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Original Article

Nitrogen removal by autotrophic ammonium oxidizing bacteria enrichment under anaerobic conditions

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Abstract

Sludge from an anoxic tank at the centralized wastewater treatment plant, Nong Khaem, Bangkok, Thailand, was inoculated in an anaerobic sequencing batch reactor (ASBR). The optimal compositions and operating conditions of the stock of autotrophic ammonium oxidizing bacteria medium were determined. The process of oxidizing ammonium with bacteria under anaerobic conditions is often referred to as the Anammox process (NO_2^- to N_2 gas, using NH_4^+ as the electron donor and NO_2^- as the electron acceptor). The startup period for the anammox culture took more than three months. With ammonium and nitrite concentration ratios of 1:1.38 and 1:1.6, the nitrogen conversion rate zero order. Fluorescent in situ hybridization (FISH) was used to identify specific autotrophic ammonium oxidizing bacteria (*Nitrosomonas* spp., Candidatus *Brocadia anammoxidans*, and Candidatus *Kuenenia stuttgartiensis*). Results from this work demonstrated a shift in the species of ammonium oxidizing bacteria from *Nitrosomonas* spp. to Candidati *Brocadia anammoxidans* and *Kuenenia stuttgartiensis*, with increased ammonium concentrations from 3 mM to 15 mM. Under NH_4^+:NO_2^- ratios of 1:1.38 and 1:1.6 the ammonium oxidizing bacteria were able to remove both ammonium and nitrite simultaneously. The specific nitrogen removal rate of the anammox bacteria (Candidati *Brocadia anammoxidans* and *Kuenenia stuttgartiensis*) was significantly higher than that of anaerobic ammonium oxidizing bacteria (*Nitrosomonas* spp.). Anaerobic ammonium oxidizing bacteria (Candidati *Brocadia anammoxidans* and *Kuenenia stuttgartiensis*) are strict anaerobes.

Keywords: nitrogen removal, anaerobic ammonium oxidizing bacteria

1. Introduction

Nitrogen from domestic wastewater is a significant pollutant source of water pollution and should be removed before it is discharged into the environment. Many nitrogen forms (e.g. ammonia (NH_4^+), nitrite (NO_2^-), and nitrate (NO_3^-)) can have deleterious effects on aquatic life, human health, and the environment. The public health concern posed by nitrate and nitrite is methemoglobinemia or blue-baby syndrome (Johnson and Kross, 1990 and USEPA, 2002). The conventional biological nitrogen removal process involving nitrification (NH_4^+ to NO_2^- to NO_3^-) followed by denitrification (NO_3^- to NO_2^- to N_2) is well known and used worldwide. However, high costs of operation occur with wastewater

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having a high nitrogen but low carbon content. The Sharon process (NH_4^+ to NO_2^- and stopping NO_2^- into NO_3^-) followed by the Anammox process (NH_4^+ as the electron donor is converted to N_2 gas with NO_2^- as the electron acceptor) is a new approach that has been proposed. The Sharon/Anammox processes bypass the formation of NO_3^-, with NO_2^- being converted directly to N_2 gas.

Although this new approach offers advantages over nitrification-denitrification processes, it has not yet been implemented and is currently poorly understood. For example, there is little information available for how to start up the bacteria for the Anammox process using culture from conventional nitrification-denitrification processes. Anammox microbes are autotrophic ammonium oxidizing bacteria. *Nitrosomonas* spp., Candidati *Brocadia anammoxidans*, and *Kuenenia stuttgartiensis* are significant microbes, which are able to denitrify nitrite by using ammonium as electron donor and nitrite as electron acceptor. The nitrogen removal rates of *Nitrosomonas* spp., Candidati *Brocadia anammoxidans*, and *Kuenenia stuttgartiensis* currently are not well understood. In the Anammox process, a stoichiometric molar ratio for NH_4^+:NO_2^- has been proposed to be 1:1.32 by van Dongen et al., 2001a. Nitrogen removal rates at concentration ratios of less or more than 1:1.32 for NH_4^+:NO_2^- are not available.

Anammox microbes are an interesting group of bacteria with very low biomass yield. For this reason, they are difficult to culture using conventional microbiological techniques (Fujii *et al.*, 2002). For studying nitrogen removal by autotrophic ammonium oxidizing bacteria, chemical analysis alone is insufficient to allow differentiation of the species of bacteria responsible. Thus, the technique of fluorescent in situ hybridization (FISH) analysis was used for this study. This analysis enabled identification of the specific ammonium and nitrite oxidizing bacteria in the autotrophic nitrification-denitrification system and in the Anammox process, as well as being both rapid and accurate.

These studies aimed to produce a vital inoculation anammox culture from conventional nitrification-denitrification processes and to compare nitrogen removal rates at molar ratios less or more than 1:1.32 (e.g. 1:0.67, 1:1.38, 1:2.18, and 1:3.05) for NH_4^+:NO_2^-. In addition, the rate of nitrogen removal by populations of *Nitrosomonas* spp., Candidati *Brocadia anammoxidans*, and *Kuenenia stuttgartiensis* was studied.

2. Materials and Methods

2.1 Stock culture

A stock culture of mixed nitrifying bacteria was maintained in an anaerobic sequencing batch reactor (ASBR). The ASBR was inoculated with activated sludge taken from the anoxic tank from the Nong Khaem wastewater treatment plant in Bangkok, Thailand. The reactor was a 1.2 L cylindrical vessel with 10.5 cm diameter, 18 cm height, and 1 L maximum working volume. The reactor contacts were mixed with a magnetic stir-bar at 250 rpm on a mag-netic stirrer. The top of the reactor was closed, but a pipeline was used to collect gas.

A manually controlled ASBR cycle consisted of five periods—fill (10 minutes), reaction time with mixer (24-46 hours), settle (1 hour), decant (10 minutes), and idle (40 minutes). The decant:recycle ratio of 1:1 was maintained by draining 500 mL of supernatant from the 1 L working volume. The remaining 500 mL was retained for the next cycle to which 500 mL of new medium was added. Argon gas (95%) and carbon dioxide (5%) were bubbled into the bottom of the ASBR after filling and before settling in order to limit dissolved oxygen (DO) content.

The composition of the stock culture medium was modified from van Dongen *et al.* (2001a,b) and Isaka *et al.* (2006), as shown in Table 1. The pH of influent and effluent was 7.6+0.4. The molar ratio of ammonium:nitrite was first started at 3:3.9 mM and increased gradually by 1 mM increments to 15:20 mM. At different ammonium:nitrite concentrations, the sample of the culture was taken to study the specific ammonium, nitrite, and nitrate removal rates.

Table 1. Composition of Growth Medium for Autotrophic Ammonium Oxidizing Bacteria

Constituent	Concentration (mM)
$NaNO_2$	3.9
$(NH_4)_2SO_4$	1.5
$KHCO_3$	12.5
KH_2PO_4	0.15
$CaCl_2$ $7H_2O$	2
$MnSO_4$ $7H_2O$	1.9
$FeSO_4$/EDTA	0.05
Trace Elements No. 1No. 2	1 ml1 ml

Modified From van Dongen *et al.*, 2001a,b and Isaka *et al.*, 2006

Trace Elements No. 1: 5g/L EDTA, 5 g/L $FeSO_4$

Trace Elements No. 2: 15g/L EDTA; 0.43 g/L $ZnSO_4$ 7 H_2O; 0.24 g/L $CoCl_2$ $6H_2O$; 0.99 g/L $MnCl_2$ $4H_2O$; 0.25 g/L $CuSO_4$ $5H_2O$; 0.22 g/L $NaMoO_4$ $2H_2O$; 0.19 g/L $NiCl_2$ $6H_2O$; 0.21 g/L, and $NaSeO_4$ 10 H_2O

2.2 Experimental approach

The effect of the ratio of ammonium and nitrite concentrations on ammonium oxidizing bacteria under anaerobic conditions was studied by measuring the rates of NH_4^+ and NO_2^- use. The concentrations of NH_4^+ and NO_2^- were 30.4:20.4; 30:41.3; 31.5:68.8; and 30.7:93.5 mg N/L or the initial molar rations were 1:0.67, 1:1.38, 1:2.18, and 1:3.05, respectively. In each experiment, 100 mL stock culture was taken directly from the ASBR. The top 90% of supernatant was discarded and the bottom 10% of supernatant and solid sediments were used. Fresh medium was added and mixed

with supernatant and solid sediments of ammonium oxidiz-ing bacteria to a total volume of mixture of 100 mL. To four 120 mL glass bottles, 100 mL mixture was transferred. Ar/CO_2 gas was bubbled into the bottom of each bottle for about 2-3 minutes. Then, each bottle was sealed by septum cap to maintain anaerobic conditions. Attempts were made to keep the quantity of biomass the same in each experiment. All glass bottles were thoroughly mixed with magnetic stirring. The pH in each experiment was the same for both influent and effluent (7.6+0.4), but no buffer control was necessary. Experiments for each ammonium:nitrite ratio were run in triplicate.

Ammonium and nitrite control without biomass but with the same synthetic feed, the same temperature, and the same mixing speed showed that ammonium and nitrite concentration did not decrease (data not shown). The rates of NH_4^+ and NO_2^- use were determined by measuring NH_4^+ and NO_2^- concentrations from 0 to 72 hours. In each experiment, NO_3^- concentration was also measured. Biomass (mg VSS/L) was determined at the beginning and the end of the experiments.

After investigating the effect of the ratio between ammonium and nitrite concentrations on ammonium oxidizing bacteria under anaerobic conditions, the stock culture was acclimated to increasing ammonium concentrations from 3 mM to 15 mM. The stock culture at 15 mM of ammonium concentration was used to study ammonium and nitrite removal over time by measuring NH_4^+, NO_2^-, and NO_3^- concentrations.

2.3 Chemical analyses

2.3.1 Ammonium, nitrite, and nitrate concentrations

The concentration of ammonium (NH_4^+) from both influent and effluent was measured by using the titration method described in Standard Methods (1995). The concentration of nitrite (NO_2^-) was measured by the colorimetric method as described in Standard Methods (1995). The concentration of nitrate (NO_3^-) was measured by 761 Compact Ion Chromatograph (Methrom, Herisau, Switzerland) equipped with a conductivity detector.

2.3.2 Biomass concentrations

The biomass of the ammonium oxidizing culture was measured as described in Standard Methods (1995). Biomass was measured at the beginning and at the end of each experiment.

2.3.3 Fluorescent in-situ hybridization (FISH)

Fluorescent in situ hybridization in this work followed the method of Amann (1995) and Daims *et al.* (1999). A sample from the anoxic tank at centralized domestic wastewater treatment plant, Nong Khaem district, Bangkok, was chosen to identify aerobic ammonium oxidizing bacteria (AerAOB, e.g. *Nitrosomonas* spp.), aerobic nitrite oxidizing bacteria (AerNOB, e.g. *Nitrobacter* spp. and *Nitrospira* spp.), and anammox bacteria (Candidatus *Brocadia anammoxidans* and Candidatus *Kuenenia stuttgartiensis*) by using fluorescent in situ hybridization (FISH). Each sample was analyzed in triplicate. To quantify relative bacteria populations in this work, the digital image analysis program *Daime* (version 1.1) was used. Oligonucleotide probes that were used in this investigation are listed in Table 2.

3. Results and Discussion

At the Nong Kheam wastewater treatment plant, Bangkok, Thailand, there were both nitrification and denitrification processes occurring. The primary study found that nitrifying bacteria were able to oxidize ammonium to nitrite

Table 2. Oligonucleotide probes used in this study.

Probe	Target	Sequence	Reference
EUB338	Most Bacteria	GCT GCC TCC CGT AGG AGT	Amann *et al.* (1990)
EUB338II	*Planctomycetales*	GCA GCC ACC CGT AGG TGT	Daims *et al.* (1999)
GAM42a	Gammaproteobacteria	GCC TTC CCA CAT CGT TT	Manz *et al.* (1992)
BET42a	Betaproteobacteria	GCC TTC CCA CTT CGT TT	Manz *et al.* (1992)
ALF1b	Alphaproteobacteria	CGT TCG YTC TGA GCC AG	Manz *et al.* (1992)
Nso 1225	Betaproteobacterial Ammonium Oxidizing Bacteria	CGC CAT TGT ATT ACG TGT GA	Mobarry *et al.* (1996)
Nso 190	Betaproteobacterial Ammonium Oxidizing Bacteria	CGA TCC CCT GCT TTT CTC C	Mobarry *et al.* (1996)
NEU	*Nitrosomonas* spp.	CCC CTC TGC TGC ACT CTA	Wagner *et al.* (1995)
NIT 3	*Nitrobacter* spp.	CCT GTG CTC CAT GCT CCG	Wagner *et al.* (1996)
NTSPA714	*Nitrospira* spp.	CCT TCG CCA CCG GCC TTC	Loy *et al.* (2002)
Amx820	Candidatus *Brocadia anammoxidans* and Candidatus *Kuenenia stuttgartiensis*	AAA ACC CCT CTA CTT AGT GCC C	Schmid *et al.* (2000)

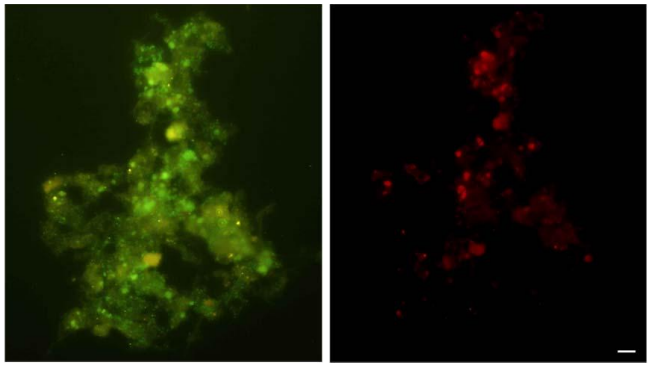
and nitrite into nitrate very well but that denitrifying bacteria in the anoxic tank were not able to oxidize nitrate to nitrogen gas because of high nitrogen loading, but low carbon content in the wastewater. For this reason, sludge from an anoxic tank at the Nong Kheam wastewater treatment plant from an anoxic tank was selected for inoculation in this work. Nongkheam's wastewater consisted of a high rich of nitrifying activity but low denitrifying bacteria activity in the anoxic tank.

A sample from the ASBR was characterized for AerAOB, AerNOB, and anammox bacteria by using fluorescent in situ hybridization (FISH). The results showed that Gammaproteobacteria were the most populous and probes (Nso 1225, Nso 190, and NEU), indicated that the most prevalent species of ammonium oxidizer bacteria was *Nitrosomonas* spp. However, no reaction was observed with the probes NIT3 and NTSPA714, indicating limited *Nitrobacter* spp. and *Nitrospira* spp., respectively. Strous *et al.* (1999) reported that *Planctomycetales* are a major group of anammox bacteria. In addition, probe EUB338II was used and this probe suggested that *Planctomycetales*, and thus perhaps some anammox bacteria, were present in the Nong Khaem sludge, but in very low numbers. It took about three months before nitrifying bacteria could start to remove ammonium under anaerobic conditions.

More than six months later after inoculation, a sample from the ASBR again was characterized for AerAOB, AerNOB, and anammox bacteria, using FISH analysis. The FISH results showed that Betaproteobacteria were the most populous (44.5+6.9%). FISH characterization of both ammonium and nitrite oxidizing bacteria in the stock culture indicated that the greatest percentage was ammonium oxidizing bacteria specific to *Nitrosomonas* spp. (30+4.1%). *Nitrobacter* spp. was not found, but *Nitrospira* spp. was found at 8+6.8%. A group of *Planctomycetales* was detected by probe EUB338II, but only at 2.1+1.9%. The FISH images of an aggregate from stock culture with probes EUB (most bacteria) and Nso1225 (*Nitrosomonas* spp.) are shown in Figure 1 a and b.

Culture at six months after inoculation was used to investigate under or below molar ratios of NH_4^+:NO_2^- at 1: 1.32. In this task the molar ratios between ammonium and nitrite concentrations that were studied were 1:0.67, 1:1.38, 1:2.18, and 1:3.05. Biomass varied slightly between 490 to 1070 mg VSS/L. The results for these ratios are shown in Figure 2 (a) to (d).

Figure 2 (a) shows that at an initial nitrite concentration of about 0.7 times the ammonium concentration, ammonium oxidizers reduced the initial nitrite concentration of 20 mg N/L to a very low concentration (0.8 mg N/L) within 22 hours while decreasing the initial ammonium concentration of 30 mg N/L to 24 mg N/L. Ammonium oxidizers reduced both ammonium and nitrite to very low concentrations (almost zero) in the same time at an initial nitrite concentration of about 1.4 times higher than the ammonium concentration (Figure 2 (b)). Figure 2 (c) shows that ammonium oxidizers completely consumed the initial ammonium concentration of 31 mg N/L within 67 hours while decreasing the initial nitrite concentration of 69 mg N/L to 19 mg N/L. At an initial nitrite concentration of 94 mg N/L/day, ammonium oxidizers also reduced the initial ammonium concentration of 31 mg N/L to very low concentration (0.5 mg N/L) within 67 hours while decreasing nitrite to 51 mg N/L/day (Figure 2 (d)). Figures 2 (c) and (d) show a possible lag time for ammonium oxidizers at high initial nitrite concentrations of 69 and 94 mg N/L. High nitrite concentration at low pH may inhibit the activity of ammonium oxidizers through the formation of free nitrous acid (HNO_2, FNA), while is known to inhibit ammonium oxidizers. For example, Prakasam and Loehr (1976) reported that FNA at 0.3 mg N/L could inhibit both *Nitrosomonas* sp. and *Nitrobacter* sp. Wong-Chong and Loehr (1978) also noted that a FNA concentration of 2.65 mg N/L completely stopped the activity of both ammonium oxidizers and nitrite oxidizers. However, free nitrous acid has a pK_a of 3.4 and at the pH of 7.6 used in this work, it could not be present as the inhibitor of ammonium oxidizing bacteria activity. Other potential reasons for this lag time are not currently known.



(a) (b)

Figure 1. Fluorescent in-situ hybridization image of an aggregate from stock culture maintained at 3 mM ammonium, stained with (a) probe EUB338 (most bacteria) and (b) probe Nso1225 (*Nitrosomonas* spp.)

This study confirmed that autotrophic ammonium oxidizing bacteria (*Nitrosomonas* spp. most populous) were able to denitrify nitrite by using NH_4^+ as electron donor and NO_2^- as electron acceptor. At molar ratios for NH_4^+:NO_2^- ratio of 1:1.38 and 1:1.6 (see Figure 2 (b)), the ammonium oxidizing bacteria were able to remove both ammonium and nitrite simultaneously and ammonium and nitrite conversion rates were 0.03 g N/g *Nitrosomonas* spp. VSS/day and 0.02 *Nitrosomonas* spp. VSS/day, respectively. The total nitrogen removal rate was 0.05 g N/g *Nitrosomonas* spp. VSS/day, comparable to the rate observed by Kuai and Verstraete (1998). They reported total nitrogen conversion rates of 0.02 g N/g VSS/day and postulated that their culture was *Nitrosomonas* spp.

Fluorescent in situ hybridization (FISH) was also

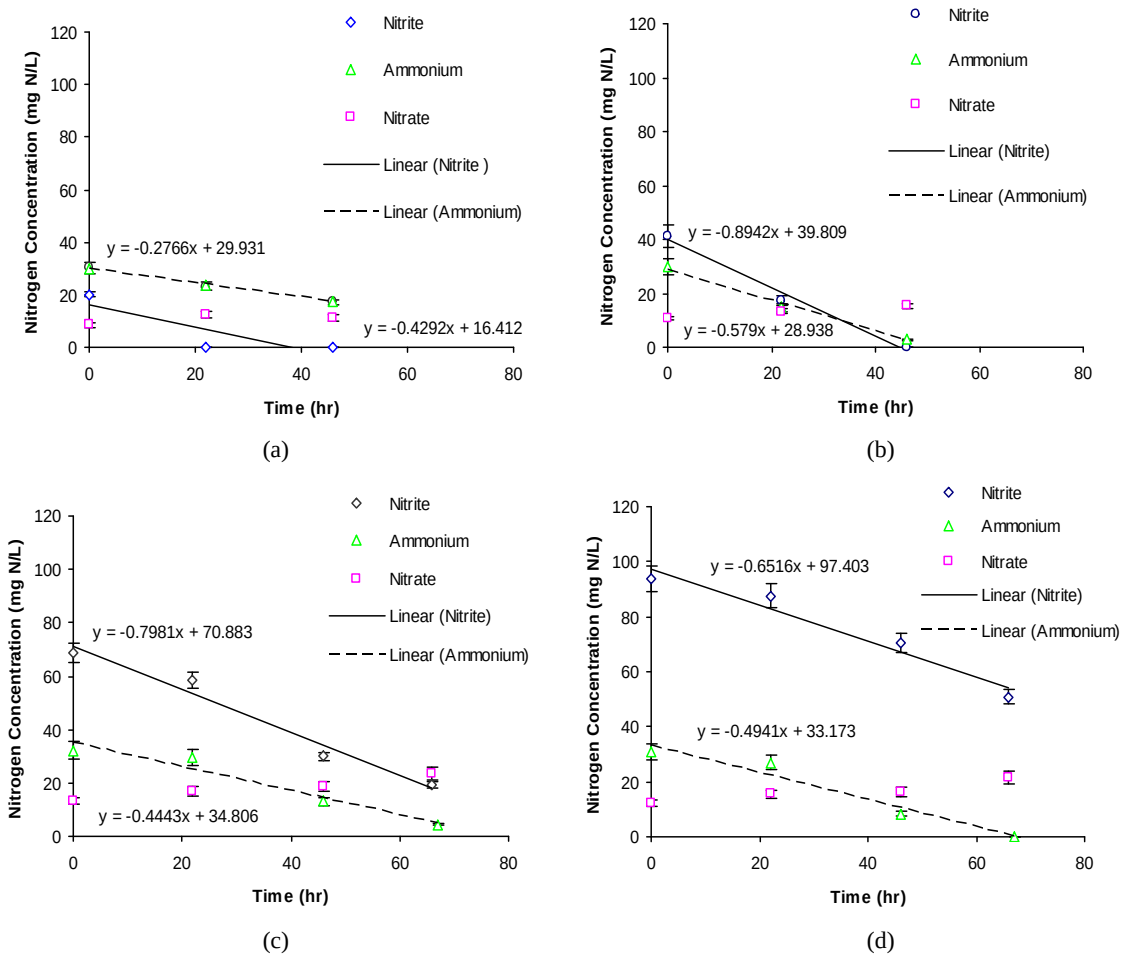
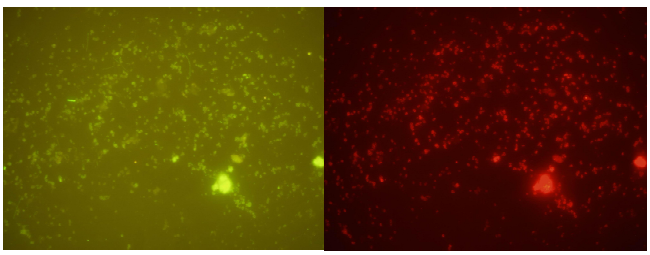


Figure 2. Nitrogen concentration *vs.* time at pH 7.6 during anaerobic ammonium oxidation for different initial ratios of ammonium and nitrite concentrations of (a) 1:0.67, (b) 1:1.38, (c) 1:2.18, and (d) 1:3.05, and biomass 490 (a), 710 (b), 925 (c), and 1070 (d) mg VSS/L

used to identify specific ammonium oxidizing bacteria in the stock culture maintained at the higher initial ammonium concentration of 15mM. FISH characterization of anammox bacteria in the stock culture using probe Amx820 indicated that the greatest percentage (85.6+7.6%) of bacteria were ammonium oxidizing bacteria, specific to Candidatus *Brocadia anammoxidans* and Candidatus *Kuenenia stuttgariensis*. Other organisms present included Gammaproteobacteria (4.4+2.8%), Betaproteobacteria (1.9+1.1%), and Alpha-proteobacteria (1.4+1.1%). *Nitrosomonas* spp., *Nitrobacter* spp., and *Nitrospira* spp. were not found. The FISH images of an aggregate from the stock culture with probes EUB and Amx820 are shown in Figure 3 a and b.

The decreasing ammonium and nitrite concentrations and increasing nitrate concentration within a single cycle of the 1-L stock culture at 74 mg N/L initial ammonium concentration are shown in Figure 4. Indicators of the anammox process were evident at the end of each cycle of ASBR.

Isaka *et al.* (2006) studied the relationship between the population of anammox and nitrogen conversion rate. FISH with probe Amx820 was used to characterize the anammox culture in the experiment. However, the nitrogen conversion rate from their work could not be directly compared with these results because Isaka *et al.* (2006) reported the nitrogen conversion rate in term of cell numbers of anammox bacteria. Their average nitrogen conversion rate was 0.2 mg N/10^7 cells/day. Kuai and Verstraete (1998)



(a) (b)

Figure 3. Fluorescent in-situ hybridization image of an aggregate from the stock culture maintained at 15 mM ammonium, stained with probes (a) EUB338 (most bacteria) and (b) Amx820 (Candidatus *Brocadia anammoxidans* and Candidatus *Kuenenia stuttgariensis)*.

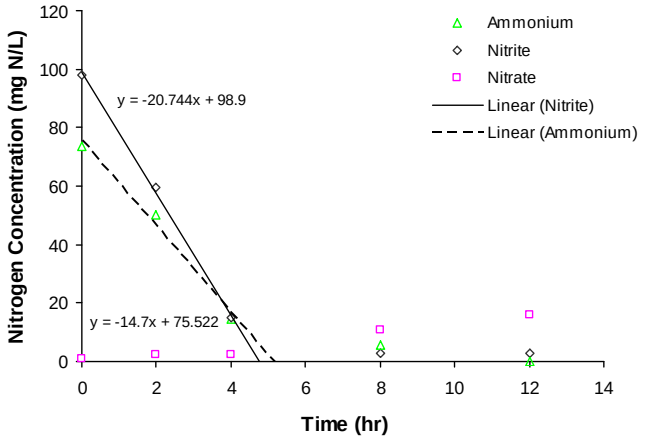


Figure 4. Nitrogen concentrations vs. time during anaerobic ammonium oxidation with initial ammonium concentration of 74 mg N/L, initial nitrite concentration of 98 mg N/L, at pH 7.6, and biomass 1494 mg VSS/L

quantified ammonium oxidizers using the most-probable-number (MPN) method and found a relationship of $2.5x10^{10}$ cells/ g VSS. Using this relationship and the relative population of anammox bacteria in this work, an average nitrogen conversion rate of 0.6 g N/g anammox VSS/day was determined (see Figure 4) in cultures maintained at 15 mM ammonium. These results are quite comparable to that observed by Isaka *et al.* and 12 times higher than the *Nitrosomonas*-specific nitrogen conversion rate described above. This study confirmed that autotrophic ammonium oxidizing bacteria (*Nitrosomonas* spp.) and anammox bacteria (Candidati *Brocadia anammoxidans* and *Kuenenia stuttgartiensis*) were able to denitrify nitrite by using ammonium as an electron donor and nitrite as an electron acceptor.

The anaerobic ammonium oxidizing culture (*Nitrosomonas* spp.) was aerated with addition of only ammonium. After only 10 hours the ammonium concentration decreased and nitrate concentration increased; nitrite was not changed. This result demonstrates that *Nitrosomonas* spp. could again, after a lag time aerobically oxidize ammonium to nitrate when oxygen was available in the system. However, this was not the case with the anaerobic ammonium oxidizing culture (Candidatus *Brocadia anammoxidans*, and Candidatus *Kuenenia stuttgartiensis*), which had no change in ammonium and nitrite concentrations following aeration, even after 10 hours. This indicates that Candidatus *Brocadia anammoxidans*, and Candidatus *Kuenenia stuttgartiensis* (anammox culture) are strict anaerobes.

4. Conclusion

The startup of anaerobic ammonium oxidation with ammonium oxidizing bacteria in an anaerobic sequencing batch reactor required a long period of time (more than three months). At molar ratios for NH_4^+:NO_2^- of 1:1.38 and 1:1.6, the ammonium and nitrite removal rates were zero order. High nitrite concentrations may inhibit the activity of ammonium oxidizers as a lag time was observed at NO_2^- concentrations of 69 and 91 mg N/L. Fluorescent in situ hybridization (FISH) identified shifts in ammonium oxidizing bacteria under different ammonium concentrations from 3 mM to 15 mM. Specific ammonium and nitrite removal rates from autotrophic ammonium oxidizing bacteria enrichment (anammox bacteria) in this culture were significantly higher than autotrophic ammonium oxidizing bacteria enrichment (*Nitrosomonas* spp.). Although the ammonium oxidizing culture (only *Nitrosomonas* spp.) was maintained under anaerobic conditions long term, these bacteria were still able to oxidize NH_4^+ into NO_3^- when oxygen was available.

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References

APHA, AWA, WPCF. 1995. Standard Methods for the Examination of Water and Wastewater. 19th edition, American Public Health Association, Washington, D.C. U.S.A.

Amann, R. 1995. In situ identification of micro-organisms by whole cell hybridization with rRNA-targeted nucleic acid probes, Akkerman, A. D. L., van Elsa, J. D., de Bruijin F.J. editor, Molecular Microbial Ecology. Kluwer Publishers, Dordrecht, Netherlands, pp.1-15.

Daims, H., Bruhl, A., Amann, R., Schleifer, K. H. and Wagner, M. 1999. The domain-specific probe EUB 338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe set. Systematic and Applied Microbiology. 22**,** 434-444.

Fujee, T., Sugino, H., Rouse, J. D. and Furukawa, K. 2002. Characterization of the microbial community in anaerobic ammonium oxidizing biofilm cultured on a nonwoven biomass carrier. Journal Bioscience Bioengineering. 94, 412-418.

Isaka, K., Date, Y., Sumino, T., Yoshie, S. and Tsuneda, S. 2006. Growth characteristics of anaerobic ammonium-oxidizing bacteria in an anaerobic biological filtrated reactor. Apply Microbiology Biotechnology. 70, 47-52.

Johnson, C. J. and Kross, B. C. 1990. Continuing importance of nitrate contamination of groundwater and wells in rural areas. American Journal Industrial Medical. 180, 450-456.

Loy, A. Lehner, A., Lee. N., Adamczyk, J., Meier, H., Ernst, J., Schleifer, K. H. and Wagner, M. 2002. Oligonucle-

otide microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment. Apply Environmental Microbiology. 68(10): 5064-5081.

Kuai, L. and Verstraete, W. 1998. Ammonium removal by the oxygen-limited autotrophic nitrification-denitrification system. Apply Environmental Microbiology. 64, 4500-4506.

Manz, W., Amann, R., Ludwig, W., Wagner, M. and Schleifer, K. H. 1992. Phylogenetic oligonucleotide probes for the major subclasses of Proteobacteria: problem and solutions Systematic and Applied Microbiology. 15, 593-600.

Mobarry, B. K., Wagner, M., Urbain, V., Rittmann, B. E. and Stahl, D. A. 1996. Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. Apply Environmental Microbiology. 62(6): 2156-2162.

Prakasam, T. B. S. and Loehr, R. C. 1976. Microbial nitrification and denitrification in concentrated wastes. Water Research. 6, 859-869.

Schmid, M., Twachtmann, U., Klein, M., Juretschko, S., Jetten, M. S. M., Metzger, J. W., Schleifer, K. H. and Wangner, M. 2000. Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation. Systematic and Applied Microbiology. 23, 93-106.

Strous, M., Fuerst, J. A., Kramer, E. H. M., Longemann, S., Muyzer, G., van de Pas-Schoonen, K. T., Webb, R., Kuenen, J. G. and Jetten, M. S. M., 1999. Missing lithotroph identified as new palntomycete. Nature. 400, 446-449.

van Dongen, J. U. and van Loosdrecht, M. C. M. 2001a. The SHARON®-anammox process for treatment of ammonium rich wastewater. Water Science and Technology. 44(1): 153-160.

van Dongen, L. G. J. M., Jetten, M. S. M. and van Loosdrecht, M. C. M., 2001b. The Combined Sharon/Anammox Process (A sustainable method for N-removal from sludge water), IWA Publishing, Alliance House, London, pp. 17-22.

Wagner, M., G. Rath, R. Amann, H. P. Koops and K. H. Schleifer. 1995. In situ identification of ammonia-oxidizing bacteria. Systematic and Applied Microbiology. 18, 251-264.

Wagner, M., Rath, G., Koops, H-P, Flood, J.and Amann, R. 1996. In situ analysis of nitrifying bacteria in sewage treatment plants. Water Science and Technology. 34 (1): 237-244.

Wong-Chong, G. M. and Loehr, R.C. (1978). Kinetics of microbial nitrification: nitrite nitrogen oxidation. Water Research. 12, 605-609.

U.S. EPA 2002. Onsite Wastewater Treatment Systems Design Manual. EPA/625/R-00/008. [February 2002]