MOLECULAR ANALYSIS OF AMMONIA-OXIDIZING BACTERIAL POPULATIONS IN AERATED-ANOXIC ORBAL PROCESSES

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ABSTRACT

Aerated-anoxic processes operate under the principle that small additions of oxygen to an anoxic reactor induce simultaneous nitrification and denitrification. In these systems, ammonia oxidation in the anoxic zone can easily account for 30-50% of the total nitrification in the reactor, even though the dissolved oxygen concentration is usually below detection limit. To investigate whether the nitrification efficiency in aerated-anoxic processes was due to the presence of specialized ammonia-oxidizing bacteria (AOB), an analysis of the AOB population in an aerated-anoxic Orbal process and a conventional nitrogen removal process was carried out using phylogenetic analyses based on the ammonia monooxygenase A (*amoA*) gene. Terminal restriction fragment length polymorphism (TRFLP) analyses revealed that *Nitrosospira*-like organisms were one of the major contributors to ammonia oxidation in a full-scale aerated-anoxic Orbal reactor. However, the relative populations of *Nitrosospira*-like and *Nitrosomonas*-like AOB were not constant and appeared to have seasonal variability. Cloning and sequence comparison of *amoA* gene fragments demonstrated that most of the AOB in the aerated-anoxic Orbal process belonged to the *Nitrosospira sp.* and *Nitrosomonas oligotropha* lineages. The abundance of *Nitrosospira*-like organisms in aerated-anoxic reactors is significant, since this group of AOB has not been usually associated with nitrification in wastewater treatment plants.

KEYWORDS

Aerated-Anoxic, Ammonia-Oxidizing Bacteria, *Nitrosomonas oligotropha*, *Nitrosospira,* Orbal, TRFLP.

INTRODUCTION

Nitrogen removal from wastewater is achieved by a combination of nitrification and denitrification. The nitrification process is catalyzed by aerobic chemoautotrophic ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). Denitrification is mediated by heterotrophic bacteria capable of utilizing nitrite and nitrate as electron acceptors in the absence of dissolved oxygen (DO). The DO concentration is a major environmental factor controlling nitrification and denitrification rates, and therefore, traditional nutrient removal treatment plants include separate anoxic and aerobic zones. Although significant nitrification is not expected at DO below 0.3 mg/L (Stenstrom and Poduska,

1980), treatment processes that promote simultaneous nitrification/denitrification can achieve up to 80% of the total nitrification under conditions involving minimal aeration (aerated-anoxic process) with no detectable DO levels (Albertson and Coughenour, 1995). Nitrification at low DO conditions contradicts the general paradigm that the DO concentration should be maintained above 1.5 mg/L for efficient nitrification (Wanner, 1997).

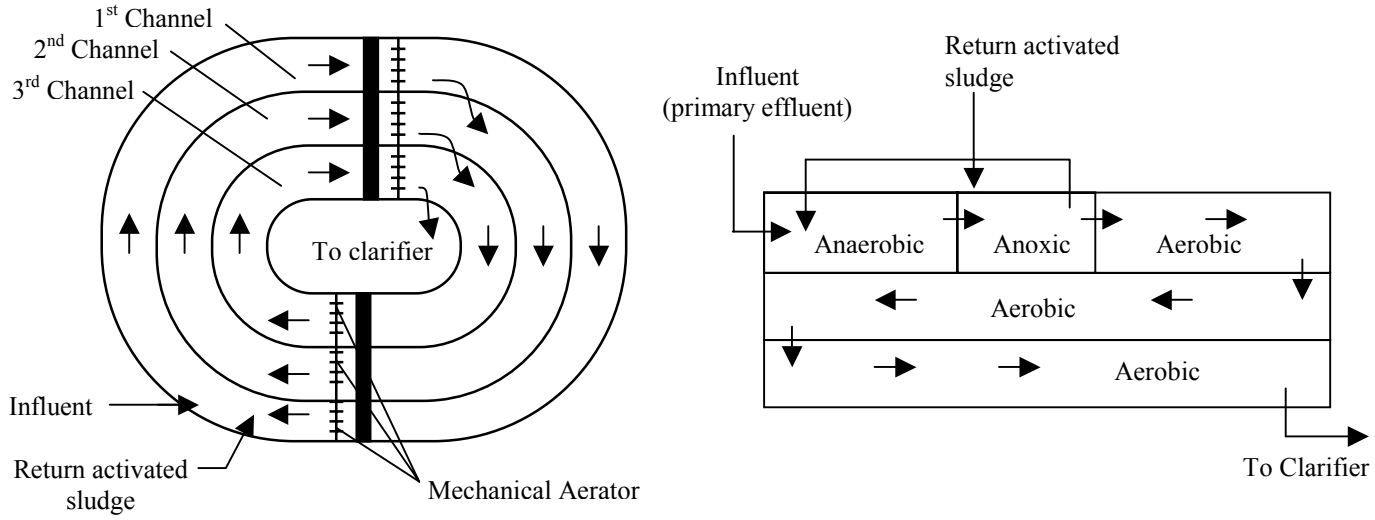
We hypothesize that the nitrification efficiency of aerated-anoxic processes could be due to the presence of AOB adapted to grow at low DO concentrations. This hypothesis is supported by recent observations of AOB with low half-saturation constants for oxygen in low DO natural environments (Bodelier *et al*., 1996). As an initial step for the evaluation of this hypothesis, we used TRFLP (terminal restriction fragment length polymorphism) analyses based on the ammonia monooxygenase A (*amoA*) gene (Horz *et al*., 2000). The analysis was complemented by cloning and sequencing the *amoA* gene fragment to detect and identify specific AOB. Using this approach, the AOB populations in an aerated-anoxic Orbal wastewater treatment plant (WWTP) and a modified University of Cape Town (UCT) process were evaluated and compared. Here we present the results of this comparison and demonstrate that indeed, the two treatment plants have different AOB populations.

MATERIALS AND METHODS

Full-scale wastewater treatment plants. Two full-scale WWTPs were sampled during this study (Figure 1). The Marshall WWTP (Marshall, WI) is an aerated-anoxic Orbal process treating 900 ~ 1,300 m^3/day of domestic wastewater. The biological reactor in this plant consists of three closed loop basins (Figure 1a). Influent wastewater and return activated sludge (RAS) discharge into the outermost channel (1st channel). Water successively flows from the 1st channel to the 2nd and 3rd channels and then to the clarifier. Each looped channel is operated at different DO concentrations, which are controlled by disc type mechanical aerators. The DO in the 1st channel is maintained near 0 mg/L, while the 2nd and 3rd channels are maintained at 0.5 ~ 5.0 mg/L DO. The operation of this plant is also characterized by a long solid retention time (SRT), generally 10 ~ 30 days, and a hydraulic retention time (HRT) of 30 ~ 43 hours. Because of the concentric channel design, 50.4% of the total reactor volume corresponds to the aerated-anoxic zone (1st channel), and 49.6% to the aerated zone (2nd and 3rd channels).

The Nine Springs WWTP (Madison, WI) is a variation of the UCT process and treats 150,000 ~ 200,000 m^3/day of domestic wastewater. Supernatant from the primary settler and mixed liquor from the end of the anoxic basin enter the anaerobic basin (Figure 1b). From the anaerobic zone, the water flows to the anoxic and then to the aerobic zones. Return activated sludge containing nitrate flows directly into the anoxic basin. The Nine Springs WWTP is operated with an SRT of 9 days and an HRT of 8.7 ~ 11 hours. The anaerobic, anoxic, and aerobic zones comprise 16.5%, 5.5%, and 78% of the total reactor volume, respectively. The DO concentration in the anaerobic and anoxic zones is below detection limit, while in the aerobic zone DO is maintained between 0.8 and 4.5 mg/L.

Grab samples of activated sludge were taken at both plants from the end of the aerobic zone. After collection, the samples were placed in a cooler and immediately transported to the laboratory for analysis.



(a) Marshall WWTP (b) Nine Springs WWTP

***Figure 1**. Schematics of Marshall and Nine Spring WWTPs.*

Genomic DNA extraction. A modification of the SDS-based extraction method (Zhou *et al*., 1996) was used to obtain genomic DNA from the activated sludge samples. Briefly, 1.5-mL samples of activated sludge were centrifuged in 1.5-mL Eppendorf tubes at 14,000 rpm for 5 min. The pellet was resuspended with 500 µL TE buffer (10mM Tris, 1mM EDTA, pH 8), 25 µL of lysozyme (50 mg/mL) were added, and the mixture was incubated at 37 °C for 1 hr. Then, 3 µL proteinase K (20 mg/mL) and 30 µL of SDS (20%) were added and the mixture was incubated for another hour at 37 °C with intermittent gentle mixing. After incubation, DNA was extracted following the phenol-chloroform-isoamyl alcohol procedure (Zhou *et al*., 1996).

PCR amplification and TRFLP analysis. Primers *amoA*-1F and *amoA*-2R were used to amplify a 491-bp fragment of the *amoA* gene, according to the protocol described by Horz *et al*. (2000). For TRFLP, *amoA*-1F was 5'-labeled with 6-carboxyfluorescein. PCR products were purified using a PCR product purification kit (Qiagen, Germany) and digested with *TaqI* restriction endonuclease. Aliquots (2µL) of digested PCR products were mixed with 1 µL of an internal length standard (GeneScan 500, TAMRA labeled) and 20 µL formamide. Samples were denatured at 95°C for 5 minutes and analyzed by capillary electrophoresis using an ABI Prism 310 Genetic Analyzer (Perkin Elmer), and the electropherograms were analyzed by GeneScan software (Perkin Elmer).

Cloning and sequencing. PCR products of unlabeled *amoA* gene fragments were ligated to the pGEM®-T vector system and transformed into JM109 *Escherichia coli* competent cells following the manufacture's protocol (Promega). Plasmids of clones were extracted by Wizard® *Plus* minipreps DNA purification system (Promega). DNA sequencing reactions were performed using ABI prism BigDye™ terminators (Applied Biosystems) and sequencing was performed at the University of Wisconsin Biotechnology Center. ClustalX (http://www-igbmc.u-strasbg.fr/BioInfo/) was used to align cloned and published *amoA* sequences. TreeView (http://www.zoology.gla.ac.uk/rod/rod.html) was used to draw phylogenetic trees.

Other analytical methods. Total Kjeldahl nitrogen (TKN), ammonia (NH_4^+-N), and nitrite/nitrate nitrogen (NO_2^--N, NO_3^--N) were measured according to standard procedures (Standard Methods, 1995).

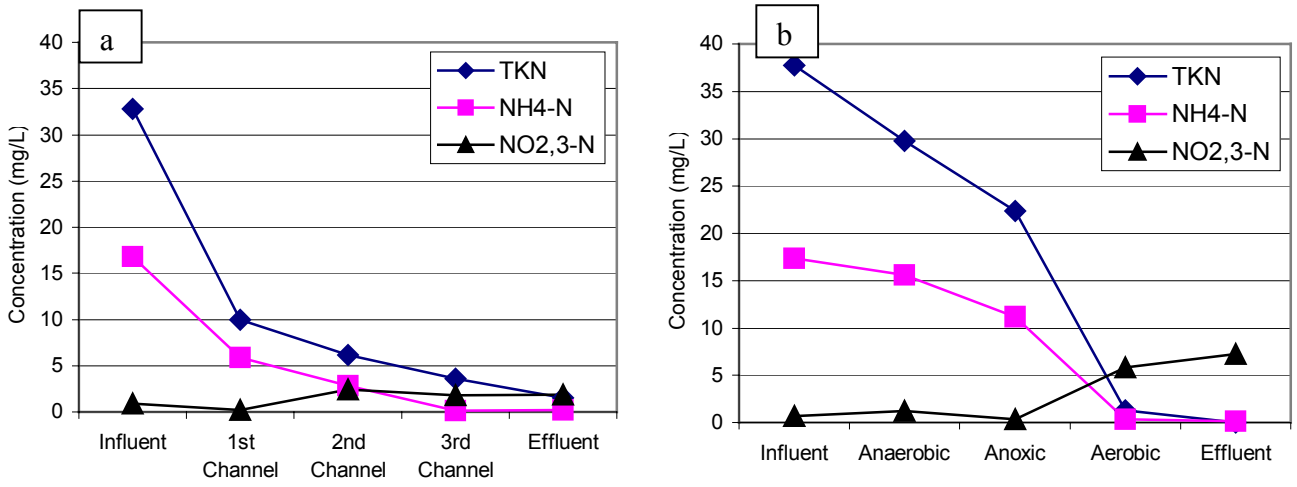


Figure 2. *Nitrogen profiles at (a) Marshall WWTP (aerated-anoxic Orbal process) and (b) Nine Springs WWTP (modified UCT process)*

RESULTS AND DISCUSSION

Nitrogen removal characteristics. The nitrogen removal characteristics of the Marshall and Nine Springs WWTPs were evaluated by measuring TKN, NH_4^+-N, and NO_2^--N/NO_3^--N (Figure 2) and performing nitrogen mass balances throughout the biological reactors (Figure 3). At Marshall, about 10.7 kgN/day of TKN were removed in the aerated-anoxic zone. This loss was attributed to nitrification under low DO conditions (10.1 kgN/day) and assimilation into cell mass (0.6 kgN/day). Significant denitrification also occurred in this reactor (14.0 kgN/day) as the concentration of nitrite/nitrate nitrogen was maintained below 0.2 mg/L. Anaerobic ammonia utilization by autotrophic denitrification (Kuai and Verstraete, 1998) was not included in the mass balance, as Littleton *et al.* (2000) demonstrated that this nitrogen removal mechanism was insignificant in Orbal WWTPs. In the 2nd and 3rd channels (aerobic zone), nitrification and assimilation into cell mass (mostly from heterotrophic growth) were assumed to be the main processes utilizing the remaining TKN. We estimated that out of 29.6 kgN/day of TKN entering the aerobic zone, about 12.1 kgN/day were nitrified, 5.4 kgN/day left the system through the treated effluent, and 6.8 kgN/day were assimilated into cell mass. About 6.7 kgN/day were unaccounted by the mechanisms included in this mass balance.

A similar mass balance analysis for Nine Springs WWTP (Figure 3) indicated that about 1200 kgN/day underwent two-step nitrification denitrification, 1800 kgN/day were assimilated into cell mass, and 1600 kgN/day left the system as effluent nitrate. About 2800 kgN/day were unaccounted by the nitrogen transformation processes included in the mass balance. On a percentage basis, two-step nitrification denitrification was similar on both plants, representing 10 ~ 14% of the total nitrogen removal. The main difference between the two plants was the TKN loss in the anoxic zones. While 30% of the nitrogen removal at Marshall occurred by simultaneous nitrification/denitrification in the

aerated-anoxic stage, TKN losses in the anaerobic and anoxic zones at Nine Springs were less than 3% of the total nitrogen.

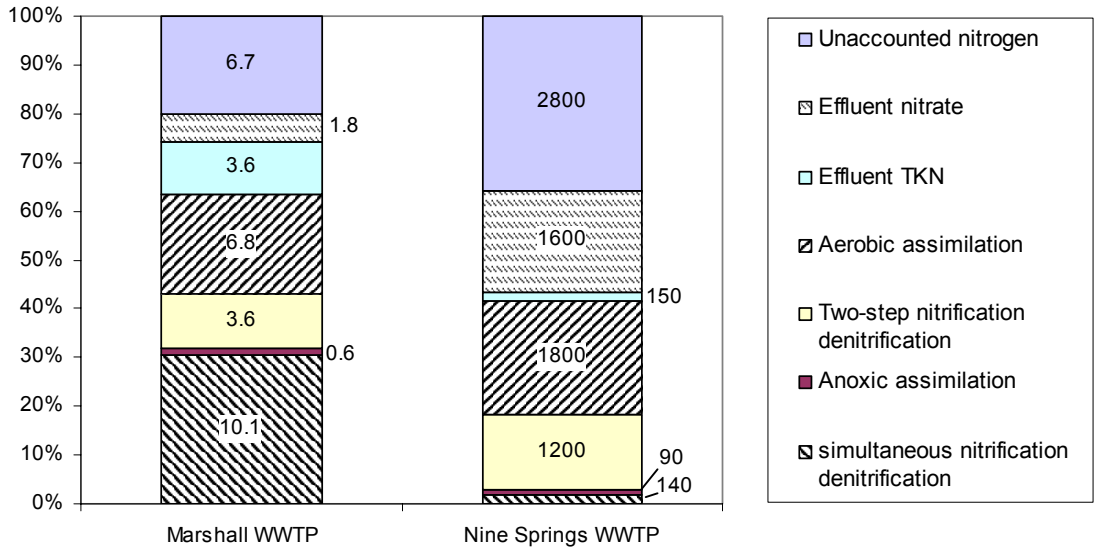


Figure 3. *Nitrogen mass balances at Marshall and Nine Springs WWTPs. Data labels indicate kgN/day.*

TRFLP analyses of AOB. AOB populations at the Marshall and Nine Springs WWTPs were evaluated using *amoA*-based TRFLP analyses based on the procedure of Horz *et al.* (2000). The expected terminal fragments and the corresponding AOB phylogenetic groups (Horz *et al.*, 2000; Purkhold *et al.*, 2000) are shown in Table 1. Although valid for the majority of the sequences available, Purkhold *et al.* (2000) recently reported on AOB clones that did not follow these group assignments.

Table 1. AOB groups and their corresponding *TaqI* terminal fragments

AOB group*	Terminal fragment size (bp)
Nitrosospira lineage	283
Nitrosomonas europaea lineage	219
Nitrosomonas oligotropha lineage	48, 354, 441, 491
Nitrosomonas cryotolerans lineage	491
Nitrosomonas marina lineage	48, 491
Nitrosomonas communis lineage	48, 354, 491
Others	48, 219

* See phylogenetic tree in Figure 5 for AOB groups

The *amoA*-based analysis of the AOB populations at Marshall and Nine Springs are presented in Figure 4, for a sampling period of six months. For all the analyses, the expected 48 bp terminal fragment could not be unequivocally detected, due to high background noise for fragments smaller

than 100 bp. Nevertheless, the TRFLP profiles from Marshall consistently contained terminal fragments at 219 bp, 283 bp, 354 bp, and 491 bp, while the samples from Nine Springs showed only two well-defined peaks at 219 bp and 354 bp. These results suggest that the AOB population at Nine Springs contains organisms related to the *Nitrosomonas europaea* lineage (219 bp peak) and the *N. oligotropha* or *N. cummunis* lineages (354 bp), while the Marshall WWTP has a more diverse AOB community, possibly encompassing all the phylogenetically distinct AOB groups shown in Table 1.

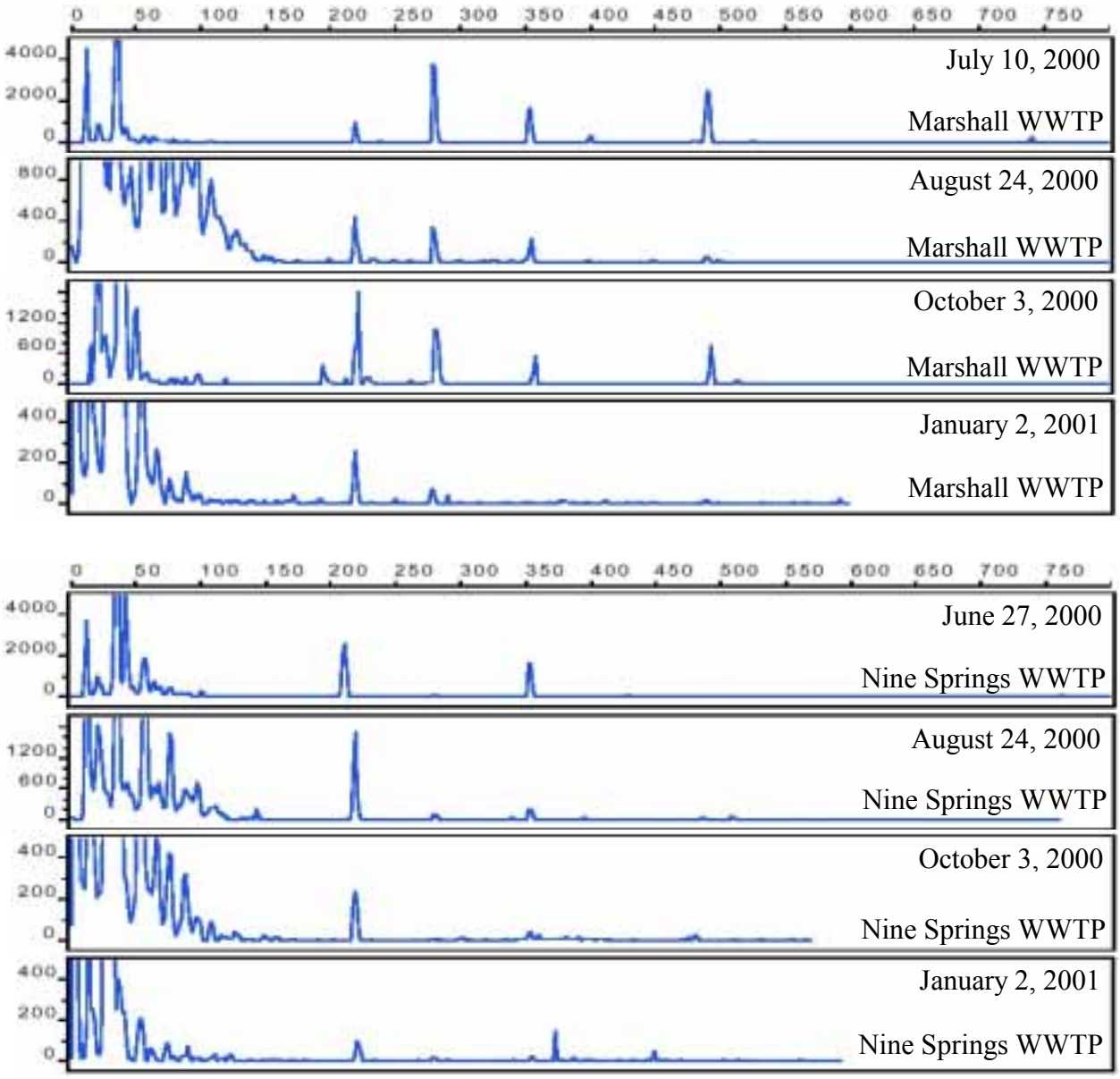


Figure 4. *amoA-based TRFLP profiles from Marshall and Nine Springs WWTPs. The x-axes indicate 5'-terminal fragment size in base pairs and the y-axes shows fluorescent intensity.*

Notably, the terminal fragment at 283 bp, which unequivocally identifies the *Nitrosospira* lineage, is significant in most of the TRFLP profiles from Marshall. AOB belonging to the *Nitrosospira* cluster appear to be ubiquitous in natural environments, especially in soil (Bothe *et al*., 2000), sediments

(Kowalchuk *et al*., 1998), rice paddies (Horz *et al*. 2000), and freshwater (Hastings *et al.*, 1998), but are not commonly reported as relevant AOB in activated sludge (Wagner *et al*., 1995, Wagner *et al*., 1996, Juretschko *et al*., 1998, Purkhold *et al*., 2000). Interestingly, environments such as rice paddies, soil, and sediments typically have low DO concentrations, and it is therefore possible that AOB belonging to the *Nitrosospira* lineage are better adapted to low DO habitats. This hypothesis would also explain their significant presence in the aerated-anoxic Orbal process studied.

Another characteristic of the TRFLP profiles from Marshall is the apparent seasonal variation in the relative intensity of the different terminal fragments. The samples taken in summer show that *Nitrosospira* relatives (283 bp) were a significant fraction of the population while *Nitrosomonas europaea* relatives (219 bp) were less important. This is in contrast to the winter samples that showed a decrease in the *Nitrosospira* population and a relative increase in the *N. europaea*-related AOB. These results suggest that *Nitrosospira* relatives may be susceptible to low temperature conditions. These observations can only be qualitatively considered, since quantification of the relative importance of the different AOB groups was not possible because of the inability to detect the expected 48 bp fragment. In addition, it is likely that population quantifications based on TRFLP analyses would have similar problems as PCR based quantifications (Von Wintzingerode *et al*., 1997).

Cloning of amoA gene fragments. To further investigate the AOB community structure in the two WWTPs, the *amoA* gene fragment flanked by the *amoA*-1F and *amoA*-2R primers was cloned and sequenced. A total of 60 clones from Marshall and 71 clones from Nine Springs were retrieved. The clone distribution according to their expected terminal fragment is summarized in Table 2. This clone distribution matched well the TRFLP results (Figure 4), although with some differences in relative ratios. In addition, the presence of AOB clones with a 48 bp terminal fragment was confirmed in both treatment plants although at low frequency.

The *amoA* sequences obtained from Marshall and Nine Springs were aligned with previously published sequences (Horz *et al.*, 2000, Purkhold *et al*., 2000) using the neighbor-joining method (Figure 5). As shown in Figure 5, most of the clones from Marshall corresponded to the *Nitrosomonas oligotropha* and the *Nitrosospira* lineages while the majority of clones from Nine Springs were related to the *Nitrosomonas europaea* cluster and a few of them were distributed among different AOB clusters. These results validated the TRFLP analyses and supported the hypothesis that the different operational conditions used in the aerated-anoxic Orbal plant at Marshall and the UCT process at Nine Springs strongly affected the AOB population structure. The low DO environment and the creation of appropriate conditions for simultaneous nitrification/denitrification in the aerated-anoxic Orbal process may be critical factors for the involvement of *Nitrosospira* relatives in wastewater treatment.

Table 2. *TaqI* terminal fragments distribution in the clone library

Sample name	Number of terminal fragment (clones)							
	48 bp	219 bp	283 bp	354 bp	441 bp	491 bp	Unknown	Total
Marshall	1	1	5	18	0	20	15	60
Nine Springs	2	37	2	17	1	4	8	71

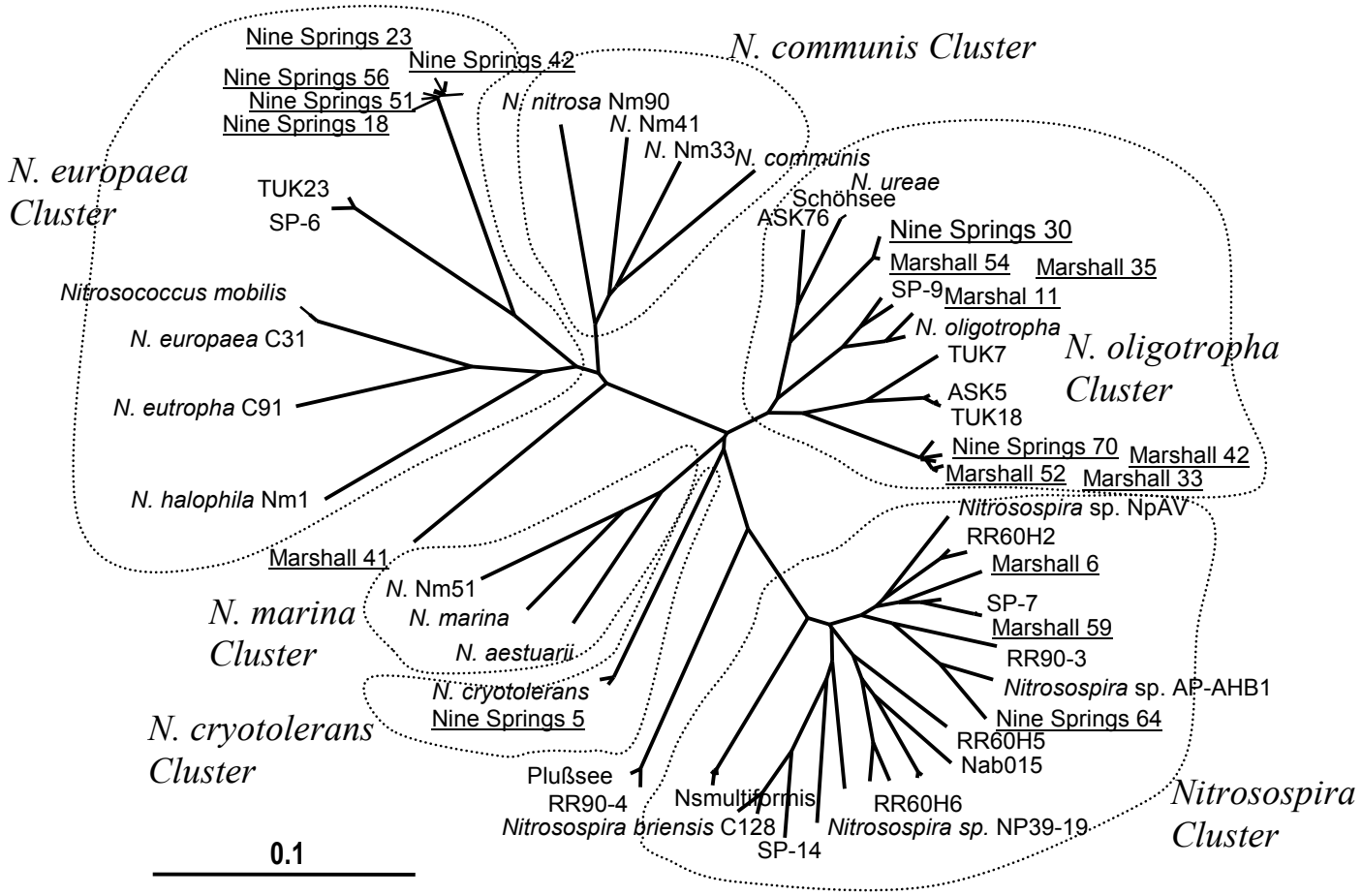


Figure 5. *AOB phylogeny based on amoA gene fragments. Clones from Marshall and Nine Springs are underlined. The scale line indicates 10% difference in nucleotide sequences.*

SUMMARY AND CONCLUSIONS

- In the aerated-anoxic Orbal WWTP at Marshall, about 30% of the TKN is removed via simultaneous nitrification/denitrification in the low-DO aerated-anoxic stage.
- The efficiency of the Marshall WWTP to nitrify at low DO conditions was correlated with the presence of a significant fraction of AOB belonging to the *Nitrosospira* and *Nitrosomonas oligotropha* lineages.
- Simultaneous nitrification/denitrification in the anoxic stage was not a significant process at the Nine Springs WWTP. Consistent with this finding, *Nitrosospira* relatives were not significantly abundant in this reactor.
- The Marshall WWTP appeared to have a greater diversity of AOB than the Nine Springs WWTP. This diversity was apparently affected by temperature.

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