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DISTRIBUTION, ABUNDANCE, AND ACTIVITY OF ANAEROBIC AMMONIUM OXIDIZING BACTERIA AND MICROBIAL COMMUNITY STRUCTURE IN MARINE SEDIMENTS

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DISTRIBUTION, ABUNDANCE, AND ACTIVITY OF ANAEROBIC AMMONIUM OXIDIZING BACTERIA AND MICROBIAL COMMUNITY STRUCTURE IN MARINE SEDIMENTS

By

Christopher Ryan Penton

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ABSTRACT

DISTRIBUTION, ABUNDANCE, AND ACTIVITY OF ANAEROBIC AMMONIUM OXIDIZING BACTERIA AND MICROBIAL COMMUNITY STRUCTURE IN MARINE SEDIMENTS

By

Christopher Ryan Penton

Anaerobic ammonium oxidation is a recently discovered nitrogen removal pathway in natural systems mediated by deep-branching members of the phylum *Planctomycetes*, the anammox bacteria. First found to be a significant nitrogen sink in the Black Sea anoxic water column in 2003, anammox has shown to be responsible for as much as 79% of N_2 production in marine sediments. Therefore, understanding the distribution, abundance, and activity of anammox communities is essential for refining estimates of N_2 removal in the marine environment. The distribution of the *Scalindua* sp. type anammox bacteria was studied in a variety of biogeochemical conditions and diverse environments, using newly designed 16S rRNA gene primers specific for their target. Since previous anammox targeted surveys used broad *Planctomycetes* primers, this was the first specific screening tool for the identification of anammox bacteria in the environment that was not based on the use of fluorescence *in-situ* hybridization. This primer set was 100% specific in the environments tested for anammox bacteria and identified the bacteria in a wide variety of environments, ranging from Siberian permafrost to shallow wetland sediments. Anammox importance in marine sediments has

been thought to increase with depth below water surface, such that the process dominates over denitrification, a result of lower reactive carbon availability. The activity and abundance of anammox bacteria was studied in the deep marine sediments of Cascadia Basin using the ^{15}N isotope pairing method and newly designed quantitative PCR primers, targeting the *Scalindua* sp. 16S rRNA gene. Anammox was found to contribute between 12 and 51% of total N_2 production in these sediments, far below the dominance that was expected based on earlier studies. The vertical distribution of anammox indicated that the bacteria were present in significant numbers at depths where oxidized N species were not found. This seemed to be indicative of bioturbation creating preferential porewater channels for NO_3^- / NO_2^- inputs, transient pulses resulting from pelagic inputs, or alternative metabolic pathways in *Scalindua* sp. that does not depend on oxidized N species. Estimated anammox abundances were highly correlated with anammox N_2 production (R^2=0.93) in these sediments, linking the catalyst with the process. Marine microbial communities in ten diverse geographical and biogeochemical sediments were studied using 16S rRNA gene targeted pyrosequencing. Geographical location, temperature, or basin did not influence the relationship among sites. There were no cosmopolitan clusters, (i.e. sequences grouped at 5% identity), found at every site. Potentially new groups of bacteria were identified that exhibited less than 85% similarity to the database, one of which comprised 25% of the community in an Arctic Barrow Canyon sediment. Rare members were more divergent from the public database, suggesting that they were not transients but rather localized divergent members that may play a minor or no role in the functional capacity of the sediment.

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time, for the tireless discussions, intriguing conference journeys, and never-ending analysis of pyrosequencing data. I would also like to apologize for those countless times my desk chair hit his. I would also like to acknowledge Erick Cardenas who has also been there throughout my stay at Michigan State for his hallway discussions and talks about life. Additional thanks goes out to Veronica Gruntzig, Peter Bergholz, Dieter Tourlousse, Deborah Rodriguez, Stephan Gantner, Deborah Himes, Brian Campbell, Patrick Chain, Shoko Iwai, and Yu Yang who have all helped in one way or another. Thanks to the members of the RDP, especially Qiong Wang and Jim Cole, for their discussions and analysis of pyrosequencing data and for those at the RTSF, especially Shari Tjugum-Holland and Jeff Landgraf for sequencing clone libraries and for quantitative PCR.

Besides the people that have contributed directly to my research, there are many people through the years that have helped me reach this point through their support and encouragement. Thank you to my family, to my mom and dad who constantly supported and loved me, who provided those early chemistry sets, who endured scrapes and bruises as I discovered physics, who nurtured me and showed me a world full of possibilities, who made sure my homework was done, who helped me at the last minute on those early science experiments, and who encouraged me through life to be my best.

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PREFACE

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CHAPTER I
GENERAL INTRODUCTION

The anammox reaction is anaerobic oxidation of ammonium coupled with nitrite reduction under anoxic conditions. This alternative nitrogen removal pathway was first proposed by Richards (1965), following observations of that indicated an ammonium deficit in anoxic marine basins. Throughout most of the twentieth century ammonium was believed to be inert under anoxic conditions. Canonical denitrification liberates ammonium from organic matter during respiration, resulting in net accumulation in the sediment/soil profile. The proposed 'anammox' pathway allows for the removal of ammonium under purely anoxic conditions. Early evidence for the presence of this reaction was provided by marine sediment porewater profiles where the simultaneous disappearance of nitrite and ammonium was observed (Codispoti and Richards 1976; Cline and Richards 1972). Broda (1977) soon proposed a new type of bacteria responsible for these observations, a "chemosynthetic bacteria that oxidize ammonia to nitrogen with O_2 or nitrate as an oxidant", which was coined one of two "lithotrophs missing in nature". It was not until 1995 that the anammox process was confirmed in a fluidized bed reactor treating wastewater effluent (Mulder et al. 1995). The anammox reaction is a chemolithotrophic process in which 1 mole of ammonium is oxidized by 1 mole of nitrite to produce N_2 gas in the absence of oxygen (Strous et al. 1999):

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$$

Compared to denitrification, this process produces twice the amount of N_2 per mole nitrite consumed and increases N_2 production in sediments where nitrification is

limited. The bacteria responsible for this process were later identified as a deep-branching planctomycete with a peculiar morphology (Strous et al. 1999). Despite the early connotation that many microbes cannot be isolated in pure culture (Winogradsky 1949), the presence of a microbially-mediated reaction that disputed the notion that ammonium was inert under anoxic conditions was initially regarded with skepticism. Since then, numerous studies have identified anammox as a key process in the global nitrogen cycle.

Anammox physiology and metabolism

All currently known bacteria capable of anaerobic ammonium oxidization belong to a deep branching lineage of the order *Planctomycetales* with high genus level diversity (Freitag and Prosser 2003; Schmid et al. 2003). The evolutionary distance among the anammox genera are large (<85% 16S rRNA gene nucleotide identity) though they share the same basic anammox metabolism and cell structure. There are currently four *Candidatus* genera whose grouping are largely based on 16S rRNA sequences: The "freshwater" *Kuenenia* (*K. stuttgartiensis*; Schmid et al. 2000) and *Brocadia* (*B. anammoxidans* (5) and *B. fulgida* (22)), and the "marine" anammox *Scalindua* (*S. sorokinii*, *S. brodae*, and *S. wagneri*; Schmid et al. 2003). The fourth *Candidatus* genus has one member, *Anammoxoglobus propionicus* (Kartal et al. 2007b) which exhibits an alternative metabolism. Anammox bacteria are characterized by a membrane-bound organelle called the anammoxosome that comprises more than 30% of the cell volume. This intracytoplasmic compartment is surrounded by unique lipids, called ladderanes (Sinninghe Damsté et al. 2002) that are unique to the anammox bacteria. Ether and ester

linkages tie the lipids to a glycerol backbone in the membrane which have historically only been found in members of the domain Archaea and may reflect an early divergence of anammox in the bacterial lineage (Brochier and Philippe 2002). Due to a very dense arrangement of carbon atoms, the ladderane lipids serve as a diffusion barrier (Sinninghe Damsté et al. 2002). This may serve to protect the bacteria from the toxic anammox reaction intermediates hydroxylamine and hydrazine (Jetten et al. 2003). Due to their unique characteristics, ladderane lipids have also been used as a biomarker for the presence of anammox bacteria (Kuypers et al. 2003).

Evidence from the genome of *Candidatus K. stuttgartiensis* (Strous et al. 2006) indicates that the anammox reaction proceeds via the following steps:

$$NO_2^- \rightarrow NO$$

$$NO + NH_4^+ \rightarrow N_2H_4 \rightarrow N_2$$

The anammox hydroxylamine oxidoreductase (HAO) enzyme is responsible for the oxidation of hydrazine to N_2 gas and is located exclusively within the anammoxosome (Lindsay et al. 2001), a possible target for future molecular studies. The highly reactive hydrazine intermediate is stored inside the anammoxosome (Sinninghe Damsté et al. 2002), which is especially important considering the slow enzymatic turnover, resulting in a doubling time of nine days in optimal conditions for the "freshwater" anammox (Strous et al. 1999). Anammox are reversibly inhibited by O_2 and reaction rates are the same after as before aeration (Jetten et al. 1999).

Anammox bacteria have been found to be metabolically flexible, exhibiting alternative metabolic pathways. For instance, anammox can reduce nitrate to nitrite to ammonium, followed by the conversion of ammonium and nitrite to N_2 through the

anammox pathway, allowing anammox bacteria to overcome ammonium limitation. Anammox bacteria are also a potential minor source N_2O production by nitric oxide detoxification (Kartal et al. 2007a). Currently the other known processes that produce N_2O are nitrification and denitrification (Fig. 1.1). As such, classical denitrification measures that depend exclusively on N_2O measures may overstate the role of denitrification in the system. Specifically, the acetylene block technique does not detect anammox N_2 production, as N_2O is not a major intermediate of the reaction. Another alternative pathway is carried out by *Candidatus* Anammoxoglobus propionicus, which has been shown to co-oxidize propionate and ammonium and out-compete denitrifiers and other anammox bacteria in the process (Kartal et al. 2007b). This supports the niche differentiation of anammox in which different "ecotypes", genetically unique populations adapted to their local environment. Lastly, iron and manganese oxides have also been found to be respired with formate as an electron donor (Strous et al. 2006), further expanding the metabolic diversity of the anammox bacteria.

This metabolic flexibility is also evident within the *Candidatus* Scalindua marine anammox which were able to use both formate and acetate as electron donors and manganese and iron as electron acceptors (van de Vossenberg et al., 2008). Temperature also appears to be a selective condition between two different closely related Scalindua phylotypes in enrichment cultures. This supports the findings that specific anammox 16S rRNA based phylotypes generally dominate in a given sediment or regional area.

Detection of anammox bacteria and activity

The isotope pairing technique (IPT) has been used as the standard measure of anammox activity, most commonly using homogenized sediments (Thamdrup and Dalsgaard 2002).

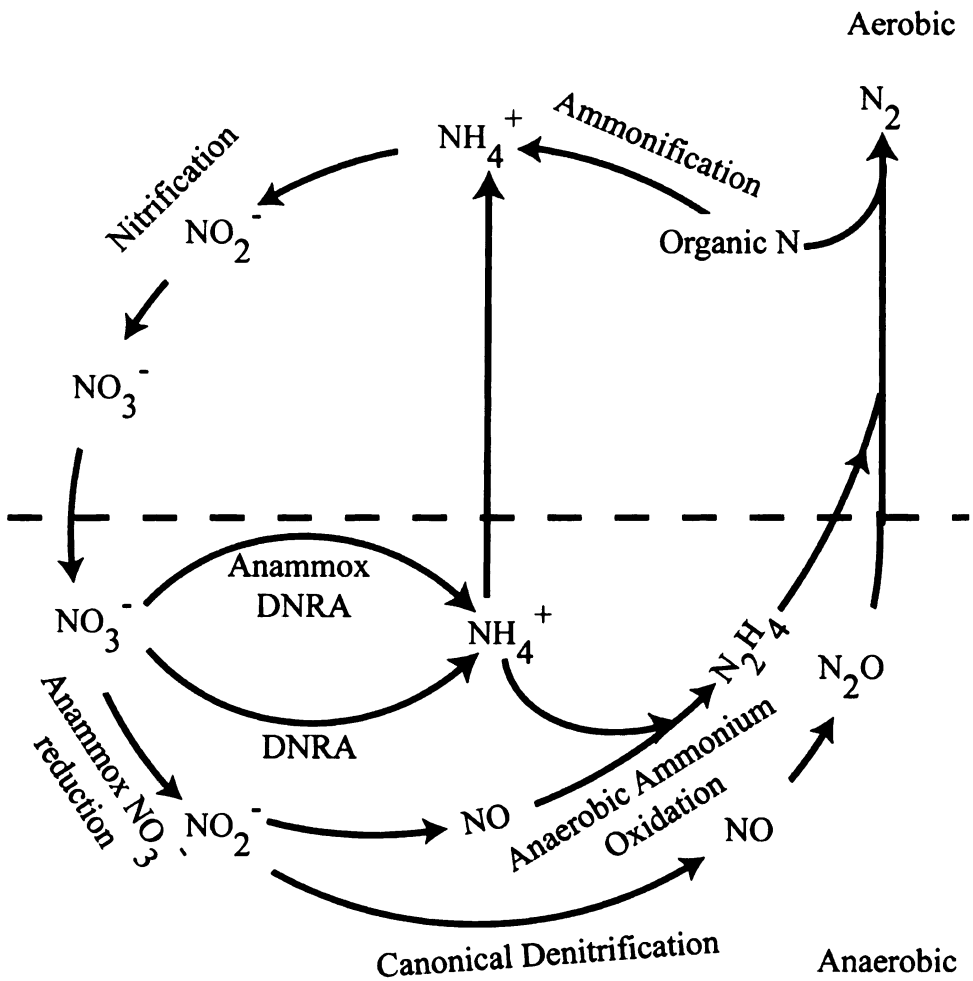

Figure 1.1. Anaerobic ammonium oxidation pathway of N removal in context of the nitrogen cycle.

Concentrations of NH_4^+, NO_3^-, and NO_2^- are first determined, the sediments are placed in airtight containers with a septum, such as Exetainer tubes, and the headspace is flushed with He for a minimum of five minutes to replace ambient O_2. Concentrations of residual NO_3^- and NO_2^- are monitored over time until all available oxidized species is removed from the incubations. Three parallel incubations are then performed: (1) $^{15}NH_4^+$ alone, (2) the combination of $^{15}NH_4^+$ and $^{14}NO_2^-$, and (3) $^{15}NO_2^-$ alone.

Reactions are stopped by the addition of $ZnCl_2$.The first incubation is used as a control to detect any oxidation of ammonium without the addition of nitrite. The lack of $^{29}N_2/^{30}N_2$ is indicative of the lack of oxidants at the end of the pre-incubations. The second treatment is used to determine if anammox activity is possible. The production of $^{29}N_2$ indicates anammox activity through the oxidation of ammonium with nitrite. The combination of the first two incubations is used to establish anammox activity. Finally, the third incubation is used to estimate anammox and denitrification rates (Figure 1.2). Anammox produces $^{29}N_2$ through the oxidation of the resident NH_4^+ pool with the added $^{15}NO_2^-$ while denitrification is measured by the production of $^{30}N_2$. However, evidence that anammox can also reduce $^{15}NO_3^-$ to $^{15}NO_2^-$ to $^{15}NH_4^+$ (Kartal et al. 2007a) results in the possibility that the anammox reaction can pair $^{15}NO_2^-$ with $^{15}NH_4^+$, and thus some proportion of measured denitrification may be partitioned to the anammox reaction. Several modifications to this protocol are promising, notably the addition of N_2O measures to more accurately quantify N_2 production and the use of intact sediment cores (Trimmer et al. 2006).

Molecular methods have been extensively utilized to identify the presence of anammox bacteria in environmental and wastewater samples. Fluorescence in-situ hybridization (FISH) targeting the 16S rRNA gene has been used extensively and is described in detail by Schmid et al. (2005). Anammox bacteria have also been identified using PCR with a variety of primers, often based on FISH probes, targeting the group as a whole or specific members (Schmid et al. 2005; Penton and Tiedje 2006). The unique ladderane lipids that constitute the anammoxosome have also been used as biomarkers for relative quantification (Kuypers et al. 2003) while distinctive hopanoid lipids may be

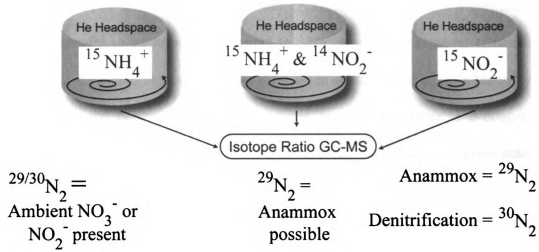

Figure 1.2. Experimental layout of the isotope pairing technique used for estimating anammox and denitrification activities.

useful in assessing relative anammox abundance in the sedimentary record (Sinninghe Damsté et al. 2004). Quantitative PCR (q-PCR) has been used for direct quantification of all known anammox-like bacteria in water columns (Hamersley et al. 2007) and wastewater enrichment cultures (Tsushima et al. 2007).

Anammox in the environment

The linkage of anammox activity with the removal of fixed inorganic nitrogen in natural systems was first confirmed in the Black Sea suboxic water column (Kuypers et al. 2003). Since then anammox has been shown to be a significant contributor to nitrogen losses in a variety of environments, responsible for 19-35% of the nitrogen loss in an anoxic coastal bay (Dalsgaard et al. 2003) and the majority of N removal in one of the most productive regions of the world's oceans, the Benguela upwelling oxygen minimal

zone (Kuypers et al. 2005). These sites exhibit characteristics of oxygen minimum zones, which are thought to be responsible for 30 to 50% of global N removal (Brandes and Devol 2002). Evidence for the anammox reaction in sediments or soils is generally first determined by the porewater N profile. Anoxic zones where there is a concomitant reduction in both nitrite/nitrate and ammonium exhibits the initial conditions necessary for anammox activity (Figure 1.3). The maximum reported contribution of anammox is 67-79%, occurring in sediments at a depth of 700m (Engström et al. 2005) which led to the hypothesis that relative anammox contributions increase with depth. Overall trends suggest that while anammox N_2 production decreases with depth, the total importance of this process in relation to denitrification increases with depth (Figure 1.4). However, these data only cover sediments ranging from 0m to 700m and do not address deeper sediments where both organic matter recalcitrance and NO_2^- / NO_3^- availability may affect the relationship between denitrification and anammox activity.

Ammonium is typically abundant in anoxic systems, provided by organic matter oxidation. Nitrate reducing or aerobic ammonium oxidizing bacteria provide the nitrite necessary for the anammox reaction. As such, organic matter availability is thought to be a major factor influencing the relative significance of anammox to total N_2 production. Greater organic matter availability creates a higher demand by denitrifiers for NO_2^- and NO_3^-, and less NO_2^- is liberated for anammox consumption. Nitrite is also an intermediate product of denitrification and the end product of nitrate respiration. The competition for NO_2^- between denitrifiers and anammox bacteria shows a non-linear response. In particular, at low NO_2^- concentrations (2-5 μM), anammox bacteria take proportionally more of the available nitrite due to a high NO_2^- affinity (Trimmer et al., 2005). Therefore,

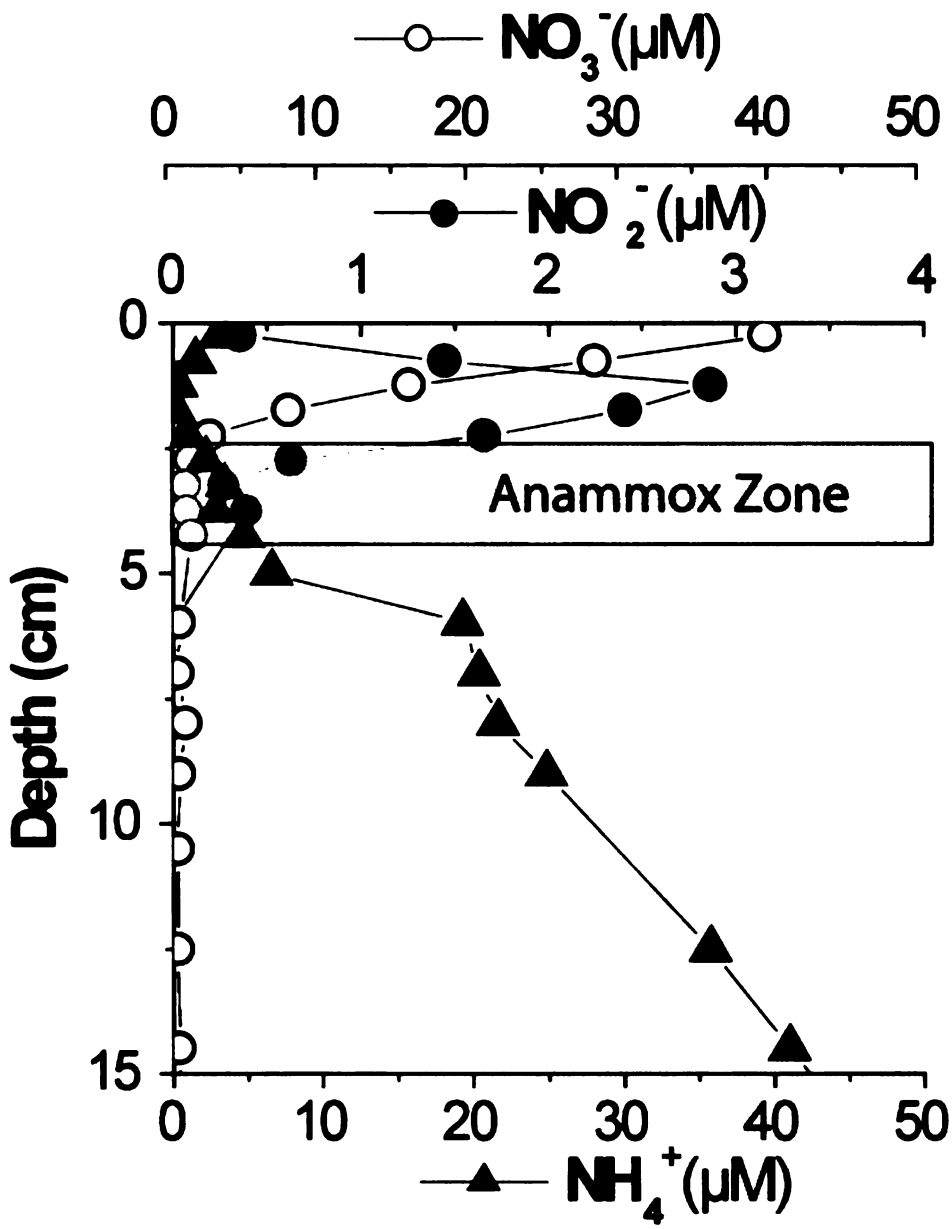

Figure 1.3. Typical porewater nitrogen profile from a deep ocean sediment indicating a possible zone of anaerobic ammonium oxidation. Figure based on data from Chapter III.

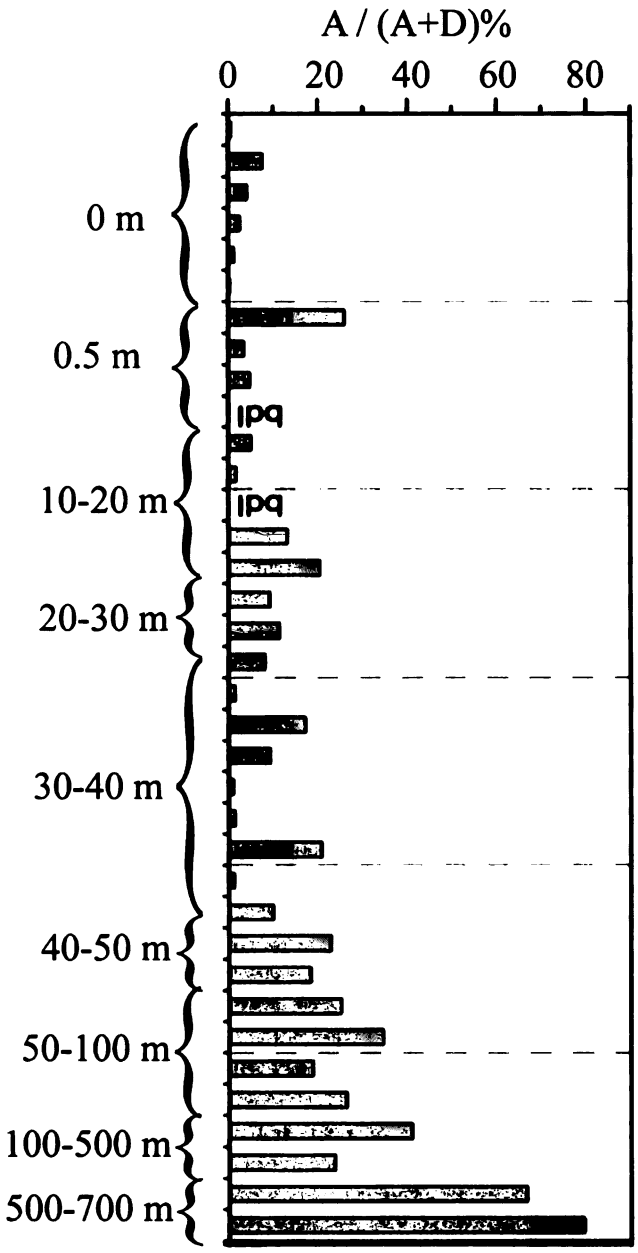

Figure 1.4. Illustration of increasing relative importance of anammox ((anammox/(anammox+denitrification)) with increasing depth in sediments up to 700m depth below water surface. Adapted from Dalsgaard et al., 2005.

in deep marine sediments, where low NO_2^- concentrations dominate due to decreased organic matter availability, anammox would be expected to dominate NO_2^- removal.

Anammox contributed 0 to 9% of total N_2 production in subtropical mangrove sediments (Meyer et al. 2005), 8% in estuarine sediments (Trimmer et al. 2003), and less than 2% in eutrophic shallow coastal bay sediments (Thamdrup and Dalsgaard 2002). Although sediment reactivity has been negatively correlated with anammox contribution to total N_2 production (Trimmer et al. 2003), absolute anammox rates appear to peak at sites with intermediate reactivity (Engström et al. 2005). Thus, a strict relationship between anammox activity and organic matter availability is not firmly established. Additionally, due to the slow growth of anammox and their inhibition by low concentrations of O_2 (if the "marine" anammox respond the same as the "freshwater" species), environmental stability may be an important controlling factor of anammox activity.

16S rRNA sequences identical or closely related to the marine anammox have been found widely distributed in marine systems, freshwater lakes, and subtropical wetlands (Penton and Tiedje 2006). Anammox 16S rRNA sequences have also been identified in Siberian frozen alluvial sandy loam, deposited in the Middle Pleistocene Epoch 300,000-400,000 years ago in the Cape Svyatoi Nos tundra zone on the Laptev Sea coast (Penton and Tiedje 2006). Anammox activity was responsible for up to 19% of total N_2 production in a stable Greenland Sea ice floe but was not detectable in annual sea ice (Rysgaard and Glud 2004). Both aerobic and anaerobic processes in microzones were found to occur simultaneously in brine pockets. This raises the possibility that anammox contributes to N_2 removal in permafrost soils.

Relatively few studies have investigated anammox activity in natural freshwater systems, although the "freshwater" anammox bacteria are the most intensively characterized due to their implementation in wastewater treatment bioreactors. Schubert et al. (2006) reported an anammox contribution of 13% in the largest freshwater anoxic lake in the world, Lake Tanganyika. Anammox 16S rRNA gene sequences with <96% sequence identity to *Candidatus* Scalindua brodae were identified in the anoxic water column and anammox cells were enumerated using FISH. Molecular analysis was used to assess the diversity of the anammox population in the Xinyi River (China) (Zhang et al. 2007). Sequences, obtained by targeted PCR, exhibited 16S nucleotide identities of 95% to *Candidatus* Brocadia anammoxidans and 95% to the *Candidatus* Scalindua species, including the sequence obtained from the Lake Tanganyika study. These findings suggest that more diverse anammox communities may exist in freshwater habitats, compared to the multitude of marine studies that indicate a single, dominant anammox phylotype.

OBJECTIVES

I undertook this work to address the following objectives and hypotheses:

Objective 1

To study the distribution and diversity of anammox bacteria in a broad range of biogeochemical conditions and geographic locations.

At the point that this study was undertaken anammox bacteria had only been identified in wastewater treatment plants (wwtps) and anoxic water columns. The

distribution of the "marine" *Scalindua* sp. type anammox was largely unknown, and was thought to be constrained to obligately anoxic environments, sufficiently stable to harbor a slow growing consortia. Therefore, it was necessary to establish what types of environments in which these bacteria may be identified as a first step in understanding their potential importance on a global scale. Primers used for anammox identification were generally based on the *Planctomycetes* specific primers, which exhibited a low specificity for anammox bacteria. It was necessary to develop new primers that could be used as a screening tool for the specific identification of anammox bacteria. Although presence does not necessarily indicate significant functionality, a widespread anammox distribution would provide evidence that anaerobic ammonium oxidation was not confined to a very selective environment. Based on these data, I tested the following hypothesis:

Hypothesis 1

Anammox bacteria will be identified in many conditions that would not appear to harbor a stable, anoxic environment due to anoxic microsites within sediment particles that would be supportive of an anammox metabolism while the sample, in general, is not a stable anoxic environment.

Objective 2

To develop a quantitative PCR method for the rapid enumeration of anammox bacteria in marine sediments in order to determine the relationship between abundance and activity.

The enumeration of anammox bacteria is based on the use of fluorescence *in-situ* hybridization (FISH), which was described as the "gold standard". However, the implementation of FISH in sediments is especially difficult due to obstacles such as non-specific hybridization and particle shielding effects, among others. One study that attempted to use FISH in sediments appeared to drastically undercount these bacteria when cell-specific activity was calculated (Tal et al., 2005). Therefore, it was necessary to develop a quantitative PCR method that would specifically target the 16S rRNA gene of the marine *Scalindua* sp. type anammox. Estimated abundances will then be directly related to anammox activity obtained through ^{15}N assays. The following exclusive hypotheses were tested:

Hypothesis 1:

Anammox bacteria will only be identified in these stable, anoxic environments where oxidized nitrogen species are identified.

Hypothesis 2:

Alternatively, anammox will be identified in depths where oxidized nitrogen species are not present. This may be due to bioturbation creating preferential porewater

channels, transient pulses of NO_2^- / NO_3^- that may penetrate deeper in the sediment column, or may reflect alternative metabolic pathways of anammox bacteria.

Objective 3

To determine the effect of increasing depth below water surface on anammox activity in order to test the hypothesis that anammox is the prevalent N_2 removal pathway in deep marine sediments.

The relative importance of anammox, compared to denitrification, showed an overall increase with increasing sediment depth below water surface (Thamdrup and Dalsgaard 2002; Risgaard-Petersen et al. 2004; Engström et al. 2005). As such, it was thought that anammox would be responsible for the majority of nitrogen removal in depths greater than 1000m (Dalsgaard et al., 2005). This was thought to be due to decreasing reactive organic matter availability, which would favor anammox over denitrification (Thamdrup and Dalsgaard 2002; Engström et al. 2005). The following hypothesis were tested:

Hypothesis 1:

Anammox activity will not be a dominate pathway in N removal in deep marine sediments. The very low organic matter availability of deep sea sediments will limit the supply of NO_2^- / NO_3^- to anammox bacteria.

Objective 4

To investigate differences in microbial community structure and diversity among marine sediments obtained in a variety of biogeochemical and geographic locations.

Surveys of microbial communities in a wide range of habitats have primarily depended on clone library analysis of 16S rRNA gene libraries. The lack of sufficient resolution or depth in many of these studies did not allow for adequate comparisons to be made, especially in regard to the rarer community members. Understanding community diversity among widely ranging geographical locations is important, especially in relation to the dogma that "everything is everywhere, the environment selects". The controls on diversity are not understood but may include environmental stability, biogeochemistry, temperature, salinity, or type of carbon input. In this study the following hypotheses were tested:

Hypothesis 1

Marine sediments from deep sites will exhibit a more similar community structure, due to the higher processing of carbon as it sinks from the pelagic zone, which reflects a more temporally stable environment.

Hypothesis 2

Community structures will be more similar in sites that share a common marine basin (e.g. Pacific sites vs. Gulf of Mexico sites).

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CHAPTER II

MOLECULAR EVIDENCE FOR THE BROAD DISTRIBUTION OF ANAMMOX IN FRESHWATER AND MARINE SEDIMENTS

ABSTRACT

Previously available primer sets for detecting anammox bacteria are inefficient, resulting in a very limited database of such sequences which limits knowledge of their ecology. To overcome this, I designed a new primer set that was 100% efficient in recovering ~700 bp 16S rRNA gene sequences with >96% homology to the "*Candidatus* Scalindua" group of anammox, and detected this group in all sites studied, including a variety of freshwater and marine sediments and permafrost soil. A second primer set was designed that exhibited greater efficiency than previous primers in recovering full length (1380 bp) sequences related to "*Ca.* Scalindua," "*Ca.* Brocadia," and "*Ca.* Kuenenia." This study provides evidence for the widespread distribution of anammox in that it detected closely related anammox 16S rRNA gene sequences in eleven geographically and biogeochemically diverse freshwater and marine sediments.

INTRODUCTION

Anaerobic ammonium oxidation (anammox) involves the direct autotrophic conversion of ammonium and nitrite to N_2 under anoxic conditions. First confirmed in a fluidized wastewater bed reactor in 1994 (Mulder et al. 1995), anammox bacteria are now

thought to form a deep branching lineage of the *Planctomycetales* with a high genus level diversity (Freitag et al. 2003, Schmid et al. 2003). The anammox reaction occurs within a specialized cell compartment, the anammoxosome, in which ammonium is oxidized via hydrazine (N_2H_4) and hydroxylamine (NH_2OH) intermediates (Schalk et al. 1998). This process produces twice the amount of N_2 per molecule of nitrite consumed as denitrification and does not require an external reducing agent (Kuai and Verstraete 1998, Strous et al. 1999).

The role of anammox bacteria in the nitrogen cycle has been investigated in only a few environments. Anammox has been proposed to be responsible for the consumption of more than 40% of the fixed N in the anoxic water in the Black Sea (Kuypers et al. 2005); 19 to 35% of the total N_2 formation in the anoxic waters of a coastal bay in Costa Rica (Dalsgaard et al. 2003); between 24 and 67% of total N_2 production in continental shelf sediments (Dalsgaard et al. 2002) and between 1 and 24% of the N lost in estuarine sediments (Risgaard-Petersen et al. 2004, Trimmer et al. 2003). In a global context, the anammox reaction may account for 30 to 70% of total oceanic N_2 production (Devol 2003), representing a major N sink.

While isotope pairing studies have been used as indicators of anammox metabolism, the detection of anammox bacteria in environmental samples has been primarily by fluorescence *in-situ* hybridization (FISH). Few environmentally derived anammox 16S rRNA gene sequences have been obtained by the use of "universal", *Planctomycetales* targeted, or anammox-specific primers. Sequences have been identified in relatively few, mostly marine, environments (Bowman and McCuaig 2003, Kirkpatrick et al. 2006, Kuypers et al. 2003, 2005, Risgaard-Peteresen et al. 2004, Tal et

al. 2005) that fall within the documented anammox group. The previously available primer sets for detecting anammox bacteria have resulted in a very limited database of such sequences and may perhaps be limiting the knowledge concerning the distribution of these important N cycling bacteria.

In this study I explored the distribution and diversity of anammox-related bacteria in sediment samples from geographically and biogeochemically distinct environments. Eleven samples from unique sites were analyzed, representing subtropical freshwater wetlands, a northern fen wetland, a hypereutrophic lake, deep ocean, continental margin, shallow marine, and Siberian permafrost.

MATERIALS AND METHODS

Sampling

Our sampling sites were chosen to represent contrasting geographical, biogeochemical, and temporal attributes. Freshwater sediment samples were collected from beneath shallow (<1 m) waters at two long-term monitoring sites at Kellogg Biological Station, Mich.: Wintergreen Lake, a small, groundwater-fed, hypereutrophic lake (42.399N 85.383W), and Sherriff's Marsh (42.422N 85.516W), a fen wetland that receives groundwater and stream flow. Sediments from subtropical wetlands were collected using a piston corer in the Florida Everglades Water Conservation Area 2A, at two sites: a high phosphorus (P) eutrophic site F1 (0.4 m depth, 26.366N 80.373W) and a reference P level oligotrophic site U3 (0.5 m depth, 26.290N 80.419W).

Marine samples were collected at six sites using a multicorer: East Hanna Shoal (160 m depth, 72.637N 158.667W) and East Hanna Shoal Deep (1450 m depth, 72.852N 158.207W) both from the Alaskan maritime in the Chuckchi Sea; Turning Basin (12 m depth, 47.088N 123.024W) and Shallow Bud Inlet (3 m depth, 47.341N 122.944W), both in Puget Sound; Washington Margin (1138 m depth, 46.575N 124.822W) and West of the Juan de Fuca Ridge (3869 m depth, 46.783N 133.667W), both in the Pacific Ocean. Siberian frozen alluvial sandy-loam deposited in the 300-400 thousand year ago Middle Pleistocene Epoch was collected from Cape Svyatoi Nos on the Laptev Sea coast tundra zone (72.917N 140.17E) as described previously (Vishnivetskaya et al. 2000).

Primer Design

Ten primers targeting the 16S rRNA genes of "*Ca.* Scalindua", "*Ca.* Brocadia", and "*Ca.* Kuenenia" were initially designed, screened against GenBank for target organisms, and tested in various sediments by PCR amplification and sequencing. The following primer sets did not achieve consistent amplification: Wag991F (5'-TAGCCAACGGTATCCAGTCC-3') and Wag1189R (5'-AAGGGCCATGATGACTTGAC-3'), targeting only "*Ca.* Scalindua wagneri"; Brod541F (5'-GAGCACGTAGGTGGGTTTGT-3') and Stut766R (5'-CGGGGTATCTAATCCCGTTT-3'), targeting *Kuenenia stuttgartiensis*; Soro155F (5'-TTGTCAATGGGAGGGTAGC-3') and Soro331R (5'-GATTCTCGACTGCAGCCTTC-3'), targeting "*Ca.* Scalindua sorokinii" and "*Ca.*

Scalindua brodae". In addition, Brod541F, Wag991F, and Soro155F were combined with the "universal" primer 1392R (5'-ACGGGCGGTGTGTAC-3').

Brod541F (5'- GAGCACGTAGGTGGGTTTGT-3') and Brod1260R (5'-GGATTCGCTTCACCTCTCGG-3') was the only primer set to consistently produce single bands and specifically target anammox bacteria (720 bp amplicons). Specifically, Brod541F exhibited 100% homology to four groups in GenBank; "*Ca.* Scalindua brodae" (Schmid et al. 2003), uncultured ammonia oxidizing bacteria (Risgaard-Petersen et al. 2004), unidentified bacteria and planctomycetes from Antarctica (Bowman and McCuaig 2003), and uncultured planctomycetes from the Black Sea (Kirkpatrick et al. 2006). Primers An7F (5'- GGCATGCAAGTCGAACGAGG-3') and An1388R (5'-GCTTGACGGGCGGTGTG-3') were designed for the capture of greater phylogenetic variability with 1380 bp amplicons.

DNA Extraction

DNA was isolated using the MoBio Ultraclean™ Soil DNA kit (MoBio Laboratories Inc.) and PCR amplifications of 16S rRNA genes targeting the anammox group were performed in a model 9600 thermal cycler (Perkin-Elmer Cetus) from 30-80 ng μL^{-1} environmental DNA extracts. Reaction conditions for the primer pair Brod541F-Brod1260R were: 10 pmol of each primer, 200 ng μL^{-1} of bovine serum albumin (Roche), 2.5 U of *Taq* polymerase (Promega), 200 μM each deoxyribonucleoside triphosphate (Invitrogen), 150 mM $MgCl_2$ (Promega), and 1/10 volume of 10X PCR buffer provided with the enzyme. The PCR was as follows: 95°C for 3 min; 30 cycles of 95°C for 45 s,

60°C for 1 min for Brod541F-Brod1260R or 63°C for 1 min for An7F-An1388R, 72°C for 1 min; and 72°C for 7 min. Clone libraries were created using TOPO TA Cloning® (Invitrogen) and clones sequenced using an ABI 3730 Genetic Analyzer or ABI Prism 3700 DNA analyzer (Applied Biosystems). Sequences were analyzed against GenBank with BLAST and the Ribosomal Database Project-II (RDP) (Cole et al. 2005). The presence of chimeric sequences was determined by using the CHIMERA_CHECK program from RDP. Sequences were aligned with ClustalW and phylogenetic trees constructed using MEGA version 3.1 (Kumar et al. 2004) using a neighbor-joining method with bootstrapping.

RESULTS AND DISCUSSION

The majority of primer sets designed for the amplification of anammox bacteria did not yield consistent results. However, the screening primer set Brod541F-Brod1260R resulted in consistent amplification in all sediments tested and was subsequently used to create clone libraries of each site. The resulting 475 sequences exhibited greater than 96% nucleotide identity to either "*Ca.* Scalindua wagneri" or "*Ca.* Scalindua brodae," and represented 100% specificity to the anammox group. Partial and redundant sequences were removed and 698 bp fragments were aligned, resulting in 287 unique sequences. These were compared against the anammox group consisting of: "*Ca.* Scalindua," "*Ca.* Brocadia," "*Ca.* Kuenenia," and "*Ca.* Jettenia asiatica" in addition to environmental and wastewater obtained sequences that fall within the anammox group. A neighbor-joining tree was constructed using the 25 sequences that remained after 98% redundancy was

removed (Figure 2.2) which excluded East Hanna Shoal Deep, Siberian Permafrost, andWashington Margin sequences. Clones generated from Sherriff's Marsh and Siberian permafrost exhibited the highest (88%) and the lowest (28%) percentage of unique sequences, respectively. There was no discernible grouping either between freshwater and marine samples (Figure 2.3). Thus, Brod541F-Brod1260R generated 16S rRNA gene sequences from the eleven different sites cluster independently although sequences from individual sites cluster together when 99% redundancy is included, reflecting a certain level of endemism.

Additional clone libraries were created to confirm that the 698 bp Brod541F-Brod1260R 16S rRNA gene sequences obtained did indeed represent full length sequences of known anammox. Primer An7F, targeting "*Ca.* Scalindua," "*Ca.* Brocadia," and "*Ca.* Kuenenia," was first combined with the universal primer 1392R. The resulting 68 clones lacked any relationship to the anammox group, as was the case with the Brod541F-1392R primer set, despite the increased specificity of the forward primers. To resolve this, An7F was combined with An1388R and two 1380 bp sequences were obtained out of 21 passing sequences. Amplification resulting from the An7F/1388R primer set is illustrated in Figure 2.4. Clone FLE12 (positions 7-1388) was 97% homologous to "*Ca.* Scalindua brodae" and 92% homologous to "*Ca.* Scalindua wagneri." Positions 541-560 and 1241-1260 were identical to the Brod541F and Brod1260R primer target sites, respectively. Clone FLC09 (positions 5-1388) was 96% homologous to "*Ca.* Scalindua brodae" and 92% homologous to "*Ca.* Scalindua

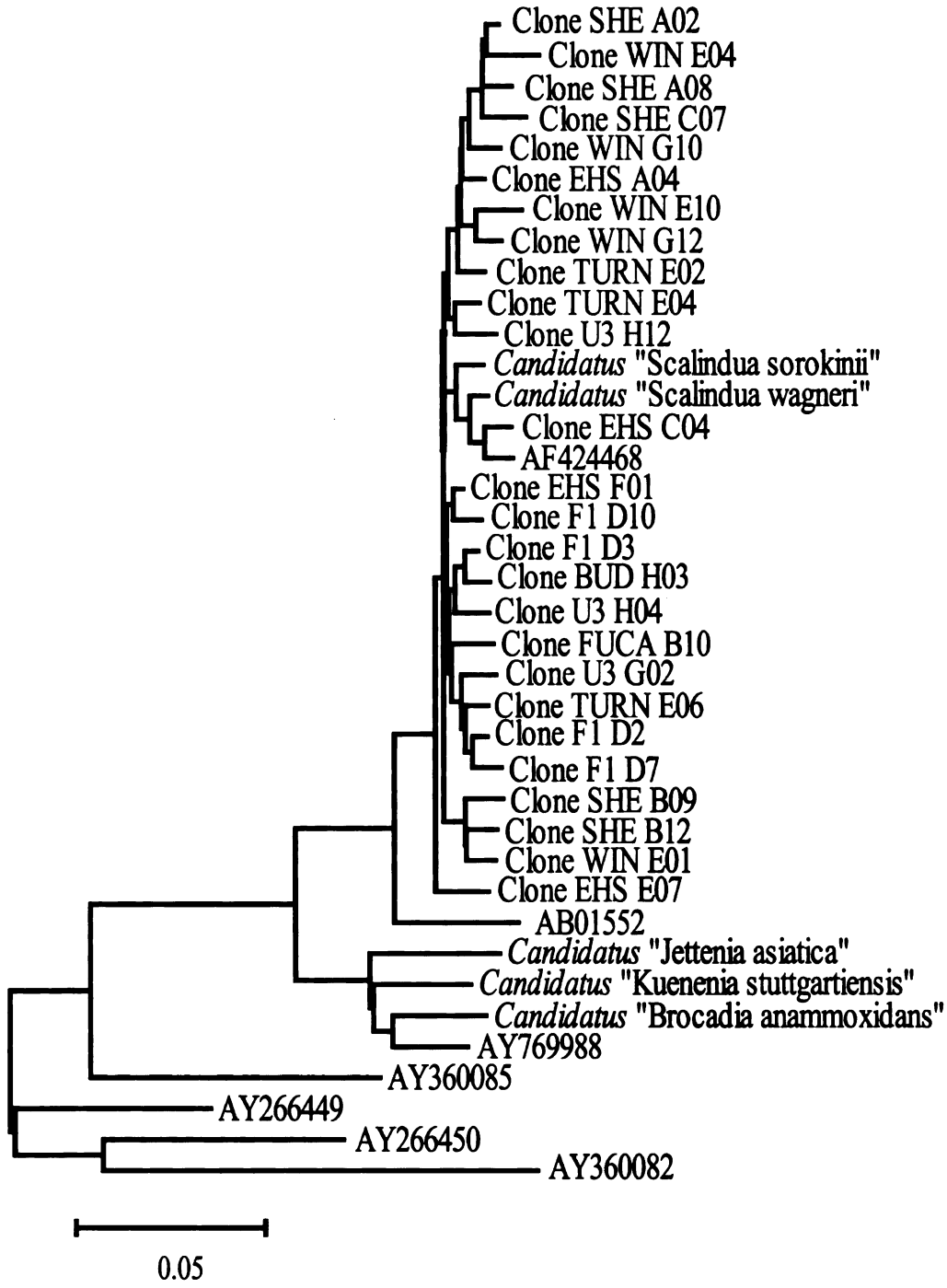


Figure 2.1. 16S rRNA gene-based bootstrapped phylogenetic tree reflecting the relationship of Brod541F-Brod1260R clones from East Hanna Shoal (EHS), East Hanna Shoal Deep (Deep), Washington margin (Wash), Turning Basin (Turn), Shallow Bud Inlet (Bud), Juan de Fuca (Fuca), Wintergreen Lake (Win), Sherriff's Marsh (She), Everglades F1 (F1), Everglades U3 (U3), and Siberian permafrost (Perm) sediments with 98% redundancy removed with the anammox group and related bacteria given as GenBank accession numbers. The bar indicates a 5% sequence divergence.

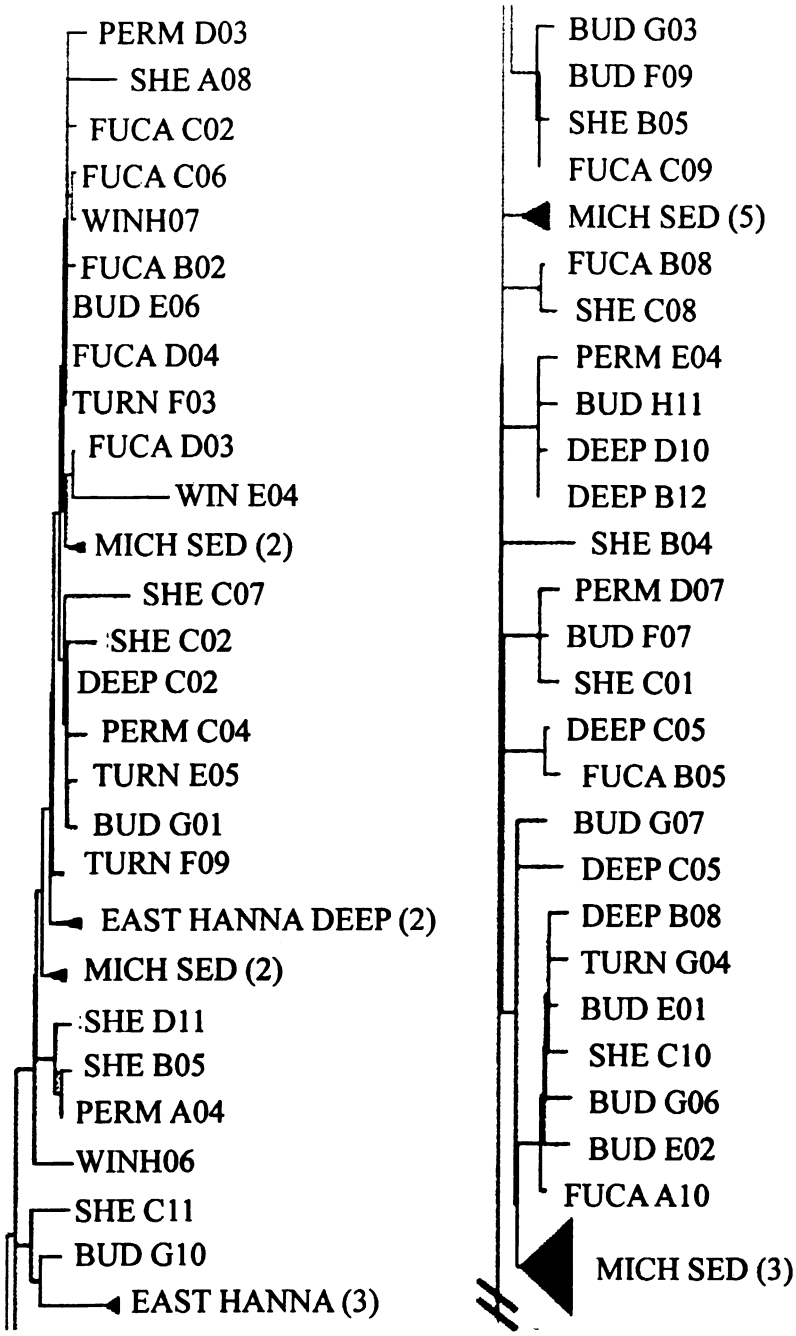
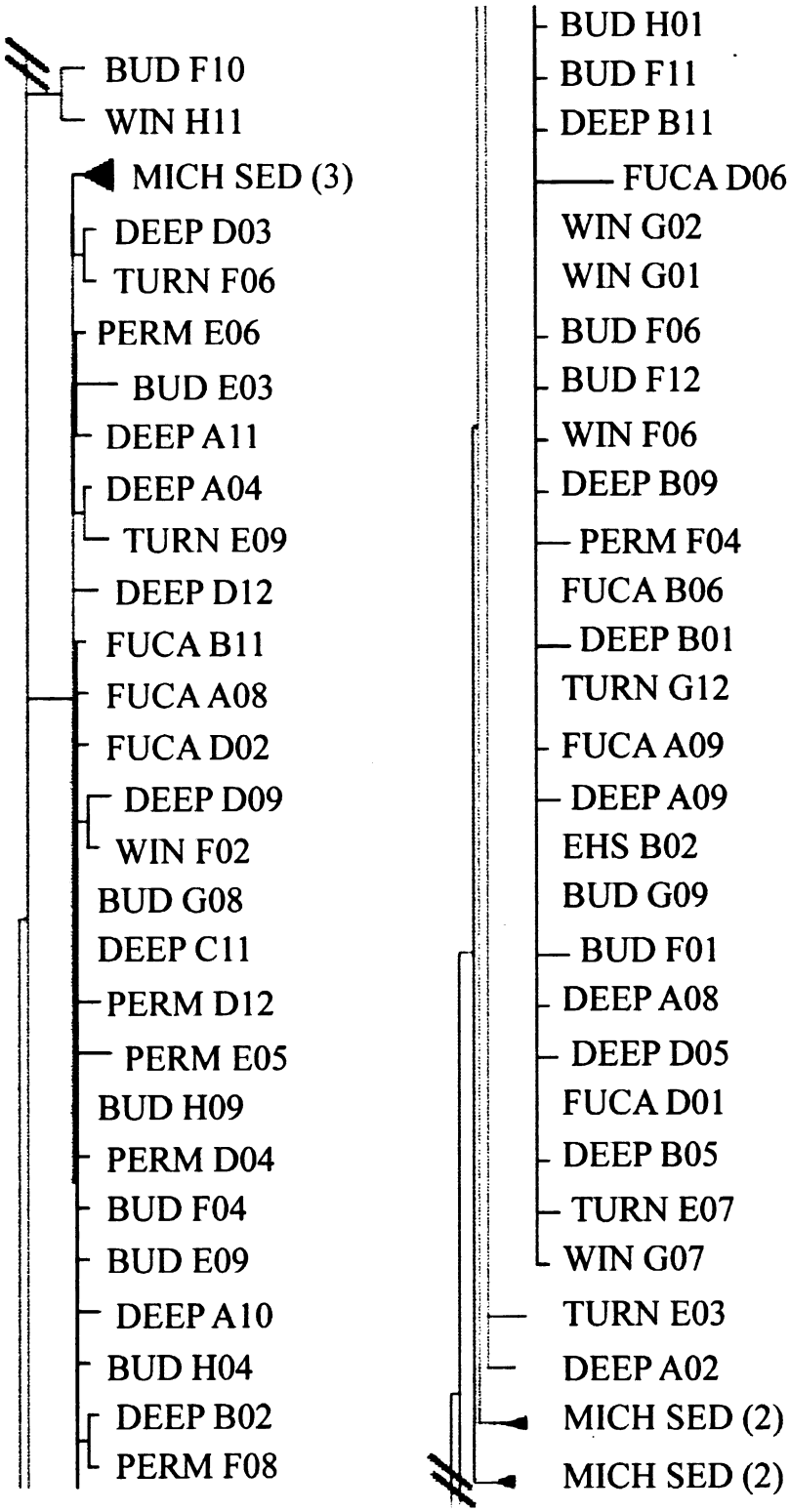


Figure 2.2. Neighbor-joining tree based on the 541F/1260R primer set derived 16S rRNA gene sequences after identical sequences were removed, illustrating the lack of phylogenetic distinctiveness between freshwater and marine samples. Right to left, top to bottom, with double bars indicating cutoff to the next tree. Groupings of Michigan sediments and Everglades samples are noted by triangles. DEEP=East Hanna Shoal Deep, Balt=four clone sequences derived from amplification of Baltimore Harbor sediments by Tal et al., 2005, Contractor=16S rRNA sequences derived from a rotating biological contractor.



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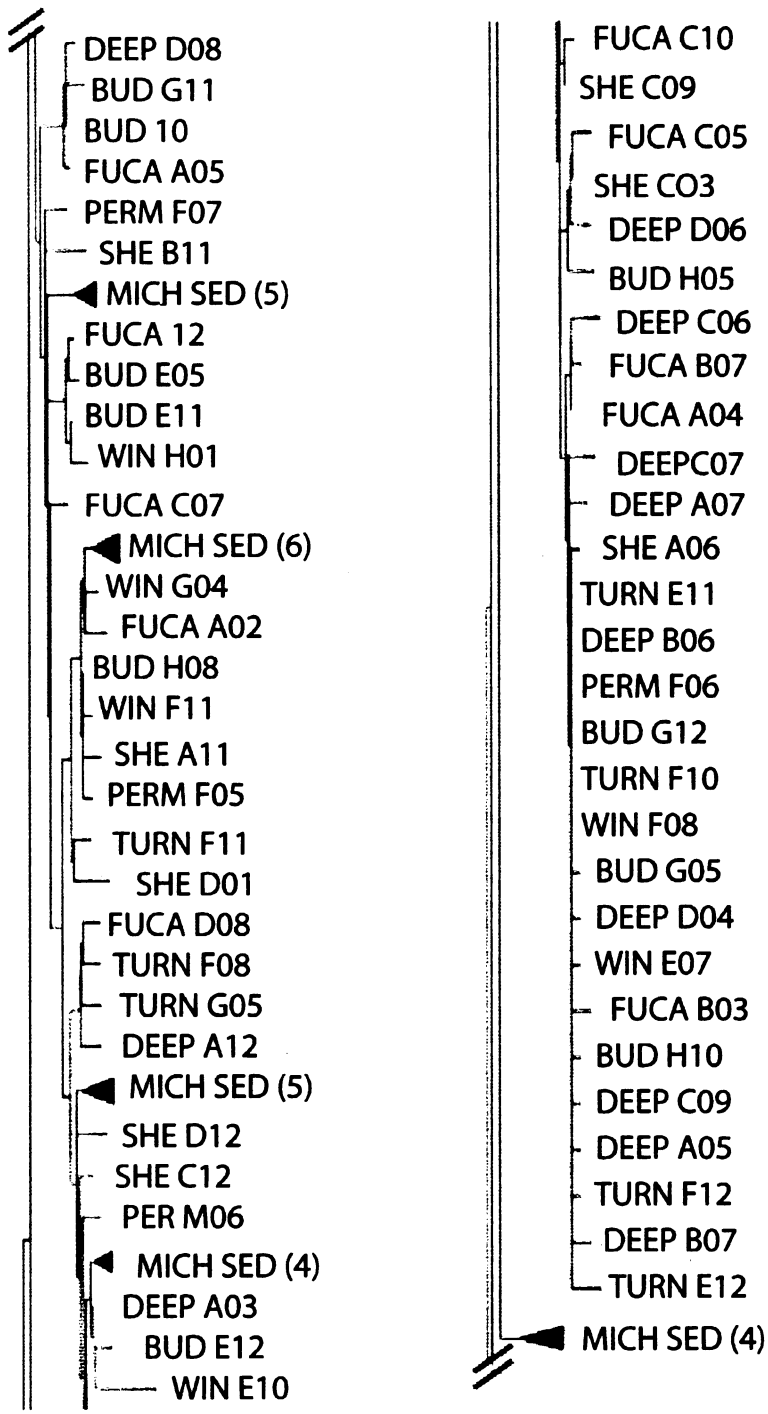

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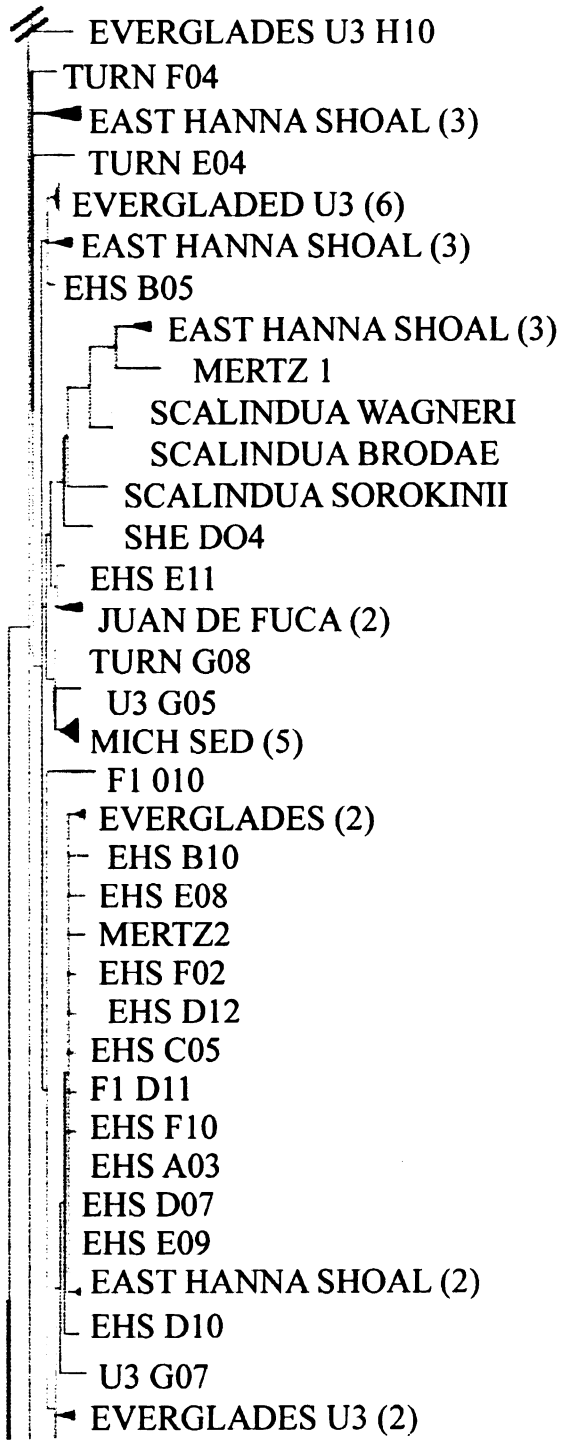

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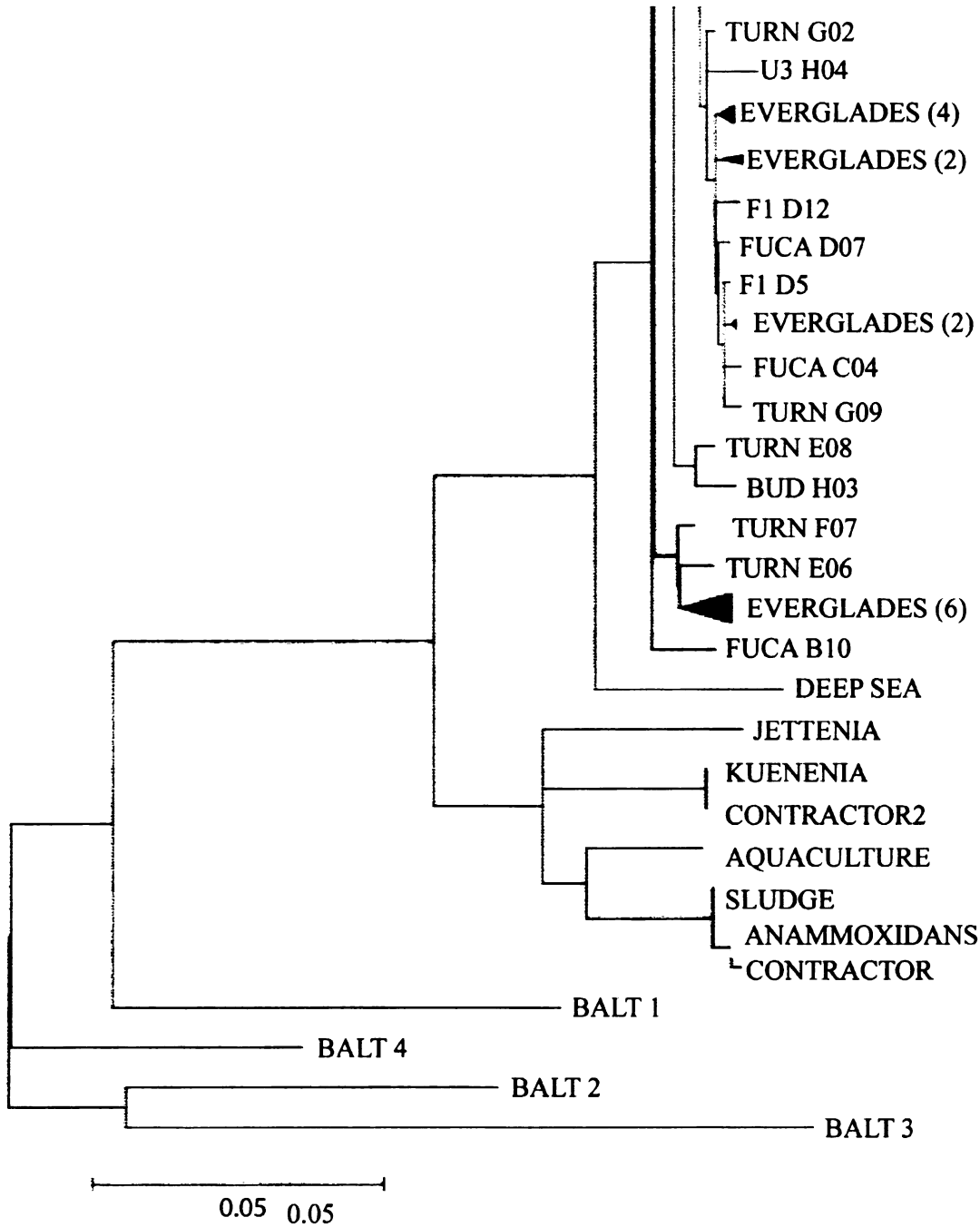

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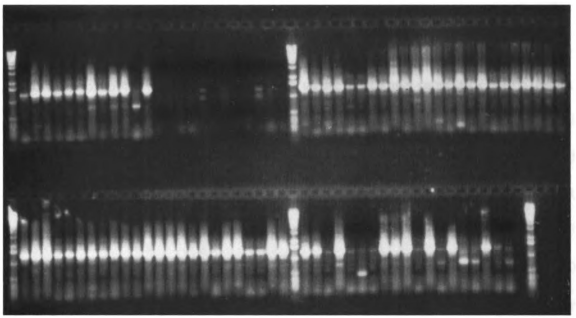

Figure 2.3. Amplicons resulting from the An7F/1388R primer set in Shallow Bud Inlet, East Hanna Shoal, and Washington Margin samples. 1kb ladders were used.

wagneri." There was one mismatch within the Brod541F and 100% homology in the Brod1260R site. A lack of homology to the *Planctomycetes* specific primer S-P-Planc-0046-a-A-18 (Pla46F) (Neef et al. 1998) was observed in both clones. Sequencing error was estimated at 4 bases in 1388 and anammox sequence targeting efficiency of this primer was estimated to be 10% of the total retrieved passing sequences. A neighbor joining tree was constructed using these full length sequences for comparison to the anammox related clones derived from GenBank (Figure 2.5).

Specific 16S rRNA sequences belonging to the anammox group have historically been difficult to recover from environmental samples. The *Planctomycetes* specific forward primer S-P-Planc-0046-a-a-18 (Pla46F), utilized as a "capture" primer for all anammox, has been used to construct clone libraries, generally in samples which show

evidence of anammox activity via ^{15}N isotope pairing studies. However, Pla46F under-represents anammox abundance in various studies (Egli et al. 2001, Schmid et al. 2000, 2003). For example, in an enriched sample where anammox represented 40% of the bacterial population, only 36% of Pla46F derived 16S rRNA clones sequenced belonged to the anammox group (Schmid et al. 2000). In environmental samples this method results in very low target sequence retrieval efficiencies, possibly due to inherent PCR template to product ratio bias (Polz and Cavanaugh 1998) or the lack of Pla46F specificity. For example, the lack of Pla46 primer annealing site homology with the 1380 bp clone FLE12 and clone FLC09 sequences in this study would have resulted in the omission of these clones from a Pla46F generated library.

In the context of this study, the novel primer set Brod541F-Brod1260R is an efficient method of screening environmental samples for the presence of *"Ca.* Scalindua"-like anammox. A previously suggested primer, S-*-BS-820-a-A-22 (Schmid et al. 2005), that targets "*Ca.* Scalindua wagneri" and "*Ca.* Scalindua sorokinii", appears to be specific for anammox bacteria with up to three mismatches but is only homologous with approximately 50% of the sequences recovered in this study. Conversely, primer S-*-Amx-0820-a-A-22 (Amx820), specific for genera "*Ca.* Brocadia" and "*Ca.* Kuenenia," (Schmid et al. 2000) exhibited at least three mismatches in approximately 80% and four mismatches in 50% of the sequences retrieved with the Brod541F-Brod1260R primer set which was expected due to our targeting of "*Ca.* Scalindua" bacteria. Lastly, primer S-*-

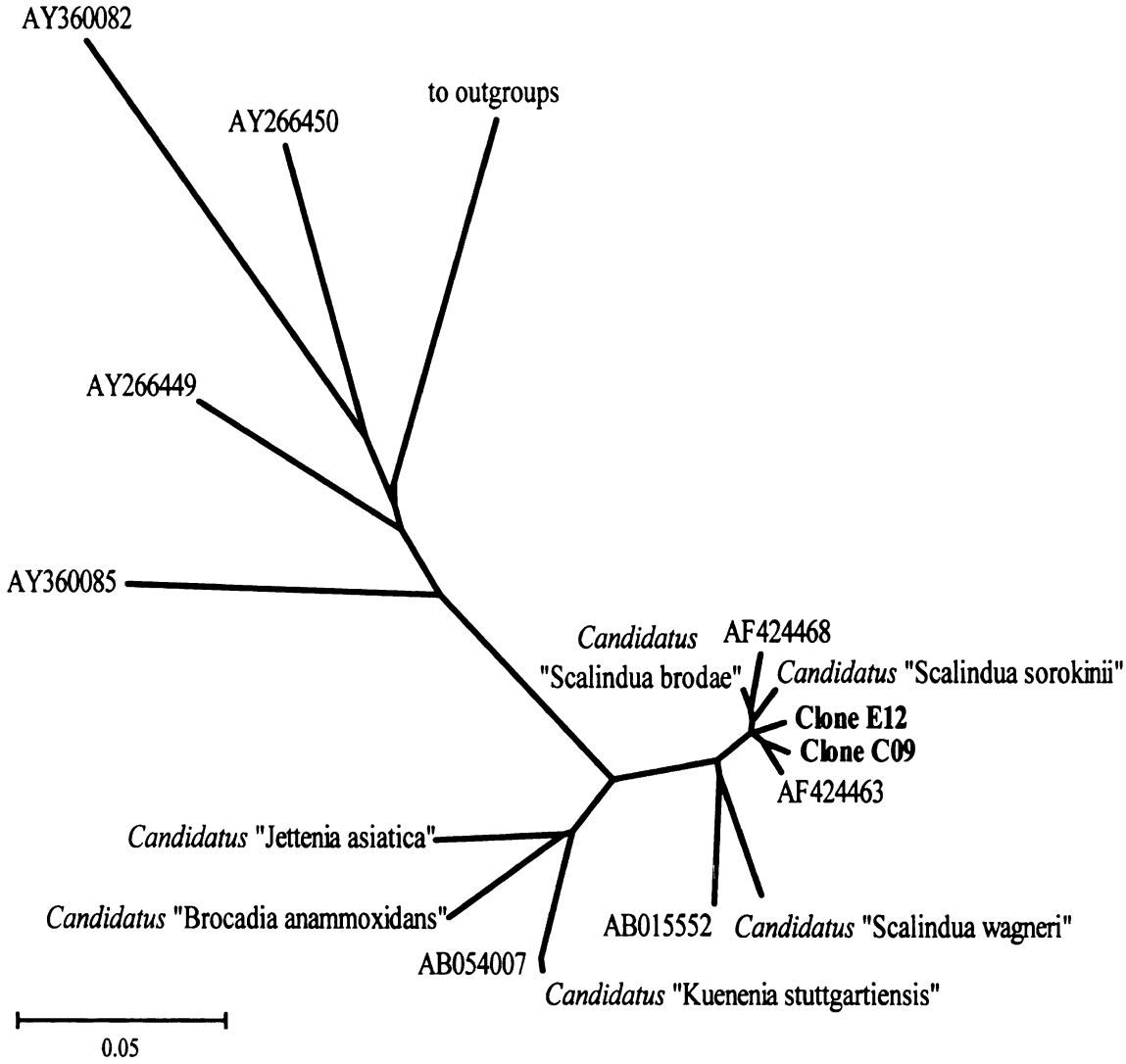

Figure 2.4. 16S rRNA gene-based bootstrapped neighbor joining tree reflecting the relationship of full length An7F-An1388R generated amplicons with the anammox group as well as other sequences derived from environmental samples given as GenBank accession numbers. The bar indicates a 5% sequence divergence.

Scabr-1114-a-A-22 (Scabr1114), specific for "*Ca.* Scalindua brodae" and recommended for use as a reverse primer with Pla46F (Schmid et al. 2003), exhibited at least 1 mismatch in 50% of the retrieved sequences. This suggests that use of these primers could result in the under-representation of anammox in environmental samples and illustrates the difficulty in specifically retrieving anammox sequences.

The identification of sequences with >96% nucleotide identity to the "*Ca.* Scalindua" anammox in all freshwater, marine, and permafrost samples tested constitute the first broad range 16S rRNA gene based molecular investigation of anammox distribution. The presence of anammox 16S rRNA gene sequences in anoxic deep sea sediments, shallow perturbed marine sediments, shallow organic rich freshwater sediments, periphyton dominated aerobic sediments, and ancient frozen permafrost sediments suggests that anammox bacteria in sediment are not constrained by conditions that appear to be unfavorable to their metabolism and thus may be more widely distributed than previously thought. While the presence of anammox sequences does not equate to *in-situ* anammox activity, they do serve to identify habitats for more intensive study and as molecular markers for better tracking candidate populations for anaerobic ammonium oxidation. The wide distribution of anammox sequences in sediments suggests that the process may not only be a significant oceanic nitrogen sink but may also be an important method of nitrogen removal from freshwater wetlands and lakes.

The GenBank accession numbers for the sequences reported here are DQ869865-DQ870184 and DQ869384-DQ869385.

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CHAPTER III

QUANTITATIVE PCR AND ACTIVITY OF ANAEROBIC AMMONIUM-OXIDIZING BACTERIA IN DEEP-PACIFIC OCEAN SEDIMENTS OF THE CASCADIA BASIN

ABSTRACT

The importance of anaerobic ammonium oxidation and the abundance of the bacteria responsible for this alternative nitrogen removal pathway were determined in deep continental margin sediments. Anammox was investigated in these 2800-3100m below water surface suboxic sediments using ^{15}N amendments coupled with porewater profiles and quantitative PCR of the *Scalindua* sp. type anammox 16S rRNA gene. Removal of NH_4^+ was indicated in the suboxic zone while anammox rates ranged from 0.065 to 1.7 nmol N mL^{-1} wet sediment h^{-1}. Estimated anammox abundances exhibited a mean density of 2 x 10^6 copies g^{-1} wet sediment and were correlated to anammox N_2 production (R^2=0.93). Significant numbers were found in the anoxic zone below the depth where NO_3^- and NO_2^- were identified. The overall contribution of anammox to total N_2 production ranged between 12 and 51%, in contrast to the general perception that anammox activity increases with depth below water surface.

INTRODUCTION

First discovered in a wastewater treatment plant (Mulder et al., 1995), the anaerobic ammonium oxidation (anammox) process was originally identified in the environment within continental shelf sediments (Thamdrup and Dalsgaard, 2002). The anammox process has been shown to be significant in ocean N removal and observations from oxygen minimal zones show that fixed N is lost exclusively through this pathway (Kuypers et al., 2005; Hamersley et al., 2007). Marine sediments are thought to be responsible for about half of the marine N_2 production and anammox activity has been detected in these environments worldwide. However, the range of reported anammox importance varies greatly, with estimates of anammox ranging between 0 and 80 percent of total N_2 production (Thamdrup and Dalsgaard, 2002; Risgaard-Petersen et al., 2004; Engström et al., 2005). Anammox has been thought to be responsible for an increasing percentage of total N_2 production as water depth increases (Dalsgaard et al., 2005), with the highest reported contribution at 700m depth in Skagerrak, Denmark (Thamdrup and Dalsgaard, 2002; Engström et al., 2005). This is thought to be due to the lower availability of reactive organic carbon in deeper sediments which would favor anammox over denitrification (Thamdrup and Dalsgaard, 2002) and would lead to anammox dominance at depths greater than 1000m (Dalsgaard et al., 2005).

The bacteria responsible for the anammox process reside within deep phylogenetic branches of the order *Planctomycetales* and have not yet been isolated in pure culture. 16S ribosomal ribonucleic acid (rRNA) gene targeted assays suggest that anammox bacteria are widespread in the environment (Penton et al., 2006), although the extent of their activity in many of these habitats is unknown. While the isotopic method

for anammox N_2 detection is now widely used for quantifying anammox N_2 production, the enumeration of anammox bacteria in sediments is performed less often. The knowledge of population depth distribution when accompanied by N_2 production rates of anammox bacteria allows for the calculation of cell-specific N_2 production. This can be further implemented to investigate correlations between anammox activity and sediment biogeochemistry. Fluorescence in-situ hybridization (FISH), targeting the 16S rRNA gene, has been the preferred method for enumerating anammox in environmental and wastewater samples (Schmid et al., 2005). FISH counts of anammox cells have ranged from 600 to 22,000 cells ml^{-1} in marine water column samples (Kuypers et al., 2003, 2005; Schubert et al., 2006). Conversely, Schmid and colleagues (2007) found $2.1x10^6$ to $1.4x10^8$ anammox cells ml^{-1} in nine diverse anoxic marine sediments while ~300 anammox cells ml^{-1} were found in Baltimore Harbor sediments (Tal et al., 2005) using the probe AMX820.

Quantitative polymerase chain reaction (Q-PCR) is a reliable indicator of bacterial numbers of targeted groups and can be an indicator of their activity (Grüntzig et al., 2001; Qiu et al., 2004; Schippers et al., 2006). Recently, the number of anammox bacteria closely related to *'Candidatus Brocadia anammoxidans'* was determined in enrichment cultures from a rotating reactor using a SYBR green Q-PCR assay targeting the 16S rRNA gene (Tsushima et al., 2007). Compared to FISH, the Q-PCR 98-well format allows for high sample throughput and eliminates investigator bias introduced by the microscopic counting technique. This method is not affected by background hybridization and the presence of dense cell clusters (Third et al., 2001) that can

influence cell counts in sediments, nor is it dependent on FISH hybridization stringencies that can vary between sediment types.

In this study we use ^{15}N isotope incubations to study benthic anammox activity at eight sites off the Washington/Oregon coast at depths of 2800-3100m We also quantitatively compare the anammox process at these sites with the catalytic bacteria, a particularly important goal for understanding the biogeochemical processes that influence anammox activity in the environment. Specifically, we developed a Q-PCR assay specific for anammox bacteria most closely related to *'Candidatus Scalindua'* (Schmid et al., 2003), the only currently recognized marine anammox bacteria.

MATERIALS AND METHODS

Site description and sediment sampling

Cascadia Basin is a sedimented basin enclosed within the North American margin, the Blanco Fracture zone and Juan the Fuca ridge, located at approximately 43-48°N and 125-130°W at an average depth of 2000-3000m. Sediment was collected at eight stations in the Cascadia Basin during a R/V. T.G. Thompson cruise (TN 198) in August 2006 (Figure 3.1). A multi corer was used to collect sediment cores (10 cm diameter) with a well-preserved sediment-water interface; on average the cores had a sediment depth of 40 cm and an overlying water column of 60 cm. Collected cores were stored on board at 4°C, in the dark.

Oxygen and porewater profiles

Three oxygen profiles were obtained from a smaller sediment core (5 cm diameter, 25 cm

length) that was sub-sampled from one of the original cores with a Clark type O_2 sensor (Diamond General Corp. Ann Arbor, United States of America (U.S.A.), type 737; Lohse et al. 1996). Oxygen profiles were measured within 1 hour of core retrieval and immediately after core sub-sampling.

A sediment core from each station was sectioned at 0.5 cm increments (0-5 cm sediment depth) and 1 cm increments (5-20 cm sediment depth) for pore water NO_2^-, NO_3^- and NH_4^+. Each section was placed in a 60 mL centrifuge tube, centrifuged for 20 min (11000 g) and supernatant water was filtered through a 0.45 μm cellulose acetate filter. All sediment slicing and centrifugation was performed at 4°C. Inorganic N samples were analysed within 5 hours from sampling, on a semi-automatic analyzer according to standard colorimetric procedures (Strickland and Parsons 1972). Porosity was estimated from sediment collected from the same profile as the inorganic N. Collected sediment was stored in pre-weighed sealed glass vials until the end of the cruise and porosity was calculated from the weight of water loss after drying the sediment (50°C) to constant weight. Samples for pore water Mn^{2+} analysis were stored frozen, acidified with HCl immediately prior to analysis and concentration was determined using a Perkin Elmer flame AAS.

Nitrate consumption and ammonium production rates in the sediment were calculated by taking the slope based on the decreasing values from the maximum NO_3^- concentration or the slope based on the increasing values from the minimum NH_4^+ concentration. Flux was then calculated using Fick's first law of diffusion, assuming a

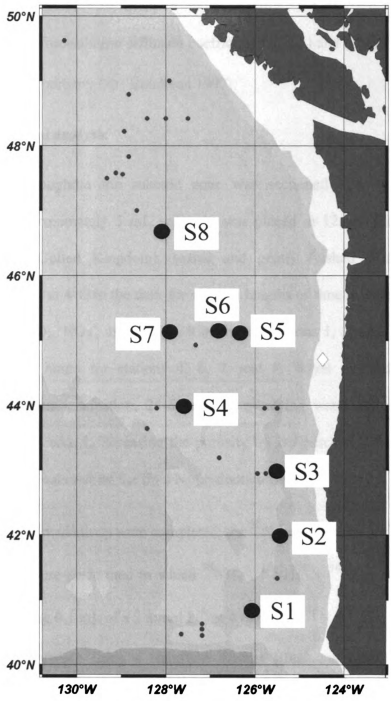

Figure 3.1. Map of the eight station locations (S1-S8) in the Cascadia Basin, North East Pacific Ocean.

steady state. The sediment diffusion coefficient (D_{sed}) used in these calculations was estimated from the free-solution diffusion coefficient (D_0; Li and Gregory 1974) and the measured sediment porosity (Φ; Boudreau 1997).

^{15}N incubations and analysis

Sediment throughout the suboxic zone was sectioned into 2 cm slices and homogenized. Approximately 5 mL sediment was placed in 12 mL Exetainer (Labco, High Wycombe, United Kingdom), sealed and gently flushed with helium. Pre-incubation followed at 4°C in the dark for varying lengths of time in order to remove the residual sediment O_2, NO_2^-, and NO_3^-: 30 hours for stations 1, 2, and 3, 50 hours for station 5, and 65 hours for stations 4, 6, 7, and 8. When the sediment was too consolidated for homogenization, 24-60 mL of overlying water collected from each respective core was added, increasing the porosity by 2-9 percent. This was taken into consideration when calculating the final N_2 production rates.

After the pre-incubations were completed, the ^{15}N isotopes were added (Figure 3.2). Three treatments were performed in which $^{15}NH_4^+$, $^{15}NH_4^+ + {}^{14}NO_2^-$ and $^{15}NO_2^-$ were added by introducing 0.1 mL of a 2 mmol L^{-1} or 4 mmol L^{-1} ^{15}N solution to the Exetainer through a rubber septum using a Hamilton syringe (final concentrations ~ 40 nmol $^{15}NH_4^+$ mL^{-1} (wet sediment), ~ 80 nmol $^{15}NH_4^+$ + 40 nmol $^{14}NO_2^-$ mL^{-1} (wet sediment), ~ 40 nmol $^{15}NO_2^-$ mL^{-1} (wet sediment). According to Trimmer et al. (2005), potential anammox rates show no response to added nitrite at concentrations over 10 μmol L^{-1} NO_2^-. A Helium flow cap was used to prevent headspace contamination with O_2 or N_2

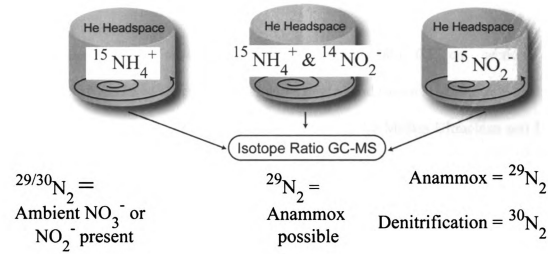

Figure 3.2. Figure illustrating the experimental setup for the determination of anammox activity.

during isotope addition. Incubations were stopped with 0.1 mL 7 M $ZnCl_2$ at regular intervals.

The $^{15}NH_4^+$ treatment was used as control to detect oxidation of ammonium in the sediment without the addition of nitrite. If all of the oxidants are removed during pre-incubation, the $^{15}NH_4^+$ treatment should not show production of $^{29/30}N_2$. In the parallel $^{15}NH_4^+$ + $^{14}NO_2^-$ treatment, $^{29}N_2$ production suggests that the added nitrite is the oxidizing agent for ammonium. The combination of these two treatments establishes anammox activity. Lastly, potential anammox and denitrification rates were estimated from the $^{29}N_2$ and $^{30}N_2$ production rates in the $^{15}NO_2^-$-only treatment according to Thamdrup and Dalsgaard (2002). Concentrations of $^{29/30}N_2$ were determined using a Finnigan-Mat 253 isotope ratio mass spectrometer and calculated as excess above natural concentrations.

DNA extraction

Extruded whole sediment cores were sliced into 0.5 cm sections and approximately 5 g of stirred sediment was sub-sampled and stored at -20°C until analysis. Genomic DNA from 0.5 g sediment was isolated using the MoBio Ultraclean soil DNA kit (MoBio Laboratories, Inc.) and further purified by electrophoresis in 0.8% Cambrex SeaPlaque low melting temperature agarose at 4°C. Genomic DNA bands were gel-extracted, treated with gelase (Epicentre) per manufacturer's instructions, and concentrated using Microcon columns. DNA concentrations were determined by absorbance at 260 nm. The number of genomes g^{-1} sediment was estimated using the conversion of 980 Mbp pg^{-1} DNA and an average genome size of 4.3 Mbp.

Primer-probe design and specificity

We were unable to identify applicable primer sequences for SYBR green quantitative anammox determination due to the relatively small number of unique homologous regions of the 16S rRNA gene for specific amplification of anammox without primer dimer formation. Hence, the Taqman assay was used to overcome these limitations. Two primers were designed for the detection of anammox 16S rRNA genes: 541FRT (5'-GAG CAC GTA GGT GGG TTT GTA AG-3') and 616RRT (5'-CCT CCT ACA CTC AAG ACT YGC AG-3'). Primer 541FRT was based on the previously described primer Brod541F, found to be 100% specific for anammox 16S rRNA genes found in various environmental samples including the Juan de Fuca Ridge and Washington Margin, both in the Pacific Ocean (Penton et al., 2006). The Taqman probe AnPrb (5'-CAG RTG TGA AAG CCT TCT GTT CAA CGG AAG-3') was labeled 5'

FAM and 3' Black Hole Quencher-1® (Integrated DNA Technologies, Coralville, Iowa). Alignments of 475 16S rRNA gene sequences obtained with Brod541F and Brod1260R as well as environmental clones related to anammox from GenBank were used to confirm primer and probe complementary regions and determine where the use of degenerative bases was necessary. The primers and probe were designed with the program PrimerExpress (PE Applied Biosystems), and verified against GenBank for specificity: 540RT, 616RT, and AnPrb respectively showed one, two, and three mismatches against '*Candidatus Scalindua wagneri*' (AY254882), none, none, and two (due to extra A's in the GenBank 16S sequence, possibly due to extra base calls) mismatches with *'Candidatus Scalindua brodae'* (AY254883), two, zero, and zero mismatches with *'Candidatus Scalindua sorokinii'*: (AY257181), and generally all had zero mismatches with the 475 clones from our previous study (Penton et al., 2006).

Quantitative PCR assays

Quantitative PCR reactions and optimizations were performed as described in the Applied Biosystems protocol for Taqman assays. Final assay concentrations of 541FRT, 616RRT, and AnPrb were 300 nmol L^{-1}, 900 nmol L^{-1}, and 250 nmol L^{-1}, respectively with 1 μL of genomic DNA added per well and annealing and extension temperatures of 61°C. The increase in fluorescence emission was monitored during PCR amplification using a 7700 Sequence Detector (PE Applied Biosystems). The threshold cycle (C_T) values obtained for each sample were compared to the standard curve to determine the initial 16S rRNA gene copy number and were expressed as μg^{-1} environmental DNA and normalized to g^{-1} wet sediment. Q-PCR assays were performed on 0.5-cm core sections while anammox incubations were performed on 2-cm sections. Five laboratory replicates

were used per 0.5 cm core section. The relationship between molecular data and ^{15}N incubation data were based on means obtained from the 0.5-cm sections per 2-cm sample.

Q-PCR sensitivity, detection limit, and accuracy

Sensitivity determination and standard curve generation were performed using an anammox 16S rRNA gene containing clone which exhibited 99% nucleotide identity to the characterized anammox *'Candidatus Scalindua wagneri'*. The clone was created using primers Brod541F/Brod1260R from various environmental samples (Penton et al., 2006) and dilutions ranging from 4 to $8x10^8$ copies were prepared in individual reaction tubes. A calibration curve was constructed to determine our ability to discriminate two-fold differences in template concentrations and the lower detection limit with assays performed in triplicate. In order to test for the presence of PCR inhibitors, $1x10^4$ clone 16S rRNA gene copies were added to representative samples from each site. Copy numbers, with and without the addition of $1x10^4$ copies, were compared and the percent recovery from each sample was determined.

RESULTS

Porewater O_2 and dissolved inorganic nitrogen

. Porewater and NH_4^+ and NO_3^- profiles from continental margin sediments in the area of this study (Lambourn et al. 1996; Stump and Emerson 2001), the western Mexican margin (Hartnett and Devol 2003), California margin (Reimers et al. 1992) and Panama Basin (Aller et al. 1998) all show a pattern that indicates ammonium removal in

the nitrate reduction zone followed by ammonium accumulation after nitrate is depleted. Another indication of nitrogen removal in these sediments is the low N:P ratios that decrease from 6.5 to <1 in the suboxic zone (5-20 cm; Station 5 and 6 in Stump and Emerson 2001).

Among the eight stations, oxygen penetrated 0.5 to 1 cm into the surface of the sediments. Nitrate penetration differed widely between sites, but were generally divided into two groups, those close to shore (S1, S2, and S3; 2.5 cm), and those farther from shore (S4, S7, S8; 7-10 cm) (Figures 3.3a and b).. The porewater Mn^{2+} distribution was measured at stations S2, S4, S6, and S8. Patterns showed an increase in Mn^{2+} in the middle of the nitrate reduction zone. Although both MnO_2 reduction and denitrification release NH_4^+ to the porewater, porewater profiles show a net ammonium removal in the suboxic zone followed by ammonium accumulation starting at the nitrate depletion zone.

^{15}N incubation experiments

There was no evidence of residual oxidized nitrogen species following depletion during pre-incubation in stations S1, S2, S3, S5, S6, and S7 as determined by the $^{15}NH_4^+$ only incubations. However, at stations S4 and S8 NO_2^- and NO_3^- were present at the zero time, identified by the detection of $^{29}N_2$. Small amounts of $^{29}N_2$ (0.5 μmol L^{-1}) accumulated after the addition of $^{15}NH_4^+$, but remained constant throughout the length of the incubation. This indicated that the initial NO_3^- / NO_2^- was the only oxidant present. Significant $^{29}N_2$ production was detected through the suboxic zone at all stations after the addition of both $^{15}NH_4^+$ + $^{14}NO_2^-$ (Figure 3.4). In the incubations where $^{15}NO_2^-$ was added, both $^{29}N_2$ and $^{30}N_2$ were formed immediately. ^{15}N-labelled N_2 could only be

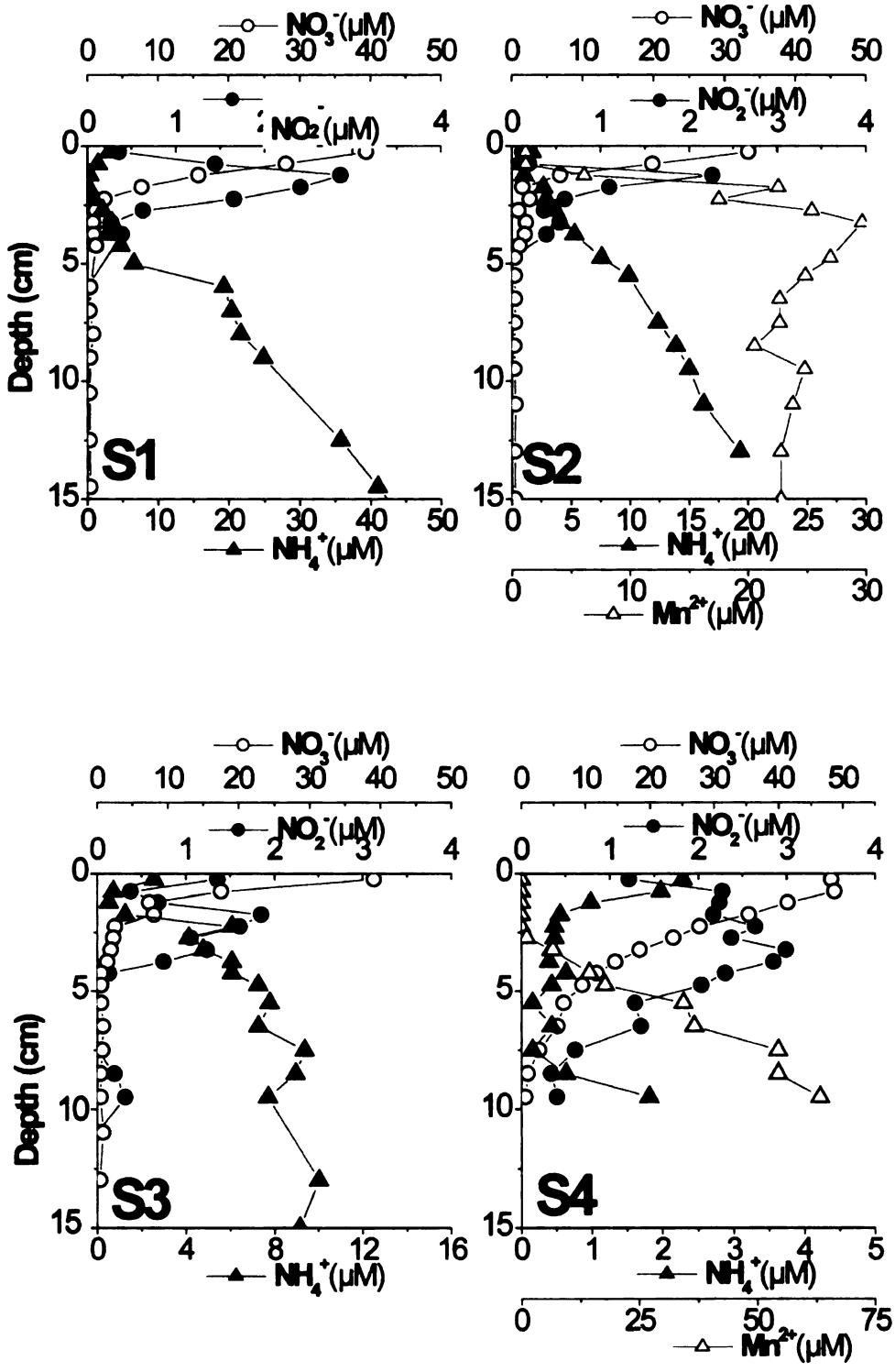

Figure 3.3a. Pore water distribution of NH_4^+(▲), NO_2^- (•) and NO_3^- (○) at stations S1-S4. At S2 and S4, Mn^{2+} (Δ) profiles are also shown. Note that the scale for ammonium and Mn^{2+} concentrations vary between stations.

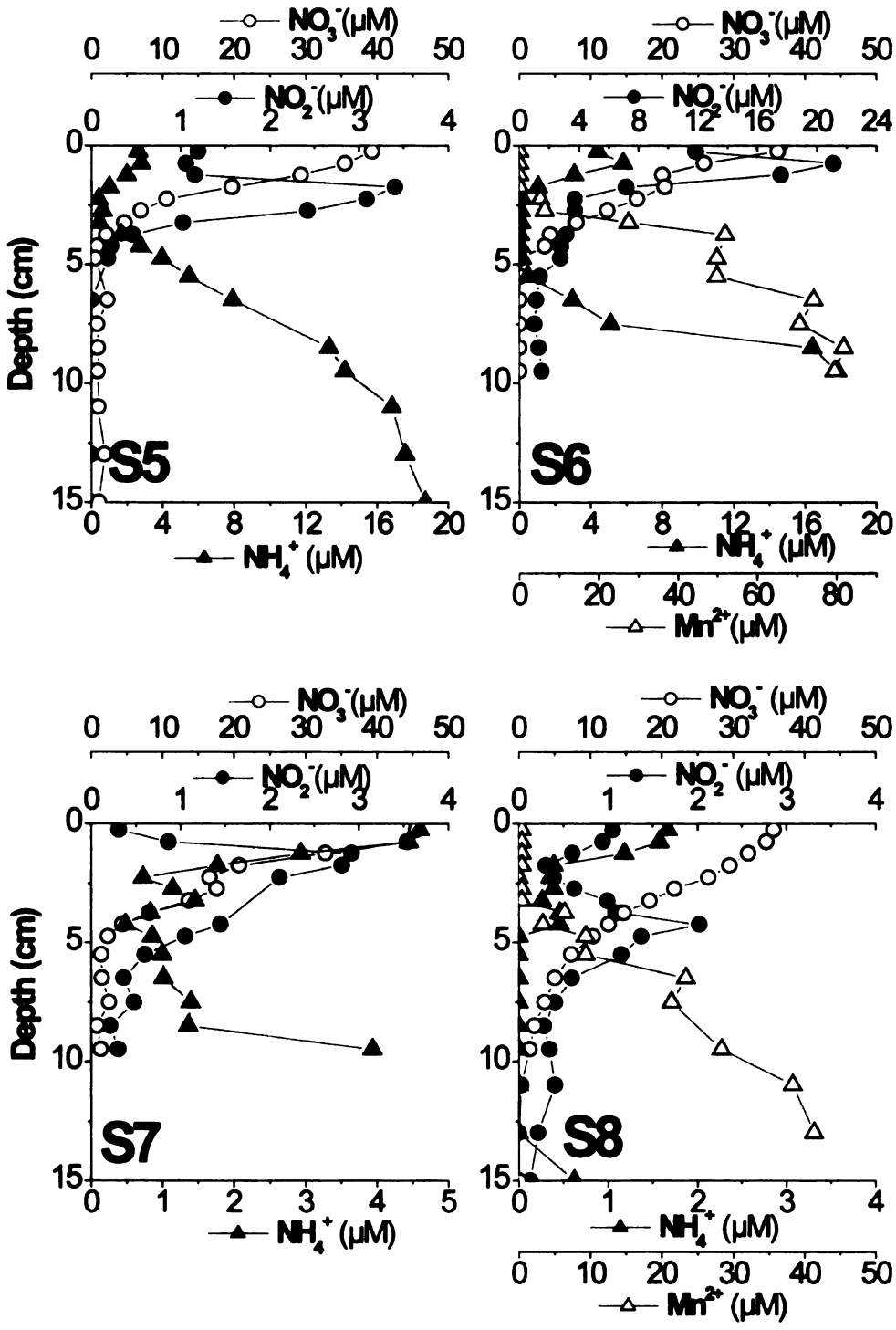

Figure 3.3b. Pore water distribution of NH_4^+(▲), NO_2^- (•) and NO_3^- (○) at stations S5-S8. At S6 and S8, Mn^{2+} (Δ) profiles are also shown. Note that the scale for ammonium and Mn^{2+} concentrations vary between stations.

detected in the presence of NO_3^- / NO_2^- which suggest that it is the dominant oxidant for anaerobic ammonium oxidation.

Total anammox contribution to total N_2 production ranged between 12-51 percent with an average of 38 ±9.9 percent. Absolute rates varied between 0.065-1.7 nmol N mL^{-1} wet sediment h^{-1} (anammox) and 0.17–1.8 nmol N mL^{-1} wet sediment h^{-1} (denitrification) (Figures 3.5a and b). Both potential denitrification and anammox activity decrease with distance from shore (Figure 3.6) with a stronger relationship with anammox activity (R^2=0.67) than denitrification (R^2=0.38). One sediment depth showed an anammox contribution of only 12 percent, due to a low anammox rate (0.065 nmol N mL^{-1} wet sediment h^{-1}) when compared to the 4-6 cm depth at S7 (0.25 nmol N mL^{-1} wet sediment h^{-1}) where the anammox contribution was 38 percent. If this value is excluded then the average anammox contribution was 40 ±7.4 percent.

Quantitative PCR Assays

Using 475 sequences with >96% homology to the anammox *'Candidatus Scalindua wagneri'* and *'Candidatus Scalindua brodae'* obtained from our previous study (Penton et al., 2006), we modified the forward primer by extension and inclusion of degenerative bases for use with the Taqman quantitative PCR system. The Q-PCR targeting of these specific anammox was further confirmed by performing GS FLX pyrosequencing (Genome Sequencer FLX System, 454 Life Sciences, Inc., Bradford, CT, USA) in station 2 surficial (0-2 cm) sediments using primers covering the 16S rRNA gene V4 hypervariable region. Using the RDP Classifier to sort sequences into the unidentified *Planctomycetes*, we identified 25 anammox sequences out of 4118, all of

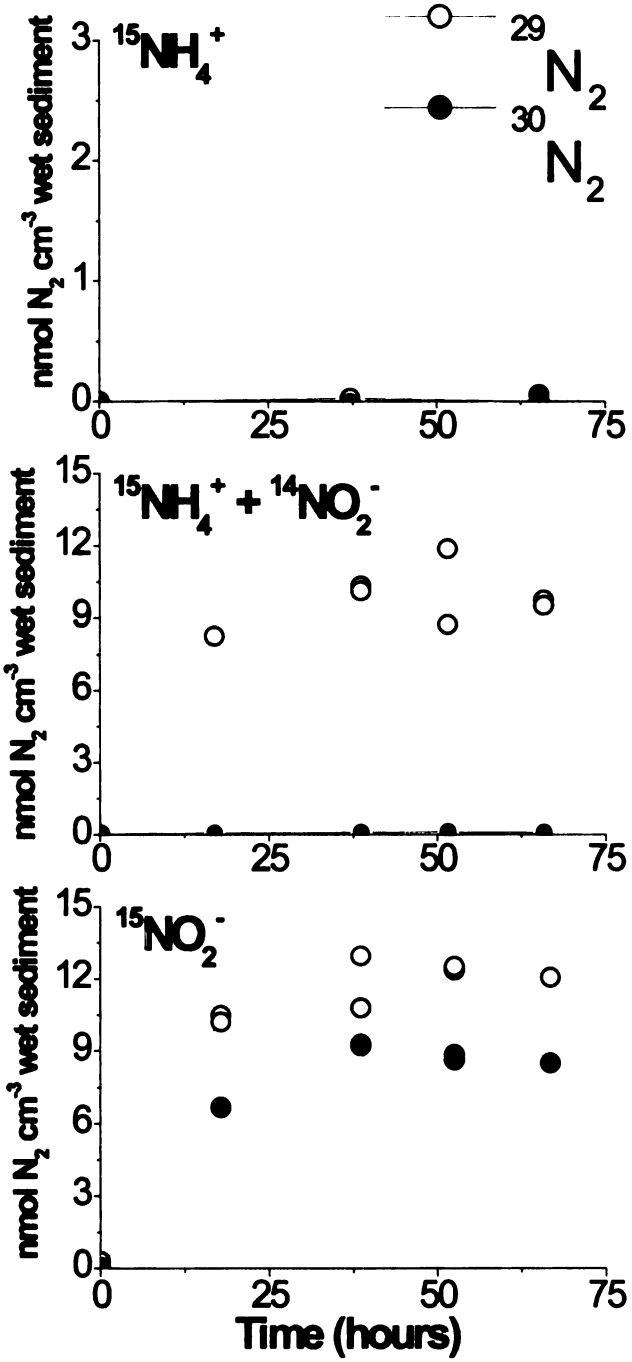

Figure. 3.4. Production of $^{29}N_2$ and $^{30}N_2$ over time in anoxic sediment incubations following additions of 40 nmol $^{15}NH_4^+$ ml^{-1} (wet sediment), 80 nmol $^{15}NH_4^+$ + 40 nmol $^{14}NO_2^-$ ml^{-1} (wet sediment) and 40 nmol $^{15}NO_2^-$ ml^{-1} (wet sediment); n=2. Data are from ^{15}N incubations at station S5, anammox and denitrification rates were estimated from $^{29}N_2$ and $^{30}N_2$ production rates in the $^{15}NO_2^-$ treatment.

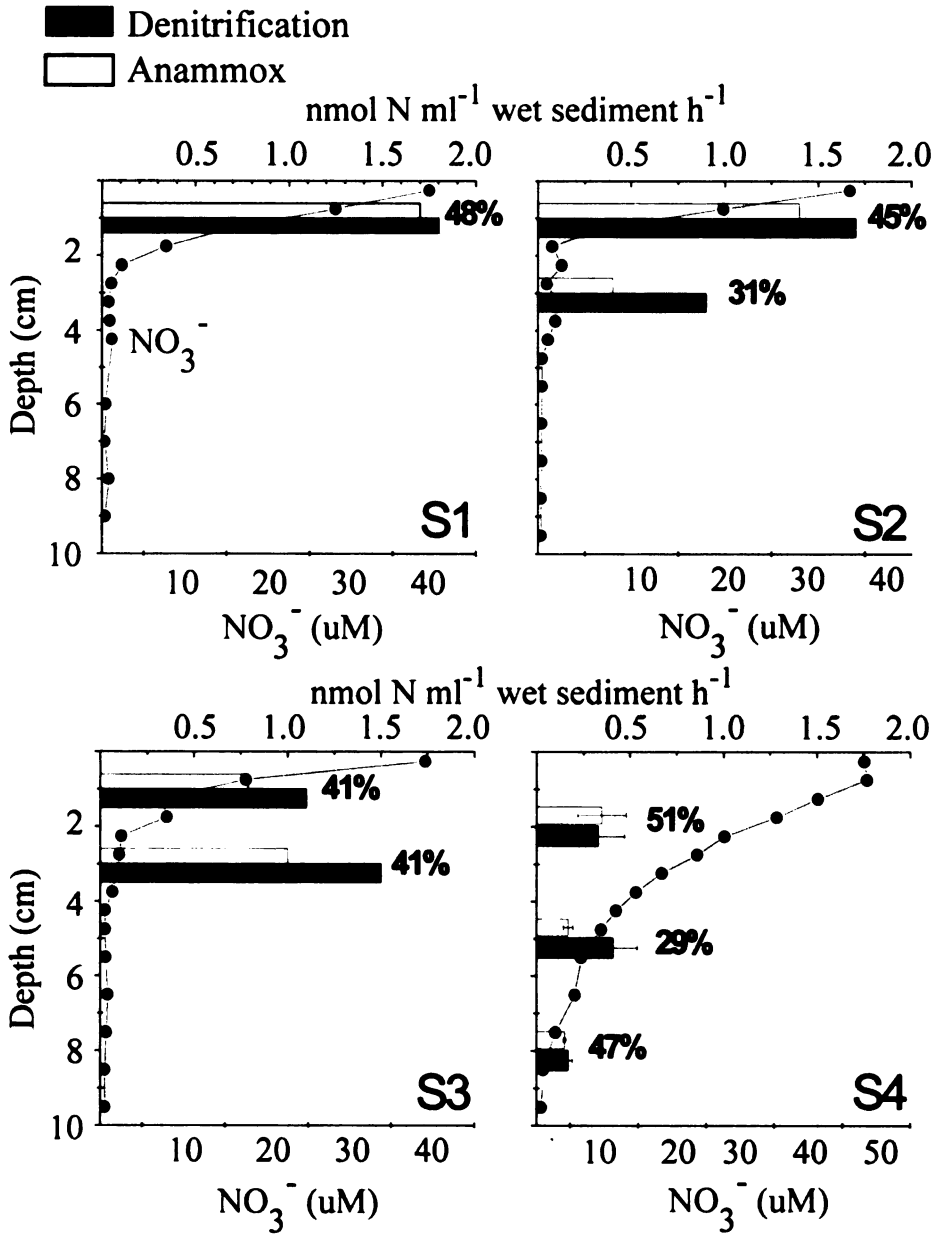

Figure 3.5a. Absolute anammox (white bar) and denitrification (black bar) rates estimated from anoxic ^{15}N sediment incubations at stations S1-S4. Error bars denote standard deviation at the stations where a replicate core could be tested (S4). Percent values show anammox contribution to total N_2 production at each depth where N_2 production rates were measured. Pore water nitrate distribution (•) indicate the suboxic zone at each site. In this study the suboxic zone is defined as the zone below oxygen penetration depth where nitrate is present.

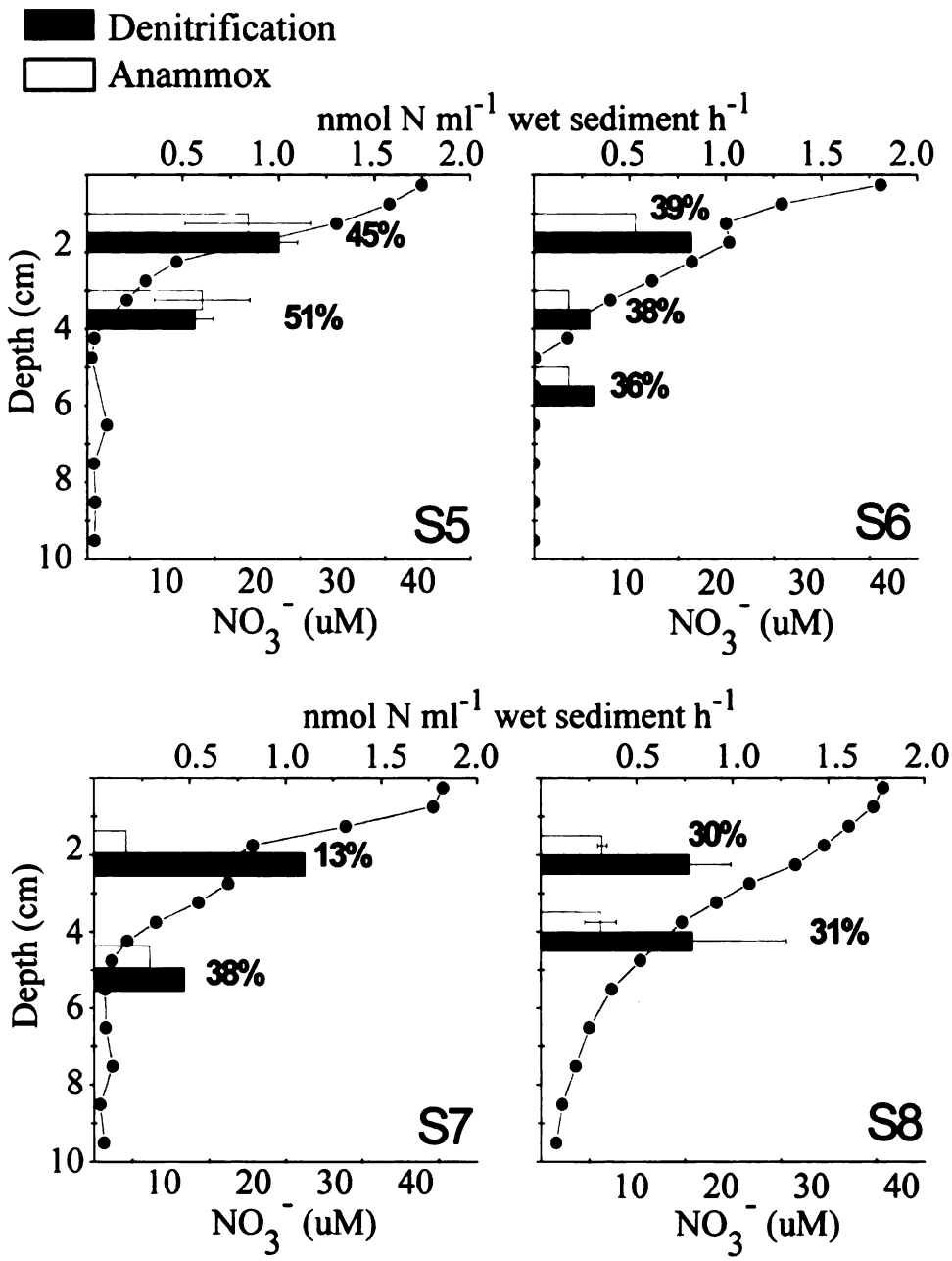

Figure 3.5b. Absolute anammox (white bar) and denitrification (black bar) rates estimated from anoxic ^{15}N sediment incubations at stations S5-S8. Error bars denote standard deviation at the stations where a replicate core could be tested (S5 and S8). Percent values show anammox contribution to total N_2 production at each depth where N_2 production rates were measured. Pore water nitrate distribution (•) indicate the suboxic zone at each site. In this study the suboxic zone is defined as the zone below oxygen penetration depth where nitrate is present.

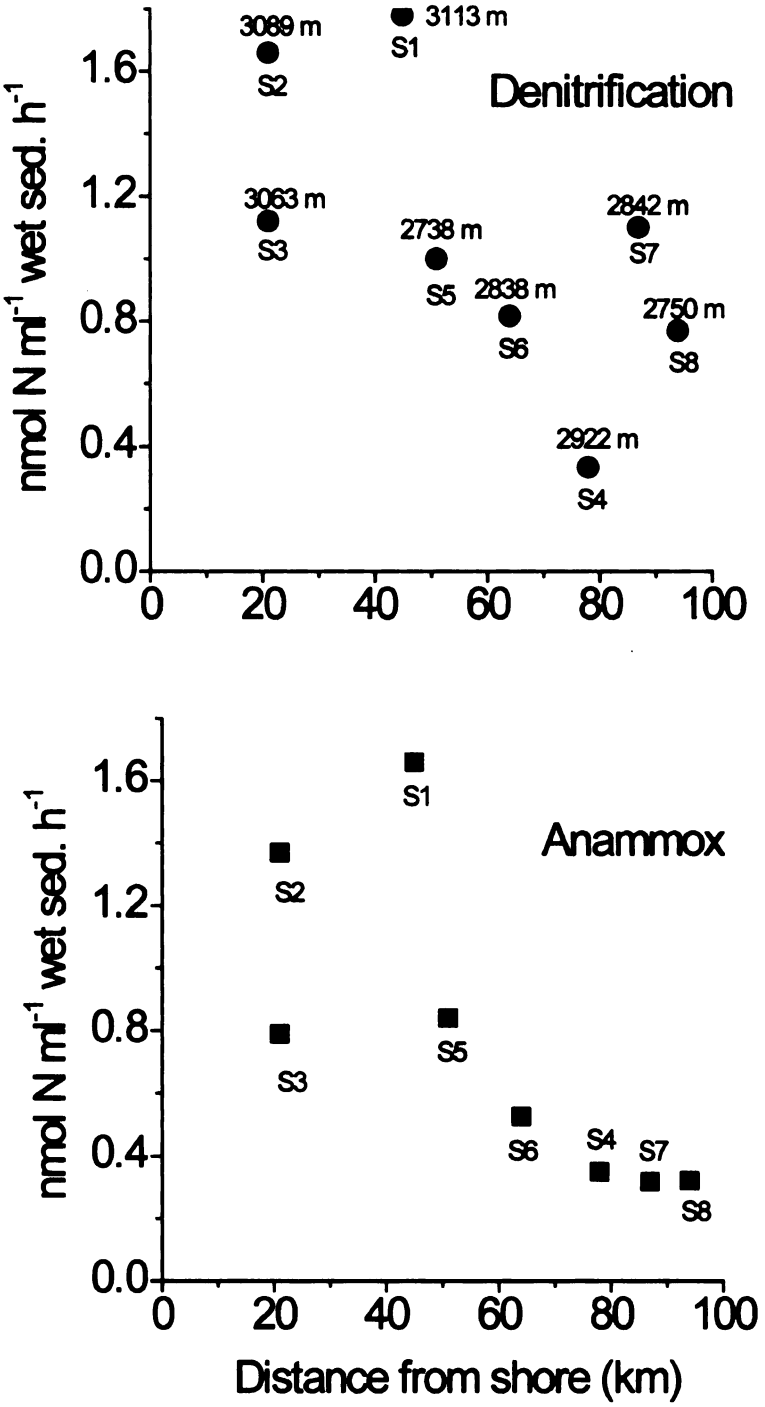

Figure 3.6. Absolute denitrification (•) and anammox (○) rates in Cascadia Basin distributed with distance from shore (km). Rates presented in the diagrams are estimated from the top 2 cm of the suboxic zone (the zone below oxygen penetration depth where nitrate is present). Distances were estimated with the GPS program Tsunami 99 from Transas.

which were >98% homologous to the *'Candidatus Scalindua'* group (Figure 3.7). No other 16S rRNA sequences related to the anammox group were identified.

Target copy number was determined using standards containing a cloned full length 16S rRNA gene sequences highly related (>98%) to *Scalindua brodae*. Amplification was monitored by the increase in fluorescence per cycle and the Ct value, the point at which fluorescence crosses the pre-determined threshold, was subsequently determined (Figure 3.8). The Q-PCR assay sensitivity and detection limit was tested using an anammox 16S rRNA gene clone diluted from $8x10^8$ to 4 copies (Figure 3.9). The calibration curve showed that fluorescence was detected as low as four copies, however precision was limited at lower copy numbers in part due to increased error at higher C_T's, similar to results from earlier studies (Grüntzig et al., 2001; Rodrigues and Tiedje, 2006). A regression of mean threshold cycles (C_T) and the template DNAs along the dilution range from 100 (the determined detection limit) to $8x10^8$ copies gave a R^2 of 0.99. The accuracy of the system was then tested by the addition of $8x10^4$ 16S rRNA gene copies to representative samples from each sediment core in the Q-PCR assay. The PCR reaction was generally not inhibited, as ten of thirteen depths exhibited 90-100% recovery with the exception of an unexplained 57% recovery at station 5 in the 2-4 cm slice (Figure 3.10).

Anammox 16S rRNA genes were distributed through Cascadia Basin sediments at a mean density of $2x10^6$ 16S rRNA gene copies μg^{-1} of total community DNA or $1.6x10^6$ copies g^{-1} wet sediment and accounted for an estimated 0.1% to 5.2% of the total bacterial genomes present (Figure 3.11) which ranged from $9x10^7$ to $6x10^8$ g^{-1} wet sediment. Using the ^{15}N measurements from the accompanying paper the activity

measures were compared with anammox population densities in each 2 cm slice. Anammox copy numbers, in μg^{-1} DNA determined by Q-PCR correlated (R^2=0.93) with anammox N_2 production (nmol N ml^{-1} wet sediment h^{-1}) (Figure 3.12). This remained after the two highest values were removed (R^2=0.68) and when all values from each 0.5 cm slice were included (R^2=0.65). Percent anammox copy number of total environmental genomes and anammox N_2 production were also linearly correlated (R^2=0.94). Cell-specific activities were calculated by dividing the anammox N_2 production by anammox 16S rRNA gene copy number for each sample and ranged from 1.3 to 11.3 fmol ammonium $cell^{-1}$ day^{-1}. There were no relationships between NO_2^-, NO_3^-, or NH_4^+ profiles and anammox bacterial abundance. A depth profile from the sediment surface to 22 cm depth at station 2 showed that anammox numbers remained relatively constant to a depth of 12 cm, ranging from $9x10^5$ to $4x10^6$ g^{-1} and then decreased to $8.8x10^4$ g^{-1} at 19 cm (Figure 3.13).

DISCUSSION

Anammox appears to be a significant pathway for sediment nitrogen removal in Cascadia Basin with an average contribution of 40 percent. This is in contrast to the trend of increasing relative anammox importance with increasing depth below water surface (bws) (Thamdrup and Dalsgaard 2002; Engström et al. 2005). When this trend is extrapolated to sediments greater than 1000m bws, anammox is suggested to dominate over denitrification (Dalsgaard et al., 2005), in conflict with these findings. The contribution of anammox of 12-51 percent is within the range of many shallow and intermediate depth sediments (40-400 m) (Dalsgaard et al., 2005).

Figure 3.7. Variable region 4 16S rRNA based UPGMA tree using sequences obtained from 454 pyrosequencing in station 2 (0-2 cm depth) sediment.

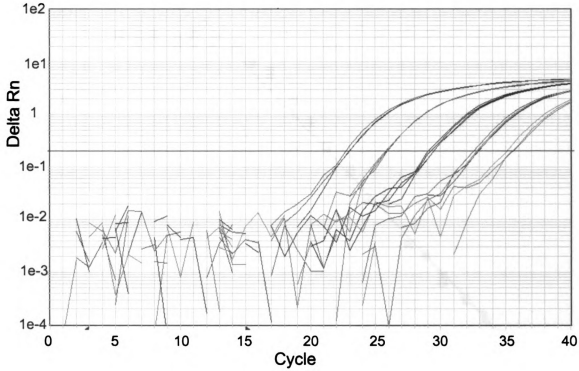

Figure 3.8. Output of SDS software for a standard curve using a clonal full length *Scalindua brodae* 16S rRNA gene with replication.

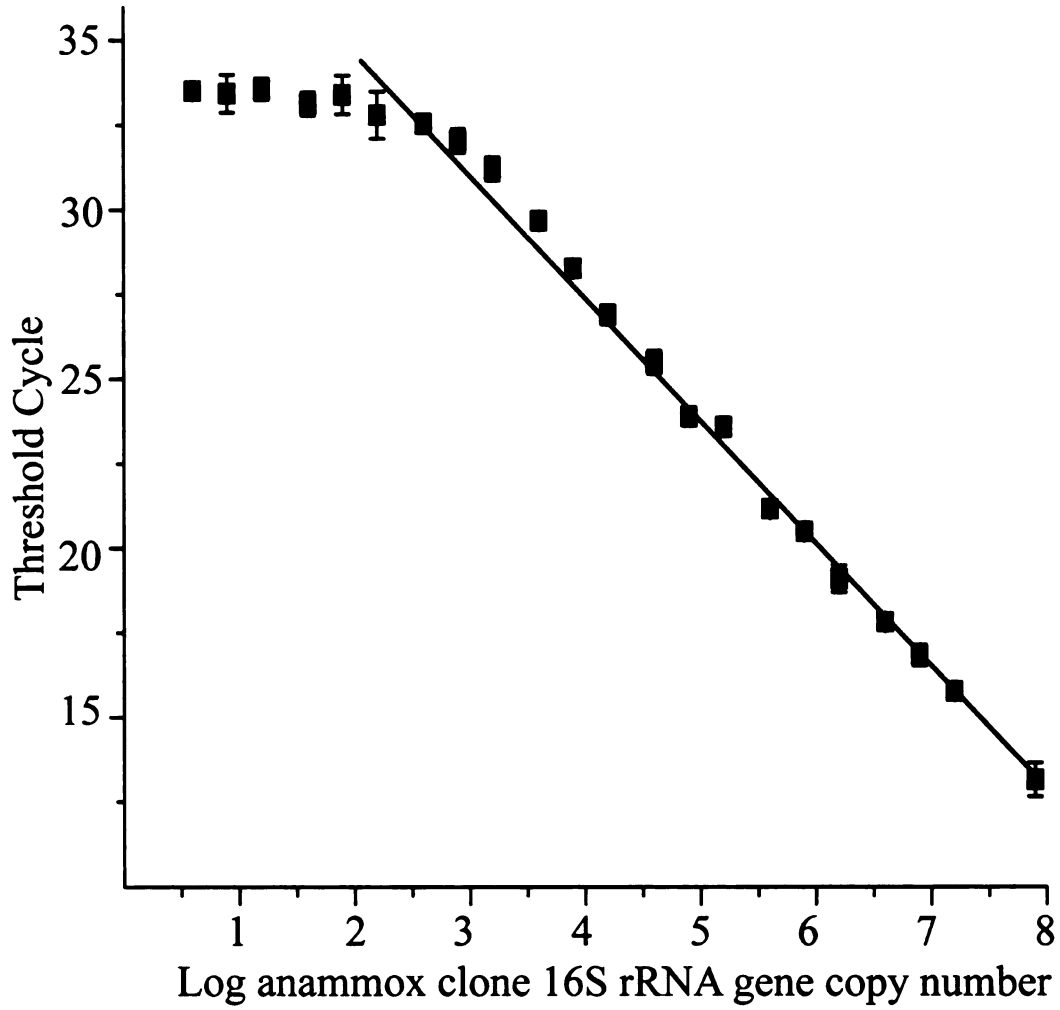

Figure 3.9. Calibration curve linear regression of anammox 16S rRNA gene copy numbers determined from a clone dilution series from 8 to $8x10^8$ copies. C_T is the threshold cycle number.

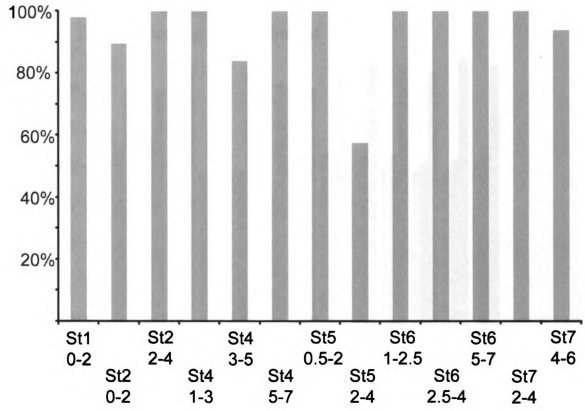

Figure 3.10. Percent recovery of 10^4 added 16S rRNA gene copies from each sediment matrix.

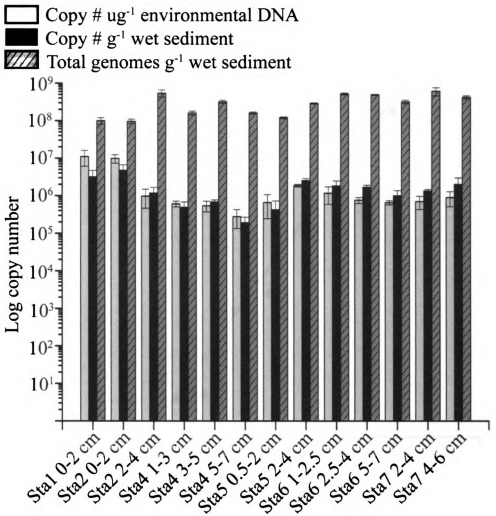

Figure 3.11. Q-PCR determined anammox copy number μg^{-1} environmental DNA, g^{-1} wet sediment, and total estimated genomes g^{-1} wet sediment at each station.

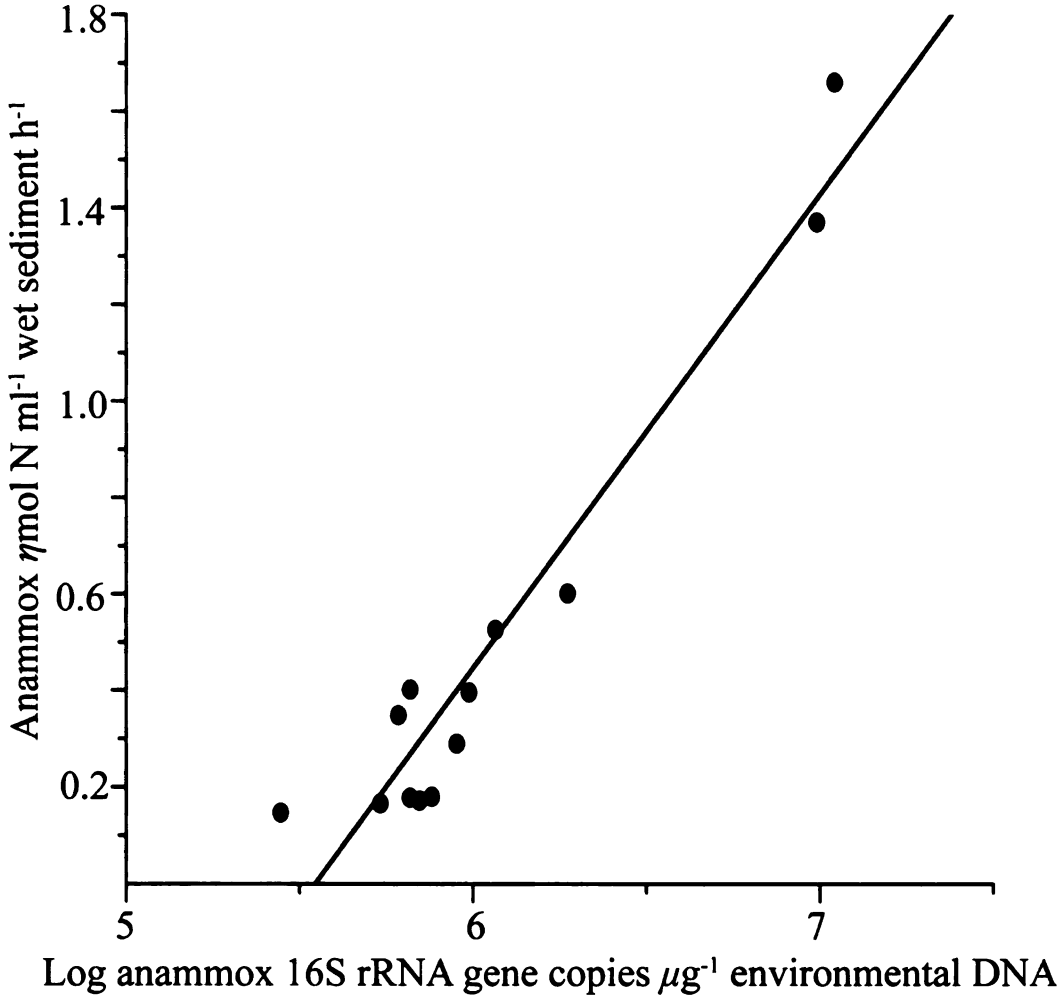

Figure 3.12. Linear regression of Q-PCR determined anammox gene copy μg^{-1} environmental DNA versus anammox N_2 production. Data reflects average copy numbers per 2 cm slice used for the determination of N_2 production

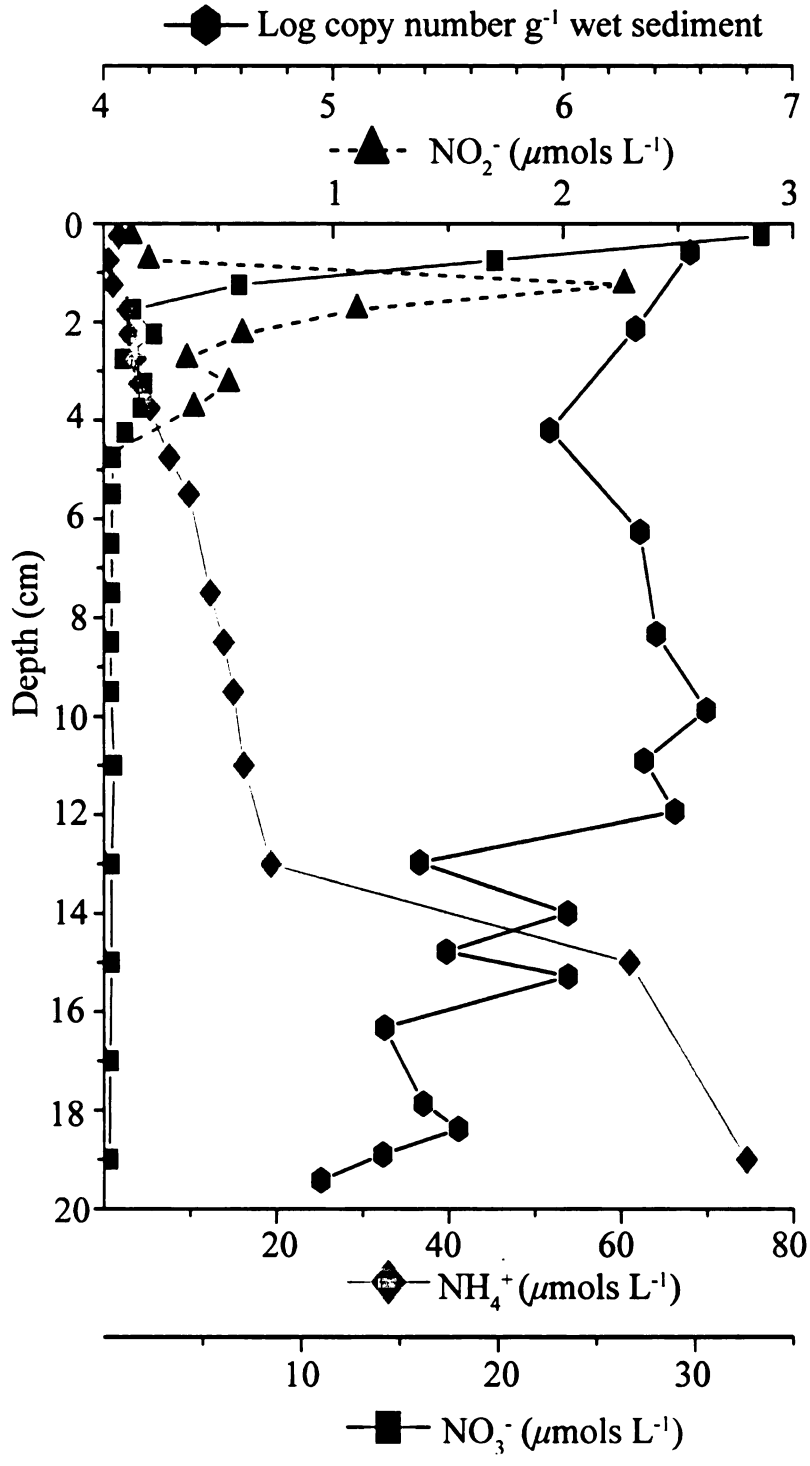

Figure 3.13. Depth relationship between anammox copy number, NO_2^-, NH_4^+, and NO_3^- at station 2.

Absolute rates

Anammox and denitrification absolute rates were significantly lower (Students t-test p=0.0002; p=0.015, respectively), at 0.065-1.7 nmol N mL^{-1} wet sediment h^{-1} and 0.17-1.8 nmol N mL^{-1} wet sediment h^{-1} respectively, than coastal sediments at 2.6-12 nmol N mL^{-1} wet sediment h^{-1} and 5.9-25 nmol N mL^{-1} wet sediment h^{-1} respectively (Hood Canal, U.S.A., 38-147 m; Tofino Bay, Canada, 70-113 m, and the Gullmar Fjord, Sweden, 118 m). Two distinct groupings occurred with the stations closer to the continental slope having the highest absolute rates (S1, S2, and S3) while the more refractive stations further from shore (S4, S6, S7, and S8) exhibited significantly lower (t-test p=0.01) anammox and denitrification activity when the top 2 cm of the suboxic zone were compared.

The decrease in anammox rates with distance from shore in this study (Figure 3.6) suggests that anammox activity is dependent on organic matter mineralization for ammonium supply. In these sediments ammonium was supplied by MnO_2 reduction and denitrification. As such, anammox N_2 production should be limited by low NH_4^+ concentrations in more refractive deeper sea sediments, such as the mid ocean or abysmal plain. Freshwater anammox bacteria have the potential to supply themselves with their own NH_4^+ from nitrate reduction (Kartal et al., 2006, 2007), a process which has not been confirmed in the marine species (Jensen et al., 2008).

A near-surface NH_4^+ peak was detected at all stations in this study from which NH_4^+ initially decreased with depth (Fig. 3.3). This decrease in NH_4^+ concentrations can be considered as a subsurface minimum in the suboxic zone due to anammox. However,

similar profiles have been observed by others who attributed them to an artifact induced by centrifugation (Berelson et al. 1990; Hammond et al. 1996) or cell lysis of microbes and meiofauna in the top surface sediment during decompression and warming when the cores are brought up to surface (Aller et al. 1998; Hall et al. 2007). In the Cascadia Basin this subsurface peak was observed at all locations deeper than 2000 m (Fig. 3.3; Stump and Emerson 2001).

Significance for the marine nitrogen budget

Denitrification rates in deep sea sediments are widely estimated using NO_3^- profiles (Berelson et al. 1990; Middelburg et al. 1996; Brunnegård et al. 2004). However, the presence of anammox confounds this calculation. N_2 production rates are calculated by the downward nitrate flux, assuming the consumption of two mole of NO_3^- per mole N_2 produced. However, anammox will consume one mole NO_2^- per mole of N_2 produced, due to the coupling with ammonium. As such, this method will potentially underestimate total N_2 production by 20 percent when an anammox contribution of 40 percent is present, as in Cascadia Basin.

Quantitative PCR

Estimated anammox abundances of 1.9×10^5 to 4.8×10^6 g^{-1} wet sediment were one to two orders of magnitude higher than previously reported for the water column using FISH. Fluorescence microscopy techniques may underestimate total abundance, especially in sediments where particle shielding and aggregates may result in lower observed numbers. However, the quantitative PCR method may also be influenced by the inherent problems associated with genomic DNA extraction and PCR biases. In

particular, FISH was used in a study that found ~300 anammox cells ml^{-1} sediment (Tal et al., 2005) using the probe AMX820. Conversely, Schmid and colleagues (2007) reported $2.1x10^6$ to $1.4x10^8$ anammox cells ml^{-1} sediment using the FISH probe BS820, which is generally within one order of magnitude of that found in our study.

Anammox percent abundance was generally below 1% of the total estimated bacterial community in our sediments and within the range found in the Black Sea anoxic water column (0.75%), Benguela upwelling (~1%), and Lake Tanganyika (~1.4%) when 4,6-diamidino-2-phenylindole (DAPI) was used for total cell counts (Kuypers et al., 2003, 2005; Schubert et al., 2006). Two exceptions were identified in the 0 to -2 cm sediments from stations 1 and 2. Here anammox comprised 5.2% and 4.6% of the total community, respectively, and was correlated to the highest two anammox N_2 production rates found (Engström et al., accompanying). The lack of significantly lower anammox abundances in the sites located farther from shore that exhibited lower anammox importance suggest that while anammox numbers remain relatively constant, their cell specific metabolism is lower at these sites. Total estimated bacterial genomes were in general agreement with $1x10^7$ to $1x10^{10}$ cells g^{-1} found in other marine sediments using Q-PCR (Schippers and Neretin, 2006) and approximately a thousand times higher than those reported in water columns (Schmidt et al., 1998). However, DNA extraction efficiency and genome size can heavily influence the calculation of total genomes present and, as such, these data should be regarded appropriately.

The linear relationship between anammox 16S rRNA gene copy numbers and ^{15}N production rates (Figure 3.12) suggest that the anammox bacteria we detected were responsible for the anaerobic ammonium oxidation occurring in these sediments. A

similar relationship was found in the suboxic Namibian shelf waters (R^2>0.8) when fluorescence in-situ hybridization (FISH) was used for enumeration (Kuypers et al., 2005). Likewise, the relationship between Q-PCR numbers of '*Candidatus Brocadia anammoxidans*' related anammox bacteria and anammox N_2 production was found (R^2=0.81) in enrichment cultures from a wastewater-seeded laboratory reactor (Tsushima et al., 2007). Cell-specific rates were comparable to those found in the Benguela upwelling (4.5 fmol of ammonium $cell^{-1}$ day^{-1}) (Kuypers et al., 2005), the Black Sea (3-4 fmol of ammonium $cell^{-1}$ day^{-1}) (Kuypers et al., 2003), and Lake Tanganyika (18 fmol of ammonium $cell^{-1}$ day^{-1}) (Schubert et al., 2006) water columns, further validating our results. However, a recent study using Q-PCR for anammox enumeration in laboratory bioreactor enrichments found a low cell-specific activity of 0.072 fmol N $cell^{-1}$ day^{-1} (Tsushima et al., 2007), possibly due to Q-PCR amplification of dead or inactive cells from substrate limiting conditions imposed by batch cultures. Cell-specific activities from a recent marine sediment study (Schmid et al., 2007) showed activities that ranged between 0.08 and 0.98 fmol N $cell^{-1}$ day^{-1}, approximately a factor of ten lower than ours despite our incubations at close to in-situ temperatures (4°C). A range of temperatures appear to harbor similar cell specific activities, 15°C in marine sediments (Dalsgaard and Thamdrup, 2002), 37°C in a wastewater treatment system (Strous et al., 2006), and our activities at 4°C. This suggests that there are redundant enzymes optimized to different temperatures or, alternatively, that there is a selective pressure on specific *Scalindua* phylotypes which has been shown in enrichment cultures (Van de Vossenberg et al., 2008).

The lack of relationship between anammox abundances and the N profile contrast with the correlation found between anammox cell number and NO_2^- in water columns (Kuypers et al., 2003, 2005). Deficits of available NO_2^- and NO_3^- below ~6 cm indicate that significant anammox numbers are present where denitrification and anammox rates should be negligible. A similar finding was reported in the Black Sea water column (Kuypers et al., 2003) where anammox lipids did not decrease wholly in concert with $^{29}N_2$ production and were present below where NO_2^- or NO_3^- was available. Anammox presence in the deeper sediments could be due to alternative metabolic pathways evident amongst other anammox lineages (Güven et al., 2005; Strous et al., 2006; Kartal et al., 2007). This was recently verified in '*Scalindua*' by van de Vossenberg (2008) and colleagues who suggested that the widespread distribution may be due to metabolic flexibility. Alternatively, sediment mixing by the significant benthic macrofauna population may allow for transient NO_3^- / NO_2^- availability or the creation of preferential porewater exchange channels for the maintenance of an anammox population.

Our results also show that Q-PCR based anammox 16S rRNA gene abundance in these deep ocean sediments was correlated with ^{15}N measurements and calculated cell-specific activities were within the range reported previously. However, this correlation significantly under-predicted measured anammox activity in productive Hood Canal (Puget Sound, Washington, United States) sediments (P. Engström, unpubl.) and significant copy numbers were also observed at depths where NO_3^- / NO_2^- was negligible. As such, extrapolation of the relationship between anammox abundance and activities to other locations other than those directly measured using ^{15}N techniques is problematic. Nonetheless, 16S rRNA-based Q-PCR provides insight into the enigmatic ecology of

these bacteria. Now that more is known about the metabolic flexibility of *'Candidatus Scalindua'* we can begin to investigate the role they play in cycling at depths where NO_3^-/ NO_2^- is not available. Eventually, gene transcripts of the anammox pathway may serve as a sensitive indicator of anammox activity.

The implementation of quantitative PCR and ^{15}N isotope pairing methods allowed for a comparison between the catalyst and the process in these deep marine sediments. The importance of anammox was lower than expected from several previous studies, and did not dominate over denitrification in these continental shelf sediments. The lack of correlation between anammox abundance and the NO_3^- / NO_2^- profile suggests that other controlling factors dominate the process and evidence from the porewater profiles suggest an ammonium control.

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CHAPTER IV

COMPARISON OF MICROBIAL COMMUNITY STRUCTURE AMONG DIVERSE MARINE SEDIMENTS USING PYROSEQUENCING

ABSTRACT

Investigations of microbial community structure in environmental samples have generally relied on the use of 16S rRNA gene based clone libraries. Sequences obtained from these studies generally only reflect the most abundant members, making comparisons that yield information about rare members difficult. We employed 16S rRNA based pyrosequencing to investigate microbial community structure among ten marine sediments. A total of 44,181 16S rRNA sequences were obtained that were placed into 8031 clusters at a 95% similarity level. The top ten clusters accounted for 12.5% of the total while 3882 clusters were represented only by one sequence in one sample. There were no cosmopolitan clusters that were identified in every site. Cluster abundance based trees showed that there was no relationship among sites based on geographic position, depth below water column, or temperature. Our data indicates that 7.9% of the 44,181 sequences examined were identical to those recovered from cultivated bacteria, challenging the notion that "less than 1% of bacteria are cultivated". Of the total, 1456 sequences were highly divergent (<85% BLAST identity) from the public database with 1063 of these occurring in one cluster in an Arctic Barrow Canyon sample. The rare sequences exhibited an overall lower identity to the database, which suggests that they are an example of microbial endemism and not transients that would have a higher

probability of being sampled in another study. To examine the effect of nutrient loading on similar sediments, we compared two Florida Bay sites that differ in phosphorus availability. The relative abundance distribution of β-*Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, and δ-*Proteobacteria* suggest that phosphorus controlled viral and grazer predation may be playing a large role in shaping the microbial community structure in these sites.

INTRODUCTION

Over the past 20 years culture independent methods have provided the breakthroughs in attempts to catalog the vast diversity of microorganisms on Earth. A biosphere has been unveiled, richer than imagined, with countless species whose metabolic roles still defy description. Yet, we are still far from classifying the vast extent of microbial diversity, as revealed by the myriad of rarefaction curves present in the literature. A large portion of microbial diversity is due to rare species whom have remained cloaked by dominant members due to the general lack of clone-based methods and inherent cloning biases. Dominant species almost surely represent the overwhelming functional component of an environment and are responsible for the highest proportion of microbial biomass. The majority of microbes in nature are represented in relatively low abundances (Pedrós-Alió, 2006; Ashby et al., 2007; Elshahed et al., 2008) and have been collectively coined the "rare biosphere" (Sogin et al., 2006).

Pyrosequencing, in which large surveys of microbial diversity are possible at a relatively low cost, is a new technique that permits rarity to become visible. Overall, the

majority of rare sequences generated by pyrosequencing are more divergent from the public database than the abundant members (Elshahed et al., 2008; Sogin et al., 2008) with some phyla exhibiting a higher dissimilarity than others. Classical sequencing fails to capture the members, even among the range of different environmental conditions. Understanding this diversity is not trivial; it is the key to discovering novel genes, drugs, and metabolic pathways. An example is the relatively recent discovery of the importance of anammox bacteria in global nitrogen cycling (Thamdrup and Dalsgaard, 2002). Only represented by a few sequences among numerous marine diversity assays, anammox bacteria were typical rare members of the biosphere, as determined by their currently low database representation. Through the use of targeted assays, they are now known to be abundant at 10^8 cells g^{-1} sediment (Schmid et al., 2007) and are responsible for up to 80% of N loss in some locations (Thamdrup and Dalsgaard, 2002). In contrast, the majority of rare species may exhibit little functional capability and not be relevant to the overall carbon and nutrient flow.

One widely held notion is that "everything is everywhere, the environment selects" (Baas Becking, 1934). Among the countless environments that have been sampled, the marine biosphere harbors some of the highest microbial diversity on Earth (Lozupone and Knight, 2007). Despite numerous studies, even the magnitude of prokaryotic diversity in the world's oceans remains unknown and subject to debate (Whitfield, 2005). Estimates have ranged from a few thousand taxa (Hagström et al., 2002), 10^3-10^4 taxa (Pommier et al., 2005), 10^6 taxa (Curtis et al., 2002), to higher numbers estimated using rarefaction curves generated from 16S rRNA gene targeted pyrosequencing assays (Sogin et al., 2006).

The persistence of species due to predation and competition in the marine environment may be important factors that influence overall diversity and the rare biosphere. Abundant members are known to be more susceptible to viral infection due to simple encounter probability (Thingstad, 2000) and grazers prey selectively on the largest and most active bacteria (Pernthaler, 2005). Environments that support higher numbers of bacterial grazers or viruses may be expected to harbor a higher diversity and more rare members, when all else remains equal. As such, there may be a survival strategy for the rare members that are less susceptible to competition and predation (Sogin et al., 2006) which otherwise may not be able to survive amongst more dominant members.

Here we report an assessment of microbial community diversity and structure among ten marine sediments using a pyrosequencing approach. Our primers target the V4 16S rRNA gene hypervariable region, which has been shown to provide high coverage, resolution, and accuracy for deep taxa classification. We further investigated the relationship among two contrasting sites representing high and low phosphorus levels in Florida Bay to test the effect of nutrient availability on diversity patterns.

MATERIALS AND METHODS

Sediment Sampling

Marine sediment samples were collected from ten sites (Figure 4.1) and the top 2 cm of sediment was used for DNA extraction unless otherwise noted: East Hanna Shoal (EHS, 160 m depth, 72.637N 158.667W) and Barrow Canyon (BC, 186 m depth, 71.607N 156.214W), both from the Alaskan maritime in the Chuckchi Sea; Washington Margin (WM, 1138 m depth, 46.575N 124.822W), West of the Juan de Fuca Ridge (JF,

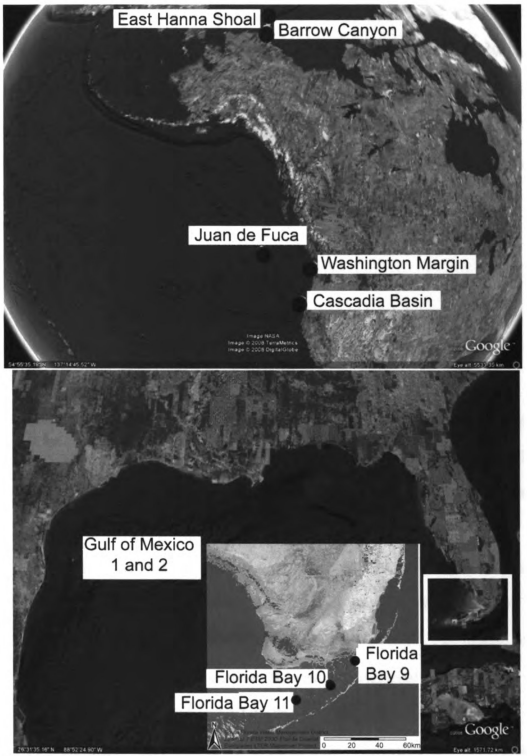

Figure 4.1. Sampling site locations in the Arctic and Pacific Ocean (top) and in the Gulf of Mexico and Florida Bay (lower). The inset shows the location of the Florida Bay sites west of the Florida Keys. Maps generated with Google™ Earth (earth.google.com). Inset from South Florida Water Management District Landsat 7 ETM 2000 Florida Coastal Everglades LTER Mapserver Project.

3869 m depth, 46.783N 133.667W), and Cascadia Basin (CB, 42.000N, 125.230 W) all in the Pacific Ocean; two samples from one site (800 m depth, 26.404N 96.064W) in the Gulf of Mexico (GM1, 0-0.5 cm) and (GM2, 4-5 cm); 3 samples from shallow marine habitats, Florida Bay 9 (FL9, 25.177N 80.490W), Florida Bay 10 (FL10, 25.025N 80.681W), and Florida Bay 11 (FL11, 24.913N 80.938W). DNA was isolated using the MoBio Ultraclean™ Soil DNA kit (MoBio Laboratories Inc.) according to the manufacturer's instructions.

SSU rRNA Amplicon Pyrosequencing

Eubacterial universal primers were used that spanned the SSU rRNA gene v4 hypervariable region, corresponding to *Escherichia coli* SSU rRNA positions 563 to 802. Primer coverage was determined by the internal alignment of perfect matches against SSU rRNA gene sequences in the Ribosomal Database Project II (RDP) (93.6% coverage) and from the Sorcerer II Global Ocean Sampling Expedition (94.6% coverage) (Rusch et al., 2007).

The forward key-tagged primers were composed of sequencing adaptor A, sample-specific 4 or 6-bp keys, and a eubacterial 563F primer (5'-GCCTCCCTCGCGCCATCAG(keys)**AYTGGGYDTAAAGVG**-3'). The reverse fusion primer consisted of sequencing adaptor B, and a eubacterial 802R primer (5'-GCCTTGCCAGCCCGCTCAG**TACNVGGGTATCTAATCC**-3'). All primers were dual HPLC-purified (Integrated DNA Technologies, Coralville, IA) to increase specificity of primers and minimize the miss-sorting of samples by primer synthesis error. PCR mixtures contained 1 μM of each primers (IDT, Coralville, IA), 1.8 mM

$MgCl_2$, 0.2 M dNTPs, 1.5 X BSA (New England Biolabs, Beverly, MA), 1 unit of FastStart High Fidelity PCR System enzyme blend (Roche Applied Science, Indianapolis, IN), and 10 ng of DNA template. Amplification conditions were 30 cycles of 95°C for 45 sec, 57°C for 45 sec, and 72°C for 1 min after an initial denaturation for 3 min at 95°C, followed a final 4 min extension at 72°C. For each sample, three replicated PCR reactions were run in parallel and excised bands in range of 270-300 bps were combined. Amplicon recovery was performed with Qiagen Gel extraction (Qiagen, Valencia, CA) followed by an extra Qiagen PCR Purification step. DNA was quantified spectrophotometrically using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and equimolar amounts of each sample were subsequently combined and subjected to pyrosequencing using Genome Sequencer FLX System (454 Life Sciences).

Pyrosequencing Analysis

Raw reads were sorted into individual samples using the assigned key sequence and filtered using two categories of sufficient read length and a less than 2bp primer edit distance. Sequences were aligned using INFERNAL version 8.1, a stochastic context-free grammar based aligner (http://infernal.janelia.org/) along with the Bacterial SSU rRNA secondary-structure model of Gutell and colleagues (Cannone et al., 2002). All sequences were then clustered by the complete-linkage method at the desired distance and assigned to bacterial taxa using the RDP Classifier trained on species-type strain sequences from the Taxonomic Outline of Bacteria (TOBA; http://www.taxonomicoutline.org) along with additional sequences for regions of bacterial diversity not covered well by TOBA. The

bootstrap confidence threshold was set to 50%. Abundance-based adjusted Jaccard and Sorensen indices (Chao et al., 2006) were calculated for each pair of samples using EstimateS (http://viceroy.eeb.uconn.edu/estimates) after clustering samples together.

RESULTS

Phylogenetic Structure

We sequenced 44,181 SSU rRNA gene V4 hypervariable region bacterial amplicons from ten sites. Operational taxonomic units (OTUs) were clustered at the 95% similarity level as this corresponds to the diversity observed between species type-strains from the same genus. These 8031 clusters were used for the generation of rarefaction curves and the calculation of Shannon diversity and Chao1 species richness estimates (Table 4.1). The highest diversity and richness estimate was found in sample FL11 while the lowest values were associated with Barrow Canyon. The difference in estimated diversity between these two contrasting sites was not illustrated in large scale changes in relative abundances among the most prolific phyla (Figure 4.2). The phylogenetic architecture (Acinas et al., 2004) indicates that there was extensive deep-lineage variation among sites (Figure 4.3).

The top ten clusters accounted for 5501 sequences or 12.5% of the total while 3882 clusters (8.8%) were singletons, only represented by one sequence in one sample. Singleton clusters were relatively uniformly distributed, accounting for ~50% of the clusters in each phylum. Two exceptions were within *candidate division* OD1 and

			0.01 (Species)			0.05 (Genus)			0.12 (Family)		
	Reads	Uncl	OTU	Chao1	H'	OTU	Chao1	H'	OTU	Chao1	H'
BC	4442	1715	1456	3221	5.80	814	1362	4.92	445	620	4.34
EHS	4153	232	1628	5183	6.30	924	2018	5.33	478	716	4.50
JF	4796	1750	1688	3616	6.44	896	1451	5.56	459	602	4.78
WM	4127	1069	2245	6178	7.24	1372	2567	6.44	710	1088	5.59
CB	3508	909	1648	4205	6.82	978	1752	6.02	519	750	5.33
GM1	4202	207	1574	4061	6.46	810	1512	5.39	417	615	4.69
GM2	4287	260	1919	5856	6.85	1079	2118	5.94	556	903	5.18
FL9	5838	1561	2793	6734	7.40	1597	2574	6.60	761	1039	5.66
FL10	4245	141	1463	4143	6.21	734	1657	5.07	365	577	4.24
FL11	4583	1285	2825	8543	7.61	1820	3568	6.95	938	1479	6.05

Table 4.1. Total sequence reads, number of unclassified (Uncl), corresponding number of clusters (OTUs), Chao1, and Shannon Diversity (H') values for three cluster cutoff dissimilarity values (0.01, 0.05, and 0.12) among sediment samples from nine sites.

Chlamydiae where singletons constituted ~67% of the clusters. Singleton distribution among sites was not uniform with FL11 expressing 17.5% singletons, while in FL10 they only comprised 5.3% of the total population. Of the 8031 clusters, 2375 were represented by tags in at least two samples. Surprisingly, there were no "cosmopolitan" clusters that were found in all ten samples. One dominant cluster accounted for 1068 sequences, of which 1063 occurred in Barrow Canyon, accounting for 24% of the sequences identified in this sample. These sequences exhibited S_ab scores of 0.26-0.29 using the RDP SEQMATCH tool to the closest known isolates and closest BLAST matches of 79-86%% to the public database of all environmental sequences.

Two independent pyrosequencing runs were performed on Cascadia Basin sediments. Chao1 values for the two runs at 0.05 dissimilarity were 1752 and 1779. The duplicate runs cladded tightly together when both were included in a Chao-Sorenson neighbor joining tree. A total of 439 clusters were shared between runs (72% of all

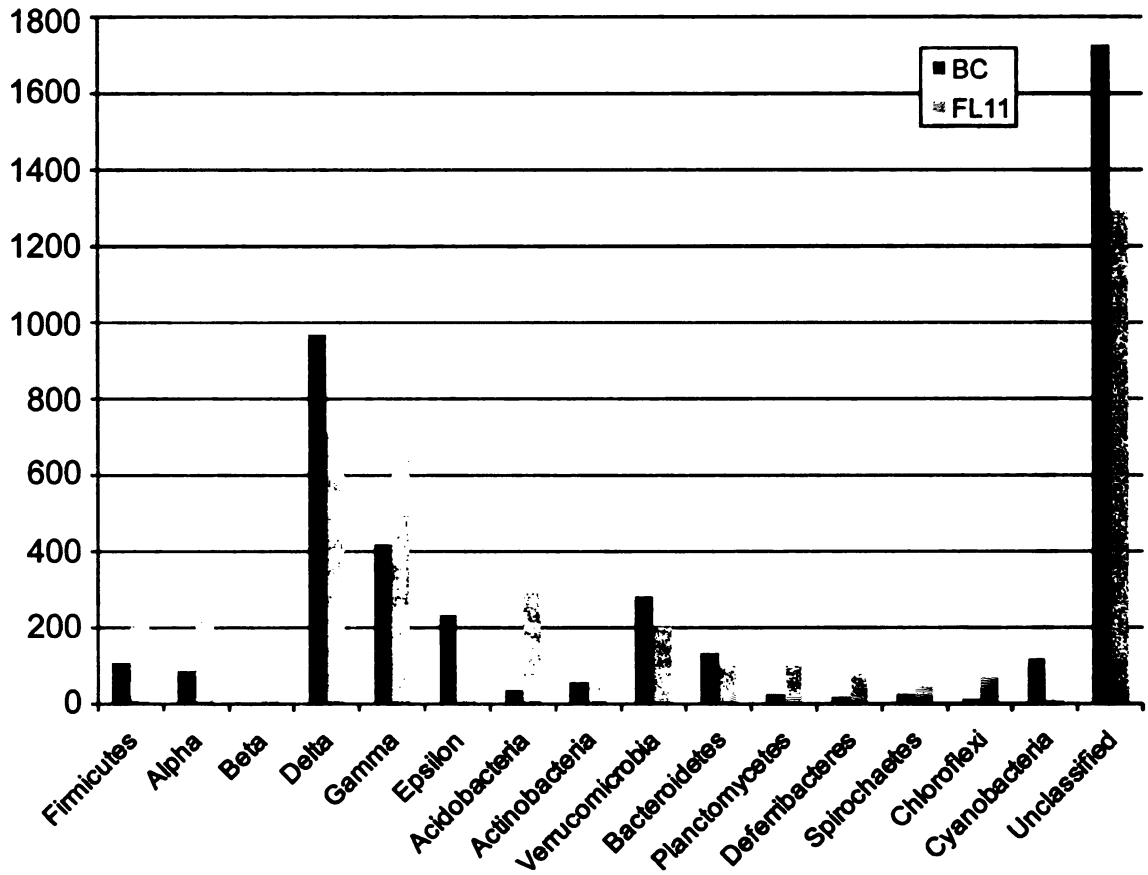

Figure 4.2. Comparison of relative abundances based on the RDP Classifier between the two most contrasting sites according to estimated richness and diversity, Florida Bay 11 and Barrow Canyon.

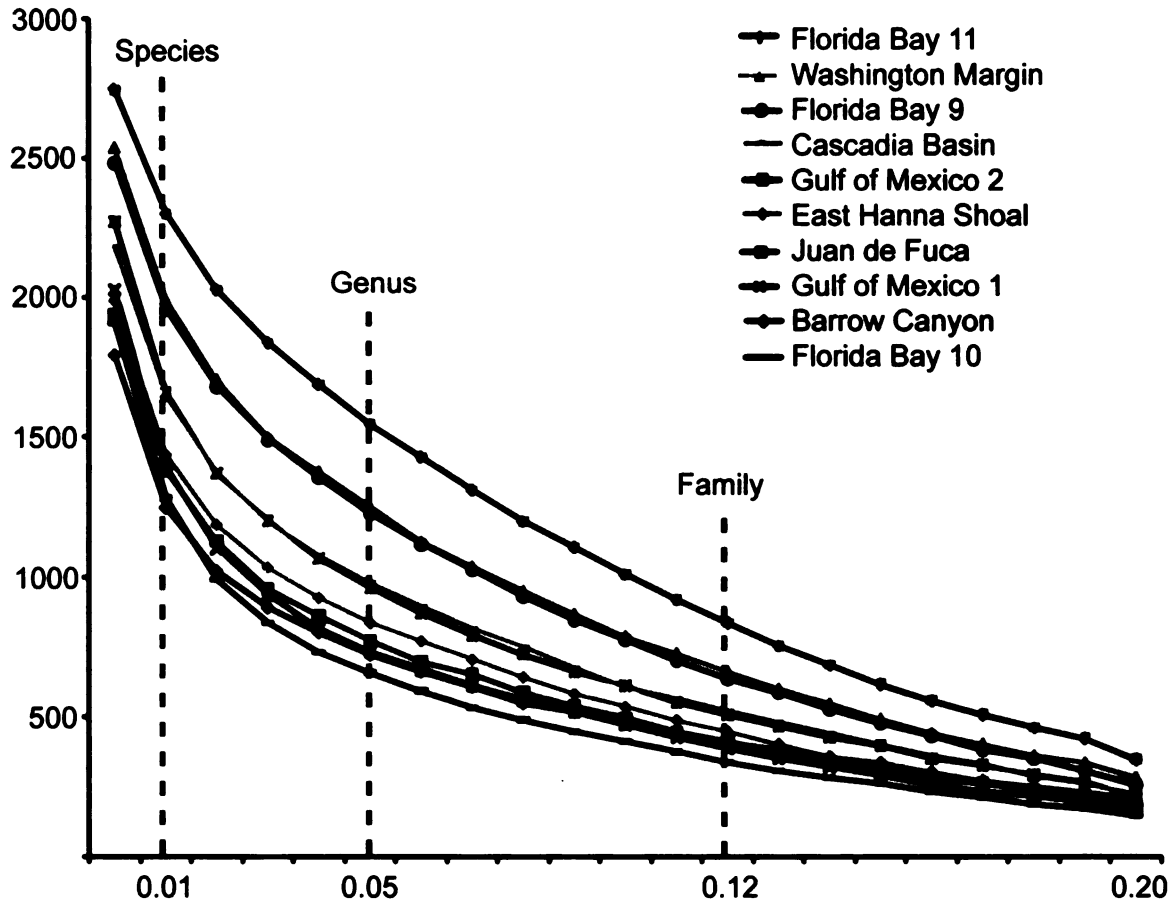

Figure 4.3. Phylogenetic architecture based on a normalized sample size of 3,500 randomly selected sequences per site. Dissimilarity values for species, genus, and family are noted on the x-axis while number of sequences are represented on the y-axis.

sequences) while 907 clusters were represented by only one sequence. The abundance of the shared clusters exhibited a standard deviation of 1.95 sequences between replicates.

Diversity Characterization

The majority of sequences belonged to the *Firmicutes*, *δ*, *γ*, *and α-Proteobacteria*, which constituted 70% of the total classified sequences (Figure 4.4). The *Acidobacteria*, *Actinobacteria*, and *Verrucomicrobia* were also relatively abundant at 15% of the sum.

According to the RDP Classifier 9181 sequences (20.8%) out of 44,181 sequences were unclassified.

Isolate best hit matches were analyzed using the RDP SEQMATCH tool for all sequences. The *Firmicutes* exhibited the highest dissimilarity from current isolates but were also represented by the highest isolate diversity, represented by the length of the x-axis (Figure 4.5) with phylum calls based on RDP classification of the best hit match. The γ-and β-*Proteobacteria* were the most extensively cultivated (12.5%) while the *Planctomycetes*, *Spirochaetes, Gemmatinomadetes, Chloroflexi*, and *Cyanobacteria* were among the most poorly cultivated.

The frequency distribution (Figure 4.6) illustrates the large γ-*Proteobacteria* crest at S_ab of 0.26-0.29 which corresponds to the single cluster of 1063 sequences found in the Barrow Canyon sample. This distribution also illustrates a bi-modal frequency, with an increase at S_ab of 0.5-0.7 (pairwise identity of 87-94%), illustrating a gap in isolate coverage. Three distinct patterns of isolate coverage were identified with high frequency exact matches and unimodal distribution in Florida Bay 10 versus Barrow Canyon which exhibited a high abundance of distant matches. A bi-modal distribution is also illustrated by the sequences from Washiington Margin, with poor coverage in the mid-range, at species-level diversity.

Cluster representative sequences were subjected to BLAST analysis according to the abundance distribution (Figure 4.7). Singletons averaged 92.5% (sd=3.6%) BLAST nearest match identity, clusters that contain <0.1% of sequences at each site without singletons averaged a 95.1% (sd=3.2%) identity while those that contain >0.5% of sequences at each site averaged a 98% (sd=1.5%) identity. A total of 1456 sequences

were highly divergent (<85% BLAST identity) from any environmental sequence. Average singleton identity ranged between Juan de Fuca (91.8%) and Florida Bay 10 (94.8%).

Chao-Sorensen estimated abundances were used for the construction of a UPGMA tree rooted in the community of a Ghana agricultural soil (data not shown) to determine the relationship among sites (Figure 4.8a). No overall spatial or depth component was apparent among sites. The two depths from the same Gulf of Mexico sample did not tree together when singletons were included. East Hanna Shoal, an Arctic sediment, grouped together with the shallow, warm Florida Bay 9 site. To determine the influence of singletons on treeing, these rare sequences were removed to construct another tree (Figure 4.8b). Three topological changes occur: Gulf of Mexico 1 and 2 group together, Cascadia Basin joins with Barrow Canyon, and Washington Margin and Juan de Fuca diverge.

In order to test whether pyrosequencing could capture anaerobic ammonium oxidizing bacteria, unclassified sequences from the RDP 10.0 release classifier were selected from each site. BLAST was performed and sequences with >90% to known anammox bacteria were selected. Sixteen anammox sequences were identified in six samples and were closely related to the *Ca. Scalindua* type anammox, most notably *Ca. Scalindua brodae* (Figure 4.9).

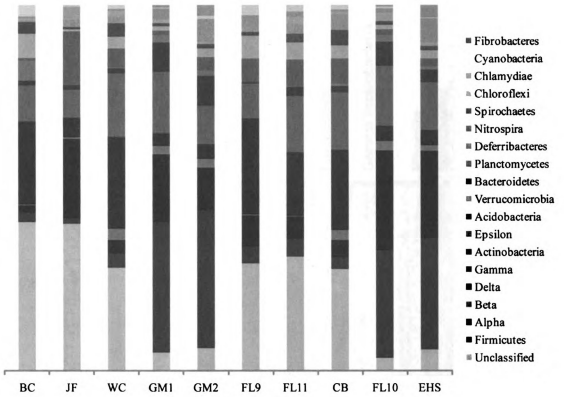

Figure 4.4. Relative abundances illustrating the phylogenetic composition of the sequences obtained at each site based on the RDP Classifier at 50% bootstrap confidence threshold.

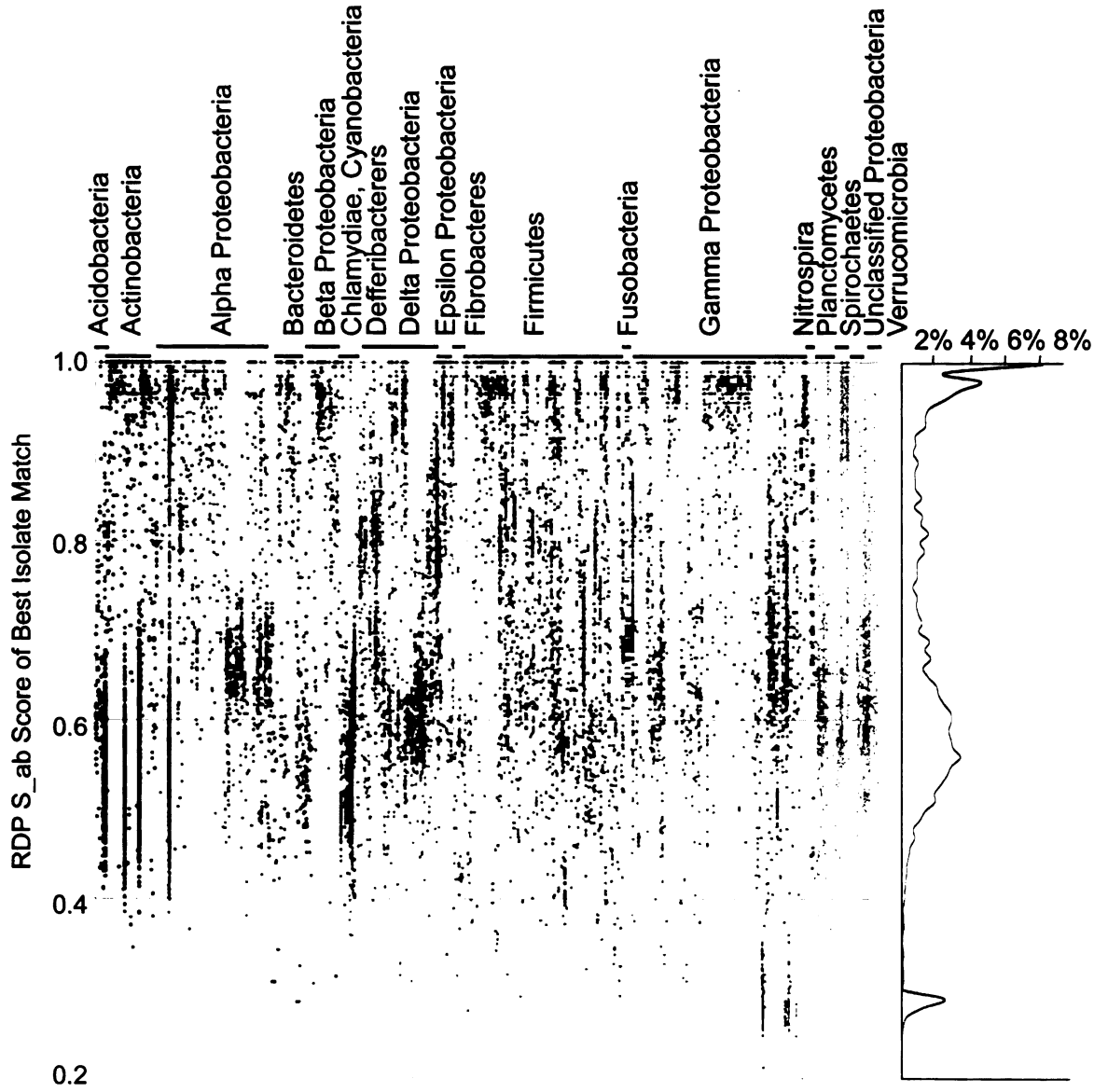

Figure 4.5. NEO plot of RDP best hit matches from all samples to isolates with S_ab score by phylum. One column represents a best match to one isolate. Percentage based frequency distribution highlighted on the right. Dashed line indicates an estimated 85% pairwise identity based on the S_ab score.

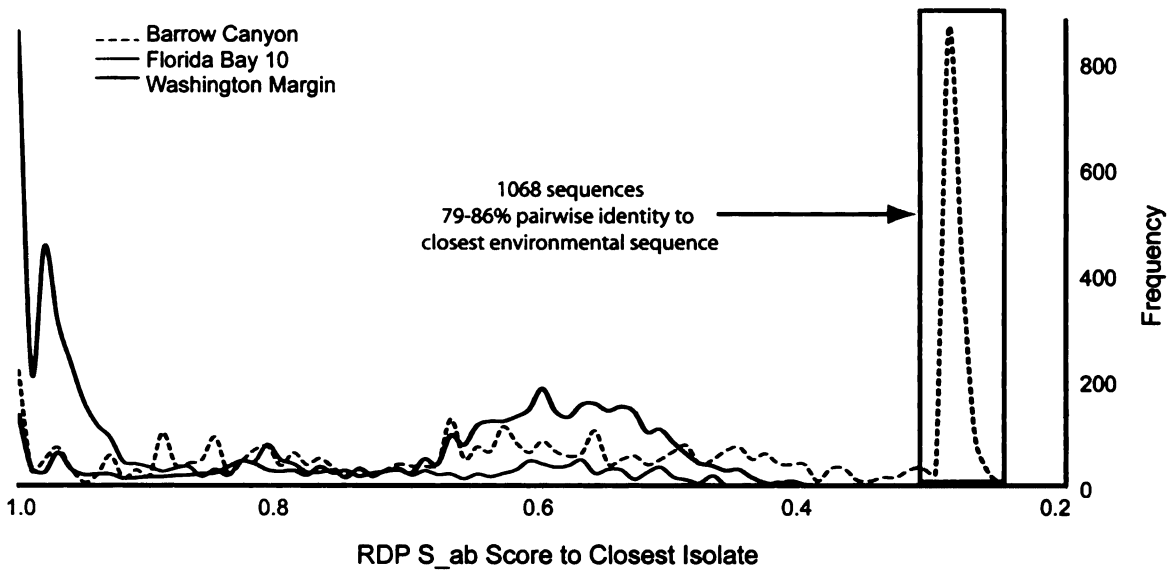

Figure 4.6. Frequency distribution of S_ab scores illustrating differences in isolate coverage among three sites. Dashed line indicates an estimated 85% pairwise identity based on S_ab scores. The 1068 sequences that comprise the peak at low RDP S_ab score consist of 1063 sequences exclusively from Barrow Canyon.

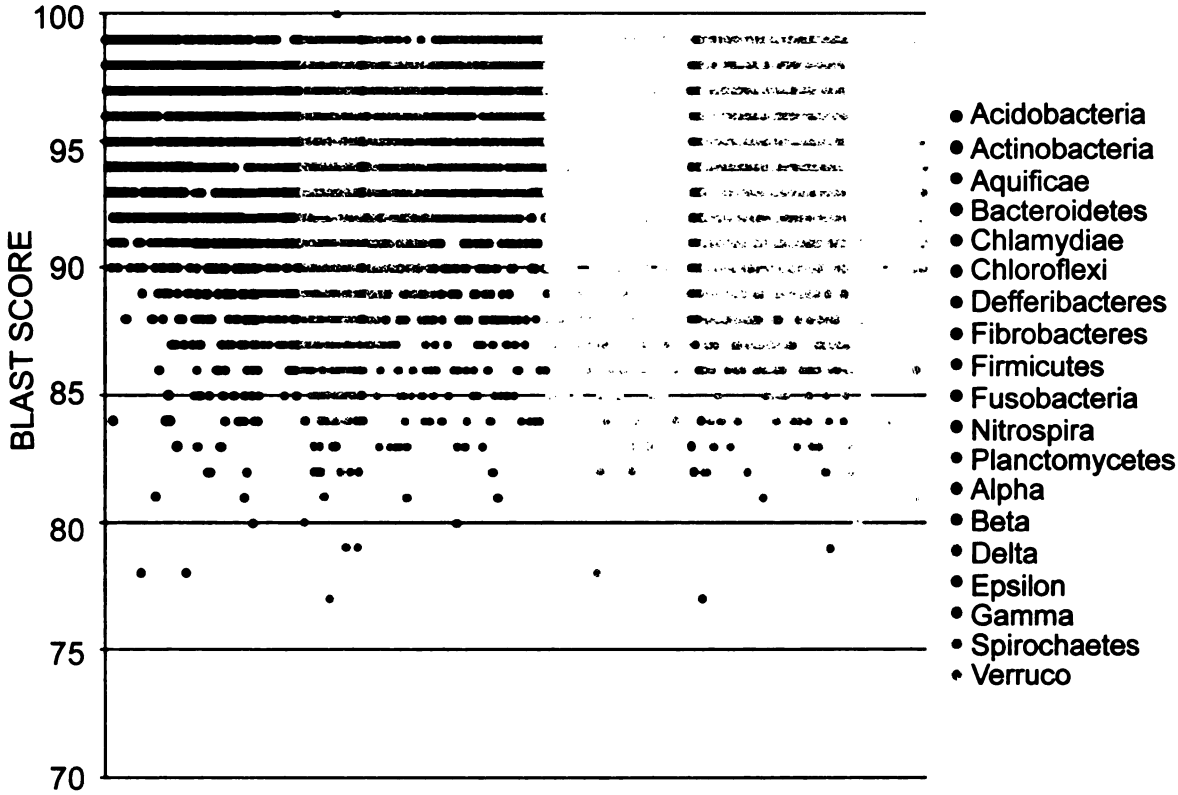

Figure 4.7. BLAST best match scores for each cluster representative sequence based on the cluster representative sequence RDP classifier results.

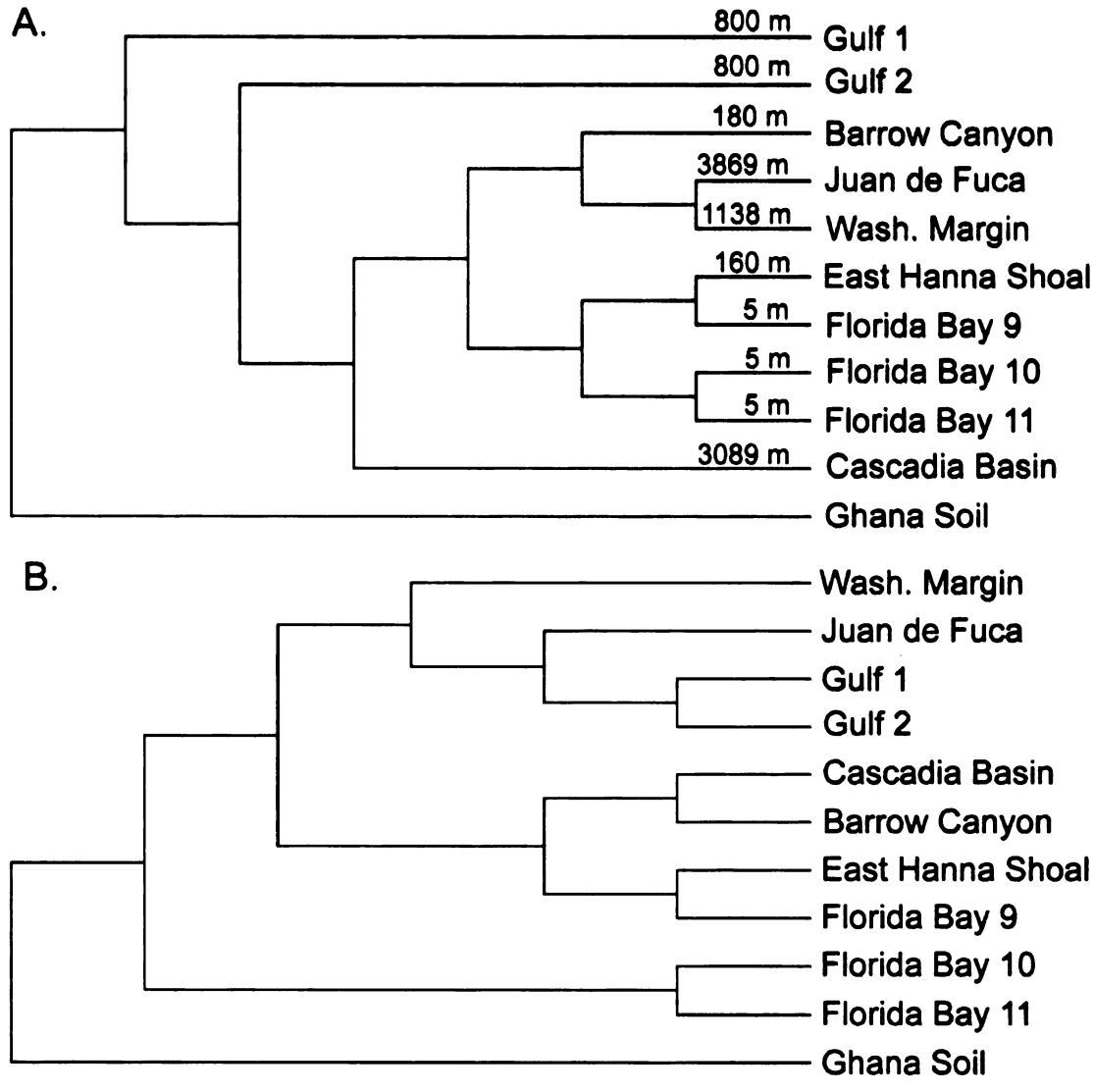

Figure 4.8. Chao-Sorensen cluster abundance based UPGMA tree at 5% dissimilarity rooted with an agricultural soil from Ghana with (A) all clusters and (B) without singletons. Depth below water surface is noted.

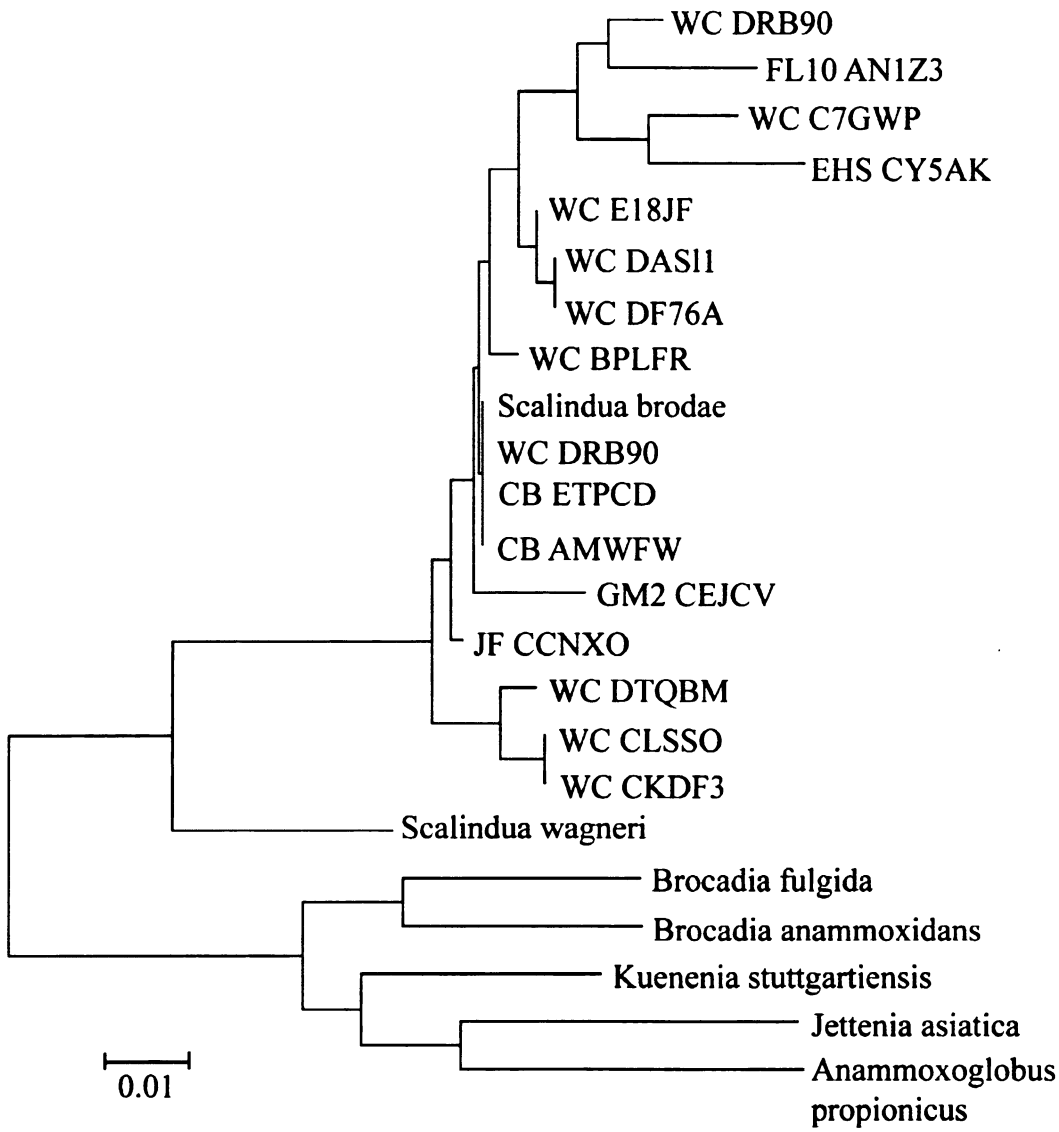

Figure 4.9. Relationship of identified sequences with >98% sequence homology to the known anammox bacteria. Site designations are referred at the beginning of each sequence name.

DISCUSSION

Community Diversity

Pyrosequencing targeting the 16S rRNA gene for community analysis is evolving into a powerful tool providing an unparalleled view into the diversity of the microbial world. The high coverage V4 hypervariable region primers used in this study are supported by *in-silico* UNIFRAC analysis (Liu et al., 2007), result in amplicons that have low insertion and deletion frequencies for simplified alignment, and have high resolution and accuracy for deep taxa classification using the RDP Classifier (Wang et al., 2007).

Our results indicate that SSU rRNA pyrosequencing can be used to assess and test hypotheses concerning microbial diversity and relative abundance among different environments. Specifically, our data indicates that 7.9% of the 44,000 sequences examined were identical to those recovered from cultivated bacteria, challenging the notion that "less than 1% of bacteria are cultivated". Among our sequences, 3.3% of were highly divergent with equal to or less than 85% BLAST identity against SSU rRNA sequences in the public database. These represent highly divergent taxa and are not uniquely rare, as the case with the large cluster in our Barrow Canyon sediment. As such, pyrosequencing appears to be a powerful method for identifying poorly characterized taxa that may play a large metabolic role in the environment.

Singletons, identified as sequences within a cluster at 5% dissimilarity that are present only in one sample, represented 8.8% of all sequences obtained in the study, compared to 18.1% found in a deep clone library from a soil sample (Elshahed et al., 2008). Both *Chlamydiae* and candidate division *OD1*exhibited the highest proportion of singletons (~67%) in our study, in comparison to the highest proportion in

Planctomycetes in the soil biosphere (Elshahed et al., 2008). The increasing sequence divergence from the database as rarity increases is also in agreement with the clone library based study and a marine sediment pyrosequencing study (Sogin et al., 2006).

The Rare Biosphere

When singletons are removed the topological changes in the cluster based tree illustrate differences between the overall diversity and the dominant "functional capacity" of the sediment. As rare members, singletons are not expected to contribute a large fraction of the total sediment metabolism. Several hypotheses have been presented to explain the presence of these rare members. They may represent a "genetic reservoir", a temporally-based population that can respond to changes in the environment and potentially become dominant when conditions are appropriate (Sogin et al., 2006). Secondly, they may be transients, in our case carried by currents, which are not currently adapted to the site conditions. Third, they may be less adapted "fuzzy" species, evolutionary remnants that are currently out-competed in all global ecosystems but are able to survive (Elshahed et al., 2008). In the first two cases we would expect that comparison to the public database would lead to identities similar to the distribution of the less rare members. Exceptions to this would be environments that are not well represented by the current database, such as the deep biosphere.

Our data shows that the rare biosphere is indeed a divergent population that is under-sampled in all environments. This does not appear to support the rare members as transient species or a genetic reservoir defined as a population that responds to changing environmental conditions. If the rare biosphere was dominated by transients we would

expect to find less unique sequences that are present only in one site and BLAST identities would be less divergent, as the database reflects a large span of biogeochemical conditions. It appears most likely that these are either bacteria suited to a specific niche that has not been well sampled, or that they are "fuzzy" species that may play a minor role in sediment nutrient cycling. However, it is impossible to discard the notion that large scale environmental changes may result in proliferation of the rare biosphere (Sogin et al., 2006), thus supporting the notion of the "genetic reservoir". For example, warming global temperatures may provide an environment that favors certain enzyme systems over others, which may currently reside in the rare biosphere. Conversely, nutrient loading may favor rare members with higher enzyme Km values that are currently out-competed for limiting resources.

While the average identities were lower among the singletons, there was a notable exception in Barrow Canyon. The most dominant overall cluster consisted of 1063 sequences with a low BLAST nearest neighbor identity of 79-86%. This highly evolutionary distinct lineage represents 24% of all sequences in BC and, due to its prevalence, may play a significant role in the microbial loop. Likely examples of microbial endemism, these dominant members are also identified in low abundance in two other sites.

Selective Effects in Florida Bay

Sampling of the two Florida Bay sites allowed us to investigate the potential causes behind different relative abundances in certain dominant phyla. Due to the influence of Gulf of Mexico waters, the otherwise oligotrophic Florida Bay is subjected

to a phosphorus gradient that is reflected in surface water and seagrass nutrient levels. Florida Bay 11 is most influenced by Gulf waters with higher phosphorus concentrations and lower N:P ratios. Conversely, Florida Bay 9 is subjected to onshore-offshore dynamics with lower P availability, higher dissolved organic carbon (DOC), and markedly lower salinity (Boyer et al., 1997; Fourqurean and Zieman, 2002). Our cluster trees, with or without singletons, show that the Florida Bay 9 and 11 communities are divergent, with FL10 most closely associated with FL11. This is in contrast with the site grouping based only on unfilitered dissolved organic matter (UDOM) characteristics in the water column (Maie et al., 2005). We expected that FL10 would be an intermediate site in terms of its impact from the Gulf of Mexico. However, FL10 has exhibited P concentrations a third less than FL9 in another study (Chambers and Pederson, 2006) which likely contributes to much lower Chao1 and Shannon Diversity values. Since this parameter falls out of the previously documented east-west trends (DOC, C:N, N:P, Salinity, etc.) we will restrict further comparisons to either end of the spectrum, FL9 and FL11.

Overall, both Florida Bay sites have the highest Chao1 and Shannon Diversity estimates of the ten sediments, suggesting that Florida Bay sediments are more taxonomically rich and diverse than many aquatic sediments, a supposition supported by an earlier study (Hewson et al., 2002). Compared to FL9, the larger FL11 Chao1 and Shannon Diversity values may be partially influenced by a higher viral abundance, as virus particles are typically higher in, and limited by P (Hewson et al., 2001; Hewson and Fuhrman, 2002). Nutrient amended mesocosms in Florida Bay have indeed shown that higher bacterial diversity and richness was accompanied by increased viral abundance

(Hewson et al., 2002), likely due to predation based on encounter probability (Thingstad, 2000). Another selective influence is the abundance of grazers, such as *Bdellovibrio*, which comprise 0.5% of the total community at FL11 with only one sequence at FL9. Therefore, increased diversity driven by predation appears to be consistent with our study where apparent viral driven and grazing selection is not overshadowed by increased P-limited microbial speciation due to constrained horizontal gene transfer (Souza et al., 2008).

The outcomes of both phosphorus and predation selective effects are illustrated in the differences in the relative abundances between sites. If predation is playing a role in shaping the microbial community structure, then we expect that changes in the relative abundances of certain bacteria would follow trends observed in previous studies. As expected, the β-*Proteobacteria* were equal in number between FL9 and FL11, as they have been shown to be opportunistic resource competitors able to compete in a variety of nutrient conditions with little grazing pressure. In contrast, *Actinobacteria* were three times more abundant in FL11, comprising 4% of the population. While *Actinobacteria* are generally not directly affected by nutrient status, they do profit from the removal of competitors through grazing pressure driven by higher P availability (Salcher et al., 2007). *Bacteroidetes*, who have been shown to profit from a high nutrient load and are triggered by grazing pressure (Salcher et al., 2007), composed 3.5% of the community at FL11, twice that of FL9. Of course, site specific nutrient conditions, other than phosphorus availability, will influence microbial community composition. One example is the overall two-fold higher relative abundance of the putative sulfur cycling δ-*Proteobacteria* at FL9, comprising 20% of all sequences at this site. Overall, the δ-

Proteobacteria are the most dominant bacteria in both sites and represent 28% and 20% of all sequences in FL9 and FL11, respectively. Overall, these correlations with earlier studies suggest that predator pressure, not uniquely phosphorus availability, is a controlling force in these shallow Florida Bay sediments and may play a role in shaping marine microbial community structure on a larger scale.

Overall there were no apparent large scale spatial or depth related trends among all sites. The grouping of the two depths of the same Gulf of Mexico core after the singletons were removed illustrates one of the issues that pyrosequencing presents. Namely, what sequencing depth do we choose in order to illustrate the grouping of sites? If an approximation of the true diversity is the goal, then the inclusion of singletons appears to be appropriate. However, if investigating the potential functional or biogeochemical relationship likely reflected by the dominant members, singletons may be excluded. These issues can be addressed in the future by comparing metatranscriptome and 16S targeted pyrosequencing based data.

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CHAPTER V

QUANTITATIVE PCR OF FRESHWATER ANAMMOX IN HAWAIIAN TARO FIELDS

ABSTRACT

The identification of anammox bacteria in a variety of freshwater habitats has suggested that anammox may be responsible for nitrogen removal in these systems. However, there is currently little evidence concerning the abundance of these microbes in flooded agricultural sediments. A previous study indicated that up to 80% of added fertilizer nitrogen could not be accounted for by volatilization, plant uptake, or denitrification, indicating that anammox activity may be responsible for the N loss. Sediments from a variety of Hawaiian taro fields were analyzed for anammox bacterial abundance using two primer sets targeting the "marine" genera *Scalindua* and the "freshwater" *Brocadia*, *Kuenenia*, and *Jettenia*. Total estimated anammox numbers were one to two orders of magnitude than those found in deep marine sediments. Among the agricultural plots, the newly flooded taro field following fertilization exhibited the highest estimated counts. Potential N removal rates calculated from the range of published cell-specific activities showed that anammox may be responsible for the removal of 2 to 763 kg^{-1} N ha^{-1} yr^{-1}. An estimated rate in Hanalei river sediment showed a much higher N removal potential, suggesting that this system may harbor a very active anammox population.

INTRODUCTION

The importance of anaerobic ammonium oxidation is becoming more well-defined in marine systems, with the process responsible for between 0 and 80 percent of total N_2 production in a variety of basins (Thamdrup and Dalsgaard, 2002; Risgaard-Petersen et al., 2004; Engström et al., 2005). However, the significance of this process in freshwater sediments including flooded agricultural soils has been largely unexplained. Only a few studies have referenced anammox bacteria or the process in these environments (Jetten et al., 2003; Trimmer et al., 2003; Schubert et al., 2006; Zhang et al., 2007). In a freshwater lake, anammox was shown to contribute up to 13% of total N_2 production in Lake Tanganyika (Schubert et al., 2006), but no literature describing anammox in flooded agricultural systems is currently available.

The contribution of anammox to total N loss in flooded agricultural systems is of great importance in determining the appropriate fertilization application amounts and methods such that N losses are mitigated. This is important not only in terms of the financial burden but also the eutrophication of surrounding waters resulting from excess fertilizer inputs. Ammonium is relatively abundant due to organic matter mineralization and fertilization, there is a thin oxic soil layer at the water/soil interface, and the subsoil remains anoxic, conditions that would appear suitable for anammox bacteria.

Anammox activity has been confirmed in rice paddies, but the overall contribution to total N_2 production (anammox + denitrification) is thought to be low (Suwa, ISME, 2008). Growing season length and treatment between cropping seasons has a direct impact on the stability of the anoxic environment. Rice has a growing season of 3 to 7 months with the soil becoming aerated in between crops. As a result, the anammox

anoxic environment is transient, which may affect not only the anammox density but the metabolic resilience of the bacteria as they are stressed. Taro is a tropical plant and a staple food in Polynesian cultures whose environment should be well suited for anammox bacteria with a long crop cycle (14-16 months) and continuous soil flooding.

In an experiment testing N availability from flooded taro fertilized with fish-bone meal on Oahu, Hawaii in 2003-2004, a rapid release of NH_4^+ was observed from the organic amendment applied to the surface soil (0-15 cm depth) immediately before flooding. More that 80% of the mineralized NH_4^+ could not be accounted for as taro plant uptake accounted for only 10% of the added N and floodwater nitrate and ammonium concentrations remained low, despite high soil NH_4^+ concentrations. This suggests that volatilization is low, which was supported by earlier studies (Cao et al., 1984; Fillery et al., 1984). Additionally, losses to denitrification appear to be low due to soil anaerobiosis which limits nitrate and nitrite production. Experiments have shown that denitrification losses are low when fertilizer was applied to already flooded soils (Mosier et al., 1998). Unlike rice systems where rhizosphere nitrification occurs in the bulk anoxic soil due to fine, diffuse roots (Arth et al., 1998), the tuber root systems of taro plants are less likely to support significant surface area for nitrification.

The determination of anammox activity in these flooded agricultural systems is of interest due to its impact on N dynamics, crop productivity, and modeling N losses. In this study seven flooded systems are tested for the presence of anammox bacteria that may be responsible for these observed N losses. Two separate quantitative PCR assays targeting the marine *Scalindua* sp. and the freshwater *Kuenenia stuttgartiensis*, *Brocadia*

anammoxidans, and *Brocadia fulgida* related anammox are used to assess their density in these soils.

METHODS

Quantitative PCR Assays

Quantitative PCR targeting the 16S rRNA gene of the freshwater anammox *Kuenenia stuttgartiensis*, *Brocadia anammoxidans*, and *Brocadia fulgida* along with the marine anammox *Scalindua sp.* was performed using the SYBR Green assay (Applied Biosystems). The primer sequences were as follows: 818F (5'-ATG GGG CAC TMR GTA GAG GGG TTT- 3') and 1066R (5'-AAC GTC TCA CGA CAC GAG CTG-3') (Tsushima et al., 2007). Each 25 uL reaction mixture contained 12.5 uL 1x Sybr Green Master Mix, 300 nM of each primer, 100 ug mL^{-1} BSA (Roche), and 2.5 uL template DNA or 10^1 to 10^8 copies of the standard plasmid carrying the target DNA of *Brocadia anammoxidans*. The reaction conditions were: 2 min 50°C, 10 min 94°C, 40 cycles at 15s at 94°C and 1 min at 60°C. Standard curves were generated using a plasmid dilution containing the 16S rRNA gene of *Brocadia anammoxidans* from 10^3 to 10^8 copies.

The Taqman assay described in Chapter III was used for the detection of anammox 16S rRNA genes using primers: 541FRT (5'-GAG CAC GTA GGT GGG TTT GTA AG-3') and 616RRT (5'-CCT CCT ACA CTC AAG ACT YGC AG-3'). The Taqman probe AnPrb (5'-CAG RTG TGA AAG CCT TCT GTT CAA CGG AAG-3') was labeled 5' FAM and 3' Black Hole Quencher-1® (Integrated DNA Technologies, Coralville, Iowa). Final assay concentrations of 541FRT, 616RRT, and AnPrb were 300 nM, 900 nM, and 250 nM, respectively with 1 μL of genomic DNA added per well and

annealing and extension temperatures of 60°C. The increase in fluorescence emission was monitored during PCR amplification using a 7700 Sequence Detector (PE Applied Biosystems). The C_T values obtained for each sample in both assays were compared to the standard curve to determine the initial 16S rRNA gene copy number g^{-1} wet sediment.

Sample Description and DNA Extraction

Soil samples were collected from seven sites in five different taro patches; flooded taro (Oahu), dry taro (Oahu), newly flooded taro (Oahu), and anaerobic taro where anaerobic conditions have been maintained for the longest period (Oahu), flooded taro (Hanalei) along with a sediment sample from the Hanalei River (Kauai), and a duck pond adjacent to the Hanalei River (Kauai).

Genomic DNA from 0.5 g sediment was isolated using the MoBio Ultraclean soil DNA kit (MoBio Laboratories, Inc.) and further purified by electrophoresis in 0.8% Cambrex SeaPlaque low melting temperature agarose at 4°C. Genomic DNA bands were gel-extracted, treated with gelase (Epicentre) per manufacturer's instructions, and concentrated using Microcon columns. DNA concentrations were determined by absorbance at 260 nm.

RESULTS AND DISCUSSION

Anammox bacteria related to *Kuenenia stuttgartiensis*, *Brocadia anammoxidans*, *Brocadia fulgida*, and *Jettenia asiatica* were enumerated using the Sybr Green assay developed by Tsushima et al. (2007). Melting curve analysis showed that there was only one observable peak, no other detectable peaks that were associated with primer-dimer

artifacts or other non-specific PCR amplification were observed. A standard curve was constructed using a *Brocadia anammoxidans* 16S rRNA gene containing plasmid dilution series (Figure 5.1). The resulting slope (-3.03) approached the value of perfect amplification efficiency (-3.3). The strong linear relationship (R^2=0.98) between threshold cycle number and target copy number demonstrated the consistency of the assay. Error increased considerably at 10^3 copies, indicating approaching detection limit. This assay also enumerates the marine anammox bacteria related to *Candidatus Scalindua brodae* and *Candidatus Scalindua wagneri* with one mismatch. Therefore, the resulting PCR efficiency of the *Scalindua* sp. is one to two orders of magnitude lower (Tsushima et al., 2007).

Marine anammox related bacteria were detected in all seven samples at a density ranging from 10^4 to 10^5 16S rRNA gene copies g^{-1} wet soil (Figure 5.2). The lowest abundances were associated with the dry and newly flooded Oahu taro patches while the highest anammox density was found in the three flooded or anaerobic Oahu taro patches. Anammox numbers in these soils were two to three orders of magnitude lower than those found in the deep marine sediments (mean=$2*10^6$ copies g^{-1} wet sediment, Chapter III) . Identification of these principally marine species in freshwater habitats is not surprising, as the marine *Scalindua* sp. type anammox were shown to be distributed widely in the environment (Penton et al., 2006). However, the presence of the freshwater anammox in flooded agricultural systems cannot be overlooked as both anammox groups have been found to co-exist in freshwater environments (Zhang et al., 2007).

Anammox bacteria counts were one to two orders of magnitude higher than in the assay that only targeted the marine *Scalindua* sp. type anammox in the same samples

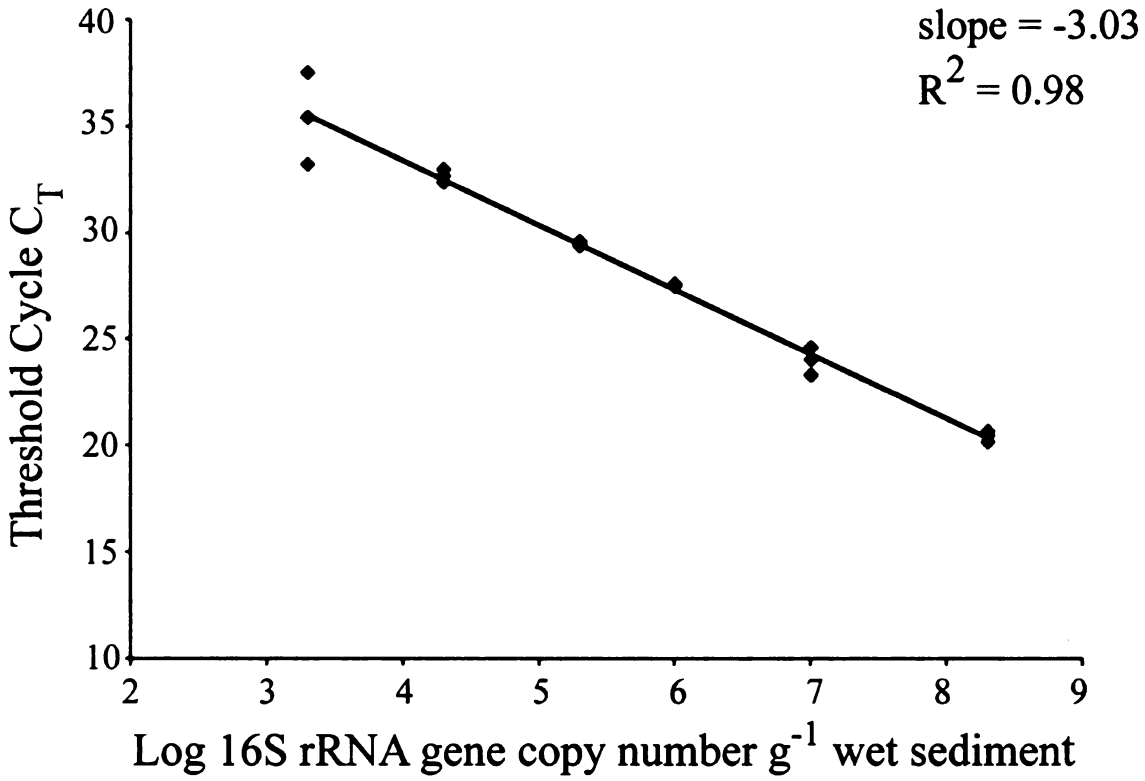

Figure 5.1. Standard curve based on a dilution series of plasmids containing the 16S rRNA gene of *Brocadia anammoxidans*.

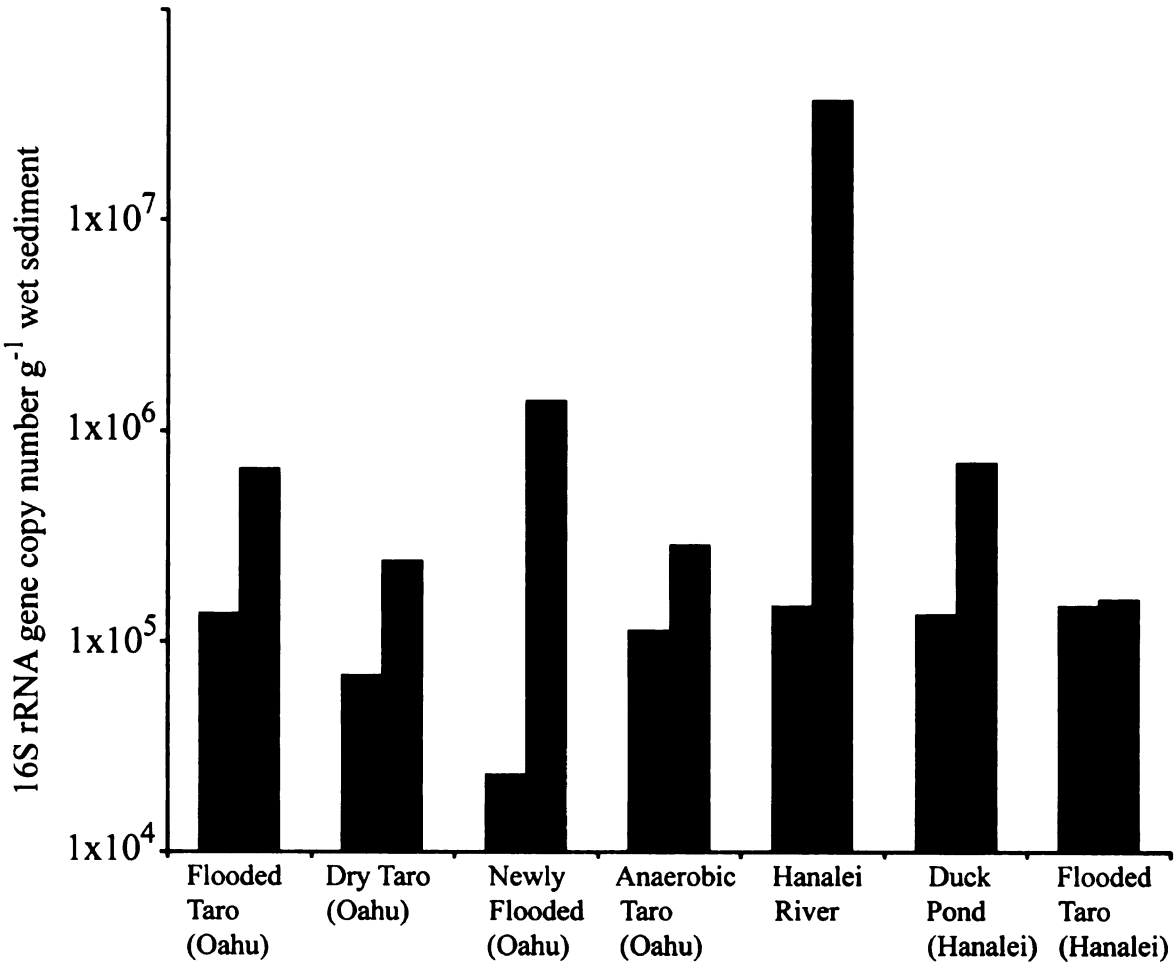

Figure 5.2. Distribution of all anammox bacteria based on the 818F/1066R primer set (dark grey) compared to the marine only *Scalindua* sp. related anammox (light grey). Abundances are normalized to 16S rRNA gene copy number g^{-1} wet sediment.

(Figure 5.2). One exception occurred in the Hanalei flooded taro field sediment where both assays detected similar numbers of bacteria. Specifically, the 818F/1066R assay detected $1.58x10^5$ versus $1.48x10^5$ 16S rRNA genes g^{-1} wet sediment in the marine *Scalindua* sp. assay. This is in contrast to the large difference between assays in the Hanalei River sediment where the broader anammox assay identified $3.68x10^7$ 16S rRNA genes g^{-1} wet sediment more anammox than the marine primer set. The range of anammox bacteria numbers were within the range reported by Tsushima et al. (2007) in batch culture enrichments where most assays identified 10^6 to 10^8 anammox 16S rRNA gene copies ml^{-1}.

The estimates of total anammox in each site were applied to the correlation between the nitrogen removal rate and cell numbers reported in a batch culture experiment (N removal rate ($\mu M\ hr^{-1}$) = 3.0×10^{-6} * (anammox 16S rRNA gene copy number ml^{-1})) (Tsushima et al., 2007). Predicted rates ranged from 0.47 to 110.95 ηmoles N hr^{-1} g^{-1} wet sediment removed (Table 5.1).This calculation assumes that 1 g sediment is equivalent to 1 ml of media in the batch reactor analysis. Potential rates were also calculated based on the range of cell specific activities for anammox bacteria from Chapter III and the literature (1-22 fmol ammonium cell day^{-1}), in a wide range of environments (Kuypers et al., 2003; Kuypers et al., 2005; Schubert et al., 2006; Strous et al., 1999). These calculated values ranged from 0.01 to 77.1 ηmoles N removed g^{-1} wet sediment hr^{-1} which can be directly compared with observed anammox rates (41 to 58 ηmol cm^{-3} hr^{-1}) in a 25 day old bioreactor enrichment using a riverine sediment (Zhang et al., 2007). Overall, the estimated rates range from 0.01 to 110.95 ηmols N consumed g^{-1} wet sediment hr^{-1} when the total anammox 818F/1066R assay was used. Estimated

nitrogen loss using these estimated anammox activities ranged from 1.8 to 763.4 kg N ha^{-1} yr^{-1}.

The marine anammox targeted assay identified that the dry and newly flooded taro fields exhibited the lowest anammox abundance, as would be expected due to the current or recent aerobiosis. However, the total anammox assay disagreed, showing the there were more anammox in the newly flooded field than the flooded taro at both the Oahu and Hanalei sites. The reason for this disparity is not apparent. The only other evidence showing the co-existence of the two anammox groups is from a study in Xinyl River, China (Zhang et al., 2007). One possible explanation is that the freshwater type anammox, who have been shown to have multiple metabolic pathways, may be able to tolerate transient aerobic conditions better than the *Scalindua* sp. type anammox. Another possibility is that an ammonium pulse resulting from the mobilization of freshly mineralized organic matter while under aerobic conditions provides a transient environment well-suited to anammox bacteria proliferation. The highest anammox numbers in the Hanalei River sediment suggests a stable, anaerobic environment that is harboring a very large anammox population almost two magnitudes larger than that observed in the deep ocean (Chapter III). Interestingly, the flooded Hanalei taro sediment had very similar anammox counts between the freshwater and total anammox assays, indicating that the marine anammox accounted for 93% of the population. In contrast they only constitute 0.4% of the community in the Hanalei River sediment. Since amplification efficiency of the marine *Scalindua* sp. anammox is one to two orders of magnitude lower in the total anammox 818F/1066R assay, it is probable that total

	Low	High	Tsushima
Flooded Taro (Oahu)	0.06	1.38	1.98
Dry Taro (Oahu)	0.02	0.50	0.72
Newly Flooded (Oahu)	0.12	2.88	4.15
Anaerobic Taro (Oahu)	0.02	0.59	0.86
Hanalei River	3.08	77.05	110.95
Duck Pond (Hanalei)	0.06	1.45	2.09
Flooded Taro (Hanalei)	0.01	0.33	0.47

Table 5.1. Estimated nitrogen removal rates based on quantitative PCR runs using the 818F/1066R primer set. The low and high columns show N removal rates calculated from literature-based cell specific activities at 1 and 20 fmol ammonium cell^{-1} day^{-1}. The last column is based on the N consumption rate calculation reported by Tsushima et al. (2007). Both calculations are normalized to the reported units of ηmols nitrogen ($NH_4^+ + NO_2^-$) consumed hr^{-1} g^{-1} wet sediment^{-1}.

	Original Values (10^5 copies g^{-1})	Corrected Values (10^5 copies g^{-1})
Flooded Taro (Oahu)	6.61	7.96
Dry Taro (Oahu)	2.40	3.09
Newly Flooded (Oahu)	13.8	1.41
Anaerobic Taro (Oahu)	2.85	3.96
Hanalei River	370	3.71
Duck Pond (Hanalei)	6.98	8.31
Flooded Taro (Hanalei)	1.58	3.04

Table 5.2. Original and corrected estimated anammox abundances based on quantitative PCR using the 818F/1066R primer set. Corrected abundances were calculated by adding the difference from a two-magnitude underestimation of the marine *Scalindua* sp. anammox, based on the estimated *Scalindua* sp. anammox abundances using the Taqman 541F/616R primer set. All values are anammox 16S rRNA gene copy number g^{-1} wet $sediment^{-1}$.

	Low*	High*	Tsushima*	Difference
Flooded Taro (Oahu)	.07	1.66	2.39	+20.4%
Dry Taro (Oahu)	.03	.64	0.93	+28.3%
Newly Flooded (Oahu)	.12	2.93	4.22	+1.7%
Anaerobic Taro (Oahu)	.03	.83	1.19	+39.0%
Hanalei River	3.09	77.35	111.39	+.4%
Duck Pond (Hanalei)	.07	1.73	2.49	+19.1%
Flooded Taro (Hanalei)	.03	.63	0.91	+92.2%

Table 5.3. Estimated corrected nitrogen removal rates based on quantitative PCR runs using the 818F/1066R primer set. Corrections were based on calculated abundances from Table 5.2 using a two magnitude underestimation of the marine *Scalindua* sp. type anammox. The low and high columns show N removal rates calculated from literature-based cell specific activities at 1 and 20 fmol ammonium cell^{-1} day^{-1}. The next to last column is based on the N consumption rate calculation reported by Tsushima et al. (2007). Both calculations are normalized to the reported units of ηmols nitrogen (NH_4^+ + NO_2^-) consumed hr^{-1} g^{-1} wet sediment^{-1}. The last column shows the percent increase in estimated N removal when corrected marine *Scalindua* sp. counts are used.

anammox numbers are slightly higher than those reported. To correct for this, anammox numbers indicated by the marine anammox 541F/616R assay were decreased two orders of magnitude and the difference was added to the total anammox 818F/1066R assay results (Table 5.2). To estimate the effect of the increase in *Scalindua* sp. type anammox counts, new estimated N removal rates were also calculated (Table 5.3). The new values reflect increases of 0.4 to 92.2% over the estimates without correction for under-reporting the *Scalindua* type anammox. Sites, such as the flooded Hanalei taro, where the marine anammox counts were close to the total anammox counts, showed the largest increase in potential N removal rates.

Overall, the estimated rates and abundances suggest that aeration status does not uniformly influence anammox bacteria. Rather, it may be driven more by N availability in these environments. For example, the newly flooded Oahu taro field may benefit from the ammonium pulse following flooding and the Duck Pond sediment may be influenced largely by the high N load resulting from duck droppings. As such, it is expected that anammox activities and numbers change throughout the taro growing season and that fluctuations will occur in direct response to fertilizer application. If this is the case then anammox may be responsible for a significant proportion of N losses in these systems where denitrification is limited due to sub-surface fertilizer applications.

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CHAPTER VI

CONCLUSIONS AND FUTURE PERSPECTIVES

At the time that this study was started, little was known about the distribution or role of anaerobic ammonium oxidation in natural systems. The only environmental evidence of anammox bacteria originated from anoxic water columns and sediments. The screening primer set showed that anammox bacteria were present in shallow freshwater systems, deep marine sediments, and Siberian permafrost. Their presence in a wide range of environments suggested that anammox bacteria were not confined to strictly anaerobic, stable habitats, which confirmed the initial hypothesis. The finding of the marine anammox in freshwater systems showed that these *Scalindua* type anammox may be found in concert with the classically "freshwater" *Brocadia*, *Kuenenia*, and *Jettenia* anammox. Furthermore, identification of anammox bacteria in these habitats also indicated bias against 16S rRNA gene amplification when universal primers are used.

The activity of anammox bacteria in the deep ocean was thought to dominate over denitrification, with anammox responsible for greater than 80% of total N_2 production. The Cascadia Basin study showed that anammox was only responsible for about half of the nitrogen loss from sediments at a depth of ~3000 m. Both denitrification and anammox activity decreased with distance from shore, a proxy for sediment reactivity. The lack of anammox dominance at low organic matter availability shows that competition between denitrifiers and anammox at low sediment reactivity is still not well understood. In fact, it appeared that ammonium availability was limiting anammox activity in these deep sediments and not direct competition for nitrite. These findings did

not wholly disprove the initial hypothesis that anammox would dominate in these deep sea sediments. Rather, the full testing of this hypothesis may require sampling of yet deeper marine sediments, the abysmal plains, which constitute the majority of the world's oceans.

The development of the quantitative PCR method allowed for the first direct correlation between anammox activity and abundance in marine sediments. The strong correlation between q-PCR determined numbers and ^{15}N isotope pairing measures of activity indicates a strong reliance of activity on the size of the anammox community. The vertical distribution of anammox down to 20 cm below sediment surface indicated that survival is possible in locations that do not receive stable oxidized nitrogen inputs, which corroborated with the alternative hypothesis. The presence of significant anammox abundances in these zones further supported the conjecture from the survey study, that the bacteria is present in environments not well suited, at least in terms of the current knowledge, for maintenance of the anammox reaction. The vertical distribution showed that these deep sea sediments were likely well-mixed due to macrofaunal bioturbation, which created transient porewater channels for NO_3^- and NO_2^- movement into the anoxic sediment.

The distribution of freshwater anammox in flooded agricultural sediments is among the least understood areas of current anammox research. Large, unexplained losses of added fertilizer N could not be accounted for in a previous study of taro fields which suggested that anammox may be a significant N sink. The combination of q-PCR primers allowed for the enumeration of both the "freshwater" and "marine" anammox bacteria. The two magnitude lower abundance of anammox in the taro cropping system

suggests that they are a less dominate member of the microbial community, as compared to the marine sediments from Cascadia Basin. However, the range of estimated anammox activities based on previous measures indicate that anammox may be responsible for N losses between 1.8 to 763.4 kg N ha^{-1} yr^{-1} in taro fields, a potentially substantial N loss where monthly fertilizer N applications are approximately 400 kg N ha^{-1} $month^{-1}$ during a nine-month cycle. The high numbers of anammox in the Hanalei River sediment indicate that anammox may be a substantial N sink in this river which is likely loaded with runoff from the surrounding taro patches. As a subject of future study, the overall importance of the anammox reaction in these sediments will require confirmation using ^{15}N isotope pairing experiments.

Sediment microbial community structure did not exhibit any correlations with geographic position, marine basin, depth below water surface, or water temperature which disproved both tested hypotheses. The high proportion of highly divergent sequences from the database demonstrated the depth obtained using 16S rRNA gene pyrosequencing. The majority of these highly divergent sequences were within the rare members at each site, suggesting that they are examples of microbial endemism and not transients. The surprising lack of any cosmopolitan clusters, when grouped at 5%, would appear to challenge the Baas-Becking hypothesis that "everything is everywhere, but the environment selects". The first part of this postulate contends that microbes are distributed universally, in which we would expect to find some bacteria present in all of our samples that share a commonality, e.g. they are all marine sediments. However, the "but the environment selects" contends with niche differentiation and proliferation of bacteria most suited to a particular environment. Clusters found at high abundance in

some sediments were not represented in other samples. This suggested that some unidentified environmental factor was selecting for these prolific bacteria, despite that all samples were from marine habitats. The high abundance of singletons suggested a very high microbial diversity, though their functional importance to sediment nutrient cycling is unknown.

The Florida Bay sediment samples provided an unique opportunity to investigate the changes in microbial community structure between a high phosphorus and low phosphorus site at long-term ecological research sites. The correlation of changes in microbial relative abundances to past mesocosm and field studies that targeted certain microbial groups showed that predation appeared to impact microbial community structure among these two sites. While phosphorus availability likely determined the coarse community structure of these sites, the relative abundances suggested that phosphorus control likely led to biased predation on the most abundant members, which allowed for a more robust rare biosphere illustrated by the highest diversity estimates at the phosphorus impacted site. However, the true impact of predation on maintaining high diversity is largely unknown and warrants further study.

Future Perspectives

As stated above, the importance of anammox in the abysmal depths of the world's oceans is currently unknown. Based on current evidence it is unlikely that anammox dominates in these sediments due to substrate limitation. However, refinement of the marine nitrogen budget requires that these questions be answered, particularly in the context of oceanic productivity and its influence on carbon sequestration. While

anammox cell numbers are known to be linked to overall anammox activities, it is unknown what the *in-situ* influences of NO_2^-, NO_3^-, and NH_4^+ concentrations have on the competition between denitrification and anammox activity on a per-cell basis. Once these influences are known it may be possible to directly link porewater measures and q-PCR determined anammox numbers to an estimated N_2 production. As of now, only a broad range of activities can be inferred from estimated cell counts.

Research into the importance of anammox in natural freshwater systems, flooded agricultural fields, and terrestrial habitats is the future of anammox research. Nothing is known regarding the activity of anammox in terrestrial systems and very little research has been done in freshwater habitats. Early evidence has suggested that anammox plays a small role in these systems, but the lack of coverage indicates that much more research is necessary to cover a range of biogeochemical conditions found in the environment. In particular, substantial anammox losses in agricultural systems would impact fertilization schemes in order to reduce both nitrogen losses and monetary expenses.

As large scale sequencing efforts are undertaken in a variety of environments, our knowledge of microbial diversity is expanding at an exponential rate. However, uncovering the underlying causes of changes in community structure is a more problematic issue. Future studies require the collection of large amounts of metadata, such as porewater nutrient analysis, redox status, q-PCR determined cell counts, and other indicators that can be correlated to relative abundances. Mesocosm studies can be utilized to determine the influence of factors such as nutrient availability on the composition and abundance of the "rare biosphere" and provide insight into the impact of predation on total community diversity. One of the more difficult questions is whether the

rare members are functionally active and play a role in the sediment nutrient cycle. A positive result would indicate that the current depth of sampling using pyrosequencing is still not adequate in order to distinguish microbial community composition among sites. On the other hand, if the rare members are not physiologically important then the current sampling depth is not revealing any additional useful information. These are some of the questions that must be addressed in the near future as sequencing capabilities continue to advance in order to manage future research expenditures while maximizing information,

APPENDIX

CANONICAL CORRESPONDENCE ANALYSIS FOR LINKING ENVIRONMENTAL VARIABLES WITH MICROBIAL COMMUNITY STRUCTURE

As data rich methods such as microbial community pyrosequencing are implemented in studies where biogeochemical parameters are also measured, it is important for microbial ecologists to begin correlating these two datasets. Depending on the goal of the study, these comparisons can be made at the level of the whole community, phyla, or specific genera. The resounding question that needs to be addressed concerns the statistical method used for comparisons. Classical methods utilized in smaller clone library studies, such as principal correspondence analysis (PCA) are not suited to the structure of pyrosequencing data sets. Abundant singletons, sequences found only in one site, can account for as much as 70% of the total data set. As such, the absence of operational taxonomic units (OTUs) creates a problem with PCA. Abundance-based distributions are also not uniform, with a significant tail that reflects the number of singletons in the dataset (Figure A-1). As such, the statistical method used for this type of analysis must be able to overcome these issues.

Canonical correspondence analysis (CCA) was first developed as a tool to investigate macrofaunal species-environment relationships. This was in order to overcome a matrix of species data that is characterized by a prevalence of zeros and a high ratio of species to samples (Clarke & Warwick, 1994). Additionally, in studies

where a specific environmental gradient is not sampled, the distribution of species would be expected to take on a unimodal response. These hump-shaped unimodal distributions cause problems in methods that assume linear response curves. However, canonical correspondence analysis is not sensitive to this distribution (ter Braak, 1986, 1994). Other canonical methods can be used where the species response to environmental variables exhibits a linear response such as redundancy analysis (Jongman et al., 1995), a constrained form of PCA, which is especially suited when sampling along environmental gradients, even if these gradients are short.

Canonical correspondence analysis is also a robust method when species exhibit bimodal ranges and unequal distributions. Additionally, it is well adapted for tables of cluster-based abundances, deals well with species absence, and is sensitive to rare species only when there is a small number of species. Specific OTUs can be identified that respond to particular environmental variables (Ramette, 2007). In particular, CCA selects ordination axes that are linear combinations of environmental variables. The best weights for each environmental variable are then chosen as to maximize the dispersion of the species scores. In CCA plots three items are displayed: species points, sample points, and environmental arrows.

Canonical correspondence analysis was implemented in the R environment using the VEGAN package. Tests of model confidence were based on the ANOVA permutation test (anova.cca) and CCA permutation test (permutest.cca). The data structure for this analysis consisted of over 290,000 16S rRNA gene sequences clustered at the 95% identity level using the Ribosomal Database Project Pyrosequencing Pipeline complete linkage clustering tool. The results show that the selected environmental variables

Available P, pH, SOC, and TN explained only 36% of the variability in the site ordination. This is the underlying foundation of CCA in contrast to PCA. Principal component analysis and other techniques perform ordination first on the community data and then attempt to secondarily relate the ordination to the environmental factors. Instead, CCA ordinates the community data based on multiple regressions on the relationships to the environmental data. Simply, it uses the environment to constrain the ordination of the community data. However, the choice of appropriate environmental variables has a substantial effect on the ordination of the community data. In the case of this study, the low percentage of variability explained by CCA is most assuredly due to the choice of explanatory environmental variables, which were also correlated to each other. In order to overcome this problem it is necessary to obtain sufficient sample metadata as to allow insightful choices into what environmental variables should be included in the analysis. Inclusion of variables that would not be expected to impact ordination introduce noise into process and diminish the significance of the results (McCune, 1997).

Stepwise linear regression was used to further partition out the responses of certain phyla to environmental variables. The interpretation of these results must be taken with caution. Linear regression is meant to be used on continuous data, of which most ecological data is not. Regardless, the absolute values of the regression coefficients and statistical significance of their inclusion in a regression model can be used to infer their overall influence in the distribution of particular phyla or other selected phylogenetic level. However, as with any multivariate statistical method, artificial axes, clusters, and variables do not necessarily correspond to actual biological or ecological conditions (Ramette, 2007).

The full R script used for this analysis follows:

```
#The Ade4 library is used to generate plots using the following script:
library(adegenet)
library(ade4)
# Switch screens only following the enter key
par(ask=T)
# 10 percent.txt is the cluster file previously arcsin transformed in Excel
fau<-read.table('10percent.txt',header=TRUE,sep=',',na.strings="NA")
# nutrient.txt is the environmental variable file
mil<-read.table('nutrient.txt',header=TRUE,sep=',',na.strings="NA")
# Log transform the nutrient data
lmil<-log(mil+1)
# Ensure that the data is still in the proper format
summary(lmil)
summary(fau)
list(lmil)
list(fau)
# Perform cca, the cluster file is listed first
iv1 <- cca(fau,lmil, scan = FALSE)
plot(iv1)
# Projections of inertia axes on PCAIV axes
s.corcircle(iv1$as)
```

```
# Species positions
s.label(iv1$c1, 2, 1, clab = 0.5, xlim = c(-4,4))
# Sites positions at the weighted mean of present species
s.label(iv1$ls, 2, 1, clab = 0.5, cpoi = 1, add.p = TRUE)
# Prediction of the positions by regression on environmental variables
s.match(iv1$ls, iv1$li, 2, 1, clab = 0.5)
# Canonical weights giving unit variance combinations
s.arrow(iv1$fa)
# Position of species by averaging
s.label(iv1$l1, 2, 1, clab = 0.5, cpoi = 1.5,add.plot=TRUE)
s.label(iv1$co, 2, 1, clab=0.5,add.plot = TRUE)
s.distri(iv1$l1, fau, 2, 1, cell = 0, csta = 0.33)
s.label(iv1$co, 2, 1, clab = 0.75, add.plot = TRUE)
# Coherence between weights and correlations
par(mfrow = c(1,2))
s.corcircle(iv1$cor, 2, 1)
s.arrow(iv1$fa, 2, 1)
par(mfrow = c(1,1))
#The vegan library is used to generate the statistics:
library(vegan)
par(ask=T)
# 3percent.csv is the cluster file which has been previously arcsin transformed
# nutrient.txt is the environmental variables file
```

```
fau<-read.table('3percent.csv',header=TRUE,sep=',',row.names=1)

mil<-read.table('nutrient.txt',header=TRUE,sep=',',na.strings="NA")

# Check the integrity of the input file:

str(fau)

# Transform the environmental variables (other transformations may be more correct,

depending on the data structure

lmil<-log(mil+1)

# Check that the data structure is still correct

summary(fau)

list(lmil)

list(fau)

# Perform canonical correspondence analysis on the two data sets

iv1 <- cca(fau,mil, scan = FALSE)

cca1.plot<-plot(iv1)

text(cca1.plot,what='sites',col="black",pch=2,cex=0.7,font=2)

goodness(iv1)

goodness(iv1, summ = TRUE)iv2 <- cca(fau$cluster

~mil$soc+mil$ph+mil$biomass)vare.ccaplot(vare.cca)

# Anova and CCA permutation tests are used to test the validity of the model

anova(iv1, alpha=0.05, beta=0.01, step=100, perm.max=10000)

permutest.cca(iv1, permutations=10000, model=c("reduced"))

# Returns the proportion of inertia accounted for by the axis
```

```
goodness(iv1,display=c("sites","species"), model = c("CA"), statistic =
c("explained"),summarize = FALSE)
iv1$CCA$alias
spec <- iv1$CCA$v
# Run regression of SOC vs cluster linear model regression
sm <- lm(percent~soc)
# How good is the model?
summary(sm)
# Perform correlations and stepwise regressions of interesting phyla versus environmental
variables
cor(percent, soc)
cor(percent,soc)^2
#What does the relationship look like?
plot(percent,soc)
# Plot the linear model
abline(sm$coeff)
title(main="Linear Model Plot")
# Returns the slope and intercept of the regression line
list(sm$coeff)
# Plot the model residuals
plot(sm$resid,)
abline (0,0)
title(main="Model residuals")
```

```
#LOCAL REGRESSIONS

#local.east <- loess(percent ~ soc)

#plot(soc,percent)

#points(soc,local.east$fitted,col="orange",pch=4)

#summary(local.east)
```

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