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Diversity and Abundance of Ammonia-Oxidizing Bacteria in Activated Sludge Treating Different Types of Wastewater

Baek, Kyunghwa[1], Chul Park[2], Hee-Mock Oh[1], Byung-Dae Yoon[1], and Hee-Sik Kim[1]*

[1]*Environmental Biotechnology Research Center, KRIBB, Daejeon 305-806, Korea*
[2]*Department of Civil and Environmental Engineering, University of Massachusetts, Amherst, MA 01003, U.S.A.*

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The diversity and abundance of ammonia-oxidizing bacteria (AOB) in activated sludge were compared using PCR–DGGE and real-time PCR assays. Activated sludge samples were collected from five different types of wastewater treatment plants (WWTPs) mainly treating textile, paper, food, and livestock wastewater or domestic sewage. The composition of total bacteria determined by PCR–DGGE was highly diverse between the samples, whereas the community of AOB was similar across all the investigated activated sludge. Total bacterial numbers and AOB numbers in the aerated mixed liquor were in the range of 1.8×10^{10} to 3.8×10^{12} and 1.7×10^{6} to 2.7×10^{10} copies/l, respectively. Activated sludge from livestock, textile, and sewage treating WWTPs contained relatively high *amoA* gene copies (more than 10^5 copies/l), whereas activated sludge from food and paper WWTPs revealed a low number of the *amoA* gene (less than 10^3 copies/l). The value of the *amoA* gene copy effectively showed the difference in composition of bacteria in different activated sludge samples and this was better than the measurement with the AOB 16S rRNA or total 16S rRNA gene. These results suggest that the quantification of the *amoA* gene can help monitor AOB and ammonia oxidation in WWTPs.

Keywords: Ammonia-oxidizing bacteria, activated sludge, PCR–DGGE, real-time PCR, wastewater

Nitrification is an important biological process in nitrogen cycling and has a significant effect on effluent quality in wastewater treatment. Nitrification occurs in two steps by two types of chemoautotrophic bacteria that are distinguishable by their substrate. The oxidation of NH_4^+ to NO_2^- is performed by ammonia-oxidizing bacteria (AOB) belonging to the β- and γ-subgroups of the Proteobacteria, whereas the oxidation of NO_2^- to NO_3^- is conducted by nitrite-oxidizing bacteria (NOB) [24]. The presence and activity of AOB was thought to be a rate-limiting step in the biological nitrification process [16]. Therefore, it is important to understand the ecology and microbial properties of AOB in wastewater treatment systems to enhance nitrification and control the treatment process.

In spite of the importance of ammonia oxidizers in wastewater treatment, their slow growth and heterogeneous nature of activated sludge have made it difficult to investigate the AOB in wastewater treatment systems using traditional culturing methods. Recently, molecular approaches based on the analysis of 16S rRNA genes have been applied to detect and identify microbial diversity in activated sludge samples [1, 14, 22, 23]. Denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), and fluorescence *in situ* hybridization (FISH) have been commonly used in analyses of ammonia-oxidizing bacterial communities [3, 8, 19]. Among these, DGGE is the most frequently used community fingerprinting method. It allows a rapid comparison of the microbial communities between the samples and is generally used to detect shifts in microbial population under different environmental conditions. More recently, real-time PCR was investigated for enumerating nitrifying bacteria in wastewater sludge and soil [10, 15]. Real-time PCR permits continuous monitoring of a sample during PCR using hybridization probes [5, 6] or DNA-binding dyes such as SYBR Green I [2, 12]. The SYBR green assay is simple, fast, and sensitive, because it relies on a fluorescence signal produced as the dye binds to double-stranded DNA during the extension step.

This experiment was performed for the comparison of the diversity and abundance of AOB in activated sludge from five different types of wastewater treatment plants (WWTPs) in February 2006. The diversity of AOB collected from an aeration tank at each facility was compared using PCR–DGGE, and the total bacterial number, AOB number,

**Corresponding author*
Phone: +82-42-860-4326; Fax: +82-42-879-8103;
E-mail: hkim@kribb.re.kr

Table 1. Characterization of activated sludge samples collected from an aeration tank at each facility in February 2006.

Activated sludge samples	pH	T-N (mg/l)	NH_4-N (mg/l)	T-P (mg/l)	MLSS (mg/l)
Livestock wastewater					
L1	7.02	54.7	35.2	7.7	2,320
L2	7.74	62	48.3	2.4	2,060
L3	8.21	92	70.3	3.1	4,240
Food wastewater					
F1	6.99	35.8	30.2	0.08	2,067
F2	7.10	95.2	77.3	3.6	5,270
F3	7.14	66.2	53.1	10.1	6,500
Paper wastewater					
P1	6.81	10.3	8.5	0.1	600
P2	6.94	59.5	48.1	3.18	2,600
P3	6.55	44.0	40.1	0.38	1,430
Sewage wastewater					
S1	6.74	14.0	10.3	6.4	1,800
S2	6.38	24.9	12.5	4.1	1,500
Textile wastewater					
T1	6.90	197	143	8.59	4,820
T2	6.95	191	151	1.49	4,890
T3	7.43	100.4	82.1	0.17	7,500

and *amoA* gene abundance were estimated by real-time PCR.

MATERIALS AND METHODS

Activated Sludge Samples

Activated sludge samples were collected from 14 different WWTPs in Korea. These facilities can be classified into five groups depending on the type of wastewater that the facility treats: wastewater from livestock, food, paper, and textile industries, and sewage wastewater. Some physicochemical properties of the activated sludge samples are presented in Table 1. All samples were collected from an aeration tank at each facility in February 2006, with the 1-l sample frozen at −20°C until use.

DNA Extraction and PCR–DGGE

Genomic DNA was isolated from activated sludge using a FastDNA SPIN kit (Q-Biogene, U.S.A.) following the manufacturer's instructions. The quantity and purity of DNA obtained from the sludge were determined by the measurement of absorbance at 260 nm and 280 nm (NanoDrop ND-1000, U.S.A.). The PCR conditions and primers used in this experiment are listed in Table 2. A nested PCR was performed for the analysis of each bacterial community in the activated sludge samples. In the first round, a nearly complete 16S rDNA fragment was amplified using the universal primer set 9F and 1392R. During the second PCR round, the obtained fragments were reamplified by using the bacterial primers 341F-GC and 518R. For the analysis of the AOB group, a three-step nested PCR was performed. In the first round, a nearly complete 16S rDNA fragment was amplified using the universal primer set 9F and 1392R. The PCR product was used as a template for a second amplification with AOB primers CTO168F and CTO654R. Finally, to generate products suitable for DGGE, a third round of amplification was performed with DGGE primers 341F-GC and 518R using the PCR product of the second round as a template.

DGGE was performed using a D-Code universal mutation system (Bio-Rad, U.S.A.) as described by Muyzer *et al.* [13]. Each sample was run on 10% polyacrylamide gels with a denature gradient of 30% to 60% for total bacteria and 30% to 40% for AOB. Each lane of the gradient gel received approximately 20 ng of PCR product. Gels were run for 15 h at 60 V and 60°C in 1×TAE (40 mM Tris-acetate, 1 mM Na-EDTA, pH 8.0) buffer, and then stained with ethidium bromide. For the analysis of DGGE band patterns, images were converted, normalized, and analyzed with the Kodak 1.0 software package (Eastman Kodak Co., U.S.A.). The Shannon–Weaver (H') index of microbial diversity [20] was calculated according to the formulas $H'=-\Sigma Pi\log Pi$, and $Pi=ni/N$, where ni is the intensity of band I in the lane, and N is the total intensity of all bands in the lane.

Real-Time PCR Assay

The abundance of AOB was estimated by real-time PCR with the DNA Engine Opticon continuous fluorescence detection system (MJ Research, U.S.A.). The 20-μl reaction mixtures contained 0.2 μM of each primer (see Table 2), 1 μl of DNA (all 1 ng/μl), and 10 μl from the DyNAmo HS SYBR Green qPCR Kit (Finzymes, U.S.A.). The real-time PCR conditions for detecting the total bacterial 16S rRNA were 5 min at 95°C, and 40 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. For the AOB 16S rRNA gene, the PCR program was 5 min at 95°C, and 40 cycles at 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min. For the *amoA* gene detection, the PCR program was 5 min at 95°C, and 40 cycles at 95°C for 1 min,

Table 2. PCR primers used in this study.

Primers	Sequence (5'→3')	Target	Annealing temp. (°C)	References
9F	GAGTTTGATCCTGGCTCAG	Bacteria	50	[5]
1392R	ACGGGCGGTGTGTAC	Bacteria	56	
1055F	ATGGCTGTCGTCAGCT	Bacteria	56	
341F[a]	TACGGGAGGCAGCAG	Bacteria	Touch-down	[12]
518R	ATTACCGCGGCTGCTGG	Bacteria	Touch-down	
CTO189f	GGAGRAAAGCAGGGGATCG	Ammonia oxidizers	56	[8]
CTO654R	CTAGCYTTGAGTTTCAACGC	Ammonia oxidizers	56	
amoA-1F	GGGGTTTCTACTGGTGGT	*amoA* gene of Betaproteobacteria	58	[16]
amoA-2R	CCCCTCTGCAAACGGTTC	*amoA* gene of Betaproteobacteria	58	

[a]A 5' GC-clamp (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG) was added for DGGE analysis.

58°C for 1 min, and 72°C for 1 min. All samples were measured three times during each assay, and negative controls without template were included in each PCR run. Because the detection by SYBR green is not fragment specific, we verified the length and quality of the amplicons by electrophoresis and melt-curve analysis.

To generate standards for each gene, each PCR product from *N. europaea* ATCC19718 was cloned into a pGEM T-easy vector (Promega Co., U.S.A.), and then prepared from serial dilution. The gene copy number was calculated as follows:

$$\text{Copy number (molecules/}\mu\text{l)} = \frac{\text{DNA conc. (g/l)} \times 6.02 \times 10^{23} \text{ molecules/mol}}{\text{Plasmid length (bp)} \times 660}$$

The number of copies of each target gene per PCR reaction was calculated by comparison of threshold cycles obtained in each PCR reaction from standard DNA. The PCR efficiency (E) was determined from the slope of the external calibration curve according to $E=10^{(1/-\text{slope})}-1$.

To confirm the specificity of the amplicons, each purified target gene product ligated into the pGEM-T easy vector was sequenced using an ABI Prism 377 automatic sequencer (Applied Biosystems, CA, U.S.A.). The sequences were compared with the GenBank database using the BLASTN function (http://ncbi.nlm.nih.gov). Sequences were aligned in ClustalX; the alignment was manually checked. Phylogenetic analyses were conducted using MEGA Version 4 [21] using the neighbor-joining method with evolutionary distances computed using the maximum composite likelihood model. Phylogeny was tested using bootstrapping with 500 replicates.

Statistical Analysis

One-way analysis of variance (ANOVA) test was performed using SPSS Version 11.0 (SPSS Inc., U.S.A.) after $\log_{10}$ transformation of the data to meet the assumption of equal variance between groups. In the ANOVA test for real-time PCR assay, the mean difference was significant at the 0.01 level. The Tukey test was used for multiple comparisons between groups.

Results and Discussion

Activated sludge samples were taken from the aeration tank of 14 wastewater treatment plants. The pH of these samples was between 6.4 and 7.7, except for L3 (Table 1). As the data in Table 1 show, ammonium concentrations varied widely depending on the types of wastewater treated and even within the same group of wastewater as well. Because of the differences in solids retention time (SRT) used in each facility, the values of mixed liquor suspended solids (MLSS) also varied widely. It is also worth noting that activated sludge from the textile wastewater treatment plant showed the highest MLSS with the highest T-N, whereas activated sludge of sewage wastewater generally showed the lowest values for both MLSS and T-N. It was found in this study that the extracted DNA concentration was correlated with the concentration of MLSS ($r^2=0.87$, $P<0.0001$).

Activated sludge consists of a complex mixture of microorganisms. For the past few decades, many researchers have examined the microbial communities of activated sludge in an effort to understand their biological roles, such as nitrification and denitrification [25], in the activated sludge process. The 16S rRNA gene fragments related to the β-proteobacteria subgroup of AOB were examined because of the critical role they play in the nitrogen removal process. Generally, the prominent members of the AOB β-proteobacteria in activated sludge were most related to *Nitrosomonas* and *Nitrosospira* [18]. To verify the specificity of the CTO primers, the PCR product obtained from genomic DNA extract of MLSS was cloned and sequenced. Randomly selected clones were mainly identified as uncultured β-proteobacteria. These sequences were 99% similar to uncultured *Nitrosomonas* sp. (AF527015), 98% similar to *N. oligotropha* (AJ298736), 97% similar to uncultured Comamonadacea bacterium (AY360635), and 96% similar to uncultured β-proteobacteria (AY921920). To confirm the specificity of the amoA primers, the PCR product obtained from MLSS was cloned and sequenced. Randomly selected clones were mostly 99% identified to be the uncultured *Nitrosomonas* sp. *amoA* gene (AY177932 and AB234567). These results demonstrate that most of the clones with AOB 16S rRNA and *amoA* gene in this study are related to *Nitrosomonas* sp. and uncultured β-proteobacteria (Fig. 1).

The structure of the AOB community in the activated sludge was characterized by DGGE analysis of 16S rDNA sequences amplified from MLSS DNA extract using a nested PCR approach (Fig. 2). The DGGE gel for the total bacteria showed highly diverse band patterns between the samples, with a few intensive bands in several lanes. In contrast, the DGGE pattern of AOB was very similar in all activated sludge samples, although each sample originated from different WWTPs. The AOB populations in all sludge samples consisted of a limited number of species. It was also proven by the Shannon diversity index (H'), which was calculated from the results of the DGGE band pattern to quantify the bacterial diversity of the different samples (Table 3). The H' value of total bacteria was between 2.73 and 3.20, and was 1.90–2.31 for AOB. However, the H' value of the total bacterial population was not significantly different in all sludge samples ($\alpha=0.05$, $P>0.20$). Furthermore, the H' value of AOB was not significantly different between groups of sludge ($\alpha=0.05$, $P>0.17$). Curtis and Craine [4] also did not find evidence of spatial, diurnal, and intra-sample variations in a WWTP. They concluded that a single sample of activated sludge from one facility was sufficient for a plant-to-plant comparison. Moreover, other researchers have proved that the communities of AOB in the activated sludge of various sewage treatment systems do not have seasonal variations, and only particular microorganisms exhibited seasonal variation in some systems [3, 11].

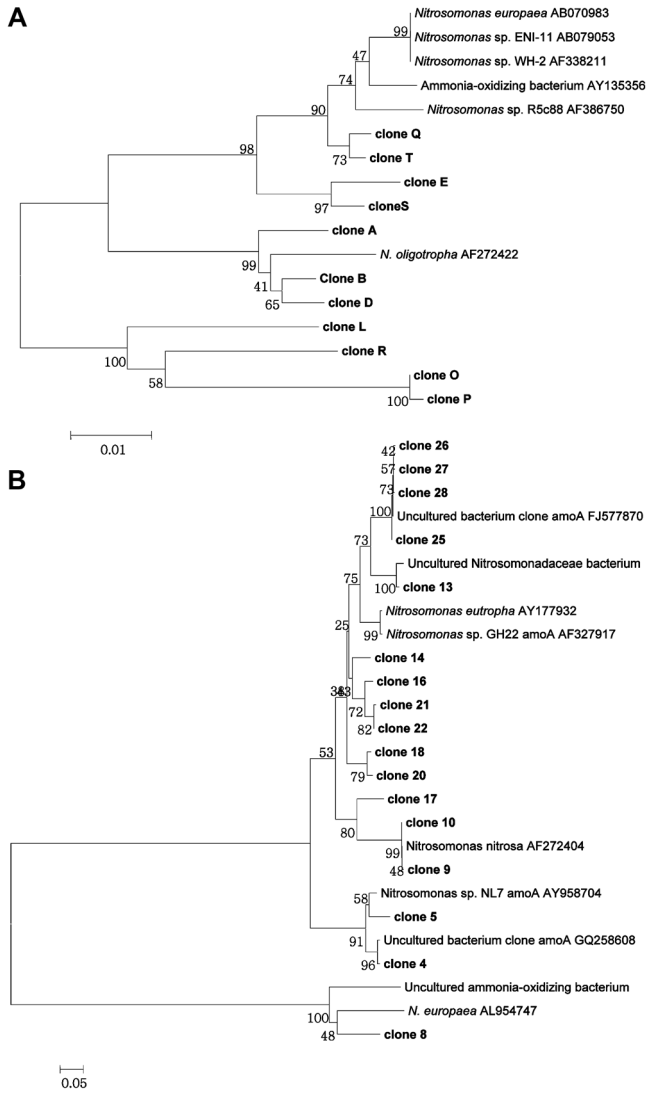

Fig. 1. Phylogenetic tree constructed from 16S rRNA gene sequences using MEGA4.
The tree was constructed using the neighbor-joining method with 16S rRNA (**A**) and *amoA* gene (**B**). The bootstrap consensus tree was inferred from 500 replicates.

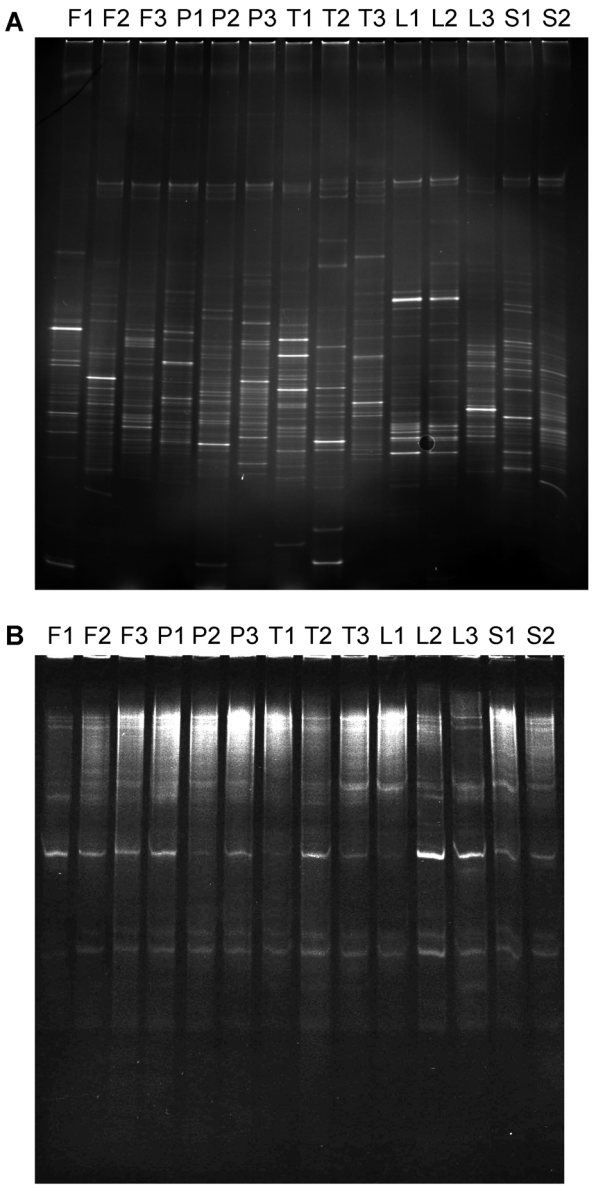

Fig. 2. DGGE profiles of PCR-amplified total bacterial (**A**) and AOB 16S rRNA genes (**B**) in the activated sludge in 14 different WWTPs.

Quantification of bacteria capable of nitrification is important for an understanding of nitrifying activity and NO_3^- fluxes in a wastewater treatment plant. DGGE analysis characterizes differences in the relative abundances of different phylotypes, but does not allow an absolute quantification of each phylotype or the number of the total bacterial group analyzed. Real-time quantitative PCR enables such analysis, because it can monitor the initial exponential phase of the PCR, and the amount of the amplified product is proportional to the concentration of the template DNA [7]. Both the 16S rRNA and *amoA* genes provide well-studied genetic markers for the characterization of AOB [17]. A functional gene, *amoA*, rather than the AOB 16S rRNA gene, was targeted to provide quantitative information about the process of ammonia oxidation [15]. For real-time PCR assay, equations of the regression for total bacterial 16S rRNA, AOB 16S rRNA, and *amoA* gene were respectively as follows: $y=-3.43x+24.92$ ($r^2=0.99$, $p=0.002$), $y=-4.0x+44.76$ ($r^2=0.98$, $p<0.002$), and $y=-3.8x+43.39$ ($r^2=0.99$, $p<0.001$). The detection limit for 16S rRNA and *amoA* was 4.4×10^2 and 8.8×10^2 target DNA copies, respectively. Total bacterial numbers in each activated sludge sample were in the range of 1.8×10^{10} to 3.8×10^{12} copies/l. Total AOB bacterial numbers were between 1.7×10^6 to 2.7×10^{10} copies/l

Table 3. The Shannon index of diversity (*H'*) for the sludge of different WWTPs.

Samples	Total bacteria	Ammonia oxidizer
Livestock wastewater		
L1	2.543	1.952
L2	2.725	2.169
L3	2.682	1.745
Food wastewater		
F1	2.654	1.688
F2	3.150	2.132
F3	3.041	1.886
Paper wastewater		
P1	2.951	2.011
P2	3.109	2.127
P3	3.108	1.760
Sewage wastewater		
S1	2.863	2.317
S2	3.027	2.230
Textile wastewater		
T1	3.072	1.849
T2	3.006	2.012
T3	3.063	1.920

and accounted for less than 0.01% to 1% of total bacterial populations (Fig. 3). Although all sludges had a similar abundance of AOB populations, the *amoA* gene abundance varied within and between different WWTPs. The *amoA* gene abundance was observed to be very low ($<10^3$ copies/l) in food and paper wastewater, except for P2 and F3. On the other hand, sludges from textile, livestock, and sewage wastewater treatment plants contained relatively high *amoA* ($>10^5$ copies/l).

In this study, the difference in the total number of bacterial 16S rRNA gene copies in each sludge sample was not significant (α=0.01, 1.8×10^{10}–3.8×10^{12} copies). The AOB population was also not significantly different between the WWTPs. However, the number of *amoA* varied within and between different WWTPs. F1, F2, P1, and P3 had less than 10^3 copies, whereas T1, L1, and S2 has more than 10^8 copies per liter of sample. Differences in the percent of AOB between the samples may reflect not only the different source of wastewater but also differences in the design and operation of each WWTP. The concentration of AOB biomass in our study may be smaller than that found in other studies, and this may be because our samples were taken during winter time.

The results of this study indicate that molecular technology, including DGGE and real-time PCR using the bacterial 16S rRNA and *amoA* gene, is useful for the comparison of population abundance in wastewater samples, offering high throughput, and analytical sensitivity and precision. The *amoA* gene, especially, provided quantitative information about the current processes. However, for better understanding of the nitrification in WWTPs, we need to estimate the NOB population and activity, as well as the AOB population and abundance. Based on these results, we will continue to monitor and enumerate total bacterial and AOB 16S rRNA and *amoA* genes using the real-time PCR assay during the full-scale operation of

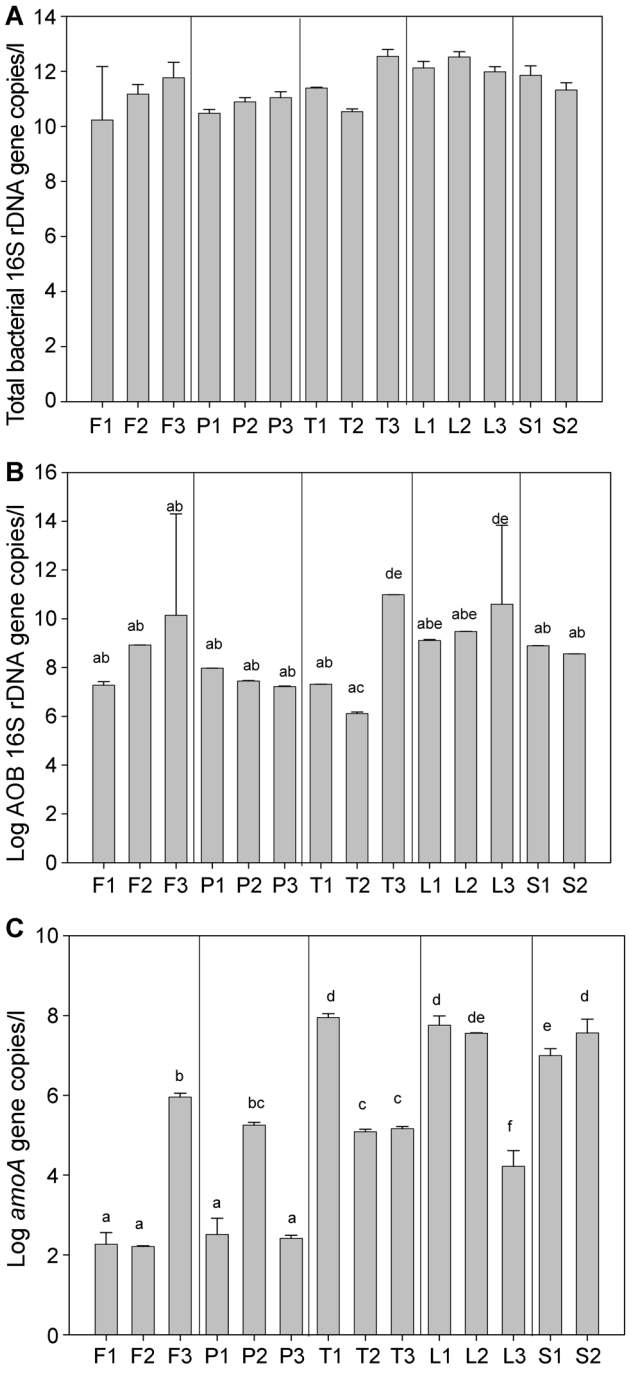

Fig. 3. Abundance of total bacterial 16S rRNA (**A**), AOB 16S rRNA (**B**), and *amoA* genes (**C**) in the activated sludge of 14 different WWTPs measured by real-time PCR assays.
Error bars represent the standard deviations of the means (n=3). Letters above bars indicate homogenous subsets by the multiple-comparison Tukey test after ANOVA (α=0.01).

wastewater treatment processes. In addition, nitrite oxidizing bacteria, as well as ammonia oxidizers in activated sludge, will be estimated at the same time.

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