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Emergence of Competitive Dominant Ammonia-Oxidizing Bacterial Populations in a Full-Scale Industrial Wastewater Treatment Plant

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Ammonia-oxidizing bacterial populations in an industrial wastewater treatment plant were investigated with *amoA* and 16S rRNA gene real-time PCR assays. *Nitrosomonas nitrosa* initially dominated, but over time RI-27-type ammonia oxidizers, also within the *Nitrosomonas communis* lineage, increased from below detection to codominance. This shift occurred even though nitrification remained constant.

Aerobic activated sludge systems are primarily designed to remove organic wastes (1). Conversion of ammonia to nitrate (nitrification) is a secondary function often exploited in municipal wastewater treatment plants (WWTPs) to reduce the ammonia toxicity of the effluent. In contrast to municipal WWTPs, nitrogen (primarily ammonia) is added to the influent in some industrial WWTPs to balance the high organic carbon waste stream and provide nitrogen for heterotrophic growth. Therefore, conversion of ammonia to nitrite and then nitrate (nitrification) is undesirable because it reduces the amount of ammonia available for heterotrophic growth (assimilatory removal).

Ammonia-oxidizing bacteria (AOB) are primarily responsible for the first step in nitrification and convert ammonia to nitrite. AOB found in WWTPs generally are nitrosomonads, which can be subdivided into lineages based on cultured representatives (12, 13). Some WWTPs may contain single AOB populations, whereas others contain multiple AOB populations (12). In a previous study, only one AOB population (*Nitrosomonas nitrosa* like) was detected by molecular methods in June of 2000 in the industrial WWTP investigated in this study (2). The goals of the present study were to monitor this AOB population in the WWTP by real-time PCR with TaqMan probes and demonstrate its persistence over time under normal plant operating conditions. The *amoA* gene was chosen as the target gene, as future conversion of the DNA-based assays to RNA-based assays using reverse transcriptase real-time PCR may lead to treatment performance indicators. Furthermore, within the nitrosomonad lineages, the *amoA* gene shows considerably more nucleotide variability between the cultured representatives than does the 16S rRNA gene. For example, the maximum 16S rRNA gene nucleotide difference within the *N. communis* lineage is around 5%, whereas the maximum *amoA* gene nucleotide difference between the same isolates is around 19% (12). Because of the higher nucleotide variability within the *amoA* gene, nucleotide assays targeting the *amoA* gene may allow more resolution between AOB populations in WWTPs than 16S rRNA-based assays. However, *amoA*-based assays may also miss more AOB populations than the 16S rRNA-based assays. Therefore, in this study measures of AOB presence other than just *amoA*-based assays, such as DNA sequencing and an AOB 16S rRNA gene real-time PCR assay, were used.

Mixed liquor samples were collected over a 13-month period (August 2000 to September 2001) from an industrial WWTP treating approximately 27 million gallons of wastewater daily, containing primarily organic acids, ethylene glycol, acetone, and alcohols (2). Both phosphorus in the form of phosphoric acid and nitrogen in the form of ammonia were added to the influent to maintain a nominal C/N ratio of 10:1 and a C/P ratio of 100:1, respectively. During the course of this investigation, the ammonia concentrations in the influent and effluent were 30 ± 12 and 1.1 ± 1.7 ppm, respectively (Fig. 1), resulting in a fairly constant ammonia removal efficiency of 96%.

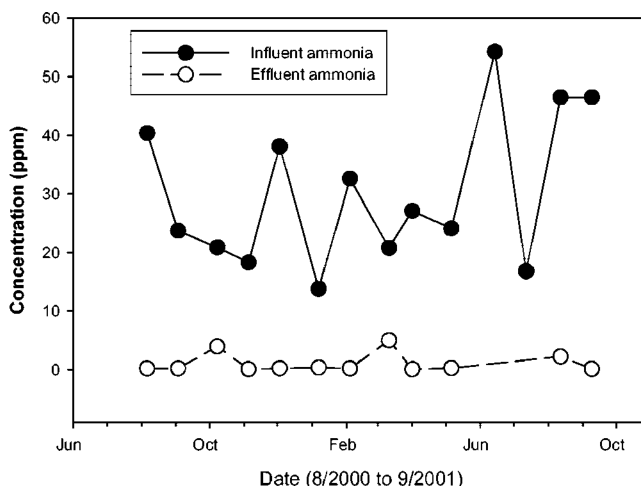

FIG. 1. Influent and effluent ammonia concentrations in the industrial WWTP over13 months.

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TABLE 1. Primers, probes, and protocols for real-time PCR assays for AOB developed in this study

Assay target	Primer or probe name, sequence (5′–3′)	Picomoles of primer or probe/reaction	PCR protocol
AOB 16S rRNA gene	AOB1149f, 5′-CTTTA(A/G)TGAGACTGCCGGTGA-3′	7.5	50°C, 2 min; 95°C, 5 min; 45 cycles of 95°C, 30 s, and 60°C, 60 s
	AOB1295r, 5′-GCGCTTTCTGAGATTAGCTCC-3′	7.5	
	AOB1225bhqr, 5′-(6-FAM)-ACGCGCCATTGTATTACGTGTGAAGC-(BHQ)3′	6.3	
N. nitrosa amoA	*Amo*NN542f, 5′-TATTGCTTTCAATGGCAGACTACA-3′	15	50°C, 2 min; 95°C, 10 min; 45 cycles of 95°C, 15 s, and 54°C, 30 s
	*Amo*NN676r, 5′-CCGCAAAGAACGCAGCAATC-3′	15	
	*Amo*NN648bhqr, 5′-(6-FAM)-ATGGCCGCCAAAGGTACGCAGTGAA-(BHQ)-3′	12.5	
Nitrosomonas sp. strain RI-27 *amoA*	*amo*RI27542f, 5′-CATTGTTATCGATGGCTGACTATA-3′	15	50°C, 2 min; 95°C, 5 min; 45 cycles of 95°C, 15 s, and 60°C, 30 s
	*amo*RI27679r, 5′-ACGCTGAGAAGAATGCTGCAAT-3′	15	
	*amo*RI27bhq651r, 5′-(6-FAM)-TGTATGACCACCGAACGTACGCAGTGAG-(BHQ)3′	12.5	

Identification of AOB in the industrial WWTP was attempted by both culture and nonculture methods. Suspended solids from the WWTP of August 2001 were used to enrich AOB by performing serial dilutions in synthetic medium no. 3 (7) and plating these enrichments over nylon membranes in the same medium supplemented with 1.5% Noble agar (Difco, Detroit, Mich.). Membranes were transferred to fresh plates every 7 days. After 8 to 12 weeks, individual brownish colonies approximately 1 to 2 mm in diameter growing on the membrane were obtained. Sequence analysis of the *amoA* gene from AOB colonies enriched from the WWTP indicated that this gene was closely related to *Nitrosomonas* sp. strain Nm148 *amoA* (99% similarity), which in turn is closely related to *N. nitrosa*, belonging to the *N. communis* lineage. Similarly, a portion of the 16S rRNA gene was amplified with primer AOB 1149f (Table 1) and reverse primer 1492r (9) and cloned. Sequence analysis of this clonal library indicated that this was not a pure culture. Three sequences types were identified; one sequence type was 99% similar to *Nitrosomonas* sp. strain Nm 148 (accession no. AY123792), whereas the other sequence types were 98% similar to *Stenotrophomonas* (accession no. AF123092) and *Oligotropha carboxidovorans* (accession no. AB09960). Cultured members of the *Nitrosomonas communis* lineage have been isolated from various environments. *N. nitrosa* Nm90 was isolated from industrial sewage in Germany (6), whereas *Nitrosomonas* sp. strain Nm 148 was isolated from a hot spring in Greece (13).

N. nitrosa-type *amoA* genes were previously detected in the WWTP sampled in June 2000 and August 2000 (2). In order to determine whether *amoA* gene sequences other than those of the *N. nitrosa* type could be detected, two new *amoA* libraries (October 2001 and December 2001) were constructed with primers *amoA*-1F and *amoA*-2R (14). All of the *amoA* clones sequenced from the October 2001 library (10 clones) and 6 clones sequenced from the December 2001 library were 99 to 100% homologous to *N. nitrosa amoA* (National Center for Biotechnology Information [NCBI] accession number AJ238495) and to the *amoA* gene isolated from the ammonia-oxidizing colony. However, three clones from the December 2001 library were 99% similar to *amoA* genes from uncultured bacterial clone RI-27 (11) (NCBI accession number AF532311) and a clone from a phosphate- and nitrogen-removing biofilm (B3-6) (4). The nucleotide similarity between the *amoA* genes from the *N. nitrosa* clones and the RI-27-type *amoA* clones was 82%. In addition, the nucleotide sequence similarities between the RI-27-type *amoA* clones and the *amoA* genes from *Nitrosomonas* strains Nm33 and Nm41 were 85 and 83%, respectively, suggesting that the RI-27-type AOB population is also part of the *N. communis* cluster (12).

Real-time PCR assays were performed with 1× QIAGEN QuantiTect probe supermix (QIAGEN, Valencia, Calif.), 1 to 10 ng of biosludge total DNA, primers, and probes as summarized in Table 1. PCR amplification and detection of the fluorescent signal were performed with the DNA Engine Opticon continuous fluorescence detection system (MJ Research, Waltham, Mass.) under the thermocycler conditions listed in Table 1. Standard curves for each assay were constructed on the basis of serial 10-fold dilutions of plasmids containing cloned gene fragments of each target molecule and ranging from 5×10^7 to 50 copies per PCR. All PCR runs, standards,

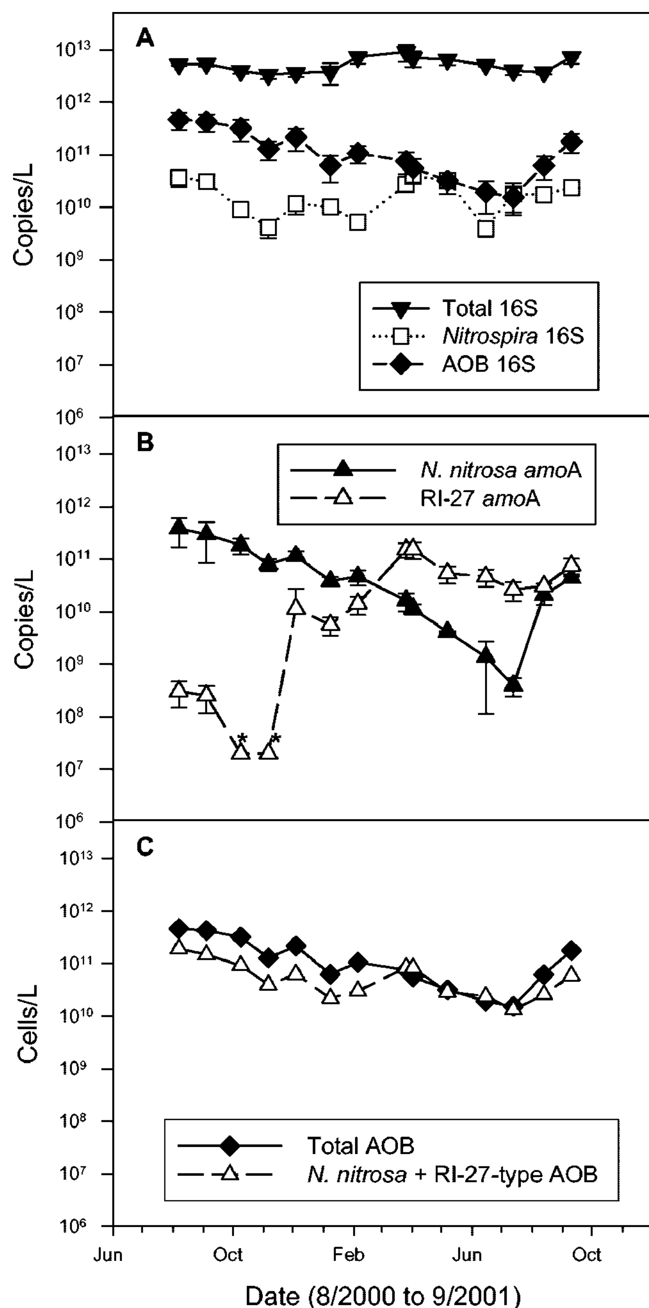

FIG. 2. Population dynamics in the activated sludge in an industrial WWTP as determined by real-time PCR assays. (A) Numbers of copies of total bacterial, nitrite-oxidizing bacterium *Nitrospira*, and β subdivision AOB 16S rRNA genes per liter of mixed liquor. The detection limits were determined on the basis of the lowest standard in gene copies routinely detected per PCR, which was 500 copies for the bacterial and AOB 16S rRNA gene assays and 50 copies for the *Nitrospira* 16S rRNA gene assay. After adjustment of the lowest number of standard copies detected for the amount of DNA recovered per milliliter of sample extracted, the DNA dilution, and the amount of DNA added per samp1e, the minimum number of gene copies per liter of mixed liquor would be 2×10^8, 2×10^7, and 2×10^8 for the total bacterial, *Nitrospira*, and AOB 16S rRNA gene assays, respectively. (B) Number of copies of *amoA* genes, *N. nitrosa*, and the RI-27 type AOB per liter of mixed liquor. The lowest number of standards routinely detected in both of the *amoA* gene assays was 50 copies per PCR or the equivalent of 2×10^7 gene copies per liter. Asterisks indicate that the number of RI-27-type *amoA* copies per liter was below the detection limit in two samples. (C) Number of cells of AOB per liter of mixed liquor determined from AOB 16S rRNA genes (panel A) assuming one copy per cell and number of *amoA* gene copies (panel B) assuming two copies per cell.

negative controls (no DNA), and biosludge samples were prepared in triplicate. For each gene assay, each sample was tested in at least two separate PCR runs.

The total bacterial population in mixed liquor samples was estimated with a real-time PCR assay previously designed to detect the 16S rRNA gene from most bacteria (5) and remained constant with a mean of 4.8 (± 2.0) $\times 10^{12}$ copies per liter (Fig. 2A). These values are consistent with the stable mixed liquor volatile suspended solid values measured at the WWTP (data not shown). *Nitrospira* 16S rRNA genes were also detected in mixed liquor samples with a previously designed real-time PCR assay (5) with a mean of 1.9 (± 1.1) $\times 10^{10}$ copies per liter (Fig. 2A).

A real-time PCR assay was developed for the quantification of the AOB 16S rRNA genes on the basis of fluorescence in situ hybridization probe NSO 1225 (10) (Table 1). Three base pairs were added to both the 5′ and 3′ ends of the NSO 1225 probe to increase the melting temperature and make it more suitable for use as a TaqMan probe. Primers and probes for the assay were developed by following the guidelines provided by Applied Biosystems (http://home.appliedbiosytems.com). Comparison of the primers and probe with AOB 16S rRNA gene sequences described in the recently published AOB phylogeny (13) suggests that the AOB 16S rRNA gene assay developed in this study would detect most of the nitrosomonad lineages, including *Nitrosomonas oligotropha*, *N. communis*, *Nitrosomonas marina*, and *Nitrosomonas* strain Nm143, as well as the *Nitrosospira* clusters. However, this assay probably would amplify neither members of the *Nitrosomonas mobilis* lineage nor half of the *Nitrosomonas europaea* lineage because of a total of three to five mismatches in the primers and probe. The application of the AOB 16S rRNA gene assay to this biological system demonstrated relatively stable concentrations of 1.37 (± 1.56) $\times 10^{11}$ AOB 16S rRNA genes per liter or 9.3 (± 8.9)% of the total bacterial population (Fig. 2A).

Separate real-time PCR assays were designed specifically for the *N. nitrosa* and RI-27-type *amoA* genes on the basis of DNA sequence alignments (Table 1). The primers and probes for both the *N. nitrosa* and RI-27 *amoA* assays were designed in the exact same locations in the *amoA* gene, but each primer and probe differed from the respective primer or probe in the other assay by 5 to 7 bp. The *N. nitrosa amoA* assay produced no fluorescent signals when tested with the RI-27 standards ranging from 5×10^7 to 50 copies, and the RI-27 *amoA* assay produced no fluorescent signals when tested with the *N. nitrosa amoA* standards ranging from 5×10^7 to 50 copies. This suggests that there was no cross-reactivity between the two *amoA* real-time PCR assays. A population change in the full-scale plant was initially identified by the approximately 100-fold decline observed in the number of *N. nitrosa*-type *amoA* copies per liter between January 2001 and July 2001 (3.8×10^{10} to 3.9×10^8) (Fig. 2B) and the considerably smaller 4-fold decline observed in the number of AOB 16S copies per liter (6.2×10^{10} versus 1.5×10^{10}) during the same time period (Fig. 2A).

The *N. nitrosa*-type organism was dominant during the first 6 months of the study, but its concentration slowly declined over this time period. Application of the RI-27 *amoA*-type gene assay to the same samples indicated that RI-27-type *amoA* increased from $<2 \times 10^7$ copies per liter to become the dominant AOB in 4 months. In order to compare the number of AOB cells per liter in the WWTP determined by the *amoA* gene assays with the number of AOB cells determined by the AOB 16S rRNA assay, the results of the *amoA* gene assays for each sample were added and divided by 2, on the basis of the assumption of two *amoA* genes per cell and one 16S rRNA operon per AOB cell (5) (Fig. 2C). The number of AOB cells determined from the *amoA* genes and the number of AOB 16S rRNA genes were similar for all months of the 13-month period.

In summary, the combination of real-time PCR assays for the AOB16S rRNA gene and the *amoA* gene allowed the detection of a change in competitive dominance between a cultured representative (*N. nitrosa*) and an uncultured representative (RI-27 type) of AOB in the *N. communis* lineage, although traditional measurements of plant performance from a nitrification standpoint (e.g., ammonia removal efficiency) remained constant. The trends seen in this WWTP with regard to the AOB populations support, on a larger scale, the concept proposed by Fernández et al. (3) that a dynamic community can maintain a stable ecosystem function. Additional research into the different ecophysiologies of nitrifying bacteria (8) may provide insight into the nonmeasured factors that may cause large-scale competitive dominance shifts in WWTPs without exerting any macroscopic effects on the biotreatment process.

Nucleotide sequence accession numbers. All of the *amoA* gene sequences from the December 2001 library containing both RI-27-type *amoA* sequences and *N. nitrosa*-type *amoA* genes were deposited in the GenBank database and assigned NCBI accession numbers AY647454 to AY647455 and AY741507 to AY41513.

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