



Characterization of a microbial community capable of nitrification at cold temperature

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ABSTRACT

While the oxidation of ammonia is an integral component of advanced aerobic livestock wastewater treatment, the rate of nitrification by ammonia-oxidizing bacteria is drastically reduced at colder temperatures. In this study we report an acclimated lagoon nitrifying sludge that is capable of high rates of nitrification at temperatures from 5 °C (11.2 mg N/g MLVSS/h) to 20 °C (40.4 mg N/g MLVSS/h). The composition of the microbial community present in the nitrifying sludge was investigated by partial 16S rRNA gene sequencing. After DNA extraction and the creation of a plasmid library, 153 partial length 16S rRNA gene clones were sequenced and analyzed phylogenetically. Over 80% of these clones were affiliated with the *Proteobacteria*, and grouped with the β - (114 clones), γ - (7 clones), and α -classes (2 clones). The remaining clones were affiliated with the *Acidobacteria* (1 clone), *Actinobacteria* (8 clones), *Bacteroidetes* (16 clones), and *Verrucomicrobia* (5 clones). The majority of the clones belonged to the genus *Nitrosomonas*, while other clones affiliated with microorganisms previously identified as having floc forming or psychrotolerance characteristics.

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1. Introduction

Liquid manure generated from livestock production is a major contributor to ammonia emissions in rural areas (Arogo et al., 2001; De Visscher et al., 2002). These emissions may produce acidification and eutrophication of coastal waters, lakes, streams, and terrestrial ecosystems, resulting in habitat degradation and a reduction in biodiversity (Vitousek et al., 1997). In municipal and industrial systems, ammonia from wastewater can be removed by a variety of physicochemical and biological processes (Metcalf & Eddy, 2002), but biological processes are preferred because they are usually more cost effective (USEPA, 1993). One such biological process is autotrophic nitrification, a reaction undertaken by ammonia-oxidizing bacteria (AOB) (Sinha and Annachhatre, 2007). It has been previously documented that nitrification by the AOB is dependent upon several environmental factors, the most critical being dissolved oxygen concentrations (Andersson and Rosen, 1990; Sharma and Ahlert, 1977), pH (Andersson and Rosen, 1990; Shammas, 1986), and temperature (Andersson and Rosen, 1990; Sharma and Ahlert, 1977; Wild et al., 1971).

The AOB consist of three genera, the marine-associated *Nitrosococcus* (γ -Proteobacteria), and the terrestrial-associated *Nitrospira* (β -Proteobacteria) and *Nitrosomonas* (β -Proteobacteria) (Head et al., 1993). The genus *Nitrosomonas* is divided into five

lineages: *N. communis*; *N. cryotolerans*; *N. europaea/eutropha*; *N. marina*; and *N. oligotropha* (Koops and Pommerening-Roser, 2001); with *N. europaea/eutropha* lineage isolates being the most commonly isolated lineage from activated sludge (Wagner et al., 2002).

The implementation of ammonia removal technology in livestock effluents is difficult due to low efficiency of nitrification in winter months. The rate of nitrification by AOB is severely affected by temperature (Characklis and Gujer, 1979; Sharma and Ahlert, 1977; Wild et al., 1971). Knowles et al. (1965) demonstrated that AOB in particular are drastically affected by temperature changes, and studies of various waste management systems employing nitrification have reported failure during winter temperatures (Ilies and Mavinic, 2001; Kim et al., 2006). Working with nitrification in activated sludge, Borchardt found an optimized activity between 15 and 35 °C and a sharp drop of nitrification rate at temperatures <15 °C, with 50% reduction at 12 °C and 100% at 5 °C (Borchardt, 1966). Similarly, Randall and Buth (1984) demonstrated that both nitrite and nitrate formation were strongly inhibited by temperatures of 10 °C or less. According to Shammas, high nitrification efficiency can only be obtained with either very long retention time or a combination of high nitrifier concentration and elevated temperature (Shammas, 1986). Increased retention time means larger reactors and capital cost. For wastewater treatment systems in areas which experience colder temperatures (<15 °C), nitrification activity rates can be addressed by inputting heat to the system and maintaining a stable temperature. This

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poses a problem for the low cost treatment of animal waste because the energy input required for heating such systems comes at a large economic expense.

Another option is to use nitrifying sludge systems which perform well under cold temperatures. We have found a microbial community with excellent $\text{NH}_3\text{-N}$ removal performance during cold weather conditions. It was isolated from a nitrifying sludge providing treatment to swine lagoon effluent. A culture of this microbial community, called acclimated lagoon nitrifying sludge (ALNS), was used repeatedly as an inoculum to start-up several nitrification bioreactors for treatment of high-ammonia livestock effluents and land-fill leachate. This ALNS was used with attachment and suspended growth nitrification processes in bench and full-scale studies, always showing rapid bioreactor start-up and excellent $\text{NH}_4\text{-N}$ removal performance during cold weather conditions in winter months (Vanotti and Hunt, 2000; Vanotti et al., 2009).

Although several studies have examined the microbial community composition of activated sludge (Hallin et al., 2005; Juretschko et al., 2002; Niu et al., 2006; Snaidr et al., 1997; Wagner et al., 2002), little information is available on the unique microbial composition of nitrifying sludges that function well at cold temperatures. Therefore, a better understanding of the structure and function of nitrifying sludge communities capable of good performance in cold temperatures may be essential for improving and optimizing wastewater treatment processes.

In this study, we evaluated the nitrification performance and characteristics of ALNS at low process temperatures. Batch nitrification tests were done under controlled laboratory conditions to reveal the relationship between nitrification activity of ALNS and temperature. Furthermore, the microbial community composition of ALNS was characterized by molecular methods.

2. Methods

2.1. Preparation and maintenance of nitrifying culture

Vanotti and Hunt have previously described the procurement of the ALNS culture using seed biofilm sludge from the surface soil of an overland field plot in a pig farm in Duplin County, NC and fill-and-draw cultivation method (Vanotti and Hunt, 2000). The overland flow provided nitrification treatment for a wastewater effluent from an anaerobic swine lagoon (Szogi et al., 2004). The obtained ALNS has been maintained for the past 12 years in an 18 L plastic aeration tank in the ARS laboratory at Florence, SC. The ALNS culture was maintained using inorganic salts medium and a fill-and-draw cultivation method, where the aeration was stopped once a week, the suspensions allowed to settle for 30 min, the 18 L of supernatant withdrawn and replaced with fresh inorganic salts medium, and aeration resumed. The inorganic salt medium used is that of Vanotti and Hunt (Vanotti and Hunt, 2000) modified with the addition of a pH 8.5 $\text{CO}_3^{2-}/\text{HCO}_3^-$ buffer to provide all the alkalinity requirements necessary for complete nitrification of 300 mg/L $\text{NH}_4\text{-N}$ and achieve stable pH. Aeration of the tank was supplied by an aquarium air pump (2000 mL/min) and porous stone that provided fine bubble aeration. Water temperature in the culture tank was routinely kept at 28–30 °C with a submersible heater. Two months prior to starting the cold temperature nitrification batch experiments, the water temperature of the 18 L culture tank was lowered to 10 °C using a water bath and chilling probe to simulate normal winter conditions in North Carolina. The cold temperature nitrification batch experiments were done in 1.2 L suspended biomass reactors (Fig. 1). For these experiments the ALNS in the 18 L culture tank was concentrated by settling to 316 mL containing 7.08 g suspended solids (5.41 g

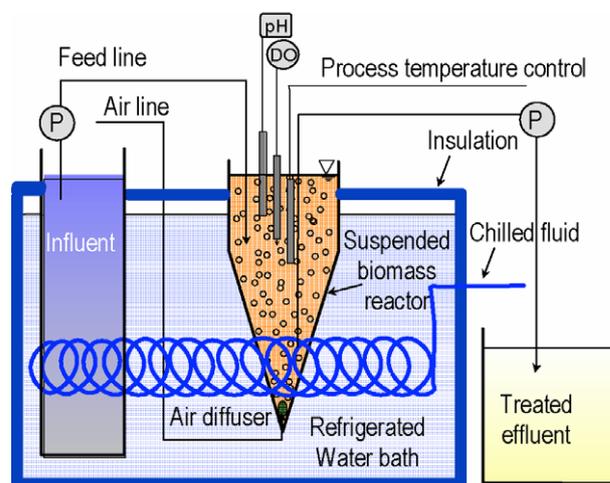


Fig. 1. Schematic diagram of suspended biomass reactor with ALNS used for batch nitrification treatment under cold water temperatures.

volatile solids) and divided into two equal portions that were transferred into the duplicate 1.2 L reactors.

2.2. Nitrification reactor configuration

Suspended biological reactors (1.2 L effective volume) were used for the nitrification batch tests under varied water temperatures (5, 10, 15, and 20 °C). Water temperature inside the reactors was controlled using a submerged temperature probe and a refrigerated circulating bath that pumped chilled car antifreeze liquid through copper coils inside another insulated water bath that contained the reactors. To ensure rapid temperature equilibration from the onset of each test, the influent synthetic wastewater was kept overnight at the same temperature of the batch test. Each batch temperature test started in the morning and lasted 6 h. The influent was of the same chemical composition of the salts medium used for culture maintenance. The air diffuser consisted of an aquarium porous stone that provided fine bubble aeration. Air was supplied at flow rates that varied from 0.6 to 1.0 L/min to ensure dissolved oxygen (DO) concentration in the mixed-liquor >3 mg/L, which is consistent with DO level recommendations for non-limiting nitrification (Metcalf & Eddy, 2002; Sharma and Ahlert, 1977). Average DO concentration obtained at the 5, 10, 15 and 20 °C tests were 13.2, 11.2, 5.8, and 4.3 mg $\text{O}_2\text{/L}$, respectively. The average concentration of mixed liquor suspended solids (MLSS) and volatile suspended solids (MLVSS) were 2115 mg/L \pm 35 and 1615 \pm 60, respectively. All experiments were duplicated.

2.3. Nitrification and oxygen activity tests at various temperatures

The effect of water temperature on nitrification activity of the ALNS was determined in batch experiments in two ways: (1) by the production of oxidized N ($\text{NO}_2\text{-N}$ and $\text{NO}_3\text{-N}$); and (2) by the consumption of oxygen during the oxidation of $\text{NH}_4\text{-N}$. At the beginning of a temperature batch experiment, the ALNS was pre-rinsed with 1 L volume of fresh inorganic medium to remove any residual $\text{NO}_3\text{-N}$ and $\text{NO}_2\text{-N}$ from a previous test. The nitrifying activity of the reactor was calculated from the rate increase of ($\text{NO}_3 + \text{NO}_2$)-N concentration during 6 h of aeration of a fresh inorganic salts medium. Water samples were taken at 0, 1, 2, 3, 4, 5, and 6 h using a 3 mL syringe. The liquid sample was immediately passed through a 0.45 μm glass microfiber filter to remove biomass particulates and transferred into a vial that contained one drop of nitrification inhibitor that used 2-chloro-6-(trichloromethyl)

pyridine (Formula 2533, Hatch Co., Loveland, CO), and kept at 4 °C until chemical analyses the following day.

For each temperature test, we also determined oxygen consumption by the ALNS. The oxygen consumption activity was calculated from the rate of decrease of DO in a closed chamber during a few minutes. This test was done at about 2.5 h into the batch nitrification test in a 300 mL glass BOD bottle. Mixed-liquor was transferred from the reactor into the BOD bottle and sealed with a DO probe (YSI, Model 52, Yellow Spring, OH). The glass bottle was jacketed with water kept at the same temperature of the reactor and contained a magnetic stir bar to suspend the biomass while the oxygen consumption of nitrification was determined. Supplemental aeration was applied for 1 min before closing the bottle to raise the initial DO to approximately 7–9 ppm when process DO in the reactor was lower than this level (15 and 20 °C runs). The decrease in DO concentration was measured with an oxygen meter (YSI, Model 52, Yellow Spring, OH) every 15–60 s for a total of 1.5–5 min until DO concentration reached <2 mg/L. Below this level the rate of O₂ consumption decreased greatly indicating nitrification inhibition and therefore not representative of the nitrification activity in the reactor. The mixed-liquor was returned to the 1.2 L reactors immediately after the O₂ test.

2.4. Water quality analysis

Wastewater analyses were performed according to APHA Standard Methods (APHA, 1998). The ammonium (NH₄-N), NO₃-N, and NO₂-N concentrations were determined in the liquid after filtration through a 0.45 µm glass microfiber syringe filter (GMF, Whatman Inc., Florham Park, NJ). NH₄-N was determined in the filtrate with the automated phenate method (Standard Method 4500-NH₃ G; APHA, 1998). NO₃-N + NO₂-N were determined by the automated cadmium reduction method (Standard Method 4500-NO₃-F; APHA, 1998). NO₂-N alone was determined using the same colorimetric method without the chemical reduction step (Technicon, 1978). Samples used for mixed liquor suspended solids (MLSS) and volatile suspended solids (MLVSS) determinations were collected in 20 mL vials from the fluidized reactors. The residue retained on a glass-fiber filter was dried to constant mass at 105 °C for suspended solids determinations and further ignited to 500 °C for volatile suspended solids (Standard Method 2540 D and E; APHA, 1998). Settling characteristics of the nitrifying sludge were determined with the sludge volume index (SVI), which is the volume in mL occupied by 1 g of a biological suspension after 30 min settling (Standard Method 2710 D; APHA, 1998). Sludge settling and compaction characteristics are rated as “excellent” (SVI < 80 mL/g), “moderate” (SVI 80 – 150 mL/g), or “poor” (SVI > 150 mL/g) (24).

2.5. DNA extraction

Prior to reducing the temperature to 10 °C, microbial cells were extracted from the 18 L culture tank to analyze the ALNS microbial community. To extract genomic DNA, bacterial cells were first exposed to bead mill homogenization. One milliliter of ALNS was centrifuged at 16,000g for 5 min and the pellet resuspended in 480 µL of 50 mM EDTA (pH 8.0). To this suspension 0.1 g of 0.1-mm glass beads (Scientific Industries, Bohemia, NY) was added and the cells homogenized with a MiniBeadbeater-8 (Biospec Products, Bartlesville, OK) using three cycles of homogenization for 60 s interspersed with incubation at 4 °C for 60 s between cycles. After homogenization, the Promega Wizard Genomic DNA Purification protocol was followed according to manufacturer specifications (Promega, Madison, WI). DNA quantity and quality were determined via Biophotometer (Eppendorf, Hamburg, Germany), and

electrophoresis on a 1% agarose gel stained with SYBR Safe (Invitrogen, Carlsbad, CA).

2.6. DNA amplification, construction of 16S rRNA gene libraries, and DNA sequencing

Based on the analysis of Wang et al. for partial 16S rRNA gene classification accuracy using a naïve Bayesian classifier and partial 16S rRNA gene sequences (≤400 bp) (Wang et al., 2007), partial DNA sequence was obtained using the universal primer 515F (TGCCAGCAGCCCGGTAA) and the bacteria-specific primer E939R (CTTGTGCGGGCCCCGTCATTC) (Baker et al., 2003). This primer set spans the variable regions V4 and V5 to amplify a ~420 bp segment of the 16S rRNA gene. The PCR reaction system consisted of 5.0 µL 10× DyNAzyme EXT PCR buffer, 1.0 µL dNTPs (10 mM), 2.0 µL of each primer (2.5 µM), 10 ng DNA template, and 2 U DyNAzyme EXT Taq polymerase (New England Biolabs, Ipswich, MA) in a final volume of 50 µL. PCR was performed under the following conditions: 94 °C/5 min denaturation step; 30 cycles of 94 °C/60 s, 55 °C/60 s, 72 °C/60 s; and a final extension step at 72 °C/10 min. The PCR products were cloned into pCR2.1, and chemically transformed into TOP10 *Escherichia coli* cells using a TOPO TA cloning kit according to manufacturer specifications (Invitrogen, Carlsbad, CA). Cells were blue/white screened on LB agar supplemented with ampicillin (100 µg/mL), and X-gal (100 µg/mL). Colonies were randomly selected and grown overnight in LB broth supplemented with ampicillin (100 µg/mL). Plasmids were then isolated using an alkaline lysis protocol (Sambrook and Russell, 2001), and sent for capillary sequencing on an ABI 3700xl sequencer (Applied Biosystems, Foster City, CA).

2.7. DNA sequence and phylogenetic analysis

A total of 154 cloned 16S rRNA gene sequences were analyzed and edited using Geneious (version 3.6.2, Biomatters Ltd. (Drummond et al., 2007)). One chimeric sequence was identified using the programs CHECK_CHIMERA (Cole et al., 2007) and Bellerophon (Huber et al., 2004), and was subsequently removed from the data set. To determine their approximate taxonomical classifications, the cloned 16S rRNA gene sequences were compared to sequences in the GenBank database by using BLAST (Basic Local Alignment Sequence Tool) (Altschul et al., 1990), and to type strain sequences, when possible, at the Ribosomal Database Project (RDP) using the RDP's naïve Bayesian Classifier (Wang et al., 2007) and Seqmatch (Cole et al., 2007). Sequence alignments were performed using the MUSCLE (Edgar, 2004) plug-in of Geneious and were analyzed using MEGA (version 4.0, (Tamura et al., 2007)). Phylogenetic reconstructions were performed in MEGA using the neighbor-joining (NJ) algorithm, with bootstrap values calculated from 5000 replicate runs. Evolutionary distances were computed using the Tajima-Nei method (Tajima and Nei, 1984), and positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons. Rarefaction curves and 95% confidence limits were calculated using Analytic Rarefaction (version 1.3, (Holland, 2003)). Sequences were grouped as operational taxonomic units (hereafter referred to as phylotypes) by using a 97% sequence similarity threshold. Coverage was calculated according to the formula $C = [1 - (n_1/N)] \times 100\%$, where n_1 represents the number of phylotypes appearing only once in the library, and N represents the total number of clones examined (Good, 1953).

2.8. Nucleotide sequence accession numbers

The 16S rRNA gene partial sequences obtained during this study were deposited in GenBank under the accession numbers FJ201190 through FJ201239.

3. Results and discussion

3.1. Characteristics of ALNS

The ALNS exhibited excellent settling and compaction properties; its sludge volume index (SVI) was only 62.2 mL/g. On the SVI index, this rating is indicative of a balanced mix of floc-forming and filamentous bacteria (Grady et al., 1999). Properly balanced activated sludge results in the formation of large, strong floc with a filamentous backbone. This leads to a non-bulking, non-foaming, easily and quickly recyclable biomass, and a quality effluent (Grady et al., 1999). On the other hand, dispersed individual bacteria and fine microbial flocs do not settle well and are often carried into the effluent, resulting in a poor quality effluent (Furukawa et al., 1993; Grady et al., 1999). The ALNS had ideal characteristics for application in various bio-treatment fields; it formed large flocs of about

4–6 mm size that settled rapidly producing a high-quality supernatant for discharge as treated effluent.

3.2. Nitrification performance of ALNS at low temperature

Although temperature significantly affected nitrification activity, the ALNS exhibited a relatively high nitrification activity at a cold water temperature zone where nitrification is known to be completely inhibited. Fig. 2A shows time courses of the $\text{NO}_2\text{-N}$ plus $\text{NO}_3\text{-N}$ concentration in the ALNS reactors during 6-h batch treatment of 300 mg/L ammonia at various temperatures. At 20 °C, the ammonia was depleted after 4 h of treatment and subsequent samplings were not considered for nitrification rate determinations. It took about 6 h at 15 °C to transform all of the ammonia (300 mg N/L). All, or nearly all, the oxidized N produced by the ALNS was $\text{NO}_2\text{-N}$, indicating nitritation was the predominant reaction. In two of the temperature tests (5 and 15 °C), very low amounts of $\text{NO}_3\text{-N}$ (2.8–7.9 mg/L) were detected at the 1 and 2 h samplings, which disappeared thereafter. In the two other tests, $\text{NO}_3\text{-N}$ was not detected. All the batch nitrification tests were well described by zero-order reactions (Fig. 2A), indicative of a lack of a lag period in nitrification, as well as no decrease in the rate of nitrification as residual $\text{NH}_4\text{-N}$ concentrations decreased (Wild et al., 1971). Oxygen consumption rates were determined at 2.5 h into the nitrification tests (Fig. 2B). These results demonstrated the effect of water temperature on nitrification activity and correlated well with the $\text{NO}_2\text{-N}$ production (Fig. 2A). The slope of the regression lines in Fig. 2A and B were used to quantify the specific nitrification activity of ALNS at various cold temperatures both in terms of $\text{NO}_2\text{-N}$ production and O_2 consumption. These nitrification activities are summarized in Table 1. In the present study, very-high-nitrification rates of 444 ± 10 mg N/L/day were confirmed at 5 °C and 813 ± 21 mg N/L/day at 10 °C. Corresponding specific activities were 11.2 and 20.5 mg N/g MLVSS/h. At 5 °C, when nitrification is known to be severely inhibited, the nitrification activity of ALNS was similar to that shown by other nitrifying sludges at 20 °C (Fig. 3) (Andersson and Rosen, 1990; Bae et al., 2001; Chiemchaisri and Yamamoto, 1993; Chudoba and Pannier, 1994; Furukawa et al., 1993; Shammas, 1986; Wild et al., 1971). At 10 °C (Fig. 3), the ALNS demonstrated a 330% increase in nitrification activity over a batch reactor system using a synthetic mineral medium (Bae et al., 2001), and a greater than 600% increase in nitrification activity over both a 2 L continuous unit (Chudoba and Pannier, 1994), and a two-stage pilot system (Wild et al., 1971), both fed on municipal wastewater (Chudoba and Pannier, 1994; Wild et al., 1971). At 20 °C (Fig. 3), the ALNS demonstrated an increase in nitrification activity of over 700% when compared to an acclimated marine nitrifying sludge tested with a synthetic inorganic medium (Furukawa et al., 1993), and over a 600% increase in nitrification activity when compared to a 5.7 L activated sludge reactor fed with anaerobic digester supernatant liquor (Shammas, 1986). Thus, the use of ALNS can offer significant advantages for biological treatment of wastewater as an alternative in situations where heating or high

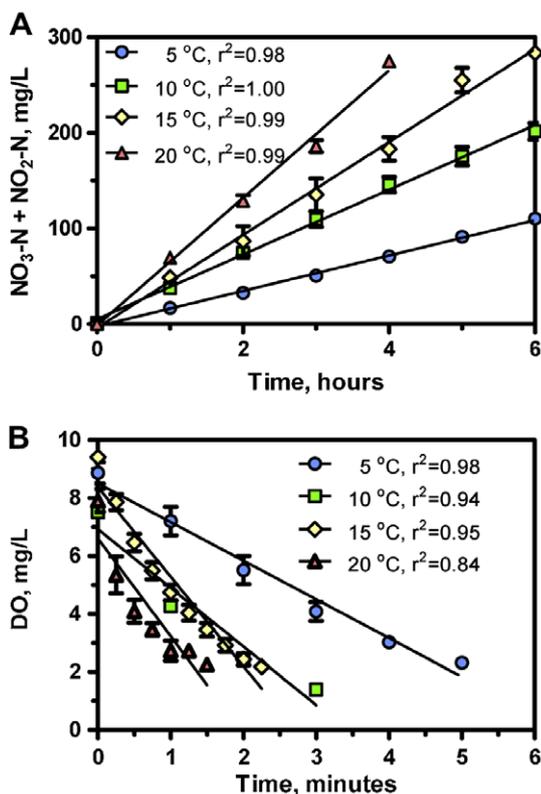


Fig. 2. The effect of water temperature on nitrification activity of ALNS. (A) Nitrate ($\text{NO}_3\text{-N}$) plus nitrite ($\text{NO}_2\text{-N}$) concentration during nitrification batch treatment of ammonia using ALNS at various water temperatures. Data are means \pm s.e. of duplicate reactors. With the exception of low amounts of $\text{NO}_3\text{-N}$ at the 1 and 2 h samplings (2.8 and 7.9 mg/L) of the 5 and 15 °C temperature tests, respectively, the measured values all correspond to $\text{NO}_2\text{-N}$. (B) Oxygen consumption during batch nitrification of ALNS at various water temperatures. Data are means \pm s.e. of duplicate reactors.

Table 1
Effect of water temperatures on nitrification activity of acclimated lagoon nitrifying bacteria.

Water temperature (°C)	Nitrite + nitrate production activity			Oxygen consumption activity		Ratio mg O_2 /mg N	
	mg N/L-reactor/h ^a	mg N/L-reactor/d	mg N/gr MLSS/h ^b	mg N/g MLVSS/h ^b	mg O_2 /L-reactor/min ^c		mg O_2 /L-reactor/d
5	18.50 \pm 0.40	444 \pm 10	8.7	11.2	1.34 \pm 0.10	1922 \pm 141	4.3
10	33.89 \pm 0.87	813 \pm 21	16.0	20.5	2.02 \pm 0.36	2915 \pm 511	3.6
15	48.59 \pm 1.78	1166 \pm 43	23.0	29.4	3.09 \pm 0.26	4454 \pm 377	3.8
20	66.67 \pm 3.03	1600 \pm 73	31.5	40.4	3.37 \pm 0.66	4914 \pm 949	3.1

^a Slope \pm s. e. from regression lines in Fig. 2A.

^b MLSS = 2115 mg/L \pm 35, MLVSS = 1615 \pm 60.

^c Slope \pm s. e. from regression lines in Fig. 2B.

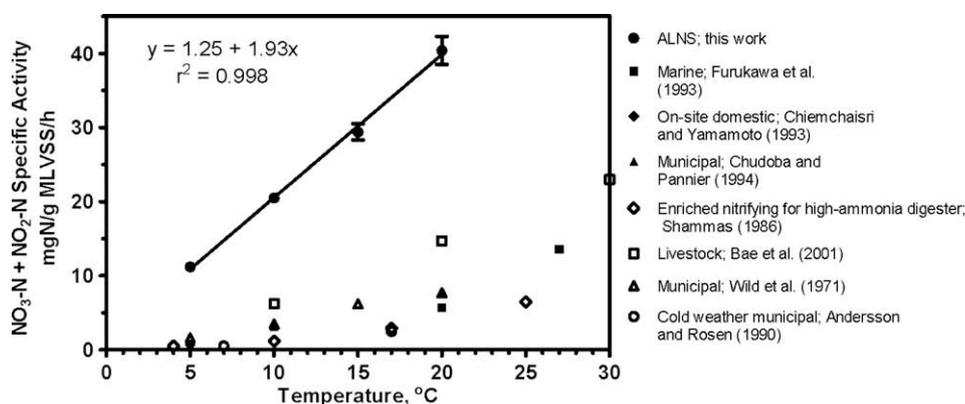


Fig. 3. Specific nitrification rates of various nitrifying sludges. Data for ALNS are means \pm s.e. of duplicate reactors. Marine and domestic on-site sludge data were reported as mg N/g MLSS/h.

retention time and large facilities are the only options for effective nitrification treatment.

Although high-nitrification rates at low temperatures were confirmed, complete nitrification to nitrate was not observed. This is probably a more desirable situation in modern wastewater treatment if the goal is to remove N through either a nitrification/denitrification cycle (Vanotti and Hunt, 2000) or a nitrification/anammox cycle (Kunz et al., 2008). This is because ammonia oxidation to nitrite and then reduction to N_2 represents an improvement over the complete oxidation to nitrate and subsequent reduction since the overall process to remove N results in less energy and time (Vanotti and Hunt, 2000).

The ratio of O_2 consumed to nitrogen oxidized was higher at the lower water temperatures (Table 1). It changed from 3.1 mg O_2 /mg N at 20 °C to 4.3 mg O_2 /mg N at 5 °C. This is an interesting finding because the expected nitrogenous oxygen demand for nitrification of 1 mg NH_3 -N is typically 3.2–3.4 (Sharma and Ahlert, 1977) and the higher consumption rate at the lower temperatures is indicative

that at low water temperatures other microbial species are also active in conjunction with the ammonia oxidizers.

3.3. 16S rRNA gene diversity

Overall, the 153 analyzed clones correspond to 50 unique sequences, and 18 phylotypes, all of which fell within previously characterized bacterial divisions (Table 2). Of the 50 unique sequences, 6 were identical to other 16S rRNA gene sequences contained in GenBank, and ALNS.25 and ALNS.28 had less than 95% similarity to 16S rRNA sequences determined in other microbial community surveys (Table 2). The remaining 42 sequences had between 96% and 99% similarity with other 16S rRNA gene sequences in GenBank (Table 2). The *Proteobacteria* are the dominant division, accounting for 80.4% (123 of 153) of the clones, 74% (37 of 50) of the unique sequences, and 50% (9 of 18) of the phylotypes, a result which is consistent with prior studies of nitrifying, activated sludge (Juretschko et al., 2002; Snaird et al., 1997). Juretschko et al.

Table 2
Closest phylogenetic affiliation of isolates based on BLASTn comparison to the GenBank database.

Isolate	Closest relative	Accession	% Similarity	Phylum/class and genus
ALNS.1 ^a	Uncultured bacterium, AnDHS-3	AB430334	99%	β -Proteobacteria, <i>Nitrosomonas</i>
ALNS.2	<i>Cryobacterium</i> sp., MSL 15	EF466127	99%	Actinobacteria, <i>Mycetocola</i>
ALNS.3	Uncultured bacterium, e01 = d01	AB241553	98%	Bacteroidetes, <i>Chitinophaga</i>
ALNS.4	Uncultured bacterium, R-23043	AJ786816	98%	γ -Proteobacteria, <i>Thermomonas</i>
ALNS.5 and 24	<i>Nitrosomonas europaea</i> , ATC 19718	AL954747	99%	β -Proteobacteria, <i>Nitrosomonas</i>
ALNS.6	Uncultured bacterium, GZKB17	AJ853512	99%	Bacteroidetes
ALNS.8, 13 and 23	Uncultured bacterium, nsmpV71	AB210047	98–99%	β -Proteobacteria, <i>Nitrosomonas</i>
ALNS.10	Uncultured bacterium, DGGE band XY-X	DQ838682	98%	γ -Proteobacteria
ALNS.11	Uncultured bacterium, TB127–31	AB196022	100%	β -Proteobacteria, <i>Nitrosomonas</i>
ALNS.12	<i>Alishewanella</i> sp., 620	EU841499	100%	γ -Proteobacteria, <i>Alishewanella</i>
ALNS.15 and 48	Uncultured bacterium, VC41	EU593781	97–98%	Bacteroidete, <i>Adhaeribacter</i>
ALNS.16	Uncultured <i>Alcaligenes</i> sp., MKC10	EF173341	100%	β -Proteobacteria, <i>Castellaniella</i>
ALNS.17	Uncultured <i>Chryseobacterium</i> sp., ChsSC	AY621829	100%	Bacteroidetes, <i>Sejongia</i>
ALNS.18	<i>Flavobacterium</i> -like sp., AY017	AF385544	100%	Bacteroidetes, <i>Kaistella</i>
ALNS.22	Uncultured bacterium, 5C231037	EU803464	99%	γ -Proteobacteria, <i>Lysobacter</i>
ALNS.25 and 28	Uncultured bacterium, FCPP479	EF516165	93%	Verrucomicrobia
ALNS.27	Uncultured <i>Bacteroidetes</i> , Bji46	AJ318151	98%	Bacteroidete, <i>Pedobacters</i>
ALNS.29 ^b	Uncultured bacterium, AnDHS-4	AB430335	99%	β -Proteobacteria, <i>Nitrosomonas</i>
ALNS.32	<i>Sphingopyxis</i> soil isolate, DCY34	EU075217	99%	α -Proteobacteria, <i>Sphingopyxis</i>
ALNS.35 and 50	Bacterium rj10	AB021328	99%	β -Proteobacteria, <i>Comamonas</i>
ALNS.37	Uncultured bacterium, FS43	EU593847	98%	Acidobacteria
ALNS.38	Uncultured soil bacterium, F5–46	EF688363	99%	Actinobacteria, <i>Subtercola</i>
ALNS.41	Bacterium rj9	AB021327	99%	β -Proteobacteria, <i>Comamonas</i>
ALNS.44	<i>Castellaniella</i> sp., DCY36	EU873313	98%	β -Proteobacteria, <i>Castellaniella</i>
ALNS.45	<i>Thermomonas</i> sp., EMB 79	DQ413155	98%	γ -Proteobacteria, <i>Thermomonas</i>
ALNS.46	Uncultured bacterium, LR A2-8	DQ988289	100%	Bacteroidetes
ALNS.49	Uncultured alpha proteobacterium, Blwii1	AJ318199	97%	α -Proteobacteria, <i>Aminobacter</i>

^a Also includes the following ALNS isolates – 7, 9, 14, 19, 20, 21, 26, 31, 34, 36, 40, 42, 43, and 47.

^b Also includes ALNS.30, ALNS.33, and ALNS.39.

(2002) reported that the *Proteobacteria* accounted for 59% of the total number of clones in the activated sludge of an industrial sewage plant, while Snaidr et al. (1997) placed the figure at 90% for sludge isolated from a municipal wastewater treatment plant. The remaining clones were affiliated with *Bacteroidetes* (16 clones; 10.4%), *Actinobacteria* (8 clones; 5.2%), *Verrucomicrobia* (5 clones; 3.3%) and *Acidobacteria* (1 clone; 0.7%). The calculated rarefaction curve of the cloned isolates approached saturation, indicating that a majority of the microbial diversity within the community was sampled (data not shown); this was confirmed by a Good's cover-

age estimate of 96.1% (Good, 1953). These data suggest that at the phylotype level, the diversity present in the ALNS community was representatively harvested.

3.4. Phylogenetic analysis of the ALNS community

The affiliations of the 50 unique sequences are demonstrated in the neighbor-joining phylogenetic trees shown in Figs. 4 and 5. Twenty-five, or half, of the unique sequences (106 of 153 clones, 69.3%) were affiliated with the ammonia-oxidizing bacterium

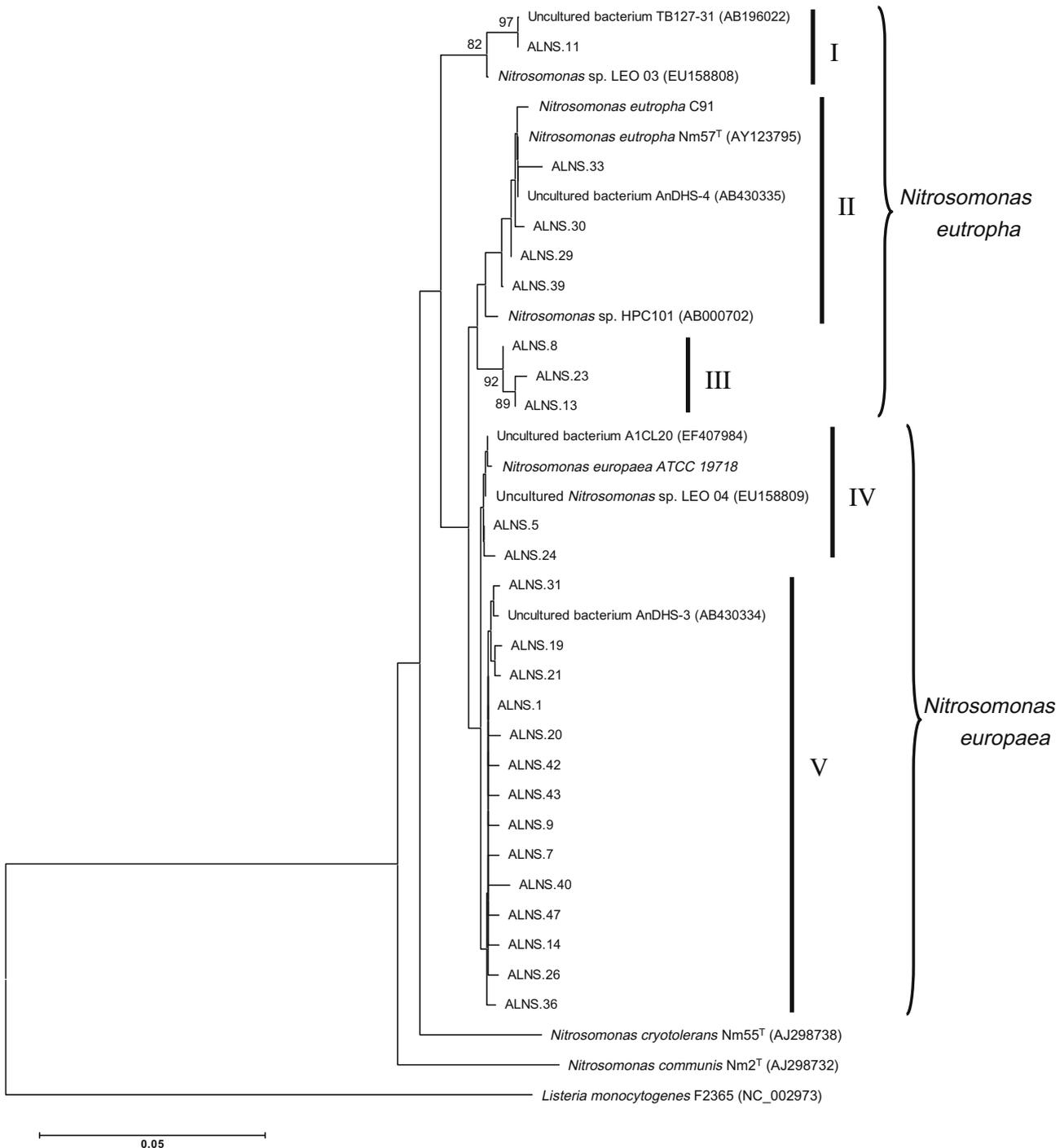


Fig. 4. Phylogenetic relationships between the 16S rRNA gene sequences of the ammonia-oxidizing bacteria (AOB) clones from the ALNS community and representatives from the β -*Proteobacteria* genus *Nitrosomonas*. The tree was constructed using partial 16S rRNA sequences and the neighbor-joining algorithm. The frequency (%) with which a given branch was recovered in 5000 bootstrap replications is shown above branches recovered in more than 70% of bootstrap replicates. Roman numerals (I–V) indicate the five monophyletic clades identified.

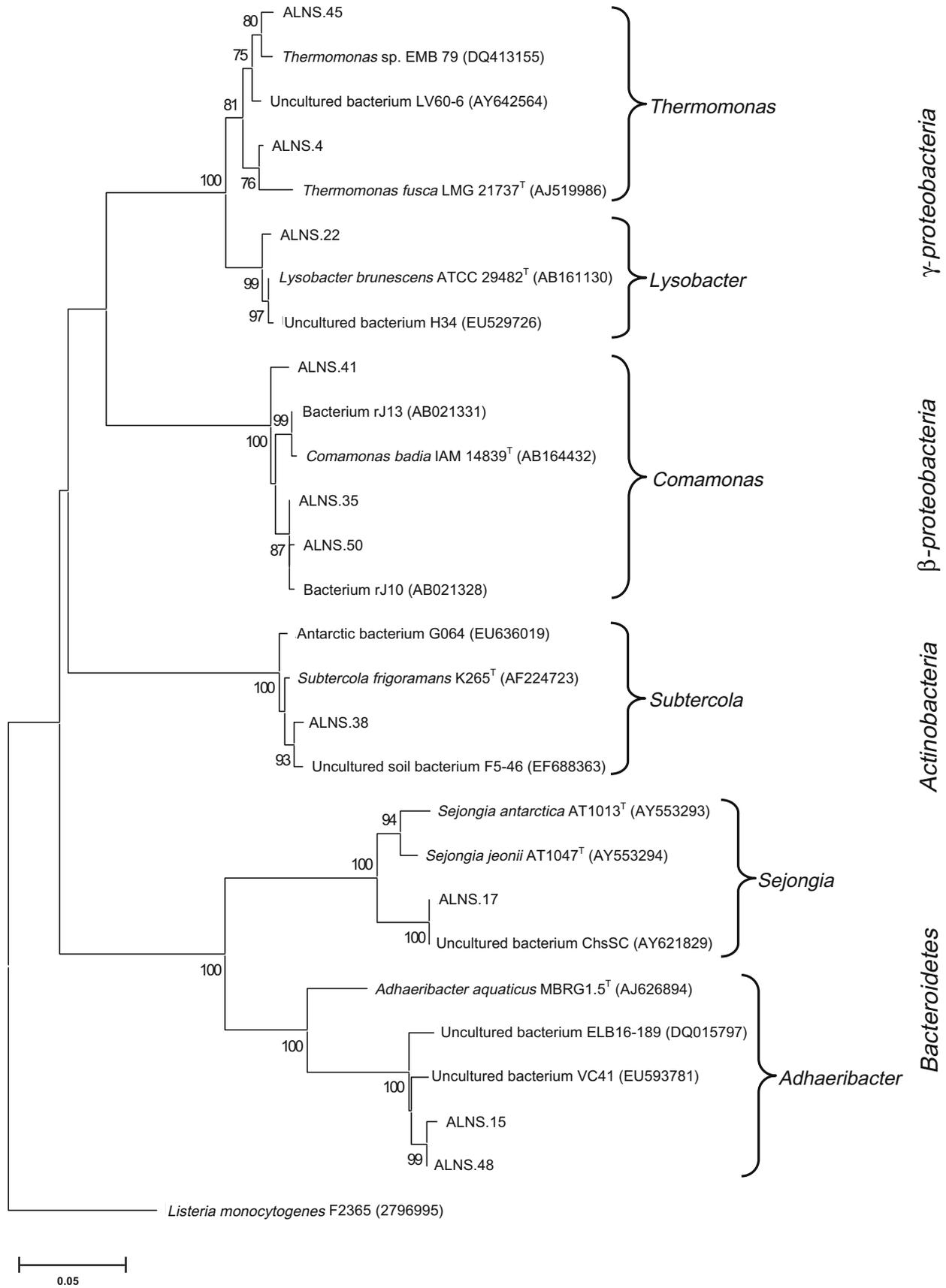


Fig. 5. Phylogenetic relationships between the 16S rRNA gene sequences from the ALNS community determined to have putative floc-forming or psychrotolerant characteristics, and representatives from the Actinobacteria, Bacteroidetes, and Proteobacteria. The tree was constructed using partial 16S rRNA sequences and the neighbor-joining algorithm. The frequency (%) with which a given branch was recovered in 5000 bootstrap replications is shown above branches recovered in more than 70% of bootstrap replicates.

(AOB), *Nitrosomonas*. The remaining 25 were affiliated with bacteria that have been identified as having characteristics that would appear to be beneficial to nitrifying sludge capable of operating at cold temperatures: such as psychrotolerance and flocculation.

3.4.1. AOB Proteobacteria

Of the 37 unique sequences affiliated with the phylum *Proteobacteria*, 30 belong to clones affiliated with the β -class (Fig. 4). Twenty-five of those unique sequences were classified as belonging to the genus *Nitrosomonas*, and specifically with the *N. europaea*/*N. eutropha* lineage. These results are congruent with prior studies which have consistently demonstrated that the *N. europaea*/*N. eutropha* lineage is the most frequently detected lineage in wastewater treatment plants and in activated sludge systems (Hallin et al., 2005; Wagner et al., 2002). While all the clones group together as one phylotype, based on 97% similarity, and classify as part of the *N. europaea*/*N. eutropha* lineage, they form five monophyletic clades (Fig. 4). Eight unique sequences, which comprise clades I, II and III, have the type strain *N. eutropha* Nm57 as their best match (99% similarity) against cultured organisms. The remaining 17 strains which comprise clades IV and V, have the sequenced *N. europaea* strain ATCC 19718 as their best match (99% similarity) against cultured organisms. These results demonstrate lineage diversity amongst the AOB populations in this activated sludge.

The 25 unique sequences, assembled from 106 clones, comprise one phylotype. All *Nitrosomonas* species examined to date have been shown to have one rRNA operon (Stein et al., 2007), and while clone frequencies are not necessarily an indication of community structure (Amann et al., 1995), every unique clone which affiliates with *Nitrosomonas* is indicative of a novel strain (Stein et al., 2007). ALNS.1 is the most often represented sequence, with 67 total clones, and therefore potentially plays the largest communal role in nitrification. ALNS.5 and ALNS.24 are found within the same clade as the uncultured *Nitrosomonas* isolate A1CL20 (GenBank accession No. EF407984), isolated from a high-strength ammonia wastewater treatment reactor. Other related sequences were uncultured *Nitrosomonas* isolates from an anammox reactor (AB430334 and AB430335), activated sludge (AB000702 and AB196022), and an aerobic/anaerobic sequential batch bioreactor (EU158808).

3.4.2. Psychrotolerant species

Two non-AOB *Proteobacteria* phylotypes and three additional phylotypes not belonging to the phylum *Proteobacteria* reveal affiliations to psychrophilic and psychrotolerant organisms putatively capable of growth below 15 °C (Fig. 5). Amongst the *Proteobacteria* ALNS.22 is affiliated (96.5% similarity) to *Lysobacter brunescens*, a microorganism capable of growth at 4 °C (Christensen and Cook, 1978); and, ALNS.4 and ALNS.45 are closely related (97.2% similarity) to *Thermomonas fusca*, which is capable of nitrite reduction and growth at 10 °C (Mergaert et al., 2003). ALNS.17 is 96.0% similar to the psychrotolerant type strain *Sejongsia jeonii* AT047^T (AY553294), a member of the genus *Bacteroidetes*, (Yi et al., 2005), and ALNS.38 is 98.8% similar to the psychrophile *Subtercola frigoramans* K265^T (AF224723), a high G + C content Gram-positive bacterium capable of growth at –2 °C (Mannisto et al., 2000). In addition, ALNS.15 and ALNS.48 both affiliate with *Adhaeribacter aquaticus*, a species capable of growth between 4 °C and 37 °C (Rickard et al., 2005). These five phylotypes from the non-AOB portion of the community which affiliate with psychrophilic and psychrotolerant organisms may explain, in part, the high rates of nitrification at colder temperatures (5 and 10 °C). While unable to oxidize ammonia themselves, these organisms may offer cold resistance to the *Nitrosomonas* isolates within the community, either acting as insulation for the floc, or by excreting chemicals which help abate the effects of colder tem-

perature. Kim and Yim previously isolated and identified seven bacterial strains from King George Island, Antarctica which were capable of producing cryoprotective exopolysaccharides, one of which, P-21653, when mixed with *E. coli*, was able to confer an increased survival to these cells over several freeze–thaw cycles (Kim and Yim, 2007).

3.4.3. Floc-forming species

Three unique sequences, represented by 6 clones, closely associate with the *Proteobacteria* β -class species from the genera *Comamonas*. ALNS.35, ALNS.41, and ALNS.50 are 98.3% similar to the type strain of *Co. badia*, IAM 14839^T, a floc-forming species of *Comamonas* that is also capable of nitrite reduction and growth at 20 °C (Tago and Yokota, 2004) (Fig. 5). *Comamonas* is a frequent microbial component of activated sludge, having been isolated from a number of activated sludge and wastewater treatment studies (Niu et al., 2006; Snaidr et al., 1997). Activated sludge with an SVI rating of “excellent” are formed by a balanced mixture of floc-forming and filamentous bacteria (Grady et al., 1999). Therefore ALNS.35, ALNS.41, and ALNS.50 may play an integral role in the excellent settling and compaction characteristics of the ALNS.

3.5. Adapted *Nitrosomonas* or community synergism?

There are two potential explanations for the excellent rate of nitrification for the ALNS community at cold temperatures. The first explanation is that the AOB population identified in this study has adapted to work well at temperatures lower than previously recorded for similar strains. Adaptability to cold temperatures has been observed in microorganisms after exposure to temperatures below those necessary for optimal growth (Membre et al., 1999). This adaptation could be the result of a number of factors: a change in fatty acid membrane composition, allowing the membrane to function normally; changes to enzyme structure; or the effect of gene expression due to low temperature stress responses (Berry and Foegeding, 1997; Jones et al., 1987). The second explanation is that other organisms in the ALNS produced an environment which allowed the AOB to maintain high levels of nitrification activity at colder temperatures. This explanation is plausible due to the number of putatively psychrotolerant and psychrophilic microorganisms identified in this study. Therefore, it is possible that excreted substances, utilized by other organisms to survive colder temperatures, have also allowed the AOB to survive and operate at colder temperatures. These organisms may also serve as insulation for the AOB, providing a degree of protection to the AOB from chemical and physical challenges.

4. Conclusions

Low nitrification rates during cold weather are often a problem for adoption of biological treatment ammonia in livestock effluents. We found a nitrifying sludge (ALNS) with excellent NH₃-N removal performance during cold weather conditions. In this study we characterized the effect of low temperature on the nitrification activity of ALNS and provided a detailed overview of its microbial community composition.

The ALNS showed ideal characteristics for application in various bio-treatment fields; it formed large flocs that settled rapidly producing a high-quality effluent. Very-high-nitrification rates of 444 ± 10 mg N/L/day were confirmed at 5 °C and 813 ± 21 mg N/L/day at 10 °C. Corresponding specific activities were 11.2 and 20.5 mg N/g MLVSS/h. These rates may be the highest reported for nitrification treatment of high-ammonia wastewater at low temperatures.

The community was dominated by AOB from the genus *Nitrosomonas*, a group of organisms responsible for the oxidation of ammonia to nitrite. This in turn may result in a symbiotic relationship with other identified organisms, most of which appear to be capable of using the accumulated nitrite for nitrogen assimilation and energy production via reduction pathways. The benefits to *Nitrosomonas* may, in part, be increased resistance to cold temperatures conferred by any of the number of identified psychrotolerant and psychrophilic organisms, and a growth matrix supplied by the putative floc-forming *Comamonas* species. This microbial community composition analysis provides a better understanding of the characteristics of a nitrifying sludge capable of high rates of nitrification at cold temperatures.

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