ABSTRACT

MOTA, CESAR. Microbial ecology of intermittently aerated reactors treating swine wastewater: molecular approaches for identifying key nitrogen-removing bacteria. (Under the direction of Dr. Francis L. de los Reyes III).

Swine farms produce large amounts of manure/wastewater that need to be treated before final disposal. Swine wastewater has traditionally been treated using anaerobic lagoons that do not remove nutrients effectively, and the treated effluent that is sprayed on cropland usually has high contents of nitrogen and phosphorus. Excess land application of animal waste can cause contamination of ground and surface waters, and may lead to eutrophication, ultimately resulting in depletion of dissolved oxygen, fish kills, and changes in water color, odor, and taste, making water unsuitable for consumption. Therefore, there is currently great demand for more effective swine wastewater treatment technologies.

One promising alternative for treatment of livestock waste consists of anaerobic digestion followed by intermittent-aeration for removal of nitrogen through nitrification and denitrification. As nitrification and denitrification are biological processes, better understanding of the ecology of the microorganisms involved and their response to key operating conditions may ultimately lead to improved performance of nitrogen removal systems.

The objective of this study was to investigate the microbial ecology of nitrogen-removing bioreactors treating swine wastewater and the effects of key operating conditions on nitrogen removal efficiency. Laboratory-scale intermittently-aerated reactors were fed anaerobically digested swine wastewater with ammonia concentrations up to 175 mg NH_3-N/L and

operated with different aeration to non-aeration (ANA) time ratios. Changes in the fractions of ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) were monitored using 16S rRNA- and amoA-based molecular approaches. *Nitrosomonas/Nitrosococcus mobilis* were the dominant AOB and *Nitrospira* were the dominant NOB in all reactors. *Nitrosomonas* sp. Nm107 was detected in all reactors, regardless of the reactor's performance. Close relatives of *Nitrosomonas europaea, Nitrosomonas* sp. ENI-11, and *Nitrosospira multiformis* were occasionally detected in all reactors. NOB were more sensitive than AOB to long anoxic periods, resulting in nitrite accumulation and lower total NOB rRNA levels. Anoxic periods of 4 h resulted in partial nitrification, followed by denitrification via nitrite, suggesting that efficient nitrogen removal can be achieved at lower operational costs due to savings related to lower oxygen and organic matter system requirements.

The difficulty in identifying active denitrifying bacteria motivated the conception of an innovative approach for identifying bacteria based on functional genes and subsequent sorting of labeled cells for proper phylogenetic identification through the use of 16S rRNA fingerprinting techniques. mRNA fluorescent in situ hybridization (FISH) was performed using tyramide signal amplification (TSA) and horse radish peroxidase (HRP)-labeled oligonucleotide probes targeting transcripts of *nirS*, the gene that codes cytochrome-containing nitrite reductase, an important enzyme that catalyzes the reduction of nitrite to nitric oxide. For the first time, the simultaneous *in situ* detection of all three groups of bacteria involved in nitrogen removal from wastewater (denitrifying, ammonia-oxidizing, and nitrite-oxidizing bacteria) was possible using the method developed in this research. Our

results revealed close spatial relations among all three groups of bacteria targeted. A number of bacterial colonies hybridized with both *nirS* mRNA and the 16S RNA of ammonia oxidizing bacteria, suggesting that members of AOB might possess and express *nirS* genes in addition to the already known *nirK* genes present in some AOB such as *Nitrosomonas europaea*.

Labeled nitrite reducers were sorted from the background microbial community using flow cytometry (FCM) for subsequent phylogenetic analysis based on 16S rRNA genes. Results suggest that the dominant *in situ* nitrite reducers were closely related to *Acidovorax* BSB421. The molecular approach developed in this research has great potential to unravel the longstanding question of which environmental processes are attributed to which microorganisms in natural and engineered habitats.

MICROBIAL ECOLOGY OF INTERMITTENTLY AERATED REACTORS TREATING SWINE WASTEWATER: MOLECULAR APPROACHES FOR IDENTIFYING KEY NITROGEN-REMOVING BACTERIA

by

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DEDICATION

I dedicate this work to Gisela, for her unconditional love, patience and encouragement.

BIOGRAPHY

Cesar Mota received his Bachelor's degree in Civil Engineering from the Federal University of Ceará, Brazil in 1998, and worked for a consulting company in Ceará on the design and implementation of water supply and wastewater treatment projects. Cesar joined the Environmental Engineering program at the University of Nevada Las Vegas, where he finished his Master of Science degree in 2001. He joined Dr. Francis de los Reyes' research group at North Carolina State University in 2002 to work on his doctoral degree studying the microbial ecology of nitrifying and denitrifying bacteria in intermittently-aerated reactors.

Cesar Mota's research interests encompass projects for the development of innovative water quality engineering processes. Specific areas of research include identification of key microorganisms in biodegradation systems based on their genetic characteristics and catabolic activity, determination of critical operating conditions affecting microbial growth and activity for optimization of biodegradation systems, and the use of cell-free enzymes embedded in membrane systems for waste treatment.

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INTRODUCTION

The swine industry in the state of North Carolina has grown remarkably in the last 20 years, and the state is now the second-ranked swine producer in the United States, behind Iowa. There are currently more hogs than people in the state of North Carolina (Economist, 1997). Swine farms produce large amounts of manure/wastewater that need to be treated before final disposal. Swine wastewater has traditionally been treated using anaerobic lagoons for removal of its high content of organic compounds and nutrients (nitrogen-N and phosphorus-P). Treated effluent from anaerobic lagoons is finally disposed of on spray fields. Anaerobic lagoons do not remove nutrients effectively and the treated effluent that is sprayed on crops usually has high contents of nitrogen and phosphorus. Excess application of animal waste to field sites can result in contamination of ground and surface waters. Nutrient enrichment of nutrient-sensitive ecosystems leads to eutrophication, resulting in dissolved oxygen depletion, fish kills, and changes in water color, odor, and taste, making water unsuitable for consumption. Therefore, there is great demand for more effective swine wastewater treatment technologies.

The most common method of nitrogen removal from wastewater combines nitrification, the aerobic oxidation of ammonia to nitrite and nitrate, and denitrification, the anoxic reduction of nitrate or nitrite to dinitrogen gas. Both processes are mediated by specific groups of microorganisms. During nitrification, ammonia is oxidized to nitrite by ammonia oxidizing bacteria (AOB), and nitrite is oxidized to nitrate by nitrite oxidizing bacteria (NOB). Denitrification is mostly catalyzed by a diverse group of heterotrophic bacteria. Nitrifying

bacteria (AOB and NOB) are slow growers and their number and physiological activity are considered the rate-limiting parameters for the bioconversion of nitrogen in biological wastewater treatment.

One promising alternative for treatment of livestock waste consists of anaerobic digestion for removal of most of the organic content, followed by intermittent-aeration (cyclic aeration/non aeration periods) for removal of nitrogen (Cheng and Liu 1996). Intermittently aerated (IA) reactors can provide appropriate environmental conditions to support growth of microorganisms carrying out nitrification and denitrification in a single reactor. The aeration to non-aeration (ANA) time ratio is a critical parameter in the operation of IA reactors, as it has a significant impact on the efficiency and total cost of the system. Ideally, IA reactors should be operated using the lowest ANA time ratio possible (longer non-aerated periods and short aerated periods), resulting in enhanced denitrification without compromising nitrification. IA reactors can potentially be optimized if used to perform partial nitrification, the oxidation of ammonia to nitrite, followed by denitrification via nitrite, resulting in reduced oxygen and organic substrate demands for ammonia removal and denitrification, respectively. Partial nitrification can be achieved by selecting AOB over NOB. Low dissolved oxygen has been identified as a strong selecting factor of AOB over NOB, and IA reactors with long anoxic periods (4 hrs) have been shown to effectively accumulate nitrite (Mota et al. 2005).

As nitrification and denitrification are biological processes, better understanding of the ecology of microorganisms involved and their response to key operating conditions may

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ultimately lead to improved performance of nitrogen removal systems. The relatively novel use of molecular techniques to help solve environmental engineering problems is a powerful approach and has allowed studies of bacterial community populations in response to varying environmental conditions. Most investigations have been based on a phylogenetic marker such as 16S rRNA, which provides information on diversity and in some cases on functional genes. The use of molecular methods allows the identification of microorganisms without the requirement of pure culture isolation. This constitutes significant progress for microbial ecology studies as it has been estimated that only a minor proportion (about 1%) of naturally occurring microorganisms have been isolated in pure culture experiments.

During the first phase of this research, we operated IA reactors treating swine wastewater and applied molecular methods based on 16S rRNA and amoA genes to determine the effects of ANA time ratios on the fraction of nitrifiers in the biomass, on the diversity of nitrifying populations, and on nitrogen removal performance. The findings from our experiments are described in two papers: (i) Paper 1 has been published in FEMS Microbiology Ecology and is shown in Chapter 1 of this dissertation; (ii) Paper 2 has been published in Applied and Environmental Microbiology and is shown in Chapter 2.

Investigations based on 16S rRNA sequences, including those described in Chapters 1 and 2, have revealed valuable information about the diversity and biomass fractions of microorganisms involved in biodegradation systems. However, 16S rRNA sequences generally provide limited information regarding the interactions and metabolic capabilities of

the microorganisms that these sequences represent. Therefore, the following pertinent question remains largely unanswered:

- *Which functions are attributable to which organisms in natural and engineered environments?*

Reaching the answer to that question is essential in biodegradation investigations. To better explain the importance of solving that long standing microbial ecology conundrum, a few papers have recently used analogies comparing biodegradation investigations to clinical investigations that are based on the well-known Koch's postulates to determine causality (Madsen 2006). Since criminal investigations are usually as popular as clinical investigations, based on the currently highest rated shows on American television, and to aim at originality I hereby propose an analogy between criminal investigations and biodegradation investigations (Table 1).

Denitrifying bacteria are a challenging group of microorganisms to study as they are highly diverse, phylogenetically unrelated, can grow on several different substrates and under various environmental conditions. The ability to denitrify is believed to have been acquired through gene transfer. Therefore, it is not possible to use molecular approaches based on the 16S rRNA gene to identify active denitrifying bacteria in environmental samples.

Chapters 3 and 4 describe an innovative approach developed in the course of the current research to identify metabolically active microorganisms in mixed cultures based on intracellular mRNA content of functional genes followed by proper phylogenetic identification based on 16S rRNA genes. The method developed during this research was

applied to identify dominant denitrifying bacteria in activated sludge collected from a denitrifying tank at a full-scale wastewater treatment plant. For the first time, the simultaneous *in situ* detection of all three groups of bacteria involved in nitrogen removal from wastewater (denitrifying, ammonia-oxidizing, and nitrite-oxidizing bacteria) was possible using the developed method. Chapters 3 and 4 are being prepared as two separate papers for submission to scientific journals.

Table 1. Analogy between criminal and biodegradation investigations.

Elements of an investigation	Criminal Investigation	Biodegradation Investigation
What would start an investigation	A missing person or a body	A pollutant has been removed from the waste during biological treatment
Pertinent question	Who is guilty of the crime?	What microorganisms are responsible for the degradation of a specific pollutant during waste treatment?
Suspects	Individuals present in the crime scene	Live microorganisms in the biomass
Eyewitnesses	People present in the crime scene that were not involved in the crime. People that saw or heard anything related to the crime and therefore can be helpful solving the crime	Microorganisms in the biomass that are not involved in the biodegradation process of interest. Not of much help as they cannot say anything. In fact, their presence in the biomass is what makes biodegradation investigations even more challenging
Motive	War, revenge, money-related, etc. Humans can kill for mundane reasons	Survival. In order to survive microorganisms need to respire. During microbial respiration pollutants are transformed into harmless compounds
Weapon	Fire gun, knife, poison, etc.	Genes that code enzymes necessary for the degradation of pollutants
Evidence of weapon use	The fact that a suspect was carrying a gun during a crime does not necessarily mean that he/she is guilty, as the gun might not have been used by him/her	The fact that a microorganism has a gene does not necessarily mean that this gene was actively producing the enzyme necessary for catalyzing the biodegradation process of interest.
How to prove that a weapon was used	Perform gunpowder residue tests on the suspects	Perform gene expression analysis using mRNA assays and/or stable isotope enrichment of nucleic acids
Consequence of a guilty verdict	Justice has been made when guilty assassins go to jail	Justice should be made, and microorganisms responsible for promoting the degradation of pollutants should be rewarded long-lasting life in biodegradation systems. For that, assays of inhibition studies, in situ activity rates, to name a few, should be performed

Concluding remarks and suggestions for future work are combined in the final section of this dissertation.

Background

Animal agriculture in the United States is advancing at unprecedented rates (Sharpley et al., 1998). Production of hogs and pigs, broilers, turkeys, and to a lesser extent cattle have increased steadily since 1980. This production generally is occurring in more localized areas and on fewer, larger farms (Sharpley et al., 1998).

Swine farms produce large amounts of manure/wastewater which have traditionally been treated using anaerobic lagoons for removal of its high content of organic compounds and, to certain extent, nutrients (nitrogen-N and phosphorus-P). Treated effluent from anaerobic lagoons is finally disposed of on land for crop irrigation (spray fields). To reduce the potential for environmental degradation due to excessive residual soil levels of nutrients derived from swine lagoon effluents, application rates must not exceed the plant and soil buffering capacities on spray fields (Stone et al., 1998). However, production of waste from swine farms is often greater than nutrient demand by local crops. Barker and Zublena (1995) reported that several counties in North Carolina produced more nitrogen in plant-available nutrients from animal manure than needed by non-legume agronomic and forage crops. Suitable application rates for animal waste are calculated based upon anticipated crop nutrient uptake (Zublena et al., 1993). These criteria determine application rates and area needed to prevent loss of excess nutrients from the soil profile. When nutrients are applied in

excess of the crop's ability to use in a harvestable product, they may be lost to the environment.

Declines in soil, water, and air quality in many of the nation's watersheds have been linked to intensification of animal production (Aschmann et al., 1999). In these watersheds, it is difficult to fully utilize the quantity of nutrients produced, and excess application of animal waste to field sites can result in contamination of shallow groundwater. In the coastal plain region of North Carolina, non-point source pollution from agriculture has been identified as a significant problem (NC Division of Water Quality, 1996; Jacobs and Gilliam, 1985). Runoff from fields with high nutrient content can increase aquatic nutrient concentrations. Problems related to nutrient leaching to groundwater are exacerbated by high rainfall, sandy textures, and low soil organic matter levels. Nitrate contamination in groundwater is of particular concern for both health and environmental quality as groundwater is the major source of drinking water for more than 90% of rural households and 75% of cities in the USA (Goodrich et al., 1991). Nutrient enrichment of nutrient-sensitive ecosystems leads to eutrophication and has been linked to Pfiesteria outbreaks in North Carolina coastal waters (Burkholder et al., 1997). Other negative effects of eutrophication include dissolved oxygen depletion, fish kills, and changes in water color, odor, and taste, making the water unsuitable for consumption.

The United States Environmental Protection Agency (EPA) has passed a rule in mid-December 2002 in the Federal Register recognizing concentrated animal feeding operations (CAFO, animal farms with more than 1000 animal units) as point source discharges. An

estimated 15,500 livestock operations nationwide will be required to apply for a clean water permit under EPA (Christen, 2003). The rule requires CAFOs to develop comprehensive nutrient management plans that set limits on how much animal manure can be applied as fertilizer on farm fields. CAFOs also must file annual reports with state permitting authorities on progress in implementing the plan. The new rule also allows the continued use of open-air lagoons for storing manure and sprayfield systems for disposing waste, although North Carolina and other states have banned such systems on new farms because of previous spills and leaks that contaminated ground and surface waters (Christen, 2003).

The environmental impacts from using anaerobic lagoons for partially treating swine wastewater are not limited only to leaching of pollutants to water bodies due to excessive nutrient application on spray fields. Raw swine wastewater and highly concentrated sludge accumulated in lagoons can leach through cracks in lagoon liners directly into the environment. In addition, major wastewater spills that can occur as a result of storms and/or hurricanes present a real threat as these storms are frequent in North Carolina. Therefore, there is great need for developing and testing more effective technologies for handling swine wastewater as substitutes for the inexpensive, but risky and inefficient anaerobic lagoons that are currently used.

REFERENCES

Aschmann, S.G., Anderson. D.P., Croft, R.J., Cassel, E.A. (1999) Using a watershed nutrient dynamics model, WEND, to address watershed-scale nutrient management challenges. Journal of Soil and Water Conservation, vol. 54, no4, p630.

Barker, J.C., Zublena, J.P. (1995) Livestock manure nutrient assessment in North Carolina. In Proc. Of the 7th Int. Symp., on Agricultural and Food Processing Wastes, Chicago, ASAE, p98.

Burkholder, J.M., Mallin, M.A., Glasgow, H.B., Larsen, L.M., McIver, M.R., Hannon, E.K. (1997) Impacts to a coastal river and estuary from rupture of a large swine waste holding lagoon. Journal of Environmental Quality, vol. 26, p1451.

Cheng, J. and B. Liu. (2001) Nitrification/denitrification in intermittent aeration process for swine wastewater treatment. J. Environ. Eng. 127: 705-711.

Christen, K. (2003) Will new manure rule improve water quality? Water Environment and Technology, vol. 15, no3, p11.

Economist, (1997) Hog's halt. vol. 344, issue 8035, p30.

Goodrich, J.A., Lykins, B.W., Clark, R.M. (1991) Drinking water from agriculturally contaminated groundwater. Journal of Environmental Quality, vol. 20, no4, p707.

Jacobs, T.C., and Gilliam, J.W. (1985) Headwater stream losses of nitrogen from two coastal plain watersheds. Journal of Environmental Quality, vol. 14, p447.

Madsen, E. (2006) Identifying microorganisms responsible for ecologically significant biogeochemical processes. Nature Reviews, vol. 3, p439.

Mota, CR., Head, M., Ridenoure, J., Cheng, J., and de los Reyes III, FL. (2005) Effects of aeration cycles on nitrifying bacterial populations and nitrogen removal in intermittently-aerated reactors, Appl. Environ. Microbiol., 71, pp. 8565-8572.

NC Division of Water Quality. (1996) Neuse river nutrient sensitive waters management strategy. Raleigh, NC.

Sharpley, A., Daniel, T.C., Sims, J.T., Pote, D.H. (1998) Determining environmentally sound phosphorus levels. Journal of Soil and Water Conservation, vol. 51, no2, p160.

Stone, K.C., Hunt, P.G., Johnson, M.H., Humenik, F.J. (1998) Impact of swine waste application on ground and stream water quality in an eastern coastal plain watershed. Transactions of the ASAE, vol. 41, no6, p1665.

Zublena, J.P., Barker, J.C., Parker, J.W., and Stanislaw, C.M. (1993) Swine manure as a fertilizer source. North Carolina Cooperative Extension Service. Publication Number: AG-439-4. Raleigh, NC: North Carolina State University.

Chapter 1.

HIGH LEVELS OF NITRIFYING BACTERIA IN INTERMITTENTLY-AERATED REACTORS TREATING HIGH AMMONIA WASTEWATER

ABSTRACT

Changes in the fractions of ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) in two laboratory-scale reactors were investigated using 16S rRNA probe hybridizations. The reactors were operated in intermittent aeration mode and different aeration cycles to treat anaerobically digested swine wastewater with ammonia concentrations up to 175 mg NH_3-N/L. High ammonia removals (>98.8%) were achieved even with increased nitrogen loads and lower aeration: non-aeration (ANA) time ratios of 1 hr: 3 hrs. *Nitrosomonas/Nitrosococcus mobilis* were the dominant AOB in the reactors. *Nitrospira*-like organisms were the dominant NOB during most of the investigation, but were occasionally outcompeted by *Nitrobacter*. High levels of nitrifiers were measured in the biomass of both reactors and AOB and NOB levels adjusted to changing ANA time ratios. The low C/N ratio and high ammonia in the influent are likely the most important factors contributing to the high fraction of nitrifiers and the stability of the nitrification process. Stable ammonia removals and no nitrite accumulation were achieved even when rRNA levels of AOB and NOB reached a minimum of 7.2% and 8.6% of total rRNA, respectively. Complete nitrification was achieved even with an ANA ratio of 1 hr: 3 hr and higher nitrogen

loads, indicating that stable performance at lower aeration requirements can be achieved. This suggests the possibility of significant savings in operational costs.

INTRODUCTION

The most common method of nitrogen removal from wastewater combines nitrification (the aerobic oxidation of ammonia to nitrite by ammonia oxidizing bacteria [AOB], followed by oxidation of nitrite to nitrate by nitrite oxidizing bacteria [NOB]), and anoxic denitrification (the reduction of nitrate or nitrite to N_2, mostly catalyzed by heterotrophic bacteria). AOB and NOB are slow growers and influenced by many environmental factors such as pH, temperature, C/N ratio, unsaturated fatty acids, dissolved oxygen, ammonia, and nitrite concentrations [1-6]. The number and physiological activity of nitrifying bacteria in wastewater treatment reactors are considered the rate-limiting parameters for the bioconversion of nitrogen in domestic wastewater [7]. In addition, the fraction of nitrifying bacteria in the biomass is important in nitrogen removal systems, since nitrifiers are considered poor competitors for oxygen compared to heterotrophs [8]. Nitrifying systems have traditionally been monitored using chemical parameters rather than microbiological data. However, the monitoring of key microorganisms by molecular methods might permit better prediction and control of process performance and efficiency [9].

Analysis of 16S rRNA gene sequences provides evidence that AOB form two monophyletic groups, one within the β- and one within the γ-*Proteobacteria*. The β-AOB comprise the genera *Nitrosomonas* and *Nitrosospira*. All members of the *Nitrosospira* cluster are very closely related to each other, whereas the *Nitrosomonas* cluster reveals six distinct lineages of descent [10]. The γ-ammonia oxidizers include the genera *Nitrosococcus*, with the

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exception of *Nitrosococcus mobilis*, which is closely related to *Nitrosomonas* and hence belong to the group of β-ammonia oxidizers. NOB form four phylogenetically distinct groups (*Nitrobacter, Nitrococcus, Nitrospina,* and *Nitrospira*). All isolates of γ-ammonia oxidizers, in addition to the members of the *Nitrococcus* and *Nitrospina* genera are obligate halophilic bacteria [11], and therefore are not expected to be dominant in municipal wastewater treatment systems. Different members of the β-ammonia oxidizers, as well as members of the *Nitrobacter* and *Nitrospira* genera have been found to dominate different wastewater treatment plants or natural ecosystems [12-15].

Biological nitrogen removal from swine wastewater is especially challenging due to the presence of high ammonia concentrations and organic matter in the wastestream, with ammonia concentrations that can be higher than 1100 mg NH_3-N/L [16]. The rapid growth of large-scale, confined animal production and the common use of anaerobic lagoons for storing manure for long periods have led to concerns about ammonia emissions, fish kills, and contaminated ground and surface water [17]. One promising alternative for treatment of livestock waste consists of anaerobic digestion for removal of most of the organic content, followed by intermittent-aeration (cyclic aeration/non aeration periods) for removal of nitrogen. Intermittently aerated (IA) reactors can provide appropriate environmental conditions to support growth of microorganisms carrying out nitrification and denitrification in a single reactor. Cheng *et al*. [16] showed that high nitrogen removal could be achieved in these reactors, with the added benefit of lower alkalinity requirements and reactor construction costs. The aeration to non-aeration (ANA) time ratio is a critical parameter in the operation of IA reactors, as it has a significant effect on the efficiency and total cost of

the system. Ideally, IA reactors should be operated using the lowest ANA time ratio possible (longer non-aerated periods), resulting in enhanced denitrification without compromising nitrification.

In this study, we assessed the effects of different ANA time ratios and ammonia loads on nitrogen removal performance and the populations of nitrifiers. The fraction of nitrifying bacteria (AOB and NOB) was monitored at the genus level using slot-blot hybridization and FISH targeting 16S rRNA.

MATERIALS AND METHODS

Reactor Design and Operation

Two 6-liter Plexiglas reactors (reactors A and B) were operated under intermittent aeration conditions. Both reactors were inoculated with the same sludge from the Neuse River Wastewater Treatment Plant (Raleigh, NC) and were operated under fixed conditions (ANA time ratio of 1:1) for 4 months before this investigation. The reactors treated anaerobically digested swine wastewater from day 1 to day 63, and a 4:1 (by volume) mixture of anaerobically digested (AD) and raw swine wastewater from day 64 to day 78 (acclimation period). Subsequently, the ammonia and COD loads were increased by feeding a 1:1 mixture of AD and raw wastewater from day 79 to day 180 (Figure 1). Wastewater was obtained from a swine lagoon at the NCSU Lake Wheeler Road Field Laboratory. The hydraulic retention time (τ) and target solids retention time (θ_c) were 3 days and 20 days, respectively, and both reactors were operated at room temperature (25^oC). The reactor design allowed biomass to settle in the clarification zone and be recycled to the aeration zone. Air cycling was controlled using a solenoid valve activated by an electronic timer (ChronTrol Corp., San

Diego, Calif.). Compressed air was regulated to 10 psi and airflow was controlled by a gas mass flow controller at 500 mL/min (Cole-Parmer Instrument Co., Vernon Hills, Ill.).

The original experimental design consisted of operating reactor A as a control, using an ANA time ratio of 1:1. However, on day 50 the gas mass flow controller in reactor A failed and aeration had to be controlled manually: turned off during the night and repeatedly turned on during the day. Chemical data from reactor A during the period with manual control (day 50 to day 120) were not collected. Aeration in reactor B was controlled using an initial ANA of 1:2 for 64 days (stage III), and an ANA time ratio of 1:3 during stage IV for 100 days, with increased ammonia loads (Figure 1).

In summary, this investigation can be separated in three stages with distinct purposes (Figure1):

Stage I: reactor A treating relatively high ammonia concentrations (average of 115 mg/L) with ANA time ratio of 1:1.

Stage II: reactor A treating higher ammonia concentrations (average of 146 mg/L) and biomass subject to long non-aerated periods (overnight) from day 80 to 120, followed by an ANA of 1:1 from day 121 to 180.

Stage III: reactor B treating relatively high ammonia concentrations (average of 115 mg/L) and biomass subject to an ANA time ratio of 1:2 from day 1 to 64.

Stage IV: reactor B treating higher ammonia concentrations (average of 146 mg/L) with longer non-aerated periods (ANA of 1:3) from day 81 to 180.

Analytical methods

Influent and effluent samples from each reactor were collected twice a week (completely mixed samples during the aerated period) and analyzed for TKN (total Kjeldahl nitrogen), NH_3-N, NO_3^--N, NO_2^--N, soluble COD (chemical oxygen demand), TOC (total organic carbon), pH, TSS (total suspended solids), and VSS (volatile suspended solids) using Standard Methods [18]. Dissolved oxygen (DO) was measured using a YSI 52 DO meter and a YSI 5739 oxygen probe (YSI Inc., Yellow Springs, Ohio).

Bacterial Cultures

Pure cultures of *Nitrosomonas europaea* (ATCC 25978, ATCC medium 2265), *Nitrosospira multiformis* (ATCC 25196, ATCC medium 929), and *Nitrobacter agilis* (ATCC 25384, ATCC medium 480) were grown aerobically in 0.5-liter flasks at 30^oC. The pH was kept at 8.0 by periodic addition of 20% Na_2CO_3. Cells were harvested by centrifugation at 3200 x g and cell pellets were processed for extraction of RNA.

Nucleic acids extraction

Mixed liquor samples (14 mL) from reactors were centrifuged at 3200 x g for 5 minutes and stored at -80^oC until extraction. RNA was extracted using a modified low-pH hot-phenol extraction procedure [19]. RNA concentrations were measured spectrophotometrically. Samples with low RNA content were not included in the analysis. DNA was extracted as previously described by Burrell *et al*. [20].

PCR, cloning, and in vitro transcription

Since pure cultures of *Nitrospira* were not available, in vitro-transcribed 16S rRNA was used as reference rRNA in membrane hybridizations. DNA was extracted from a reactor sample and amplified using bacterial primers (S-D-Bact-0011-a-S-17 and S-D-Bact-1492-b-a-16).

PCR products were purified using a High Pure PCR Product Purification Kit (Roche, Indianapolis, IN) before amplification with specific primers. A set of PCR primers (Fw-Ntspa-311 and Rv-Ntspa-1463, Table 2) was designed to amplify DNA of microorganisms in the *Nitrospira moscoviensis* subgroup (Table 1). PCR was performed using a thermal cycler (Eppendorf Scientific Inc., Westbury, NY) under the following conditions: 94°C for 5 min, 30 cycles of 92°C for 1.0 min, 61°C for 1.0 min, and 72°C for 1.0 min, and a final extension at 72°C for 7.0 min. PCR product was evaluated using agarose gel electrophoresis. Final PCR product was purified and then cloned using a TA Cloning Kit (Invitrogen, Carlsbad, CA), as previously described [21]. The rRNA was produced by transcribing the selected insert using an AmpliScribe T7 In Vitro-Transcription Kit (Epicentre, Madison, WI), according to the manufacturer's instructions. Transcripts were purified with RNase-free DNase (Ambion Inc., Austin, TX). The in vitro-transcribed RNA had a total length of 1,173 base pairs (GenBank accession number <u>AY741094</u>), with a region perfectly matching probe S-G-Ntspa-0685-a-A-22.

Oligonucleotide probes

Oligonucleotide probes targeting the 16S rRNA of AOB and NOB, as well as universal probes were used in fluorescence in situ hybridizations (FISH) and membrane hybridizations (Table 2). Unlabeled probes were obtained from Sigma-Genosys (The Woodlands, TX) and probes prelabeled with Cy3 were obtained from Integrated DNA Technologies (Coralville, IA).

Slot-Blot and In-Situ Hybridizations

Mixed liquor samples (3 mL) were collected from reactors and fixed with 3 volumes of PFA fixative on ice for 2 hours, as previously described [12]. Fluorescent in situ hybridization

(FISH) was performed as previously described [27] using Cy-3 labeled probes (Table 2). Images were visualized with a Nikon Optiphot epifluorescence microscope (Nikon, Japan). Images were captured with a Sensys charge coupled device (CCD) camera (Photometrics, Newington, VA). To estimate the fraction of nitrifiers in the biomass using FISH, 30 pictures were taken per data point (3 pictures per well, 5 wells per slide for each of the specific probes and for DAPI). The total area of pixels with signal above background was determined using the image analysis software Metamorph (Universal Image Corporation, Downingtown, PA) after manual thresholding. The results were expressed as percentages of the DAPI stained area.

For slot-blot membrane hybridizations, oligonucleotide probes were 5'-end labeled with $[\gamma^{32}$-P]ATP (ICN Radiochemicals, Irvine, California) and T4 polynucleotide kinase (Promega Corp., Madison, Wisconsin) and purified with a Quickspin Oligo column (Roche Molecular Biochemicals, Indianapolis, Indiana). Membranes with immobilized RNA were hybridized as previously described [27] and washed at the appropriate wash temperatures (Table 2). The results were expressed as percentages of the total rRNA as measured with the universal probe.

RESULTS

Performance of Reactors

The average influent ammonia and TKN concentrations for stage I (Figure 1) were 115 mg NH_3-N/L and 166 mg TKN/L, respectively (Table 3). During stages II and IV, nitrogen loads in the influent were increased by mixing anaerobically digested (AD) and raw wastewater, resulting in average influent concentrations of 146 mg NH_3-N/L and 209 mg

TKN/L. These ammonia concentrations are approximately 5 times the mean ammonia concentrations in municipal wastewater. Average influent VSS concentrations increased two-fold, whereas influent TOC/TKN and soluble COD/TKN ratios remained unaffected when the influent changed from anaerobically digested (stages I and III) to a mix of anaerobically digested and raw swine wastewater (stages II and IV).

Table 4 shows mean effluent concentrations and removal efficiencies for reactor A during stage I and the period in stage II when aeration was controlled at 1:1 and the reactor was receiving higher nitrogen loads. For both periods, average ammonia and TKN removal efficiencies were higher than 98% and 89%, respectively; and effluent nitrite concentrations were negligible, indicating practically complete nitrification for both stages. Similar results were observed for reactor B (Table 5) using ANA ratios of 1:2 and 1:3 (stages III and IV). Average ammonia and TKN removal efficiencies were higher than 98% and 90%, respectively. Nitrite accumulation was also negligible for both stages. Considerably lower effluent nitrate concentrations were observed for stage II (reactor A) and stage IV (reactor B), both when the influent had higher nitrogen and VSS contents. For all stages, removal of soluble COD was not substantial (average of 32 %), whereas average TOC removal efficiencies exceeded 57%, suggesting that degradable compounds from the conversion of particulate organic carbon were the source of energy for the denitrifying heterotrophic biomass.

Fraction of Nitrifiers in the Biomass

The fraction of nitrifiers in the biomass on different days was monitored using membrane hybridizations and the average values and respective standard deviations during each period

are shown in Figures 2 and 3 (results expressed as % 16S rRNA). Additionally, a sample from each period was randomly selected and the fraction of nitrifiers in the biomass was estimated using FISH (results expressed as % DAPI area). Both membrane hybridization and FISH results for the selected days are also shown in Figures 2 and 3.

In reactor A, total AOB in the β-Proteobacteria group (measured with probe Nso1225) averaged 18% of total rRNA in the biomass during stage I. *Nitrosomonas/Nitrosococcus mobilis* (measured with probe Nsm156) were the dominant AOB with an average of 9.5% of total 16S rRNA, and *Nitrosospira* (measured with probe Nsv443) were only occasionally detected in low levels, averaging 1.4% of total 16S rRNA. *Nitrospira* was the dominant NOB, with an average of 15% 16S rRNA. *Nitrobacter* was also detected in considerable levels, averaging 7% of 16S rRNA. High standard deviations indicate significant variability in the fraction of nitrifiers in reactor A for that period. During the initial 40 days of stage II, aeration was controlled manually: turned off during the night and repeatedly turned on during the day. Although the biomass was exposed to long periods (overnight) without aeration, fractions of nitrifiers remained high, as indicated by an average of 22% for total β-AOB 16S rRNA and averages of 16% and 8% for *Nitrospira* and *Nitrobacter*, respectively. Changing the aeration to an ANA time ratio of 1:1 seemed to have a positive effect on the fraction of AOB, as suggested by an average of 28% of β-AOB rRNA, with *Nitrosomonas* still as the dominant β-AOB. *Nitrosospira* levels remained low, with an average of 3% of 16S rRNA. Levels of *Nitrospira* decreased to an average of 7%, and *Nitrobacter* levels increased to an average of 11% of 16S rRNA, indicating a shift in the NOB population.

Longer non-aeration periods (stage IV) seemed to have slightly impacted the fraction of nitrifiers in reactor B (Figure 3). Total fraction of β-AOB decreased from an average of 28% to an average of 22% for ANA time ratios of 1:2 to 1:3, respectively. Fractions of NOB did not appear to be affected, as levels of *Nitrospira* averaged 11% and 8% and *Nitrobacter* levels averaged 3% and 5% for ANA time ratios of 1:1 and 1:3, respectively.

The combined signal of *Nitrosomonas* and *Nitrosospira* remained within one standard deviation from the signal of total β-AOB for most of the samples. However, there were exceptions, suggesting that there might be AOB in the biomass that hybridize with probe Nso1225, but not with either probe Nsm156 or probe Nsv443.

The lowest AOB and NOB levels measured were 7.2% of total rRNA and 8.6% of total rRNA (6.1% as *Nitrospira*, and 2.5% as *Nitrobacter*), respectively. These fractions were sufficient to allow stable nitrification performance, as there was no significant ammonia or nitrite accumulation during the entire investigation.

Levels of rRNA from membrane hybridizations and area percentages from FISH remained within one standard deviation for most of the samples, except for β-AOB data points for stage III.

DISCUSSION

The average levels of nitrifying bacteria measured in the IA reactors using membrane hybridizations ranged from 18 to 30 % for β-AOB; 6 to 15% for *Nitrospira*; and 5 to 12% for *Nitrobacter*. These levels of nitrifiers are generally higher than most fractions reported.

Previous studies in bench and full-scale activated sludge systems using membrane and in-situ hybridizations reported AOB and NOB fractions in the ranges of 5 to 20% and 1.5 to 12 %, respectively [6-8, 28-30]. Although FISH and membrane hybridization measure biomass fractions in different ways, both methods yielded comparable measurements of the fraction of nitrifiers for most samples analyzed. Stoichiometric calculations based on operating parameters (COD, TOC, and TKN removed) and previously determined kinetic parameters [8] indicated that the AOB levels in the current study should range from 40 to 49 % of total biomass (SRT of 20 days; data not shown). Such high fractions of nitrifiers are potentially possible because of high ammonia concentrations and low C/N ratio in the influent, which gives nitrifiers competitive advantages over heterotrophs. One possible reason for the discrepancy between the estimated and measured fractions of AOB is that significant carbon solubilization occurred in the reactors and neither soluble COD nor TOC removals fully represented the total amount of electrons available for heterotrophic growth. Therefore, solubilization of particulate organic matter plays an important role in the microbial ecology of single sludge IA reactors treating swine wastewater, and estimation of AOB fractions and correlations with TKN and soluble COD removals may not apply to wastewaters with non-traditional organic matter characteristics (i.e., not like municipal wastewater). More information on the amount of particulate and soluble organic matter removed would be necessary for better estimates of the fraction of nitrifiers based on stoichiometric calculations.

Stoichiometrically, the oxidation of ammonia to nitrite (catalyzed by AOB) yields more electron equivalents than the oxidation of nitrite to nitrate (catalyzed by NOB). Therefore,

one would expect a single sludge system receiving ammonia and organic N as the sole source of nitrogen to comprise higher levels of AOB than NOB. However, NOB levels (including *Nitrospira* and *Nitrobacter*) were at least the same as AOB levels in 21% of the samples analyzed (data not shown). We hypothesize that low levels of oxygen during the initial period of non-aerated cycles and the existence of anoxic microenvironments within flocs could allow for concurrent nitrate reduction to nitrite and nitrite oxidation by NOB. This hypothesis is supported by the low K_s of NOB for oxygen and nitrite. Thus, NOB could obtain substrate from two different sources: from ammonia oxidation during the aeration period, and from nitrate reduction during the initial period of non-aerated cycles. In addition, pure culture studies have indicated that *Nitrospira moscoviensis* [31] and *Nitrobacter agilis* [32] have the ability to simultaneously incorporate CO_2 and pyruvate, an organic carbon source, in the presence of oxygen. This ability might give NOB competitive advantages in wastewater treatment systems.

The β-AOB population was dominated by *Nitrosomonas/Nitrosococcus mobilis* in both reactors for all operating conditions. *Nitrosospira* were only occasionally detected in low levels, which suggests that this group of AOB probably played a negligible role in ammonia oxidation in the reactors. These results are in agreement with a number of previous studies that have suggested that *Nitrosomonas* can outcompete *Nitrosospira* in environments with high nitrogen and dissolved oxygen concentrations because of *Nitrosomonas'* higher maximum growth rates [31, 32]. Several molecular ecological investigations have led to the suggestion that members of the *Nitrosospira* cluster generally are the most ubiquitously distributed AOB in nature [1, 11]. *Nitrosospira* are thought to act as K_s strategists,

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competing well in environments with limited ammonia concentrations [32]. There is evidence that niche differentiation occurs not only at the genus level of AOB, but also at the species level, as the substrate affinities (K_s values) differ significantly among the AOB species. Within the genus *Nitrosomonas*, different K_s values reflect well the phylogenetically definable groups [11]. *Nitrosomonas/Nitrosococcus mobilis* are well known to occur in wastewater treatment plants. Purkhold *et al.* [33] analyzed samples from 11 nitrifying wastewater treatment plants and determined that in all but two plants only nitrosomonads could be detected. *Nitrosomonas europaea* has frequently been reported as the dominant AOB in activated sludge [15, 32]. Dionisi *et al*. [28] used competitive PCR to study nitrifiers in a full-scale wastewater treatment plant and determined that *Nitrosomonas oligotropha* was the dominant AOB. Juretschko *et al*. [30] used FISH to investigate nitrifying bacteria in an industrial wastewater treatment plant with high ammonia concentrations and determined that *Nitrosococcus mobilis* was the dominant AOB in the activated sludge.

Ballinger *et al.* [4] showed that increasing the C/N ratio from 2 to 5 resulted in a reduction of 50% in nitrification rates, and ultimately resulted in washout of *Nitrosomonas* and *Nitrosospira*. The detrimental effect of high C/N ratios on nitrification activity is due to the lower ability of nitrifiers to compete with heterotrophs for ammonia and oxygen. Therefore, the C/N ratio has contradictory effects in single-sludge systems performing nitrification and denitrification: low C/N ratios favor nitrification activity but hamper denitrification due to limited availability of organic matter. In our study, the relatively low influent C/N ratio (approximately 1.7 as TKN/sol. COD and 1.08 as TKN/TOC) was probably a strong factor contributing to the stability of nitrifying activity. Further research is needed to determine

optimum combinatorial ranges of C/N ratios and aeration cycles that would result in stable and adequate nitrification and denitrification.

Although levels of *Nitrospira* were generally higher than those of *Nitrobacter*, the results show that both genera of NOB coexisted, suggesting that both contributed to nitrite oxidation in the reactors. Recent studies have also reported coexistence of *Nitrospira* and *Nitrobacter* in biofilms and activated sludge. Daims *et al*. [14] used FISH to study the NOB community of a sequencing biofilm batch reactor treating wastewater with high ammonia and salt concentrations. In addition to *Nitrospira,* they detected smaller numbers of *Nitrobacter*. Liebig *et al*. [34] investigated the nitrifier community composition of a chemostat treating sludge reject water using FISH and detected *Nitrospira* and *Nitrobacter* at similar levels. Coskuner and Curtis [35] detected both NOB genera in a full scale activated sludge plant. *Nitrobacter* is thought to outcompete *Nitrospira* in environments with high substrate concentrations (such as in culture media) due to their higher maximum growth rates, whereas *Nitrospira* are better competitors in environments with low substrate concentrations as a result of their higher affinity for nitrite and oxygen [31]. *Nitrospira* has been regarded as the key NOB in wastewater treatment due to frequent reports of quantitative dominance in activated sludge and biofilms [13, 30, 31]. However, factors selecting for *Nitrospira* or *Nitrobacter* remain unknown and deserve further investigation [14]. From the perspective of performance stability, it might be prudent to choose conditions favoring a more complex community of nitrifiers [6, 14, 15, 36], as differences in environmental sensitivity among functionally similar species give stability to ecosystem processes [37]. In reactor A, the shifts in NOB community seemed to be related to the change in aeration conditions. During

the period with manual control, the air supply was off during long periods (overnight) and lower oxygen conditions seemed to favor *Nitrospira* presumably because of their higher affinity for oxygen. When automated aeration control was restored at 1hr ON: 1hr OFF, higher oxygen levels seemed to favor *Nitrobacter*.

Nitrification performance was not negatively affected by the shifts in the AOB and NOB levels, as high ammonia removals and no significant nitrite accumulation were observed. Under the conditions evaluated in this study, the lowest AOB levels of 7.2% of total rRNA and lowest NOB levels of 8.6% of total rRNA (6.1% as *Nitrospira*, and 2.5% as *Nitrobacter*) were sufficient for stable nitrification performance. However, it has not been determined whether these relatively low levels of nitrifiers can sustain the same performance for extended periods.

Our results provide an insight into the nitrifier populations in reactors treating high-nitrogen wastewater in response to changing operating conditions. The fraction of nitrifers in the biomass varied considerably in spite of steady nitrification performance throughout the experiment. The solids content in the influent is believed to have a major impact on the fraction of nitrifiers in the biomass, as the solubilization of particulate organic during the treatment process gives heterotrophs competitive advantages over AOB and NOB, resulting in lower fraction of nitrifiers in the biomass. High levels of nitrifiers were measured in the biomass of both reactors using ANA time ratios with non-aerated times up to 3 hours. Low C/N ratio and high ammonia in the influent are likely the most important factors contributing to the high fraction of nitrifiers and consequently the stability of the nitrification process.

Although slightly lower levels of total β-AOB were measured after the increase in the non-aeration period and change in the influent characteristics, ammonia removal efficiencies were not significantly affected. This illustrates that stable nitrification performance at lower aeration requirements can be achieved, resulting in significant savings in operational costs.

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REFERENCES

[1] Stephen, J.R., Kowalchuk, GA, Bruns, MA, McCaig, AE, Phillips, CJ, Embley, TM, and Prosser, JI. (1998) Analysis of the β-subgroup ammonia oxidizer populations in soil by DGGE analysis and hierarchical phylogenetic probing. Appl. Environ. Microbiol. 64: 2958-2965.

[2] Svenson, A., Sanden, B., Dalhammar, G., Remberger, M., and Kaj, L. (2000) Toxicity identification and evaluation of nitrification inhibitors in wastewaters. Environ. Toxicol. 15: 527-532.

[3]   Burrell, P.C., Phalen, C.M., and Hovanec, T.A. (2001)   Identification of bacteria responsible for ammonia oxidation in freshwater aquaria. Appl. Environ. Microbiol. 67: 5791-5800.

[4] Ballinger, S.J., Head, I.M., Curtis, T.P., and Godley, A.R. (2002) The effect of C/N ratio on ammonia oxidizing bacteria community structure in a laboratory nitrification-denitrification reactor. Water Sci. Technol. 46: 543-550.

[5] Avrahami, S., and Conrad, R. (2003) Patterns of community change among ammonia oxidizers in meadow soils upon long-term incubation at different temperatures. Appl. Environ. Microbiol. 69: 6152-6164.

[6] Egli, K., Langer, C., Siegrist, H-R, Zehnder, A.J.B., Wagner, M., and van der Meer, J.R. (2003)   Community analysis of ammonia and nitrite oxidizers during start-up of nitrification reactors. Appl. Environ. Microbiol. 69: 3213-3222.

[7] Wagner, M., Rath, G., Amann, R., Koops, H.-P., and Schleifer, K.-H. (1995) In situ identification of ammonia-oxidizing bacteria. Syst. Appl. Microbiol. 18: 251-264.

[8] Rittmann, B., Laspidou, C.S., Flax, J., Stahl, D.A., Urbain, V., Harduin, H., van der Waarde, J.J., Geurkink, B., Henssen, M.J.C., Brouwer, H., Klapwijk, A., and Wetterauw, M. (1999) Molecular and modeling analysis of the structure and function of nitrifying activated sludge. Water Sci. Technol. 39: 51-59.

[9] Hall, S.J., Keller, J., Blackall, L.L. (2003) Microbial quantification in activated sludge: the hits and misses. Water Sci. Technol. 48: 121-126.

[10] Purkhold, U, Wagner, M., Timmermann, G., Pommerening-Roser, A., and Koops, H-P. (2003)   16S rRNA and *amo*A-based phylogeny of 12 novel betaproteobacterial ammonia-oxidizing isolates: extension of the dataset and proposal of a new lineage within the nitrosomonads. Int. J. Syst. Evol. Microbiol. 53: 1485-1494.

[11] Koops, H.P., and Pommerening-Roser, A. (2001) Distribution and ecophysiology of the nitrifying bacteria emphasizing cultured species. FEMS Microbiol. Ecol. 37: 1-9.

[12] Wagner M., Rath G., Koops H.P., Flood J. and Amann R. (1996) In situ analysis of nitrifying bacteria in sewage treatment plants. Water Sci. Tech. 34: 237-244.

[13] Schramm A., De Beer D., Wagner M. and Amann R. (1998) Identification and activities in situ of *Nitrosospira* and *Nitrospira* spp. as dominant populations in a nitrifying fluidized bed reactor. Appl. Environ. Microbiol. 64: 3480-3485.

[14] Daims, H., Purkhold, U., Bjerrum, L., Arnold, E., Wilderer, P.A., and Wagner, M. (2001) Nitrification in sequencing biofilm batch reactors: lessons from molecular approaches. Water Sci. Technol. 43: 9-18.

[15] Gieseke, A., Bjerrum, L., Wagner, M., and Amann, R. (2003) Structure and activity of multiple nitrifying bacterial populations co-existing in a biofilm. Environ. Microbiol. 5: 355-369.

[16] Cheng, J. and B. Liu. (2001) Nitrification/denitrification in intermittent aeration process for swine wastewater treatment. J. Environ. Eng. 127: 705-711.

[17] Burkholder, J.M., M.A. Mallin, H.B. Glasgow, Jr., L.M. Larsen, M.R. McIver, G. C. Shank, N.Deamer-Melia, D.S. Briley, J. Springer, B.W. Touchette and E.K. Hannon. (1997) Impacts to a coastal river and estuary from rupture of a large swine waste holding lagoon. J. Environ Qual. 26: 1451-1466

[18] Clesceri, L.S., Greenberg, A.E., and Eaton, A.D. (1995) Standard Methods for the Examination of Water and Wastewater. 20th ed. APHA, AWWA, and WEF. Baltimore, MD. United Book Press, Inc.

[19] Stahl, D.A., Flesher, B., Mansfield, H., and Montgomery, R. (1988) Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. Appl. Environ. Microbiol. 54: 1079-1084.

[20] Burrell, P.C., Keller, J., and Blackall, L.L. (1998) Microbiology of a nitrite-oxidizing bioreactor. Appl. Environ. Microbiol. 64: 1878-1883.

[21] de los Reyes III, F.L., Rothauszky, Raskin, L. (2002) Microbial community structures in foaming and non-foaming full-scale wastewater treatment plants. Water Environ. Res. 74: 437-449.

[22] Zheng D., Alm E. W., Stahl D. A. and Raskin L. (1996) Characterization of universal small-subunit rRNA hybridization probes for quantitative molecular microbial ecology studies. Appl. Environ. Microbiol. 62: 4504-4513.

[23] Mobarry B. K., Wagner M., Urbain V., Rittmann B. E. and Stahl D. A. (1996) Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. Appl. Environ. Microbiol. 62: 2156-2162.

[24]   Mobarry B. K., Wagner M., Urbain V., Rittmann B. E. and Stahl D. A. (1997) Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. Appl. Environ. Microbiol. 63: 815a.

[25]   Hovanec T. A., Taylor L. T., Blakis A. and DeLong E. F. (1998) *Nitrospira*-like bacteria associated with nitrite oxidation in freshwater aquaria. Appl. Environ. Microbiol. 64: 258-264.

[26]   Alm, E.W., Oerther, D.B., Larsen, N., Stahl, D.A., and Raskin, L. (1996) The Oligonucleotide Probe Database. Appl. Environ. Microbiol. 62: 3557–3559.

[27]   de los Reyes III, F.L., W. Ritter, and L. Raskin (1997) Group-specific small subunit hybridization probes to characterize filamentous foaming in activated sludge systems. Appl. Environ. Microbiol. 63: 1107-1117.

[28]   Dionisi, H. M., Layton, A. C., Harms, G., Gregory, I. R., Robinson, K. G. and Sayler, G. S. (2002) Quantification of *Nitrosomonas oligotropha*-like ammonia-oxidizing bacteria and *Nitrospira* spp. from full-scale wastewater treatment plants by competitive PCR. Appl. Environ. Microbiol. 68: 245-253.

[29]   Urbain, V, Mobarry, B, de Silva, V, Stahl, D.A., Rittmann, B.E., and Manem, J. (1998) Integration of performance, molecular biology and modeling to describe the activated sludge process. Water Sci. Tech. 37: 223-229.

[30]   Juretschko, S., Timmermann, G., Schmid, M., Schleifer, K.-H., Pommerening-Roser, A., Koops, H.-P., and Wagner, M. (1998) Combined molecular and conventional analysis of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. Appl. Environ. Microbiol. 64: 3042-3051.

[31] Schramm, A., de Beer, D., vanden Heuvel, J., Ottengra, S., and Amann, A. (1999) Microscale distribution of populations and activities of *Nitrosospira* and *Nitrospira* spp. along a macroscale gradient in a nitrifying bioreactor: quantification by in situ hybridization and the use of microsensors. Appl. Environ. Microbiol. 65: 3690 – 3696.

[32] Schramm A., De Beer D., Gieseke, A., and Amann R. (2000) Microenvironments and distribution of nitrifying bacteria in a membrane-bound biofilm. Environ. Microbiol. 2: 680-686.

[33] Purkhold, U., Pommerening-Röser, A., Juretschko, S., Schmid, M.C., Koops, H-P., Wagner, M. (2000) Phylogeny of All Recognized Species of Ammonia Oxidizers Based on Comparative 16S rRNA and *amoA* Sequence Analysis: Implications for Molecular Diversity Surveys. Appl. Environ. Microbiol. 66: 5368-5382.

[34] Liebig, T., Wagner, M., Bjerrum, L., and Denecke, M. (2001) Nitrification performance and nitrifier community composition of a chemostat and a membrane-assisted bioreactor for the nitrification of sludge reject water. Bioprocess Biosyst. Eng. 24: 203-210.

[35] Coskuner, G. and Curtis, T.P. (2002)   In situ characterization of nitrifiers in an activated sludge plant: detection of Nitrobacter spp. J. Appl. Microbiol. 93: 431-437.

[36] Ballinger, S.J., Head, I.M., Curtis, T.P., and Godley, A.R. (1998) Molecular microbial ecology of nitrification in an activated sludge process treating refinery wastewater. Water Sci. Technol. 37: 105-108.

[37] Chaplin III, F.S., Walker, B.H., Hobbs, R.J., Hooper, D.U., Lawton, J.H., Sala, O.E., and Tilman, D. (1997) Biotic Control over the functioning of ecosystems. Science 277: 500-504.

Table 1. Forward and reverse primers used to amplify *Nitrospira moscoviensis*-like DNA.

Primer	Sequence (5' to 3')	Target organisms
Fw-Nspa311	ACACTGGCACTGCGACA	*Nitrospira moscoviensis*
Rv-Nstpa1463	TTCACCCCAATCATCGGTCA	*Nitrospira moscoviensis subgroup*

Table 2. Oligonucleotide probes used in slot blot and in-situ hybridizations.

Probe		Target Site[3]	Sequence (5' to 3')	Wash Temp (ºC)	% Formamide	Target organisms	Ref.
Name[1]	Abbrev.						
S-*-Univ-1390-a-A-18	Univ1390	1390 - 1407	GACGGGCGGTGTGTACAA	44	0	All organisms	[22]
S-G-Nsv-0444-a-A-19	Nsv443	444 - 462	CCGTGACCGTTTCGTTCCG	52	30	*Nitrosospira* spp.	[23-24]
S-G-Nsm-0156-a-A-19	Nsm156	156 - 174	TATTAGCACATCTTTCGAT	46	5	*Nitrosomonas* spp., *Nitrosococcus mobilis*	[23-24]
S-G-βAOB-1224-a-A-20	Nso1225	1224-1243	CGCCATTGTATTACGTGTGA	51	35	Betaproteobacterial ammonia-oxidizing bacteria	[23-24]
S-G-Nbac-1000-a-A-15	Nb1000	1000 - 1014	TGCGACCGGTCATGG	42	---	*Nitrobacter* spp.	[23-24]
S-G-Nbac-1035-a-A-18[2]	NIT3	1035 - 1052	CCTGTGCTCCATGCTCCG	---	40	*Nitrobacter* spp.	[12]
S-G-Ntspa-0685-a-A-22	Ntspa685	664 – 685	CACCGGGAATTCCGCGCTCCTC	63	---	*Nitrospira moscoviensis, Nitrospira marina*	[25]

[1]Probe names have been standardized according to OPD [26].

[2]Used with unlabeled competitor probe NIT3-Competior in an equimolar ratio.

[3]*E. coli* numbering.

Table 3. Average concentrations in the influent wastewater. (Standard deviations in parentheses).

Stage	Days	Mean Influent Concentrations (mg/L)[*]								
		VSS	TKN	NH_3.N	NO_3-N	NO_2-N	Sol. COD	TOC	Sol.COD / TKN	TOC/ TKN
I and III	1-64	336 ($\pm$113)	166.0 ($\pm$8.4)	114.9 ($\pm$5.7)	3.8 ($\pm$3.7)	0.0 ($\pm$0.0)	280.2 ($\pm$27.5)	182.2 ($\pm$37.8)	1.7 ($\pm$0.14)	1.1 ($\pm$0.2)
II and IV	81-180	733 ($\pm$457)	208.7 ($\pm$44.5)	146.4 ($\pm$27.5)	0.4 ($\pm$0.7)	0.0 ($\pm$0.0)	362.5 ($\pm$73.0)	208.1 ($\pm$47.0)	1.8 ($\pm$0.3)	1.0 ($\pm$0.4)

[*]A total of 10 data points each for stages I and III, and 13 points each for stages II and IV were computed.

Table 4. Average effluent concentrations and removal efficiencies for reactor A. (Standard deviations in parentheses).

Stage	Days	*Effluent Concentrations (mg/L)					*Removal Efficiency (%)				
		VSS	TKN	NH$_3$-N	NO$_2$-N	NO$_3$-N	TKN	NH$_3$-N	TIN	TOC	Soluble COD
I	1 – 50	2475 (±881)	18.0 (±7.1)	1.4 (±2.6)	0.4 (±0.6)	66.3 (±26.8)	89.1 (±4.3)	98.8 (±2.3)	41.9 (±23.7)	57.9 (±14.0)	33.2 (±12.8)
II	121 – 180	719 (±336)	12.0 (±7.9)	0.9 (±2.2)	0.3 (±0.4)	23.7 (±16.1)	94.6 (±4.0)	99.4 (±1.4)	83.7 (±12.0)	64.6 (±14.7)	29.5 (±28.5)

*A total of 8 data points were computed for each period.

Table 5. Average effluent concentrations and removal efficiencies for reactor B. (Standard deviations in parentheses).

Stage	Days	*Effluent Concentrations (mg/L)					*Removal Efficiency (%)				
		VSS	TKN	NH$_3$-N	NO$_2$-N	NO$_3$-N	TKN	NH$_3$-N	TIN	TOC	Soluble COD
III	1 – 64	1202 (±358)	16.1 (±8.1)	1.6 (±2.6)	0.2 (±0.2)	54.4 (±10.4)	90.34 (±4.7)	98.6 (±2.3)	52.6 (±9.1)	58.6 (±10.4)	32.9 (±9.6)
IV	81 – 180	1628 (±469)	13.7 (±6.2)	0.4 (±0.6)	0.4 (±0.7)	16.6 (±13.2)	93.77 (±3.0)	99.7 (±0.4)	83.8 (±18.8)	68.1 (±9.4)	46.1 (±10.7)

*A total of 9 data points were computed for each period.

	Stage I		Reactor A	Stage II	
Aeration	1 hr ON : 1 hr OFF		Manual Control	1 hr ON : 1 hr OFF	
Influent Wastewater	Anaerobically Digested (AD)	80% AD 20% raw	50% AD + 50% raw		
(day)	20 40	60	80 100 120 140 160 180		

	Reactor B				
Aeration	1 hr ON : 2 hr OFF		1 hr ON : 3 hr OFF		
Influent Wastewater	Anaerobically Digested (AD)	80% AD 20% raw	50% AD + 50% raw		
(day)	20 40 60		80 100 120 140 160 180		

Stage III   Stage IV
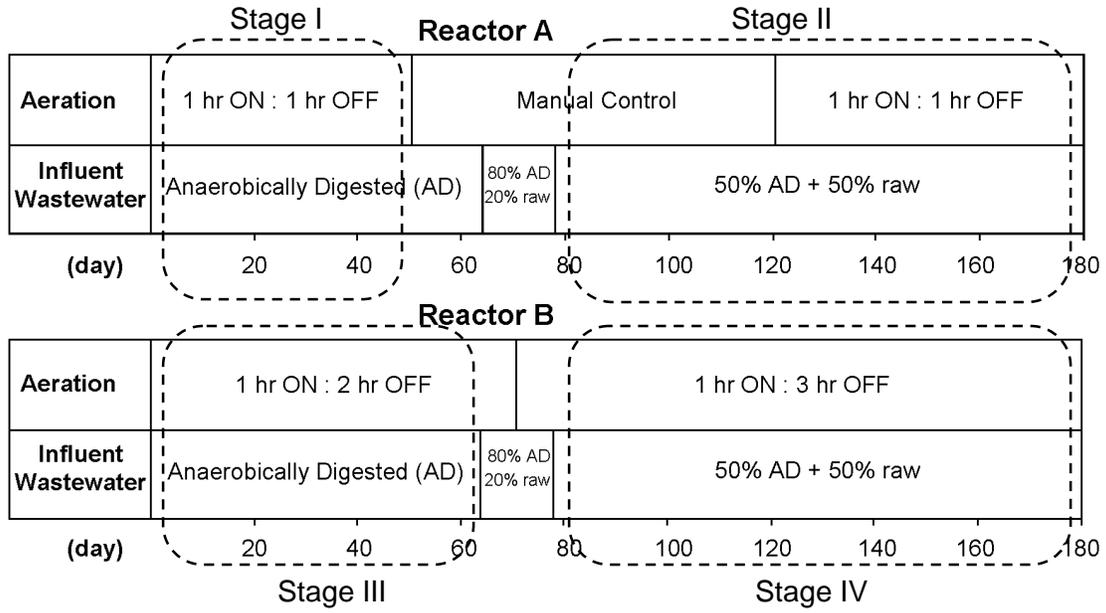
Figure 1.   Diagram of operational conditions showing changes in influent wastewater composition and aeration cycles.

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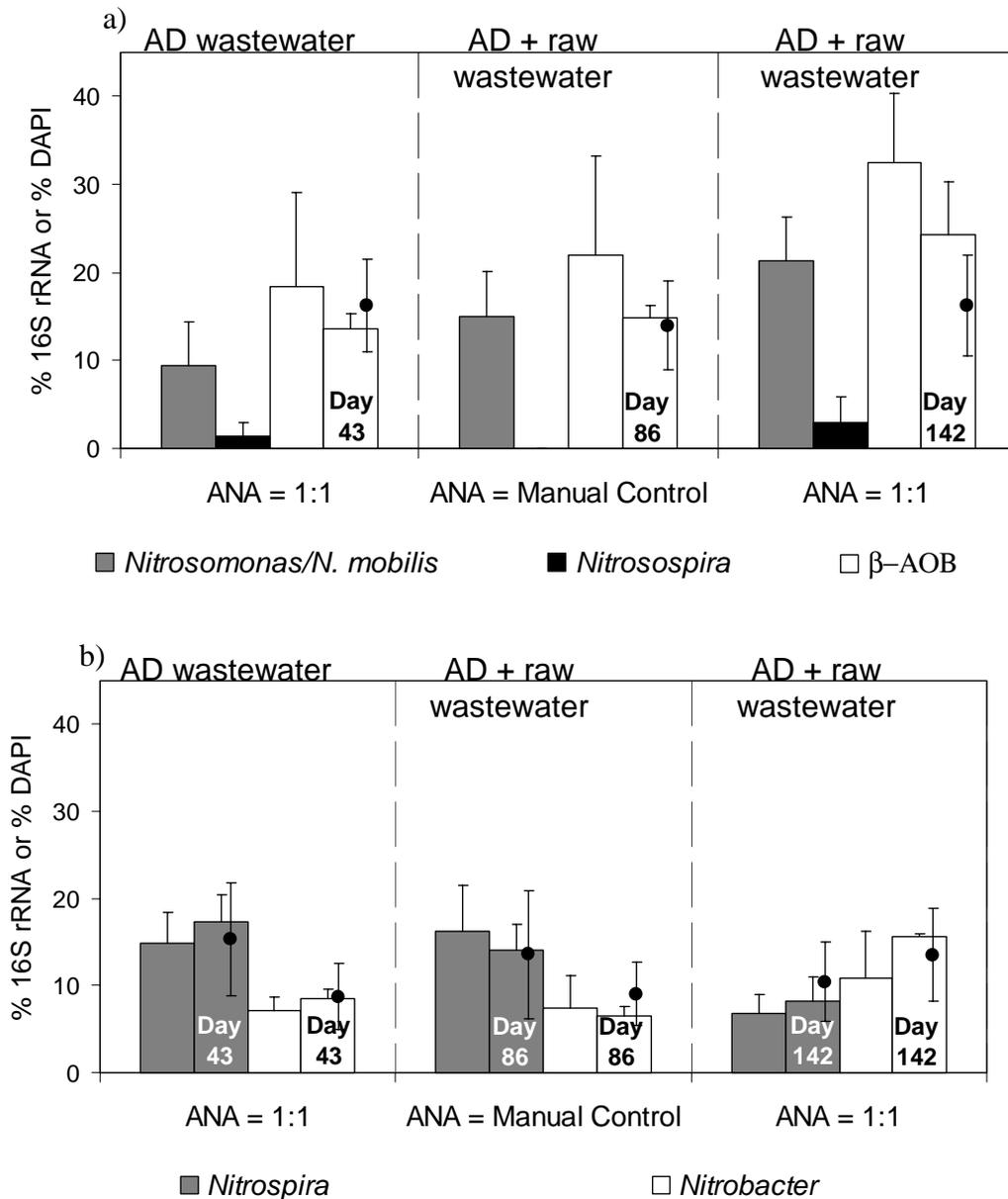
Figure 2. a) Average fractions of total β-AOB, *Nitrosomonas* and *Nitrosospira*; b) average fractions of *Nitrospira* and *Nitrobacter* for reactor A (stages I and II). Bars represent average data from membrane hybridizations (expressed as %16S rRNA), with respective standard deviations. The bar with a day label corresponds to the membrane hybridization data for that specific day, and the error bar represents the standard deviation for the triplicate measurements. (●) correspond to FISH data for the same day (expressed as % DAPI area), with respective error bar. AD stands for anaerobically digested swine wastewater.
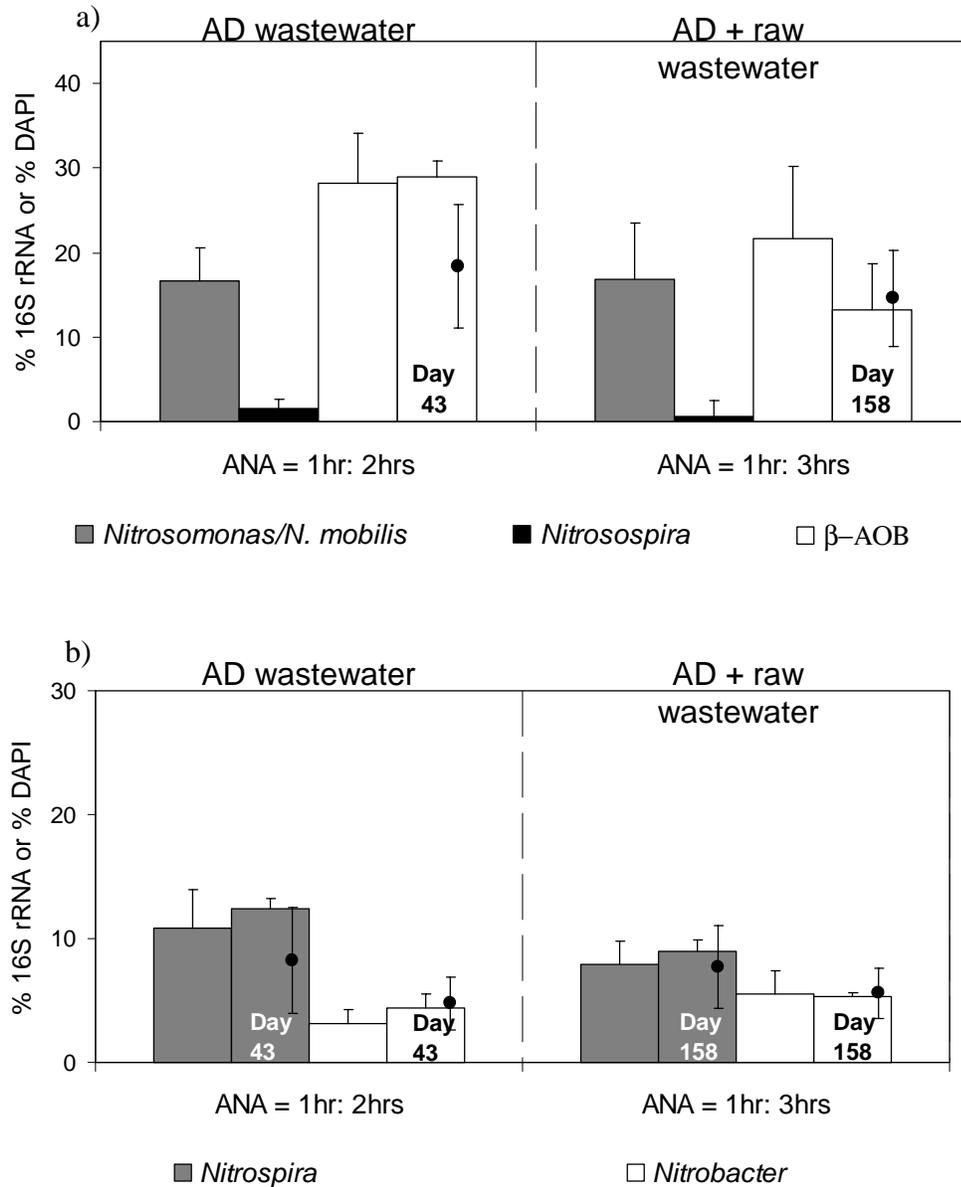
Figure 3. a) Average fractions of total β-AOB, *Nitrosomonas* and *Nitrosospira*; b) average fractions of *Nitrospira* and *Nitrobacter* for reactor B (stages III and IV). Bars represent average data from membrane hybridizations (expressed as %16S rRNA), with respective standard deviations. The bar with a day label corresponds to the membrane hybridization data for that specific day, and the error bar represents the standard deviation for the triplicate measurements. (●) correspond to FISH data for the same day (expressed as % DAPI area), with respective error bar. AD stands for anaerobically digested swine wastewater.

Chapter 2.

EFFECTS OF AERATION CYCLES ON NITRIFYING BACTERIAL POPULATIONS AND NITROGEN REMOVAL IN INTERMITTENTLY-AERATED REACTORS

ABSTRACT

The effects of the length of aeration and non-aeration periods on nitrogen removal and the nitrifying bacterial community structure were assessed in intermittently-aerated (IA) reactors treating digested swine wastewater. Five IA reactors were operated in parallel with different aeration to non-aeration (ANA) time ratios. Populations of ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) were monitored using 16S rRNA slot-blot hybridizations. AOB species diversity was assessed using *amoA* gene DGGE (denaturant gradient gel electrophoresis). *Nitrosomonas/N. mobilis* were the dominant AOB and *Nitrospira* were the dominant NOB in all reactors, although *Nitrosospira* and *Nitrobacter* were also detected at lower levels. Reactors operated with the shortest aeration time (30 min) showed the highest rRNA levels of *Nitrosospira* and reactors operated with the longest anoxic periods (3 and 4h) showed the lowest levels of *Nitrobacter* as compared to the other reactors. *Nitrosomonas* sp. Nm107 was detected in all reactors, regardless of the reactor's performance. Close relatives of *Nitrosomonas europaea, Nitrosomonas* sp. ENI-11, and *Nitrosospira multiformis* were occasionally detected in all reactors. Biomass fractions of

AOB and effluent ammonia concentrations were not significantly different among the reactors. NOB were more sensitive than AOB to long non-aeration periods as nitrite accumulation and lower total NOB rRNA levels were observed for ANA of 1h:4h. The reactor with the longest non-aeration time of 4 h performed partial nitrification, followed by denitrification via nitrite, whereas the other reactors removed nitrogen through traditional nitrification and denitrification via nitrate. Superior ammonia removal efficiencies were not associated with levels of specific AOB species or with higher AOB species diversity.

INTRODUCTION

There is increasing interest in biological nitrogen removal technologies that use low levels of oxygen to achieve partial nitrification, the oxidation of ammonia to nitrite by ammonia oxidizing bacteria (AOB), and subsequent denitrification via nitrite, the reduction of nitrite to dinitrogen gas by heterotrophic denitrifiers. Alkalinity and oxygen demands are lower for partial nitrification and organic substrate requirements are lower for denitrification via nitrite as compared to the traditional nitrification/denitrification process, resulting in substantial operational savings (2). Partial nitrification relies on the selection of AOB over nitrite oxidizing bacteria (NOB), which allows the accumulation of nitrite. Sustained nitrite accumulation can be accomplished by controlling solids retention time, temperature, free ammonia and hydroxylamine concentrations, or dissolved oxygen conditions (43, 19, 2, 12, 23, 18).

The key to efficient and robust biological wastewater treatment relies on knowing the microorganisms involved and how they respond to different operating conditions (42). Several microbial diversity studies of activated sludge and biofilms based on 16S rRNA gene

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libraries have been reported in the last decade. Denaturant gradient gel electrophoresis (DGGE) has been used to separate amplified 16S rRNA genes and determine the effects of ammonia and dissolved oxygen concentrations on community composition of nitrifiers (36, 25, 17). However, commonly used 16S rRNA primers for AOB studies have limited specificity and the high similarity among 16S rRNA genes of AOB makes it impossible to resolve and identify closely related AOB species (32). Alternatively, the functional gene encoding the α-subunit of ammonia monooxygenase (*amoA*), the enzyme responsible for the conversion of ammonia to hydroxylamine found in all AOB, has been used as a specific molecular marker in environmental studies of AOB using DGGE (4, 5, 33, 40) and RT-PCR (16).

Intermittently aerated (IA) reactors have been successfully used for nitrogen removal from digested swine manure by achieving complete nitrification during aerated periods, followed by denitrification during non-aerated periods (9, 31). Digested swine manure usually contains high ammonia concentrations and a low carbon/nitrogen (C/N) ratio. These characteristics impose challenges for the traditional nitrification/denitrification approach due to: (i) the high oxygen demand for complete nitrification of ammonia to nitrate and (ii) the relatively low organic substrate content available for complete denitrification. IA reactors can potentially be optimized if used to perform partial nitrification followed by denitrification via nitrite, resulting in reduced oxygen demand for ammonia removal and reduced organic substrate for denitrification. We hypothesized that aeration cycles with sufficiently short aerated periods or sufficiently long non-aerated periods can provide appropriate conditions for partial nitrification and denitrification via nitrite. In the present

study, we assess the effects of different aeration cycles on nitrogen removal performance and the community composition of AOB and NOB using *amoA* DGGE and quantitative slot-blot hybridizations based on 16S rRNA.

MATERIALS AND METHODS

Laboratory scale reactors

Five identical 6-liter Plexiglas reactors (A, B, C, D, and E) were operated under intermittent aeration conditions each with a different aeration to non-aeration (ANA) time ratio: 1h:1h (reactor A); 1h:3h (reactor B); 0.5h:1.5h (reactor C); 0.5h:2h (reactor D); and 1h:4h (reactor E). The reactors were fed with anaerobically digested swine wastewater with the following average concentrations: 197 ± 111 mg NH_3-N L^{-1} (total ammonia), 296 ± 150 mg Total Kjeldahl Nitrogen L^{-1}(TKN), 344 ± 83 mg Soluble Chemical Oxygen Demand L^{-1} (Sol. COD), and 305 ± 114 mg Total Organic Carbon L^{-1} (TOC). The influent flow rate was 2 L day^{-1}, with substrate inflow for 20 min every 60 min. The target hydraulic retention time (HRT) and mean cell residence time (MCRT) were 3 days and 20 days, respectively. All reactors were operated at room temperature (25^oC). Mean pH in all reactors ranged from 7.6 to 7.8, and alkalinity addition was not necessary.

Reactors A and B were inoculated with activated sludge from the Neuse River Wastewater Treatment Plant (Raleigh, NC). Waste sludge from reactors A and B was stored at 4^oC and used for inoculating reactors C, D, and E. Wastewater was obtained biweekly from a swine lagoon at the NCSU Lake Wheeler Road Field Laboratory and stored at 4^oC. The reactor design allowed biomass settling in the clarification zone and recycling to the aeration zone. Air cycling was controlled using a solenoid valve activated by an electronic timer (ChronTrol

Corp., San Diego, Calif.). Compressed air was regulated to 10 psi and airflow was controlled

by a gas mass flow controller at 500 mL min^{-1} (Cole-Parmer Instrument Co., Vernon Hills,

Ill.).

Analytical methods

Grab samples of the influent and the effluent during the aerated phase were collected from

each reactor and analyzed for TKN, NH_3-N (total ammonia), NO_3^--N, NO_2^--N, soluble COD,

TOC, pH, TSS (total suspended solids), and VSS (volatile suspended solids) using Standard

Methods (10). Dissolved oxygen (DO) was measured using an YSI 52 DO meter and an YSI

5739 oxygen probe (YSI Inc., Yellow Springs, Ohio). Oxidation-reduction potential (ORP)

measurements were taken with an Accumet metallic combination platinum/Ag/AgCl

electrode (EID Corp., Bridgeport, Connecticut).

Bacterial Cultures

Pure cultures of *Nitrosomonas europaea* (ATCC 25978, ATCC medium 2265), *Nitrosospira*

multiformis (ATCC 25196, ATCC medium 929), and *Nitrobacter agilis* (ATCC 25384,

ATCC medium 480) were grown aerobically in 0.5-liter flasks at 30°C. The pH was kept at

8.0 by periodic addition of 20% Na_2CO_3. Cells were harvested by centrifugation at 3200 x g

and cell pellets were processed for extraction of RNA.

Nucleic acids extraction and in vitro transcription

Two sets of mixed liquor samples (14 mL each sample) were centrifuged at 3200 x g for 5

minutes and stored at –80°C for RNA and DNA extractions. RNA was extracted using a

modified low-pH hot-phenol extraction procedure (39). DNA was extracted using a

PowerSoil™ DNA isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA) according to the

manufacturer's instructions. Nucleic acids concentrations were measured spectrophotometrically.

Since pure cultures of *Nitrospira* were not available, in vitro-transcribed 16S rRNA was used as reference rRNA in membrane hybridizations as previously described (31).

DGGE and cloning

amoA gene fragments were amplified using a semi-nested approach in which products of an initial round of PCR with primers AmoA-1F and AmoA-2R-TC (37, 32) were agarose purified and used as template for a second round of PCR using the same primers, except that the forward primer (AmoA-1F) had a GC clamp attached to its 5'-end. Agarose purification of PCR products was performed by coring bands of the correct size using a sterile plastic pipette tip and transferring the excised core into a sterile PCR tube for the second round of PCR. Amplifications were performed using 0.5 µM of each primer, 25 µL of FailSafe PCR System Reaction Mix E (Epicentre Technologies, Madison, WI), 1 µL DNA extract, and sterile pure water to a total reaction volume of 50 µL. PCR was performed using a thermal cycler (Eppendorf Scientific Inc., Westbury, NY) under the following conditions: 94°C for 5 min, 28 cycles of 92°C for 1.0 min, 58°C for 1.0 min, 72°C for 1.0 min, and a final extension at 72°C for 45 min.

Amplified *amoA* gene fragments (491 bp) were separated on 8% (wt/vol) acrylamide-bisacrylamide gels with a denaturant gradient of 20 to 60% urea-formamide at 60°C and 80 V for 16 h using a D-Code System (Biorad Laboratories, Hercules, CA). The gels were stained with SYBR Gold (Molecular Probes, Inc., Eugene, OR), visualized with a Dark Reader

transilluminator (Clare Chemical Research, Inc., Dolores, CO) and photographed with a Canon Powershot A70 digital camera (Canon USA, Inc., Chesapeake, VA).

Sterile syringe needles were used to core DGGE bands (approximately a third of the band width) and excised cores were transferred into sterile PCR tubes for re-amplification and subsequent DGGE for checking efficacy of band isolation. This procedure was repeated 3 to 5 times. Nonetheless, some bands appeared pure while others were still "contaminated" with co-migrating bands. To resolve band purity and obtain reliable sequences, "isolated" bands were re-amplified using unclamped primers, ligated into pCR2.1 vectors, and transformed into *E. coli INVaF'* competent cells using a TA cloning kit (Invitrogen, Carlsbad, CA). White colonies were picked from agar plates (10 colonies from each ligation sample) and plasmid inserts were amplified using primers AmoA-1F-GC clamp and AmoA-2R-TC. A final DGGE was required before sequencing of the bands to confirm that amplified inserts and originally excised bands had the same migrating patterns. To check reproducibility, two pairs of bands with identical migrating patterns but from different environmental samples were also sequenced.

Sequencing

Selected clones were sequenced at the Duke University DNA Sequencing Facility using a Perkin Elmer Dye Terminator Cycle Sequencing system with AmpliTaq DNA Polymerase combined with ABI 3730 and 3100 PRISM DNA sequencing instruments and BigDyeTMv1.1 terminator.

Oligonucleotide probes and slot-blot hybridizations

The oligonucleotide probes targeting the 16S rRNA of nitrifiers used in slot-dot hybridizations are listed in Table 1. The probes were obtained from Sigma-Genosys (The Woodlands, TX). Probes were 5'-end labeled with [γ^{32}-P]ATP (ICN Radiochemicals, Irvine, California) and T4 polynucleotide kinase (Promega Corp., Madison, Wisconsin) and purified with a Quickspin Oligo column (Roche Molecular Biochemicals, Indianapolis, Indiana). Membranes with immobilized RNA were hybridized as previously described (27) and washed at the appropriate wash temperatures (Table 1). The results were expressed as percentages of the total rRNA as measured with the universal probe.

RESULTS

Reactor performance

The influent wastewater was the same for all reactors. However, biomass was subjected to unique NH_3 and oxygen concentrations in each reactor, as influent feeding was semi-continuous and NH_3 accumulation occurred during non-aerated periods. The nitrogen, pH, DO, and ORP profiles for the reactors described in this study have been previously discussed (Head et al., submitted for publication). A summary of the data for nitrogen and DO profiles is presented in Table 2. Reactors B (ANA 1h:3h) and E (ANA 1h:4h), showed nitrite accumulation up to 5.4 mg NO_2^--N L^{-1} and 7.8 mg NO_2^--N L^{-1}, respectively, while the other reactors exhibited negligible nitrite accumulation during aeration periods. Only reactor E, with the longest non-aeration period, showed substantial nitrite reduction during non-aeration periods as indicated by effluent nitrite concentrations of 0.8 mg NO_2^--N L^{-1} at the end of non-

aeration periods. Nitrite reduction in reactor B was minor, as indicated by effluent nitrite concentration of 4.9 mg NO_2^--N L^{-1} at the end of non-aeration periods.

Long-term operation of reactors and membrane hybridizations

Table 3 shows average effluent concentrations during aeration periods from the five reactors for 1 year of operation. The reactors showed stable performance for most of the days sampled. However, short periods of efficiency instability occurred in all reactors, resulting in relatively high standard deviations for the effluent data shown in table 3. Reactor C had the highest mean effluent ammonia concentrations (32 mg NH_3-N L^{-1}) and reactor B had the lowest mean effluent ammonia concentrations (22 mg NH_3-N L^{-1}). However, mean effluent ammonia concentrations were not significantly different among reactors (ANOVA P-value 0.7212, $\alpha = 0.5$), indicating that the AOB communities in all reactors could oxidize ammonia to similar levels during aerated periods. Effluent nitrite and nitrate concentrations were significantly different among reactors (ANOVA P-values of 0.003 and 0.004 for nitrite and nitrate, respectively; $\alpha = 0.5$). Reactor A, with the shortest non-aerated period, had the lowest mean effluent nitrite concentration. Reactor E, with the longest non-aerated period, had the highest mean effluent nitrite concentration. These findings suggest that NOB are more sensitive than AOB to longer non-aerated periods. Reactor B had the lowest mean effluent nitrate concentration and reactor C had the highest mean effluent nitrate concentration.

The fraction of AOB (total β-AOB) and NOB (including *Nitrobacter* and *Nitrospira*) in the biomass were also monitored and the average values are shown in Table 4. The mean fraction of AOB was not significantly different among reactors (ANOVA P-value of 0.376, α

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= 0.5). *Nitrosomonas/Nitrosoccocus* were the dominant AOB in all reactors, accounting for more than 70% of the total AOB fraction. Reactors C and D, both with the shortest aeration period (0.5h), showed the lowest *Nitrosomonas/Nitrosoccocus* mean percentage of total β-AOB among the reactors. *Nitrospira* was the dominant group of NOB in all reactors, accounting for more than 73% of total NOB. Reactors B and E, both with the longest non-aeration periods (3 and 4h), showed the lowest mean *Nitrobacter, Nitrospira* and total NOB (*Nitrobacter + Nitrospira*) fractions among the reactors, suggesting that long non-aeration periods have a strong effect on the biomass levels of NOB. Reactors C and D, both operated with 0.5h aeration, did not show significantly different total NOB biomass fractions as compared to reactors with longer aeration periods, suggesting that short aeration periods did not have considerable impacts on the NOB populations in the reactors.

amoA DGGE profiles

The community structure of AOB and removal efficiencies of TKN and ammonia were monitored in each reactor throughout the experiment (Figure 1). This approach was used to: (i) visually assess the stability of AOB community structure, and (ii) investigate whether ammonia and TKN removal efficiencies could be related to AOB diversity or to specific AOB species. *amoA* DGGE profiles showed frequent shifts in composition and diversity of AOB communities, as indicated by the appearance and disappearance of certain bands with time. Five bands were successfully isolated and sequenced. BLAST analysis of the sequences revealed that the sequence of band 1 (Figure 1) shared 99% sequence similarity to the *amoA* gene of *Nitrosomonas* sp. Nm107 (Table 5). The sequences of bands 2 and 3 shared 91 and 95% sequence similarity to the *amoA* sequence of *Nitrosomonas europaea*, respectively. The sequence of band 4 was 91% similar to that of *Nitrosomonas* sp. ENI-11

and the sequence of band 5 shared 92% sequence similarity to the *amoA* gene of *Nitrosospira multiformis*.

Comparison between sequences of band pairs with identical migrating patterns but from different environmental samples resulted in identical sequences for one pair and a total of 2 mismatches for the other pair of bands (data not shown).

Although environmental conditions in the reactors were considerably different, practically all *amoA* DGGE profiles showed the same two dominant bands (bands 1 and 2). Band 3 was particularly strong in virtually all *amoA* DGGE profiles of reactor C (0.5h:1.5h) and was occasionally present in the other reactors. Bands 4 and 5 were only occasionally detected in all reactors and usually with faint signals, except for panels h and j (Figure 1) that show band 4 as a major band. Throughout the experiment, *amoA* DGGE profiles showed a total of 9 different bands, with increasing AOB species diversity (based on the number of bands) toward the end of the experiment.

High ammonia and TKN removal efficiencies were observed for samples with various *amoA* DGGE profiles, including samples with high and low AOB species diversity. Similarly, *amoA* DGGE profiles showing high AOB diversity, as well as *amoA* DGGE profiles with low AOB diversity, were shown for days with poor ammonia and TKN removal performance.

DISCUSSION

Nitrite accumulation is a result of the ammonia oxidation rate exceeding that of nitrite oxidation. Differences in oxidation rates can be attributed to AOB outcompeting NOB due to a number of factors including free ammonia inhibition, dissolved oxygen concentration, organic matter and volatile fatty acids concentrations, temperature, and pH (43, 35, 12, 2). Accumulation of nitrite at low DO is usually attributed to the difference in the saturation constant for DO (K_O) between AOB and NOB (15). Values for the K_O of AOB and NOB in activated sludge in the literature range from 0.25 to 0.5 mg O_2 L^{-1} and 0.34 to 2.5 mg O_2 L^{-1}, respectively (6). Hanaki et al. (15) reported that AOB remained unaffected in a suspended growth reactor operated at low DO (<0.5 mg L^{-1}), while NOB were strongly inhibited, resulting in accumulation of nitrite to 60 mg L^{-1}. In addition, AOB have been found to more readily recover from periods of substrate starvation than NOB, resulting in occasional nitrite accumulation after ammonia becomes available again (13, 41).   Peng et al. (34) reported that AOB developed an ability to endure DO fluctuations, but not NOB. In this study, NOB and AOB were found to adjust well to varying DO levels when non-aeration cycles were below 2h. NOB were only affected by long non-aeration periods of 3 and 4h, resulting in lower NOB biomass fractions and nitrite accumulation during aeration periods. Short aeration periods (0.5h) did not affect NOB populations significantly and the nitrification process was carried all the way to nitrate during aeration. Therefore, not only the dissolved oxygen concentration but also the length of non-aeration periods is important in the selection of AOB over NOB in intermittently-aerated reactors.

High ammonia concentrations have been shown to select AOB over NOB, resulting in nitrite accumulation. Generally, AOB have lower ammonia inhibition constants and can survive at elevated ammonia concentrations (43). Since influent feeding of the reactors was semi-continuous, ammonia accumulation occurred during non-aeration periods and biomass in reactors with different aeration cycles was subjected to unique ammonia concentrations. However, ammonia inhibition of NOB was likely not important, since usually only free ammonia is taken into consideration in the context of inhibition, and free ammonia is negligible at the pH range (7.6 – 7.8) and total ammonia concentrations (1.2 – 64.0 mg NH_3-N) maintained in this study.

DGGE based on *amoA* genes has been previously used to study the AOB communities in soil, biofilm, and activated sludge (4, 28, 33). For most groups of AOB, a high consistency has been found when comparing phylogenetic trees based on 16S rRNA and *amoA* sequences (1). We used methods targeting two AOB genes: 16S rRNA membrane hybridizations to estimate the biomass fraction of AOB at the genus level and *amoA* DGGE to monitor AOB community structure at the species level. 16S rRNA membrane hybridizations and *amoA* DGGE results corroborated each other for the ammonia-oxidizing bacteria, as both methods indicated the dominance of *Nitrosomonas/N. mobilis* in the AOB populations assessed. Although using DGGE has many advantages and applications, problems may arise due to PCR bias, heterogeneity of copy number of 16S rRNA among species and the fact that single DGGE bands do not always represent a single bacterial strain (38). However, it was shown in this study that sequences of bands from different environmental samples and identical migrating patterns were identical or differed by only two base pairs. Avrahami and Conrad

(5) have shown that sequences of *amoA* bands that migrated identically but originated from different environmental samples were always found to be identical by amino acid sequence and only occasionally showed differences at no more than 2 base-pairs. Bias caused by multiple copies of *amoA* within one organism can be excluded because only non-degenerate *amoA* primers were used in this study. In addition, previous amplification of the *amoA* genes from 31 pure cultures with the same primers resulted in unambiguous sequences (32, 5, 1).

Membrane hybridizations showed that the biomass fraction of *Nitrosomonas/N. mobilis* far exceeded that of *Nitrosospira* in all reactors, suggesting that the former were responsible for most of the ammonia oxidation. These results concur with a number of previous studies that have suggested that *Nitrosomonas* can outcompete *Nitrosospira* in environments with high nitrogen loads (14, 16, 21). *amoA* DGGE profiles showed that a close relative to *Nitrosomonas* sp. Nm107 corresponded to the dominant band in virtually all samples analyzed. *Nitrosomonas* sp. Nm107 was first isolated from an activated sludge rendering plant and is believed to be a strain of *Nitrosococcus mobilis* based on its 16S rRNA (36). In addition, the *amoA* sequences of *Nitrosomonas* sp. Nm107 and *Nitrosococcus mobilis* were shown to be identical (36). *Nitrosococcus mobilis* was isolated from a sample of brackish water and is characterized by an obligate salt requirement, being considered a moderate halophilic AOB (24). The maximum growth rate of *Nitrosococcus mobilis* reported for pure cultures is close to that of *N. europaea/oligotropha* (14, 24). Juretschko et al. (21) used fluorescent in situ hybridization (FISH) to confirm that *N. mobilis* was the numerically dominant AOB in the nitrifying activated sludge of an industrial wastewater treatment plant receiving sewage with high ammonia concentrations. Gieseke et al. (14) detected

coexistence of *Nitrosococcus mobilis* and *Nitrosomonas europaea/N. oligotropha* in a biofilm from a nitrifying pilot-scale sequencing batch reactor treating nitrogen-rich wastewater loads. Although *Nitrosomonas* sp. Nm107 corresponded to the dominant band in practically all *amoA* DGGE profiles, it may not have been the numerically dominant or the most active AOB in the reactors due to the inherent qualitative rather than quantitative feature of PCR-DGGE.

Regardless of the significant differences in the environments created by each of the aeration cycles, comparison of *amoA* DGGE profiles showed that AOB speciation was rather similar among the reactors. This suggests that factors other than the operating parameters tested had a stronger influence on AOB speciation. The concomitant increase in AOB species diversity in all reactors during the last phase of the investigation suggests that AOB species diversity was affected by a common environmental factor to all reactors. Given that temperature, pH, and aeration cycles remained unchanged, influent characteristics are probably the strongest factor affecting AOB speciation. Since the influent was collected from an anaerobic lagoon treating swine wastewater, it was impossible to control numerous potential factors affecting AOB speciation, such as ammonia concentrations, wastewater salinity, metals concentrations, organic matter, and solids content. Previous studies have shown that the microbial community in identically operated laboratory-scale reactors can differ significantly while showing similar performances (22). Therefore, when assessing the effect of environmental parameters on the microbial communities in laboratory-scale experiments, it is desirable to test reproducibility by either running duplicates or operating the reactors for long periods (total of 1 year in this study).

In the process of denitrification in intermittently-aerated reactors, the length of the sequential aeration and non-aeration periods within the residence time is also of significance (35). As the induction of the different denitrification enzymes proceeds sequentially, the different intermediates of denitrification accumulate temporarily during non-aeration periods. Oxygen inhibits the synthesis of nitrate reductase only partially, while the synthesis of nitrite reductase is completely suppressed (35). At the start of anoxic conditions after an aerobic period in intermittently-aerated reactors, only the nitrate reduction takes place, leading to a temporary nitrite accumulation (26). Therefore, the length of non-aeration periods in intermittently-aerated reactors is also important to achieve complete denitrification. Baumann et al. (7) assessed mRNA and the enzymes involved in denitrification in activated sludge reactors operated with intermittent aeration and concluded that denitrifying bacteria were not able to completely synthesize the enzymes for the denitrification process in appropriate amounts when subjected to non-aerated stages shorter than 3h, resulting in the accumulation of nitrite during non-aeration periods. In the current study, only the reactor with a 4h non-aeration period showed considerable nitrite reduction during non-aeration periods.

Comparison of *amoA* DGGE profiles and TKN and ammonia removal efficiencies suggest that superior ammonia and TKN removal efficiencies did not seem to be associated with specific AOB species or with higher AOB species diversity. These results seem to contradict the generally accepted concept that higher functional diversity is beneficial for performance stability (11, 8). However, this concept is necessarily related to the diversity of the

microorganisms that are metabolically active in the process and not merely the species diversity of microorganisms present in the biomass. Although *amoA* is one of the functional genes involved in the oxidation of ammonia, PCR amplification of environmental *amoA* genes does not reveal gene expression or microbial activity. Rather, amplification of the *amoA* gene was used only as an alternative method for identifying AOB and hence can only give an indication of species diversity and not of functional diversity. A more appropriate approach for testing correlation of functional diversity and performance stability would involve methods that can determine which processes are catalyzed by which microorganisms and to what extent.

ACKNOWLEDGEMENTS

REFERENCES

1. **Aakra, A., Utaker, J.B., and Nes, I.F.** 2001. Comparative phylogeny of the ammonia monooxygenase subunit A and 16S rRNA genes of ammonia-oxidizing bacteria. FEMS Microbiol. Lett., **205**: 237-242.

2. **Abeling, U. and Seyfried, C.F.** 1992. Anaerobic-aerobic treatment of high-strength ammonium wastewater – nitrogen removal via nitrite. Wat. Sci. Technol., **26**: 1007-1015.

3. **Alm, E.W., Oerther, D.B., Larsen, N., Stahl, D.A., and Raskin, L.** 1996. The Oligonucleotide Probe Database. Appl. Environ. Microbiol. **62**: 3557–3559.

4. **Avrahami, S., Conrad, R., and Braker, G.** 2002. Effect of soil ammonium concentration on N_2O release and on the community structure of ammonia oxidizers and denitrifiers. Appl. Environ. Microbiol. **68**:5685-5692.

5. **Avrahami, S. and Conrad, R.** 2003. Patterns of community change among ammonia oxidizers in meadow soils upon long-term incubation at different temperatures. Appl. Environ. Microbiol., **69**: 6152-6164.

6. **Barnes, D. and Bliss, P.J.** 1983. Biological control of nitrogen in wastewater treatment. E. & F.N. Spon, New York, NY.

7. **Baumann, B., Snozzi, M., van der Meer, J.R., and Zehder, A.J.B.** 1997. Development of stable denitrifying cultures during repeated aerobic-anaerobic transient periods. Water Res. **31**: 1947–1954.

8. **Briones, A., and Raskin, L.** 2003. Diversity and dynamics of microbial communities in engineered environments and their implications for process stability. Curr. Opin. Biotechnol. **14**: 270-276.

9. **Cheng, J. and B. Liu.** 2001. Nitrification/denitrification in intermittent aeration process for swine wastewater treatment. J. Environ. Eng. **127**: 705-711.

10. **Clesceri, L.S., Greenberg, A.E., and Eaton, A.D.** 1995. Standard Methods for the Examination of Water and Wastewater. 20th ed. APHA, AWWA, and WEF. United Book Press, Inc., Baltimore, MD.

11. **Dejonghe, W., Boon, N., Seghers, D., Top, E.M., and Verstraete, W.** 2001. Bioaugmentation of soils by increasing microbial richness: missing links. Environ. Microbiol. **3**: 649-657.

12. **Garrido, J.M., Van Benthum, W.A.J., Van Loosdrecht, M.C.M., and Heijnen, J.J.** 1997. Influence of dissolved oxygen concentration on nitrite accumulation in a biofilm airlift suspension reactor. Biotechnol. Bioeng. **53**: 168-178.

13. **Gerards, S., Duyts, H., and Laanbroek, H.J.** 1998. Ammonium-induced inhibition of ammonium-starved *Nitrosomonas europaea* cells in soil and sand slurries. FEMS Microbiol. Ecol. **26**: 269-280.

14. **Gieseke, A., Bjerrum, L., Wagner, M., and Amann, R.** 2003. Structure and activity of multiple nitrifying bacterial populations co-existing in a biofilm. Environ. Microbiol. **5**: 355-369.

15. **Hanaki, K, Chalermraj, W., and Shinichiro, O.** 1990. Nitrification at low levels of dissolved oxygen with and without organic loading in a suspended-growth reactor. Water Res. **24**: 297–302.

16. **Harms, G., Layton, A., Dionisi, H., Gregory, I., Garret, V., Hawkins, S., Robinson, K., and Sayler, G.** 2003. Real-time PCR quantification of nitrifying bacteria in a municipal wastewater treatment plant. Environ. Sci. Technol. **37**: 343-351.

17. **Hastings, R.C., Saunders, J.R., Hall, G.H., Pickup, R. W., and McCarthy, A.J.** 1998. Application of molecular biological techniques to a seasonal study of ammonia oxidation in a eutrophic freshwater lake. Appl. Environ. Microbiol. **64**: 3674-3682.

18. **Han, D.W., Chang, J.S., and Kim, D.J.** 2002. Nitrifying microbial community analysis of nitrite accumulating biofilm reactor by fluorescence in situ hybridization. Water Sci. Technol. **47**: 97-104.

19. **Hellinga, C., Schellen, A.A.J.C., Mulder, J.W., Van Loosdrecht, M.C.M., and Heijnen, J.J.** 1998. The Sharon process: an innovative method for nitrogen removal from ammonium-rich wastewater. Water Sci. Technol. **37**: 135-142.

20. **Hovanec T. A., Taylor L. T., Blakis A. and DeLong E. F.** 1998. *Nitrospira*-like bacteria associated with nitrite oxidation in freshwater aquaria. Appl. Environ. Microbiol. **64**: 258-264.

21. **Juretschko, S., Timmermann, G., Schmid, M., Schleifer, K-H, Pommerening-Röser, A., Koops, H-P., and Wagner, M.** 1998. Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. Appl. Environ. Microbiol. **64**: 3042-3051.

22. **Kaewpipat, K. and Grady Jr., CPL** 2002. Microbial population dynamics in laboratory-scale activated sludge reactors. Water Sci. Technol. **46**: 19-27.

23. **Kim, D.J., Chang, J.S., Lee, D.I., Han, D.W., Yoo, I.K., and Cha, G.C.** 2003. Nitrification of high strength ammonia wastewater and nitrite accumulation characteristics. Water Sci. Technol. **47**: 45-51.

24. **Koops, H.-P. and H. Harms.** 1985. Deoxyribonucleic acid homologies among 96 strains of ammonia-oxidizing bacteria. Arch. Microbiol. **141**: 214-218.

25. **Kowalchuk, G.A., Bodelier, P.L.E., Heilig, G.H.J., Stephen, J.R., and Laanbroek, H.J.** 1998. Community analysis of ammonia-oxidizing bacteria in relation to oxygen availability in soils and root-oxygenated sediments using PCR, DGGE, and oligonucleotide probe hybridization. FEMS Microbiol. Ecol. **27**: 339-350.

26. **Krul, J.M. and Veeningen, R.** 1977. The synthesis of the dissimilatory nitrate reductase under aerobic conditions in a number of denitrifying bacteria, isolated from activated sludge and drinking water. Water Res. **11**: 39–43

27. **Liao, J., Lou, I., and de los Reyes III, F.L.** 2004. Relationship of species-specific filament levels to filamentous bulking in activated sludge. Appl. Environ. Microbiol. **70**: 2420-2428.

28. **Mendum, T.A., Hirsch, P.R.** 2002. Changes in the population structure of β-group autotrophic ammonia oxidizing bacteria in arable soils in response to agricultural practice. Soil Biol. Biochem. **34**: 1479-1485.

29. **Mobarry B. K., Wagner M., Urbain V., Rittmann B. E. and Stahl D. A.** 1996. Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. Appl. Environ. Microbiol. **62**: 2156-2162.

30. **Mobarry B. K., Wagner M., Urbain V., Rittmann B. E. and Stahl D. A.** 1997. Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. Erratum. Appl. Environ. Microbiol. **63**: 815a.

31. **Mota, C.R., Ridenoure, J., Cheng, J., de los Reyes III, F.L.** 2005. High levels of nitrifying bacteria in intermittently-aerated reactors treating high ammonia wastewater. FEMS Microbiol. Ecol., in press.

32. **Nicolaisen, M.H., Ramsing, N.B.** 2002. Denaturing gradient gel electrophoresis (DGGE) approaches to study the diversity of ammonia-oxidizing bacteria. J. Microbiol. Meth., **50**:189-203.

33. **Oved, T., Shaviv,A., Goldrath,T., Mandelbaum, R.T., and Minz, D.** 2001. Influence of effluent irrigation on community composition and function of ammonia-oxidizing bacteria in soil. Appl. Environ. Microbiol., **67**: 3426-3433.

34. **Peng, D.C., Bernet, N., Delgenes, J.P., and Moletta, R.** 2000. Effects of oxygen supply methods on the performance of a sequencing batch reactor for high ammonium nitrification. Water Environ. Res. **72**: 195-200.

35. **Philips, S., Laanbroek, H.J., and Verstraete, W.** 2002. Origin, causes and effects of increased nitrite concentrations in aquatic environments. Rev. Environ. Sci. Biotech. **1**: 115-141.

36. **Purkhold, U., Pommerening-Röser, A., Juretschko, S., Schmid, M.C., Koops, H-P., Wagner, M.** 2000. Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and *amoA* sequence analysis: implications for molecular diversity surveys. Appl. Environ. Microbiol. **66**: 5368-5382.

37. **Rotthauwe, J.-H., Witzel, K.-P., and Liesack, W.** 1997. The ammonia monooxygenase structural gene *amoA* as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl. Environ. Microbiol. **63**: 4704-4712.

38. **Sekiguchi, H, Tomioka, N., Nakahara, T., Uchiyama, H.** 2001. A single band does not always represent single bacterial strains in denaturing gradient gel electrophoresis analysis. Biotechnol. Lett. **23**: 1205-1208.

39. **Stahl, D.A., Flesher, B., Mansfield, H., and Montgomery, R.** 1988. Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. Appl. Environ. Microbiol. **54**: 1079-1084.

40. **Stephen, J.R., Kowalchuk, G.A., Bruns, M.A., McCaig, A.E., Phillips, C.J., Embley, T.M., and Prosser, J.I.** 1998. Analysis of the β-subgroup ammonia oxidizer populations in soil by DGGE analysis and hierarchical phylogenetic probing. Appl. Environ. Microbiol. **64**: 2958-2965.

41. **Tappe, W., Laverman, A., Bohland, M., Braster, M., Rittershaus, S., Groeneweg, J., and van Verseveld, H.W.** 1999. Maintenance energy demand and starvation recovery dynamics of *Nitrosomonas europaea* and *Nitrobacter winogradsky* cultivated in a retentostat with complete biomass retention. Appl. Environ. Microbiol. **65**: 2471–2477.

42. **Wagner, M., and Loy, A.** 2002. Bacterial community composition and function in sewage treatment systems. Curr. Opin. Biotechnol. **13**: 218-227.

43. **Wyffels, S., Boeckx, P., Pynaert, K., Verstraete, W., and Van Cleemput, O.** 2003. Sustained nitrite accumulation in a membrane-assisted bioreactor (MBR) for the treatment of ammonium-rich wastewater. J. Chem, Technol. Biotechnol. **78**: 412-419.

44. **Zheng D., Alm E. W., Stahl D. A. and Raskin L.** 1996. Characterization of universal small-subunit rRNA hybridization probes for quantitative molecular microbial ecology studies. Appl. Environ. Microbiol. **62**: 4504-4513.

Table 1. Oligonucleotide probes used in slot blot hybridizations.

| Probe | | Target Site[2] | Sequence (5' to 3') | Wash Temp (ºC) | Target organisms | Ref. |
Name[1]	Abbrev.					
S-*-Univ-1390-a-A-18	Univ1390	1390 - 1407	GACGGGCGGTGTGTACAA	44	All organisms	44
S-G-Nsm-0156-a-A-19	Nsm156	156 - 174	TATTAGCACATCTTTCGAT	46	*Nitrosomonas* spp., *Nitrosococcus mobilis*	29
S-G-βAOB-1224-a-A-20	Nso1225	1224-1243	CGCCATTGTATTACGTGTGA	51	Betaproteobacterial ammonia-oxidizing bacteria	29, 30
S-G-Nbac-1000-a-A-15	Nb1000	1000 - 1014	TGCGACCGGTCATGG	42	*Nitrobacter* spp.	29
S-G-Ntspa-0685-a-A-22	Ntspa685	664 – 685	CACCGGGAATTCCGCGCTCCTC	63	*Nitrospira moscoviensis, Nitrospira marina*	20

[1]Probe names have been standardized according to OPD (3).

[2]*E. coli* numbering.

Table 2. Summary of data for nitrogen and DO profiles during aeration cycles.

Reactor	ANA time ratio (h ON:h OFF)	*Max. DO conc. During aeration (mg L^{-1})	NH_3 conc. (mg N L^{-1})		NO_2^- conc. (mg N L^{-1})	
			Beginning of aeration	End of aeration	Beginning of non-aeration	End of non-aeration
A	1h:1h	5.0	3.7	0.2	0.1	0.1
B	1h: 3h	4.2	8.0	1.2	5.4	4.9
C	0.5h: 1.5h	4.3	10.0	6.0	2.3	3.8
D	0.5h: 2h	3.2	6.3	0.2	0.1	0.9
E	1h: 4h	1.4	12.0	3.6	7.8	0.8

*DO concentrations reached levels below 0.3 mg L^{-1} during non-aeration periods in all reactors.

Table 3. Mean effluent concentrations of nitrogen species during aeration cycles.

Reactor	ANA time ratio (h ON:h OFF)	[1]Mean Effluent Concentrations (mg L^{-1})			
		NH_3-N	NO_2-N	NO_3-N	TKN
A	1h:1h	22.8 (± 39.5)	0.7 (±0.7)	14.9 (±20.1)	40.5 (± 45.4)
B	1h: 3h	22.0 (± 33.0)	1.9 (± 1.8)	10.3 (± 6.3)	51.5 (± 41.9)
C	0.5h: 1.5h	31.9 (± 43.5)	2.5 (± 2.4)	22.7 (± 22.0)	55.4 (± 50.8)
D	0.5h: 2h	23.7 (± 36.5)	2.5 (± 2.3)	16.9 (± 10.9)	48.0 (± 43.6)
E	1h: 4h	23.9 (± 37.4)	2.8 (± 4.6)	15.7 (± 10.8)	49.3 (± 45.2)
[2]ANOVA: P-value		0.721	0.003	0.004	0.597

[1] A total of at least 40 data points were used to calculate mean effluent concentrations. Standard deviations are in parentheses.

[2] Single-factor ANOVA.

Table 4. Mean fraction of nitrifying bacteria measured with slot-blot hybridizations and results of ANOVA analysis.

| Target Microorganism	[1]Average Levels of Nitrifiers (% Total rRNA)					[2]P-value
	A(1h:1h)	B(1h:3h)	C(0.5h:1.5h)	D(0.5h:2h)	E(1h:4h)	
AOB						
Nitrosomonas + *N. mobilis* (Nsm 156/Uni)	9.1 (3.3)	11.3 (5.1)	12.9 (5.0)	10.0 (4.1)	13.6 (5.1)	0.130
AOB (Nso 1225/Uni)	11.4 (4.4)	13.2 (9.0)	17.2 (9.7)	14.4 (5.7)	15.2 (6.1)	0.376
Nitrosomonas + *N. mobilis*/β-AOB	83.3 (11.6)	85.9 (15.7)	78.9 (18.2)	71.9 (18.7)	89.2 (15.1)	0.095
NOB						
Nitrospira (Ntsp 685/Uni)	7.0 (1.6)	5.3 (2.1)	5.9 (2.9)	6.4 (2.8)	4.8 (1.8)	0.168
Nitrobacter (Nb 1000/Uni)	2.6 (2.3)	1.6 (0.9)	2.2 (1.3)	2.2 (2.1)	1.1 (0.4)	0.158
Tot. NOB (Ntsp 685 + Nb 1000/Uni)	9.5 (3.1)	6.9 (2.5)	8.1 (3.7)	8.6 (4.0)	5.8 (2.0)	0.049
Nitrospira/Tot. NOB	75.5 (14.1)	77.0 (9.7)	73.2 (12.5)	75.0 (13.4)	81.4 (5.5)	0.497

[1]A total of 12 data points were used to calculate mean rRNA levels. Standard deviations are in parentheses. All membrane hybridization measurements were performed in triplicate.

[2] P-values from single-factor ANOVA analysis.

Table 5. Nearest GenBank relatives of *amoA* gene fragments retrieved in this study.

DGGE band	GenBank accession no.	Phylotype with highest sequence similarity	Accession number of phylotype	% Seq. Similarity
1	DQ088390	*Nitrosomonas sp. Nm107*	AF272407	99
2	DQ088391	*Nitrosomonas europaea ATCC 19718*	BX321863	91
3	DQ088392	*Nitrosomonas europaea ATCC 19718*	BX321863	95
4	DQ088393	*Nitrosomonas sp. ENI-11*	AB079055	91
5	DQ088394	*Nitrosospira multiformis*	AY177933	92

Figure 1.   Comparison of populations of ammonia oxidizing bacteria (AOB), biomass fractions of AOB, and ammonia and TKN removal efficiencies in intermittently-aerated reactors. (a to e): (◊) TKN removal efficiency; (■) Ammonia removal efficiency; (●) rRNA fraction of AOB in the βProteobacteria subgroup. (f to j): *amoA* DGGE profiles; Sequence analysis of bands numbered 1 to 5 is discussed in the text and summarized in Table 5. (a and f) correspond to day 78; (b and g) correspond to day 170; (c and h) correspond to day 226; (d and i) correspond to day 281; (e and j) correspond to day 310.
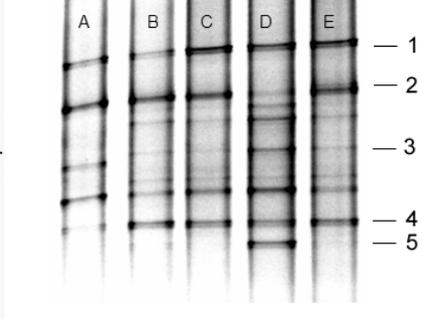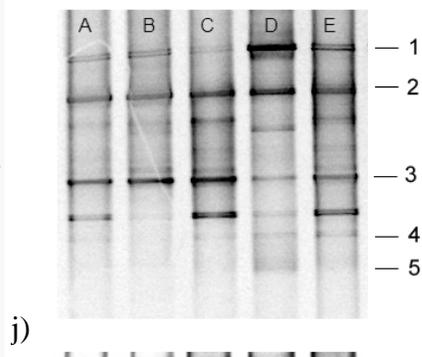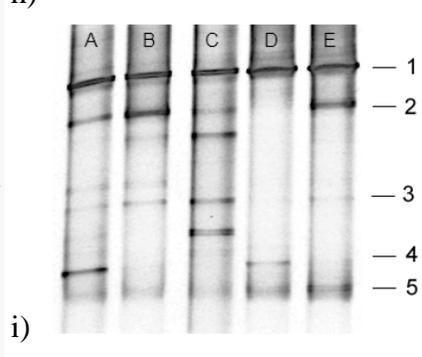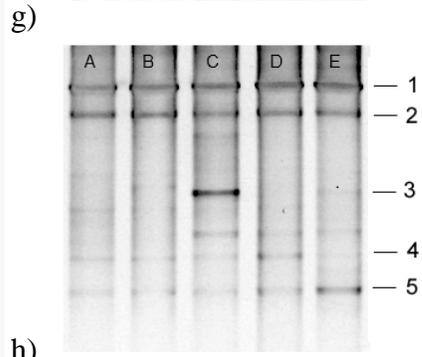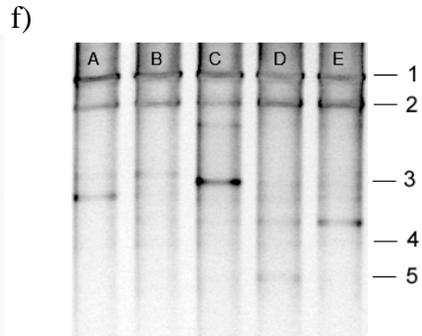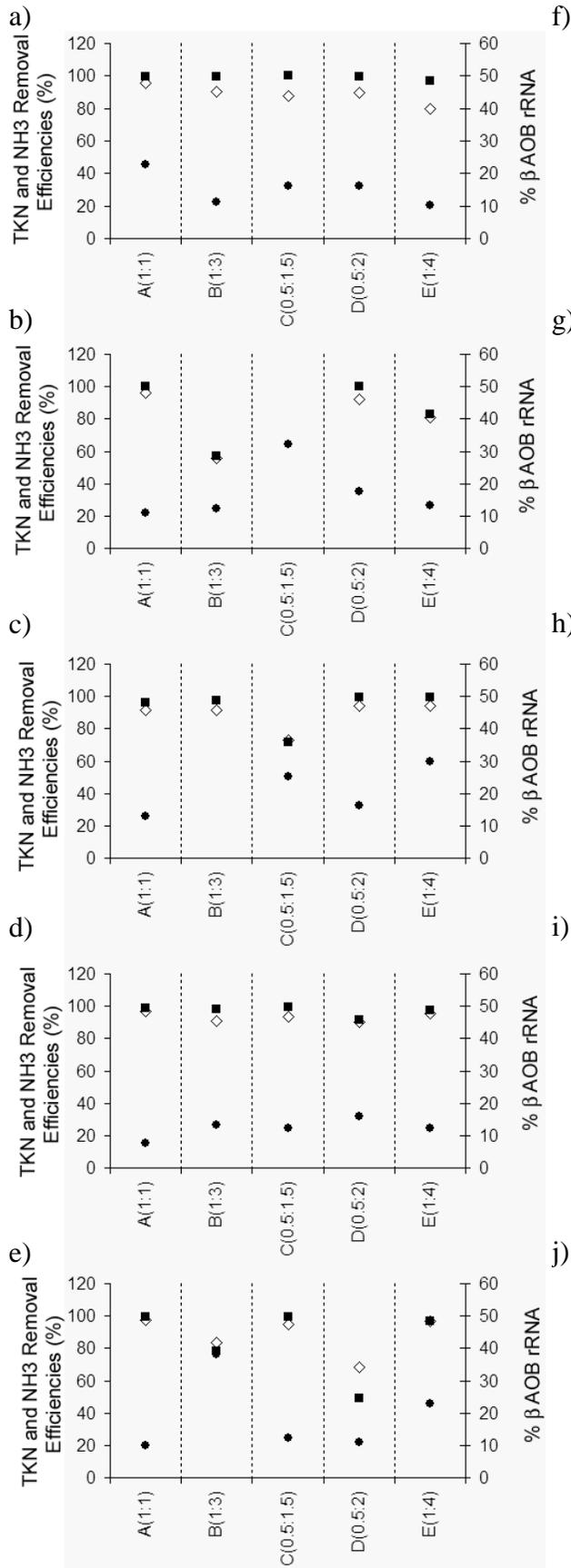
Chapter 3.

IN SITU DETECTION OF ACTIVE NITRITE REDUCERS IN ACTIVATED SLUDGE USING mRNA FISH

ABSTRACT

Active nitrite reducers in activated sludge samples were identified *in situ* using an oligonucleotide probe labeled with horse radish peroxidase (HRP) targeting mRNA of *nirS*, the gene responsible for coding nitrite reductase. Nitrite reductase is the enzyme that catalyzes the reduction of nitrite to nitric oxide. To detect low levels of mRNA in environmental samples, tyramide signal amplification (TSA) was used with a fluorescent TMR dye. Expression clones were built to allow the strict regulation of mRNA content by using IPTG as a gene expression inducer. The TSA FISH method was first tested and fine-tuned using bacterial expression clones. Activated sludge samples were then processed and analyzed using the same methodology. In addition, all three groups of bacteria involved in nitrogen removal from wastewater (ammonia-oxidizing bacteria, nitrite-oxidizing bacteria, and denitrifying bacteria) were simultaneously fluorescently labeled using simultaneous mRNA and 16S rRNA FISH. Results showed close spatial relationships among all three groups of bacteria targeted. A number of bacterial colonies hybridized with both *nirS* mRNA and the 16S RNA of ammonia oxidizing bacteria, suggesting that members of AOB might possess and express *nirS* genes in addition to the already known *nirK* genes present in some AOB such as *Nitrosomonas europaea*.

INTRODUCTION

The ability to denitrify is widespread among a variety of phylogenetically unrelated organisms and was presumably acquired through horizontal gene transfer (Braker and Tiedje, 2003). There are approximately 130 denitrifying bacterial species found within more than 50 genera (Zumft, 1992). This makes the identification and quantification of important denitrifiers in engineered systems a challenging task. In fact, we still do not know which microorganisms are important *in situ* denitrifiers in wastewater treatment plants (Wagner, M. and Loy, A., 2002). The classic approach of using 16S rRNA gene sequences to detect and analyze bacterial communities in environmental samples without isolation and cultivation is not possible when studying denitrifying bacteria (Hallin and Lindgren, 1999). Instead, the phylogenetic diversity of denitrifiers suggests the use of functional probes and PCR primers based on functional genes to detect denitrifying bacteria in general. Primer sets targeting functional genes coding key enzymes of denitrification have been designed to detect *narG* (Gregory et al., 2000), *narH* (Petri and Imhoff, 2000), *nirK* and *nirS* (Braker et al., 1998, Hallin and Lindgren, 1999), *norB* (Braker and Tiedje, 2003), and *nosZ* (Scala and Kerkhof, 1998), the genes coding the enzymes nitrate, nitrite, nitric oxide, and nitrous oxide reductase, respectively. Molecular studies based on denitrification genes are generally limited to assessing the diversity of the targeted genes rather than the phylogenetic groups of bacteria responsible for the process. In addition, the detection of denitrification genes alone does not necessarily mean that the denitrifers detected are metabolically active.

One approach for assessing active bacterial populations catalyzing a specific process *in situ* is to monitor mRNA transcribed from the gene of interest. mRNA is the intracellular

molecule that carries genetic information from DNA to ribosomes, leading to the synthesis of new proteins. The detection of mRNA provides evidence that the organism is actively coding the respective protein and levels of mRNA can sometimes directly correlate with specific metabolic activities (Azam et al., 1999, Ball et al., 1992).

In situ detection of mRNA with labeled probes is a typical technique for studying gene expression in eukaryotic tissues, which has allowed researchers to detect rare mRNA at the single cell level (Speel, 1999, van de Corput et al, 1998, Yang et al., 1999). Recently, Chen et al. (2004) described an *in situ* identification method in which five different fluorochrome-labeled oligonucleotide probes were used to hybridize to mRNA of *fis* and *dps* (genes that regulate cell growth) in enterobacteria. Pernthaler and Amann (2004) described a detailed method for detecting mRNA in prokaryotic cells using long DIG-labeled transcript probes, and were able to detect active methane-oxidizing bacteria in sediments and pure cultures using riboprobes targeting mRNA of *pmoA*, the gene coding the membrane bound particulate methane monooxygenase enzyme (pMMO).

In the current study we were able to identify active nitrite reducers in activated sludge samples using one oligonuleotide probe targeting mRNA of *nirS*, the gene responsible for coding nitrite reductase, the enzyme that catalyzes the reduction of nitrite. To be able to detect low levels of mRNA in environmental samples we used tyramide signal amplification (TSA) with a fluorescent TMR dye. Expression clones were built to allow the strict regulation of intracellular mRNA content by using IPTG as a gene expression inducer. Once appropriate method adjustments were made using bacterial expression clones, activated

sludge samples were processed and analyzed using the same methodology. In addition, we were also able to simultaneously identify all three groups of bacteria involved in nitrogen removal from wastewater: ammonia oxidizing bacteria, nitrite oxidizing bacteria, and denitrifying bacteria.

MATERIALS AND METHODS

Bacterial Cultures

Pseudomonas stutzeri (ATCC 14405) were grown aerobically on 250-ml Erlenmeyer flasks containing nutrient broth in an incubator shaker at 26°C and 120rpm. The *nirS* gene of *P. stutzeri* was induced by transferring aerobically grown cells into pressure tubes, adding 100mg l^{-1} $NaNO_2$-N, and capping the tube with rubber stoppers and aluminum clamps. Cells were harvested by centrifugation at 2000g for 3min and processed for DNA extraction. In addition, cell pellets were fixed for mRNA FISH.

Sample Collection and Fixation

Activated sludge samples were collected from the denitrification tank of the North Cary wastewater treatment plant and from an intermittently-aerated laboratory-scale reactor (air turned on for 1hr and turned off for 4hr) performing nitrification and denitrification in a single tank (Mota et al., 2005). Samples were immediately fixed with chilled 4% paraformaldehyde (by weight) in nuclease-free 1 X phosphate-buffered saline (PBS) (pH 7.4, Ambion Inc., Austin, TX) for 3hr, washed twice with 1 X PBS, resuspended in absolute ethanol, and stored at -20°C.

DNA Extraction and Amplification

DNA was extracted using a PowerSoil DNA isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions. Nucleic acid concentrations

were measured spectrophotometrically. Full-length *nirS* fragments of *Pseudomonas stutzeri*

were amplified using forward (5' - ATCGACATATGAGCAATGTTGGT - 3') and reverse

(5' - ATTATGGATCCTTAGTACACGTCGTT- 3') primers designed in this study. The

primers included extra bases with restriction sites for Nde I and Bam H I, respectively, to

guarantee attachment of restriction enzymes and correct insertion during subsequent

expression cloning. PCR was performed using a thermal cycler (Eppendorf Scientific Inc.,

Westbury, NY) under the following conditions: $94^{o}C$ for 5 min, 28 cycles of $92^{o}C$ for 1.0

min, $56^{o}C$ for 1.0 min, and $72^{o}C$ for 1.0 min, and a final extension at $72^{o}C$ C for 7.0 min.

PCR products were evaluated using 1% (by weight) low melting point agarose gel

electrophoresis.

nirS Expression Clones

nirS from *P. stutzeri* was first cloned using a TA Cloning Kit (Invitrogen, Carlsbad, CA),

according to the manufacturer's instructions. Plasmids were extracted from clones and

purified using a UltraClean™ 6 Minute Mini Plasmid Prep Kit (MoBio Laboratories Inc.,

Carlsbad, CA). Purified plasmids and vector pET16b (EMD Biosciences, Inc., San Diego,

CA) were subjected to a sequential restriction digestion: first with NdeI (Promega Corp.,

Madison, WI) for 1hr at $37^{o}C$, followed by enzyme inactivation for 15min at $70^{o}C$, digestion

with BamHI for 1hr at $37^{o}C$, and final inactivation for 15min at $70^{o}C$. Restriction reactions

were performed using 0.5-ml sterile PCR tubes in a thermal cycler (Eppendorf Scientific Inc.,

Westbury, NY). Digested plasmids and vector were loaded to a 1% agarose gel for

electrophoresis at 80V for 45min. Gel bands of correct size were cored using sterile syringe

needles and purified using a Wizard® SV Gel and PCR Clean-Up System (Promega Corp.,

Madison, WI). Inserts were then ligated to vector pET16b using T4 DNA ligase (Fisher

Bioreagents, Rockville, MD) and incubated for 14hr at 14°C. Ligation reactions were diluted

5 times in sterile water and transformed into E. coli BL21 chemically competent cells (New

England Biolabs Inc., Ipswich, MA) according to manufacturer's instructions. Colonies were

screened using PCR with T7 promoter and T7 terminator primers (Integrated DNA

Technologies, Coralville, IA). PCR was performed as described above.

Oligonucleotide Probes

Throback et al. (2004) designed and assessed several primers for the detection of denitrifying

genes in pure cultures and environmental samples. The *nirS* R3cd primer [5'-GA(C/G) TTC

GG(A/G) TG(C/G) GTC TTG A-3'] was chosen among those primers to be used as an

oligonucleotide probe, as it showed superior sensitivity when compared to other primers. To

be used as a probe, R3cd primer was labeled with horseradish peroxidase (HRP) (Biomers,

Germany).

Oligonucleotide probes targeting the 16S rRNA of AOB and NOB were used in 16S rRNA

FISH (Table 1). Fluorescence-labeled oligonucleotides were synthesized by Integrated DNA

Technologies (Coralville, IA). The oligonucleotides were labeled with Oregon Green, TMR,

or Coumarin dyes.

Table 1. Oligonucleotide probes used in 16S rRNA FISH.

Probe		Target Site[2]	Sequence (5' to 3')	% Formamide	Target organisms
Name[1]	Abbrev.				
S-F-βAOB-1224-a-A-20	Nso1225	1224-1243	CGCCATTGTATTACGTGTGA	35	Betaproteobacterial ammonia-oxidizing bacteria
S-G-Nsr-1156-a-A-22	Nsr1156	1156-1173	CACCGGGAATTCCGCGCTCCTC	30	*Nitrospira moscoviensis, freshwater Nitrospira spp.*

[1]Probe names have been standardized according to OPD.
[2]*E. coli* numbering.

mRNA FISH

mRNA FISH experiments were performed according to the following protocol:

1. Cell fixation and immobilization: Cells were fixed in 4% paraformaldehyde in PBS for 3hr at 40°C, and stored in absolute ethanol at -20°C. Cells were immobilized by transferring 3µl of fixed cells onto microscope slides, dehydrated in absolute ethanol for 3min, and air-dried.

2. Cell permeabilization: Cells were permeabilized by adding 20 µl lysozyme (5mg ml^{-1}) on each well and incubating for 30min at room temperature (RT). Slides were washed with sterile water. Two drops of blocking reagent were added onto each well and slides were incubated at room temperature for 10min. Cells were washed in sterile water and dehydrated in absolute ethanol for 3min.

3. Hybridization: 20ul of hybridization buffer (0.9M NaCl, 50mM Na$_2$HPO$_4$, 0.1% wt/vol SDS, 0.5mg yeast tRNA ml^{-1}, 10X Denhardt's solution) containing 0.05ng/ul of HRP-labeled probe were pipetted onto each well, and slides were incubated overnight at 46°C in humidified chambers.

4. Washing: Slides were washed in wash buffer (0.9M NaCl, 50mM Na$_2$HPO$_4$, 0.1% wt/vol SDS) for 20min at 46°C and then washed in water at RT for 1min.

5. Signal Amplification: 12µl of amplification buffer containing fluorescent tyramide (1:50 dilution by volume) (PerkinElmer Life And Analytical Sciences Inc., Wellesley, MA) were added onto each well and slides were incubated for 5min at RT. Cells were washed 3 times in PBS for 5min at RT, in sterile water for 1min, and absolute ethanol for 3min.

16S rRNA FISH

16S rRNA fluorescent in situ hybridization (FISH) was performed as previously described (de los Reyes et al. 1997). For the simultaneous detection of nitrifying and denitrifying bacteria, 16S rRNA FISH was first performed using probes targeting AOB and NOB, followed by mRNA FISH using a *nirS*-targeted probe.

Fluorescence Microscopy

Images were visualized with a Nikon Optiphot epifluorescence microscope (Nikon, Japan) and a Leica Laser Scanning Confocal Microscope (Leica Microsystems Inc., Bannockburn, IL) located at the NCSU Cellular and Molecular Imaging Facility.

RESULTS

Induced and Uninduced Cells

Expression clones containing the *nirS* gene of *Pseudomonas stutzeri*, a known nitrite reducer*,* were built to allow the strict regulation of intracellular mRNA content by using IPTG as a gene expression inducer. Clones grown without IPTG did not express the inserted *nirS* gene. Since the inserted *nirS* gene was not induced, *nirS* mRNA was not produced and therefore uninduced clones could be used as absolute negative controls after cell fixation (Figure 1). Clones grown on IPTG produced high amounts of *nirS* mRNA and were fixed and used as positive controls (Figure 1). Once method adjustments were made using absolute positive and negative controls, *Pseudomonas stutzeri* cells that were grown aerobically were used as

an additional negative control, as cells were not expressing the *nirS* gene. *Pseudomonas stutzeri* that were grown under anoxic conditions and with nitrite as electron acceptor were used as additional positive controls (Figure 1).

Probe Titration

Serial probe dilutions ranging from 0.005 to 50 ng l^{-1} (probe concentration in the hybridization buffer) were tested on both induced and uninduced *nirS* clones. Uninduced clones showed detectable fluorescence at probe concentrations of 0.5 ng l^{-1} and higher. Induced clones showed strong fluorescent signals even at probe concentrations as low as 0.01 ng l^{-1}. The adopted probe concentration for all hybridizations on environmental samples and on clones was 0.05 ng l^{-1}.

Selection of Fluorophore

Tyramides labeled with coumarin, fluorescein, Cy3, Cy5, and TMR were tested in this study. Significant signal was detected when using all mentioned dyes, except for Cy5, which did not show distinguishable signals between negative and positive controls using a confocal laser scanning microscope (CLSM). TMR showed the strongest signal on both epifluorescent and confocal laser scanning microscopes when used in hybridizations of clones and activated sludge cells (data not shown) and hence was the preferred dye for *in situ* mRNA detection.

Sense and Antisense Probes

mRNA FISH experiments using TSA for improved sensitivity require the use of a sense probe, which is the reverse complement of the antisense, mRNA-targeted probe. A sense probe should not target any mRNA but adding it to cells delivers HRP molecules to samples and helps identify non-specific probe binding and false-positive cell detection. HRP-labeled sense probes were tested at different concentrations on induced and uninduced clones, as well

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as on fixed activated sludge samples. The use of sense probes at concentrations higher than 2ng l^{-1} yielded detectable fluorescent signal on induced clones, uninduced clones, and activated sludge samples. When used at 0.05 ng l^{-1}, the same as the optimal concentration determined for the antisense probe, the sense probe did not show significant signal on environmental samples, induced and uninduced clones (Figure 1).

Figure 1. Epifluorescence micrographs of hybridized cells using *nirS* mRNA–targeted HRP-labeled probes and TMR-labeled tyramides. a) *P. stutzeri* cells containing *nirS* mRNA (grown under anoxic conditions and using nitrite as electron acceptor) and hybridized with *nirS* antisense probe. b) *P. stutzeri* cells without *nirS* mRNA (grown aerobically and without nitrite) and hybridized with *nirS* antisense probe. c) Induced *nirS* clones hybridized with *nirS* antisense probe. d) Induced *nirS* clones hybridized with *nirS* sense probe. e) Induced *nirS* clones hybridized with *nirS* antisense probe. f) Uninduced *nirS* clones hybridized with *nirS* antisense probe. g) Combined micrograph of fluorescent signal and phase contrast of activated sludge hybridized with antisense *nirS* probe. h) phase contrast of activated sludge (same field view as g.).

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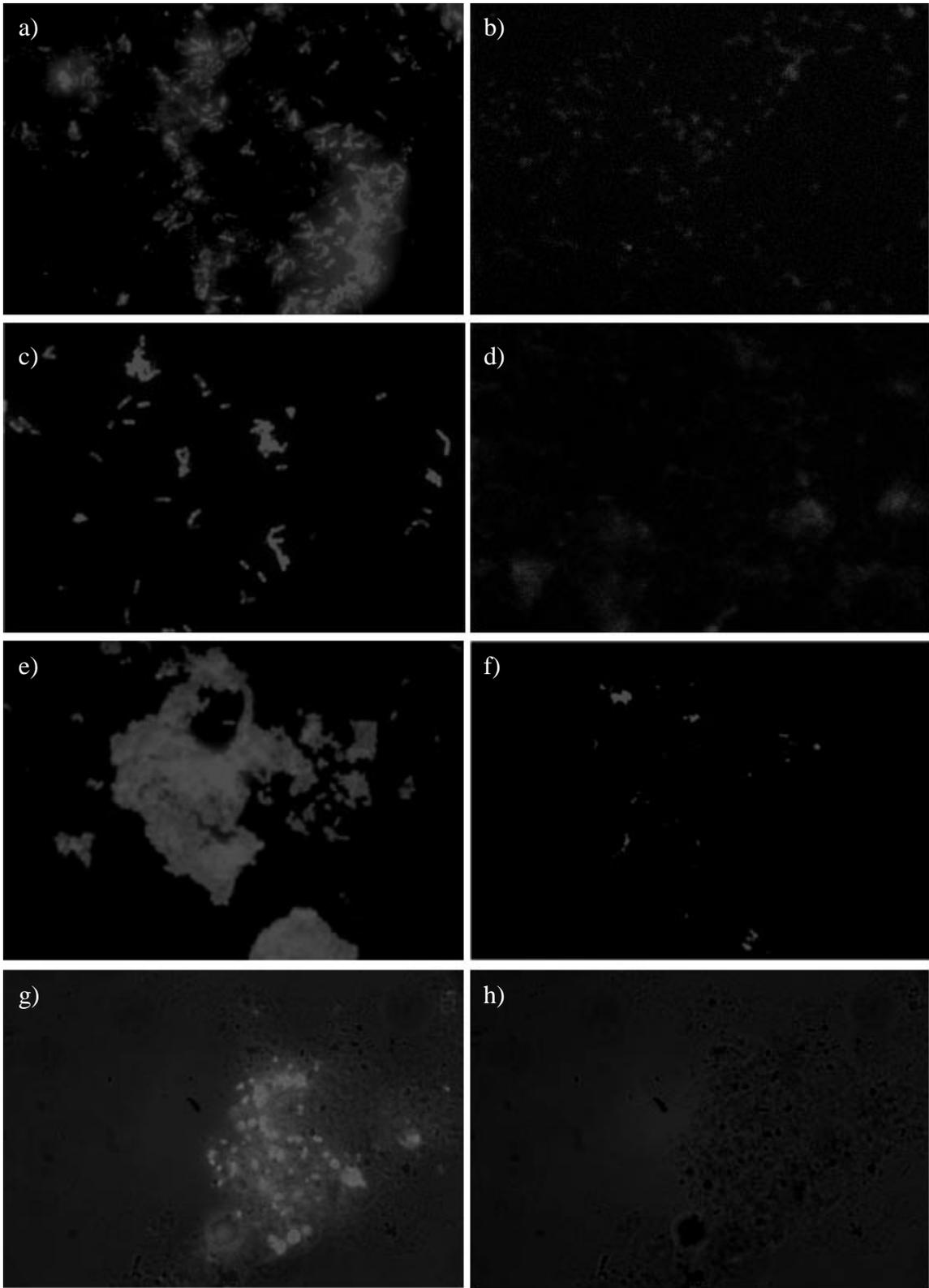
Probe Specificity

To validate newly-designed probes for use on traditional 16S rRNA FISH experiments it is required to perform probe specificity studies using non-target microorganisms that are phylogenetically closely related to the target organisms (usually showing 1, 2, and 3 mismatches with the probe sequence). We tried to apply the same principle and tested probe specificity for targeting mRNA by building expression clones with 2 mismatches with the probe sequence. Expression clones with 2 mismatches were obtained applying the same method used for building zero-mismatch clones, except that two mismatches were added to the reverse primer for the PCR amplification of *P. stutzeri* before insertion into expression plasmids. Clones with 2 mismatches were grown in the presence of IPTG for the expression of the inserted *nirS* gene. Figure 2 shows the quantification of fluorescent signals performed during probe specificity studies. Induced clones with zero-mismatch with the *nirS* probe showed high fluorescent signals with formamide concentrations up to 50% in the hybridization buffer. At 0% formamide concentration in hybridization buffer, average fluorescent signals for activated sludge samples were almost double the signals measured for induced clones with 2-mismatches, suggesting that *in situ* nitrite reducers could be specifically targeted in a mixed community using the described protocol.
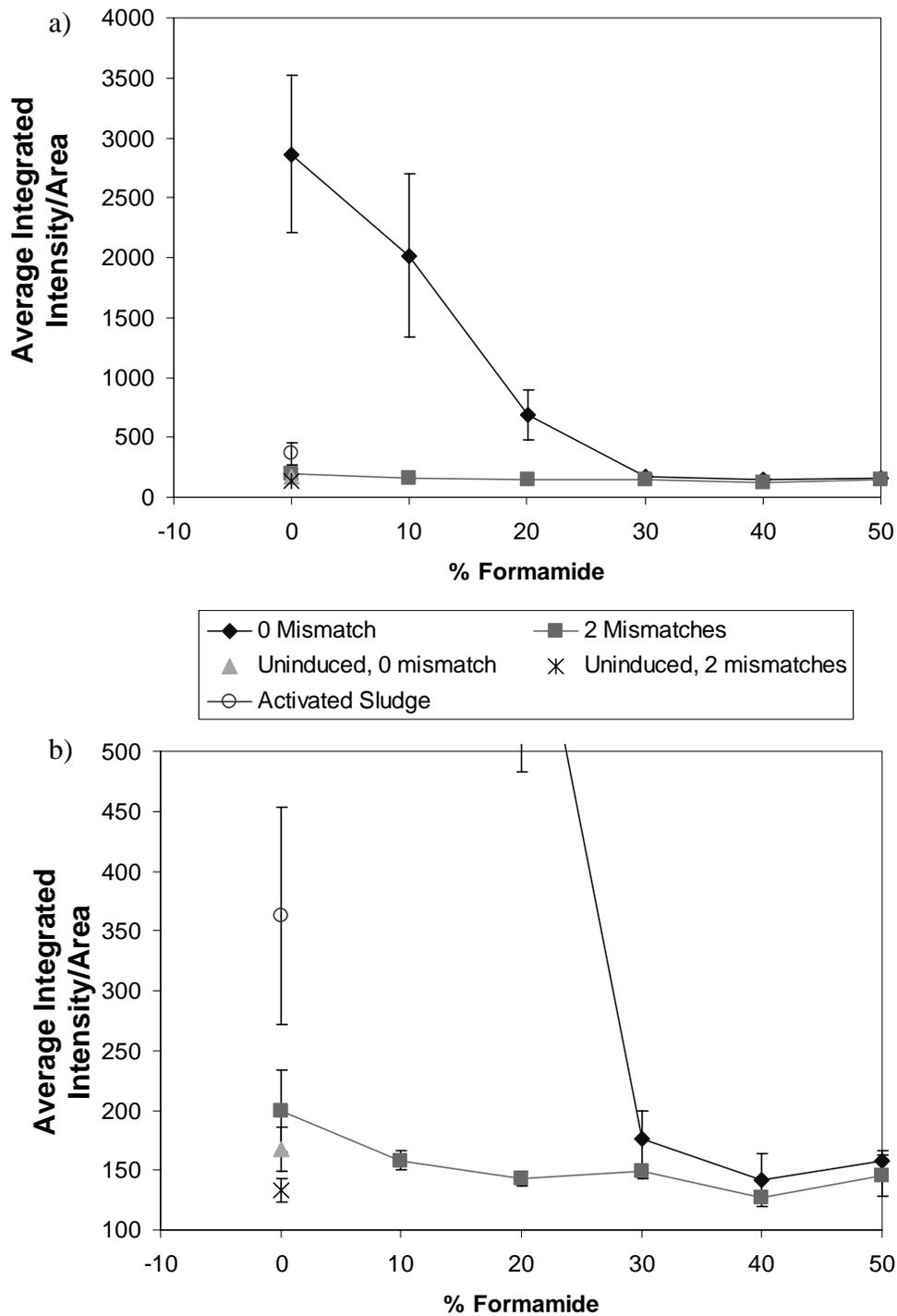
Figure 2. Probe specificity study using induced and uninduced expression clones containing

zero and two mismatches with the *nirS* probe sequence used.

Simultaneous in situ identification of denitrifying, ammonia-oxidizing, and nitrite-oxidizing bacteria

Once the mRNA FISH protocol was optimized and all controls and environmental samples were tested successfully, the method was used in combination with traditional 16S rRNA FISH targeting ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) in activated sludge collected from intermittently-aerated reactors treating swine wastewater (Figure 3). A number of colonies that hybridized to the probe targeting the 16S rRNA of AOB also hybridized to the probe targeting *nirS* mRNA. No overlap of fluorescent signal was detected when NOB-targeted and *nirS* mRNA-targeted probes were used simultaneously. The morphology of cells that hybridized with the *nirS* mRNA probe was diverse, including cocci-, rod-, and filamentous-shaped cells as well as entire colonies inside activated sludge flocs.

Figure 3. Confocal laser scanning micrographs showing the simultaneous in situ identification of nitrite reducing-, nitrite oxiding-, and ammonia-oxidizing bacteria in activated sludge. a) Ammonia-oxidizing bacteria shown in green and nitrite-reducing bacteria shown in red. b) Nitrite-oxidizing bacteria shown in green and nitrite-reducing bacteria shown in red. c) Ammonia-oxidizing bacteria shown in green, nitrite-oxidizing bacteria shown in red and nitrite-reducing bacteria shown in blue.
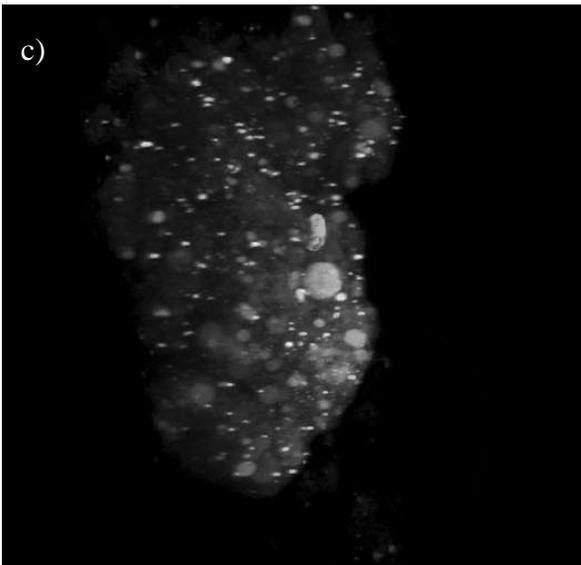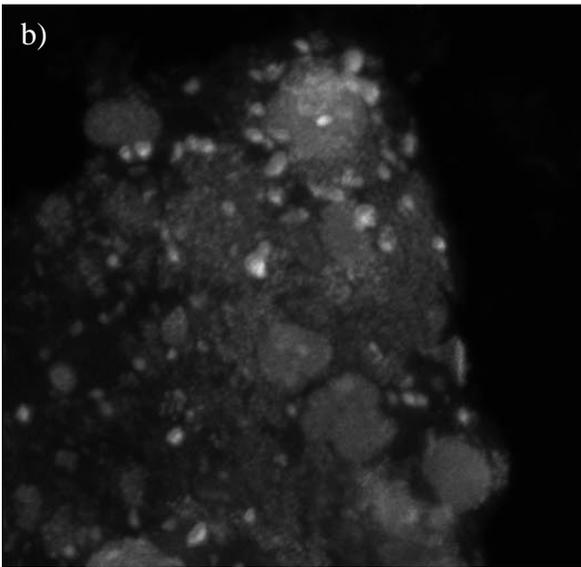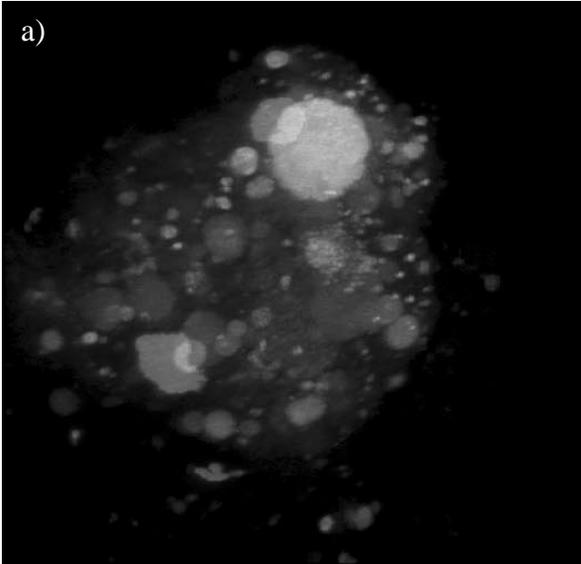
DISCUSSION

This study focused on the *in situ* identification of microorganisms expressing *nirS*, the gene that codes cytochrome cd_1- nitrite reductase. A different nitrite reductase, containing copper rather than cytochrome, is coded by *nirK* gene. These two nitrite reductases are evolutionarily unrelated and structurally different, but functionally equivalent, and catalyze the reduction of nitrite to nitric oxide (Glockner et al. 1993). *nirS* and *nirK* genes seem to occur mutually exclusively in a given strain, but both types have been found in different strains of the same species. *nirS* is more widely distributed, whereas *nirK* is found in only 30% of the denitrifiers studied so far. However, *nirK* is found in a wider range of physiological groups (Braker et al. 1998). Nitrite reductases play a key role among the four dissimilatory reductases of the denitrifying pathway, as this is the step where losses of fixed nitrogen from soil and water into the atmosphere become irreversible (Pinho et al., 2004).

The successful *in situ* detection of mRNA is directly related to the copy number and the integrity of the target molecules. Therefore, any study based on mRNA should use nuclease-free reagents and laboratory apparatus to avoid degradation of mRNA. To circumvent the limitations related to the low copy number of target molecules, we used tyramide signal amplification (TSA). TSA is a powerful technique and has been reported to increase fluorescent signal 20-fold as compared to single fluorophore approaches (Schonhuber et al. 1997). With such high possible signal intensity, it is essential to have appropriate methodological controls to sort out false positive cell detections. Of particular importance is the probe concentration in the hybridization buffer, because the addition of excessive

amounts of probe (and consequently HRP molecules attached to oligos) may result in non-specific binding and fluorescent detection of false-positive cells.

Significant effort was made to ensure that the mRNA FISH method described in the present study was highly sensitive and specific, minimizing false-positive cell detection. When *nirS* mRNA FISH was used in combination with AOB-targeted 16S rRNA FISH, a number of bacterial colonies in activated sludge samples showed significant overlap of signals from both FISH methods. This suggests the presence of *nirS* genes in some members of the β-*Proteobacteria* AOB. The presence of nitrite reductase in *Nitrosomonas europaea*, the most studied ammonia oxidizer, is known from early biochemical work (Hooper 1968). Further studies revealed that the nitrite reductase of *N. europaea* is a copper-containing enzyme, coded by a *nirK* gene, with biochemical similarities to the copper-containing nitrite reductases of traditional heterotrophic denitrifiers (Miller and Nicholas 1985, Ritchie and Nicholas 1974). Casciotti and Ward (2001) have recently discovered the presence of *nirK* genes in several marine ammonia oxidizing bacteria. Although our results show significant evidence supporting the presence of *nirS* in AOB, these findings are still speculative and more extensive studies are needed for final confirmation. If the presence of *nirS* genes in AOB is confirmed, it is likely that *nirS* sequences retrieved from the environment include sequences from ammonia oxidizing bacteria.

The in situ identification of denitrifiers based on mRNA FISH was combined with FISH targeting the 16S rRNA of ammonia and nitrite oxidizers. This approach has great potential for unraveling the spatial and metabolic interactions between the microorganisms involved in

nitrification and denitrification processes. Of particular interest is the simultaneous *in situ* identification of bacteria performing dissimilatory reduction of nitrite and bacteria performing nitrite oxidation in wastewater treatment. The distinction between these two groups of bacteria is important because only the former (nitrite reducers) are capable of producing enzymes that can convert toxic, soluble nitrite into innocuous nitrogen gas, whereas nitrite oxidizers can only convert soluble nitrite to soluble nitrate (which is also a water pollutant). Although nitrite reduction and nitrite oxidation occur under anoxic and aerobic conditions, respectively, microniches in activated sludge flocs and temporal changes in environmental conditions (such as intermittent aeration) allow both processes to occur simultaneously. From the engineering perspective, it is advantageous to inhibit nitrite oxidizing bacteria while maintaining nitrite reducers active. The availability of methods capable of making that distinction can be very valuable if combined with studies aimed at determining the key environmental factors affecting the biomass ratio of both groups of nitrite-metabolizing bacteria.

A similar approach can potentially be used for identifying denitrifiers actively reducing nitrate, nitric oxide, and nitrous oxide by using labeled probes targeting the genes coding the respective enzymes of each process. These probes could be used simultaneously, and the distribution of microorganisms capable of partial and/or full denitrification in activated sludge flocs could be resolved by using different fluorescent labels for each probe. In addition, this approach can be a powerful tool for investigating not only denitrifying bacteria, but also microorganisms involved in virtually all biodegradation processes, as long as there is

enough information on the functional gene of interest available and the levels of mRNA are

not below detection limit.

ACKNOWLEDGEMENTS

REFERENCES

Azam, T., Iwata, A., Nishimura, A., Ueda, S., and Ishihama, A. (1999) Growth phase-dependent variation in protein composition of the *Escherichia coli* nucleoid. J. Bacteriol., 181, pp. 6361-6370.

Ball, C.A., Osuma, R., Ferguson, K.C., and Johnson, R.C. (1992) Dramatic changes in Fis levels upon nutrient upshift in Escherichia coli. J. Bacteriol., 174, pp. 8043-8056.

Braker, G., A. Fesefeldt, and K.-P. Witzel. (1998) Development of PCR primer systems for amplification of nitrite reductase genes (*nirK* and *nirS*) to detect denitrifying bacteria in environmental samples. Appl. Environ. Microbiol. 64, pp. 3769-3775.

Braker, G., and Tiedje, J.M. (2003) Nitric oxide reductase (*norB*) genes from pure cultures and environmental samples. Appl. Environ. Microbiol., 69, No 6, pp. 3467-3483.

Casciotti, K., and Bess, BB. (2001) Dissimilatory nitrite reductase genes from autotrophic ammonia-oxidizing bacteria. Appl. Environ. Microbiol., 67, No. 5, pp. 2213-2221.

Chen, H., Ponniah, G., Salonen, N., and Blum, P. (2004) Culture-independent analysis of fecal enterobacteria in environmental samples by single-cell mRNA profiling. Appl. Environ. Microbiol., 70, No. 8, pp. 4432-4439.

de los Reyes III, F.L., W. Ritter, and L. Raskin (1997) Group-specific small subunit hybridization probes to characterize filamentous foaming in activated sludge systems. Appl. Environ. Microbiol. 63: 1107-1117.

Glockner, A. B., Jungst, A., Zumft, W.G. (1993). Copper-containing nitrate reductase from *Pseudomonas aurofaciens* is functional in a mutationally cytochrome cd_1 free background (*nirS*) of *Pseudomonas stutzeri*. Arch. Microbiol., 160, pp. 18-26.

Gregory, L. G., A. Karakas-Sen, D. J. Richardson, and S. Spiro (2000) Detection of genes for membrane-bound nitrate reductase in nitrate-respiring bacteria and in community DNA. FEMS Microbiol. Lett. 183, pp. 275-279.

Hallin, S., and Lindgren, P.-E. (1999) PCR detection of genes encoding nitrite reductase in denitrifying bacteria. Appl. Environ. Microbiol., 65, No. 4, pp. 1652-1657.

Hooper, AB., (1968) A nitrite-reducing enzyme from *Nitrosomonas europaea*: preliminary characterization with hydroxylamine as electron donor. Biochem. Biophys. Acta, 162, pp. 49-65.

Miller, DJ., and Nicholas, DJD. (1985) Characterization of a soluble cytochrome oxidase/nitrite reductase from *Nitrosomonas europaea*. J. Gen. Microbiol., 131, pp. 2851-2854.

Mota, CR., Head, M., Ridenoure, J., Cheng, J., and de los Reyes III, FL. (2005) Effects of Aeration Cycles on Nitrifying Bacterial Populations and Nitrogen Removal in Intermittently-Aerated Reactors, Appl. Environ. Microbiol., 71, pp. 8565-8572.

Pernthaler, A. and Amann, R. (2004) Simultaneous fluorescence in situ hybridization of mRNA and rRNA in environmental bacteria. Appl. Environ. Microbiol., 70, No. 9, pp. 5426-5433.

Petri, R., and J. Imhoff (2000) The relationship of nitrate reducing bacteria on the basis of *narH* gene sequences and comparison of *narH* and 16S rDNA based phylogeny. Syst. Appl. Microbiol. 23, pp. 47-57.

Pinho, D., Besson, S., Brondino, C., Castro, B., and Moura, I. (2004) Copper-containing nitrite reductase from *Pseudomonas chlororaphis* DSM 50135. Eur. J. Biochem. 271, pp. 2361-2369.

Ritchie, GAF., and Nicholas, DJD. (1974) The partial characterization of purified nitrite reductase and hydroxylamine oxidase from *Nitrosomonas europaea*. Biochem. J., 126, pp. 471-480.

Scala, D. J., and L. J. Kerkhof (1998) Nitrous oxide reductase (*nosZ*) gene-specific PCR primers for detection of denitrifiers and three *nosZ* genes from marine sediments. FEMS Microbiol. Lett. 162, pp. 61-68.

Schonhuber, W., Fuchs, B., Juretschko, S., Amann, R. (1997) Improved sensitivity of whole-cell hybridization by the combination of horseradish peroxidase-labeled oligonucleotides and tyramide signal amplification. Appl. Environ. Microbiol. 63, pp. 3268– 3273.

Speel, E.J.M. (1999) Detection and amplification systems for sensitive, multiple target DNA and RNA in situ hybridization: looking inside cells with a spectrum of colors. Histochem. Cell Biol., 112, pp. 89-113.

Throbäck, IN., Enwall, K, Jarvis, Å., and Hallin, S. (2004) Reassessing PCR primers targeting *nirS*, *nirK* and *nosZ* genes for community surveys of denitrifying bacteria with DGGE. FEMS Microbiol. Ecol., 49, pp. 401-417.

van de Corput, M.P.C., Dirks, R.W., van Gijlswijk, R.P.M., van de Rijke, F.M., and Raap, A.K. (1998) Fluorescence in situ hybridization using horse-radish peroxidase-labeled oligonucleotides and tyramide signal amplification for sensitive DNA and RNA detection. Histochem. Cell Biol., 110, pp. 431-437.

Wagner, M., and Loy, A. (2002). Bacterial community composition and function in sewage treatment systems. Current Opinion in Biotechnology, 13, pp. 218-227.

Yang, H., Wanner, I.B., Roper, S.D., and Chaudhari, N. (1999) An optimized method for in situ hybridization with signal amplification that allows the detection of rare mRNAs. J. Histochem. Cell Biol., 47, pp. 431-445.

Zumft, W. G. (1992). The denitrifying prokaryotes. The Prokaryotes, Edited by A. Balows, H. G. Truper, M. Dworkin, W. Harder and K.-H. Schleifer. New York: Springler Verlag.

Chapter 4.

mRNA FISH AND FLOW CYTOMETRY FOR THE PHYLOGENETIC IDENTIFICATION OF BACTERIAL CELLS SORTED BASED ON ACTIVITY OF FUNCTIONAL GENES

ABSTRACT

In the current study we describe a method for the phylogenetic identification of metabolically active microorganisms responsible for biodegradation of specific pollutants sorted from mixed cultures based on mRNA content. Bacterial cells in activated sludge were fixed *in situ* using paraformaldehyde for subsequent hybridization with a *nirS* mRNA-targeted oligonucleotide probe labeled with horse radish peroxidase (HRP). Following hybridization and cell washes, fluorophore-tagged tyramides were added to the cells and the cleavage product that was deposited in the proximity of HRP molecules conferred a strongly fluorescent signal only to cells that were actively producing *nirS* mRNA, the precursor to nitrite reductase, the enzyme responsible for the dissimilatory reduction of nitrite to nitric oxide. Fluorescent cells were sorted from the background microbial community using flow cytometry (FCM), and were subsequently submitted to phylogenetic analysis based on 16S genes isolated using denaturant gradient gel electrophoresis (DGGE). Phylogenetic analysis of sequences from bands isolated from DGGE profiles of sorted cells indicated that the dominant in-situ nitrite reducers were closely related to *Acidovorax* BSB421. We believe that the described molecular approach has great potential to unravel the longstanding

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microbial ecology challenge of determining which environmental processes are attributed to which microorganisms in natural and engineered habitats.

INTRODUCTION

Phylogenetic studies derived from rRNA sequences have revealed a remarkably vast microbial diversity in biological wastewater treatment systems. However, this genetic marker generally provides little direct evidence regarding the interactions and metabolic capabilities of the microorganisms that these sequences represent. Therefore, the pertinent question of which functions are attributable to which organisms in natural and engineered environments remains largely unanswered. Only recently have a number of studies addressed this question by first establishing the biological process and then identifying the microorganisms that are involved using isotope analysis of nucleic acids (Radajewski et al., 2000, Manefield et al. 2002, McGregor et al. 2002), amino acids (Pelz et al. 1998), and fatty acids (Roslev and Iversen 1999), as well as isotope array (Adamczyk et al. 2003), and microautoradiography combined with FISH (MAR-FISH) (Ouverney and Fuhrman 1999, Hesselsoe et al. 2005).

We have recently described an mRNA FISH protocol for the *in situ* identification of bacteria transcribing genes of interest in activated sludge (Mota and de los Reyes 2006). In brief, mixed bacterial communities are fixed *in situ* using paraformaldehyde for subsequent hybridization with mRNA-targeted gene probes labeled with horse radish peroxidase (HRP). Following hybridization and cell washes, fluorophore-tagged tyramides are added to the cells and the cleavage product is deposited in the proximity of HRP molecules, conferring a

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strongly fluorescent signal only to cells that are actively producing the targeted enzymes responsible for the biodegradation process of interest.

Once cells of interest are labeled with a fluorescent dye targeting the transcripts of the relevant gene, cells can be quantified by optical means and physically sorted using flow cytometry (FCM). During flow cytometry, cell suspensions (alive or fixed at the time of measurement) are passed single-file through a laser beam by continuous flow of a fine stream of the suspension. Each cell scatters some of the laser light, and also emits fluorescent light excited by the laser. The instrument electrically charges droplets containing cells of interest so they can be deflected into a separate test tube. Wallner et al. (1997) described a FCM method for sorting bacteria with fluorescein-labeled rRNA from activated sludge, lake water, and lake sediment. Cell enrichments up to 280-fold were achieved and almost full-length 16S ribosomal DNA fragments from sorted microbial cells could be successfully amplified and sequenced. Bernard et al. (2001) used FCM to sort viable cells from mixed culture cell suspensions using the direct viable count (DVC) method and were able to assess the phylogenetic diversity of the sorted, previously viable cells using DGGE.

In the current study, active nitrite reducing bacteria were identified by hybridizing a *nirS* mRNA-targeted oligonucleotide probe to activated sludge fixed *in situ*. Labeled cells were subsequently sorted using flow cytometry (FCM) and submitted to denaturant gradient gel electrophoresis (DGGE) for proper phylogenetic identification of active nitrite reducers *in situ*. Limitations of the method and potential applications are discussed.

MATERIAL AND METHODS

Sample Collection and Fixation

Activated sludge samples were collected from a denitrification tank of the North Cary wastewater treatment plant. Samples were immediately fixed with chilled 4% paraformaldehyde (by weight) in nuclease-free 1 X phosphate-buffered saline (PBS) (pH 7.4, Ambion Inc., Austin, TX) for 3hr, washed twice with 1 X PBS, resuspended in absolute ethanol, and stored at -20°C until ready for mRNA FISH.

mRNA FISH and Flow Cytometry

mRNA FISH was performed on activated sludge and expression clones containing the *nirS* gene of *Psudomonas stutzeri* according to the protocol described by Mota and de los Reyes (2006) with minor modifications. Fluorescein-tagged tyramides were used rather than TMR-tagged tyramides, and fixed cells were mixed with 20µl 0.1% agarose (low melting point) per slide well for optimum cell immobilization to and subsequent elution from microscope slides. In addition, activated sludge flocs were broken by repeated pumping (at least 10 times) of cells through a 1-ml syringe containing a 23-gauge needle prior to cell immobilization to microscope slides.

After the mRNA FISH protocol was completed, cells were eluted from microscope slides by adding 30µl sterile PBS buffer pre-warmed to 65°C. Warm buffer was pipetted in and out of slide wells several times to melt the agarose and resuspend hybridized cells for subsequent FCM analysis. Resuspended cells were transferred into sterile FCM tubes, 1ml sterile PBS buffer was added to each tube, and cells were submitted to FCM analysis within 4 hours after resuspension. Flow cytometric analsysis and cell sorting were performed using a Dako

Cytomation MoFlo (Cytomation Inc., Fort Collins, CO) cytometer located at the North Carolina State University College of Veterinary Medicine. The flow cytometer was equipped with an iCyte 200mw Blue Sappjire 488nm laser, a Melles Groit Helium-Neon laser, and a UV laser. Cells were sorted directly into sterile 15-ml centrifuge tubes and sterile PBS buffer was used as sheath fluid.

PCR and DGGE

Sorted cells were centrifuged at 5000 x g for 5 min, washed with sterile water twice, resuspended in 30µl sterile water, and frozen at -80°C. Cells were thawed on a heated block at 65°C for 5 min, transferred to -80°C for 5 min, transferred back to 65°C for 5 min, and placed on ice for PCR amplification (2µl of thaw-freeze treated cells were used per PCR reaction). 16S rRNA gene fragments were amplified using a nested approach in which products of an initial round of PCR with primers Bact8F and Univ1492 were agarose purified and used as template for a second round of PCR using primers Bact341 (with a GC clamp attached to its 5'-end) and Bact926. Agarose purification of PCR products was performed by coring bands of the correct size using a sterile plastic pipette tip and transferring the excised core into a sterile PCR tube for the second round of PCR. Amplifications were performed using 0.5 µM of each primer, 25 µL of FailSafe PCR System Reaction Mix E (Epicentre Technologies, Madison, WI), and sterile water to a total reaction volume of 50 µL. PCR was performed using a thermal cycler (Eppendorf Scientific Inc., Westbury, NY) under previously described conditions (Mota et al. 2005).

Amplified 16S rRNA gene fragments were separated on 6% (wt/vol) acrylamide-bisacrylamide gels with a denaturant gradient of 30 to 60% urea-formamide at 60°C and

200V for 6 h using a DCode System (BioRad Laboratories, Hercules, CA). The gels were stained with SYBR Gold for 30 min (Molecular Probes, Inc., Eugene, OR), visualized with a Dark Reader transilluminator (Clare Chemical Research, Inc., Dolores, CO) and photographed with a Canon Powershot A70 digital camera (Canon USA, Inc., Chesapeake, VA). Sterile syringe needles were used to excise DGGE bands (approximately a third of the band width) and excised bands were transferred into sterile PCR tubes for re-amplification and subsequent DGGE for checking band purity before sequencing.

Sequencing

Purified and re-amplified bands were sequenced using reverse primer Bact926 at the Duke University DNA Sequencing Facility using a Perkin Elmer Dye Terminator Cycle Sequencing system with AmpliTaq DNA Polymerase combined with ABI 3730 and 3100 PRISM DNA sequencing instruments and BigDyeTMv1.1 terminator.

RESULTS

Flow Cytometry

Induced clones, uninduced clones, and activated sludge cells fixed *in situ* were hybridized with a *nirS* mRNA-targeted oligonucleotide probe and labeled with fluorescein-tagged tyramides. Agarose concentrations of 1%, 0.5%, and 0.1% were mixed with fixed cells prior to mRNA FISH to minimize cell loss during mRNA FISH and to test for proper elution of cells from microscope slides using warm PBS buffer for cells resuspension and subsequent flow cytometric analysis. Addition of 1% agarose resulted in almost complete loss of cells from microscope slides during mRNA stringent washes. 0.5% and 0.1% agarose showed adequate cell retention during mRNA FISH and cell elution prior to FCM analysis (data not shown). The use of 0.5% agarose resulted in decreased fluorescent signal during FCM

analysis as compared to 0.1% agarose. Therefore, 0.1% agarose was used for all mRNA FISH experiments for FCM analysis.

Sorting gates were first set based on FCM analysis of induced and uninduced clones and the same gates were then used to sort activated sludge sample (Figure 1). Cells showing strong fluorescence were gated as positive and cells with weak fluorescence were gated as negative. Cells showing intermediate fluorescence were not gated. 2.7% of uninduced clones were gated as positive and 27.5% were gated as negative. 31.0% of induced clones were gated as positive and 51.5% were gated as negative. 9.3% of activated sludge cells were gated as positive and 22.3% were gated as negative. A total of 4.4 x 10^5 cells were analyzed from the hybridized activated sludge sample. Approximately 100000 cells were gated as positive and 250000 cells were gated as negative. The remainder of the cells was not sorted.

Figure 1. Cytograms of induced *nirS* clones (a), uninduced *nirS* clones (b), and activated sludge (c) hybridized with a *nirS* mRNA-targeted oligonucleotide probe and labeled with fluorescein-tagged tyramides.
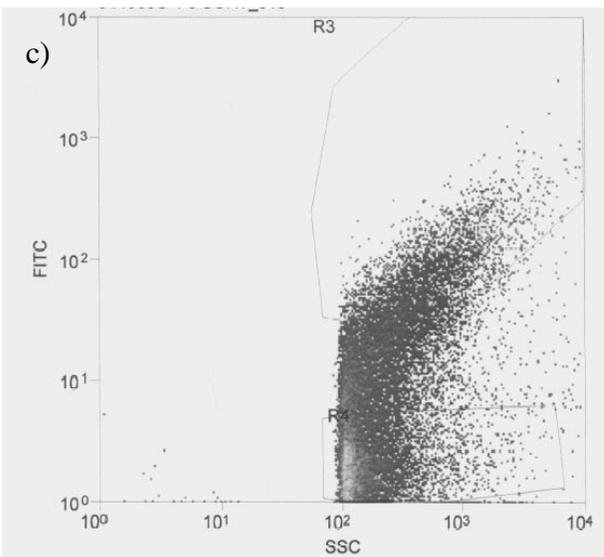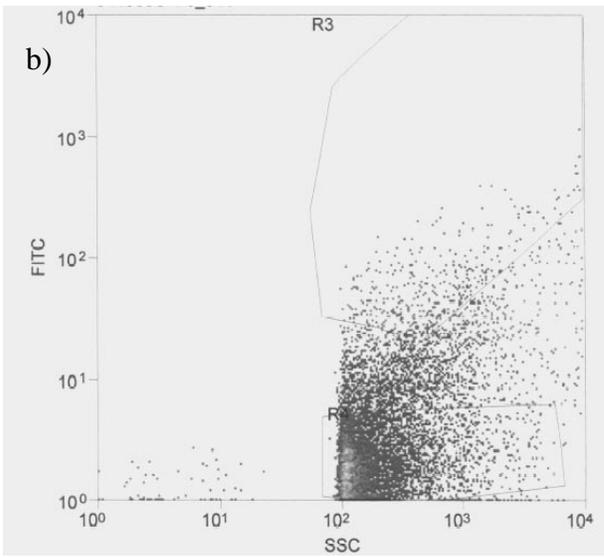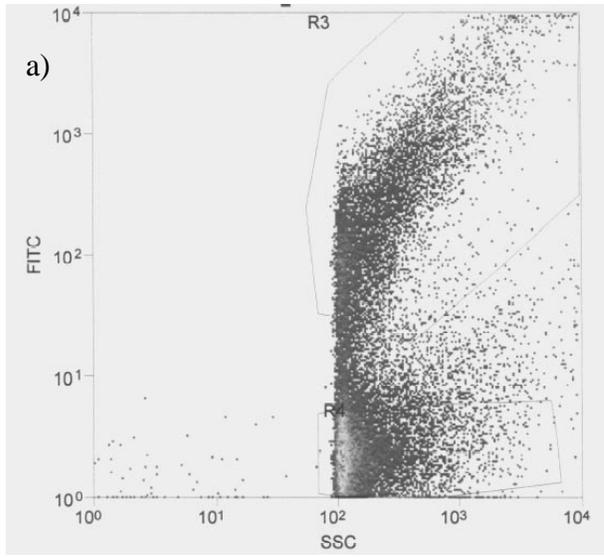
DGGE Profiles and Band Sequences

Figure 2 shows the DGGE profiles of positively and negatively gated activated sludge cells sorted by FCM. The DGGE profile of positively gated cells, which should consist mostly of nitrite reducing bacteria, shows two clear dominant bands that are also present at the DGGE profile of negatively gated cells but at much lower intensity, suggesting that the microorganisms represented by those bands are the dominant nitrite reducing bacteria *in situ*. Phylogenetic analysis of sequences retrieved from DGGE bands (Table 1) indicates that the two dominant bands in the positively gated profile correspond to close relatives of *Brevundimonas* sp. 52AD23 (85% sequence similarity, band 3) and *Acidovorax* BSB421 (100% sequence similarity, band 4) (Table 1). Bands 6 and 7, which represent close relatives of *Deinococcus* sp. CC-FR2-10 (88% sequence similarity) and *Methylobacterium* sp. RKT-5 (99% sequence similarity), respectively, also appeared stronger on the DGGE profile of positively gated cells as compared to the profile of negatively gated cells.

Table 1. Nearest GenBank relatives of 16S rRNA gene fragments retrieved in this study.

| DGGE band	Phylotype with highest sequence similarity	% Seq. Similarity
1	*Bacterium* 7B4	99
	Flavobacterium sp. TSBY-11	96
2	*Brevundimonas nasdae*	98
3	*Brevundimonas* sp. 52AD23	85
4	*Acidovorax* sp. BSB421	100
5	*Comamonas testosterone*	98
6	*Deinococcus* sp. CC-FR2-10	88
7	*Methylobacterium* sp. RKT-5	99
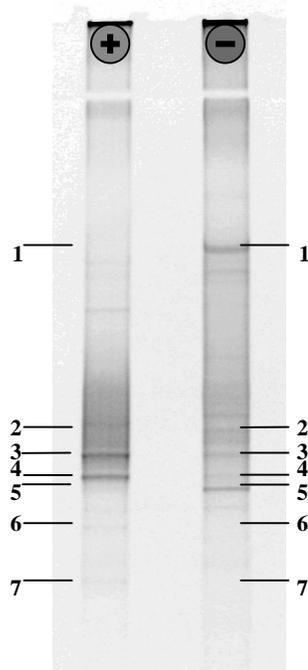
Figure 2. DGGE profiles of FCM sorted activated sludge cells based on fluorescein signal after *nirS* mRNA FISH. Excised bands are numbered and corresponding phylotypes with highest sequence similarity are shown in Table 1. (+) indicates the DGGE profile of positively gated cells during FCM sorting, rich in *nirS*-expressing bacteria. (-) indicates the DGGE profile of negatively gated cells sorted by FCM.

DISCUSSION

Flow cytometry (FCM) is the measurement of physicochemical characteristics of cells as they flow through an observation channel. 16S rRNA FISH (with monolabled fluorophores or using tyramide signal amplification, also known as catalyzed reporter deposition fluorescence in situ hybridization - CARD-FISH, for increased sensitivity) followed by FCM has been successfully used on a wide range of biomedical, biotechnological, and environmental microbiology studies (Gruden et al. 2004). FCM requires that cells be in suspension, whereas CARD-FISH is usually performed in membrane- or microscope slide-immobilized cells. Therefore, the usual CARD-FISH protocols cannot be readily used for

subsequent FCM analysis. Sekar et al. (2004) recently developed a method for CARD-FISH on membrane filters and subsequent high-recovery resuspension of cells from the filters. In this study we described an alternative solution for the same problem. Cells were mixed with 0.1% agarose prior to immobilization to microscope slides. The benefits of agarose mixing to cells were two-fold: (i) to minimize cell loss during mRNA FISH protocol, and (ii) to allow elution of cells from microscope slides once agarose is melted by adding warm PBS buffer for subsequent FCM analysis.

One limitation of FCM for microbial ecology studies is the fact that FCM does not separate microbial aggregates, such as activated sludge flocs, during cell sorting. Rather, fluorescent cells inside activated sludge flocs are sorted together with non-fluorescent cells that are attached to them. Therefore, it is important to break activated sludge flocs prior to FCM analysis to minimize sorting of false positive cells. In this study, fixed activated sludge flocs were broken by the repeated pumping of cells through a 1-ml syringe containing a 23-gauge needle. Although this technique significantly decreased average activated sludge floc size (data not shown), sorting of false-positive cells during FCM analysis could not be ruled out. Therefore, phylogenetic analysis of FCM sorted activated sludge cells should focus on significant differences, rather than absolute differences, between phylogenetic data corresponding to positively and negatively gated cells.

For the phylogenetic analysis of positively and negatively sorted cells, DGGE was preferred over clone libraries, because dominant microorganisms can be easily distinguished by comparing band intensities between two or more DGGE profiles. In this study, the DGGE

profile of positively gated cells showed two clearly dominant bands (bands 3 and 4) that were also present in the DGGE profile of negatively gated cells, but at much lower intensities, suggesting that the microorganisms which those two bands represent are the dominant nitrite reducing bacteria *in situ.* Sequencing of bands 3 and 4 showed that the bands correspond to bacteria closely related to *Brevundimonas* sp. 52AD23 (85% sequence similarity) and *Acidovorax* BSB421 (100% sequence similarity), respectively.

Acidovorax sp. BSB421 was isolated from activated sludge treating municipal wastewater using agar plates after serial dilution (Schulze et al. 1999). Currently, the GenBank database has only one nucleotide entry for *Acidovorax* sp. BSB421, which corresponds to its 16S rRNA gene. *Acidovorax* spp. (formally *Pseudomonas* spp.) are gram-negative, rod-shaped, polybetahydroxybutyrate (PHB)-accumulating bacteria that belong to the *beta* subclass of *Proteobacteria*. A new 16S rRNA probe has been recently designed to target all *Acidovorax* species (including *Acidovorax* sp. BSB421) and FISH experiments using the new probe have shown that these bacteria are highly abundant in municipal wastewater treatment plants (Amann et al. 1996, Schulze et al. 1999). *Acidovorax* sp. BSB421 has been detected in denitrifying fluidized bed reactors (Hwang et al. 2005), in a continuous-upflow fixed-bed reactor for denitrification of drinking water with bacterial polyester as carbon source (Mergaert et al. 2001), in nitrate- and uranium-contaminated groundwater (Yan et al. 2003), and in a poly(3-hydroxybutyrate-co-3-hydroxyvalerate)-degrading denitrifying community in activated sludge (Khan et al. 2002). More recently, *Acidovorax* sp. BSB421 has been isolated from a diverse halobenzoate-degrading denitrifying bacterial consortium in soil and sediments (Song et al. 2000) and from denitrifying activated sludge (Heylen et al 2006).

Although there are numerous reports suggesting that *Acidovorax* sp. BSB421 are denitrifiers, the GenBank database does not show any protein entry that would clarify whether *Acidovorax* sp. BSB421 can reduce nitrate and/or nitrite, and whether it has *nirK* or *nirS* genes. However, the GenBank database has a total of 8 entries corresponding to nitrite reductase genes of other species of the *Acidovorax* group, which are close relatives to *Acidovorax.* sp. BSB421. *Acidovorax* sp. R-25052, *Acidovorax* sp. R-24613, *Acidovorax* sp. R-24614, *Acidovorax* sp. R-24336, *Acidovorax* sp. R-25075, and *Acidovorax* sp. R-25076 have *nirK* genes for nitrite reductases. *Acidovorax* sp. R-25212 and *Acidovorax* sp. 2FB7 have *nirS* genes for nitrite reductases. The *nirS* genes of *Acidovorax* sp. R-25212 and *Acidovorax* sp. 2FB7 have 2 mismatches and 1 mismatch, respectively, with the *nirS* probe used in this study. Although this provides additional evidence that the described mRNA FISH/FCM method correctly identified *Acidovorax* sp. BSB421 as the dominant nitrite reducer *in situ*, sequencing of the *nir* gene of *Acidovorax* sp. BSB421 would provide undisputable evidence. We have asked the research group originally responsible for isolating and classifying *Acidovorax* sp. BSB421 (Stefan Spring, previously at the University of Munich and currently at DSMZ) for the bacterial culture for sequencing of its *nir* gene.

Brevundimonas are α-*Proteobacteria* of the family of *Caulobacteraceae*. *Brevundimonas* sp. 52AD23 has been recently isolated from tomato leaves. The literature does not show any evidence that members of the *Brevundimonas* group are denitrifiers. The only related GenBank database entry corresponds to the pathogenic strain *Brevundimonas pseudomallei* K96243, which has a *nirB* gene similar to that of *E. coli*. The *nirB* gene of *E. coli* codes a

soluble cytoplasmic nitrite reductase that reduces nitrite to nitric oxide but is not capable of dissimilatory nitrite reduction and therefore is not a denitrifier that would be of significance in WWT. Thus, it is possible that *Brevundimonas* sp. 52AD23 is a false positive cell detected using mRNA FISH/FCM. To test whether *Brevundimonas* sp. 52AD23 is really a false positive, these microorganisms have to be grown in pure culture and their *nirS* gene fragments amplified. If the result is a PCR product of the expected size, the amplified nucleic acid should be purified and sequenced for confirmation of *nirS* probe target sites.

Alternatively, if inocula are not available or if cells cannot be grown in pure cultures, oligonucleotide probes targeting the 16S rRNA of *Brevundimonas* sp. 52AD23 (a probe would need to be designed) and *Acidovorax* sp. BSB421 (a probe has already been designed and tested for FISH applications by Schulze et al. 1999) could be used in combination with *nirS* mRNA-FISH. The overlapping of fluorescent signals conferred by 16S rRNA FISH and *nirS* mRNA-FISH would provide additional evidence supporting the findings reported in this study.

In spite of the discussed limitations, we believe that the mRNA FISH/FCM molecular approach has great potential to unravel the longstanding microbial ecology challenge of determining which environmental processes are attributed to which microorganisms in natural and engineered habitats.

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REFERENCES

Adamczyk, J., Hesselsoe, M., Iversen, N., Lehner, A., Nielsen, PH., Schloter, M., Roslev, P., and Wagner, M. (2003) The isotope array, a new tool that employs substrate-mediated labeling of rRNA for determination of microbial community structure and function. Appl. Environ. Microbiol., 69, pp. 6875-6887.

Bernard, L., Courties, C., Duperray, C., Schafer, H., Muyser, G., and Lebaron, P. (2001) A new approach to determine the genetic diversity of viable and active bacteria in aquatic ecosystems. Cytometry, 43, pp. 314-321.

de los Reyes III, F.L., W. Ritter, and L. Raskin (1997) Group-specific small subunit hybridization probes to characterize filamentous foaming in activated sludge systems. Appl. Environ. Microbiol. 63: 1107-1117.

Gruden, C., Skerlos, S., and Adriaens, P. (2004) Flow cytometry for microbial sensing in environmental sustainability applications: current status and future prospects. FEMS Microbiol. Ecol., 49, pp. 37-49.

Hesselsoe, M., Nielsen, JL., Roslev, P., and Nielsen, PH. (2005) Isotope labeling and microautoradiography of active heterotrophic bacteria on the basis of assimilation of $^{14}CO_2$. Appl. Environ. Microbiol., 71, pp. 646-655.

Heylen, K., Vanparys, B., Wittebolle, L., Verstraete, W., Boon, N., and De Vos, P. (2006) Cultivation of denitrifying bacteria: optimization of isolation conditions and diversity study. Appl. Environ. Microbiol., 72, pp. 2637-2643.

Khan, ST., Horiba, Y., Yamamoto, M., and Hiraishi, A. (2002) Members of the family *Comamonadaceae* as primary poly(3-hydroxybutyrate-co-3-hydroxyvalerate)-

degrading denitrifiers in activated sludge as revealed by a polyphasic approach. Appl. Environ. Microbiol., 68, pp. 3206-3214.

MacGregor, B. J., V. Bruchert, S. Fleischer, and R. Amann. (2002) Isolation of small-subunit rRNA for stable isotopic characterization. Environ. Microbiol. 4, pp. 451-464.

Manefield, M., Whiteley, AS., Griffiths, RI., and Bailay, MJ. (2002) RNA stable isotope probing, a novel means of linking microbial community function to phylogeny. Appl. Environ. Microbiol., 68, pp. 5367-5373.

Mergaert, J., Boley, A., Cnockaert, M.C., Müller, W-R., and Swings, J. (2001) Identity and Potential Functions of Heterotrophic Bacterial Isolates from a Continuous-Upflow Fixed-Bed Reactor for Denitrification of Drinking Water with Bacterial Polyester as Source of Carbon and Electron Donor. System. Appl. Microbiol, 24, pp. 303-310.

Mota, CR., Head, M., Ridenoure, J., Cheng, J., and de los Reyes III, FL. (2005) Effects of Aeration Cycles on Nitrifying Bacterial Populations and Nitrogen Removal in Intermittently-Aerated Reactors, Appl. Environ. Microbiol., 71, pp. 8565-8572.

Mota, CR., Hyman, M., and de los Reyes III, FL. (2006) In situ detection of active nitrite reducers in activated sludge using mRNA FISH. (in preparation).

Ouverney, C., and Fuhrman, JA. (1999) Combined microautoradiography-16S rRNA probe technique for determination of radioisotope uptake by specific microbial cell types in situ. Appl. Environ. Microbiol., 65, pp. 1746-1752.

Pelz, O., Cifuentes, LA., Hammer, BT., Kelley, CA., and Coffin, RB. (1998) Tracing the assimilation of organic compounds using δ13C analysis of unique amino acids in the bacterial peptidoglycan cell wall. FEMS Microbiol. Ecol., 25, pp. 229-240.

Radajewski, S., Ineson, P., Parekh, NR., and Murrell, JC. (2000) Stable-isotope probing as a tool in microbial ecology. Nature, 403, pp. 646-649.

Roslev, P., and Iversen, N. (1999) Radioactive fingerprinting of microorganisms that oxidize atmospheric methane in different soils. Appl. Environ. Microbiol., 65, pp. 4064-4070.

Schulze, R., Spring, S., Amann, R., Huber, I., Ludwig, W., Schleifer, K-H., and Kampfer, P. (1999) Genotypic diversity of *Acidovorax* strains isolated from activated sludge and description of *Acidovorax defluvii* sp. nov. System. Appl. Microbiol., 22, pp. 205-214.

Sekar, R., Fuchs, B., Amann, R., and Pernthaler, J. (2004) Flow sorting of marine bacterioplankton after fluorescence in situ hybridization. Appl. Environ. Microbiol., 70, pp. 6210-6219.

Song, B., Palleroni, N., and Haggblom, MM. (2000) Isolation and characterization of diverse halobenzoate-degrading denitrifying bacteria from soil and sediments. Appl. Environ. Microbiol., 66, pp. 3446-3453.

Wallner, G., Fuchs, B., Spring, S., Beisker, W., and Amann, R. (1997) Flow sorting of microorganisms for molecular analysis. Appl. Environ. Microbiol., 63, No. 11, pp. 4223-4231.

Yan, T., Fields, MW., Wu, L., Zu, Y., Tiedle, J., Zhou, J. (2003) Molecular diversity and characterization of nitrite reductase gene fragments (*nirK* and *nirS*) from nitrate- and uranium-contaminated groundwater. Environ. Microbiol., 5, pp. 13-24.

Chapter 5.

CONCLUSIONS AND RECOMMENDATIONS

The objective of this study was to investigate the microbial ecology of nitrogen-removing bioreactors treating swine wastewater and the effects of key operating conditions on nitrogen removal efficiency. We believe that better understanding of the microbial ecology of engineered biodegradation systems can ultimately lead to improved process efficiency and stability. In this study it was demonstrated, using chemical and microbiological data, that improved performance of nitrogen removing systems could be achieved at lower costs by minimizing the amount of air added to the system. In addition, a pioneering approach was developed based on novel molecular methods to identify microorganisms responsible for catabolic transformations of particular pollutants in natural and engineered systems. The identification of microorganisms based on catabolic traits presents great potential for improving biodegradation systems

Changes in the fractions of ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) were monitored in laboratory-scale intermittently aerated reactors operated with different aeration cycles using 16S rRNA- and *amoA*-based molecular approaches. *Nitrosomonas/N. mobilis* were found to be the dominant AOB and *Nitrospira* were the dominant NOB in all reactors. *Nitrosomonas* sp. Nm107 was detected in all reactors, regardless of the reactor's performance. Close relatives of *Nitrosomonas europaea, Nitrosomonas* sp. ENI-11, and *Nitrosospira multiformis* were occasionally detected in all reactors. NOB were more sensitive than AOB to long anoxic periods, and nitrite

accumulation and lower total NOB rRNA levels were observed for reactors with long anoxic periods. Anoxic periods of 4 h resulted in partial nitrification, followed by denitrification via nitrite, suggesting that efficient nitrogen removal can be achieved at lower operational costs due to savings related to lower oxygen and organic matter system requirements.

An innovative approach was developed in this study for the identification of bacteria based on the presence and activity of functional genes. This consists of performing mRNA-FISH targeting functional genes of interest and subsequent flow cytometric sorting of labeled cells for proper phylogenetic identification based on 16S rRNA genes. mRNA fluorescent in situ hybridization (FISH) was performed using tyramide signal amplification (TSA) and horse radish peroxidase (HRP)-labeled oligonucleotide probes targeting transcripts of *nirS*, the gene that codes cytochrome-containing nitrite reductase, an important enzyme that catalyzes the reduction of nitrite to nitric oxide. For the first time, the simultaneous *in situ* detection of all three groups of bacteria involved in nitrogen removal from wastewater (denitrifying, ammonia-oxidizing, and nitrite-oxidizing bacteria) was possible using the method developed in this research. Our results revealed close spatial relations among all three groups of bacteria targeted. A number of bacterial colonies hybridized with both *nirS* mRNA and the 16S RNA of ammonia oxidizing bacteria, suggesting that members of AOB might possess and express *nirS* genes in addition to the already known *nirK* genes present in some AOB such as *Nitrosomonas europaea*.

Labeled nitrite reducers were sorted from the background microbial community using flow cytometry (FCM) phylogenetic studies on sorted cells suggested that the dominant *in situ*

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nitrite reducers were closely related to *Acidovorax* BSB421. We believe that the molecular approach developed in this research has great potential to answering the longstanding question of which environmental processes are attributed to which microorganisms in natural and engineered habitats.

Recommendations for Future Work

1) Oligonucleotide probes targeting the 16S rRNA of *Brevundimonas* sp. 52AD23 and *Acidovorax* sp. BSB421 should be used in combination with *nirS* mRNA-FISH to investigate whether the described mRNA FISH/FCM method accurately identified the dominant nitrite reducers *in situ*. The overlapping of fluorescent signals conferred by 16S rRNA FISH and *nirS* mRNA-FISH would provide additional evidence supporting the findings reported in this study.

2) To examine whether Acidovorax sp. BSB421 is a nitrite reducer and to determine what type of nitrite reductase it produces, primers targeting the *nirK* and *nirS* genes should be used for the amplification of *nir* genes from pure cultures of Acidovorax sp. BSB421, followed by cloning or PCR product purification, and sequencing.

3) To determine the identity of *nirS*-containing AOB, activated sludge should be hybridized with Oregon Green-labeled probes targeting the 16S rRNA of AOB and TMR-tagged probes targeting *nirS* transcripts in cells fixed *in situ*. Cells showing both Oregon Green and TMR

signals should be sorted. DGGE of sorted cells and sequencing of bands could reveal the phylogenetic identity of *nirS*-containing AOB.

4) The detection limit of the described mRNA FISH protocol could be determined if mRNA concentrations are measured. This could be achieved using RT-PCR targeting *nirS* mRNA of expression clones induced with different amounts of IPTG and showing distinct average fluorescent signal intensities under fluorescent microscopy. The detection limit of mRNA FISH is believed to be the most important factor affecting the application of the method to target microorganisms responsible for catalyzing biodegradation of other important pollutants in water, soil, air, and solid waste treatment.

5) Use HRP-labeled probes and different fluorophores targeting the *nirS, nirK, nar, nor*, and *nos* genes of activated sludge for the in situ identification of the entire denitrifying bacterial population.

6) Monoclonal antibodies can be produced for nitrite reductase expressed using the *E. coli* clones built in this study for the in situ identification of the NIR enzyme, rather than the *nirS* mRNA. This approach presents potential problems related to specificity. However, if validated, this approach would allow sorting of live microorganism, rather than fixed cells, and would permit study of the physiology of isolated microorganisms in pure or enriched cultures.