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Redundancy Analysis Demonstration of the Relevance of Temperature to Ammonia-Oxidizing Bacterial Community Compositions in a Full-Scale Nitrifying Bioreactor Treating Saline Wastewater

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Although salt is known to influence the performance of nitrification significantly, it has not been well reported on how salt affects ammonia-oxidizing bacterial (AOB) community compositions and dynamics in wastewater treatment bioreactors. In this study, these questions were evaluated in a full-scale bioreactor treating saline wastewater. Clone library analysis for the ammonia monooxygenase subunit A gene revealed that AOB belonging to the *Nitrosomonas europaea* and the *N. oligotropha* lineages inhabited in the bioreactor. Terminal restriction fragment length polymorphism analysis for monthly samples demonstrated a fluctuation pattern among AOB populations, although AOB within the *N. europaea* lineage were dominant during the test period. Correlation analysis between patterns of terminal restriction fragments and environmental variables suggested that sodium, chloride, and sulfate were less important; rather, temperature was the most significant factor affecting the AOB community in the bioreactor.

Keywords: Nitrification, ammonia-oxidizing bacteria, terminal restriction fragment length polymorphism, wastewater treatment, salt inhibition

It is well reported that nitrogen discharge to water bodies stimulates the growth of phototrophic microorganisms (*e.g.*, free-living algae) and leads to depletion of dissolved oxygen [1], which pollutes drinking water resources as well as harms aquatic organisms. Because significant amounts of nitrogen contamination in water bodies originate from municipal wastewater [17], nitrogen discharge from wastewater treatment plants is strictly regulated in many countries. The biological nitrogen removal (BNR) process is a stable and economical option to remove nitrogen from nitrogen-containing wastewater [8]. BNR processes incorporate microbiological unit operations such as nitrification (*i.e.*, conversion of ammonia to nitrate) and denitrification (*i.e.*, conversion of nitrate to dinitrogen gas) to remove nitrogen from wastewater.

Two groups of chemolithoautotrophic bacteria, named ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB), are largely responsible for the nitrification in BNR processes [8], although other types of nitrifying bacteria are sporadically found in some wastewater treatment bioreactors (*e.g.*, heterotrophic ammonia-oxidizing bacteria [14] and ammonia-oxidizing archaea [12]). AOB and NOB have metabolic ability to oxidize ammonia to nitrite and nitrite to nitrate, respectively. In many cases of BNR processes, it is likely that ammonia oxidation is the rate-limiting step to determine the overall efficiency of nitrification [17]. The activity of AOB is affected by several environmental and operational conditions including temperature, pH, dissolved oxygen levels, toxic chemicals, retention time, and aeration/mixing intensities [11, 19].

Salt is also known to be a factor affecting the activity of AOB [4] as well as the efficiency of BNR processes [5, 10, 18].

Table 1. The characteristics of influent and effluent of the Pohang wastewater treatment bioreactor.

	Influent	Effluent
Temperature, °C	Not available	21±5
Soluble chemical oxygen demands, mg/l	94±41	76±38
Biomass (mixed liquor suspended solids), mg/l	Not available	2,770±720
Total kjeldahl nitrogen, mg N/L	24.5±14.4	Not available
NH_4^+-N, mg N/l	10.1±4.7	6.7±8.4
NO_3^--N, mg N/l	Not available	8.1±7.2
Na^+, mg/l	2,890±250	2,220±1,210
Cl^-, mg/l	5,416±560	4,110±2,390
SO_4^{2-}, mg/l	880±220	760±390

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According to the study of Dincer and Kargi [2], the nitrification rate decreased to 20% in wastewater containing 5% NaCl in a laboratory-scale bioreactor. Panswad and Anan [10] also reported similar results on the inhibition of nitrification by saline wastewater. Moussa *et al.* [9] studied the effect of salt on nitrification with more systematic experiments using laboratory-scale sequencing batch reactors and found that *Nitrosomonas europaea* was the dominant AOB adapted to high salt conditions. Most of the previous works have been conducted at laboratory levels and focused on the salt effects on the activity of nitrification and/or snap-shot information of nitrifying bacteria. It has not been well reported about the dynamics of nitrifying bacteria in the presence of salt and the correlation between nitrifying population dynamics and salt concentrations. Thus, the objective of this study was to investigate AOB community compositions, AOB population

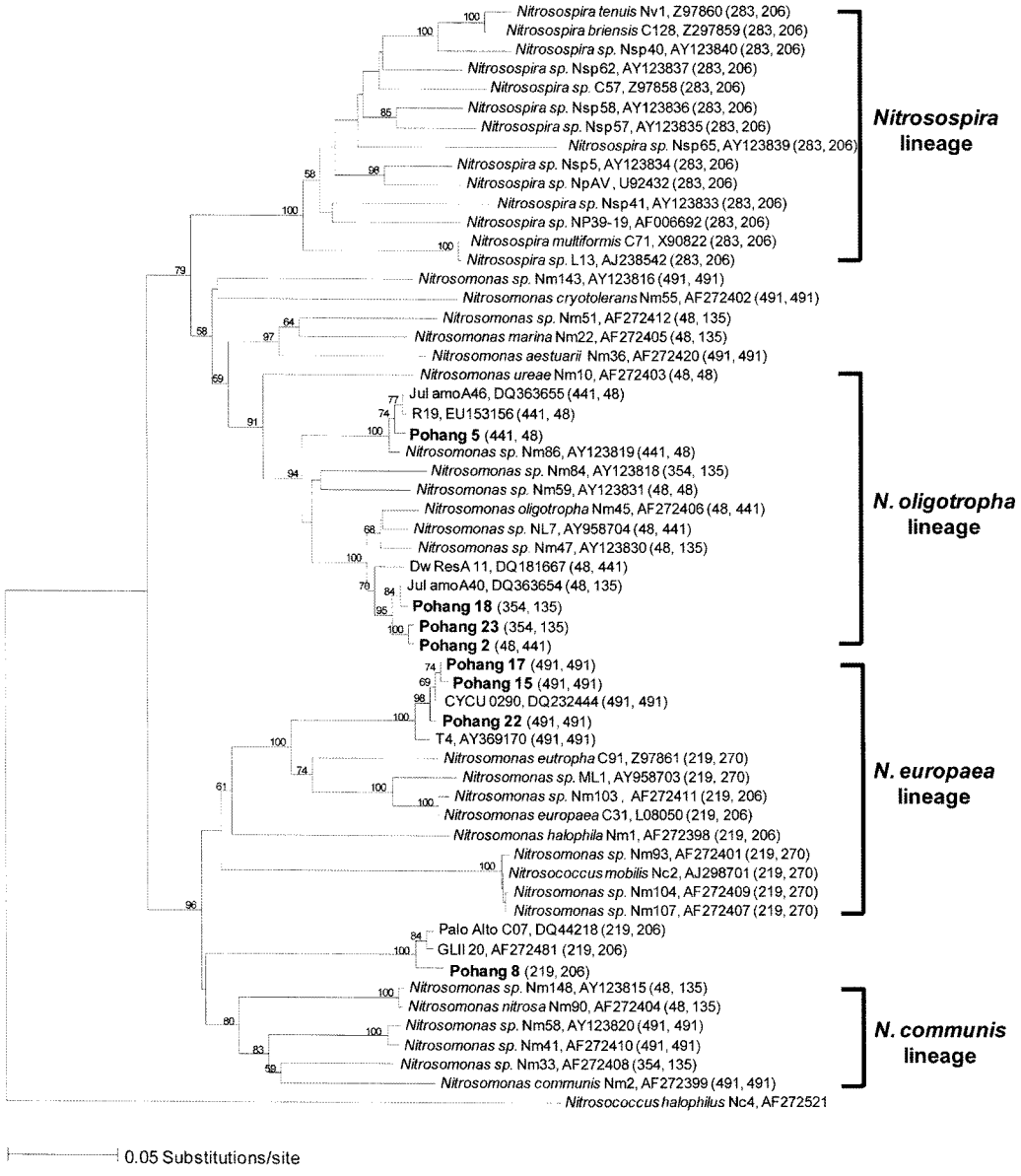

Fig. 1. Neighbor-joining tree based on *amo*A gene sequences retrieved from this study (boldface type), pure-culture AOB strains, and other environmental samples.

The sizes of terminal restriction fragments (t-RFs) digested with TaqI restriction enzyme were determined *in silico* and are indicated beside sequence names in parentheses. Bootstrap values were determined based on 100 trials and shown at nodes greater than 50. Sampling, DNA extraction, PCR, cloning, and phylogenetic analysis were conducted following protocols introduced in the previous publication [11]. Sequences determined in this study have been deposited in the GenBank database under accession numbers EU708492 to EU708499. Sequences encoding the *amo*A gene were PCR-amplified from the genomic DNA using the primer set *amo*A-1F (5'-GGGGTTTCTACTGGTGGT-3') and *amo*A-2R (5'-CCCCTCKGSAAAGCCTTCTTC-3') [13]. Both the sequence alignment and the phylogenetic analysis were conducted using the ClustalX software [16].

dynamics, and correlation between AOB population dynamics and salt concentrations in a full-scale bioreactor treating saline wastewater.

In this study, ammonia oxidation performances, AOB community compositions, and AOB population dynamics were monitored from the Pohang municipal wastewater treatment plant located in Pohang, Korea (36.004° N latitude, 129.350° E longitude). The plant treats ~80,000 m^3 of municipal sewage per day and discharges its treated effluent to the East Sea. The plant operates its bioreactor using a conventional activated sludge treatment system [8] and removes mainly organic and particulate matters. The treatment plant also induced nitrification during the test periods when the average nitrification rate was 73%. Influent wastewater contained high salt concentrations due to frequent infiltration of seawater to its sewer system. The characteristics of influent and effluent of the treatment bioreactor are indicated in Table 1.

AOB community composition in the bioreactor was investigated from May 2005 samples by constructing a clone library for an AOB-specific functional gene encoding ammonia monooxygenase subunit A (*amo*A). Eight Pohang *amo*A sequences were retrieved based on a restriction fragment length polymorphism analysis. These sequences, 42 pure-culture AOB *amo*A sequences and 8 environmental *amo*A sequences, were used to generate a phylogenetic tree (Fig. 1). Environmental sequences were selected based on close identity to the Pohang clone sequences, which was conducted by searching sequences from a nucleotide database provided by NCBI (http://www.ncbi.nlm.nih.gov). The phylogenetic analysis revealed that most of the Pohang sequences were distributed to the two *Nitrosomonas* lineages (*i.e.*, *N. oligotropha* and *N. europaea* lineages) except for one clone (*i.e.*, Pohang 8 clone). Four Pohang sequences (Pohang 2, 5, 18, and 23) belonged to the *N. oligotropha* lineages. These sequences were similar to sequences retrieved from drinking water treatment systems (DW ResA 11 and Jul-amoA 40), whereas Pohang 5 was similar to a pure-culture AOB isolated from a river (Nm 86) and to sequences retrieved from a biofilm reactor (R19) and a drinking water system (Jul-amoA 46). Three Pohang sequences (Pohang 15, 17, and 22) were related to the *N. europaea* lineages. These sequences were similar to sequences retrieved from activated sludge (T-4) and membrane bioreactors (CYCU 0290). Pohang 8 was quite different from the sequences belonging to the *N. europaea* or the *N. communis* lineages, which was also supported by bootstrapping. Although there is no pure-culture AOB sequence available that is similar to Pohang 8, sequences retrieved from activated sludge bioreactors (*i.e.*, Palo Alto C07 and GLII 20) were closely related to Pohang 8.

Dynamic changes of AOB community compositions were traced using a fingerprinting method called dual-labeled terminal restriction fragment length polymorphism (t-RFLP) analysis based on the *amo*A gene [11] for monthly (June to November 2005) samples (Fig. 2). The method generates two

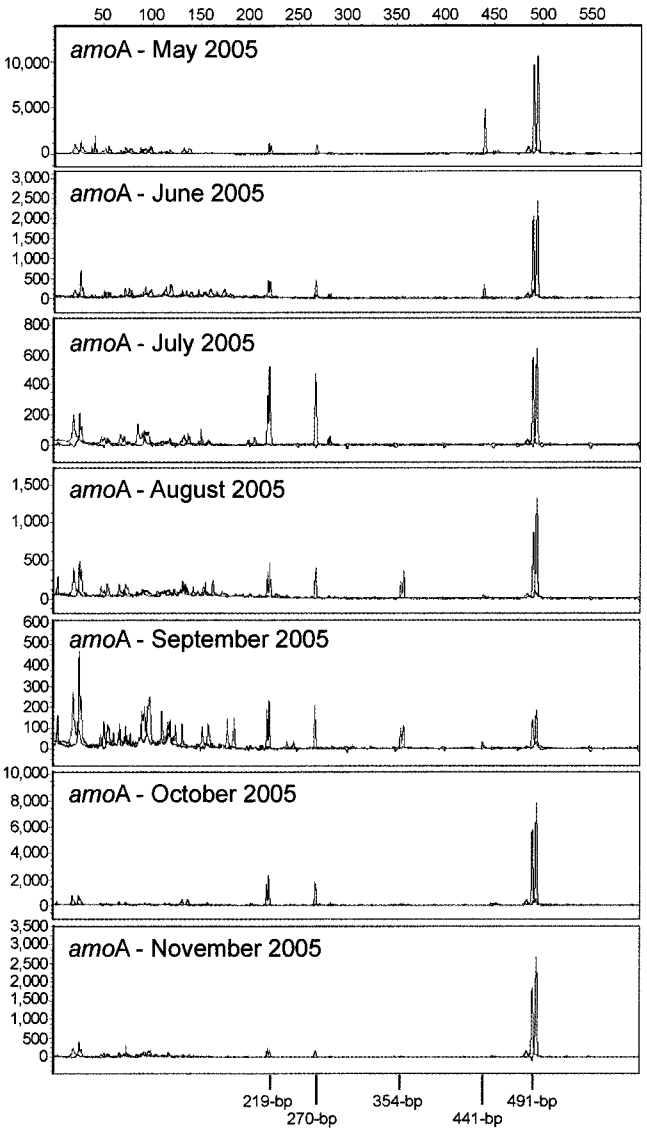

Fig. 2. T-RFLP electropherograms based on the *amo*A gene for samples collected from the Pohang wastewater treatment bioreactor. X- and Y-axes of each eletropherogram indicate size of terminal fragment in base pairs and fluorescence intensity in fluorescence units, respectively. Sampling, DNA extraction, PCR, restriction digestion, and analysis of terminal fragments were conducted following the protocols published in the previous publication [11]. In the PCR reactions, a HEX-labeled forward primer (*amo*A-1F) and a 6-FAM-labeled reverse primer (*amoA*-2R) to amplify 491-bp dual-labeled *amoA* gene PCR products were used. Samples were run through an ABI 310 DNA sequencer (Applied Biosystems, Foster City, U.S.A.) and analyzed using the GeneMarker software version 1.6 (SoftGenetics, State College, U.S.A.). *In silico* t-RFLP analysis based on this study (Fig. 1) as well as the previous publication [11] demonstrated that forward t-RF sized in 48, 219, 354, 441, and 49-bp and reverse t-RF sized in 48, 135, 270, 441, and 491 bp were frequently monitored AOB signatures. Thus, in this study, t-RFs sized in other lengths were regarded as backgrounds or as signatures originated from non-AOB.

different sets of terminal restriction fragments (t-RFs): Green and blue peaks originate from forward t-RFs and reverse t-RFs, respectively. Electropherograms represented in Fig. 2

demonstrated that the 491-bp forward t-RF (*i.e.*, green peak) and 491-bp reverse t-RF (*i.e.*, blue peak) were dominant AOB signatures for all Pohang samples. It is likely that these signatures correspond to sequences similar to Pohang 15, 17, and 22 (*N. europaea* lineage) according to the clone library and *in silico* t-RFLP analyses (Fig. 1). In July, August, September, and October samples, 219-bp forward t-RF and reverse 270-bp t-RF were significant. Although these AOB signatures were not found in the clone library, probably due to the small number of clones evaluated in this study, they are frequently found in sequences within the *N. europaea* lineage (Fig. 1). The 354-bp forward t-RF and 441-bp reverse t-RF (probably AOB signatures belonging to the *N. oligotropha* lineage) were sporadically monitored for some samples, but their magnitudes (*i.e.*, peak areas) were insignificant. AOB signatures like the Pohang 8 clone (*i.e.*, 219-bp forward t-RF and 206-bp reverse t-RF) were not evident in the electropherograms, which indicated that such AOB were not significant in the full-scale bioreactor. The t-RFLP results as well as the clone library analysis (Fig. 1) confirmed that AOB belonging to the *N. europaea* lineage were the important organisms in the bioreactor.

The correlations between environmental variables (*i.e.*, temperature, biomass, NH_4^+, NO_3^-, Na^+, Cl^-, and SO_4^{2-}) and AOB community structures (*i.e.*, the patterns of t-RFs) were evaluated using a multivariate statistical method called redundancy analysis (RDA). RDA is a choice of method for identifying important environmental variables to determine community structures in ecological data sets, assuming linear species response to the underlying environmental gradient [7]. Fig. 3 shows the RDA triplot, which represents the three parameters (*i.e.*, t-RFs, environmental variables, and samples) in a same plot and correlates among them. The plot suggested that the 491-bp forward t-RF/491-bp reverse t-RF was proportional to ammonia and biomass levels and was inversely proportional to temperature and nitrate levels. Likewise, the 354-bp forward t-RF/135-bp reverse t-RF was proportional to temperature and nitrate levels and was inversely proportional to ammonia and biomass levels. Salts (*i.e.*, Na^+, Cl^-, and SO_4^{2-}) and COD levels were related to the 219-bp forward t-RF/270-bp reverse t-RF and were not related to the 491-bp forward t-RF/491-bp reverse t-RF or 219-bp forward t-RF/270-bp reverse t-RF. The statistical importance of these correlations was tested using a forward selection method [15], which revealed that temperature was the most important variable ($P<0.05$) out of the seven environmental variables. Salts appeared to be not so significant ($P>0.05$) in determining AOB community structure in our study.

The work presented here is the first report on a detailed description of AOB community compositions and dynamics for a full-scale bioreactor treating saline wastewater. The results suggested that AOB belonging to the *N. europaea*

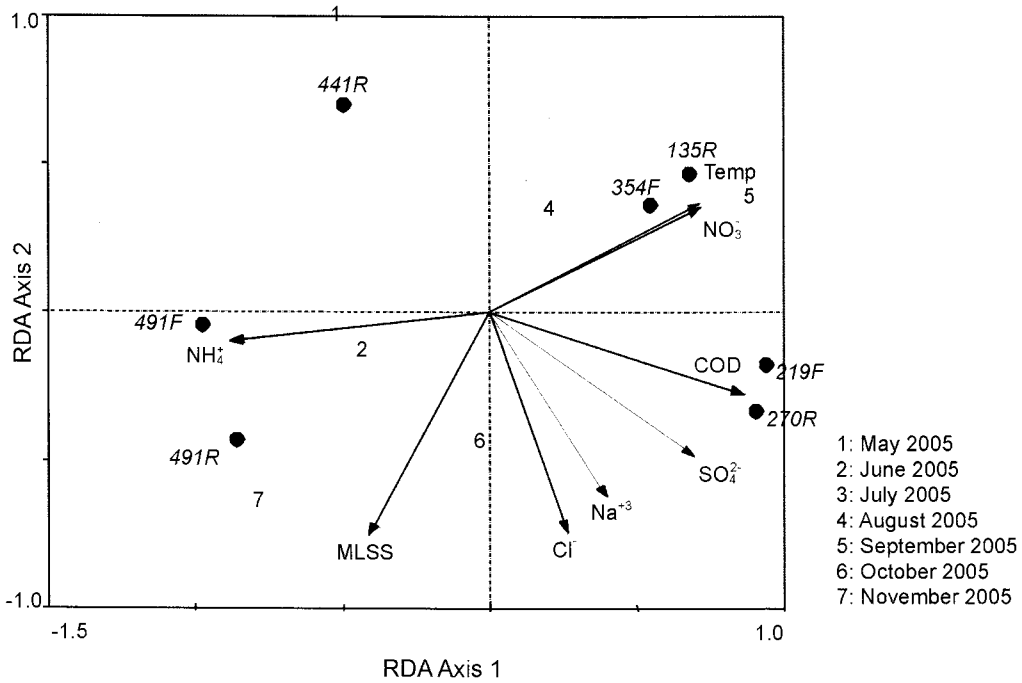

Fig. 3. RDA analysis of monthly t-RFLP patterns for the Pohang wastewater treatment bioreactor and correlation with environmental variables.

The length of an arrow indicates the relative importance of the explanatory variables to the t-RF patterns, and the angle of an arrow indicates whether the explanatory variable increases or decreases in magnitude with respect to the indicated t-RF length. Legends: arrows (environmental variables), closed circles (terminal fragments), and numbers (monthly samples). RDA analyses were conducted focusing on interspecies distances using the Canoco statistical software (Plant Research International, The Netherlands). A Monte Carlo permutation was used to test statistically important explanatory variables using the same software.

lineage were the major ammonia oxidizers in the saline wastewater bioreactor (see Figs. 1 and 2). Although AOB belonging to the *N. oligotropha* lineage were detected in the clone library analysis (Fig. 1), they were not likely to be the significant ammonia oxidizers in the saline wastewater bioreactor, which was evidenced by the fingerprint data (Fig. 2). These results agree with the report of Moussa *et al.* [9], who observed the dominance of *N. europaea* over *N. oligotropha* when the level of Cl^- increased from 0 to 10 g/l in a laboratory-scale reactor. *N. europaea* is also known as a halotolerant or moderately halophilic ammonia oxidizer [6] and was found in marine habitats [3] as well as in wastewater treatments, which supports the idea that *N. europaea* is an ammonia oxidizer treating saline wastewater. However, it is not yet clear whether salt is the selective pressure enriching *N. europaea*-related AOB in saline wastewater. A correlation analysis between AOB community structures and environmental variables (Fig. 3) revealed that salts were not significant factors affecting the AOB community structure; rather, temperature was more important. One possible explanation for this is that the fluctuations of salt in our study were not high enough to induce changes in the AOB community structures. During the test period, the concentrations of Na^+, Cl^-, and SO_4^{2-} varied from 2,590 to 3,140 mg/l, from 4,860 to 6,190 mg/l, and from 660 to 1,160 mg/l, respectively.

Salt is a factor inhibiting nitrification efficiency in wastewater treatment [5, 10, 18]. In order to design and operate a bioreactor treating saline wastewater, it is crucial to identify what AOB can survive in saline condition and determine their activity. In this study, we identified that a specific AOB belonging to the *N. europaea* lineage was dominant in a full-scale bioreactor treating saline wastewater, although other types of AOB belonging to the *N. oligotropha* and a novel lineage were found sporadically. The AOB populations fluctuated with operational time in the bioreactor and were significantly affected by temperature rather than by salts. The findings would be helpful for us to better understand nitrification in saline wastewater.

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