Molecular assessment of ammonia- and nitrite-oxidizing bacteria in full-scale activated sludge wastewater treatment plants

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Abstract Nitrification was assessed in two full-scale wastewater treatment plants (WWTPs) over time using molecular methods. Both WWTPs employed a complete-mix suspended growth, aerobic activated sludge process (with biomass recycle) for combined carbon and nitrogen treatment. However, one facility treated primarily municipal wastewater while the other only industrial wastewater. Real time PCR assays were developed to determine copy numbers for total 16S rDNA (a measure of biomass content), the *amo*A gene (a measure of ammonia-oxidizers), and the *Nitrospira* 16S rDNA gene (a measure of nitrite-oxidizers) in mixed liquor samples. In both the municipal and industrial WWTP samples, total 16S rDNA values were approximately 2–9 × 10^{13} copies/L and *Nitrospira* 16S rDNA values were 2–4 × 10^{10} copies/L. *amoA* gene concentrations averaged 1.73 × 10^{9} copies/L (municipal) and 1.06 × 10^{10} copies/L (industrial), however, assays for two distinct ammonia oxidizing bacteria were required.
Keywords Activated sludge; molecular analysis; nitrification; real time PCR

Introduction

Current practices for municipal wastewater treatment utilizing activated sludge are hindered by the lack of specific microbial population information. Recent advances in molecular biology have yielded unprecedented opportunities for insight into the community structure of activated sludge. Together with traditional microbial analyses, these techniques offer the prospect of improved process design/control and the development of more efficient biotreatment strategies.

A key process in the removal of nitrogen during wastewater treatment is through the 2-step oxidation of ammonia to nitrate via microbial mediated nitrification. This biological process requires the coordination of two distinct chemolithotrophic groups: the ammonia-oxidizing bacteria (AOB) and the nitrite-oxidizing bacteria (NOB). The failure of wastewater treatment plants (WWTPs) to establish stable nitrification is generally attributed to the slow growth of these bacteria and their sensitivity to environmental factors (Okabe *et al*., 1999).

The ammonia-oxidizing bacteria convert NH_4^+ to NO_2^- via a two-step enzymatic process encoded by the ammonia monooxygenase (*amo*) and hydroxylamine oxidoreductase (*hao)* genes (Bock *et al*., 1992). A robust phylogenetic analysis of the ammonia-oxidizing bacteria indicates that the majority of species fall into the beta-subclass *Proteobacteria* within 6 clusters spanning the *Nitrosomonas* and *Nitrosospira* genera (Purkhold *et al*., 2000). Recently published studies indicate that wastewater treatment plants vary in ammonia oxidizing bacteria species diversity and in species richness (Rotthauwe *et al*., 1997; Purkhold *et al*., 2000). While some plants are dominated by a single ammonia-oxidizing species, other plants harbor at least four different ammonia-oxidizing species.

The nitrite-oxidizing bacteria belong to several phylogenetic groups including *Nitrobacter* (alpha-subclass *Proteobacteria*), *Nitrococcus* (gamma-subclass *Proteobacteria*)

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and *Nitrospira* (*Nitrospira* phylum). Until the late-1990s, it was generally assumed that *Nitrobacter* were the key nitrite-oxidizing bacteria in wastewater treatment (Mobarry *et al*., 1996; Bitten, 1999). 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*., 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 (Bitten, 1999). Many of these models are based on *Nitrobacter* growth rates and activities. 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 (Bitten, 1999). Traditionally, enumeration of bacteria relied on sample dilution most probable number (MPN) methods or spread plate inoculation. However, ammonia-oxidizing bacteria 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). Additionally, *Nitrospira* is extremely difficult to cultivate (Erlich *et al*., 1995) and cannot be quantified by culture methods.

Due to the problems associated with quantification of nitrifying bacteria by cultivation techniques, several culture-independent molecular methods have been investigated including quantitative dot-blot hybridization (Mobarry *et al*., 1996), PCR based methods (Kowalchuk *et al*., 1999; Mendum *et al*., 1999; Stephen *et al*., 1999; Phillips *et al*., 2000; Ivanova *et al*., 2000), and fluorescent in-situ hybridization (FISH) using oligonucleotide probes (Okabe *et al*., 1999; Schramm *et al*., 1999). Recently, Dionisi *et al*. (2002) developed PCR assays to quantify a *Nitrosomonas oligotropha*-type *amoA* gene (an indicator of ammonia oxidizers present) and the 16S rDNA gene from *Nitrospira* (an indicator of nitrite oxidizers present) in activated sludge bioreactors.

Reliable quantification of both AOB and NOB during activated sludge treatment utilizing molecular techniques can improve process performance and control in WWTPs by increasing our understanding of the microbiology and ecology of these systems. This research attempted to utilize recently developed real time PCR assays to quantify total bacteria, AOB and NOB in two full-scale, combined carbon/nitrogen activated sludge treatment systems receiving chemically distinct influents.

Materials and methods

Full scale WWTPs

Mixed liquor samples were collected over time from the aeration basins of a municipal and an industrial WWTP. Both WWTPs employed a complete-mix suspended growth, aerobic activated sludge process (with biomass recycle) for treatment of dilute waste streams. Bioreactors were operated in the neutral pH range and in the mesophilic temperature range. However, the chemical composition of the influent wastewater, in addition to various operational/environmental parameters, differed considerably for each facility.

The municipal WWTP biologically treated an average flow of 151,400 m^3/d (40,000,000 gal/d) in a tank reactor with a 6 h hydraulic retention time. The wastewater consisted primarily of sanitary sewage collected from a local municipality, however, small industrial and hospital discharges contributed to the overall flow. Typical influent 5-day biochemical oxygen demand (BOD_5) and ammonia nitrogen (NH_4^+-N) levels were 200 and 20 mg/L, respectively. Organic carbon oxidation and nitrification occurred in the same treatment reactor that operated at an average solids retention time (SRT) of 10 days. The

average level of ammonia removed in the plant during sample collection was 86.7% ± 7.8%.

The industrial WWTP treated approximately 102,200 m^3/d (27,000,000 gal/d) with a hydraulic retention time of 24 h. Wastewater flows resulted from the manufacture of fibers, plastics, and chemicals and consisted mainly of acetic acid, propionic acid, *n*-butyric acid, ethylene glycol, ethanol, methanol, isopropanol and acetone. No sanitary sewage was discharged to the WWTP. The carbonaceous influent was supplemented with both nitrogen (ammonia) and phosphorus (phosphoric acid). Average influent BOD_5 and NH_4^+-N levels were 750 and 45 mg/L, respectively. The WWTP operated at a SRT between 12 and 14 days. The average level of ammonia removed in the plant during sample collection was 98.4% ± 1.8%.

DNA extraction

Genomic DNA was extracted in triplicate from 2 ml of mixed liquor using a FastDNA™ kit (BIO 101, Vista, CA) with minor modifications: two washes of the binding matrix/DNA complex using ethanol 80% (vol/vol) were added after the recommended salt ethanol wash step, and the DNA was eluted in 100 µl of 10 mM Tris-HCl, pH 8.0 (Dionisi *et al*., 2002). The three extractions were combined before analyzing the DNA. The integrity of the DNA samples was analyzed by electrophoresis in 0.8% (wt/vol) agarose (Fisher Scientific, Pittsburgh, PA), 1X TBE and 1X GelStar® nucleic acid gel stain (FMC Corporation, Rockland, ME).

Real-time PCR assays

Real-time PCR assays, using an internal fluorescent probe, were developed and optimized for quantification of total bacterial population, *Nitrospira* spp. and *N. oligotropha*-type AOB in mixed liquor samples. Three TaqMan probes were developed from guidelines provided by Applied Biosystems (Foster City, CA, USA) and were synthesized by Sigma Genosys (Woodlands, TX, USA). Real-time PCR reactions were run on a Bio-Rad iCycler with the iCycler iQ™ fluorescence detector and iCycler software (Bio-Rad, Hercules, CA, USA).

Real time PCR for quantification of total 16S rDNA

Amplification of total bacterial 16S rDNA was performed with primers 1055f and 1392r (Lane, 1991). Slight modifications were made in 1055f in order to adapt it for use during real-time PCR using a TaqMan probe. The TaqMan probe 16STaq1115f was modified from the 1114f primer (Lane, 1991). The PCR mix contained Platinum® SuperMix with 5 mM $MgCl_2$, 15 pmol primers 1055f and 1392r, 6.25 pmol TaqMan probe 16STaq1115f, and 3.2 to 7.0 ng of sample DNA or dilutions of plasmid 931 as a standard (from 4.5×10^3 to 4.5×10^8 copies). The PCR program was 2 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 were previously designed and tested using genomic DNA extracted from municipal and industrial MLSS as templates (Dionisi *et al*., 2002). The TaqMan probe NSR1143fTaq was designed for *Nitrospira* 16S rDNA. The probe sequence was derived from a conserved sequence region within the primer pair NSR1113f and NSR1264r. Real-time PCR assays for quantification of *Nitrospira* 16S rDNA with NSR1143fTaq were performed in a total volume of 25 µl with 5 mM $MgCl_2$, Platinum® Quantitative PCR SuperMix-UDG (Life Technologies, Inc., Gaithersburg, Md.), 15 pmol of primers NSR1113f and NSR1264r, 6.25 pmol TaqMan

probe NSR1143fTaq, and 3.2 to 7.0 ng of sample DNA or dilutions of the standard 151 bp *Nitrospira* 16S rDNA obtained by PCR from plasmid 931 (from 3×10^1 to 3×10^6 copies per reaction). 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.

Real time PCR for quantification of *N. oligotropha*-like *amoA* gene

The ammonium monooxygenase gene *amoA* primers *amo*No550D2f and *amo*No754r were chosen from an alignment of *amoA* gene sequences using the CLUSTAL W program. Alignments consisted of *amoA* sequences from clonal libraries obtained from a municipal WWTP (Dionisi *et al*., 2002), four bench-scale municipal wastewater treatment systems, and *amoA* sequences available in GenBank (Purkhold *et al*., 2000). The primer pair was designed to target the *amoA* gene of *Nitrosomonas marina*/*Nitrosomonas oligotropha* type AOB as a predominant group in mixed liquor from full-scale WWTPs. PCR amplification of the *amoA* gene using these primers was performed in 25 µl using Ready-To-Go PCR Beads (Amersham Pharmacia, Piscataway, NJ), 10 pmol of each primer and 2 nmol genomic DNA from biosolid samples as template. The concentration of $MgCl_2$ in the reactions was 5 mM. The program used for amplification was 5 min at 94°C, then 40 cycles consisting of 30 s at 94°C 30 s at 56°C, and 30 s at 72°C; and a final cycle of 15 min at 72ºC. In order to verify specificity of the new primers, PCR product obtained with total DNA extracted from mixed liquor suspended solids of the municipal WWTP as template was cloned and sequenced. The TaqMan probe *amo*NoTaq729r, designed for the *amoA* gene, included a probe sequence derived from a conserved sequence region within the primer pair *amo*No550D2f and *amo*No754r. The PCR mix contained TaqMan® Universal PCR Master Mix (PE Applied Biosystems, Foster City, Calif.) with > 5 mM $MgCl_2$, 22.5 pmol primers *amo*No550D2f and *amo*No754r, 2.5 pmol TaqMan probe *amo*NoTaq729r, and 3.2 to 7.0 ng of sample DNA or dilutions of *amoA* gene produced from plasmid M-20 (Dionisi *et al*., 2002) as standard DNA (from 2.3×10^1 to 2.3×10^6 copies). PCR amplification consisted of 2 min at 50°C, 10 min at 95°C, 55 cycles at 95°C for 30 s, 56°C for 60 s.

Results and discussion

Quantification of total bacterial populations in WWTP mixed liquor

Two overall measures of biomass content (mixed liquor volatile suspended solids (MLVSS) and total 16S rDNA) were assessed in this study. Both measures were higher in the industrial mixed liquor samples than the municipal samples which reflected the higher COD loading to the industrial facility. A mean MLVSS value of 1,970 mg/L (±180 mg/L) was obtained for the municipal WWTP from mixed liquor samples collected over a 12-month period (one sample/month). The industrial WWTP had an average MLVSS of 2,860 mg/L (±510 mg/L) using mixed liquor samples collected over a 16 month period (one sample/month). Total 16S rDNA values for the same samples were $1.91 \pm 0.68 \times 10^{13}$ copies/L (municipal) and $8.89 \pm 2.35 \times 10^{13}$ copies/L (industrial). The higher MLVSS and 16S rDNA levels measured at the industrial facility were the result of higher biomass inventories which were maintained in the activated sludge system at this WWTP.

The conversion of mass (MLVSS) and total 16S rDNA copy number to cell numbers is desirable in order to determine specific activities per cell. Assuming a constant value of 2.8×10^{-13} g/cell (Madigan *et al*., 1997), average biomass concentrations of 7.03×10^{12} cells/L (municipal) and 1.02×10^{13} cell/L (industrial) were calculated. However, this conversion factor assumes constant growth factors under all physical, chemical and environmental conditions. The conversion of total 16S rDNA copy number to a cell number is also problematic because bacteria may vary in ribosomal operons from 1 to 15 (Condon *et al*., 1995; Klappenbach *et al*., 2000). It is hypothesized that bacteria with faster growth rates or the

ability to respond rapidly to environmental changes have higher ribosomal operon numbers than slow growing bacteria that are sensitive to environmental changes (Condon *et al*., 1995, Klappenbach *et al*., 2000). Therefore, it is possible that the industrial WWTP may select for microbial populations with a different number of ribosomal operons than the municipal WWTP. The reported ribosomal operon copy numbers for bacterial species found in wastewater treatment range from a high of 6 in *Burkholderia* to 1 in *Nitrosomonas* (Klappenbach *et al*., 2001). Given that the majority of species in wastewater treatment contain a relatively low number of ribosomal operons, the current 3.6 average operon number for all bacterial species (Klappenbach *et al*., 2001) was used to obtain values of 5.3×10^{12} cells/L (municipal) and 2.5×10^{13} cells/L (industrial) from the total 16S rDNA data. Cell concentration values using MLVSS or total 16S rDNA yielded similar values for biomass in each WWTP.

Quantification of ammonia oxidizing bacteria in WWTP mixed liquor

Ammonia oxidizing bacteria in mixed liquor samples were quantified in DNA extracts using real-time PCR methods targeting the *amo*A gene from *N. oligotropha*-type organisms. Gene copies averaged $1.73 \pm 2.1 \times 10^9$ copies/L in the municipal WWTP over the 12 month sample period. Assuming that one cell of AOB has 2 copies of the *amoA* gene (Klappenbach *et al*., 2001), the average number of *N. oligotropha*-like AOB measured in the municipal mixed liquor was 8.65×10^8 cells/L. In the industrial samples, the *amo*A PCR products were not quantifiable using the molecular probes developed. This was unexpected because influent ammonia concentrations were higher in the industrial wastewater than the municipal wastewater. Additional research indicated that the activated sludge system at the industrial WWTP contained a different ammonia oxidizing bacterium (*N. nitrosa*-type) than the municipal WWTP (*N. oligotropha*-type). The *amo*A genes from these two organisms are sufficiently different that the real-time PCR assay developed for the municipal plant does not detect AOB in the industrial plant. Therefore, a second real-time PCR assay was developed and validated to quantify AOB in the industrial WWTP.

N. nitrosa-type *amo*A primers and probe were designed based on sequence analysis of an *amo*A clonal library from the industrial WWTP and the *amo*A sequence from an ammonia-oxidizing bacterium isolated from the industrial WWTP. The *amoA* gene from this bacterium was 100% similar to a *N. nitrosa* previously isolated from an industrial sewage in Marl and a pond in Saudi Arabia.

Using a PCR product of the *amoA* gene cloned from the isolated organism, a standard curve was generated in the range of $1 \times 10^3 - 1 \times 10^9$ target gene copies. The quantification of known amounts of a plasmid containing the *amoA* gene of *N. nitrosa* indicated a good correlation between actual and determined copies of the target (Figure 1). In addition, the *N. nitrosa amo*A real-time PCR assay amplified specific products from DNA extracted from the industrial WWTP but not from DNA extracted from the municipal WWTP or *N. europaea* genomic DNA (Figure 2). The optimized real-time PCR assay was used to quantify the *N. nitrosa*-like population in mixed liquor of the industrial WWTP sampled during a 14-month period. The average concentration of *amoA* genes (*N. nitrosa*-type) was $1.06 \pm 1.31 \times 10^{10}$ copies/L in the industrial WWTP which corresponds to 5.3×10^9 cells/L (assuming 2 *amoA* gene copies/cell).

The AOB levels measured in the two WWTPs using the *amoA* assays represent approximately 0.009–0.012% of the total rDNA population present in the mixed liquors. Although very low, the average concentration of *amoA* genes was approximately 60% greater in the industrial WWTP (*N. nitrosa*-type) than the municipal WWTP (*N. oligotropha*-type). The industrial facility receives over twice as much influent ammonia (on average) as the municipal facility although N-synthesis is greater in the industrial WWTP due to the higher

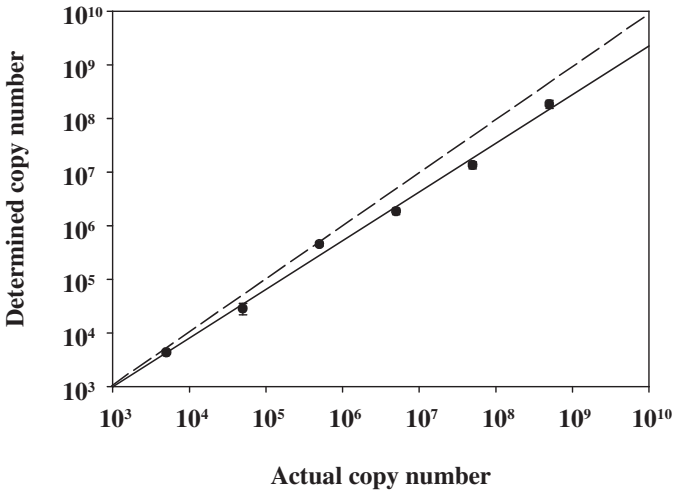

Figure 1 Actual versus determined copy number of a plasmid containing the *amoA* gene from *N. nitrosa*. Error bars indicate the standard deviation of the means (n = 3 determinations). Dotted line, 1:1 ratio between actual and determined copy numbers

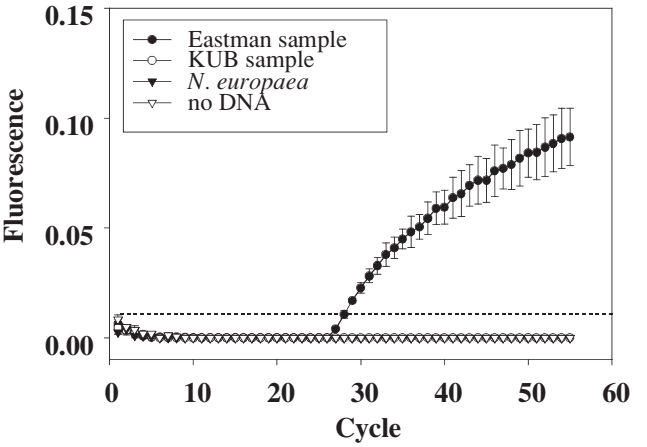

Figure 2 Amplification of *N. nitrosa amo*A from DNA extracted from the industrial WWTP (Eastman), the municipal WWTP (KUB) and *N. europaea* genomic DNA (*N. europaea*). A negative control without DNA was also assessed. The average and standard deviation of three replicates are shown. The dotted line depicts threshold fluorescence intensity

MLVSS levels maintained in the bioreactor. The number of *amo*A copies decreased over time in the industrial WWTP; however, decreases in ammonia-oxidizing activity (as measured by the MPN method) also occurred over the same period.

The decrease was greater in the MPN method than in the molecular method. It is important to note that the MPN method is a direct measurement of the nitrifying activity, whereas real-time PCR measures the total amount of ammonia-oxidizing cells in the mixed liquor.

Quantification of *Nitrospira* spp. in WWTP mixed liquor

Nitrospira 16S rDNA concentrations were similar in both WWTPs. Average concentrations were $3.74 \pm 3.2 \times 10^{10}$ copies/L (municipal) and $1.80 \pm 1.20 \times 10^{10}$ copies/L (industrial). The *Nitrospira* levels measured represent approximately 0.02–0.2% of the total rDNA population present in the mixed liquor from the two WWTPs. Additionally, the *Nitrospira* concentrations measured were, on average, higher than measured *amo*A concentrations at each facility. Higher cell numbers of nitrite oxidizers to ammonia oxidizers is consistent

with values from nitrifying fluidized bed reactors using fluorescence *in situ* hybridization (FISH) with 16S rRNA-targeted oligonucleotide probes for ammonia and nitrite oxidizers (Schramm *et al*., 1999).

Conclusions

The real-time PCR-based techniques used in this study represent a viable approach for quantification of targeted bacterial populations present in activated sludge. The total 16S rDNA and *Nitrospira* 16S rDNA assays developed can be used to quantify total bacterial populations and the nitrite-oxidizing bacterium *Nitrospira* in both industrial and municipal WWTPs. However, quantification of ammonia-oxidizing bacteria will require multiple assays to include the diverse subpopulations that can apparently exist within activated sludge systems treating chemically distinct waste streams.

The low levels of ammonia- and nitrite-oxidizing bacteria measured (relative to total biomass levels) suggests that either autotrophic bacteria in wastewater treatment systems represent a smaller percentage of the total population than values typically used in current design models or that additional important groups within the AOB and NOB populations were not measured by the newly developed assays.

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