Ammonia oxidizing bacterial community composition and process performance in wastewater treatment plants under low temperature conditions

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ABSTRACT

Nitrification can be difficult to maintain at wastewater treatment plants (WWTPs) during cold periods resulting in disrupted nitrogen removal. The aim of this study was to relate nitrification process performance to abundance and composition of the ammonia oxidizer communities in two closely located municipal WWTPs in Sweden during an eight month period covering seasonal changes and low temperature conditions. Both facilities showed lower NH_4^+-N removal efficiency and nitrification rates as temperature decreased. However, one of the plants had a more stable nitrification rate and higher ammonia removal efficiency throughout the entire period. The differences in performance was related to a shift in the composition of the bacterial ammonia oxidizing community from a *Nitrosomonas oligotropha*-dominated community to a mixed community including also *Nitrosomonas ureae*-like ammonia oxidizers. This was likely a response to differences in NH_4^+-N and organic loading.

Key words | activated sludge, ammonia-oxidizing bacteria and archaea, ammonium removal, low temperature, nitrification

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INTRODUCTION

Nitrification coupled with denitrification is a microbial process used to remove excess nitrogen from wastewater to prevent eutrophication and toxicity in the recipients. It is a two-step process, in which ammonia (NH_3) is first oxidized aerobically to nitrite (NO_2^-) by chemolithoautotrophic ammonia-oxidizing bacteria (AOB). Nitrite is subsequently converted by nitrite-oxidizing bacteria (NOB) to nitrate (NO_3^-) that is readily reduced to nitrogen gas by the denitrifying bacteria. Recent studies have demonstrated that members of the Crenarchaeota are also capable of ammonia oxidation (Könneke *et al.* 2005; Leininger *et al.* 2006a, b), but the contribution of ammonia oxidizing archaea (AOA) to ammonia oxidation in municipal wastewater treatment plants (WWTPs) seems limited (Wells *et al.* 2009).

Low temperature slows down or inhibits the growth and functioning of the ammonia oxidizers, which can result in nitrification process failure (Eighmy & Bishop 1989). Wastewater treatment facilities situated in the Nordic countries face severe problems to maintain nitrification during the cold season or snow and ice melting periods when the temperature of the wastewater may drop below 10 °C. Different populations of the AOB community normally co-exist in WWTPs, but changes in temperature can alter the composition of the AOB community (Siripong & Rittmann 2007). Moreover, temperature has been reported as the most significant factor affecting the AOB community structure when compared with other environmental variables (Park *et al.* 2009). In addition to the influence of temperature, the growth rate and activity of AOB can be negatively affected by other environmental factors and process parameters such as decreased pH, low dissolved oxygen (DO) concentration, toxic compounds, sludge retention time (SRT) and high organic load (Prosser 1989; Wagner *et al.* 1995; Hallin *et al.* 2005). Furthermore, changes in the AOB community diversity have been related to SRT (Yu *et al.* 2010), concentration of NH_3 (Limpiyakorn *et al.* 2007; Lydmark *et al.* 2007), and salt concentration (Park *et al.* 2009). The AOB community composition has been related

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to nitrification efficiency (Layton *et al.* 2005; Zhang *et al.* 2009) although changes in the AOB community composition occur even when nitrification is stable (favorable process performance), as reported by Layton *et al.* (2005).

Studying community composition in well-functioning nitrogen removal activated sludge processes at low temperatures could provide clues for how to prevent nitrification failure. Therefore, the aim of the present work was to compare two full-scale municipal WWTPs with activated sludge processes for which differences in ammonium removal efficiencies and nitrification rates have been observed during winter time. The two facilities are closely located and have similar process configurations. The hypothesis was that differences in the community composition of the ammonia oxidizers could explain differences in performance between the two WWTPs. The process performance was monitored over an eight-month period starting in September and ending in May. On two occasions within this time period, the abundance and community composition were determined based on the signature gene *amoA* encoding the catalytic subunit A of the ammonia monooxygenase, which catalyzes the first step in ammonia oxidation.

MATERIALS AND METHODS

Full-scale activated sludge processes

Two closely located full-scale WWTPs in Sweden were selected for this study. The WWTP in Västerås currently treats 125,000 person equivalents (p.e.). The biological treatment system consists of a modified contact stabilization activated sludge process with pre-denitrification. Periodically, an industrial effluent characterized by high ammonium as nitrogen (NH_4^+-N) concentration is discharged into this facility. Methanol and/or glycol are added to the activated sludge to enhance denitrification. The WWTP in Eskilstuna has a working load of 75,500 p.e. The biological step consists of a fully aerobic activated sludge process with pre-denitrification. In this plant, further denitrification is achieved in a constructed wetland located after the secondary sedimentation system.

Processes performance

Data on wastewater temperature, DO, flow rates, suspended solids, mixed liquor suspended solids and influent NH_4^+-N, as well as effluent NO_2^--N and NO_3^--N were obtained from the on-line measurements from each WWTP during eight months, including a period of three months before and after sample collection for AOB community analysis, in order to compare the process characteristics at both facilities. The influent wastewater biological oxygen demand (BOD) was analyzed according to Swedish standard methods (SS-EN 1899 1998). Additionally, key parameters indicating process performance, such as nitrification rates and NH_4^+-N removal efficiencies were calculated.

Sampling for AOB community analysis and DNA extraction

Activated sludge samples were collected from the aerated activated sludge basins in Västerås and Eskilstuna WWTPs on the 16th of December 2008 and the 26th of February 2009. No more than 1 hour was spent from the sampling to arrival at the laboratory. 500-μL portions of activated sludge sample were centrifuged at 7,500 r.p.m for 10 min. After discarding the supernatant, the pellet was stored at −20 °C until DNA extraction was performed. Genomic DNA was extracted from 500 μL of fresh activated sludge samples using DNeasy® Blood and Tissue Kit (QIAGEN Nordic, Solna, Sweden) following the manufacturer's protocol for Gram-positive bacteria.

Real-time PCR quantification of AOB and AOA amoA genes

Real-time quantitative PCR of the amoA genes was performed in duplicate to estimate the abundance of the ammonia oxidizing bacterial and archaeal communities. The primers amoA-1F (5′-GGGGTTTCTACTGGTGGT-3′) and amoA-2R (5′-CCCCTCKGSAAAGCCTTCTTC-3′) were used for bacteria generating a 491-bp fragment (Rotthauwe *et al.* 1997); and CrenamoA23f (5′-ATGGTCTGGCTWAGACG-3′) and CrenamoA616r (5′-GCCATCCATCTGTATGTCCA-3′) were used for archaea generating a 628-bp fragment (Tourna *et al.* 2008). Quantification was based on the fluorescence intensity of the SYBR Green dye in a total volume of 20 μL using Absolute QPCR SYBR Green Rox (ABgene, Courtaboeuf, France), 1 μmol/L of each primer, 10 ng of soil DNA and 0.5 μg T4Gp32 (QBiogene, Illkrich, France), and reactions were performed in two independent PCR reactions in an ABI7900HT thermal cycler (Applied Biosystems, Carlsbad, CA, USA). All reactions were finished with a melting curve starting at 80 °C with an increase of 0.5 °C up to 95 °C. The PCR efficiency ranged between 83 and 100%. Standard curves were obtained using serial dilutions of linearized plasmids (pGEM-T) containing cloned *amoA* genes.

PCR amplification, cloning and sequencing of AOB

The primers amoA-1F and amoA-2R (Rotthauwe *et al.* 1997) were used to amplify a 491 bp fragment of the *amoA* gene using a T3 thermocycler (Biometra Gmbh, Göttingen, Germany). A mixture containing 0.4–0.5 μmol/L of each primer, 75 μmol/L of each dNTP, 4 mmol/L $MgCl_2$, 10% GeneAmp 10× PCR Buffer II (Applied Biosystems), 0.5 U *Taq* DNA polymerase (QIAGEN Nordic, Solna, Sweden) and 20–50 ng template DNA was prepared in 25 μL volume reaction. The PCR thermal program was structured as follows: 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 46 °C for 30 s and 72 °C for 45 s, and a final elongation step of 10 min at 72 °C.

One clone library per sample was generated and analyzed. Gel electrophoresis was performed using 150 μg of each PCR product. Purification of the PCR products before cloning was carried out through the PCR-M™ Clean Up System (Viogene, Sunnyvale, CA, USA) following the manufacturer's protocol. The TOPO TA Cloning® Kit for sequencing (Invitrogen, Carlsbad, CA, USA) was utilized for the chemical transformation of the pCR®2.1-TOPO® Vector into One Shot® TOP 10F' (Invitrogen) competent *Escherichia coli* cells according to the manufacturer's protocol. Cells were grown overnight on Luria-Bertani solid medium plates with 75 μg mL^{-1} ampicillin. Per sample, 20 transformed cells were picked and analyzed by PCR for correct insert size using the M13 reverse and M13 forward primers. The PCR was performed using Illustra™PuReTaq™ Ready-To-Go™ PCR beads (GE Healthcare UK Ltd) and run with 29 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 45 s preceded by 94 °C for 10 min and a final elongation step of 10 min at 72 °C.

All 80 screened clones had the correct insert size and were sequenced at KIGene genetic analysis facility (Center for Molecular Medicine, CMM) at the Karolinska University Hospital (Solna, Sweden) using an ABI 3730 sequencer (AME Bioscience, Toroed, Norway). The 25 μL sequencing reaction consisted of 2 μL BigDye v.3.1 (Applied Biosystems), 3 μL of 5 × dilution buffer (Applied Biosystems), 1 μL M13 forward primer at 5 pmol concentration and 15 μL of 50 times diluted PCR product. The PCR-sequencing thermal program was run with 21 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min, after one cycle of 96 °C for 2 min as an initial denaturation step. The DNA was ethanol precipitated and the 96-well plates were sealed, vortexed and placed on ice for 15 min. After several centrifugation steps to remove excess reaction the plates were left in the dark to air-dry. The pellets were dissolved in 20 μL milliQ water and 11 μL of was used for sequencing.

Phylogenetic analysis

All 80 nucleotide sequences were assigned as *amoA* genes when compared with *amoA* sequences from the GenBank (NCBI) database using the BLASTn software (www.ncbi.nlm.nih.gov). In total, 57 *amoA* gene sequences of good quality were retrieved and included in the phylogenetic analysis together with the most similar sequences from GenBank. The sequences were trimmed to 451 bp by excluding the primer sites and aligned using MUSCLE (Edgar 2004). Phylogenetic trees were generated based on maximum likelihood analysis using the RAxML software version 7.2.3 (Stamatakis *et al.* 2008) on the CIPRES computer cluster from the online servers at the San Diego Supercomputing Center (www.phylo.org). The *amoA* gene sequence from *Nitrosospira multiformis* was used as outgroup. Branch support was determined using the fast bootstrapping option in RAxML with 1,000 bootstrap replicates.

Accession numbers for nucleotide sequences

The partial *amoA* gene sequences obtained were deposited in the GenBank nucleotide sequence database under accession numbers HQ425721–HQ425777.

RESULTS AND DISCUSSION

Temperature, ammonia removal and nitrification performance

The temperature decreased from September to March and increased from March to May at both WWTPs (Figure 1(a)). Nevertheless, the temperature was on average 3 °C lower at the Västerås plant during the experimental period (Figure 1(a)). This difference in temperature was not expected due to the geographic proximity of both WWTPs, but the explanation could be that the volume of storm water processed at Västerås was higher than at Eskilstuna, resulting in colder wastewater. The lower wastewater temperature recorded at Västerås did not influence the ammonium removal efficiency negatively in comparison with the Eskilstuna plant during the cold season. Instead, the removal of NH_4^+-N in Västerås was stable around 90% with low effluent NH_4^+-N concentrations, while Eskilstuna displayed a continuous decrease from 91 to 55% from September to February with increasing effluent NH_4^+-N concentrations (Figure 1(b) and (c)). During

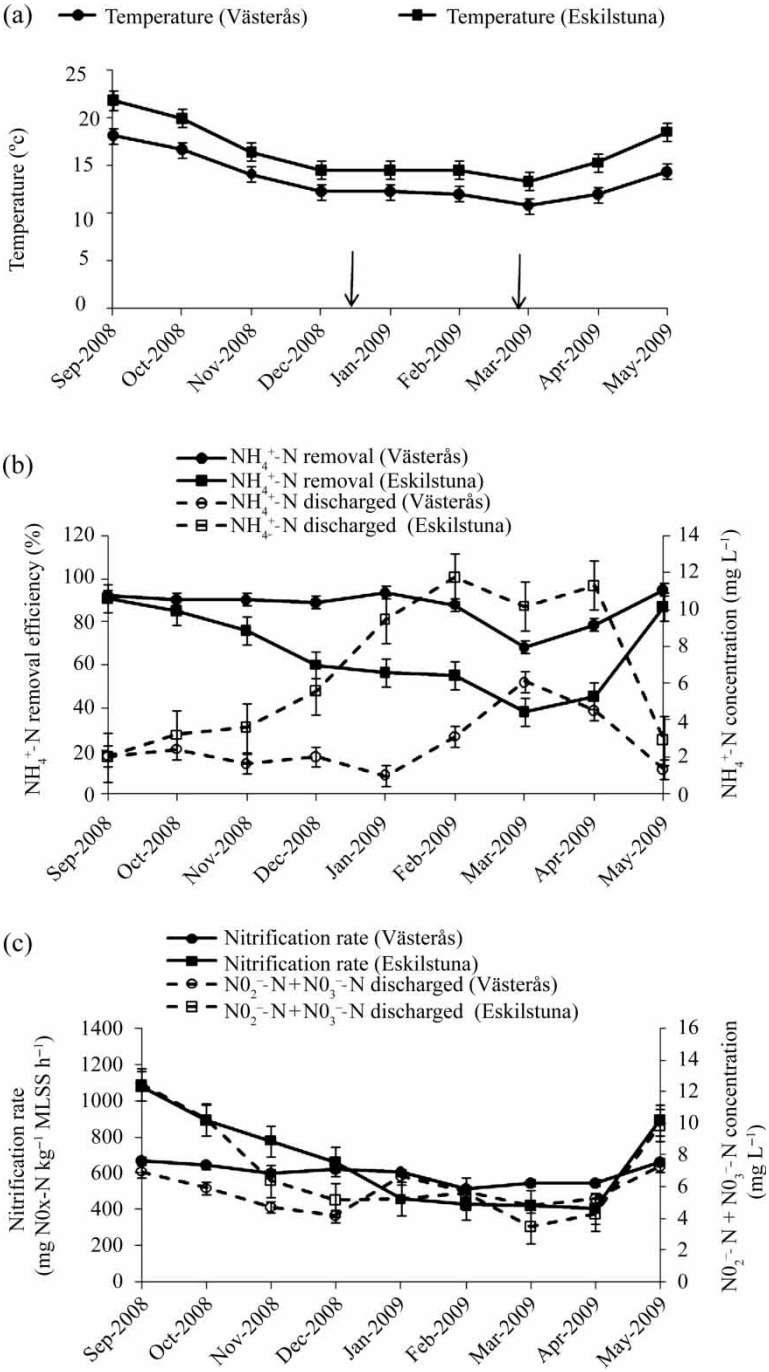

Figure 1 | (a) Wastewater temperature (arrows represent the sampling dates for the analyses of abundance and composition of ammonia oxidizing communities) and activated sludge processes performance in terms of (b) NH_4^+-N removal efficiency and effluent NH_4^+-N concentrations and (c) nitrification rates and effluent NO_2^--N + NO_3^--N concentrations monitored at Västerås and Eskilstuna WWTPs over eight months.

the warm periods in September and May, the ammonium removal efficiencies were similar with low effluent NH_4^+-N concentrations at both Västerås and Eskilstuna. As expected, the highest nitrification rates in both WWTPs were reached during the warmest periods and vice versa, although Västerås showed higher nitrification rates than Eskilstuna (Figure 1(c)). Similar to the NH_4^+-N removal, the nitrification rates at Eskilstuna decreased from September to April, whereas the nitrification rates at Västerås were stable during the entire period. The nitrification rates by and large mirrored the

effluent NO_3^--N and NO_2^--N concentrations at both WWTPs (Figure 1(c)).

Ammonia oxidizing communities

The AOB were more abundant than the AOA at Västerås and Eskilstuna WWTPs on both sampling occasions (Table 1). A lower relative abundance of AOA compared with AOB in municipal WWTPs has previously been demonstrated (Wells *et al.* 2009), which fits with other studies in which the AOB have been proposed to be functionally more important in nitrogen rich environments as compared with the AOA (Di *et al.* 2009; Jia & Conrad 2009; Zhang *et al.* 2010). In agreement, although AOA are the predominating terrestrial ammonia oxidizers, AOB were shown to be more abundant than AOA in a sewage influenced marsh (Höfferle *et al.* 2010). Altogether, this is also supported by recent works reporting that AOA are adapted to low nutrient conditions (Erguder *et al.* 2009; Martens-Habbena *et al.* 2009).

As the activated sludge basins under study were dominated by AOB rather than AOA, the analysis of the ammonia oxidizing community composition was only considering the bacterial counterpart. The majorities of AOB clones from Västerås and Eskilstuna were similar to *Nitrosomonas oligotropha*-related bacteria (Figure 2). Small differences between the plants were observed as indicated by the Västerås and Eskilstuna sub-clusters. There were no differences in the AOB community at Eskilstuna between the two sampling occasions. However, at Västerås WWTP some clones were found in the *N. marina*/*N. ureae* cluster in February while *N. oligotropha*-related clones were still dominating. These results suggest a seasonal shift in the ammonia oxidizing community at Västerås between December and February resulting in increased diversity. Park *et al.* (2009) highlighted the relevance of temperature in terms of AOB community diversity and variations in the AOB community composition were reported as one of the reasons for nitrification failure (Park *et al.* 2008). However, at Västerås the nitrification rates remained constant even though the AOB community changed. This is in accordance with the results reported by Layton *et al.* (2005) showing that changes in AOB community composition can occur even when nitrification rates remain the same. The opposite, with stable AOB communities and changing nitrification rates, has also been observed (Hallin *et al.* 2005). Nevertheless, the higher ammonium removal efficiency combined with stable nitrification rates at Västerås as compared with Eskilstuna WWTP under low temperature could be related to the AOB community composition that changed from only comprising the *N. oligotropha*-related AOB to also include those belonging to the *N. marina*/*N. ureae* cluster in February. Accordingly, increased diversity has been shown to be an important factor in terms of microbial process stability and efficiency (Daims *et al.* 2001; Bell *et al.* 2005; Akarsubasi *et al.* 2009; Wittebolle *et al.* 2009).

Ammonium and organic content can affect the composition of the AOB community in activated sludge (Bollmann & Laanbroek 2001; Okabe *et al.* 2005) and both these factors differed over time and between plants in the present study. Average influent NH_4^+-N loads during the experimental period were similar at Västerås and Eskilstuna receiving 1,170 and 1,000 kg day^{-1}, respectively. However, the influent NH_4^+-N load fluctuated in Västerås during December and February with influent values ranging from 780 to 1,440 kg day^{-1}. By contrast, the NH_4^+-N load remained constant at around 1,000 kg day^{-1} at Eskilstuna WWTP. Several studies have shown the presence of *N. oligotropha* in WWTPs with low influent NH_4^+-N concentrations (Bollmann & Laanbroek 2001; Koops & Pommerening-Röser 2001; Limpiyakorn *et al.* 2007), although Wang *et al.* (2010) found *N. oligotropha* at WWTP's with influent NH_4^+-N concentrations close to 40 mg L^{-1}. Isolated cultures of *N. ureae* have been proven more tolerant than *N. oligotropha* to high NH_4^+-N concentrations in a laboratory investigation (Koops & Moeller 1992). Thus, the NH_4^+-N concentration may select for certain ammonia oxidizers, which could be due to different sensitivities against the toxic effects of NH_4^+-N or to the better competitive abilities of some members under certain conditions (Bollmann & Laanbroek 2001). In this study, the influent concentration of NH_4^+-N at Västerås and Eskilstuna was similar when the AOB were studied (data not shown), but it could be hypothesized that the fluctuating NH_4^+-N load at Västerås during the winter period could have resulted in co-occurrence of *N. ureae*-like bacteria with the otherwise dominating *N. oligotropha* population.

Table 1 | Abundance of AOB and archaea (AOA) and the ratio between these groups in Västerås and Eskilstuna WWTPs in December and March

		***amoA* gene abundance (10^3 copies ng DNA^{-1})**		
Month	**WWTP**	**AOB**	**AOA**	**AOB:AOA**
December:	Västerås	1.1	0.10	11.0
	Eskilstuna	1.1	0.16	6.8
February:	Västerås	1.3	0.40	3.3
	Eskilstuna	1.1	0.19	5.8

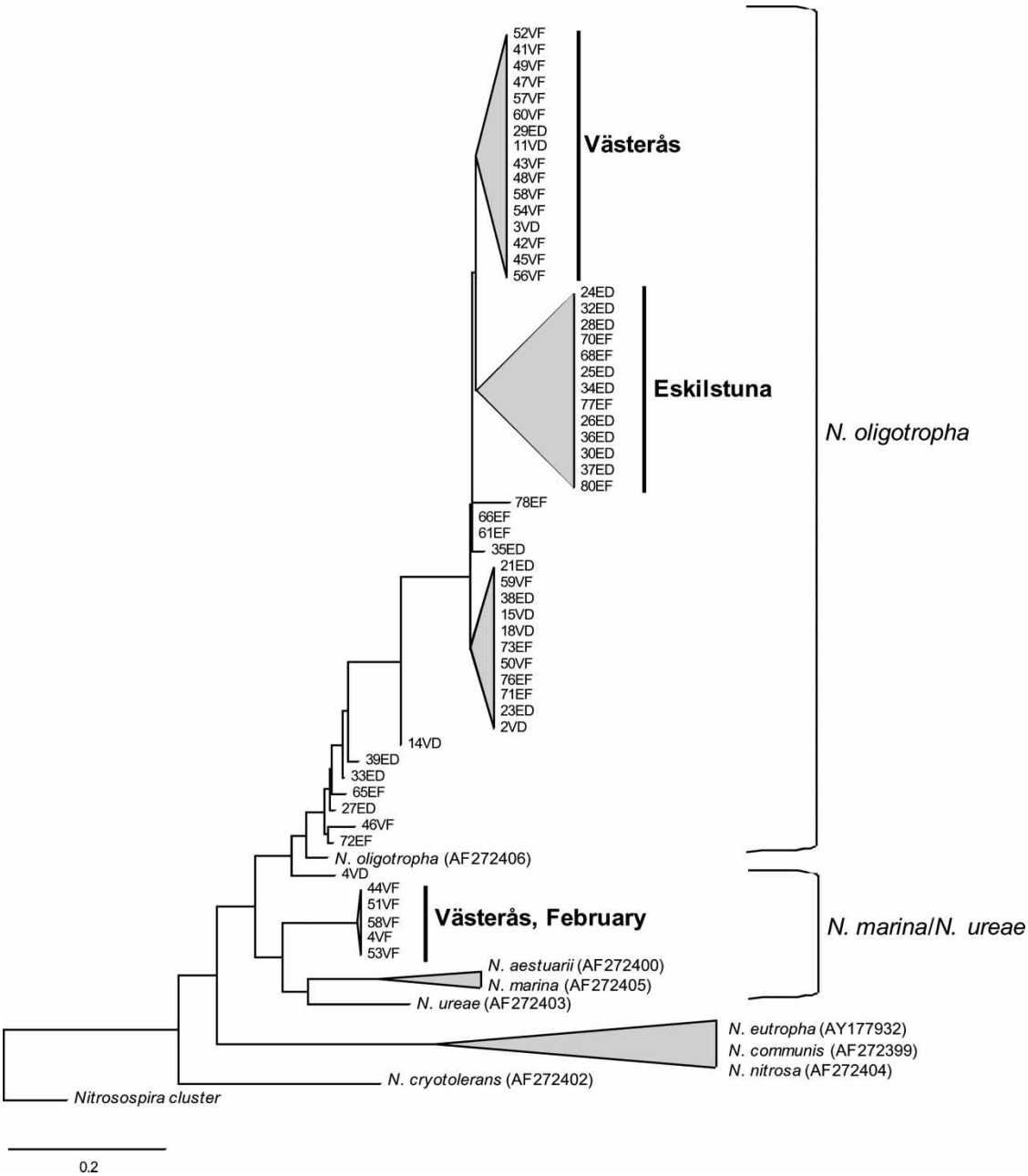

Figure 2 | Maximum likelihood tree inferred from analysis of partial *amoA* sequences from AOB within the genus *Nitrosomonas* recovered from Västerås (V) and Eskilstuna (E) WWTPs in December (D) and February (F). Sequences from pure cultures are included with accession numbers in parenthesis. Bootstrap values higher than 50 are indicated at the nodes (•, n = 1,000).

The organic content of the wastewater at Västerås and Eskilstuna was measured in terms of BOD_7 and ranged between 57 and 119 mg L^{-1} and 18 and 42 mg L^{-1}, respectively. The difference between the two plants was biggest after January, being on average 64 mg L^{-1} higher at Västerås. These differences in BOD_7 were due to additional methanol and glycol periodically used at Västerås to increase denitrification rates, whereas no external carbon sources were added at Eskilstuna WWTP. The source of organic carbon is known to affect the heterotrophic bacterial populations in the activated sludge that co-exist with the AOB and compete for space and oxygen (Nogueira *et al*. 2002). A certain inhibition of the AOB activity under high C:N ratios could therefore be expected (Okabe *et al*. 1996; Michaud *et al*. 2006). However, a few studies have given a different perspective on the ecological relationship between heterotrophic bacteria and the AOB, highlighting the importance of substrate exchange among phylogenetic groups as an

eco-physiological interaction in complex environments (Okabe *et al*. 2005). For example, a recent study suggested a symbiotic relationship between the AOB and heterotrophic bacteria (Racz *et al*. 2010). The influence of two different organic sources (peptone and glucose) on the composition of these communities was demonstrated in a mixed culture in relation to altered nitrification kinetics. An earlier investigation showed that ammonia oxidation increased when *Nitrosomonas* spp. were grown together with heterotrophic bacteria (Jones & Hood 1980). In this context, the addition of external methanol and glycol at Västerås WWTP might have enhanced the interaction between heterotrophic bacteria and the AOB, resulting in different AOB communities and/or higher nitrification rates. However, further research is needed to confirm this hypothesis.

CONCLUSIONS

Västerås WWTP performed better than Eskilstuna in terms of ammonium removal efficiency in addition to maintaining stable nitrification rates under low temperature conditions. Both plants were dominated by *N. oligotropha*-like AOB both in December and February but *N. ureae*-like bacteria were also detected at Västerås WWTP in February. This difference between the two plants could be a response of the AOB community to a combination of factors such as influent NH_4^+-N load and organic carbon. The shift in the AOB community composition likely played a pivotal role for the maintained nitrification activity throughout the winter period. However, further research is needed in order to ascertain causal relationships between process performance and bacterial dynamics in activated sludge systems.

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