Quantification of *Nitrosomonas oligotropha* and *Nitrospira* spp. Using Competitive Polymerase Chain Reaction in Bench-Scale Wastewater Treatment Reactors Operating at Different Solids Retention Times

H. M. Dionisi, A. C. Layton, K. G. Robinson, J. R. Brown, I. R. Gregory, J. J. Parker, G. S. Sayler

ABSTRACT: The effect of solids retention time (SRT) on ammonia- and nitrite-oxidizing bacteria was measured by *Nitrosomonas oligotropha*-like *ammonia monooxygenase* A and *Nitrospira* 16S rDNA competitive polymerase chain reaction assays in a complete-mix, bench-scale, activated-sludge system. During steady-state operation, nitrification was complete in the 20- and 10-day SRT reactors, nearly complete in the 5-day SRT reactor, and incomplete in the 2-day SRT reactor (76% ammonia oxidation and 85% nitrite oxidation). Total microbes, measured by dot-blot hybridizations, ranged from 3×10^{11} to 3×10^{12} cells/L, and increased with increasing SRTs. The concentration of the ammonia-oxidizer *N. oligotropha* dropped 100-fold from the 20-day SRT (5×10^9 cells/L) to the 2-day SRT ($\leq 4 \times 10^7$ cells/L). Thus, *N. oligotropha* became a much smaller fraction of the total biomass in the poorly performing 2-day SRT reactor. The concentration of *Nitrospira* cells also decreased (10-fold) as the SRT was reduced from 20 days to 2 days. However, the number of *Nitrospira* cells was always greater than the number of *N. oligotropha* cells measured in each reactor (10- to 60-fold). While *Nitrospira* comprised 1 to 2% of the biomass, *N. oligotropha* represented only 0.04 to 0.27% of the total population. This low percentage suggests that *N. oligotropha* was not a dominant ammonia oxidizer in the bench-scale systems. *Water Environ. Res.*, **74,** 462 (2002).

KEYWORDS: competitive polymerase chain reaction, *Nitrosomonas*, *Nitrospira*, *ammonia monooxygenase* A (*amo*A), 16S rDNA, nitrification.

Introduction

Removal of ammonia from municipal wastewater is important because its release into receiving waters may result in ammonia toxicity, oxygen depletion, and eutrophication of surface waters (Bitton, 1999). The key process in ammonia removal during wastewater treatment is through the two-step oxidation of ammonia to nitrate via microbial-mediated nitrification. Biological oxidation of ammonia to nitrate occurs primarily through the coordination of two distinct chemolithotrophic groups of bacteria: ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). The slow growth rate of these bacteria and their sensitivity to environmental factors including temperature, pH, and oxygen concentration influence the minimum solids retention time (SRT) required to establish stable nitrification during wastewater treatment (Okabe et al., 1999). Nitrifying bacteria are characterized by low half-saturation constants that are typically approximately around 1.0 mg N/L (Grady et al., 1999). Consequently, low ammonia concentrations can be achieved in bioreactors whenever the SRT is long enough to ensure stable nitrifier growth. However, the ammonia concentration rises rapidly as the SRT is decreased to the point of washout (Benefield and Randall, 1980), giving rise to the reputation of nitrification as an "all or nothing" phenomenon (Grady et al., 1999).

The AOB convert ammonia (NH_3) to nitrite (NO_2^-) via a two-step enzymatic process encoded by the ammonia monooxygenase *(amo)* and hydroxylamine oxidoreductase (*hao)* genes (Bock et al., 1992). To date, all aerobic AOB strains isolated from nonmarine ecosystems fall within the β-subclass *Proteobacteria* (Kowalchuk and Stephen, 2001). Recently published studies indicate that wastewater treatment plants vary in AOB species diversity and in species richness (Purkhold et al., 2000; Rotthauwe et al., 1997). While some plants are dominated by a single ammonia-oxidizing species, other plants harbor at least four different ammonia-oxidizing species. In studies by Dionisi et al. (2002), only *amo*A sequences closely related to *Nitrosomonas oligotropha* were found in a local municipal treatment plant, suggesting that this was the dominant ammonia-oxidizing species in the facility.

The NOB convert nitrite to nitrate (NO_3^-) and belong to several phylogenetic groups including *Nitrobacter* (α-subclass *Proteobacteria*), *Nitrococcus* (γ-subclass *Proteobacteria*), and *Nitrospira* (*Nitrospira* phylum). Until the late 1990s, it was generally assumed that *Nitrobacter* were the key NOB in wastewater treatment (Bitton, 1999; Mobarry et al., 1996). However, several molecular studies demonstrated that *Nitrospira* occurred more frequently and at higher concentrations than *Nitrobacter* in nitrifying reactors (Burrell et al., 1998; Hovanec et al., 1998; Okabe et al., 1999; Schramm et al., 1998, 1999), suggesting that *Nitrospira* plays a larger role in nitrite oxidation than *Nitrobacter* in wastewater treatment systems.

A number of steady-state design and kinetic simulation models have been developed to describe nitrification in single-stage, activated-sludge systems (Bitton, 1999). Many of these models are based on *Nitrobacter* growth rates and activities. Furthermore, these models have led to the assumptions that the rate-limiting step in nitrification is the conversion of ammonia to nitrite because *Nitrobacter* has a higher growth rate than *Nitrosomonas*, and that the ammonia oxidizers (i.e., *Nitrosomonas*) are present at higher numbers than the nitrite oxidizers (i.e., *Nitrobacter*) in wastewater

treatment systems (Bitton, 1999). Traditional enumeration of bacteria relies on sample dilution most probable number (MPN) methods or spread-plate inoculation. However, AOB are difficult to cultivate and cultivation methods undoubtedly result in an underestimation of both number (Phillips et al., 2000) and diversity (Juretschko et al., 1998). *Nitrospira* is also extremely difficult to cultivate (Ehrich et al., 1995) and cannot be quantified by culture methods.

Because of the problems associated with quantification of nitrifying bacteria by cultivation techniques, several culture-independent molecular methods have been pursued including quantitative dot-blot hybridization (Hovanec and Delong, 1996; Mobarry et al., 1996; Rittmann et al., 1999; Urbain et al., 1998), polymerase chain reaction (PCR)-based methods (Ivanova et al., 2000; Kowalchuk et al., 1999; Mendum et al., 1999; Phillips et al., 2000; Stephen et al., 1999), and fluorescent in situ hybridization (FISH) using oligonucleotide probes (Gieseke et al., 2001; Okabe et al., 1999; Schramm et al., 1999). These methods are based on the fact that all organisms have some conserved and some unique DNA sequences. In the PCR method, a unique DNA fragment (target) comprising a very small percentage of the total DNA is exponentially amplified in vitro. Thus, the amount of DNA of interest is increased to a level that can be detected or identified by gel electrophoresis, sequencing, and other detection methods. The main drawback of the original PCR method is that it is not quantitative. During the past 10 years, variations of PCR such as competitive PCR (cPCR) have been developed to allow quantification of specific targets present in a complex sample. Briefly, in the cPCR technique, a fixed (and unknown) amount of target DNA is amplified with a dilution series of a competitor DNA (Zimmermann and Mannhalter, 1996). After the PCR reaction, the products of the target and the competitor are separated by agarose gel electrophoresis and quantified. When the amount of target and competitor DNA are equivalent in the starting PCR reaction, the same amount of their products will accumulate. Because the concentration of competitor added to each tube at the start of the PCR reaction is precisely known, the initial concentration of target DNA in the sample can be calculated. Recently, Dionisi et al. (2002) developed cPCR assays to quantify the *N.*

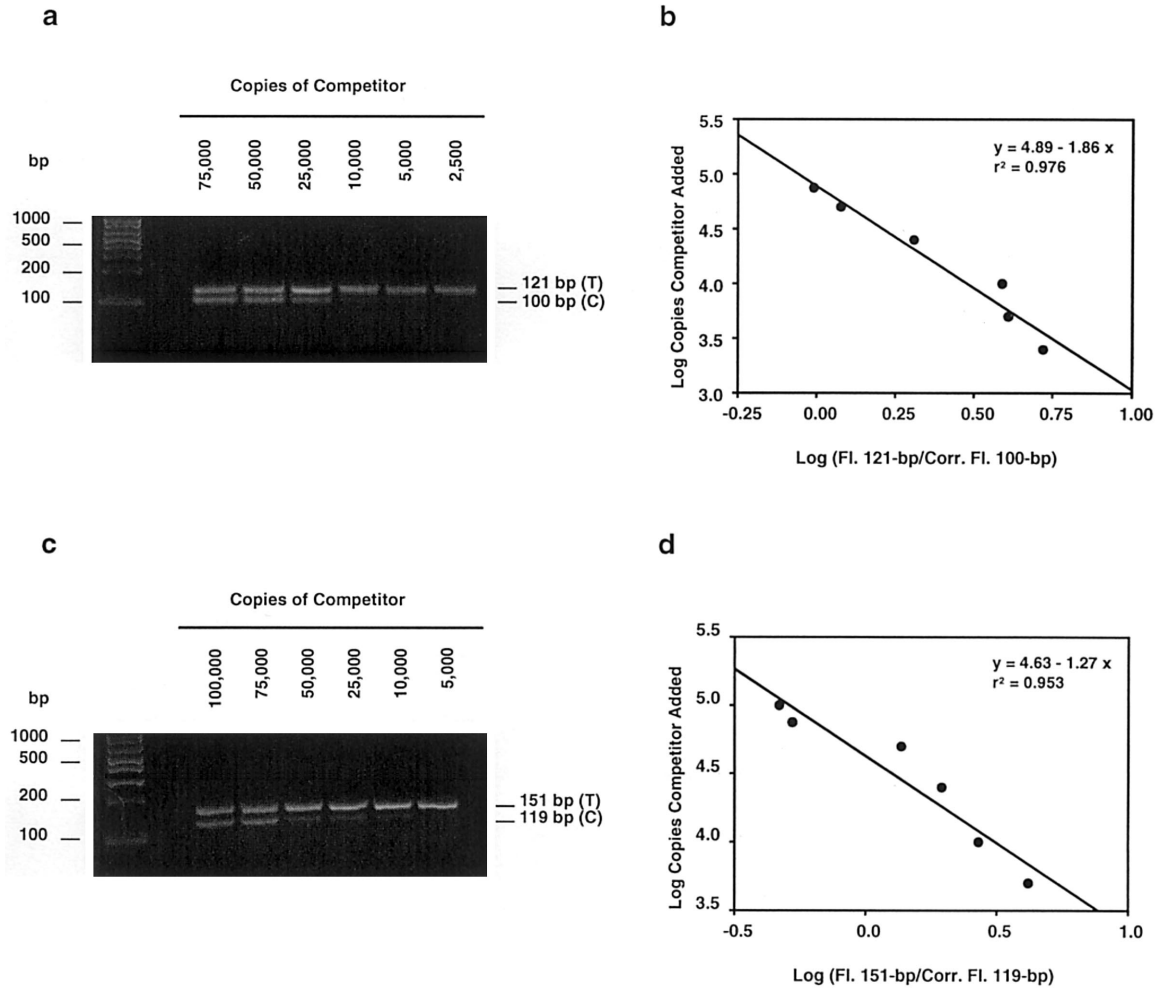

Figure 1—Competitive PCR assays for *amo*A and *Nitrospira* 16S rDNA: (a) agarose gel of competitive PCR reactions for the *amo*A gene using 27.5 ng of DNA extracted from a 20-day SRT sample (day 28); (b) determination of the competition equivalence point for the data shown in panel (a); (c) agarose gel of competitive PCR reactions for the *Nitrospira* 16S rDNA gene from 2.75 ng of DNA from the same reactor sample extracts as in panel (a); (d) determination of the competition equivalence point for the data shown in panel (c) (T = target, C = competitor, Fl. = fluorescence, and Corr. Fluor. = corrected fluorescence).

Table 1—Performance of reactors under steady-state conditions.

	Average operational parameters at steady state[a]			
SRT (d)	MLVSS (mg/L)	COD removal (%)	Ammonia oxidation (%)	Nitrite oxidation (%)
2	367 ± 76	79.2 ± 10.4	76.1 ± 6.8	84.9 ± 4.9
5	718 ± 208	80.5 ± 10.3	100	97.4 ± 1.7
10	1462 ± 367	83.5 ± 10.1	100	100
20	2172 ± 247	83.4 ± 12.0	100	100

[a] Nine sample measurements during the 2-month steady-state period were averaged for each SRT.

oligotropha-type *amo*A gene and the 16S rDNA gene from *Nitrospira* present in the activated sludge of a municipal treatment plant. Thus, the nitrifying populations in this municipal facility were quantified without cultivation or traditional MPN assays.

The goals of this study were to assess the overall utility of the cPCR method in determining the number of AOB and NOB present in an activated-sludge process and to relate the structural (AOB and NOB quantities) and functional (treatment performance) analyses. Because SRT is the principle factor determining whether an activated-sludge process will support nitrification (Cheremisinoff, 1996), bench-scale reactors operating at different SRTs between 2 and 20 days were sampled, and specific populations of AOB and NOB were quantified using cPCR methods targeting the *amo*A gene from the AOB *N. oligotropha* and the 16S rDNA from the NOB *Nitrospira*. The molecular analysis was compared to the treatment efficiency in each reactor as determined by traditional measures.

Materials and Methods

Bench-Scale, Activated-Sludge Reactors. The complete-mix, bench-scale, activated-sludge system was composed of four reactors (10 L in volume) with external clarifiers operated at SRTs of 20, 10, 5, and 2 days. Influent was collected from a large municipal treatment plant (primary clarifier effluent) and fed to the reactors at a rate of 19 mL/min. The average ammonia concentration in the influent feed was 17 ± 1.5 mg N/L, while the nitrite and nitrate levels were consistently below the detection limit of 0.1 mg/L. The dissolved oxygen concentration was maintained at 2 to 3 mg/L except for the last sampling date, which had a dissolved oxygen concentration of 1.5 mg/L. Solids retention time was maintained via direct wastage from the aerated reactors. Alkalinity remained within a suitable range (85 to 181 mg/L as calcium carbonate) for all reactors during the course of the experiments (APHA et al., 1992). Chemical oxygen demand (COD) was measured using Micro-COD test kits (Bioscience, Inc., Bethlehem, Pennsylvania). Ammonia concentration in the influent and effluent was measured using a selective probe (method 4500-NH_3F, *Standard Methods*, APHA et al., 1992). Nitrite and nitrate concentrations were measured according to *Standard Methods* (method 4110 B, APHA et al., 1992) using an ion chromatograph (model DX500, Dionex, Sunnyvale, California) outfitted with a 4-mm anion column (Ionpac AS4A, Dionex). Mixed liquor volatile suspended solids (MLVSS) were measured following *Standard Methods* (method 2540 E, APHA et al., 1992).

Molecular Methods. Total DNA from nine activated-sludge samples in each reactor was extracted in duplicate from 2 to 25 mL of mixed liquor, depending on the SRT and MLVSS values, using a FastDNA kit (BIO 101, Vista, California) with modifications as described by Dionisi et al. (2002). Total 16S rDNA measurements were performed by dot-blot hybridization with the ^{32}P-labeled 1392r probe as described by Dionisi et al. (2002) and Applegate et al. (1995). Two cPCR assays (one for the detection of *amo*A in *N. oligotropha*-type AOB and one for the detection of *Nitrospira* 16S rDNA) were performed as described by Dionisi et al. (2002).

The cPCR technique consists of coamplification of a target sequence with known concentrations of an internal standard, called "the competitor" (Chandler, 1998; Zimmerman and Mannhalter, 1996). In these assays, the competitor is identical to the target, except for a deletion that allows the target and competitor PCR products to be differentiated in an agarose gel by size. In essence, the competitor and the intended target DNA compete for the primers (small DNA fragments used to initiate the PCR reaction) and other reagents in the PCR reaction mix. Quantification is achieved by comparing the amount of product amplified from the target sequence with the amount of product amplified from known concentrations of the competitor (Figures 1a and 1c). At the competition equivalence point, the ratio between the fluorescence of the target and the competitor should equal 1 (after the fluorescence of the competitor is corrected because of differences in sizes between both PCR products). The logarithm (base 10) of the fluorescence intensity of the target product divided by the fluorescence intensity of the competitor (Figures 1b and 1d, *x*-axis) is plotted as a function of the logarithm (base 10) of the copies of competitor added (Figures 1b and 1d, *y*-axis). The interpolation on this linear plot for an *x* value of 0 gives the number of copies of the target present in the PCR reaction.

In the *N. oligotropha*-like *amo*A cPCR assay, a 100-base pair (bp) oligonucleotide corresponding to the target DNA (121 bp of the *amoA* gene with an internal deletion of 21 bp) was used as the competitor (Dionisi et al., 2002). Fluorescent band intensities were quantified using a documentation and analysis system (Alpha Imager 1220, Alpha Innotech Corporation, San Leandro, California). The fluorescence of the competitor product was corrected by a factor of 121:100 to compensate for the difference in size between these two products. In the *Nitrospira* cPCR assay, a 119-bp oligonucleotide consisting of a 32-bp deletion in the 151-bp target sequence of the *Nitrospira* spp. 16S rDNA was used as the competitor (Dionisi et al., 2002). The fluorescence of the competitor product was corrected by a factor of 151:119 as previously described. An additional factor of 1.74 was used to correct for unequal amplification of the target and competitor.

Results and Discussion

Treatment Performance as Determined by Traditional Measures. After the bench-scale reactors had been in operation for 103 days, a 2-month time period was selected to begin evaluation of steady-state treatment performance parameters. Steady state was based on stability of operational parameters, particularly biomass concentration (as measured by MLVSS) and ammonia removal efficiency. The COD removal efficiencies for these reactors were similar (Table 1). In the 20- and 10-day SRT reactors, chemolithotrophic oxidation of ammonia and nitrite resulted in complete nitrification during steady-state operation (Figure 2). Ammonia oxidation was determined via measurement of influent and effluent concentrations (netting out biomass-$N_{new\ growth}$), whereas nitrite oxidation (%) was determined using the following equation:

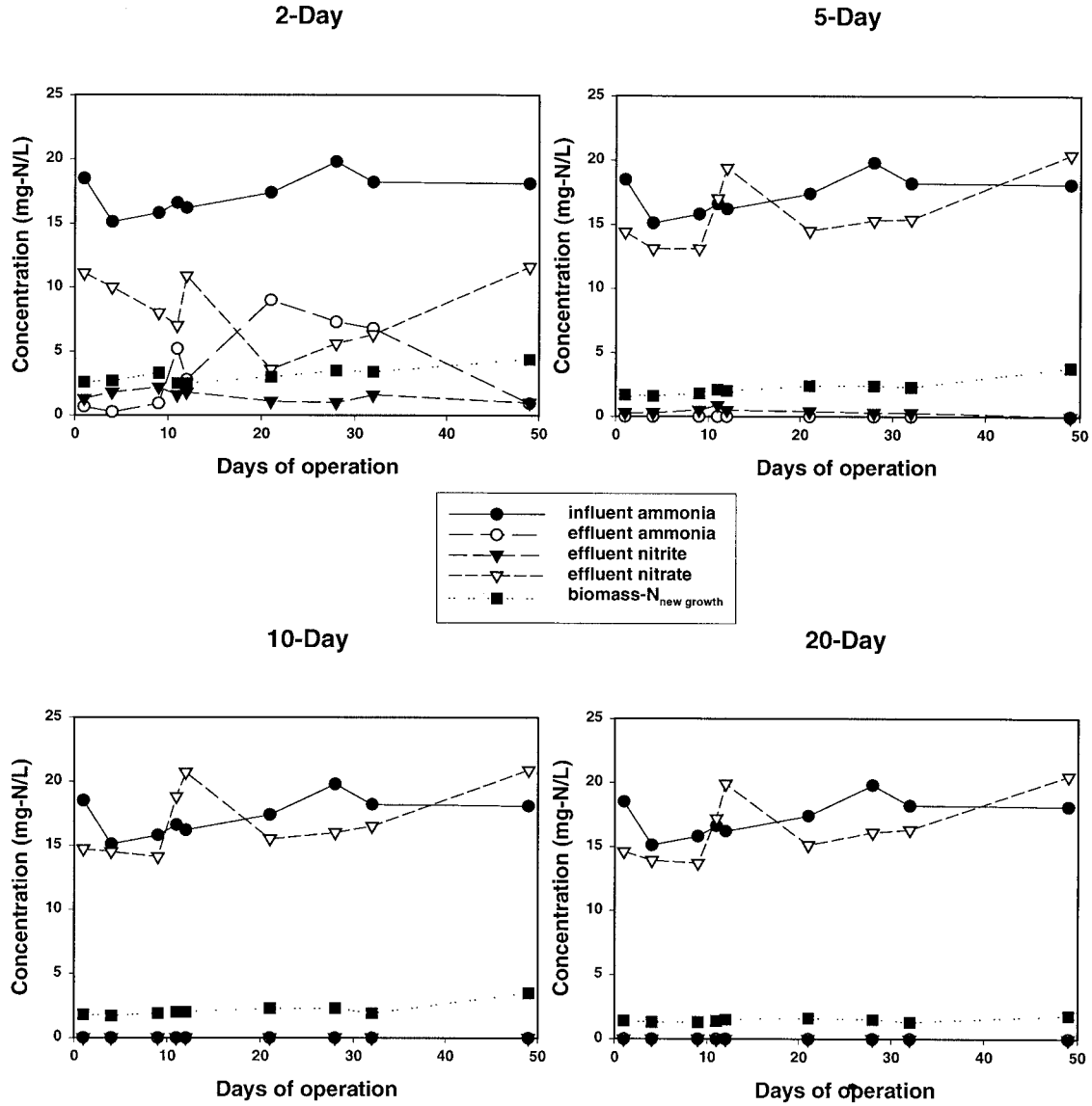

Figure 2—Nitrogen partitioning in the 2-, 5-, 10-, and 20-day SRT reactors. All concentrations are calculated with respect to nitrogen.

NO_2^- Oxidation

$$= \frac{(NH_4^+)_{in} - (\text{biomass-N}_{\text{new growth}}) - (NH_4^+)_{eff} - (NO_2^-)_{eff}}{(NH_4^+)_{in} - (\text{biomass-N}_{\text{new growth}}) - (NH_4^+)_{eff}} \quad (1)$$

Where

$(NH_4^+)_{in}$, $(NH_4^+)_{eff}$ = ammonia concentrations (mg N/L) in the influent and effluent, respectively;

$(NO_2^-)_{eff}$ = effluent nitrite concentration (mg N/L); and

biomass-$N_{\text{new growth}}$ = amount of nitrogen used by new cells (mg N/L) as defined in eq 2.

In the 5-day SRT reactor, ammonia oxidation was complete and nitrite oxidation was nearly complete (97%) (Table 1). Nitrification was unstable in the 2-day SRT reactor, which resulted in as much as 9 mg N/L of ammonia detected in the effluent (Figure 2). Although a large percentage of the nitrite produced from ammonia oxidation was oxidized in the 2-day SRT reactor (Table 2), 1 to 2 mg N/L of nitrite was routinely measured in the effluent (Figure 2).

Table 2—Extraction of DNA from mixed liquor reactor samples.[a]

SRT (d)	MLVSS (mg/L)	DNA extraction (μg DNA/mL mixed liquor)	DNA extraction (μg DNA/mg biomass)
2	367 ± 7	0.20 ± 0.17	0.52 ± 0.41
5	718 ± 20	0.27 ± 0.26	0.41 ± 0.42
10	1460 ± 367	0.78 ± 0.41	0.52 ± 0.20
20	2170 ± 246	2.02 ± 1.46	0.92 ± 0.65

[a] Nine sample measurements during the 2-month steady-state period were averaged for each SRT.

Table 3—Total microbes, *N. oligotropha* AOB, and *Nitrospira* NOB in bench-scale reactors operating under various SRT steady-state nitrification conditions.

	Normalized bacterial population[a] (copies/μg DNA)			Microbial concentrations[a,b] (cells/L)		
SRT (d)	Total 16S rDNA	*N. oligotropha amo*A	*Nitrospira* 16S rDNA	Total microbes	*N. oligotropha* (AOB)	*Nitrospira* (NOB)
2	$7.3 (\pm 9.2) \times 10^9$	$7.2 (\pm 6.0) \times 10^5$ [c]	$1.0 (\pm 0.4) \times 10^7$	$3.1 (\pm 3.3) \times 10^{11}$	$3.8 (\pm 2.3) \times 10^7$	$2.0 (\pm 1.7) \times 10^9$
5	$6.4 (\pm 4.0) \times 10^9$	$4.1 (\pm 4.6) \times 10^6$	$1.4 (\pm 1.2) \times 10^7$	$3.4 (\pm 1.9) \times 10^{11}$	$4.4 (\pm 4.2) \times 10^8$	$3.4 (\pm 1.9) \times 10^9$
10	$6.3 (\pm 1.8) \times 10^9$	$2.0 (\pm 1.4) \times 10^6$	$6.1 (\pm 7.8) \times 10^7$	$1.3 (\pm 0.5) \times 10^{12}$	$6.6 (\pm 4.9) \times 10^8$	$3.2 (\pm 2.2) \times 10^{10}$
20	$6.3 (\pm 2.9) \times 10^9$	$6.7 (\pm 3.7) \times 10^6$	$2.5 (\pm 1.9) \times 10^7$	$3.7 (\pm 3.2) \times 10^{12}$	$5.4 (\pm 3.4) \times 10^9$	$3.8 (\pm 2.6) \times 10^{10}$

[a] Nine sample measurements during the 2-month steady-state period were averaged for each SRT.
[b] Cells/L = Copies/μg DNA × μg DNA/L × Cell/Copies.
[c] *amo*A copies/μg DNA in a 2-day SRT; in six samples, *amo*A copies were below the detection limit and, therefore, calculated using the minimum detection value of 5000 copies/μL DNA extract.

Information concerning the mass of new cells produced on a daily basis and the amount of nitrogen used by the new growth was needed to establish nitrogen partitioning in each reactor. New growth was estimated from the solids wasted and lost daily from each reactor because an amount of biomass equivalent to the new growth must be removed each day to maintain steady-state solids levels at each SRT. The amount of nitrogen uptake by the new cells (biomass-$N_{new\ growth}$) was estimated using the following relationship:

$$\text{biomass-N}_{\text{new growth}} = \left[\frac{\text{MLVSS} \times N \times V_{\text{waste}}}{Q}\right] + \left[\text{ESS} \times \left(\frac{\text{MLVSS}}{\text{MLSS}}\right) \times N\right] \quad (2)$$

Where

V_{waste} = wastage volume from the reactor (L/d),
Q = flowrate through the reactor (L/d),
ESS = effluent suspended solids (mg/L),
MLSS = mixed liquor suspended solids (mg/L), and
N = nitrogen content of solids (%).

Nitrogen content was determined by total Kjeldahl nitrogen analysis of representative MLSS samples from each reactor (average nitrogen content for the 20-, 10-, 5-, and 2-day reactors was found to be 3.65, 4.03, 4.25, and 4.63%, respectively). Based on eq 2, new cells used approximately 2 to 4 mg/L of the incoming nitrogen for anabolic processes (Figure 2).

Molecular Analysis and Comparison to Traditional Analysis of Nitrification. Assays of DNA were used to calculate the total microbes (dot-blot hybridization), *N. oligotropha* (cPCR), and *Nitrospira* (cPCR) in mixed liquor samples from the bench-scale reactors. The concentration of DNA extracted from mixed liquor samples (micrograms of DNA per milliliter) increased with increasing SRTs (Table 2). This was expected because longer SRTs had higher biomass (MLVSS) concentrations. Although the extracted DNA concentration increased with SRT, the amount of DNA extracted per mass of solids was similar for all SRTs (Table 2), suggesting that DNA extraction efficiency was comparable in all four reactors. In addition, total 16S rDNA copies per mass of DNA were constant in all samples at approximately 7×10^9 copies/μg DNA (Table 3). These results suggest that calculation of copies per mass of DNA provides a method independent of DNA extraction efficiency and total biomass to compare specific microbial populations.

Traditional physical and chemical analysis of treatment performance clearly indicated that nitrification in the 2-day SRT reactor was less efficient than nitrification in the 10- and 20-day SRT reactors (Figure 2). Therefore, it was hypothesized that the *N. oligotropha amo*A and *Nitrospira* 16S rDNA copies per microgram of DNA would be less for the shorter SRT than the longer SRTs. Because the normalized total bacterial population remained relatively constant in all four reactors (as measured by total 16S rDNA copies per mass of DNA), fewer copies of the AOB and NOB target molecules would indicate fewer ammonia- and nitrite-oxidizing organisms in the treatment reactors relative to the total bacterial population. The average number of *N. oligotropha amo*A copies per microgram of DNA in the 2-day SRT samples was lower than the number in the longer SRT samples (Table 3). This finding was consistent with the 24% reduction in ammonia oxidation observed in the 2-day SRT reactor (Table 1). The lower AOB population at the shortest SRT may be a reflection of the slow growth rate and the tendency of these chemolithotrophic bacteria to wash out at low SRTs. The normalized number of *N. oligotropha amo*A copies was similar in the 5-, 10-, and 20-day SRT samples, which is consistent with the similar ammonia-oxidation rates (greater than 97%) in these reactors. While the amount of nitrite oxidized was lower in the 2-day SRT reactor, a corresponding change in *Nitrospira* levels (copies per microgram of DNA) was not observed (Table 3). It may be that a much lower nitrite-oxidation efficiency is needed to better document changes in normalized *Nitrospira* copy numbers.

To better compare the molecular and traditional information collected, data conversion to a cell-per-liter (mixed liquor) basis was attempted. The following assumptions were made for the conversion of the molecular data. First, the average number of 16S rDNA copies per cell in activated sludge was assumed to be 3.6 (Dionisi et al. 2002), which is the average operon number for all bacterial species (Klappenbach et al., 2001). Second, because the number of *Nitrospira* 16S rDNA copies per cell is unknown, it was assumed to be 1 based on the average copies of 16S rDNA per cell in other nitrifying bacteria, such as *Nitrosomonas* and *Nitrobacter* (Klappenbach et al., 2001; Navarro et al., 1992). Third, *N. oligotropha*-type ammonia-oxidizing bacteria were assumed to have 2 copies of the *amo*A gene per cell (McTavish et al., 1993). For comparative purposes, total microbial cell concentrations were

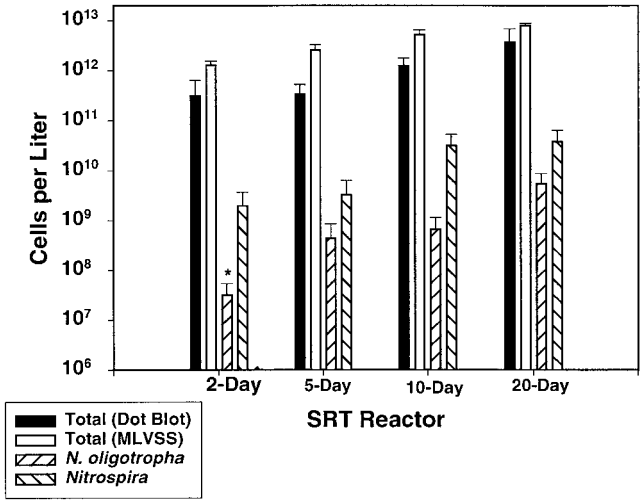

Figure 3—Total 16S rDNA, *Nitrospira* 16S rDNA (NOB), *N. oligotropha amo*A (AOB), and mixed liquor volatile suspended solids (MLVSS) concentrations in reactors operating at 2-, 5-, 10-, and 20-day SRTs (average ± standard deviation of nine samples). *In the 2-day reactor, some samples had *amo*A copies below the detection limit (4×10^7 copies/L) and, thus, this represents a maximum value.

calculated from the MLVSS data using a weight-to-cell conversion value of 2.8×10^{-13} g/cell (Madigan et al., 1997). This conversion assumes constant growth factors between the high- and low-SRT reactors and constant cell mass.

Total microbial concentrations (cells per liter) followed expected trends: the highest cell concentrations were present in the 20-day SRT samples and the lowest cell concentrations were present in the 2-day SRT samples (Figure 3). In addition, the total cell concentration values obtained by the molecular dot-blot assay and conversion of the MLVSS values were positively correlated (Figure 4, linear correlation).

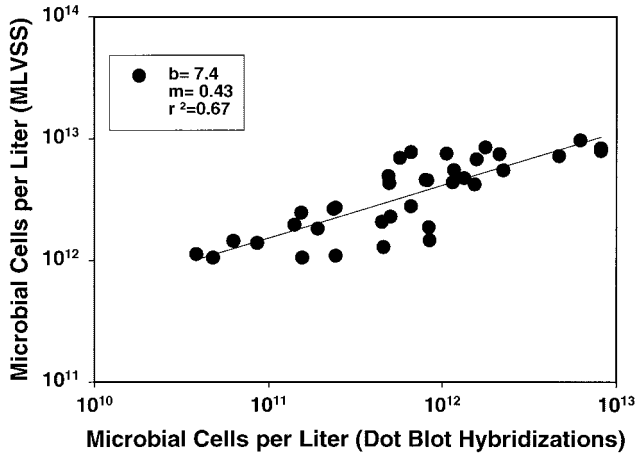

Figure 4—Comparison of total microbial populations as determined from MLVSS and dot-blot hybridization data for all reactor samples (linear correlation).

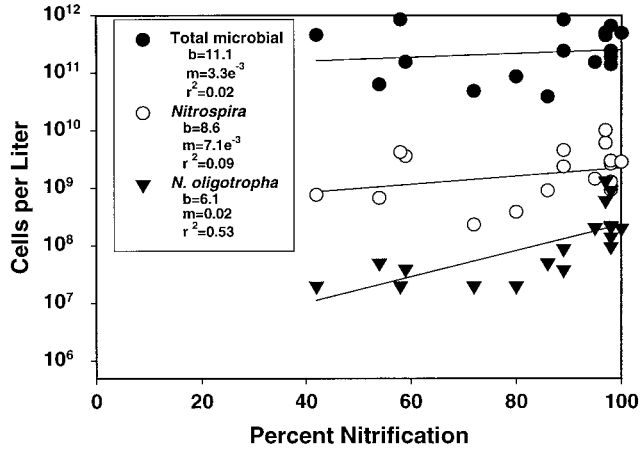

Figure 5—Comparison of percent nitrification to *N. oligotropha*, *Nitrospira,* and total microbial cell concentrations in mixed-liquor samples from the 2- and 5-day SRT reactors.

The AOB and NOB concentrations also followed the same general trend as those for total cells with higher cell concentrations in the 20-day SRT reactor than the 2-day SRT reactor (Figure 3). However, larger differences were measured in the AOB concentration (100-fold) between the 2- and 20-day SRTs than in the NOB and total microbial concentrations (approximately 10-fold) (Table 3). The AOB and NOB levels measured in the 5-, 10-, and 20-day SRT reactors were within the ranges of 0.9×10^8 to 8×10^8 cells/L for *N. oligotropha* and 0.6×10^{10} to 3×10^{10} cells/L for *Nitrospira* reported for the full-scale municipal treatment plant activated sludge (approximately 10-day SRT) used to seed these reactors (Dionisi et al., 2002). In addition, the *Nitrospira* cell concentrations detected in these reactors were similar to those recently reported (3×10^9 copies/L) in laboratory-scale reactors by Yuan and Blackall (2002).

The *N. oligotropha*, *Nitrospira,* and total microbial cell concentrations for the 2- and 5-day SRT reactors (nine samples each) were plotted against percent nitrification to assess any relative relationships using the following formula:

$$\% \text{ Nitrification} = \frac{(NH_4^+)_{in} - (NH_4^+)_{eff} - (NO_2^-)_{eff}}{(NH_4^+)_{in}} \times 100\% \quad (3)$$

As expected, *N. oligotropha* cell concentrations increased with increasing nitrification (Figure 5). No clear relationship was seen between the percent nitrification and *Nitrospira* or total microbial cell concentrations.

For all SRTs, *Nitrospira* levels were higher (10- to 60-fold) than *N. oligotropha* levels (Table 4). Based on calculated cell values in this study, *Nitrospira* represented 1 to 2% and *N. oligotropha* represented 0.037 to 0.27% of the total microbial population present in these reactors (Table 4). Other studies using oligonucleotide-probe hybridizations with RNA isolated from biosolids or FISH report AOB values in the 5 to 10% range (Daims et al., 2001; Rittmann et al., 1999; Urbain et al., 1998). In addition, stoichiometric calculations based on typical kinetic parameters (Rittmann and McCarty, 2001) suggest that the fraction of biomass corresponding to ammonia oxidizers should be 5 to 10% for ammonia oxidizers (2- and 20-day SRTs, respectively) and 0.6 to 2.4% (2-

Table 4—Percent of total microbial population consisting of *N. oligotropha* and *Nitrospira* and ratio of *Nitrospira* to *N. oligotropha* cells in the SRTs.

SRT (d)	*N. oligotropha* (%)[a]	*Nitrospira* (%)[b]	Ratio of *Nitrospira* to *N. oligotropha*[c]
2	0.037 (±0.041)	1.1 (±0.89)	61 (±67)
5	0.17 (±0.20)	0.93 (±0.53)	11 (±9.1)
10	0.055 (±0.041)	2.1 (±1.2)	56 (±54)
20	0.27 (±0.23)	1.5 (±1.1)	9.9 (±9.8)

[a] Percent = *N. oligotropha* (AOB) cells/total microbes × 100%.
[b] Percent = *Nitrospira* (NOB) cells/total microbes × 100%.
[c] Ratio of *Nitrospira* to *N. oligotropha* was calculated for each individual sample before averaging.

and 20-day SRTs, respectively) for nitrite-oxidizers. In this study, the percentage of *N. oligotropha* obtained was significantly lower than expected, but the percentage of *Nitrospira* was in the estimated range. The *amo*A assay was designed to specifically detect *amo*A from *N. oligotropha*, which was the dominant AOB in the municipal treatment plant activated sludge used to seed these reactors (Dionisi et al., 2002). The extremely low percentage of ammonia oxidizers measured in this study suggests that AOB other than *N. oligotropha* were dominant in the bench-scale systems. Because multiple ammonia-oxidizing organisms exist (at least 15 or 16) (Purkhold et al., 2000), additional work is needed to identify all the significant ammonia oxidizers.

Conclusions

The molecular-based analysis used in this study represents a new approach for the quantification of *N. oligotropha* and *Nitrospira* bacteria. The calculation of total 16S rDNA, *Nitrospira* 16S rDNA, and *amo*A gene copies normalized to mass of DNA allowed for comparison between the different SRT reactors independent of DNA extraction efficiency. The 2-day SRT reactor had partial loss of nitrification and showed a significant drop (100-fold) in *N. oligotropha*, although *Nitrospira* did not decline so dramatically (10-fold) with respect to longer retention times. The low percentage of *N. oligotropha* cells in the mixed liquor suggests that this bacterium may not be the dominant AOB in this bench-scale system, although it was the AOB detected in the full-scale municipal treatment plant activated sludge used to seed this system.

Acknowledgments

Credits. This work was funded, in part, by the U.S. Environmental Protection Agency (Washington, D.C.) through cooperative agreement no. CR825237-01 with the Water Environment Research Foundation (Alexandria, VA), and by the Waste Management Research and Education Institute at the University of Tennessee (Knoxville). Hebe M. Dionisi is a recipient of a postdoctoral fellowship from CONICET (Argentinean National Research Council, Buenos Aires, Argentina). The authors acknowledge Arthur Meyers (Eastman Chemical Company, Tennessee Eastman Division, Kingsport, Tennessee) for technical advice.

Authors. Hebe M. Dionisi is a postdoctoral research associate in the Center for Environmental Biotechnology at the University of Tennessee, Knoxville. Alice C. Layton is a research assistant professor in the Department of Microbiology; Kevin G. Robinson is an associate professor, Janalyn R. Brown is a graduate research assistant, and Jack J. Parker is a graduate research assistant in the Department of Civil and Environmental Engineering; and Igrid R. Gregory is a laboratory manager in the Center for Environmental Biotechnology at the University. Gary S. Sayler is a distinguished professor in the Department of Microbiology and is the director of the Center for Environmental Biotechnology. Correspondence should be addressed to Gary S. Sayler, Center for Environmental Biotechnology, 676 Dabney Hall, Knoxville, TN 37996; e-mail: sayler@utk.edu.

Submitted for publication October 14, 2002; revised manuscript submitted May 21, 2002; accepted for publication May 24, 2002.

The deadline to submit Discussions of this paper is January 15, 2003.

References

American Public Health Association; American Water Works Association; Water Environment Federation (1992) *Standard Methods for the Examination of Water and Wastewater,* 18th ed.; Washington, D.C.

Applegate, B. M.; Matrubutham, U.; Sanseverino, J.; Sayler, G. S. (1995) Biodegradation Genes as Marker Genes in Microbial Ecosystems. In *Molecular Microbial Ecology Manual 6.1*; Kluwer Academic Publishers: Netherlands; Chapter 8, pp 1–14.

Benefield, L. D.; Randall, C. W. (1980) *Biological Process Design for Wastewater Treatment;* Prentice Hall: Englewood Cliffs, New Jersey.

Bitton, G. (1999) *Wastewater Microbiology,* 2nd ed.; Wiley-Liss: New York.

Bock, E.; Loops, H. P.; Ahlers, B.; Harms, H. (1992) Oxidation of Inorganic Nitrogen Compounds as Energy Source. In *The Prokaryotes,* 2nd ed.; Balows, A., Trüper, H. G., Dworkin, M., Harder, W., Schleifer, K.-H., Eds.; Springer-Verlag: New York; pp 414–430.

Burrell, P. C.; Keller, J.; Blackall, L. L. (1998) Microbiology of a Nitrite-Oxidizing Bioreactor. *Appl. Environ. Microbiol*., **64,** 1878.

Chandler, D. P. (1998) Redefining Relativity: Quantitiative PCR at Low Template Concentrations for Industrial and Environmental Microbiology. *J. Industrial Microbiol. Biotechnol,* **21,** 128.

Cheremisinoff, N. P. (1996) *Biotechnology for Waste and Wastewater Treatment;* Noyes Publications: Wentwood, New Jersey.

Daims, H.; Ramsing, N. B.; Schleifer, K-H.; Wagner, M. (2001) Cultivation-Independent, Semiautomatic Determination of Absolute Bacterial Cell Numbers in Environmental Samples by Fluorescence In Situ Hybridization. *Appl. Environ. Microbiol*., **67,** 5810.

Dionisi, H. M.; Layton, A. C.; Harms, G.; Gregory, I. R.; Robinson, K. G.; 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.

Ehrich, S.; Behrens, D.; Lebedeva, E.; Ludwig, W.; Bock, E. (1995) A New Obligately Chemolithoautotrophic, Nitrite-Oxidizing Bacterium, *Nitrospira moscoviensis* sp. nov. and its Phylogenetic Relationship. *Arch. Microbiol.,* **164,** 16.

Gieseke, A.; Purkhold, U.; Wagner, M.; Amann, R.; Schramm, A. (2001) Community Structure and Activity Dynamics of Nitrifying Bacteria in a Phosphate-Removing Biofilm. *Appl. Environ. Microbiol.,* **67,** 1351.

Grady, C. P. L.; Daigger, G. T.; Lim, H. C. (1999) *Biological Wastewater Treatment,* 2nd ed.; Marcel Dekker: New York.

Hovanec, T. A.; DeLong, E. F. (1996) Comparative Analysis of Nitrifying Bacteria Associated with Freshwater and Marine Aquaria. *Appl. Environ. Microbiol.,* **62,** 2888.

Hovanec, T. A.; Taylor, L. T.; Blakis, A.; Delong, E. F. (1998) *Nitrospira*-Like Bacteria Associated with Nitrite Oxidation in Freshwater Aquaria. *Appl. Environ. Microbiol.,* **64,** 258.

Ivanova, I.; Stephen, J. R.; Chang, Y.-J.; Bruggemann, J.; Long, P. E.; McKinley, J. P.; Kowalchuk, G. A.; White, D.C.; Macnaughton, S. J. (2000) A Survey of 16S rRNA and *amo*A Genes Related to Autotro-

phic Ammonia-Oxidizing Bacteria of the β-Subdivision of the Class Proteobacteria in Contaminated Groundwater. *Can. J. Microbiol.,* **46,** 1012.

Juretschko, S.; Timmermann, G.; Schmid, M.; Schleifer, K.-H.; Pommerening-Röser, A.; Koops, H. P.; 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.

Klappenbach, J. A.; Saxman, P. R.; Cole, J. T.; Schmidt, T. M. (2001) rrndb: The Ribosomal RNA Operon Copy Number Database. *Nucleic Acids Res.,* **29,** 181.

Kowalchuk, G. A.; Naomenko, Z. S.; Derikx, P. J.; Felske, A.; Stephen, J. R.; Arkipchenko, I. A. (1999) Molecular Analysis of Ammonia-Oxidizing Bacteria of the Beta Subdivision of the Class Proteobacteria in Compost and Composted Material. *Appl. Environ. Microbiol.,* **65,** 396.

Kowalchuk, G. A.; Stephen, J. R. (2001) Ammonia-Oxidizing Bacteria: A Model for Molecular Microbial Ecology. *Annu. Rev. Microbiol.,* **55,** 485.

Madigan, M. T.; Martinko, J. M.; Parker, J. (1997) *Brock Biology of Microorganisms,* 8th ed.; Prentice-Hall: New York.

McTavish, H.; Fuchs, J. A.; Hooper, A. B. (1993) Sequence of the Gene Coding for Ammonia Monooxygenase in *Nitrosomonas europaea*. *J. Bacteriol*., **175,** 2436.

Mendum, T. A.; Sockett, R. E.; Hirsch, P. R. (1999) Use of Molecular and Isotopic Techniques to Monitor the Response of Autotrophic Ammonia-Oxidizing Populations of the β Subdivision of the Class Proteobacteria in Arable Soils to Nitrogen Fertilizer. *Appl. Environ. Microbiol.,* **65,** 4155.

Mobarry, B. K.; Wagner, M.; Urbain, V.; Rittmann, B. E.; Stahl, D. (1996) Phylogenetic Probes for Analyzing Abundance and Spatial Organization of Nitrifying Bacteria. *Appl. Environ. Microbiol*., **62,** 2156.

Navarro, E.; Fernandez, M. P.; Grimont, F.; Clays-Josserand, A.; Bardin, R. (1992) Genomic Heterogeneity of the Genus *Nitrobacter*. *Int. J. Syst. Bacteriol.,* **42,** 554.

Okabe, S.; Satoh, H.; Watanabe, Y. (1999) In Situ Analysis of Nitrifying Biofilms as Determined by In Situ Hybridization and the Use of Microelectrodes. *Appl. Environ. Microbiol.,* **65,** 3182.

Phillips, C. J.; Paul, E. A.; Prosser, J. I. (2000) Quantitative Analysis of Ammonia Oxidizing Bacteria Using Competitive PCR. *FEMS Microbiol. Ecol.,* **32,** 167.

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 *amo*A Sequence Analysis: Implications for Molecular Diversity Surveys. *Appl. Environ. Microbiol*., **66,** 5368.

Rittmann, B. E.; Laspidou, C. S.; Flax, J.; Stahl, D. A.; Urbain, V.; Harduin, H.; van der Waarde, J. J.; Geurkink, B.; Henssen, M. J. C.; Bouwer, H.; Klapwijk, A.; Wetterauw, M. (1999) Molecular and Modeling Analysis of the Structure and Function of Nitrifying Activated Sludge. *Water Sci. Technol.,* **39,** 51.

Rittmann, B. E.; McCarty, P. L. (2001*) Environmental Microbiology: Principles and Applications.* McGraw-Hill: Boston, Massachusetts.

Rotthauwe, J.; Witzel, K.; Liesack, W. (1997) The Ammonia Monooxygenase Structural Gene *amo*A as a Functional Marker: Molecular Fine-Scale Analysis of Natural Ammonia-Oxidizing Populations. *Appl. Environ. Microbiol.,* **63,** 4704.

Schramm, A.; de Beer, D.; Wagner, M.; 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.

Schramm, A.; de Beer, D.; Van Den Heuvel, J. C.; Ottengraf, S.; Amann, R. (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.

Stephen, J. R.; Chang, Y. J.; Macnaughton, S. J.; Kowalchuk, G. A.; Leung, K. T.; Flemming, C. A.; White, D. C. (1999) Effect of Toxic Metals on Indigenous Soil Beta-Subgroup Proteobacterium Ammonia Oxidizer Community Structure and Protection Against Toxicity by Inoculated Metal-Resistant Bacteria. *Appl. Environ. Microbiol.,* **65,** 95.

Urbain, V.; Mobarry, B.; de Silva, V.; Stahl, D. A.; Rittmann, B. E.; Manem, J. (1998) Integration of Performance, Molecular Biology and Modeling to Describe the Activated Sludge Process. *Water Sci. Technol.,* **37,** 223.

Yuan, Z.; Blackall, L. L. (2002) Sludge Population Optimization: A New Dimension for the Control of Biological Wastewater Treatment Systems. *Water Res.,* **36,** 482.

Zimmermann, K.; Mannhalter, J. W. (1996) Technical Aspects of Quatitative Competitive PCR. *Bio/Techniques,* **21,** 268.