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Nitrifying Bacterial Community Structure of a Full-Scale Integrated Fixed-Film Activated Sludge Process as Investigated by Pyrosequencing

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Nitrifying bacterial community structures of suspended and attached biomasses in a full-scale integrated fixed-film activated sludge process were investigated by analyzing 16S rRNA gene sequences obtained from pyrosequencing. The suspended biomass had a higher number of ammonia-oxidizing bacterial sequences (0.8% of total sequences) than the attached biomass (0.07%), although most of the sequences were within the *Nitrosomonas oligotropha* lineage in both biomasses. *Nitrospira*-like nitrite-oxidizing bacterial sequences were retrieved in the suspended biomass (0.06%), not in the attached biomass, whereas the existence of *Nitrobacter*-like sequences was not evident. The suspended biomass had higher nitrification activity (1.13 mg N/TSS/h) than the attached biomass (0.07 mg N/TSS/h). Overall, the results made it possible to conclude the importance of the suspended biomass, rather than the attached biomass, in nitrification in the wastewater treatment process studied.

Keywords: IFAS, pyrosequencing, AOB, NOB, activated sludge

Improperly treated municipal wastewater increases the concentration of nutrients such as nitrogen and phosphorus in receiving waters (*i.e.*, eutrophication), which stimulates the growth of phototrophic organisms and affects the water environment negatively [8, 23]. For example, an abrupt increase in biomass of phytoplankton deteriorates water quality, making it difficult to use the water for potable, agricultural, and recreational purposes [8, 23]. Occasionally, the toxins produced by phytoplankton threaten the lives of aquatic organisms [7]. Thus, the nitrogen level in the wastewater effluent has been strictly regulated to avoid such harmful effects [8, 23].

Biological nitrogen removal (BNR) processes are commonly used to remove nitrogen in wastewater treatment. The processes adopt the metabolic potentials of microorganisms, including nitrification and denitrification. Nitrification is oxidation of ammonia to nitrate *via* nitrite, and is conducted by two groups of aerobic chemolithoautotrophic bacteria, named ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) [13]. Ammonia can also be oxidized by ammonia-oxidizing archaea [12], some heterotrophic bacteria [21], and anaerobic ammonia-oxidizing bacteria [22]. However, these nitrifiers are known to be relatively unimportant compared with AOB and NOB in wastewater treatment bioreactors [25]. Denitrification is a series of reduction processes in which oxidized nitrogen species (*e.g.*, nitrite and nitrate) are transformed into dinitrogen as the final product for removal [8]. Generally, nitrification is regarded as the limiting factor in BNR processes, because the nitrification rate is significantly lower than the denitrification rate and is affected by many environmental and process operational factors [8, 23].

The integrated fixed-film activated sludge (IFAS) process is a modification of the conventional activated sludge wastewater treatment process, in which solid biomass support materials (*e.g.*, suspended plastic pieces or fixed synthetic mesh) are integrated into a suspended growth activated sludge bioreactor [3, 9]. The incorporation of biomass support materials provides a surface for the attachment of biomass and increases the biomass concentrations in the bioreactor [8]. Owing to the increased biomass concentration, the IFAS process has several advantages over conventional activated sludge processes, including higher treatment rates, tolerance to fluctuating organic and hydraulic loads, and improved settling of activated sludge [3, 8, 19]. In particular, an enhanced nitrification rate is the prominent feature of the IFAS system and would allow retrofitting of the conventional treatment processes to the BNR process [9, 14].

Nitrification of IFAS processes has been evaluated by several researchers with pilot-scale [4, 17, 20] and full-scale experiments [9, 14]. In addition, an enhanced nitrification rate has been regarded as being the result of increased

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nitrifying organisms of the IFAS process compared with conventional activated sludge systems [3], as demonstrated by results of quantitative PCR [4] and fluorescent *in situ* hybridization [9]. Nevertheless, not a great deal of information is available on the phylogenetic identities of the nitrifying organisms in the IFAS processes. An investigation of the diversity and compositions of nitrifying organisms is also required, which would be the basis for a better understanding of nitrification in the IFAS processes. Thus, the main objective of this study was to analyze nitrifying bacterial communities in a full-scale IFAS process and to characterize them by comparing with the nitrifying bacterial communities that are found in conventional activated sludge systems.

The Guri wastewater treatment plant (WWTP) located in Guri, Gyeonggi-Do, Korea, was used in this study. The Guri WWTP has a capacity to treat 160,000 m^3 of domestic wastewater per day and operates using an IFAS process. The bioreactor of the WWTP consists of anaerobic, anoxic, and aerobic basins to facilitate enhanced biological phosphorus removal [8] as well as BNR. In the aerobic basin, synthetic fiber media made of acryl and polyester are integrated (surface area of media per volume of aerobic basin = 1.52). The bioreactor facilitated active nitrification as well as denitrification at the sampling time based on profiles of ammonia, nitrite, and nitrate (Supplemental Fig. 1).

In order to investigate AOB communities in the Guri WWTP, AOB-like sequences were retrieved from total bacterial 16S rRNA gene sequences obtained in our previous study using a high-throughput pyrosequencing in the Guri WWTP [6]. Suspended and attached biomass samples were collected in June 2009 by grab sampling at three different points in the aerobic tank of the Guri WWTP and pooled together for the sequencing experiments, respectively. DNA extraction, PCR, pyrosequencing, and phylogenetic analysis were conducted following the protocols introduced in the previous publications [6, 25]. As a first step, the sequences within family Nitrosomonadaceae were extracted using the RDP Classifier (http://rdp.cme.msu.edu) at the 50% confidence level. After constructing a phylogenetic tree, sequences not affiliated with AOB were removed for further analysis. As a result, a total of 189 AOB-like sequences were retrieved out of 23,536 sequences (0.80%) in the suspended sample, whereas only 31 sequences out of 44,003 sequences (0.07%) showed affinity with AOB in the attached sample. The AOB sequences retrieved from the suspended sample were more diverse than those retrieved from the attached sample: for the suspended and attached samples, 26 and 3 unique operational taxonomic units (OTUs), respectively, were observed (3% cutoff) and 31 and 3 OTUs, respectively, were estimated by Chao 1 richness (3% cutoff), as determined by Mothur [16]. A phylogenetic analysis was conducted to investigate the evolutionary relationship between the retrieved AOB-like sequences and sequences in the NCBI database (http://www.ncbi.nlm.nih.gov/) as presented in Fig. 1. Most of the sequences retrieved from the suspended sample (186 sequences) showed affinity with the *Nitrosomonas oligotropha* lineage, and only three sequences were within the *N. marina* lineage. *N. oligotropha* is known to grow well in low ammonia concentrations (<50 mM) and is frequently found in wastewater treatment plants [5]. The sequences within the *N. oligotropha* lineage were grouped into three clusters: 84, 70, and 30 sequences were observed in the Clusters 1, 2, and 3, respectively. Clusters 1 and 2 included sequences from the activated sludge and nitrification bioreactor, but there were no reported sequences (>98% identity) in Cluster 3. For the attached sample, all of the sequences (31 sequences) were found with the *N. oligotropha* lineage, and most of the sequences were clustered with Cluster 2 (28 sequences).

Similar analyses were conducted for NOB-like sequences. The sequences within family Nitrospiraceae were extracted using the RDP Classifier at the 50% confidence level. A total of 13 *Nitrospira*-like sequences were identified in the suspended sample (0.06%), but there was no *Nitrospira*-like sequence in the attached sample. With regards diversity, 8 OTUs were observed and 16 OTUs were estimated by Chao1 richness index. A phylogenetic analysis (Fig. 2) demonstrated that all of the *Nitrospira*-like sequences were grouped in Cluster 1, which included *Candidatus Nitrospira defluvii* [18] and sequences retrieved from the activated sludge, biofilm, and anammox reactor. A total of 26 *Nitrobacter*-like sequences (3 in the suspended sample and 23 in the attached sample) were identified within genus *Nitrobacter* (50% confidence level) by the RDP Classifier, but all of the sequences were not closely affiliated with known *Nitrobacter* sequences phylogenetically, including *N. hamburgensis*, *N. wibogradsky*, *N. alkalicus*, and *N. vulgaris* (Supplemental Fig. 2). Although those sequences might be related to unknown *Nitrobacter* species, it appears that those sequences were originated from different species irrelevant to NOB, based on clustering distinct from the *Nitrobacter* cluster (bootstrap value distinguishing the cluster = 100%) and low sequence identities with pure-culture *Nitrobacter* species (95–97%). Therefore, NOB within the genus *Nitrobacter* appear to be unimportant in the Guri WWTP. Recent reports have demonstrated the importance of *Nitrospira* rather than *Nitrobacter* in nitrite oxidation in WWTPs [9, 11, 24]. *Nitrospira* is known to have a higher affinity for substrate (*i.e.*, nitrite), but a lower growth rate than *Nitrobacter* and is suited to a low nitrite environment such as a WWTP [24]. The concentration of nitrite was detected to be less than 0.1 mg N/l in the Guri WWTP (Supplemental Fig. 1).

It is well known that oxygen is the terminal electron acceptor for nitrifying bacteria, and the level of dissolved oxygen (DO) affects their growth rate [8]. In addition, DO

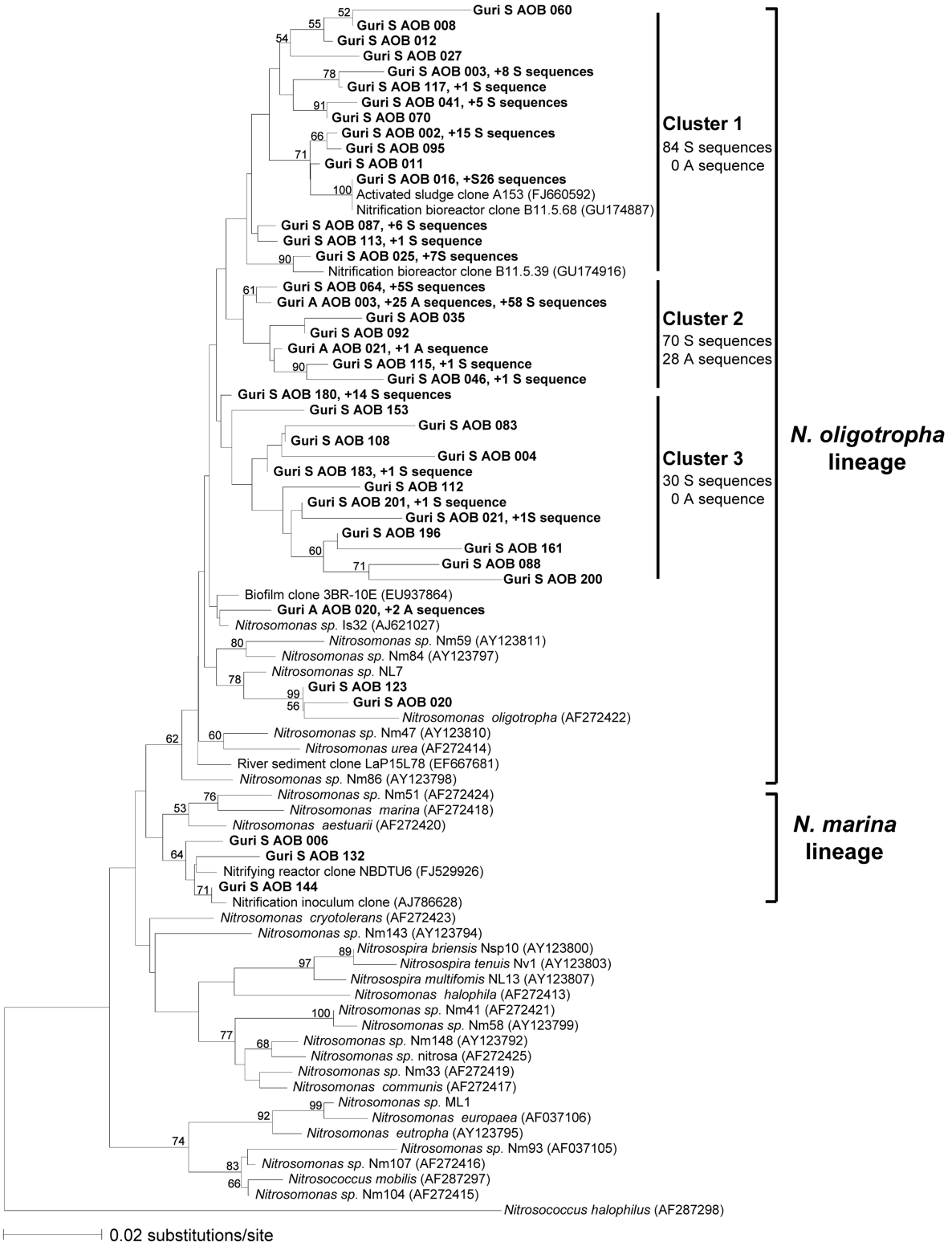

Fig. 1. Neighbor-joining tree of AOB based on 16S rRNA gene sequences retrieved from this study (boldface type), pure-culture strains, and environmental samples.

Guri WWTP sequences retrieved from the suspended biomass are indicated as Guri S, and those retrieved from the attached biomass are indicated as Guri A. Sequences showing >98% identities are indicated as a representative sequence. The tree was rooted with the *Nitrosococcus halophilus* Nc4 16S rRNA gene sequence. Bootstrap values were determined by 1,000 trials and were indicated on branch nodes greater than 50%. Sequences determined for the analysis were deposited in the GenBank database under accession numbers from HQ269884 to HQ270114.

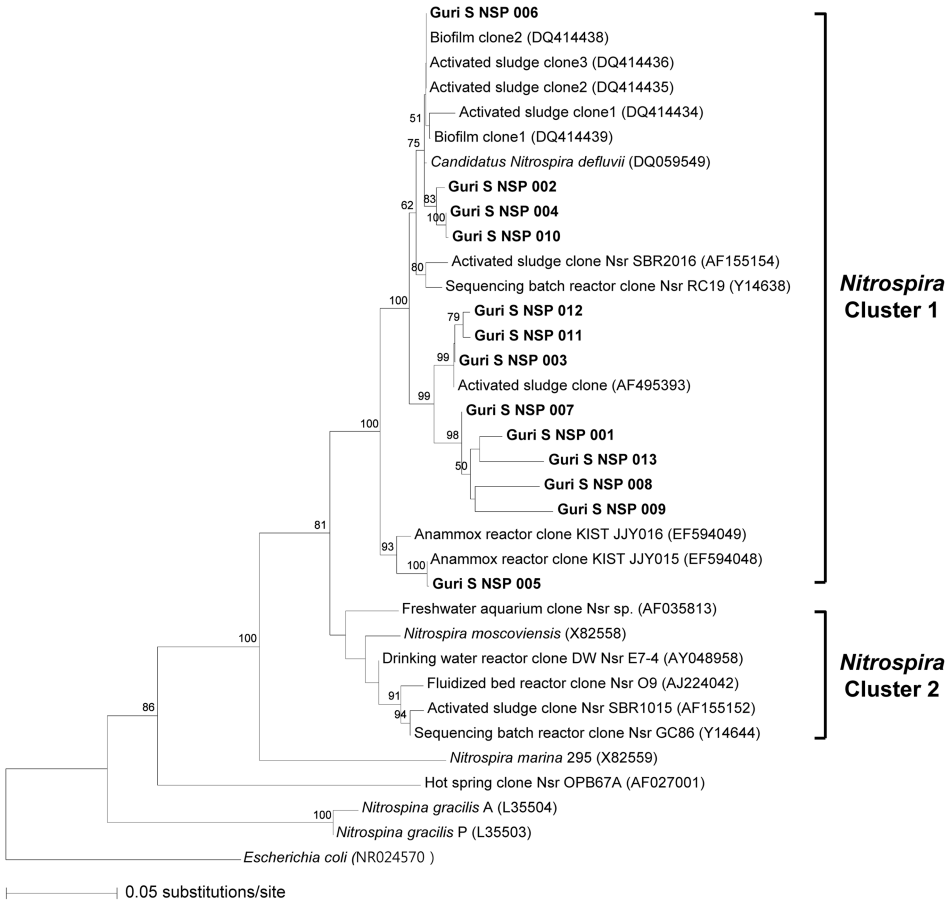

Fig. 2. Neighbor-joining tree of *Nitrospira* based on 16S rRNA gene sequences retrieved from this study (boldface type), pure-culture strains, and environmental samples.
Guri WWTP sequences retrieved from the suspended biomass are indicated as Guri S, and those retrieved from the attached biomass are indicated as Guri A. The tree was rooted with the *Escherichia coli* 16S rRNA gene sequence. Bootstrap values were determined by 1,000 trials and were indicated on branch nodes greater than 50%. Sequences determined for the analysis were deposited in the GenBank database under accession numbers from HQ269845 to HQ269857.

appears to influence the distribution of nitrifying bacteria in natural [2] and engineering environments [10, 11]. The suspended sample was in a higher DO condition (>2.0 mg DO/L) than the attached sample in the Guri WWTP. Although the DO concentration of the attached sample was not measured in this study, DO was expected to be very low because the DO concentration is reported to reach near 0 mg/l below the depth of a 200-mm biofilm [15]. Therefore, the distinct distribution of nitrifying bacterial community composition in the Guri WWTP, such as the exclusive detection of *Nitrospira* in the suspended sample, could be partly explained by the difference of DO conditions between the suspended and attached samples.

The nitrification activity of the Guri WWTP was investigated by determining ammonia oxidation rates, as shown in Fig. 3. The rates were 1.13±0.44 (average±95% confidence interval) and 0.07±0.04 mg N/total suspended solids (TSS)/h for the suspended and attached samples,

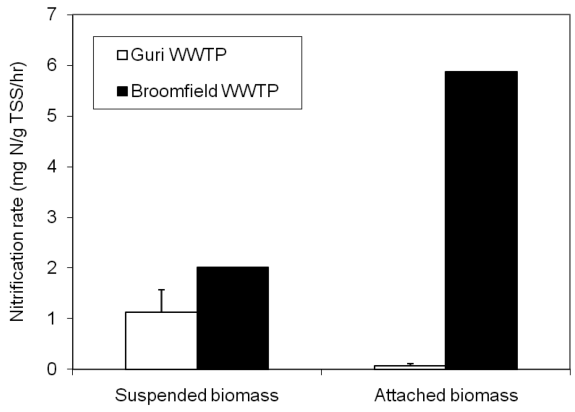

Fig. 3. Nitrification rates of suspended and attached biomasses in the IFAS processes: the Guri WWTP (open bars) and the Broomfield WWTP (closed bars).
The error bars of the Guri WWTP represent 95% confidence intervals of the rates. The rates of the Broomfield WWTP were obtained from a publication of Onnis-Hayden *et al*. [9].

Table 1. Characteristics of the two IFAS wastewater treatment plants (WWTPs).

	Guri WWTP	Broomfield WWTP
Location	Guri, Gyeonggi-Do, Korea	Broomfield, Colorado, USA
Capacity (m^3/day)	160,000	30,000
Media type	Synthetic fiber	Suspended plastic piece
Media material	Acryl and polyester	Polyethylene
Influent biochemical oxygen demand (mg/l)	120.5	145.8
Influent total nitrogen (mg N/l)	4	46.0
Hydraulic retention time (h)	5.9	5.5
Solids retention time (day)	8.5	4.7

respectively. The values are 1.2–59% of the measurements obtained by Onnis-Hayden *et al*. [9], who conducted similar experiment with a full-scale IFAS process. The result suggested a low nitrification activity of the Guri WWTP and explained the significant ammonium concentration in the effluent (5.0 mg N/l, Supplemental Fig. 1). Another feature of the Guri WWTP was a lower nitrification rate of the attached biomass than that of the suspended biomass, an observation that was in contrast to that of Onnis-Hayden *et al*. [9]. The differences in nitrification rate also accords with the numbers of AOB sequences in the Guri WWTP, where 0.80% and 0.07% of total sequences were AOB-like sequences in the suspended and attached biomasses, respectively. These results suggest that the suspended biomass contributed more to nitrification than did the attached biomass in the Guri WWTP. In terms of the nitrifying bacterial community, Onnis-Hayden *et al*. [9] reported the predominance of AOB within the *N. europaea* lineage as well as those within the *N. oligotropha* lineage, and exclusive detection of NOB within the genus *Nitrospira* but not within the genus *Nitrobacter*. The distinct nitrification rates and distribution of nitrifying bacteria between the two WWTPs appear to reflect characteristics of the two treatment plants, such as different operational conditions, influent conditions, and media used for biomass attachment (Table 1).

This study reports the diversity and phylogenetic affiliation of AOB-like and NOB-like sequences as well as nitrification rates of the suspended and attached biomasses in a full-scale IFAS process. The information made it possible to deduce the relative importance of the two biomasses in the studied WWTP. Unlike other WWTPs using the IFAS process [4, 9], the studied WWTP had a much lower nitrification activity in the attached biomass. This suggests that the studied WWTP did not take advantages of attached biomass in nitrification, and needs operational (*e.g*., aeration strategy) and/or design modifications (*e.g*., locations of the biomass support materials) to increase the nitrifying biomass (or nitrification activity) attached to biomass support materials. These modifications should be studied in the future. Furthermore, this study used sequences obtained from pyrosequencing instead of clone sequences for the phylogenetic study of nitrifying bacteria, and this involved skipping the cloning step and possibly omitted a bias conferred by cloning.

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