ABSTRACT

SMITH, JENNIFER MARY. Detection of Ammonia-Oxidizing β-Proteobacteria in Swine Waste Treatment Systems. (Under the Direction of John Classen and Sarah Liehr).

In order to obtain supporting evidence for biological denitrification in anaerobic lagoons degenerate β-Proteobacterial AOB primers were used to create and sequence clone libraries to detect the presence of ammonia-oxidizing bacteria at three field sites. Although there were PCR products from almost all samples, the clone libraries that were created show that not all PCR reactions produce only PCR products from ammonia-oxidizing bacteria. However, these primers did verify the presence of ammonia-oxidizers at one site, although their presence was not verified at the other sites. The presence of ammonia-oxidizers at the Battelle site implies that aerobic ammonia-oxidation is occurring. Clones were created and sequenced that were significantly different from other known sequences and tended to form very closely related phylogenetic groups. These phylogenetic groups were not isolated to one field site, and often more than one site had representatives in a closely related group. Future research in this field includes the design of new primer sets based on the sequences of the nitrifying bacteria clones reported in this research, creation of enrichment cultures, and use of new primers for fluorescent in situ hybridization.

DETECTION OF AMMONIA-OXIDIZING β-PROTEOBACTERIA IN SWINE WASTE TREATMENT SYSTEMS

by

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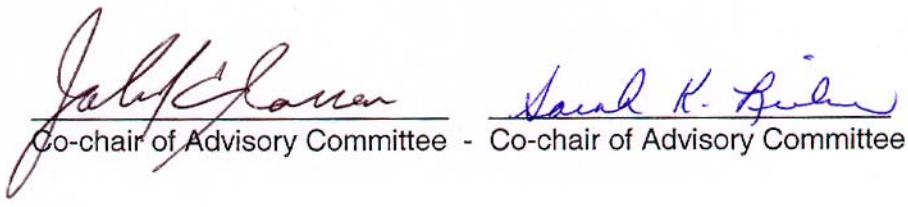

Co-chair of Advisory Committee - Co-chair of Advisory Committee

DEDICATION

This thesis is dedicated to my parents, David and Mary Smith, for giving me the opportunity to go to college and giving me the encouragement to pursue my career goals. It is also dedicated to Gareth Jones for providing me with motivation to finish, and for support, financial and otherwise. Finally, it is dedicated to my brother, Andrew Smith.

BIOGRAPHY

Jennifer Smith completed Bachelor's degrees in Ecology, Evolution, and Conservation Biology (B.S.) and Anthropology (B.A.) at the University of Washington in Seattle in 1998. She then worked in Lima, Peru before coming to North Carolina in 1999. She worked at North Carolina State University until 2002 when she entered the Master's program in Biological and Agricultural Engineering. This paper represents the final requirement for a M.S. in Biological Engineering at North Carolina State University. Jennifer will begin her Ph.D. in the fall in at NCSU in the Bioprocess Engineering area of Biological and Agricultural Engineering.

ACKNOWLEDGEMENTS

Many people have contributed to this thesis project and I would like to acknowledge thank them for their assistance. Dr. John Classen, Dr. Sarah Liehr and Dr. James Brown have provided direction, guidance and advice for this research project. I would also like to thank Chris Ellis and Jeff Barnes for innumerable days of help in the lab. Dr. Pedro Luna and Mr. Doug Williams of NC State University provided much needed assistance collecting samples in the field. Ms. Rachel Huie and Ms. Tracey Whiteneck of the Biological Engineering Department at North Carolina State University provided laboratory analysis of liquid samples. Tracey Whiteneck also initially provided valuable help with molecular biology problems. Finally, I would like to thank the North Carolina Department of Environment and Natural Resources, the North Carolina Agricultural Foundation, Inc., and the Battelle Memorial Institute for providing funding this research.

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1.1 Swine Farms in North Carolina

The number of swine farms in the United States has decreased from almost 600,000 hog operations in 1981 to less than 100,000 in 2003 (National Agricultural Statistics Service, 2002).

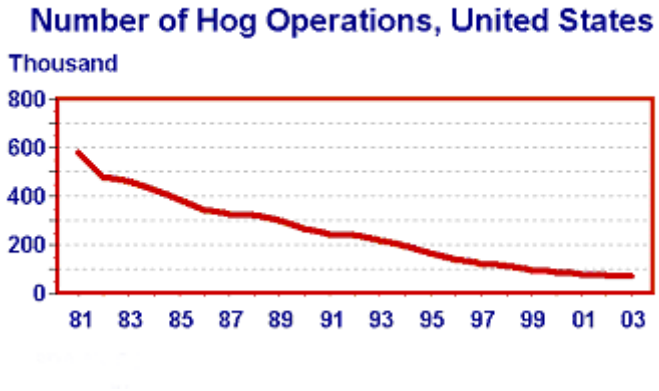

Figure 0-1. Number of Hog Operations, United States. United States Department of Agriculture, National Agricultural Statistics Service, April 30th, 2004.

The decrease in operations has not led to a decrease in the number of hogs in the United States, which has steadily increased from the mid 1980's to the present. This decrease in farms coincides with an increase in the concentration of animals on farms.

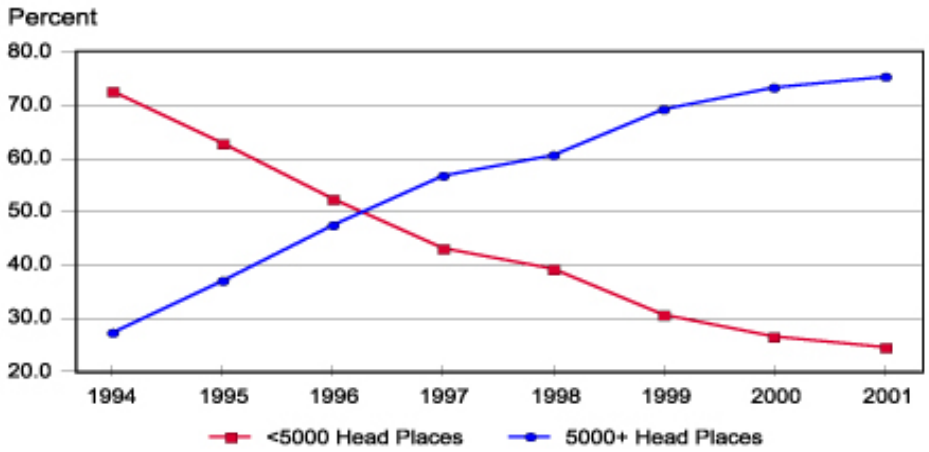

Figure 0-2. U.S. Annual Distribution, Head of Swine. From USDA-NASS, 2002.

In 1994 only 27% of swine were produced from farms with greater than 5,000 head. By 2001, farms with more than 5,000 head of livestock in an operation accounted for more than 75% of the total pig production in the U.S (Figure 1-2). This is attributed to a decrease in the amount of smaller less efficient farms, and an increase in farms with 1,000 to 5,000 animals at each operation.

North Carolina ranks as the state with the second largest hog population in the United States after Iowa (North Carolina Department of Livestock and Consumer Services, 2003) with 9.9 million head in North Carolina out of total 59.3 million head in the U.S. North Carolina therefore has 16.7% of the total hog population in the U.S (National Agricultural Statistics Service, 2004).

Swine housing trends in North Carolina mimic nationwide trends given that 76.2% of hogs were located in operations with 5000 or more head (North Carolina Department of Livestock and Consumer Services, 2003). Most hog operations in North Carolina use a water wash animal waste treatment system where the animal waste is mixed with water and stored in a large earthen pit dug into the ground (this is often termed an anaerobic lagoon, for more discussion see below). The waste is then left for a period of time for treatment and the liquid is then land applied to cropland for nutrient uptake (EPA, 1997). Hog waste in North Carolina in 1997 amounted to approximately 9.5 million tons per year (EPA, 1997). Animal waste is high in many nutrients, especially nitrogen (EPA, 1997; Harper and Sharpe, 1998), and in hog operations with a high concentration of animals, high levels of nitrogen are present in the waste in the form of ammonia (EPA, 1997; Harper and Sharpe, 1998).

1.2 Problems with Excess Nitrogen in Environmental Systems

Excess nitrogen in aquatic and terrestrial systems is the cause of several environmental problems. Aquatic systems can be contaminated by high levels of ammonia and nitrate that seep from animal waste lagoons and from runoff from land application of animal waste to agricultural fields. In many places ground water has become contaminated with NO_3^- from over-application. Nitrate in water can cause methemoglobinemia or "blue baby syndrome" where nitrates interact with hemoglobin and oxygen transportation around the body is inhibited. Nitrate-contaminated well water has been reported as the cause of this syndrome where nitrate can originate from livestock operations, agricultural runoff, or other facilities that generate high levels of nitrogen (NBExtension, 1995). Additionally, high levels of nitrogen in groundwater and surface water can cause eutrophication and algal blooms in streams and rivers (Kowalchuk and Stephen, 2001). Increased phototrophic growth can use up dissolved oxygen in surface waters, leading to anoxic conditions (Kowalchuk and Stephen, 2001).

In terrestrial systems, excess ammonia can also lead to high concentrations of ammonium in places where sandy forest soil has low levels of nitrification. Ammonium deposition can also cause acidification of soils leading to higher concentration of dissolved metals such as Al^{3+} that can be toxic. These two factors lead to the decline of forests and may destabilize the forest ecosystem.

1.3 Nitrogen Cycle

The nitrogen cycle describes the transformation of nitrogen to and from both inorganic and organic forms. Ammonia oxidation is the first step in nitrification and is often performed by two groups of ammonia-oxidizing bacteria (AOB), the γ- and the β–Proteobacteria, which convert ammonia to nitrite (Figure 1-3). This step is also perceived to be the most crucial

because it is thought to be rate limiting – nitrite is not often found to accumulate in the environment, and it is thought to be converted quickly to nitrate and other nitrogen forms, while ammonia can be more difficult to convert to other forms than nitrite (Kowalchuk and Stephen, 2001; McCaig et al., 1999). Ammonia-oxidation was previously thought to occur only under aerobic conditions however there is new evidence that nitrification may occur in low oxygen environments and ammonia-oxidizers may be able to survive for periods in anoxic environments (Bernet et al., 2001; Bodelier et al., 1996).

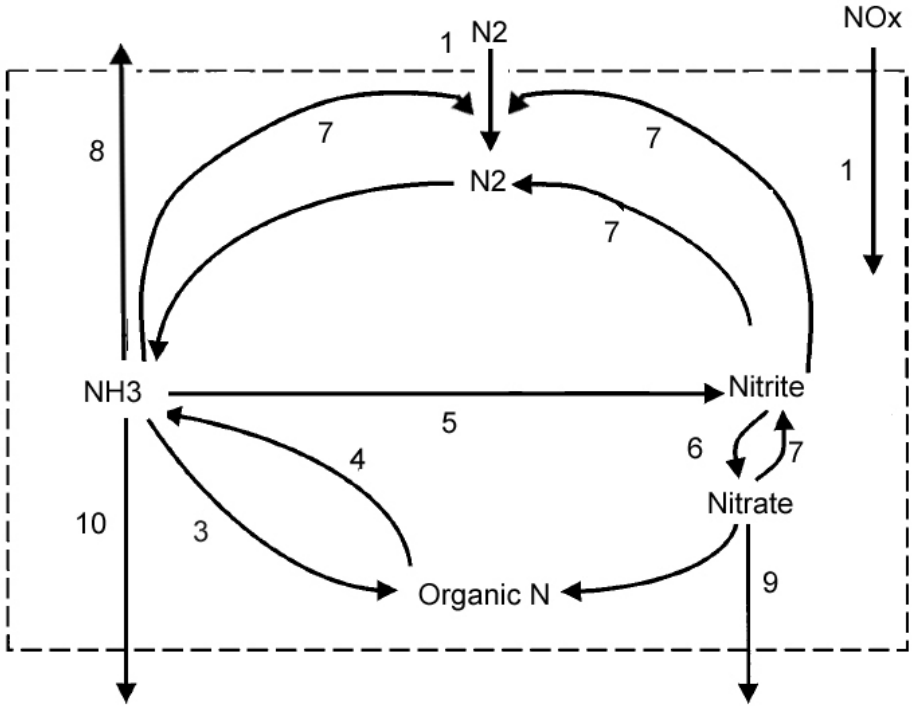

Figure 0-3. The nitrogen cycle: 1, atmospheric input; 2, nitrogen fixation; 3, immobilization (assimilation); 4, mineralization (ammonification); 5, aerobic ammonia oxidation; 6, nitrite oxidation; 7, denitrification; 8, volatilization; 9, nitrate leaching; 10, ammonia leaching (Tietema et al., 1992)

Denitrification (Step 7 in Figure 1-3) is the process of reducing oxidized nitrogen forms such as nitrite and nitrate. Denitrification ultimately converts nitrogen to dinitrogen gas, N_2, which can be released benignly into the atmosphere, and is the main source of loss of

nitrogen for many environmental systems (Kowalchuk and Stephen, 2001). Denitrification is performed by many different diverse phylogenetic groups of bacterial species, unlike ammonia oxidizers, which are a monophyletic group. There have also been several reports of nitrogenous gas production from groups of ammonia oxidizing bacteria, and denitrification from NO_2^- to N_2 gas is also reported to have occurred from pure cultures of *Nitrosomonas europaea* and *Nitrosomonas eutropha* –both known ammonia oxidizers – under low oxygen conditions (Jetten et al., 1997; Kuai and Verstraete, 1998).

1.4 Anaerobic Lagoons and Ammonia Emissions

Anaerobic lagoons are common waste treatment systems for swine farms in eastern North Carolina. An anaerobic lagoon is a large earthen pit dug into the ground with no aeration system and where the surface is left exposed to the atmosphere, precipitation, climate, and wind disturbances. However, the term “anaerobic” is really misleading as there is some surface transfer of oxygen into the liquid from the atmosphere. It is thought that this creates a system where there is a low level of dissolved oxygen, perhaps below detection limits. In this case the liquid may not be totally devoid of oxygen (anaerobic), but there may be anaerobic areas within some parts of the water column and in bacterial flocs (Jones et al., 2000).

It has been a widely held belief that nitrogen loss in lagoons is due to ammonia volatilization, and nitrite leakage into surrounding soils, groundwater and surface waters (Krapac et al., 2002). Recently there has been new evidence that nitrogen loss may not be due to ammonia volatilization and instead a significant portion may be due to chemical or biological denitrification (Harper and Sharpe, 1998; Harper et al., 2000). Their field data from a primary swine lagoon suggests that the emission of dinitrogen gas is greater than the emissions of volatilized ammonia (Harper and Sharpe, 1998).

1.5 Alternative Technologies - Swine Waste Treatment Systems

Alternative technologies have been developed in order to treat traditional swine waste lagoons by aeration. Surface aeration of liquid swine waste treatment systems involves using high rate aerators that mix air and water to increase the level of dissolved oxygen in the water. Although these systems are effective in creating aerated conditions in the liquid portion of the treatment system, high electricity costs are associated with their use. Surface disk aerators have a lower level of aeration however, but have lower electricity costs than traditional surface and diffused aeration devices.

1.6 Ammonia-Oxidizing Bacteria

Ammonia-oxidizing bacteria (AOB) are slow-growing chemolithotrophic aerobic bacteria (Wagner and Loy, 2002). *Nitrosococcus halophilus* and *Nitrosococcus oceani* and closely related species belong to the γ-Proteobacteria. The remaining majority (14 known species) of the ammonia-oxidizers form a monophyletic group in the gram-negative β-subclass of Proteobacteria (Juretschko et al., 1998; Purkhold et al., 2003; Purkhold et al., 2000).

Ammonia-oxidizing bacteria are thought to be obligate aerobic bacteria although there is some evidence that AOB can live in low oxygen systems and survive anoxic conditions (Bodelier et al., 1996; Kowalchuk and Stephen, 2001). AOB are obligate chemolithotrophs that obtain energy and reducing power from the oxidation of ammonia. This step is catalyzed by ammonia mono-oxygenase (AMO) (Hyman and Arp, 1992), an enzyme unique to ammonia-oxidizers. AMO is composed of three subunits: AmoA, a 27- to 30-kDa membrane-bound protein containing the active site of AMO (Hyman and Arp, 1992), AmoB, of 38- to 43-kDa (Bergmann and Hooper, 1994), and (Klotz et al., 1997).

1.7 AOB Populations in Different Systems

Ammonia-oxidizing bacterial populations vary immensely due to differing environmental conditions (Cebron et al., 2003; Kowalchuk and Stephen, 2001; McCaig et al., 1999; Purkhold et al., 2003; Rowan et al., 2002). Recent studies have also found that different wastewater treatment plants sustain different populations of AOB and that these differences are dependent on reactor type, reactor configuration, and the source and type of influent that is provided (Daims et al., 2001; Juretschko et al., 1998; Rowan et al., 2003a; Rowan et al., 2003b). This implies that systems with different configurations and different influent characteristics will have somewhat different ammonia-oxidizer populations.

There has not been as much study on AOB populations of agricultural treatment systems as there has been of industrial and domestic waste water treatment plants – therefore much less is known about their population compositions. Due to different influent waste characteristics and different waste treatment technologies employed on swine farms it is possible that there are different AOB community compositions at different treatment sites and that there are novel species that have not yet been found.

1.8 Culture vs. Molecular Techniques to determine Bacterial Population Identities

Cell culture has long been the standard for the determination of species identity (Winogradsky, 1892). However, it is now thought that only 10% of bacterial species can be found in culture (Hugenholtz et al., 1998). Many novel species have been found using new molecular techniques and systems that were thought to be well described are being re-examined. For instance, it was thought that *Nitrosomonas europaea* species were the dominant species found in wastewater treatment plants because they grow well in culture,

however molecular methods have shown that *Nitrosococcus mobilis* is more common (Juretschko et al., 1998; Wagner and Loy, 2002).

In the last 20 years the development of molecular techniques and a change towards using 16S rRNA and other molecular markers to identify species has caused a shift in thinking about bacterial population composition in various environments. This has lead to the replacement of the belief of the differentiation between prokaryotic and eukaryotic life, with principle of three divisions of life – Archeae, Bacteria, and Eukaryea (Hugenholtz et al., 1998). This information based on 16S rRNA has also led to a change in the classification of bacterial species – not based on environmental and physical characteristics but on genetic information. It has also led to the realization that culturing bacteria can create biases by selecting for certain members of the population that may or may not be representative of population in the environment. In many cases, including those of ammonia-oxidizing bacteria, environmental samples have proven to have different community structures than was previously suggested by culturing studies due to differences in the culturing media and environmental substrates (Juretschko et al., 1998). Additionally ammonia-oxidizing bacteria are known for their low growth rates that can complicate their isolation and distort the true picture of the population.

Molecular techniques are superior in detecting the true population structure because the scientist can sample directly from the environment with no time lapse, and she can find species that may be abundant in the environment but do not grow well in culture or have difficult or unknown culturing requirements. This has been the case in studies involving ammonia oxidizing bacteria (Juretschko et al., 1998; Purkhold et al., 2000).

Molecular techniques do have their own biases, however. Polymerase chain reaction (PCR) can have preferential primer annealing to different DNA targets. PCR from environmental samples can be complicated by the concentration (and therefore availability) of DNA especially in mixed cultures, and because of inhibitors that may be present (Harms

et al., 2003). Different DNA extraction efficiencies can favor some species over others, which in turn could affect the efficacy and the use of PCR as a balanced reflection of the population (Juretschko et al., 1998; Wagner and Loy, 2002). However, even though there are these possible difficulties with molecular methods, they are less severe than the numerous biases that are created by culturing environmental samples.

1.9 The 16S rRNA Gene

Molecular methods based on the 16S rRNA gene analysis have provided unique insights into the breadth of microbial diversity (Hugenholtz et al., 1998). This sequence is universally present in all bacteria and contains highly conserved regions interspersed with variable regions, thereby facilitating the design of primers and probes for analysis of bacterial phylogeny at different levels of specificity (i.e., family, genus, or species level) (Amann et al., 1995; Cilia et al., 1996). Additionally, the size of the 16S rRNA gene is extremely constant. This however limits its use in gel-based community-fingerprinting techniques, and 16S rRNA gene sequences may be too highly conserved to permit analysis of phylogenetic relationships between closely related species (Aakra et al., 1999; Cilia et al., 1996).

1.10 Gaps in the Literature and Objectives

Based on this review of the literature there are some areas of research that need to be addressed. In their 1998 paper, Harper and Sharpe suggest that either chemical or biological denitrification is responsible for the dinitrogen emission from lagoons that they have recorded. They suggest that at some sites denitrification is chemical rather than biological in nature, but neither give a reason, nor site evidence to support this statement.

One way to find supporting evidence for denitrification in “anaerobic” lagoons is to look for biological verification by using degenerate β-Proteobacterial AOB primers to amplify environmental samples and then create clone libraries. Identification of AOB in waste treatment systems would help to support claims that nitrification can occur in them because AOB live by aerobically converting ammonia to nitrite, though they may survive in anoxic conditions.

Because ammonia-oxidizers can live in systems with lower oxygen than was previously thought, it is unknown if they can live in other low-oxygen systems. Moreover, if it unknown what (or if) AOBs live in differently aerated hog waste treatment system. In every environmental system it can be expected that different environmental conditions produce different population compositions, therefore if detectable differences in AOB population structures can be distinguished based on aeration levels, this would also be of interest.

Finally although clone libraries will identify organisms, this method is not quantitative, albeit in the most broad way. It is of note that although making one clone library can find the most prominent members of a population, it is not exhaustive and does not show all members of the population. Because of this there may be prominent members of a community that will not be identified, however, a clone library will give data about the exact members of a population, can definitively identify them, and will identify previously unknown members of communities.

Based on the gaps in the literature and the research needs due to the gaps, the objectives of this study are twofold. The first objective is to determine if ammonia-oxidizing bacteria are present in differentially aerated swine waste treatment systems. The second objective is to determine if these AOB populations differ in the population composition at three different waste treatment systems.

2 Materials and Methods

2.1 Description of Sample Collection Areas and Sampling

Samples were collected from treatment systems of three different swine production units. All swine farms are located in the Coastal Plains of North Carolina. Swine houses were flushed with recycled water from the treatment pond at each farm. Additional water in the treatment system comes from precipitation, water from routine cleaning, and spilled water.

Samples were taken in clean 500 ml HDPE wide mouth Nalgene bottles in all field sites. At all field sites samples were taken 15 cm below the surface, from the sludge layer and from a floating surface scum layer. Samples were taken by hand 15 cm below the surface and samples from the sludge layer were taken with a dredge and pulled up to the surface. Sludge samples were taken from the center of the dredge and surface scum was sampled from one location at each treatment site and collected in 500 ml bottles. Samples were immediately stored on ice and transported to the laboratory. Lab samples were stored at 4°C until analyzed.

The Battelle waste treatment system is located at a nursery swine production unit in Richlands, North Carolina. The Battelle waste treatment system is comprised of a non-aerated treatment section and an aerated treatment section with a rotating disk aerator. A barrier separates the aerated section from the non-aerated section. Samples were taken from one location within the non-aerated section and at three locations within the aerated section. At all locations samples were taken 15 cm below the surface and from the sludge layer. A sample of the surface scum was also taken at this site.

The Hoffland animal waste treatment system is located in Fremont, North Carolina and is comprised of a solids separator, followed by an aeration basin with four surface aerators, and a final storage pond. Animal waste leaves the swine houses and is directed to a

clarifier. The liquid portion of the waste goes to the aeration pond where four surface aerators oxygenate the pond. It then travels to another clarifier, and then finally to the storage pond. Samples from the Hoffland waste treatment site were taken in the center of the aeration pond, the side of the aeration pond and in the storage pond. Samples were taken 15 cm below the surface and from the sludge layer at each of these three locations. A sample of the surface scum was also taken at this site.

The third sampling location, Farm 10, is located in Faison, North Carolina, described by Harper and Sharpe (1998). Farm 10 has a rectangular "anaerobic" lagoon treatment system that covers approximately 2.7 hectares (6.6 acres). Samples were taken from the lagoon in two locations – from the center of the lagoon and from near the recycle pump inlet. At these two locations samples were taken 15 cm below the surface and from the sludge layer. Samples of surface scum were also taken from this site.

2.2 Environmental Analysis

Physical and chemical measurements were taken at each location in each treatment site. Measurements of dissolved oxygen, oxidation-reduction potential, temperature, and pH were all taken at the surface at each location. A portion of each sample taken was also sent to the NC State Biological and Agricultural Engineering Environmental Analysis Laboratory for analysis to determine NO_3^--N, NO_2^--N, total ammoniacal nitrogen (TAN = NH_3-N + NH_4^+-N), chemical oxygen demand (COD), and total organic carbon (TOC).

Averages of the environmental parameter from different locations (e.g. center and side) were taken and standard deviations were calculated. Locations were averaged within the aerated sections of the treatment systems at the Battelle and Hoffland sites, and in the main treatment system at Farm 10. All averaged samples were taken from the same depth - 15 cm below the surface - and were taken on the same day and depth as samples for DNA extraction.

2.3 DNA Extraction and Purification

DNA was extracted from samples on the same day that samples were collected. Two liquid and sludge samples from each location were put into 28 ml sterilized Nalgene Oak Ridge Centrifuge Tubes and were spun at 12,000 rpm for 8 minutes. This produced two pellets from which 0.25-0.4g of material was obtained. DNA was extracted using the MoBio UltraClean Fecal DNA Kit (Carlsbad, CA, USA) following the standard protocol. DNA was stored at -20°C until it was used.

2.4 PCR amplification of 16S rRNA genes

PCR of 16S rDNA was performed using primers Nso190 (3' GGAGAAAAGCAGCCC ATCG 5') and Nso1225 (5' CGCGATTGTATTACGTGTGA 3') specific for the Proteobacterial β-subclass ammonia-oxidizers (Mobarry et al., 1997). PCR products were approximately 1000 base pairs. PCR was performed with an Eppendorf Mastercycler thermocycler (Hamburg, Germany) with heated lid. The 50 μl PCR reaction was carried out according to the manufacturer's instructions using the BD Biosciences Clontech Advantage 2 PCR Kit (Palo Alto, CA, USA) in 0.3 ml tubes. PCR cycling consisted of an initial denaturing of 94°C for 120 seconds, followed by 31 cycles of denaturation at 92°C for 90

seconds, an annealing step at 55°C for 90 seconds, an elongation step at 72°C for 90 seconds. This cycle was performed 31 times, followed by a final elongation step at 72°C for 10 minutes. PCR products were kept at 4°C in the thermocycler until frozen at -20°C. PCR products were run on a 1% agarose gel (Fisher Scientific, Fairlawn, NJ, USA), stained with Ethidium Bromide, and DNA band sizes and intensities were analyzed using imaging and analysis software.

2.5 Construction and Sequencing of Clone Libraries

Clone libraries were constructed for three sites – the Battelle, Farm 10 and Hoffland waste treatment systems. PCR products amplified from samples taken 15 cm below the surface at Battle, Hoffland and Farm 10 were extracted from low melting-point agarose gels with Qiagen QIAquick Gel Extraction Kit (Hilden, Germany) and eluted in Tris-EDTA buffer. The 1000 base pair DNA fragment was ligated into a Promega P-gem T Easy Vector (Madison, WI, USA) according to the manufacturer's instructions (at room temperature for one hour). Electro-competent DH5α *E. coli* cells were transformed using a Gibco BRL Electroporator (Carlsbad, CA, USA), grown in Luria-Bertani (LB) broth for one hour, and then plated on LB/Ampicillin (20 mg/ml) plates. Clones were tested for vectors with insert with blue/white screening. Clones with plasmid and insert were grown in LB/Ampicillin overnight. 60 clones from Battelle, 30 clones from Farm 10, and 23 clones from the Hoffland waste treatment facility were grown up. Plasmids were extracted from these clones with the Qiagen QIAprep Spin Miniprep Kit (Hilden, Germany). Clones were single-sequenced by MWG Biotech's Value-Read sequencing service (High Point, North Carolina).

Clones were created from PCR products amplified from DNA taken 15 cm below the surface in the aerated section of the treatment system in the Battelle and Hoffland sites and in the anaerobic lagoon of Farm 10. This corresponds to samples B1, F0-1 and H1 from the

Battelle, Farm 10 and Hoffland sites, respectively. Farm 10 PCR products were intensified for cloning by running five simultaneous PCR reactions and the combined PCR products from these five reactions were used in the cloning reaction.

2.6 Phylogenetic Analysis

Phylogenetic analysis was performed using the Phylip (Seattle, WA, USA) interface of the Ribosomal Database Project (RDP) (Cole et al., 2003). Multiple sequence alignments for each of the clones were made by uploading each clone sequence and using the RDP's site to align each of the clones to the 16S sequences of most closely related species (neighbors) in the RDP's database (Figure 2-3).

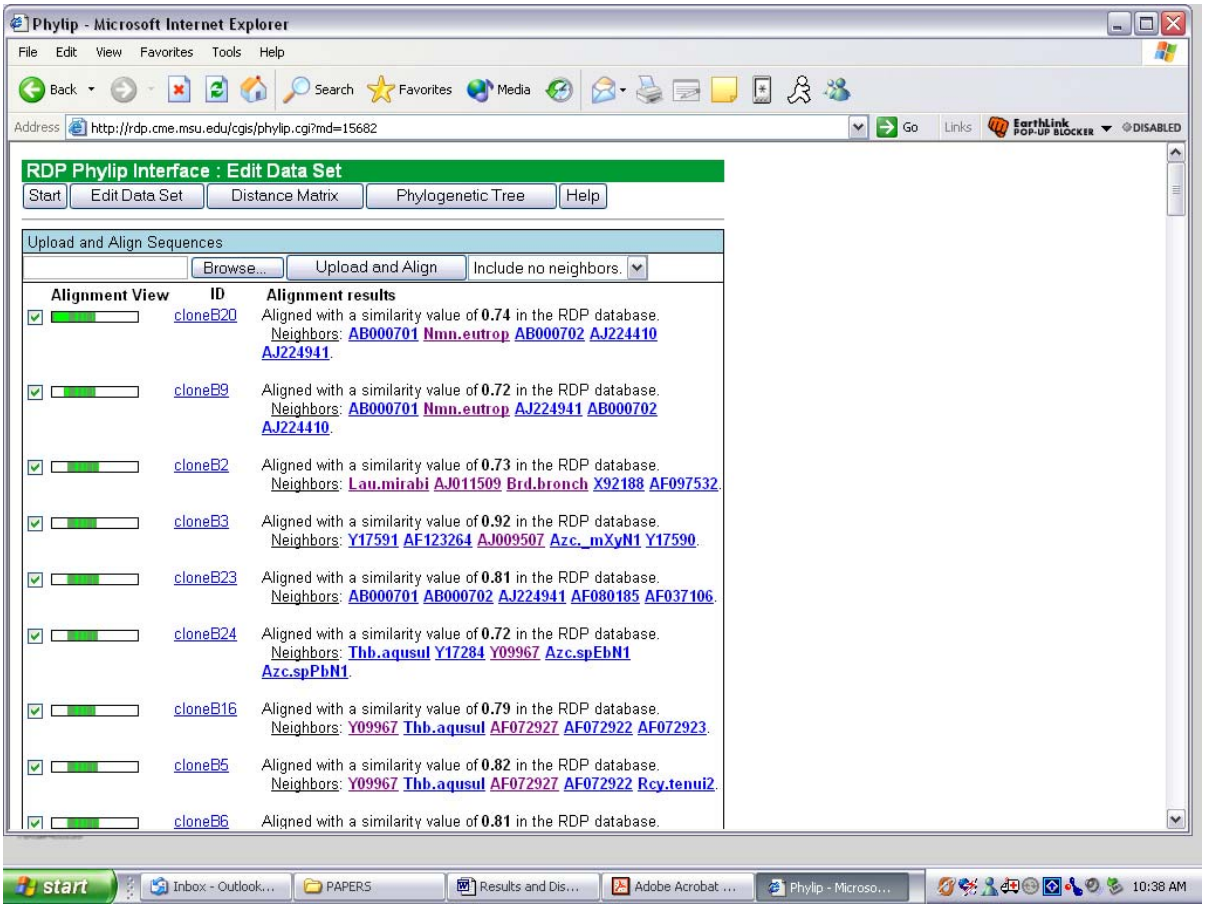

Figure 2-1. Uploading sequences to the Ribosomal Database Project's Phylip Interface.

A matrix of was created by the RDP's software (Cole et al., 2003), using the Maximum Likelihood method. Only bases that were shared among all clones and species that were compared could be used to create the matrix. The quality and length of DNA sequence and the degree of relatedness of clones and neighboring species limits the matrix that can be created (Figure 2-4).

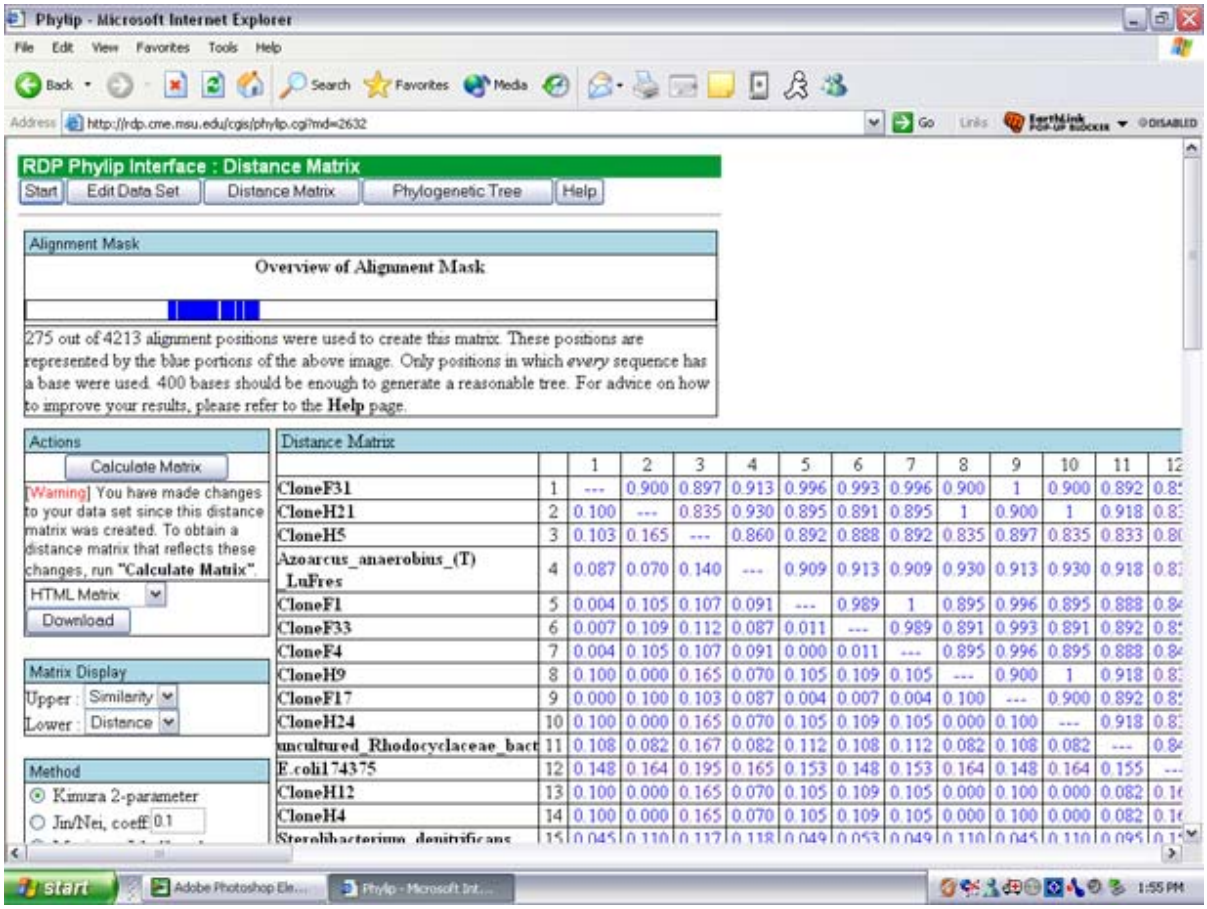

Figure 2-2. RDP Phylip Interface, creation of distance matrix.

Phylogenetic trees were then created using the 16S gene sequence from *Escherichia coli* (gi 174375) from the NCBI database as the out-group in order to root the tree (Figure 2-5).

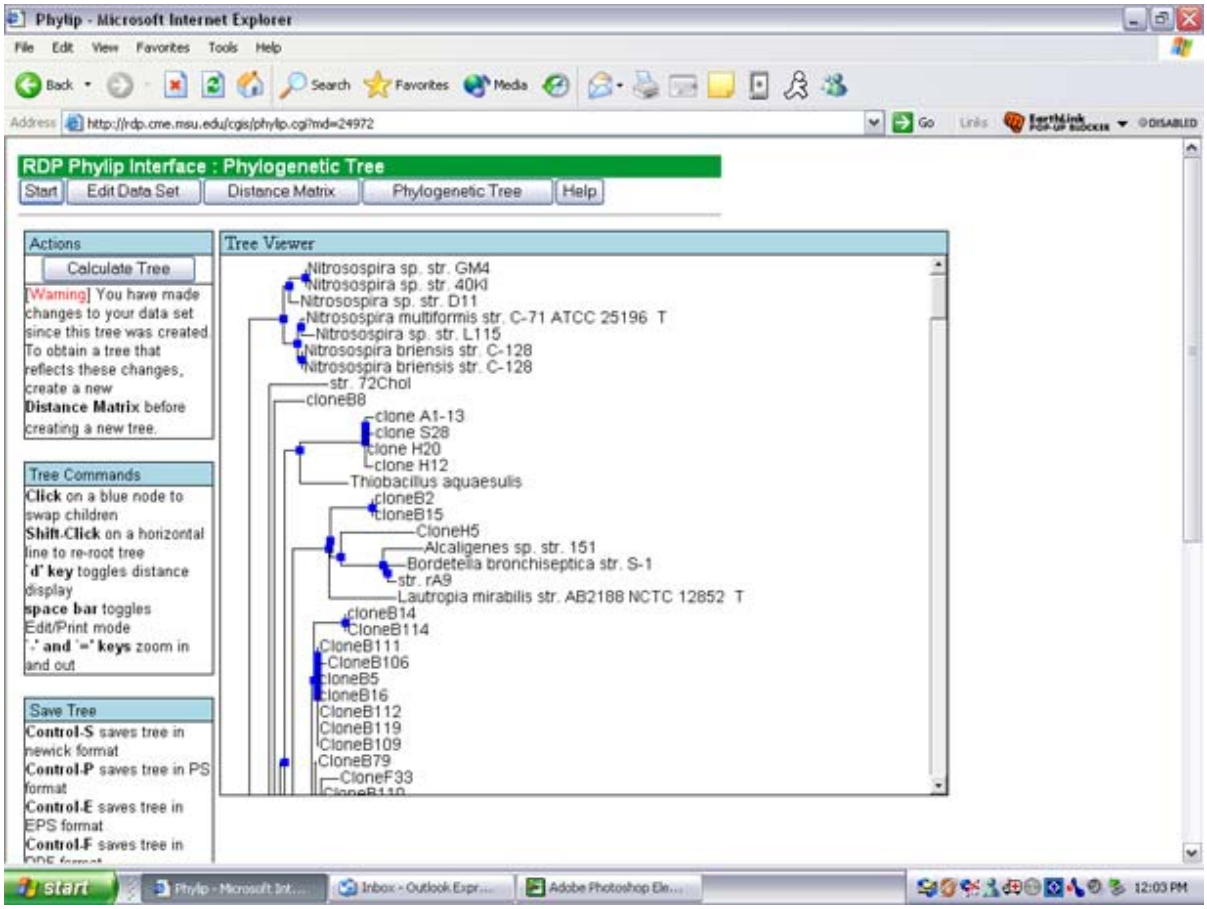

Figure 2-3. RDP Phylip Interface, creating distance matrix.

Phylogenetic trees were created by the RDP in Newick format that can be viewed in Treeview (Seattle, WA, USA) and Adobe Acrobat (San Jose, CA, USA).

Multiple phylogenetic trees were made based on groups of closely related clones. If clone groups did not share many sequence alignments with neighboring species, clone numbers were reduced in that particular analysis in order to maximize the number of bases that were found in common among the compared species. This method of maximizing base comparison was employed to create the most accurate phylogenetic trees. Phylogenetic trees were created by individual field site, by closely related phylogenetic groups at one site,

by closely related groups across multiple sites, and by a complete clone set from all field sites.

3 Results/Discussion

3.1 Environmental Conditions at Each Site

The environmental parameters measured were dissolved oxygen (DO), oxidation-reduction potential (ORP), pH, temperature, total ammoniacal nitrogen (TAN, NH_3-N + NH_4^+-N), NO_3^--N, NO_2^--N, chemical oxygen demand (COD) and total organic carbon (TOC). The average and standard deviation of each environmental parameter was calculated as discussed in the Materials and Methods section above.

Environmental conditions at each of the sites varied, especially at the Battelle site. Conditions at the Hoffland and Farm 10 sites appeared to be more similar to each other than either was to the Battelle site.

Dissolved oxygen was not statistically different at all sites, based on the amount of variation between field measurements as can be seen in Figure 3-1.

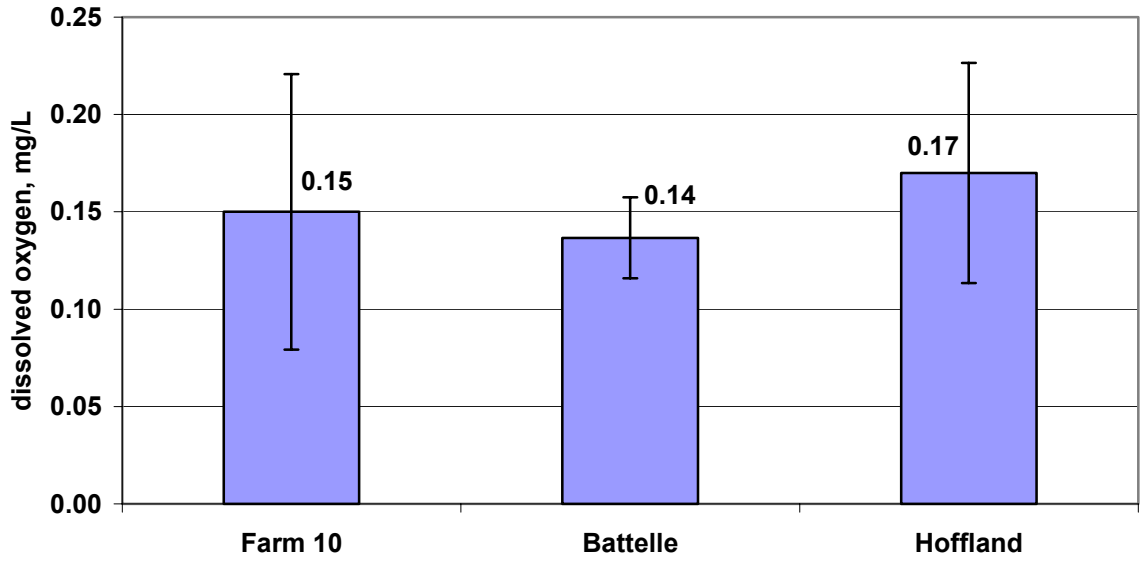

Figure 3-1. Average DO with standard deviation bars at Farm 10, Battelle, and Hoffland, in treatment lagoons, 15 cm below surface, July, 2003.

Average TAN (total ammoniacal nitrogen) was found to be much lower in the Battelle site than in either the Farm 10 or Hoffland sites. The Hoffland field site had the highest average TAN concentration and the Farm 10 site fell between the two other sites (Figure 3-2).

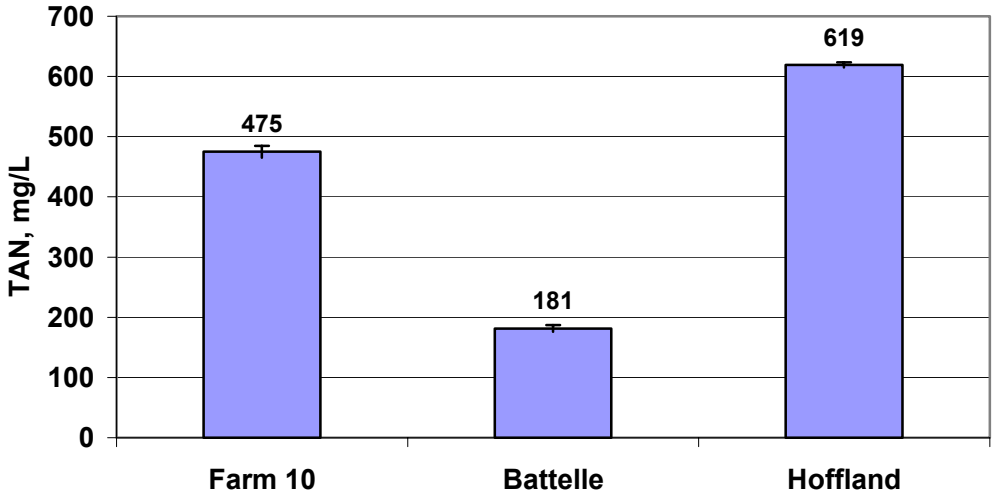

Figure 3-2. Average TAN with standard deviation bars; samples from treatment lagoons, 15 cm below surface, July, 2003.

When comparing the levels of TAN to NO_3^--N and NO_2^--N at the three field sites, there appeared to be a difference between these levels at the Battelle site and at the Hoffland and Farm 10 sites (Figure 3-3). The levels of TAN at the Battelle field site were lower, while the levels of the NO_3^--N and NO_2^--N were considerably higher at Battelle site that at the two other field sites.

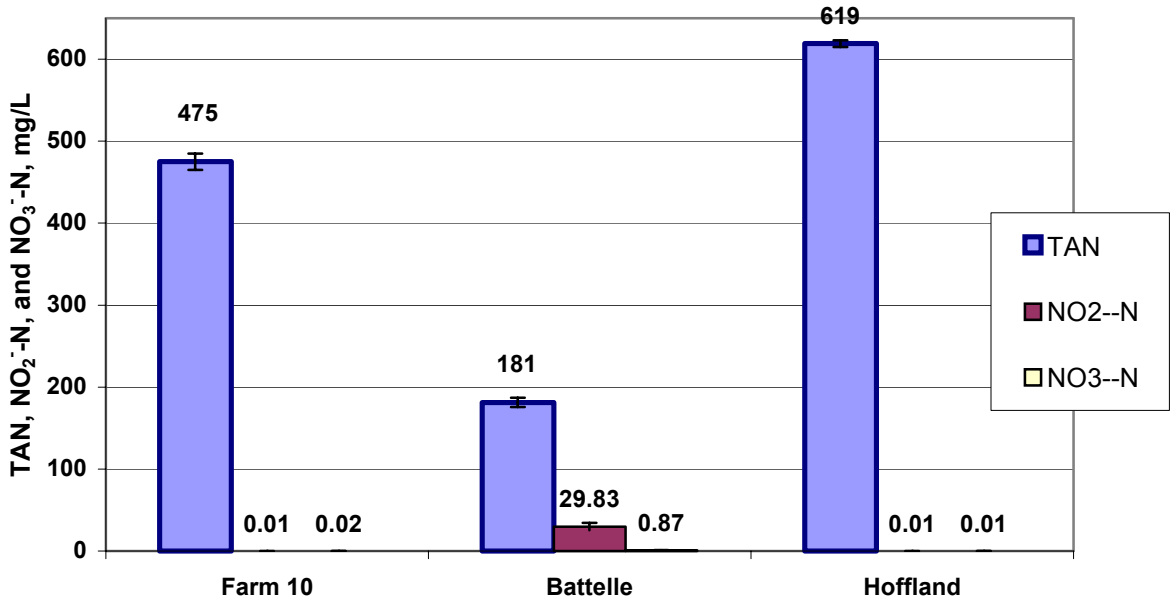

Figure 3-3. Averaged TAN, NO3--N, & NO2--N with standard deviation error bars, all treatment systems, 15 cm below surface, July, 2003.

The nitrate-N and nitrite-N levels at the Hoffland and Farm 10 field sites were so low (<0.01) and their standard deviations were so large that there was no statistical difference in their levels. Similarly, the nitrite-N level was significantly higher than the nitrate-N level at the Battelle field site (Figure 3-4).

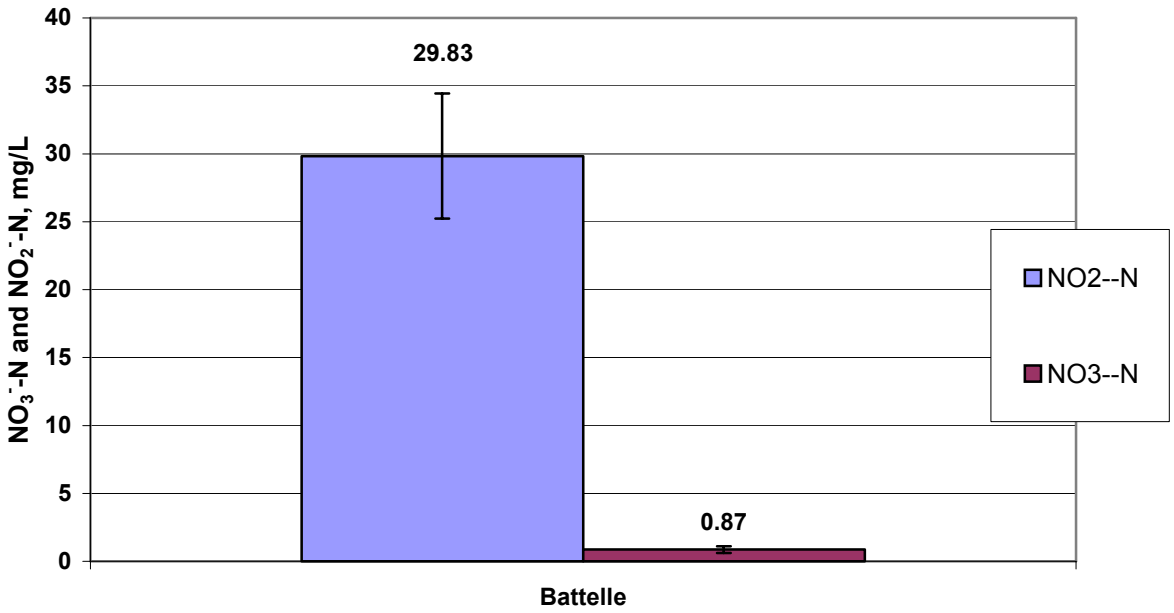

Figure 3-4. Battelle averaged NO3--N and NO2--N concentrations with standard error bars, samples taken from 15 cm below surface, July, 2003.

Although the nitrate-N level was significantly lower than the nitrite-N level at Battelle, it was still significantly higher than the nitrate-N and nitrite-N levels at both the Farm 10 and Hoffland field sites (Figures 3-4). Nitrate-N levels do not appear to be significantly different from nitrite-N levels at Farm 10 and Hoffland field sites (data not shown).

In summary, we find that the levels of TAN appear to be significantly different at the three sites. The Hoffland field site has the highest concentration of TAN, the Farm 10 field site has the second highest, and the Battelle field site had the lowest. The Battelle field site had by far the highest concentration of NO_2^--N while the Farm 10 and Hoffland sites had much lower concentrations. NO_3^--N levels were much higher at the Battelle site than at either of the other two field sites.

COD and TOC followed generally the same pattern as total ammoniacal nitrogen. Battelle had the lowest concentrations of COD and TOC, Farm 10 had intermediate concentrations, and the Hoffland site had the highest concentrations (Figure 3-5).

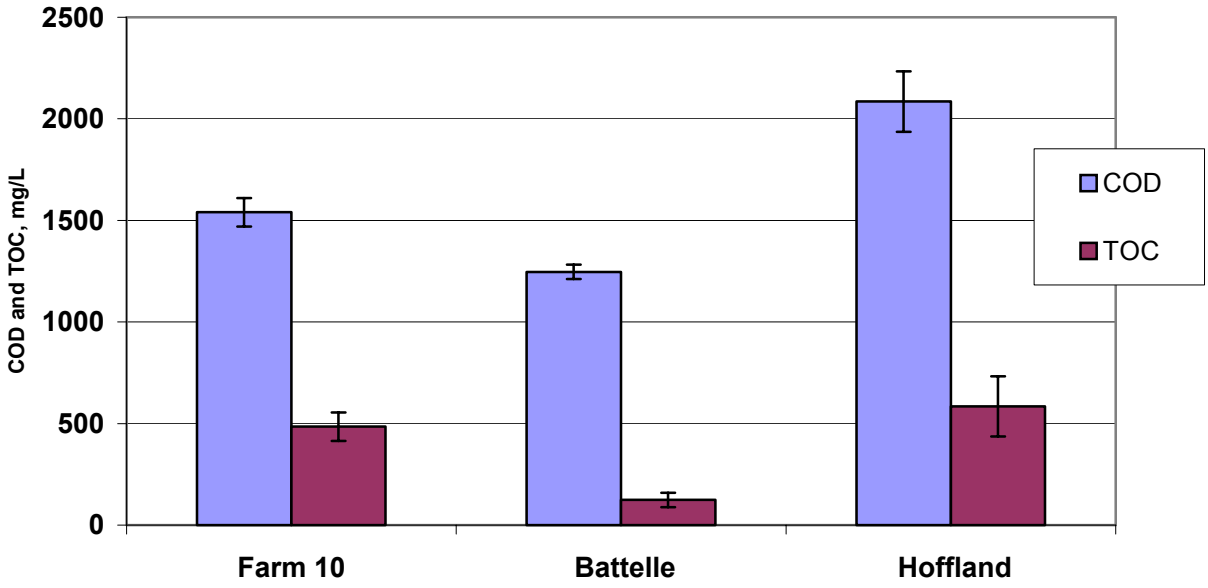

Figure 3-5. Average chemical oxygen demand (COD), total organic carbon (TOC), and total carbon (TC) with standard deviation bars for Farm 10, Battelle and Hoffland field sites, July 2003.

Temperature did not appear to be significantly different at the three field sites (data not shown). However pH seemed to vary from site to site on the day sampled (Figure 3-6).

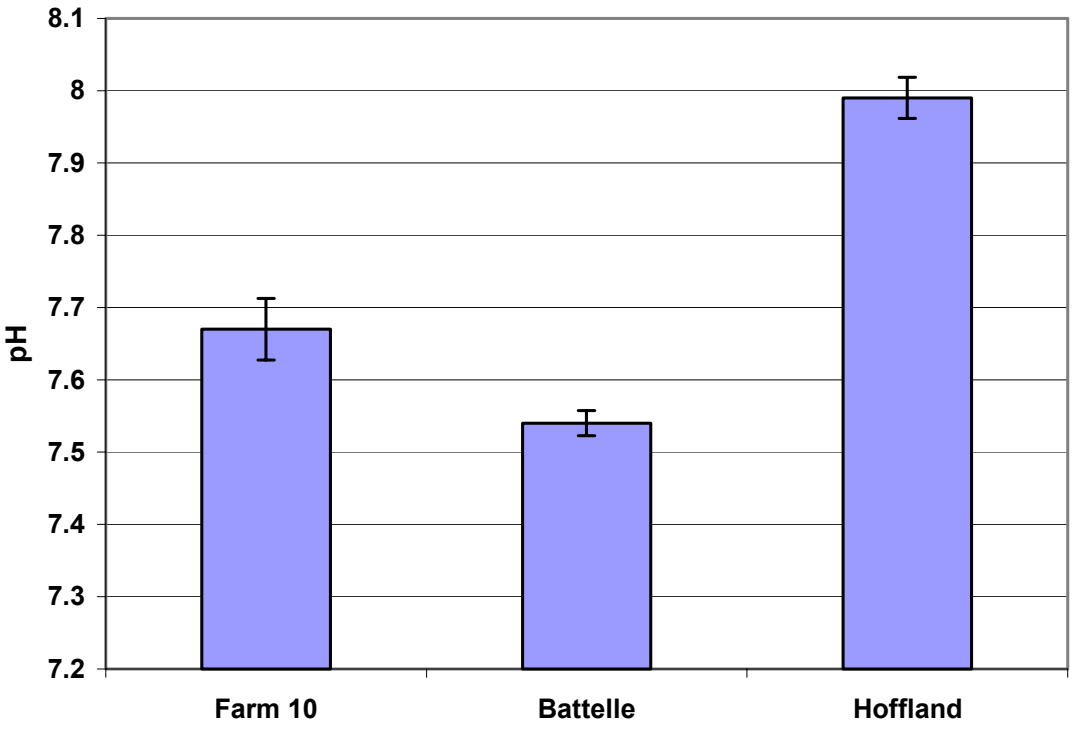

Figure 3-6. Average pH with standard deviation bars, July, 2003.

Oxidation-reduction potential does appear to be significantly greater at the Hoffland field site than at the other two field sites (data not shown). These data appear to suggest that there is a general trend where the Hoffland field site has highest levels of TAN, COD, TOC followed by the Farm 10 and Battelle sites, respectively. The Battelle site appears to have a significantly higher concentration of NO_3^--N and NO_2^--N than the other two sites, and the significantly lowest concentration of TAN.

3.2 DNA Extraction, PCR and Cloning

DNA was successfully extracted from all samples. PCR was performed with both universal and nitrifying primers on all samples, and PCR products were obtained from almost all samples. However, a PCR product was visible for all samples that were cloned. Figures 3-1, 3-2, and 3-3 show that almost all DNA samples taken from the field site treatment systems in July, 2003 produced 16S rDNA PCR fragments of the expected size (1000 base pairs).

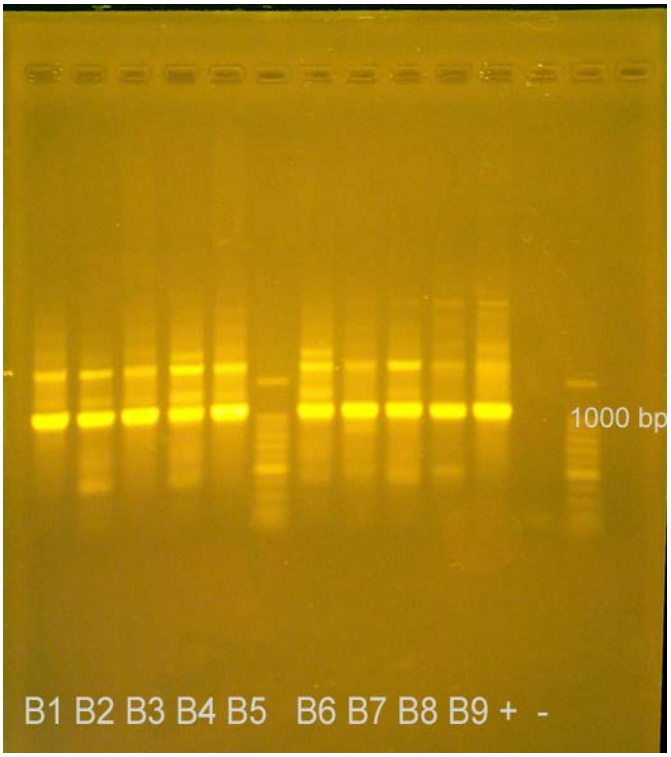

Figure 3-7. PCR products from Battelle samples taken on July 25th, 2003 (5 ml run on a 1% agarose gel). PCR was performed with nitrifying primers Nso190F and Nso1225R.

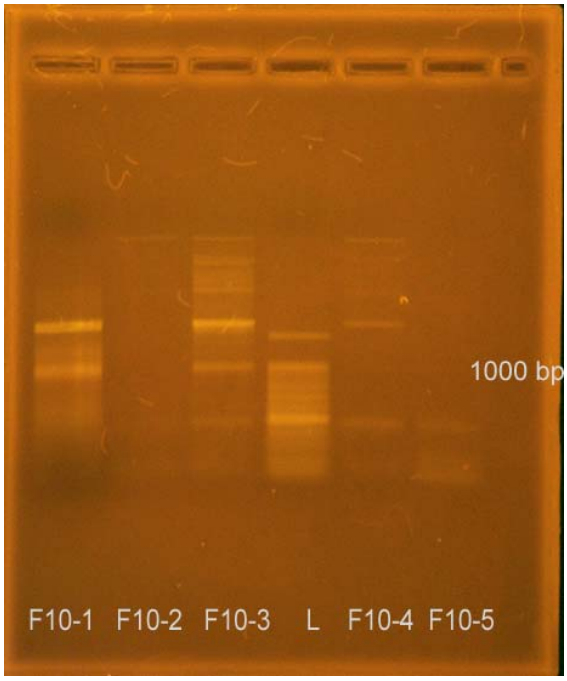

Figure 3-8. PCR products from Farm 10 samples taken on July 7th, 2003 (5 ml run on a 1% agarose gel). PCR was performed with nitrifying primers Nso190F and Nso1225R.

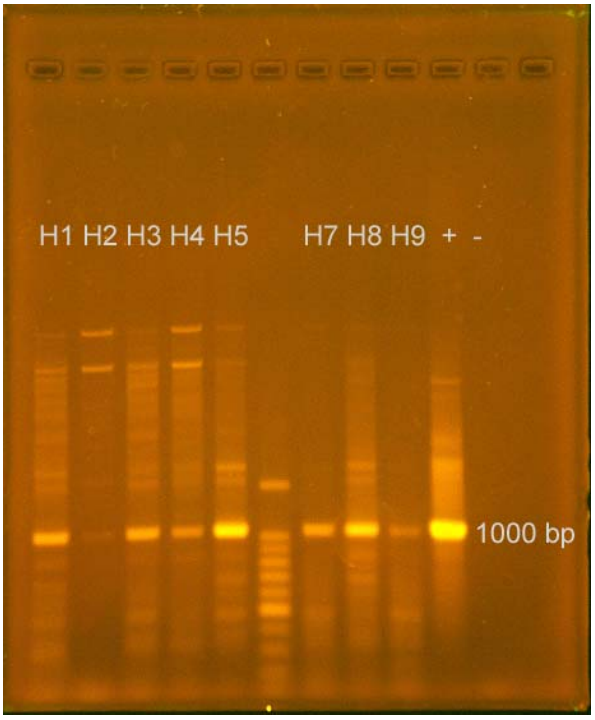

Figure 3-9. PCR products from Hoffland samples taken on July 22nd, 2003 (5 ml run on a 1% agarose gel). PCR was performed with nitrifying primers Nso190F and Nso1225R.

Clones were created from PCR products of samples taken 15 cm below the surface in the aerated section of the treatment system in the Battelle and Hoffland sites and in the anaerobic lagoon of Farm 10. Approximately 500-700 bases of sequence was produced when each readable clone was sequenced, however not all clones gave readable sequence. 52 of the 60 Battelle clones that were sequenced, 23 of 30 Farm 10 were sequenced, and 14 of the 23 Hoffland clones were sequenced. Although clones H27 and H23 had clear sequence, they were not related to the β-Proteobacteria (primers had non-specific binding to eukaryotic DNA) and were therefore eliminated from the analysis.

3.3 Result of Phylogenetic Analysis/Trees

3.3.1 Non-Specific Primer Binding

AOB primers are difficult to design because wastewater treatment systems contain many species of AOB (Juretschko et al., 1998). Additionally, many AOB are phylogenetically more closely related to other activated sludge bacteria within the β- Proteobacteria (Harms et al., 2003). This makes primer design difficult because the level of differentiation between 16S genes is very fine. In other studies, AOB primers have regularly hybridized with non-AOB species that are closely related to give false positives (Harms et al., 2003).

In our study, although primers were theoretically specific to the ammonia-oxidizing bacterial subset of the β-Proteobacteria, phylogenetic analysis of cloned sequences showed more non-specific primer binding to AOB occurred than specific binding. Only four of the 87 cloned sequences clones were identified as AOB. These sequences were only from Battelle site DNA. No clones were found from AOB in either the Farm 10 or Hoffland sites. All

clones did fall within the β-Proteobacteria, however they were not identified as ammonia-oxidizers but were related to denitrifying bacteria. Because of this non-specific binding, PCR products of the expected size could not be used as a diagnostic tool for identifications of AOB.

One explanation for the lack of AOB sequences is simply that there were not many (or no) ammonia-oxidizers present in these treatment systems. Another explanation is that if primers were non-specific to AOB (as has been shown) and if denitrifiers greatly outnumbered AOBs in the lagoon systems, the statistical chance that an AOB 16S is amplified during PCR would be greatly reduced.

The *amo-A* gene can be used as alternative phylogenetic marker because it is only found in AOB bacteria, and primers can be created based on these protein-encoding genes. However, the *amo-A* gene has higher sequence variability between AOB than 16S rDNA, making it more difficult to design one pair of primers to detect all ammonia-oxidizing bacteria (Harms et al., 2003). Additionally, creating a tree based on protein sequence information is more difficult and can be less accurate than creating one based on16S information (Brown, 2004). Finally, creating a new set of primers would have been problematic because due to time constraints they could not have been sufficiently tested.

3.3.2 Sequence Homology to Known 16S Sequences

When the cloned sequences were uploaded to the Ribosomal Database Project (Cole et al., 2003), none matched sequences in the database 100%. The similarity of Battelle clone sequences to the RDP database ranged from 64% (clone B15) to 98% (B120) (see Figure 2-1 and 2-2). Only one clone sequence (B15) had lower than 70% homology to sequences in the RDP database, 16 sequences had homology ranging from 70-80%, 27 sequences had a homology ranging from 80-90%, and 8 sequences had a homology ranging from 90-

100%. Although there is no definitive standard for species and genus levels of similarity, the lack of homology to known sequences indicates that these sequences may not be of the same species or genus as previously known organisms.

Farm 10 clones ranged in homology to the RDP 16S sequence database from 50% (clone F27) to 92%. Of the 23 Farm 10 clones that were sequenced, only one clone had lower than 70% homology to the RDP 16S sequence database, (clone F27), while 17 clones had a 70-80% homology, four clones had a 80-90% homology, and only one clone had a greater and 90% homology with the 16S sequences in the RDP database. This shows that the clones from Farm 10 are not the same species, (only one is a candidate for similar species) and many may be of unknown genera within the β-Proteobacteria.

Hoffland clones ranged in homology from 69% (clone H5) to 95% (clones H9, H22 and H24). However out of 12 clones that had readable sequence, 11 had homologies to the database above 90% while only one (clone H5) had a 69% homology. There were no clones that had homologies between 70 and 89%.

3.3.3 Phylogenetic Analysis of Battelle Clones

All Battelle clone sequences were located phylogenetically within the β-Proteobacteria, however only four of the 52 clones that were sequenced were related to known ammonia-oxidizing bacteria. These four are phylogenetic offshoots of the β-Protebacterial Nitrosomonads, which are lithotrophic, oxidize ammonia to nitrite, and assimilate carbon as CO_2. Three clones appear to be phylogenetic offshoots of several *Nitrosomonas* species, while one is related more closely to *Nitrosospira* species.

Clones B23, B9 and B20 formed a group that was related to Nitrosomonads such as *Nitrosomonas eutropha* (Figure 3-10). *Nitrosomonas eutropha* is a lithotrophic ammonia-oxidizing bacteria, a member of the β-Proteobacteria. The molecular sequences of

Nitrosomonas eutropha species are derived from the cultured strains – most from wastewater treatment plants, where they are often the dominant species (Daims et al., 2001; Juretschko et al., 1998; Koops, 2001).

Clone B8 was most closely related to *Nitrosospira* strains Ka3, SM16, and D11. *Nitrosospira* is a nitrifying ammonia-oxidizing β-Proteobacteria. The genus Nitrosospira contains species with many phenotypes that are often pleomorphic (Koops, 2001). Isolates come from soil, terrestrial and marine systems but rarely have been detected in wastewater treatment systems.

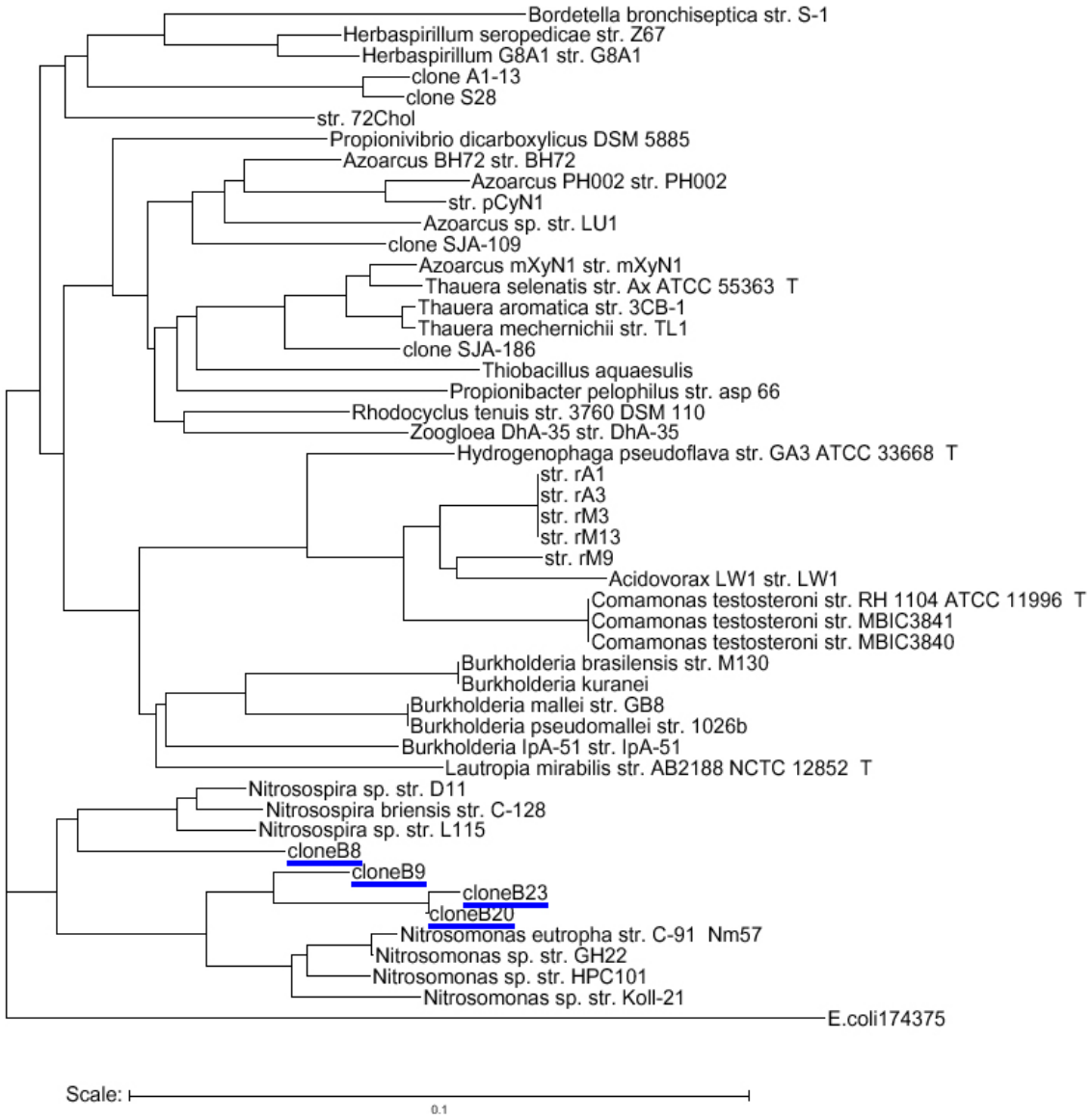

Figure 3-10. Clones B8, B9, B20, and B23.

All other Battelle clones were most closely related to denitrifying β-Proteobacteria. Most Battelle clones were usually clustered into closely related groupings or clusters, but did not have a high level of homology to known sequences on the RDP database. Clones B18, B76, B77, B78, B86, B92, B93, B98, and B122 appear to be most closely related to *Comamonas* strains *Comamonas testosteroni* and *Comamonas denitrificans* (Figure 3-11). *Comamonas testosteroni* is capable of NO_3^--N reduction, but cannot reduce NO_2^-, while *C. denitrificans* can reduce NO_3^- to N_2. *Comamonas* is an aerobic respirator and are able to degrade a wide range of aromatic compounds. Testosterone can be used as sole carbon source by *C. testosteroni*. *Comamonas* species are found frequently in the environment and have been isolated from mud, soil, water and activated sludge. *Comamonas testosteroni* has also been reported in activated sludge (Boon et al., 2000). *Comamonas denitrificans* (Gumaelius et al., 2001) has only been reported once as a member of the microbial denitrifying component in activated sludge (Willems and De Vos, 2002). Although these Battelle clones seem to be most closely related to *Comamonas*, they are not so closely related that they appear to be either the same species or perhaps the same genera (Figure 3-12).

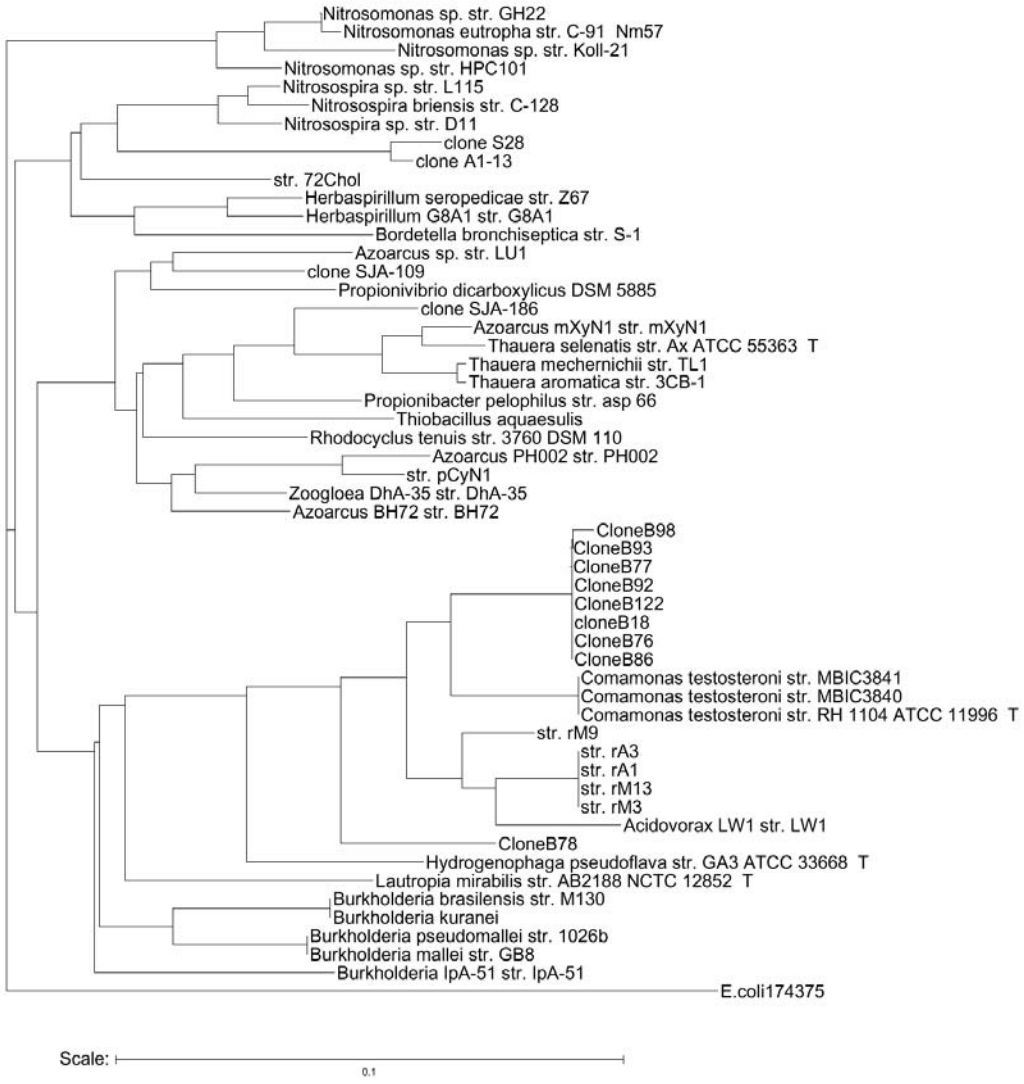

Figure 3-11. Cluster of Clones related to *Comamonas* species.

Clone B117 appears to be most closely related to *Burkholderia psuedomallei* and *B. mallei* Strain GB8 (Figure 3-12). *Burkholderia* is a β-Proteobacteria that is usually found on plant roots, in the rizosphere or in wet environments. The group is diverse in that it contains many pathogens to animals and humans but it also contains species that promote plant growth and help in bioremediation (Woods and Sokol, 2000). Clones B15 and B2 appear to be most closely related to a strain of *Lautropia mirabilis*. *Lautropia mirabilis* can reduce NO_3^- and NO_2^- and is anaerobic. *Lautropia mirabilis* was originally isolated from the human mouth and is most closely related to *Burkholderia* but is its own genera. Clones B15 and B2 appear to be closely related to each other but not as closely related to *Lautropia*, possibly forming their own genera (Figure 3-12).

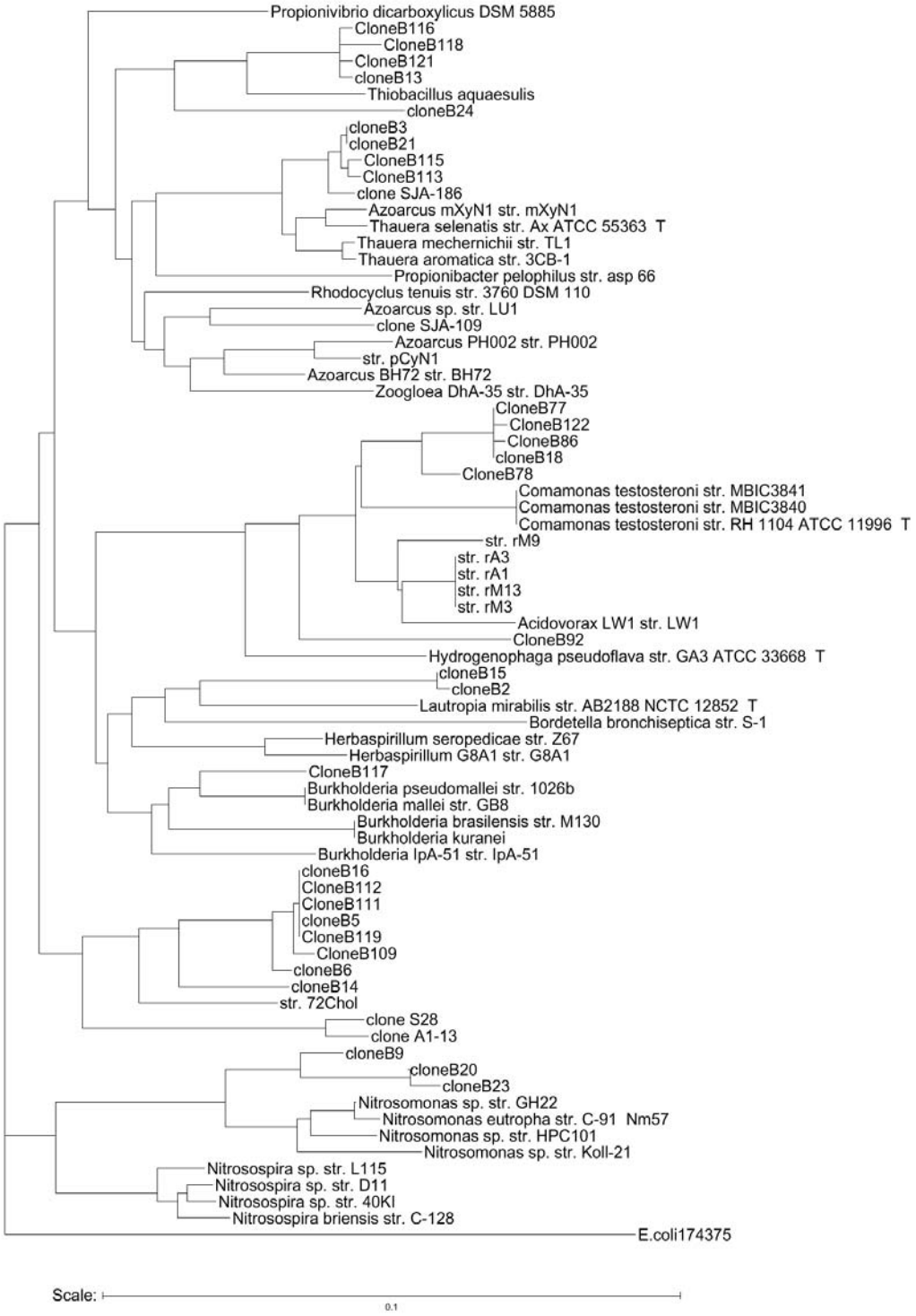

Figure 3-12. Clones related to *Burkholderia*, *Lautropia mirabilis*, and Strain 72Chol.

Clones B16, B112, B111, B5, B119, B109, B6 and B14 are most closely related to Strain 72Chol (Figure 3-12), denitrifying bacterium that grows on cholesterol with oxygen or NO_3^- as electron acceptor (Harder and Probain, 1997). This strain reduces NO_3^- to NO_2^- during anaerobic growth, and at low NO_3^- concentrations, NO_2^- was further reduced to nitrogen gas. Ammonia can be assimilated by this strain. Again, although this cluster of clones is most closely related to this strain, they are not the same species or necessarily the same genera.

Clones B82, B115, B95, B113, B120, B96, B3 and B21 are most closely related to *Thauera* and *Azoarcus* (Figure 3-13). Some members of *Thauera* are heterotrophic nitrifier/aerobic denitrifier such as *Thauera mechernichensis* while others are anaerobic denitrifiers like *T. aromatica* and *T. selenatis* (Lukow and Diekmann, 1997; Scholten et al., 1999).

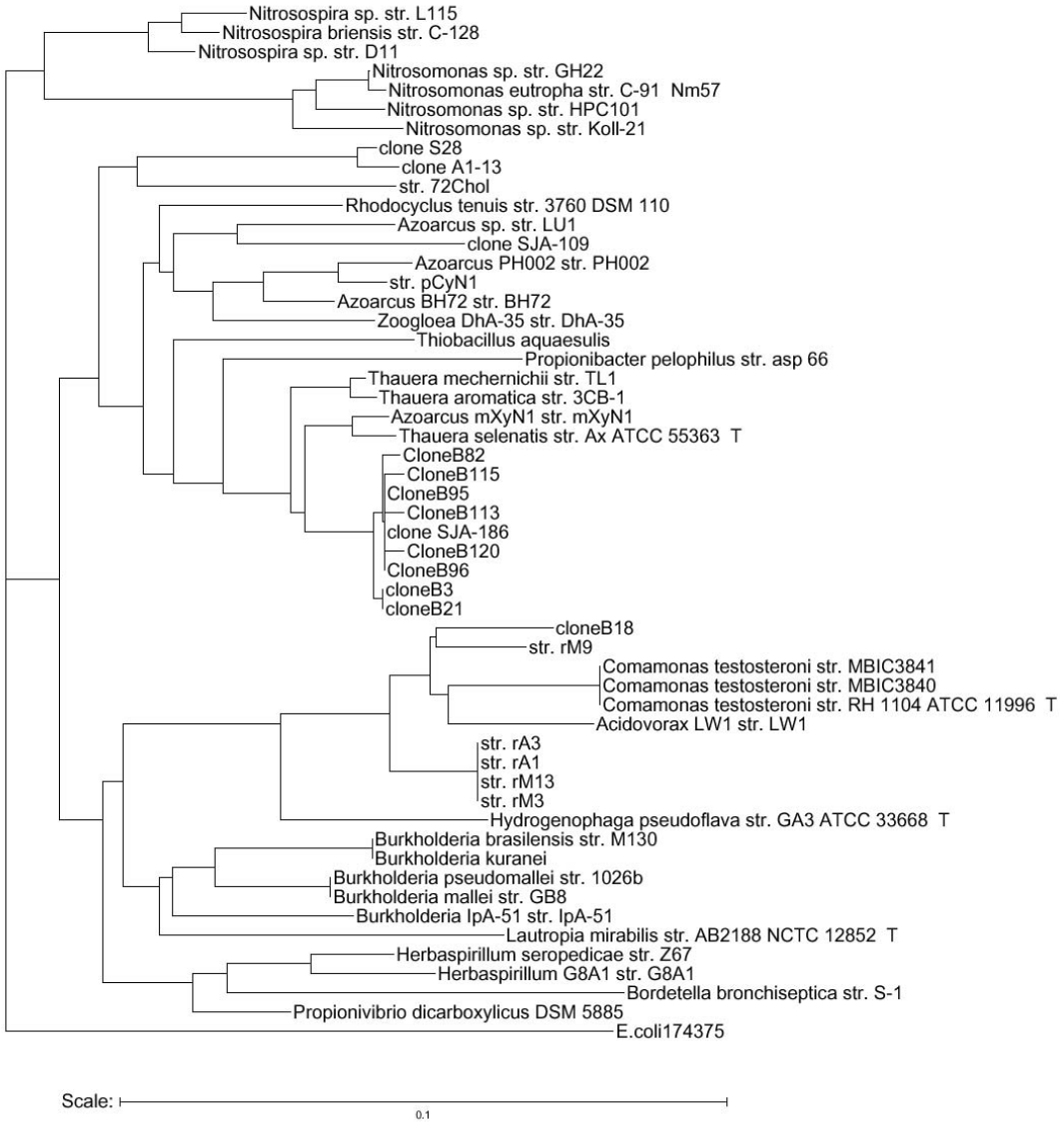

Figure 3-13. Groups related to *Thauera* and *Azoarcus*.

3.3.4 Phylogenetic Analysis of Hoffland and Farm 10 Clones

Hoffland and Farm 10 clones showed much of the same groupings as Battelle clones but with one important distinction. No Hoffland or Farm 10 clones appear to be ammonia-oxidizing bacteria or nitrifiers. They all however, appear to be β-Proteobacterial denitrifiers that are closely related to one another in clusters, but are not as closely related to known species. Most of the Farm 10 clones appear to be related to Strain 72Chol with the exception of Clone F12 and F16, which appear to be more closely related to *Thauera* species and clone SJA-186, respectively (Figure 3-15). Clone SJA-186 comes from a fluidized bed reactor (FBR) inoculated with a trichlorobenzene-transforming consortium (von Wintzingerode et al., 1998). Universal primers for the domain *Bacteria* were used to amplify DNA from the FBR. Clone SJA-186 was most closely related to *Thauera aromatica*.

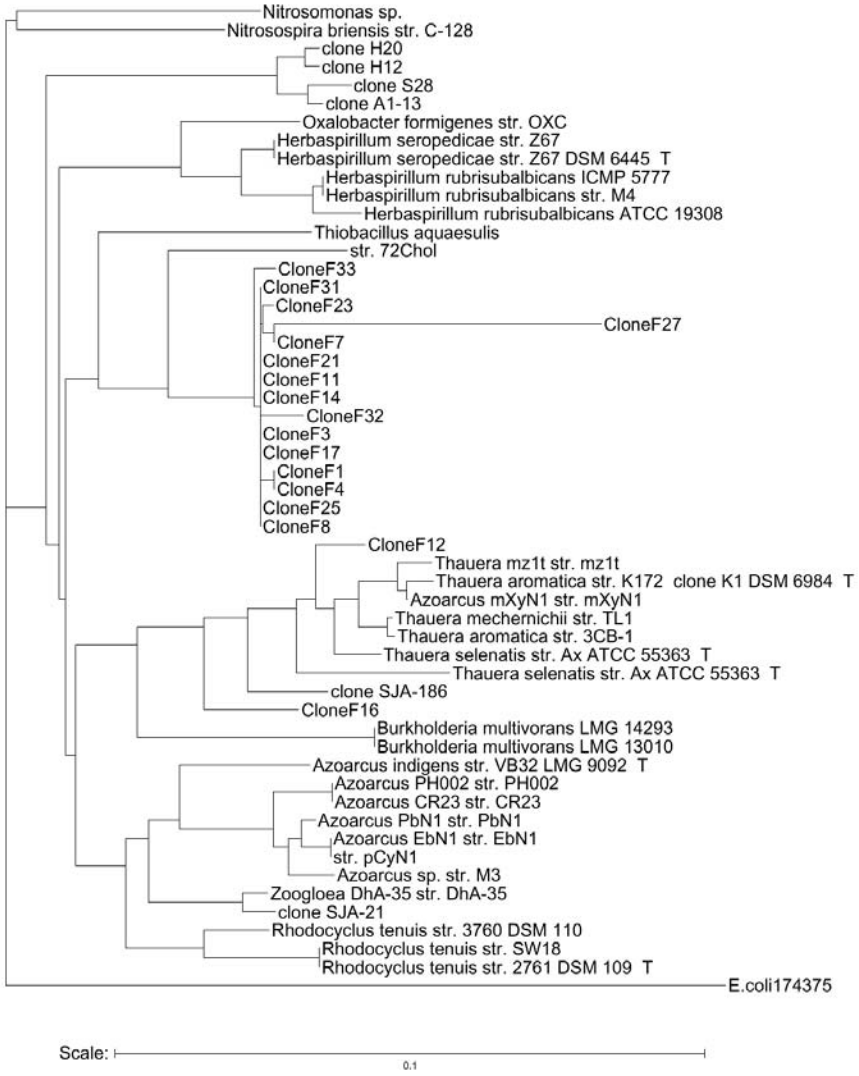

Figure 3-14. Clones from Farm 10 phylogenetic tree.

Hoffland clones appear to be very closely related to SJA-186 with the exception of clone H5 which appears to be somewhat more closely related to *Lautropia mirabilis*, and clone H19 which is more closely related to *Comamonas* strain D22 (Figure 3-16).

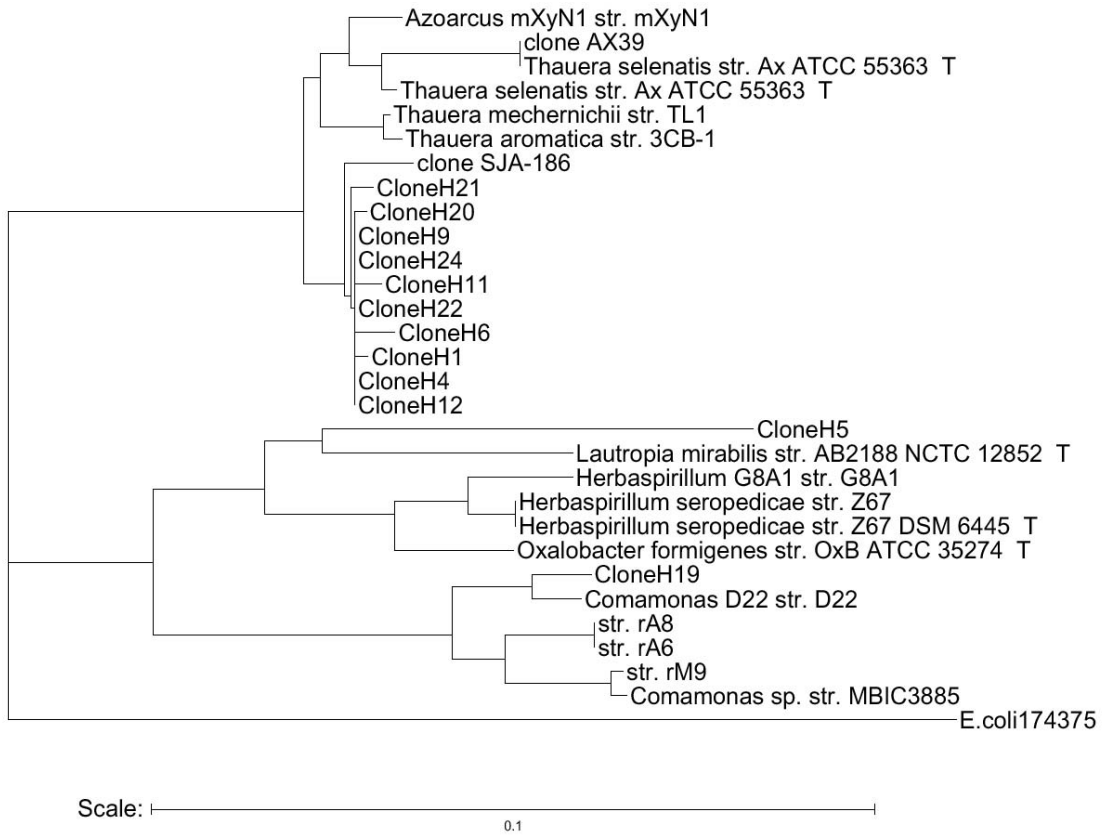

Figure 3-15. Phylogenetic tree of Hoffland clones.

Both the Hoffland and Farm 10 Clones are grouped in tight clusters and are not very closely related to other known sequences or species in the Ribosomal Database Project. Interestingly, when clones from Hoffland, Battelle and Farm 10 (even though this is not as phylogenetically accurate because of the low sequence homology between all groups) are grouped on the same tree together, clones from different systems assemble into the same clusters of clones, even though they come from diverse systems (Figure 3-17). For example, the Hoffland and Battelle clones that are closely related to *Thauera* are also closely related to one another. The Battelle and Farm 10 clones that are closely related to Strain 72Chol are also closely related to one another.

Figure 3-16. Phylogenetic tree of Battelle, Farm 10 and Hoffland Clones.

3.4 Environmental Parameters and Phylogenetic Analysis

There is no evidence of ammonia-oxidizing bacteria at the Hoffland and Farm 10 sites, however evidence exists for the presence of AOB at the Battelle site. The lack of evidence at the Hoffland and Farm 10 sites does not exclude the possibility of their existence at these sites, however, it does point to some shortcomings of the methods used in this research and to future research that should be conducted. Due to the lack of specificity of the primers, most β-Proteobacteria that were found were not ammonia-oxidizers. In many systems where both nitrification and denitrification occurs, denitrifiers frequently greatly outnumber nitrifiers (Bodelier et al., 1996). If this was the case in these field sites, then nitrifiers may have been unable to be detected due to the overwhelming numbers of β-Proteobacterial denitrifiers. It is possible that at the Battelle site the percentage of AOBs (in relation to total β-Proteobacteria) were higher than at the other sites, making them detectable by cloning. Additionally it is possible that at low DO levels AOB may be able to out-compete nitrite-oxidizers due to their higher affinity for oxygen as Bernet et al. (2001) suggest, although further research is needed in order to verify this theory.

Due to the lack of evidence of AOB in the Farm 10 and Hoffland field sites, it is impossible to say whether their presence or absence can be correlated to environmental conditions. The only thing we can say with certainty about the environmental conditions was that on the sampling date the Battelle site clearly had by far the lowest levels of TAN and by far the highest levels of nitrite-N and nitrate-N. The high levels of nitrite-N (as compared to nitrate-N) at Battelle are interesting. The presence of heterotrophic nitrifiers would explain the disparity between nitrite-N and nitrate-N levels. It is possible that heterotrophic nitrifiers are present, like *Thauera merchernichii* strain TL1 (Lukow and Diekmann, 1997) as clones were present that were similar to these strains. However, much more research would be

needed on this topic in order to confirm the presence of heterotrophic nitrifiers in these treatment systems.

Future research could clarify the relationship between the environmental conditions and the bacterial populations that live there. Using the sequences that were obtained from the clones that belong to nitrifying bacteria, new primers can be created to more specifically search for those bacteria. Enrichment cultures should be created from samples taken from the field sites in order to select and isolate these species. Using these new, more specific primers, enrichment cultures can be tested with these probes by fluorescent in situ hybridization (FISH). Once these primer sets are determined to work, environmental samples could be tested with FISH to qualitatively analyze the samples. If this is successful, quantitative analysis of AOB populations over time could be correlated with it to determine a relationship between environmental conditions and quantity and species of AOB.

4 Conclusions

In order to obtain supporting evidence for biological denitrification in “anaerobic” lagoons degenerate β-Proteobacterial AOB primers were used to amplify DNA. Clone libraries were then created and sequenced to look for the presence of ammonia-oxidizing bacteria. Because AOB live by aerobically converting ammonia to nitrite, identification of AOB in waste treatment systems would help to support claims that nitrification can occur in these systems.

Although there were PCR products from almost all samples, the clone libraries that were created show that not all PCR reactions produce only PCR products from ammonia-oxidizing bacteria. It appears from cloning that the majority of PCR products produced were not from AOB. Therefore using primers Nso190F and Nso1225R cannot be used as a diagnostic tool alone to test for the presence of AOB. However, these primers did verify the presence of ammonia-oxidizers in the Battelle site, although their presence was not verified at the Hoffland and Farm 10 sites. The presence of ammonia-oxidizers at the Battelle site implies that aerobic ammonia-oxidation is occurring. Non-specific binding appeared to be a problem with the primer pair, and most clones that were sequenced were more closely related to denitrifying β-Proteobacteria.

Clones were created and sequenced that were significantly different from other known sequences and tended to form very closely related phylogenetic groups. These phylogenetic groups were not isolated to one field site, and often more than one site had representatives in a closely related group.

Future research in this field includes the design of new primer sets based on the sequences of the nitrifying bacteria clones reported in this research. Enrichment culture studies will also be important to isolate and better understand these new species. Fluorescent in situ hybridization of enrichment cultures and environmental samples will

verify their presence and aid in their quantification. The use of FISH over time will show how community population structure is correlated with environmental data, and how the two are related.

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