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Ammonia-oxidizing microbial communities in reactors with efficient nitrification at low-dissolved oxygen

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Abstract

Ammonia-oxidizing microbial communities involved in ammonia oxidation under low dissolved oxygen (DO) conditions (<0.3 mg/L) were investigated using chemostat reactors. One lab-scale reactor (NS_LowDO) was seeded with sludge from a full-scale wastewater treatment plant (WWTP) not adapted to low-DO nitrification, while a second reactor (JP_LowDO) was seeded with sludge from a full-scale WWTP already achieving low-DO nitrification. The experimental evidence from quantitative PCR, rDNA tag pyrosequencing, and fluorescence in situ hybridization (FISH) suggested that ammonia-oxidizing bacteria (AOB) in the *Nitrosomonas* genus were responsible for low-DO nitrification in the NS_LowDO reactor, whereas in the JP_LowDO reactor nitrification was not associated with any known ammonia-oxidizing prokaryote. Neither reactor had a significant population of ammonia-oxidizing archaea (AOA) or anaerobic ammonium oxidation (anammox) organisms. Organisms isolated from JP_LowDO were capable of autotrophic and heterotrophic ammonia utilization, albeit without stoichiometric accumulation of nitrite or nitrate. Based on the experimental evidence we propose that *Pseudomonas*, *Xanthomonadaceae*, *Rhodococcus*, and *Sphingomonas* are involved in nitrification under low-DO conditions.

Keywords

Nitrification; Low dissolved oxygen; Ammonia oxidizing bacteria; Ammonia oxidizing archaea; Activated sludge

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Contributors

CMF, PC, and JZO performed experiments, data analysis, contributed to writing the manuscript. DRN directed the research and contributed to data analysis and manuscript preparation. All authors have approved the final article.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.watres.2014.11.041>.

1. Introduction

Achieving energy independence has become a critical component of sustainability goals in the wastewater treatment industry. Simultaneously, the industry has to respond to pressures from increasingly stringent effluent regulations. In current practice, conventional biological nutrient removal (BNR) systems rely on high dissolved oxygen (DO) concentrations in portions of the treatment plant to accomplish oxidation of organic matter, nitrification, and phosphorus removal. Since aeration in BNR plants can account for nearly half of the energy used in BNR systems (Tchobanoglous et al., 2003), decreasing oxygen supply is one way to reduce energy consumption provided that reactors operated with lower DO can meet discharge regulations. Oxidation ditch-type reactors are one example of existing processes where nitrification occurs at low-DO and phosphorus removal can be established by cycling between low-DO and aerobic conditions (Zilles et al., 2002b).

It has been well documented that stable nitrifying reactors can be operated at DO concentrations below 0.5 mg/L (Bellucci et al., 2011; Liu and Wang, 2013; Park and Noguera, 2004), but it remains unclear which organisms are responsible for nitrification in such low-DO environments. Initial studies (Park and Noguera, 2004) focused only on ammonia oxidizing bacteria (AOB). However, the first study of ammonia-oxidizing archaea (AOA) in activated sludge (Park et al., 2006a) demonstrated their presence in full-scale oxidation-ditch type reactors with long solids retention times (SRTs) and having large portions of the activated sludge basin operated with low DO to achieve simultaneous nitrification and denitrification (SND). The same study did not find detectable amounts of AOA in treatment plants operated with conventional high-DO nitrification stages, opening up the possibility that AOA may play a role in low-DO nitrification. In other environments, studies comparing the relative abundance of AOB and AOA suggest that AOA may be more abundant than AOB when the concentrations of oxygen and ammonia are low (Erguder et al., 2009; Francis et al., 2005; Labrenz et al., 2010), in agreement with the initial observations in activated sludge (Park et al., 2006a). Nevertheless, recent studies regarding low-DO nitrification (Bellucci et al., 2011; Liu and Wang, 2013) have shown undetectable levels of AOA in low-DO lab-scale reactors. Thus, the comparative analyses of AOB and AOA in activated sludge have not produced consistent results of the effect of oxygen on the populations of AOB and AOA.

Other microorganisms that could be functionally important for nitrification in low-DO reactors, but have not been studied in detail, are anaerobic ammonia-oxidizing (anammox) bacteria and heterotrophic nitrifiers. Treatment processes that take advantage of the anaerobic metabolism of anammox organisms have been primarily developed for sidestreams with high ammonia concentrations (Strous et al., 1997), although there is interest in developing mainstream processes in which anammox contribute to nitrification in low-DO reactors (De Clippeleir et al., 2011). The influence of heterotrophic nitrification in activated sludge remains poorly understood. Most research regarding heterotrophic nitrification has focused on understanding whether specific heterotrophs have the ability to oxidize ammonia (Kim et al., 2005; Papen et al., 1989; Zhang et al., 2011), and very little research has focused on the influence heterotrophic nitrifiers may have in full-scale engineered systems.

In this study we aimed at investigating the microorganisms responsible for ammonia oxidation in low-DO reactors that received ammonia as the sole energy source. Specifically, we used quantitative PCR (qPCR) targeting the ammonia monooxygenase gene (*amoA*) to evaluate the relative abundance of AOA and AOB in low-DO lab-scale reactors seeded with sludge from different full-scale plants. In addition, we used tag pyrosequencing targeting the small subunit rRNA gene to gain a more comprehensive understanding of the communities in the reactors, and culturing to search for novel organisms potentially playing a role in low-DO nitrification.

2. aterial and methods

2.1. Chemostat operation

Two chemostat reactors were operated in this study. Each reactor consisted of a 2 L glass vessel loosely sealed with parafilm. The influent flow rate in each reactor was 8.33 mL/h, producing 10-day solids and hydraulic retention times. DO was monitored and recorded every 5 min using a portable meter and an optical probe (WTW Multi 3410 Multiparameter Meter). The DO was maintained in the reactors below 0.3 mg/L by constantly flushing the headspace with a mixture of air and compressed nitrogen gas, and facilitating oxygen diffusion into the liquid by gentle mixing with a magnetic stirrer. Adjustments to the flow rate of each gas into the headspace were performed daily to ensure the desired DO was achieved. The reactors were fed a synthetic medium containing 30 mg $NH_4^+ - N/L$, as described elsewhere (Park and Noguera, 2004). Under these conditions, the concentration of total suspended solids was always less than 10 mg/L (Park and Noguera, 2004). The reactors were operated at room temperature (between 21 and 23 °C).

2.2. Seed sludge

One of the ammonia-fed reactors (NS_LowDO) was seeded with sludge from the Nine Springs wastewater treatment plant (WWTP) (Madison WI), which uses a modified University of Cape Town process designed to achieve biological phosphorus removal (Zilles et al., 2002a). The plant is not operated to achieve significant denitrification, and therefore, an internal nitrate recycle flow is not used. The Nine Springs WWTP has approximately a 10-day SRT and 11 h hydraulic retention time. In the aerobic stage, DO reaches concentrations greater than 2 mg/L (Park et al., 2006b).

The other ammonia-fed reactor (JP_LowDO) was seeded with sludge from the Jefferson Peaks WWTP in Oak Ridge, NJ. Jefferson Peaks uses an MBR operated to achieve SND, in which greater than 90% of the treatment system has DO less than 0.2 mg/L (Littleton et al., 2013).

2.3. Sample collection and analytical tests

Biomass samples from the reactors were saved weekly by filtering between 50 and 100 mL of the cultures through 0.22- μ m membrane filters (Millipore Laboratories, Billerica, MA) and then storing at -80 °C until DNA extraction. Culture supernatants were collected weekly, filtered through 0.45- μ m membrane filters (Millipore Laboratories, Billerica, MA) and stored at -80 °C until analysis.

DNA was extracted using UltraClean® Soil DNA Isolation Kits (MoBIO Laboratories, Carlsbad, CA) and subsequently purified via ethanol precipitation. Extracted DNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and stored at -80°C . Multiple extraction methods were tested since some degree of bias in community structure can be associated with the DNA extraction and purification method used (Bergmann et al., 2010; Guo and Zhang, 2013; Vanysacker et al., 2010). Previous studies have indicated that the presence of a mechanical cell lysis step significantly reduces the biases associated with DNA extraction (Guo and Zhang, 2013). The extraction method chosen included mechanical lysis via bead beating and provided the most favorable combination of DNA yield and purity.

Culture supernatants were analyzed for total ammonia (NH_3 plus NH_4^+), hydroxylamine, nitrite and nitrate concentrations. Total ammonia concentrations were analyzed using the salicylate method (Method 10031, Hach Company, Loveland, CO). Nitrite and nitrate were measured using high pressure liquid chromatography on a Prevail™ Organic Acids (Discovery Sciences, Deerfield, IL) column with a mobile phase consisting of 25 mM KH_2PO_4 , pH adjusted to 2.5 with phosphoric acid, and detection by UV at 214 nm. Hydroxylamine was measured by the 8-hydroxyquinoline method (Magee and Burris, 1954).

2.4. Enrichment and isolation conditions

Isolations under autotrophic and heterotrophic conditions were performed from JP_LowDO sludge to obtain representative ammonia oxidizing cultures. To culture heterotrophic organisms, we used dilution-to-extinction techniques in agar plates with one-fifth strength modified R2A medium, as has been described for the isolation of novel heterotrophic organisms that grow under oligotrophic conditions (Im et al., 2012). To isolate autotrophic ammonia oxidizers, we used agar plates with the same synthetic medium used in the lab-scale reactors, which contained ammonia as the sole electron donor. After several transfers of single colonies to new agar plates, culture purity was confirmed by a combination of light microscopy and small clone libraries of the 16S rRNA gene. Selected isolates were screened for their potential involvement in autotrophic or heterotrophic nitrogen transformations by incubating them in liquid cultures using the ammonia medium for autotrophic growth and the same medium supplemented with sodium pyruvate (130 mg/L) for heterotrophic conditions. Total ammonia, nitrate, nitrite and hydroxylamine concentrations in the supernatant were measured after 3 weeks of incubation.

2.5. Quantitative polymerase chain reaction (qPCR)

qPCR targeting the bacterial and archaeal *amoA* gene was used to quantify the relative abundance of AOA and AOB. Thermocycling conditions for all qPCR assays were performed according to published methods associated with each of the following primers. The bacterial *amoA* gene was amplified using the primer set amoA-1F (5'-GGGGTTTCTACTGGTGGT-3') and amoA-2R (5'-CCCCTCKGSAAAGCCTTCTTC-3') (Rotthauwe et al., 1997). Since recent publications suggest that the most common archaeal *amoA* primer set (Francis et al., 2005) does not have sufficient coverage of the currently known AOA diversity (Park et al., 2008; Zhang et al., 2009), different primers sets were tested (Francis et al., 2005; Hallam et al., 2006; Park et al., 2008) with activated sludge

samples from several lab-scale and full-scale reactors. The selected primer set, A26F (5'-GACTACATMTTCTAYACWGAYTGGGC-3') and A416R (5'-GGKGTCA TRTATGG WGGYAA YGTTGG-3') (Park et al., 2008), provided the most consistent results and best amplification of activated sludge samples known to contain AOA. All qPCR reactions were run on a CFX96 Touch™ Real-Time PCR Detection System (BioRAD Laboratories, Hercules, CA). Each reaction was 20 µL and contained 10 µL iQ™ SYBR® Green Supermix (BioRAD Laboratories, Hercules, CA), 0.8 µL each of 10 µM forward and reverse primer, 2.4 µL nuclease free water, 2 µL of 1 mg/mL bovine serum albumin (Fisher Bio-Reagents, Waltham, MA), and 4 µL of sample. All samples were run in duplicate or triplicate and each plate contained non-template and known-addition controls. Calibration curves for each target gene were obtained with clones available for each specific amplification.

2.6. Cloning and sequencing

Amplicon cloning was performed using the TOPO® TA Cloning® Kit for Sequencing, with pCR™4-TOPO® Vector, One Shot® TOP10 Chemically Competent *Escherichia coli* (Life Technologies, Grand Island, NY). Sequencing was performed at the University of Wisconsin Biotechnology Center, using Big Dye® chemistry.

The obtained sequences of archaeal and bacterial *amoA* genes were transferred into MEGA 5.0 software package and combined with the most similar *amoA* gene sequences downloaded from GenBank to construct neighbor-joining phylogenetic trees with 1000 bootstrap tests for every node to estimate the confidence of tree topologies (Tamura et al., 2011).

2.7. Ribosomal RNA-based tag pyrosequencing

Tag pyrosequencing was performed using a Roche GS Jr instrument targeting the small subunit of the rRNA gene. Archaea specific primers (Arch-340F, 5'-CCCTAHGGGGYGCASCA-3' and Arch-915R, 5'-GWGCYCCCCCGYCAATTC-3'), targeting the V3–V5 region (Pinto and Raskin, 2012) and universal primers (926F, 5'-AACTYAAAKGAATTGRCGG-3' and 1392R, 5'-ACGGGCGGTGTGTRC-3') targeting the V6–V9 region (Kozubal et al., 2012) were used. DNA was amplified using Phusion® High Fidelity polymerase (Thermo Fisher Scientific, Waltham, MA). Each 20-µL reaction consisted of 0.8 µL each of 1 µM forward and reverse primer (final concentration of 40 nM each primer), 0.2 µL of Phusion DNA polymerase, 4 µL 5X Phusion High Fidelity buffer, 0.4 µL of 10 mM DNTP mix (Promega, Madison, WI), and 11.6 µL of nuclease free water. The remaining 3 µL consisted of a mixture of the sample and nuclease free water that resulted in between 10 and 50 ng of genomic DNA per reaction. Universal amplifications were performed in triplicate. The 1392R primer contained the 454 key tag and adaptor and sample-specific 5-mer multiplex identifier (MID) tags. The 926F primer contained the 454 adaptor sequence (Integrated DNA Technologies, Coralville, Iowa). Thermocycling conditions were: 98° C for 5s followed by 30 cycles of 98° C for 15s, 55/53° C (universal/archaea) for 30s and 72° C for 15s, followed by 72° C for 3 min. One of the replicates was used to verify quality of the PCR product. The remaining two samples were pooled and a 500-700bp section was purified using gel extraction. For Archaea amplifications, a dilution

series from 1 to 100 ng per reaction over 7 wells was amplified and the two brightest bands ranging from 600 to 900bp, were purified. PCR amplicons were first purified using Qiagen QIAquick Gel Extraction kit (Qiagen, Venlo, Netherlands) followed by a second purification using Qiagen QIAquick PCR purification kit (Qiagen, Venlo, Netherlands). Purified DNA was checked for quality using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and quantified using the Qubit® Fluorometer (Life Technologies, Grand Island, NY).

Sequencing was performed using the Titanium series reagents for the Roche GS Jr. instrument following a slightly modified version of the standard protocol. This modified method was previously shown to provide higher yields than the Roche Standard GS Junior protocol (Hanshaw et al., 2013).

Pyrosequencing data was analyzed using Mothur (Schloss et al., 2011). Trimmed sequences were classified with a modified SILVA taxonomy file (Pruess et al., 2007). The modified taxonomy file included all of the SILVA Bacteria, Archaea, and Eukaryota taxonomies with the addition a supplementary taxonomy file that included the names and sequences of uncultured organisms known to have a role in engineered systems (See Supplementary Table S1). All samples were separately aligned with the Silva Bacteria, Archaea, and Eukaryota alignments to ensure proper classification. All chimeras were removed using UCHIME (Edgar et al., 2011) followed by DECIPHER (Wright et al., 2012) to reduce the effects of sequencing errors on downstream analysis. Sequences were clustered using the average neighbor method and operational taxonomic units (OTUs) were constructed with a 3% distance cutoff.

2.8. Fluorescence in situ hybridization

Biomass samples were fixed with 4% paraformaldehyde and FISH analysis were carried out according to established protocols (Amann et al., 1990). Controls without the addition of probes were performed to examine autofluorescence. Negative controls were also performed using the complement to the EUB probe (nonEUB; 5'- ACTCCTACGGGA GGCAGC-3') to assess nonspecific oligonucleotide binding (Wallner et al., 1992).

Hybridization experiments were carried out with the mixture of EUB probes (Eub338: 5'- GCTGCCTCCCGTAGGAGT-3'; Eub338-II: 5'-GCAGCCACCCGTAGGTGT-3'; Eub338-III: 5'-GCTGCCACCCGTAGGTGT-3') specific for the Bacteria domain (Daims et al., 1999), the Nso1225 probe (5'- CGCCATTGTATTACGTGTGA -3'), specific for ammonia oxidizing bacteria in the Betaproteobacteria (Mobarry et al., 1996), and Cren499 (5'- CCAGRCTTGCCCCCGCT -3'), targeting most Crenarchaeaota (Burggraf et al., 1994).

2.9. Statistical analyses

In order to statistically represent the influence that DO has on the microbial communities, the Pearson r statistic was used to correlate DO to the relative abundance of each OTU. To remove rare OTUs, samples were sub-sampled 1000 times at the smallest sample size of 1085 sequences. The Z score for each OTU and for DO in each sample was calculated according to Equation (1), where X_i is the relative abundance of an OTU or the DO

concentration in sample i , μ is the average relative OTU abundance or DO concentration in all samples, and σ is the standard deviation of the corresponding metric in all samples.

$$Z_i = \frac{(X_i - \mu)}{\sigma} \quad (1)$$

The Pearson correlation coefficient (r) between DO and each OTU was then calculated according to Equation (2), where Z_{OTU_i} is the Z score for an individual OTU, Z_{DO_i} is the Z score for DO, and n is the number of samples ($n = 7$ in this study). For this analysis, r values were considered significant if the value was greater than 0.4 or smaller than -0.4.

$$r = \frac{\sum_{i=1}^n (Z_{OTU_i} \times Z_{DO_i})}{n - 1} \quad (2)$$

2.10. Analysis of archived samples

In addition to analyzing the reactors described above, we also used DNA archived from a previous study of low-DO nitrification (Park, 2005; Park and Noguera, 2004) to increase the dataset related to nitrification at low-DO conditions and also include samples from reactors operated under similar conditions, but high DO (8.7 mg/L). One set of reactors (NS_LowDO_2004, NS_HighDO_2004) was seeded from the Nine Springs WWTP, the same treatment system used to seed reactor NS_LowDO (Park and Noguera, 2004). The other set of reactors (M_LowDO_2004, M_HighDO_2004) was seeded from the Marshall WWTP (Marshall, WI), which operates an aerated-anoxic Orbal process (Park et al., 2002).

2.11. Nucleotide accession numbers

Sequences have been deposited into GenBank under accession numbers KJ669693: KJ669721 and KM243922:KM243926 for *amoA* and 16S rRNA gene sequences, respectively. Pyrosequencing data was deposited into BioProject under accession number PRJNA212116.

3. Results

3.1. Reactor performance

Complete nitrification, with accumulation of nitrate was obtained in the two ammonia-fed chemostat reactors operated at low-DO conditions (Fig. 1). In these reactors, the average DO was 0.24 mg/L and 0.20 mg/L for NS_LowDO and JP_LowDO, respectively. The JP_LowDO reactor, which was seeded from sludge already adapted to low-DO conditions, had no delay in the onset of nitrification and only had a minor amount of nitrite build up between days 5 and 10, demonstrating the presence of an efficient low-DO ammonia- and nitriteoxidizing community. In contrast, the NS_LowDO, seeded from sludge not adapted to low-DO conditions, exhibited a significant delay in the onset of nitrification. Substantial nitrification was not observed until after day 35, and a transient build up of nitrite was observed. These results indicate that efficient low-DO ammonia- and nitrite-oxidizing organisms were not abundant in the seed sludge, but were enriched in the reactor over the

first 100 days of operation. Furthermore, although efficient conversion of ammonia to nitrate took place, nitrate recovery in the effluent of both reactors during the last 10 days of operation was between 80 and 85%, consistent with the possibility of some denitrification activity taking place in the reactors.

3.2. AOB and AOA quantification

Quantitative PCR was used to estimate the relative enrichment of known AOB and AOA in the reactors (Fig. 2). The NS_LowDO reactor experienced an enrichment of AOB during the first 100 days of operation, going from 1.95×10^3 gene copies/mL at day 8 to 2.54×10^6 copies/mL at day 81. In contrast, qPCR showed that although AOA appeared to be present, they were not enriched during the low-DO operation. Thus, qPCR results indicated that after 81 days of operation the concentration of AOB was three orders of magnitude higher than AOA. Although AOB and AOA were both apparently present in the JP_LowDO reactor, neither group was enriched during low-DO operation and both remained at average concentrations lower than 10^3 copies/mL. Therefore, while both reactors were achieving efficient low-DO nitrification under identical operational conditions (Fig. 1), the NS_LowDO reactor had three orders of magnitude higher concentration of quantifiable ammonia-oxidizing prokaryotes than the JP_LowDO reactor.

The discrepancy in quantifications in both reactors prompted us to clone and sequence amplicons from these reactions to verify that the amplification products were indeed *amoA* fragments of AOB and AOA. Between 16 and 24 clones were sequenced from each reactor and each target gene amplification. For the NS_LowDO reactor, 54% of the recovered AOB-*amoA* clones had sequences that indeed corresponded to AOB, while the remaining sequences were PCR artifacts. As for AOA, only 25% of the recovered clones were related to known AOA. Likewise, sequencing of the JP_LowDO reactor showed only 9% of clones from the AOB reaction matching the AOB-*amoA* gene and 44% of the AOA-*amoA* clones correctly classified as AOA. These results suggest that even though presence of AOB and AOA was confirmed by cloning and sequencing, qPCR with the *amoA* primer sets used in this study was overestimating their concentration in the reactors.

The classification of the recovered clones that matched to AOB or AOA is presented in Fig. 3. In the NS_LowDO reactor, AOB clones were grouped into two distinct clusters. One set of 11 sequences corresponded to the *Nitrosomonas oligotropha* clade, with 97% identity to *Nitrosomonas* sp. NL7, a strain previously isolated from low-DO reactors seeded with the same sludge used in this study (Park and Noguera, 2007). The second set of sequences were related to *N. eutropha* and *N. europaea*, with 3 sequences having 86% identity to *Nitrosomonas* sp. LT-2, and one sequence having 99% identity to *Nitrosococcus* sp. LT-3. Interestingly, the *amoA* sequences of *Nitrosomonas* sp. LT-2 and *Nitrosococcus* sp. LT-3 were both obtained from a completely autotrophic nitrogen removal over nitrite (CANON) process, which operates under oxygen-limited conditions (Third et al., 2001). The AOB-*amoA* sequences retrieved from the JP_LowDO reactor were also classified as *N. oligotropha*, with 96% identity to *Nitrosomonas* sp. NL7. The AOA-*amoA* sequences retrieved from both reactors clustered with the *Nitrosopumilus* clade (Pester et al., 2012), with 85% identity to *Nitrosopumilus maritimus* and *Ca. Nitrosoarchaeum koreensis* MY1.

3.3. AOA were not abundant in the low-DO nitrifying reactors

Tag pyrosequencing of the 16S rRNA gene was performed to more comprehensively investigate the microbial communities in both reactors. The sequencing occurred over four barcoded sequencing runs (Table S2) that included not only samples from the two reactors, but also samples from the seed sludge, archived DNA samples from an earlier low-DO nitrification study (Park, 2005; Park and Noguera, 2004), and control samples from other full-scale activated sludge reactors (Tables S3 and S4). The primer pair 926F/1392R, previously described as specific for archaeal and bacterial 16S rDNA (Kozubal et al., 2012) was used. Domain level classification of the sequences obtained from the chemostats and seed samples revealed mostly bacterial and eukaryotic sequences (Fig. 4). Analysis of the eukaryotic sequences in NS_LowDO or JP_LowDO indicated that greater than 95% of them corresponded to rotifers, whose presence was confirmed by microscopy (data not shown). More importantly, archaeal sequences were not found in NS_LowDO or JP_LowDO and were in low abundance (<0.6% of all sequences) in the seed sludge, although most of these were euryarchaeal sequences, and thus, not related to known AOA. Only the Jefferson Peak's sludge contained crenarchaeal sequences, in low abundance (0.06% of sequences).

DNA samples from an earlier low-DO nitrification study using similar chemostat reactors (Park, 2005; Park and Noguera, 2004) were also included in the sequencing runs (Table S3). In that study, two reactors were seeded with sludge from the Nine Springs WWTP, the same plant used to seed NS_LowDO, and two reactors were seeded from an Orbal-type oxidation ditch in Marshall, WI (Park et al., 2002). For each seed, one reactor was operated at high DO (~5 mg/L) while the other reactor was operated under low-DO conditions (<0.3 mg/L). AOA were also undetected in all of these samples.

Activated sludge samples from full-scale reactors were also analyzed as control tests (Table S4). Archaeal sequences were detected in most of these samples, with one of them having 37.4% of the sequences classified as Archaea. This provides confidence that the DNA extraction procedure was not systematically biased against archaeal sequences. As an independent test to verify the low abundance of archaeal sequences in the chemostat reactors and the seed sludge, a subsequent run was performed using primers arch340f/arch915r, recently described as being specific to Archaea (Pinto and Raskin, 2012). With this primer set the recovery of good quality sequences was poor, with the majority of sequences not passing quality control filters and likely corresponding to unspecific amplification when the template has mostly bacterial DNA and has an insignificant abundance of archaeal DNA. Therefore, we conclude from the pyrosequencing results that although some specific AOA-*amoA* sequences were obtained by cloning, neither of the low-DO reactors, nor the seed sludge, had significant populations of AOA.

3.4. Presence of known nitrifying organisms

The bacterial sequences obtained with pyrosequencing (Table S3) provide additional information of known ammonia oxidizers in the reactors (Table 1). About 1.0% of the sequences from NS_LowDO were classified as *Nitrosomonas* and formed two separate OTUs. No other known AOB (in either the Beta or Gamma subclasses of the Proteobacteria) nor sequences corresponding to anammox were detected. In addition, 2.3% of the sequences

were nitrite-oxidizing bacteria (NOB), most of them within a single OTU related to *Candidatus Nitrospira defluvii* (99% identity). In the seed sludge, 0.78% of the sequences belonged to one of the two *Nitrosomonas* OTUs identified in the reactor and no other AOB or anammox were detected. Furthermore, 1.3% of the sequences were NOB, classified in the same *Nitrospira* OTU as in the reactor. Therefore, the identification of *Nitrosomonas* is consistent with the *amoA*-based identification of AOB in this reactor (Fig. 3), and the presence of NOB agrees with the complete oxidation of ammonia to nitrate (Fig. 1).

In contrast, sequences corresponding to known AOB or anammox were not found in JP_LowDO, although 20% of the sequences were NOB (99% identity to *Nitrospira moscoviensis*). In the seed sludge only 0.06% of the sequences were related to AOB. These observations are consistent with the poor recovery of AOB-*amoA* sequences from this reactor.

FISH was used to further evaluate the abundance of AOB and AOA in the reactors. Probe Nso1225, targeting AOB in the Betaproteobacteria, hybridized to a small number of cells in NS_LowDO but not in JP_LowDO. However, the number of positive cells was too small to allow an adequate quantification. Probe Cren499, targeting most Crenarchaeota, did not show any positive response with samples from either reactor.

Taken together, the qPCR, pyrosequencing, and FISH results demonstrate an insignificant population of anammox and AOA in either reactor, an insignificant population of AOB in JP_LowDO, and low abundance of AOB in NS_LowDO. Thus, the results are consistent with the hypothesis that ammonia oxidation in these reactors, and in particular in JP_LowDO, was carried out by yet uncharacterized ammonia-oxidizing organisms.

3.5. Searching for unrecognized ammonia-oxidizing prokaryotes

With the recognition that the known ammonia-oxidizing prokaryotes were not dominant in the low-DO nitrifying enrichment cultures, we set out to isolate representative autotrophic and heterotrophic organisms from JP_LowDO to test for their potential participation in ammonia oxidation. This culturing exercise resulted in five pure cultures, as seen in Table 2. Of the five pure cultures, three were isolated under heterotrophic conditions and two were isolated under autotrophic conditions.

Culturing of these isolates in autotrophic liquid medium, with ammonia as the sole electron donor resulted in the removal of approximately one third of the total nitrogen after three weeks of incubation (Table 2). While this indicated utilization of ammonia under autotrophic conditions by all cultures, 4 out of the 5 cultures did not have noticeable growth in the autotrophic medium. Only one culture (strain 110.1) had a noticeable increase in cell density under these conditions. Thus, ammonia oxidation in most of these cultures does not appear to be linked to autotrophic growth, as has been shown to be the case for some methane-oxidizing bacteria (Trotsenko and Murrell, 2008). When the isolates were incubated in the same liquid medium, but supplemented with pyruvate as an organic carbon source, the ammonia removal was higher than the expected nitrogen assimilation based on pyruvate consumption and the assumption that 20 mg COD utilized would require 1 mg nitrogen for biomass growth (Metcalf and Eddy, 1991). While inorganic carbon was

available in the media and could potentially serve as a carbon source for autotrophic growth, the observations of pyruvate utilization (Table 2) and visible growth in all of the cultures were consistent with cells growing heterotrophically. These results suggest that the isolated bacteria have the ability to take up ammonia under both autotrophic and heterotrophic conditions at levels higher than expected from assimilation alone.

Remarkably, no more than a 0.3% of the ammonia removed by these microorganisms was oxidized to nitrite and although the amount of nitrogen converted to nitrate was higher, these oxidized nitrogen species do not account for the total nitrogen removed by the pure cultures. A possible explanation to this observation is the accumulation of hydroxylamine, an intermediary in the ammonia oxidation reaction. Previous studies have shown that under low DO, hydroxylamine may accumulate (Bock et al., 1994; Yang et al., 2011). However, hydroxylamine measurements after one day of incubation showed undetectable levels of this compound in the cultures; only after three weeks of incubation a minimal production of hydroxylamine was detected (Table 2). Thus, it is possible that either hydroxylamine is not an intermediate, or that given the inherent instability of hydroxylamine it is disproportionated as soon as it is formed (Pacheco et al., 2011) leading to loss of nitrogen as a gas product.

Alternatively, the lack of stoichiometric accumulation of oxidized nitrogen species could be attributed to the isolates performing aerobic denitrification, as has been suggested for some isolates that show heterotrophic nitrification (Kim et al., 2005; Zhang et al., 2011). However, this metabolic feature would not explain the absence of a stoichiometric nitrogen balance in the autotrophic cultures, where an organic electron donor was not present.

Interestingly, the lack of significant accumulation of nitrite by the pure cultures (Table 2) is in contrast with the chemostats showing almost complete oxidation to nitrate (Fig. 1). This discrepancy could be explained by NOB, which will catalyze the rapid oxidation of any nitrite produced in the reactors. Thus, further studies of the isolates in co-culture with NOB may provide additional insight to their potential role in low-DO nitrification in mixed communities where substrate competition is prevalent. Nevertheless, although more research is needed to elucidate the fate of nitrogen in these cultures, the observed high ammonia removal supports the hypothesis that organisms not related to known AOB or AOA are involved in ammonia removal in the low-DO reactors.

3.6. Taxonomic units enriched under Low-DO conditions

To further identify organisms potentially involved in low-DO nitrification in the chemostat reactors we analyzed the pyrosequencing results (Table S3) from NS_LowDO, JP_LowDO, plus the four reactors from the earlier study, two operated with low DO (NS_LowDO_2004, M_LowDO_2004) and two with high DO (NS_HighDO_2004, M_HighDO_2004). A Pearson correlation analysis of the relative abundance of OTUs in the reactors was performed (Figure S1). Table 3 summarizes the OTUs that had significant correlations to either low DO ($r < -0.4$) or with high DO ($r > 0.4$), and whose abundance was greater than 1% of all the sequences in at least on sample.

From this analysis it can be seen that OTUs related to the *Pseudomonas*, *Flexibacter*, and *Dokdonella* genera, and to two unclassified Bacteria are most correlated to Low-DO conditions. The OTUs that correlated with high DO conditions include *Nitrosomonas*, *Nitrospira defluvii*, and unclassified members of the *Alphaproteobacteria*, *Gammaproteobacteria*, and *Bacteroidetes*.

4. Discussion

4.1. AOA were not abundant in low-DO reactors

We studied the microbial communities in two ammonia-fed reactors operated with DO concentrations below 0.3 mg/L. The initial motivation for this study was to investigate whether AOA were functionally important in activated sludge reactors operated with low-DO conditions, since low DO and low ammonia concentrations had been suggested as favoring AOA over AOB in different environments (Erguder et al., 2009; Francis et al., 2005; Labrenz et al., 2010). Although presence of AOA in both reactors was confirmed by *amoA* sequences recovered from the reactors, all other analyses (qPCR, pyrosequencing, FISH) strongly suggested that AOA were not abundant, and therefore, not functionally important in these reactors. The main evidence for this conclusion was the lack of amplification of archaeal sequences when either universal or Archaea-specific primers were used with 454 pyrosequencing. When universal primers were used, bacterial and eukaryotic sequences were amplified, indicating that DNA from these domains was more abundant than archaeal DNA. Increasing the specificity of the primers to the domain Archaea did not improve amplification of the domain. We observed a very poor recovery of high quality sequences and the amplified sequences we were able to recover corresponded to bacterial and eukaryotic cells, instead of archaeal cells. This can occur if targeted templates are not abundant in the sample and non-targeted templates that inefficiently bind to the primers are present at concentrations that are several orders of magnitude higher than the targeted template. Under these circumstances, if a mismatched template is amplified in one cycle, the product becomes a perfectly matched template in future cycles thus yielding false-positive amplifications (Wright et al., 2014).

The observation that AOA were not abundant in the low-DO reactors agrees with recent studies of low-DO nitrification (Bellucci et al., 2011; Liu and Wang, 2013), which reported that AOA were not detected in their enrichments. In both of these studies, bench scale reactors were operated under oxygen concentration below 0.5 mg/L and a range of solids retention times from 3 to 40 days. PCR experiments did not detect AOA in these systems using primers sets specific for the *amoA* gene of AOA (Francis et al., 2005). *Nitrosomonas*-like AOB were described as the dominant ammonia oxidizers by means of T-RFLP analysis and sequencing of clone libraries, respectively.

Although we conclude that AOA were not a dominant part of the community, and therefore, not major contributors to the observed ammonia oxidation activity in the low-DO reactors, anammox were also not present, and the AOB population size alone is not sufficient to explain the observed ammonia oxidation. This is particularly apparent in the JP_LowDO reactor. While *amoA*-based qPCR showed an enrichment of AOB in NS_LowDO that could potentially explain the observed ammonia oxidation activity in that reactor, AOB were

apparently not enriched in the JP_LowDO reactor (Fig. 2). Moreover, the poor recovery of good quality AOB *amoA* sequences from JP_LowDO suggests that the *amoA*-based quantification of AOB in this reactor may have also suffered from the problem of amplification of non-targeted sequences when perfectly matched templates are not abundant or present (Wright et al., 2014).

Additional evidence suggesting that recognized AOB may not be the main ammonia-oxidizing organisms in the JP_LowDO reactor comes from the pyrosequencing data of archived DNA samples (Table S3) from reactors operated under different DO conditions (Park, 2005; Park and Noguera, 2004). One set of reactors in that study was seeded with sludge from the Marshall WWTP (Park, 2005). In those reactors, which exhibited complete nitrification of ammonia to nitrate, AOB represented 12% of all sequences in a high DO condition and only 0.55% in a low-DO reactor, suggesting the potential enrichment of alternative ammonia-oxidizing organisms in the low-DO reactor. Noteworthy, neither AOA nor anammox were detected by pyrosequencing in these samples.

A similar trend of higher AOB in high-DO conditions was observed in the reactors seeded with sludge from the Nine Springs WWTP (Park and Noguera, 2004), where AOB accounted for 6% in the high-DO reactor and 2.3% in the low-DO reactor (Table S3), whereas AOA or anammox were not detected. The decrease in AOB abundance was not as significant as in the Marshall seeded reactors, which is an indication that AOB may still play an important role in low-DO reactors seeded with sludge from the Nine Springs WWTP, similar to the observations in NS_LowDO, in which AOB accounted for 1% of the sequences and qPCR results indicated an enrichment of AOB in the reactor (Fig. 2).

Therefore, the data suggests that the role of AOB in low-DO nitrification may be more significant in reactors seeded with sludge from the Nine Springs WWTP, than in reactors seeded with sludge from either the Jefferson Peaks WWTP or the Marshall WWTP. The latter two are full-scale reactors operating with SND, in which a large portion of the nitrification occurs at low-DO conditions, whereas at the Nine Springs WWTP nitrification occurs in a high-DO environment, and therefore, it is plausible that the sludge from Marshall and Jefferson Peaks is already enriched in efficient, albeit yet unidentified, ammonia oxidizing organisms with high affinity for oxygen.

4.2. Potential role of other organisms in low-DO nitrification

The culturing exercise from the JP_LowDO reactor, which did not have a significant population of known ammonia-oxidizing prokaryotes, resulted in the isolation of several bacterial strains that were tested for nitrogen transformation under autotrophic and heterotrophic conditions. All strains removed ammonia at levels that were higher than anticipated from assimilation alone, and some produced small non-stoichiometric amounts of nitrite and nitrate (Table 2). Combining the results from culturing, pyrosequencing and the correlation analysis of microbial communities with DO conditions provides insights into potential nitrifiers active in JP_LowDO.

This study suggested that members of the *Xanthomonadacea* family within the Gammaproteobacteria may be involved in low-DO nitrification. On one hand, the 16S rRNA

sequence of Strain 110.1, isolated under autotrophic conditions, had 97.5% identity to *Luteibacter yejuensis*, a member of the *Xanthomonadaceae* family. Although none of the *Luteibacter* species have been described as either capable of autotrophic growth or of ammonia oxidation (Johansen et al., 2005; Kampfer et al., 2009; Wang et al., 2011), we showed that this organism can grow autotrophically using ammonia as the sole energy source, and also to consume higher than expected ammonia during heterotrophic growth (Table 2). Interestingly, the most abundant sequence obtained from tag pyrosequencing of the JP_LowDO reactor (23% of sequences), had 95.4% identity to Strain 110.1, was also classified within the *Xanthomonadaceae* (*Dokdonella* genus), and was one of the top taxonomic units with a statistically significant correlation to low-DO conditions (OTU 6 in Table S3).

The second organism isolated autotrophically, Strain 103.1, also exhibited ammonia consumption under autotrophic and heterotrophic conditions, with small, non stoichiometric production of nitrite and nitrate under autotrophic conditions (Table 2). This strain was classified as a member of the *Rhodococcus* genus within the Actinobacteria. Sequences in this genera were also present in the tag pyrosequencing results from the JP_LowDO reactor (OTU 65 in Table S3). The involvement of *Rhodococcus* species in nitrification has been previously suggested, although the literature on nitrification by *Rhodococcus* has been associated with heterotrophic nitrification (Chen et al., 2012; Cuangya et al., 2003).

The Pearson's correlation analyses (Table 3) of reactors receiving ammonia as the only energy source but with different aeration conditions suggested *Pseudomonas* as an additional organism associated with low-DO conditions. *Pseudomonas*-related sequences were present in all low-DO reactors with the exception of M_LowDO_2004 (Table 3). *Pseudomonas stutzeri*, a species with 100% sequence identity to the sequences found in the low-DO reactors, has been shown to have the ability to perform heterotrophic nitrification, and a specific strain (*P. stutzeri* YZN-001) isolated from swine waste has been shown capable of heterotrophic nitrification under the environmental conditions found in activated sludge (Zhang et al., 2011).

Furthermore, the three strains heterotrophically isolated also showed high levels of ammonia consumption under autotrophic and heterotrophic conditions (Table 2). Among them, it is interesting to note that *Sphingomonas* has been recently suggested as an organism capable of nitrification in chloraminated drinking water distribution systems (Krishna et al., 2013), where ammonia oxidation cannot always be associated with known ammonia-oxidizing prokaryotes and the involvement of heretofore unrecognized ammonia oxidizers is hypothesized (Krishna et al., 2013; Noguera et al., 2008).

5. Conclusions

- The inoculum source defined the ammonia oxidizing community in the low-DO chemostats. No known ammonia oxidizing prokaryotes were identified at a significant concentration in the reactor inoculated with sludge from a full-scale WWTP already operating to achieve low-DO nitrification (JP_LowDO). AOB were identified as the known ammonia oxidizing prokaryotes in the reactor inoculated

with sludge from a full-scale WWTP operating with high-DO nitrification (NS_LowDO).

- AOA were not a significant part of the ammonia oxidizing community in either reactor.
- The characterization of the ammonia-oxidizing community in the reactors via qPCR, FISH, and tag pyrosequencing strongly suggested that ammonia oxidation in the reactors was carried out by heretofore unrecognized ammonia-oxidizing organisms. We conclude that current understanding of aerobic nitrification is incomplete, and further work is needed to unravel the microbiological puzzle of low-DO nitrification. For instance, stable isotope probing with ^{13}C -labeled bicarbonate could help identify novel autotrophic organisms in the low-DO nitrifying enrichments.
- Based on preferential enrichment in low-DO nitrifying reactors, experiments with isolates from JP_LowDO, and the existing literature, we suggest that *Pseudomonas*, *Xanthomonadaceae*, *Rhodococcus*, and *Sphingomonas* have the potential to participate in ammonia oxidation in low-DO systems, either as heterotrophic nitrifiers, or via autotrophic nitrification through yet uncharacterized pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Nitrification performance in lab-scale chemostat reactors operated at low DO conditions. (A) Reactor NS_LowDO, seeded with activated sludge from a full-scale plant that performs nitrification at high DO, (B) Reactor JP_LowDO, seeded with activated sludge from a full-scale membrane bioreactor that performs nitrification at low DO.

Fig. 2.
Quantification of AOB and AOA with *amoA*-based qPCR. (A) NS_LowDO reactor; (B) JP_LowDO reactor.

Fig. 3. Neighbor-joining phylogenetic trees of bacterial and archaeal *amoA* gene sequences reflecting the relationship between sequences obtained from the NS_LowDO and JP_LowDO reactors and reference organisms. Sequences were clustered using a 90% identity cutoff, and the number of sequences of each cluster is shown in parenthesis. Accession numbers for the reference sequences also shown in parenthesis. Bootstrap values are indicated at branch points. Branch lengths correspond to sequence differences as indicated by the scale bar. (A) Bacterial *amoA*, and (B) archaeal *amoA* phylogenetic trees.

Fig. 4.

Pyrosequencing domain recovery with universal primer pair 927f-1392r and archaea specific primer pair Arch340f-915r. JP = Jefferson Peaks; NS = Nine Springs; M = Marshall.

Table 1

Relative abundance of known ammonia oxidizers in low-DO chemostat reactors as determined by 16S rRNA-targeted pyrosequencing.

Highest classification	Relative abundance			
	NS_LowDO	NS_Seed sludge	JP_LowDO	JP seed sludge
AOB Total	1.02	0.78	0.00	0.06
<i>Nitrosomonas</i> , OTU 1	0.59	0.78	0.00	0.00
<i>Nitrosomonas</i> , OTU 2	0.43	0.00	0.00	0.00
Other <i>Nitrosomonadales</i>	0.00	0.00	0.00	0.06
Anammox	0.00	0.00	0.00	0.00
NOB	2.34	1.34	20.46	7.11
AOA	0.00	0.00	0.00	0.00

Table 2

Pure cultures isolated from JP_LowDO under autotrophic and heterotrophic conditions and results of nitrogen removal when incubated in liquid medium under autotrophic and heterotrophic conditions.

Isolation condition	Strain	Phylum/class	Closest organism	% Identity	Incubated under autotrophic conditions					Incubated under heterotrophic conditions				
					% N-NH ₃ ^a	% N-NO ₂ ^b	% N-NO ₃ ^b	% N-H ₃ NO ^b	% C ₃ H ₃ O ₃ ^c	% N-NH ₃ ^a	% N-NO ₂ ^b	% N-NO ₃ ^b	% N-H ₃ NO ^b	
Heterotrophic Conditions	001.1	Proteobacteria/Alphaproteobacteria	<i>Sphingomonas aquatilis</i> ISS-7 (NR_024997)	98.7%	34.0%	ND	2.9%	0.1%	97.0%	32.7% (12.4%)	0.04%	1.9%	0.1%	
	003.2	Firmicutes/Bacilli	<i>Bacillus (Brevibacterium) frigoritolerans</i> DSM 8801 (NR_042639)	99.5%	21.5%	0.3%	0.8%	0.1%	98.0%	28.8% (12.5%)	0.3%	0.2%	0.04%	
Autotrophic Conditions	004.1	Proteobacteria/Alphaproteobacteria	<i>Bosea thiooxidans</i> DSM 9653 (NR_041994)	97.2%	22.5%	ND	0.6%	0.2%	71.0%	27.7% (9.1%)	ND	0.5%	0.04%	
	103.1	Actinobacteria/Actinobacteria	<i>Rhodococcus yunnanensis</i> YIM 70056(NR_043009)	99.2%	19.6%	0.3%	0.8%	0.2%	99.0%	27.1% (12.6%)	ND	ND	0.05%	
	110.1	Proteobacteria/Gammaproteobacteria	<i>Luteibacter anthropi</i> CCUG 25036 (NR_043618)	99.2%	29.5%	0.2%	0.6%	0.1%	46.0%	32.6% (5.8%)	ND	1.4%	ND	

ND- Compound not detected.

^aPercentage of N–NH₃ removed in autotrophic/heterotrophic liquid media. In parenthesis: calculated percentage of N–NH₃ assimilated by the cultures based on pyruvate consumption (COD:N ratio of 100:5) in heterotrophic liquid media.

^bPercentage of N–NH₃ oxidized to N–NO₂, N–NO₃ or N–H₃NO in autotrophic/heterotrophic liquid media.

^cPercentage of pyruvate consumed by the cultures in heterotrophic liquid media.

Table 3

Operational taxonomic units (OTUs) with significant correlation to oxygen concentration.

Silva taxonomy	OTU	Selected cultured match in RDP	Accession number	r value	p-value	Relative abundance (%)							
						NS_High DO 2004	M_High DO 2004	NS_LowDO2004	NS_LowDO	M_Low DO 2004	JP_LowDO	NS-full scale	fp-Full scale
Low DO													
Genus <i>Pseudomonas</i>	13	<i>Pseudomonas stutzeri</i> (T)	KC935382	-0.66	0.15	0.09	0.00	5.35	3.69	0.09	2.67	0.09	0.28
Genus <i>Nitrospira</i>	8	<i>Nitrospira moscoviensis</i> (T)	X82558	-0.49	0.33	0.28	0.18	1.66	0.00	32.90	17.33	0.00	7.00
Genus <i>Flexibacter</i>	16	<i>Ohtaekwangia koreensis</i> (T)	GU117702	-0.49	0.33	0.28	0.00	2.30	0.00	2.95	0.18	3.87	0.28
<i>unclassified Bacteria</i>	86	<i>Phycisphaera mikarensis</i> (T)	AB447464	-0.48	0.34	0.00	0.00	0.00	1.47	2.49	0.00	0.00	0.00
Genus <i>Dokdonella</i>	6	<i>Dokdonella soli</i> (T)	EU685334	-0.42	0.41	0.83	0.00	6.82	0.92	0.09	22.86	1.84	1.29
<i>unclassified Bacteria</i>	36	<i>Fimbriimonas ginsengisoli</i> Gsoil 348	GQ339893	-0.40	0.44	0.65	0.37	0.18	3.59	3.41	0.00	0.00	0.00
High DO													
Class <i>Alphaproteobacteria</i>	243	<i>Sphingomonas</i> sp. <i>BXN7-9</i>	EU423301	0.60	0.21	0.00	2.03	0.09	0.00	0.18	0.00	0.00	0.00
Family <i>Chitinophagaceae</i>	124	<i>Niabella tibetensis</i> (T)	GU291295	0.61	0.20	0.00	3.87	0.28	0.00	0.09	0.00	0.65	0.00
Family <i>Chitinophagaceae</i>	56	<i>Sediminibacterium salmonicum</i> (T)	EF407879	0.62	0.19	0.00	3.41	0.00	0.18	0.00	0.00	3.04	0.00
Family <i>Chitinophagaceae</i>	34	<i>Eubacterium</i> sp. <i>11-14</i>	EU571161	0.62	0.19	0.00	24.24	0.00	1.01	0.00	0.00	0.00	0.00
Class <i>Gammaproteobacteria</i>	41	<i>Thiobacillus prosperus</i> ;Y6	EU653290	0.63	0.18	6.82	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<i>unclassified Bacteria</i>	180	<i>Mycoplasma sualvi</i> (T)	AF412988	0.63	0.18	0.00	3.96	0.00	0.00	0.00	0.00	0.00	0.00
Family env. <i>OPS_17</i>	25	<i>Tuber borchii</i> symbiont b-17BO	AF233293	0.67	0.15	0.18	2.30	0.00	0.09	0.18	0.00	0.00	0.00
Genus <i>Nitrospira</i>	4	<i>Candidatus Nitrospira defluvia</i>	NR_074700	0.72	0.11	52.63	12.90	15.94	1.66	2.03	1.75	1.84	0.00
Family <i>Chitinophagaceae</i>	17	<i>Ferruginibacter alkalilientus</i> (T)	FJ177530	0.86	0.03	2.58	2.21	0.46	0.00	1.11	1.38	0.65	0.83
Genus <i>Nitrosomonas</i>	27	<i>Nitrosomonas</i> sp. <i>Nm86</i>	AY123798	0.90	0.01	5.99	11.80	2.30	0.37	0.18	0.00	0.55	0.00
Family <i>PHOS-HE51</i>	54	<i>Adhaeribacter aerophilus</i> (T)	GQ421850	0.93	0.01	1.29	2.03	0.46	0.09	0.28	0.00	0.00	0.00
Dissolved oxygen (mg/L)				-	-	8.7	8.7	0.13	0.27	0.13	0.19	3	0.2
Nitrogen source				-	-	NH3	NH3	NH3	NH3	NH3	NH3	WW	WW
Carbon source				-	-	None	None	None	None	None	None	WW	WW