 37°C and autoclaved after the treatment to eliminate DEPC residues. All glassware was oven baked overnight at 240°C, and plasticware was thoroughly rinsed with 0.1 M NaOH, 1 mM EDTA, and DEPC treated dH_2O.

3.4 Bacterial Cell permeabilization

3.4.1 Enzyme treatment

An aliquot of 200 µl of the paraformaldehyde fixed cells was pelleted by centrifugation for 10 min. at 7,000 rpm. The cells were washed twice by resuspending them in 1X PBS, centrifuging between washes as described before. Pellet was resuspended in 90 µl of 1X PBS after the two washes. Cell wall permeabilization was achieved by treatment with lysozyme at a final concentration of 1 mg/ml for 15 minutes at room temperature. Lysozyme was removed by three consecutive washes with 1X PBS, and the pellet was resuspended in 90 µl after the last wash. Permeabilization was

completed by treatment with proteinase K at a final concentration of 1 µg/ml for 10 minutes at room temperature. Protease was inactivated by heating the samples for 2 minutes at 92°C and removed by 3 consecutive washes with 1X PBS. (Hodson et al., 1995). For in situ RT-PCR, the enzyme solutions were prepared in RNase free water and PBS was treated with DEPC as mentioned earlier.

3.4.2 Electroporation

We grew the bacterial cells in 500 ml of media using the same conditions described before. The cells were grown overnight up to an optical density of 0.5-0.6 OD. (8 x 10^9 cells/ml) and harvested by centrifugation at 4°C for 20 minutes at 3,000 rpm. This was followed by two washes with 50 ml of ice cold sterile water by shaking gently. Centrifuging between washes was done for 20 minutes at 4,200 rpm and 4°C. We proceeded by resuspending the cells in 1 ml of ice cold water if they were going to be used fresh or in 10% glycerol if they were going to be used stored. Fifty micro liters aliquots were then stored at -80°C in microcentrifuge tubes. The PCR master mix (40µl) and 10µl of the cells were mixed in an electroporation cuvette. Cell transformation was done using an electroporation apparatus (BIO-RAD Gene Pulser®, Hercules, CA), which was set to 2.5kV and 25µF and the pulse controller to 200 or 400 ohms according to the company specifications (www.biorad.com), in order to introduce the PCR reagents into the bacterial cells. After electroporation the 50µl of transformed cells and PCR reaction mixture were transferred to a thin-walled PCR tube and subjected to thermal cycling as described in section 3.5.1. Since fluorescein labeled nucleotides (direct IS-PCR) were

used with this permeabilization procedure, the slides were examined by fluorescent microscopy after thermal cycling.

3.4.3 Calcium Chloride ($CaCl_2$)

The culture conditions and the media, used for the calcium chloride permeabilization procedure, are exactly identical to the conditions mentioned previously. For this application the cells were grown overnight up to a 0.3-0.4 OD (4×10^9) and centrifuged for 7 minutes at 3,000 rpm (1,600 x g), at 4°C on a Beckman centrifuge (Model GS-15R). The cells were then resuspended gently in 50 ml of ice cold 1M $CaCl_2$. We kept the cells on ice for 30 minutes, after which they were spun down at 2500 rpm (1100 x g) for 5 minutes at 4°C. Cells were well, but carefully, resuspended in ice cold $CaCl_2$ before dispensing in microcentrifuge tubes in 25 µl aliquots which were immediately frozen. We mixed 10 µl of the treated cells with 40 µl of the PCR reaction mix and place the cells and the reaction mix on ice for 10 minutes. A heat shock was applied by incubating at 42°C, after which the cells were instantly placed on ice (Seidman et al., 1997).

3.5 *In situ* Polymerase Chain Reaction (IS-PCR)

In situ PCR was performed with all the cell samples and using the three methods of permeabilization described before. Two different approaches were used during the optimization trials of this technique which are discussed next. They were performed in PCR thin walled tubes (MicroAmp-Perkin Elmer) and on IS-PCR designed glass slides

(Applied Biosystems, Foster City, CA) using AmpliCover™ Discs and AmpliCover™ Clips to seal the reaction mixture.

3.5.1 Direct IS-PCR (One stage) and Indirect IS-PCR

For a more specific and / or efficient amplification of the *nirS* gene different sets of primers and primers combinations were tested (Table 2). The reactions for direct IS-PCR were prepared in a total volume of 50 µl. The reaction mixture contained 5 µl of 10X PCR buffer, 2.5 µl of PCR Fluorescein labeling Mix (Roche Molecular Biochemical, Indianapolis, IN), 5 µl bovine serum albumin (20 mg/ml, Sigma, St. Louis, MO), 10 pmol of each primer (Integrated DNA Technologies, Coralville, IA), 1 µl Taq polymerase (Promega, Madison, WI) and sterile water up to 50 µl. The reaction mixture and 10 µl of each of the fixed and permeabilized cells were heated separately at 70°C for 2 minutes. For the reaction in tubes, the cells were mixed with the reaction mixture in thin walled PCR tubes. The PCR reactions were done in an automated thermal cycler (Gene Amp PCR System 2400 Perkin Elmer, Norwalk, CT) using the following temperature and time profiles: 94°C for 5 min for initial denaturation, followed by 10 cycles of melting at 94°C for 30 sec, annealing at 56°C for 40sec (decreasing 0.5°C/cycle), extension at 72°C for 40 sec; this was followed by 25 additional cycles with the same conditions except the annealing temperature which was 54°C and a final extension step at 72°C for 7 min. After PCR cells were washed twice with PBS and resuspended in 50-100 µl of PBS depending on the amount of cells remaining. Two to five microliters of the cells were spotted onto microscope slides and allowed to air dry. Cells were serially dehydrated in

ethanol (50, 80, and 96%; each for 3 min) and dried at 50°C before viewing under epifluorescence microscopy. Alternatively, reactions were also done in slides, were 5-10 µl of the fixed cells were spotted onto the slides and air dried. Permeabilization treatment was performed on the slides as described before and then dehydrated in ethanol as done earlier. The heated reaction mixture was added to the cells on the slide and sealed using the assembly tool (Perkin Elmer, Norwalk, CT). The reactions were done in an automated in situ PCR thermal cycler (Gene Amp In Situ PCR System 1000 Perkin Elmer, Norwalk, CT) using the same PCR conditions previously described. Following completion of the PCR procedure cells were washed twice in PBS and the slides were viewed under an epifluorescence microscope (Olympus BH2-RFCA, Olympus, Japan). Photographs were taken using a digital SPOT Insight Color Camera and the corresponding SPOT Advanced software V 3.2 (Diagnostic Instruments, USA).

For the indirect IS-PCR procedure the same methodology used for direct IS-PCR was applied except that the deoxynucleotides triphosphate were not labeled, and an additional fluorescent *in situ* hybridization (FISH) detection step was required.

Table 2. Primers and Probe sequences and positions used for *in situ* amplification of *nirS gene* fragments and FISH.

Primer/ Probe[a]	Position[b]	Oligonucleotide Sequence (5'-3')	Reference
NIRS 1F	763-780	CCT AYT GGC CGC CRC ART	Braker et al., 1998
NIRS 6R	1638-1653	CGT TGA ACT TRC CGG T'	Braker et al., 1998
PSTUT	1251-1267	TTC CTB CAY GAC GGC GG[c]	Braker et al., 1998

[a] The primers are indicated by NIRS for the *nirS* gene; forward and reverse primers are indicated by the last letters F and R, respectively.
[b] Positions in the *nirS* gene of *Pseudomonas stutzeri* ZoBell EMBL X56813

3.6 *In situ* Reverse Transcription Polymerase Chain Reaction (*In situ* RT-PCR)

The bacterial strains used for the optimization of the in situ RT-PCR procedure are listed in Table 1. Pseudomonas G-179 was used as *nir*S negative control. Bacterial cultures were grown in Nitrate Broth at 28°C with constant shaking at 150 rpm until gas production was visible. Cells were pelleted by centrifugation at 7000 rpm for 10 min at 4°C. Fixation of the cells was done with RNase free materials as described before.

Fixed cells were permeabilized under RNase free conditions, and spotted into RNase free *in situ* PCR glass slides. Permeabilization was done only by enzyme treatment as shown in section 3.4.1. The samples were spotted into the slides, air dried, and dehydrated serially in RNase free ethanol. The bacterial samples were sealed with the RT-PCR mixture (Section 3.5.1) and *in situ* RT-PCR was performed using the QIAGEN® OneStep RT-PCR Kit.

Fluorescent *In Situ* Hybridization was done after *in situ* RT-PCR following the procedure described in the next section (Figure 5). Hybridization was carried out on the PCR thermal cycler for 3 hours at 40°C. All post-hybridization washes were done as previously described as well as the DAPI counterstaining step described later. and the slides were analyzed under epifluorescence microscopy.

3.7 Detection of amplified gene product by fluorescent *in situ* hybridization (FISH)

` After indirect IS-PCR or *in situ* RT-PCR, cells were washed and dehydrated on a microscope slide. Samples were subjected to a denaturation step by treatment with denaturation buffer (0.5M NaOH/1.5 M NaCl) at room temperature for 10 min, and subsequently neutralized using neutralizing buffer (1M Tris-HCl (pH 8.0) 1.5M NaCl)

for 5 min at room temperature. The cell spots were covered with a 1:10 dilution of a 50ng/µl cyanine dye Cy3™ (Integrated DNA Technologies) labeled probe (Table 2) in Dig-Easy hyb solution (Boehringer Mannheim GmbH, Germany). The hybridization mixture was then sealed using the assembly tool (Perkin Elmer, Norwalk, CT) to prevent evaporation of the reaction mixture and incubated in an in situ PCR thermal cycler (Gene Amp In Situ PCR System 1000 Perkin Elmer, Norwalk, CT) for 2 hours at 40°C. After hybridization, the slides were washed twice in 50 ml of pre-warmed hybridization buffer (900 mM NaCl, 20 mM Tris-HCl (pH 7.2) and 0.01% SDS) at 48°C for 20 min. Finally slides were washed in 50 ml of washing buffer (900mM NaCl/100 mM Tris-HCl (pH 7.2)) for 5 min at room temperature. Samples were counterstained in the dark with 4', 6-diamidino-2-phenylidole dihydrochloride (DAPI) at a final concentration of 1µg/µl for 5 min (Sigma, St. Louis, MO). Following the 5 min period slides were rinsed in dH_2O and 80% ethanol. Results were then observed by epifluorescence microscopy. For DAPI staining visualization an UG-1 filter (Olympus, Japan) was used. An XF108-2 filter set (Omega Optical, Brattleboro, Vermont) was used to visualize Cy3™ stained cells.

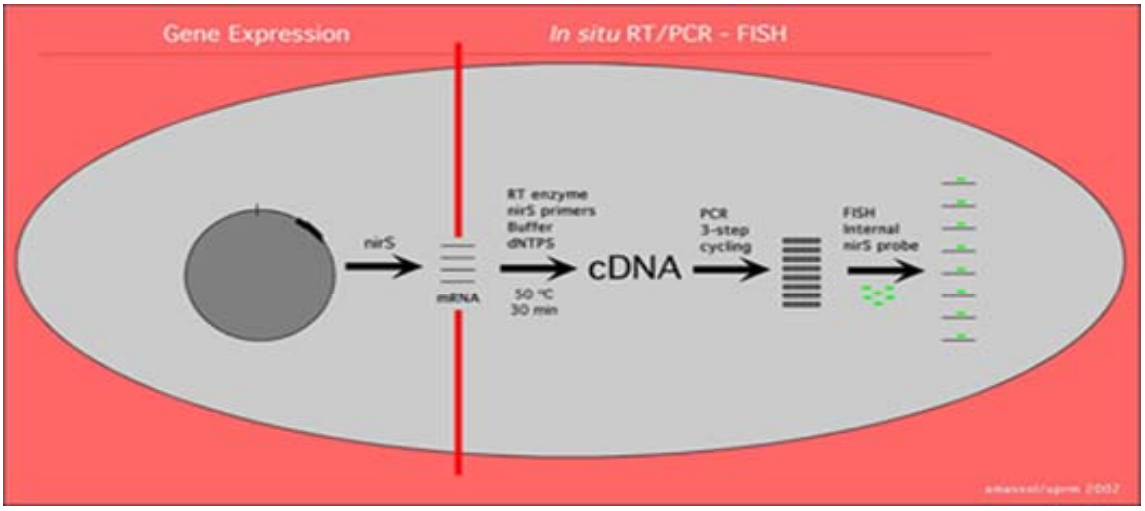

Figure 5. Graphical representation of the *in situ* RT-PCR reaction with indirect detection by FISH (Massol, 2002).

3.8 Marine sediment microcosms assembly

In order to isolate novel denitrifying bacteria various microcosms systems were prepared. Microcosms were assembled in autoclaved glass vials. They contained 10g (wet weight) of marine sediment from the Puget Sound Area in the Washington coast margin (Figure 4) and the headspace was filled with seawater from the same location. Water from the same location was autoclaved and amended with various concentrations of DMSO and nitrate (Table 3). Microscope slides were inserted in some of the microcosms for later RT-PCR analysis. After filling the microcosm vials they were air stripped with nitrogen gas and sealed with rubber stoppers and aluminum seals. Two sets of microcosms could be differentiated based on their incubation temperatures. One set was incubated at room temperature (25°C) and the other at 4°C for about 6 months. A total of 18 microcosms were assembled all incubated in the dark (Table 3).

3.9 Isolation of novel denitrifying bacteria

After a 6 month incubation period, the microcosms were shaken for 1 min. and left still for approximately 5 min. to let the large sediment particles settle. Samples of 3cc were withdrawn from 6 of the microcosms using a sterile syringe and replaced with 3cc of the respective amended sterile seawater. The microcosms used were selected based on visible gas production. A volume of 2.9cc was used for DNA extraction and a clone library generation. The remaining 0.1cc was used for the isolation of denitrifying bacteria. Serial dilutions were performed from 10^{-1} to 10^{-5} in microcentrifuge tubes containing 0.9ml of sterile seawater. Isolation was achieved by the spread plate method by plating the 10^{-2} to 10^{-6} dilutions in Nitrate Agar (Difco, Detroit, MI) at a 7.6 pH.

Plates with Nitrate Agar using seawater were also used for the isolation of denitrifying strains by four way streaking from the 10^{-2} to 10^{-5} serial dilutions in duplicates incubated at 25°C and 4°C.

3.10 Determining nitrate reduction capabilities of isolates

After isolation, the nitrate reduction capability of the isolates was determined. Culture tubes with 10 ml of Nitrate Broth (Difco, Detroit, MI) at pH 7.6 were prepared with an inverted Durham tube for gas production visualization. All isolates were incubated with constant shaking at 150 rpm at 28°C for 24 hours to one week. After the incubation period a nitrate reduction test was performed which consisted of the addition of 2-3 drops of reagent A (0.8% solution of sulfanilic acid in 5N acetic acid) and 2-3 drops of reagent B (0.5% solution of dimethyl-α-naphtylamnine in 5N acetic acid) to approximately 5 ml of each of the isolate cultures. Immediate formation of a red color demonstrates the presence of nitrite; this is indicative of the reduction of nitrate to nitrite. The Durham tube was observed for gas (nitrogen) production. To the tubes in which results were negative a small amount of zinc dust was added and observed for red color development (indicates a negative reaction for nitrate reductase). Samples that remained uncolored after addition of zinc dust indicated that nitrate was reduced beyond the nitrite stage to either ammonia or nitrogen gas (Atlas et al., 1995).

Table 3. Marine sediment microcosm systems for the isolation of novel denitrifying bacteria.

Microcosm ID	Incubation Temperature (°C)	Sample[a] depth (cm)	Carbon Source (DMSO %)[b]	Nitrogen Source (NO_3^- %)[b]
T5 a	25°C	2 - 2.5	_____	0.1 %
T5 b	25°C	2 - 2.5	5 %	0.1 %
T11 a	25°C	5 - 5.5	_____	0.1 %
T11 b	25°C	5 - 5.5	5 %	0.1 %
T14 a	25°C	8 - 8.5	5 %	_____
T14 b	25°C	8 - 8.5	5 %	0.1 %
T5 a	4°C	2 - 2.5	_____	0.1 %
T5 b	4°C	2 - 2.5	5 %	0.1 %
T5 c	4°C	2 - 2.5	5 %	0.5 %
T5 d	4°C	2 - 2.5	5 %	_____
T11 a	4°C	5 - 5.5	_____	0.1 %
T11 b	4°C	5 - 5.5	5 %	0.1 %
T11 c	4°C	5 - 5.5	5 %	0.5 %
T11 d	4°C	5 - 5.5	5 %	_____
T14 a	4°C	8 - 8.5	_____	0.1 %
T14 b	4°C	8 - 8.5	5 %	0.1 %
T14 c	4°C	8 - 8.5	5 %	0.5 %
T14 d	4°C	8 - 8.5	5 %	_____

[a] All sediment and water samples used to prepare the microcosms are from Turning Basin (Puget Sound, WA). Ten grams (wet weight) of sediment were added to each microcosm.
[b] DMSO and NO_3^- percentages are the final concentration in the microcosm systems.

3.11 Amplified ribosomal DNA restriction analysis

Nucleic acid was extracted from cell pellets of bacterial isolates were gas production was visible and a positive result for nitrate reduction was observed, using the QIAGEN DNeasy^{TM} Tissue Kit (QUIAGEN Inc, Valencia, CA). Genes encoding for the 16S rRNA were amplified from the extracted DNA via PCR using universal primers with the following sequences, forward primer 8F (5'-AGA GTT TGA TCM TGG CTC AG-3'), and the reverse primer 1392R (5'-ACG GGC GGT GTG TAC A-3'). The forward primer corresponds to positions 8-27 in *E. coli* 16S rRNA, and the reverse primer is complementary to positions 1392-1376 in *E. coli* 16S rRNA.

In vitro amplification was carried out in a total reaction volume of 100 µl containing 10 µl of 10X PCR buffer, 6 µl of 25 mM $MgCl_2$, 1µM of each primer, 250 µM of each deoxynucleotide triphospate (TaKaRa Biomedicals, Japan), approximately 100 ng of template DNA and 2.5 U of Taq polymerase (Promega, Madison, WI). The PCR reactions were done in an automated thermal cycler (2400 Perkin Elmer Cetus) using the following temperature and time profiles: 92°C for 1 min 30 sec, for initial denaturation, followed by 30 cycles of melting at 92°C for 1 min 30 sec, annealing at 51°C for 30 sec, extension at 72°C for 1min 30 sec and final extension at 72°C for 7 min (Rodríguez, 1998). In general aliquots containing 12-16 µl of the PCR products were digested with *Hinf* I, *Hae* III, and *Rsa* I restriction enzymes (Promega, Madison, WI). Digests were carried out at 37°C for 3 hours and the resulting DNA fragments were electrophoresed at 60V in a 3% Metaphor (FMC, Rockland, Maine) agarose gel made in 1X TAE. Gels were stained in a 1 µg/ml ethidium bromide solution for 30 min and destained for 30 min in 1X TAE.

The size of the bands obtained from the digestions were estimated using a λ / Hind III molecular size marker and corroborated using the Gel-Pro® Analyzer 3.1 software. Band patterns obtained from the bacterial isolates were compared to each other and to six well-characterized isolates in terms of the number and size of the bands generated by the enzymes for each digestion. The Microsoft® Excel 2002 software was used to organize the ARDRA band patterns based on the absence or presence of a band. A scale of band size in base pairs was done in 50 –100 bp increments ranging from 100 bp to 1600 bp. A value of 1 was assigned as positive for the presence of a band for a given band size, and a zero for absence of a band for a given band size. These binary data was then analyzed using the SYSTAT® 9 software to perform a hierarchical cluster analysis to construct a dendogram. Reference strains used for this analysis are shown in Table 1.

3.12 Analysis of 16S rDNA sequences

Genomic DNA was extracted from several of the denitrifying isolates (I-19, I-49, I-54, I-60, I-62A, I-73, I-74, I-75, I-78, and I-80). The 16S rDNA gene was amplified as described earlier. The PCR products were sent, overnight in dry ice, to the Genomics Technology Support Facility (GTSF) at Michigan State University. The PCR products were sequenced with an automated ABI PRISM® 3100 Genetic Analyzer. Sequences were directly accessed through the GTSF website (www.gemonics.msu.edu). The isolates were phylogenetically characterized by gene sequence comparison with the online database, of the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov), using the Basic Local Alignment Search Tool (BLAST®). In

addition, these 16S rDNA sequences were evaluated with the PHYLIP® interface program of the Ribosomal Database Project II (www.rdp.cme.msu.edu).

IV. RESULTS

4.1 Isolation of novel denitrifying bacteria from marine sediment microcosms

A total of 18 Puget Sound marine sediment microcosm systems were visually evaluated for denitrifying activity based on gas production. Out of those 18 microcosms, 6 were selected for further characterization including T5b, T11b, T5c, T11c, T14b, and T14c (Table 3).

A total of 82 strains were isolated using nitrate agar prepared with distilled water and nitrate agar prepared with marine water from the sampling site. Out of the 82 isolates, 58 were positive for the nitrate reduction test. Eighteen of the 58 isolates reduced nitrate to nitrite or further (six of them passed nitrite reduction) with concurrent gas production observed inside the Durham tubes, therefore these cultures could be considered as true denitrifiers (Nogales et al. 2002). The other 40 strains, which are capable of nitrate reduction without gas production, could be considered as nitrate reducers or ammonifiers (Table 4). Eight strains were isolated at 4°C all on nitrate agar prepared with sterile seawater from Puget Sound, from which 2 could be considered as denitrifiers and the remaining 6 as nitrate reducers or ammonifiers according to the previous analysis. The remaining 74 strains were isolated at room temperature, 11 of them with nitrate agar prepared with Puget Sound seawater.

Table 4. Bacterial Isolates from marine sediment microcosms and determination of nitrate reduction capabilities.

Isolate ID[d]	Microcosm	NO_3^- reduction test [c]	Gas production
I-1	T5b	+	-
I-2	T5b	+	-
I-3	T5b	+	-
I-4	T5b	+	-
I-5	T5b	-	-
I-6	T5b	-	-
I-7	T5b	+	-
I-8	T5b	-	-
I-9	T5b	+	-
I-10	T5b	+	-
I-11	T5b	-	-
I-12	T5b	+	-
I-13	T5b	+	-
I-14	T5b	-	-
I-15[b]	T5b	-	-
I-16[b]	T5c	+	-
I-17[b]	T5c	-	-
I-18[b]	T5c	+	-
I-19	T5c	++	+
I-20	T5c	-	-
I-21	T5c	-	-
I-22	T5c	+	-
I-23	T5c	+	-
I-24	T5c	+	-
I-25	T5c	+	-
I-26	T5c	-	-
I-27	T5c	+	-
I-28	T11b	+	-
I-29	T11b	-	-
I-30	T11b	+	-
I-31	T11b	+	-
I-32	T11b	+	-
I-33	T11b	-	-
I-34	T11b	+	-
I-35	T11b	+	-
I-36	T11b	-	-
I-37	T11b	-	-
I-38	T11b	+	-
I-39	T11b	**N/G**	**N/G**
I-40	T11b	-	-
I-41	T11b	-	-
I-42	T11b	-	-
I-43	T11b	+	-
I-44	T11c	+	-
I-45	T11c	-	-
I-46	T11c	+	-

I-47	T11c	-	-
I-48	T11c	+	-
I-49	T11c	+	+
I-50	T11c	+	-
I-51	T11c	+	-
I-52[b]	T11c	+	-
I-53[b]	T11c	+	-
I-54	T11c	+	+
I-55	T11c	+	+
I-56[b]	T14b	**N/G**	**N/G**
I-57[b]	T14b	+	-
I-58	T14b	-	-
I-59	T14b	+	-
I-60A	T14b	++	+
I-60B	T14b	++	+
I-61	T14b	+	+
I-62A	T14b	+	+
I-62B	T14b	+	+
I-63	T14b	-	-
I-64[b]	T14c	+	+
I-65[b]	T14c	++	+
I-66[b]	T14c	+	+
I-67	T14c	++	+
I-68	T14c	++	+
I-69	T14c	+	-
I-70	T14c	+	+
I-71	T14c	**N/G**	**N/G**
I-72	**T14c**	++	+
I-73[a, b]	T5c	+	-
I-74[a, b]	T5c	+	+
I-75[a, b]	T5c	+	-
I-76[a, b]	T5c	+	-
I-77[a, b]	T5c	+	+
I-78[a, b]	T5c	+	-
I-79[a, b]	T11c	+	-
I-80[a, b]	T14b	+	-

[a] Strains isolated at 4°C, the rest of the isolates were grown at room temperature.
[b] Grown in Nitrate agar prepared with seawater. The others were grown in Nitrate agar prepared with distilled water
[c] ++ = nitrate reduction passed nitrite; N/G = No growth
[d] Isolates used for ARDRA analysis are indicated in red (bold)

4.2 ARDRA and 16S sequence analysis of bacterial isolates and control strains

Genomic DNA was extracted from 23 of the denitrifying isolate cultures and the 6 reference strains were provided by the CME (Michigan State University, East Lansing, MI)(Table 4; Figure 6). The DNA was of high molecular mass (> 23.1 kb) amplifiable and digestible by restriction endonucleases. The rDNA of each strain (control strains and denitrifying isolates) was amplified as described earlier and a band of approximately 1.5-kb (Figure 6) was observed as determined by visual comparison with the pGEM® DNA marker (Promega, Madison, WI). Each of the reference strains showed a characteristic restriction fragment pattern with the amplified rDNA when digested with each of the restriction enzymes: *Rsa* I, *Hinf* I, and *Hae* III (Figure 7). Furthermore, the rDNA of each isolated strain was digested as well with the same endonucleases. Various distinctive restriction fragment patterns were observed for the isolates (Figure 8 and 9).

The combined *Rsa* I, *Hinf* I, and *Hae* III restriction patterns of the amplified 16S rDNA region of the isolates and denitrifying control strains, were used for cluster analysis using the SYSTAT® 9 software. From the cluster analysis 8 groups could be differentiated within the 25°C isolates and 6 groups within the 4°C denitrifying isolates (Figure 10). As demonstrated by the cluster analysis, it seems that the microcosm isolates are considerably different from the reference strains. And a higher diversity could be observed within the 4°C isolates were 8 strains were grouped in 6 clusters. On the other hand the 25°C isolates consisted of 15 strains distributed between 8 clusters. The dendogram also showed a clear distinction between 4°C and 25°C groups of isolates. All

25°C isolates were grouped relatively close together as well as the 4°C isolates. Isolate 19 (I-19) was the only 25°C strain grouped within the 4°C isolates.

The partial rDNA gene sequence of representative denitrifying isolates was obtained (Appendix 1). A phylogenetic analysis was performed by using the PHYLIP® interface program of the Ribosomal Database Project II (RDP II). The phylogenetic analysis results of both, the isolated strains and reference cultures are presented in Figure 11. These results confirmed the information obtained by ARDRA, even though three of the isolates were more closely related to *P. stutzeri* (I-60, I-62A and I-80) than previously thought. The isolated cultures at 25°C and 4°C were grouped in different clusters. Strain I-19 was phylogenetically related to other strains isolated at 4°C, while I-80 closely grouped with the 25°C isolates. More importantly, most of the isolates were considerably different from any of the reference strains, as 5 of 10 sequences were identified as previously uncultured bacteria.

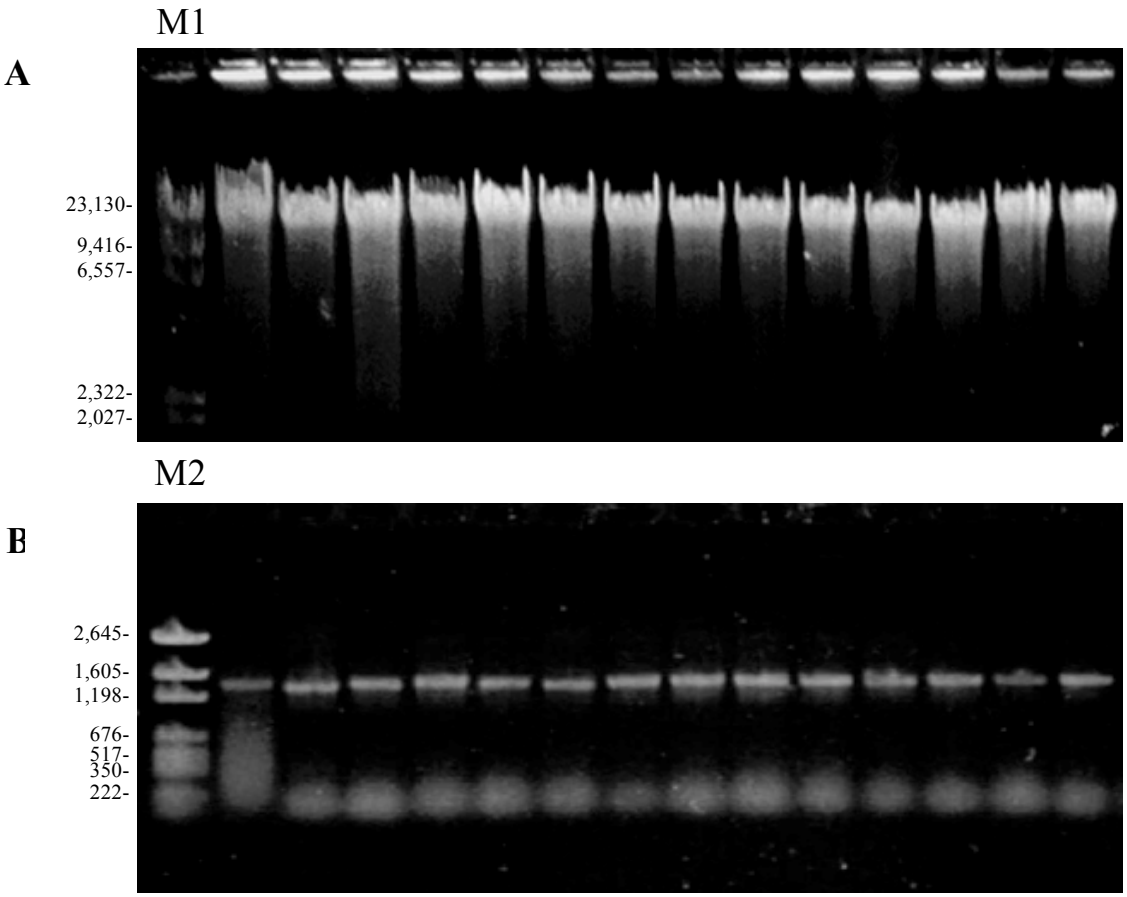

Figure 6. (A) Agarose gel electrophoresis of genomic DNA isolated from denitrifying isolates. Lane M1 corresponds to lambda/*Hind* III DNA marker (Promega). (B) Amplification of the 16S rRNA gene from the isolates extracted DNA. Lane M2 corresponds to pGEM® DNA Marker (Promega). The sizes of marker DNA fragments (in base pairs) are indicated on the left.

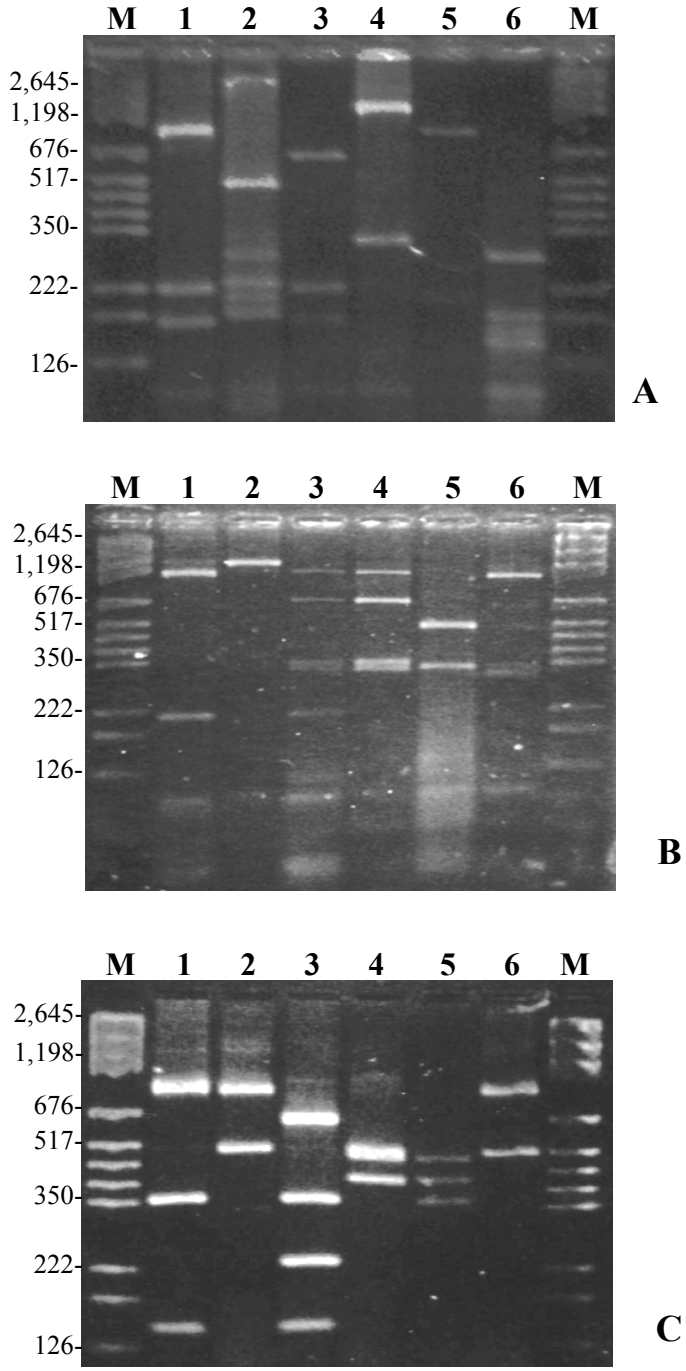

Figure 7. ARDRA patterns of the reference strains obtained with (A) *Hae* III, (B) *Hinf* I, and (C) *Rsa* I. Lanes 1 to 6 correspond to *P. stutzeri, Pseudomonas* sp. G-179, *P. aeruginosa, P. denitrificans, R. eutropha, A. brazilense*, respectively. Lanes M corresponds to the pGEM® DNA marker. The sizes of marker DNA fragments (in base pairs) are indicated on the left.

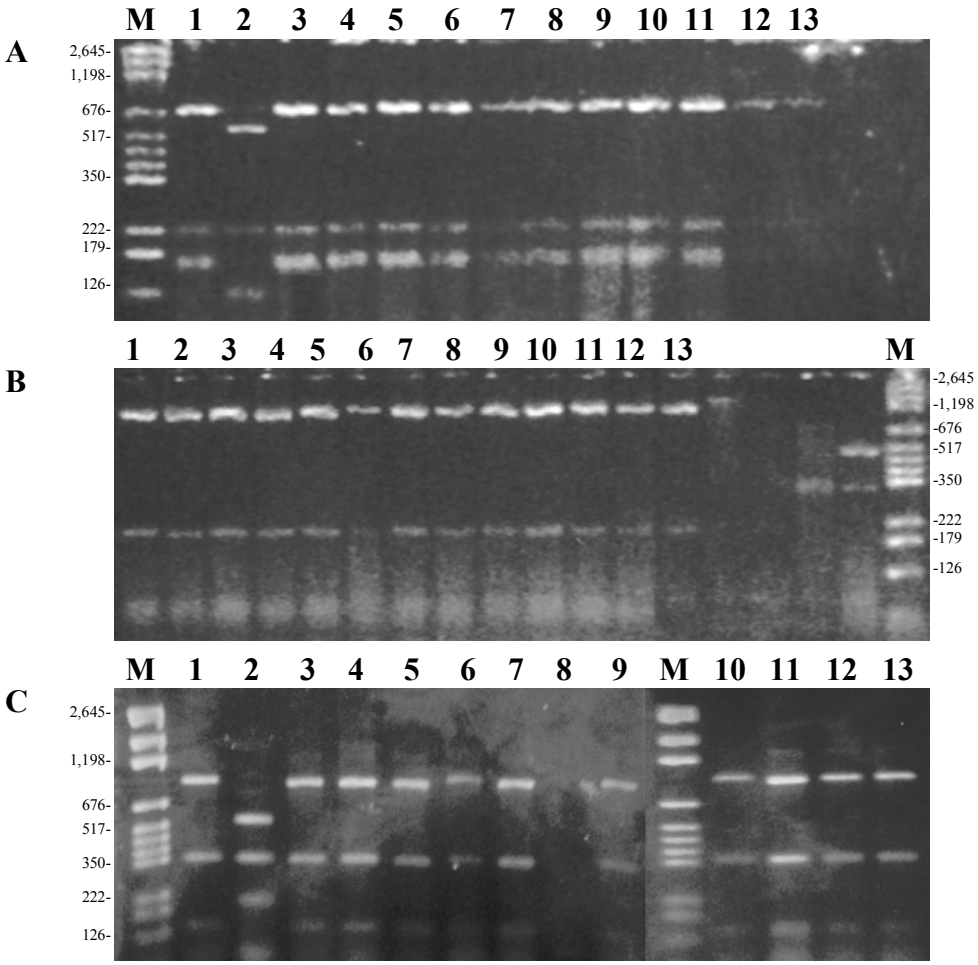

Figure 8. ARDRA patterns of the bacterial isolates from the 25°C microcosms digested with *Hae* III (A), *Hinf* I (B) and *Rsa* I (C). For gel (A) samples 1-13 correspond to I-49, I-54, I-55, I-60A, I-60B, I-61, I-62A, I-64, I-66, I-67, I-68, I-70, and I-72 respectively. For gel (B) samples 1-13 correspond to samples I-49, I-54, I-55, I-60A, I-60B, I-61, I-62A, I-64, I-66, I-67, I-68, I-70, and I-72 respectively. For gel (C) samples 1-13 correspond to samples I-49, I-54, I-60A, I-60B, I-61, I-62A, I-64, I-65, I-66, I-67, I-68, I-70, and I-72 respectively. Lanes (M) correspond to the pGEM® DNA Marker (Promega). The sizes of the marker DNA fragments (in base pairs) are indicated on the side of the gels.

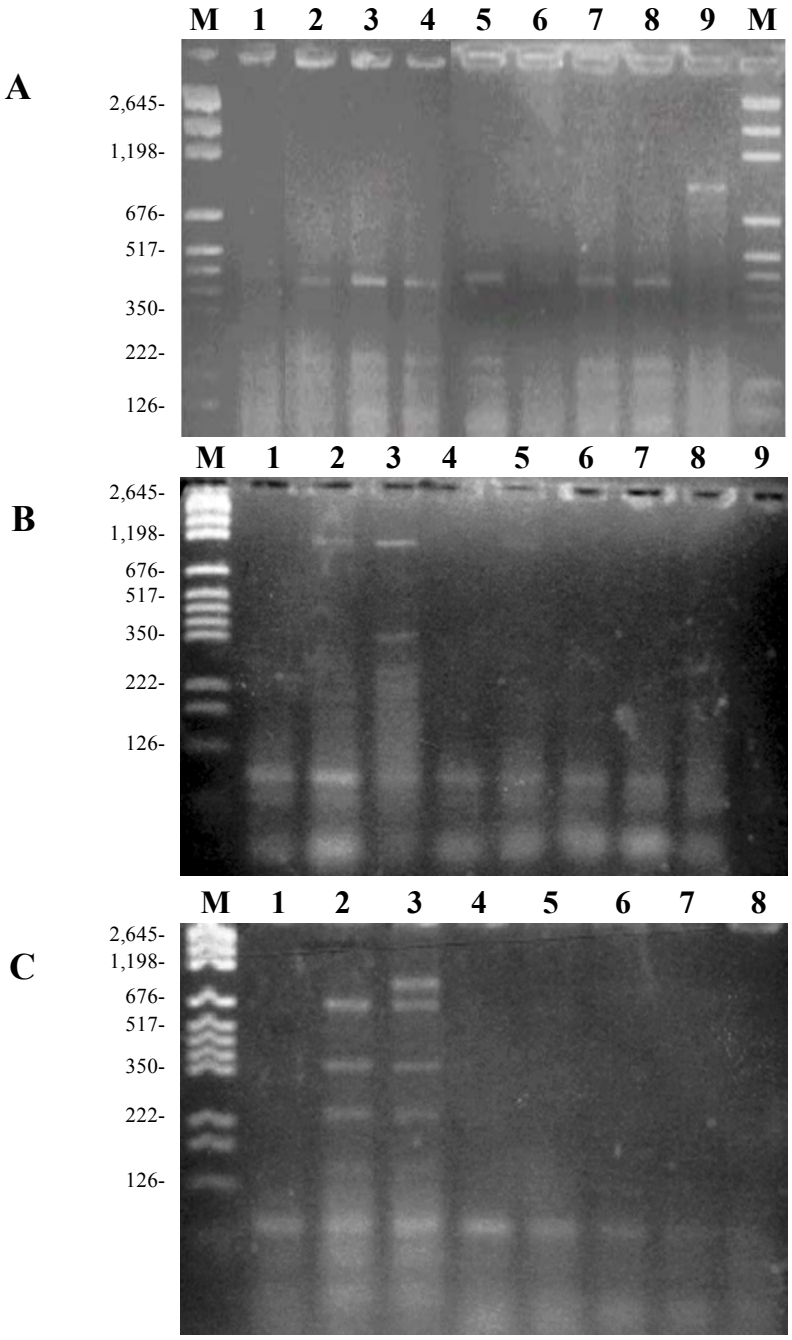

Figure 9. ARDRA patterns of isolates from 4°C microcosms digested with *Hae* III (A), *Hinf* I (B) and *Rsa* I (C). Samples 1-9 correspond to I-19, I-73, I-74, I-75, I-76, I-77, I-78, I-79, and I-80 respectively. All except I-19 were isolated at 4°C. Lanes (M) correspond to the pGEM® DNA Marker (Promega). The sizes of marker DNA fragments (in base pairs) are indicated on the left.

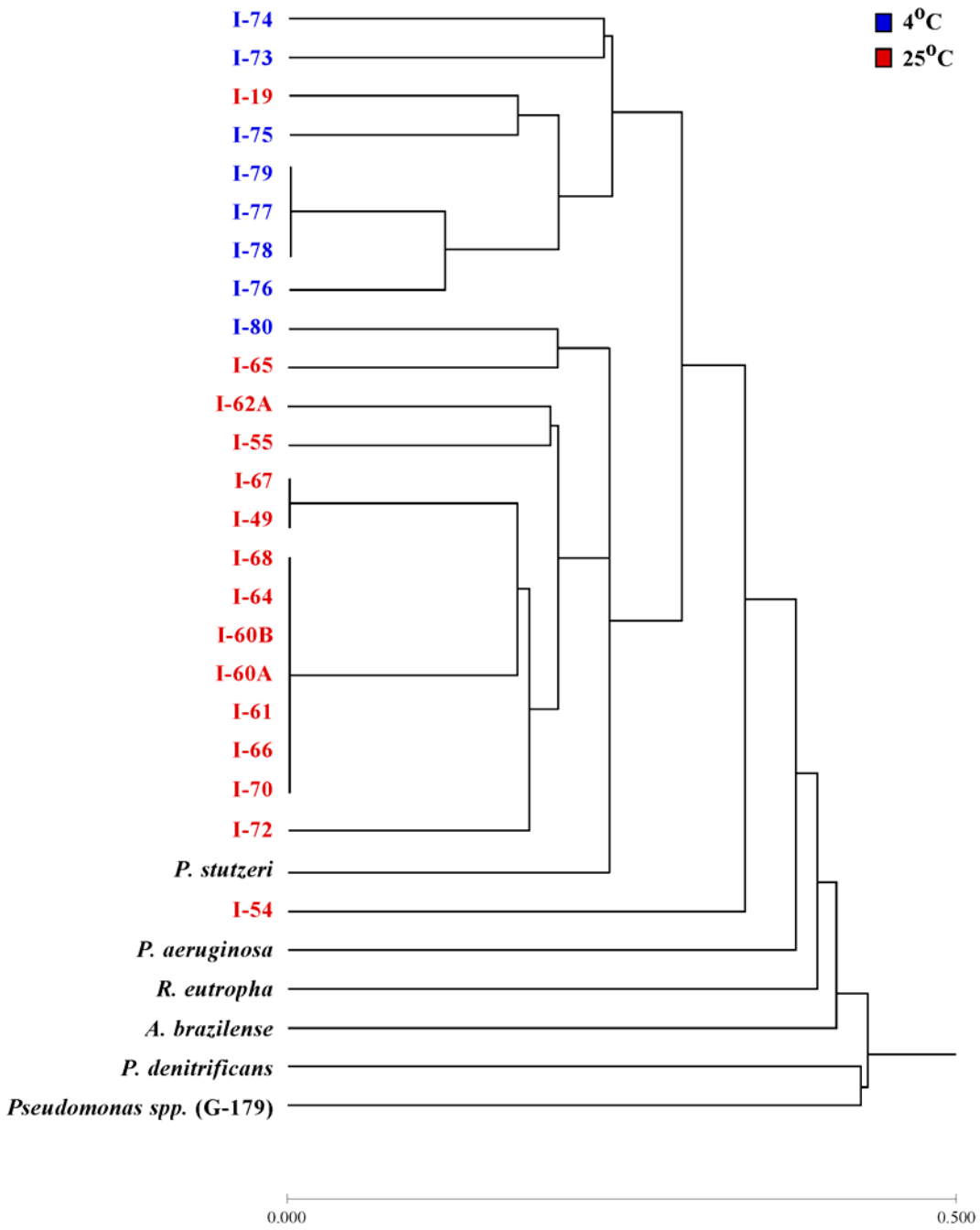

Figure 10. Results of cluster analysis generated from the 16S ARDRA patterns.

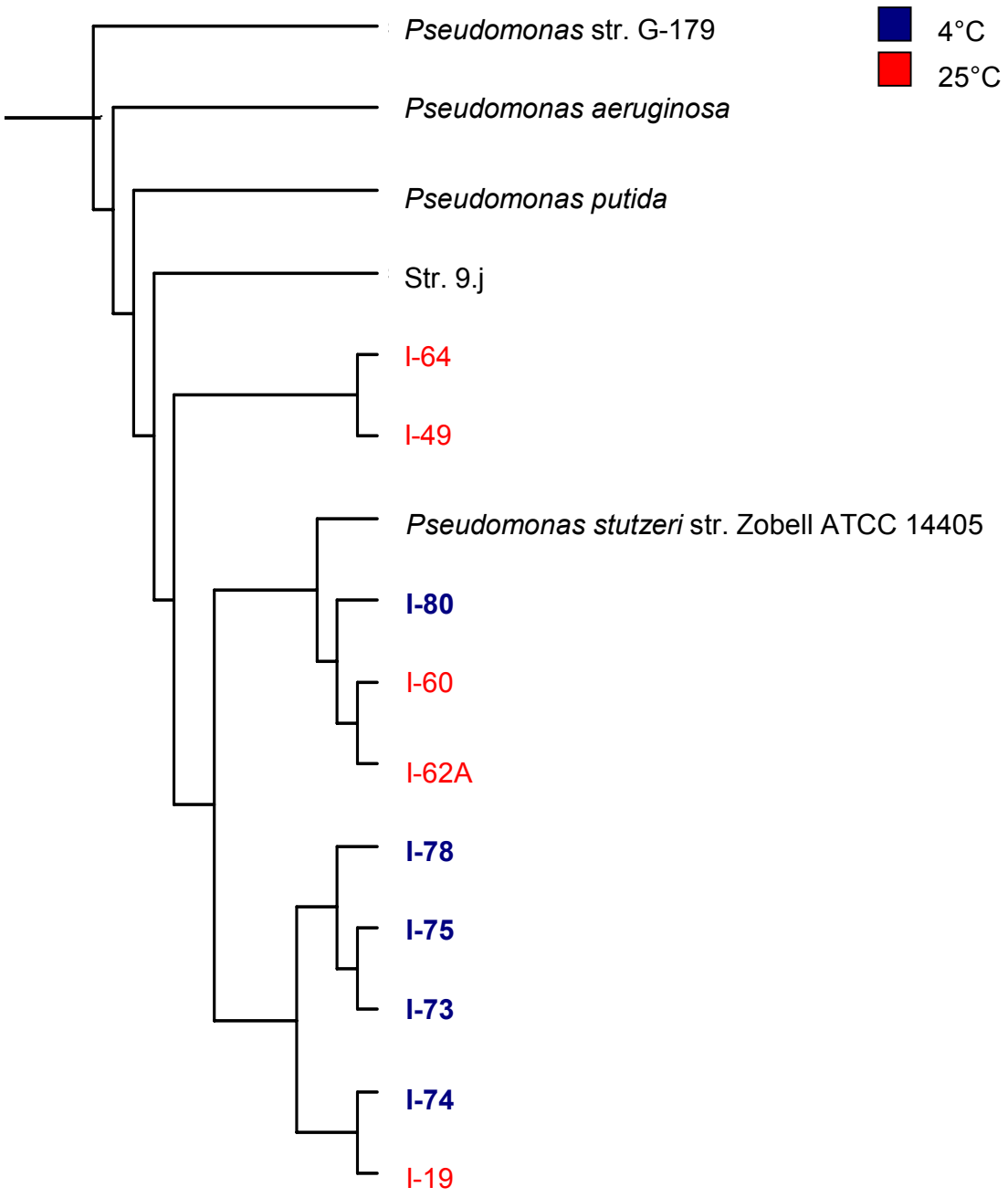

Figure 11. Cluster analysis generated with the 16S rDNA sequences.

4.3 *In situ* gene amplification

Prior to any *in situ* PCR procedure, control bacterial strains were used to test the NIRS 1F and NIRS 6R primer set for *nirS* gene amplification with pure DNA. A PCR product of 890 bp was obtained after PCR amplification of positive *nirS* gene bacterial samples using the NIRS 1F and NIRS 6R (Braker et al., 1998) primers and also unspecific amplification was observed in *E. coli* cells (Data not shown). Considerable homology was observed between NIRS 1F and NIRS 6R primers with pUC 19 sequences (Figure 12). After this drawback was identified new primer sets and probes were designed by Ricardo Maggi and tested with nirS positive cells and negative controls.

The use of fluorescein labeled nucleotides was the first approach taken in order to optimize the *in situ* PCR conditions. This approach is referred to as direct *in situ* PCR. All results showed a relatively weak and rapidly fading signal while false positive results were also commonly observed (Figure 13). A new approach had to be taken in order to get a more specific detection.

The PSTUT internal probe labeled at the 5' terminal with the Cy3 fluorochrome was used for hybridization with extracted DNA fixed to a microscope slide, after amplification with the NIRS 1F and NIRS 6R primers. This step was performed to determine if the low specificity observed with the *in situ* reactions was a result of the cellular components, which might interfere with the *in situ* reaction. The results demonstrated that DNA extracts essays were more specific probably because of less interference of other materials on the PCR and *in situ* hybridization reactions. Positive results were observed for several of the *nirS* containing strains and negative results were

observed for pUC19 DNA (Data not shown). These results showed that an *in situ* hybridization step must be performed in order to achieve a more specific detection.

An indirect *in situ* PCR procedure was used in order to increase the detection specificity. Regular nucleotides were used for the *in situ* PCR reaction and a FISH step with 5' labeled fluorescent probe(s) internal to the PCR amplicon was performed after the PI-PCR amplification. Three different permeabilization methods were tested to introduce the PCR reaction reagents inside the cells: (1) enzyme treatment, (2) electroporation and (3) calcium chloride treatment (Section 3.4). Results showed that all the permeabilization approaches worked, but false positive results were obtained with all of them (Figure 14). Up to this moment enzyme treatment was the on permeabilization method commonly used for *in situ* PCR applications. Our results showed that other methods, like electroporation, could be further optimized for faster and more economic permeabilization procedures. Calcium chloride permeabilization seemed to be as efficient as electroporation for *in situ* PCR permeabilization.

The false positive results made us think about the possibility that the labeled probe was just being retained inside the cells instead of being hybridized to an unspecific target. To test this hypothesis, *nirS* positive and negative cells were subjected to electroporation with a solution containing only the labeled probe. These cells were not treated for denaturation to be sure the probe was not being retained inside the cells. There was no signal for any of the cells after fluorescent microscopy examination (Data not shown).

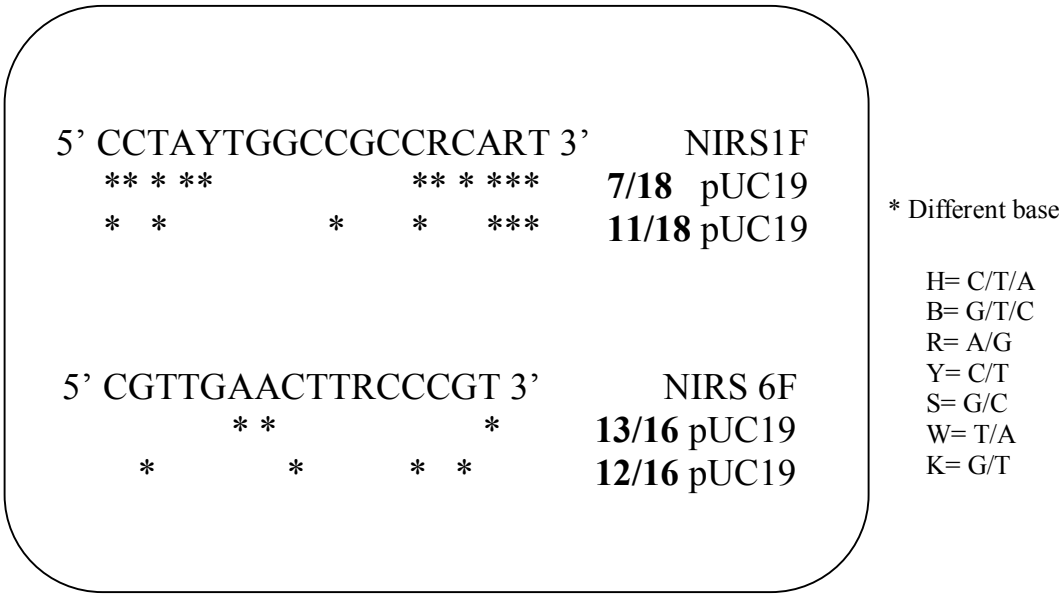

Figure 12. Sequence comparison of the NIRS 1F and NIRS 6R degenerate primers with pUC19. The lack of specificity resulted in unspecific amplification when trying to amplify the nitrite reductase (Nirs) gene.

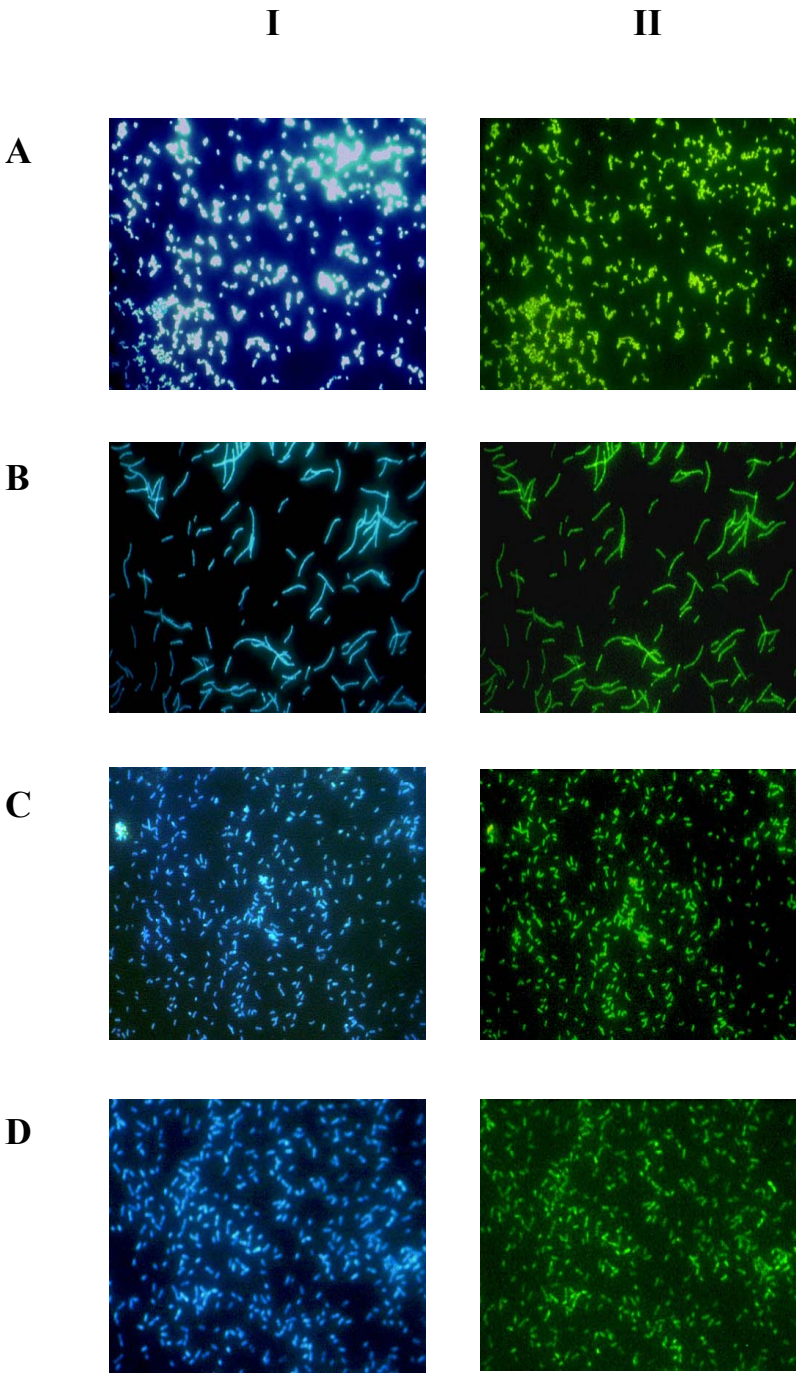

Figure 13. DAPI counterstained cells (column I) and direct IS-PCR detection of the *nirS* gene using fluorescein labeled nucleotides (column II) of denitrifying bacterial cultures. (A) *P. denitrificans*, (B) *R. eutropha*, (C) *P. stutzeri*, and (D) *Pseudomonas sp.* (G-179), which does not have the *nir*S gene. Images on columns I and II correspond to the same view field.

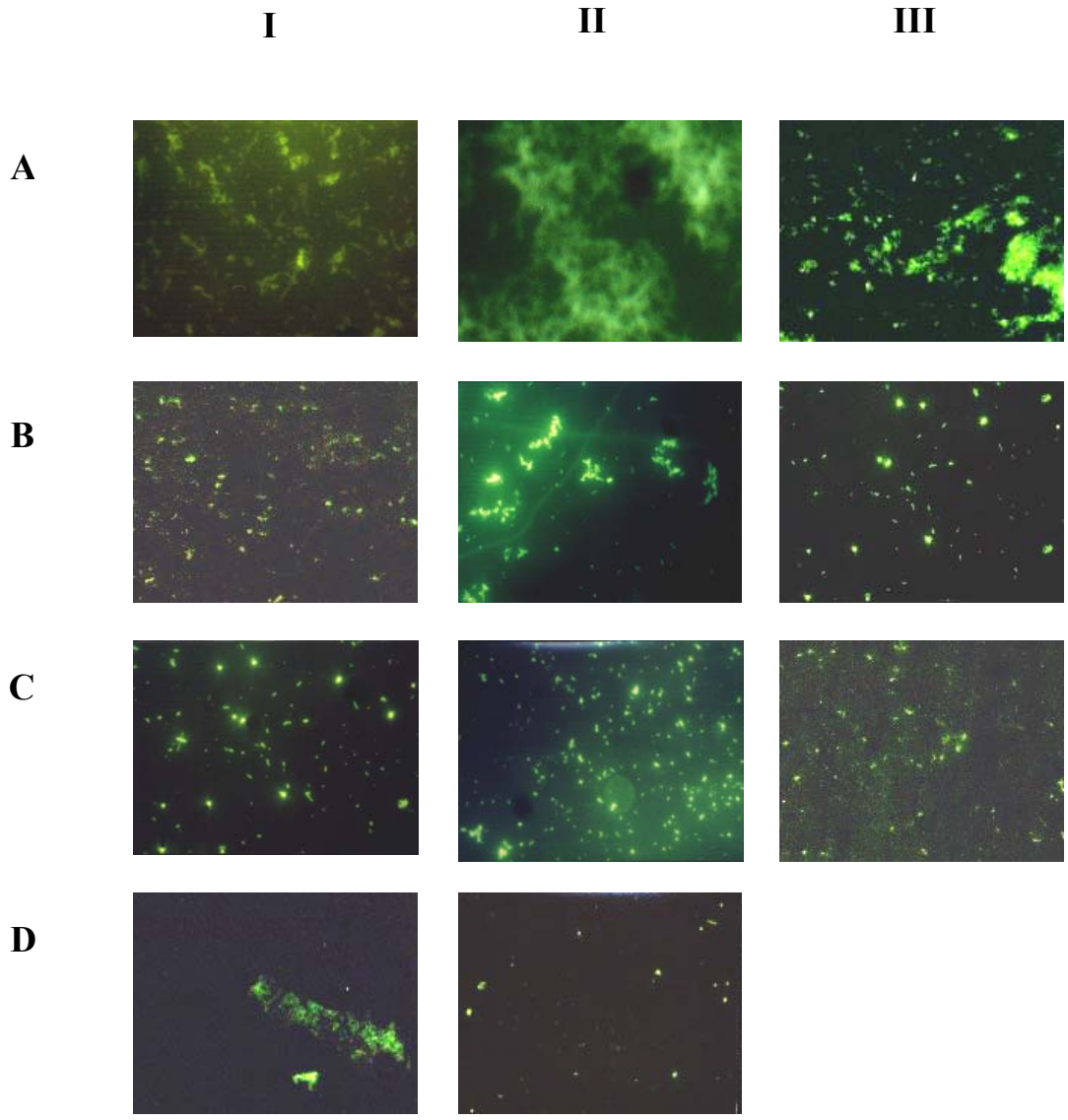

Figure 14. **Direct IS-PCR of the *nirS* gene using three different permeabilization procedures: enzyme treatment (I), electroporation (II) and calcium chloride (III). The denitrifying isolate E4-2 (A) and the *E. coli* clones pA5 and pA17 (B and C respectively) carried the *nirS* gene. Sample (D) corresponds to *E. coli* cells with pUC19 which does not have the *nirS* gene.**

4.4 *In situ* RT-PCR

Since *in situ* PCR resulted in relatively weak and inconsistent results, the *in situ* RT-PCR approach was used in order to test if the amount of starting template material affected the signal strength and consistency of the technique results. A step of *in situ* reverse transcription PCR converted all the mRNA into cDNA which served as template for *in situ* PCR amplification with the NIRS primers. The *in situ* RT-PCR was followed by FISH with the Cy3 labeled PSTUT probe. Five different bacterial species containing the *nirS* gene were used for the optimization of this process along with a negative control that has the *nirK* gene (Table 1). With this approach amplification was successful for various denitrifying cultures and no fluorescence signal or a weak signal compared to the positive cells was observed for the negative controls (Figure 15). It was observed for various strains (*P. denitrificans* and *P. aeruginosa*) that a large number of the cells as determined by fluorescence microscopy were expressing the *nirS* gene after 48 hours of incubation when gas production was already observed. After a week of incubation a duplicate culture was analyzed the same way resulting in a clear decrease of the number of cells expressing the gene. Counterstaining with DAPI allowed us to determine or compare the total amount of cells (blue fluorescence) present in a view field and the cells expressing (yellow-orange fluorescence) the *nirS* gene. After DAPI counterstaining it was clearly distinguishable that not all the cells present in the sample (blue) were expressing the *nirS* gene when compared to the Cy3 fluorescing cells (orange yellow). This detection strategy allowed a clearer distinction between *nirS* mRNA containing cells and cells without *nirS* mRNA, mostly because of a noticeable increase in signal strength.

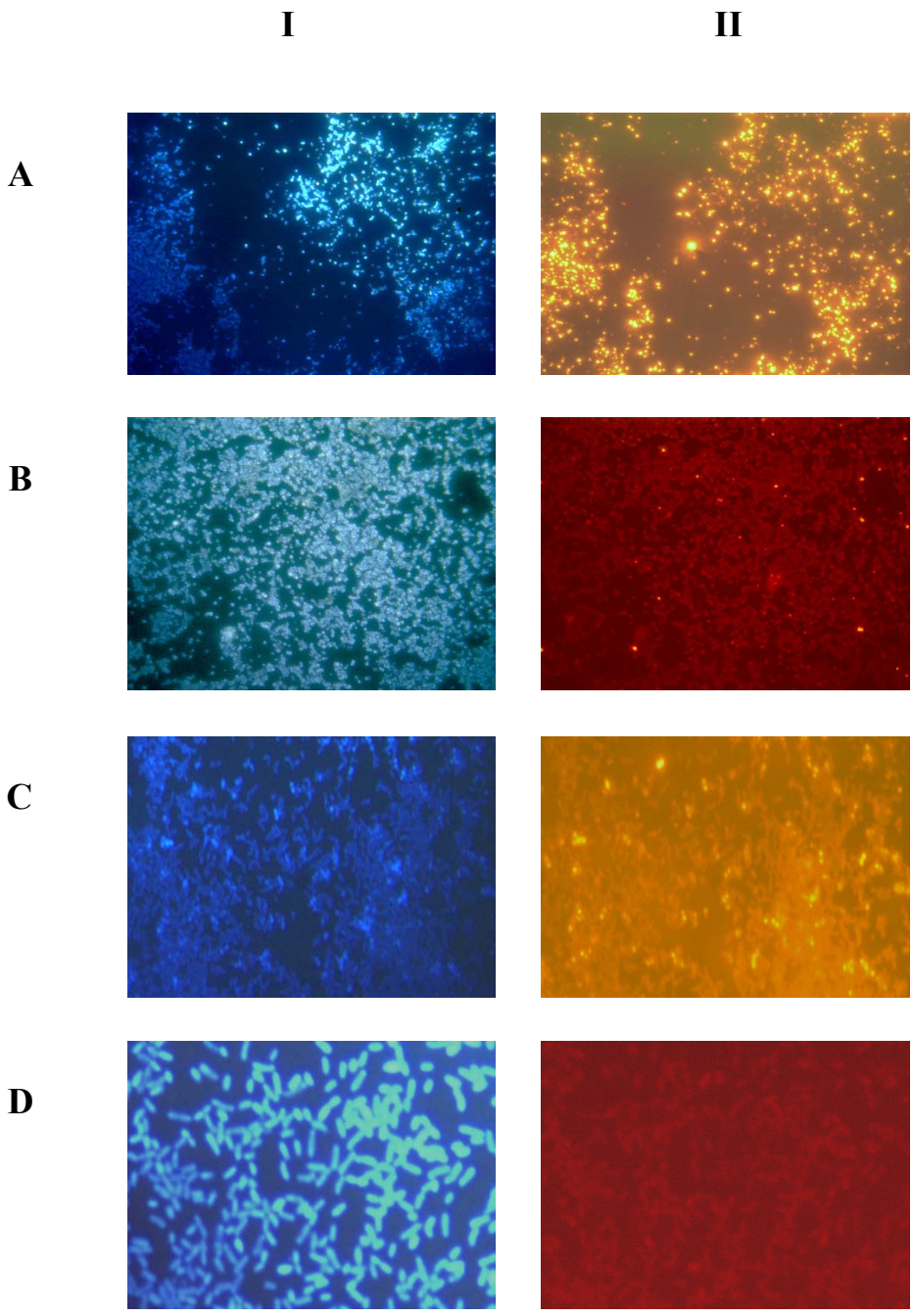

Figure 15. **DAPI counterstained cells (column I) and indirect *in situ* RT-PCR of active denitrifying bacterial cultures detected by FISH with a Cy3 labeled probe (column II). Rows (A), (B), and (C) correspond to *P. denitrificans* fixed after 48h, 1 week and *P. aeruginosa* respectively. Row (D) corresponds to *Pseudomonas* sp. (G-179) which does not have the *nirS* gene.**

V. DISCUSSION

5.1 Isolation of denitrifying bacteria from marine sediments

Denitrifiers are among the most successful physiological groups of microorganisms in nature. In agricultural soils, the denitrifier population is often 1-5 x 10^6 organisms/g of soil. Even though more is known about denitrifiers in agricultural soil, they are also prevalent in a variety of other environments (Gamble et al., 1977; Tiedje et al., 1982; Zumft, 1997). Approximately 22% of the isolates from our marine sediment microcosms showed denitrifying potential. Previous research showed that nitrate broth and nitrate agar gave reliable and equivalent counts compared to soil extract supplemented with yeast extract and nitrate or tryptic soy broth. Therefore, because of their ease of preparation, we used nitrate broth/agar throughout the isolation procedure (Gamble et al., 1977). Similar results were obtained by Gamble et al. (1977), using nitrate broth/agar, they isolated 1,500 organisms of which approximately 17% were identified as denitrifiers.

5.2 Analysis of 16S rDNA from bacterial isolates and control strains

Diverse populations of denitrifying bacteria were isolated from Puget Sound marine sediment microcosms indicating that the denitrifying potential is present in this environment. The presence of microbial populations capable of denitrifying activity has been described as widely distributed in nature, including marine sediments (Payne, 1973;

Gamble et al., 1977; Tiedje et al., 1982). Based on ARDRA, genetic relationships among the isolated strains and 6 reference strains were established. A total of 14 clusters were obtained grouping a total of 23 isolates. Fifteen of these isolates were from 25°C microcosms and were grouped into 8 clusters. Nine of the fifteen 25°C isolates were clustered between two groups, one composed of seven members and the other of two members. These strains grouped together generated similar ARDRA patterns, which could mean that they are the same or closely related populations. The remaining isolates were clustered individually. The 8 isolated strains from the 4°C microcosms grouped into six clusters. Three strains grouped into one cluster while the remaining five (5) isolates were distinct from one another and the control strains. As observed in Figure 10, higher diversity was obtained from the 4°C microcosms. As seen in related studies (Ruder and Nedwell, 1994), *P. stutzeri* may have out competed most of the slower growing denitrifying bacteria in the sample at 25°C. When grown at 4°C, a lag phase induction in *P. stutzeri* after a temperature shift down favored the coexistence with the slower growing bacterial strains. This could be a major factor that explained the higher diversity or lower redundancy observed within strains isolated at 4°C. Furthermore, both ARDRA and sequence analysis suggest, that all the isolated strains are not *P. stutzeri* or closely related to the reference strains. Therefore, some of these cultures could be considered as novel denitrifying strains.

On the other hand, it seems possible that for certain groups of bacteria, the information derived from ARDRA might result limited in comparison to data obtained from 16S rDNA sequencing analysis. ARDRA analyses are performed using the presence or absence of specific restriction sites within two 16S rRNA genes as the criteria

for estimating genetic diversity. This approach uses a very limited amount of data (only restriction sites) from 16S rDNA molecules in comparison to sequence alignments of 16S rRNA genes to establish genetic relationships. In addition, it is difficult to establish whether ARDRA bands of similar sizes of two unknown 16S rDNA molecules originated from restriction sites having the same location in both sequences. Consequently, ARDRA analyses are performed based on the assumption that fragments having the same size are generated from restriction sites that are conserved among the 16S rDNA molecules being compared (Rodríguez, 1998). In order to overcome this limitation, the 16S rDNA gene of various representative isolates was sequenced. The 16S rDNA gene of five (5) of the 25°C and five (5) of the 4°C isolated strains were sequenced at the GTSF (MSU –East Lansing, MI). The BLAST® analysis, performed with the sequence information confirmed most of the observations derived by ARDRA. Only three of the isolates (I-60, I-62A and I-80) were identified to be closely related to *P. stutzeri*. This could be a result of the greater resolution and specificity of the sequencing approach. The other seven (7) isolates were distantly related to the Ribosomal Database Project. These results are consistent with ARDRA in determining the novelty of the isolated denitrifying strains. The sequences were compared with over 69,000 bacterial 16S sequences in the Ribosomal Database Project..

Our results also showed that not all the isolates 16S rDNA was readily digestible by all the restriction enzymes. It has been previously shown that the type of tetrameric restriction enzymes (TREs) used for the screening of genetic diversity affects the discriminatory capability of the ARDRA technique.

Results from a computer-simulated restriction fragment length polymorphism (RFLP) analysis of 16S rRNA genes, indicate that a particular combination of TREs may be more efficient in discriminating among a specific group of bacteria than among others (Moyer et al., 1996). Moyer et al. (1996) demonstrated the effective use of the ARDRA methodology by using three TREs (*Bst* U I, *Hha* I, and *Rsa* I) in reconstructing the phylogenetic affiliations of 106 16S rDNA sequences of closely and distantly related bacteria whose taxonomic relationships were known. These three enzymes had the capacity to distinguish and correctly classify 80% of the tested sequences in comparison to 91% when using ten restriction enzymes. Rodríguez (1998) also used ARDRA effectively in the characterization of toluene-degrading bacterial isolates diversity in a study with tropical soils.

The limitations of the discriminatory capacity of restriction enzymes with respect to the extensive diversity of naturally occurring organisms, is another factor that could cause some inconsistencies in the topology of the dendogram. Therefore, results obtained from ARDRA may be used as a measure of the diversity among the tested organisms and should be used for a presumptive identification and differentiation due to the limitations of this approach (Rodríguez, 1998).

Despite the potential flaws that ARDRA might have when dealing with distantly related organisms, the method has been effectively used in the characterization of related bacterial populations (Vaneechoutte et al., 1992; Laguerre et al., 1994; Rodríguez, 1998). Since this study was focused on denitrifying bacterial populations it was feasible to accurately group closely related microorganisms with this technique.

5.3 *In situ* PCR and *In situ* RT-PCR

Unlike traditional PCR in which extracted DNA or RNA is used as a template for amplification, *in situ* PCR/RT-PCR is performed using DNA or RNA still inside intact permeabilized cells where the cell membrane acts like a sac to retain the amplified products. Although several variations of *in situ* amplification have been developed, the basic procedures of IS-PCR/RT-PCR involve cell fixation, cell permeabilization, *in situ* amplification and signal detection (Chen et al., 1998). Even though *in situ* PCR has been previously shown to be sensitive enough to amplify a single copy gene target sequence in prokaryotes (Hodson et al., 1995; Tani et al., 2002; Hoshino et al., 2001), our experienced showed otherwise. Neither the direct nor the indirect *in situ* PCR approaches were consistently successful for the detection of a single copy of the *nirS* gene. The direct *in situ* PCR approach (Figure 3), using fluorescein labeled nucleotides (Boehringer), resulted in multiple false positive results detecting even *Pseudomonas* sp (G-179) which has the *nirK* gene and *E. coli* with pUC19 as having the *nirS* gene (Figure 11 and 12). These false positive results could be a consequence of non-specific amplification (Figure 10) given the sequence homologies between the primers and sequences of non-denitrifying cultures. Other studies have identified similar difficulties with the direct detection approach of IS-PCR amplification products. It has been shown that, despite controlled fixation and permeabilization, the direct detection yields significantly less reliable results than indirect *in situ* PCR (Komminoth and Long, 1993, 1995; Chen et al., 1999). False positive results and background fluorescent signal are the two most common problems encountered when applying direct *in situ* PCR/RT-PCR.

Background signal is caused by binding of fluorescent label onto the surface of the microscope slide, while false positive results could probably be due to:

1. Incorporation of labeled nucleotides into cellular DNA by the repair mechanism of DNA polymerase.
2. Mispriming, were added primers bind to nontarget sequence regions
3. Endogenous priming, in which endogenous DNA or RNA fragments act as primers for PCR amplification.
4. Unstable binding between fluorochrome and dUTP during heating and cooling cycles of PCR which releases fluorochrome that might act like a general stain for all cells.
5. Binding of fluorescently-labeled dUTP to the cellular components inside cells due to high temperatures reached during PCR (Komminoth and Long, 1995; Chen et al., 1999).

Cell fixation is another key step upon successful *in situ* amplification. Prolonged fixation have shown to cause cross-linking of proteins and nucleic acids which, can have two opposing effects on the intact cells: (1) prevention of diffusion (out of the cell) of the amplified DNA and (2) inhibition of entry of key reagents for PCR (Nuovo, 1994a; Chen et al., 1998). An ideal IS-PCR protocol will minimize diffusion of amplification product(s) while causing little or no inhibition to the reagents entry into the cell (Chen et al., 1998). It also has been shown, that prolonged permeabilization times increases the risk of false positives in experiments using direct *in situ* PCR (Komminoth and Long, 1995; Chen et al., 1998; Nuovo, 1994a).

Indirect IS-PCR has been shown to generate more specific results (Long et al., 1993).

The indirect approach consists of an *in situ* hybridization (ISH) step after the *in situ*

amplification. However, our results when using the indirect *in situ* PCR (IS-PCR) approach to amplify the *nirS* gene resulted in a weak and rapidly fainting signal that was not very reliable or reproducible. The lack of consistency and low signal strength of these trials could be a result of the small starting template concentration and low accessibility.

In order to augment the success probability an indirect *in situ* RT-PCR approach was attempted. Here *in situ* reverse transcription and subsequent amplification of the *nirS* mRNAs is followed by FISH detection step. Successful detection of *in situ* RT-PCR reactions was observed after FISH with a Cy-3 labeled probe. Recent studies showed that IS-PCR/RT-PCR followed by FISH provided the most consistent results (Chen et al., 1998). It was possible to detect denitrifying strains based on the reverse transcribed mRNAs of the *nirS* gene. Analysis of mRNAs as an indicator of gene expression could enhance our understanding of active functional groups in the environment. Also, detection of mRNAs with short half-life could provide a strong indication of specific gene expression at the time of sampling that can be correlated with the physicochemical conditions. Therefore this approach could be used as a viable method to detect denitrifying activity at an individual cell level. This cellular activity could be consequently associated with other important microbial groups. Our results showed that when *in situ* RT-PCR was performed with denitrifying cultures, it was not possible to detect all the cells on the sample (Figure 15). False negative results have also been observed by Long et al. (1993), where fewer positives cells than expected were detected in experiments with high densities of "positive" cells. Some of the possible reasons for these false negative results include cell to cell variations in the amplification efficiency

due to differences in membrane permeability, nuclear proteins, and other causes of non-accessibility of DNA, and loss of amplification products during washing steps in the detection procedures (Liu et al., 1992; Long et al., 1993). When DAPI stained cells (total cells) are compared to the Cy3 stained cells (actively denitrifying cells) it was clearly visible that a larger number of denitrifying cells could be detected in the 48 hours culture compared to the 1 week grown cells. Neither all the DAPI detected cells, of the 48 hours culture, were visible with Cy3 (Figure 15). These results showed that only actively denitrifying cells with the *nirS* gene were being detected since the *nirK* strain used as a negative control was not detected by FISH. The large decrease of *P. denitrificans* cells detected between the 48 hours and 1-week cultures is supported by previous data (Holmstrom et al., 1999) where the short half-life of the *nirS* mRNAs seem to be a major reason for this decrease in cell detection. Also the expression of *nirS* in *P. stutzeri* is maintained at a low oxygen tension as long as nitrate or nitrite is present, while a decline in the number of transcripts is observed within the time determined by their half-lives (approximately 13 min) (Nogales et al., 2002).

Our results also showed that other permeabilization approaches could be a feasible alternative (Figure 14). Electroporation and calcium chloride permeabilization could be further optimized for faster and more economic permeabilization procedures. Both permeabilization alternatives seemed to be as efficient as electroporation for *in situ* PCR permeabilization. Enzyme permeabilization is the traditional method used for permeabilization and the only one found on published literature up to this moment.

The *in situ* amplification protocols were successful only when used with pure culture samples. While the methods (IS-PCR and *in situ* RT-PCR) appear to be difficult

to use in natural sediments, they appear to be much more feasible with enrichment cultures or simple communities. This would also represent a logical progression in optimizing the methods use in a simpler matrix before applying it to fresh sediments.

Important differences between the methods employed and the results obtained suggest that there is no generally applicable single *in situ* PCR protocol. Small methodological differences between similar protocols can significantly affect the outcome of *in situ* PCR studies (Komminoth and Long, 1993, 1995). All these technical differences include: type of starting material, type and copy number of target sequence, DNA amplification method, and detection systems (Long et al., 1993). Despite of these technical difficulties of *in situ* amplification, it is a new molecular tool with important potential in various research and diagnostics areas (Long et al., 1993; Teo and Shaunak, 1995; Nuovo, 1994a).

VI. CONCLUSIONS

1. Novel denitrifying organisms have been isolated given their significant differences of ARDRA profiles as compared to the profiles of reference denitrifying cultures. This was confirmed by 16S rDNA sequencing.
2. Greater diversity of denitrifying bacteria was obtained from slow growth enrichment cultures incubated at 4°C. Enrichments at 25°C favored *P. stutzeri* growth which seems to out compete other denitrifying strains under these isolation conditions.
3. Neither direct nor indirect IS-PCR approaches were efficient procedures for the detection of a single copy of the *nirS* gene.
4. *In situ* RT-PCR was a successful approach for the detection of denitrifying bacteria, as multiple target genes (mRNA) were available.
5. *In situ* amplification methods could provide very useful information on a molecular and/or individual cell (organism) level, for a better understanding of microbial communities.

VII. RECOMMENDATIONS

1. Regular stimulation of denitrifying activity through discontinuous nitrate addition to the microcosms before isolation. Different novel denitrifying microorganisms could be isolated by using a different selective media like the Minimal Media for Denitrifying Bacteria (Difco Manual).
2. Use the 16S rDNA gene sequence to further characterize the phylogenetic affiliation of novel cultures.
3. Since *in situ* amplification procedures are an emerging technique, they should be further tested and optimized with simple bacterial communities.
4. A constitutive gene's mRNA or a more stable mRNA molecule should be targeted to overcome the degradation of unstable mRNA or requiring an activation period for the gene to be studied.
5. Non traditional methods such as: electroporation and calcium chloride could be further evaluated to improve current permeabilization protocols.
6. After a nitrate pulse, *in situ* PCR glass slides were inserted into the marine sediment microcosms. *In situ* RT-PCR could be performed after removal and cell fixation on these slides. These could give us a better understanding of the spatial distribution and interactions of the denitrifying bacteria present in this sediment samples.

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APPENDIX 1. Partial sequences of the 16S rDNA gene of representative denitrifying bacterial isolates.

Partial 16S rDNA gene sequence of strain I-19

Positions: 122-487

atacgtcctacgggagggaggggggaccttcgggcctcgcgccattagatgagcctag
gtcggattagctagttggtgaggtaacggctcaccaaggcgacgatccgtaactggtct
gagaggatgatcagtcacactggaactgagacacggtccagactcctacgggaggcagc
agtggggaatattggacaatgggcgcaagcctgatccagccatgccgcgtgtgtgaaga
aggtcttcggattgtaaagcactttaagttgggaggaagggcagtaagcgaataccttg
ctgttttgacgttagcgacagaataagcaccggctaactctgtgccagcagcccgcggt
aatacagagggtggcaaggttaatcggaattactgggcg

BLAST closest match: Uncultured gamma proteobacterium, clone Gitt-KF-104.
363/368 (98%)

Partial 16S rDNA gene sequence of strain I-49

Positions: 87-362

gtgagtaatgcctaggaatctgcctgatagtgggggacaacgcttcgaaaggaacgcta
ataccgcatacgtcctacgggagaaagcaggggaccttcgggccttgcgctatcagatg
agcctaggtcggattagctagttggtgaggtaacggctcaccaaggcgacgatccgtaa
ctggtctgagaggatgatcagtcacactggaactgagacacggtccagactcctacagg
aggcagcagtggggaatattgcacaatgggcgaaagcctg

BLAST closest match: *Pseudomonas chloritidismutans.* 273/276 (98%)

Partial 16S rDNA gene sequence of strain I-54

Positions: 89-635

gagtaatgcctaggaatctgcctgatagtgggggacaacctctcgaaaggaacgctaat
accgcatacgtcctacgggagaaagcaggggaccttcgggccttgcgctatcagatgag
cctaggtcggattagctagttggtgaggtaacggctcaccaaggcgacgatccgtaact
ggtctgagaggatgatcagtcacactggaactgagacacggtccagactcctacgggag
gcagcagtggggaatattggacaatgggcgaaagcctgatccagccatgccgcgtgtgt
gaagaaggtcttcggattgtaaagcactttaagttgggaggaagggcattaacctaata
cgttagtgttttgacgttaccgacagaataagcaccggctaacttcgtgccagcagccg
cggtaatacgaagggtgcaagcgttaatcggaattactgggcgtaaagcgcgcgtaggt
ggtttgttaagttgaatgtgaaagccccgggctcaacctgggaactgcatccaaaactg
gcaagctagagtatgg

BLAST closest match: *Pseudomonas chloritidismutans.* 545/547 (99%)

Partial 16S rDNA gene sequence of strain I-60

Positions: 47-653

gtgagtaatgcctaggaatctgcctattagtgggggacaacgcctcgaaaggaacgcta
ataccgcatacgtcctacgggagaaagcaggggaccttcgggccttgcgctaatagatg
agcctaggtcggattagctagttggtgaggtaaaggctcaccaaggcgacgatccgtaa
ctggtctgagaggatgatcagtcacactggaactgagacacggtccagactcctacggg
aggcagcagtggggaatattggacaatgggcgaaagcctgatccagccatgccgcgtgt
gtgaagaaggtcttcggattgtaaagcactttaagttgggaggaagggcagtaagttaa
taccttgctgttttgacgttaccgacagaataagcaccggctaacttcgtgccagcagc
cgcggtaatacgaagggtgcaagcgttaatcggaattactgggcgtaaagcgcgcgtag
gtggttcgttaagttggatgtgaaagccccgggctcaacctgggaactgcatccaaaac
tggcgagctagagtatggcagagggtggtggaatttcctgtgtagcggtgaaatgcgta
gatataggaaggaacac

BLAST closest match: *Pseudomonas stutzeri* strain IrT-JG-7. 605/607 (99%)

Partial 16S rDNA gene sequence of strain I-62A

Positions: 47-633

gtgagtaatgcctaggaatctgcctattagtgggggacaacgcctcgaaaggaacgcta
ataccgcatacgtcctacgggagaaagcaggggaccttcgggccttgcgctaatagatg
agcctaggtcggattagctagttggtgaggtaaaggctcaccaaggcgacgatccgtaa
ctggtctgagaggatgatcagtcacactggaactgagacacggtccagactcctacggg
aggcagcagtggggaatattggacaatgggcgaaagcctgatccagccatgccgcgtgt
gtgaagaaggtcttcggattgtaaagcactttaagttgggaggaagggcagtaagttaa
taccttgctgttttgacgttaccgacagaataagcaccggctaacttcgtgccagcagc
cgcggtaatacgaagggtgcaagcgttaatcggaattactgggcgtaaagcgcgcgtag
gtggttcgttaagttggatgtgaaagccccgggctcaacctgggaactgcatccaaaac
tggcgagctagagtatggcagagggtggtggaatttcctgtgtagcggtgaaatgc

BLAST closest match: *Pseudomonas stutzeri* strain IrT-JG-7. 585/587 (99%)

Partial 16S rDNA gene sequence of strain I-73

Positions: 32-623

gagtaatgcctaggaatctgcctggtggtgggggataacgttcggaaacggacgctaat
accgcatacgtcctacgggagaaagcgggggaccttcgggcctcgcgccattagatgag
cctaggtcggattagctagttggtgaggtaacggctcaccaaggcgacgatccgtaact
ggtctgagaggatgatcagtcacactggaactgagacacggtccagactcctacgggag
gcagcagtggggaatattggacaatgggcgaaagcctgatccagccatgccgcgtgtgt
gaagaaggtcttcggattgtaaagcactttaagttgggaggaagggcagtaagcgaata
ccttgctgttttgacgttaccgacagaataagcaccggctaactctgtgccagcagccg
cggtaatacagagggtgcaagcgttaatcggaattactgggcgtaaagcgcgcgtaggt
ggttggttaagttggatgtgaaagccccgggctcaacctgggaactgcatccaaaactg
gccagctagagtacagtagagggtggtggaatttcctgtgtagcggtgaaatgcgtaga
ta

BLAST closest match: Uncultured gamma proteobacterium, clone Gitt-KF-67.
589/592 (99%)

Partial 16S rDNA gene sequence of strain I-74

Positions: 32-667

agagtaatgcctaggaatctgcctagtggtgggggatatcgctcggaaacggacgctaa
taccgcatacgtcctacgggagaaagcgggggaccttcgggcctcgcgccattagatga
gcctaggtcggattagctagttggtgaggtaacggctcaccaaggcgacgatccgtaac
tggtctgagaggatgatcagtcacactggaactgagacacggtccagactcctacggga
ggcagcagtggggaatattggacaatgggcgcaagcctgatccagccatgccgcgtgtg
tgaagaaggtcttcggattgtaaagcactttaagttgggaggaagggcagtaagcgaat
accttgctgttttgacgttaccgacagaataagcaccggctaactctgtgccagcagcc
gcggtaatacagagggtgcaagcgttaatcggaattactgggcgtaaagcgcgcgtagg
tggttggttaagttggatgtgaaagccccgggctcaacctgggaactgcatccaaaact
ggccgactagagtacggtagagggtggtggaatttcctgtgtagcggtgaaatgcgtag
atataggaaggaacaccagtggcgaaggcgaccacctggactgatac

BLAST closest match: Uncultured gamma proteobacterium, clone Gitt-KF-67.
632/636 (99%)

Partial 16S rDNA gene sequence of strain I-75

Positions: 32-674

gagtaatgcctaggaatctgcctggtggtgggggataacgttcggaaacggacgctaat
accgcatacgtcctacgggagaaagcgggggaccttcgggcctcgcgccattagatgag
cctaggtcggattagctagttggtgaggtaacggctcaccaaggcgacgatccgtaact
ggtctgagaggatgatcagtcacactggaactgagacacggtccagactcctacgggag
gcagcagtggggaatattggacaatgggcgaaagcctgatccagccatgccgcgtgtgt
gaagaaggtcttcggattgtaaagcactttaagttgggaggaagggcagtaagcgaata
ccttgctgttttgacgttaccgacagaataagcaccggctaactctgtgccagcagccg
cggtaatacagagggtgcaagcgttaatcggaattactgggcgtaaagcgcgcgtaggt
ggttggttaagttggatgtgaaagccccgggctcaacctgggaactgcatccaaaactg
gccagctagagtacagtagagggtggtggaatttcctgtgtagcggtgaaatgcgtaga
tataggaaggaacaccagtggcgaaggcgaccacctggactgatactgacact

BLAST closest match: Uncultured gamma proteobacterium, clone Gitt-KF-67.
640/643 (99%)

Partial 16S rDNA gene sequence of strain I-78

Positions: 32-483

agagtaatgcctaggaatctgcctggtggtgggggataacgcccggaaacggacgctaa
taccgcatacgtcctacgggagaaagcgggggaccttcgggcctcgcgccattagatga
gcctaggtcggattagctagttggtgaggtaacggctcaccaaggcgacgatccgtaac
tggtctgagaggatgatcagtcacactggaactgagacacggtccagactcctacggga
ggcagcagtggggaatattggacaatgggcgaaagcctgatccagccatgccgcgtgtg
tgaagaaggtcttcggattgtaaagcactttaagttgggaggaagggcagtaagcgaat
accttgctgttttgacgttaccgaccgaataagcaccggctaactctgtgccagcagcc
gcggtaatacagagggcgcaagcgttaatcggaattactg

BLAST closest match: Uncultured gamma proteobacterium, clone Gitt-KF-67.
447/452 (98%)

Partial 16S rDNA gene sequence of strain I-80

Positions: 7-686

acggatgaagagagcttgctctctttctcagcggcggacgggtgagtaatgcctaggaa
tctgcctattagtgggggacaacgtttcgaaaggaacgctaataccgcatacgtcctac
gggagaaagcaggggaccttcgggccttgcgctaatagatgagcctaggtcggattagc
tagttggtgaggtaaaggctcaccaaggcgacgatccgtaactggtctgagaggatgat
cagtcacactggaactgagacacggtccagactcctacgggaggcagcagtggggaata
ttggacaatgggcgaaagcctgatccagccatgccgcgtgtgtgaagaaggtcttcgga
ttgtaaagcactttaagttgggaggaagggcagtaagttaataccttgctgttttgacg
ttaccgacagaataagcaccggctaacttcgtgccagcagccgcggtaatacgaagggt
gcaagcgttaatcggaattactgggcgtaaagcgcgcgtaggtggttcgttaagttgga
tgtgaaagccccgggctcaacctgggaactgcatccaaaactggcgagctagagtatgg
cagagggtggtggaatttcctgtgtagcggtgaaatgcgtagatataggaaggaacacc
agtggcgaaggcgaccacctgggctaatactg

BLAST closest match: *Pseudomonas stutzeri* strain IrT-JG-7. 677/680 (99%)