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ARTICLE *in* ENVIRONMENTAL SCIENCE AND TECHNOLOGY · FEBRUARY 2003

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Environ. Sci. Technol. **2003**, *37,* 343−351

Real-Time PCR Quantification of Nitrifying Bacteria in a Municipal Wastewater Treatment Plant

GERDA HARMS,[†,§] ALICE C. LAYTON,[†,§] HEBE M. DIONISI,[†,§] IGRID R. GREGORY,[†,§] VICTORIA M. GARRETT,[†,§] SHAWN A. HAWKINS,[‡,§] KEVIN G. ROBINSON,[‡,§] AND GARY S. SAYLER*[,†,§]

Department of Microbiology, Department of Civil and Environmental Engineering, and Center for Environmental Biotechnology, The University of Tennessee, Knoxville, Tennessee, 37996

Real-time PCR assays using TaqMan or Molecular Beacon probes were developed and optimized for the quantification of total bacteria, the nitrite-oxidizing bacteria *Nitrospira*, and *Nitrosomonas oligotropha*-like ammonia oxidizing bacteria (AOB) in mixed liquor suspended solids (MLSS) from a municipal wastewater treatment plant (WWTP) using a single-sludge nitrification process. The targets for the real-time PCR assays were the 16S rRNA genes (16S rDNA) for bacteria and *Nitrospira* spp. and the *amoA* gene for *N. oligotropha*. A previously reported assay for AOB 16S rDNA was also tested for its application to activated sludge. The *Nitrospira* 16S rDNA, AOB 16S rDNA, and *N. oligotropha*-like *amoA* assays were log-linear over 6 orders of magnitude and the bacterial 16S rDNA real-time PCR assay was log-linear over 4 orders of magnitude with DNA standards. When these real-time PCR assays were applied to DNA extracted from MLSS, dilution of the DNA extracts was necessary to prevent PCR inhibition. The optimal DNA dilution range was broad for the bacterial 16S rDNA (1000-fold) and *Nitrospira* 16S rDNA assays (2500-fold) but narrow for the AOB 16S rDNA assay (10-fold) and *N. oligotropha*-like *amoA* real-time PCR assay (5-fold). In twelve MLSS samples collected over one year, mean cell per L values were $4.3 \pm 2.0 \times 10^{11}$ for bacteria, $3.7 \pm 3.2 \times 10^{10}$ for *Nitrospira*, $1.2 \pm 0.9 \times 10^{10}$ for all AOB, and $7.5 \pm 6.0 \times 10^{9}$ for *N. oligotropha*-like AOB. The percent of the nitrifying population was 1.7% *N. oligotropha*-like AOB based on the *N. oligotropha amo*A assay, 2.9% total AOB based on the AOB 16S rDNA assay, and 8.6% nitrite-oxidizing bacteria based on the *Nitrospira* 16S rDNA assay. Ammonia-oxidizing bacteria in the wastewater treatment plant were estimated to oxidize 7.7 ± 6.8 fmol/hr/cell based on the AOB 16S rDNA assay and 12.4 ± 7.3 fmol/hr/cell based on the *N. oligotropha amo*A assay.

* Corresponding author phone: (865)974-8080; fax: (865)974-8086; e-mail: sayler@utk.edu.
† Department of Microbiology.
‡ Department of Civil and Environmental Engineering.
§ Center for Environmental Biotechnology.

1. Introduction

Chemolithotrophic nitrification is a two-step process involving two groups of bacteria: ammonia-oxidizing bacteria (AOB) oxidize NH_3 to NO_2^- and nitrite-oxidizing bacteria (NOB) oxidize NO_2^- to NO_3^- (*1*). Nitrification is an important process in biogeochemical nitrogen cycling and in controlling effluent toxicity in wastewater treatment. The physiological activity and abundance of these organisms in wastewater processing is critical in the design and operation of waste treatment systems, particularly since these organisms display low growth rate and high sensitivity to environmental disturbances and inhibitors (*2*). An important aspect of activity relates to reactor design. Single sludge wastewater treatment designs accomplish nitrification concurrent with removal of carbonaceous oxygen demand in one reactor; activated sludge in these reactors contains both heterotrophs and nitrifiers that necessarily compete for resources such as oxygen (*3*). Single sludge reactors are a dominant design in the United States (*4*). Alternatively, nitrification can be accomplished using a series of reactors, the first dedicated to carbonaceous oxygen demand removal and the second to nitrification. To date, molecular quantification of nitrifying populations in activated sludge from full-scale wastewater treatment plants (WWTPs) has been performed on samples obtained from facilities with high nitrogen loads (*5*) or two-stage reators (*6, 7*). Given that single-sludge nitrification processes may contain fewer nitrifiers and are more susceptible to plant upsets, attempts at quantification of nitrifiers in these plants is warranted.

In recent studies, a competitive Polymerase Chain Reaction (cPCR) procedure was developed to quantify nitrifying bacteria by PCR amplification of ammonia monooxygenase (*amoA*) and *Nitrospira* spp. 16S rRNA genes (16S rDNA), respectively (*8, 9*). The cPCR procedure is a well established quantitative method that has been used to enumerate both culturable and nonculturable organisms, including nitrifying bacteria (*10*−*13*). This method relies on the measurement of PCR products at the endpoint, after gel electrophoresis, and it has a log-linear detection range of only 2 to 3 orders of magnitude (*14, 15*). The cPCR technique is robust due to the presence of a stringent internal control. However, cPCR is difficult to use in routine process monitoring of populations in wastewater treatment processes because it is labor- and cost-intensive with low throughput.

In the current study, real-time PCR was investigated for applications in monitoring nitrifying populations in wastewater treatment because it combines high throughput with high analytical sensitivity and precision, offering a dynamic detection range of 6 orders of magnitude or more (*15, 16*). Although real-time PCR has been widely applied in medical research, its application to environmental research has been slower (*17*−*21*). In real-time PCR, amplicons are detected by measurement of a fluorescence signal without post-PCR sample processing such as gel electrophoresis (Figure 1). Several different fluorescent probes can be used in real-time PCR including TaqMan (*22*) or Molecular Beacon (*23, 24*). A TaqMan probe is a linear oligonucleotide complementary to a target nucleic acid sequence, with a fluorophore attached to the 5′end and a quencher to the 3′end (*22*) (Figure 1). The TaqMan probe is cleaved by the 5′ exonuclease activity of *Taq* DNA polymerase as the primer is extended, resulting in the separation of the reporter dye from quencher dye and an increase in fluorescence signal emitted by the reporter. In each cycle, additional reporter dye molecules are cleaved, resulting in an increase in fluorescence intensity proportional to the amount of amplicon produced (*22*). In contrast, a

10.1021/es0257164 CCC: $25.00
Published on Web 12/04/2002

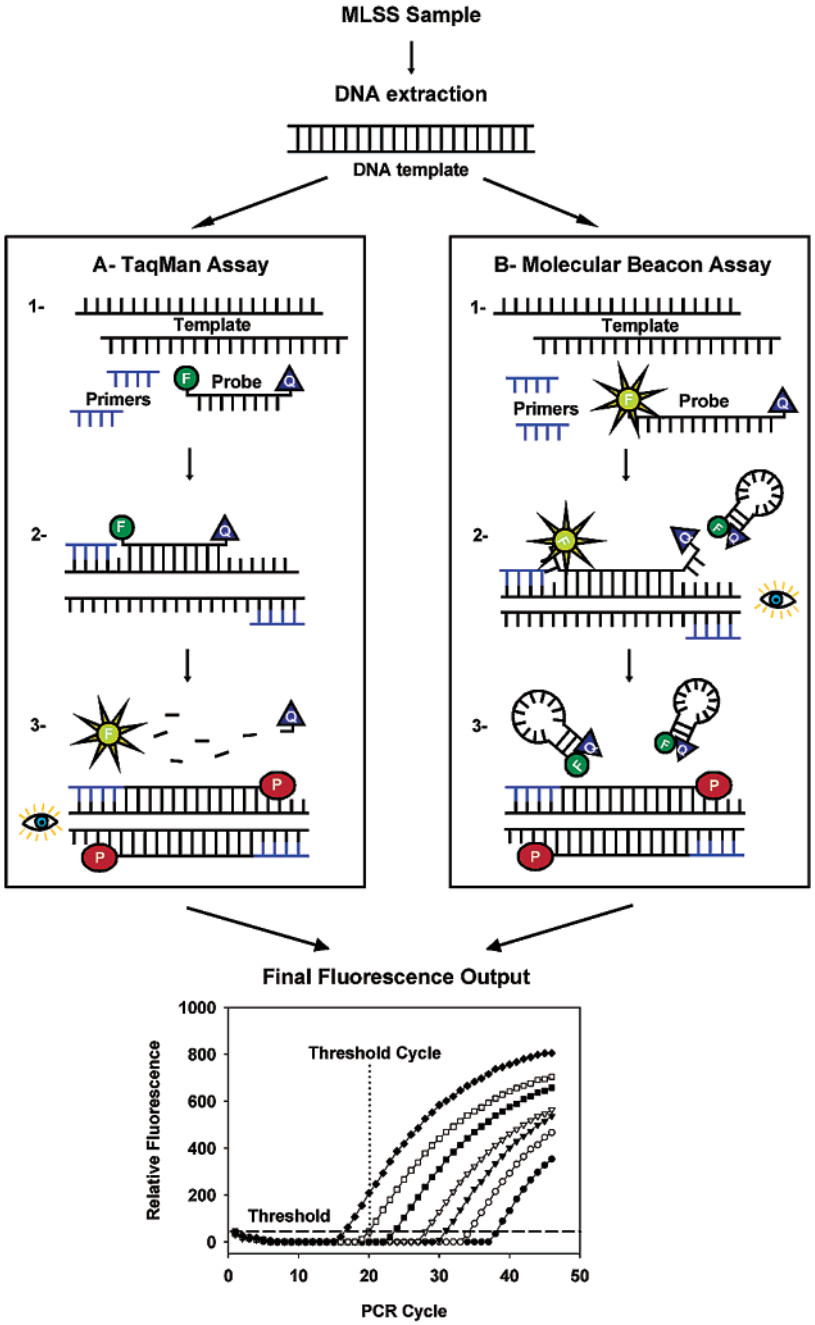

FIGURE 1. Schematic representation of the quantification of specific targets from mixed-liquor suspended solids (MLSS) samples. After DNA extraction, the DNA template is used in real-time PCR assays. (A) TaqManprobe assay. A TaqMan probe is a linear oligonucleotide probe complementary to the target sequence, with a fluorescent dye (green circle, F) attached to the 5′end and a quencher (blue triangle, Q) to the 3′end. The proximity of these two dyes quenches the signal. Steps: (1) Denaturation of the DNA at 95 °C separates the template. (2) Annealing of primers (usually 18−22 nucleotides) and probe to target DNA at temperatures of 50 °C to 60 °C. (3) Extension and synthesis of the DNA strand results in the 5′ exonuclease activity by *Taq* DNA polymerase (red oval, P) separating the fluorescent dye from the quencher resulting in an increase in fluorescence. Steps 2 and 3 are often performed simultaneously. The spectrofluorimetric thermal cycler measures the relative fluorescence at the end of step 3 (eye). Steps 1−3 are repeated 45 to 50 times. In each cycle, additional reporter dye molecules are cleaved, resulting in an increase in fluorescence intensity proportional to the amount of amplicon produced (*22*, *43*). (B) Molecular beacon assay. A Molecular beacon probe has a stem and loop structure. The loop section of the probe is complementary to the target and the stem results from the annealing of artificially designed arm sequences (*23*, *24*, *43*). A fluorescent dye (green circle, F) and a quencher (blue triangle, Q) are attached to the arm sequences. Step: (1) Denaturation at 95 °C results in the separation of the template DNA and separation of the stem on the molecular beacon probe, separating the quencher from the dye allowing fluorescence. (2) Annealing at 60 °C allows the probe to hybridize to the template resulting in fluorescence. Alternatively, if the probe does not hybridize with the template the stem-and-loop structure is reformed and fluorescence is quenched. The relative fluorescence is measured at the beginning of this step (eye) and is proportional to the number of target sequences. (3) Extension and synthesis of DNA at 72 °C results in dissociation of the probe from the target and fluorescence is quenched. Steps 1−3 are repeated 45 to 50 times. The final output for both assays shows relative fluorescence as a function of cycle number. Amplification curves for template concentrations between 10^7 and 10 copies per PCR reaction are shown (left to right). The threshold is calculated as 10 times the standard deviation of the background fluorescence (dashed line). The point where the fluorescence signal crosses the threshold is the threshold cycle (C_T) (dotted line) and is lower when more copies of the template are present at the beginning of the reaction.

TABLE 1. Primers and Probes Used in This Study

assay	target	primer/probe[a]	sequence (5′-3′)[b]	T_M (°C)[c]	reference
bacterial 16S rDNA	bacterial 16S rDNA	1055f	5′-ATGGCTGTCGTCAGCT-3′	57.7	(*25*)
		1392r	5′-ACGGGCGGTGTGTAC-3′	58.9	(*26*)
		16STaq1115	5′-(6-FAM)-CAACGAGCGCAACCC-(TAMRA)-3′	62.9	this study
N. oligotropha-like *amo*A	*N. oligotropha amoA* gene	*amo*No550D2f	5′-TCAGTAGCYGACTACACMGG-3′	55.4	this study
		*amo*No754r	5′-CTTTAACATAGTAGAAAGCGG-3′	55.0	this study
		*amo*NoTaq729	5′-(6-FAM)-CCAAAGTACCACCATACGCAG-(TAMRA)-3′	64.1	this study
AOB	ammonia-oxidizing bacterial 16S rDNA	CTO 189fA/B	5′-GGAGRAAAGCAGGGGATCG-3′	63.5	(*20*)
		CTO 189fC	5′-GGAGGAAAGTAGGGGATCG-3′	62.3	(*20*)
		RT1r	5′-CGTCCTCTCAGACCARCTACTG-3′	62.8	(*20*)
		TMP1	5′-(6-FAM)-CAACTAGCTAATCAGRCATCRGC-CGCTC-(TAMRA)-3′	71.0	(*20*)
Nitrospira 16S rDNA	*Nitrospira* spp. 16S rDNA	NSR1113f	5′-CCTGCTTTCAGTTGCTACCG-3′	64.7	(*9*)
		NSR1264r	5′-GTTTGCAGCGCTTTGTACCG-3′	67.8	(*9*)
		NSR1143Taq	5′-(6-FAM)-AGCACTCTGAAAGGACTGCCCAGG-(TAMRA)-3′	73.9	this study
		NSR1143Beac	5′-(6-FAM)-GCTGCACC|AGCACTCTGAAAGG-ACTGCCCAGG|GGTGCAGC-(DABCYL)-3′		this study

[a] Primer/probe abbreviations: f = forward primer, r = reverse primer, Taq = TaqMan probe, Beac = beacon probe. [b] 5′-Fluorophore-probe-quencher-3′ in case of TaqMan probe; 5′-fluorophore-arm|probe|arm-quencher-3′ in case of Molecular Beacon. 6-FAM = 6-carboxyfluorescein; TAMRA = carboxytetramethylrhodamine; DABCYL = 4-(4-dimethylaminophenyl)azo)benzoic acid. [c] Melting temperatures were calculated using the oligo calculator from Sigma Genosys (http://www.genosys.com/cgi-win/oligo_calconly.exe; Sigma Genosys, The Woodlands, TX).

Molecular Beacon probe has a stem and loop structure, with the loop section of the probe complementary to the target and the stem formed by the annealing of an artificially designed arm sequence (*24*). A fluorescent dye and a quencher are attached to both ends of the molecule (Figure 1). When free in solution the Molecular Beacon adopts a hairpin structure, which results in fluorophore quenching. In the presence of a complementary target, the hairpin structure unfolds and the separation of the fluorophore and the quencher leads to emission of fluorescence. Using either a TaqMan or a Molecular Beacon probe the threshold cycle of a sample is inversely proportional to the logarithm of the amount of template DNA initially added to the PCR reaction for both types of probe (*22*−*24*). Although the design, chemistry and portion of the PCR cycle in which fluorescence is detected differ for these two probes, similar results should be obtained in the enumeration of molecules.

The primary objectives of the current study were to develop methods and to quantify ammonia-oxidizing bacteria and nitrite-oxidizing bacteria (*Nitrospira*) in a single-stage type municipal WWTP. Real-time PCR assays for *Nitrospira* 16S rDNA and *Nitrosomonas oligotropha amo*A genes were developed from existing cPCR assays for monitoring nitrifying bacteria, and a real-time PCR assay developed to enumerate AOB 16S rDNA in soil (*20*) was applied to mixed-liquor suspended solids (MLSS). A bacterial 16S rDNA TaqMan assay was also developed to monitor total biomass in the MLSS samples. Secondary objectives of this study were to compare real-time PCR assays using different probe designs (molecular beacon versus TaqMan), compare real-time PCR assays directed toward ribosomal RNA genes versus catabolic genes (*amo*A), and validate real-time PCR assays by comparison with previous data in copies per liter obtained by dot-blot hybridization and competitive PCR. The results of this study indicate that real-time PCR can be implemented as a tool to facilitate molecular monitoring and quantification of critical sub-populations, such as nitrifying bacteria, in wastewater treatment processes.

2. Methodology

Samples. MLSS samples were collected monthly for one year from a local municipal WWTP treating mainly municipal wastewater. The WWTP employs single stage reactors for carbon (biological oxygen demand (BOD_5)) and nitrogen (NH_4^+-N) removal designed with average and peak flow capacities of 40 million gallons per day (MGD) and 70 MGD, respectively. In the year 2000, this single stage reactor system was operated at an average solids retention time of 12 days. The reactor system consists of six identical basins arranged in parallel, each basin being 183 feet long, 32 feet wide, and unusually deep at 33 feet. The basins are in turn sub-divided into five compartments in series along the flow path. Each compartment is aerated with ceramic fine bubble diffusers that provide for complete mixing. The basins are fed via a common influent channel. Gravity overflow from the basins first combines and then splits to flow to a series of circular clarifiers. MLSS samples were collected from the upwell at the center of one of the clarifiers prior to the effluent flow entering the clarifier quiescent zone. Reactor specific flow and influent ammonia data along with plant effluent data was obtained. The mean influent BOD_5 was 302 (± 47) mg/L, and mean mixed-liquor volatile suspended solids (MLVSS) was 1971 ± 178 mg/L over the one-year period. The mean monthly influent temperature for the year was 17 °C, with a low of 9 °C and a high of 25 °C. Genomic DNA was extracted in triplicate from 2 mL of MLSS samples using a FastDNA kit (BIO 101, Vista, CA) with minor modifications as described by Dionisi et al. (*9*).

Real-Time PCR Assays. Real-time PCR assays were developed for the quantification of bacterial 16S rDNA, *Nitrospira* spp. 16S rDNA, and *N. oligotropha*-like *amoA*. Three TaqMan probes, 16sTaq1115, *amo*NoTaq729, and NSR1143-Taq, were designed (Table 1) using the guidelines provided by Applied Biosystems (http://home.appliedbiosystems.com; Applied Biosystems, Foster City, CA). The primers and probes were synthesized by Sigma Genosys (Sigma Genosys, The Woodlands, TX). A Molecular Beacon probe, NSR1143Beac, was designed using the guidelines provided at http://www.molecular-beacons.org/protocol.html and synthesized by Stratagene (Stratagene, La Jolla, CA). The optimal artificial arm sequences were determined by using the Zuker DNA folding program (mfold) (http://bioinfo.math.rpi.edu/~mfold/dna/form1/cgi).

The real-time PCR assay for AOB used two forward primers CTO 189A/B and CTO189C, one reverse primer RT1r and the TaqMan probe TMP1 (Table 1) as described by Hermansson and Lindgren (*20*).

Real-Time PCR for Quantification of Bacterial 16S rDNA. Bacterial 16S rDNA was amplified using primers 1055f (*25*) and 1392r (*26*) (Table 1). The TaqMan probe 16STaq1115 was modified from the 1114f primer (*26*). The PCR mix with a total volume of 25 μL contained Platinum Quantitative PCR SuperMix-UDG (Life Technologies, Inc., Gaithersburg, MD) with 5 mM $MgCl_2$, 15 pmol primers 1055f and 1392r, 6.25 pmol TaqMan probe 16STaq1115, 3.2 to 7.0 ng of sample DNA or dilutions of plasmid pCR2.1 vector (Invitrogen, Carlsbad, CA) carrying a 16S rRNA gene for *Nitrospira* (GenBank accession number AF420301) (*9*) as standard (from 4.5×10^3 to 4.5×10^8 copies of the 16S rDNA gene). The PCR program was 3 min at 50 °C, 10 min at 95 °C, 45 cycles at 95 °C for 30 s, 50 °C for 60 s, and 72 °C for 20 s.

Real-Time PCR for Quantification of *Nitrospira* 16S rDNA. The *Nitrospira* 16S rDNA primers NSR1113f and NSR1264r (Table 1) were previously designed and tested using genomic DNA extracted from municipal and industrial MLSS as templates (*9*). The TaqMan probe NSR1143Taq (Table 1) was derived from a conserved sequence region between the primers NSR1113f and NSR1264r. The probe portion of the Molecular Beacon NSR1143Beac (Table 1) is identical to that one used in the TaqMan probe. The probe region of NSR1143Beac has a T_M of 68.0 °C, and the stem region has a T_M of 70.2 °C (with 5.0 mM $MgCl_2$).

Real-time PCR assays using NSR1143Taq were performed in a total volume of 25 μL with 5 mM $MgCl_2$, Platinum SuperMix, 15 pmol of primers NSR1113f and NSR1264r, 6.25 pmol TaqMan probe NSR1143Taq, and 3.2 to 7.0 ng of sample DNA or 30 to 3×10^7 copies of the standard (151 bp fragment of *Nitrospira* 16S rDNA from AF420301) (*9*). PCR amplification consisted of 2 min at 50 °C, 10 min at 95 °C, 55 cycles at 95 °C for 30 s, 63 °C for 60 s.

The Molecular Beacon assay contained Platinum SuperMix with 5 mM $MgCl_2$, 25 pmol of primers NSR1113f and NSR1264r, 8.5 pmol Molecular Beacon probe NSR1143Beac, 3.2 to 7.0 ng of sample DNA or 30 to 3×10^6 copies of standard DNA in 25 μL. PCR amplification consisted of 5 min at 95 °C, 55 cycles at 95 °C for 30 s, 60 °C for 60 s, and 72 °C for 10 s.

Real-Time PCR for Quantification of *N. oligotropha*-like amoA Gene. The primers *amo*No550D2f and *amo*No754r (Table 1) were designed to target the *amoA* gene of ammonia-oxidizing bacteria found in the full-scale municipal WWTP (*9*) based on alignment of *amoA* gene sequences using the CLUSTAL W program (*27*). Alignments consisted of *amoA* sequences from clonal libraries obtained from the WWTP (*9*), four bench-scale municipal wastewater treatment systems (*8*), and *amoA* sequences available in GenBank (*28*). The forward primer *amo*No550D2f contained two degenerate bases in order to amplify all *amoA* clones from the libraries, as well as *N. urea* (AJ388585) and *N. oligotropha* (AF272406) *amoA* genes. The TaqMan probe *amo*NoTaq729 was derived from a conserved sequence region within the primer pair *amo*No550D2f and *amo*No754r (Table 1). The 25 μL PCR mix contained TaqMan Universal PCR Master Mix (PE Applied Biosystems, Foster City, CA) with 7.5 pmol primers *amo*No550D2f and *amo*No754r, 6.25 pmol TaqMan probe *amo*NoTaq729, 0.3 to 0.7 ng of sample DNA. Standards consisted of the plasmid pCR2.1 carrying the M-20 *amoA* gene (GenBank accession number AF420299) (*9*) adjusted to 10 to 1.0×10^7 copies per PCR. PCR amplification consisted of 3 min at 50 °C, 10 min at 95 °C, 55 cycles at 95 °C for 30 s, 56 °C for 60 s.

Real-Time PCR for Quantification of AOB 16S rRNA Gene. Real-time PCR assays were performed as described by Hermansson and Lindgren (*20*) in a total volume of 25 μL with Universal PCR Master Mix (PE Applied Biosystems), 7.5 pmol of a 2:1 ratio of primers CTO 189fA/B and CTO 189fC, 7.5 pmol of the reverse primer RT1r, 3.125 pmol TaqMan probe TMP1, and 0.3 to 0.7 ng of sample DNA or 60 to 6×10^7 copies of the standard (*Nitrosomonas europea* 16S rDNA cloned into pCR2.1). PCR amplification consisted of 2 min at 50 °C, 10 min at 95 °C, 40 cycles at 95 °C for 30 s, 60 °C for 60 s.

Acquisition and Data Analysis. Real-time PCR assays for bacterial 16S rDNA and *Nitrospira* 16S rDNA were run on a Bio-Rad iCycler with the iCycler iQ fluorescence detector and iCycler software version 2.3 (Bio-Rad, Hercules, CA). Plate well factors were determined prior to each PCR run to normalize background fluorescence intensities from each single well. *amoA* and AOB 16S rDNA real-time PCR assays were run using a DNA Engine Opticon Continuous fluorescence Detection System (MJ Research, Waltham, MA). The threshold was determined by the computer software as 10 times the standard deviation of the background fluorescence averaged over at least 5 cycles at the start of the run. The threshold cycle (C_T) of each PCR reaction was automatically determined by detecting the cycle at which the fluorescence exceeded the calculated threshold. For the Molecular Beacon probe, the data window was adjusted to capture roughly 10% of all data, chosen among the data points in the beginning area of the annealing step. During each PCR run, the C_T values obtained from the DNA standards were used for the construction of standard curves.

Data for bacterial 16S rDNA, *Nitrospira* 16S rDNA, and *N. oligotropha*-like *amoA* was previously collected for the same set of samples used in this study using dot-blots and hybridization with a P^{32} labeled universal 16S rDNA probe and competitive PCR assays for the *Nitrospira* 16S rDNA and *amoA* genes (*9*). These data were compared to the data obtained by real-time PCR for each gene using paired samples t-tests. Paired samples t-tests compute the differences between the values of the two variables for each case and tests whether the average differs from zero (SPSS version 11.01, SPSS Inc., Chicago, IL). The null hypothesis was that there were no significant differences between the copies/L on each sample date obtained by the real-time PCR assay and the analogous competitive PCR assay or dot-blot hybridization.

Application of Real-Time PCR Assays to MLSS Samples. All real-time PCR assays were performed using three replicates per sample, and all PCR runs included control reactions without template. The effect of sample concentration on PCR performance was determined using dilutions of sample DNA (initial concentration of 50 ng/μL) containing 5 pg to 50 ng in sterile water followed by real-time PCR analysis for 16S rDNA, *Nitrospira* 16S rDNA, AOB 16S rDNA, and *N. oligotropha*-like *amoA* as described above.

Gene copies were initially calculated by comparison of threshold cycles obtained in each PCR run from known standard DNA concentrations. To reduce variability between PCR runs, data were recalculated using a second standard curve generated from 11 standard curves for bacterial 16S rDNA ($r^2 = 0.94$), 22 standard curves for *Nitrospira* 16S rDNA ($r^2 = 0.84$), 3 standard curves for AOB 16S rDNA ($r^2 = 0.98$), and 5 standard curves for *N. oligotropha amoA* ($r^2 = 0.90$). In the case of the *Nitrospira* 16S rDNA and bacterial 16S rDNA assays, one universal standard curve was applied for calculations, because both assays were shown to function alike with standard plasmid AF420301 as template (Figure 2).

3. Results

Development and Optimization of Real-Time PCR Assays. The real-time PCR assays were validated using known concentrations of standard DNA. The linear range of detection for the real-time PCR assay for bacterial 16S rDNA was 4 orders of magnitude, from 4.5×10^4 to 4.5×10^8 copies per PCR and the detection limit for this assay was 4.5×10^3 target DNA copies. The linear range of detection for the real-time PCR assays for *Nitrospira* 16S rDNA, *N. oligotropha amoA*,

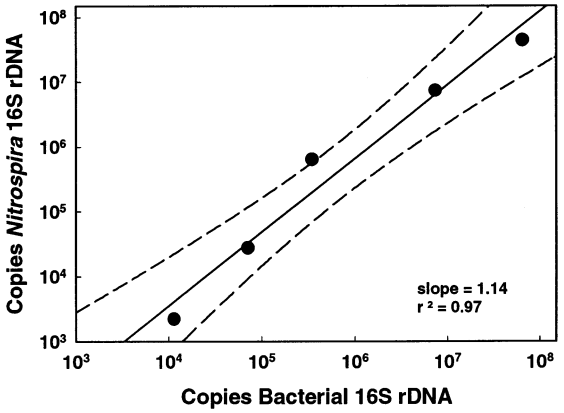

FIGURE 2. Comparison of bacterial 16S rDNA and *Nitrospira* 16S rDNA real-time PCR TaqMan assays. The plasmid containing *Nitrospira* 16S rDNA was used as the target for both assays. The dashed line represents the 95% confidence interval. Error bars indicate the standard deviation of 3 PCRs and are smaller than the symbols.

and AOB 16S rDNA were at least 6 orders of magnitude, from 30 to 3.0×10^7, 10 to 1.0×10^7, and 60 to 6×10^7 copies per PCR, respectively. The regression coefficient (r^2) values for standard curves for all real-time PCR assays in each run were always above 0.90. In addition, copies per PCR calculated for the standard plasmid AF420301 by the bacterial 16S rDNA and *Nitrospira* 16S rDNA assays were highly correlated and demonstrated a linear relationship ($r^2 = 0.97$) with a slope of 1.14, indicating that the assays for bacterial 16S rDNA and *Nitrospira* 16S rDNA were functioning alike (Figure 2).

Application of Real-Time TaqMan PCR Assays to WWTP Samples. In environmental samples, PCR techniques can be biased by the presence of inhibitory compounds that co-purify with the DNA or low target concentrations in a high background of heterologous DNA (*15*, *29*). Inhibition effects by the environmental samples on the real-time PCR assays were tested using serial dilutions of genomic DNA extracted from MLSS. PCR amplification was completely inhibited in the undiluted sample at 50 ng per PCR assay for all four real-time PCR assays and also in diluted samples containing 10 ng per PCR assay for the bacterial 16S rDNA and the *N. oligotropha*-like *amoA* assays. C_T values were proportional to DNA at concentrations ranging from 5 pg to 5 ng for the bacterial 16S rDNA ($r^2 = 0.98$, slope $= -3.87$), at concentrations ranging from 5 pg to 5 ng for the *Nitrospira* 16S rDNA ($r^2 = 0.98$, slope $= -3.45$), at concentrations from 250 pg to 2.5 ng for the AOB 16S rDNA ($r^2 = 0.98$, slope $= -3.15$), and at concentrations ranging from 200 pg to 1 ng for the *N. oligotropha*-like *amoA* assay ($r^2 = 0.97$, slope $= -3.01$) (Figure 3A). In the no-template control reactions no C_T values were obtained for the *Nitrospira* 16S rDNA, AOB 16S rDNA, and *N. oligotropha*-like *amoA* assays. In the no template control reactions using the 16S rDNA assay a C_T value of 29.7 ± 0.43 was obtained. Conversion of the C_T value to copies per PCR based on the standard curve indicated that there were $3.8 \pm 0.9 \times 10^3$ copies bacterial 16S rDNA in the control reaction without sample DNA. This background value may result from bacterial DNA contamination of the *Taq* enzyme or other reagents in the PCR mix. The MLSS sample containing greater than 5 pg DNA per PCR assay was 20-fold higher than the detection limit (Figure 3B) so the effect of background contamination on the calculated value would be less than 5% in the most dilute samples. Conversion of the C_T values to copies per PCR for the other 3 assays indicated that the detection limit for quantifying these targets in MLSS samples were 180 copies for *Nitrospira* 16S rDNA, 1.2×10^3 for *N. oligotropha*-like *amoA*, and 2.6×10^3 AOB 16S rDNA assays (Figure 3 B).

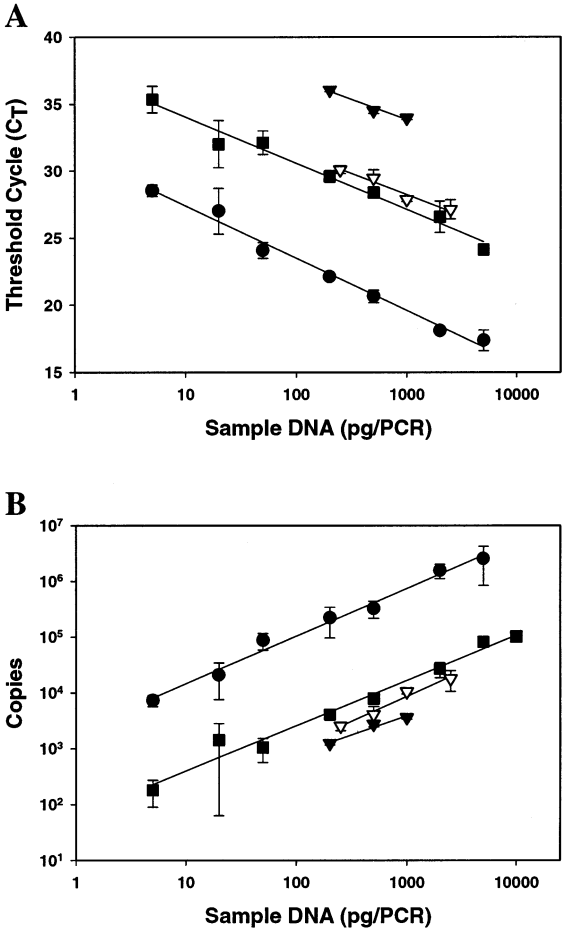

FIGURE 3. (A) Threshold cycle (C_T) measurements in diluted DNA from a MLSS sample and (B) calculated copies of target genes in diluted DNA from a MLSS sample. TaqMan real-time PCR assays shown are Bacterial 16S rDNA (●), *N. oligotropha*-like *amoA* gene (▼), AOB 16S rDNA (▽), and *Nitrospira* 16S rDNA (■). Error bars indicate the standard deviation of 3 PCRs.

Comparison of TaqMan and Molecular Beacon Probes in the Nitrospira 16S rDNA Real-Time PCR Assay. Fluorescence obtained with NSR1143Taq was twice as high as the fluorescence obtained with NSR1143Beac (Figure 4a), indicating a higher signal-to-noise ratio using the TaqMan probe. The r^2 values of standard curves obtained using NSR1143Beac and NSR1143Taq were similar, ranging from 0.97 to 0.99 and 0.92 to 0.99, respectively. When MLSS samples were analyzed using both probes, the copies obtained with the Molecular Beacon probe were up to 12.5 times lower than those obtained with the TaqMan probe, except for the last 3 samples in which the values were almost equivalent (Figure 4b). *Nitrospira* 16S rDNA copies per liter in the municipal WWTP ranged from 1.7×10^{10} to 1.2×10^{11} using the TaqMan probe and from 2×10^9 to 2.1×10^{11} using the Molecular Beacon probe. In a paired t-test the differences in results obtained by the Molecular Beacon probe and the TaqMan probe assays were not significant ($t = -0.125$, $p = 0.903$) (Table 2).

Validation of *N. oligotropha amoA*, *Nitrospira* 16S rDNA, and Bacterial 16S rDNA Real-Time PCR Assays. To verify the specificity of the *amoA* primers, PCR product obtained with genomic DNA extracted from MLSS of the municipal WWTP as template was cloned and sequenced as described previously (*9*). Amplification of genomic DNA from suspended solids using the primers *amo*No550D2f and *amo*No754r produced a product with the expected size of approximately 205 bp (data not shown). In a clonal library,

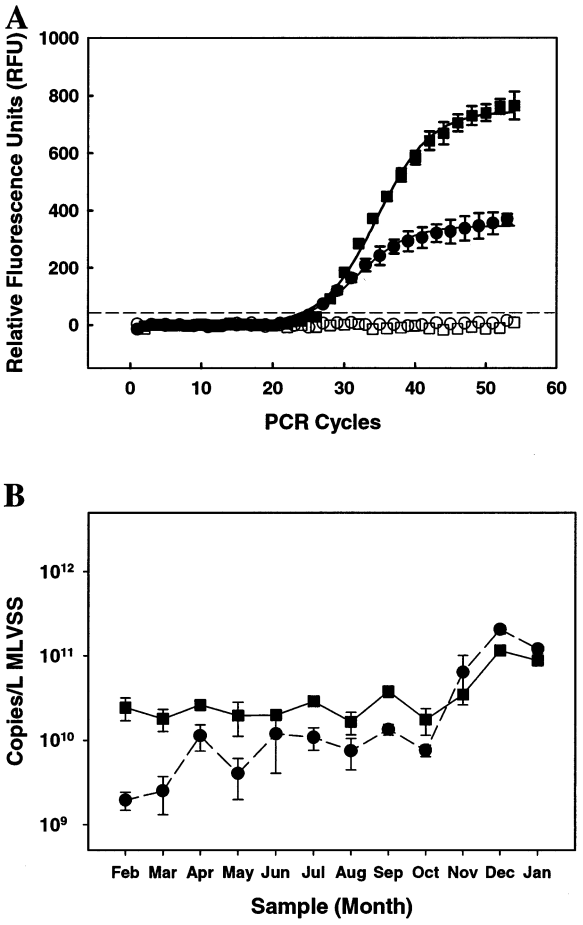

FIGURE 4. (A) Measurements of *Nitrospira* 16S rDNA copies during real-time PCR using TaqMan probe NSR1143Taq (■) or molecular beacon probe NSR1143Beac (●) in DNA extracted from MLSS. The fluorescence intensity was measured during the annealing step of each temperature cycle. No-template controls are shown as (□) and (○) for the TaqMan and molecular beacon probes, respectively. Error bars indicate the standard deviation of 3 reactions. The dashed line represents the threshold value (42 RFU) calculated for the molecular beacon assay. The threshold value for the TaqMan assay was 20 RFU. (B) *Nitrospira* 16S rDNA copies per liter as determined by real-time PCR using TaqMan probe NSR1143Taq (■) or Molecular Beacon probe NSR1143Beac (●) in DNA extracted from MLSS.

TABLE 2. Comparison of Real-Time PCR Assays with Other Molecular Methods Using Paired Samples t-Test

compared groups	mean paired differences	t	df	significance (2-tailed)
microbial 16S dot-blot[a] vs bacterial 16S TM[b]	2.2×10^{13}	10.79[h]	11	0.000
amoA[c] TM vs *amoA* cPCR[d]	1.5×10^{10}	4.324[h]	11	0.001
Ntspa[f] TM vs Ntspa cPCR	1.4×10^{10}	1.456	11	0.173
Ntspa MB[g] vs Ntspa cPCR	1.5×10^{10}	0.813	11	0.433

[a] dot-blot = dot-blot hybridization. [b] TM = TaqMan based real-time PCR assay. [c] *amoA* = *N. oligotropha*-like *amoA*. [d] cPCR = competitive quantitative PCR. [e] AOB = ammonia-oxidizing bacteria 16S rDNA. [f] Ntspa = *Nitrospira* spp. 16S rDNA. [g] MB = Molecular Beacon based real-time PCR assay. [h] Significant at the prescribed α.

23 randomly selected clones were identified as *amoA* sequences. These sequences were 89−94% similar to clone M20 and 92−94% similar to clone M379, which were *amoA* clone sequences previously isolated from this WWTP (*9*). *N. oligotropha*-like *amoA* gene copies ranged from 2.6×10^9 to 4.3×10^{10} per L MLSS of the municipal WWTP. In contrast, no amplification was observed when DNA isolated from an industrial WWTP, where no *N. oligotropha*-like *amoA* sequences were detected (*9*), was used as template (data not shown).

Paired samples t-tests were used to determine whether gene copies obtained in the real-time PCR assays were equivalent to the values previously obtained using dot-blot hybridization and competitive PCR assays for *Nitrospira* 16S rDNA and *N. oligotropha*-like *amoA* (Table 2). The mean bacterial 16S rDNA copies per liter previously obtained by dot-blot hybridization was $2.3 \pm 0.7 \times 10^{13}$ (*9*). In this study, the average 16S rDNA copies per liter obtained by real-time PCR was 1 order of magnitude lower at $1.6 \pm 0.7 \times 10^{12}$ and the differences in values obtained by these two methods were statistically significant (Table 2). The mean *Nitrospira* 16S rDNA copies per liter previously obtained by competitive PCR was $2.4 \pm 1.3 \times 10^{10}$ compared to $3.7 \pm 3.2 \times 10^{10}$ and $3.9 \pm 6.4 \times 10^{10}$ copies per liter obtained using the *Nitrospira* TaqMan and Molecular Beacon assays, respectively. The differences in the values obtained by the three assays were not statistically significant (Table 2). The mean values obtained using the *N. oligotropha*-like *amoA* competitive PCR and the real-time PCR were $3.4 \pm 2.3 \times 10^8$ and $1.5 \pm 1.2 \times 10^{10}$ copies per liter, respectively. The differences in the mean values obtained by the two assays were statistically significant and may reflect the differences in the primers used for the two assays.

Calculation of Cells/Liter from Copies/Liter and Ammonia-Oxidizing Activity in MLSS Samples. The number of total bacteria, AOB, *N. oligotropha*-like AOB and *Nitrospira* cells per liter MLSS were calculated from copies per liter using several assumptions regarding gene copies per cell (Table 3, Figure 5). First, the average 16S rDNA copies per genome in bacterial cells were assumed to be 3.6 copies based on the average 16S rDNA copies found in cultured bacteria (*30*). Second, one cell of *N. oligotropha* was assumed to contain 2 copies *amoA* based on the copies reported for *N. europaea* (*31*). Third, both AOB and *Nitrospira* were assumed to contain 1 copy 16S rDNA per cell based on copies 16S rDNA found in *Nitrobacter* and the AOB *Nitrosomonas and Nitrosospira* (*32, 33*).

In the 12 monthly samples, total bacteria ranged from 2.3×10^{11} to 8.4×10^{11} and *Nitrospira* spp. ranged from 1.7×10^{10} to 1.2×10^{11} cells per liter MLSS and were relatively constant through the year (Figure 5). The number of ammonia-oxidizing bacteria as determined using the AOB 16S rDNA and *N. oligotropha amoA* assays were not as constant with a drop in AOB in the August, September and October samples (Figure 5). Although the number of AOB calculated using the 16S rDNA assay were about 2-fold the values calculated using the amo*A* assays, the assays were significantly correlated with a Pearsons coefficient of 0.901.

Ammonia-oxidizing activity per cell·hour was calculated from the AOB cell number and WWTP plant data using the following formula:

$$\text{activity} = \frac{(NH_{4IN}^{+} - NH_{4UP}^{+} - NH_{4OUT}^{+}) \times Q}{AOB}$$

$NH_4^+{}_{IN}$ is the average ammonia concentration (measured in mg nitrogen/L) in the secondary influent into the WWTP reactor. $NH_4^+{}_{OUT}$ is the average ammonia concentration in the plant effluent. $NH_4^+{}_{UP}$ is used to account for ammonia assimilation, because ammonia is removed by both assimilation into cells for cell growth and by autotrophic ammonia oxidation by AOB, and equals $0.20 \times NH_4^+{}_{SI}$. Ammonia assimilation values of 0.1 and 0.2 were previously used by Daims et al. (*6, 34*). In this WWTP an ammonia assimilation value of 0.3 was estimated based on a Monte Carlo analysis (*35*) using the steady-state ASM1 model (*36*).

TABLE 3. Conversion of Copies/L to Cells/L, Cells/g, and Percent of Biomass

target cells[a]	copies/L	cells/L[b]	cells/g[c]	% biomass[d]
bacteria 16S rDNA	$1.6 \pm 0.7 \times 10^{12}$	$4.3 \pm 2.0 \times 10^{11}$	$2.2 \pm 0.97 \times 10^{11}$	100
N. oligotropha amoA	$1.5 \pm 1.2 \times 10^{10}$	$7.5 \pm 6.0 \times 10^{9}$	$3.8 \pm 3.0 \times 10^{9}$	1.7
AOB 16S rDNA	$1.2 \pm 0.9 \times 10^{10}$	$1.2 \pm 0.9 \times 10^{10}$	$6.1 \pm 4.7 \times 10^{9}$	2.9
Nitrospira 16S rDNA	$3.7 \pm 3.2 \times 10^{10}$	$3.7 \pm 3.2 \times 10^{10}$	$1.9 \pm 1.6 \times 10^{10}$	8.6

[a] Values are averaged for all 12 samples. [b] Cells/L = copies/L ÷ gene copy number/cell. Assumed gene copy number/cell is 3.6 for bacterial 16S rDNA, 1 for *Nitrospira* 16S rDNA, and 2 for *amoA* gene. [c] Cells/g = cells/L ÷ 1.97 g/L (mean mixed liquor volatile suspended solids (MLVSS)). [d] Bacterial 16S rDNA provides normalization for comparison to the subpopulations and is set at 100%.

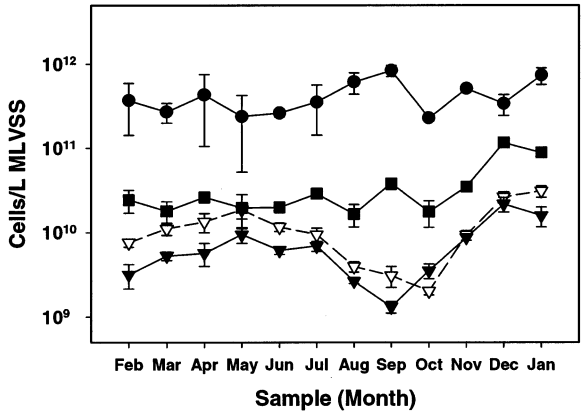

FIGURE 5. Total bacteria (●), *N. oligotropha* (▼), AOB (▽), *Nitrospira* (■) cells per L MLSS in a municipal WWTP determined using the bacterial 16S rDNA, *N. oligotropha amoA*, AOB 16S rDNA and *Nitrospira* real-time PCR Taqman assays. Error bars indicate the standard deviation of 3 reactions. Quantitative detection limits for each assay were as follows: bacterial 16S rDNA, 1.0×10^{10}, *N. oligotropha*-like *amoA*, 1.5×10^{9}, AOB 16S rDNA 2.6×10^{9} and *Nitrospira* 16S rDNA, 9×10^{7} cells per L.

An intermediate value of 0.2 was used for activity calculations. Q is the average influent flowrate to the WWTP reactor (L/hr). AOB is the total AOB cell number in the basin as determined by real-time PCR.

Mean monthly ammonia-oxidizing activity based on the *N. oligotropha amoA* cell calculations ranged from 3.5 to 56.2 fmol/hr/cell. The highest activity value, which was greater than twice the standard deviation of the data set, was excluded resulting in a mean activity of 12.4 ± 7.3 fmol/hr/cell. The mean ammonia-oxidizing activity based on the AOB 16S rDNA cell values was 7.7 ± 6.8 fmol/hr/cell.

4. Discussion

Real-time PCR using a fluorescent internal probe combines high throughput with high analytical sensitivity for the detection of specific genes present in low concentrations in complex and variable DNA mixtures, such as those extracted from environmental samples. The log-linear detection range using standards in the real-time PCR assays was very broad, with up to 6 orders of magnitude and detection limits of 10 to 60 copies for the *Nitrospira* 16S rDNA, 16S AOB rDNA, and *N. oligotropha amoA* assays. These values were comparable to other real-time PCR assays (*15*−*20*). The bacterial 16S rDNA assay showed a log-linear detection range across 4 orders of magnitude. The higher detection limit (4.5×10^{3} copies per PCR) in the bacterial assay was comparable to the detection limit for other bacterial real-time PCR assays (*21*, *37*). These high detection limits in bacteria real-time PCR assays are generally attributed to DNA contamination of the *Taq* DNA polymerase enzyme or other sources such as water and plasticware (*37*). The high detection limit in the bacterial real-time PCR assay did not affect the use of this assay in DNA extracted from MLSS because these samples contained approximately 10^{8} bacterial 16S rDNA copies per μg of DNA.

The bacterial 16S rDNA real-time PCR assay was designed to be a broad-based assay for the estimation of total bacterial biomass in the MLSS. This assay may also serve as an internal standard, since bacterial 16S rDNA in the MLSS remained relatively constant. Although the PCR primers and probes used in this study have zero base pair mismatches with more than 9000 bacterial 16S rDNA sequences found in GenBank, the number of bacterial cells calculated needs to be considered an estimate because some bacterial strains/and or species may not hybridize to the primers or probes and thus may not be detected. The cell/L values obtained using the real-time PCR assay were 10-fold less than those obtained by dot-blots using a single probe. This may result from the reduction of background due to the combined specificity levels of the primers and probe used for the 16S rDNA assay. In addition the probe 1392r used for the dot-blot hybridizations (*9*) is a universal probe that hybridizes to eukaryotic DNA found in the MLSS and measures total microbial population including both eukaryotic and bacterial cells. The bacterial real-time PCR assay used in this study has not been compared to other bacterial 16S rDNA real-time PCR assays (*21*, *37*, *38*).

Application of PCR assays to environmental samples is complicated by several factors such as low concentration of targets and the possible presence of PCR inhibitors. The inhibitory effects of complex genomic DNA at concentrations of 10 to 50 ng/ul and other PCR inhibitors can be minimized by dilution of the DNA extracts. However, over-dilution may result in low total DNA and target concentrations resulting in high variability and potential over- or under-estimation of the target. In this study, the acceptable DNA per PCR range varied between the assays with the bacterial 16S rDNA and *Nitrospira* 16S rDNA assays producing similar results over a broad DNA concentration range (1000 to 2500-fold). In contrast, the *N. oligotropha*-like *amoA* and AOB 16S rDNA assay produced consistent results over a narrow DNA concentration range (5 to 10-fold). These results suggest that in DNA extracts from environmental samples real-time PCR assays may need to be performed using several dilutions. Other factors that may affect real-time PCR assays are a loss in fluorescence signal due to a large excess of complex DNA or an overestimation of target DNA due to limited probe specificity (*15*).

The design of specific PCR assays for *Nitrospira* 16S rDNA was straightforward because regions of the *Nitrospira* 16S rDNA are well conserved between *Nitrospira* species and distinct from nearest phylogenetic groups (*39*, *40*). The primers used for the *Nitrospira* real-time PCR assays in this study were previously demonstrated to be specific for *Nitrospira* sp. in the cPCR assay (*9*). The Molecular Beacon and TaqMan probes were designed using the same target sequence used in the cPCR assay. The differences in values obtained previously in individual MLSS samples using cPCR and more recently using the Molecular Beacon and TaqMan real-time PCR assays were statistically insignificant. This suggests that quantitative PCR methods are robust and that real-time PCR assays can be adapted from cPCR assays. Slight differences in numbers obtained with cPCR and real-time

PCR using the TaqMan probes have also been reported for the quantification of *Mycobacterium tuberculosis* DNA in sputum (*41*).

In contrast to the *Nitrospira* assay, designing a real-time PCR assay to detect AOB is more complicated for two reasons. First, wastewater treatment plants may contain multiple species of AOB (*28*). Second, most AOB are phylogenetically closely related to other activated sludge bacteria within the beta-subdivision class of Proteobacteria.

Both the 16S rDNA and *amo*A gene provide well-studied genetic markers for the characterization of AOB (*28*). DNA probes and primers targeting AOB 16S rDNA have been used to detect essentially all known beta-subdivision AOB species (*2*, *5*−*7*, *11*, *12*, *28*). The 16S rDNA assay described by Hermansson and Lindgren (*20*) is broad based and can quantify a range of AOB species including *N. oligotropha*, the presumptive AOB species at this WWTP. One potential problem with probes and primers designed to target AOB 16S rDNA is that they may hybridize with closely related non-AOB species resulting in false positives. Indeed, BLAST analysis of the primers and probe used in the AOB 16S rDNA assay indicated that one of the degenerate permutations of each of the RT1r and TMP1 sequences had zero mismatches to *Ralstonia eutropha*, a non-AOB species. However, in real-time PCR assays, 3 oligonucleotides (2 primers and 1 probe) must bind for efficient amplification and detection, so the effect of nonspecific hybridization by one primer or probe is reduced.

The *amoA* gene can be used as an alternative phylogenetic marker or target molecule for the detection of AOB (*28*). The *amo*A gene is found only in AOB and thus serves as a more specific marker than the 16S rDNA. However, the *amo*A gene has higher sequence variability between AOB than the 16S rDNA, thus making it more difficult to design a single assay to detect all ammonia oxidizing bacteria. In this study, the real-time PCR assay was designed to detect *N. oligotropha*-like bacteria because it was the only AOB previously found within the municipal WWTP used in this study (*9*). The primers *amo*No550D2f and *amo*No754r were redesigned from the previously published cPCR primers based on additional sequence information obtained from *amoA* clonal libraries made to MLSS obtained from a bioreactor system (*8*). The specificity of the primer pair was confirmed by additional sequence analysis of clonal libraries obtained after PCR amplification and cloning of the product. When compared to the cPCR assay, the real-time PCR with the redesigned primers detected about 50-fold more *amoA* copies in the municipal MLSS samples. The specificity of the *N. oligotropha*-like *amoA* real-time PCR assay was confirmed by the lack of amplification found in industrial MLSS samples which lack *N. oligotropha* but contain *N. nitrosa* AOB (*9*).

Because the AOB 16S rDNA assay has the potential to produce false positives and the *N. oligotropha amo*A assay has the potential to produce false negatives, the use of the two assays in these samples provides complimentary data for the detection of AOB. The 16S rDNA assay and the *amo*A assay were highly correlated and both assays indicated a drop in AOB during August, September, and October suggesting that both assays were useful for AOB quantification. The reason for the drop in AOB is unclear as the percent nitrification remained constant and there was no apparent link to the basin temperature. The AOB cell numbers calculated by the 16S rDNA assay were approximately 2-fold higher than the *N. oligotropha* cell numbers using the *amo*A assay. Although AOB have not been cultured from this plant, these results suggest that *N. oligotropha*-like AOB comprise at least 50% of the AOB population in this WWTP.

The percent of the AOB population determined using either the AOB 16S rDNA assay (2.9%) or the *N. oligotropha amo*A assay (1.7%) were 3−4-fold lower than reported for an activated sludge sample obtained from the second stage of a 2-stage WWTP measured by fluorescence in situ hybridization (FISH) (8.4%) (*6*). Additionally, the AOB percent was approximately 2−3-fold lower than reported for a sludge sample from an industrial plant connected to a rendering factory (7%) (*7*). Differences in the percent of ammonia-oxidixing bacteria between these studies may reflect the differences in the operation and design of the WWTPs, e.g. single-sludge nitrification versus two-stage rectors or sludge with a high nitrogen load. Alternatively, the differences may reflect differences in the methods, FISH versus real-time PCR. Interestingly, the percent *Nitrospira* (8.6%) calculated in this study is consistent with values obtained by FISH in the sludge from an industrial plant connected to a rendering factory at 9% to 12% *Nitrospira* population (*7*, *40*).

The ammonia-oxidizing activity per cell per hour was calculated based on the estimated number of ammonia-oxidizing cells in the basin and the estimated amount of ammonia oxidized per hour. The estimated ammonia-oxidizing rate of 7.7 fmol/hr/cell based on AOB 16S rDNA, or 12.4 fmol/hr/cell based on the *N. oligotropha amoA*, were in the range of values reported using FISH (2.3 ± 0.4 fmol/hr/cell) (*6*), a cPCR assay (16 to 43 fmol/hr/cell) (*34*) and for pure cultures (4 to 23 fmol/hr/cell) (*42*). Given that the estimated ammonia-oxidizing rates are in the expected range, it is likely that the real-time PCR assays used in this study detects one of, if not, the major organisms mediating ammonia oxidation in the WWTP under study.

The results of this study indicate that real-time PCR technology is a valuable tool for quantification of uncultivable or difficult to culture microbes in environmental samples, offering high throughput, analytical sensitivity, and precision. The bacterial 16S rDNA assay and the *Nitrospira* 16S rDNA assays described in the study and the previously published AOB 16S rDNA may have broad utility to other wastewater treatment plants and environmental samples. The *N. oligotropha amoA* assay will be more useful in wastewater systems where the *N. oligotropha* is a known member of the AOB population. The *amo*A assay may also prove useful in developing mRNA based reverse transcriptase real-time PCR assays to measure physiological responses of *N. oligotropha* to changes in environmental conditions.

Acknowledgments

This work was funded by a Water Environment Research Foundation research grant (WERF project #98-CTS-2) and by the University of Tennessee, Waste Management Research and Education Institute. H.D. is a recipient of a postdoctoral fellowship from CONICET. We thank Arthur Meyers of Eastman Chemical Company (Kingsport, TN) for technical advice and Neil Quigley at the Molecular Biology Resource Facility (University of Tennessee, Knoxville, TN) for DNA sequencing. We thank Knoxville Utilities Board (KUB, Knoxville, TN) for providing samples.

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Received for review April 12, 2002. Revised manuscript received October 1, 2002. Accepted October 18, 2002.

ES0257164